The changing face of rhizobial systematics

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In the present scenario where we need to feed enormous population of estimated five billion, legume–rhizobium association has gained importance. Biological nitrogen fixation (BNF) is an important attribute of symbiotic association of legume host with rhizobia. To achieve maximum BNF out of any legume–rhizobium association it is necessary to properly characterize and identify rhizobia before they are made commercially available for field application. Bacterial taxonomy is in the evolutionary phase with the advent and use of molecular tools in the characterization and identification of the isolates. Nowadays polyphasic approach is most reliable in classifying microorganisms. This has led to the reorganization of the already described species of rhizobia as also identification of new species from the nodules of already explored and large number of yet unexplored legume plants. We discuss here the evolutionary systematics of the group Rhizobia.

SYMBIOSIS between leguminous plants and soil bacteria commonly referred to as rhizobia is of considerable environmental and agricultural importance, since they are responsible for an estimated 180×10^6 tonnes per year of biological nitrogen fixation worldwide[1], which is equivalent to generation of resource equivalent to US $ 160–180 billion. The symbiotic component alone contributes about 120×10^6 metric tonnes year^{-1} to global nitrogen economy; this represents more than 65% of the nitrogen used in agriculture[2] and is several-fold greater than the input of nitrogen from N fertilizers, which is estimated at 65×10^6 tonnes per annum.

Rapid industrialization is associated with land degradation. The available statistics reveals that the situation is alarming. The annual rate of abandonment of dry lands in India due to land degradation is to the tune of 9 to 11 m ha. Legume–*Rhizobium* associations have potential application in ecological restoration of such degraded lands. Considering the potential of legume–*Rhizobium* associations, rhizobial inoculants have been used to improve plant and soil health for more than a century now. Inherent with the use of bioinoculants is the problem of variability in field performance and successful establishment of introduced strain(s) on account of competition with the indigenous rhizobacterial population.

Bioinoculant formulations are usually based on laboratory screening followed by appropriate trials in the field. Isolates with enhanced growth promotion and sustenance in soil are targetted as potential candidates for technology development and commercialization.

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Pathogenic forms in rhizosphere and nodules

In recent years, effective plant growth-promoting rhizobacteria (PGPR), including rhizobia have been characterized based on molecular fingerprinting and other tools that have changed our perception of the available diversity and heterogeneity. Tripathi *et al.*[3] have grouped salt-tolerant (3% NaCl) bacteria of rice rhizosphere in four clusters based on ARDRA and RAPD, and assigned these clusters to *Alcaligenes xylosoxidans*, *Ochrobactrum anthropi*, *Pseudomonas aeruginosa* and *Serratia marcescens*. Surprisingly, all these four species are potential human pathogens that can infect immunocompromised patients. Chen *et al.*[4] have identified isolates from *Mimosa* nodules and in sputum of cystic fibrosis patients as *Ralstonia taiwanensis*. From India, Tripathi[5] has reported *R. eutropha* in *Mimosa* nodules. It is pertinent to note that several species of genus *Ralstonia* are opportunistic pathogens of humans and plants, prominent being *R. mannitolilytica*[6], *R. paucula*[7], *R. solanacearum*[8] and *R. taiwanensis*[4].

In the present scenario where rhizobial inoculants have gained significance but where pathogenic isolates are being reported from nodules and rhizosphere, there is an urgent need to properly characterize and identify the rhizobacteria before they are made available for field applications.

Rhizobial systematics: An evolution

Classical systematics of Rhizobia

Rhizobia are classically defined as symbiotic bacteria capable of eliciting and invading root and stem tissue-forming nodules on leguminous plants where they undertake symbiotic nitrogen fixation. Beijerinck[9] had, for the first time, isolated a bacterium from root nodules of legumes and named it *Bacillus radicicola*. This was subsequently renamed *Rhizobium*[10]. The earliest classification of rhizobia was based on specificity of symbiotic plant range of bacterial species. Fred *et al.*[11] recognized six species in the genus *Rhizobium*, viz. *R. japonicum* (*Lathyrus*, *Lens*, *Pisum* and *Vicia*), *R. lupini* (*Lupinus*), *R. meliloti* (*Melilotus*, *Medicago*, *Trigonella*), *R. phaseoli* (*Phaseolus*) and *R. trifolii* (*Trifolium*) based on their host range,

though they also described certain morphological and physiological properties of the identified species. Based on growth rate, bacteria were grouped as fast growers and slow growers, but were still placed in the genus *Rhizobium* till Jordan[12] coined the new genus *Bradyrhizobium*. A single species, *Bradyrhizobium japonicum,* was described for isolates of *Glycine max*. Norris[13] observed that fast growers and slow growers differed in their symbiotic affinity. Accordingly, alkali-producing slow growers were associated with the tropical legumes and acid-producing fast growers with the temperate legumes; exceptions to this general observation, however, are known. Temperate legumes like *Corollina* and *Lupinus*[14] are infected by slow-growing rhizobia whereas tropical legumes, e.g. *Acacia*, *Leucaena* and *Sesbania* are infected by fast-growing rhizobia[15,16]; indeed, fast and slow-growing rhizobia have been isolated from the same legume species, e.g. *G. max*[17] or even from the same plant, e.g. *Acacia*[18], *Lupin*[19] and *Prosopis*[20]. Thus it is clear that classification of rhizobia on the basis of host range and physiological properties does not reflect the true phylogeny of the group.

Modern molecular tools: Reorganization of the group Rhizobia

In the first edition of *Bergey's Manual of Systematic Bacteriology*[21], two rhizobial genera (*Bradyrhizobium* and *Rhizobium*) and four species were described based solely on growth rate and symbiotic host ranges. These include *Bradyrhizobium japonicum* for slow-growing isolates of *G. max*, *Rhizobium leguminosarum* for isolates nodulating *Lathyrus*, *Lens*, *Phaseolus vulgaris*, *P. augustifolius*, *P. multiflorus*, *Pisum* and *Trifolium*[22], *R. meliloti* nodulating *Medicago*, *Melilotus* and *Trigonella*[23] and *R. loti* for isolates nodulating *Anthyllis*, *Caragana arborescens*, *Cicer arietinum*, *Leucaena leucocephala*, *Lotus* sp., *Lupinus* and *Mimosa*.

Since early nineties, sequence comparison of 16S rRNA genes and genetic fingerprinting methods based on the use of PCR have been used extensively for characterizing rhizobia. The topology of the phylogenetic trees obtained from aligned sequences and those obtained from ARDRA patterns have been found to be well correlated[24]. Phylogenies resulting by sequencing of only 300 bp variable region of 16S rRNA genes are not congruent with those from the complete sequence of certain rhizobial species[25–27] (Figure 1). Oligonucleotide probes are also not reliable as until now no single probe is specific for rhizobia[28]. The above arguments support the use of sequencing of full 16S rRNA gene and different PCR tools as most reliable to ascertain the phylogeny of rhizobial isolates.

Several workers[25,29,30] have divided rhizobia into three genera: *Azorhizobium*, *Bradyrhizobium* and *Rhizobium* based on 16S rRNA sequence alignment. Subsequently, the genus *Rhizobium* has been further divided into two new genera, *Mesorhizobium*[31] and *Sinorhizobium*[15]. However, no single or multiple phenotypic characteristics have been reported for *Allorhizobium*, *Rhizobium* and *Sinorhizobium* by which these taxa can be differentiated as genera.

Wang and coworkers[32] characterized rhizobial populations nodulating *Leucaena leucocephala*, *Mimosa affinis* and *Sesbania herbacea* through PCR–RFLP of 16S rRNA genes and observed that isolates from a single legume species were dispersed under various species of the same genus or different genera. A population of 150 isolates of *L. leucocephala* was clustered into 18 rDNA types corresponding to *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*[32]. Similarly, 50 isolates from *M. affinis* were clustered into two groups, one corresponding to *R. etli* and the other, a novel group within the genus *Rhizobium* in which isolates from *L. leucocephala* and *M. affinis* were intermingled on the basis of results of PCR–RFLP of SSU rRNA genes, multilocus enzyme electrophoresis and

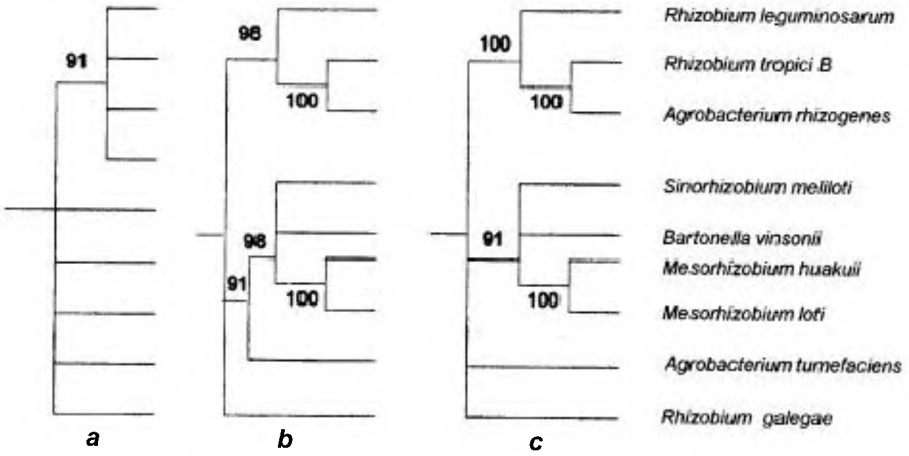


Figure 1. Parts of SSU gene sequence analysis support inconsistent phylogenies. Trees are based on (***a***) the first 300 bases, (***b***) the rest of the gene, or (***c***) the whole sequence[27].

DNA–DNA homology[33]. Further extension of this work by the same group led to the proposal of a new biovar, bv. *mimosae* within *R. etli* to encompass isolates from *M. affinis* which can effectively nodulate both *L. leucocephala* and *P. vulgaris*; they differed from *R. etli* strains (originated from *P. vulgaris*) in *nif H* gene sequence and organization, melanin production and host specificity. Four rDNA types corresponding to *Rhizobium* sp., *R. huautlense*, *R. tropici* type-B and *M. plurifarium* were identified among isolates of *Sesbania herbacea* from Mexico[32] however, earlier *Sinorhizobium terangae* and *Sinorhizobium saheli* have been identified in nodules of *Sesbania* from Africa[15].

On the basis of current taxonomy for root nodule bacteria, greater than 70% DNA–DNA homology coupled to distinctive phenotypic characters, is one of the main criteria for defining species. However, in a few cases, strains sharing lower homology (40–60%) have been found within a single species, viz. *R. tropici* type-A and B[34] and *Mesorhizobium plurifarium*[35]. At present, 36 rhizobial species distributed among seven genera of rhizobia are recognized based on the polyphasic approach (Table 1).

Description of different genera of Rhizobia

The genus *Allorhizobium* has been proposed[36] for nitrogen-fixing species, *A. undicola,* accommodating isolates which effectively nodulate *Neptunia natans* as an outlying branch of *Agrobacterium–Rhizobium* cluster; however, this genus has since been reclassified[37]. Genus *Bradyrhizobium* with a single species, *B. japonicum*, was proposed for symbionts of soybean[12]. Later, Hollis and coworkers[38] separated *B. japonicum* into three DNA homology groups with species *B. elkanii* for one group[39] and *B. liaoningense* for another group comprising extra-slow growing *Glycine* isolates[40], retaining the name *B. japonicum* for slow-growing isolates of *G. max*. On the basis of 16S rDNA similarities and total DNA homology values, *B. elkanii* is considered distinct from *B. liaoningense* and could represent a separate genus[41]; *B. liaoningense* is phylogenetically closer to *B. japonicum* which is closer to genera *Afipia*, *Agromonas*, *Blastobacter*, *Nitrobacter* and *Rhodopseudomonas*. All slow-growing isolates nodulating legumes other than soybean are known as *Bradyrhizobium* sp., followed by the genus of legume host in parentheses, e.g. *Bradyrhizobium* sp. (*Acacia*)[42], *Bradyrhizobium* sp. (*Aeschynomene* sp.)[43] and *Bradyrhizobium* sp. (*Lupinus*)[44].

Willems *et al.*[45] have proposed a separate genus for all photosynthetic *Bradyrhizobium* isolates alongwith *Agromonas oligotrophica* and *Blastobacter denitrificans*. Chen *et al.*[46] proposed the name *Mesorhizobium* to include all rhizobial species with growth rate intermediate between *Bradyrhizobium* and typical fast-growing *Rhizobium* strains. Young[47] raised the status of the group to denote phylogenetic position of rhizobia intermediate between *Bradyrhizobium* and *Rhizobium*. Formally, at the tenth International Congress on Nitrogen Fixation, genus *Mesorhizobium* was created to include all the species of *R. loti* rRNA branch[48,31], isolated from a diverse range of

Table 1. Current status of rhizobial taxonomy

Genus	Species	Host	Reference
Allorhizobium	*A. undicola*	*Neptunia natans*	36
Azorhizobium	*Az. caulinodans*	*Sesbania rostrata*	73
Bradyrhizobium	*B. elkanii*	*Glycine max*	74
	B. japonicum	*G. max*	12
	B. liaoningense	*G. max*	40
Mesorhizobium	*M. amorphae*	*Amorpha fruticosa*	49
	M. chacoense	*Prosopis alba*	50
	M. ciceri	*Cicer arietinum*	75
	M. huakuii	*Astragalus*	76
	M. loti	*Loti*	
	M. mediterraneum	*Cicer arietinum*	77
	M. plurifarium	*Acacia, Leucaena*	35
	M. tianshanense	*Glycyrrhiza, Sophora* and *Glycine*	46
Methylobacterium	*M. nodulans*	*Crotalaria pedocarpa*	62
Rhizobium	*R. etli*	*Phaseolus vulgaris*	51
	R. galegae	*Galega*	52
	R. gallicum	*P. vulgaris*	53
	R. giardinii	*P. vulgaris*	53
	R. hainanense	*Centrosema, Desmodium, Stylosanthes, Tephrosia*	55
	R. huautlense	*Sesbania herbacea*	56
	R. leguminosarum	*Trifolium, Vicia*	10
	R. mongolense	*Medicago ruthenica*	57
	R. phaseoli	*P. vulgaris*	23
	R. sullae	*Hedysarum hedysari*	59
	R. tropici	*Leucaena, P. vulgaris*	34
	R. trifolii	*Trifolium*	23
	R. yanglingense	*Amphicarpaea, Trisperma Corollina varia* and *Gueldenstaedtia multiflora*	
Sinorhizobium	*S. arboris*	*Acacia senegal Prosopis chilensis*	78
	S. fredii	*G. max*	60
	S. kostiense	*A. senegal, P. chilensis*	78
	S. medicae	*Medicago* spp.	79
	S. meliloti	*Medicago sativa*	15
	S. saheli	*Sesbania*	15
	S. terangae	*Acacia, Sesbania*	15
	S. xinjiangense	*G. max*	60

host legumes, including *Anthyllis*, *Astragalus*, *Caragena*, *Cicer*, *Genista*, *Leucaena*, *Lotus*, *Lupinus*, *Ornithopus*, *Ononis* and *Mimosa*. Later, two new species, *M. plurifarium* for isolates from *Acacia*, *Leucaena*, *Prosopis* and *Chamaecrista*[35], and *M. amorphae* for isolates of *Amorpha fruticosa*[49] and recently based on LMW RNA profiles, *M. chacoense* has been proposed for isolates of *Prosopis alba* from Argentina[50]. On the basis of clustering analysis of phenotypic characters and DNA–DNA reassociation data, all fast-growing, acid-producing, symbiotic nitrogen-fixing bacteria are placed in the genus *Rhizobium*[12]. *Rhizobium leguminosarum*, the type species (amalgamating the former species *R. leguminosarum*, *R. phaseoli* and *R. trifolii*) has three biovars on the basis of distinct host ranges; biovar *phaseoli* nodulates *Phaseolus*, biovar *trifolii* nodulates *Trifolium* and biovar *vicae* nodulates *Lathyrus*, *Lens*, *Pisum* and *Vicia*. Until 1996 (ref. 27), two additional species *R. etli*[51] and *R. tropici*[34] were placed in this genus. *R. galegae*[52] which was a separate entity until 1992 (ref. 47), was included as a separate species in the genus *Rhizobium*[53].

During the period 1997–2002, based on PCR–RFLP of SSU rRNA gene, 16S sequencing, analysis of LMW RNA molecules, whole-cell protein profiles, plasmid profiles and host specificity for nodulation, seven new species have been proposed within this genus. These are *R. gallicum* and *R. giardinii* both for isolates of *P. vulgaris* from various locations in France. *R. gallicum* sp., was related to *R. etli* and *R. tropici*, whereas *R. giardinii* clustered with *R. galegae* and *Agrobacterium* sp. Each of these species is subdivided into two biovars based on organization of *nif H* genes[54]. *R. hainanense*[55], *R. huautlense*[56], *R. mongolense*[57], *R. yanglingense*[58] and *R. sullae*[59] (formerly *R. hedysari*) are also placed in the genus *Rhizobium*. *R. sullae* have been proposed for coherent set of symbionts of *Hedysarum coronarium* which were distinct from strains of other *Hedysarum* spp[59]. *Sinorhizobium* was originally proposed by Chen *et al.*[60] to differentiate *R. fredii* from *R. meliloti*, but further study carried out by Jarvis and coworkers[61] showed that *R. fredii* and *R. meliloti* are similar and finally, de Lajuidie *et al.*[15] placed it on a separate branch that includes *R. fredii* and *R. meliloti*. Subsequently, six more species have been described in the genus *Sinorhizobium*. Recently, Sy *et al.*[62] have identified isolates from nodules of African *Crotalaria* spp. as *Methylobacterium nodulans*, a bacterial species which is a part of novel 16S rDNA *Methylobacterium* branch, a fourth branch of α-proteobacteria (Figure 2). These isolates have a unique property of growing on one carbon compound such as methanol and formaldehyde.

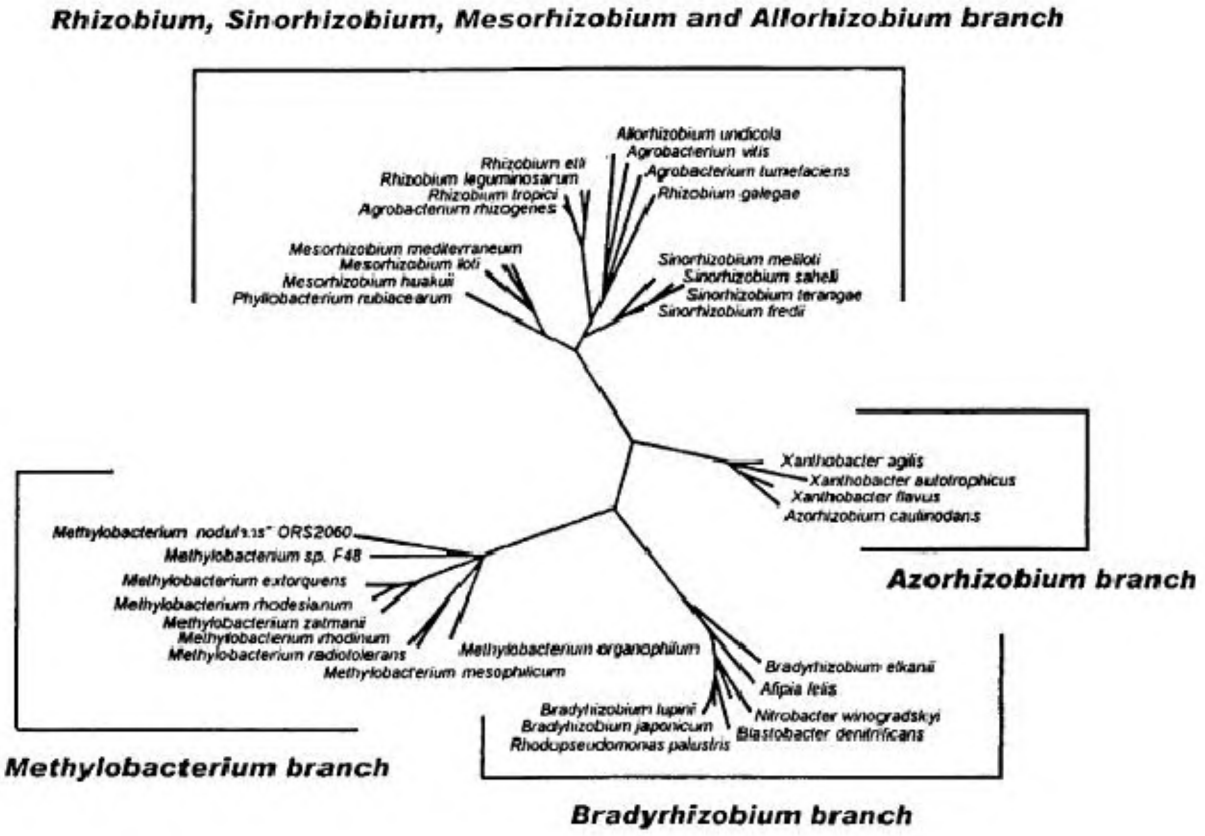
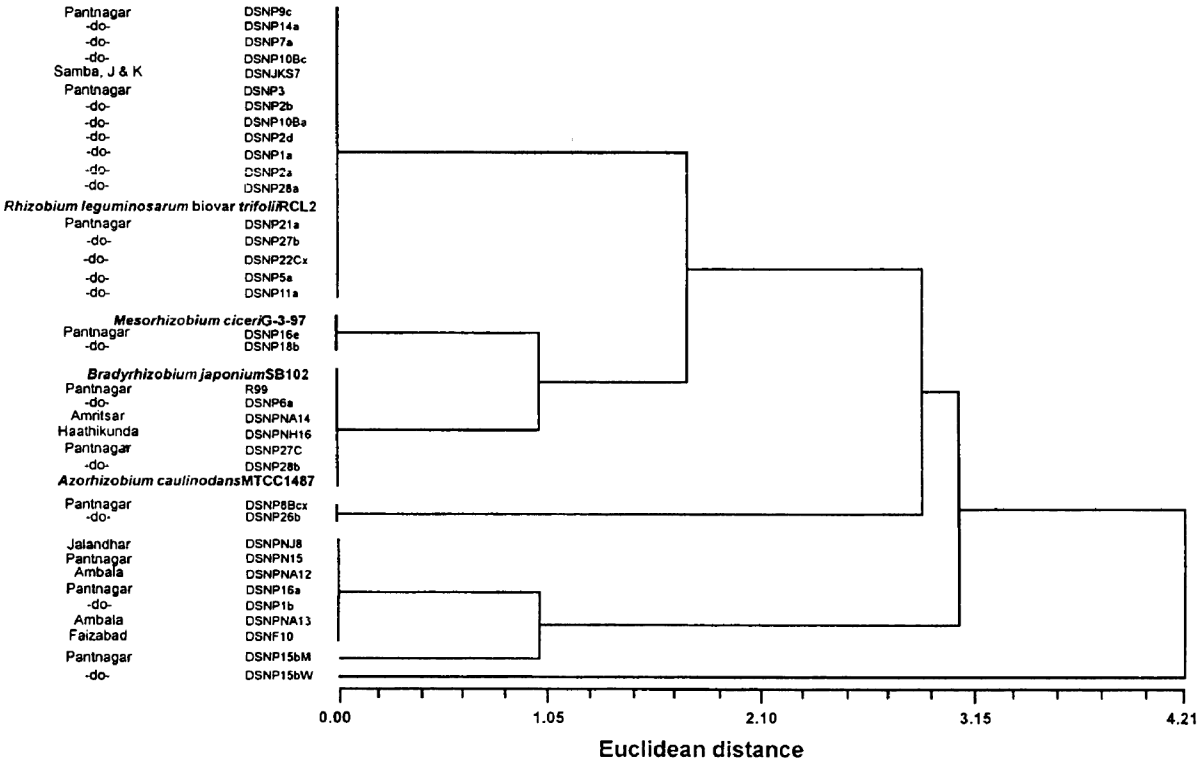


Figure 2. Unrooted phylogenetic tree (neighbour-joining) showing different rhizobial branches in the α-subdivision of Proteobacteria[62].



Figrue 3. UPGMA cluster of *Dalbergia sissoo* nodule isolates on the basis of 16S ARDRA with *Sau*3A.

Changes and surprises

The genera *Azorhizobium*, *Bradyrhizobium* and *Mesorhizobium* which have been included in the Family Rhizobiaceae[63] are allocated in the forthcoming second edition of the *Bergey's Manual of Systematic Bacteriology*[64] in the new families Azorhizobiaceae, Bradyrhizobiaceae and Phyllobacteriaceae respectively. The revised family Rhizobiaceae has two genera *Rhizobium* and *Sinorhizobium*, is genotypically related to Bartonellaceae, Brucellaceae and Phyllobacteriaceae[37]. Families Azorhizobiaceae and Bradyrhizobiaceae are closely related to each other, but are distant from other families in the group based on 16S rDNA sequence alignment. In addition to reorganization at family level, the genus *Rhizobium* has also been extensively revised. All the species of genera *Agrobacterium* and *Allorhizobium* have been placed in the genus *Rhizobium* with amended names; *Rhizobium radiobacter* (amalgamating *Ag. radiobacter*[65] and *Ag. tumefaciens*[66]), *R. rhizogenes* (Syn. *Ag. rubi*)[67], *R. undicola* (Syn. *Allorhizobium undicola*)[46] and *R. vitis* (Syn. *Ag. vitis*)[68]. This was based on an unifying property to exchange large Sym and Ti plasmids within the members of *Agrobacterium* and *Rhizobium*[69–71] as also comparative analysis of 16S rDNA sequence data. The new systematics of rhizobial genera is available at the site: http://www.cme.msu.edu/bergey's, p. 7).

Recently, proteobacteria from β-subclass have also been identified in the nodules of legumes. Bacterial isolates from nodules of *Aspalathus carnosa* and *Machaerium lunatum* have been identified as *Burkholderia*[72]. Subsequently, *Ralstonia taiwanensis* has been identified in the nodules of *Mimosa* from China[4] and *Ralstonia eutropha* in *Mimosa* nodules from India[5] based on polyphasic taxonomic approach.

ARDRA analysis of rhizobia carried out in our laboratory with isolates from nodules of *Dalbergia sissoo* representing various ecozones of northern India revealed the existing diversity (Figure 3). Though a limited number of reference strains were included, none of the seven rDNA types were identical to known species. Considering the vast availability of cultivated and wild legumes in the country and the changing scenario of rhizobial taxonomy, it is an opportune moment to study the nodulating isolates in an exhaustive manner.

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