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REVIEW

The changing paradigm of rhizobial taxonomy and its systematic growth upto postgenomic technologies

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Abstract

Rhizobia are a diazotrophic group of bacteria that are usually isolated form the nodules in roots, stem of leguminous plants and are able to form nodules in the host plant owing to the presence of symbiotic genes. The rhizobial community is highly diverse, and therefore, the taxonomy and genera-wise classification of rhizobia has been constantly changing since the last three decades. This is mainly due to technical advancements, and shifts in definitions, resulting in a changing paradigm of rhizobia taxonomy. Initially, the taxonomic definitions at the species and sub species level were based on phylogenetic analysis of 16S rRNA sequence, followed by polyphasic approach to have phenotypic, biochemical, and genetic analysis including multilocus sequence analysis. Rhizobia mainly belonging to α- and β-proteobacteria, and recently new additions from γ-proteobacteria had been classified. Nowadays rhizobial taxonomy has been replaced by genome-based taxonomy that allows gaining more insights of genomic characteristics. These omics—technologies provide genome specific information that considers nodulation and symbiotic genes, along with molecular markers as taxonomic traits. Taxonomy based on complete genome sequence (genotaxonomy), average nucleotide identity, is now being considered as primary approach, resulting in an ongoing paradigm shift in rhizobial taxonomy. Also, pairwise whole-genome comparisons, phylogenomic analyses offer correlations between DNA and DNA re-association values that have delineated biologically important species. This review elaborates the present classification and taxonomy of rhizobia, vis-a-vis development of technical advancements, parameters and controversies associated with it, and describe the updated information on evolutionary lineages of rhizobia.

Keywords Rhizobia · Rhizobial taxonomy · Bacterial phylogeny · Root nodules · 16S ribosomal RNA · Genotaxonomy

Introduction

Rhizobia are members of the family Rhizobiaceae, classically recognized as symbiotic bacteria of leguminous plants that have the characteristic feature of fixing atmospheric nitrogen (Hellriegel and Wilfarth 1888). The group comprises a large number of genera that nodulate more than 750 genera of legumes (Wojciechowski et al. 2004). The taxonomic status of rhizobia remain dynamic, as new rhizobial species are being identified on regular basis, and also, the known genera had been re-assigned or re-classified (Hernandez-Lucas et al. 1995). Rhizobial species so far identified are very diverse and exhibited phylogenetically distinct groups. Previously, widespread phylogenetic diversity of nitrogen-fixing legume symbionts and their taxonomy had been reported (Rivas et al. 2009). Until 1980s, all symbiotic rhizobia isolated from leguminous plants were classified as belonging to *Rhizobium* genus (Zakhia and de Lajudie 2001), however in 1984; the taxonomy changed and continues to evolve till today. Rhizobial taxonomic studies have currently led to a total of 21 genera (Chen et al. 2021; Kuzmanovic et al. 2022) and progress in taxonomy is due to increasing numbers of effective techniques available for characterization of bacteria (Ormeno-Orrillo et al. 2015; Lassalle et al. 2021). While 16S rRNA gene sequence is

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considered as benchmark for description of rhizobial species (Graham et al. 1991), yet other technological developments in genetic analysis including DNA fingerprinting techniques, Polymerase chain reaction (PCR) analysis using large number of genes, Restriction fragment length polymorphism (RFLP), had contributed to defining and differentiating the closest strains of rhizobia (Ramirez-Bahena et al. 2008). Recently, next generation sequencing (NGS) techniques have been assimilated in rhizobial taxonomy with strategies such as—comparative genomics (Ormeno-Orrillo et al. 2015), average nucleotide identity (ANI) of genome comparisons (Rashid et al. 2015), core genome phylogeny, core-proteome average average amino acid identity (cpAAI) (Kuzmanovic et al. 2022), high throughput sequencing of 16S rRNA for bacterial diversity (Zheng et al. 2020). In fact, advancement in molecular biology techniques has facilitated considerable changes and proposal of new rhizobial species.

Recently, the post genomics technologies have encouraged creation of several algorithms that are introducing new genome-based definitions for the taxonomy of prokaryotes. These algorithms have been widely accepted and provide valuable insights of microbial speciation and genomic diversity (Zong 2020). NGS technologies has led to the discovery of microbial phylogenetic novelty and enable the researchers to (re-)classify and (re-)name organisms and explore diverse natural microbial communities and their uncultivated taxa (Sanford et al. 2021). Theory on prokaryotic genome evolution has been progressed with comparative genome analysis covering a wide range of evolutionary distances and this could change the concepts of prokaryotic taxonomy (Koonin et al. 2021), including rhizobia. Phylogenomics gives exact strategies to depict species and permits us to derive the phylogeny at higher ordered taxonomic positions, as well as those at the subspecies level.

There had been some interesting reviews, which have discussed the taxonomy of legume nodulating bacteria (Berrada and Fikri-Benbrahim 2014; Shamseldin et al. 2017). But as the number of new genera had been reported, or reclassified, an updated description is required. Here in this review, we summarize the various constant developments in identification of rhizobia using recent techniques including genomics-based strategies. New approaches have led to the reclassification of several genera resulting in considerable changes in taxonomy and nomenclature of rhizobia. The postgenomics technologies are significantly changing current scientific classification of rhizobia. Therefore, this review describes the developments in rhizobial taxonomy, considering the technological advancements and progress in molecular perspectives, and also presents the currently recognized classification of different genera of rhizobia.

Historical antecedents: The historical perspectives in rhizobial taxonomy can be categorized under two sections (i) initial classification based on culture attributes, and (ii) numerical taxonomy based on phenotypic characteristics, as summarized below:

Culture attributes

Young and Haukka (1996) described isolation and culturing of root-nodule bacteria by Beijerinck, (1888), (as cited in Young and Haukka 1996) which was named as *Bacillus radicicola*, and later it was renamed as *Rhizobium leguminosarum* by Frank (1889) as a type of strain of the *Rhizobium* genus (Willems 2006). The original genus *Rhizobium* underwent several changes that gave rise to numerous taxa. Until 1980s, all symbiotic nitrogen fixing bacteria were identified as *Rhizobium*, classified into six species (*R. leguminosarum*, *R. trifolii*, *R. meliloti*, *R. phaseoli*, *R. japonicum* and *R. lupine*) (Fred et al. 1932; Jordan and Allen 1974). Jordan (1984) classified the second genus *Bradyrhizobium* based on slow and fast-growing rhizobia, this led to transfer of *Rhizobium japonicum* to the genus *Bradyrhizobium*. Baldwin and Fred (1929) developed cross-inoculation tests to assess the specificity of *Rhizobium* with their host plants. This aided to classify rhizobia into two categories depending on their growth rates viz. fast-growers and slow growers (Lohnis and Hansen 1921; Fred et al. 1932). These two groups of rhizobia had been shown to exhibit intragenic and intergenic diversity (Elkan 1992). Both groups exhibited metabolic diversity, as fast-growing bacteria could utilize mannitol and sucrose (Alien and Allen 1950), while slow-growing bacteria utilize arabinose (Fred et al. 1932) as their carbon source. This principle becomes less acceptable to classify rhizobial taxonomy and so Wilson (1944) provided evidence to abandon cross inoculation group concept. This was also not helpful due to the possibility of transfer of symbiotic plasmids among soil bacteria (Nakatsukasa et al. 2008). While position of symbiotic genes was used to differentiate between fast and slow growers, as these are located on chromosome for slow growing bradyrhizobia and for fast growers they are located on plasmids. It was reported that a strain of *Bradyrhizobium* DOA9 carry symbiotic genes on a megaplasmid (Teamtisong et al. 2013). In fact, rhizobial genes for symbioses in legumes are not as stable as those present in chromosome, rather they are located on large plasmids. In most of the *Rhizobium* strains, genes encoding root hair adhesion, nitrogen fixation, infection thread formation and host specificity are found on one plasmid species (Djordjevlc et al. 1982) and these genes are located on one segment of this *Sym* plasmid that range approximately 20–30 kilobase pairs (kb) (Homnbrecher et al. 1981). Thus, rhizobial symbiotic plasmids play an important role in symbiosis and contains core symbiosis genes (*nod* and *nif*/*fix*) involved in functioning of nitrogen fixation and nodulation. Wang et al. (2018) compared 24 rhizobial symbiotic plasmids which showed significant different topological structures when

compared to phylogenetic trees constructed using *nodCIJ* and *fixABC* genes. Rhizobial symbiotic plasmids retain a mosaic structure due to transposition, horizontal gene transfer and plasmid DNA recombination (Lopez-Guerrero et al. 2012a, b), because of which, such plasmid borne functions are avoided for taxonomic purpose (Saidi et al. 2014).

In the second half of the twentieth century, traditional phenotypic methods such as morphophysiological characteristics, growth kinetics, and pH of the growth medium were used to identify rhizobia (Vincent and Humphrey 1970). Major changes in the nomenclature of rhizobia occurred when rhizobia were classified with other methods such as polyphasic approach that includes phenotypic, genotypic, and phylogenetic analysis, serology, RNA/DNA or DNA/DNA hybridization, and/or plasmid analysis, since previous methods (host-range nodulation and growth rates) were inconsistent (Vandamme et al. 1996; Rao et al. 2018). As a result, the number of rhizobial species increased rapidly (Table S1) (Zakhia and Lajudie 2001).

Phylogenetic analysis of 16S rRNA gene led to division of genus *Rhizobium*, placing *Rhizobium*, *Agrobacterium* and *Allobacterium* in a group whereas *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Azorhizobium* formed separate clusters (Willems, 2006). Multi Locus Sequencing Analysis (MLSA) using housekeeping genes, which had been used to identify and delineate at species level, was also recognized for rhizobia (Rivas et al. 2009; Aserse et al. 2012). Before the delineation of new generic names, rhizobial species such as *Sinorhizobium* and *Mesorhizobium* were placed under *Rhizobium* (Lindstrom et al. 2015). There was proposal to integrate closely related genera *Agrobacterium* and *Allorhizobium* into the genus *Rhizobium*, and the merger of *Sinorhizobium* with *Ensifer*, which has been much debated (Young et al. 2001; Willems et al. 2003).

However, analytical methods have improved since the last 30 years and the emergence of whole genome sequence analysis now facilitates recognition of a novel species, which is being used presently as a powerful tool to study taxonomy of rhizobia as revealed from comparative genome sequence studies. Measuring the genomic relatedness aid in demarcation of genus and it allows delineation of closely related species into separate genera. Taxonomy based on genome sequencing (genotaxonomy) offer a clear concept of identification of correct species as well as explore novel rhizobial species that are yet to be isolated from different legume species. A schematic timeline diagram is given (Fig. 1) to illustrate the major breakthroughs vis-a-vis technical advances, in rhizobial taxonomy.

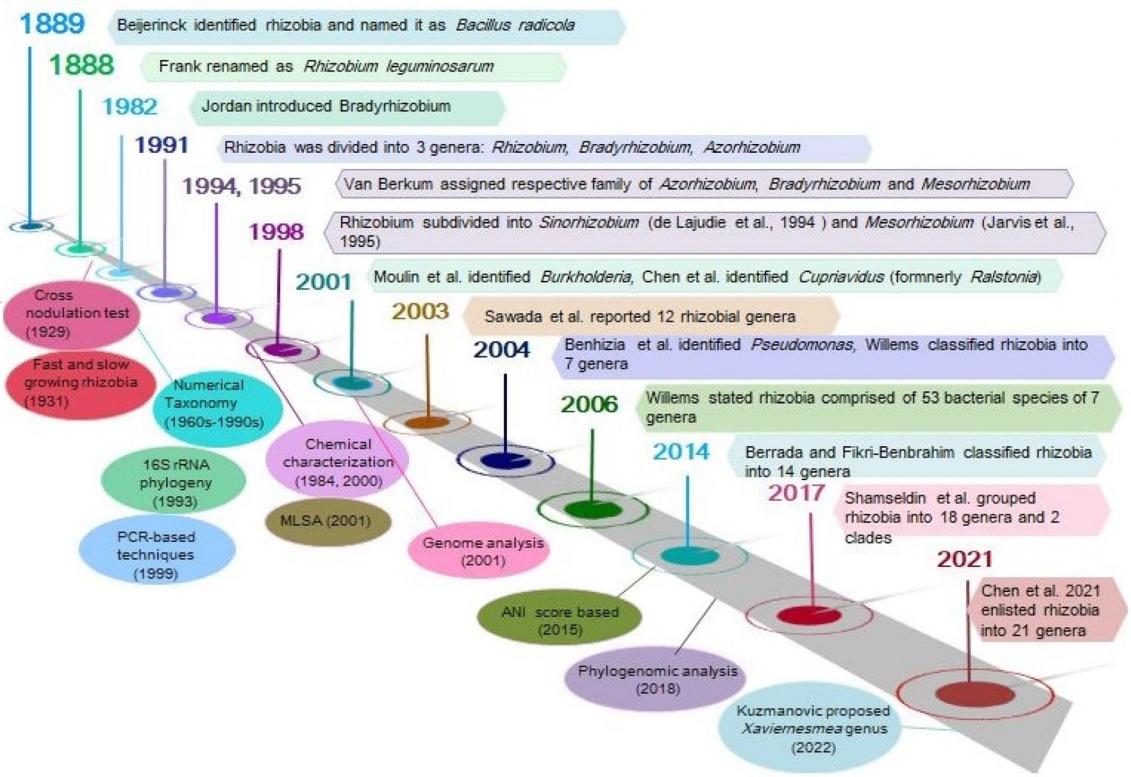

Fig. 1 A schematic diagram to illustrate major breakthroughs vis-a-vis technical advances, in rhizobial taxonomy

Numerical taxonomy of rhizobia based on phenotypic characteristics

The numerical taxonomy approach was applied for rhizobia, based on phenotypic characteristics including morphology, physiological, serological analysis, symbiotic characteristics, utilization of carbon and nitrogen sources, metabolic features and other abiotic growth factors (Graham et al. 1991). *Azorhizobium*, a new genus was discovered using numerical taxonomy approach, as it was found to have different characteristics from other fast-growing rhizobia. It could utilize numerous carbohydrates and exhibited a separate branch from *Rhizobium* and *Bradyrhizobium* (Dreyfus et al. 1988). Also, three *Rhizobium* strains (*R. leguminosarum*, *R. phaseoli* and *R. trifolii*) were classified under the same species by numerical taxonomy approach; previously classified based on cross-nodulation. *R. japonicum* and *R. lupini* were clustered in a phenotypic group and fast growers (*R. leguminosarum*, *R. phaseoli*, *R. trifolii*, *R. meliloti*) were observed to be similar to *Agrobacterium.* Following this numerical taxonomy, *Rhizobium* classification was then re-organized that resulted to the identification of another rhizobial genus *Sinorhizobium* (Chen et al. 1988). Based on the physiological features, utilisation of carbon sources of alcohols, sugars, organic acids, and enzyme activities, *Sinorhizobium xinjiangense* was reclassified into a separate species which was previously classified with *Sinorhizobium fredii* (Chen et al. 1988). Genus *Mesorhizobium* was classified based on phenotypic characteristics including nodulation and physiological properties, and the five *Rhizobium* species (*R. huakuii*, *R. ciceri*, *R. tianshanense*, *R. loti*, and *R. mediterraneum*) had shifted to *Mesorhizobium*. It was revealed to be phylogenetically different from other rhizobia such as *Rhizobium*, *Sinorhizobium*, *Agrobacterium* and related groups. *Mesorhizorium* was described to exhibit intermediate growth between fast-grower and and slow grower. The population of this genus utilize glucose, rhamnose and sucrose with acid end products (Jarvis et al. 1997).

Most of the rhizobial population were classified under Proteobacteria, mainly belonging to the Class Alpha-proteobacteria (α-proteobacteria), Beta-proteobacteria (β-proteobacteria) and Gamma-proteobacteria (γ-proteobacteria) (Shiraishi et al. 2010). In α-Proteobacteria, six families, comprising Bradyrhizobiaceae, Brucellaceae, Hyphomocrobiaceae, Methylobacteriaceae, Phylobacteriaceae, and Rhizobiaceae belonging to the order Rhizobiales were defined. Rhizobial genera were increased to 12 with 44 species (Sawada et al. 2003), and soon after again revised to 53 rhizobial species belonging to *Allorhizobium*, *Agrobacterium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* (Willems 2006). Later Berrada and Fikri-Benbrahim (2014), reported 14 rhizobia genera with 98 species, Suneja et al. (2017) listed 17 rhizobial genera with 168 validly published species, which was updated to 238 species distributed among 18 rhizobial genera as described by Shamseldin et al. (2017). Chen et al. (2021) detailed 20 genera of rhizobia of different families: Rhizobiaceae [*Allorhizobium*, *Agrobacterium*, *Ensifer* (syn. *Sinorhizobium*), *Neorhizobium*, *Pararhizobium*, *Rhizobium*, *Shinella*], Bradyrhizobiaceae (*Bradyrhizobium*), Brucellaceae (*Ochrobactrum*), Hyphomicrobiaceae (*Devosia*), Xanthobacteraceae (*Azorhizobium*), Phyllobacteriaceae (*Aminobacter*, *Mesorhizobium*, *Phyllobacterium*), Methylobacteriaceae (*Methylobacterium*, *Microvirga*), Burkholderiaceae (*Paraburkholderia*, *Trinickia*, *Cupriavidus*), Pseudomonadaceae (*Pseudomonas*). Also, Kuzmanovic et al. (2022) proposed formation of new rhizobial genus *Xaviernesmea*. The second class β-Proteobacteria was found to be less diverse, as it included one family—Burkholderiales, consisting of three genera *Paraburkholderia*, *Cupriavidus* and *Trinickia* (Estrada-de los Santos et al. 2018). Initially *Cupriavidus* was described as *Ralstonia* (Chen et al. 2001), and *Paraburkholderia* and *Trinickia* were formerly described as some species of *Burkholderia* (Dobritsa and Samadpour 2016; Estrada-de los Santos et al. 2018).

Phylogeny and taxonomy of rhizobia based on small subunit (SSU) ribosomal RNA

Submolecular phylogenetics emerged as a powerful tool to decipher evolutionary relationships between bacteria, by utilizing molecular data (DNA and rRNA or protein sequences) (Dai et al. 2012). 16S rRNA gene is regarded as the phylogenetic marker in the field of microbial taxonomy (Stackerbrandt and Goebel 1994). 16S rRNA based grouping of fast and slow growing rhizobia were clearly segregated in genetic phyla, as these groups were found to be less related to each other, rather than to their nonsymbiotic relatives. For instance, *Rhizobium* was found to be closely related to *Agrobacterium*, while slow growing rhizobia had close relationship with *Pseudomonas palustris* (Young and Johnston 1989). 16S rRNA sequence alignment, clearly distinguished rhizobia into three respective genera as was already described by previous methods—*Azorhizobium*, *Bradyrhizobium* and *Rhizobium* (Young et al. 1991; Willems and Collins 1993). 16S rRNA gene sequence analysis was in agreement to the classification of rhizobia at genus level, with previous strategies, but was more definitive. Classification of rhizobia into five genera i.e., *Rhizobium*, *Azorhizobium Sinorhizobium*, *Meshorhizobium* and *Bradyrhizobium* was supported by analysing 16S rRNA sequences of recognised seventeen species of four rhizobium genera (Young and Haukka 1996). Phylogenetic analysis of 16S rRNA sequence led to the division of *Rhizobium* genus and its relatives of α-Proteobacteria.

Different species of *Agrobacterium*, *Allorhizobium undicola* clustered together with all species of *Rhizobium* according to 16S rDNA analyses. Hence, *Rhizobium*, *Allorhizobium* and *Agrobacterium* (Rhizobiaceae) were merged with the *Rhizobium* genus due to the monophyletic nature and their common phenotypic generic contraint (Young et al. 2001). Whereas *Azorhizobium* (Hyphomicrobiaceae), *Bradyrhizobium* (Bradyrhizobiaceae), *Mesorhizobium* (Phyllobacteriaceae), *Sinorhizobium* (Rhizobiaceae) formed separate clusters (Willems 2006). The genus *Rhizobium* had incorporated both *Allorhizobium* and *Agrobacterium* genera, while *Chelatobacter* was renamed as *Aminobacter* (Young et al. 2001; Kampfer et al. 2002) and *Sinorhizobium* have been known as *Ensifer* (Young 2010) based on 16S rDNA sequence analysis. *Rhizobium* and *Sinorhizobium* showed close relationship with *Agrobacterium* while distantly related with *Bradyrhizobium* (Garrity et al. 2005) and *Phyllobacterium* (Mergaert and Swings 2006). Later, isolation and identification of *Agrobacterium* species resulted in changes of nomenclature of rhizobial species (Slater et al. 2013). *Agrobacterium rhizogenes*, an old species was retained as *Rhizobium rhizogenes*, and also a new species *Rhizobium tumorigenes* was included that induce plant tumours (Kuzmanovic et al. 2018). The controversy was moderated by reclassification of *Agrobacterium larrymoorei* as *Rhizobium larrymoorei* (Young 2004).

Ensifer (*Sinorhizobium*), *Mesorhizobium* and *Rhizobium*, fall under α-proteobacteria and *Burkholderia* and/or *Paraburkholderia*, *Cupriavidus*, belong to β-Proteobacteria (Andrew and Andrews 2017). Many rhizobial species had been reported to share high homology (> 97%) or else they were almost similar with 16S rRNA sequence (Moura et al. 2020). Based on 16S rRNA sequence similarity, rhizobia were reported to belong to three main distinct phylogenetic subclasses i.e., α, β and γ-Proteobacteria (Zakhia and de Lajudie 2001). In Fig. 2, the phylogeny of the rhizobial species belonging to three distinct subclasses, with representative species of rhizobial genera had been shown.

The usage of 16S rRNA gene sequence as phylogenetic marker in rhizobia presented some challenges as well, as some of the bacterial genomes possess multiple copies of the sequence and was suggested to develop vulnerability to horizontal gene transfer (van Berkum et al. 2003; Gevers et al. 2005). For instance, symbiotic rhizobia isloted from *Mimosa* spp. were highly specific, and the phylogenies based on 16S rRNA, and housekeeping gene sequences were observed to be different. Further, housekeeping gene sequences were reported to represent the diversity, in line with the symbiosis genes for *Burkholderia* (isolated from Brazil) and *Rhizobium*/*Ensifer* (isolated from Mexico) (Bontemps et al. 2010, 2016). Therefore, the efficacy of 16S rRNA was criticized for rhizobial taxa and other housekeeping genes were being given preference in delineating new species of rhizobia (Aserse et al. 2012), as also it cannot be used to differentiate among the closest *Rhizobium* species (Ramirez-Bahena et al. 2008). Further, 16S rRNA gene sequence of α- and β-proteobacteria are highly conserved, so discrimination of diverse species remains challenging, therefore other complementary approaches were used (Azevedo et al. 2015) as discussed below.

Taxonomy based on housekeeping genes

Several housekeeping gene sequences had been used to identify of rhizobia at the genus level and delineate rhizobial species with high relatedness (Rivas et al. 2009). This included nitrogen fixation genes (*nif*, *fix*, x genes) and nodulation genes (*nodABCIJ* genes) that are located within genomic regions or symbiotic plasmids in most of the α-rhizobia groups (Suominen et al. 2001). Diversity of rhizobial population had been assessed by analysing *nodC* and *nifH* gene (Dubey et al. 2010). Analysis of combination of other gene sequence such as *dnaK* (Stepkowski et al. 2003), *glnII* (Stepkowski et al. 2005), *atpD* and *recA* (Vinuesa et al. 2005) had elucidated the rhizobial phylogenetic relationship. Genes such as *atpD*, *recA* and *glnII* help in differentiation of closely related species of *R. leguminosarum* sv. *trifolii*, *R. leguminosarum* sv. *phaseoli* and *R. leguminosarum* sv. *viceae* (Ribeiro et al. 2009). *recA* gene screening was found to resolve and define rhizobial strains at genus and species level (Lindstrom et al. 2015; Peix et al. 2015). In rhizobial taxa, *recA* gene which code for DNA recombination and repair system had demonstrated to be similar with the small subunit rRNA genes (Gaunt et al. 2001; Vinuesa et al. 2005). Further, phylogenetic analysis of *recA* in bacteria had been observed to be consistent with the corresponding phylogeny of 16S rRNA gene (Eisen 1995). Figures 2, 3 and 4 in this review present Maximum Likelihood (ML) phylogenetic trees that dipict the evolutionary relationships among rhizobial genera based on analysis of the 16S, *recA* and *atdD* genes respectively. The gene sequences were retrieved from GenBank and trees were constructed based on Tamura–Nei model (1993), and drawn to scale with branch lengths measured in the number of substitution per site. The *recA* and *atpD* gene have been sequenced in all the rhizobial strains of all genera and they had been used to differentiate between rhizobial species for those species whose 16S rRNA had been found to be closely related (Valverde et al. 2006; Ramirez-Bahena et al. 2008). Young et al. (2001) classified *Agrobacterium* as genus *Rhizobium* based on phylogenetic relationship of *rrs* gene sequence which endured a conflict, and were disapproved by different scientist (Farrand et al. 2003). Therefore, the taxonomic classification of *Agrobacterium* was reformed (Mousavi et al. 2014), based on *rrs*, *recA*, *atpD* and *rpoB* gene sequences. Subsequently, some *Rhizobium* species (*R.*

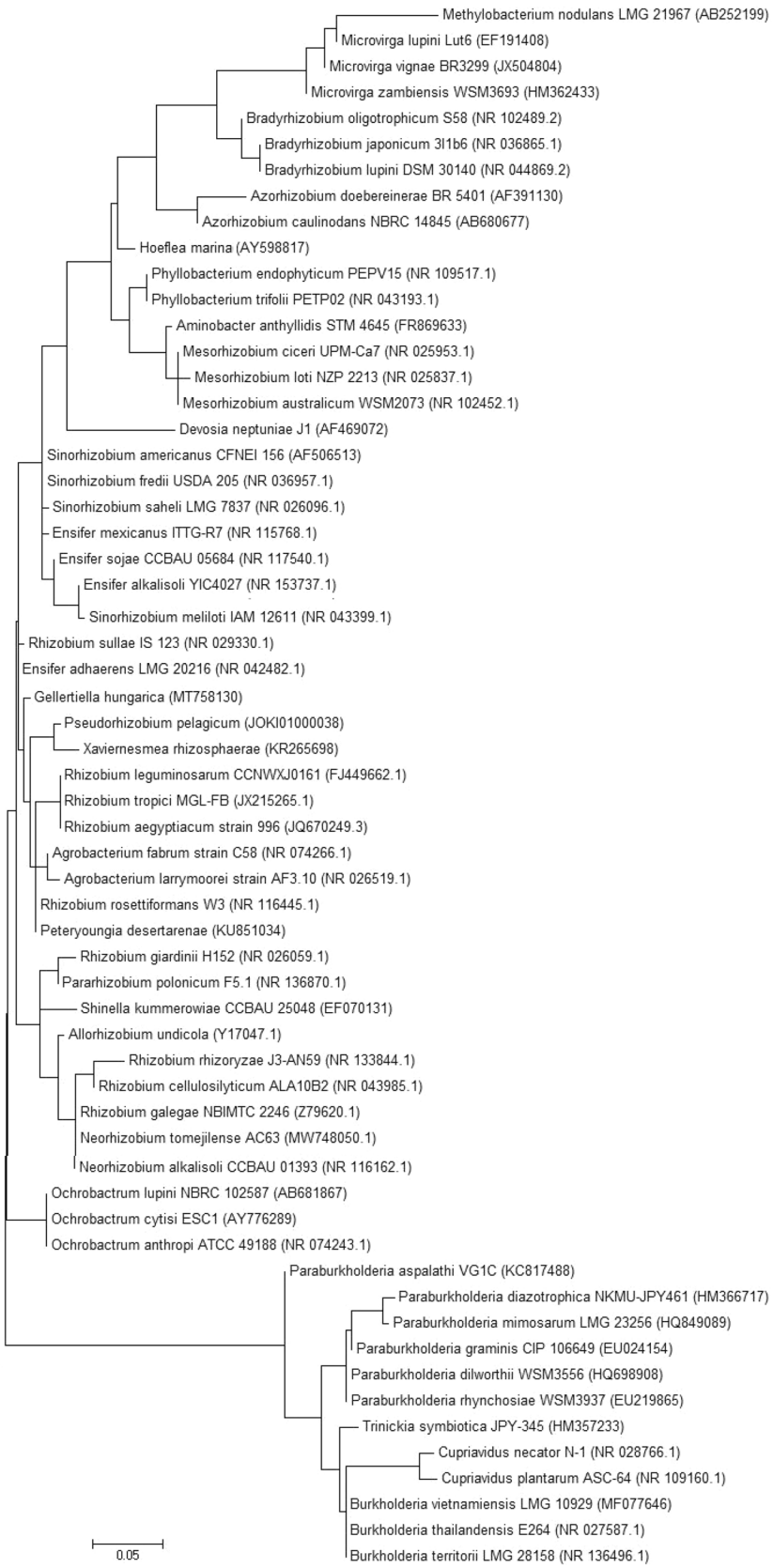

Fig. 2 Maximum likelihood phylogenetic tree of 16S rRNA gene of 61 representative species of 26 genera of *Rhizobia*. Scale bar (0.05) indicates estimated nucleotide substitution per site

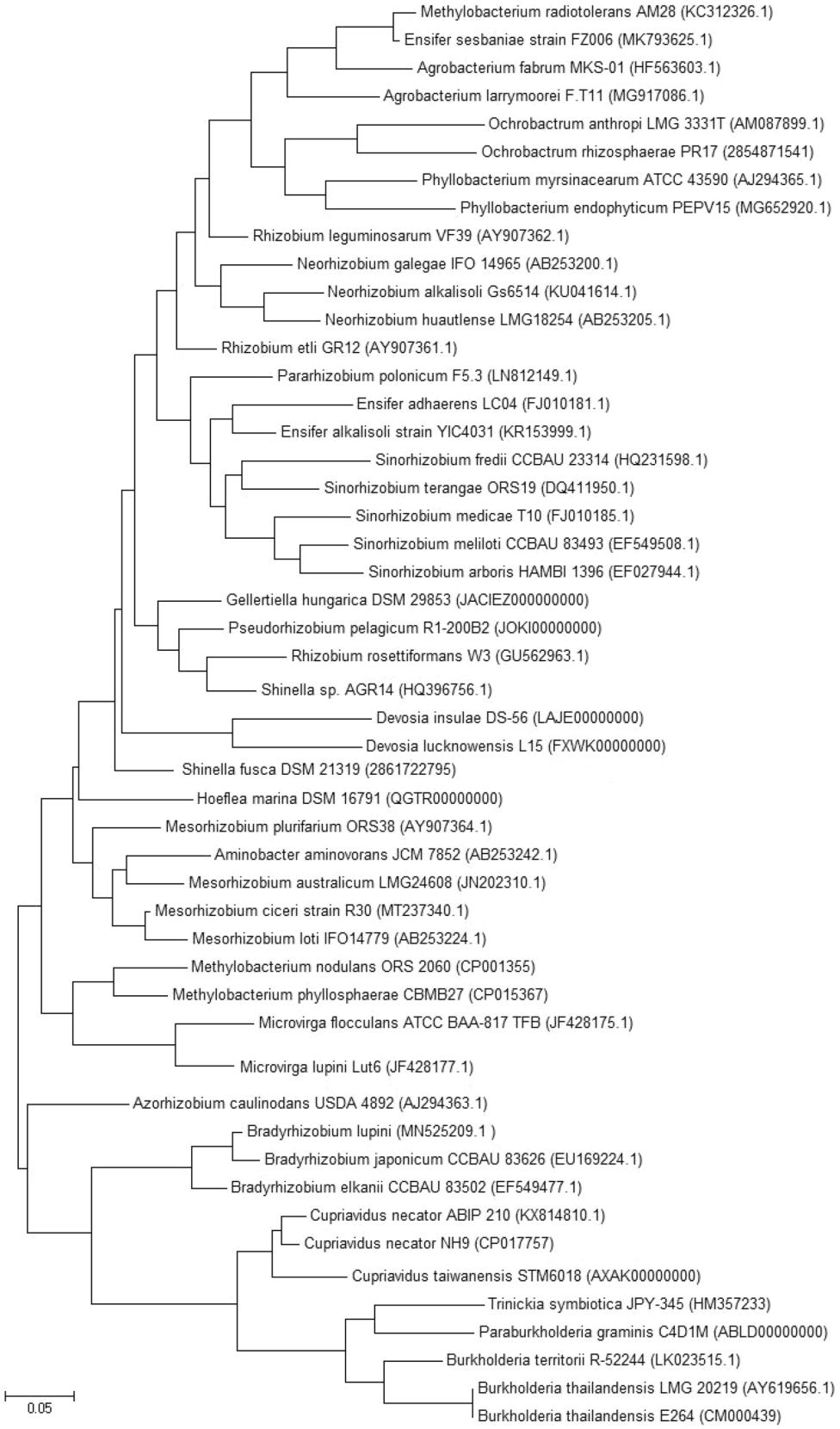

Fig. 3 Maximum likelihood phylogenetic tree of *recA* gene of 51 representative species of 23 genera of *Rhizobia*

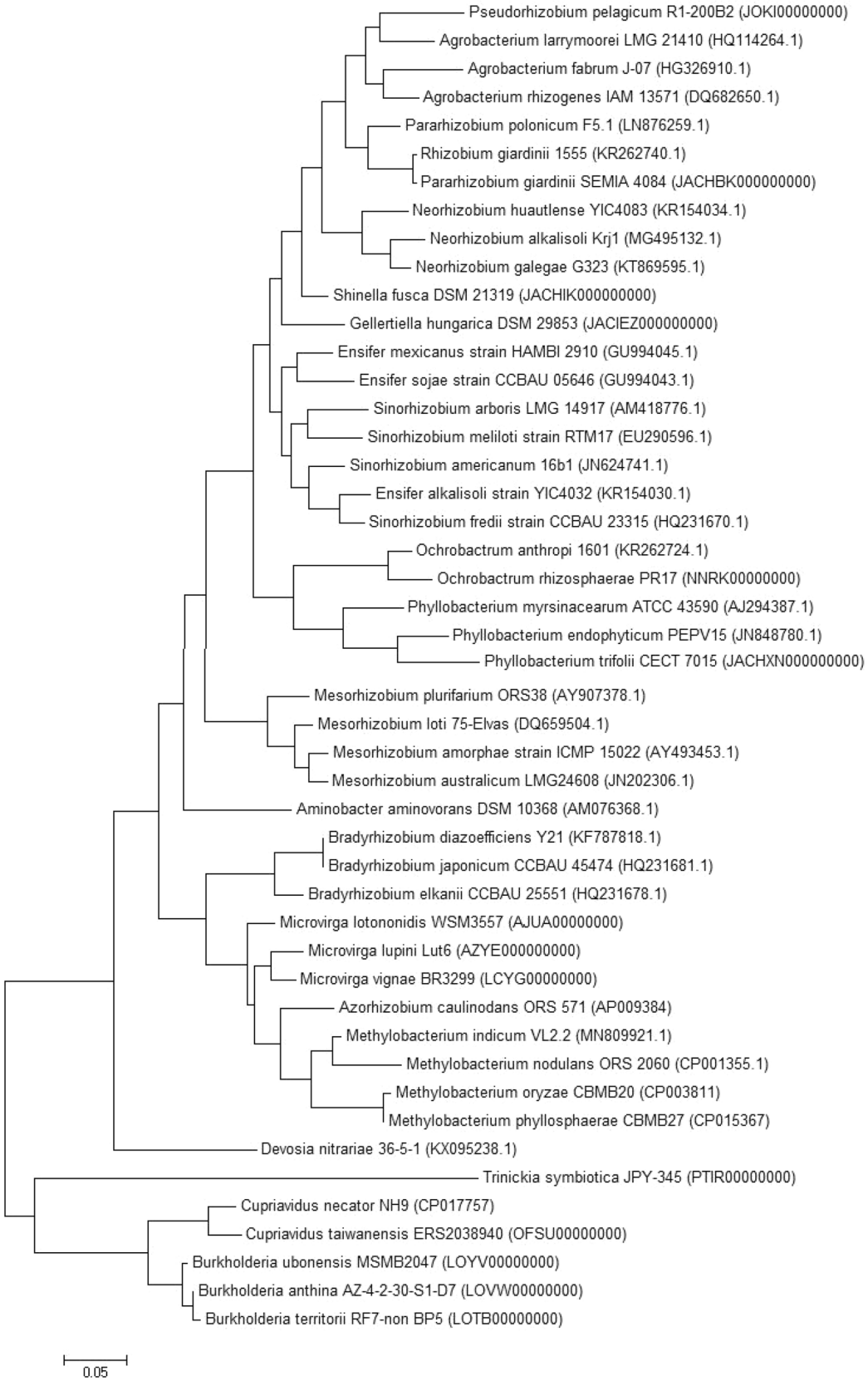

Fig. 4 Maximum likelihood phylogenetic tree of *atpD* gene of 47 representative species of 21 genera of *Rhizobia*

pusense, *R. skierniewicense*, and *R. nepotum*) were shifted to genus *Agrobacterium* and *R. vitis* (primarily *A. vitis*) was shifted to genus *Allorhizobium* (Oren and Garrity 2016). Furthermore, other housekeeping genes of rhizobia such as *dnaK*, *gap*, *glnA*, *gltA*, *gyrB*, *pnp*, *recA*, *rpoB*, and *thrC* had been used to identify precisely (Aoki et al. 2013). On the otherhand, *nodA* gene sequences of *Cupriavidus* rhizobia isolated from Uruguay were reported to be inconsistent with the housekeeping gene sequences however they were placed in the same clade which indicated several species of the group acquired symbiosis genes through horizontal gene transfer (Platero et al. 2016). The symbiosis gene sequences (*nodA*, *nodC*, *nifH* and *nifHD*) of *Burkholderia* (*Paraburkholderia*) sp. and *Pseudomonas* sp. were found to be identical to other rhizobial species which indicated that the genes had acquired by horizontal gene transfer (Shiraishi et al. 2010). Careful analysis of these housekeeping genes of each genus revealed incongruent phylogenetic relationships among these loci that lead to improve identification and characterization of rhizobia (Werner et al. 2015).

PCR-based techniques in rhizobial taxonomy

The use of PCR-based techniques such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), and random amplified fragment polymorphic DNA (RAPD) have facilitated in determining the genetic variation in rhizobia (Silva et al. 2012; Onyango et al. 2015; Boakye et al. 2016). Universal and specific primers including 16S–23S rRNA ITS (Internally Transcribed Space) region of different rhizobial strains had been used in amplification and sequencing to distinguish taxonomic positions of different rhizobial isolates (Gronemeyer et al. 2014). Rahmani et al. (2011) analysed common-bean nodulating rhizobia by PCR–RFLP technique and reported that the isolates had shown large genetic variation and comprised 43 ITS genotypes that showed clustering into ten groups at a similarity of 64%. PCR and amplified ribosomal DNA restriction analysis (ARDRA) methods of 41 rhizobial isolates from root nodules of beans categorized them into nine separate morphotypes (Koskey et al. 2018). RAPD-PCR was used by Harrison et al. (1992) in defining strains of *R. leguminosarum* and Niemann et al. (1997) to characterize among indigenous *S. meliloti* strains.

Similarly, different PCR fingerprinting techniques such as 16S rDNA PCR–RFLP, rep-PCR and RAPD analysis had shown considerable diversity among eighteen soybean nodule isolates. RFLP patterns indicated that the isolates were different from *Bradyrhizobium elkani* and *Sinorhizobium fredii* and showed close relatedness with *Bradyrhizobium japonicum* (Sikora and Redzepovic 2003). Ogutcu et al. (2009) characterized *R. leguminosarum* subsp. *ciceri* isolates associated with chickpea species and revealed high intraspecies diversity among the strains using different PCR techniques. Characterization of exopolysaccharide producing *R. leguminosarum* species using PCR-based methods could discriminate among *R. leguminosarum* strains, *R. etli* and *R. gallicum* (Janczarek et al. 2009). Genetic relationship and diversity of rhizobial isolates from *Lembotropis nigricans* displayed great heterogeneicity, as out of 33 rhizobia, AFLP techniques could demarcate 32 genotypes and BOX-PCR could identify 27 genotyes and identified root nodule symbionts belong to *Bradyrhizobium japonicum* (Wojcik et al. 2019). Bayesian inference of phylogeny of of *atpD* and *recA* sequences were estimated to study the taxonomic classification of *Sesbania* rhizobia, while the identification of the isolates at species level was evaluated using *rrs* plus *rrl* PCR-RFLPs and *Sesbania* isolates were identified as *Mesorhizobium pluriformis* or *Rhizobium huautlense*. The study revealed geographic distribution of *M. pluriformis* and the analysis showed *R. galegae* and *R. huautlense* belong to same lineages and synonym of *R. gallicum*, *R. mongolense* and *R. yanglingense* (Vinuesa et al. 2005).

The use of various molecular markers has greater ability to discriminate between species. The phenotypic and molecular characterization of the rhizobial isolates with fingerprint markers including BOX, ERIC, REP and BOX-PCR could discrimate the rhizobia from indigenous tree legumes (*Mimosa tenuiflora*, *Piptadenia stipulacea* and *M. caesalpiniifolia*). However, amplification technique by duplex PCR with *nifH* and *nodC* genes could result in false-positive data as these genes are highly pleomorphic between species and biovars. Therefore, it was discouraged and rather, use of larger molecular markers which could provide safer knowledge on the taxonomy and diversity of rhizobia was recommended (Lyra et al. 2019).

Taxonomy of rhizobia based of polyphasic approach

Polyphasic approach had been used as a powerful technique in identifying and resolving the Rhizobiaceae family (Cardoso et al. 2012). A combination of phenotypic and phylogenetic classification of 16S rRNA and 23S rRNA gene sequences in polyphasic approach were employed to classify rhizobia (Vandamme et al. 1996). This technique had provided in studying the generic relationships of *Bradyrhizobium* and *Rhizobium* (Graham et al. 1991), also, *Azorhizobium* was discreetly segregated with one species *Azorhizobium caulidans* (Dreyfus et al. 1988). The polyphasic study incorporates various other techniques and it was useful in identifying 52 rhizobia isolated from *Acacia* spp. and *Sesbania* spp. which could identify two clusters by SDS-PAGE, which were genotypically and phenotypically different belonging to *Rhizobium meliloti* and *R.*

fredii and a third cluster was found to branch with *R. loti*. This polyphasic taxonomy was used to emend genus *Sinorhizobium*, which was previously classified as *Rhizobium meliloti* for *Sinorhizobium meliloti* com. nov. Further two other species of the genus namely, *S. saheli* and *S. terungu* were proposed for the strains isolated from Senegal (de Lajudie et al. 1994). Rhizobia that could nodulate wild legumes were classified using polyphasic taxonomy including other tools such as profiling fatty acid content with analysis of whole cell protein pattern that led to the classification of 20 strains into 12 strains of *R. leguminosarum*, 5 strains of *S. meliloti* and 3 strains of *Rhizobium* spp. (Zahran et al. 2003). Fatty acid methyl ester analysis (FAME) had been reported to use as a taxonomic marker for rhizobia classification and it is also considered as a part of polyphasic technique to identify a new species (Zahran 1997). Fatty acid profiles were used to classify 600 rhizobial strains belonging to genera *Rhizobium*, *Agrobacterium*, *Sinohizobium*, *Bradyrhizobium*, and *Mesorhizobium* (Tighe et al. 2000). Diouf et al. (2000) used polyphasic approach to classify 58 rhizobial strains isolated from West Africa and the different phenotypic and genotypic techniques employed led to the classification of isolates into two main groups that belong to *R. tropici* type B and *R. etli*. The isolates belonging to *R. etli* exhibited different electrophoretic type which was indicative of internal heterogeneity within the strains as analysed by multilocus enzyme electrophoresis (MLEE). The heterogeneicity was further examined by host-plant specificity, intergenic spacer region (ITS) PCR–RFLP, and SDS–PAGE which revealed genetic variation in the isolates. Using the polyphasic approach including phenotypic and genetic analyses, Pinto et al. (2007) characterized *R. tropici* strains from Brazil and found that the *R. tropici* strains consisted high variability in ribosomal genes, but higher similarity in *nifH* and *nodC* genes as confirmed by RFLP-PCR, with inference that there might be possibility to divide *R. tropici* into two different species (Pinto et al. 2007). Based on polyhasic approach, de Lajudie et al. (1998) detailed rhizobia into seven genera (*Rhizobium*, *Allorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Mesorhizobium*, *Methlylorhizobium*, *Sinorhizobium*). Indigenous rhizobial community chickpea had been reported to exhibit heterogeneity at different locations with different methods of characterization methods (Dudeja and Singh 2008; Nandwani and Dudeja 2009; Rai et al. 2012). Polyphasic approach has advantages in classifying microorganisms into precise genera and species, as it utilizes phylogenetic, phenotypic, genomic, and chemotaxonomic methods for characterization.

Taxonomy based on multilocus sequence analysis (MLSA)

As already explained, 16S rRNA based phylogeny exhibited low resolution among highly related species, as the gene sequences is too conserved for separation of closely related species. In such cases, MLSA of housekeeping genes aid in resolving taxonomic issues and discriminate the species into subspecies (Werner et al. 2015). Description of new genera upto species and sub species levels were provided by analysis of symbiotic genes such as nodulation genes (*nodABCIJ*), and nitrogen fixation genes (*nifDK*, *nifH*, *fix* and *x*) genes. Glutamine synthetase (GSI, GSII), *recA* and *atpD* that lead to appropriate taxonomy and systematics of rhizobia nodulating legumes (Zeze et al. 2001, Suominen et al. 2001; Ribeiro et al. 2009). MLSA analysis of four housekeeping genes (16S *rRNA*, *atpD*, *recA* and *rpoB*) supported the separation of *Rhizobium giardinii* which represents a novel genus *Pararhizobium* (Mousavi et al. 2015).

The MLSA was used to study the symbiovars (symbiotic variety) of *Mesorhizobium* nodulating chickpea. It revealed the existence of one new chickpea *Mesorhizobium* species and one novel symbiovar, *M. opportunistum* sv. *ciceri* by analysing phylogenetic relationship of core genes and *nodC* symbiotic gene (Laranjo et al. 2012). Based on the MLSA of six protein-coding housekeeping genes in 114 rhizobial taxa, novel species had been reclassified into different genera namely, *Allorhizobium*, *Agrobacterium*, *Rhizobium*, *Pararhizhobium* and *Neorhizobium* (Mousavi et al. 2014, 2015).

Omics technology in rhizobial taxonomy

Advances in whole genome sequencing techniques facilitate to classify rhizobia based on ANI of the genomes, and species of *Rhizobium* was found to be comprised of numerous genomic lineages (Acosta et al. 2011; Santamaria et al. 2017). Whole genomes enable reconstruction of phylogenomic trees on the basis of thousands of genes that represent evolutionary relationships that replaced phylogeny based on few markaers including 16S rRNA genes. Different strains of *R. etli* exhibiting low recombination rate indicated that distinguished genomic lineages could involve a given species or multiple species (Acosta et al. 2011).

Phylogenomic analysis of the genome sequence led to the identification of *Allorhizobium* and distinguished *Agrobacterium* from Rhizobiaceae family. Genome phylogeny had supported the inclusion of *Rhizobium vignae*

in *Neorhizobium* group, although ANI values were found to be less than 91%, it was considered as *Neorhizobium vignae*. Further, this technique also revived *Allorhizobium* as a genus and included *Allorhizobium vitis* (formerly *Agrobacterium vitis*) and *Allorhizobium taibaishanense* (formerly *Rhizobium taibaishanense*). Also, closely related species of *Rhizobium* leguminosarum were found within tropici group and designed as *Rhizobium rhizogenes* which was previously known as *Agrobacterium rhizogenes* (Ormeno-Orrillo et al. 2015). Gonzalez et al. (2019) suggested that phylogenomic clades represent evolutionary continuum within the species defined by genomic clusters. This phylogenomic relationship based on core genome markers and complete sets of ribosomal proteins discovered the main lineages of *Rhizobium*.

New bioinformatics tools that reduce the technical confinements of classical DNA hybridization measurements to delineate prokaryotic species are now being utilized routinely. At present, the primary approach in the taxonomy of the rhizobia is based on genomic average nucleotide identity (ANI) between the genome sequences of the strains (Ormeno-Orrillo et al. 2015). This gives an array of sequence similarity between sets of genomes (designated the query and reference genome) and computes this value for areas in the genome. ANI values of 95–96% 16S rRNA gene sequence similarity have been described to delineate species-level similarities. ANI values of concatenated sequences of partial sequences of core genes are employed to delineate rhizobial species (de Lajudie et al. 2019). According to this criteria, nodulating bacteria *R. aegyptiacum* (Shamseldin et al. 2017), *R. esperanzae* (Cordeiro et al. 2017) and *R. ecuadorense* (Ribeiro et al. 2015) had been defined as species. However, it is noticable that ANI scores between a query and reference genome are regularly asymmetric considering contrasts in gene complements and genome sizes. This asymmetry is not completely surprising as it was regularly seen in reciprocal hybridization studies about utilizing marked DNA tests in the past. ANI also has restricted utility in characterizing species, subspecies, and strain-level relationships. It is suggestive of genomic clusters; its values can range within species that lead to division or fusion of species based on the cut-off used, therefore phylogenomic and genetic measures of population could delineate species significantly (Fraser et al. 2009).

Classification based on the whole genome sequence comparisons are termed as genotaxonomy. *Rhizobium* spp. nodulating common-bean and *R. leguminosarum* nodulating clover were comprised of diverse genomic clusters of related strains (Kumar et al. 2015; Perez-Carrascal et al. 2016). Based on the genomic comparison, common bean-nodulating rhizobial strains assigned to *R. etli* and *R. phaseoli* were suggested to be resembling in independent species within the same environment (Miranda-Sanchez et al. 2016; Santamaria et al. 2017). Gan et al. (2019) analysed the genome sequence of *A. radiobacter* NCPPB3001$_T$ and *A. tumefacien* B6$_T$ and compared with *A. radiobacter* LMG140$_T$ and determined that the type strains of *A. tumefacien* and *A. radiobacter* illustrate two subspecies from the same species.

Draft genome sequence of a rhizobial strain NAU-18$_T$ was reported to consist of 6588 protein-coding genes. Phylogenetic analysis showed the strain was similar with *Neorhizobium alkalisoli* CCBAU 01393$_T$ and *Rhizobium oryzicola* ZYY136$_T$ and clustered with *R. oryzicola* based on 16S rRNA gene sequences. The strain represented a novel species of *Rhizobium* and classified as *Rhizobium terrae* sp. nov. NAU-18$_T$ (Ruan et al. 2020). Gonzalez et al. (2019) studied the genomic clusters to establish the significance of phylogeny of *Rhizobium* at species level. Rhizobial species that resemble *R. etli* and *R. leguminosarum* were inversely correlated and displayed genomic clusters with ANI > 95%. The pan-genome of the *Rhizobium* revealed the presence/absence of the gene profiles both in chromosomes and plasmids that follow the phylogenomic pattern of species divergence which may be due to inter-strain gene transfer. *Rhizobium* genome cluster may be a part of evolutionary divergence for formation of species. Considering the dynamics of genome evolution in bacteria, accessory genes are the determining factor for adaptation and specialization. These genes comprise mobile genetic elements, including phages and transposons, which are generally termed as symbiosis-related genes. Genomic islands are the mobile elements that are flanked by tRNA genes (Young et al. 2006). The bacterial genome had revealed to have symbiosis islands which were closely related to *Mesorhizobium loti* of Phyllobacteriaceae family (Kaneko et al. 2000).

Also, lateral gene transfer has been suggested to play an important role for genome evolution in *Agrobacterium*/*Rhizobium* and *Ensifer*/*Sinorhizobium* (Young et al. 2006). It had been stated that stable taxonomy is specified by core genes present in the chromosome and involved in housekeeping processes. Also, the specificity of different host by same bacterial species is due to the presence of different accessory genes; in case of rhizobia, or "nodulation genes", which determine the host specificity (Young et al. 2006). The accessory genes aid in discriminating closely related species, while other core genes *recA* and *atpD* had been used to specify relationship among different mesorhizobia. These accessory genes deliver important properties other than nodulation such as pathovar (that defines specificity of the plant pathogen), serovar (that defines antigenic properties of cell surface of the bacteria (Berrada and Fikri-Benbrahim 2014). Comparative genomic analysis of 29 rhizobia (21 *Rhizobium*, 4 *Ensifer*, 4 *Bradyrhizobium*) showed horizontal gene transfer ensued at plasmid despite the high plasticity of symbiosis genes. This revealed symbiosis and housekeeping genes played

important role in rhizobial evolution that led to expand the diversity of bean-nodulating *Rhizobium* strains. Further phylogenetic analysis of 191 HGT genes showed consistent in the taxonomy of bacterial species. Dispersion of symbiosis genes was suggested to be unusual between rhizobial genera whereas within the same genus expansion of genes was common that could result in formaton of multi-symbiovars. Comparative genomic analysis of *Ensifer* and *Bradyrhizobium* exhibited diverse symbiotic regions and had shown symbiotic compatibility between soybean and common bean microsymbionts (Tong et al. 2020).

Comparative genome analysis of strains of Rhizobiaceae family had indicated replicons varied involving single chromosomes, extrachromosomal replicons (ERs) (or chromids) and plasmids (Slater et al. 2009). ERs genes are genus-specific genes that functions as accessory activities (Harrison et al. 2010). The chromids in *Agrobacterium*/*Rhizobium* and *Ensifer*/*Sinorhizobium* genomes represented half of these genomes. It had been stated that the nodulation genes and genes for nitrogen fixation may perhaps reside in these chromids (Lopez-Guerrero et al. 2012a, b; Althabegoiti et al. 2014) and their presence make these species capable to grow faster in culture (Harrison et al. 2010).

Taxonomic classification based on whole-genome sequence, core genome phylogeny, and chemotaxonomic comparison of group of *Rhizobium* species had resulted in a novel genus—*Pseudorhizobium*. This led to the reclassification of *Rhizobium flavum*, *R. endolothicum*, *R. halotolerans*, *R. marium*, as *P. flavum* comb. nov., *P. endolothicum* comb. nov., *P. halotolerans* sp. nov., and *P. marium* comb. nov. respectively. Resolution of taxonomic classification was improved and supported by genomic basis of phenotytic traits, fatty acid, protein, and metabolic profiles. Phylogenetic analysis of the pan-genome of *Pseudorhizobium* indicated divergence of each species within this genus to adapt their ecological niches (Lassalle et al. 2021). *Bradyrhizobium* and *Azorhizobium* of α-rhizobia has single chromosome (Kaneko et al. 2002; Lee et al. 2008) while *Mesorhizobium* have megaplasmid along with the chromosome (Kaneko et al. 2000). *Sinorhizobium* and *Rhizobium* have highly divided genome structures i.e., *R. leguminosarum* harbors seven replicons (Young et al. 2006) whereas *S. meliloti* genome has more than half size on the chromosome. α-Proteobacteria rhizobia genomes follow the phylogenetic relatedness of these species (Galibert et al. 2001). Pan-genome could indicate the genomic intraspecies diversity (Vernikos et al. 2015) and rhizobia have been reported to have large pangenomes that comprise of thousands of genes that contributed to the phenotypic diversity of the rhizobia. The pan-genome of *S. meliloti* had over 20,000 genes (Sugawara et al. 2013) and *Bradyrhizobium* had 35,000 genes (Tian et al. 2012). The location of classical symbiotic genes (i.e., *nif*, *nod* and *fix* genes) had used as a genotypic tool to classify fast- and slow-growing species of *Rhizobium*. These genes are found on the large symbiotic plasmids or megaplasmids in α- and β-rhizobia (Rosenberg et al. 1981; Teamtisong et al. 2013).

Kuzmanovic et al. (2022) proposed delineation of genus of family Rhizobiaceae, in which genera were separated from related species utilizing core-proteome average average amino acid identity (cpAAI) and the genera were defined as monophyletic groups based on core genome phylogeny. They proposed that genomic or phylogenetic data could help in division of species into separate genera and reclassified *Rhizobium rhizosphaerae* and *R. oryzae* into *Xaviernesmea* gen. nov. The study also provided data for the formation of *Endobacterium yantingense* comb. nov., *Mycoplana azooxidifex* comb. nov. *Neorhizobium petrolearium* comb. nov., *Pararhizobium arenae* comb. nov., *Peteryoungia aggregate* comb. nov., *Pseudorhizobium tarimense* comb. nov. Using genomic, phenotypic data, and cpAAI values (> 86%) of all *Ensifer* and *Sinorhizobium* species, they proposed to consider these two genera as separate genera. Previously, ANI values of strains of *Ensifer fredii* USDA 257 and NGR 234 were reported to be low as compared with type strains of *E. fredii* and other *Sinorhizobium americanum* strains which indicated that NGR 234 corresponds to a separate species (Lloret et al. 2007).

Current classification of rhizobia

Most of the rhizobia bolong to the class α-proteobacteria with wide distribution among the host plants, β-proteobacteria are mainly isolated from root nodules of *Mimosa* sp. (Liu et al. 2020). Alpha-proteobacteria of Rhizobiaceae family are diverse and has undergone several revisions and recently 21 genera consisting of *Allorhizobium*, *Agrobacterium*, *Carbophilus*, *Cicerbacter*, *Ensifer*, *Endobacterium*, *Georhizobium*, *Gellertiella*, *Hoeflea*, *Liberibacter*, *Lentilitoribacter*, *Mycoplana*, *Martelella*, *Neorhizobium*, *Neopararhizobium*, *Pseudorhizobium*, *Peteryoungia*, *Rhizobium*, *Sinorhizobium*, *Shinella*, *Xaviernesmia* has been classified (Kuzmanovic et al. 2022), (https://lpsn.dsmz.de/). Taxonomical description of various rhizobial genera that forms nodules on different hosts are enlisted in Table S2. The different genera of rhizobia which are able to induce nodulation in their respective hosts are discussed below in Table 1.

Today, taxonomic classification of bacteria is based on accessible genomic data of sequenced prokaryotic genomes. A decade back, genome sequencing remained costly and tedious, however the advent of NGS strategies presented after 2005 has made it a lot less expensive and quicker. The genome sequences deposited in public database are easily available for phylogenomics

Table 1 Different genera of classified rhizobia

Genera	Description	Host	References
Class: α-proteobacteria			
Rhizobium Family: Rhizobiaceae	Established by Beijerinck in 1888 and later classified by Frank in 1889. Previously there were 12 *Rhizobium* genera with 44 recognized bacterial species, currently this genus constitutes around seventy-six (76) species	*Phaseolus vulgaris*, *Mimosa pudica*, *Vicia faba*, *Pisum sativum*, *Lens culinaris*, *Glycine max* L., *Cicer arietinum* L.	Sawada et al. (2003)
Neorhizobium Family: Rhizobiaceae	The genus was segregated from the *Rhizobium* *Neorhizobium alkalisoli*, *N. galegae* and *N. huautlense* were reported to be similar to the genera *Rhizobium* and *Agrobacterium* and currently consists of five (05) species	*Astragalus*, *Galega*, *Caragana intermedia*, *Medicago*, *Lotus Sesbania herbacea* and *Vigna*	Osterman et al. (2014), Mousavi et al. (2014)
Pararhizobium Family: Rhizobiaceae	The genus is newly delineated from *Rhizobium.* The type species of this genus is *P. giardinii* and the genus currently consists of six (06) validly identified species	*Phaseolus vulgaris*, *Sphaerophysa salsula*, stone fruit rootstocks tumours and eutrophic forest pond	Mousavi et al. (2015)
Ensifer Family: Rhizobiaceae	Published in 1982 to describe type strain *E. adhaerans* and subsequently the genus *Sinorhizobium* was described in 1988. Based on the similarities of 16S rRNA and *recA* sequences and MLSA approach suggested to consolidate these genera and named the priority genus *Ensifer*. However based on the distinct phenotypic and genomic characteristics, it was suggested that the symbiotic and non-symbiotic members combined in the genus *Ensifer* should be seperated back into previously existing two genera	*Medicago sativa*, *Melilotus officinalis*, *Trigonella foenum-graecum*, *Glycine max*, *Vigna radiata*, *Pisum sativum*, *Cajanus cajan*, legume crops	Young (2003), Willems et al. (2003), Fagorzi et al. (2020)
Sinorhizobium Family: Rhizobiaceae	Described in 1988 while *Rhizobium fredii* was reclassified as *Sinorhizobium fredii*. The Judicial Commission (2008) admitted that type strain *Sinorhizobium adhaerens* is not validly published and transfer the members of *Sinorhizobium* to the synonym genus *Ensifer* and affirmed to conserve the genus *Ensifer* Casida 1982. Based on genome analysis *Ensifer* and *Sinorhizobium* represent a separate taxon, as *E. adhaerens* was found within the nonsymbiotic clade and *S. fredii* was found within the symbiotic clade	Soybean	Chen et al. (1988), Willems et al. (2003), Kuzmanovic et al. (2022)
Shinella Family: Rhizobiaceae	Isolated by An et al. (2006) and proposed as novel genus *Shinella zoogloeoides*. It currently consists of eight (8) species	*Kummerowia stipulacea*, sludge, rhizosphere soil and domestic compost, root nodules and from polluted soil	An et al. (2006)

Table 1 (continued)

Genera	Description	Host	References
Endobacterium Family Rhizobiaceae	Genomic and phylogenetic characteristics of the type strain *Endobacterium cerealis* RZME27T was reported to be clustered with *Pseudorhizobium pelagicum* and *Neorhizobium galegae* of Rhizobiaceae family. ANI value and other phenotypic variation of *E. cerealis* RZME27T with *N. galegae* and *P. pelagicum* supported to propose it as a type strain of this novel genus and species of Rhizobiaceae family	*Zea mays*	Menendez et al. (2020)
Gellertiella Family Rhizobiaceae	*G. hungarica* RAM11T was proposed as a type strain of this genus and species. Its 16S rRNA gene sequence was reported to be 96–97% similar with *E. adhaerens* Casida A, *E. americanus* CFNEI 156T and *R. azooxidifex* Po 20/26T	Pool water of thermal bath in Budapest, Hungary	Toth et al. (2017)
Ciceribacter Family Rhizobiaceae	This bacterium forms bluish black colonies on yeast malt agar and they fix nitrogen. This genus represents close relative of the genus *Ensifer*. Phylogenetic analysis of 16S rRNA, *recA*, *dnaK* and *thrC* genes represent a distinct outgroup from identified genera of Rhizobiaceae family. Also, genotypic, phenotypic, and chemotaxonomic characters supported to propose *Ciceribacter lividus* as a novel species	Rhizospheric soil of *Cicer arietium* L.	Kathiravan et al. (2013)
Pseudorhizobium Family Rhizobiaceae	Two *Pseudorhizobium pelagicum* strains R1-200B4T and R2-400B4 were isolated and *Pseudorhizobium* was proposed as new genus with new species named as *P. pelagicum*. Phylogenetic analysis of 16S rRNA gene demonstrated close relationship with members of Alphaproteobacteria of family Rhizobiaceae	Mediterranean Sea off the coast of Alicante	Kimes et al. (2015)
Hoeflea Family Phyllobacteriaceae	Genus *Hoeflea* (strain LMG 128T) was initially identified as a species belonging to *Agrobacterium ferrugineum* later based on 16S rRNA gene sequence it was found to belong to a new genus and reclassified as a new genus *Hoeflea*, the type strain of this genus is named as *Hoeflea marina* LMG 128T	Marine ecosystem	Peix et al. (2005)

Table 1 (continued)

Genera	Description	Host	References
Peteryoungia Family Rhizobiaceae	The genus was initially placed with members of genera *Rhizobium* and *Ciceribacter*. Phylogenetic analysis and average amino acid identity demonstrated that it clustered with five species of *Rhizobium* which is different from the cluster of genera *Rhizobium* and *Ciceribacter*. This led to the reclassification of *R. ipomoeae*, *R. wuzhouense*, *R. rosettiformans* and *R. rhizophilum* into a new genus *Peteryoungia.* The type species of the genus is *P. ipomoeae*	Saline desert soil	Rahi et al. (2021)
Aminobacter Family: Phyllobacteriaceae	The genus comprises seven species: *A. aganoensis*, *A. aminovorans*, *A. anthyllidis*, *A. carboxidus*, *A. ciceronei*, *A. lissarensis*, *A. niigataensis*. The genus showed close phylogenetic relationship with genus *Chelatobacter* consisting of single species *Chelatobacter heintzii*. Therefore, *C. heintzii* was regarded as synonymous with *Aminobacter aminovorans*	*Anthyllis vulneria*	Kampfer et al. (2002), Notification List IJSEM (2002), Maynaud et al. (2012)
Phyllobacterium Family: Phyllobacteriaceae	*Phyllobacterium* was established by Knosel in the year 1962 isolated from the leaf nodules of tropical ornamental plants and they were found to adapt legume roots than the leaf nodules This genus currently comprises of thirteen (13) species	*Trifolium pratense*, *Lotus corniculatus*, *Argyrolobium uniflorum*, *Astragalus algerianus*, *Lathyrus numidicus*, *Sophora flavescens*	Mergaert et al. (2002), Jiao et al. (2015)
Methylobacterium Family Methylobacteriaceae	This genus was established by Patt et al. (1976) describing the methylotrophic bacteria. The strains of this genus are known as pink-pigmented facultative methylotrophs except a single strain *M. nodulans*. It currently consists of sixty (60) validated species	They are found at a wide range of habitats such soil, sea water, plant rhizospheres, etc.and they exist as free-living organisms and isolated from *Lotononis bainesii*	Green and Bousfield (1983)
Microvirga Family Methylobacteriaceae	This genus was coined by Kanso and Patel in the year 2003 while searching for a thermophile from a deep thermal aquifer. The members of this genus exhibited common features in cell appearances and their cell-wall composition. It currently has seventeen (17) species	Found in different types of soil alongwith some leguminous plant rhizospheres	Kanso and Patel (2003), Li et al. (2020)
Ochrobactrum Family *Brucellaceae*	The genus had been described as root nodule forming bacteria and derived from *Achromobacter*. This genus currently comprises of nineteen (19) validated species	*Cystisus scorparius*, *Lupinus albus*	Trujillo et al. (2005), Zurdo-Pineiro et al. (2007)

Table 1 (continued)

Genera	Description	Host	References
Azorhizobium *Family: Hyphomicrobiaceae*	Isolated by Dreyfus et al. (1988). The unique feature of one of the species, *A. caulinodans* is that it can fix nitrogen in both free-living and symbiotic conditions and does not require plant metabolites to make nitrogenase. It currently comprises of only three (3) species	*Sesbania rostrata*	Dreyfus et al. (1988)
Devosia *Family: Hyphomicrobiaceae*	Established by Nakagawa et al. in (1996) and reclassified from *Pseudomonas riboflavina* as *Devosia riboflavina*. Aquatic legume, *Neptunia natans*-nodulating rhizobia isolated from India was reported to symbiotically nodulate and named the species as *D. neptuniae*. It currently comprises of twenty-five (25) validly published species	Leguminous plants, contaminated sites and polluted waters	Rivas et al. (2003)
Bradyrhizobium Family: Bradyrhizobiaceae	Established by Jordan in the year (1982) by transferring type species *Bradyrhizobium japonicum* from *Rhizobium japonicum*. *Bradyrhizobium* strains are also photosynthetic, that produces active phytohormones in plants leading to plant growth promotion of leguminous plants and they need no *Nod* factors to induce nodules. It comprises of fifty-five (55) validly published species	Soybean, peanuts	Steenkamp et al. (2008), Delamuta et al. (2013)
Class β-proteobacteria			
Burkholderia *Family: Burkholderiaceae*	*B. vietnamiensis* was the first species of this genus found to fix nitrogen. Different species of *Burkholderia* were reported to be produce root-nodule. Currently, it comprises of one hundred twenty-five (125) validated species	*Mimosa* and *Daldergia* legumes	Bournaud et al. (2013)
Paraburkholderia Family: Burkholderiaceae	*Paraburkholderia* was sub-divided from the genus *Burkholderia.* The genus formed a separate clade with the *Burkholderia* group, and the type species of the genus is *Paraburkholderia graminis.* Eleven symbiotic nitrogen fixing *Burkholderia* species had been transferred to the genus *Paraburkholderia* and reclassified 16 *Paraburkholderia* species. The genus comprises seventy-eight (78) validly published species	*Mimosoideae* symbionts, *Mimosa pudica* and *Phaseolus vulgaris*	Dobritsa and Samadpour (2016), Paulitsch et al. (2020)
Cupriavidus Family: Burkholderiaceae	This genus was established by Makkar and Casida (1987). Initially, nodulating species of *Mimosa* spp. were published as *Ralstonia taiwanensis* and it was later renamed as *Cupriavidus taiwanensis*. It is the most studied species of this genus for its plant growth promoting attributes and currently it comprises of nineteen (19) species	*Mimosa* spp.	Vandamme and Coeyene (2004)

Table 1 (continued)

Genera	Description	Host	References
Trinickia Family: Burkholderiaceae	The genus was described for some former *Burkholderia* species, later it revealed it was divergent fom other β-rhizobia groups and occupy a separate lineage, in terms of *nif* and *nod* genes	*Mimosa* species	Estrada-de los Santos et al. (2018)

and therefore, overall genome based indices has replaced DNA-DNA hybridization (DDH) for its low cost and quality of genomic information (Sentausa and Fournier 2013). As explained above, this has also impacted the taxonomy of rhizobia. Parks et al. (2018) had recently proposed a standardized bacterial taxonomy (GTDB taxonomy, http://gtdb.ecogenomic.org/), which is based on phylogeny of bacterial genomes, by analyzing the amino acid sequences of 120 proteins encoded by 120 universal genes. While this strategy utilized concatenated protein phylogeny for prokaryotic classification, which conservatively removes polyphyletic groups, it would be interesting to see if this may resolve up to inter-genus level, as in this case, for the diversity for rhizobia.

Conclusion

Constant development in identification of new legume nodulating bacteria resulted in considerable changes in the taxonomy and nomenclature of rhizobia. Phylogenetic studies using the 16S rRNA gene determine the taxonomic position of rhizobia, while polyphasic approach were used as it became the most reliable method that delineate at species level. Sequence analysis of 16S rRNA, 16–23S rRNA and other housekeeping genes, advances in molecular biology techniques and the use of bioinformatics techniques have facilitated to identify, classify, and discriminate rhizobia to species and subspecies levels. Most of the symbiotic nitrogen fixing bacteria belongs to the main Phylum Proteobacteria of which α-Proteobacteria are most widely distributed in the environment and host plants, while β-Proteobacteria are less widely distributed and found in specific legumes and γ-Proteobacteria are reported for some isolates in temperate legume tree. Genomics analyses have revolutionized which deliver a significant impact in the rhizobial taxonomy. The rhizobial genomes harbors whole spectrum from unichrosomal to highly multipartite, while some strains encode single chromosome and a megaplasmid as well. This approach could describe the main features of Rhizobiaceae genomes, bacterial chromid/ER gene, plasmids, and significance of horizontal gene transfer. The genetic material and genome organization of rhizobia represent evolutionary process of multipartite genomes, which would deliver valuable models for understanding the significance of genome organization in environment adaptation. Comparative genome sequence analysis coupled with ANI could describe new species and it has completely replaced the wet lab DDH values in species characteriation. For accurate characterization of taxonomy, it is better to characterize with different parameters such as phenotypic, genotypic, chemotaxonomic as well as genome sequence analysis.

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Author contributions PK and JR prepared and compiled the original manuscript. SD provided the suggestions. PP and DKM conceptualized, reviewed the work and PP, wrote, and edited the final manuscript.

Declarations

Competing interests The authors declare that they have no competing interests in any financial or personal relationships that could have appeared to influence the work reported in this paper.

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