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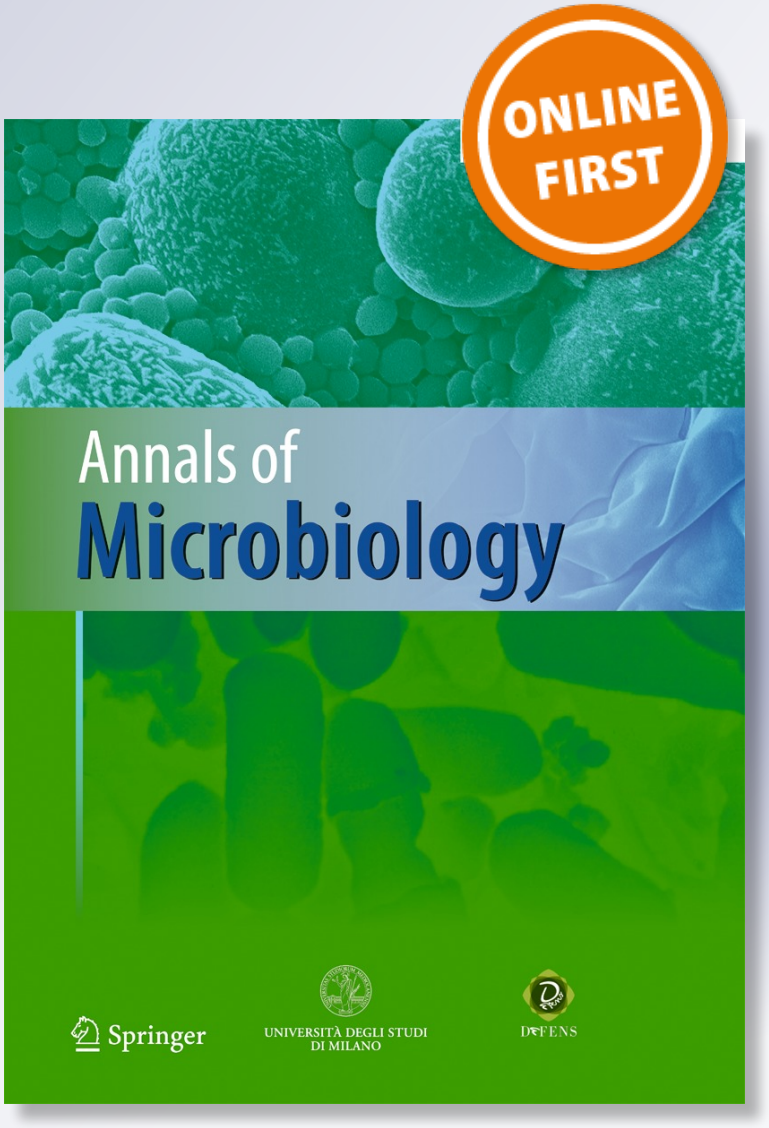

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ORIGINAL ARTICLE

Diversity and phylogeny of soybean rhizobia in central India

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Abstract Soybean is the most important crop legume with the highest share of biological nitrogen fixation among cultivated legumes. During the early years following the introduction of soybean cultivation in India, the effects of rhizobial inoculation were impressive, but they have declined over time due to naturalization of the introduced strains. We have characterized the diversity of soybean rhizobia, mainly those from central India, for phenotypic features, such as utilization of carbon sources and intrinsic resistance to antibiotics. The PCR-amplified 16S rRNA gene products of the strains were sequenced to study genetic diversity and phylogenetic relationships. We found that the rhizobia comprised both slow and fast growers, with the former having a higher Shannon–Weiner Diversity Index (H=2.93 and 3.00 for carbon utilization and intrinsic antibiotic resistance, respectively) than the latter (H=2.62 and 1.90, respectively). There were two 16S rRNA sequence types among the slow growers—*Bradyrhizobium* spp. (99.4–99.8 % sequence homology) and *Rhizobium radiobacter* (96.1–99.7 %). In contrast, the fast growing strains belonged exclusively to *R. radiobacter* (98.9–99.7 %). *Bradyrhizobium japonicum* strain USDA 110, which was originally introduced on a large scale in Indian soils more than four decades ago, shared 34–81 % phenotypic and 63–83 % genotypic similarity with the other Indian rhizobial isolates characterized. There was conservation of 16S rRNA gene sequences among rhizobia in various soybean-growing areas and the evolution of native rhizobial strains among slow and fast growers. These results on the biodiversity of soybean rhizobia are important for strain selection, which is crucial for the design of successful inoculation programs.

Keywords *Bradyrhizobium* · Bioinoculants · Carbon utilization · Central India · Intrinsic antibiotic resistance · Microbial ecology

Introduction

Soybean is the most important legume crop since it fixes 16 Tg of atmospheric nitrogen each year, which represents nearly 77 % of the nitrogen biologically fixed by crop legumes (Herridge et al. 2008). Most of the soybean cultivation in India is concentrated in about 10.3 million ha in central and western India, an area of predominantly Vertisol soils. The large-scale cultivation of soybean in India began in the late 1960s with the use of inoculants prepared from *Bradyrhizobium japonicum* cultures imported from USA. Several acclimatized strains derived from the imported strains were subsequently used for the production of inoculants. The effects of inoculation during the early years of soybean cultivation were very convincing (Dube 1975), but the response gradually declined over time (Rawat et al. 2008). Hungria and Vargas (2000) observed a similar declining trend of response to introduced rhizobial strains in Brazil and attributed this to the naturalization (evolution of native or indigenous strains) of the introduced strains—namely, the indigenously adapted rhizobia were more competitive in terms of nodulation occupancy than the introduced strains.

Soybean has been reported to be nodulated worldwide by the slow-growing *Bradyrhizobium japonicum* strains. It has also been reported to be nodulated by the extra-slow-growing *B. liaoningense* and fast-growing *Sinorhizobium fredii* strains

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in China (Xu et al. 1995; Li et al. 2011; Zhang et al. 2011). Nodulation of soybean by fast-growing rhizobia has also been reported by Hungria et al. (2001) in Brazil and Sharma et al. (2010) in central India. Chen et al. (2002) isolated native strains of soybean rhizobia in Paraguay that resembled *Agrobacterium* sp. and which were effective in nodulating soybean. Sullivan et al. (1996) and Santos et al. (1999) concluded that large diversities of soybean rhizobia (in countries where soybean was an exotic crop) might have arisen due to the transfer of symbiotic genes from the strains used in the introduced inoculants to the indigenous non-symbiotic rhizobia. Consequently, the rhizobial population structure in soils is an ultimate resultant of not only the selection imposed by the host plant and the environmental factors, but also the genetic interactions among the introduced and native rhizobia. A comprehensive knowledge of the biodiversity of rhizobia in soils is thus important for strain selection in inoculant programs.

In a review on rhizobial diversity, Lindstrom et al. (2010) concluded that while intensive research on the molecular diversity of rhizobia has been carried out, there have as yet been no attempts to link this knowledge to the more practical agronomic requirement of searching for strains of rhizobia that are more competitive when used as inoculants. This is especially true in situations where there is extensive adaptation of introduced rhizobia to the local conditions that lead to the evolution of native rhizobia. Several reports on the molecular diversity and biogeography of soybean rhizobia have recently been published (Vinuesa et al. 2008; Li et al. 2011; Zhang et al. 2011; Adhikari et al. 2012; Qi et al. 2012), including a report on soybean rhizobia in Indian soils (Appunu et al. 2008). Surveys carried out by the All India Network Project on Biofertilizers of the Indian Council of Agricultural Research on soybean rhizobia in central India during 2002–2007 (Saxena et al., personal communication) found that about 85 % of the isolates contained the 900-bp RSα gene fragment, indicating the predominance of the slow-growing *B. japonicum* group. Studies by Appunu et al. (2008) revealed eight haplotypes of soybean bradyrhizobia in India based on PCR-restriction fragment length polymorphism (RFLP) analysis of 16S rRNA and intergenic spacer (IGS) region between 16S and 23S rRNA. These authors found that genetic diversity was conserved across regions and was wider than expected. Sequence analysis of the IGS region and four other functional genes, including *nif*H, revealed three groups of rhizobia, *Bradyrhizobium liaoningense*, *B. yuanmingense* and a third group different from other described species but with the same symbiotic genotype as *B. liaoningense* and *B. japonicum* bv. *glycinearum* (Appunu et al. 2008).

In earlier studies on soybean rhizobia in Indian soils including those in Vertisols (Annapurna et al. 2007; Sharma et al. 2010) the sequences of the 16S rRNA gene were not reported, and in the study of Appunu et al. (2008), phenotypic diversity was not studied. The study reported here is the first to focus on the genetic diversity of soybean rhizobia based on both 16S rRNA gene sequences and phenotypic diversity. Also, in none of the previous studies was there an attempt to distinguish the native adapted, slow- and fast-growing rhizobia from the slow-growing strains introduced in the 1960s from the USA. Since the originally introduced slow-growing strains of *B. japonicum* and the native slow- and fast-growing Indian strains have all interacted with the soybean host plants for several decades during cultivation in the same geographical region of central India, we expected that there would be varying degrees of homology between these strains, which has important implications when selecting rhizobial strains for inoculant production for soybean. Therefore, the objectives of our study were to assess the characteristics of native soybean rhizobia in the Vertisols of central India in terms of catabolic versatility, intrinsic antibiotic resistance (IAR) and 16S rRNA gene homology and to compare these characteristics with those of the originally introduced strains from the U.S. Department of Agriculture (USDA) collection in order to understand the variations and nature and extent of soybean rhizobial diversity.

Materials and methods

Soil and nodule samples

Root nodules were sampled from healthy soybean plants grown in experimental and farmers' fields in Vertisols in Madhya Pradesh, central India (Table 1; Fig. 1) and stored at 4 °C. Soil samples (depth 0–15 cm) were air-dried and ground to pass through a 2-mm sieve for determination of pH and electrical conductivity (in 1:2 soil:water). Organic carbon was determined by the Walkley–Black method (Hesse 1971). Soil samples were also taken from other traditional soybean-growing areas belonging to Vertisols and other soil orders in India, including those from the experimental farms of G. B. Pant University of Agriculture and Technology (GBPUAT; Pantnagar, North India) and Marathwada Agricultural University (MAU; Parbhani, Western India), and from some non-traditional areas, such as the experimental farms of Punjab Agricultural University (PAU; Ludhiana) and Himachal Pradesh Krishi Vishwavidyalaya (HPKV; Palampur), both in northern India. At these four sites, soil was sampled between soybean plants and used to isolate rhizobia using soybean as 'trap plants'. These soils were chosen in diverse geographical regions within a specific context of very early introduction (Palampur, Himachal Pradesh; acid Alfisol), early introduction (Pantnagar, Uttarakhand; Mollisol) and recent introduction (Parbhani, Maharashtra; Vertisol) of soybean cultivation.

Table 1 Location and salient soil properties (depth 0–15 cm)[a] of the soybean rhizobial isolation sites

Strain	District	Farm/village	pH	Electrical conductivity (mS/cm)	Organic carbon content (%)
Central India					
R1	Bhopal	IISS general farm, Bhopal	7.8	0.24	0.39
R2	Rajgarh	Farmer field, Geelakhedi, Narsinghgarh	7.7	0.85	0.77
R3	Anuppur	Deptt. Agric. farm, Pondi, Pushprajgarh	6.8	0.38	0.69
R4	Betul	KVK farm, Betul Bazaar	6.8	0.36	0.39
R5	Betul	Beej Nigam farm, Betul Bazaar.	7.0	0.52	0.40
R6	Chhattarpur	Farmer field, Rajnagar	7.9	0.17	0.55
R7	Chhattarpur	Farmer field, Brijpura	7.4	0.20	0.78
R8	Chhindwara	ZARS farm, Chhindwara	6.4	0.23	0.97
R9	Dewas	Farmer field, Bherua, Tonk Khurd	7.4	0.10	0.42
R10	Dhar	Farmer field, Delmi	8.1	0.19	0.73
R11	Dhar	Farmer field, Delmi	7.9	0.14	0.64
R12	Harda	Farmer field, Pidagaon	7.9	0.16	0.68
R13	Indore	Farmer field, Keshati, Agra/Sanver, Depalpur	7.1	0.10	0.65
R14	Narsinghpur	Farmer field, Choti Barman, Kareli	8.2	0.15	0.76
R15	Rajgarh	Farmer field, Geelakhedi, Narsinghgarh	7.8	0.13	0.88
R16	Rajgarh	Farmer field, Geelakhedi, Narsinghgarh	7.7	0.14	0.89
R17	Rajgarh	Farmer field, Geelakhedi, Narsinghgarh	7.8	0.15	0.72
R18	Seoni	Beej Nigam farm, Sikara, Lakhnadon	7.3	0.26	0.80
R19	Tikamgarh	Agric. College and Farmer field, Tikamgarh, Chandrapur/ Tikamgarh, Orchha	7.5	0.20	0.59
R20	Vidisha	Farmer field, Rangai, Vidisha	7.7	0.22	1.16
R21	Dhar	Farmer field, Betma, Dhar	8.1	ND	0.44
R22	Dhar	Farmer field, Betma, Dhar			
R23	Dhar	Farmer field, Utavad, Dhar			
R24	Sehore	Farmer field, Daulatpur, Sehore	7.8	ND	0.64
R25	Sehore	Farmer field, Daulatpur, Sehore			
R26	Sehore	MP Oil Fed Centre, Amlah, Sehore			
R33	Vidisha	Farmer field, Sanchi, Vidisha	7.9	0.66	1.04
R34	Vidisha				
R35	Vidisha				
R36	Vidisha				
Rest of India[b]					
R50	Parbhani	MAU farm, Parbhani	7.7	0.87	1.19
R51	Pantnagar	GBPUAT farm, Pantnagar	8.1	0.48	0.38
R52	Palampur	HPKV farm, Palampur	5.1	0.44	1.16
R53	Ludhiana	PAU farm, Ludhiana	8.0	0.61	0.39

ND Not determined

Strains R21–R26 were obtained from the Directorate of Soybean Research, Indore; see ESM 1 for original strain codes

[a] Soil properties of pooled samples for strains R21-R26 are from Sharma et al. (2010)

[b] See text in section Soil and nodule samples for definition of the farm abbreviations

Rhizobial strains

Healthy pink nodules were surface sterilized, and rhizobia were isolated in Congo-red yeast extract medium and characterized by morphological, physiological and nodulation tests as per standard procedures (Vincent 1970). Soybean isolates R1, R2 and R32–R36) are from the IISS culture collection, isolates R3–R20 are from the AICRP-BNF survey collection at Jawaharlal Nehru Krishi Vishwavidyalaya, Jabalpur, isolates R21–R26 are from the Directorate of Soybean Research,

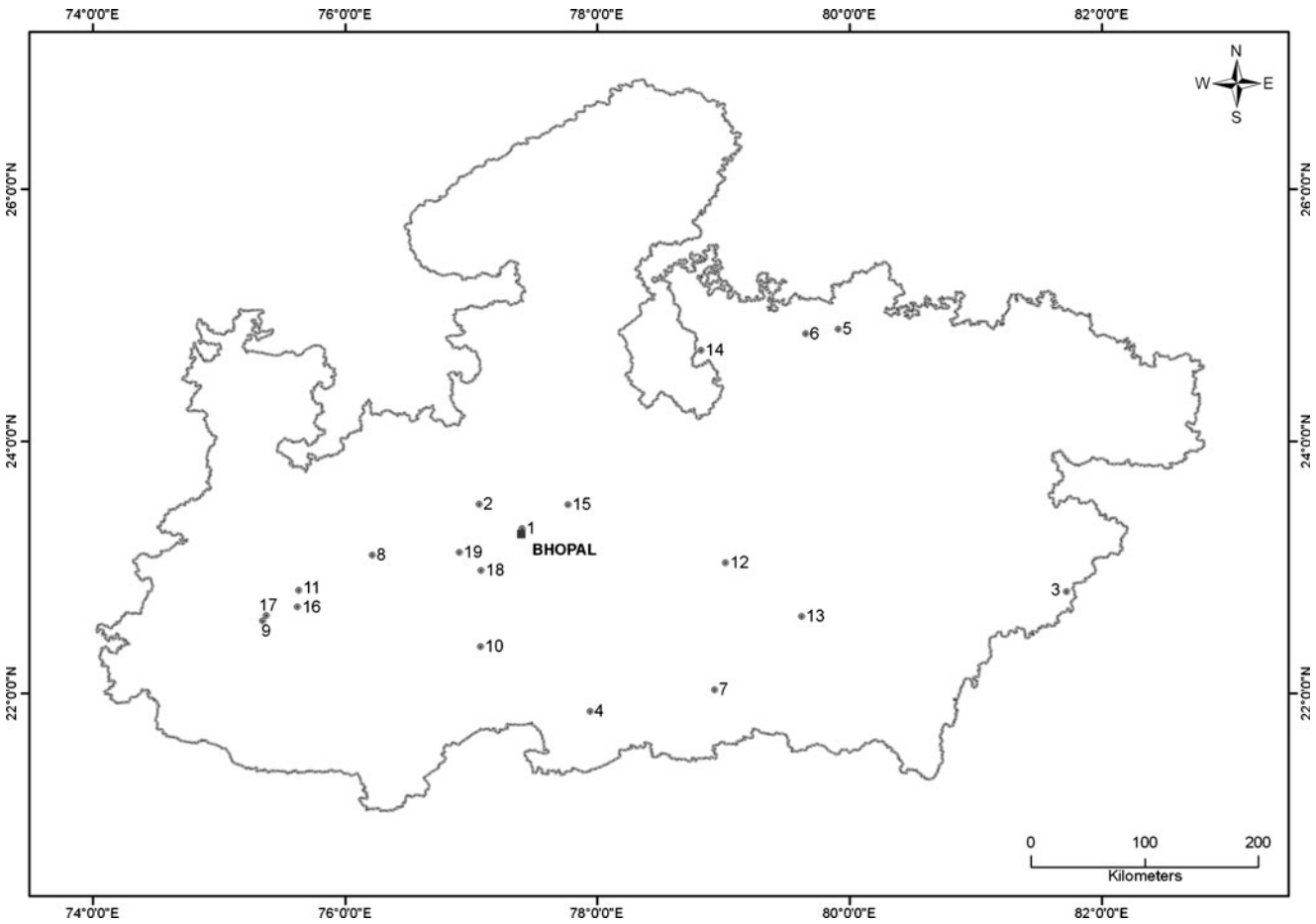

Fig. 1 Map showing the locations of rhizobia sampling in Madhya Pradesh, central India. *Numbers in parenthesis* Strain numbers: *1* Bhopal (R1), *2* Rajgarh (R2, R15, R16, R17), *3* Anuppur (R3), *4* Betul (R4, R5), *5* Chhattarpur (R6), *6* Chhattarpur (R7), *7* Chhindwara (R8), *8* Dewas (R9), *9* Dhar (R10, R11), *10* Harda (R12), *11* Indore (R13), *12* Narsinghpur (R14), *13* Seoni (R18), *14* Tikamgarh (R19), *15* Vidisha (R20, R33, R34, R35, R36), *16* Dhar (R21, R22), *17* Dhar (R23), *18* Sehore (R24, R25), *19* Sehore (R26)

Indore, isolates R30–R31 were isolated from a commercial liquid inoculant, R32 is a re-isolate from a commercial inoculant after plant passage and isolates R50–53 are isolates from soils. Reference strains *Bradyrhizobium japonicum* USDA 110 (R27), *B. japonicum* USDA 122 (R28) and *B. elkanii* USDA 31 (R29) were also included in our analysis. Working stocks of the cultures were maintained on agar slopes at 4 °C; for long-term preservation, the isolates were maintained as glycerol stocks at −20 °C.

Phenotypic diversity

Utilization of carbon sources

The Hi-Carbohydrate kit (Hi Media Laboratories, Mumbai, India), a standardized colorimetric identification system with 35 carbohydrate utilization tests pre-dispensed in wells in three aseptically sealed units, was used to analyze the carbon sources. Each unit of this kit has 12 wells containing agar growth medium, with a different carbon source in each well, and one control. The tests are based on the principle of pH change and substrate utilization, and scored as positive or negative based on the results as defined on the interpretation chart. The carbon sources were monosaccharides and their derivatives (adonitol, L-arabinose, D-arabinose, dextrose, dulcitol, fructose, galactose, glucosamine, glycerol, α- methyl-D- glucoside, inositol, mannose, mannitol, α-methyl-D-mannoside, rhamnose, ribose, sorbose, sorbitol, xylose, xylitol), disaccharides and their derivatives [cellobiose, lactose, maltose, melibiose, ortho-nitrophenyl-galactoside (ONPG), salicin, sucrose, trehalose], trisaccharides and their derivatives (melezitose, raffinose), polysaccharides (inulin) and some others (sodium gluconate, esculin, citrate, malonate). A 5-μl sample of each rhizobial culture suspension (OD 0.5 at 620 nm) was inoculated into each well, and the units were incubated at 28 °C for 48 h. Changes in color were taken as indicating a positive result for utilization: for the carbohydrate fermentation test, from red to yellow; for ONPG, from colorless to yellow; for esculin, from cream to black; for citrate, from yellowish green to blue; for malonate, from light green to blue.

Intrinsic antibiotic resistance

Nineteen antibiotics of different groups were tested: ß-lactams (ampicillin 10 μg, carbenicillin 100 μg); aminoglycosides (streptomycin 25 μg, kanamycin 30 μg, gentamicin 10 μg, neomycin 30 μg, amikacin 30 μg, novobiocin 30 μg); tetracyclines (tetracycline 30 μg); macrolides (erythromycin 15 μg); polypeptides (polymyxin B 300 units); rifamycin (rifampicin 5 μg); glycopeptides (vancomycin 30 μg); quinolones (ciprofloxacin 5 μg, nalidixic acid 30 μg); sulfonamides (trimethoprim 5 μg); aminocyclitol (spectinomycin 100 μg); others (chloramphenicol 30 μg, co-trimoxazole 25 μg). Twenty-four-hour-old cultures of *Rhizobium* were used to make bacterial lawns on yeast extract-mannitol agar medium in plates. Impregnated antibiotic discs (Hi Media Laboratories) were pressed onto the agar surface. The petri plates were incubated at 28 ° C, and the clearing zone around the discs (2–5 days incubation for fast growers and 7–10 days for slow growers) were measured using the Hi-Antibiotic zone scale supplied by the manufacturer.

Diversity indices

Two indices of species richness were calculated: Margalef's Index [$D_{Mg}=(S-1)/\ln N$] and Menhinick's Index ($D_{Mn}=S/\sqrt{N}$; Whittaker 1977) where S=number of different species (i.e. no. of different carbon utilization or antibiotic resistance patterns) and N=total number of individuals in the sample (i.e. total isolates screened). The Shannon–Weiner Diversity Index (H) was calculated as:

$$H = -\sum_{i=1}^{s} (P_i * \ln P_i)$$

where p_i is the fraction of individuals belonging to the i-th species (Pielou 1969; Saeki et al. 2008). Evenness (E) is a measure of how similar the abundances of different patterns were among the rhizobial strains sampled. Using species richness (S) and H, we computed the evenness (E) of carbon utilization and antibiotic resistance as: $E=H/\ln(S)$.

Cluster analysis

The data on carbon utilization (35 sources) by the rhizobial strains were used for the cluster analysis. A binary matrix was constructed where 0 denotes inability and 1 denotes ability to catabolize a particular source. Similarly, the IAR (19 antibiotics) of the rhizobial strains was also used to assess diversity, with 0 denoting sensitivity and 1 denoting resistance to a particular antibiotic. The data were analyzed using the SIMQUAL (Similarity for Qualitative data) subroutine of NTSYS (Numerical Taxonomic and Multivariate Analysis System). The isolates were grouped by the unweighted paired group method using arithmetic means (UPGMA) and depicted in a dendrogram. The data on IAR and carbon utilization were combined in a final matrix containing all 54 datasets (35 carbon sources, 19 antibiotics), and similarity was analyzed to construct a combined dendrogram.

Genotypic diversity

In the analysis of genotypic diversity we selected a subsample of 17 strains from among all of the slow- and fast-growing strains to represent various growing zones in Madhya Pradesh and in the rest of India (10 fast growing and seven slow growing; 14 from central India, two from the rest of India and one reference strain) (Table 1).

16S rRNA gene analysis

Genomic DNA was isolated from exponentially growing cultures of the rhizobia using a genomic DNA isolation kit (Axygen Scientific Inc., Union City, CA). PCR amplification was performed in a thermal cycler (Eppendorf Master Cycler Gradient; Eppendorf, Hamburg, Germany) in 25-μl reaction mixtures containing 2.5 μl of 10× buffer, 1.5 μl of 25 mM $MgCl_2$, 2.5 μl of 2 mM dNTPs, 13.55 μl PCR water, 1.0 μl of forward primer (100 pmol) 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′), 1.0 μl of reverse primer (100 μl) 1492R (5′-ACG GCT ACC TTG TTA CGA CTT-3′), 0.1 μl RNAase 5U/μl, 0.3 μl Taq-DNA polymerase (5U/μl) and 2.55 μl of bacterial DNA. The temperature and cycling conditions used were one cycle at 95 °C for 5 min; 35 cycles at 94 °C for 1 min, 53.9 °C for 1 min and 72 °C for 2 min), with a final extension at 72 °C for 10 min. The PCR products and their concentrations were verified by electrophoresis of a 5-μl sample of each product on a 1 % agarose gel and staining with ethidium bromide. A molecular mass marker was included to estimate the length of the amplicons. The amplified products of the near full-length sequences were purified using a PCR purification kit of Promega (Madison, WI) and custom sequenced using the BDT v3.1 Cycle Sequencing kit on an ABI 3,730×l Genetic Analyzer (Applied Biosystems, Foster City) at Xcelris Labs Ltd., Ahmedabad, Gujarat, India.

Genbank accession numbers

The 16S rRNA gene sequences of the rhizobial strains were deposited in the National Center for Biotechnology Information (NCBI) GenBank database and accession number obtained (Table 1). Nearest identities of the strains were obtained by comparing sequences of the isolated 16S

rRNA gene with available sequences in GenBank (http://www.ncbi.nlm.nih.gov/) using the BLASTn program.

Phylogenetic analysis

The 16S rRNA gene sequences of the isolated strains and the sequence of the reference strains in GenBank were aligned using Molecular Evolutionary Genetics Analysis software (MEGA, ver. 5.0). A phylogenetic tree was constructed by the neighbor-joining method using Kimura's two-parameter model. Confidence in the nodes was assessed using bootstrap proportions (500 replicates). For relating the 16S rRNA gene sequences, we included the sequences of another 17 reference strains of rhizobia in the analysis. These latter strains were representatives of the genera *Agrobacterium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*.

Divergence analysis

The on-line software of Chun et al. (2007) was used to match each 16S rRNA gene sequence of the 17 soybean rhizobial strains with type strains of *Rhizobium radiobacter*, *Bradyrhizobium japonicum*, *B. liaoningense*, *B. yuanmingense*, *B. elkanii*, *Mesorhizobium ciceri*, *M. loti*, *Rhizobium ciceri*, *R. leguminosarum*, *R. lupini*, *R. phaseoli*, *R. pisi*, *Sinorhizobium fredii* and *S. meliloti* to determine the level of similarity.

Results

Phenotypic diversity

Utilization of carbon sources

The morphological, physiological and biochemical traits of the 40 rhizobial strains are given in Electronic Supplementary Material (ESM) 1, and the utilization of carbon sources is presented in ESM 2. About 80 % of the soybean rhizobial strains utilized mannose, L-arabinose, ribose or sucrose while about 45 % utilized esculin. All of the carbon sources tested were utilized by at least one of the rhizobial strains. Among the 20 monosaccharides tested, mannose was utilized by the majority of strains (29), followed by L-arabinose (28) and ribose (27 strains); all the monosaccharides were utilized by at least one the rhizobial strains. Of the eight disaccharides tested, sucrose was utilized by the majority of strains (28) while the others were utilized by at least one other rhizobial strain. Of the five polysaccharides tested, esculin was utilized by the majority of strains (18). Interestingly, there was no rhizobial strain among the 40 tested that could utilize all of the 35 carbohydrate sources. The number of sources utilized by any one strain ranged from 0 to 29 (mean 15). The slow-growing bradyrhizobia had greater catabolic versatility since they utilized all of the 35 carbohydrate sources while the fast-growing rhizobia utilized only 30 sources. The fast growers could not utilize xylitol, α-methyl-D-mannoside, sorbose and sodium gluconate. The better utilization of trehalose and raffinose by fast growers compared to slow growers (87 vs. 73 % and 35 vs. 30 %, respectively) coupled with the poorer ability of fast growers to utilize glucosamine [33 vs. 75 % (slow growers)] distinguished the fast growers from the slow growers.

Intrinsic antibiotic resistance

Of the 20 slow-growing bradyrhizobia, 15 were resistant to trimethoprim, 16 to nalidixic acid, 13 to polymyxin B, 14 to vancomycin and nine to novobiocin (ESM 3). Of the 15 fast-growing rhizobia, ten were resistant to trimethoprim, nine to nalidixic acid and eight to novobiocin. These results indicate that the slow growers were more resistant to antibiotics. Both slow- and fast-growing rhizobia showed maximum resistance to nalidixic acid and trimethoprim (slow-growing rhizobia: 80 % and 75 %, respectively; fast-growing rhizobia: 60 and 67 %, respectively). All of the slow- and fast-growing rhizobia were sensitive to gentamycin and tetracycline, and the slow-growing rhizobia were also sensitive to spectinomycin. Compared to the slow growers, the fast growers were more sensitive to seven antibiotics, namely, gentamycin, amikacin, neomycin, tetracycline, kanamycin, co-trimoxazole and streptomycin. A larger proportion of slow growers were resistant to vancomycin, polymyxin-B and rifampicin (70, 65 and 55 % respectively) compared to fast growers (13, 7 and 7 %, respectively). There was only one slow-growing strain [R26 from Sehore (Indore 9b)] and two fast-growing strains [R18 from Seoni and R24 from Sehore (Indore 7.1)] that were completely sensitive to all 19 antibiotics. One strain with intermediate growth, R7 from Chattarpur, was completely sensitive to all antibiotics.

Diversity indices

As shown in Table 2, the Margalef Index of species richness (D_{Mg}) was slightly higher for all of the Indian strains than for only the central Indian strains (9.07 vs. 8.24 for carbon utilization and 6.80 vs. 5.88 for IAR, respectively). The Menhinick Index of species richness (D_{Mn}) showed a similar trend, being higher for all of the Indian strains than for the central Indian strains only [5.66 vs. 5.29 (carbon utilization) and 4.26 vs. 3.83 (antibiotic resistance), respectively]. Slow growers also showed a higher degree of species richness than fast growers. For carbon utilization, the D_{Mg} was 6.00 for slow growers versus 4.80 for fast growers, and the D_{Mn} was 4.25 for slow growers versus 3.61 for slow growers. For the IAR, the D_{Mg} was 6.34 for slow growers versus 2.95 for fast growers, and the D_{Mn} was 4.47 for slow growers versus 2.32 for fast growers. Particularly with respect to IAR, the reduction in the diversity of fast growers was marked.

Table 2 Species richness, evenness and Shannon–Weiner Diversity Index of soybean rhizobia[a]

Region and growth rate	Carbohydrates					Antibiotics				
	S	D_{Mg}	D_{Mn}	*E*	*H*	*S*	D_{Mg}	D_{Mn}	*E*	*H*
Region[b]										
Strains from central India (30)	29	8.24	5.29	0.996	3.35	21	5.88	3.83	0.923	2.81
All strains (34)	33	9.07	5.66	0.997	3.49	25	6.80	4.29	0.934	3.01
Growth rate										
Slow (20)	19	6.00	4.25	0.994	2.93	20	6.34	4.47	1.000	3.00
Fast (15)	14	4.80	3.61	0.991	2.62	9	2.95	2.32	0.864	1.90

[a] Diversity indexes of species richness: Margalef's Index (D_{Mg}); Menhinick's Index (D_{Mn}). *S* is number of different species (i.e. species richness: no. of different carbon utilization or antibiotic resistance patterns); *H* is the Shannon–Weiner Diversity Index; *E* is the evenness of carbon utilization and antibiotic resistance. For more detailed description, see section Diversity indices

[b] Number of strains analyzed is given in parenthesis

The Shannon–Weiner Diversity Index (*H*) of soybean rhizobial strains in central India based on utilization of carbon sources was 3.35 and more discriminatory than that based on IAR, which was 2.81 (Table 2). As expected, *H* was higher in the strains from all of India (3.49 and 3.01 for carbon utilization and IAR, respectively). The evenness of the rhizobial species strains from central or all of India or in slow and fast growers was similar (0.92–1.00) except in the case of fast growers for IAR where the evenness was clearly lower (0.86) (Table 2).

Cluster analysis

The dendrogram of the combined cluster analysis based on IAR and carbon utilization showed five clusters at the 52 % level of similarity (Fig. 2). Cluster 5 at the top contained the majority of the strains (25 of 40). The distribution of these strains was as follows: 19/32 from central India, 4/4 from the rest of India and 2/4 reference strains. All of the strains in this major cluster shared 62 % similarity. *Bradyrhizobium japonicum* strain USDA 110, the strain originally introduced in Indian soils from the USA, fell in this major cluster and shared 34–81 % similarity with the rest of the Indian isolates (Fig. 2). This major cluster included 16 of the 26 strains that were effective in the field screenings for grain yield promotion (data not shown; a strain was considered effective if it increased grain yield by 15 %). Of these 16 effective strains, 13 strains were from central India (two slow growers, one intermediate grower, 10 fast growers), two were from rest of India and one was a reference strain (all slow growing).

These results show that fast growers had a greater similarity among themselves (63–88 %) than slow growers (48–69 %). This difference was also evident from the dendrogram in which the former strains grouped themselves in the upper part of the dendrogram and showed a higher coefficient of similarity than the slow growers that grouped themselves on the lower part of the dendrogram and showed a lower coefficient of similarity. In other words, there was greater diversity among the slow-growing bradyrhizobia (31–52 %) than among the fast-growing rhizobia (12–37 %).

It is significant that of the 15 fast-growing strains, 13 fell in cluster 5 and one strain (R20) was a nearest neighbor in cluster 4; the only exception was strain R18 which fell in cluster 1 at the bottom of the dendrogram. It is also significant that of the five strains which showed intermediate growth, four fell in this cluster and another strain with intermediate growth (R21) was the nearest neighbor in cluster 4. Thus, of the 20 strains that showed either a fast or intermediate growth rate, 19 (95 %) clustered together at the top of the dendrogram. Additionally, within mega cluster 5, there was minor sub-cluster of six soybean rhizobia (R4, R10, R22, R12, R14, R11) that were all fast growing and from in a very intensive soybean growing region of the Malwa plateau and Narmada river valley in central India in a 400-km belt; these six strains shared 88 % similarity. There was also a minor sub-cluster of two slow-growing strains [R27; USDA 110) and R 29 (USDA 31)] that shared 90 % similarity among themselves. Their nearest neighbors, i.e. R52 (Palampur) and R50 (Pantnagar) were also slow growers, but shared only 72 % similarity.

Interestingly, cluster 3 contained five strains that were all slow-growing. Of these, four are reference strains from the USA that shared 64 % similarity among themselves and 54 % similarity with the fifth strain in the cluster (R2) that was isolated from Geelakhedi village located near Rajgarh in central India. Cluster 2 contained five strains that shared 53 % similarity. Of these, three (R33, 34, 35) were from the same field sampled at the same time in a 1-m^2 area. These strains shared 53 % similarity among themselves. The highest similarity was only 67 % (between R33 and R 35). Cluster 1 contained three strains, namely, R17 (Rajgarh), R18 (Seoni) and R19 (Tikamgarh), which shared 63 % similarity.

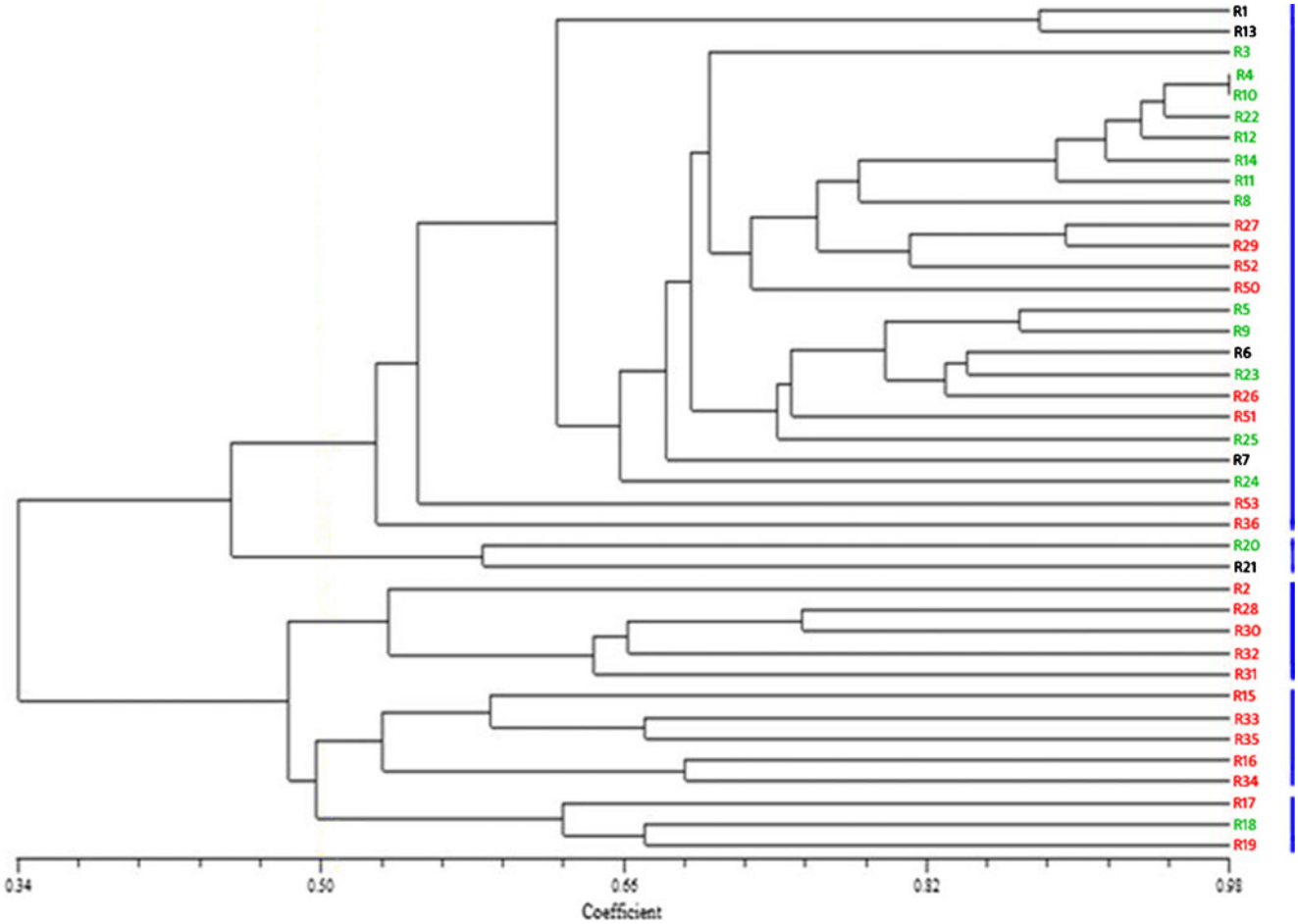

Fig. 2 Combined cluster diagram of the fast-growing (*red font*) and slow-growing (*green font*) soybean rhizobia based on intrinsic antibiotic resistance patterns and utilization of carbohydrates. *Breaks in dark-blue vertical line* (*right*) denote the 5 clusters*bottom* cluster 1,*top* cluster 2

Genotypic diversity

16S rRNA gene identity

The results of our analyses of the similarity of the 16S rRNA gene sequences of the 17 soybean rhizobial strains of this study with other sequences in Genbank are shown in Tables 3, 4 and 5. The slow-growing soybean bradyrhizobial strains tested in our study showed 98.2–99.7 % similarity with the *Bradyrhizobium japonicum* group (*B. japonicum*, *B. liaoningense*, *B. yuanmingense*) (Table 4) and 96.8–97.2 % similarity with *B. elkanii*. However, they showed only 87.7–89.5 % similarity with *Sinorhizobium fredii* and *Rhizobium radiobacter*, which are fast growing, and 88.1–88.9 % with *Rhizobium ciceri* and *Mesorhizobium ciceri*, which are of intermediate growth (Table 5). The fast-growing soybean rhizobial strains in our study showed 99.1–99.7 % similarity with *R. radiobacter* (Table 3) and 94.8–95.4 % similarity with *S. fredii* (Table 5), both of which are reported to be fast growing. They had 90.1–93.3 % similarity with *R. ciceri* and *M. ciceri*, both of which are of intermediate growth, and a low similarity of 85.1–88.9 % with the slow-growing *B. japonicum* group (Table 5).

Cluster analysis

The phylogram of the 16S rRNA gene sequence of 17 soybean rhizobial strains showed that all of these had 63 % similarity among themselves (Fig. 3). Two strains, R5 (Betul) and R12 (Harda), showed a greater similarity (83 and 78 %, respectively) with the originally introduced strain. Thus, *Bradyrhizobium japonicum* strain USDA 110 shared 63–83 % similarity with the rest of the Indian strains.

Five major clusters were evident in the phylogram (Fig. 3). In agreement with the diversity pattern based on IAR and carbon source utilization, the fast-and slow-growing strains fell in separate clusters which were then followed by clusters which had a mixture of slow- and fast-growing strains. The fast growers grouped themselves in the upper part of the dendrogram in cluster 5 and shared 63 % similarity among themselves. The slow growers grouped in cluster 4 also shared 63 % similarity among themselves. Cluster 5 consisted of four fast-growing strains while cluster 4 comprised two slow-growing strains. A slow-growing strain from Parbhani in western India (R50) clustered together with *B. liaoningense*

Table 3 16S rRNA gene sequence similarity of soybean rhizobial strains and their accession numbers

Code	Geographical origin	Growth rate[a]	Acid production	Strain	No. of base pairs	GenBank 16S rRNA gene accession number	Pairwise similarity (EzTaxon) (%)[b]
R3	Anuppur	F	Acid	*Rhizobium* sp.	1,452	JQ514071	99.508
R4	Betul	F	Acid	*Rhizobium* sp.	1,456	JQ514072	99.297
R5	Betul	F	Acid	*Rhizobium* sp.	1,469	JQ514073	99.719
R8	Chhindwara	F	Acid	*Rhizobium* sp.	1,460	JQ514074	98.946
R9	Dewas	F	Acid	*Rhizobium* sp.	1,469	JQ514075	99.368
R10	Dhar	F	Alkali	*Rhizobium* sp.	1,464	JQ514076	99.649
R11	Dhar	F	Acid	*Rhizobium* sp.	1,467	JQ514077	99.649
R12	Harda	F	Acid	*Rhizobium* sp.	1,468	JQ514078	99.368
R14	Narsinghpur	F	Acid	*Rhizobium* sp.	1,449	JQ514079	99.648
R16	Rajgarh	S	Alkali	*Bradyrhizobium* sp.	1,468	JQ514080	99.584
R19	Tikamgarh	S	Acid	*Rhizobium* sp.	1,486	JQ514081	96.143
R22	Dhar (Indore 3c)	F	Acid	*Rhizobium* sp.	1,463	JQ665272	99.297
R32	Liphatech reisolate	S	Alkali	*Rhizobium* sp.	1,459	JQ665273	99.719
R33	Vidisha	S	Alkali	*Bradyrhizobium* sp.	1,457	JQ665274	99.363
R34	Vidisha	S	Alkali	*Bradyrhizobium* sp.	1,486	JQ514083	99.792
R50	Parbhani[c]	S	Alkali	*Rhizobium* sp.	1,447	JQ514084	99.719
R51	Pantnagar[d]	S	Alkali	*Rhizobium* sp.	1,470	JQ514085	99.368

[a] F, Fast growing; S, slow growing

[b] EzTaxon is a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences

[c] West India

[d] North India; all others are from central India

at 71.5 % similarity in cluster 4, even though its sequence was similar to *Rhizobium radiobacter*.

Cluster 3 consisted of six strains of which three were fast growers and three were slow growers and in which *B. japonicum* strain USDA 110 was also present. The strain R5 (Betul) was closest to the *B. japonicum* strain USDA 110, showing 83 % similarity. Cluster 2 had four strains of which two were slow growers and two were fast growers. The outlier R4, a fast grower from Betul in central India, fell on a separate branch in cluster 1, most distant from the other fast growers and diverged from the root itself. This represented a unique niche-divergent evolution of strain R4 since strain R5, which was sampled only 500 m from it, fell in cluster 3 with which it shared only 63 % similarity. The high bacteriocin-producing, effective strain R33 (data not shown) fell in cluster 2, in the same group of R32 (Liphatech reisolate). Two other very effective strains, R16 and R34 (data not shown), also fell in same cluster as USDA 110 in cluster 3.

Discussion

Knowledge of the biodiversity of rhizobia is important for selecting the best strains for inoculation. The indigenization

Table 4 Identity of slow-growing soybean rhizobia based on 16S rRNA gene homology as per EzTaxon

Strain no.	Code	Organism	Pairwise similarity (%)
1	R16; Rajgarh	*Bradyrhizobium liaoningense*	99.584
		Bradyrhizobium japonicum	99.509
		Bradyrhizobium yuanmingense	99.508
2	R33; Vidisha	*Bradyrhizobium liaoningense*	99.363
		Bradyrhizobium japonicum	99.356
		Bradyrhizobium yuanmingense	99.355
3	R34; Vidisha	*Bradyrhizobium liaoningense*	99.792
		Bradyrhizobium japonicum	99.719
		Bradyrhizobium yuanmingense	99.719

Table 5 Homology of soybean rhizobial strains with type strains (details in ESM 4) based on 16S rRNA sequences

Soybean type strains	Bradyrhizobia (%)	Rhizobia (%)
Rhizobium radiobacter	87.7–88.1	99.1–99.7
Mesorhizobium ciceri	88.1–88.2	90.1–93.0
Rhizobium ciceri	88.5–88.9	90.2–93.3
Sinorhizobium fredii	89.2–89.5	94.8–95.4
Bradyrhizobium japonicum	98.2–98.6	85.1–88.6
Bradyrhizobium japonicum (*India*)	98.8–99.1	85.1–88.3
Bradyrhizobium liaoningense (*India*)	98.8–99.1	88.0–88.3
Bradyrhizobium liaoningense	99.3–99.7	85.3–88.6
Bradyrhizobium yuanmingense	99.5–99.7	85.1–88.4
Bradyrhizobium elkanii	96.8–97.2	85.8–88.9

and adaptation of soybean rhizobia in soils of central India was judged from the divergence of the 16S rRNA gene sequences as well as on the phenotypic diversity (carbon utilization and antibiotic resistance patterns) of the present day isolates from that of the originally introduced strain from the USA.

Phenotypic diversity

Slow-growing bradyrhizobia are the predominant population in soybean rhizobia. Until recently, six bradyrhizobia species have been identified (Xu et al. 1995; Yao et al. 2002; Rivas et al. 2004; Vinuesa et al. 2005), among which *Bradyrhizobium japonicum*, *B. elkanii* and *B. liaoningense* were originally isolated from soybean. Investigations on soybean bradyrhizobia isolated from the USA (van Berkum and Fuhrmann 2000; de Fatima Loureiro et al. 2007), tropical Africa (Abaidoo et al. 2002), Japan, Southeast Asia (Sameshima et al. 2003) and China (Yang et al. 2006) have demonstrated that these latter species are more diverse than the three species mentioned above. Two more recent additions to the list of bradyrhizobia that nodulate soybean include *B. huanghuaihaiense* (Zhang et al. 2012) and *B. daqingense* (Wang et al. 2013).

Nodulation by 'native saprophytic bacteria' has been reported in soybean (Santos et al. 1999; Chen et al. 2002), and strains 'resembling *Agrobacterium* spp.' have been isolated in effectively nodulated soybean fields (Chen et al. 2002). In our study, we also isolated effective fast-growing rhizobial isolates from soybean nodules in Vertisols at a low frequency (2–3 out of every 32 isolations). Of the 35 isolates used in our study 20 were slow growing and 15 were fast growing. Sharma et al. (2010) found the majority of soybean isolates in central India to be fast growing (4 slow growing, 18 fast growing). In our study, with one exception, all of the slow growers were alkali producers, and all of the fast growers were acid producers. All of the fast growers had a high similarity to *Rhizobium radiobacter* (99 %), while only three of the seven slow growers had a high similarity to *Bradyrhizobium* sp. (99 %), with the remaining four showing a high similarity to *R. radiobacter* (99 %) All were keto-lactose negative and nod positive and showed effective nodulation with the soybean host.

Based on IAR, carbon source utilization and DNA homology, Gao et al. (1994) reported that there was more diversity among fast-growing rhizobia than among slow-growing rhizobia in a region. However, in contrast, we found that slow growers showed more diversity than fast growers. For example, the Shannon–Weiner Diversity Index (*H*) was 2.93 for slow growers and 2.62 for fast growers for carbon utilization and 3.00 and 1.90, respectively, for IAR. Also, it can be seen from Fig. 2 that diversity among slow growers was 31–52 % but it was only 12–37 % among the fast growers. Species richness and evenness (*E*) were also less among fast growers (and hence there was more similarity, i.e. lesser diversity). The species richness and diversity indices were also slightly more in strains from all of India than in those from only central India.

Among the phenotypic criteria, carbon utilization was more discriminating than IAR. An interesting observation was that there was a clear pattern to the clustering of the phenotypes. The fast growers clustered at the top of the dendrogram in cluster 5, followed by slow growers in cluster 4, then followed by a mixture of fast and slow growers in the other clusters below (Fig. 2). This pattern was also obtained with genotypic clustering based on 16S rRNA gene analysis (Fig. 3). Other researchers have also reported that clusters obtained with the IAR profile are similar to the species groups generated with molecular methods (16S rDNA RFLP analysis, direct amplified polymorphic DNA, sodium dodecyl sulfate-polyacrylamide gel electrophoresis) (Abaidoo et al. 2002). The IAR-based groups did not show a relationship to the geographic origin of the isolates. These results may indicate a chromosomal location of antibiotic resistance genes and suggest that IAR is species-related (Abaidoo et al. 2002). In our studies, five clusters of rhizobia were obtained based on IAR profiles and carbohydrate utilization, and these did not reflect any geographic specificity, which is similar to the findings of Alexandre et al. (2006).

At least one very resistant native isolate has been identified for each of the antibiotics tested (Abaidoo et al. 2002). However, we found that there was no strain that was resistant to gentamycin and tetracycline. The resemblance between IAR clustering and 16S rRNA-based phylogeny (Alexandre et al. 2006) suggests that rhizobia of the same species may have acquired similar genes for IAR but only when subjected to identical specific environmental conditions. These conditions could include free DNA (from native microorganisms resistant to naturally occurring antibiotics) that rhizobia could acquire by transformation. Overall, our results suggest that

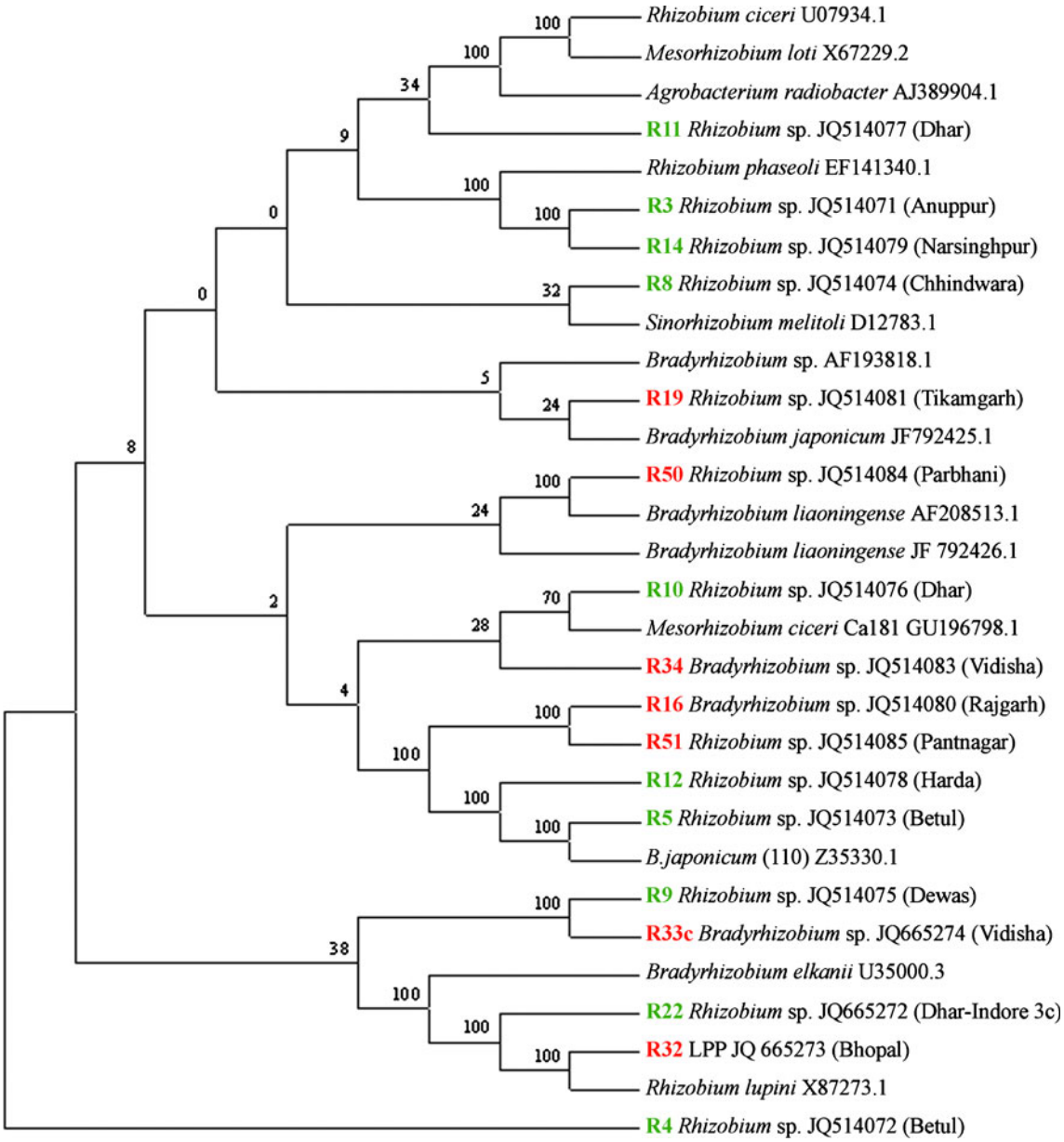

Fig. 3 Phylogenetic relationships of the fast- and slow-growing soybean rhizobia in tropical Vertisols based on 16S rRNA gene sequences. *Bottom* Cluster 1, *Top* cluster 5. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal 1973). The optimal tree with the sum of branch lengths=8.87163649 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown *above the branches* (Felsenstein 1985). The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar 2000) and are in the units of the number of base differences per site. The analysis involved 30 nucleotide sequences. All positions containing gaps, and missing data were eliminated. There were a total of 1,406 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.0 (Tamura et al. 2011)

rhizobial IAR is related both to species genetic determinants (maximum strains were resistant to nalidixic acid, novobiocin and trimethoprim) and to local conditions.

Genotypic diversity

Comparison of 16S rRNA gene sequences is useful for assigning species to genera and for defining the inter- and intra-generic relationships although they cannot resolve the relationships between close species (Young and Haukka 1996). Novel metabolic capabilities are conferred to the genome through horizontal gene transfer which enables the recipient organism to explore new ecological niches (Dutta and Pan 2002). Functional or essential genes, such as the ribosomal genes, are not usual candidates for horizontal gene transfer, but it does occur (Turner and Young 2000). All of the

fast-growing rhizobia without exception showed 16S rRNA gene sequence similarity with *Rhizobium radiobacter* (*Agrobacterium radiobacter*). This was evident across all soybean-growing regions. Some of slow-growing bradyrhizobia showed sequence similarity with bradyrhizobia group (*B. japonicum*, *B. liaoningense*, *B. yuanmingense*), while other slow-growing rhizobia (R50 and R51) showed sequence similarity with *R. radiobacter*. The two areas from where R50 and R51 were sampled (Pantnagar and Parbhani) have a long history of cultivation of not only soybean but also many other legumes nodulated by the cowpea miscellany group and would thus be expected to have a high diversity of native rhizobia. These two rhizobial strains may represent an example of horizontal gene transfer which needs further investigation. Another interesting strain is the fast grower R4 *Rhizobium radiobacter* from Betul, which is an outlier and did not cluster together with any other fast-growing *R. radiobacter*.

A high diversity of indigenous strains of soybean rhizobia has been reported from other tropical areas, such as Kenya (Wasike et al. 2009). The phylogenetic analysis of the 16S rRNA gene sequences performed by these authors showed that all indigenous strains belonged to the genus *Bradyrhizobium*, with *B. elkanii*-, *Bradyrhizobium* sp.- and *B. japonicum*-related strains being the most predominant and accounting for 37.9, 34.5 and 20.7 % of those identified at two combined sites, respectively, while *B. yuanmigense*-related strains accounted for 6.9 %. Chen et al. (2000) found a high level of genetic diversity in Paraguay, reporting that most rhizobia were slow growing, produced an alkaline reaction in medium and clustered with strains representative of the *B. japonicum* and *B. elkanii* species based on 16S rRNA gene sequence; they were also highly polymorphic in relation to reference strains and represented native bradyrhizobia. In our study, soybean rhizobia shared 63 % similarity in 16S rRNA gene sequences among themselves, and the extent of similarity with the originally introduced USDA 110 strain was 63–83 % (i.e. divergence of only 17–37 % from original strain despite four decades of adaptation to tropical environments). However, in terms of phenotypic similarity the range was 34–81 %—i.e. divergence was 19–66 %. This result shows that genotypic diversity was conserved while allowing variation in phenotypes based on environmental pressure.

Conclusions

Both slow- and fast-growing rhizobial strains were found in central India and elsewhere in India. Based on 16S rRNA gene similarity they were of three types: (1) slow-growing bradyrhizobia with 16S rRNA gene homology to *Bradyrhizobium japonicum* group; (2) slow-growing rhizobia with homology to *Rhizobium radiobacter*; (3) fast-growing rhizobia with homology to *Rhizobium radiobacter*. The rhizobial strain originally introduced on a large scale in Indian soils more than four decades ago, namely, *Bradyrhizobium japonicum* strain USDA 110, shared 34–81 % phenotypic and 63–83 % genotypic similarity with the native isolates. The results showed conservation of gene sequences while allowing variations in phenotype in various soybean-growing areas and the evolution of native strains of rhizobia among slow- and fast-growing soybean rhizobia.

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Conflict of interest The authors declare that they have no conflict of interest with the funding organization, the National Bureau of Agriculturally Important Microorganisms, Mau, India.

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