Chapter 3
Current Systematics of Rhizobia

3.1 Current Methodology for Studying Diversity and Taxonomy

In the investigation of rhizobia, research on diversity forms a basis for studies of other kinds, since diversity studies offer characterised strains that serve as resource for further studies of taxonomy, genetics, biochemistry, evolution, ecology, application and so on. In general, biodiversity includes diversity at three levels: genetic, species and ecosystem. Among these three levels, diversity of species is closely related to the methods or criteria for species definition. In the past century, criteria for definition of bacterial species have changed depending on the development of biological and computer sciences, which also affected the taxonomy of rhizobia. The definition of rhizobial species was at one time based on the host specificity of rhizobial strains (1932–1982) (Fred et al. 1932); on numerical taxonomy and DNA-DNA or DNA-RNA relatedness (1980s–1990s) (Chen et al. 1988; Dreyfus et al. 1988; Jordan 1982); on phylogeny of the 16S rRNA gene combined with numerical taxonomy and DNA-DNA relatedness (Chen et al. 1995; Young et al. 1991); on polyphasic characterisation and multilocus sequence analysis (de Lajudie et al. 1994; Martens et al. 2007); and most recently on genome analysis (Román-Ponce et al. 2016; Wang et al. 2016a). With the addition and shifting of methods, the system of rhizobial taxonomy has been greatly improved (Tak et al. 2017), while the species definition is more related to their evolutionary relationships.

Currently, some of the traditional methods, such as nodulation tests and biochemical and biophysical analysis, are still in use, while some molecular techniques have been replaced by other more recent (reliable and convenient) methods (see Chapter 16 for details). For example, rRNA-DNA hybridisation was replaced by 16S rRNA gene sequencing for determining phylogenetic relationships, and MLSA has been used to replace the 16S rRNA sequence analysis for species definition. Recent studies of rhizobial diversity have generally used a polyphasic approach, usually including genomic analysis, phylogenetic analysis and phenotypic analysis.

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Distinct combinations of the analyses can be selected depending on the purpose of investigation. Based upon our experience and related references, the following methods and thresholds are recommended.

3.1.1 General Strategy for Research on Rhizobial Diversity

During the last three decades, extensive studies on the diversity of rhizobia have been performed worldwide, including the serial studies on the rhizobia of China. These serial studies have been organised (1) for some special regions, like Xinjiang Region which is a vast area with dry continental climate, dramatically varying altitude (from −154 m in the Turpan Basin to 8600 m in the mountains of Karakoram) (Chen et al. 1988, 1995; Han et al. 2008a, b, 2009, 2010; He et al. 2011; Jia et al. 2008; Peng et al. 2002; Tan et al. 1997; Yan et al. 2000); (2) according to the hosts, such as soybean grown in different regions (Chen et al. 2017; Yan et al. 2014, 2016, 2017b; Yang et al. 2018; Zhang et al. 2014b); and (3) for special host species in special regions, such as rhizobia associated with peanut in Guangdong Province (Chen et al. 2016b), or with chickpea in Xinjiang (Zhang et al. 2012a, b, 2014a, 2018a).

As mentioned above, the investigation of rhizobial diversity formed a basis for other kinds of studies; therefore, some subsequent studies can be performed after the strains are characterised. In the last four decades, the study of rhizobia has been developed gradually from resource collection and characterisation (Chen et al. 1988, 1991; Gao et al. 1994) to description of novel taxa (Chen et al. 1988, 1991, 1995, 1997); biogeography of rhizobia or interaction among the rhizobia, host plants and environment (soil characters) (Cao et al. 2014; Gu et al. 2007; Han et al. 2009; Tian et al. 2007; Wang and Martínez-Romero 2000; Yan et al. 2014, 2017b; Zhang et al. 2011a, b); rhizobial genetics and evolution (Guo et al. 2014; Ji et al. 2015; Ruan et al. 2018; Tang et al. 2007; Yao et al. 2014; Yan et al. 2017a; Zhang et al. 2014a); rhizobial genomics (Wang et al. 2018); and inoculant selection and rhizobial application (Jia et al. 2008, 2013; Yang et al. 2018). A general strategy for these studies is shown in Fig. 3.1.

3.1.2 Nodule Sampling and Rhizobial Isolation Strategy

When starting a diversity study, it is important to consider the sampling strategy, which must fit the objective of the study. The first is selection of the host legume(s) and the region(s). When the objective of a study is to clarify the rhizobia associated with a certain legume, the economic importance, the distribution or cultivation area, the prior record of investigation, etc. could be considered for the host selection. For instance, soybean (*Glycine max*), originating in China, and bean (*Phaseolus vulgaris*), originating in Mesoamerica, have been cultivated world-

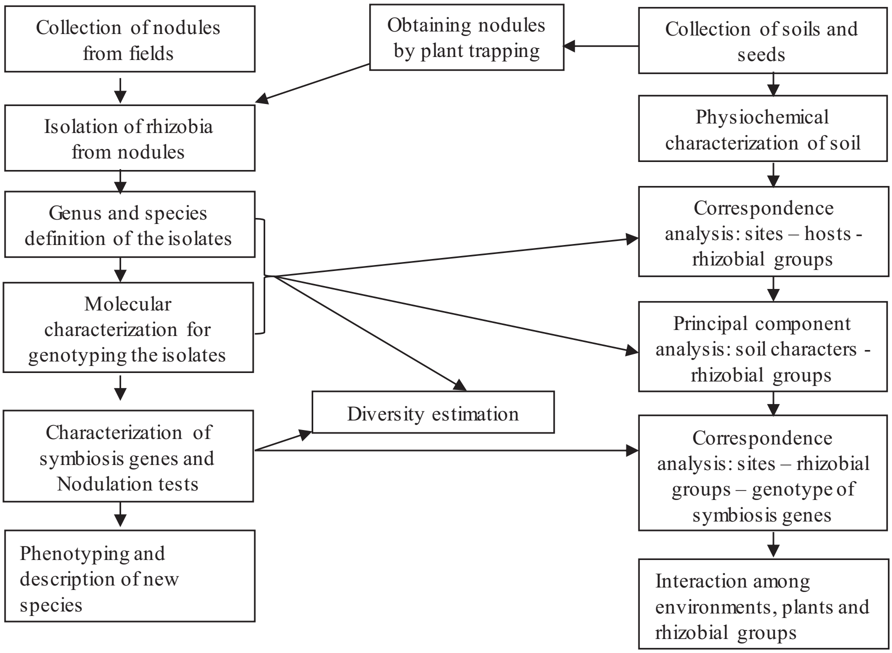

Fig. 3.1 General strategy for studies on diversity and biogeography of rhizobia

wide as grain crops, while the diversity and geographic distribution of their rhizobia have been extensively studied both worldwide and in their original centre. However, there are still some novel groups being described for their rhizobia. To date, *Bradyrhizobium japonicum*, *B. elkanii*, *B. yuamingense*, *B. liaoningense*, *Ensifer fredii*, *E. soyae*, *E. glycinis*, *Mesorhizobium tianshanense* and others have been reported to nodulate soybean, while *Rhizobium etli*, *R. phaseoli*, *R. gallicum*, *Ensifer meliloti*, *E. americanus*, *Burkholderia phymatum* and others nodulate bean plants. Even so, their diversity is still not completely explored, since these legumes are cultivated in diverse regions that lead to the formation of distinct combinations among the rhizobial species (genomic lineages) and the symbiosis genes under the combined selection by soil factors and the host plants, as we have emphasised in previous reports (Han et al. 2009; Li et al. 2011a; Zhang et al. 2011a). The importance of this is confirmed by the discovery of *Mesorhizobium muleiense*, which harbours symbiosis genes similar to those of *Mesorhizobium ciceri* and nodulates chickpea in alkaline soils in China (Zhang et al. 2012b), and of *Rhizobium acidisoli*/*R. hidalgonense* in acid soil and *E. americanus* in alkaline soil in Mexico, which harbour symbiosis genes similar to those of other bean-nodulating rhizobia (Román-Ponce et al. 2016; Verástegui-Valdés et al. 2014; Yan et al. 2017c).

After the target legume plants are chosen, the sampling region(s) might be the determining factor for the discovery of novel rhizobia. As mentioned previously, the association of rhizobial species with a host plant is a result of interactions among the bacteria, plant and the soil factors (Han et al. 2009; Li et al. 2011a; Zhang et al. 2011a), so it is better to sample nodules from plants growing in sites with distinct soil types, especially soil pH. Therefore, soil samples and seeds should be collected simultaneously whenever it is possible. For soil sampling at a site or a field, the cross (X) sampling strategy is usually used, e.g. the soils are sampled from four corners and the centre of the field, which are then mixed and used for subsequent physiochemical characterisation or for trapping rhizobia.

Currently, two kinds of nodule sampling strategies are used: the first is to collect the root nodules from the field plants; the other is to grow the legume plants in a greenhouse in pots filled with soil (without dilution) collected from the root zone of the targeted species, i.e. plant trapping. This latter method is especially used for tree legumes and some perennial herbaceous legumes, for which nodules are very difficult to find in the fields, except on seedlings of the current season. Previously, it has been shown that the rhizobial communities obtained by these two isolation strategies were very similar (Duodu et al. 2006; Harrison et al. 1987; Odair et al. 2006; Van Cauwenberghe et al. 2016), but the rhizobial population composition can be changed in the nodules of legumes inoculated with soil dilutions, resulting in increased or decreased genetic diversity (depending on the host plants) (Duodu et al. 2006; Odair et al. 2006). Depending on the legume species, the trapping plants can be cultivated for 1 month (soybean, bean, *Leucaena*, etc.) or a couple of months (*Acacia*, *Prosopis*, etc.). The previously described procedures of nodule collection and the culture of trapping plants have been regularly applied as the standard methods (Vincent 1970), although some minor modifications can be found, such as the use of plastic cups as the pots. Usually, we use five plant individuals from a field, and five nodules from each plant are randomly selected and used for rhizobial isolation.

At the beginning of the diversity study, the second consideration is how many strains should be used. In general, it is believed that the more strains are studied, the more exact diversity may be revealed. However, the strain number should be appropriate for the capacity of a graduate student or a researcher for a certain period (2–4 years) and enough to fit the objective of the study. In our laboratory, 60–200 strains are used, depending on the aims of the studies. The strain number may be lesser if it is focused on rhizobia associated with a certain host in a certain area (Zhang et al. 2012a, b, 2014a, 2018a), while the strain numbers should be greater for studies on diversity of rhizobia associated with the legume community in a region (Chen et al. 1988; Gao et al. 1994; Han et al. 2008b) or rhizobia associated with a certain host in different regions (Gu et al. 2007; Man et al. 2008). In any case, the rarefaction or coverage of the species or genotypes can be estimated to verify if the strain numbers are adequate for revealing the real diversity (McInnes et al. 2004), although this analysis is rarely used in rhizobial studies (Date and Hurse 1991; Handley et al. 1998).

For rhizobial isolation, the best way is from surface-sterilised fresh nodules, although some procedures using dehydrated nodules have been suggested previously (Vincent 1970). Traditionally, the medium of yeast extract-mannitol agar (YMA) supplied with Congo red (yeast extract, 1.0 g; mannitol, 10 g; K_2HPO_4, 0.5 g;

$MgSO_4$, 0.2 g; NaCl, 0.1 g; Congo red, 0.025 g; agar, 20 g; pH 6.8±0.2) is used for isolation of rhizobia. In this medium, growth of Gram-positive bacteria is inhibited, and the rhizobial colonies are described as white, translucent, glistening and elevated, with entire margins (Allen and Allen 1950). Another traditional medium used for rhizobial isolation and identification is YMA supplied with 5 ml per litre of 0.4% bromothymol blue (BTB). This medium was used for differentiating the fast-growing acid producing *Rhizobium* species (also the current *Sinorhizobium*/*Ensifer*, *Mesorhizobium*, etc.) from the slowly growing, alkali-producing *Bradyrhizobium* (Jordan 1982). For rhizobial isolation, the third medium is TY medium (tryptone, 5 g; yeast extract, 3 g; $CaCl_2$, 0.7 g; agar, 20 g; distilled water, 1.0 L; pH 7.0) (Beringer 1974) or PY medium in which tryptone is replaced with peptone (Poupot et al. 1995). TY or PY medium is recommendable for rhizobial isolation in diversity studies because they are media allowing the growth of diverse bacteria, so it is more possible to obtain some unknown rhizobia, such as those in *Betaproteobacteria*. In addition, attention should be paid to the unusual but dominant colonies, such as those with colour. In general, the isolates from nodules need to be incubated 3 to 15 days for fast-growing (*Rhizobium*, *Ensifer*, etc.) and slow-growing (*Bradyrhizobium* and *Mesorhizobium*) rhizobia. However, a longer time of incubation is recommended if no growth occurs on the medium after 15 days.

3.1.2.1 Molecular Characterisation Strategy

In rhizobial investigation, many molecular methods have been used to reveal the diversity at genetic, strain, species, genus or higher levels. However, some of them have lost their value since other more convenient methods have been developed as a result of the progress in technology. For example, multilocus sequence analysis (MLSA) (Martens et al. 2008) is currently widely used in estimation of genetic diversity and species definition of rhizobia to replace multilocus enzyme electrophoresis (MLEE) (Wang et al. 1998, 1999a), PAGE of total bacterial proteins (SDS-PAGE) (Diouf et al. 2000), two-dimensional electrophoresis of total bacterial proteins (Roberts et al. 1980), amplified fragment length polymorphism (AFLP) (Gao et al. 2001; Terefework et al. 2001), amplified 16S rDNA restriction analysis (ARDRA, or PCR-RFLP of 16S rRNA gene) (Wang et al. 1998, 1999a) and amplified 16S-23S intergenic spacer (IGS) RFLP analysis (Tan et al. 2001; Vinuesa et al. 1998). Also, MLSA has been suggested to replace DNA-DNA hybridisation (Martens et al. 2007, 2008).

The strategy or combinations of molecular methods for investigation of rhizobial diversity may vary among different studies since distinct methods may play the same role in differentiation of genotypes, strains, species, etc. (Bala and Giller 2006; Jiao et al. 2015a; Wolde-Meskel et al. 2005; Yan et al. 2014). Bala and Giller (2006) studied diversity of rhizobia associated with *Calliandra calothyrsus*, *Gliricidia sepium* and *Leucaena leucocephala* grown in four soils, with ARDRA, PCR-RFLP of IGS and full-length 16S rDNA sequencing, and reported four genospecies related to *R. tropici*, *R. etli*, *Sinorhizobium* and *Agrobacterium*.

Wolde-Meskel et al. (2005) investigated the genetic diversity of 195 rhizobial strains associated with 18 agroforestry species in Ethiopia, by using PCR-RFLP of 16S rRNA gene, 23S rRNA gene and ITS region between the 16S rRNA and 23S rRNA genes and 16S rRNA gene partial sequence (800 and 1350 bp) analyses. They delineated 87 genotypes, in which 46 16S rRNA gene sequence types (12 identical to those of described species and 34 novel, with 94–99% similarity to those of recognised species) were assigned to the genera *Agrobacterium*, *Bradyrhizobium*, *Mesorhizobium*, *Methylobacterium*, *Rhizobium* and *Sinorhizobium*.

Jiao et al. (2015a) studied 269 rhizobial isolates obtained from nodules of *Sophora flavescens* grown in three ecoregions. They firstly grouped the isolates in 17 genotypes with *recA* gene sequence analysis. A subset of 35 representative isolates was further characterised with MLSA of housekeeping genes *atpD*, *glnII* and *recA*, which identified the 17 genospecies into genera *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Rhizobium* and *Phyllobacterium*. Yan et al. (2014) used a similar strategy to characterise 280 nodule isolates, but five housekeeping genes, *glnII*, *atpD*, *dnaK*, *gyrB*, and *rpoB*, in addition to *recA*, were amplified and sequenced to identify them into *Bradyrhizobium japonicum* and three novel genospecies. These four examples demonstrated a trend that the PCR-RFLP analyses of ribosomal operons have been replaced by the sequence analysis of housekeeping genes for identifying the species.

We recommend the following strategies for rhizobial diversity study:

1. **Screening by *recA* phylogeny**. Screening the isolates with *recA* amplification and sequence analysis to group them into genotype, species and genus, as done by Jiao et al. (2015b, c) and Yan et al. (2014). Genotypes are defined for isolates that shared identical *recA* gene sequences, while the threshold of 97% sequence similarity can be used to differentiate species. The advantage of using this gene is that its phylogeny can simultaneously determine the genus and species of the rhizobial strains, while sequence analysis of 16S rRNA genes cannot, because many rhizobial species within a genus share very similar (>97% similarity) or even identical sequence of 16S rRNA.
2. **Phylogenetic analyses of housekeeping genes**. Amplification and sequence analyses of 16S rRNA genes and of two (*atpD*, *glnII*) or more housekeeping genes (such as *dnaK*, *gap*, *glnA*, *gltA*, *gyrB*, *pnp*, *rpoB* and *thrC*) (Martens et al. 2008) can be used for further characterisation of representative strains of each *recA* genotype. The 16S rRNA gene sequences are used to reconstruct the phylogenetic tree together with those from the defined species, while sequences of *atpD* and *glnII* together with that of *recA* or the other genes mentioned above will be concatenated and used to construct a phylogenetic tree for confirming the species affiliation of the strains (Martens et al. 2008). In some cases, the genospecies defined by the concatenated sequence analysis can be used for calculation of alpha diversity with the Shannon index and for correspondence and principal component analyses in combination with the sampling sites and soil factors (Han et al. 2009; Zhang et al. 2011a). These data are adequate for preparing a paper about the diversity and biogeography of rhizobia.

3. **BOX-PCR**. For investigating genetic diversity within a species or a novel genospecies, Eric-PCR or rep-PCR (BOX-PCR) fingerprinting is a recommendable method, which is more convenient, discriminative and reproducible compared with random amplified polymorphic DNA (RAPD) analysis (Agius et al. 1997). After the amplification, the PCR products (amplicons) are subjected to electrophoresis in agarose gel (1%, w/v), and the amplicon patterns are visualised (Agius et al. 1997). The electrophoretic patterns can be standardised and used for clustering analysis. Isolates sharing the same BOX-PCR amplicon patterns are identified as clones of the same strain. However, this method is not adequate for defining species or genera (Binde et al. 2009), and strain groups belonging to different species may be intermingled.
4. **Phenotypic characterisation**. This is for revealing phenotypic diversity among the rhizobial populations and for searching distinctive features for the novel species (Mazur et al. 2013). In current bacterial taxonomy, the novel groups different from the defined species by DNA sequence analysis are initially named genospecies. In the past, it was considered necessary that phenotypic features differentiating the genospecies from the defined species were found before the novel genospecies could be described as species (Graham et al. 1991). In rhizobial study, the phenotypic traits covered symbiotic, cultural, morphological, and physiological traits (Graham et al. 1991); however, we consider the symbiosis traits as a separate item and discuss these later. In general, the colony and cell morphology (including mobility and flagellation) are observed at the isolation and purification stage. The cultural features normally covered the range and optimal pH, temperature and salinity for growth, while resistance to antibiotics, heavy metals and some other chemicals may also be analysed depending on the study purpose (Gao et al. 1994). For the physiological traits, the normal analyses are utilisation of sole carbon source of sugars, alcohols, organic acids, etc. that can be obtained by using the Biolog GN2 microplates and enzyme activities that could be estimated with the API 20NE kit (bioMérieux) (Chen et al. 2017; McInroy et al. 1999). From these data, a dendrogram can be generated by numerical taxonomy (Graham 1964; Gao et al. 1994), but the grouping results may be not consistent with the species definition or the phylogenetic relationships, as in the case of *Sinorhizobium xinjiangense*, originally defined by numerical taxonomy (Chen et al. 1988), which has been merged into *Sinorhizobium fredii* based upon the phylogenetic analyses (Martens et al. 2008).
5. **Chemical taxonomy**. For description of novel species of rhizobia, some analyses of chemical composition of cells are used currently. These include, but not limited to, the composition of cellular fatty acids (Tighe et al. 2000; Chen et al. 2017), protein composition (Ahnia et al. 2018), respiratory quinones and polar lipids (Choma and Komaniecka 2003; Miller et al. 1990; Minder et al. 2001; Orgambide et al. 1993; Wang et al. 2013a, 2013b). Another traditional feature is the G+C mol% of the genomic DNA, for which both the chemical-HPLC method (Peyret et al. 1989) and denaturation-spectrophotometric method (De Ley 1970) were developed. But now, it is more often to estimate G+C mol% from the genome sequence data (Aserse et al. 2017a, b; Zhang et al. 2018b). Some of the

chemical taxonomic data also can be used for cluster analysis (Goodacre et al. 1991; Jia et al. 2015), although the results may be not corresponding to the species affinities. In fact, these data are not so valuable for species differentiation but served as description characters for the species.

6. **Phylogeny of symbiosis genes and symbiotic specificity**. These features are specific for rhizobia, since they are symbiotic bacteria with host specificity. With the host specificity, rhizobial strains can be grouped into symbiovars (sv), which may cross the border of species, even genera. For example, symbiovar phaseoli covered the bean-nodulating strains within the species *Rhizobium etli*, *Rhizobium leguminosarum*, *Rhizobium gallicum*, *R. acidisoli*, *R. hidalgonense*, *Pararhizobium giardinii*, etc. (Amarger et al. 1997; Verástegui-Valdés et al. 2014). For nodulation test, a list of host legumes including *Medicago sativa*, *Pisum sativum*, *Phaseolus vulgaris*, *Trifolium repens*, *Lotus corniculatus*, *Glycine max*, *Vigna unguiculata*, *Leucaena leucocephala*, *Macroptilium atropurpureum* and *Galega officinalis* and standard methods were suggested by Graham et al. (1991). However, the diversity of rhizobia has been enlarged dramatically during the past decades, and the host spectrum of rhizobia also greatly increased. More symbiovars have been described, like sv. *mimosae* in *R. etli* that nodulates *Mimosa* species (Wang et al. 1999b) and sv. *mediterranense* in *Ensifer* (*Sinorhizobium*) *meliloti* and *E. americanum* that nodulates bean plants (Verástegui-Valdés et al. 2014). Therefore, some new hosts for cross-nodulation tests should be added. Laguerre et al. (1996) reported the correspondence between symbiosis gene genotyping and the host range of rhizobia, which has been further evidenced by the symbiosis gene phylogeny (Rogel et al. 2014; Verástegui-Valdés et al. 2014). Therefore, the cross-nodulation relationships can be estimated from the phylogeny of symbiosis genes, and the host species used in cross-nodulation tests for new rhizobial species can be selected according to its symbiovar.
7. **Genome analysis**. Since the 1960s, DNA-DNA hybridisation, which estimates the genome similarities between the bacterial species, has been used as a standard method for species definition, and 70% relatedness was suggested as the species threshold (Graham et al. 1991). Correspondingly, different methods have been developed for DNA-DNA hybridisation, such as measurement of renaturation rates (De Ley et al. 1975), and membrane hybridisation with radioactively labelled DNA (Jarvis et al. 1980; Wedlock and Jarvis 1986). These methods were widely used and played key role in rhizobial species definition (Chen et al. 1991; Jordan 1982; Li et al. 2011b; Wang et al. 1998, 1999a, b). However, there are some obvious disadvantages of DNA-DNA hybridisation methods: they require large amount of DNA and are labour-intensive and time-consuming; the results depend on the exact equipment used and are unreliable for low level of relatedness; and the results are pairwise and cannot be accumulated for database construction (Goris et al. 2007). With the development of genome sequence analysis, the DNA-DNA hybridisation (DDH) methods have been replaced by average nucleotide identity (ANI) and digital hybridisation of genome sequences in the description of novel species and genera (Grönemeyer et al. 2017; Safronova et al. 2018).

8. **Description of novel species and genus** The final step of a study is writing a paper for publication. For diversity studies, it is convenient in some cases to prepare manuscripts separately for diversity and for description of new taxa. For description of a new taxon, the first consideration is nomenclature, which must follow the rules of bacterial nomenclature (Lapage et al. 1992). For naming new rhizobial genera, "rhizobium" has been used as suffix to combine with a prefix demonstrating (1) the important phenotypic feature, like *Bradyrhizobium* (slow-growing rhizobia) (Jordan 1982), *Mesorhizobium* (moderately growing rhizobia) (Jarvis et al. 1997) and *Azorhizobium* (free-living nitrogen-fixing rhizobia) (Dreyfus et al. 1988); (2) the geographic origin of the bacteria, like *Sinorhizobium* (rhizobia from China) (Chen et al. 1988); and (3) the relation to *Rhizobium* (genus similar to *Rhizobium*), like *Allorhizobium* (de Lajudie et al. 1998a), *Neorhizobium* (Mousavi et al. 2014), *Pararhizobium* (Mousavi et al. 2015) and *Pseudorhizobium* (no symbiotic rhizobia) (Kimes et al. 2015). For naming species, the most common specific epithets are the name (genus) of host legume, the geographic origin or the name of a person who has made an important contribution to rhizobial study, for example, *Ensifer* (*Sinorhizobium*) *meliloti* (from *Melilotus*), *Rhizobium etli* (from "etl" = bean in Nahuatl language), *Mesorhizobium mediterraneum* (from Mediterranean Basin), *Sinorhizobium fredii* (in memory of Dr. Edwin B. Fred) and *Mesorhizobium huakuii* (in memory of Dr. Huakui Chen). Other epithets can be ecological location (*Rhizobium rhizosphaerae*, *Rhizobium endophyticum*, *Azorhizobium caulinodans*, *Rhizobium alkalisoli*) or notable characteristics of the species (*Rhizobium metallidurans*).

The International Committee on Systematics of Prokaryotes has a Subcommittee for the Taxonomy of Rhizobia and Agrobacteria that holds regular meetings to discuss relevant issues and keep track of newly published species and genera. Its minutes are published (de Lajudie and Young 2017, 2018, 2019), and it maintains a web site (https://sites.google.com/view/taxonomyagrorhizo/home), and these resources should be consulted by those planning to describe new taxa. Importantly, the subcommittee publishes recommendations for the description of new species and genera of rhizobia and agrobacteria, and authors are expected to follow these guidelines. Until very recently, the only available guidelines were very out of date (Graham et al. 1991), but new guidelines have just been published (de Lajudie et al. 2019). A notable change is that genomic comparisons will form the main basis for taxonomy in future, and a genome sequence of the type strain is now required for the publication of a new species.

3.2 Phylogeny and Systematics of Rhizobia

Based upon biogeographic and genetic studies, we can conclude that rhizobial diversity depends on four factors: their long evolutionary history, environmental selection for their survival (for chromosomal genes), host selection for nodulation (for symbiosis genes) and lateral transfer of symbiosis genes (novel combinations of chromosome and symbiosis genes).

Currently, all the symbiotic nitrogen-fixing bacteria are found in the phylum *Proteobacteria*, within the classes *Alphaproteobacteria* (α-rhizobia), *Betaproteobacteria* (β-rhizobia) and maybe also *Gammaproteobacteria* (γ-rhizobia), with about 180 nodulating species in 21 genera at the time of writing. Among them, α-rhizobia are the most common group with a very wide distribution in geography and host plants, and beta-rhizobia are also well established though less widely distributed. The existence of γ-rhizobia remains controversial: there have been a number of claims, of which the isolation of *Pseudomonas* strains from nodules by Shiraishi et al. (2010) is perhaps the strongest, though their status as rhizobia is not fully proven.

It has been estimated that nitrogen fixation is an ancient feature that evolved when the planet was anoxic (2000 million years ago), while *Bradyrhizobium* may most closely resemble the ancestor of all the rhizobia (Lloret and Martínez-Romero 2005). According to the phylogenetic relationships (substitution of amino acids) estimated from GSI and GSII (glutamine synthetase I and II), *Bradyrhizobium* originated 553 million years (m. y) ago, before terrestrial plants arose (438 m. y) on the planet; then the other rhizobial genera (*Mesorhizobium*, *Rhizobium*, *Sinorhizobium*) evolved 400–324 m. y ago, still long before the first legumes (70 m. y ago) (Lloret and Martínez-Romero 2005). These estimations are also supported by some phenomena of rhizobia; for example, free-living nitrogen fixation has been detected in some strains of *Azorhizobium* and *Bradyrhizobium*, two lineages that are very distant from the other rhizobial genera, which may be evidence for their ancestral state.

From the comparison of symbiosis gene phylogeny and 16S rRNA gene phylogeny, it is clear that some of the nodule symbiotic bacteria or rhizobia evolved by acquiring the symbiosis genes from other rhizobial species, like the beta-rhizobia (see Sect. 3.4). Based on the lateral transfer, it could be estimated that more novel rhizobia might be found in further studies on rhizobial diversity. In our previous studies, *nifH* gene similar to that of *R. leguminosarum* was detected in an endophytic *Bacillus* isolate (not published), which might be also a result of lateral gene transfer. Lateral transfer of symbiosis genes is also found among rhizobial species with close phylogenetic relationships, such as *Mesorhizobium* species nodulating *Lotus* species (Sullivan and Ronson 1998), *Sinorhizobium*/*Bradyrhizobium* associated with soybean (2011a) and bean rhizobia in the genera *Rhizobium* and *Sinorhizobium* (Verástegui-Valdés et al. 2014).

3.3 Alpha-Rhizobia

The symbiotic bacteria in Class *Alphaproteobacteria* are the most common rhizobia, which are distributed in 16 genera of seven families: *Agrobacterium*, *Allorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Neorhizobium*, *Pararhizobium*, *Rhizobium* and *Shinella* in family *Rhizobiaceae*; *Aminobacter*, *Phyllobacterium* and *Mesorhizobium* in *Phyllobacteriaceae*; *Bradyrhizobium* in *Bradyrhizobiaceae*; *Microvirga* and *Methylobacterium* in *Methylobacteriaceae*; *Ochrobactrum* in *Brucellaceae*; *Devosia* in *Hyphomicrobiaceae*; and *Azorhizobium* in *Xanthobacteraceae*. All of them are members of the order *Rhizobiales*, in which the families *Bartonellaceae*, *Beijerinckiaceae*, *Cohaesibacteraceae*, *Methylocystaceae*, *Rhodobiaceae* and *Roseiarcaceae* are also included.

3.3.1 Family **Rhizobiaceae** *Conn (1938)*

The family *Rhizobiaceae* accommodates 12 genera and a *Candidatus* to date, among which symbiotic nitrogen-fixing bacteria have been found in *Agrobacterium*, *Allorhizobium*, *Ensifer*, *Neorhizobium*, *Pararhizobium*, *Rhizobium* and *Shinella*. The phylogenetic relationships of these symbiotic bacteria are shown in Fig. 3.2. At all taxonomic levels, they are intermingled with non-symbiotic bacteria, such as the rhizosphere bacteria in genus *Pseudorhizobium* (Kimes et al. 2015), endophyte *Rhizobium zeae* (Celador-Lera et al. 2017) and non-symbiotic strains in *Rhizobium leguminosarum* (Laguerre et al. 1993).

In general, the rhizobial species in this family, like those in the genera *Ensifer* and *Rhizobium*, harbour their symbiosis genes in plasmids, the so-called symbiosis

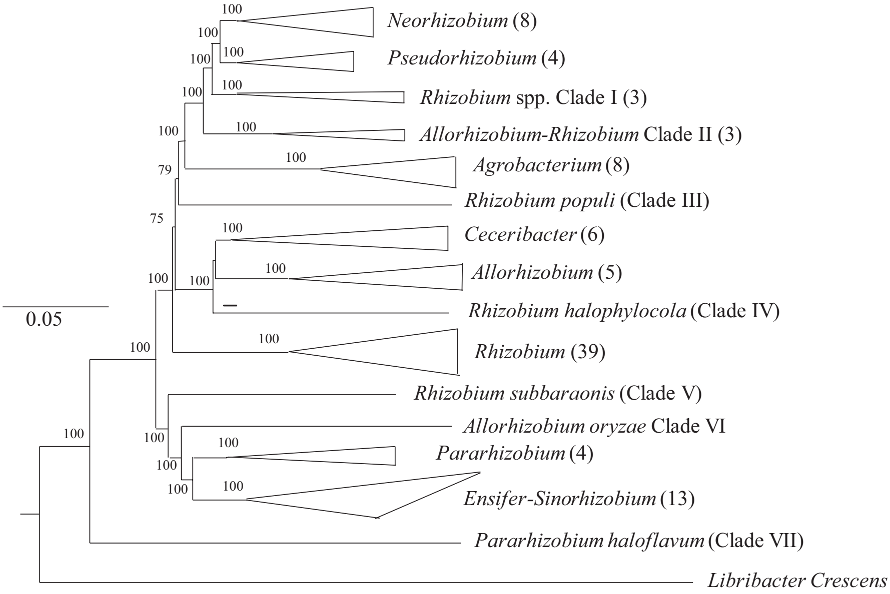

Fig. 3.2 Phylogenetic tree of 96 *Rhizobiaceae* strains constructed with GET_HOMOLOGUES software based on the concatenated amino acid sequences deduced from 316 core genes. A total of 17 clades (genera) were defined at the threshold of 75% of ANI. The scale bar represents 5% of the substitution of amino acids. (Provided by Dr. Y. Li)

plasmid or pSym. According to their sizes, the pSym may be classified as (1) a megaplasmid (≥1000 kbp) with size similar to that of the chromosome in the case of *Ensifer meliloti* (Lagares et al. 2014) and *Neorhizobium galegae* (Wang et al. 1998) and (2) a large plasmid with various sizes, like 400 kbp in *Neorhizobium huautlense*, 600 kbp in *Rhizobium etli* sv. *mimosae*, etc. (Wang et al. 1998).

Considering the generation time, the symbiotic bacteria in this family are termed fast-growing rhizobia, with generation time about 2–4 h, and their colonies in YMA appear with a diameter of 2–5 mm after 3 day incubation at 28°C. Three copies of 16S rRNA gene have been detected in some strains as revealed by RFLP and genome sequence analyses.

3.3.1.1 Genus *Agrobacterium* (Smith and Townsend 1907) Conn (1942)

Agrobacterium was originally described for phytopathogens that cause tumours on roots and stems of some plants, and three biovars were defined for these phytopathogens based on their physiological and biochemical properties (Kerr and Panagopoulos 1977). Later, the specific names *A. tumefaciens*, *A. rhizogenes* and *A. rubi* were designed for biovars 1, 2 and 3, respectively (Holmes and Roberts 1981). Subsequently, species *Agrobacterium vitis* for biovar 3 strains from grapevines (Ophel and Kerr 1990) and *Agrobacterium larrymoorei* for *Ficus benjamina* aerial tumour-inducing pathogens (Bouzar and Jones 2001) were described. In addition, some marine star-shaped-aggregate-forming bacteria were described as *Agrobacterium atlanticum*, *Agrobacterium ferrugineum*, *Agrobacterium gelatinovorum*, *Agrobacterium meteori*, *Agrobacterium stellulatum* and *Agrobacterium kieliense* based on phenotypic analyses, DNA G+C content, DNA-DNA hybridisation and low-molecular-weight RNA (5s rRNA and tRNA) electrophoretic analysis (Rüger and Hofle 1992). Later, based on the phylogeny of 16S rRNA gene, these marine *Agrobacterium* species were transferred into genera *Ahrensia*, *Pseudorhodobacter*, *Ruegeria* and *Stappia* in the order *Rhodobacterales* (Uchino et al. 1998).

Based on the 16S rRNA gene phylogeny, the pathogenic strains in *Agrobacterium* species were intermingled with the symbiotic strains in *Rhizobium*. Considering these relationships, and the fact that both the tumour-inducing genes in *Agrobacterium* and the nodule-inducing genes in *Rhizobium* were plasmid genes, Young et al. (2001) proposed the transfer of all the *Agrobacterium* and *Allorhizobium* species into *Rhizobium*, which was a controversial reclassification (Farrand et al. 2003) and was not widely applied in the related investigations. Subsequently, *Agrobacterium* was reclassified according to the genomic data (Mousavi et al. 2015), in which the species *Agrobacterium radiobacter* (synonymous with *A. tumefaciens*), *A. fabrum* (represented by the former *A. tumefaciens* strain C58), *A. larrymoorei* and *A. rubi* were maintained, and the previously reported phytopathogens *Rhizobium nepotum* and *Rhizobium skierniewicense*, as well as the rhizosphere and human pathogen *Rhizobium pusense*, were transferred into *Agrobacterium*. The root tumour-inducing *Rhizobium rhizogenes* was, however, retained in *Rhizobium*. Currently, about ten species are included in this genus: *A. bohemicum*, *A. rosae*, *A. rubi*, *A. larrymoorei*, *A. nepotum*, *A. pusense*, *A. radiobacter*, *A. salinitolerans*, *A. arsenijevicii* and *A. skierniewicense*. More species should be added with further study on more isolates, like the recently described species *Agrobacterium deltaense* for endophytic bacteria of *Sesbania cannabina* (Yan et al. 2017d).

The most important feature of this genus is that the strains in *Agrobacterium radiobacter* (*tumefaciens*) harbouring the Ti plasmid are the unique natural vector to transfer genes between the bacteria (procaryotes) and host plants (Eucaryotes). Because of this, they have been used as an important tool for genetic engineering. Although none of the *Agrobacterium* species were originally described for symbiotic bacteria, symbiotic ability has been evidenced in some strains in different species. This fact demonstrates that symbiotic character is present but not widely distributed in *Agrobacterium* strains.

Agrobacterium pusense **Symbiotic Strain** In this species, IRBG74 has been reported as the only symbiotic strain that harboured a symbiosis plasmid and fixed nitrogen in root nodules of *Sesbania cannabina* (Aguilar et al. 2017; Cummings et al. 2009) and infected rice endophytically (Tan et al. 2001). No tumour-inducing plasmid was detected, and a symbiosis plasmid pIRBG74a exists in this strain that contains *nifH* and *nodA* genes similar to those in other *Sesbania* rhizobia, like the bv. *sesbaniae* in *E. saheli* and *E. terangae* (de Lajudie et al. 1994; Boivin et al. 1997). The pIRBG74a is a *repABC* family plasmid containing many symbiosis genes like *nod*, *nif* and *fix* genes. It is suggested that this plasmid has been acquired by lateral transfer (Crook et al. 2013).

Agrobacterium radiobacter **Symbiotic Strains** This species covered most of the strains in the formerly named species *Agrobacterium tumefaciens* (Mousavi et al. 2015). Chen et al. (2000) investigated the soybean rhizobial diversity in Paraguay and obtained five strains corresponding to *A. radiobacter* in 16S rRNA phylogeny, for which the nodulation ability was confirmed for two representative strains PRY 60 and PRY 62.

Other Symbiotic Strains in *Agrobacterium* Wang et al. (2016b) reported an *Agrobacterium* genospecies (sp. III) with 11 isolates originating from bean nodules that harboured *nifH* and *nodC* similar to those of *R. etli*. Since a nodulation test on bean plants failed, it was suggested that they were recently evolved symbiotic bacteria with unstable nodulation ability.

3.3.1.2 Genus *Allorhizobium*

This genus was first described for the symbiotic bacteria associated with the aquatic plant *Neptunia natans* in Senegal (de Lajudie et al. 1998a), and it was combined into the genus *Rhizobium* (Young et al. 2001) based on the 16S rRNA phylogeny. Later, it was emended with accumulation of more genome data (Mousavi et al. 2014), by covering *Allorhizobium undicola*, *Allorhizobium vitis* (the former *Agrobacterium vitis*), *Allorhizobium taibaishanense* (former *Rhizobium taibaishanense* as endophytes of *Kummerowia striata* root nodules), *Allorhizobium borbori* (aniline-degrading bacteria isolated from activated sludge), *Allorhizobium oryzae* (rice endophyte), *Allorhizobium paknamense* (endophyte of lesser duckweeds *Lemna aequinoctialis*), *Allorhizobium pseudoryzae* (from rhizosphere of rice), *Rhizobium capsici* (from root tumour of green bell pepper *Capsicum annuum* var. *grossum*), *Rhizobium tarimense* (soil of the ancient Khiyik River) and so on. In addition, the recently described rice endophyte *Rhizobium oryziradicis* (Zhao et al. 2017a) should be renamed as *Allorhizobium oryziradicis* based on its close phylogenetic relationships with *Al. vitis* and *Al. taibaishanense*. According to the comparative study of genome sequences (Fig. 3.2), as well as the 16S rRNA gene phylogeny, *Al. oryzae* and *Al. pseudoryzae* should be removed from the genus.

Among them, only *Al. undicola* and *Al. oryzae* were symbiotic bacteria. Therefore, symbiotic feature is a character for some species in this genus. *Al. undicola* is the type species of the genus described by de Lajudie et al. (1998a). The strains of this species are nitrogen-fixing microsymbionts of the aquatic legume *Neptunia natans*. *Al. oryzae* strains were originally isolated as endophytes of rice, but they were able to effectively nodulate *Phaseolus vulgaris* and *Glycine max* (Peng et al. 2008)

3.3.1.3 Genus *Ensifer* (formerly *Sinorhizobium*)

Ensifer was first described for a group of bacterial predators of bacteria (Casida 1982). For two decades, only the type species *Ensifer adhaerens* was reported in this genus. Meanwhile, the genus *Sinorhizobium* was described (Chen et al. 1988) for the fast-growing soybean microsymbionts, including *S. fredii* (formerly named *Rhizobium fredii*) and *S. xinjiangense.* Subsequently, this genus was emended (de Lajudie et al. 1994), and more species were defined in it: *S. americanum*, *S. arboris*, *S. chiapanecum*, *S. fredii*, *S. kostiense*, *S. kummerowiae*, *S. medicae*, *S. meliloti*, *S. mexicanum*, *S. morelense* and *S. terangae*. However, later studies on phylogeny revealed that *Sinorhizobium* and *Ensifer* species shared high similarities of 16S rRNA genes (Chen et al. 2017) and they could be the same genus. Since *Ensifer* is the earlier heterotypic synonym and it takes priority, Young (2003) proposed the combination of *Sinorhizobium* and *Ensifer*, by renaming all the *Sinorhizobium* species as *Ensifer* species. Willems et al. (2003) suggested to maintain the genus *Sinorhizobium* by transferring *E. adhaerens* to *S. adhaerens* to avoid confusion in the literature and in databases, which was rejected later according to the Bacteriological code (Lindström and Young 2009; Young 2010). However, this change caused great controversy in rhizobial studies other than in taxonomy. Although this change has been accepted in taxonomic work, including the description of new species *Ensifer shofinae* (Chen et al. 2017) and *Ensifer collicola* (Jang et al. 2017), the names *Sinorhizobium fredii* and *Sinorhizobium meliloti* are still used in studies of genetics, ecology, biochemistry, biophysiology and so on (Jiao et al.

2018; Lehman and Long 2018; van Loo et al. 2018; Xue and Biondi 2018). Based on the phylogeny of 318 core genes, *E. sesbaniae*, *E. adhaerens* and *Ensifer* sp. 4180 formed a subgroup separated from the other species, implying the possibility of emendating the genus *Sinorhizobium* later.

The controversy caused by the change of *Sinorhizobium* into *Ensifer* (Young 2003), also the combination of *Agrobacterium*-*Allorhizobium* with *Rhizobium* (Young et al. 2001) and the recent split/revision of these three genera (Mousavi et al. 2014, 2015), drove a question for taxonomists: it is better to keep the nomenclature of bacteria relatively stable, since a name is always linked to a lot of history literature and a good taxonomy should be convenient for the people to use the bacteria in studies of varied fields. Otherwise, taxonomy will become a game only for the small group of taxonomists. Currently, 24 species have been described in the genus *Ensifer* (*Sinorhizobium*) (Table 3.1), in which most species, except *E. collicola* (Jang et al. 2017) and *E. morelensis* (*S. morelense*) (Wang et al. 2002, 2016a), contain symbiotic strains nodulating with distinct legume plants.

According to current knowledge, most of the species in the genus *Ensifer* are symbiotic bacteria; some important and common features related to their symbiosis abilities are described here. (1) They harbour the symbiosis genes in large plasmids or megaplasmids, so-called symbiosis plasmids (Galibert et al. 2001; Jiao et al. 2018; Schmeisser et al. 2009; Vinardell et al. 2015), on which most genes (58–59%) are related to the specific symbiosis, followed by genes common for the species (23–25%), strain-specific genes (11–13%) and common genes for the genus (5%) (Jiao et al. 2018). The symbiosis plasmids can be transferred into other, non-symbiotic, species or can be lost. An example could be *E. morelensis* strains: seven strains of *E. morelensis* were isolated from root nodules of *Leucaena leucocephala*, and a symbiosis plasmid with 670 kbp was detected in these strains and also in several other *Leucaena*-nodulating rhizobia (Wang et al. 1999c). However, after storage, their symbiosis plasmid was lost, accompanied by a failure of nodulation on the host of origin (Wang et al. 2002). (2). It is worth mentioning that different symbiovars exist in some of the *Ensifer* species, such as the bean-nodulating strains of sv. *mediterranense* in *E. meliloti* (Mnasri et al. 2007; Zurdo-Piñeiro et al. 2009) and *E. americanus* (Mnasri et al. 2012; Verástegui-Valdés et al. 2014).

3.3.1.4 Genus *Neorhizobium* Mousavi et al. (2014)

Neorhizobium (Mousavi et al. 2014) covered several species formerly described as members of *Rhizobium*, namely, *Rhizobium galegae* (Lindström 1989), *R. huautlense* (Wang et al. 1998), *R. alkalisoli* (Lu et al. 2009a) and *R. vignae* (Ren et al. 2011a), which were microsymbionts of *Galega* species, *Sesbania herbacea*, *Caragana intermedia* and multiple legume species, respectively. Based upon the phylogeny of 16S rRNA, these species formed a divergent lineage in the genus *Rhizobium*, and a possible separation of them as an independent genus was discussed (Lindström and Young 2011; Young and Haukka 1996). However, the separation of this lineage from *Rhizobium* was not realised during a long period until the study of Mousavi

Table 3.1 *Ensifer* (*Sinorhizobium*) species and their host spectra

No.	Species	Host legumes or origin of isolation	Type or reference strain	References
1	“*S. abri*”[a]	*Abrus precatorius*	HA-1^{T} (JCM 10305^{T})	Arora (2003)
2	*E. adhaerens*	*Lotus arabicus*, *Sophora tormentosa*	Genovar A ORS 529, RIOP 231	Merabet et al. (2010) and Toma et al. (2017)
3	*E. alkalisoli*	*Sesbania cannabina* in saline-alkaline soils	YIC4027^{T} (=HAMBI 3655^{T}=LMG 29286^{T})	Li et al. (2016c)
4	*E. americanus*	*Acacia* spp. sv. *mediterranense*: *P. vulgaris*	CFNEI 156^{T} (*Acacia*), 23C40 (*Paseolus*)	Mnasri et al. (2012), Toledo et al. (2003) and Wang et al. (2016b)
5	*E. arboris*	*Acacia senegal*, *Prosopis chilensis*	HAMBI 1552^{T} = LMG 14919^{T}	Nick et al. (1999) and Young 2003
6	“*S. chiapanecum*”[a]	*Acaciella angustissima*	ITTG S70^{T}	Rincón-Rosales et al. (2009)
7	*E. collicola*	Soil in South Korea	Mol12^{T} =KCTC 42816^{T} =JCM 31049^{T}	Jang et al. (2017)
8	*E. fredii*	*Glycine max*, *Glycine soyae* sv. *mediterranense*: *P. vulgaris*	USDA 205^{T} = ATCC 35423^{T} = PRC 205^{T}	Chen et al. (1988), Young (2003) and Zurdo-Piñeiro et al. (2009)
9	*E. garamanticus*	*Argyrolobium uniflorum*, *Medicago sativa*	ORS 1400^{T} (=LMG 246925^{T}=CIP 109916^{T})	Merabet et al. (2010)
10	*E. glycinis*	*Glycine*	CCBAU 23380^{T} (=LMG 29231^{T} =HAMBI 3645^{T})	Yan et al. (2016)
11	“*S. indiaense*”[a]	*Sesbania rostrata*	Ra-3^{T} (=JCM10304^{T})	Arora (2003)
12	*E. kostiensis*	*Acacia senegal*, *Prosopis chilensis*	HAMBI 1489^{T} = LMG 15613^{T}	Nick et al. (1999) and Young (2003)
13	*E. kummerowiae*	*Kummerowia stipulacea*	CCBAU 71042^{T} = AS 1.3045^{T}	Wei et al. (2002) and Young (2003)
14	*E. medicae*	*Medicago truncatula*	A 321^{T}	Rome et al. (1996) and Young (2003)
15	*E. meliloti*	*Medicago*, *Melilotus*, *Trigonella* sv. *mediterranense*: *P. vulgaris*	USDA 1002^{T}	de Lajudie et al. (1994), Mnasri et al. (2007) and Young (2003)
16	*E. mexicanus*	*Acacia angustissima*	ITTG R7^{T} (=CFN ER1001^{T}, HAMBI 2910^{T}, DSM18446^{T})	Lloret et al. (2007)
17	*E. morelensis*	Nodules of *Leucaena leucocephala*	Lc04^{T} = LMG 21331^{T} = CFN E1007^{T}	Wang et al. (2002)
18	*E. numidicus*	*Argyrolobium uniflorum*, *Lotus creticus*	ORS 1407^{T} (=LMG 246905^{T}=CIP 109850^{T})	Merabet et al. (2010)
19	*E. psoraleae*	*Psoralea corylifolia*	CCBAU 65732^{T} (=LMG 26835^{T}=HAMBI 3286^{T})	Wang et al. (2013c)

(continued)

Table 3.1 (continued)

No.	Species	Host legumes or origin of isolation	Type or reference strain	References
20	*E. saheli*	*Sesbania* spp.	ORS 609^T = LMG 7837^T	de Lajudie et al. (1994) and Young (2003)
21	*E. sesbaniae*	*Sesbania cannabina*	CCBAU 65729^T (=LMG 26833^T=HAMBI 3287^T)	Wang et al. (2013c)
22	*E. shofinae*	*G. max*	CCBAU 251167^T (=ACCC 19939^T=LMG 29645^T)	Chen et al. (2017)
23	*E. sojae*	*Glycine* spp.	CCBAU 05684^T (= LMG 25493^T= HAMBI 3098^T)	Li et al. (2011b)
24	*E. terangae*	*Acacia* spp., *Sesbania* spp.	ORS 1009^T = LMG 7854^T	de Lajudie et al. (1994) and Young (2003)

[a]These species have not been valid published

et al. (2014), because of the consideration of maintaining the taxonomy stable and waiting for more data or related taxa. The oscillation of phylogenetic position of *R. galegae* in single gene analyses (16S rRNA gene and *dnaK* gene) between the clades of *Agrobacterium* and *Rhizobium* (Eardly et al. 2005; Wang et al. 1998) implied the immaturity of the nomenclature change, as was discussed by Mousavi et al. (2014).

After the sister species (Lu et al. 2009a; Ren et al. 2011a; Wang et al. 1998) and some other rhizobia related to *R. galegae* (Li et al. 2012; Zakhia et al. 2004) were reported for strains from multiple hosts, Mousavi et al. (2014) analysed the phylogenetic relationships of the "*R. galegae* complex" with *Agrobacterium*, *Allorhizobium* and *Rhizobium* by analysis of six concatenated housekeeping loci (*atpD*, *glnA*, *glnII*, *recA*, *rpoB* and *thrC*). In the concatenated MLSA tree, the strains of *R. galegae* complex formed a unique monophyletic group closer to the clade of *Agrobacterium* than to the other *Rhizobium* species. Combined with the previous results and suggestions (Lindström and Young 2011; Martens et al. 2007, 2008; Vinuesa et al. 2005a; Young and Haukka 1996), Mousavi et al. (2014) suggested the separation of the "*R. galegae* complex" from other *Rhizobium* species by describing them a novel genus, *Neorhizobium*, and this description is well supported by the core gene phylogeny (Fig. 3.2).

N. galegae includes two symbiovars (sv.), such that sv. *orientalis* and sv. *officinalis* nodulate with *Galega orientalis* and *G. officinalis*, respectively (Radeva et al. 2001). The symbiosis genes in the type strain HAMBI 540^T are located on megaplasmid (Kaijalainen and Lindström 1989; Novikova and Safronova 1992; Wang et al. 1998). Some strains isolated from *Anthyllis henoniana* (HAMBI 2502), *Argyrolobium uniflorum* (HAMBI 3144, HAMBI 3145, HAMBI 3146), *Astragalus cruciatus* (HAMBI 3141), *Glycyrrhiza uralensis* (HAMBI 3429) and *Medicago* *truncatula* (HABMI 3140) were included in this species. Therefore, the strains in this species are microsymbionts for multiple hosts belonging to tribes of Galegeae, Phaseoleae, Desmodieae, Loteae, Astragaleae, Genisteae and Trifolieae.

N. huautlense was described originally for rhizobia associated with *Sesbania herbaceae* (Wang et al. 1998), which was dominant in flooded soil (Wang and Martínez-Romero 2000). Most of the symbiotic strains in this species harboured a symbiosis plasmid of 400 kbp (Wang et al. 1998). In addition to the symbiotic strains, a plant growth-promoting strain T1-17 was also identified as *N. huautlense*; it could significantly immobilise Cd and Pb in solution and increased the biomass and vitamin C content of hot pepper fruits (Chen et al. 2016a).

N. alkalisoli was proposed for several strains isolated from nodules of *Caragana intermedia* grown in saline-alkaline soils (Lu et al. 2009a). Their *nodC* genes were a unique lineage most similar to those of *Rhizobium loessense* and *Rhizobium mongolense* that nodulate *Astragalus* species (Lu et al. 2009a).

N. vignae is considered as the fourth symbiotic species in the genus. In the description of *Neorhizobium*, the former species *R. vignae*, containing rhizobia from *Astragalus dahuricus*, *Astragalus oxyglottis*, *Vigna radiata* and *Desmodium microphyllum*, was transferred into the species *N. galegae*, since the *R. vignae* strains are intermingled with those defined as *N. galegae*, although they showed low DNA-DNA relatedness and some other phenotypic differences (Wang et al. 1998). However, the later MLSA for describing non-symbiotic species *Neorhizobium tomejilense* isolated from soil in southern Spain (Soenens et al. 2018) showed that both *N. tomejilense* and *R. vignae* are independent species. Our recent genome analyses also demonstrated a clear separation between *R. vignae* and *N. galegae* (Fig. 3.2). Therefore, the species *R. vignae* should be reemended as *Neorhizobium vignae*.

Apart from the four defined species in this genus, there is also an unnamed species, *Neorhizobium* sp., covering several symbiotic stains isolated from *Medicago marina* and *Anthyllis henoniana* (Mousavi et al. 2014).

In addition to *N. tomejilense*, the currently named non-symbiotic *Rhizobium* species, *R. petrolearium* from oil-contaminated soil (Zhang et al. 2012c) and *R. pakistanensis* (Khalid et al. 2015) from nodules of peanut, are also closely related to *N. galegae* in 16S rRNA gene phylogeny and in comparative analysis of genome sequences (Fig. 3.2). So, these two species should be transferred to *Neorhizobium*.

3.3.1.5 Genus *Pararhizobium* Mousavi et al. (2015)

Like *Neorhizobium*, *Pararhizobium* was also described (Mousavi et al. 2015) by transferring several species of *Rhizobium*, including *R. giardinii* (Amarger et al. 1997), *R. herbae* (Ren et al. 2011b), *R. sphaerophysae* (Xu et al. 2011) and *R. helanshanense* (Qin et al. 2012), as well as the species *Blastobacter capsulatus* (Hirsch 1985). This transfer was based upon MLSA of four housekeeping genes (16S rRNA, *atpD*, *recA* and *rpoB*), as well as analyses of cellular fatty acids and phenotypic relationships (Tighe et al. 2000). And it is supported by the comparative analysis of genomes (Fig. 3.2). These species have been reported as *P giardinii*, *P. capsulatum*, *P. herbae* and *P. sphaerophysae*, as well as "*P. helanshanense*". According to the current data, this genus contains symbiotic species *P. giardinii*, *P. herbae*, *P. sphaerophysae* and *P. helanshanense* isolated from different hosts, a saprophytic species *P. capsulatum* isolated from fresh water (Hirsch 1985) and a phytopathogenic (*P. polonicum*) isolated from tumours on stone fruit rootstocks (Puławska et al. 2016).

The species ***P. giardinii*** was proposed for a group of rhizobia associated with bean plants, and it was the most divergent lineage, distantly related to the other species in *Rhizobium* and *Agrobacterium* (Amarger et al. 1997). Symbiosis plasmids were detected in strains of this species, and two symbiovars (sv. *giardinii* and sv. *phaseoli*) were described according to their symbiotic characters (Amarger et al. 1997). Later, this species was also identified as microsymbiont of *Desmanthus illinoensis* (Beyhaut et al. 2006), *Arachis hypogaea* (Ibañez et al. 2008), *Caragana sinica*, *Albizia kalkora* and *Kummerowia stipulacea* (Ren et al. 2011b).

P. herbae (*R. herbae*) was described for rhizobia from *Astragalus membranaceus* and *Oxytropis cashmiriana* (Ren et al. 2011b). ***P. sphaerophysae*** (*R. sphaerophysae*) (Xu et al. 2011) and ***P. helanshanense*** (Qin et al. 2012) were proposed for root nodule bacteria of *Sphaerophysa salsula*.

3.3.1.6 *Rhizobium* (Frank 1889)

Among the rhizobial genera, *Rhizobium* is the one with the longest history and forms the mother for several other genera, since *Bradyrhizobium* (Jordan 1982), *Sinorhizobium* (now *Ensifer*) (Chen et al. 1988; de Lajudie et al. 1994), *Mesorhizobium* (Jarvis et al. 1997), *Neorhizobium* (Mousavi et al. 2014) and *Pararhizobium* (Mousavi et al. 2015) were all proposed for some former *Rhizobium* species, such as *R. japonicum* to *B. japonicum*, *R. meliloti* to *S. meliloti*, *R. loti* to *M. loti*, *R. galegae* to *N. galegae* and *R. giardinii* to *P. giardinii*. In addition, some of the former *Rhizobium* species have been moved into other genera, such as the pathogenic species *R. nepotum* and *R. skierniewicense* have been renamed as *Agrobacterium nepotum* and *Ag. skierniewicense,* and *R. taibaishanense* and *R. oryzae* have been renamed as *Allorhizobium taibaishanense* and *Al. oryzae.*

Currently, more than 90 species are described in this genus (Tables 3.2 and 3.3), in which 40 (Table 3.3) showed phylogenetic relationships closer to *Allorhizobium*, *Agrobacterium*, *Neorhizobium*, *Pararhizobium*, *Pseudorhizobium* and *Shinella* (Fig. 3.2) (Kuzmanović et al. 2018), which were mainly isolated as non-symbiotic endophytic/rhizospheric bacteria or bacteria from different environments (marine, freshwater, soil, reactors and so on) (Table 3.3). Therefore, further taxonomic revisions of this genus are still possible by changing the nomenclature of the divergent species in the genus, such as the strains in Group V (Fig. 3.2, Table 3.3), which might be a novel genus. According to the phylogeny of 16S rRNA genes, a total of 53 species are confidential species of *Rhizobium* (Table 3.2), among them 48 are symbiotic species or species containing symbiotic strains.

Table 3.2 Species in the genus *Rhizobium* Frank 1889 that form a monophyletic group in the phylogeny of 16S rRNA gene (Kuzmanović et al. 2018)

No.	Species	Host plants or resource of isolation	References
1	*R. acidisoli*	*Phaseolus vulgaris*	Román-Ponce et al. (2016)
2	*R. aegyptiacum*	*Trifolium alexandrinum*	Shamseldin et al. (2016)
3	*R. aethiopicum*	*P. vulgaris*	Aserse et al. (2017a)
4	*R. alkalisoli*	*Caragana intermedia*	Lu et al. (2009a)
5	*R. altiplani*	*Mimosa*	Baraúna et al. (2016)
6	*R. anhuiense*	*Lathyrus maritimus*, *Pisum sativum*, *Vicia faba*	Li et al. (2016a)
7	*R. azibense*	*P. vulgaris*	Mnasri et al. (2014)
8	*R. bangladeshense*	*Lens culinaris*, *Trifolium alexandrinum*	Rashid et al. (2015) and Shamseldin et al. (2016)
9	*R. binae*	*Lens culinaris*	Rashid et al. (2015)
10	*R. calliandrae*	*Calliandra grandiflora*	Rincón-Rosales et al. (2013)
11	*R. cauense*	*Kummerowia stipulacea*	Liu et al. (2012)
12	*R. ecuadorense*	*P. vulgaris*	Ribeiro et al. (2015)
13	*R. esperanzae*	*P. vulgaris*	Cordeiro et al. (2017)
14	*R. etli*	*P. vulgaris*	Segovia et al. (1993)
15	*R. fabae*	*Vicia faba*	Tian et al. (2008)
16	*R. favelukesii*	*Medicago sativa*	Torres Tejerizo et al. (2016)
17	*R. freirei*	*P. vulgaris*	Dall'Agnol et al. (2013)
18	*R. gallicum*	*P. vulgaris*	Amarger et al. (1997)
19	*R. grahamii*	*Dalea leporina*, *Leucaena leucocephala*, *Clitoria ternatea*	López-López et al. (2012)
20	*R. hainanense*	*Stylosanthes*, *Centrosema*, *Desmodium*, *Tephrosia*, *Acacia*, *Zomia*, *Macroptilium*, etc.	Chen et al. (1991)
21	‘*R. hidalgonense*’	*P. vulgaris*	Yan et al. (2017c)
22	*R. indigoferae*	*Indigofera* spp.	Wei et al. (2002)
23	*R. jaguaris*	*Calliandra grandiflora*	Rincón-Rosales et al. (2013)
24	*R. laguerreae*	*Vicia faba*	Saïdi et al. (2014)
25	*R. leguminosarum*	*Pisum*, *Trifolium*, *Vicia*, *Lens*, *Lathyrus*, *P. vulgaris*	Jordan (1984)
26	*R. lentis*	*Lens culinaris*	Rashid et al. (2015)
27	*R. leucaenae*	*Leucaena* spp., *Gliricidia sepium*, *P. vulgaris*	Ribeiro et al. (2012)
28	*R. loessense*	*Astragalus*, *Lespedeza*	Wei et al. (2003)
29	*R. lusitanum*	*P. vulgaris*	Valverde et al. (2006)
30	*R. mayense*	*Calliandra grandiflora*	Rincón-Rosales et al. (2013)

(continued)

Table 3.2 (continued)

No.	Species	Host plants or resource of isolation	References
31	*R. mesoamericanum*	*P. vulgaris*, siratro, cowpea, *Mimosa pudica*	López-López et al. (2012)
32	*R. mesosinicum*	*Albizia*, *Kummerowia*, *Dalbergia*	Lin et al. (2009)
33	*R. metallidurans*	*Anthyllis vulneraria*	Grison et al. (2015)
34	*R. miluonense*	*Lespedeza*	Gu et al. (2008)
35	*R. mongolense*	*Medicago ruthenica*	van Berkum et al. (1998)
36	*R. multihospitium*	*Alhagi*, *Astragalus*, *Caragana*, *Halimodendron*, *Lathyrus*, *Lotus*, *Oxytropis*, *Robinia*, *Sophora*, *Vicia*	Han et al. (2008a)
37	*R. oryzae*	*P. vulgaris*, also endophyte for rice	Peng et al. (2008)
38	*R. paranaense*	*P. vulgaris*	Dall'Agnol et al. (2014)
39	*R. phaseoli*	*P. vulgaris*	Ramirez-Bahena et al. (2008)
40	*R. pisi*	*Pisum sativum*	Ramirez-Bahena et al. (2008)
41	*R. rhizogenes*	*P. vulgaris*, also tumour inducers	Velázquez et al. (2005)
42	*R. sophorae*	*Sophora flavescens*	Jiao et al. (2015b)
43	*R. sophoriradicis*	*S. flavescens*	Jiao et al. (2015b)
44	*R. sullae*	*Hedysarum coronarium*	Squartini et al. (2002)
45	*R. tibeticum*	*Trigonella archiducis-nicolai*	Hou et al. (2009)
46	*R. tropici*	*P. vulgaris*, *Leucaena*	Martínez-Romero et al. (1991)
47	*R. tubonense*	*Vigna unguiculata*, *Medicago sativa*	Zhang et al. (2011b)
48	*R. vallis*	*P. vulgaris*	Wang et al. (2011)
No symbiotic species			
49	‘*R. aquaticum*’	Lake water	Máthé et al. (2018)
50	*R. viscosum*	Soil	Flores-Félix et al. (2017)
51	*R. alamii*	Rhizosphere of *Arabidopsis*	Berge et al. (2009)
52	*R. endophyticum*	Seeds of *Phaseolus vulgaris*	López-López et al. (2010)
53	*R. tumorigenes*	Tumour of thornless blackberry	Kuzmanović et al. (2018)

From the data in Table 3.2, it could be observed that *Rhizobium* includes symbiotic, phytopathogenic, endophytic and environmental bacteria, but most of the *Rhizobium* species are symbiotic bacteria. The fact that at least 19 species are able to nodulate *Phaseolus vulgaris* might imply important effects of host geographic distribution on the diversification of rhizobia. It also demonstrates the possible dispersion of nodulation genes among related species by lateral transfer. Vice versa, the nodulation ability of strains in a single species (such as *R. hainanense* or *R. multihospitium*) with multiple legume species in the same geographic regions (Hainan Province or Xinjiang Region) suggests the importance of symbiotic ability for their survival in nature.

Table 3.3 Species currently in *Rhizobium* but phylogenetically related to other genera

No.	Species	Related group		Resource of isolation	References
		Genome[a]	16S rRNA gene		
1	‘*R. albus*’	–	*Allorhizobium*	Lake water	Li et al. (2017)
2	*R. azooxidifex*	–	*Allorhizobium*	Soil	Behrendt et al. (2016)
3	*R. capsici*	–	*Allorhizobium*	Root tumour of *Capsicum*	Lin et al. (2015)
4	*R. halophytocola*[b]	Clade IV	*Allorhizobium*	*Rosa rugosa* root endosphere	Bibi et al. (2012)
5	*R. hedysari*[c]	–	*Allorhizobium*	*Hedysarum multijugum* nodules	Xu et al. (2017)
6	*R. helianthi*	Clade II	*Allorhizobium*	Rhizosphere of sunflower	Wei et al. (2015)
7	*R. lemnae*	–	*Allorhizobium*	Rhizosphere of *Lemna*	Kittiwongwattana and Thawai (2014)
8	*R. oryzicola*	–	*Allorhizobium*	Rhizosphere of rice	Zhang et al. (2015)
9	*R. oryziradicis*	*Allorhizobium*	*Allorhizobium*	Rice root endosphere	Zhao et al. (2017a)
10	*R. rhizoryzae*	Clade II	*Allorhizobium*	Rhizosphere of rice	Zhang et al. (2014c)
11	‘*R. rhizosphaerae*’	–	*Allorhizobium*	Rice rhizosphere	Zhao et al. (2017b)
12	*R. straminoryzae*	–	*Allorhizobium*	Rice straw	Lin et al. (2014)
13	*R. subbaraonis*	Clade V	*Allorhizobium*	Beach sand	Ramana et al. (2013)
14	*R. tarimense*	*Pseudorhizobium*	*Allorhizobium*	Soil	Turdahon et al. (2013)
15	*R. endolithicum*	–	*Allorhizobium*/*Neorhizobium*	Beach sample	Parag et al. (2013)
16	*R. flavum*	*Pseudorhizobium*	*Allorhizobium*/*Neorhizobium*	Soil	Gu et al. (2014)
17	‘*R. halotolerans*’	–	*Allorhizobium*/*Neorhizobium*	Soil	Diange and Lee (2013)
18	*R. pakistanense*	*Neorhizobium*	*Allorhizobium*/*Neorhizobium*	Nodule of peanut	Khalid et al. (2015)
19	*R. aggregatum*	*Ciceribacter*	*Agrobacterium*	Fresh water	Kaur et al. (2011)

20	‘*R. arsenicireducens*’	–	*Agrobacterium*	Groundwater	Mohapatra et al. (2017)
21	*R. daejeonense*	*Ciceribacter*	*Agrobacterium*	Cyanide degrading bioreactor	Quan et al. (2005)
22	*R. ipomoeae*	–	*Agrobacterium*	Water	Sheu et al. (2016)
23	*R. naphthalenivorans*	–	*Agrobacterium*	Sediment	Kaiya et al. (2012)
24	*R. wuzhouense*	*Ciceribacter*	*Agrobacterium*	Roots of *Oryza officinalis*	Yuan et al. (2018)
25	*R. rosettiformans*	*Ciceribacter*	*Agrobacterium*	Groundwater	Kaur et al. (2011)
26	*R. selenitireducens*	*Ciceribacter*	*Agrobacterium*	Bioreactor	Hunter et al. (2007)
27	*R. populi*	Clade III	*Agrobacterium*	Endosphere of *Populus*	Rozahon et al. (2014)
28	*R. cellulosilyticum*	Clade I	*Neorhizobium*	Sawdust of *Populus alba*	García-Fraile et al. (2007)
29	*R. smilacinae*	Clade I	*Neorhizobium*	Leaf of *Smilacina japonica*	Zhang et al. (2014d)
30	*R. soli*	–	*Neorhizobium*	Soil	Yoon et al. (2010)
31	*R. wenxiniae*	–	*Neorhizobium*	Endosphere of maize root	Gao et al. (2017a, b)
32	*R. yantingense*	Clade I	*Neorhizobium*	Weathering rock	Chen et al. (2015)
33	*R. zeae*	–	*Neorhizobium*	Endosphere of maize root	Celador-Lera et al. (2017)
34	*R. gei*	–	*Pararhizobium*	Endosphere of *Geum*	Shi et al. (2016)
35	*R. marinum*	*Pseudorhizobium*	*Pseudorhizobium*	Seawater	Liu et al. (2015)
36	*R. alvei*	–	*Shinella*	Fresh water	Sheu et al. (2015a)
37	*R. arenae*	*Pararhizobium*	Unique group	Sand	Zhang et al. (2017)
38	‘*R. kunmingense*’	–	Unique group	Rhizosphere of *Camptotheca acuminata*	Shen et al. (2010)
39	*R. petrolearium*	*Neorhizobium*	Unique group	Soil	Zhang et al. (2012c)
40	‘*R. phenanthrenilyticum*’	–	Unique group	Petroleum residue treatment system	Wen et al. (2011)
41	*R. puerariae*	–	Unique group	Endosphere of *Pueraria candollei* root nodules	Boonsnongcheep et al. (2016)

[a] See Fig. 3.2

[b] Strains form nodule symbiosis with *Vigna unguiculata* and *Pisum sativum*

[c] This species is nodule symbiont for its host

3.3.1.7 Genus *Shinella* An et al. (2006)

Genus *Shinella* was first described for some environmental bacteria characterised by Gram-negative, aerobic, motile and oxidase- and catalase-positive features (An et al. 2006). Currently, it contains *Shinella granuli* (type strain Ch06^T=KCTC 12237^T=JCM 13254^T), *Shinella zoogloeoides* (type strain ATCC 19623^T=IAM 12669^T=I-16-M^T), *Shinella curvata* (type strain C3^T =KEMB 2255-446^T=JCM 31239^T), *Shinella daejeonensis* (type strain MJ02^T=KCTC 22450^T=JCM 16236^T), *Shinella fusca* (type strain DC-196^T=CCUG 55808^T=LMG 24714^T), *Shinella yambaruensis* (type strain MS4^T=NBRC 102122^T=DSM 18801^T) and *Shinella kummerowiae* (type strain CCBAU 25048^T=JCM 14778^T =LMG 24136^T). In addition, the name '*Shinella alba*' was proposed for a bioflocculant-producing strain xn-1, but no species description was offered (Li et al. 2016b). Most *Shinella* species/strains were studied because of their ability of biodegradation, especially hydrocarbon degradation.

Among these species, only ***Shinella kummerowiae*** was proposed as a symbiotic nitrogen-fixing bacterium (Lin et al. 2008), which was isolated from root nodules of *Kummerowia stipulacea*, but it only formed nodules on *Phaseolus vulgaris*. So, it was suggested that the *Shinella kummerowiae* strain was an endophyte in *Kummerowia* nodules.

3.3.2 Rhizobia in Family Phyllobacteriaceae

In this family, about 50 species within two genera *Mesorhizobium* (46 species) and *Phyllobacterium* (4 species) have been reported as symbiotic bacteria, which nodulate with diverse legumes distributed in various regions.

3.3.2.1 *Mesorhizobium* Jarvis et al. (1997)

The genus name *Mesorhizobium* was first suggested by Wen Xin Chen when the species *Rhizobium tianshanense* was proposed (Chen et al. 1995), based on the phylogenetic separation of *Rhizobium loti*, *Rhizobium huakuii* and *R. tianshanense* from the other *Rhizobium* species in analysis of partial 16S rRNA gene sequences and on their intermediate growth rate compared with the fast-growing rhizobia (*Rhizobium* and *Sinorhizobium*) and slow-growing *Bradyrhizobium.* However, this suggestion was rejected at that moment since more related species and more information were expected. Later, Jarvis et al. (1997) formally suggested this genus name, and several *Rhizobium* species were transferred to this genus. It currently consists of more than 50 rhizobial species, including several names that are not validly published, and 5 non-symbiotic species (Table 3.4). In general, the species in *Mesorhizobium* form a monophyletic group, and close relationships have been observed among them (Zhang et al. 2018b).

Table 3.4 List of current *Mesorhizobium* species and their representative hosts

No.	Species	Type strain	Host or resource of isolation	Reference
1	*M. abyssinicae*	AC98c^T =LMG 26967^T=HAMBI 3306^T	*Acacia abyssinica*	Degefu et al. (2013)
2	*M. acaciae*	RITF741^T=CCBAU 101090^T=JCM 30534^T	*Acacia melanoxylon*	Zhu et al. (2015)
3	*M. albiziae*	CCBAU 61158^T=LMG 23507^T=USDA 4964^T	*Albizia kalkora*	Wang et al. (2007)
4	*M. alhagi*	CCNWXJ12-2^T=ACCC 15461^T=HAMBI 3019^T	*Alhagi sparsifolia*	Chen et al. (2010)
5	*M. amorphae*	ACCC 19665^T	*Amorpha fruticosa*	Wang et al. (1999a)
6	*M. australicum*	WSM2073^T=LMG 24608^T=HAMBI 3006^T	*Biserrula pelecinus*	Nandasena et al. (2009)
7	*M. calcicola*	ICMP 19560^T = LMG 28224^T = HAMBI 3609^T	*Sophora* spp.	De Meyer et al. (2016)
8	*M. camelthorni*	CCNWXJ 40-4^T=HAMBI 3020^T=ACCC 14549^T	*Alhagi sparsifolia*	Chen et al. (2011)
9	*M. caraganae*	CCBAU 11299^T=LMG 24397^T=HAMBI 2990^T	*Caragana* spp.	Guan et al. (2008)
10	*M. cantuariense*	ICMP 19515^T=LMG 28225^T=HAMBI 3604^T	*Sophora microphylla*	De Meyer et al. (2015)
11	*M. chacoense*	LMG 19008^T=CECT 5336^T	*Prosopis alba*	Velázquez et al. (2001)
12	*M. ciceri*	UPM-Ca7^T	*Cicer arietinum*	Nour et al. (1994)
13	*M. delmotii*	STM4623^T=LMG 29640^T=CFBP 8436^T	*Anthyllis vulneraria*	Mohamad et al. (2017)
14	*M. erdmanii*	USDA 3471^T=CECT 8631^T= LMG 17826t2^T	*Lotus corniculatus*	Martínez-Hidalgo et al. (2015)
15	*M. gobiense*	CCBAU 83330^T=LMG 23949^T=HAMBI 2974^T	*Oxytropis glabra*	Han et al. (2008c)
16	*M. hawassense*	AC99b^T=LMG 26968^T=HAMBI 3301^T	*Sesbania sesban*	Degefu et al. (2013)
17	*M. helmanticense*	CSLC115N^T= LMG 29734^T=CECT 9168^T	*Lotus corniculatus*	Marcos-García et al. (2017)
18	*M. huakuii*	CCBAU 2609^T=IFO 15243^T	*Astragalus sinicus*	Chen et al. (1991)
19	*M. japonicum*	MAFF 303099^T=LMG 29417^T=CECT 9101^T	*Lotus* spp.	Martínez-Hidalgo et al. (2016)
20	*M. jarvisii*	ATCC 33669^T=CECT 8632^T=LMG 28313^T	*Lotus corniculatus*	Martínez-Hidalgo et al. (2015)

(continued)

Table 3.4 (continued)

No.	Species	Type strain	Host or resource of isolation	Reference
21	*M. kowhaii*	ICMP 19512^T=LMG 28222^T=HAMBI 3603^T	*Sophora microphylla*	De Meyer et al. (2016)
22	*M. loti*	NZP 2213^T	*Lotus corniculatus*	Jarvis et al. (1982)
23	*M. mediterraneum*	UPM-Ca36^T	*Cicer arietinum*	Nour et al. (1995)
24	*M. metallidurans*	STM 2683^T=CFBP 7147^T=LMG 24485^T	*Anthyllis vulneraria*	Vidal et al. (2009)
25	*M. muleiense*	CCBAU 83963^T=HAMBI 3264^T=CGMCC 1.11022^T	*Cicer arietinum*	Zhang et al. (2012b)
26	*M. newzealandense*	ICMP 19545^T=LMG 28226^T=HAMBI 3607^T	*Sophora prostrata*	De Meyer et al. (2016)
27	*M. olivaresii*	CPS13^T=LMG 29295^T=CECT 9099^T	*Lotus corniculatus*	Lorite et al. (2016)
28	*M. opportunistum*	WSM2075^T=LMG 24607^T=HAMBI 3007^T	*Biserrula pelecinus*	Nandasena et al. (2009)
29	*M. plurifarium*	LMG 11892^T	*Acacia senegal*	de Lajudie et al. (1998b)
30	*M. prunaredense*	STM4891^T=LMG 29641^T=CFBP 8437^T	*Anthyllis vulneraria*	Mohamad et al. (2017)
31	*M. qingshengii*	CCBAU 33460^T=CGMCC 1.12097^T=LMG 26793^T=HAMBI 3277^T	*Astragalus sinicus*	Zheng et al. (2013)
32	*M. robiniae*	CCNWYC 115^T=ACCC 14543^T=HAMBI 3082^T	*Robinia pseudoacacia*	Zhou et al. (2010)
33	*M. sangaii*	SCAU7^T=HAMBI 3318^T=ACCC 13218^T	*Astragalus luteolus*	Zhou et al. (2013)
34	*M. septentrionale*	SDW014^T=CCBAU 11014^T=HAMBI 2582^T	*Astragalus adsurgens*	Gao et al. (2004)
35	*M. shangrilense*	CCBAU 65327^T=LMG 24762^T=HAMBI 3050^T	*Caragana bicolor*	Lu et al. (2009b)
36	*M. shonense*	AC39a^{T}LMG 26966^T=HAMBI 3295^T	*Acacia abyssinica*	Degefu et al. (2013)
37	*M. silamurunense*	CCBAU 01550^T=HAMBI 3029^T=LMG 24822^T	*Astragalus membranaceus*	Zhao et al. (2012)
38	*M. sophorae*	ICMP 19535^T=5LMG 28223^T=HAMBI 3606^T	*Sophora microphylla*	De Meyer et al. (2016)
39	*M. tamadayense*	Ala-3^T=CECT 8040^T=LMG 26736^T	*Anagyris latifolia*	Ramírez-Bahena et al. (2012)
40	*M. tarimense*	CCBAU 83306^T=LMG 24338^T=HAMBI 2973^T	*Lotus frondosus*	Han et al. (2008c)
41	*M. temperatum*	SDW018^T=CCBAU 11018^T=HAMBI 2583^T	*Astragalus adsurgens*	Gao et al. (2004)
42	*M. tianshanense*	A-1BS^T=CCBAU3306^T	*Glycyrrhiza pallidiflora*	Chen et al. (1995)

(continued)

Table 3.4 (continued)

No.	Species	Type strain	Host or resource of isolation	Reference
43	*M. waitakense*	CMP 19523^T=LMG 28227^T=HAMBI 3605^T	*Sophora microphylla*	De Meyer et al. (2016)
44	*M. waimense*	ICMP 19557^T = LMG 28228^T = HAMBI 3608^T	*Sophora longicarinata*	De Meyer et al. (2015)
45	*M. wenxiniae*	WYCCWR 10195^T=HAMBI 3692^T=LMG 30254^T	*Cicer arietinum*	Zhang et al. (2018b)
46	*'M. zhangyense'*	23-3-2^T=CGMCC 1.15528^T=NBRC 112337^T	*Thermopsis lanceolata*	Xu et al. (2018)
Non-symbiont				
47	*'M. hungaricum'*	UASWS1009^T	Sewage sludge	Crovadore et al. (2016)
48	*M. oceanicum*	B7^T=KCTC 42783^T=MCCC 1K02305^T	Deep seawater	Fu et al. (2017)
49	*M. sediminum*	YIM M12096^T=CCTCC AB 2014219^T=KCTC 42205^T	Deep sea sediment	Yuan et al. (2016)
50	*M. soli*	NHI-8^T=KEMB 9005-153^T=KACC 17916^T=JCM 19897^T	Rhizosphere soil of *Robinia pseudoacacia*	Nguyen et al. (2015)
51	*M. thiogangeticum*	SJT^T=LMG 22697^T= MTCC 7001^T	Rhizosphere soil of *Clitoria ternatea*	Ghosh and Roy (2006)

In China, as well as in other regions of the world, it seems that the diversity of *Mesorhizobium* is greater in the temperate regions than in the tropical regions. This phenomenon might be related to the geographic distribution of their host plants. In Table 3.4, except *Acacia* spp., *Biserrula pelecinus*, *Prosopis alba* and *Sesbania sesban* (hosts for *M. abyssinicae*/*M. acaciae*/*M. plurifarium*, *M. australicum*/*M. opportunistum*, *M. chacoense* and *M. hawassense*, respectively), most of the hosts are temperate plants. Therefore, it could be suggested that *Mesorhizobium* species might be more adapted to temperate regions and they have greatly diversified in the temperate regions in association with their host plants.

It has been reported that the *Mesorhizobium* species harbour two gene copies of 16S rRNA, which differs from the *Bradyrhizobium* species (one copy) and *Rhizobium* and *Ensifer* (*Sinorhizobium*) (three copies). In addition, the symbiosis genes are located in symbiosis plasmids in *M. amorphae* (930 kbp) (Wang et al. 1999a) and *M. huakuii* (Hu et al. 2010) or in the chromosome in *M. loti*, *M. mediterraneum*, *M. tianshanense*, etc. (Wang et al. 1999a). These observations were also confirmed by the recent genome sequence analyses.

3.3.2.2 Genus *Phyllobacterium* (ex Knösel 1962) Knösel (1984)

As reviewed by Mantelin et al. (2006), the first *Phyllobacterium* strain was isolated by A. Zimmermann, and *Phyllobacterium* as the genus name was first used in 1962 by D. H. Knösel for the endophytic bacteria in leaf nodules of some tropical plants.

For a long period, only *Phyllobacterium myrsinacearum* and *Phyllobacterium rubiacearum* were described in this genus, based upon the phenotypic characterisation, and *P. rubiacearum* was later merged into the type species *P. myrsinacearum* on the basis of molecular characteristics (Mergaert et al. 2002). The genus description has been emended twice with the description of more species in the genus (Mantelin et al. 2006; Mergaert et al. 2002).

Currently, 11 species have been described in this genus (Table 3.5), including four symbiotic species *P. salinisoli* (León-Barrios et al. 2018), *P. sophorae* (Jiao et al. 2015c), *P. trifolii* (Valverde et al. 2005) and *P. zundukense* (Safronova et al. 2018), which were isolated from the root nodules of *Lotus lancerottensis*, *Sophora flavescens*, *Trifolium pratense* and *Oxytropis triphylla*, respectively. Based upon a study on the nodulation specificity of *Lupinus*-nodulating rhizobia, two symbiovars were differentiated, and a strain *P. sophorae* LmiT21 was denominated as sv. *mediterranense* (Msaddak et al. 2018).

Most of the other *Phyllobacterium* species or isolates were also plant-associated bacteria, especially root or nodule endophytes (Table 3.5). For instance, they were isolated in rhizosphere, rhizoplane, endosphere and root nodules of diverse plants (Mantelin et al. 2006). In addition, the existence of free-living bacteria in soil (*Phyllobacterium catacumbae*) and in water, as well as strains associated with unicellular organisms (Mantelin et al. 2006), demonstrated that the *Phyllobacterium* members are also adapted to other environments. It is clear that the symbiotic species or strains could help their host plant, while many of the plant-associated non-symbiotic *Phyllobacterium* strains are also plant growth-promoting bacteria (PGPB) or potential agents for bioremediation (Mattarozzi et al. 2017, Teng et al. 2017). In addition, their occupation of the endosphere of nodules makes them candidates for novel rhizobia, since they have more opportunities to acquire the symbiosis genes by lateral transfer from the symbionts present inside nodules, as described elsewhere (Andrews et al. 2018).

3.3.3 Symbiotic Bacteria in Bradyrhizobiaceae

This family currently covers more than ten genera distributed in diverse habitats, including the endophytes of root nodules in the genus *Tardiphaga*, animal pathogens in *Afipia*, soil bacteria in *Nitrobacter*, aquatic and phototrophic bacteria in *Rhodopseudomonas*, etc. Among them, only *Bradyrhizobium* contains symbiotic nitrogen-fixing bacteria.

Genus *Bradyrhizobium* was described by Jordan (1982) based upon its phylogenetic divergence from the species within the genus *Rhizobium*. Bacteria in this genus have slow growth rates, with generation times from 8 h to 90 h, and form single colonies with diameter ≤1 mm after incubation on YMA for 7 days or even 2 weeks. They have a single copy of the 16S rRNA gene in the chromosome. The

Table 3.5 *Phyllobacterium* species and their isolation origins

Species	Type strain	Host legume or origin of isolation	References
P. bourgognense[a]	STM 201^T = CFBP 5553^T = LMG 22837^T	Root of *Brassica napus* cv. Eurol	Mantelin et al. (2006)
P. catacumbae	CSC19^T=CECT 5680^T=LMG 22520^T	Roman catacombs	Jurado et al. (2005)
P. brassicacearum[a]	STM 196^T = CFBP 5551^T = LMG 22836^T	Root of *Brassica napus* cv. Eurol	Mantelin et al. (2006)
P. endophyticum	PEPV15^T =LMG 26470^T=CECT 7949^T	Root nodule endophyte of *Phaseolus vulgaris*	Flores-Félix et al. (2013)
P. ifriqiyense[a]	STM 370^T = CFBP 6742^T = LMG 22831^T	Root nodule of *Lathyrus numidicus*	Mantelin et al. (2006)
P. leguminum[a]	ORS 1419^T = CFBP 6745^T = LMG 22833^T	Root nodule of *Astragalus algerianus*	Mantelin et al. (2006)
P. loti	S658^T=LMG 27289^T=CECT 8230^T	Nodule endophyte of *Lotus corniculatus*	Sánchez et al. (2014)
P. myrsinacearum	ATCC 43590^T = DSM 5892^T = JCM 20932^T	Leaf-nodules of Myrsinaceae	Mergaert et al. (2002)
P. salinisoli	LLAN61^T=LMG 30173^T = CECT 9417^T	*Lotus lancerottensis*	León-Barrios et al. (2018)
P. sophorae	CCBAU 03422^T=A-6-3^T=LMG 27899^T=HAMBI 3508^T	*Sophora flavescens*	Jiao et al. (2015b)
P. trifolii	PETP02^T=LMG 22712^T=CECT 7015^T	*Trifolium pratense*	Valverde et al. (2005)
P. zundukense	Tri-48^T=LMG 30371^T=RCAM 03910^T	*Oxytropis triphylla*	Safronova et al. (2018)

[a]Nodulation was not improved

symbiosis genes are normally located in the chromosome as a symbiosis island and rarely in a plasmid (Okazaki et al. 2015; Okubo et al. 2016). In addition, nodulation that is independent of *nod* genes has been reported in several *Bradyrhizobium* strains associated with *Aeschynomene* (Giraud et al. 2007). Therefore, two infection mechanisms exist in *Bradyrhizobium* (Bonaldi et al. 2011), even in the same strain (Gully et al. 2017), depending on the host (*Aeschynomene*) (Chaintreuil et al. 2018). From the evolutionary point of view, it has been proposed that, among current rhizobia, *Bradyrhizobium* is the most similar to the ancestral form of rhizobia (Lloret and Martínez-Romero 2005).

Currently *Bradyrhizobium* consists of 48 symbiotic species and two non-symbiotic species, *Bradyrhizobium betae* and *B. oligotrophicum*, that were isolated from roots of *Beta vulgaris* and rice paddy soil, respectively (Table 3.6). The association of *Bradyrhizobium* strains is more common with tropical plants than with those in the temperate regions.

Table 3.6 Summary information for *Bradyrhizobium* species

No.	Species	Type strain	Host legume or origin of isolation	References
1	*‘B. algeriense‘*	RST89=LMG 27618=CECT 8363	*Retama sphaerocarpa*	Ahnia et al. (2018)
2	*B. americanum*	CMVU44=LMG 29514=CECT 9096	*Centrosema* spp.	Ramírez-Bahena et al. (2016)
3	*‘B. arachidis’*	CCBAU 051107=HAMBI 3281=LMG 26795	*Arachis hypogaea*	Wang et al. (2013a)
4	*B. betae*[a]	PL7HG1=LMG 21987=CECT 5829	Roots of *Beta vulgaris*	Rivas et al. (2004)
5	*‘B. brasilense’*	UFLA03-321=LMG 29353=CBAS645	*Vigna unguiculata*	Martins da Costa et al. (2017)
6	*B. cajani*	AMBPC1010=LMG 29967=CECT 9227	*Cajanus cajan*	Araújo et al. (2017)
7	*B. canariense*	BTA-1=LMG 22265=CFNE 1008	*Chamaecytisus*, *Teline*, *Lupinus*	Vinuesa et al. (2005b)
8	*B. centrosematis*	A9=LMG 29515=CECT 9095	*Centrosema* spp.	Ramírez-Bahena et al. (2016)
9	*‘B. centrolobii‘*	BR 10245=HAMBI 3597	*Centrolobium paraense*	Michel et al. (2017)
10	*B. cytisi*	CTAW11=LMG 25866=CECT 7749	*Cytisus villosus*	Chahboune et al. (2011)
11	*B. daqingense*	CCBAU 15774=LMG 26137=HAMBI 3184	*Glycine max*	Wang et al. (2013b)
12	*B. denitrificans*[b]	LMG 8443=IFAM 1005	*Aeschynomene indica*	Van Berkum et al. (2006)
13	*B. diazoefficiens*	USDA 110=IAM 13628=ACCC 15034	*Glycine max*	Delamuta et al. (2013)
14	*B. elkanii*	USDA 76=MLG 6134	*Glycine max*	Kuykendall et al. (1992)
15	*B. embrapense*	CNPSo 2833=CIAT 2372=BR 2212=LMG 2987	*Desmodium heterocarpon*	Delamuta et al. (2015)
16	*B. erythrophlei*	CCBAU 53325=HAMBI 3614=CGMCC 1.13002	*Erythrophleum fordii*	Yao et al. (2015)
17	*B. ferriligni*	CCBAU 51502=HAMBI 3613=CGMCC 1.13001	*Erythrophleum fordii*	Yao et al. (2015)
18	*B. ganzhouense*	RITF806=CCBAU 101088=JCM 1988	*Acacia melanoxylon*	Lu et al. (2014)
19	*‘B. forestalis’*	INPA54B=LMG 10044	*Inga* sp., *Swartzia* sp.	Martins da Costa et al. (2018)
20	*B. guangdongense*	CCBAU 51649=CGMCC 1.15034=LMG 28620	*Arachis hypogaea*	Li et al. (2015)
21	*B. guangxiense*	CCBAU 53363=CGMCC 1.15035=LMG 28621	*Arachis hypogaea*	Li et al. (2015)

(continued)

Table 3.6 (continued)

No.	Species	Type strain	Host legume or origin of isolation	References
22	*B. huanghuaihaiense*	CCBAU 23303=LMG 26136=HAMBI 3180	*Glycine max*	Zhang et al. (2012d)
23	*B. icense*	LMTR 13=HAMBI 3584=CECT 8509	*Phaseolus lunatus*	Durán et al. (2014b)
24	*B. ingae*	BR 10250=HAMBI 3600	*Inga laurina*	da Silva et al. (2014)
25	*B. iriomotense*[c]	EK05=NBRC 102520=LMG 24129	Tumour-like root of *Entada koshunensis*	Islam et al. (2008)
26	*B. japonicum*	USDA 6=LMG 6138	*Glycine max*	Jordan (1982)
27	*B. jicamae*	PAC68=LMG 24556=CECT 7395	*Pachyrhizus erosus*	Ramírez-Bahena et al. (2009)
28	*B. kavangense*	14-3=DSM 100299=LMG 28790=NTCCM 0012	*Vigna* spp.	Lasse Grönemeyer et al. (2015)
29	*B. lablabi*	CCBAU 23086=LMG 25572=HAMBI 3052	*Lablab purpureus*, *Arachis hypogaea.*	Chang et al. (2011)
30	*B. liaoningense*	2281=USDA 3622=LMG 18230	*Glycine max*	Xu et al. (1995)
31	*'B. macuxiense'*	BR 10303=HAMBI 3602	*Centrolobium paraense*	Michel et al. (2017)
32	*B. manausense*	BR 3351= HAMBI 3596	*Vigna unguiculata*	Silva et al. (2014)
33	*B. mercantei*	SEMIA 6399=CNPSo 1165=LMG 30031	*Deguelia costata*	Helene et al. (2017)
34	*B. namibiense*	5-10=LMG 28789=DSM 100300=NTCCM0017	*Lablab purpureus*	Grönemeyer et al. (2017)
35	*B. neotropicale*	BR 10247=HAMBI 3599	*Centrolobium paraense*	Zilli et al. (2014)
36	*B. oligotrophicum*	LMG 10732=JCM 1494=ATCC43045	Rice paddy soil	Ramírez-Bahena et al. (2013)
37	*B. ottawaense*	OO99=LMG 26739=HAMBI 3284	*Glycine max*	Yu et al. (2014)
38	*B. pachyrhizi*	PAC48=LMG 24246=CECT 7396	*Pachyrhizus erosus*	Ramírez-Bahena et al. (2009)
39	*B. paxllaeri*	LMTR 21=DSM 18454=HAMBI 2911	*Phaseolus lunatus*	Durán et al. (2014b)
40	*B. retamae*	Ro19=LMG 27393=CECT 8261	*Retama* spp.	Guerrouj et al. (2013)
41	*B. rifense*	CTAW71=LMG 26781=CECT 8066	*Cytisus villosus*	Chahboune et al. (2012)
42	*'B. sacchari'*	BR 10280	Sugarcane root, but nodulating *C. cajan*	De Matos et al. (2017)
43	*B. shewense*	ERR11=HAMBI 3532=LMG 30162	*Erythrina brucei*	Aserse et al. (2017b)

(continued)

Table 3.6 (continued)

No.	Species	Type strain	Host legume or origin of isolation	References
44	*B. stylosanthis*	BR 446=CNPSo 2823=HAMBI 3668=H-8	*Stylosanthes* spp.	Delamuta et al. (2016)
45	*B. subterraneum*	58 2-1=DSM 100298=LMG 28792=NTCCM0016	*Vigna subterranea*	Grönemeyer et al. (2015)
46	*B. tropiciagri*	CNPSo 1112=SMS 303=BR 1009=LMG 28867	*Noenotonia wightii*	Delamuta et al. (2015)
47	*'B. valentinum'*	LmjM3=CECT 8364=LMG 2761	*Lupinus mariae-josephae*	Durán et al. (2014a)
48	*B. vignae*	7-2=LMG 28791=DSMZ 100297=NTCCM 0018	*Vigna*, *Arachis*	Gronemeyer et al. (2016)
49	*B. viridifuturi*	SEMIA 690=CNPSo 991=BR 1804=LMG 28866	*Centrosema*, *Acacia*	Helene et al. (2015)
50	*B. yuanmingense*	CCBAU 10071= CFNEB 101	*Lespedeza* spp.	Yao et al. (2002)

[a] This effectively nodulates *Macroptilium atropurpureum*

[b] *This is a combination of the former Blastobacter denitrificans and a group of photosynthetic stem nodule symbionts*

[c] This was originally named *Agromonas oligotrophica*

3.3.4 Rhizobia in Family Methylobacteriaceae

This family was proposed over 10 years ago (Garrity et al. 2005), and most of its species were isolated from environmental samples, with capacities to utilise methane and other one-carbon compounds. However, some strains were also plant-associated bacteria, for instance, *Microvirga ossetica* in root nodules of *Vicia alpestris* (Safronova et al. 2017), *Methylobacterium mesophilicum* in the phytoplane or endosphere of plants (Araújo et al. 2015). In this case, they could improve the growth of the associated plants and have the chance to get nodulation ability by lateral gene transfer. Just recently, some strains belonging to the genus *Microvirga* have been reported to be rhizobia associated with *Lupinus* (Msaddak et al. 2017a, b).

3.3.4.1 Rhizobia in Genus *Methylobacterium* Patt et al. (1976)

Currently, this genus consists of about 50 species, most living in water and soils, with capacity of oxidising methane or methyl compounds, as well as associating with plants. Sy et al. (2001) reported that some symbiotic bacteria isolated from legume species in *Crotalaria* belonged to a unique group in the *Methylobacterium* genus. After further study, these rhizobia were named *Methylobacterium nodulans* (Jourand et al. 2004), and that is the only facultative methylotrophic symbiotic nitrogen-fixing bacterium associated with legume root nodules so far. The strains in this species have been isolated from nodules of some tropical legumes, including

Crotalaria juncea and *Sesbania aculeata* (Madhaiyan et al. 2009), *Lotononis bainesii*, *L. listii* and *L. solitudinis* (Ardley et al. 2009; Jaftha et al. 2002).

The symbiosis genes *nodA* and *nifH* in different strains of this species were closely related to *Bradyrhizobium nodA* (Sy et al. 2001) or to *Azospirillum brasilense nifH* (Jaftha et al. 2002), respectively, suggesting that their symbiosis genes were acquired by lateral gene transfer.

Some very unusual features have been observed in the nodulation process of *M. nodulans* on *Crotalaria podocarpa*. In general, they presented root hair-independent infection without the formation of infection threads, and their bacteroids were spherical shaped, and all the cells were infected in the nitrogen-fixing zone of the multilobed indeterminate nodules. The other unusual features are (1) starch storage within the cells filled by bacteroids in the fixation zone and (2) complete lysis of apical tissues of the nodule where the bacteria could realise their methylotrophic metabolism and become free-living (Renier et al. 2011).

3.3.4.2 Rhizobia in Genus *Microvirga* Kanso and Patel (2003)

The genus *Microvirga* was first described for hot spring isolates, and its description has been emended several times based upon the addition of more species (Safronova et al. 2017). Currently, this genus contains 14 species, most isolated from different environmental samples, like water-, soil- and heavy metal-contaminated environments. Since 2012, four *Microvirga* species, *M. lotononidis* (type strain WSM3557^T =LMG 26455^T =HAMBI 3237^T), *M. lupini* (type strain Lut6^T =LMG 26460^T=HAMBI 3236^T), *M. zambiensis* (type strain WSM3693^T=LMG 26454^T=HAMBI 3238^T) (Ardley *et al.* 2012) and *M. vignae* (type strain BR3299^T=HAMBI 3457^T) (Radl *et al.* 2014), have been reported as nodule-forming nitrogen-fixing bacteria associated with *Listia* (*Lotononis*) *angolensis*, *Lupinus texensis*, *Listia angolensis* and *Vigna unguiculata*, respectively. In addition, *Microvirga ossetica* was reported as a rhizobial species isolated from root nodules of *Vicia alpestris* (Safronova et al. 2017), but it failed to nodulate its host of origin, and the common nodulation genes *nodABC* were absent in the genome, though it harboured the symbiosis genes *nodG*, *nodM*, *nifU*, *fixAB*, *fixJL* and *fixR*. Since there is no evidence that it can nodulate any host legume, it is not currently regarded as a rhizobium (de Lajudie and Young, 2018).

3.3.5 *Rhizobia in Family* Hyphomicrobiaceae

In this family, more than 20 species have been reported, and rhizobia have been found in two genera, *Azorhizobium* and *Devosia.*

3.3.5.1 *Azorhizobium* Dreyfus et al. (1988)

When *Azorhizobium* was first described, the ability to effectively nodulate roots and stems of the legume *Sesbania rostrata* and of free-living nitrogen fixation under microaerobic conditions with supplement of vitamins were reported as the descriptive feature for the genus (Dreyfus et al. 1988). Only the type species *A. caulinodans* (type strain ORS 571^T = LMG 6465^T) was reported in the genus until the woody legume *Sesbania virgata* root-nodulating species *Azorhizobium doebereinerae* (type strain UFLA1-100^T =BR5401^T =LMG9993^T =SEMIA 6401^T) was described (de Souza Moreira et al. 2006). After that, a phytopathogenic species *Azorhizobium oxalatiphilum* (type strain NS12^T=DSM 18749^T=CCM 7897^T) was described for some free-living nitrogen-fixing bacteria isolated from macerated petioles of *Rumex* sp. (Lang et al. 2013). The genus description should, therefore, be emended to include the root nodule bacteria from other legume species as well as non-symbionts.

3.3.5.2 *Devosia* Nakagawa et al. (1996)

Currently, 25 formally described species are listed in this genus, isolated from soil, water, sediments, clinical samples, rhizosphere and so on. So far, rhizobia have only been reported in one species, *D. neptuniae* (LMG 21357^T =CECT 5650^T), which is associated with the aquatic legume *Neptunia natans* (Rivas et al. 2003). Symbiosis plasmids of ca. 170 kb were detected in two strains J1 and J2, and their symbiosis genes *nodD* and *nifH* were phylogenetically related to those of *R. tropici* CIAT 899^T (Rivas et al. 2002).

3.3.6 Rhizobia in Family Brucellaceae

In this family, rhizobia have been only found in the genus *Ochrobactrum,* in which 19 species have been described for bacteria originating from environmental, plant, animal and clinical samples. Some of them were from the rhizosphere or endosphere of plants, such as *O. endophyticum* and *O. oryzae*, while two species are rhizobia.

Ochrobactrum cytisi (type strain ESC1^T =LMG 22713^T=CECT 7172^T) (Zurdo-Piñeiro et al. 2007) was proposed for two strains isolated from root nodules of *Cytisus scoparius*, which harboured the symbiosis genes in a megaplasmid. Their symbiosis genes *nodD* and *nifH* presented high similarities with those of the rhizobia nodulating *Phaseolus*, *Leucaena*, *Trifolium* and *Lupinus*.

Ochrobactrum lupini (type strain: LUP21^T =LMG 20667^T) was described for two fast-growing strains (LUP21^T and LUP23) isolated from nodules of *Lupinus honoratus* (Trujillo et al. 2005). They could reinfect their host plant of origin.

Symbiosis plasmids were detected in these strains, and their *nodD* and *nifH* gene sequences were closely related to the corresponding genes of *R. etli*.

In addition to the species mentioned above, *Ochrobactrum ciceri* (type strain Ca-34^T =DSM 22292^T =CCUG 57879^T) was also described for a strain isolated from a chickpea nodule (Imran et al. 2010), but its symbiosis phenotype was not reported.

3.4 Beta-Rhizobia and Gamma-Rhizobia

Compared with the rhizobia in the class *Alphaproteobacteria*, the symbiotic bacteria in *Betaproteobacteria* and *Gammaproteobacteria* were found much later (Moulin et al. 2001, Shiraishi et al. 2010) and are less diverse, including about 20 species in four genera: *Cupriavidus*, *Paraburkholderia* and *Trinickia* (Estrada-de los Santos et al. 2018) belonging to the family *Burkholderiaceae* and *Herbaspirillum* in the family *Oxalobacteraceae* (Chen et al. 2001; Moulin et al. 2001) (Table 3.7, Fig. 3.3). Both the genera *Paraburkholderia* and *Trinickia* were described for some former *Burkholderia* species (Estrada-de los Santos et al. 2018), and the symbiotic species in *Cupriavidus* was first described as *Ralstonia* (Chen et al. 2003). These findings changed the dogma that only the bacteria within *Alphaproteobacteria* could form nitrogen-fixing nodule symbiosis with legume plants. After that, the terms alpha-rhizobia and beta-rhizobia were used to represent the symbionts in the former two classes (Gyaneshwar et al. 2011). To date, beta-rhizobia were mainly isolated from nodules of some tropical legumes, like *Mimosa* species (Taulé et al. 2012), *Phaseolus vulgaris* (Dall'Agnol et al. 2017), *Podalyria calyptrata* (Lemaire et al. 2016), *Hypocalyptus* spp. and *Virgilia oroboides* (Steenkamp et al. 2015). The gamma-rhizobia in *Pseudomonas* were isolated from the temperate legume tree *Robinia pseudoacacia* (Shiraishi et al. 2010). The sequences of symbiosis genes (*nodA*, *nodC*, *nifH* and *nifHD*) of rhizobia in *Pseudomonas* sp. and *Burkholderia* (*Paraburkholderia*) sp. isolated from *Robinia* were very similar to those of rhizobial species, indicating that they might have acquired these genes by lateral transfer (Shiraishi et al. 2010). An alternative explanation is that these observations were based on mixed cultures of a relatively slow-growing *Mesorhizobium* that had the symbiosis genes and formed the nodules and a very fast-growing *Pseudomonas* or *Burkholderia* that was good at colonising the nodules. Critical additional evidence is needed, including microscopy to show that the bacteroids are labelled with a *Pseudomonas* marker and a genome assembly to demonstrate that the symbiosis genes are integrated into a *Pseudomonas* genome. Until such studies are completed, the existence of gamma-rhizobia remains unproven.

In addition to the species listed in Table 3.7, several species in the mentioned genera were also isolated from the root nodules of legume plants, but their nodulation abilities were not confirmed, such as *Burkholderia aspalathi* isolated from *Aspalathus abietina* (Mavengere et al. 2014) and *Herbaspirillum robiniae* isolated from *Robinia pseudoacacia* (Fan et al. 2018). Platero et al. (2016) reported some

Table 3.7 Symbiotic bacterial species currently defined as beta-rhizobia (Estrada-de los Santos et al. 2018)

No.	Genus and species	Type strain	Host plant	References
Herbaspirillum				
1	*H. lusitanum*	P6-12 =LMG 21710 =CECT 5661	*Phaseolus vulgaris*	Valverde et al. (2003)
Cupriavidus				
2	*C. taiwanensis*	LMG 19424 =CCUG 44338	*Mimosa putida*	Chen et al. (2001) and Vandamme and Coenye (2004)
	C. necator	UYMM14A[R]	*Mimosa*, *Parapiptadenia*	Platero et al. (2016)
Paraburkholderia				
3	*P. caballeronis*	TNe-841 = LMG 26416 = CIP 110324	*Phaseolus vulgaris*	Martínez-Aguilar et al. (2013)
4	*P. caribensis*	TJ182[R]	*Mimosa* sp.	Chen et al. (2005)
5	*P. diazotrophica*	JPY461=LMG 26031=KCTC 23308	*Mimosa* spp.	Sheu et al. (2013)
6	*P. dipogonis*[a]	ICMP 19430 =LMG 28415 =HAMBI 3637	*Dipogon lignosus*	Sheu et al. (2015b)
7	*P. dilworthii*	WSM3556 =LMG 27173=HAMBI 3353	*Lebeckia ambigua*	De Meyer (2014)
8	*P. fynbosensis*	WSM4178 =LMG 27177 =HAMBI 3356	*Lebeckia ambigua*	De Meyer et al. (2018)
9	*P. kirstenboschensis*	Kb15=LMG 28727=SARC 695	*Hypocalyptus* spp., *Virgilia oroboides*	Dobritsa and Samadpour (2016) and Steenkamp et al. (2015)
10	*P. mimosarum*	PAS44 =LMG 23256 =BCRC 17516	*Mimosa* spp.	Chen et al. (2006) and Sawana et al. (2014)
11	*P. nodosa*	Br3437 =LMG 23741 =BCRC 17575	*Mimosa bimucronata*, *Mimosa scabrella*	Chen et al. (2007) and Sawana et al. (2014)
12	*P. phenoliruptrix*	BR3459a[R]	*Mimosa flocculosa*	de Oliveira Cunha et al. (2012)
13	*P. rhynchosiae*	WSM3937=LMG 27174=HAMBI 3354	*Rhynchosia ferulifolia*	De Meyer (2013b)
14	*P. phymatum*	LMG 21445	*Mimosa* spp.	Sawana et al. (2014) and Vandamme et al. (2002)
15	*P. piptadeniae*	STM 7183 =DSM 101189 =LMG 29163	*Piptadenia gonoacantha*	Bournaud et al. (2017)
16	*P. ribeironis*	STM 7296 =DSM 101188 =LMG 29351	*Piptadenia gonoacantha*	Bournaud et al. (2017)
17	*P. sabiae*	Br3407 =LMG 24235 =BCRC 17587	*Mimosa caesalpiniifolia*	Sawana et al. (2014)

(continued)

Table 3.7 (continued)

No.	Genus and species	Type strain	Host plant	References
18	*P. sprentiae*	WSM5005 =LMG 27175 =HAMBI 3357	*Lebeckia ambigua*	De Meyer (2013a)
19	*P. tuberum*	LMG 21444	*Mimosa* spp.	Sawana et al. (2014) and Vandamme et al. (2002)
20	*P. xenovorans*	LB 400 = LMG 21463 =CCUG 46959	PCB-contaminated soil[b]	Goris et al. (2004)
Trinickia				
21	*T. symbiotica*	JPY-345 =LMG 26032 =BCRC 80258	*Mimosa* spp.	Sheu et al. (2012)

R reference strain was given here since the type strain of the species is non-symbiotic bacterium

[a] This species is not included in Estrada-de los Santos et al. (2018)

[b] Nodulation has not been reported, but symbiosis genes are detected (Estrada-de los Santos et al. 2018)

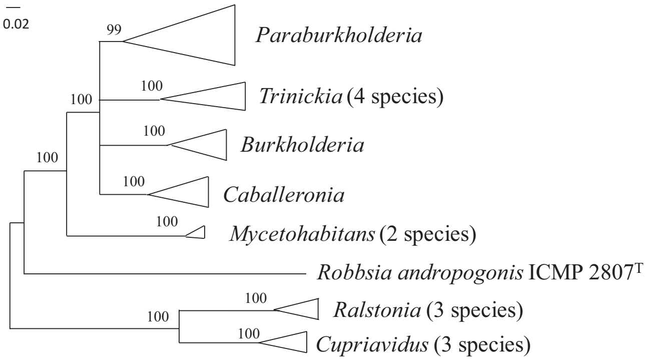

Fig. 3.3 Simplified phylogeny constructed with the maximum likelihood (ML) based on the amino acid sequences of 106 concatenated genes showing the relationships of the β-rhizobia. The scale bar represents number of changes per site. The numbers at nodes are bootstrap values estimated with 1000 pseudo-replicates. Symbiotic species are found in *Paraburkholderia*, *Trinickia* and *Cupriavidus*. Deduced from Estrada-de los Santos et al. (2018)

symbiotic strains belonging to the defined species *C. necator* and to a novel genospecies isolated from *Mimosa ramulosa*, *M. magentea* and *M. reptans*, which formed unique phylogenetic group related to *Cupriavidus basilensis*, *C. numazuensis* and *C. pinatubonensis*. So, some new symbiotic species will be defined with further study of more isolates and more host plants.

It is interesting to note that the symbiotic *Paraburkholderia* species have *nif* genes similar to those of their free-living relatives but quite different from those of other symbiotic bacteria in α-rhizobia and *Herbaspirillum* (β-rhizobia) (Estrada-de los Santos et al. 2018). In *nodA* gene phylogeny, all the strains isolated from the

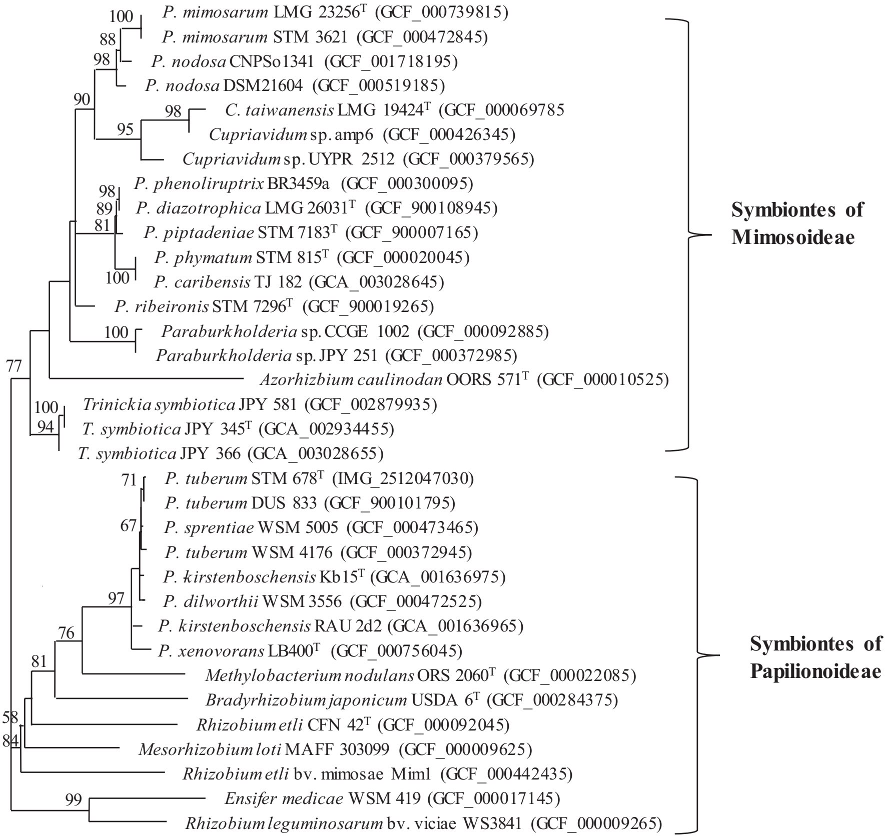

Fig. 3.4 Phylogenetic tree of *nodA* genes constructed with the method of maximum likelihood showing the differences between microsymbionts of Mimosoideae and Papilionoideae. Bootstrap values (based on 100 nonparametric bootstrap calculations) greater than 50% are indicated at the nodes. (Deduced from Estrada-de los Santos et al. (2018)

papilionoid legumes are closely related to the α-rhizobia and *Herbaspirillum* (β-rhizobia), and the strains isolated from mimosoid legumes form a unique group (Fig. 3.4). These results demonstrate that the *nif* and *nod* genes in mimosoid-nodulating *Paraburkholderia* have evolved independently, while the *nif* and *nod* genes in papilionoid-nodulating *Paraburkholderia* have different evolutionary history and their *nod* genes may have acquired by horizontal gene transfer (Estrada-de los Santos et al. 2018).

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