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Preface

Traditionally, the term ‘rhizobia’ refers to a special group of soil bacteria that can induce root and/or stem nodules on legume plants and reduce dinitrogen (N_2) into ammonia (NH_3) inside the nodules. The nodule symbiosis of rhizobia-leguminous plants is the most efficient biological nitrogen-fixing system on the planet. Based upon their important ecological and economic impacts, rhizobia have been studied for about one and half centuries, and the legume-rhizobium symbiosis has been applied for improving the growth of plants since the ancient time, even before rhizobia were recognised. According to the ancient literature, the legume-rhizobium symbiosis was applied 2000 years ago in China for maintaining the soil fertility by rotation or intercropping of the legumes (soybean, green gram and red mung bean) with the grain crops (wheat, rice or millet), although the mechanism (biological nitrogen fixation) was unknown. Currently, it is known that rhizobia can not only fix nitrogen in the symbiosis with legumes, but can also colonise many plant species other than their symbiotic hosts as endophytes to improve the growth and productivity of the plants by different mechanisms, like solubilizing mineral compounds, production of phytohormones, etc. Therefore, rhizobia are one of the most important bioresources and a good model for investigation of the ecological interactions among the bacteria, plants and environmental factors.

As with many other scientific researches, the development of study on rhizobia was dependent on the production requirements and was closely related to the developments of other scientific disciplines. To understand the mechanism by which legumes could improve the soil fertility, the early scientists in the 1800s performed studies and noticed the relation between root nodules on the legume crops and their fertility impact. Due to the development of microbial culturing, Beijerink (1888) isolated the first nodule bacterium from pea nodules. Related to the petroleum crisis erupted in the 1970’s, biological nitrogen fixation was a hot point for investigation, and many studies have investigated the diversity, genetics, physiochemical properties, ecology and application of rhizobia. By the previous studies accompanying the application of novel methodology in different eras, the mechanisms of rhizobial specificity for host legumes (nodulation genes and their expression/regulation) and

of their capability for nitrogen fixation (nitrogen-fixing genes and their expression/regulation, structure and activity of nitrogenase) have been clearly described at the molecular level. Also, knowledge about the diversity and evolution of rhizobia has been greatly improved; for example, more than 100 symbiotic rhizobial species have been described in about 15 genera belonging to *Alphaproteobacteria* and *Betaproteobacteria* classes.

The previous studies on rhizobia have answered many scientific questions but also generated some new ones. Furthermore, the current ecological problems, such as the global climate change and environmental contamination caused by the excessive application of chemical fertilisers and mining activities, require more investigations into the ecology and application of rhizobia. During the past 40 years, our research groups have worked continuously on rhizobial diversity, ecology and application. In this book, we would like to make a resume of our work and share all of our experience and ideas for future study on rhizobia. To make this happen, I am happy to have my former PhD students and colleagues Prof. En Tao Wang (Chaps. 1, 2, 3 and 11), Assoc. Prof. Wen Feng Chen (Chaps. 5, 7, 8, 9 and 10), and Prof. Chang Fu Tian (Chaps. 4 and 6) to be involved in preparing this book. I would also like to thank my longtime friend Prof. J. Peter W. Young for his contribution to this book (Chaps. 4, 6 and book review).

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1.1.1 Rhizobia and Biological Nitrogen Fixation

Definition and Importance The term of rhizobia, or root nodule bacteria, is the common collective name for diverse symbiotic nitrogen-fixing soil bacteria that can induce root and/or stem nodules on the legume plants (Fig. 1.1). Although this common name was derived from the genus name *Rhizobium* (Frank 1889), bacteria distributed in diverse species within different genera, families and classes have been confirmed as microsymbionts of nodules of distinct legume species; therefore, the word "rhizobia" has lost its taxonomic meaning but refers to the symbiotic nitrogen-fixing bacteria associated with nodules of legumes. Inside the nodules, the cells of these bacteria differentiate into bacteroids, which are polymorphic cells with modified cell walls, enclosed by plant cell membrane to form a structure called the symbiosome. This structure provides the conditions that allow the function of nitrogenase, the enzyme that reduces elemental nitrogen to ammonia in the bacteroids. From this association, both the legumes and the rhizobia can benefit: the host plants supply the rhizobia with C4-dicarboxylic acids as carbon and energy source, and the rhizobia offer the plants nitrogen nutrients produced by reducing atmospheric nitrogen and incorporating it into amino acids such as alanine (Poole and Allaway 2000).

It is believed that nitrogen fixation is one of the most important biological processes on Earth, ranking closely behind photosynthesis (Sylvia et al. 2005). Unlike photosynthesis, the function of biological nitrogen fixation (BNF) is restricted to a small number of prokaryotes. Although nitrogen makes up about 78% of the atmosphere, this gaseous nitrogen (N_2) is chemically inert due to its triple bonds, and its reduction is an energy-expensive procedure. Therefore, nitrogen is always the most limited nutrient in soil for supporting plant growth. In addition to the Haber-Bosch process used in industrial production of fertiliser, three natural mechanisms of N_2

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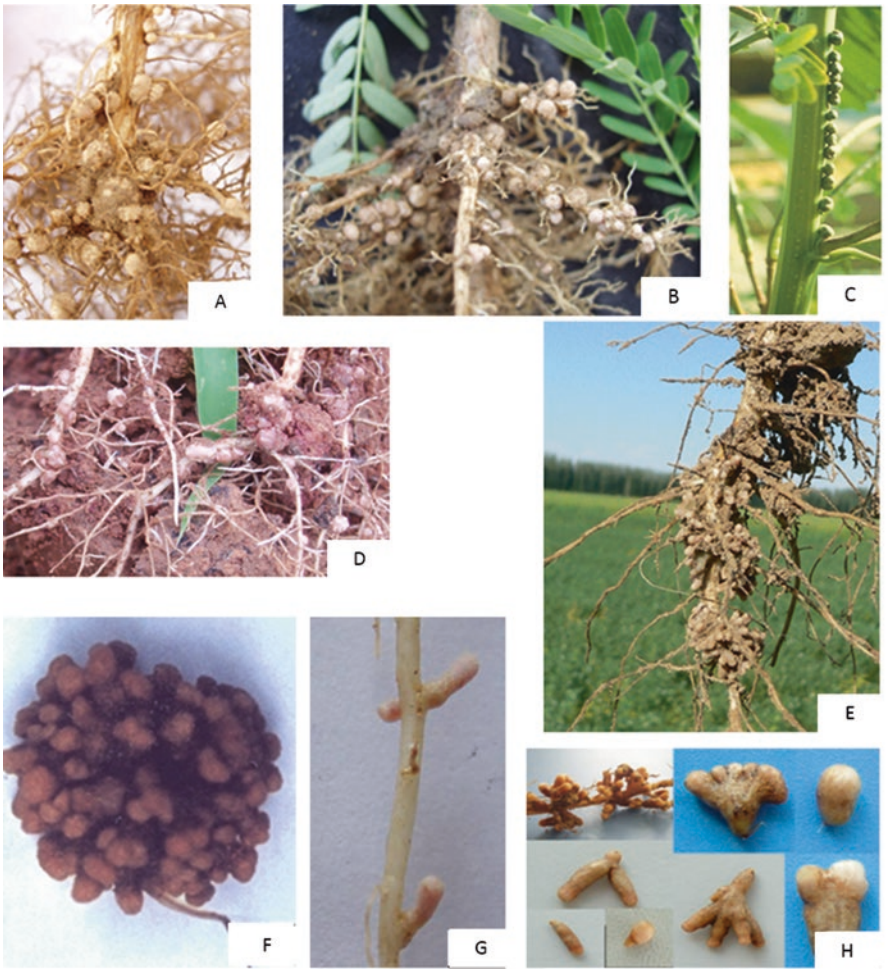

Fig. 1.1 Root and stem nodules of legumes induced by rhizobia (From Chen and Wang 2014). Determined root-nodules (A, B, D) of *Glycine max* (by Zhang YZ), *Sesbania cannabiana* (by Chen WF), and *Vigna radiata* (by Chen WF); Stem-nodules (C) of *Sesbania rostrata* (Masson-Boivin et al. 2009); Indeterminated root-nodules (E-H) of *Cicer arietinum* (by Chen WF & Zhang JJ), *Robinia pseudoacacia* (by Wang ET), *Medicago sativa* (by Chen WF) and *Caragana* spp. (by Yan XR)

fixation are documented, including lightning strikes, combustion and the biological procedure realised by some prokaryotes (bacteria and archaea).

In combustion processes (particularly in internal combustion engines), some nitrogen oxides are produced as nitric oxide (NO) at concentrations of 100–1000 mg/m^3, NO_2 at 10–100 mg/m^3 and N_2O at 1–10 mg/m^3 or less than 5 ppm in the flue gas (Linak et al. 1990). Lightning strikes produce nitrogen oxides, and it has been estimated that lightning could fix about 14.4×10^6 tonnes of NO_2 per year (Hill et al. 1980). This NO_2 will precipitate to soil with rain, where it can be further converted into nitrate (NO_3^-) by microorganisms (Dunfield and Knowles 1997) and then used by plants.

Among the three natural processes, BNF contributed the largest proportion of annual nitrogen input in the planet. Since it functions in conditions without high temperature and high pressure, it has attracted a lot of attention in the last century. In general, BNF can be performed by free-living diazotrophs, by symbiotic diazotrophic bacteria and by endophytic diazotrophic bacteria (Sylvia et al. 2005). The global amount of N fixed by the diazotrophs in both agricultural and natural systems is about 122 Tg N per year as estimated by Burris (1980), which is comparable with the scale of the global carbon (C) cycle. The most effective N_2-fixing system is the symbiotic association between legumes and rhizobia: with 2.95 Tg and 18.5 Tg fixed N per year for the pulses and the oilseed legumes, respectively (Herridge et al. 2008). In relation to their high efficiency in BNF, rhizobia have been studied extensively for about one and a half centuries. According to the ancient Chinese literature, increase in soil fertility by cultivation of legumes was noticed 2000 years ago, and rotation or intercropping of legumes (soybean, green bean, *Vigna angularis*) with grain crops (wheat, rice or millet) was applied traditionally in agricultural practice to enhance grain production, although the mechanism was unknown. More recently, it has been shown that rhizobia not only form symbiosis with their host legumes but also could be endophytes to improve the growth and productivity of other plants by various mechanisms, including solubilising mineral compounds, producing phytohormones, etc. (Gopalakrishnan et al. 2015). Therefore, rhizobia are one of the most important bioresources and a good model for investigation of the ecological interactions among bacteria, plants and environmental factors.

Brief History The history of study on rhizobia has been summarised in several reviews (Fred 1932; Long 2001; Ramírez-Bahena et al. 2016; Sessitsch et al. 2002; Willems 2006). Studies on rhizobia can be divided into several stages depending on the knowledge and the development of biotechnology.

1. **Evidencing the relationship between rhizobia and nodulation-nitrogen fixation of legumes**. Although the beneficial effects of legumes on the soil fertility were recorded in ancient Chinese literature 2000 years ago, root nodules were first reported in 1675 by Marcello Malpighi (Leigh 2004) for common bean and pea plants. Later, the relationship between nodules of legumes and nitrogen fixation was demonstrated by Hellriegel and Wilfarth in 1888 (Evans and Russell 1971), who then linked the nodulation ability of legumes to the soil microorganisms by comparing the growth of plants in sterilised and natural soils. In the same year, Beijerinck (Fred et al. 1932) isolated the first nodule bacteria from pea root nodules, using a medium prepared with plant leaf, aspartic acid, sucrose and gelatine. He named his isolates *Bacillus radicicola.* One year later, Prazmowski (Fred et al. 1932) successfully obtained nodules by inoculating pure culture isolated from legume nodules and first used the name *Rhizobium* for the nodule-inducing bacteria. In 1889, Frank suggested using *Rhizobium* as the genus name to accommodate all the nodule-inducing bacteria (Fred et al. 1932).
2. **Definition of cross-nodulation groups**. After isolation of bacteria from root nodules, specificity between the rhizobial strains and their host legumes was found,

and a lot of cross-nodulation tests were performed. Based upon these results, many cross-nodulation groups were defined for strains that could nodulate with the same legumes species, and six main cross-nodulation groups were described as species within the genus *Rhizobium* (Fred et al. 1932). This taxonomic system was used for about 50 years, although many cases of nodulation across the boundaries of species (cross-nodulation groups) were recorded (Wilson 1944).

3. **Stage of biochemistry and physiology.** This stage began in the 1950s and was very active until the 1990s, accompanying the development of techniques in bacteriology, biochemistry, genetics, computers and electron microscopy. During this period, the energy (petroleum) crisis in the 1970s stimulated studies on BNF, including diversity, biochemistry and genetics of rhizobia, such as the structure and function of nitrogenase (Smith 1977). Standard methods for isolation, identification and application of rhizobia were established (Vincent 1970) in this stage, including the acetylene reduction method used for evaluation of nitrogen-fixing efficiency of root nodules (Shah et al. 1975). In this period, the computer-based grouping analysis called numerical taxonomy and some techniques of molecular biology such as DNA-DNA hybridization and 16S rRNA-DNA hybridization were introduced in rhizobial taxonomy, which resulted the modification of the taxonomic system of rhizobia (Jordan 1982), in which the cross-nodulation group as a basis for species definition was abandoned and the slow-growing, alkaline-producing rhizobia were designed as an independent genus, *Bradyrhizobium*, separated from the fast-growing, acid-producing rhizobia in the genus *Rhizobium*.
4. **Molecular stage.** This stage started in the 1980s and has overlapped the subsequent genome stage until the present day. In response to the effects of the energy crisis in the 1970s, biological nitrogen fixation was a focus for investigation, and the diversity, genetics, physiochemical properties, ecology and application of rhizobia developed rapidly since then (see reviews of Long 1989; Young and Haukka 1996; Zahran 1999). Nodulation genes of rhizobia were found and sequenced, meanwhile the Nod factors were detected, and their structures and functions were revealed in this period, which opened the gate for investigating the molecular interactions between rhizobia and their host legumes (Geurts and Bisseling 2002). In addition, the symbiosis plasmid and symbiosis island, as well as their lateral transfer among different species and genera, were discovered in this period (Schofield et al. 1987; Sullivan et al. 1995; Sullivan and Ronson 1998). During these years, the systematics and diversity of rhizobia were greatly improved as a result of the development of taxonomic techniques and theory in bacteriology and the participation of more scientists. Briefly, the taxonomic techniques were improved following the route from numerical taxonomy based upon the large number of biochemical and biophysical data, DNA-DNA hybridization, 16S rRNA sequence analysis and later polyphasic taxonomy including all the previous analyses plus the housekeeping gene or multilocus sequence analysis (MLSA) (de Lajudie et al. 1994; Martens et al. 2008). Also, different research groups in various countries, like the laboratories in Belgium (Laboratorium voor Microbiologie, Universiteit Gent), China (*Rhizobium* Research Center, China Agricultural University), etc., did a lot of work to investigate the diversity of rhizobia.

5. **Genomic stage.** This period started at the end of last century and continues today. With the comparative study on genomics of rhizobial strains, it was found that only 30% of the genes were included as core genome and the majority of genes were responsible for the adaptation of rhizobia to the environments (Young et al. 2006). This finding could explain why different *Bradyrhizobium* and *Sinorhizobium* species harbouring various nodulation genotypes were dominant in different soil types in association with soybean (Han et al. 2009; Zhang et al. 2011). In addition, the phylogenetic relationships among them were further clarified, and the previously described genera *Rhizobium*, *Allorhizobium* and *Agrobacterium* were reclassified, while novel genera *Pararhizobium* and *Neorhizobium* were described (Mousavi et al. 2014, 2015). Genome comparisons are increasingly forming the main basis for taxonomy, replacing the earlier polyphasic approach (de Lajudie et al. 2019).

Diversity To date, all the described rhizobia are Gram-negative, non-spore-forming rods, moving with one or more polar or peritrichous flagella. Taxonomically, they are distributed in about 100 species of 15 genera belonging to the classes *Alphaproteobacteria* and *Betaproteobacteria*. Taxonomically, symbiotic bacteria and non-symbiotic (endophytic, saprophytic and pathogenetic) bacteria are intermingled in the classes, orders, families, genera and even in the same species. For example, the symbiotic species *Rhizobium leguminosarum* (Mousavi et al. 2014), the endophytic species *R. endophyticum* (López-López et al. 2010), the phytopathogenic species *Rhizobium rhizogenes* (previous *Agrobacterium rhizogenes*) (Mousavi et al. 2014) and the saprophytic species *Rhizobium halotolerans* (Diange and Lee 2013) are included in the genus *Rhizobium*. In another case, saprophytic, endophytic, phytopathogenic and symbiotic strains have been reported in the single species *Agrobacterium radiobacter* (Saïdi et al. 2011), even in a single strain (Velázquez et al. 2005).

Physiology and Biochemistry Despite their phylogenetic/taxonomic diversity, all the rhizobia are mesophilic aerobic bacteria with optimal temperature around 28 °C, and their pH range, salinity range, carbon source spectrum and nitrogen source spectrum varied according to the species, even strains. All of them can fix nitrogen in the symbiosis via the activity of the nitrogenase complex. It is known that the Mg^{2+}-dependent nitrogenase complex is composed of nitrogenase (EC 1.19.6.1) and nitrogenase reductase (EC 1.18.6.1). As in other (free-living) nitrogen-fixing bacteria, the nitrogenase of rhizobia comprises two subunits with Fe-Mo cofactor and two subunits with Fe (see review of Hoffman et al. 2014). The nitrogenase reductase contains two equal subunits with Fe (Hoffman et al. 2014). These enzymes carry on the nitrogen reduction as presented in the following formula:

$$N_2 + 16ATP + 8e^- + 8H^+ \xrightarrow{\text{Nitrogenase}} 2NH_3 + H_2 + 16ADP + 16Pi$$

From this formula, it can be seen that biological nitrogen fixation consumes a large amount of ATP and hydrogen is a characteristic byproduct of this procedure.

In some rhizobial strains (hup$^+$), hydrogen produced in nitrogen fixation can be recycled by hydrogenase (Baginsky et al. 2002; Evans et al. 1987).

Biogeography Rhizobia have been isolated from host legumes in diverse environments. Some rhizobia cause nodulation on the roots of legumes grown in acid soils with pH 4.5 (Anyango et al. 1995; Vargas and Graham 1988; Wood and Shepherd 1987) or in saline-alkaline soils with pH 9.0 (Appunu et al. 2008). Some of them form root/stem nodules with aquatic legumes, like *Sesbania* (Dreyfus et al. 1988; Wang et al. 1998), *Neptunia natans* (Rivas et al. 2002; Subba-Rao et al. 1995) and *Aeschynomene* (Molouba et al. 1999). They are also associated with legumes grown in soils highly contaminated by heavy metals (Abd-Alla et al. 2012; Grison et al. 2015). Corresponding to their wide geographic distribution, rhizobial strains present varied resistance to antibiotics, heavy metals and other toxic chemicals and can use different ranges of carbon and nitrogen sources. Some of them can grow by nitrate respiration under anoxic conditions, like *Neorhizobium huautlense* (Wang et al. 1998).

Life Cycle Despite the taxonomic differences, the life cycle of all rhizobial species includes different states: saprophytic (in soil), symbiotic (in nodule of the host legume) and endophytic (in roots and stems of plants other than its host) (Fig. 1.2). Many data have revealed that symbiotic bacteria can also live inside the tissues of plants other than their hosts, for example, *R. leguminosarum* in rice (Yanni et al. 1997), *R. etli* in maize (Gutiérrez-Zamora and Martínez-Romero 2001), *R. endophyticum* in wild rice (Peng et al. 2008) and several rhizobial species in tobacco tissues (Ji et al. 2010).

Phylogeny and Genomics The mixing of symbiotic and non-symbiotic bacteria in the taxa at different levels has been supported by their phylogenetic relationships (Fig. 1.3). According to the results of genetic studies, the symbiotic or phytopatho-

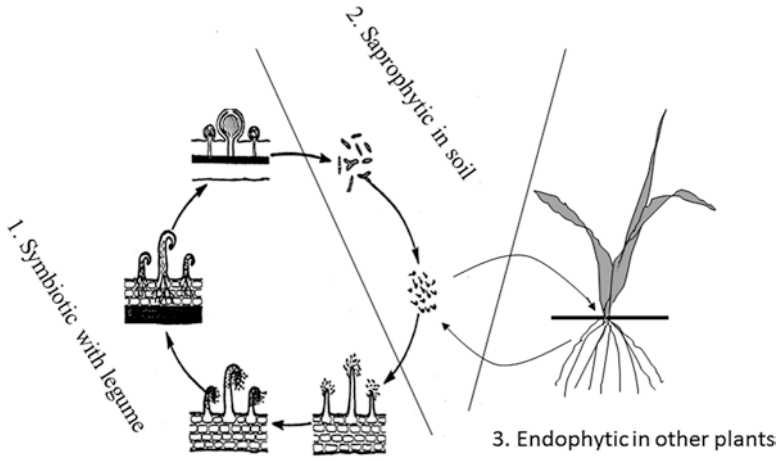

Fig. 1.2 The three living stages of rhizobia (modified from Hardoim et al. 2008)

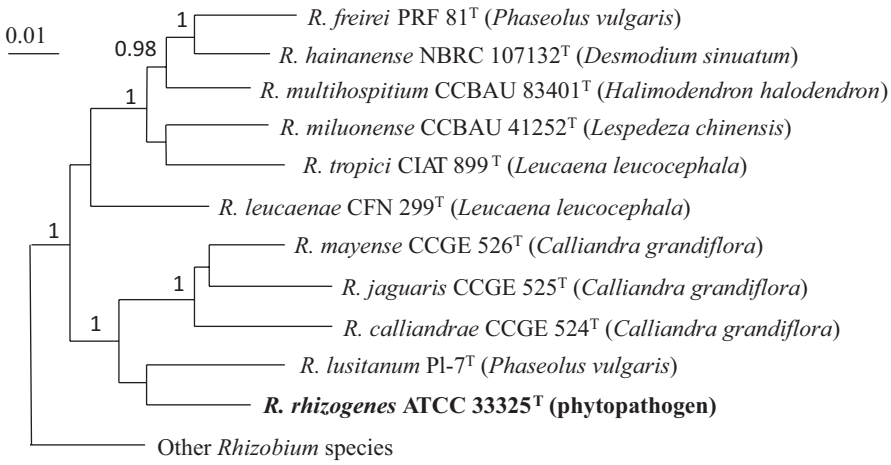

Fig. 1.3 Phylogenetic tree of the concatenated genes 16S *rRNA*, *atpD*, *recA* and rpoB genes showing the close relationships between the symbiotic and phytopathogen rhizobial species. The tree was constructed based on a Bayesian inference analysis. The posterior probabilities ≥0.95 are shown at the corresponding nodes. The type strains are marked by a superscript "T" at the end of the strain code. Scale bar represents 1% substitutions of nucleotides (simplified from Mousavi et al. 2015)

genic abilities of these bacteria are determined by the nodule- or tumour-inducing (*nod* or *vir*) genes in plasmids or gene islands; both are genomic elements transferable by conjugation among closely related bacteria. Therefore, the strains harbouring symbiosis genes are able to induce nodulation on legume hosts; the strains harbouring the Ti plasmid are able to induce tumours on the plants, and both the symbiotic and phytopathogenic bacterial strains could lose their symbiotic or phytopathogenic ability by curing the plasmids/gene islands, thereby becoming saprophytic/endophytic bacteria. In special cases, a strain even could harbour both the symbiotic and phytopathogenic plasmids that make it able to cause nitrogen-fixing nodules with a legume and induce tumours on another plant (Velázquez et al. 2005). In addition, some *A. tumefaciens* (*radiobacter*) strains (B6, C58, NT1) are also free-living nitrogen-fixing bacteria (Kanvinde and Sastry 1990) or able to form symbiosis with some legumes (Wang et al. 2016).

Corresponding to their complex life cycle (habitats), the symbiotic rhizobia have chromosomes larger than those of most other bacteria, because they need more genes to adapt to different habitats. Among the genome of rhizobia, most genes (about 70%) are related to the adaption (accessory genes), and only about 30% are core genes (Young 2006). Among the accessory genes in rhizobia, the symbiosis genes are responsible for the symbiosis, including the nodulation genes and nitrogen-fixing genes.

In conclusion, rhizobia include diverse bacteria in the *Alphaproteobacteria* and *Betaproteobacteria* that can induce nodulation on roots and/or stems of some leguminous plants.

1.1.2 Symbiosis Between Rhizobia and Legumes

1.1.2.1 Characteristics of the Symbiosis Between Rhizobia and Legumes

Definition of Symbiosis Symbiosis is a universal phenomenon in nature and played a key role in the evolution of organisms. Although different definitions of symbiosis exist, they can be classified into two categories: one refers to all kinds of close associations between two distinct organisms, in which negative or positive effects can be obtained by one or both the participants; another is a more restrictive definition that refers only the mutualistic associations between two organisms, in which both the symbionts obtain benefits (see review of Martin and Schwab 2013). In addition, the microorganisms living inside the plant tissues can be classified into three categories according to their effects on the plants: microsymbionts that form symbiosis with plants and benefit the hosts, phytopathogens that cause damage or negative effects on the hosts and endophytes that living inside the plant tissues without visible damage or negative effects on the plants. In this book, we use the restrictive definition, and the association between rhizobia and their host legumes is a kind of symbiosis.

Leguminosae Leguminosae (Fabaceae) is the third largest family of the angiosperms, widely distributed or cultured throughout the world. It is classified in the eurosid I clade, together with the other higher plants with nitrogen-fixing root nodules and many that lack nodules (Azani et al. 2017; Soltis et al. 2000). Originating 60 million years ago, about 19,700 species within 750 genera have been reported in the family Leguminosae, of which 1485 species within 172 genera have been recorded in China. A recent taxonomic revision has established six subfamilies within the Leguminosae (Azani et al. 2017). Nodulation is absent in the four basal subfamilies but found in the majority of genera and species in the two sister subfamilies Papilionoideae and Caesalpinioideae (Doyle 2016). In the Papilionoideae, nodulation is found in all members of the core papilionoid clade and the tribe Swartzieae. In the Caesalpinioideae, nodules are found in all members of the mimosoid clade and some members of various smaller clades. Since the core papilionoids (12,000 species) and the mimosoids (3300 species) include the great majority of legume genera and species, it is true to say that most legumes have root nodules.

Many of the legumes are important grain or economic crops, like soybean and peanuts cultured in China, the USA and South America, common bean cultured in Mexico and chickpea in the Mediterranean countries. Other species like alfalfa, clover and *Desmodium* are cultured or naturally distributed in the world as excellent forages and plants to protect the soil from erosion. Some legumes, like *Alhagi sparsifolia* and *Caragana* spp. growing in the temperate desert regions, are plants of high resistance to environmental stresses (such as saline-alkaline soil and drought) and of high efficiency in nitrogen fixation that are suitable candidates for sand fixation and reforestation in the desert regions. Legumes like *Robinia pseudoacacia*, *Glycyrrhiza uralensis*, *Astragalus membranaceus*, *Trigonella* spp. and others are important resources for honey, herbal medicine and compounds for industrial appli-

cation. In addition, some species like *Wisteria sinensis*, *Cercis chinensis* Bunge and *Lathyrus odoratus* are valuable ornamental plants in the streets and gardens. The wide distribution and adaptation of legumes are directly related to their symbiotic association with rhizobia, because the root and/or stem nodules provide them with 23–94% of their nitrogen nutrition in the field, depending on the soil fertility and geographic regions (Kermah et al. 2018). This symbiosis has some unique characters, as mentioned subsequently.

Firstly, the symbiosis of rhizobia with legumes is the most efficient biological nitrogen-fixing system and provides about 65% of the total nitrogen fixed by BNF. Other systems contribute the remaining 35%, including free-living diazotrophs (like *Azotobacter*), plant-associated diazotrophs (like *Azospirillum* associated with herbaceous plants), symbiosis between *Cyanobacteria* and some plants (like *Nostoc* associated with *Azolla*) and the nodule symbiosis between *Frankia* and some angiosperms (like *Alnus*). Annual legumes, like soybean and common bean, have been estimated to fix 50–300 kg N $year^{-1}$ ha^{-1}, while the perennial legumes like clover and alfalfa can fix 100–500 kg N $year^{-1}$ ha^{-1} (Wani et al. 1995). Therefore, this symbiotic system is of high potential for improving the soil fertility, for applying in bioremediation, for use as foliage and for supplying proteins to humans.

Secondly, most of the rhizobia only fix nitrogen in symbiosis with their legume hosts. However, the efficiency of nitrogen fixation varied dramatically strain by strain, which may be related to the genomic background of the rhizobia. For example, the mutation or deletion of some nodulation genes may cause the decrease of nodulation ability and/or nitrogen fixation efficiency. In another way, the nodulation and nitrogen fixation efficiency are also affected by combinations among the strains and the plants species/varieties and the soil factors (Jia et al. 2013). Among the rhizobia, some strains within the genera *Bradyrhizobium* and *Azorhizobium* can fix nitrogen in free-living conditions in defined media (Dreyfus et al. 1988; Tjepkema and Evans 1975).

Thirdly, a specific association exists in the symbiosis between rhizobia and legumes. In the early stage of study on rhizobia, it was believed that the specificity was between the rhizobial species and the legume species, e.g. a legume species only nodulated with a certain rhizobial species. In this case, all the plants that could share their rhizobial strains were defined as a cross-nodulation group, and their rhizobia were classified as a species, such as *R. leguminosarum* for pea rhizobia, *Rhizobium trifolii* for clover rhizobia and *Rhizobium phaseoli* for common bean rhizobia (Fred 1932). However, subsequent studies have revealed that the specificity of rhizobia is determined by the nodulation genes and these genes are transferable between different bacteria. In fact, different rhizobial species harbouring the same or similar nodulation genes, called a symbiovar (sv.), could nodulate the same legume species, like the common bean-nodulating rhizobia *Rhizobium etli* sv. phaseoli, *Rhizobium leguminosarum* sv. phaseoli, *Pararhizobium giardinii* sv. phaseoli and *Sinorhizobium meliloti* bv. mediterranense (Verástegui-Valdés et al. 2014). Therefore, host specificity is no more used as a taxonomic feature.

Fourthly, the combination between rhizobial species and legume species can vary according to the soil conditions. This phenomenon is clear for soybean- and common

bean-nodulating rhizobia. Previous studies showed that common bean nodulated with *R. etli* in American soils (Silva et al. 2003) and in neutral-alkaline semi-arid soils in several other countries (Tamimi and Young 2004), with *R. leguminosarum* in slightly acid soil in China (Cao et al. 2014; Wang et al. 2009), with *Rhizobium acidisoli* in acid soil (pH 5.0) (Roman-Ponce et al. 2016; Verástegui-Valdés et al. 2014) and with *S. meliloti* and *S. americanum* in alkaline soil at Mexico and other countries (Mnasri et al. 2012; Verástegui-Valdés et al. 2014; Zurdo-Pineiro et al. 2009), respectively. As to soybean, *Bradyrhizobium japonicum* and *Bradyrhizobium elkani* are predominant symbionts in neutral soils (Man et al. 2008; Zhang et al. 2011), *S. fredii* is dominant in saline-alkaline soils (Han et al. 2009; Zhang et al. 2011) and *Bradyrhizobium liaoningense* is dominant in alkaline soil (Appunu et al. 2008). Based upon these reports, it is clear that the symbiotic combination is determined by the interaction among the rhizobia, host legumes and soil factors.

Lastly, vertical and horizontal transfers of symbiosis genes among the species and genera are two aspects of rhizobial evolution. In relation to their complex life cycle, rhizobia, especially the strains in *Bradyrhizobium*, have large genome sizes compared with most other bacteria (MacLean et al. 2007), in which the major parts are adaptation genes, including the symbiosis genes (Tian et al. 2012). In many cases, the phylogenetic positions of the symbiosis genes, especially the nodulation genes, are different from those of the chromosomal (housekeeping) genes, and these cases are believed to be evidence for horizontal transfer of symbiosis genes (Zhao et al. 2008). However, in some studies of large numbers of wild-type rhizobial strains, it appeared that the chromosomal genes and the symbiosis genes had coevolved, or the symbiosis genes were vertically transferred, in most cases, and horizontal transfer happened rarely. For example, the high degree of correlation between the phylogenetic positions of housekeeping genes and the nodulation genes in most of the rhizobia associated with different wild legumes in Xinjiang, China, indicated the vertical transfer of nodulation genes, but horizontal transfer was also evident in several cases (Han et al. 2010). Similarly, the phylogenies of core genes and symbiosis genes were largely congruent in *Burkholderia* species nodulating endemic *Mimosa* species in Brazil, implying that there had been little horizontal transfer between them (Bontemps et al. 2010). Therefore, in most cases, the symbiosis genes are mainly maintained in the rhizobia by vertical transfer, but horizontal transfer is also an important mechanism for the diversification of symbiotic bacteria.

1.2 Importance of Research on Rhizobia

1.2.1 Economic and Ecological Importance

Currently, the huge population of human beings in the world generates a strong pressure on the food supply, and this pressure will increase in the future since the population, which already exceeds 7.5 billion, is predicted to reach 11 billion by

2100 (United Nations, Department of Economic and Social Affairs, Population Division 2017). To feed the increased population of the world, application of synthetic fertilisers has played a great role during the last 60 years by enhancing the food production. However, this agricultural practice generated a great ecological problem since the long-term and excess application of chemical fertilisers has caused the degradation of soil fertility and environmental contamination, especially the eutrophication of water bodies (Norse 2005; Tetteh 2015). Because of their nitrogen fixation ability, studies of rhizobia are important for improving the production of leguminous crops and for exploring the symbiosis as natural resource to reduce the application of chemical fertilisers. In general, nitrogen fixation occurs in two natural ways, including geochemical processes such as lightning and explosion of volcanos and BNF by prokaryotes (bacteria and archaea), in addition to the industrial process. BNF, catalysed by the nitrogenase complex (EC 1.18.6.1, EC 1.19.6.1), has a long evolutionary history, probably originating before oxygen levels rose in the atmosphere and before the split between the bacteria and archaea (3.2 billion years ago) (Stüeken et al. 2015). Before the industrial nitrogen fixation increased in the 1920s, BNF was the main supply of nitrogen source for all life on the planet. Symbiotic nitrogen fixation by the legumes contributes two-thirds of the amount of biologically fixed nitrogen, and almost the same amount is currently provided by synthetic ammonia production (Gruber and Galloway 2008). The ecological and economic importance of the BNF by the rhizobia-legume symbiosis has been reported elsewhere (do Vale Barreto Figueiredo et al. 2013).

In the last decades, there have been many studies on the selection of highly efficient (nitrogen-fixing) inoculants for different legumes, and some of them have led to application in field production. A very successful example is the inoculation of soybean plants with *Bradyrhizobium* inoculants in the USA, Brazil, Argentina, etc., where the soybean was introduced as an alien plant and no native bacterium could form nodules with this plant. In this case, the inoculation of rhizobia could significantly improve the production of soybean and greatly reduced the application of chemical fertilisers in these countries (Ulzen et al. 2016). Currently, these three countries are the first three producers of soybean, with production of 115.8, 107.0 and 57.0 million tonnes, and provide 37%, 34.2% and 18.2% of the world's soybean production, respectively, in 2016 (http://www.globalsoybeanproduction.com). Another case of successful application of rhizobial inoculant was achieved in New Zealand where *Lotus corniculatus* was introduced as forage in the early 1900s (Scott and Charlton 1983), and the inoculation with rhizobial strains specific to this plant helped its colonisation in the introduced regions (Patrick and Lowther 1992). In relation to the application of rhizobial inoculants, studies on the diversity, specificity, competence, biogeography, etc. could offer the bioresource of rhizobia for the effective strain selection (Jia et al. 2013) and also guide strain selection according to the adaptation of rhizobial populations to the environmental conditions (biogeography).

1.2.2 Value as Model for Interactions Among the Microbes, Plants and Environmental Factors

The interactions among microbes, plants and environmental factors are determinants for the colonisation and development of microorganisms and plants in ecosystems. These interactions can be reflected in different aspects, such as variations in cellular morphology, changes in physiochemical traits, exchange and conversion of metabolites, molecular dialog, gene transfer, etc., and drive the diversification or genotype selection of the organisms. In addition, the colonisation and development of organisms in the environment are largely dependent on the species diversity and adaptation, since high functional redundancy in a microbial community could increase the competitive and adaptation abilities of the community and inhibit the possible invasion of non-native species. Previously, it has been concluded that the symbiotic associations between rhizobia and leguminous plants in a certain area are the result of a coevolution of both the rhizobia and the legume plants under environmental selection (Han et al. 2009; Zhang et al. 2011). As a model of symbiosis, studies of the association between rhizobia and legumes have greatly improved our knowledge of microbe-plant interactions, such as the molecular basis for recognition between microsymbionts and their host legumes (Long 2001), production of secondary metabolites and siderophores against environmental stresses (Datta and Chakrabartty 2014; Guerinot 1991), quorum sensing systems for regulating expression of genes related to conjugation and nodulation (Wisniewski-Dye and Downie 2002) and cellular transduction signalling (Wener 2008). Therefore, study of rhizobia and the symbiosis of rhizobia with legumes is important for basic science, including evolution of lifestyle, plant-microbe interaction and interactions among bacteria, plants and environmental factors, as well as for agricultural production and phytoremediation.

In the subsequent chapters of this book, more detailed information will be presented to explain how the interactions among rhizobia, legume hosts and soil characters determine the rhizobial community structure, diversity and their distribution in different geographic regions, how the rhizobia have diversified and adapted in diverse environments, etc.

1.3 Concluding Remarks and Perspectives

Like many other scientific research areas, the development of studies on rhizobia depended on practical needs and was closely related to the developments in other scientific disciplines. To understand the mechanism by which legumes could improve soil fertility, the early scientists in the 1800s performed studies and noticed the relation between root nodules on the legume crops and their fertility impact. Thanks to the development of microbial culturing, Beijerinck (1888) was able to isolate the first nodule bacteria from pea nodules. By the application of

novel methodologies as they became available, the mechanisms of rhizobial specificity for host legumes (nodulation genes and their expression/regulation) and of nitrogen fixation (nitrogen-fixing genes and their expression/regulation, structure and activity of nitrogenase) have been clearly described at the molecular level. Also, knowledge of the diversity and evolution of rhizobia has been greatly improved, and more than 100 symbiotic rhizobial species have been described in about 15 genera.

Previous studies on rhizobia have answered many scientific questions but also generated some new ones. Furthermore, the current ecological problems, such as global climate change and environment contamination caused by the excessive application of chemical fertilisers and mining activities, require more investigations into the ecology and application of rhizobia. During the past 40 years, our research groups have worked continuously on rhizobial diversity, ecology and application. In this book, we would like to make a resume of our work and share all of our experience and ideas for future study of rhizobia.

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Part II
Diversity and Evolution of Rhizobia

Chapter 2
History of Rhizobial Taxonomy

2.1 Conception and Importance of Biodiversity

Conception Biological diversity or biodiversity refers to the wide variety of living things on Earth, including the diversity of ecosystems, diversity of species and diversity of genes. Although studies of biodiversity have a long history, the term "biodiversity" was first used in 1986 by Walter G. Rosen in *National Forum on Biodiversity* (de Andrade Franco 2013). Ecosystem diversity is the largest scale of biodiversity that reveals variations in ecosystems on Earth such as the terrestrial ecosystem, the aquatic ecosystem, agricultural ecosystems, forestry ecosystems, etc., in which the organisms colonise and interact in trophic chains. The diversity of ecosystems can be measured in terms of variation in the complexity of communities, such as trophic levels, niche types/numbers, productivity and biotransformation efficiencies, etc., that depend on both species and genetic diversity (Ives and Carpente 2009). Species diversity is related to the numbers of species represented in the ecosystems or communities and considers both species richness and their relative abundance (species evenness) (Hill 1973). Gene or genetic diversity is usually applied to the biodiversity within species, relating to the total number of genetic characteristics in their chromosomes. This diversity allows microbial populations or species to adapt different environments. A greater gene diversity in a population or species means the existence of more alleles that offer the population and species a greater chance to adapt to variations in the environment and to maintain the population. It has been estimated that about 5.3×10^{31} megabases (Mb) of DNA exist on Earth (Landenmark et al. 2015), which form a huge gene pool for diverse metabolic pathways and for diversification of the species. In conclusion, biodiversity was defined by Wilson (1992) as "… all hereditarily based variation at all levels of organization, from the genes within a single local population, to the species composing all or part of a local community, and finally to the communities themselves that compose the living parts of the multifarious ecosystems of the world".

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Importance The importance of biodiversity can be estimated from three aspects: the ecological aspect, economic aspect and scientific aspect. For the ecological aspect, biodiversity guarantees the wellness and equilibrium of ecosystems, since diverse organisms occupy different niches and functions and form trophic chains that drive the flux of energy and materials. However, the relationship between biodiversity and ecosystem function may be positive, but it can be inconsistent across scales and systems (Harrison et al. 2014), depending on the species functional traits in the ecosystem (Hooper et al. 2005). Usually, the more biological diversity present in an ecosystem, the more stability exists in the ecosystem; therefore, biodiversity is the basis for long-term sustainability of ecosystems in the face of environmental changes; however, not all ecosystems with higher biodiversity are more stable. The effects of biodiversity on stability of ecosystems may be through the following mechanisms: (1) species respond to environmental fluctuations asynchronously; (2) species respond at different speeds; and (3) species diversity reduces the strength of competition (Loreau and de Mazancourt 2013). The economic importance of biodiversity arises because the diverse organisms are bioresources for food production, for useful enzyme and metabolite (antibiotics, etc.) production as well as for biotransformation of components (nitrogen fixation, bioremediation, etc.). From the scientific aspect, "biodiversity is inherently multidimensional, encompassing taxonomic, functional, phylogenetic, genetic, landscape and many other elements of variability of life on the Earth" (Naeem et al. 2016). Indeed, as result of long-term evolution, each species is unique in nature and occupies its special position in phylogeny. In this case, the extinction of a species might mean a loss of irreplaceable genetic information and a breakdown of biological interaction in an ecosystem. The development of genome analysis offers a powerful tool and abundant genetic information for reconstructing the evolution of organisms. A recent study estimated the number of genes that possibly existed in the last universal common ancestor of cellular organisms (LUCA, or the progenote), based on an investigation of all 6.1 million protein-coding genes from prokaryotic genomes, and identified 355 protein families in LUCA, which "depict LUCA as anaerobic, CO_2-fixing, H_2-dependent with a Wood–Ljungdahl pathway, N_2-fixing and thermophilic" (Weiss et al. 2016). With the phylogenies of these 355 genes, clostridia and methanogens were recognised as basal among bacteria and archaea, respectively. "LUCA inhabited a geochemically active environment rich in H_2, CO_2 and iron" (Weiss et al. 2016). These data offered genomic evidence for the autotrophic origin of life in a hydrothermal environment.

2.2 Bacterial Diversity and Taxonomy

Bacterial Diversity Bacteria, together with archaea, are prokaryotes with single cells typically in shapes of sphere, rod, vibrio or spiral and size in the order of micrometers. They exist in almost all environments, including water bodies, soils, deep surfaces and endosphere of macroorganisms, and they are the living things with the longest history on Earth. It is estimated that the unicellular bacteria and archaea occurred on Earth about 4 billion years ago, and they occupied the Earth for about 3 billion years as the dominant, even the only forms of life on Earth (DeLong and Pace 2001; Schopf 1994).

To estimate the evolution and systematic relationships of macroorganisms, fossil data are essential (Benton 2015), but fossil records do not allow us to retrace the origin and evolution of bacteria (Dodd et al. 2017; Schmidt and Schäfer 2005) because of the tiny and simple forms of bacteria. However, the development of gene sequence analysis (both the sequencing techniques and computer estimation) opened the gate to reconstruct bacterial phylogeny and to estimate their evolutionary history (Brown and Doolittle 1997; Di Giulio 2003). Based on sequence analysis, it is estimated that bacteria and archaea were split at proximately 4 billion years ago (Battistuzzi et al. 2004).

As a result of their long-term evolution and diverse distribution in nature, it is believed that very diverse bacteria exist on Earth, with more than 10^{12} species of microbes altogether (bacteria, archaea and microscopic eukaryotes) (Locey and Lennon 2016; Pike et al. 2018) and about 4–6 × 10^{30} bacterial and archaeal cells (Whitman et al. 1998). The diverse and abundant bacteria play important roles in nature based upon their huge metabolic diversity. Some metabolic functions are only found in bacteria and archaea, such as chemoautotrophy (nitrification, sulphur oxidation, hydrogen bacteria), photoheterotrophy (purple and green non-sulphur bacteria), anoxygenic photosynthesis (purple and green sulphur bacteria), biological nitrogen fixation (diazotrophs such as *Azotobacter*, *Rhizobium*), anaerobic respiration (denitrification, sulphate reduction, arsenate reduction), methanogenesis, etc. These capabilities make prokaryotes a unique bioresource in biodegradation and biotransformation, especially in biodegradation of the xenobiotic compounds like the polychlorinated biphenyls (Borja et al. 2005). In addition, some bacteria also can be pathogens for human being, for animals and for plants (Boyd et al. 2013; Vouga and Greub 2016). Based on their great economic and ecological importance, as well as their scientific value in study of the origin and evolution of life (Errington 2013), taxonomy of bacteria or bacterial systematics is needed by both the scientific and wider human communities.

Bacterial Taxonomy Among the three hierarchical levels of biodiversity, the species diversity is directly and closely related to taxonomy. Taxonomy is a classic and basic branch of science concerning with putting organisms into a ranking system and giving each taxon (taxonomic unit) a scientific name, which is used in communication for biological investigation, application and education. Therefore, bacterial taxonomy organises the organisms in a hierarchical system. Based upon taxonomy, *Escherichia coli* as the unique name for the same bacterial group has been used for a hundred years in all the world since it has been reported (Castellani and Chalmers 1919).

Taxonomy of bacteria is the study to accommodate (group) the bacteria into a hierarchical system (classification) based on their similarities or relationships, to give the bacterial groups scientific names (nomenclature) according to the International Code of Nomenclature of Bacteria (Parker et al. 2019) and to clarify the taxonomic affiliation of newly isolated or detected bacteria (identification). The principal purpose of bacterial taxonomy is to give the study of bacteriology a comparable basis, e.g. to make sure that the same scientific name used in the world refers to the same bacterial identity (species). In bacterial classification, the system of Carl Linnaeus is used, in which species is the basic taxonomic unit (with a genus name and a specific epithet – the so-called binary nomenclature) to construct a

hierarchy of ranks from lower to higher levels: genus, family, order, class, phylum and kingdom. Based upon the molecular phylogeny of ribosomal RNA (rRNA), all cellular organisms are classified into three kingdoms or domains: eukaryotes, bacteria and archaea (Woese et al. 1990). Besides the phylogenetic division, bacteria can be differentiated in phenotype from the other two domains: the absence of nuclei in cells and sensitivity to antibiotics distinguishes prokaryotes (bacteria and archaea) from eukaryotes, while the presence of peptidoglycan and ester composed of unbranched fatty acids and glycerol in the membrane differentiates bacteria from archaea, the latter having ethers formed by saturated isoprenoids and glycerol.

Currently, the bacterial taxa at genus and higher levels are mainly based on phylogenetic relationships estimated from sequencing of small-subunit ribosomal RNA (SSU rRNA) genes. A sequence similarity of 94.5% or lower is evidence for distinct genera (Yarza et al. 2008). Qin et al. (2014) proposed a "genus boundary for the prokaryotes based on genomic insights" and suggested the use of "percentage of conserved proteins (POCP) between two strains to estimate their evolutionary and phenotypic distance". They suggested that "a prokaryotic genus can be defined as a group of species with all pairwise POCP values higher than 50%".

With the development of culture-independent methods, such as deep sequencing of environmental samples and single-cell genomics, and their application in research of bacterial diversity, the number of bacterial phyla has expanded from 6 (Woese 1987) to more than 70 based on culture-independent metagenomic analysis (Pace 2009; Yarza et al. 2014) and then 99 based upon the phylogenetic analysis of 94,759 bacterial genomes (Parks et al. 2018). Further novel phyla are still reported as a result of analyses of metagenomes (Brown et al. 2015; Eloe-Fadrosh et al. 2016) (Fig. 2.1).

Concept of Bacterial Species Species is the basic unit in taxonomy. In contrast to the definition of animal and plants, interspecific reproductive isolation has not usually been a criterion to separate bacterial species from each other. In *Bergey's Manual of Systematic Bacteriology*, a bacterial species was defined as "a distinct group of strains that have certain distinguishing features and that generally bear a close resemblance to one another in the more essential features of organization" (Brenner et al. 2005). Other definitions of bacterial species have also been given in several publications (Doolittle and Zhaxybayeva 2009; Konstantinidis et al. 2006; Rosselló-Mora and Amann 2001; Staley 2006). In general, only cultured bacteria can be described as species. Until recently, the bacterial species definition has been based on polyphasic analysis (Gillis et al. 2015; Prakash et al. 2007; Vandamme et al. 1996), including phenotypic characterisation and taxonomy (Sneath 1995; Willcox et al. 1980), chemical taxonomy (Brondz and Olsen 1986), multilocus sequence analysis (MLSA) of housekeeping genes (Glaeser and Kämpfer 2015) and some kind of genomic analysis like average nucleotide sequence identity (ANI) or average amino acid sequence identity (AAI) (Garrity 2016; Thompson et al. 2013). Among these analyses, some thresholds for the species definition have been suggested: 80% phenotypic similarity (Austin et al. 1978), 70% DNA-DNA relatedness (Wayne et al. 1987), 97% sequence similarity of 16S rRNA gene (Vandamme et al. 1996), 96–97% similarity in MLSA (Glaeser and Kämpfer 2015) and 95–96% of ANI (Goris et al. 2007; Richter and Rosselló-Móra 2009; Tindall et al. 2010).

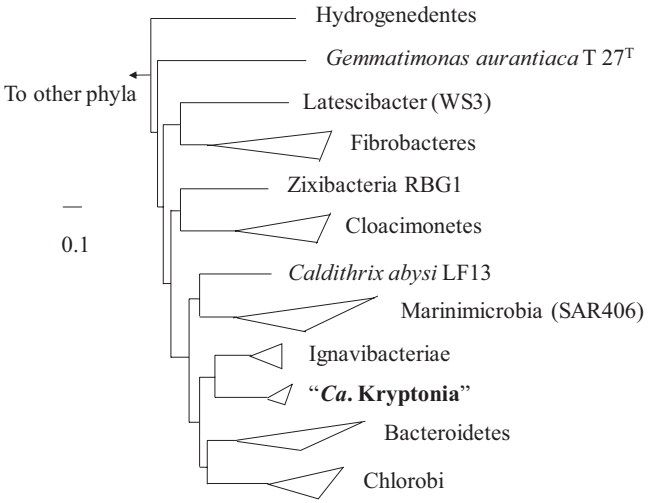

Fig. 2.1 Maximum likelihood phylogeny of bacterial phyla based on concatenation of 56 conserved marker proteins, showing the novel phylum "**Ca. Kryptonia**". Scale bar, 10% of the protein sequences. [Deduced from Eloe-Fadrosh et al. (2016)]

The exact features or methods in the polyphasic approach may vary in the taxonomy of different bacterial groups. In taxonomy of the genus *Streptomyces* and related bacteria, the polyphasic approach included numerical taxonomy based on a large number of phenotypic traits and chemotaxonomy based on analyses of fatty acids; whole-cell analysis with Curie-point pyrolysis mass spectrometry (PyMS); biochemical (enzymatic) analyses; serotype, phage type and protein profiling; genomic analyses for DNA-DNA hybridisation; low-frequency restriction fragment analysis (LFRFA) of total chromosomal DNA and randomly amplified polymorphic DNA (RAPD) PCR assays; and phylogenetic (nucleic acid sequence) comparisons like rRNAs, elongation factors and ATPase subunits (Anderson and Wellington 2001). For polyphasic taxonomy of genus *Shewanella*, growth features, haemolysis, tolerance to NaCl in different concentrations, iron reduction, anaerobic respiration with nitrate, etc. were included in phenotypic characterisation; fatty acid analysis and quinone analysis were used for chemical taxonomy; sequence analyses of 16S rRNA and *gyrB* were performed for phylogenetic study, while DNA-DNA hybridisation was applied for genomic characterisation (Venkateswaran et al. 1999).

With the development of molecular biology, especially the application of metagenomic analysis in the studies on biodiversity, more than three million 16S rRNA gene sequences have accumulated in databases (Quast et al. 2013), and many distinct 16S rRNA gene sequences that share sequence similarities less than 97% with those of the defined species have been detected from environmental samples, either by cloning-sequencing procedures or by the high-throughput sequencing methods. These 16S rRNA sequences undoubtedly represent different bacterial species, but the related bacteria have not been cultured and isolated. Therefore, the concept of genomic-phylogenetic species (GPS) has been suggested for the taxonomy of pro-

karyotes (Staley 2006), which refers to the bacterial group represented only by the 16S rRNA sequences or genome sharing similarities ≥97%. Indeed, this concept is also applicable for isolates, before distinctive features are found. In taxonomy, species that have not been cultured, but for which there is evidence based on sequences and other observations, can be described as *Candidatus* (Murray and Schleifer 1994).

Up to date, about 5×10^4 cultured bacterial species, 10^5 potential species (without cultured strain) represented only by 16S rRNA sequences and 10^7 phylogenetic species detected by high-throughput sequencing from metagenomic DNAs isolated from different sites have been reported (Locey and Lennon 2016). Given the enormous estimated number of bacterial species (10^{12}) in the world (Locey and Lennon 2016), as well as the existence of primer specificity-related "blind spots" (Evguenieva-Hackenberg 2005) in high-throughput sequencing, the diversity of bacterial species is far from sufficiently explored.

2.3 History of Studies on Rhizobial Diversity and Taxonomy

The diversity and taxonomy of rhizobia have been studied since root nodule bacteria were first isolated 100 years ago. Based upon biogeographic and genetic studies, we can deduce that rhizobial diversity depends on four factors: their long evolutionary history, environmental selection for their survival (chromosome genes), host selection for nodulation (symbiosis genes) and symbiosis gene lateral transfer (creating novel combinations of chromosome and symbiosis genes). As a functional group, rhizobia present great diversity at the species level, with about 100 species within many genera.

As reviewed previously (Parker 2001; Willems 2006), the nodules on roots of legumes were recognised by Malpighi in 1675 on common bean (*Phaseolus vulgaris*) and on faba bean (*Vicia faba*); the ability of these nodules to fix atmospheric nitrogen was demonstrated by Hellriegel and Wilfarth in 1888. In the same year, Beijerinck isolated for the first time the rod bacteria from root nodules of pea plants (*Pisum sativum*) and named them *Bacillus radicicola.* Furthermore, he related them to the nitrogen fixation process. In 1889, Frank suggested the genus name *Rhizobium* to accommodate the root nodule bacteria and described the only species *Rhizobium leguminosarum*. After that, the name *Rhizobium* has been used up to the present time (http://doi.namesforlife.com/10.1601/nm.1280), although knowledge of the diversity and taxonomy of rhizobia has developed dramatically.

2.3.1 Cross-Nodulation Groups and the Early Definition of Rhizobium *Species*

The enormous agricultural and economic value of rhizobia made their symbiotic properties important in the development of rhizobial taxonomy and diversity studies. Therefore, host specificity was given a lot of weight in the early studies on rhizobia,

Table 2.1 *Rhizobium* species defined based upon the cross-nodulation groups (Fred et al. 1932)

Species name	Cross-nodulation group	Representative of host plants
Rhizobium leguminosarum^T	Peas	*Pisum* spp., *Vicia* spp., *Lathyrus* spp., *Lens* spp.
Rhizobium phaseoli	Beans	*Phaseolus vulgaris*
Rhizobium trifolii	Clovers	*Trifolium* spp.
Rhizobium meliloti	Alfalfas	*Medicago* spp., *Melilotus* spp., *Trigonella* spp.
Rhizobium lupini	Lupins	*Lupinus* spp.
Rhizobium japonicum	Soybean	*Glycine max*
Rhizobium spp.	Cowpea miscellaneous	*Vigna unguiculata, Crotalaria* spp.

T Type species

which led the definition of rhizobial species based upon the cross-nodulation groups for about 80 years.

In the beginning of the twentieth century, nodulation of diverse leguminous species was extensively studied, and the specificity of the symbiosis between the rhizobial isolates and the host plants was recognised. Based upon the specificity, cross-nodulation groups were described for the rhizobia isolated from a spectrum of leguminous species, in which the plants can share their microsymbionts for nodulation. Then six main cross-nodulation groups were defined as six species within the genus *Rhizobium*, and some other strains in the cowpea miscellaneous group were named as *Rhizobium* spp. Strains of *R. leguminosarum*, *R. phaseoli*, *R. trifolii* and *R. meliloti* grew fast and produced acid on YMA medium, while strains of *R. lupini*, *R. japonicum* and those in the cowpea miscellaneous group (*Rhizobium* spp.) grew slowly and produced alkali on YMA (Table 2.1) (Fred et al. 1932).

After its establishment, the species definition based on cross-nodulation groups was frequently thrown in doubt by the results of subsequent studies, such as many nodulation cases that crossed the boundary of cross-nodulation groups (Wilson 1944) and the high phenotypic similarities among the strains of different cross-nodulation groups (Graham 1964). Although the concept of cross-nodulation group as a basis for species has been abandoned, the specificity between rhizobial strains and leguminous species or preference of legume species for some rhizobial species is still an important feature for rhizobial application or inoculation practice.

2.3.2 Rhizobial Classification by Numerical Taxonomy

In the 1960s, numerical taxonomy was introduced into the taxonomy of rhizobia (Graham 1964). This is a technique that uses computers for comparison of a substantial number of phenotypic characters, covering morphological, growth conditions (pH, temperature and salinity ranges), spectra of C and N resources, metabolic

features (acid/alkali production, respiration/fermentation) and resistance to antibiotics and other chemicals (Graham 1964; Moffet and Colwell 1968; 'tMannetje 1967). During the 1960s–1990s, numerical taxonomy based on phenotypic features played a key role in improving the *Rhizobium* classification. In the same period, serological analyses (Graham 1963; Vincent and Humphrey 1970) and DNA G+C mol% (De Ley and Rassel 1965) were also used for rhizobial classification.

The earlier numerical taxonomy studies revealed that (1) strains in the three cross-nodulation groups of peas, beans and clovers (*R. leguminosarum*, *R. phaseoli*, *R. trifolii*) might be the same species since they were grouped in a single phenon (phenotypic group); (2) *R. japonicum* and *R. lupini* might be the same species; and (3) the fast-growing rhizobia (*R. leguminosarum*, *R. phaseoli*, *R. trifolii*, *R. meliloti*) were more related to *Agrobacterium* than to the slow-growing rhizobia (*R. japonicum*, *R. lupini*, cowpea miscellaneous group). These findings were considered by Jordan and Allen (1974), who proposed the Family *Rhizobiaceae* consisting of the genera *Agrobacterium, Chromobacterium* and *Rhizobium*, while the six *Rhizobium* species were maintained and divided into the fast and slow groups. Later, the slow-growing rhizobia were transferred into a novel genus, *Bradyrhizobium*, by combining the results of numerical taxonomy with those of other analyses including DNA and rRNA (Jordan 1982).

In the 1980s–1990s, numerical taxonomy, alone or together with other methods, was widely applied for investigating phenotypic similarities among rhizobial strains, which greatly enlarged the diversity of rhizobia and led to the description of a third rhizobial genus, *Sinorhizobium*, for fast-growing soybean rhizobia (Chen et al. 1988). In addition, several numerical taxonomy studies alone defined a unique phenon for the photosynthetic rhizobia nodulating *Aeschynomene* species (Ladha and So 1994), two unique phenotypic groups for *Rhizobium* strains from legumes of the temperate zone (Novikova et al. 1994) and *Bradyrhizobium* members for *Sarothamnus scoparius* rhizobia (Sajnaga and Malek 2001). Subsequently, some of the phena resulting from numerical taxonomy were confirmed by polyphasic analyses, including 16S rRNA gene sequence, such as the emendation of genus *Sinorhizobium* (De Lajudie et al. 1994) and the description of *Rhizobium hainanense* (Gao et al. 1994; Chen et al. 1997). However, the results of numerical taxonomy do not have phylogenetic significance, i.e. the phena defined in numerical taxonomy do not reflect evolutionary relationships among the rhizobia. For example, the photosynthetic rhizobia nodulating *Aeschynomene* species were identified as members of the defined *Bradyrhizobium* genus (Molouba et al. 1999), although they formed a unique group (phenon) in numerical taxonomy (Ladha and So 1994).

In addition to the phenotypic features mentioned above, the method of numerical taxonomy was also applied to data of qualitative coding of immunodiffusion reactions (Dudman and Belbin 1988), cross-nodulation patterns of legumes and *Rhizobium* (Lieberman et al. 1985) and chemical taxonomy such as cellular fatty acids (Dunfield et al. 2001). This method is also a basis for grouping biochemical

and genetic fingerprint data, such as the patterns of multilocus enzyme electrophoresis (MLEE) (Wang et al. 1998, 1999), SDS-PAGE of total cellular proteins (Dupuy et al. 1994; Doignon-Bourcier et al. 1999), PCR-based restriction fragment length polymorphism (RFLP) of 16S rRNA genes (Wang et al. 1998, 1999) and IGS genes (Laguerre et al. 1996), BOX- or rep-PCR, Eric-PCR (De Bruij 1992; Laguerre et al. 1996), random amplified polymorphic DNA (RAPD) (Dooley et al. 1993; Harrison et al. 1992), amplified fragment length polymorphism (AFLP) (Terefework et al. 2001) and so on.

2.3.3 DNA and Phylogenetic Analyses

After the 1970s, many DNA sequence analyses were introduced into taxonomic studies of rhizobia. These analyses were used for estimating genetic and phylogenetic relationships at the genetic level, the species level and the genus and higher taxonomic levels. To define the genetic diversity among the strains within the species, DNA fingerprinting methods like BOX- or rep-PCR, Eric-PCR (De Bruij 1992; Laguerre et al. 1996) and RAPD (Dooley et al. 1993; Harrison et al. 1992) have been widely used, and BOX-PCR is still used currently based upon the reproducibility of its PCR patterns and sensitivity to distinguish closely related strains (Chidebe et al. 2018; Dall'Agnol et al. 2014).

For species definition, DNA-DNA hybridisation (Rosselló-Mora 2006), PCR-based RFLP of 16S rRNA genes (Wang et al. 1998, 1999), PCR-based RFLP of 16S–23S rRNA IGS (Laguerre et al. 1996) and AFLP (Terefework et al. 2001) were used. Among these methods, AFLP was not so popular since it is a complicated and labour-consuming procedure. With the development of DNA sequencing techniques, sequencing has become more convenient, economic and rapid than the PCR-based RFLP of 16S rRNA gene and IGS. In addition, sequence data are available for repeated use, so the RLFP analyses have been almost replaced by sequencing analyses of the corresponding DNA fragments. Sequence similarity of 97% for the16S rRNA gene has been used as the threshold for species definition (Wayne et al. 1987), and this threshold was later modified to 98.7% (Stackebrandt and Ebers 2006). However, rhizobial strains sharing very high or even identical 16S rRNA gene sequences have been divided into distinct species (Román-Ponce et al. 2016; Yan et al. 2017). DNA-DNA hybridisation was formerly used as a gold criterion for the definition of bacterial species, and 70% relatedness was used as the species threshold (Graham et al. 1991), but it has been made unnecessary, firstly by the sequencing and phylogenetic analysis of housekeeping genes, the so-called multilocus sequence analysis (MLSA) (Gaunt et al. 2001; Martens et al. 2008), and more recently by genome sequence analyses such as average nucleotide identity (ANI) (Ormeño-Orrillo et al. 2015; Zhang et al. 2014) or in silico DNA-DNA hybridisation based on genome sequences (Meier-Kolthoff et al. 2013; Ormeño-Orrillo et al. 2015).

For the definition of genera and higher taxonomic levels, DNA-rRNA hybridisation (De Smedt and De Ley 1977; Wang 1990), rRNA catalogues (Hennecke et al. 1990; Jarvis et al. 1986), 16S ribosomal DNA restriction fragment length polymorphism (Molouba et al. 1999) and rRNA gene sequencing (So et al. 1994) were used. Currently, the phylogeny of 16S rRNA gene is widely used as the primary criterion for definition of rhizobial species, genera (Graham et al. 1991) and higher taxonomic levels (Yanagi and Yamasato 1993). In general, 95% identity of the 16S rRNA gene sequence has been used as the threshold for a bacterial genus (Rossi-Tamisier et al. 2015). Based upon the data of 16S rRNA gene phylogeny, *Allorhizobium* (de Lajudie et al. 1998), *Azorhizobium* (Dreyfus et al. 1988), *Bradyrhizobium* (Jordan 1982), *Mesorhizobium* (Jarvis et al. 1997) and *Sinorhizobium* (Chen et al. 1988; de Lajudie et al. 1994) were reported or emended as novel genera for some new strains or for species previously described within the genus *Rhizobium*.

These genera were further transferred into different families: the *Bradyrhizobium* branch located in family *Bradyrhizobiaceae*, the *Rhizobium-Sinorhizobium-Allorhizobium* branch affiliated in *Rhizobiaceae*, the *Mesorhizobium* branch classified as a member of *Phyllobacteriaceae* and the *Azorhizobium* branch in *Xanthobacteraceae* (Sy et al. 2001; van Berkum and Eardly 1998). Sequence similarities of 16S rRNA genes greater than 92% were detected among the genera within the family *Rhizobiaceae* (Yanagi and Yamasato 1993); therefore, 92% similarity of 16S rRNA gene sequence might be a reference threshold for family definition.

Recently, the species within the genus *Rhizobium* were further reclassified based upon the phylogeny of 16S rRNA gene and MLSA (Mousavi et al. 2014, 2015), which led to the proposal of *Neorhizobium* and *Pararhizobium*, as well as the emendation of *Agrobacterium* and *Allorhizobium*. However, the existence of several lineages without affiliation to the defined genera implies the necessity for further improvement of the taxonomy of rhizobia.

2.3.4 Rhizobial Taxonomy in Genome Era

Until recently, the definition of rhizobial species was mainly based on the polyphasic approach, covering the estimates of evolutionary relationships from the gene sequence data (16S rRNA gene and housekeeping genes), chemotaxomic, physiological and cultural features. However, the development of whole-genome sequencing has opened a new era of bacterial taxonomy in general (Coenye et al. 2005; Thompson et al. 2013), as well specifically for rhizobial taxonomy (Tong et al. 2018; Wang et al. 2016). Thompson et al. (2013) suggested a unified species definition based on genomics: “strains from the same microbial species share >95% Average Amino Acid Identity (AAI) and Average Nucleotide Identity (ANI), >95%

identity based on multiple alignment genes, <10 in Karlin genomic signature, and > 70% *in silico* Genome-to-Genome Hybridization similarity (GGDH)". With these values, it is convenient to define genomic groups or species; when distinctive phenotypic features are found, the genomic group could be described as a species. In addition, Qin et al. (2014) found that two species within a genus shared a pairwise percentage of conserved proteins (POCP) higher than 50%, which could be used as the boundary of genus. Therefore, the integration of whole-genome data into the taxonomy of rhizobia might also help the genus definition and delimitation of these symbiotic bacteria. In fact, genome analysis has already been involved in the taxonomy of rhizobia, and it is now recommended as the primary approach (de Lajudie et al. 2019). Wang et al. (2016) reported that both ANI and core-genome phylogenetic trees revealed similar relationships among rhizobial strains. In many publications, the standard ANI value of 95–96% has been applied for species threshold (Richter and Rosselló-Móra 2009), while 75% and 70% could be the thresholds for genus and family, respectively (Wang et al. 2016). With analysis of genome data of 45 strains representing the genera *Agrobacterium*, *Allorhizobium*, *Bradyrhizobium* and *Sinorhizobium*, 24 defined species and a putative novel genus represented by *Agrobacterium albertimagni* AOL15 were distinguished (Wang et al. 2016).

It is worthy to note that ANI and/or POCP values between rhizobial genera or species greater than the suggested threshold for genus (75% ANI, 50% COCP) and species (95% ANI) have been detected. For example, ANI values >75% were reported between *Pararhizobium* and *Sinorhizobium* (75.3–77.9%), or between *Neorhizobium* and *Ensifer* (72.7–75.2%); while 74.5–81.5% ANI among the species in *Allorhizobium* and 48.2–86.9% POCP among the species in *Rhizobium* were observed (our unpublished study). The existence of values crossing the thresholds demonstrated that both ANI and POCP thresholds have their limits in taxonomy of rhizobia, similar with the other methods used in bacterial taxonomy. So, genome analysis may replace some other genomic analysis, such as DNA-DNA hybridisation and G + C mol% determination, but it is still a method in the polyphasic taxonomy.

In addition, genome data also offered the possible phenotyping of the strains, for example, arsenite-oxidising and antimonite tolerant genes were detected in ten strains of *Agrobacterium radiobacter* and of two distinctive *Sinorhizobium* genomic species. In another study, Tong et al. (2018) investigated the species diversity of bean and clover rhizobia by comparative genome sequence analysis. In this study, 28 clusters were defined among 69 *Rhizobium* strains based on genome ANI, digital DNA-DNA hybridisation and phylogenetic analysis of 1458 single-copy core genes. The grouping results in this study were consistent with the species affiliation based on of *atpD*, *glnII* and *recA*. Therefore, the MLSA could be a cheaper and more rapid method for grouping rhizobial strains at species level, and genome analysis of at least the types strains of each species could be used for genus definition as revealed by both Tong et al. (2018) and Wang et al. (2016).

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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-

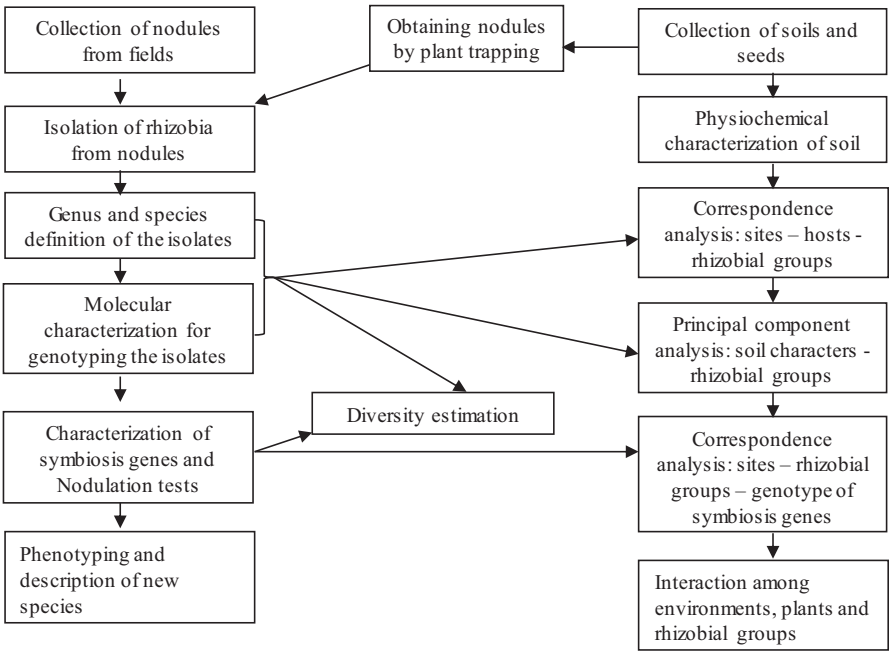

Fig. 3.1 General strategy for studies on diversity and biogeography of rhizobia

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

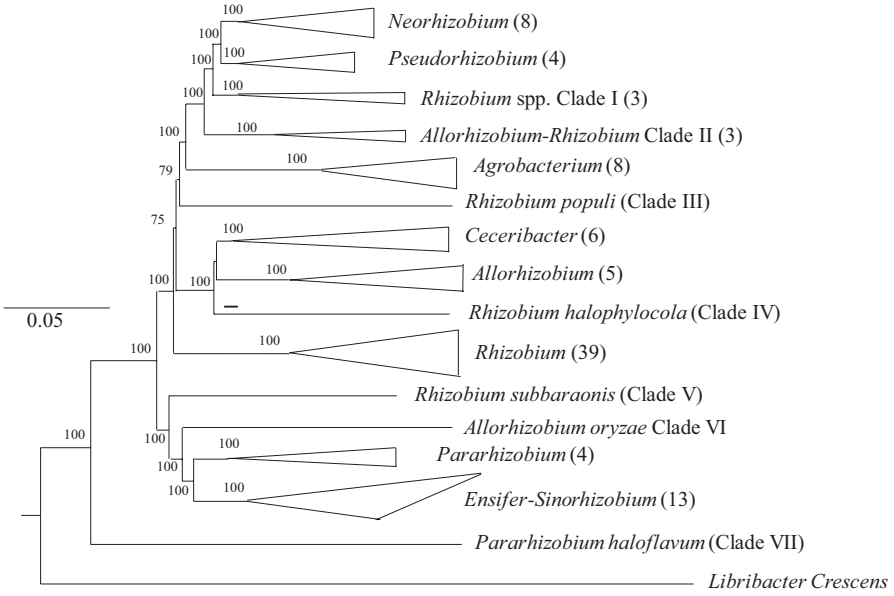

Fig. 3.2 Phylogenetic tree of 96 *Rhizobiaceae* strains constructed with GET_HOMOLOGUES software based on the concatenated amino acid sequences deduced from 316 core genes. A total of 17 clades (genera) were defined at the threshold of 75% of ANI. The scale bar represents 5% of the substitution of amino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

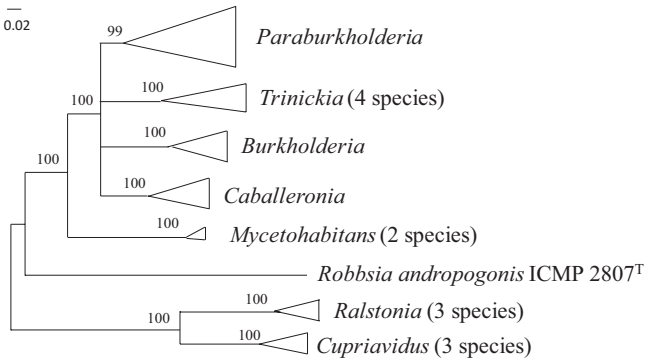

Fig. 3.3 Simplified phylogeny constructed with the maximum likelihood (ML) based on the amino acid sequences of 106 concatenated genes showing the relationships of the β-rhizobia. The scale bar represents number of changes per site. The numbers at nodes are bootstrap values estimated with 1000 pseudo-replicates. 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 geno-species 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

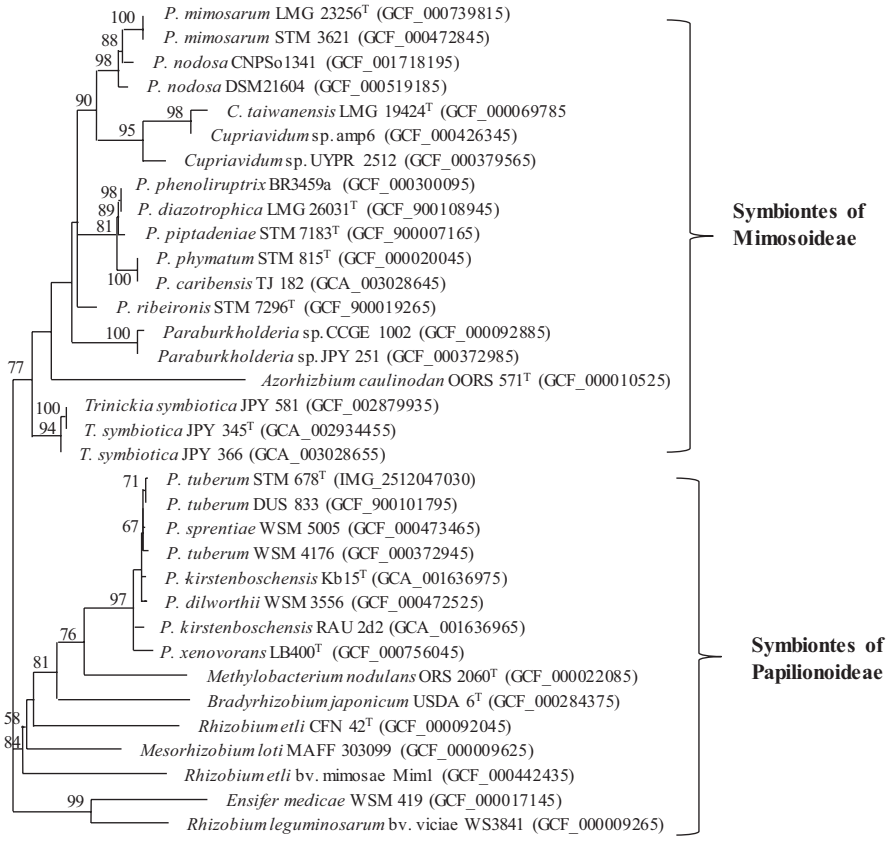

Fig. 3.4 Phylogenetic tree of *nodA* genes constructed with the method of maximum likelihood showing the differences between microsymbionts of Mimosoideae and Papilionoideae. Bootstrap values (based on 100 nonparametric bootstrap calculations) greater than 50% are indicated at the nodes. (Deduced from 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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Chapter 4
Genomics and Evolution of Rhizobia

4.1 The General Organisation of Rhizobial Genomes

In the first decade of the twenty-first century, people working on rhizobia had been very excited about the release of the complete genomes of model strains including *Mesorhizobium loti* MAFF303099 (reclassified as *M. japonicum*) (Kaneko et al. 2000), *Sinorhizobium meliloti* 1021 (Galibert et al. 2001), *Bradyrhizobium japonicum* USDA110 (reclassified as *B. diazoefficiens*) (Kaneko et al. 2002), *Rhizobium etli* CFN42 (González et al. 2006), *Rhizobium leguminosarum* bv. *viciae* 3841 (Young et al. 2006), *Bradyrhizobium* sp. BTAi1 and ORS278 (Giraud et al. 2007), *Azorhizobium caulinodans* ORS571 (Lee et al. 2008), *Cupriavidus taiwanensis* LMG19424 (Amadou et al. 2008), *Sinorhizobium* sp. NGR234 (Schmeisser et al. 2009) and *Sinorhizobium medicae* WSM419 (Reeve et al. 2010). Notably, all of these genomes were sequenced using the Sanger platform, and these valuable earlier efforts have provided us essential information regarding general features of rhizobial genomes. For example, symbiosis genes are intensively clustered in a symbiosis island or a symbiosis plasmid, and genome organisation and gene content can vary drastically between different species. These features have been further validated by more than 100 complete rhizobial genomes obtained later on using next-generation sequencing platforms such as Illumina, Roche 454, Ion Torrent and PacBio. As shown in Fig. 4.1, there is a great variation in genome size of different strains/species within each genus, indicating diverse metabolic abilities of rhizobial germplasms.

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Strain	Genus	Genome Size (Mb)	BioProject
B.diazoefficiens NK6	*Bradyrhizobium*	10.48	PRJDB3027
B. japonicum J5	*Bradyrhizobium*	10.14	PRJNA347501
Mic. ossetica V5/3M	*Microvirga*	9.63	PRJNA329489
B. japonicum E109	*Bradyrhizobium*	9.22	PRJNA270102
B. japonicum USDA 6	*Bradyrhizobium*	9.21	PRJDA67463
B. diazoefficiens USDA 122	*Bradyrhizobium*	9.14	PRJNA298974
B. diazoefficiens USDA 110	*Bradyrhizobium*	9.11	PRJNA17
Methy. nodulans ORS 2060	*Methylobacterium*	8.84	PRJNA20477
Para. phymatum STM815	*Paraburkholderia*	8.68	PRJNA17409
R. leguminosarum Vaf10	*Rhizobium*	8.57	PRJNA316801
B. sp. BTAi1	*Bradyrhizobium*	8.49	PRJNA16137
R. leguminosarum Vaf-108	*Rhizobium*	8.45	PRJNA316801
B. icense LMTR 13	*Bradyrhizobium*	8.32	PRJNA325367
B. sp. SK17	*Bradyrhizobium*	8.29	PRJNA420598
B. oligotrophicum S58	*Bradyrhizobium*	8.26	PRJDB684
R. leguminosarum BIHB	*Rhizobium*	7.95	PRJNA395792
Burk. sp. *CCGE1002*	*Burkholderia*	7.88	PRJNA37719
R. leguminosarum UPM791	*Rhizobium*	7.84	PRJNA417467
B. sp. CCGE-LA001	*Bradyrhizobium*	7.83	PRJNA172908
Para. sprentiae WSM5005	*Paraburkholderia*	7.83	PRJNA344837
B. sp. ORS 285	*Bradyrhizobium*	7.80	PRJEB20226
R. leguminosarum Norway	*Rhizobium*	7.79	PRJNA417364
R. leguminosarum 3841	*Rhizobium*	7.75	PRJNA344
Methy. sp. 4-46	*Methylobacterium*	7.74	PRJNA18809
S. meliloti RU11/001	*Sinorhizobium*	7.69	PRJEB4559
Para. phenoliruptrix BR3459a	*Paraburkholderia*	7.65	PRJNA174166
M. japonicum MAFF 303099	*Mesorhizobium*	7.60	PRJNA18
B. sp. BF49	*Bradyrhizobium*	7.55	PRJEB10689
M. loti NZP2037	*Mesorhizobium*	7.48	PRJNA325064
R. gallicum IE4872	*Rhizobium*	7.47	PRJNA293856
B. sp. ORS 278	*Bradyrhizobium*	7.46	PRJNA19575
R. leguminosarum	*Rhizobium*	7.42	PRJNA20097
M. amorphae CCNWGS0123	*Mesorhizobium*	7.34	PRJNA318467
R. gallicum R602	*Rhizobium*	7.31	PRJNA227036
R. etli 8C-3	*Rhizobium*	7.31	PRJNA293876
R. sp. NXC24	*Rhizobium*	7.30	PRJNA415482
S. meliloti M270	*Sinorhizobium*	7.28	PRJNA388336
B. sp. S23321	*Bradyrhizobium*	7.23	PRJDA72425
S. meliloti USDA1157	*Sinorhizobium*	7.21	PRJNA388336
M. sp. WSM1497	*Mesorhizobium*	7.20	PRJNA323416
R. etli Mim1	*Rhizobium*	7.20	PRJNA200310
S. meliloti SM11	*Sinorhizobium*	7.17	PRJNA41117
S. meliloti Rm41	*Sinorhizobium*	7.15	PRJEB436
S. meliloti USDA1021	*Sinorhizobium*	7.15	PRJNA388336
S. meliloti AK83	*Sinorhizobium*	7.14	PRJNA41993
S. meliloti GR4	*Sinorhizobium*	7.14	PRJNA175860
S. meliloti Rm41	*Sinorhizobium*	7.14	PRJNA388336
S. fredii CCBAU 83666	*Sinorhizobium*	7.08	PRJNA353922
R. sp. 10195	*Rhizobium*	7.07	PRJNA399130
R. sp. IE4771	*Rhizobium*	7.06	PRJNA230502
S. meliloti T073	*Sinorhizobium*	7.04	PRJNA388336
R. etli bv. *phaseoli* IE4803	*Rhizobium*	7.00	PRJNA231318
S. meliloti HM006	*Sinorhizobium*	7.00	PRJNA388336
S. meliloti BL225C	*Sinorhizobium*	6.98	PRJNA42477
S. meliloti KH46	*Sinorhizobium*	6.98	PRJNA388336
R. sp. N324	*Rhizobium*	6.97	PRJNA293118
R. leguminosarum BIHB	*Rhizobium*	6.96	PRJNA395793
M. ciceri CC1192	*Mesorhizobium*	6.94	PRJNA317272
R. phaseoli N671	*Rhizobium*	6.91	PRJNA293118
R. phaseoli N771	*Rhizobium*	6.91	PRJNA293118
R. leguminosarum	*Rhizobium*	6.90	PRJNA62289

Strain	Genus	Genome Size (Mb)	BioProject
S. fredii NGR234	*Sinorhizobium*	6.89	PRJNA21101
M. opportunistum WSM2075	*Mesorhizobium*	6.88	PRJNA33861
M. ciceri WSM1284	*Mesorhizobium*	6.88	PRJNA317273
R. leguminosarum	*Rhizobium*	6.87	PRJNA20179
R. phaseoli N841	*Rhizobium*	6.86	PRJNA293118
S. americanum CCGM7	*Sinorhizobium*	6.85	PRJNA222537
R. sp. Kim5	*Rhizobium*	6.82	PRJNA29555
S. medicae WSM419	*Sinorhizobium*	6.82	PRJNA16304
S. meliloti M162	*Sinorhizobium*	6.81	PRJNA388336
R. etli NXC12	*Rhizobium*	6.76	PRJNA383588
R. phaseoli N831	*Rhizobium*	6.75	PRJNA293118
R. phaseoli R630	*Rhizobium*	6.75	PRJNA293118
R. phaseoli N931	*Rhizobium*	6.75	PRJNA293118
S. americanum CFNEI 73	*Sinorhizobium*	6.75	PRJNA298565
S. meliloti KH35c	*Sinorhizobium*	6.75	PRJNA388336
R. phaseoli N261	*Rhizobium*	6.74	PRJNA293118
R. phaseoli R723	*Rhizobium*	6.74	PRJNA293118
R. phaseoli R650	*Rhizobium*	6.73	PRJNA293118
S. meliloti RMO17	*Sinorhizobium*	6.73	PRJNA244712
R. phaseoli N161	*Rhizobium*	6.72	PRJNA293118
S. meliloti USDA1106	*Sinorhizobium*	6.72	PRJNA388336
R. leguminosarum CB782	*Rhizobium*	6.70	PRJNA67103
R. phaseoli R611	*Rhizobium*	6.70	PRJNA293118
S. meliloti CCMM B554	*Sinorhizobium*	6.70	PRJNA369312
M. ciceri WSM1271	*Mesorhizobium*	6.69	PRJNA48991
R. tropici CIAT 899	*Rhizobium*	6.69	PRJNA42391
R. sp. NXC14	*Rhizobium*	6.69	PRJNA383589
S. meliloti 1021	*Sinorhizobium*	6.69	PRJNA19
R. phaseoli Brasil 5	*Rhizobium*	6.67	PRJNA29557
R. sp. CIAT894	*Rhizobium*	6.66	PRJNA29565
R. phaseoli R620	*Rhizobium*	6.63	PRJNA293118
R. sp. N1341	*Rhizobium*	6.56	PRJNA293118
R. sp. N741	*Rhizobium*	6.56	PRJNA293118
S. fredii NXT3	*Sinorhizobium*	6.56	PRJNA415486
R. etli CFN 42	*Rhizobium*	6.53	PRJNA13932
R. sp. N113	*Rhizobium*	6.52	PRJNA293118
C. taiwanensis LMG 19424	*Cupriavidus*	6.48	PRJNA15733
R. etli N561	*Rhizobium*	6.48	PRJNA293118
R. sp. N871	*Rhizobium*	6.48	PRJNA293118
S. meliloti B399	*Sinorhizobium*	6.47	PRJNA361265
N. galegae HAMBI 540	*Neorhizobium*	6.46	PRJEB1950
R. etli CIAT 652	*Rhizobium*	6.45	PRJNA28021
R. sp. N621	*Rhizobium*	6.43	PRJNA293118
R. sp. N6212	*Rhizobium*	6.42	PRJNA293118
N. galegae HAMBI 1141	*Neorhizobium*	6.41	PRJEB1951
R. sp. TAL182	*Rhizobium*	6.40	PRJNA294178
S. sp. CCBAU 05631	*Sinorhizobium*	6.39	PRJNA353922
R. sp. ACO-34A	*Rhizobium*	6.28	PRJNA384228
R. sp. N731	*Rhizobium*	6.27	PRJNA293118
R. sp. N1314	*Rhizobium*	6.27	PRJNA293118
P. sp. Tri-48	*Phyllobacterium*	6.21	PRJNA329489
M. australicum WSM2073	*Mesorhizobium*	6.20	PRJNA47287
S. meliloti B401	*Sinorhizobium*	6.14	PRJNA361251
S. sojae CCBAU 05684	*Sinorhizobium*	6.09	PRJNA353922
R. sp. S41	*Rhizobium*	5.52	PRJNA327265
R. sp. IRBG74	*Rhizobium*	5.46	PRJEB4411
A. caulinodans ORS 571	*Azorhizobium*	5.37	PRJDA19267
M. sp. B7	*Mesorhizobium*	5.32	PRJNA354894
R. sp. Y9	*Rhizobium*	5.32	PRJNA351913
O. anthropi ATCC 49188	*Ochrobactrum*	5.21	PRJNA19485
R. sp. NT-26	*Rhizobium*	4.58	PRJEB4806

Fig. 4.1 122 complete rhizobial genomes. Genomes retrieved on March 30, 2018, from BioProject as indicated are ordered according to the genome sizes. *Bradyrhizobium*, *Rhizobium*, *Mesorhizobium* and *Sinorhizobium* are coloured

4.1.1 Replicons: Chromosome, Chromid and Plasmid

4.1.1.1 Replicons

Although some archaea harbour a chromosome with the multiple-origin mode of replication (Lindås and Bernander 2013), all DNA molecules of bacterial genomes studied to date have a single replication origin. Consequently, replicon has been widely used to refer such a DNA molecule in bacterial genomes (Harrison et al. 2010; diCenzo and Finan 2017). Among the 122 complete rhizobial genomes from 12 genera accessible till March of 2018 (Fig. 4.1), 107 genomes from 11 genera have two or more replicons (Fig. 4.2a), a genome organisation feature described as a multipartite genome. The multipartite genome organisation is apparently overrepresented in rhizobia compared to an estimated 10% for bacterial genomes (diCenzo and Finan 2017).

The multipartite genome architecture is scarce in *Bradyrhizobium* and not found in the genus *Azorhizobium*, from which only one complete genome is available (Fig. 4.2a). More replicons per genome do not necessarily lead to a larger genome size (Fig. 4.2b). Genome sizes of *Bradyrhizobium* strains are among the largest in rhizobia (Figs. 4.1 and 4.3), even though most *Bradyrhizobium* strains have only one replicon. In a recent global exploration of the soil microbiome, *Bradyrhizobium* was identified as one of the most ubiquitous phylotypes of bacteria (Delgado-Baquerizo et al. 2018). It would be interesting to investigate the relationship between the genome size and the adaptation ability of different *Bradyrhizobium* strains with a great variation in their genome sizes (such as from 7.23 to 10.48 Mb; Fig. 4.1). The size of individual replicons in a multipartite genome can be smaller than the single replicon found in *Bradyrhizobium* and *Azorhizobium*, and the total genome size of a rhizobial strain is generally above 4.5 Mb (Fig. 4.3). This number is several times larger than

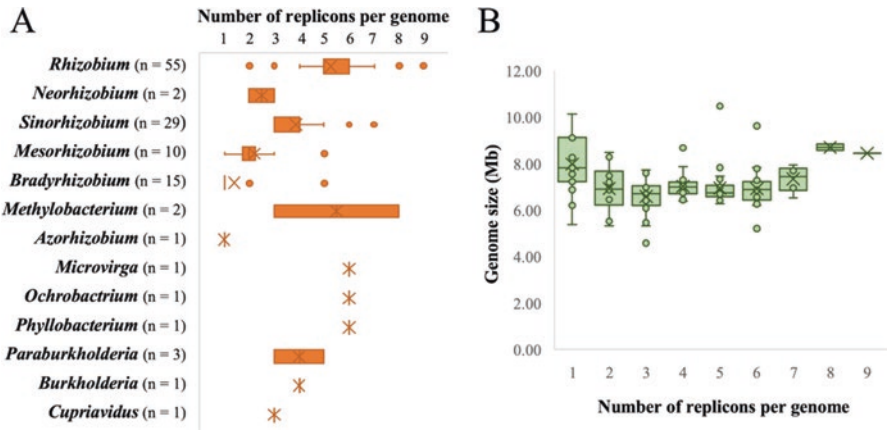

Fig. 4.2 The number of replicons in rhizobial genomes. (**a**) Box plot of the number of replicons within individual complete genomes available in the corresponding genus. The number of analysed genomes is indicated in brackets. (**b**) Box plot of the genome size in rhizobia with different numbers of replicons. One hundred twenty-two rhizobial genomes were analysed

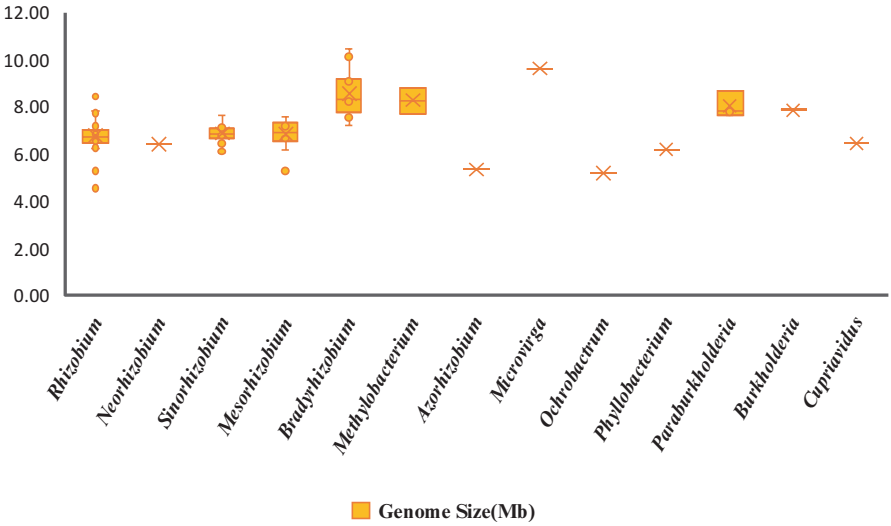

Fig. 4.3 Box plot showing the size of rhizobial genomes. The numbers of analysed genomes are the same as those indicated in Fig. 4.2a for each genus

those of animal-associated obligate endosymbionts (Toft and Andersson 2010), which cannot be cultivated in the laboratory. It is also larger than the average and median bacterial genome sizes, 3.87 Mb and 3.65 Mb, respectively (diCenzo and Finan 2017).

4.1.1.2 Chromosome, Chromid and Plasmid

The single replicon or the largest replicon in a multipartite genome is called a chromosome. In most cases for a multipartite genome, information genes such as rRNA genes and highly conserved housekeeping genes and most essential genes are located on the chromosome. But the presence of a sole rRNA operon in a nonchromosomal replicon has been reported for a plant-associated α-proteobacterium *Aureimonas* sp. AU20 (Anda et al. 2015). In addition to the chromosome, secondary chromosome, chromid, megaplasmids (above 350 kb in size) and plasmids are terms that have been proposed to classify different replicons present in a multipartite genome (diCenzo and Finan 2017). Among them, secondary chromosome and chromid refer to a secondary replicon harbouring some essential genes (either under all conditions or environmentally) (Harrison et al. 2010; diCenzo and Finan 2017). The name "secondary chromosome" was used to indicate that it resulted from a split of the ancestor chromosome into two (Fig. 4.4). With the increasing number of genomes in the database, convincing evidence for this split event is however rare (diCenzo and Finan 2017) and not easily obtained for researchers not fully involved in this specific field. On the other hand, nearly all secondary replicons with essential genes are considered to be chromids evolved from plasmids (Harrison et al. 2010; diCenzo

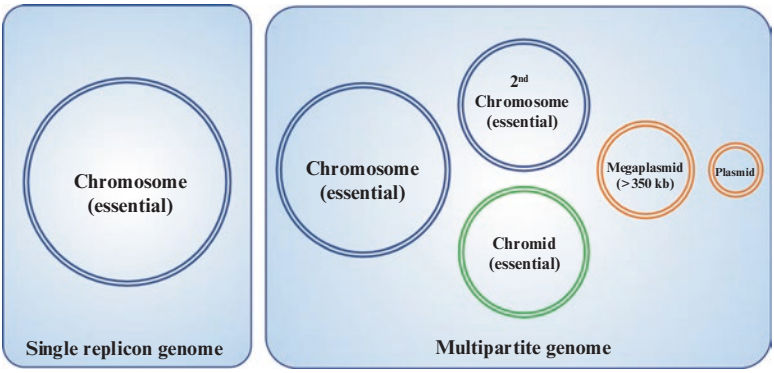

Fig. 4.4 Classification of bacterial replicons. A single replicon genome (left) and a multipartite genome (right) are shown. Different replicons in a multipartite genome are ordered according to their sizes from left to right, but the diameter of each replicon is not scaled to actual size (e.g. >350 kb for megaplasmid). The same colour of second chromosome and the primary chromosome indicates that two replicons result from a split of the ancestor chromosome into two. Essential genes are harboured by the corresponding replicon as indicated. Notably, a chromid smaller than megaplasmids has been reported in a non-rhizobial strain *Deinococcus deserti* VCD115. (Harrison et al. 2010)

Table 4.1 General features of different replicons

Characteristic	Chromosome	Chromid	(Mega)plasmid
Size	The largest	Usually secondary replicons	Smallest replicons
G + C	Varies	Usually within 1% of chromosome	Often >1% lower than chromosome
Maintenance and replication systems	Chromosome-type	Plasmid-type	Plasmid-type
Core genes	Most essential genes	Some essential genes	Few genes shared at any phylogenetic level
Phylogenetic distribution of genes	Wide conservation of genes and synteny between genera	Gene conservation and shared synteny only within genus	Genes specific to strain or species

Adapted from Harrison et al. (2010)

and Finan 2017). Megaplasmids (>350 kb) and plasmids are used to refer replicons lacking essential genes but enriched with dispensable genes and characterised with biased sequence features such as lower GC content and biased codon usage compared to the chromosome. By contrast, the sequence features including GC content and codon usage of chromids are similar to those of the chromosome (Harrison et al. 2010). Distinct characteristics of different replicons have been excellently reviewed by Harrison et al. (2010) and listed herein in Table 4.1.

4.1.2 Symbiosis Plasmid and Symbiosis Island

Despite the great diversity of alpha- and beta-rhizobia (Peix et al. 2015), most rhizobia have a cluster of key symbiosis genes, including *nod*, *nif* and *fix*, localised within a symbiosis island in the chromosome or in the symbiosis plasmid. These genes are specifically involved in nodulation and nitrogen fixation processes during the symbiosis with compatible legumes. Deletion of the key nodulation genes usually leads to a complete loss of symbiotic ability of rhizobia associated with either specific (such as *Medicago sativa*) or promiscuous (such as *Sophora flavescens*) legume hosts (Marvel 1985, 1987; Horvath et al. 1986; Liu et al. 2018). Some *Bradyrhizobium* strains do not require canonical *nod* genes and typical lipochitooligosaccharidic Nod factors for symbiosis with certain *Aeschynomene* species (Giraud et al. 2007; Miche et al. 2010); nevertheless the *nif* and *fix* genes are clustered in a 45-kb island in their genomes (Giraud et al. 2007).

As expected from Table 4.1, symbiosis plasmids have a lower GC content compared to the chromosome in a multipartite rhizobial genome. Here we take *Sinorhizobium* strains associated with soybeans as examples (Fig. 4.5a, b). *S. fredii* CCBAU45436 is an epidemic and efficient soybean microsymbiont in alkaline soils (Zhang et al. 2011; Tian et al. 2012). Five replicons were identified in its multipartite genome (Jiao et al. 2018): chromosome (cSF45436), chromid (pSF45436b), symbiosis plasmid (pSF45436a) and two accessory plasmids (pSF45436d and pSF45436e) (Fig. 4.5a). The symbiosis plasmid pSF45436a is a megaplasmid of 0.42 Mb, which is around 10% and 20% of the size of the chromosome (cSF45436) and chromid (pSF45436b), respectively (Fig. 4.5a). Its GC% (59.9%) is at least 3% lower than those of chromid and chromosome (Fig. 4.5a). Although the replicon size of the symbiosis plasmid varies in different *S. fredii* strains nodulating soybeans, such as 0.40–0.74 Mb in CCBAU45436, CCBAU25509 and CCBAU83666, the average GC% varies little among symbiosis plasmids of *Sinorhizobium* spp. nodulating soybeans (Fig. 4.5b). By contrast, the GC% of chromid is only slightly (0.5%) but also significantly lower than that of chromosome (Fig. 4.5b). Another notable feature of the symbiosis plasmid in these *Sinorhizobium* strains is the enrichment of insertion sequences (ISs), particularly those high-copy ones, compared to chromosome and chromid (Zhao et al. 2018). Although transposable elements had been considered as junk and selfish components in genomes, accumulative evidence supports their critical roles in the evolution of both eukaryotes and prokaryotes (Biémont 2010). A recent experimental evolution study demonstrated that insertion mutation of type three secretion system (T3SS) genes by parallel transpositions of ISs, enriched on the same symbiosis plasmid, is the major mutagenesis mechanism during adaptive evolution of symbiotic compatibility of *Sinorhizobium* associated with soybeans (Zhao et al. 2018). It should be noted, however, that the symbiosis plasmid is not essential for the free-living stage of its rhizobial host, as experimentally demonstrated in *S. meliloti* (diCenzo et al. 2014, 2018). Transcriptomics analyses recurrently show that most genes on the symbiosis plasmid of diverse rhizobia are specifically induced during nodulation and nitrogen fixation, but not under free-living conditions lacking a compatible host or its

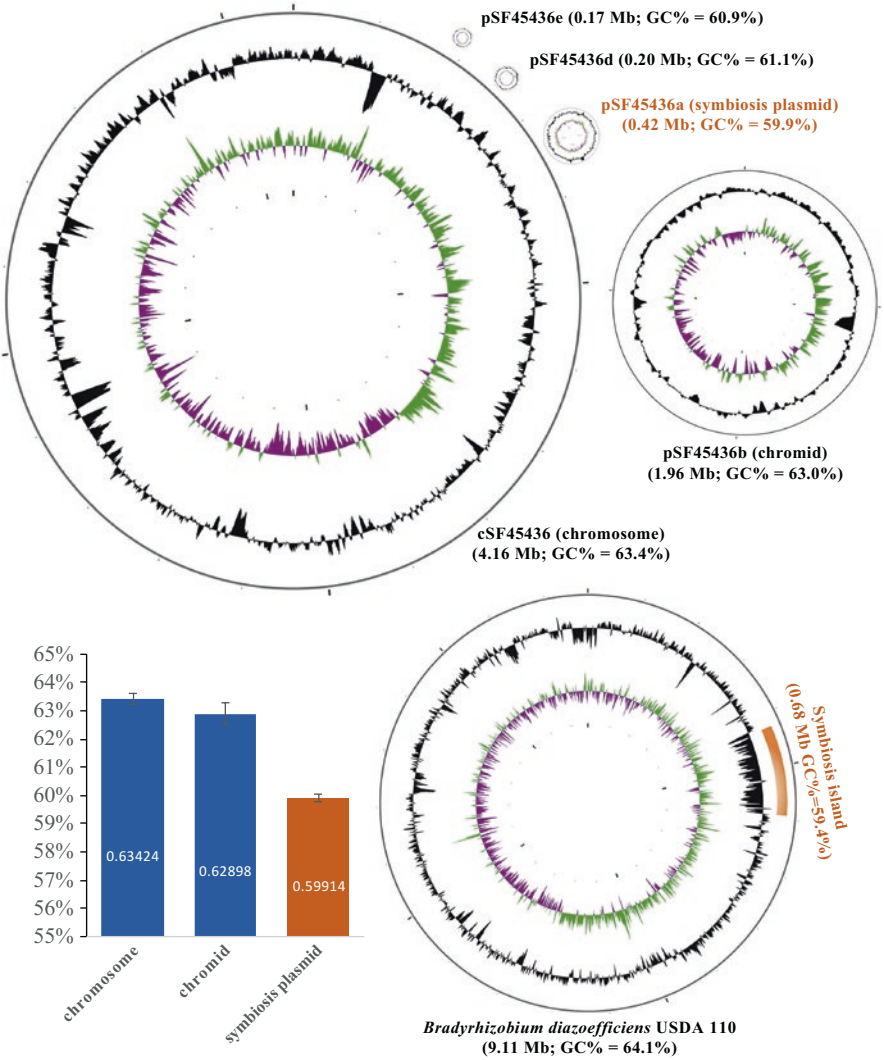

Fig. 4.5 Representative symbiosis plasmid and symbiosis island. (**a**) Five replicons including the symbiosis plasmid pSF45436a in the genome of *Sinorhizobium fredii* CCBAU45436 nodulating soybeans. (**b**) Average GC% of three major replicons in soybean microsymbionts belonging to *Sinorhizobium* (*S. fredii* CCBAU45436, *S. fredii* CCBAU25509, *S. fredii* CCBAU83666, *S. sojae* CCBAU05684, *Sinorhizobium* sp. CCBAU05631). Significant GC% difference of chromid or symbiosis plasmid compared to that of chromosome is shown (T-test; ∗, $p < 0.05$; ∗∗∗, $p < 0.001$). (**c**) The genome of *Bradyrhizobium diazoefficiens* USDA 110 nodulating soybeans. The size and GC% of the symbiosis island are indicated. (**a** and **c**) GC content (black ring) and GC skew (the ring in green and purple) are shown. The genome size of USDA 110 in (**c**) is at a scale of one third of the CCBAU45436 genome in (**a**). A window size of 10,000 and a step of 100 were used in GC content and GC skew analyses for USDA 110, cSF45436 and pSF45436b, while a size of 1000 and a step of 10 were used for pSF45436a, pSF45436d and pSF45436e

symbiotic signal molecules (Ampe et al. 2003; Capela et al. 2006; Vercruysse et al. 2011; Li et al. 2013; Jiao et al. 2016).

In rhizobia with a single replicon and some rhizobia (such as certain *Mesorhizobium* strains) with multiple replicons, key symbiosis genes are found on the chromosome. As shown in the genome of *Bradyrhizobium diazoefficiens* USDA 110 (Fig. 4.5c), a genomic island of six hundred eighty-one kilobyte in length is characterized by its lower GC% (59.4%) than the genomic average (64.4%).

Six hundred eighty-one kilobyte in length is characterised by its lower GC% (59.4%) than the genomic average (64.4%). This island contains key symbiosis genes *nod*, *nif* and *fix* (Göttfert et al. 2001; Kaneko et al. 2002) and many uncharacterised genes, which are highly transcribed in soybean nodules (Pessi et al. 2007). Similarly, a genomic island of 611 kb containing *nod/nif* genes was identified on the chromosome of *Mesorhizobium japonicum* MAFF303099, which harbours two more replicons (plasmids) (Kaneko et al. 2000). Consequently, "symbiosis island" has been used to refer this kind of genomic island (Sullivan and Ronson 1998). As in symbiosis plasmids, there is an overrepresentation of ISs in symbiosis islands, such that 60% of the ISs of *B. diazoefficiens* USDA 110 were localised in this island (Kaneko et al. 2002). The symbiosis island of *M. japonicum* is also characterised by its enrichment of transposable elements compared to the chromosome background and the two plasmids (Kaneko et al. 2000).

More than 20 years ago, it was demonstrated that the symbiosis island of *Mesorhizobium loti* can be transferred into non-symbiotic mesorhizobia under field and lab conditions and integrated into a phe-tRNA gene (Sullivan et al. 1995; Sullivan and Ronson 1998). Recently, Ling et al. provided evidence that the symbiosis island of *Azorhizobium caulinodans* is an integrative and conjugative element that can be transferred to a specific site in a gly-tRNA gene of other rhizobial genera (Ling et al. 2016). Moreover, the horizontal transfer frequency of this symbiosis island increased in the legume rhizosphere or in the presence of plant flavonoids (Ling et al. 2016), highlighting an intriguing host-dependent evolutionary scenario of rhizobia. As shown in Table 4.2, one or two conserved met-tRNA gene(s) can be identified in symbiosis plasmids but not other extrachromosomal replicons of *Sinorhizobium* strains nodulating soybeans. These data imply that integration into a tRNA gene may have played an important role in the horizontal transfer of symbiosis genes in many rhizobial genera in the long run. It is noteworthy that the symbiosis plasmid itself can be subject to conjugative transfer, as demonstrated in *Rhizobium* and *Sinorhizobium* (Danino et al. 2003; Perez-Mendoza et al. 2004, 2005). This is in line with the finding that extremely similar symbiosis plasmids were found in different *Rhizobium* species associated with common bean (Perez Carrascal et al. 2016). If we look at the alignment of symbiosis plasmids from *Sinorhizobium* strains associated with soybeans (Fig. 4.6), a similar conclusion can be drawn for certain *S. fredii* and *S. sojae* strains (CCBAU45436, CCBAU25509 and CCBAU05684). Although highly conserved locally collinear blocks can also be found in *S. fredii* CCBAU83666 and *Sinorhizobium* sp. CCBAU05631, extensive rearrangement and the presence of other accessory sequences can be found in symbiosis plasmids of these two strains (Fig. 4.6).

Table 4.2 Distribution of tRNA genes in multipartite genomes of *Sinorhizobium* nodulating soybeans

	S. sojae	*S. fredii*			*Sinorhizobium* sp.
Replicon	CCBAU05684	CCBAU45436	CCBAU25509	CCBAU83666	CCBAU05631
Chromosome	51	53	53	53	50
Chromid	0	0	0	0	0
Symbiosis plasmid	1 met-tRNA	1 met-tRNA	1 met-tRNA	2 met-tRNA	2 met-tRNA
Other plasmids	0	0	0	0	0

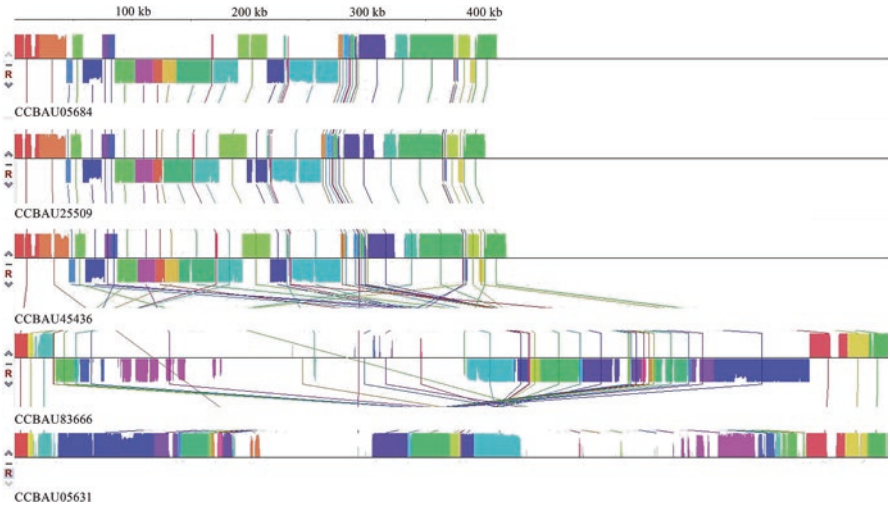

Fig. 4.6 Progressive Mauve alignment of symbiosis plasmids from *Sinorhizobium* microsymbionts of soybean. From the first to fifth row: *S. sojae* CCBAU05684, *S. fredii* CCBAU25509, *S. fredii* CCBAU45436, *S. fredii* CCBAU83666 and *Sinorhizobium* sp. CCBAU05631. Locally collinear blocks conserved between different strains are indicated in the same colour and connected

4.2 Evolution of Core and Accessory Genes

4.2.1 Characteristics of Core and Accessory Genes

In the previous Sect. (4.1), intraspecies and intra-genus variation in rhizobial genome size at the scale of Mb can be observed. If we simply take 1 kb as the average length of a gene, the difference in gene number can be up to several thousand between strains. This phenomenon is widespread in prokaryotes. In 2005, a term "pan-genome" ("pan" – "**παν**" in Greek – means "whole") was introduced to refer the gene repertoire accessible to any given species (Medini et al. 2005; Tettelin et al. 2005). The pan-genome is composed of a "core genome" containing genes present

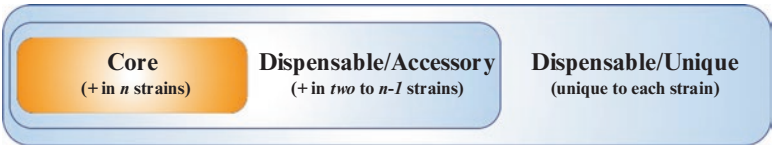

Fig. 4.7 A schematic diagram illustrating the partition of a pan-genome for n strains of a given taxonomic unit. +, present

in all strains and a "dispensable genome" (also called accessory, flexible or adaptive) with genes present in a subset of strains (Medini et al. 2005) (Fig. 4.7). The dispensable genome can be further divided into two elements: genes shared by some but not all strains (named "accessory" genes in some publications to distinguish it from "core" and "unique" elements) and genes unique to each strain (Medini et al. 2005, 2008; Rouli et al. 2015) (Fig. 4.7). Although the species is usually considered to be an evolutionary unit, the pan-genome concept has been extended to higher taxonomic units (Lapierre and Gogarten 2009). This is biologically meaningful, since accessory gene functions may provide adaptive advantages for their host cells in a specific niche and the pan-genome analysis of different species inhabiting the same niche can provide novel insight into the evolutionary mechanisms underlying their adaptation and competition. For example, *S. sojae* CCBAU05684, *Sinorhizobium* sp. CCBAU05631 and *S. fredii* CCBAU45436 share certain wild soybean hosts (Li et al. 2011; Zhang et al. 2011; Liu et al. 2017; Zhao et al. 2018). A pan-genome analysis followed by reverse genetics has revealed that an accessory gene cluster present in CCBAU45436 and CCBAU05631 but absent in CCBAU05684 is essential for effective symbiosis of its host strains (Liu et al. 2017).

It has been estimated that the pan-genome of the bacterial domain is of infinite size, likely due to numerous niches on earth (Lapierre and Gogarten 2009; McInerney et al. 2017), i.e. the number of new genes grows indefinitely with the number of sequenced strains. An "open" pan-genome is used to refer this pattern (Medini et al. 2005). By contrast, if the size of a pan-genome quickly saturates to a limiting value, a "closed" pan-genome can be proposed (Medini et al. 2005). A closed pan-genome has been reported for species living in isolated niches with limited access to the global microbial gene pool, such as *Bacillus anthracis*, *Mycobacterium tuberculosis* and *Chlamydia trachomatis* (Medini et al. 2005). As facultative microsymbionts, rhizobia are expected to have a large pan-genome to cope with fluctuating biotic and abiotic stimuli in soils and during symbiosis with legumes. Indeed, rhizobia such as the model species *Sinorhizobium meliloti* associated with *Medicago* and *S. fredii* nodulating soybeans have a typical open pan-genome (Tian et al. 2012; Galardini et al. 2013). The same conclusion can be drawn for species belonging to *Rhizobium*, *Mesorhizobium* and *Bradyrhizobium* (Tian et al. 2012; Kumar et al. 2015; Perez Carrascal et al. 2016; Porter et al. 2017).

A genome-wide average nucleotide identity (ANI) value of 95% has been widely used to determine if two prokaryotic strains can be considered to be the same spe-

cies (Richter and Rossello-Mora 2009), and a discontinuity in ANI space is observed around this boundary (Konstantinidis and Tiedje 2005; Richter and Rossello-Mora 2009). This gap in sequence space has also been reported in several independent analyses of rhizobia using either a fixed number of shared core genes or a genome scale alignment (Tian et al. 2012; Zhang et al. 2012; Kumar et al. 2015). Therefore, it is established that core genome determines the taxonomy of rhizobia, as for other prokaryotes (Ormeno-Orrillo et al. 2015). By contrast, representative features used in polyphasic taxonomy in pre-genomics studies only capture a tiny fraction of the inter-species variation, and it is not uncommon that these features can also vary at the intraspecies level (Ormeño-Orrillo and Martínez-Romero 2013; Kumar et al. 2015; Vernikos et al. 2015; Young 2016), thus blurring the species boundary. Comparative genomics of rhizobia from 8 genera suggested that the phyletic distribution of 887 functional genes with experimental evidence can reflect the species phylogeny of test strains, while the distribution of the whole pan-genome could not (Tian et al. 2012). This highlights that accessory genes in the open pan-genome of rhizobia are differentially integrated with the genome backgrounds of individual species. As typical accessory genes, key nodulation and nitrogen fixation genes within symbiosis islands or symbiosis plasmids of rhizobia determine the symbiovar and hence the corresponding legume host, rather than the bacterial species assignments (Rogel et al. 2011). These key symbiosis genes provide adaptive advantage for rhizobia in the presence of compatible legumes, while many other accessory genes can be adaptive in diverse niches in soils. For example, in contrast to *Sinorhizobium*, *Bradyrhizobium* strains are enriched with accessory genes involved in secondary metabolism, which may explain the high global abundance of *Bradyrhizobium* in soils (Tian et al. 2012; Delgado-Baquerizo et al. 2018).

4.2.2 Main Evolutionary Forces Shaping the Diversity of Core and Accessory Genes

It is estimated that the divergence of rhizobial genera predates the origin of legumes (Turner and Young 2000), and transferable accessory symbiosis genes can be considered "microsymbionts" that have spread across diverse bacteria (Remigi et al. 2016). That is to say, these symbiosis genes succeed, regarding their wide phyletic distribution in at least two bacterial orders, by improving the adaptation of their host strains. This regime has largely dominated the evolutionary study of rhizobia in past decades.

With the burst of new rhizobial species being documented in the literature and the development of sequencing technology, our knowledge of rhizobial core genes has been extended from information on the 16S rRNA gene and few housekeeping genes (such as *atpD*, *glnII*, *recA*, *rpoB*, etc.) to hundreds and thousands of core genes. It is notable that both intragenic and intergenic recombination, in addition to

point mutation, have played a substantial role in creating the observed diversity of chromosomal housekeeping genes in rhizobial species such as *Bradyrhizobium canariense*, *B. japonicum*, *B. elkanii*, *B. liaoningense*, *B. yuanmingense*, *B. diazoefficiens*, *Rhizobium gallicum* sensu *lato*, *Rhizobium leguminosarum* bv. *viciae* and *Sinorhizobium fredii* (Vinuesa et al. 2005, 2008; Silva et al. 2005; Tian et al. 2010; Zhang et al. 2014; Guo et al. 2014). This view has been further verified in a comparison of individual core gene trees to the species tree based on 295 core genes in alpha- and beta-rhizobia (Tian et al. 2012). Around 90% of these core genes have undergone horizontal gene transfer or intergenic recombination, and only 20 out of 295 genes in test strains were free of either inter- or intragenic recombination (Tian et al. 2012). Therefore, strict vertical evolution is rare in rhizobial chromosomal core genes.

The multipartite architecture of many rhizobial genomes (Fig. 4.2 and Table 4.1) provides a unique opportunity to investigate the evolution of core and accessory genes. Extrachromosomal replicons thought to be essential for the saprophytic lifestyle in soils and rhizospheres usually show higher rates of recombination than the chromosomes, as demonstrated in *Rhizobium* and *Sinorhizobium* (Bailly et al. 2011; Guo et al. 2014; Perez Carrascal et al. 2016). The chromid of *Sinorhizobium* species such as *S. meliloti* and *S. fredii* is characterised by its distinct role in intraspecies differentiation and enrichment with accessory genes (Galardini et al. 2013; Jiao et al. 2018). Moreover, the chromid is a hot spot for positively selected genes such as those involved in the synthesis of polysaccharides (Bailly et al. 2011; Galardini et al. 2013), which can influence diverse aspects including host range and phage tolerance (Campbell et al. 2003; Parada et al. 2006; Staehelin et al. 2006; Müller et al. 2009; López-Baena et al. 2016). Horizontal gene transfer has a greater effect on gene content of symbiosis plasmids/islands than of chromids or chromosomes (Bailly et al. 2011; Zhang et al. 2014; Guo et al. 2014; Kumar et al. 2015; Perez Carrascal et al. 2016). Symbiosis plasmids are more prone to share a gene pool with accessory plasmids, as reported in *S. meliloti* strains (Nelson et al. 2018). A low frequency of horizontal gene transfer on chromosomes does not equal none. Although accessory genes can be interspersed throughout the chromosome, most are concentrated in flexible genomic islands (fGIs) (Rodriguez-valera and Lo 2016). This phenomenon can be clearly identified in the example of *S. fredii* strains (Fig. 4.8). Several fGIs are present in locally collinear blocks. These fGIs may contribute to intraspecies variation and increase the adaptation potential of populations. For example, an accessory operon encoding a multidrug efflux system in *S. fredii* CCBAU45436 is located within a fGI on the chromosome (indicated in Fig. 4.8) and is essential for efficient symbiosis of CCBAU45436 with soybeans (Jiao et al. 2018).

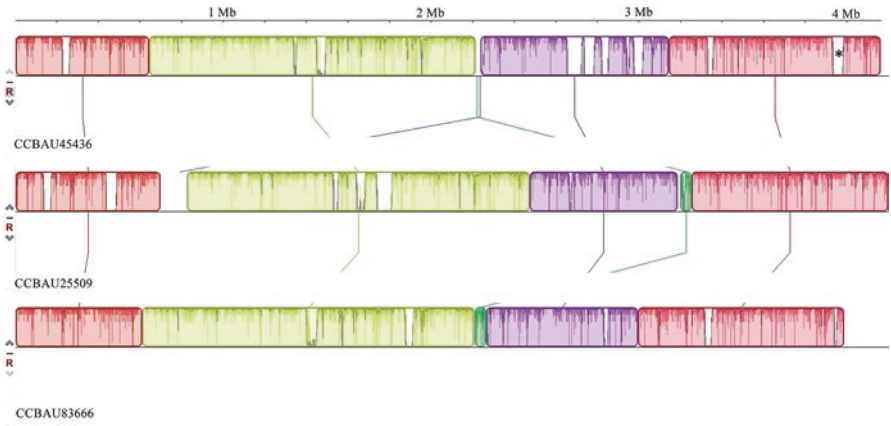

Fig. 4.8 Progressive Mauve alignment of chromosomes from soybean microsymbionts of *Sinorhizobium fredii*. From the first to third row: *S. fredii* CCBAU45436, CCBAU25509 and CCBAU83666. Locally collinear blocks conserved between different strains are indicated in the same colour and connected. ∗ indicates a flexible genome island containing genes encoding a multidrug efflux system that is essential for effective symbiosis of CCBAU45436 with soybeans. (Jiao et al. 2018)

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Part III
Diversity and Evolution of Rhizobial Symbiosis Genes

Chapter 5
Symbiosis Genes: Organisation and Diversity

Abstract With only specific exceptions, almost all the symbiotic rhizobia have a set of symbiosis genes that include nodulation- and N_2-fixing-related genes. Organisation of the symbiosis genes and their roles in the synthesis of Nod factors (LCOs) and nitrogen fixation are first illustrated and compared. Then the diversity and phylogeny of the nodulation gene *nodC* are discussed in detail in various rhizobia with narrow or broad host ranges. The relationship between the *nodC* phylogeny and the rhizobial host range is explored in detail.

5.1 The Organisation of Symbiosis-Related Genes in Rhizobial Genomes

5.1.1 Nodulation-Related Genes

To establish the symbiotic relationship, legumes secrete flavonoid compounds (daidzein, luteolin, naringenin, genistein, apigenin, etc.) that inducing rhizobia to produce the nodulation factors (NFs), which are modified lipochitooligosaccharides (LCOs). In return, the NFs can be perceived by the receptor of the host legumes and stimulate the root hairs to deform. Almost all the symbiotic rhizobia need NFs to trigger nodule initiation in most legumes, except that NFs are not necessarily involved in the *Bradyrhizobium-Aeschynomene* symbiosis (Giraud et al. 2007).

The basic structure of LCOs consists of two parts: three to five units of N-acetylglucosamine (GlcNAc) and a long-chain (C_{16} to C_{20}) saturated or unsaturated fatty acid linked to the sugar at the non-reduced end of this oligosaccharide (Fig. 5.1). Disruption of either of the two parts of the LCO will lead to the failure of nodulation on legumes.

There are more than 30 different *nod*, *nol* and *noe* genes involved in the synthesis and secretion of the LCOs (Table 5.1, Figs. 5.2, 5.3, and 5.4). Common nodulation genes (*nodABC*, *nodD*, *nodIJ*) exist in all symbiotic rhizobia except some *Bradyrhizobium* strains (Giraud et al. 2007). (Iso)flavonoids from legumes diffuse across the membrane of the rhizobia and induce the synthesis of NodD protein to

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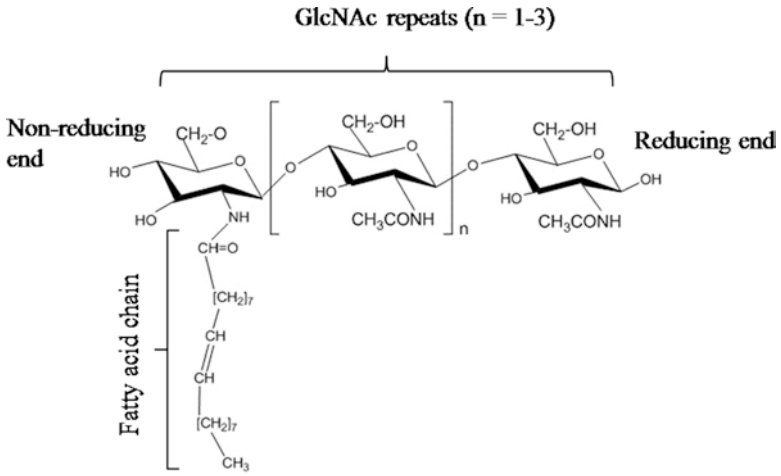


Fig. 5.1 Basic structure of LCOs

activate transcription of other nodulation genes involved in the production of LCOs and their modification (Figs. 5.3 and 5.4).

The *nodABC* genes, usually existing in an operon (Fig. 5.2), encode for the proteins required to synthesise the basic structure of the LCO. This is then modified by species-specific enzymes resulting in various substitutions on both the reducing and non-reducing end, including glycosylation, sulphation and methylation (Fig. 5.4) (Long 1996). The substitutions are specific for each host legume and offer a certain level of symbiotic specificity (Long 1996; Lewin et al. 1990). The specific structure of LCOs is known to be essential for recognition by specific host NF receptors (NFRs), which are receptor kinases containing lysine motifs (LysM) (Nelson and Sadowsky 2015). A plant may have one or more different NFRs. For example, the promiscuous legume *Sophora flavescens* may have distinct NFRs because it can be nodulated by different *nodC*-specific rhizobia secreting different NFs (Jiao et al. 2015a; Liu et al. 2018a).

The functions of various nodulation genes involved in the synthesis and modification of LCOs are shown in Fig. 5.4 and Table 5.1.

5.1.2 Nitrogen-Fixing-Related Genes in Rhizobia

Relatively inert atmospheric nitrogen (N_2) in air cannot be utilised directly by plants and animals. In nature, only some prokaryotic microorganisms, termed diazotrophs, can convert N_2 into the more reactive nitrogen compound ammonia (NH_3) through the enzyme nitrogenase (or dinitrogenase), with consumption of ATP and release of hydrogen (H_2) (Fig. 5.5a). Ammonia is then delivered to α-ketoglutarate/glutamate

Table 5.1 Nodulation gene products required for biosynthesis and secretion of LCOs

Protein/ enzyme	Function
Regulation of LCO synthesis-related genes	
NodD[a]	Transcriptional regulator of common *nod* genes, induced by plant (iso)flavonoids
Biosynthesis of backbone of GlcNAc oligosaccharide	
NodB[a]	Deacetylase, involved in the deacetylation of the non-reducing end of glucosamine oligosaccharide
NodC[a]	N-acetyl-glucosamine transferase, involved in the synthesis of backbone of glucosamine oligosaccharide
NodM	Glucosamine synthase, involved in biosynthesis of the basic unit of GlcNAc
Biosynthesis and transfer of fatty acid motif to non-reducing end	
NodA[a]	Acyltransferase, involved in N-acylation of deacetylated non-reducing terminus of the oligosaccharide
NodE	β-Ketoacyl synthase, involved in the formation of acyl-ACP
NodF	Acyl carrier protein, carrying fatty acid group to GlcNAc repeat
Modification on non-reducing end	
NodS	Methyl transferase
NodU	Carbamoyl transferase
NolO	Carbamoyl transferase
NodL	*O*-acetyl transferase, *O*-acetylates at R_5
Modification on reducing end	
NodP, Q	ATP (adenosine triphosphate) sulphurylase and APS (adenosine-5′-phosphosulphate) kinase, provide activated Sulphur for sulphated LCOs
NodH	Sulphotransferase
NoeE	Sulphotransferase involved in sulphation of fucose
NolK	GDP (guanosine diphosphate) fucose synthesis
NodZ	Fucosyl transferase
NolL	*O*-acetyltransferase; involved in acetyl-fucose formation
NodX	*O*-acetyltransferase, specifically *O*-acetylates the 6-C of the terminal non-reducing sugar of the penta-*N*-acetylglucosamine of *Rhizobium leguminosarum* TOM
NoeI	2-*O*-methyltransferase involved in 2-*O*-methylation of fucose
Secretion of LCOs	
NodI[a]	ABC transporter component, carrying an ATPase domain
NodJ[a]	ABC transporter subunit, LCO transporter, involved in the secretion of LCO to outside of rhizobial cell

[a]Present in all symbiotic rhizobia except some strains of *Aeschynomene*-associated *Bradyrhizobium*

to form glutamate/glutamine and is further transmitted to other amino acids and N-containing compounds in N metabolism (Fig. 5.5b).

The nitrogenase complex is composed of two main functional subunits, dinitrogenase reductase (NifH, γ_2 homodimeric azoferredoxin, Fe protein) and dinitrogenase (NifD/K, $\alpha_2\beta_2$ heterotetrameric molybdoferredoxin, MoFe) (Hageman and Burris 1978; Kneip et al. 2007). The activity of nitrogenase is positively and negatively regulated by NifA and NifL proteins, respectively. NifA, in conjunction with RpoN (σ^{54} transcriptional factor), activates the transcription of nitrogen fixation

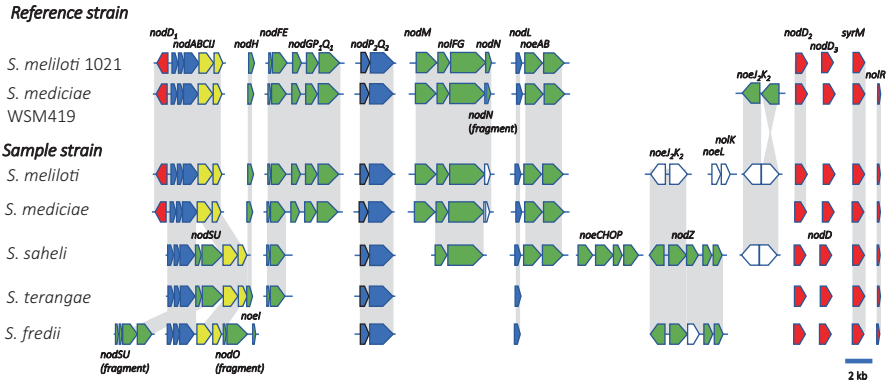


Fig. 5.2 Gene organisation and correlation of Nod factor biosynthetic genes in some *Sinorhizobium* species (Sugawara et al. 2013). Blue arrows indicate the genes encoding enzymes for Nod factor synthesis commonly detected in all tested *Sinorhizobium* strains. Yellow arrows indicate the genes involved in Nod factor secretion. Green arrows indicate specifically detected genes involved in Nod factor synthesis in an individual species. Red arrows indicate the genes encoding transcriptional regulators of nodulation genes. White arrows indicate genes involved in Nod factor biosynthesis that are not in common. Many different strains in the five representative species (*S. meliloti*, *S. medicae*, *S. saheli*, *S. terangae* and *S. fredii*) were used to compare the two reference strains (*S. meliloti* 1021 and *S. medicae* WSM419) (Sugawara et al. 2013)

genes, such as the *nifHDKE* and *fixABCX* operons (Jimenez-Guerrero et al. 2017). Moreover, FixK also induces the transcription of other nitrogen fixation genes, such as the *fixNOQP* and *fixGHIS* operons (Jimenez-Guerrero et al. 2017). At least 15 proteins are involved in the maturation, stability and activity of nitrogenase. Another eight proteins participate in the synthesis of FeMo cofactor (FeMo-co, containing iron and molybdenum used for transporting electron to molecular N_2) (Table 5.2). Additionally, electron donor and transport are necessary to provide electron to nitrogenase (Table 5.2).

The organisation of *nif* and *fix* genes of *Sinorhizobium meliloti* and *Bradyrhizobium japonicum* (now *B. diazoefficiens*) (Fischer 1994) is shown in Fig. 5.6. These nitrogen fixation genes are organised in distinct clusters whose structure and genomic location are species specific (Fig. 5.6) (Fischer 1994). For a detailed description of the organisation and location, refer to the review paper of Fischer (Fischer 1994).

5.1.3 Symbiosis-Related Functions: Exopolysaccharides, Secretion Systems and Others

Besides the genes directly related to the nodulation and nitrogen fixation mentioned above, there are many other genes or determinants in rhizobia that are involved in symbiosis (Table 5.3) (Shamseldin 2013; Liu et al. 2018b). Mutation of these genes

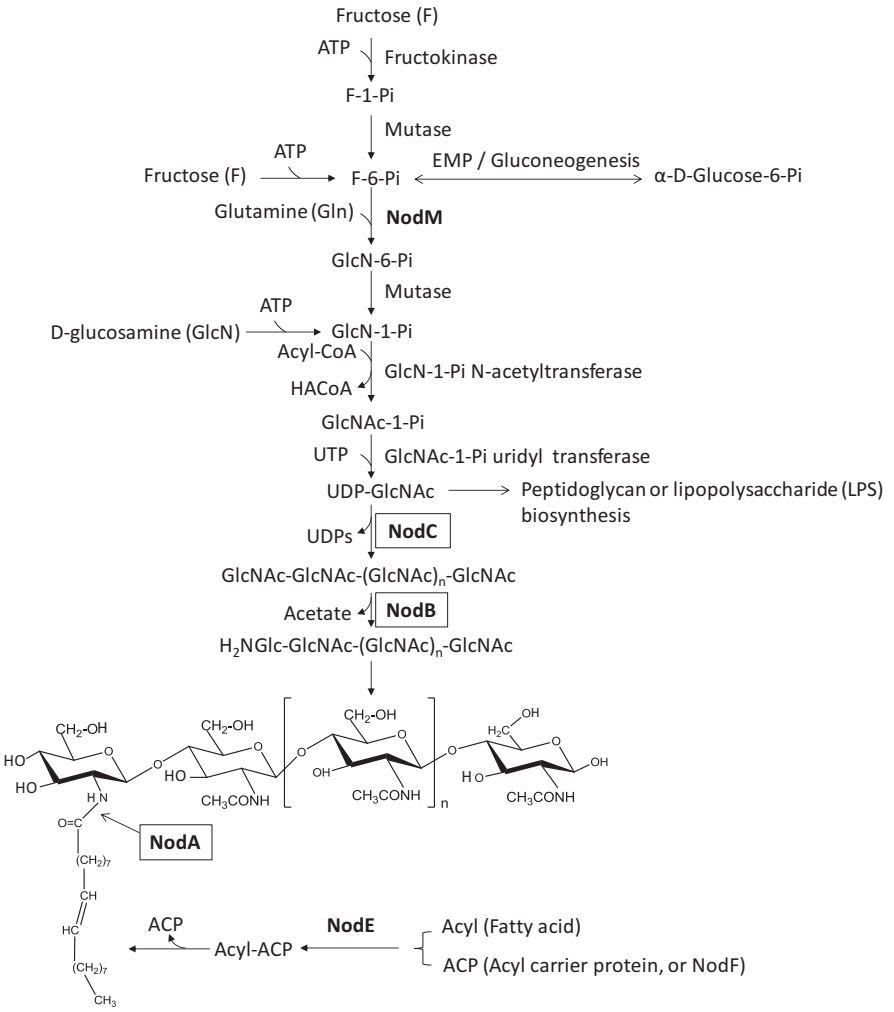


Fig. 5.3 The central pathway of basic LCO biosynthesis and the enzymes involved (Liu et al. 2018a)

in different rhizobia will lead to a change of nitrogen fixation efficiency or an alteration in specificity for host plants.

Mutation of genes related to the synthesis of exopolysaccharides (*exo*) in *Sinorhizobium meliloti* resulted in ineffective nodules on alfalfa containing no bacteroids (Leigh et al. 1985). MucR1, an ancestral zinc finger regulator, is essential for supporting nitrogen fixation of *Sinorhizobium fredii* CCBAU 45436 within soybean nodules and regulates the production of exopolysaccharides of this strain under free-living conditions (Jiao et al. 2016).

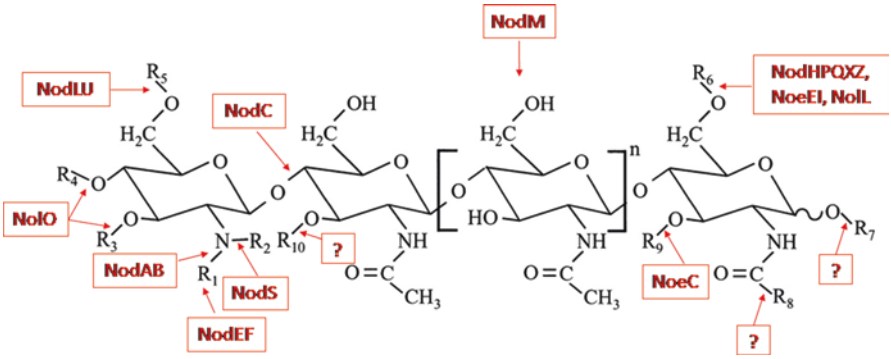


Fig. 5.4 Various substitutions (R_1–R_{10}) and enzymes (boxed) responsible for the synthesis and modification of LCOs produced by rhizobia (Revised based on D'Haeze and Holsters (2002)). R_1 = fatty acid acyl chain; R_2 = methyl (CH_3-) or hydrogen (H-); R_3 = H- or carbamoyl (NH_2CO-); R_4 = H-, NH_2CO- or acetyl (CH_3CO-); R_5 = H-, NH_2CO- or (CH_3CO-); R_6 = fucosyl, sulphate ester, H- or methyl fucosyl, etc.; R_7 = H- or mannosyl; R_8 = CH_3-, H- or $HOCH_2$-; R_9 = H-, arabinosyl or fucosyl; R_{10} = H-, fucosyl or acetyl. n = 0, 1, 2. The functions of enzymes are shown in Table 5.1

A: $N_2 + 8\,H^+ + 8\,e^- + 16\,ATP \rightarrow 2NH_3 + H_2 + 16\,ADP + 16\,Pi$

B:

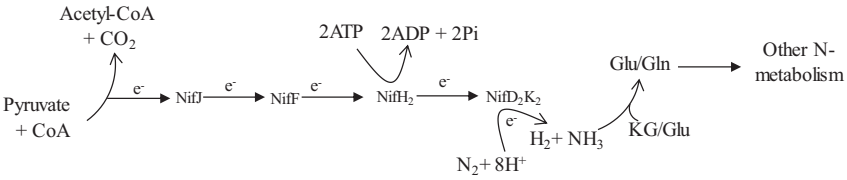


Fig. 5.5 Reaction and molecular mechanism of biological nitrogen fixation (Revised based on Kneip et al. (2007)). (**a**) General reaction of molecular nitrogen fixation. (**b**) Schematic structure and operation of the nitrogenase enzyme complex and subsequent metabolism of nitrogen. Functions of enzymes involved are listed in Table 5.2. KG = ketoglutarate; Glu = glutamate; Gln = glutamine

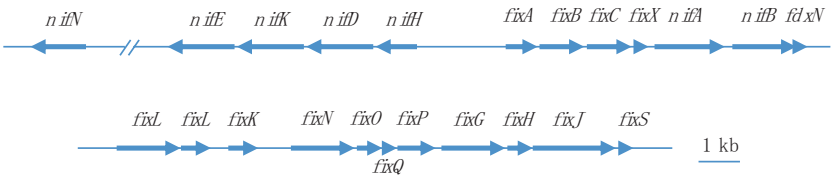
Different rhizobia use different secretion systems – type III, type IV and type VI – to transport effector proteins into host cells (Nelson and Sadowsky 2015). These secretion systems have an effect on rhizobial host specificity and the nodule number on legumes (Nelson and Sadowsky 2015). Abolition of type III secretion systems (TTSS or T3SS) can affect nodule formation in different ways, ranging from no effect to a reduction or an increase in nodule number (Marie et al. 2001). The proteins secreted through TTSS may induce or suppress plant defence responses and thereby prevent or increase symbiotic efficiency (Marie et al. 2001; Nelson and Sadowsky 2015). A T3SS mutant of *Bradyrhizobium elkanii* USDA61 could overcome nodulation restriction in a soybean variety carrying the *Rj4* allele, implying that the incompatibility is partly mediated by effector-triggered immunity (Faruque et al. 2015).

Table 5.2 Nitrogen-fixing-related genes in rhizobia (Shamseldin 2013; Fischer 1994)

Protein/ enzyme	Function
Main components of nitrogenase	
NifH	35 kDa dinitrogenase reductase, Fe protein. Obligate electron donor to dinitrogenase during dinitrogenase turnover. Also is required for FeMo-co biosynthesis and apodinitrogenase maturation
NifD	56 kDa α-subunit of dinitrogenase. Forms $\alpha_2\beta_2$ tetramer with β-subunit (NifK) interface. FeMo-co, the site of substrate reduction, is buried within the α-subunit of dinitrogenase
NifK	β-Subunit of dinitrogenase, 60 kDa
Regulation of nitrogenase activity	
NifA	Positive regulation element of *nif*, *fix* and additional genes
NifL	Negative regulatory element
NifM	Required for the maturation of NifH
NifW	Involved in stability of dinitrogenase. Proposed to protect dinitrogenase from O_2 inactivation
NifZ	Nitrogenase MoFe maturation protein
FixABCX	Required for nitrogenase activity
FixNOQP	Microaerobically induced, membrane-bound cytochrome oxidase
FixLJ	Oxygen-responsive two-component regulatory system involved in positive control of FixK and NifA (Fischer 1994)
FixK/FixK2	Positive regulator of FixNOQP, NifA, RpoN and "nitrate respiration"; negative regulator of NifA and FixK (Fischer 1994)
NfrA	Regulation of NifA (Fischer 1994)
FeMo-co and Fe-S cluster synthesis	
NifB	Required for FeMo-co synthesis. Metabolic product. NifB-co is the specific Fe and S donor to FeMo-co
NifE	40 kDa forms $\alpha_2\beta_2$ tetramer with NifN, required for FeMo-co synthesis
NifN	50 kDa, required for FeMo-co synthesis
NifS	Involved in mobilisation of S for Fe-S cluster synthesis and repair
NifQ	Involved in FeMo-co synthesis. Proposed to function in early MoO_4^{2-} processing
NifU	Involved in mobilisation of FeMo-co cluster synthesis and repair
NifV	Homocitrate synthesis involved in FeMo-co synthesis
NifX	Involved in FeMo-co synthesis
Electron transport	
NifF	17 kDa flavodoxin, electron donor to NifH
NifJ	120 kDa, pyruvate flavodoxin (ferredoxin) oxidoreductase involved in electron transport to nitrogenase
FdxN	Ferredoxin serves as electron donor to nitrogenase

Mutation of several specific genes (Table 5.3) involved in metabolic pathways, transporters, chemotaxis and mobility in strain *B. diazoefficiens* USDA 110 can change its host range from soybean to *Sophora flavescens*, a promiscuous legume (Liu et al. 2018b). In addition, the nitrogen efficiency of these mutants inoculated on soybean decreased to some extent (Liu et al. 2018b).

A. *Sinorhizobium meliloti*



B. *Bradyrhizobium japonicum* (now *B. diazoefficiens*)

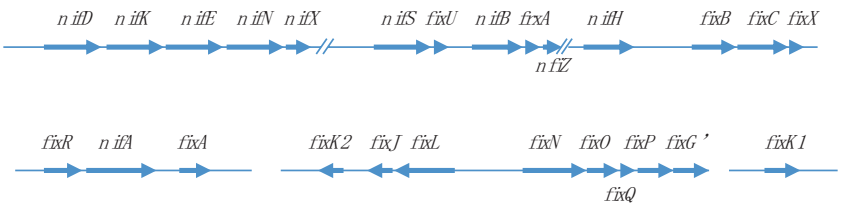


Fig. 5.6 Organisation of *nif* and *fix* gene clusters in *S. meliloti* (**a**) and *B. japonicum* (now *B. diazoefficiens*) (**b**) (Fischer 1994)

5.2 Phylogenetic Diversity of Symbiosis Gene *nodC*

5.2.1 Phylogenetic Diversity of the Nodulation Gene nodC

As described in Sect. 5.1 of this chapter, the *nodC* gene, as well as other common genes, is conserved in all symbiotic rhizobia except some bradyrhizobia associated with *Aeschynomene*. The presence or not of the common genes is the essential characteristic of symbiotic rhizobia. Besides, phylogenetic positions and genetic diversity of *nodC* genes in rhizobia can reflect the host specificity and host range to some extent.

5.2.1.1 Specific Legumes and Rhizobia Bearing Highly Distinct *nodC* Genes

Some legumes only select specific rhizobia (or symbiotic varieties, abbr. sv.) with highly conserved and distinct *nodC* gene sequences. Common examples of these kinds of legumes include chickpea (*Cicer arietinum*), Chinese milk vetch (*Astragalus sinicus*), *Amorpha fruticosa* and *Trifolium* spp.

Four species, first *Mesorhizobium ciceri* (Nour et al. 1994) and *M. mediterraneum* (Nour et al. 1995), which were described as *Rhizobium* before the genus *Mesorhizobium* was created (Jarvis et al. 1997), and more recently *M. muleiense* (Zhang et al. 2012) and *M. wenxiniae* (Zhang et al. 2018a), were isolated from root

Table 5.3 Symbiosis-related genes other than those mentioned in Tables 5.1 and 5.2

Gene	Function	Gene	Function
hsn	Host specificity nodulation	*iol*	Inositol catabolism (competitiveness)
gsn	Genotypic-specific nodulation	*tfx*	Trifolitoxin (competitiveness)
exo	Exopolysaccharides	*ppsA*(blr4655)[a]	Phosphoenolpyruvate synthase
hup	Hydrogen uptake	blr3848[a]	Hypothetical membrane protein
gln	Glutamine synthase	bll6035[a]	Hypothetical protein
dct	Dicarboxylate transport	*flgE*(bll5854)[a]	Flagellar hook-basal body protein
nfe	Nodulation formation efficiency	bll0096[a]	Chemotaxis protein
ndv	β-1,2-glucans	*qor*(bll1503)[a]	NAD(P)H-quinone oxidoreductase
lps	Lipopolysaccharide	blr3961[a]	NAD(FAD)-utilising dehydrogenase
bacA	Bacteroid development	bll2373[a]	Hypothetical protein
tts	Type III secretion system	blr0767[a]	SH3-like domain-containing protein
virB	Type IV secretion system	*corA*(blr2622)[a]	Magnesium transporter
pur	Purine biosynthesis	*ivd*(bll7899)[a]	Isovaleryl-CoA dehydrogenase (IVD)
rosR/ mucR	C_2H_2 zinc finger-bearing transcriptional regulator[b]	*dnaC*(bll4072)[a]	Replicative DNA helicase
acds/rtx	Inhibition of plant ethylene biosynthesis	*moc*	Rhizopine catabolism (competitiveness)
ntrX	Regulator of succinoglycan production and motility[c]		

Note: The data are cited from (Shamseldin 2013) except as indicated
[a]Mutation of these genes in *Bradyrhizobium diazoefficiens* USDA 110 led to inefficient nitrogen fixation in soybean (Liu et al. 2018b)
[b]Reference: Jiao et al. (2016)
[c]Reference: Wang et al. (2013a)

nodules of chickpea, but certain isolates of several other species, including *M. tianshanense*, *M. amorphae* (Rivas et al. 2007) and *M. opportunistum* (Laranjo et al. 2012), also nodulate chickpea. All these chickpea symbionts have highly similar *nodC* gene sequences, indicating that a single symbiovar, sv. *ciceri*, has been transferred among multiple species. Detailed discussion of chickpea mesorhizobia and *nodC* gene phylogeny can be found in Chap. 7 of this book.

Astragalus sinicus is another highly specific legume. It differs from other nodulating species of this genus in that it is only nodulated by mesorhizobia (*M. huakuii*, *M. qingshengii* and *M. jarvisii* sv. *astragali*) that have a specific and conserved *nodC* gene sequence (Zhang et al. 2018b), as seen in the phylogenetic tree (Fig. 5.7). The majority of isolates from root nodules of *A. sinicus* grown in acidic soils of Xinyang, central China, were classified as *M. jarvisii* (Zhang et al. 2018b). The *nodC* genes of these isolates were almost identical to the *nodC* genes in previously

described *A. sinicus* mesorhizobia in *M. huakuii* and *M. qingshengii* (Zhang et al. 2018b) and different from that of the type strain (ATCC 33669^T) of *M. jarvisii*, which was isolated originally from *Lotus corniculatus*. Therefore, a novel symbiotic variety *M. jarvisii* sv. *astragali* was proposed (Zhang et al. 2018b). The highly conserved *nodC* genes among these different mesorhizobia provide more evidence for lateral gene transfer in rhizobia and high selection pressure by the host legume.

No other sequences were found to be close to the *nodC* gene sequence of *M. amorphae* type strain ACCC 19665^T associated with *Amorpha fruticosa* (Fig. 5.7). No evidence was obtained that this strain ACCC 19665^T could form symbioses with any other host plant except for its host plant *A. fruticosa*, further confirming the specific symbiosis between *A. fruticosa* and *M. amorphae* in both China and America (Wang et al. 1999, 2002). Even the promiscuous legume *Sophora flavescens* could not be nodulated by this specific rhizobial strain ACCC 19665^T (Jiao et al. 2015a).

Various *Mesorhizobium* species were isolated from *Caragana* spp., shrubby legumes mainly growing in the arid and semi-arid regions of Asia and Eastern Europe. Analyses of the *nodC* genes of these different *Caragana*-associated mesorhizobia showed that they had more than 93% sequence similarity (Chen et al. 2008). In addition, the *nodC* genes of these mesorhizobia showed close phylogenetic relationship with those of other rhizobia isolated from legumes belonging to same tribe Galegeae (Ji et al. 2015). Selection of distinct *nodC* types of different mesorhizobia by *Caragana* spp. was also demonstrated previously by Li et al. (Li et al. 2012). For further details on the symbiotic relationship between *Caragana* and different rhizobia, the reader should refer to Chap. 8 of this book.

Another specific symbiosis with evidence for selection pressure by legumes is the partnership between various mesorhizobia and various endemic species of *Sophora* growing in New Zealand. All the rhizobia from *Sophora* growing there belong to *Mesorhizobium* and bear highly similar *nodC* (and *nifH*) gene sequences (Nguyen et al. 2017). This is very different from the very diverse rhizobia of multiple genera isolated from *Sophora flavescens* grown in different regions in China (Fig. 5.7) (Jiao et al. 2015a). However, the two species, *M. cantuariense* and *M. waimense*, isolated from *Sophora* spp. in New Zealand, had high *nodC* gene sequence similarities to those of some of the mesorhizobia isolated from China (Fig. 5.7). Therefore, the *nodC* gene corresponding to *Sophora* mesorhizobia in New Zealand and China may have a common origin. Detailed discussion of *Sophora* rhizobia can be found in Chap. 7 of this book.

Other specific rhizobial species/symbiotic variety (sv.) symbioses include *Galega officinalis* (*Neorhizobium galegeae* sv. *officinalis*), *Galega orientalis* (*Neorhizobium galegeae* sv. *orientalis*), *Hedysarum coronarium* (*Rhizobium sullae*), *Medicago laciniata* (*Sinorhizobium/Ensifer meliloti* sv. *medicaginis*), *Medicago rigiduloides* (*Sinorhizobium/Ensifer meliloti* sv. *rigiduloide*s) and *Trifolium ambiguum* (*Rhizobium leguminosarum* sv. *trifolii*) (Andrews and Andrews 2017). These rhizobial symbiotic varieties possess distinct *nodC* genes different from other rhizobia (Fig. 5.7), and they do not cross-nodulate with other legumes.

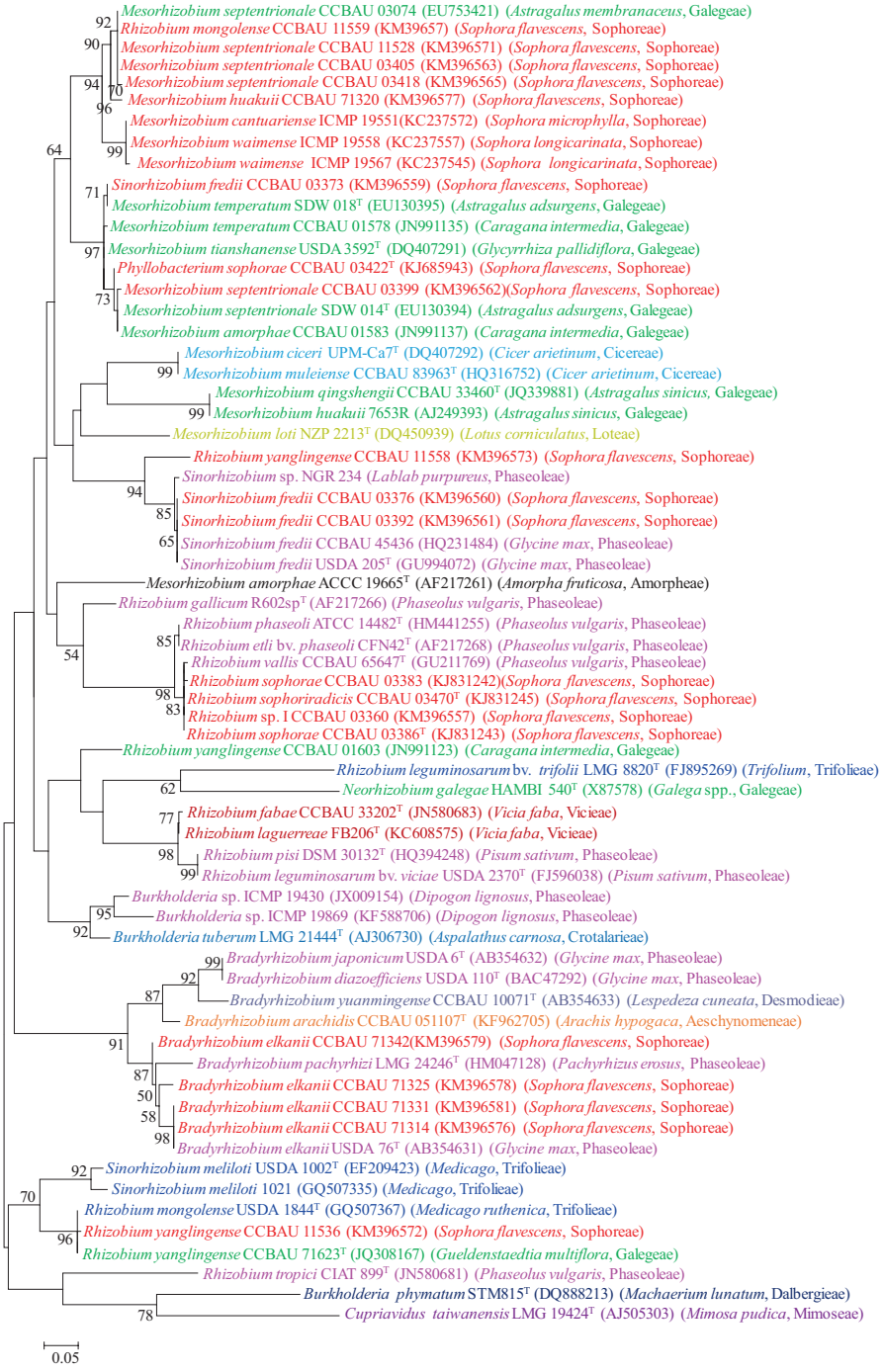


Fig. 5.7 Maximum likelihood phylogenetic tree based on *nodC* genes (Revised from Jiao et al. (2015a)). The model T92+G+I was used to construct the tree. Bar, 5% nucleotide substitution per site. Strains isolated from same tribe of plants were printed in same colour

Gene transfers laterally among different genera and species in rhizobia are common. Identical *nodC* genes were found among different strains of *Mesorhizobium septentrionale* and *Rhizobium mongolense* CCBAU 11559, in *Sinorhizobium fredii* CCBAU 03373 and *Mesorhizobium temperatum* SDW 018^T (Fig. 5.7) (Jiao et al. 2015a). The *nodC* gene was apparently also transferred into the *Aminobacter* strain BA135 from *Mesorhizobium* (Estrella et al. 2009). Gene transfers among different rhizobia are further discussed in Chap. 6 of this book.

5.2.1.2 Promiscuous Legumes and Highly Diverse *nodC*-Gene-Bearing Rhizobia

Besides the specific symbioses between specific legumes and certain rhizobial species with distinct *nodC* genes, mentioned above, there are many non-specific or promiscuous legumes that can be nodulated by various rhizobia bearing different *nodC* genes. Legumes of this kind include soybean (*Glycine max*) (Zhang et al. 2011), wild soybean (*Glycine soja*) (Wu et al. 2011), *Sophora flavescens* (Jiao et al. 2015a) and *Sophora alopecuroides* (Zhao et al. 2010), common bean (*Phaseolus vulgaris*) (Wang et al. 2016; Laguerre et al. 2001), *Astragalus* spp. (Zhao et al. 2008), *Caragana* spp. (Lu et al. 2009; Yan et al. 2017), peanut (*Arachis hypogaea*) (Chen et al. 2016), *Centrosema* (Ramírez-Bahena et al. 2013), *Lotus* spp. (Estrella et al. 2009; Lorite et al. 2018; Sullivan et al. 1996) and others.

Soybean and Its Rhizobia

Soybean can be nodulated by two genera of rhizobia, *Bradyrhizobium* and *Sinorhizobium* (syn. *Ensifer*) (Zhang et al. 2011; Tian et al. 2012). Phylogeny of the *nodC* genes of these soybean rhizobia assigned them to three branches: I to III (Fig. 5.8). Branches I and II include several species in the genera *Sinorhizobium/*

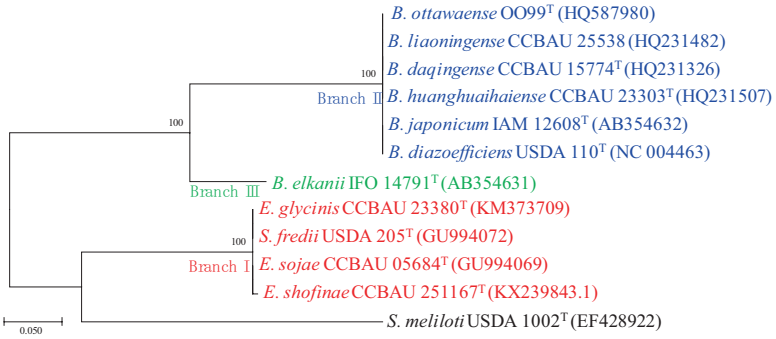


Fig. 5.8 Maximum likelihood phylogenetic tree based on *nodC* genes of soybean rhizobia. The tree was constructed based on the Kimura two-parameter model using Mega 7 software. Bar, 5% nucleotide substitution per site. *Sinorhizobium* (*S.*) *meliloti* USDA 1002^T was used as an outgroup. T in superscript, type strain. Bootstraps over 50 are shown at each branch node

Ensifer and *Bradyrhizobium*, respectively. Different species within each of these two branches (I and II) have identical or almost identical *nodC* gene sequences. Only *Bradyrhizobium elkanii* has distinctly different *nodC* gene sequences, belonging to Branch III, which are not close to the other bradyrhizobia and fast growers in *Sinorhizobium/Ensifer* (Fig. 5.8).

The identical LCOs secreted by different soybean-nodulating *Sinorhizobium* spp. (Wang et al. 2018; Bec-Ferte et al. 1994), *B. diazoefficiens* (formerly *B. japonicum*) USDA 110 and *B. elkanii* USDA 61 (Liu et al. 2018b; Sanjuan et al. 1992; D'Haeze and Holsters 2002) may allow these rhizobia to have a common host plant, soybean, despite their distinct *nodC* gene sequence and phylogenetic position. All the LCOs of soybean rhizobia have a common substituent group (2-*O*-methyl fucosyl) on the reducing terminal (D'Haeze and Holsters 2002), though the deletion of this residue does not affect nodulation on soybean or on the promiscuous legume *Sophora flavescens* (Liu et al. 2018b).

Sophora and Its Rhizobia

Comparably, the promiscuous legumes *S. flavescens* and *S. alopecuroides* can be nodulated by more than five genera of rhizobia (Jiao et al. 2015a; Zhao et al. 2010) (detailed discussion can be found in Chaps. 7 and 8 of this book). Various rhizobia bearing dissimilar *nodC* gene sequences and originating from different cross-nodulation groups can effectively nodulate *S. flavescens* (Fig. 5.7) (Jiao et al. 2015a). Mutants of the *nodC* gene in different representative rhizobial species failed to nodulate either *S. flavescens* or their usual host plants, demonstrating the indispensability of the *nodC* gene or the Nod factor in launching root nodule formation (Liu et al. 2018a). Furthermore, abolition of Nod factor-decorative genes did not change nodulation activity, although it did decrease or increase N_2-fixing efficiency (Liu et al. 2018a).

Surprisingly, although identical Nod factors were produced by *S. fredii* CCBAU 45436 and *B. diazoefficiens* USDA 110 and they had common host range, the latter could not nodulate *S. flavescens* (Jiao et al. 2015a). Several mutants were selected from a Tn5 library of USDA 110, and they altered the host range from soybean to *S. flavescens* (Liu et al. 2018a, b). However, these mutated genes were not related directly to the structural genes of Nod factor synthesis but were involved in metabolic pathways, transporters, chemotaxis and mobility (Liu et al. 2018b). These mutants may have lost their immunostimulation of the *S. flavescens* plant, so that they were allowed to enter the nodule cells and form functional nodules.

Common Bean (*Phaseolus vulgaris*) and Its Rhizobia

Common bean (*Phaseolus vulgaris*) is another promiscuous legume that can be nodulated mainly by species in genus of *Rhizobium*, including *R. etli* (Segovia et al. 1993), *R. leguminosarum* (García-Fraile et al. 2010; Mulas et al. 2011), *R. lusitanum*

(Valverde et al. 2006), *R. gallicum* and *R. giardinii* (Amarger et al. 1997), *R. phaseoli* (Ramírez-Bahena et al. 2008), *R. tropici* (Amarger et al. 1994; Martinez-Romero et al. 1991), *R. leucaenae* (Ribeiro et al. 2012), *R. paranaense* (Dall'Agnol et al. 2014), *R. vallis* (Wang et al. 2011) and *R. sophoriadicis* (Ormeño-Orrillo et al. 2018; Jiao et al. 2015b). Additionally, minor isolates in genera of *Agrobacterium* (Wang et al. 2016), *Bradyrhizobium* (Cao et al. 2014), *Ensifer* (Wang et al. 2016) and non-nodulating *Phyllobacterium* (Flores-Félix et al. 2012) were reported to be isolated from root nodules of *P. vulgaris* grown in China, Mexico and Spain.

The phylogenetic pattern of *nodC* genes of different *P. vulgaris*-nodulating rhizobia is highly host-specific and mainly consisted of two clusters: I and VI, corresponding to symbiovar (sv.) *phaseoli* and sv. *tropici*, respectively (Fig. 5.9). Some strains (Y21, SX1660, SX1597, SX1647 and SX1555) in clusters V and VII had *nodC* genes highly similar (even identical) to those of soybean-nodulating *Bradyrhizobium* and *Sinorhizobium/Ensifer* bacteria (Fig. 5.9), suggesting gene lateral transfers coming different rhizobial species. Three clusters (II, III and IV) were distinct and far from the two clusters I and VI (Fig. 5.9).

In the cluster sv. *phaseoli*, two species of *R. sophorae* and *R. sophoriradicis* isolated from *Sophora flavescens* had highly similar (and even identical) *nodC* genes to those strains isolated from *P. vulgaris* (Fig. 5.9). Cross-nodulation demonstrated that these two species could induce effective nodules on *P. vulgaris* and their original host plant (Jiao et al. 2015b), further indicating the coevolution of *nodC* gene in *P. vulgaris*-rhizobia and the host plant (Aguilar et al. 2004).

The conservation of *nodC* gene sequence in sv. *phaseoli* and sv. *tropici* and the selection pressure from *P. vulgaris* on their rhizobial *nodC* genes were supported by the identical *nodC* sequence possessed by different *Rhizobium* species distributed around the world (Fig. 5.9).These events suggest that these *nod* genes in *Rhizobium* spp. sv. *phaseoli* and sv. *tropici* evolved from their respective common ancestors.

Other Promiscuous Legumes

The promiscuous legume genera *Caragana* and *Astragalus* and their various rhizobia are discussed in Chap. 7 of this book. Though great diversity was observed in the nodulation genes (*nodA*, *nodC*, *nodD*, *nodG*, *nodP*) of *Caragana-Astragalus-Glycyrrhiza*-nodulating rhizobia and most representative strains presented unique nodulation gene types, they all clustered in a large group (Ji et al. 2015; Chen et al. 2008). The type strains for species *Mesorhizobium metallidurans*, *M. amorphae* and *M. mediterraneum*, isolated from *Anthyllis*, *Amorpha* and *Cicer*, respectively, formed three deep branches deviating from the strains isolated from the genera *Caragana, Astragalus*, *Glycyrrhiza* and *Oxytropis*, all belonging to Tribe Galegeae (Ji et al. 2015). This point suggested a consanguineous affiliation of rhizobial nodulation genes in the rhizobia nodulating with the same legume genus or tribe (Li et al. 2012). In addition, effects of geographic isolation on the divergence of the nodulation genes have been observed (Ji et al. 2015).

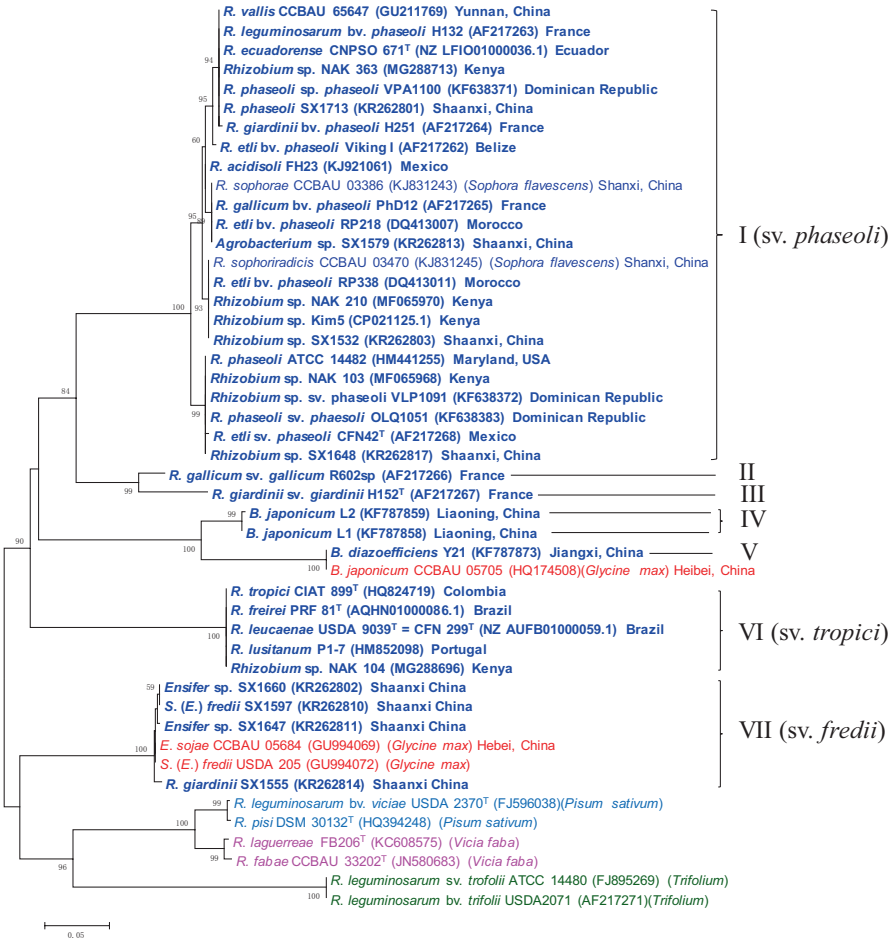


Fig. 5.9 Neighbour-joining (NJ) phylogenetic tree based on *nodC* genes of rhizobia mainly isolated from root nodules of common bean (*Phaseolus vulgaris*). Rhizobia isolated from common bean are shown in blue and boldface. Rhizobia from plants other than common bean are printed in other colours. T in superscript, type strain. Bootstraps over 50 are shown at each branch node. Bar, 5% nucleotide substitution per site

A comparison of the genome of *Rhizobium yanglingense* strain CCBAU 01603 with those of *Caragana-Astragalus*-nodulating *Mesorhizobium* spp. led to the interesting observation that these rhizobia had evolutionarily conserved *nodE*, *nodO*, T1SS and hydrogenase systems, allowing them to have common host ranges (Yan et al. 2017).

Peanut (*Arachis hypogaea*) is another promiscuous legume that can be nodulated by different rhizobial species, although all the effective peanut rhizobia belong to slow-growing *Bradyrhizobium* (see Chap. 7). Comparison of *nodC* (and *nodA*) sequences also indicated the high diversity of peanut isolates (Santos et al. 2017;

Chen et al. 2016). The *nodC* genes of different *B. arachidis* strains are not completely identical, and strain CCBAU 33067 is far from the other three strains (Fig. 5.10) (Wang et al. 2013b). Surprisingly, two bradyrhizobial species, *B. guangdongense* CCBAU 51649 and *B. guangxiense* CCBAU 53363, which were isolated from peanut grown in different provinces, had completely identical and distinct *nodC* genes occupying a separate branch in the phylogenetic tree (Fig. 5.10), suggesting the independent origin of their *nodC* genes. Phylogenetic analyses based on 16S rRNA genes and housekeeping genes of these two peanut bradyrhizobial species confirmed the dissimilarities between *B. guangdongense* and *B. guangxiense*, and they differ from other known species (Li et al. 2015).

One strain, CCBAU 23160, isolated from a peanut nodule, had *nodC* and *nifH* genes identical to those of the type strain of *B. lablabi* CCBAU 23086^{T}, suggesting that these two strains may have the same host spectrum (Chang et al. 2011).

Like peanut, *Centrosema* is also a promiscuous legume nodulated by various *Bradyrhizobium* species (Ramírez-Bahena et al. 2013). The *nodC* genes of the strains associated with *Centrosema* spp. were also divergent among themselves and found in different branches (Fig. 5.10) (Ramírez-Bahena et al. 2013).

From the extensive literature, we see that *Lotus* rhizobia are dispersed among nearly 20 species in 5 genera (*Mesorhizobium*, *Bradyrhizobium*, *Rhizobium*, *Ensifer*/*Sinorhizobium* and *Aminobacter*) (Lorite et al. 2018). However, the majority of the *Lotus tenuis* isolates appeared to be in the genus *Mesorhizobium*, with some in *Rhizobium* (Estrella et al. 2009). All the mesorhizobia from *Lotus tenuis* had *nodC* genes similar to narrow host range strains of *Mesorhizobium japonicum* MAFF303099^{T} and R7A but far from broad host range strain *M. loti* NZP2037 (Estrella et al. 2009). *Aminobacter aminovorans* strain BA135 was first isolated from *L. tenuis*, but it had a *nodC* gene sequence identical to those of some *Mesorhizobium* species, suggesting lateral transfer between the genera (Estrella et al. 2009).

5.3 Concluding Remarks and Perspectives

The existence of nodulation genes (*nodC* and others) is an essential feature of almost all symbiotic rhizobia. Specific legumes prefer distinct *nodC*-bearing rhizobia for their partners. The *nod* genes are often tightly linked in the genome, and they can be located on transmissible elements such as plasmids in many fast-growing rhizobia or transposon-like elements in *Mesorhizobium loti*. The sequence and phylogeny of *nodC* gene is a good molecular marker for the rhizobial host plant range, and this is determined by strong selection by the host plant.

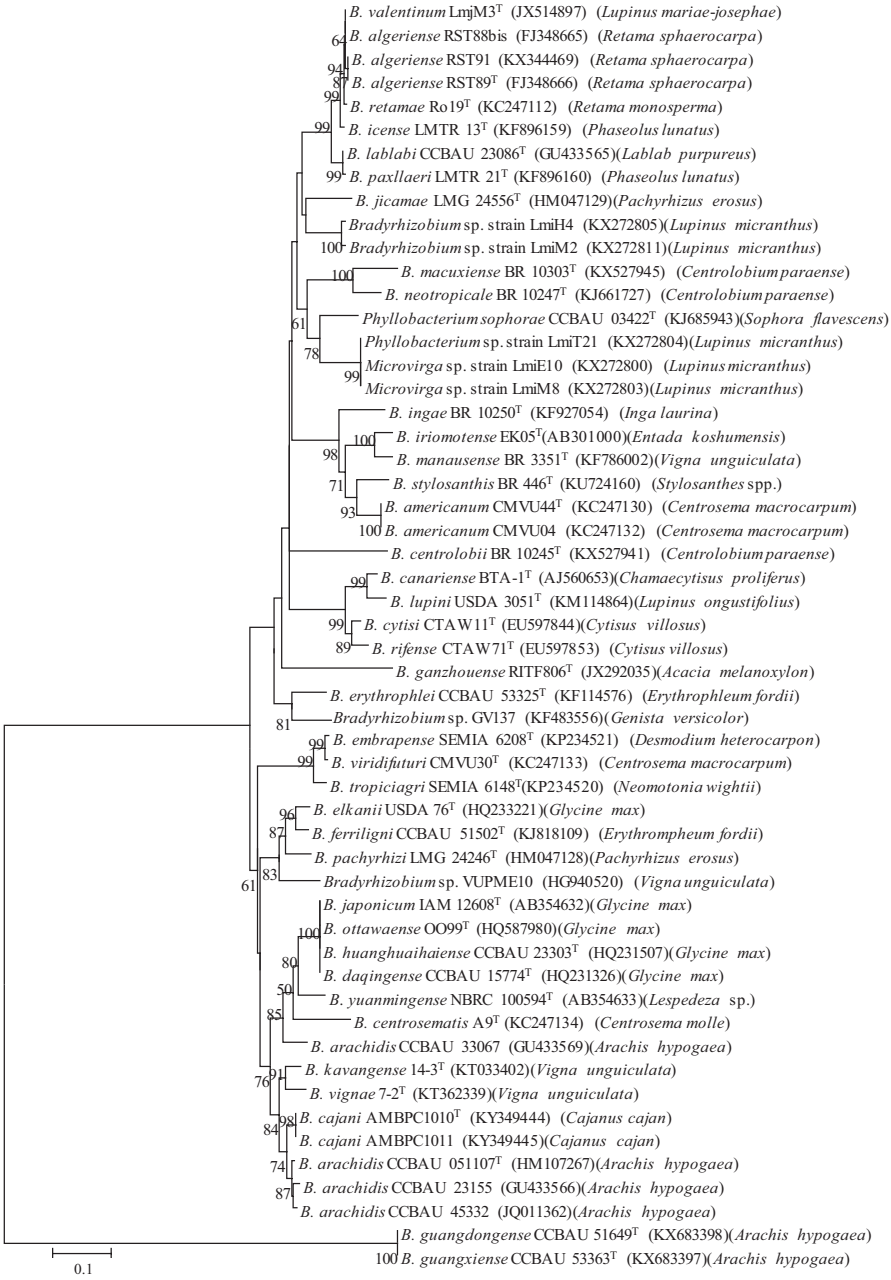


Fig. 5.10 NJ phylogenetic tree based on *nodC* genes of bradyrhizobia. T in superscript, type strain. Bootstraps over 50 are shown at each branch node. Bar, 1% nucleotide substitution per site. GenBank accession No. and rhizobial host plants were shown after the strain numbers

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Chapter 6
Evolution of Symbiosis Genes: Vertical and Horizontal Gene Transfer

6.1 The Origin and Transfer of Symbiosis Genes

Conserved nitrogen fixation genes *nifHDK* and *nifENB* have been found in 14 phyla from either bacteria or archaea (Dos Santos et al. 2012), while nodulation function is restricted to *Alphaproteobacteria* and *Betaproteobacteria*. Consequently, nitrogen fixation is proposed to predate nodulation function. Although *nif* genes can be co-transferred with *nod* genes among rhizobia (Sullivan et al. 1995; Sullivan and Ronson 1998), the symbiosis-specific *nif* genes can have a close relationship with homologues from non-symbiotic loci or non-rhizobial strains of the same genus (Bontemps et al. 2010; Okubo et al. 2016), indicating potential replacement of transferred *nif* genes by indigenous ones. Moreover, the flavonoid-induced transfer of a symbiosis island harbouring *nod* but not *nif* genes from *Azorhizobium caulinodans* to other rhizobia has been reported (Ling et al. 2016). Therefore, the evolutionary history of nitrogen fixation genes can be disassociated from that of nodulation genes (Fig. 6.1).

The origin of *nod* remains elusive. Genome sequencing showed that genes homologous to canonical rhizobial *nodABC* and *nodH* are present in certain isolates of cultivated *Frankia* and these *Frankia nod* genes are more deeply rooted than those from alpha- and beta-rhizobia (Persson et al. 2015; Nguyen et al. 2016; Ktari et al. 2017). Moreover, the *nodC* gene from *Frankia* Dg1 can partially complement a *nodC* mutant of *Rhizobium leguminosarum* A34 (Persson et al. 2015). These findings imply that the ancestors of these *Frankia* versions could be candidates for the origin of *nod* genes of rhizobia. Notably, *nod* genes are only widespread in *Frankia* associated with poorly studied *Ceanothus americanus*, and most actinorhizal plants are possibly infected through the nod-independent pathway (Ktari et al. 2017). In line with their rhizobial homologues, *nod* genes in *Frankia* are of lower GC content than the genome average (Ktari et al. 2017). The potential role of GC content of *nod* genes in functional adaptation was highlighted by a study of *nodI*

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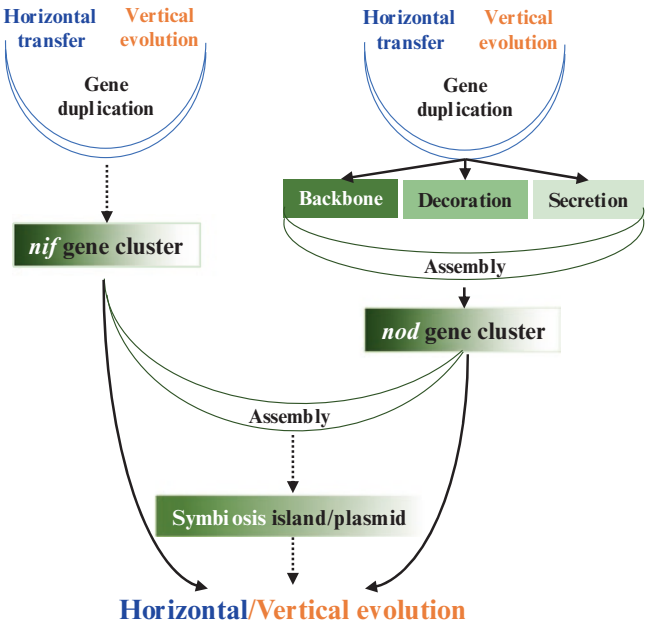

Fig. 6.1 Simplified evolutionary model of key symbiosis genes. Genes involved in backbone synthesis, decoration and secretion of Nod factors can have different origins and are postulated to be assembled into a gene cluster named *nod*. The *nod* and *nif* gene clusters can be further assembled in a symbiosis island or symbiosis plasmid. The *nod* and *nif* genes can undergo either distinct or similar evolutionary history as implied by the observed diversity of these symbiosis genes in extant rhizobial germplasms and experimental evolution studies. Refer to the main text for more details

and *nodJ* involved in the secretion of Nod factors (Aoki et al. 2013). DRA-ATPase/permease genes coexist with their paralogous *nodIJ* genes in beta-rhizobia but were not found in alpha-rhizobia, and the evolutionary rates of *nodIJ* were accelerated relative to their homologues regarding GC content rather than nonsynonymous/synonymous substitutions or transition/transversion rates (Aoki et al. 2013). This finding also suggests that *nodIJ* may have originated in *Betaproteobacteria* via gene duplication and then been transferred into *Alphaproteobacteria* (Aoki et al. 2013).

Given the great diversity of alpha- and beta-rhizobia (Peix et al. 2015), it has been hypothesised that horizontal transfer of key symbiosis genes between bacteria has largely accounted for an observed expanding collection of rhizobial germplasms (Masson-Boivin et al. 2009; Remigi et al. 2016). Under lab conditions, plant flavonoids can induce the transfer of an integrative and conjugative element harbouring *nod* genes from *Azorhizobium caulinodans* to certain strains of *Mesorhizobium* and *Sinorhizobium* but not *Rhizobium* or *Bradyrhizobium* (Ling et al. 2016). There is also evidence of horizontal transfer of symbiosis genes under field conditions from *Bradyrhizobium japonicum* inoculant to indigenous

Sinorhizobium fredii (Barcellos et al. 2007). It should be noted that transfer events across different genera are rare in rhizobial germplasms. It is more commonly found that different species of the same genus can have closely related key symbiosis genes and form effective nodules on the same legume host, such as those *Rhizobium* spp. nodulating *Pisum sativum* and *Vicia faba* (Tian et al. 2010; Kumar et al. 2015; Xiong et al. 2017) and *Bradyrhizobium* strains associated with soybeans (Tian et al. 2012; Zhang et al. 2014). Although both *Bradyrhizobium* and *Sinorhizobium* strains have been recurrently identified as microsymbionts of soybeans, the evolutionary history of key symbiosis genes is distinct between the two genera (Tian et al. 2012; Zhang et al. 2014; Guo et al. 2014). It is also reported that symbiosis genes of *Mimosa* microsymbionts form deep *Burkholderia*-specific clades corresponding to individual species complex without substantial horizontal gene transfer between species complexes (Bontemps et al. 2010). Therefore, the extant diversity of symbiosis genes is shaped by both horizontal and vertical evolutionary processes depending on both genomic backgrounds of rhizobia and the associated legume species.

6.2 Integration of Symbiosis Genes with Genomic Backgrounds

Experimental evolution studies performed in the lab of Catherine Masson-Boivin suggest that receiving key symbiosis genes does not guarantee a pathogenic *Ralstonia solanacearum* strain an effective symbiosis with legumes (Marchetti et al. 2010). Inactivation and recruitment of lineage-specific virulence functions in the *R. solanacearum* recipient are required for efficient nodulation and intracellular infections (Marchetti et al. 2010; Guan et al. 2013; Capela et al. 2017), though the evolved clones obtained so far were not able to fix nitrogen. Genetics studies on rhizobial strains associated with diverse legumes showed that many conserved or lineage-specific functions are involved in optimising the symbiotic efficiency of rhizobia (Mao et al. 2005; Amadou et al. 2008; Masson-Boivin et al. 2009). For example, Aap/Bra involved in importing branched amino acids are essential for efficient symbiosis of *Rhizobium leguminosarum* associated with pea or common bean but dispensable in the *Sinorhizobium meliloti-Medicago* symbiosis (Lodwig et al. 2003; Prell et al. 2009, 2010). The conserved high-affinity phosphate transporter Pst is required for symbiosis of *S. fredii* within soybean nodules but unnecessary for *S. meliloti* within *Medicago* nodules (Yuan et al. 2006; Hu et al. 2018). The *exo* genes involved in biosynthesis of exopolysaccharides are essential for infection of *S. meliloti* (Leigh 1985; Gonzalez et al. 1996), but no orthologs of these genes were found in other rhizobial genera (Amadou et al. 2008; Tian et al. 2012). A T3SS and its effector proteins determine compatibility of *Sinorhizobium*

strains associated with different soybean cultivars, whereas they are absent in most strains of *S. meliloti* and *S. medicae* nodulating *Medicago* (Sugawara et al. 2013). More recently, comparative genomics provided a general picture of the phyletic distribution of symbiosis-related genes in alpha- and beta-rhizobia (Tian et al. 2012), showing that the clustering patterns based on the presence and absence of these symbiosis-related genes can reflect the species phylogeny of rhizobia. These studies imply that integration of key symbiosis functions (nodulation and nitrogen fixation) is a lineage-specific process. Different genomic backgrounds can provide distinct support to make the installed "symbiosis app" work.

As mentioned above, most genetic evidence of the differential role of core genes and involvement of lineage-specific genes came from model strains associated with different legume hosts. However, the major obstacles restricting the efficiency of rhizobial inoculants involve competitive nodulation and variation in efficiency of nitrogen fixation among closely related strains associated with the same host (Brockwell and Bottomley 1995). Although the underlying mechanisms have not been well addressed, limited evidence supports a strain-specific integration between key symbiosis genes and genomic backgrounds harbouring both core and accessory functions.

The T3SS is conserved in *Sinorhizobium* strains nodulating soybeans but can be either a positive or negative player in nodulation (Jimenez-Guerrero et al. 2015; Zhao et al. 2018), implying a potential variation in the pool of effector proteins among different strains. It was recently reported that variation in the effector NopP can account for the contrasting compatibility of different *Bradyrhizobium* strains associated with soybeans that have the *Rj2* gene (Sugawara et al. 2018). In a more extreme example, *Bradyrhizobium elkanii* USDA61 uses a T3SS to activate soybean nodulation signalling by bypassing Nod factor recognition (Okazaki et al. 2013). Moreover, the T3SS structural genes, the positive regulator gene *ttsI* or the effector gene *nopP* can be inactivated in *Sinorhizobium* strains by parallel transpositions of insertion sequences (ISs) that are enriched on the symbiosis plasmid where the T3SS gene cluster is located, and these resultant clones can extend their host range to a commercial soybean cultivar of *rj2(Rfg1)* genotype (Zhao et al. 2018). These ISs were more efficient than introduced Tn*5* or point mutation (Zhao et al. 2018), indicating that these abundant "selfish" or "junk" elements on the symbiosis plasmid can be evolutionarily important in the adaptive evolution of rhizobia. In this way, without genetic manipulation, incompatible *Sinorhizobium* strains can evolve into compatible microsymbionts of soybeans. This can be very important for ecological success of rhizobia under field conditions and in wild ecosystems in the long run. In addition to receiving *nod* genes from rhizobial inocula, indigenous strains of poor nitrogen fixation ability may also evolve in this way and to some extent interfere with the competitive nodulation of introduced rhizobial inocula.

The pangenome of rhizobia is highly flexible partially due to their life cycle as typical facultative microsymbionts, and symbiosis genes are just a tiny portion of the accessory gene pool. It is reasonable to speculate that other flexible "apps" should also be integrated into the genomic backgrounds of different strains for proper functioning. The key symbiosis app essential for nodulation and nitrogen fixation may interact with other accessory apps either directly or indirectly. For example, the nitrate-reduction gene cluster, harbouring *nap*, *nir*, *nor*, *hemN*, etc., is present on the symbiosis plasmid of *S. meliloti* 1021 and *Sinorhizobium* sp. CCBAU05631, on the chromosome of *Sinorhizobium fredii* but absent in *S. sojae* CCBAU05684 (Liu et al. 2017). The high degree of synteny between nitrate-reduction gene clusters found in these *Sinorhizobium* species and the presence of copies on the mobile symbiosis plasmids in certain species imply that this gene cluster may have undergone horizontal transfer and inter-replicon translocation. Components of this accessory gene cluster exerted a lineage-dependent contribution to optimisation of the *Sinorhizobium* symbiosis with soybeans regarding nodulation, nitrogen fixation and modulation of nitrogen assimilation by plants (Liu et al. 2017). This indicates differential integration of the "nitrate-reduction app" and the "symbiosis app" in test strains. Another example of integration between the "T3SS app" and the "*nod* app" has been observed in *S. fredii*. The T3SS is involved in pathogenic interactions in other bacteria (Lindeberg et al. 2012), and its positive regulator gene *ttsI* in *S. fredii* is induced by NodD1, which can sense compatible legume flavonoids and activate transcription of *nod* genes.

It was recently proposed that symbiosis genes can be considered as microsymbionts within the genomes of rhizobia, i.e. symbiosis within symbiosis (Remigi et al. 2016). In fact, it can be more generally postulated that all accessory functions are like apps in smartphone-like bacterial cells (Young 2016). Different apps should be compatible with the operating systems, and potential negative interference between apps should be minimised. It is well known that most bacteria have a limited size of genome compared to plants and animals. Therefore, there seems to be an intrinsic regulation of the storage space in bacteria, and different accessory apps may compete with each other in the app market, which can be the metagenome available in a community. If popular apps such as WeChat, Alipay, WhatsApp and Apple Pay have been used too often to be replaced in a particular period of the developing world, other accessory apps or functions can benefit by establishing a compatible connection with these popular apps. In line with this smartphone phenomenon, recent comparative transcriptomics of *S. fredii* strains under free-living and symbiotic conditions showed that the more conserved a gene is, the higher its transcriptional plasticity and gene connectivity are (Jiao et al. 2018). The abovementioned integration of the symbiosis app with the genomic backgrounds of the rhizobial pangenome is summarised in a simplified model in Fig. 6.2.

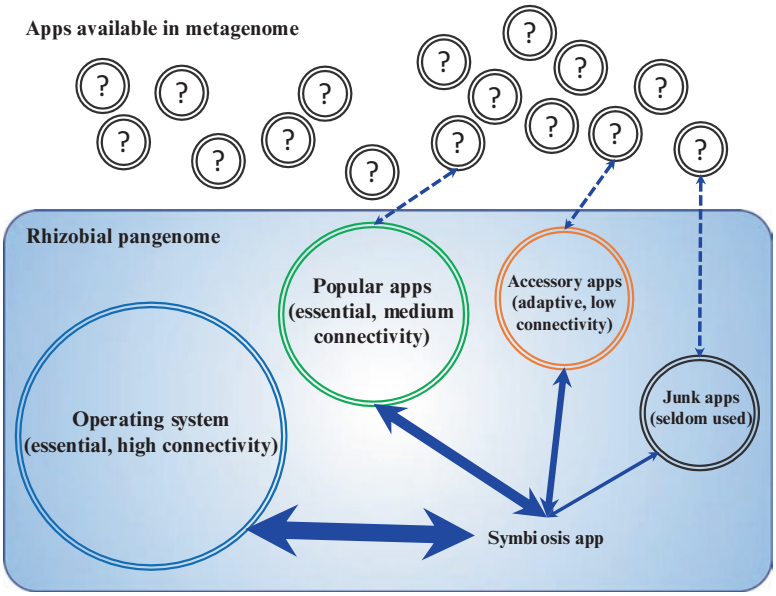

Fig. 6.2 Simplified working model of the integration of the "symbiosis app" with genomic backgrounds in the rhizobial pangenome. The relative levels of connectivity for genes belonging to the operating system, popular apps, adaptive apps and junk apps are indicated in brackets. The symbiosis app is presumably differentially connected with the core operating system and apps of different conservation levels. The width of arrows represents average degree of gene connectivity. Rhizobia can take more apps from metagenome of the microbial community as indicated by dashed arrows, and they can also supply diverse apps for the community

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Chapter 7
Diversity of Interactions Between Rhizobia and Legumes

Abstract The relationship between rhizobia and legumes can be specific or promiscuous. Some rhizobia can nodulate a diverse range of legumes, while other rhizobia only infect specific legumes. From the plant perspective, some legumes can be nodulated only by specific rhizobia, while other legumes can be nodulated by different genera of rhizobia. Various interactions between rhizobia and legumes are reviewed in this chapter. In addition, different infection patterns, nodule morphology and development and types of bacteroids in nodule cells are briefly discussed.

7.1 Rhizobia with Broad Host Ranges

Every rhizobium has its own host range. Some rhizobia like *Sinorhizobium meliloti* can nodulate with only specific host legume, *Melilotus* and *Medicago*, and these rhizobia are classified as narrow host-range rhizobia. In contrast, some rhizobia can infect and nodulate many different kinds of legumes, and they are described as broad or wide host-range rhizobia.

Strain NGR234 (NGR is the abbreviation for New Guinea *Rhizobium*) was the only fast-growing strain among 30 isolates prepared from *Lablab purpureus* nodules grown in Papua New Guinea (soil pH 8.5) by Trinick in 1965 (Pueppke and Broughton 1999). The bacterium NGR234 nodulates a very wide range of hosts, with more than 112 genera of legumes (Pueppke and Broughton 1999) as well as the nonlegumes *Parasponia andersonii* (Op den Camp et al. 2012), *Parasponia rugosa* and *Parasponia parviflora* in the family Cannabaceae (Behm et al. 2014). The hosts infected by strain NGR234 are evolutionarily divergent legumes with fundamental differences in nodule morphology and physiology. Strain NGR234-infected nodules of different legumes include both determinate and indeterminate nodules. The common legumes forming determinate nodules infected by NGR234 are represented by *Lablab purpureus* (Li et al. 2013), *Vigna unguiculata* (Li et al. 2013; Pueppke and Broughton 1999) and *Lotus japonicus* (Pueppke and Broughton 1999), while indeterminate nodules formed by NGR234 are exemplified by its well-studied interactions with *Leucaena leucocephala* (Li et al. 2013) and *Sophora flavescens* (Jiao et al. 2015b).

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However, the broad host-range strain NGR234 cannot nodulate some widely used and studied legumes such as *Medicago sativa*, *Medicago truncatula*, *Pisum sativum*, *Vicia faba*, *Cicer arietinum*, *Astragalus sinicus*, *Arachis hypogaea*, *Glycine max* (only some varieties could be nodulated by NGR234 and the nodules formed are inefficient), *Trifolium repens* and *Phaseolus vulgaris*. The inability to nodulate these important model legumes restrict its use in some extensive and deep studies, but NGR234 has nevertheless been used in some important studies of the symbiotic relationship between rhizobia and legumes (Li et al. 2013; Müller et al. 2001; Xin et al. 2012; Xu et al. 2018; Zamani et al. 2017).

NGR234 is a *Sinorhizobium*, closely related to *S. fredii* but perhaps representing a distinct species (Tian et al. 2012). Besides strain NGR234, other rhizobial strains have also been found to have wide host ranges. Examples are various strains of *Sinorhizobium fredii*: HH103, USDA257 and CCBAU 45436. Strain HH103 can nodulate *Lotus burttii*, *Glycine max* (Acosta-Jurado et al. 2016), *Glycyrrhiza uralensis* (Crespo-Rivas et al. 2016) and *P. vulgaris* (Sadowsky et al. 1988). Strain USDA257 can form nodules with 79 legume plant genera (Pueppke and Broughton 1999; Schuldes et al. 2012). Strain CCBAU 45436 can establish effective symbiosis with *G. max* (determinate nodules) and *Cajanus cajan* (indeterminate nodules) (Li et al. 2016).

7.2 Rhizobia Associated with Symbiotically Specific Plants

7.2.1 Mesorhizobia and Chickpea

Belonging to the tribe Cicereae, chickpea (*Cicer arietinum*) is, after soybean, the second most widely grown legume crop. It is grown in nearly 50 countries around the world (Varshney et al. 2013), particularly in India, Australia and arid and semi-arid countries around the Mediterranean Basin. In relation to its symbiotic nitrogen fixation (SNF) with rhizobia and its ability to grow on depleted soils with low rainfalls, chickpea cultivation presents both huge agricultural significance and environmental security, decreasing fertiliser input, promoting the growth of chickpea itself and the crops rotated with it and providing human dietary protein, especially in developing counties. Through SNF, chickpea can supply nitrogen to the soil ranging from 80 to 141 kg ha^{-1} when inoculated with an efficient rhizobial strain (Herridge et al. 1995). Rhizobial population richness, genetic diversity, geographic distribution, symbiotic matching, soil properties and inoculation with an effective strain can influence the effectiveness of SNF between the two symbiotic partners.

Chickpea rhizobia were first assigned to the genus *Rhizobium* by Nour et al. with two species described, *Rhizobium ciceri* in 1994 (Nour et al. 1994) and *Rhizobium mediterraneum* in 1995 (Nour et al. 1995). However, it became apparent that these species were sufficiently diverged to require the creation of a new genus, and because the intermediate growth speed of chickpea rhizobia is slower than that of fast growers like *Rhizobium* species but faster than the slow growers like *Bradyrhizobium* species, a novel genus *Mesorhizobium* (meso-, middle, intermediate) was proposed,

and these two species were subsequently transferred to *Mesorhizobium* gen. nov. (Jarvis et al. 1997), with *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum* as their new combined names in 1997. In 2012, a third novel species *Mesorhizobium muleiense* was proposed by Zhang et al. (2012a) when they studied the distinct chickpea rhizobia in Xinjiang China (Zhang et al. 2012b). An additional species was isolated from root nodules of chickpea grown in Gansu Province of Northwest China (Zhang et al. 2017) and has now been named *Mesorhizobium wenxiniae* (Zhang et al. 2018). There are reports of other potential novel *Mesorhizobium* species nodulating chickpea in southern and central Ethiopia (Tena et al. 2017) and in Indian soils (Nandwani and Dudeja 2009).

Besides the above four species, chickpea can be nodulated by many other *Mesorhizobium* that were originally described from hosts other than chickpea, including *M. amorphae*, *M. tianshanense* and *M. opportunistum* isolated from chickpea nodules in Spain and Portugal (Laranjo et al. 2012; Rivas et al. 2007) and *M. tarimense*, *M. gobiense*, *M. tamadayense*, *M. opportunistum*, *M. loti* and *M. amorphae* isolated from chickpea nodules in eastern Algeria (Dekkiche et al. 2017).

Endophytic bacteria, including *Sinorhizobium* (*Ensifer*) spp., were also isolated from chickpea nodules in some arid regions. In a survey of chickpea rhizobia in Algeria, 41 isolates were assigned to *Ensifer meliloti*, while 19 isolates could firmly be considered as *Mesorhizobium* strains (Dekkiche et al. 2017). Of the 15 randomly chosen *Ensifer* isolates, only 2 could form root nodules and were verified to be identical to the original isolated strain (Dekkiche et al. 2017). However, *nodC* gene could not be amplified from any of the 41 *E. meliloti* isolates, though 9 isolates had a *nifH* gene closely related to the *nifH* gene in *E. meliloti* and *E. medicae* (Dekkiche et al. 2017). Other studies also reported the existence of *Ensifer* spp. in chickpea nodules (Aouani et al. 2001; Ben Romdhane et al. 2007). Some other bacteria, including *Agrobacterium* sp., *Ochrobactrum* sp. (Naseem et al. 2005) and *Rhizobium* (Ogutcu et al. 2009), have also been isolated from chickpea nodules. The efficiency of nitrogen fixation of these endophytic non-*Mesorhizobium* isolates on chickpea needs to be checked. The role of these apparently non-symbiotic endophytic bacteria is unclear, but they might be helping in the process of nodulation (Hameed et al. 2004) or have the function of increasing phosphate solubilisation (Hameed et al. 2005).

From studies on the phylogeny of symbiosis genes (*nodC* and *nifH*) of chickpea rhizobia, it is clear that all these different chickpea-nodulating *Mesorhizobium* spp. have very similar symbiosis genes and these differ from the genes of rhizobia associated with non-chickpea legumes (Fig. 7.1). This could explain the highly host specificity of chickpea (Dadarwal 1980), and all these chickpea-nodulating rhizobia should be assigned to the symbiovar *ciceri*, i.e. they are *Mesorhizobium* spp. sv. *ciceri*. The distinct symbiosis genes are found in different *Mesorhizobium* spp. in different countries, indicating strong positive selection by chickpea. However, specific chickpea *Mesorhizobium* species were found only in specific regions. Retention and horizontal transfer of the chickpea-specific symbiosis genes among different mesorhizobia may be common to keep the specific symbiosis with chickpea.

Fig. 7.1 Phylogenetic tree based on *nodC* genes of different sv. *ciceri* strains nodulating *Cicer arietinum* and *Cicer canariense*. *Sinorhizobium medicae* A-321 was used as outgroup. Strains from different continents (Europe, Asia, Africa) were highlighted with different colours. Bar, 5% nucleotide substitution

So far, only *M. muleiense* and *M. wenxiniae* have been found in nodules of chickpea in China. *M. ciceri* is, however, found to be widely distributed in the Mediterranean, North Africa, North America, Indian subcontinent and Russia (Nour et al. 1995). The wide distribution of *M. ciceri* may be due to its adaption to soils with a wide range of pH from 5.0 to 10.0 and its temperature resistance up to 40 °C (Jarvis et al. 1997). *M. muleiense* from chickpea nodules has only been found in China, especially in alkaline soils (pH 8.46–9.16) in Xinjiang Province, where chickpea has been cultivated for over 2500 years (Zhang et al. 2012a, b, 2014). Ten strains isolated from nodules of *Astragalus* in Ningxia and Shanxi Provinces were classified as *M. muleiense*, but their symbiosis genes (*nodC* and *nifH*) were different from those of sv. *ciceri* (Yan et al. 2016a). The complete genome sequence of *M. ciceri* sv. *biserrulae* strain WSM1271 (isolated from root nodules of the pasture legume *Biserrula pelecinus* introduced into Australian soil in 1994) (Nandasena

et al. 2014) and the ongoing genome sequencing (unpublished) of four sv. *ciceri* strains belonging to different species (*M. ciceri*, *M. mediterraneum*, *M. muleiense*, *M. wenxiniae*) will help scientists to elucidate the distinct origin of their symbiosis genes and their transfer among different *Mesorhizobium* species.

Cicer canariense is a perennial wild chickpea endemic to the Canary Islands, and nine *Mesorhizobium* genospecies, including *M. ciceri*, were found to nodulate this wild plant (Armas-Capote et al. 2014). The other eight species included *M. caraganae*, *M. opportunistum*, *M. tamadayense*, *M. australicum*, *M. loti* and three (*M. tianshanense*/*M. gobiense*/*M. metallidurans*) that could not be unambiguously assigned based on 16S rRNA gene phylogeny (Armas-Capote et al. 2014). Only a small group of isolates had *nodC* sequences identical to those of sv. *ciceri*, and these were all *M. ciceri* (Armas-Capote et al. 2014). The other isolates have two distinct *nodC* lineages, one close to *Lotus* symbionts such as *M. loti* NZP2213^T and the other differing from all known mesorhizobia (Armas-Capote et al. 2014). Besides these *Mesorhizobium* spp., some fast-growing strains belonging to *Rhizobium leguminosarum* whose symbiotic *nodC* gene was not close to bv. *ciceri* but close to *R. leguminosarum* bv. *trifolii* ATCC 14480 phylogenetically have been reported to nodulate *C. canariense* effectively (Martinez-Hidalgo et al. 2015). A LacZ-labelled derivative of strain *R. leguminosarum* bv. *trifolii* RCCHU01 was used to confirm the effective nodulation on *C. canariense* and *Trifolium repens* (Martinez-Hidalgo et al. 2015). These results indicate that the domesticated chickpea (*C. arietinum*) is more selective in its choice of rhizobia than the wild chickpea (*C. canariense*).

7.2.2 Sinorhizobium/Ensifer *and Alfalfa*

It is considered that the *Sinorhizobium* (*Ensifer*) *meliloti* is the most effective and widely distributed rhizobial species in interacting with alfalfa (*Medicago sativa* L.) in symbiotic nitrogen-fixing (Wang et al. 2018), though the other minor species of *S. medicae* (Rome et al. 1996), *Rhizobium tibeticum* (Hou et al. 2009) and *R. favelukesii* (Torres Tejerizo et al. 2016) were reported to nodulate alfalfa.

Results from the analyses of 581 nodule isolates of alfalfa grown in Tibetan Plateau have been shown, 579 of them were classified as *S. meliloti*, only 1 isolate was identified as *S. medicae*, and 1 isolate was identified as *Rhizobium* sp. (close to *R. mongolense* and *R. yanglingense* based on *nodC* phylogeny) (Wang et al. 2018), supporting the extremely wide distribution and preponderance of *S. meliloti* in Tibetan Plateau. Similarly, the dominant species from alfalfa nodules in Serbian soil was also *S. meliloti*, and only one strain was identified as *S. medicae*, two strains as *R. tibeticum* and one strain as *Rhizobium* sp. (Stajković-Srbinović et al. 2012). This is also true in Argentina and Uruguay (Segundo et al. 1999).

In summary, alfalfa is a highly selective and specific legume that prefers *S. meliloti* as its effective symbiont around the world.

7.2.3 Mesorhizobium amorphae *and* Amorpha fruticosa

Amorpha fruticosa is a shrub legume indigenous to North America (Allen and Allen 1981) that was introduced to China as a windbreak and as soil cover for erosion control. The majority of rhizobia forming an effective symbiosis with *A. fruticosa* growing in different regions of China were characterised as a sole species of *Mesorhizobium*, *M. amorphae* (Wang et al. 1999). The type strain of *M. amorphae*, ACCC 19665^T, only nodulates its host plant *A. fruticosa* in cross-nodulation tests (Wang et al. 1999). In 2002, Wang et al. (2002) reported that *A. fruticosa* in its native North America was also nodulated by *M. amorphae*, which was probably transmitted from the USA to China.

In 2007, Gu et al. identified several rhizobial strains isolated from *Lespedeza* species as *M. amorphae* (Gu et al. 2007), and two strains (CCBAU 45224 and CCBAU 25056) had *nodC* and *nifH* genes completely identical those of type strain ACCC 19665^T. Cross-nodulation tests among the *M. amorphae* populations isolated, respectively, from *A. fruticosa* and *Lespedeza* spp. would be helpful to reveal if they can nodulate both the host plants.

7.3 Rhizobia Associated with Promiscuous Plants

7.3.1 *Various Rhizobia and Soybean (*Glycine max*)*

Two genera, the *Bradyrhizobium* and *Sinorhizobium* (syn. *Ensifer*), are the publicly recognised rhizobia that can establish effective symbiosis with soybean (*Glycine max*). Two isolates from nodules of soybean grown in Xinjiang were previously classified as *Rhizobium* (now *Mesorhizobium*) *tianshanense* (Chen et al. 1995), but their authentication could not be verified later (our unpublished observations). The current subsection only focuses on the two well-known genera of soybean rhizobia.

In the genus *Bradyrhizobium*, seven rhizobial species including *B. elkanii* (Kuykendall et al. 1992), *B. japonicum* (Jordan 1982), *B. diazoefficiens* (Delamuta et al. 2013), *B. daqingense* (Wang et al. 2013a), *B. liaoningense* (Xu et al. 1995), *B. huanghuaihaiense* (Zhang et al. 2012c) and *B. ottawaense* (Yu et al. 2014) have been described until now to nodulate soybean. In the phylogenetic tree based on six concatenated housekeeping gene sequences (Fig. 7.2), these seven soybean bradyrhizobial species occupied their distinct positions. However, only two *nodC* gene types were found among these seven species, with *B. elkanii* as a sole type; the other six species had identical *nodC* gene sequences (see Chap. 5).

In the genus *Sinorhizobium*, four fast-growing species of soybean symbionts have been established: *S.* (*E.*) *fredii* (de Lajudie et al. 1994; Jarvis et al. 1992), *E. sojae* (Li et al. 2011), *E. glycinis* (Yan et al. 2016b) and *E. shofinae* (Chen et al. 2017). Based on the maximum likelihood phylogenetic tree of three housekeeping genes (Chen et al. 2017), the four fast-growing soybean rhizobia occupy distinct positions among the other known species in *Ensifer/Sinorhizobium* (Fig. 7.3).

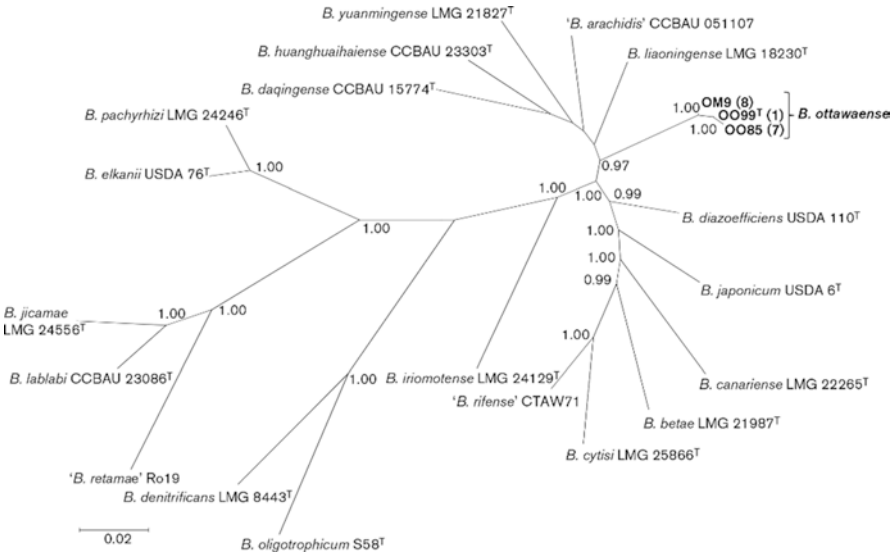

Fig. 7.2 Bayesian phylogenetic tree of *atpD-glnII-recA-gyrB-rpoB-dnaK* concatenated gene sequences for species in genus *Bradyrhizobium* (Yu et al. 2014)

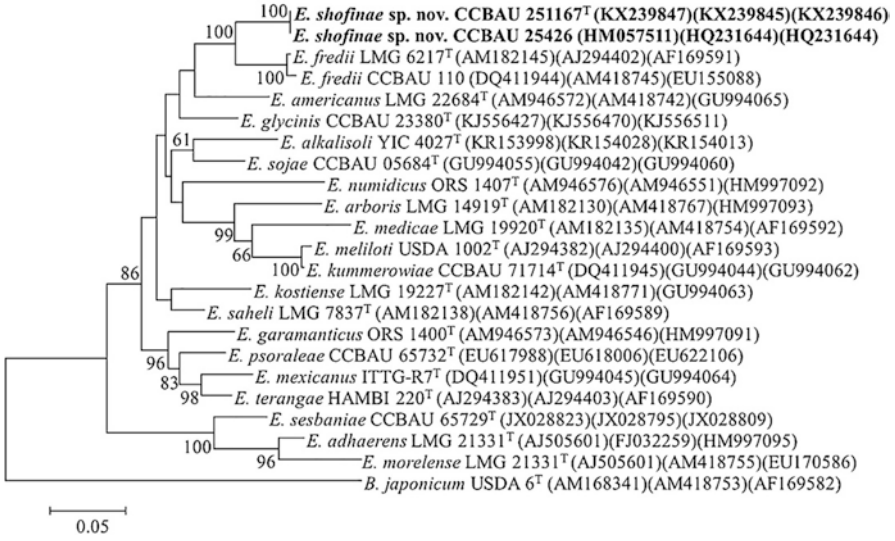

Fig. 7.3 Maximum likelihood (ML) phylogenetic tree based on concatenated sequences of *recA*, *atpD* and *glnII* showing the genetic relationship among type strains of species in genus *Ensifer* (*Sinorhizobium*) (Reprint from Chen et al. (2017))

As with the *nodC* gene in *Bradyrhizobium* species, all the four *Ensifer* species had identical *nodC* genes, too (see Chap. 5). In addition, the common components and structure of Nod factors (LCOs) secreted by these soybean-associating *Bradyrhizobium* and *Sinorhizobium/Ensifer* species may endow them with same host range (see Chap. 5).

7.3.2 Various Rhizobia and Sophora

Sophora is a leguminous genus in the tribe Sophoreae, the subfamily Faboideae. This genus contains about 50 species (Sprent 2009) of shrubs and small trees and is distributed worldwide. About 19 species in the genus *Sophora* have been documented to have nitrogen-fixing nodules (Sprent 2009), and for some species, their rhizobial diversity and taxonomy have been studied in detail since 1995 when Chen et al. described a novel species, *Rhizobium tianshanense* (Chen et al. 1995) (now renamed *Mesorhizobium tianshanense* (Jarvis et al. 1997)), from *Sophora alopecuroides* growing in Xinjiang, China. Another two rhizobial species, *Bradyrhizobium elkanii* (Han et al. 2008a) and *Rhizobium multihospitium* (Han et al. 2008b) were isolated from *S. alopecuroides* by Han et al. Five rhizobial strains isolated from other *Sophora* species growing in New Zealand were defined as groups related to two *Mesorhizobium* species and one *Rhizobium* species (Weir et al. 2004). Seventy-five nodule isolates of *S. alopecuroides* grown in China's Loess Plateau were clustered into nine genospecies in five genera, *Mesorhizobium*, *Agrobacterium*, *Phyllobacterium*, *Rhizobium* and *Sinorhizobium* (Zhao et al. 2010), revealing the promiscuous symbiosis of this legume. Cross-nodulation tests further confirmed that not only alpha-rhizobia (α-rhizobia) but also beta-rhizobia (β-rhizobia) could nodulate *Sophora flavescens* (Jiao et al. 2015a), another extremely promiscuous host plant, sister of *S. alopecuroides*. The extreme diversity of *nodC* types of various rhizobia that nodulate *S. flavescens* has been confirmed (Jiao et al. 2015a; Liu et al. 2018a).

The rhizobial species isolated from nodules of *Sophora* species are listed in Table 7.1, and they are classified into five genera as mentioned above. Altogether, 15 rhizobial (geno)species were clustered into the genus *Mesorhizobium*, isolated from 6 *Sophora* species. Fourteen rhizobial (geno)species were classified into the genus *Rhizobium*; five species were found in *Sinorhizobium/Ensifer*; two species in each of *Phyllobacterium* and *Bradyrhizobium*; and only one species was in the genus *Agrobacterium* (Table 7.1). Clearly, the predominant rhizobia of *Sophora* belong to the genera *Mesorhizobium* and *Rhizobium*.

The phylogeny of *nodC* and *nodA* symbiosis genes of the *Sophora*-associated rhizobia revealed diverse branches (Jiao et al. 2015a; Zhao et al. 2010). Five clades (13 types) of *nodA* genes were found in different *Sophora* rhizobia by Zhao et al. (Jiao et al. 2015a). Seven types of *nodC* genes were detected among different *Sophora* rhizobia in the study of Jiao et al. (Jiao et al. 2015a). Clearly, *Sophora* can be nodulated by rhizobia with phylogenetically different symbiosis genes. Interestingly, all the 51 *Mesorhizobium* strains belonging to 7 different species col-

Table 7.1 Various rhizobia isolated from root nodule of *Sophora* species

Rhizobial genus/ species	*Sophora* spp.	Geographic origin	References
***Mesorhizobium* (15 spp.)**			
M. tianshanense	*S. alopecuroides*	Xinjiang, China	Chen et al. (1995) and Jarvis et al. (1997)
M. alhagi	*S. alopecuroides*	Gansu, Ningxia and Shaanxi, China	Zhao et al. (2010)
M. gobiense	*S. alopecuroides*	Gansu and Ningxia China	Zhao et al. (2010)
M. amorphae	*S. alopecuroides*	Ningxia, China	Zhao et al. (2010)
M. calcicola	*S. longicarinata*; *S. flavescens*	Marlborough, New Zealand Shaanxi, China	De Meyer et al. (2016) and Jiao et al. (2015a)
M. waitakense	*S. microphylla*	Otago, New Zealand	De Meyer et al. (2016)
M. sophorae	*S. microphylla*	Westland, New Zealand; Shaanxi, China	De Meyer et al. (2016) and Jiao et al. (2015a)
M. newzealandense	*S. prostrata*	Marlborough, New Zealand	De Meyer et al. (2016)
M. kowhaii	*S. microphylla*	Canterbury, New Zealand	De Meyer et al. (2016)
M. waimense	*S. longicarinata*	Waima/Ure River, Marlborough, New Zealand	De Meyer et al. (2015)
M. cantuariense	*S. microphylla*	Canterbury, New Zealand	De Meyer et al. (2015)
M. ciceri	*S. tetraptera*	New Zealand	Weir et al. (2004)
M. huakuii	*S. flavescens*	Shaanxi, China	Jiao et al. (2015a)
M. septentrionale	*S. flavescens*	Liaoning, Shanxi, China	Jiao et al. (2015a)
M. sp. ICMP 14330	*S. microphylla*	New Zealand	Weir et al. (2004)
***Rhizobium* (14 spp.)**			
R. indigoferae	*S. alopecuroides*	Ningxia and Gansu, China	Zhao et al. (2010)
R. giardinii	*S. alopecuroides*	Ningxia, China	Zhao et al. (2010)
R. leguminosarum	*S. chathamica*	New Zealand	Weir et al. (2004)
R. multihospitium	*S. alopecuroides*	Xinjiang, China	Han et al. (2008b)
R. sophorae	*S. flavescens*	Shanxi, China	Jiao et al. (2015c)
R. sophoriradicis	*S. flavescens*	Shanxi, China	Jiao et al. (2015c)
R. sp. II CCBAU 03429	*S. flavescens*	Shanxi, China	Jiao et al. (2015a)
R. sp. I CCBAU 03360	*S. flavescens*	Shanxi, China	Jiao et al. (2015a)
R. sp. III CCBAU 03419	*S. flavescens*	Shanxi, China	Jiao et al. (2015a)
R. sp. V CCBAU 11560	*S. flavescens*	Liaoning, China	Jiao et al. (2015a)
R. lusitanum	*S. flavescens*	Shanxi, China	Jiao et al. (2015a)
R. yanglingense	*S. flavescens*	Liaoning, China	Jiao et al. (2015a)
R. mongolense	*S. flavescens*	Liaoning, China	Jiao et al. (2015a)
R. pusense	*S. tomentosa*	Rio de Janeiro, Brazil	Toma et al. (2017)

(continued)

Table 7.1 (continued)

Rhizobial genus/ species	*Sophora* spp.	Geographic origin	References
***Sinorhizobium* (5 spp.)**			
S. fredii	*S. alopecuroides*; *S. flavescens*	Ningxia, Shanxi, China	Jiao et al. (2015a) and Zhao et al. (2010)
S. meliloti	*S. alopecuroides*	Gansu, China	Zhao et al. (2010)
S. (*E.*) *adhaerens*	*S. tomentosa*	Rio de Janeiro, Brazil; Taiwan, China	Hung et al. (2005) and Toma et al. (2017)
S. mexicanus	*S. tomentosa*	Rio de Janeiro, Brazil	Toma et al. (2017)
S. chiapanecum	*S. tomentosa*	Rio de Janeiro, Brazil	Toma et al. (2017)
***Agrobacterium* (1 spp.)**			
A. tumefaciens	*S. alopecuroides*	Gansu, China	Zhao et al. (2010)
***Phyllobacterium* (2 spp.)**			
P. trifolii	*S. alopecuroides*	Gansu and Ningxia, China	Zhao et al. (2010)
P. sophorae	*S. flavescens*	Shanxi, China	Jiao et al. (2015d)
***Bradyrhizobium* (2 spp.)**			
B. elkanii	*S. alopecuroides*; *S. flavescens*	Xinjiang, Shaanxi, Gansu, China	Han et al. (2008a) and Jiao et al. (2015a)
B. sp. I CCBAU 03416	*S. flavescens*	Shanxi, China	Jiao et al. (2015a)

lected from New Zealand had almost identical *nodC* genes, and they clustered closely with the mesorhizobia collected from China (Jiao et al. 2015a; Nguyen et al. 2017), indicating their common origin. Furthermore, identical *nodC* genes were found in isolates of two different genera, *R. mongolense* CCBAU 11559 and *M. septentrionale* CCBAU 11523, supporting the lateral gene transfer of nodulation genes among different rhizobial genera (Jiao et al. 2015a).

To test the promiscuous nodulation of *Sophora*, more rhizobial species belonging to different genera with different *nodC* phylogeny in the classes *Alphaproteobacteria* and *Betaproteobacteria* were chosen and cross-inoculated on *Sophora.* This identified another 26 rhizobial species that could nodulate *S. flavescens* (Table 7.2) (Jiao et al. 2015a). Consistent with the observed diversity of rhizobial species and symbiosis genes among isolates from *S. flavescens*, the cross-nodulation tests further confirmed the promiscuity of this plant, in that not only does it not select strictly the rhizobial species, but it does not select strictly the symbiosis gene types and accepts rhizobia originally isolated from different cross-nodulation groups. For example, *S. meliloti* and *M. huakuii* are two very host-specific rhizobial species, only selecting *Medicago* and *Astragalus sinicus*, respectively, as their host plant, but both of them can nodulate *S. flavescens* (Jiao et al. 2015a).

Although *S. flavescens* is nodulated promiscuously by many different rhizobia, there are still some rhizobial strains that cannot nodulate it, including the following: *Bradyrhizobium japonicum* USDA 6, *B. diazoefficiens* USDA 110, *B. yuanmingense* CCBAU 10071, *Bradyrhizobium* sp. strain ORS278, *B. oligotrophicum* LMG 10732, *Ochrobactrum lupini* LMG 22726, *M. muleiense* CCBAU 83963, *M. amor-*

Table 7.2 Cross-nodulation on *Sophora flavescens* by different rhizobia

Rhizobia	Original host	Nodulation
Alphaproteobacteria		
Bradyrhizobium elkanii USDA76	*Glycine max*	Yes
B. arachidis CCBAU 051107	*Arachis hypogenea*	Yes
Rhizobium yanglingense CCBAU 71623	*Gueldenstaedtia multiflora*	Yes
R. yanglingense CCBAU 01603	*Caragana intermedia*	Yes
R. fabae CCBAU 33202	*Vicia faba*	Yes
R. leguminosarum sv. *viciae* USDA 2370	*Pisum sativum*	Yes
R. tropici CIAT 899	*Phaseolus vulgaris*	Yes
R. pisi DSM 30132	*Pisum sativum*	Yes
R. laguerreae FB206	*Vicia faba*	Yes
R. vallis CCBAU 65647	*Phaseolus vulgaris*	Yes
R. etli bv. *phaseoli* CFN42	*Phaseolus vulgaris*	Yes
R. leguminosarum sv. *trifolii* LMG 8820	*Trifolium pratense*	Yes
Sinorhizobium meliloti USDA 1002	*Medicago sativa*	Yes
S. meliloti 1021	*Medicago sativa*	Yes
S. sp. NGR234	*Lablab purpureus*	Yes
S. fredii CCBAU 45436	*Glycine max*	Yes
Mesorhizobium loti NZP 2213	*Lotus corniculatus*	Yes
M. huakuii 7653R	*Astragalus sinicus*	Yes
M. qingshengii CCBAU 33460	*Astragalus sinicus*	Yes
M. tianshanense USDA 3592	*Glycyrrhiza pallidiflora*	Yes
M. temperatum CCBAU 01578	*Caragana intermedia*	Yes
M. amorphae CCBAU 01583	*Caragana intermedia*	Yes
M. septentrionale CCBAU 03074	*Astragalus membranaceus*	Yes
Phyllobacterium sophorae CCBAU 03422	*Sophora flavescens*	Yes
Betaproteobacteria		
Burkholderia tuberum LMG 21444	*Aspalathus carnosa*	Yes
Bur. sp. ICMP 19869	*Dipogon lignosus*	Yes
Bur. dipogonis ICMP 19430	*Diphgon lignosus*	Yes
Cupriavidus taiwanensis LMG 19424	*Mimosa pudica*	Yes

phae ATCC 19665, *R. galegae* HAMBI 1174, *Burkholderia nodosa* LMG 23741 and *Burkholderia phymatum* STM 815 (Jiao et al. 2015a). One of these specific rhizobia, USDA 110, a strain isolated from soybean (*Glycine max*), was studied further to find the molecular mechanism determining its host range (Liu et al. 2018a). Tn5 transposon mutants of USDA 110 were constructed, and 14 mutants were able to form normal nodules on *S. flavescens*, while wild-type USDA 110 only induced small pseudonodules with no bacteroids on this plant (Liu et al. 2018a). Analyses of the mutated genes found that they were involved in metabolism, transportation and chemotaxis and did not participate in the biosynthesis of Nod factors directly (Liu et al. 2018a). These results indicate that mutation of some nonessential genes could expand rhizobial host ranges (Brewin et al. 1980) and that promiscuity or specificity is dependent both on host and rhizobia (Liu et al. 2018a).

Ensifer adhaerens, a bacterial predator attacking and lysing *Micrococcus luteus* cells, was originally isolated from Hagerstown silty clay loam soil (Casida 1980, 1982). Controversy over the nomenclature of *E. adhaerens* and *Sinorhizobium adhaerens* is not our concern here, but *S. adhaerens* was previously isolated from root nodules of *Sophora tomentosa* grown in Brazil and Taiwan and China (Hung et al. 2005; Toma et al. 2017). Although *S.* (*E.*) *adhaerens* was reported to be a predominant occupant in nodules of *S. tomentosa* (Toma et al. 2017), the capability of nodulation and nitrogen fixation of this species need to be further checked carefully. Other endophytic bacterial species belonging to the genera *Bacillus*, *Brevibacillus* and *Paenibacillus* were also found within *S. tomentosa* nodules (Toma et al. 2017), further supporting the importance of nodulation tests with *S.* (*E.*) *adhaerens*.

7.3.3 *Rhizobia and Common Bean (*Phaseolus vulgaris*)*

As mentioned in Chap. 5, the promiscuous legume common bean (*Phaseolus vulgaris*) can be nodulated by various species mainly in the genus *Rhizobium*, including *Rhizobium etli* (Aguilar et al. 2004), *R. leguminosarum* (García-Fraile et al. 2010; Mulas et al. 2011), *R. lusitanum* (Valverde et al. 2006), *R. gallicum* (Amarger et al. 1997), *R. phaseoli* (Ramírez-Bahena et al. 2008), *R. tropici* (Amarger et al. 1994; Martinez-Romero et al. 1991), *R. leucaenae* (Ribeiro et al. 2012), *R. paranaense* (Dall'Agnol et al. 2014), *R. vallis* (Wang et al. 2011) and *R. sophoriradicis* (Jiao et al. 2015c; Ormeño-Orrillo et al. 2018), as well as *R. giardinii* (Amarger et al. 1997), which is now *Pararhizobium giardinii* (Mousavi et al. 2015). In addition, some isolates containing symbiosis genes (*nod* and *nif*) were found in the genera *Agrobacterium* (Wang et al. 2016), *Bradyrhizobium* (Cao et al. 2014) and *Ensifer* (Wang et al. 2016), and one non-nodulating species *Phyllobacterium endophyticum* (Flores-Félix et al. 2012) was reported to be isolated from root nodules of *P. vulgaris* grown in different soils of China, Mexico and Spain.

The phylogeny of *nodC* symbiosis genes of the rhizobia associated with common bean is discussed in Chap. 5 of this book.

7.3.4 *Bradyrhizobia and Peanut (*Arachis hypogaea*)*

Most of the effective rhizobia associated with peanut (*Arachis hypogaea*) belong to the genus *Bradyrhizobium* (Chen et al. 2003, 2016; El-Akhal et al. 2009; Santos et al. 2017; Taurian et al. 2006), though some effective fast-growing rhizobia have also been described (El-Akhal et al. 2009; Jaiswal et al. 2017; Osei et al. 2018; Taurian et al. 2006). *Bradyrhizobium arachidis* (Wang et al. 2013b), *B. guangdongense* and *B. guangxiense* (Li et al. 2015) have been described as novel species, and the type strains of these species were isolated originally from nodules of peanut. Strain CCBAU 23160, isolated from peanut grown in Anhui province, China, was

identified as *B. lablabi*, though the type strain of *B. lablabi*, CCBAU 23086^T, was isolated from nodules of *Lablab purpureus* (Chang et al. 2011).

Bacteria from Ghana, closely related to *B. yuanmingense*, were reported to be effective peanut microsymbionts (Osei et al. 2018). In the same study, fast-growing isolates KNUST 1003 and 1007 were isolated that were highly similar to *Rhizobium tropici* (Osei et al. 2018). In another study, *Bradyrhizobium* isolates obtained from native peanut grown in Córdoba, Argentina, were classified as *B. iriomotense* and *B. yuanmingense* based on phenotypic and phylogenetic comparisons (Muñoz et al. 2011). Other isolates, also from Córdoba, were identified as *B. japonicum*, *Bradyrhizobium* sp., *R. gardinii* and *R. tropici* (Taurian et al. 2006).

Other potentially novel species of peanut symbionts are being described by researchers at China Agricultural University (personal communication). The symbiosis genes of peanut bradyrhizobia are described in Chap. 5 of this book.

7.3.5 Bradyrhizobia and Erythrophleum fordii

Erythrophleum fordii, commonly known as ironwood tree, is an evergreen legume in the subfamily Caesalpinioideae indigenous to the south of China and the north of Vietnam (Sein and Mitlöner 2011; Yao et al. 2014). Previously, only one rhizobial strain closely related to *B. elkanii* was reported to be a symbiotic bacterium of *E. fordii* (Lu et al. 2011). Later, systematic studies on the rhizobia of this ironwood tree were carried out by Yao et al. (2014, 2015).

A total of 166 bacterial isolates were obtained from the nodules of *E. fordii* growing in Guangdong and Guangxi provinces in southern China. All these isolates were found to be in the genus *Bradyrhizobium*, with 22 genotypes. Based on multilocus sequence analyses, five genospecies were identified: *B. elkanii* and *B. pachyrhizi* comprising the dominant symbionts and *B. yuanmingense*, *B. erythrophlei* and *B. ferriligni* comprising the minor symbionts (Yao et al. 2014, 2015).

7.4 Rhizobial Infection Through Root Hair Entry or Crack Entry

The establishment of symbiosis between legumes and rhizobia starts from the molecular recognition and interaction between them, followed by infection and entry of rhizobia into the leguminous cell. To form an efficient nitrogen-fixing nodule, live rhizobia must enter the root cell of legume through a specific pathway. Two major infection patterns can be found commonly: root hair infection thread (Fig. 7.4a) and crack entry (Fig. 7.4b). In addition, intercellular infection (Fig. 7.4c) was observed in some other legumes. Most legumes have only one of the infection patterns, while certain legumes can have two kinds of infection patterns.

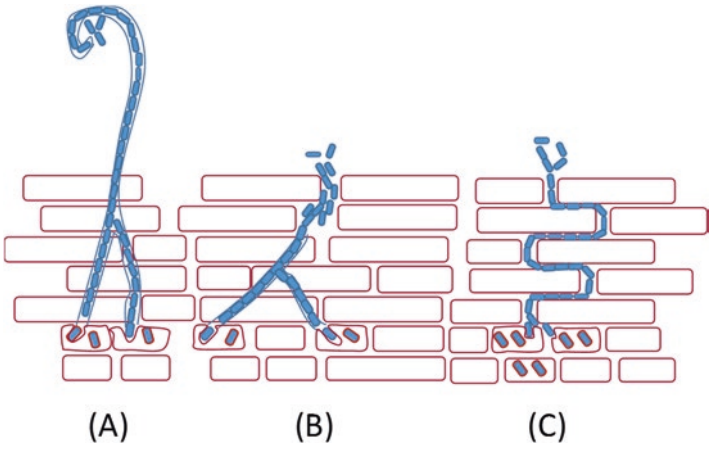

Fig. 7.4 Infection patterns. (**a**) Root hair infection thread. (**b**) Crack entry with or without infection threads. (**c**) Intercellular infection. The lower part of the figure shows endocytosis into cortical cells and the host-derived peribacteroid membrane surrounding the rhizobial bacteroids. (Figure redrawn from Madsen et al. (2010))

The root hair infection thread pattern (Fig. 7.4a) is found in most well-studied legumes, such as alfalfa (*Medicago sativa*) (Gage 2004), *Medicago truncatula* (Fournier et al. 2008), soybean (*Glycine max*) (Rao and Kerster 1978), pea (*Pisum sativum*), *Lotus japonicus* (Markmann et al. 2012; Xie et al. 2012) and *Sophora flavescens* (Jiao et al. 2015a). Rhizobial entry by the crack pattern (Fig. 7.4b) is found in certain legumes including peanut (*Arachis hypogaea*) (Boogerd and vanRossum 1997), joinvetch (*Aeschynomene americana*) (Grant and Trese 1996) and *Adesmia bicolor* (Bianco 2014), as well as the nonlegume, *Parasponia andersonii* (Bender et al. 1987). Besides these two major patterns of infection, other distinct patterns are observed in some legumes. A woody legume, tagasaste (*Chamaecytisus proliferus* ssp. *proliferus* var. *palmensis*), forms N_2-fixing indeterminate nodules in response to infection by strains of *Bradyrhizobium* sp. (*Chamaecytisus*) (Vega-Hernandez et al. 2001). The infection process in tagasaste starts with deformed root hairs, but the infection aborts early, and, instead, the rhizobia use the crack entry mode to move along the intercellular route, eventually entering the altered cell walls directly (Vega-Hernandez et al. 2001). In *Lotus japonicus*, two alternative intercellular infection modes, crack entry and infection thread independent single cell infection, were discovered using symbiotic mutants (Markmann et al. 2012). Similar to tagasaste, the semiaquatic legume *Sesbania rostrata* is also infected via an intercellular invasion; the bacteria colonise epidermal fissures at lateral root bases and trigger cortical cell death for infection pocket formation and subsequent intercellular and intracellular infection thread progression towards the primordium (Capoen et al. 2010).

In a study of the infection pattern of *Sophora flavescens* by different rhizobial strains (Fig. 7.5), the authors found the distinguished deformation of the root hairs infected by different rhizobia, leading to different morphologies (Fig. 7.5b–h) (Jiao et al. 2015a). Though different deformed root hairs, the rhizobia invade the root through infection thread clearly (Fig. 7.5h).

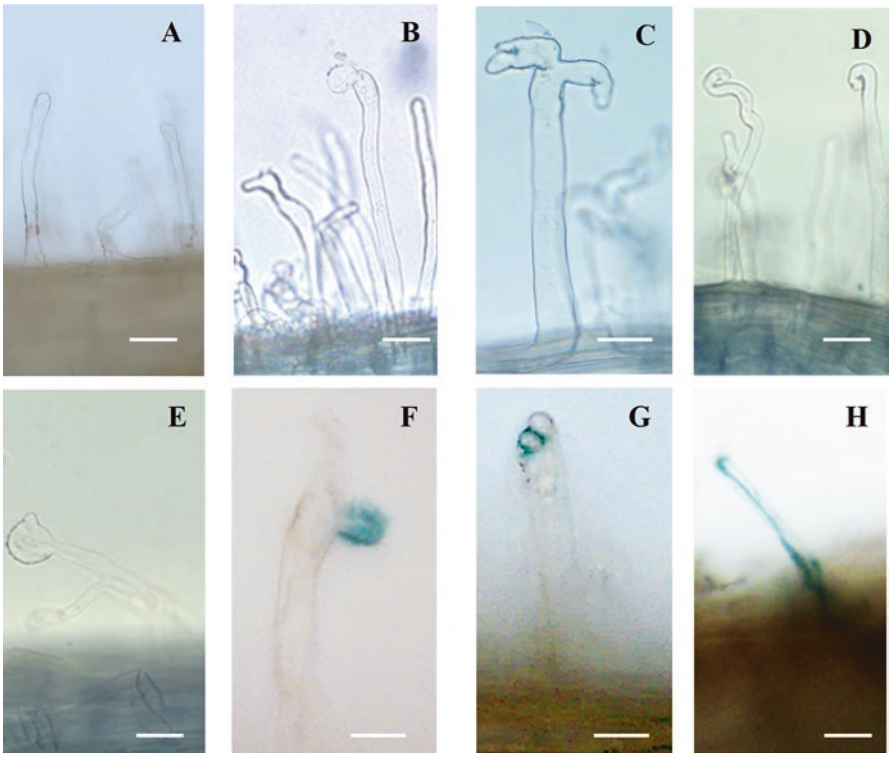

Fig. 7.5 Light micrograph of root hair, infection thread and nodule primordium of *Sophora flavescens* inoculated by different rhizobia (Jiao et al. 2015a). (**a**) Uninoculated root hairs at 6 days. (**b** and **c**) Root hairs, 6 days after inoculation (DAI) with *Sinorhizobium fredii* CCBAU 45436. (**d** and **e**) Root hairs, 6 DAI with *Rhizobium yanglingense* CCBAU 01603. (**f**) Aggregating cells of the *lacZ*-labelled *Sinorhizobium fredii* CCBAU 45436 surrounding the tip of a biforked root hair, 10 DAI. (**g**) Infection pocket formed by the *lacZ*-labelled *R. yanglingense* CCBAU 01603, 10 DAI. (**h**) Infection thread induced by *lacZ*-labelled *Sinorhizobium fredii* CCBAU 45436, 11 DAI. Bars: 40 μm in (**a**), (**b**), (**d**), (**e**) and (**h**); 20 μm in (**c**), (**f**) and (**g**)

7.5 Determinate or Indeterminate Root Nodules

Two major types of nodules, indeterminate (Fig. 7.6a, b) and determinate (Fig. 7.6c, d), are found on the roots of legumes. Types of nodules are classified according to their mode of development. The indeterminate type is characterised by a persistent nodule meristem (Fig. 7.6b), while the determinate nodule type lacks such a meristem (Fig. 7.6d). Therefore, indeterminate nodules are often clavate and elongated, while determinate nodules are usually spherical and have limited size.

Determinate nodules are found on root of cowpea (*Vigna unguiculata*) (Fig. 7.6c, d), soybean (*Glycine max*), peanut (*Arachis hypogaea*), mungbean (*Vigna radiata*), common bean (*Phaseolus vulgaris*), *Lotus japonicus*, etc. Legumes having indeterminate nodules include alfalfa (*Medicago sativa*), *Medicago truncatula*, white clover (*Trifolium repens*), red clover (*Trifolium pratense*), common vetch (*Vicia sativa*), broad bean

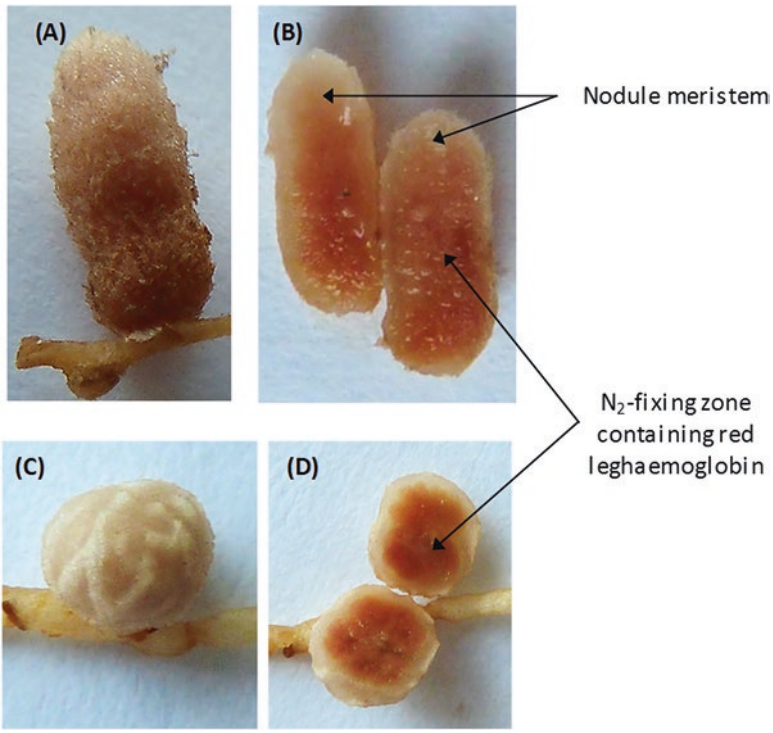

Fig. 7.6 Determinate and indeterminate nodules. (**a** and **b**) Indeterminate nodules of *Leucaena leucocephala*; (**c** and **d**) determinate nodules of cowpea (*Vigna unguiculata*). (**b**) and (**d**) are nodule sections showing the dark red N_2-fixing zone containing leghaemoglobin

(*Vicia faba*) and *Leucaena leucocephala* (Fig. 7.6a, b), pea (*Pisum sativum*) and *Sophora flavescens* (Jiao et al. 2015a, d). The type of nodule is determined by the host plant, not by the rhizobial strain (itself). The phenomenon is well demonstrated by the observation of root nodules of *S. flavescens* that only indeterminate nodules are formed regardless of the different rhizobial strains originally isolated from determinate nodule-forming legumes or the indeterminate nodule-forming legumes (Jiao et al. 2015a, d). Club-shaped pseudonodule, that no rhizobial bacteroid inside the nodule, could produce by infection of wild-type *Bradyrhizobium diazoefficiens* (formerly *Bradyrhizobium japonicum*) USDA 110 on *S. flavescens* (Liu et al. 2018b). Also nodule could initialise and develop induced only by the existence of Nod factor, lipooligosaccharide (LCO) (Stokkermans and Peters 1994). Therefore, the initiation of nodule primordium could be independent on the alive rhizobia but is dependent on LCO of rhizobia (D'Haeze and Holsters 2002). Another study showed that even without the LCO as well as rhizobia, the mutants of *Lotus japonicus* could form spontaneous nodules (Tirichine et al. 2006).

The biology of these two types of nodules has been fully described in a previous Tansley review (Hirsch 1992). Readers could refer this review for the comparison and differences of these two types of nodules.

7.6 Swollen or Non-swollen Bacteroids

Inside the legume nodule cells, rhizobia differentiate into N_2-fixing bacteroids which are physiologically and morphologically different from the free-living bacteria. Two kinds of bacteroids can be recognised: terminal or swollen bacteroids and nonterminal or non-swollen bacteroids. The size and shape of non-swollen bacteroids are similar to those of free-living bacteria, and these bacteroids can reproduce when they are re-isolated from the nodule cells. On the other hand, the swollen or terminal bacteroids have lost their reproductive capacity and no longer divide normally when they are re-isolated from the nodules (Zhou et al. 1985).

Typically, the differentiation of bacteroids to swollen or non-swollen types is determined by the host plant. Swollen/terminal bacteroids are observed usually in nodules of pea (*Pisum sativum*) (Oono and Denison 2010), alfalfa (*Medicago sativa*), peanut (*Arachis hypogaea*) (Fig. 7.7a, c) and clover (*Trifolium repens*) (Zhou et al. 1985). Branched, "Y"-shaped terminal bacteroids are found in pea, and

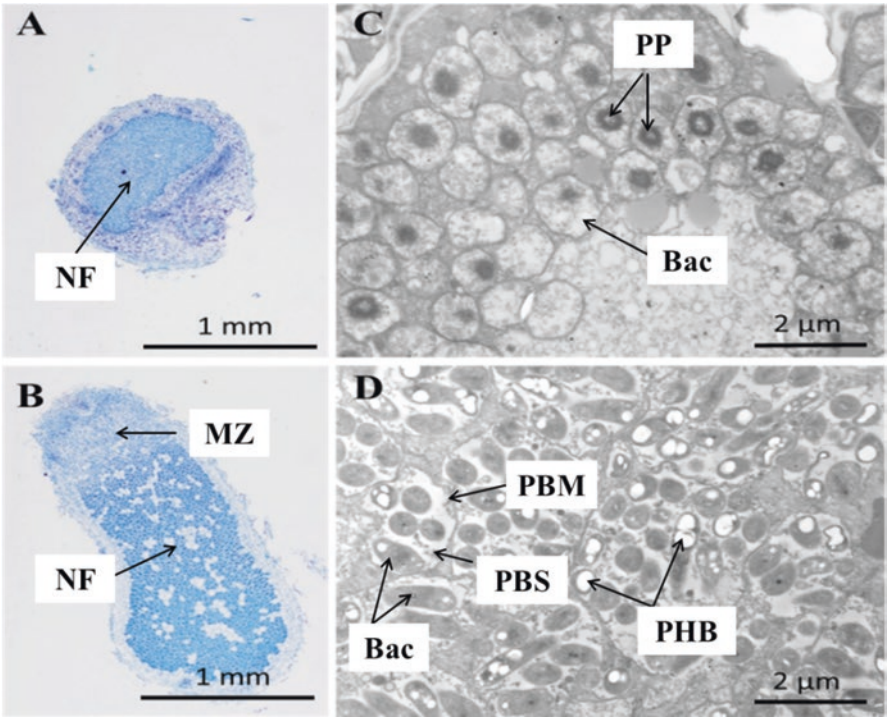

Fig. 7.7 Nodule section and transmission electron microscope of ultrathin nodule sections photographed by Xiang-Fei Meng. General light microscope of nodule sections of *Arachis hypogaea* (**a**) and *Sophora flavescens* (**b**). Transmission election microscope of ultrathin nodule sections of *Arachis hypogaea* (**c**) and *Sophora flavescens* (**d**). *Bac* bacteroid, *PBS* peribacteroid space, *PBM* peribacteroid membrane, *PP* electron-opaque polyphosphate inclusions, *MZ* meristem zone, *NF* nitrogen fixation zone, *PHB* poly-β-hydroxybutyrate

spherical bacteroids (Fig. 7.7c) are found in peanut. Non-swollen/nonterminal bacteroids are observed in nodules of soybean (*Glycine max*) (Zhou et al. 1985), bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*) (Oono and Denison 2010) and *Sophora flavescens* (Fig. 7.7b, d).

Swollen bacteroids have higher fixation efficiency as well as greater benefit to the legume host than non-swollen ones (Florian et al. 2018; Oono and Denison 2010).

7.7 Concluding Remarks and Perspectives

In this chapter, we have reviewed the diversity of interaction between rhizobia and legumes. Some rhizobia, like NGR234, can nodulate more than a hundred genera of legumes. However, this strain cannot nodulate some common leguminous crops, such as soybean, peanut, alfalfa and chickpea. Chickpea selects only *Mesorhizobium* with a specific *nodC* gene as its effective microsymbionts. In contrast, *Sophora flavescens* is an extremely promiscuous legume and can nodulate with different genera of rhizobia in the *Alphaproteobacteria* and *Betaproteobacteria*. The infection pattern, nodule and bacteroid development are also diverse in different symbioses.

With further investigation of the relationship between rhizobia and legumes, more novel rhizobia will be discovered. The range of symbiotic relationships among them may become more complex, but it will be illuminated more clearly.

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Part IV
Interaction of Rhizobia, Environments and Legumes

Chapter 8
Geographical Distribution of Rhizobia

Abstract Distribution and geography of rhizobia is affected not only by the geographic origin but also by the host plants. In this chapter, biogeography of rhizobia associated with soybean, *Caragana*, *Astragalus*, faba bean, peanut, *Sophora*, *Phaseolus vulgaris* and alfalfa are discussed in details. Clearly, in some places, only specific rhizobia are found to nodulate specific legume.

8.1 Biogeography of Soybean Rhizobia

Two genera of rhizobia, *Bradyrhizobium* and *Sinorhizobium* (syn. *Ensifer*), are the publicly recognised soybean microsymbionts. Genus *Bradyrhizobium* was established early in 1982, and, at the time of writing, 39 species have been described including seven soybean-associating slow-growing rhizobia: *B. elkanii*, *B. japonicum*, *B. diazoefficiens*, *B. daqingense*, *B. liaoningense*, *B. huanghuaihaiense* and *B. ottawaense*. The genus *Sinorhizobium* was defined in 1988 to describe the fast-growing soybean rhizobia, with *Sinorhizobium fredii* as the type species (Chen et al. 1988). The genus name *Sinorhizobium* was judged a later synonym of *Ensifer* by the Judicial Commission of the International Committee on Systematics of Prokaryotes (2008) because the two genera formed a single group in the 16S rDNA dendrogram of the α-*Proteobacteria* as well as in the phylogenetic analysis of partial *recA* gene sequence (Willems et al. 2003). Since then, three novel soybean-associating fast-growing rhizobial species have been described: *E. sojae* (Li et al. 2011), *E. glycinis* (Yan et al. 2016b) and *E. shofinae* (Chen et al. 2017). Despite the priority of the name *"Ensifer"*, "*Sinorhizobium*" is still widely used in the literature, perhaps because of the clear implication contained in the suffix "rhizobium".

The distribution of soybean rhizobia is clearly affected by the soil pH level. The soybean rhizobia in the genus *Bradyrhizobium* are widely distributed around the world mainly in acid soils, while the fast-growing soybean rhizobia in the genus *Sinorhizobium* are distributed in Asia in alkaline soils (Zhang et al. 2011). Of the seven soybean bradyrhizobia, *B. elkanii*, *B. japonicum*, *B. diazoefficiens* and *B. ottawaense* are widely distributed in different countries, while *B. daqingense*, *B. liaoningense* and *B. huanghuaihaiense* have only been found in some regions of

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China. In addition, soybean-nodulating *B. yuanmingense* was found in some regions of China (Zhang et al. 2011). Of the four fast-growing soybean rhizobial species, *S.* (*E.*) *fredii* is distributed mainly in Xinjiang and Huanghuaihai regions of China (Tian et al. 2012), while the other three species were only found in specific sampling sites in Huanghuaihai region of China. The distinct distribution of the two genera of soybean rhizobia is associated with their genomic characteristics (Tian et al. 2012).

8.2 Biogeography of Rhizobia Associated with *Caragana*

8.2.1 Brief Introduction to the Legume Genus Caragana

Caragana Lam. belongs to tribe Caraganeae (Ranjbar and Karamian 2003), subfamily Papilionoideae. The generic name came from the Latinised version of *karaghan*, the Mongolian name for these plants (Allen and Allen 1981).

Plants in this genus are shrubs or small trees having paripinnate, often fascicled, leaves. Colour of flowers is usually yellow, rarely white or pink (Allen and Allen 1981). The legume genus *Caragana* contains about 100 species native to northeastern Europe, Russia, Central Asia, the Himalayas and China (Allen and Allen 1981). They are remarkable for their cold- and drought-resistant properties and nitrogen-fixing ability in barren soil and sand. Approximately 70 species have been recorded in China, and most of them grow on hills, plains and plateaus, as well as in gullies and thickets (Zhang 2005). The *Caragana* species serve as forage resources for wild animals and are preferable plants for reforestation against desertification and erosion (Fan et al. 2007; Su et al. 2005). In addition, their flowers are a good source of honey production, and their seeds are used as an herbal medicine in China (Xiang et al. 2005).

8.2.2 Nodulation of the Legume Caragana *spp.*

Nodulation and isolation of slow-grower rhizobia from *Caragana arborescens* was first described by Beijerinck as early as 1888 (Allen and Allen 1981). Besides this species, another ten nodulated species were recorded by Allen and Allen (Allen and Allen 1981). In 2009, Sprent updated the records of nodulated *Caragana* to 23 species (Sprent 2009). In the survey of the nodulation of legumes in China, we reported more than 31 nodulated *Caragana* species (Chen et al. 2011). The nodulated species of *Caragana* are listed in Table 8.1.

Table 8.1 List of the nodulated *Caragana* species

Species	References
C. arborescens Lam.	Allen and Allen (1981)
C. aurantiaca Koehne (= *C. sinica* Rehder)	Allen and Allen (1981)
C. frutescens Medik	Allen and Allen (1981)
C. frutex Koch	Allen and Allen (1981)
C. maximowicziana Kom.	Allen and Allen (1981)
C. microphylla Lam.	Allen and Allen (1981)
C. pekinensis Kom.	Allen and Allen (1981)
C. sophoraelia Tausch	Allen and Allen (1981)
C. tragacanthoides Poir.	Allen and Allen (1981)
C. turkestanica Kom.	Allen and Allen (1981)
C. ambigua Stocks	Sprent (2009)
C. arborescens Lam.	Sprent (2009)
C. aurantiaca Koehne	Sprent (2009)
C. brevispina Benth.	Sprent (2009)
C. decorticans Hemsl.	Sprent (2009)
C. erinacea Kom.	Sprent (2009)
C. frutescens (L.) Medik. = *C. frutex,*	Sprent (2009)
C. frutex (L.) K. Koch	Sprent (2009)
C. intermedia Kuang & H.C. Fu = *C. korshinskii*	Sprent (2009)
C. korshinskii Kom.	Sprent (2009)
C. leveillei Kom.	Sprent (2009)
C. maximowicziana Kom. = *C. erinacea, C. microphylla* Lam.	Sprent (2009)
C. pekinensis Kom. = *C. zalbruckneri* var. *pekinesis*	Sprent (2009)
C. pleiophylla (Regel) Pojark.	Sprent (2009)
C. polourensis Franch.	Sprent (2009)
C. pruinosa Korn.	Sprent (2009)
C. rosea Turcz. ex Maxim.	Sprent (2009)
C. sinica (Buc'hoz) Rehder	Sprent (2009)
C. sophoraelia x Tausch	Sprent (2009)
C. tragacanthoides Poir.	Sprent (2009)
C. turkestanica Kom.	Sprent (2009)
C. versicolor Benth.	Sprent (2009)
C. zalbruckneri var. *pekinesis* (Kom.) Yakovle	Sprent (2009)
C. acanthophylla Kom.	Chen et al. (2011)
C. bicolor Kom.	Chen et al. (2011)
C. bongardiana (Fisch. & Mey.) Pojark.	Chen et al. (2011)
C. brevifolia Kom.	Chen et al. (2011)
C. erinacea Bunge ex Kom.	Chen et al. (2011)
C. franchetiana	Chen et al. (2011)
C. hololeuca Bunge ex Kom.	Chen et al. (2011)
C. jubata	Chen et al. (2011)

(continued)

Table 8.1 (continued)

Species	References
C. kansuensis Pojark.	Chen et al. (2011)
C. leucophloea Pojark.	Chen et al. (2011)
C. leveillei Kom.	Chen et al. (2011)
C. opulens Kom.	Chen et al. (2011)
C. potaninii	Chen et al. (2011)
C. pygmaea (Linn.) DC.	Chen et al. (2011)
C. roborovskyi Kom.	Chen et al. (2011)
C. stenophylla Pojark.	Chen et al. (2011)
C. tibetica Kom.	Chen et al. (2011)
C. arborescens Lam.	Chen et al. (2011)
C. frutex (Linn.) Koch.	Chen et al. (2011)
C. intermedia Kuang & Fu	Chen et al. (2011)
C. korshinskii Kom.	Chen et al. (2011)
C. microphylla Lam.	Chen et al. (2011)
C. pruinosa Korn. Tan	Chen et al. (2011)
C. rosea Turcz. ex Maxim.	Chen et al. (2011)
C. sinica (Buc'hoz) Rehd.	Chen et al. (2011)
C. versicolor Zeng	Chen et al. (2011)
C. polourensis	Chen et al. (2011)
C. pumila	Chen et al. (2011)
C. camilli-schneideri	Chen et al. (2011)
C. turkestanica	Chen et al. (2011)
Caragana spp. (grown in Tianshan and Yili, Xinjiang Province)	Chen et al. (2011)

8.2.3 *Diversity and Gene Characterisation of* Caragana *Rhizobia*

The *Caragana* rhizobia were always considered slow-growing cowpea–soybean–lupine types. Through the last two decades of studies, it is now clear that both the slow-growing and fast-growing rhizobia, in genera *Mesorhizobium* and *Rhizobium*, could establish symbiosis with *Caragana* species. Besides, endophytes belonging to *Bradyrhizobium*, *Agrobacterium* and *Burkholderia* were also isolated from the root nodules of *Caragana* species (Gao et al. 2002; Li et al. 2012; Yan et al. 2007).

The predominant rhizobia associated with *Caragana* belong to the genus *Mesorhizobium,* with extensive species diversity. *Mesorhizobium caraganae* (Guan et al. 2008) and *Mesorhizobium shangrilense* (Lu et al. 2009c) are the only two species whose original host plants are *Caragana*. However, different mesorhizobia besides these two species could be isolated from root nodules of *Caragana* species, including *Mesorhizobium septentrionale*, *M. amorphae*, *M. gobiense*, *M. mediterraneum*, *M. temperatum*, *M. huakuii*, *M. loti*, *M. tianshanense* and twelve other unidentified genospecies (Chen et al. 1995; Ji et al. 2015).

Though the mesorhizobia are diverse, all the nodulation genes of the strains share the highest similarity and are clustered into one big branch, indicating strong selection by the host plant. The phylogenetic relationships of the concatenated sequences of the nodulation genes (*nodA*, *nodC*, *nodD*, *nodG* and *nodP*) were significantly different from those of the core (*atpD*, *glnII*, *gyrB*, *recA* and *rpoB*) and heat shock factor genes (*clpA*, *clpB*, *dnaK*, *dnaJ*, *grpE* and *hlsU*) (Ji et al. 2015).

Besides the mesorhizobia, fast-growing *Rhizobium alkalisoli* (Lu et al. 2009a) and *Rhizobium yanglingense* are found to be the main microsymbionts of *Caragana*, especially the latter (Ji et al. 2017; Li et al. 2012).

To find shared genes of *R. yanglingense* and *Mesorhizobium* spp. in determining the association with *Caragana* species, genome sequences of *R. yanglingense* CCBAU 01603 and six mesorhizobia of *Caragana* were sequenced and compared (Yan et al. 2017). Results showed that genes *nodE* and *nodO*, type I secretion systems (T1SS) and a hydrogenase system were evolutionarily conserved in all these *Caragana* rhizobia. Moreover, the highest similarity of *nodO* genes among the *R. yanglingense* strain and these mesorhizobial strains was found, endowing them to nodulating common host range, *Caragana* and *Astragalus membranaceus*. In addition, this *nodO* gene could be used as an indicator relating to nodule formation and nitrogen fixing efficiency when the rhizobia interact with *Caragana* (Yan et al. 2017).

8.2.4 Geography of Caragana *Rhizobia*

As mentioned above, *Mesorhizobium* is the predominant genus of the *Caragana* rhizobia. Therefore, different *Caragana* mesorhizobia could be found in nodules of *Caragana* in various environments.

In a survey of the genetic diversity of rhizobia associated with *Caragana* grown in Liaoning Province, northeast of China, the authors isolated 112 symbiotic bacteria, and they were clustered into 11 putative species in genus *Mesorhizobium* (Yan et al. 2007). Based on further multilocus sequence analysis (MLSA) of *recA*, ITS and 16S rRNA genes, these putative species were classified as three known species, *M. amorphae*, *M. septentrionale* and *M. huakuii*, and eight unknown species (Chen et al. 2008). Of these eight unknown species, *Mesorhizobium* sp. III (represented by strains CCBAU 11226, 11,229, 11,300) was the predominant species distributed in Liaoning Province, and it was identified as *M. caraganae* by Guan et al. (Guan et al. 2008), together with three strains (CCBAU 01519, 01528, 01502) isolated from the neighbouring region, Inner Mongolia.

The correlation of the geographic origin and rhizobial genospecies of *Caragana* was clearly observed among the three regions: Eastern Inner Mongolia (ecoregion A), Northern Shanxi (ecoregion B) and Northwestern Yunnan (ecoregion C) Provinces (Lu et al. 2009b). *Mesorhizobium* genospecies I, II, IV and VII contained the strains isolated from ecoregion A, while the genospecies *M. temperatum*, *M. tianshanense*, *M. septentrionale*, *R. yanglingense* and *Rhizobium* sp. V were mainly

from ecoregion B. *M. plurifarium* and *Mesorhizobium* genospecies VI and VIII and *Rhizobium* sp. IV were from Yunnan (ecoregion C). *M. amorphae* was isolated from both ecoregion A and B, while *M. temperatum* was found in both ecoregion B and C.

Significant genetic divergence was observed among the genospecies of *Caragana* mesorhizobia sampled from the semi-fixing desert belt, north of China (ecoregion A), and mountain forest region in Yunnan Province, southwest of China (ecoregion C), meaning their close relationship is related to the environmental conditions and geographic distance. Gene flow occurred more frequently among the genospecies in areas of the semi-fixing desert belt (ecoregion A) than between area of Yunnan Province (ecoregion C). Recombination occurred among strains more frequently for heat shock factor genes than the other nodulation and core genes. The results conclusively showed that the *Caragana*-associated mesorhizobia had divergently evolved according to their geographic distribution and have been selected not only by the environmental conditions but also by the host plants (Ji et al. 2015).

The type strain of *Rhizobium yanglingense* CCBAU 71623 was isolated from a root nodule of *Gueldenstaedtia multiflora* growing in Gansu Province, northwest of China (Tan et al. 2001). In our survey of *Caragana* rhizobia in the arid and semi-arid alkaline deserts of Inner Mongolia, north of China, we found *R. yanglingense* was one of the predominant species in this region (Li et al. 2012). Furthermore, this species was isolated from *Sophora flavescens* growing in Liaoning Province, northeast of China (Jiao et al. 2015a).

The competition between *R. yanglingense* strain 01603 and five mesorhizobia (*M. amorphae* CCBAU 01583; *M. temperatum* CCBAU 01582; *M. caraganae* CCBAU 01502; and *M. septentrionale* CCBAU 01570) under different environmental conditions (vermiculite or sandy soil) was evaluated systematically (Ji et al. 2017). Interesting results showed that, depending on the environment, *Caragana* could select its preferential rhizobial partner from the mixture of the above different strains in order to establish basic symbiosis. Because *Caragana* is a perennial legume, it can be nodulated by different rhizobial strains in a sequential process. When the most favourable rhizobial strain was available to infect *Caragana* first, the strain was dominant in the nodules, regardless of the existence of other rhizobial strains in the rhizosphere. Other rhizobial strains had an opportunity to establish symbiosis with the plant when the most favourable rhizobial strain was not present in the rhizosphere. Nodule occupancy rates of the most favourable rhizobial strain depended on the competitiveness of other rhizobial strains in the rhizosphere and the environmental adaptability of the favourable rhizobial strain (in this case, to mild vermiculite or hostile sandy soil). To produce high nodulation and efficient nitrogen fixation, the most favourable rhizobial strain should be selected and inoculated into the rhizosphere of legume plants under optimum environmental conditions.

8.3 Biogeography of Rhizobia Associated with *Astragalus*

8.3.1 Nodulation and Diversity of Astragalus *Rhizobia*

The genus *Astragalus* is the largest one (nearly one-third of all legume species) in the Leguminosae, containing about 2500 species distributing widely in geographic habitats ranging from frigid to warm and from wet to arid (Liston and Wheeler 1994; Sprent 2009). About 119 species have been recorded to have root nodules summarised by Allen and Allen (Allen and Allen 1981). In the book, *Chinese Rhizobia*, 43 *Astragalus* species were recorded to have nodules found in China (Chen et al. 2011), including plants of *Astragalus aksuensis*, *A. arpilobus*, *A. basiflorus*, *A. beketovii*, *A. bhotanensis*, *A. capillipes*, *A. chrysopterus*, *A. confertus*, *A. dahuricus*, *A. discolor*, *A. ellipsoideus*, *A. filicaulis*, *A. floridus*, *A. galactites*, *A. henryi*, *A. kifonsanicus*, *A. laceratus*, *A. lioui*, *A. mahoschanicus*, *A. melilotoides*, *A. moellendorffii*, *A. monadelphus*, *A. nivalis*, *A. polycladus*, *A. przewalskii*, *A. puberulus*, *A. tataricus*, *A. tatsienensis* var. *kangrenbuchiensis*, *A. yunnanensis*, *A. adsurgens*, *A. complanatus*, *A. coronilloides*, *A. danicus*, *A. lehmannianus*, *A. mongholicus*, *A. miniatus*, *A. oxyglottis*, *A. scaberrimus*, *A. sinicus*, *A. striatus*, *A. tanguticus* and *A. skythropos*. Of these nodulated *Astragalus* species, 29 species (underlined) were first registered to have nodules found in China (Chen et al. 2011). Li et al. reported another two species of *A. luteolus* and *A. ernestii* that bear nodules growing in Sichuan Province of China (Li et al. 2009; Zhou et al. 2013). In addition, effective symbiosis between *A. chrysopterus* and *Rhizobium* sp. CCNWTB 701 was recorded in a mining tailing region in Shaanxi Province, northwest of China.

Although different rhizobial species and genera have been isolated from root nodules of different species of the genus *Astragalus* (Chen et al. 2011; Fan et al. 2007), the overwhelming majority of rhizobia reported belong to the genus *Mesorhizobium* (Ampomah et al. 2017; Gao et al. 2001; Li et al. 2009; Yan et al. 2016a), followed by *Rhizobium* and *Sinorhizobium* (Mahdhi et al. 2016; Zhao et al. 2008).

Eleven different rhizobial species in the genus *Mesorhizobium* (Table 8.2), including *M. amorphae*, *M. ciceri*, *M. huakuii*, *M. mediterraneum*, *M. muleiense*, *M. qingshengii*, *M. sangaii*, *M. septentrionale*, *M. silamurunense*, *M. temperatum* and *M. tianshanense*, were reported to be isolated from root nodules of *Astragalus* spp., and they are the main microsymbionts of this plant genus. Of these mesorhizobial species, *M. huakuii* (Chen et al. 1991) and *M. qingshengii* (Zheng et al. 2013) are highly host-specific microsymbionts of Chinese milk vetch (*Astragalus sinicus*), though the cross-nodulation results showed that they can nodulate other legumes, such as Lespedeza (Gu et al. 2007) and the promiscuous medicinal legume *Sophora flavescens* (Jiao et al. 2015a; Liu et al. 2018). Similarly, *M. amorphae* (Wang et al. 2002), *M. ciceri* (Nour et al. 1994), *M. muleiense* (Zhang et al. 2012) and *M. mediterraneum* (Nour et al. 1995) are also host-specific rhizobia, while they could be

Table 8.2 Bacteria isolated from root nodules of *Astragalus* species

Rhizobial species	Host plant	References
Mesorhizobium amorphae	*Astragalus* spp., *Lespedeza* and *Amorpha fruticosa*	Gnat et al. (2015, 2016), Zhao et al. (2008), Gu et al. (2007) and Wang et al. (1999, 2002)
Mesorhizobium ciceri	*Astragalus* spp. and *Cicer arietinum*	Gnat et al. (2015, 2016), Yan et al. (2016a), Nour et al. (1994) and Wdowiak and Malek (2000)
Mesorhizobium huakuii	*A. sinicus*, *Sophora flavescens*, *Lespedeza*	Chen et al. (1991), Jarvis et al. (1997), Zhao et al. (2008), Wdowiak and Malek (2000) and Gu et al. (2007)
Mesorhizobium mediterraneum	*A. cicer* and *Cicer arietinum*	Wdowiak and Malek (2000) and Nour et al. (1995)
Mesorhizobium muleiense	*Astragalus* spp. and *Cicer arietinum*	Yan et al. (2016a) and Zhang et al. (2012)
Mesorhizobium qingshengii	*Astragalus sinicus*	Zheng et al. (2013)
Mesorhizobium sangaii	*A. luteolus* and *A. ernestii*	Zhou et al. (2013)
Mesorhizobium septentrionale	*Astragalus* spp.	Gao et al. (2004), Gnat et al. (2015, 2016) and Yan et al. (2016a)
Mesorhizobium silamurunense	*A. membranaceus*	Zhao et al. (2012)
Mesorhizobium temperatum	*Astragalus* spp.	Gao et al. (2004) and Yan et al. (2016a)
Mesorhizobium tianshanense	*Astragalus* spp.	Yan et al. (2016a) and Wdowiak and Malek (2000)
Sinorhizobium fredii	*A. membranaceus* and *A. scaberrimus*	Zhao et al. (2008)
Sinorhizobium meliloti		Zhao et al. (2008) and Yan et al. (2016a)
Sinorhizobium spp.	*Astragalus* spp.	Zhao et al. (2008) and Mousavi et al. (2015)
Ensifer (syn. *Sinorhizobium*) *glycinis*	*A. mongholicus* and soybean (*Glycine max*)	Yan et al. (2016b)
Bradyrhizobium japonicum	*A. henryi*	Zhao et al. (2008)
Bradyrhizobium elkanii	*A. melilotoides*	Zhao et al. (2008)
Agrobacterium tumefaciens	*A. mongholicus*	Yan et al. (2016a)
Rhizobium alamii	*A. mongholicus*	Yan et al. (2016a)
Rhizobium gallicum	*Astragalus* spp.	Zhao et al. (2008)
Rhizobium giardinii	*Astragalus* spp.	Yan et al. (2016a)
Rhizobium leguminosarum	*Astragalus* spp.	Zhao et al. (2008) and Yan et al. (2016a)
Rhizobium loessense	*Astragalus* spp. and *Lespedeza* spp.	Wei et al. (2003) and Zhao et al. (2008)
Rhizobium mongolense	*Astragalus* sp. and *Medicago ruthenica*	van Berkum et al. (1998) and Mousavi et al. (2015)

(continued)

Table 8.2 (continued)

Rhizobial species	Host plant	References
Rhizobium multihospitium	*A. aksuensis*, *A. betetovii* and others	Han et al. (2008c)
Rhizobium yanglingense	*Astragalus*, *Caragana*, *S. flavescens*	Yan et al. (2016a, 2017), Li et al. (2012) and Jiao et al. (2015a)
Rhizobium sp. (close to *Rhizobium mongolense* and *R. gallicum*)	*A. chrysopterus*	Wei et al. (2008) and Zhao et al. (2008)
Rhizobium sp. I CCBAU 75186 (close to *Rhizobium cellulosilyticum*)	*A. mongholicus*	Yan et al. (2016a)
Neorhizobium galegae	*Galega* spp. and *A. cruciatus*	Mousavi et al. (2014), Zakhia et al. (2004) and Yan et al. (2016a)
Nocardioides astragali	*A. chrysopterus*	Xu et al. (2018)
Pararhizobium herbae	*A. membranaceus* and *Oxytropis cashmiriana*	Ren et al. (2011), Mousavi et al. (2015) and Zhao et al. (2008)
Phyllobacterium leguminum	*A. algerianus* and *Argyrolobium uniflorum*	Mantelin et al. (2006)
Phyllobacterium ifriqiyense	*A. algerianus* and *Lathyrus numidicus*	Mantelin et al. (2006)

isolated from different species of *Astragalus* (Gnat et al. 2016; Wdowiak and Malek 2000; Yan et al. 2016a). However these specific mesorhizobia from *Astragalus* have distinct *nodC* genes phylogenetically differing from the *nodC* genes possessed by the type strains of the species, whose original host plants are not *Astragalus* (Yan et al. 2016a; Zhang et al. 2018).

Rhizobium is the second genus that includes many species that can nodulate *Astragalus*. *Rhizobium herbae* (now reclassified as *Pararhizobium herbae*) strain CCBAU 83011^T can nodulate *A. membranaceus* well (Ren et al. 2011; Zhao et al. 2008). *Rhizobium yanglingense* strain CCBAU 01603 was isolated from *Caragana intermedia* (Li et al. 2012) and can also nodulate *Astragalus membranaceus* (Yan et al. 2017) as well as *Caragana microphylla* (Ji et al. 2017). Results from comparative genomes and cross-nodulation tests showed that evolutionarily conserved *nodE* and *nodO* genes, T1SS (type 1 secretion system) and hydrogenase system (*hup*) among the *A. membranaceus* and *C. intermedia* (AC)-associated rhizobia including *M. silamurunense*, *M. septentrionale*, *M. amorphae*, *M. caraganae* and *M. temperatum* in genus *Mesorhizobium* and the phylogenetically distinct species, based on MLSA, *R. yanglingense* CCBAU 01603 (Li et al. 2012), were responsible for their common host ranges (Yan et al. 2017). *Rhizobium mongolense* USDA 1844^T was first isolated from nodules of *Medicago ruthenica*, but it only induced inefficient nodules on *M. ruthenica.* In our studies, we found that the strain *R. mongolense* USDA 1844^T (van Berkum et al. 1998), as well as another species isolated from alfalfa (*Medicago sativa* L.), *R. favelukesii* LPU83^T (Tejerizo et al. 2016), could nodulate *A. membranaceus* well (personal records). The *Rhizobium* sp. strain CCNWTB701 isolated from *A. chrysopterus* was first reported to have very active

phenol-degrading ability (Wei et al. 2008), and this strain formed effective pink nodules on its host plant. Studies revealed that *Rhizobium* strains isolated from *A. cicer* and wild desert plants effectively nodulated *Medicago sativa* and *Phaseolus vulgaris* (Zhao et al. 1997). The nodulation ability of other *Rhizobium* species (Table 8.2) was not confirmed solidly.

Other genera of bacteria isolated from root nodules of *Astragalus* are minor groups. In nodulation tests, some strains belonging to *Sinorhizobium/Ensifer* (Dai et al. 2012; Yan et al. 2016a), *Agrobacterium* (Mahdhi et al. 2016), *Nocardioides astragali* (Xu et al. 2018) and others could not nodulate *Astragalus* spp., indicating their opportunistic entry as endophytes into root nodules of *Astragalus* species. On the other hand, bacteria isolated from root nodules of plants other than *Astragalus* species may nodulate *Astragalus* well. For example, *Ensifer* (syn. *Sinorhizobium*) *shofinae* CCBAU 251167^T was isolated from a root nodule of soybean, but it can nodulate *A. membranaceus* and fix nitrogen efficiently (Chen et al. 2017). In contrast, the type strain CCBAU 23380^T of *Ensifer* (syn. *Sinorhizobium*) *glycinis* was isolated from a root nodule of *Astragalus mongholicus*; it could not nodulate its host plant but can nodulate soybean (*Glycine max*) (Yan et al. 2016b). The highly similar symbiotic gene sequences (*nodC* and *nifH*) among species of *E. shofinae*, *E. glycinis* and *E. fredii* (sym. *S. fredii*) may explain why they have common host, soybean (Chen et al. 2017). However they are still different species with a low ANI (average nucleotide identity) values (<90.5%) among them and different nodulation properties (Chen et al. 2017).

Two species in the genus *Phyllobacterium*, *P. leguminum* and *P. ifriqiyense* were reported to be isolated from root nodules of *A. algerianus* and *A. uniflorum* (Mantelin et al. 2006), but nitrogen fixation was not clearly stated and demonstrated.

8.3.2 Geography of Astragalus *Rhizobia*

Extensive studies conducted on the geography of *Astragalus*-associated rhizobia focused on the rhizobia isolated from *A. membranaceus*, *A. mongholicus* and *A. adsurgens* in north and northwest China (Gao et al. 2001; Yan et al. 2016a; Zhao et al. 2008). Therefore, the focus here is on the rhizobial geography of these *Astragalus* species in China, and these studies also could represent the characteristics of *Astragalus* rhizobia around the world.

Different *Mesorhizobium* species were the extremely predominant microsymbionts of the two medicinal legumes *A. membranaceus* and *A. mongholicus* and the sand-fixing plant *A. adsurgens* growing in Shanxi, Shaanxi, Ningxia and Liaoning provinces. Of these rhizobial genospecies, *M. temperatum*, *Mesorhizobium septentrionale* and *M. muleiense* were the three most widely distributed rhizobial genospecies, and they could be found in root nodules in different regions (Fig. 8.1). Minor strains belonging to *M. tianshanense* and *M. ciceri* were only found in root nodules in some regions.

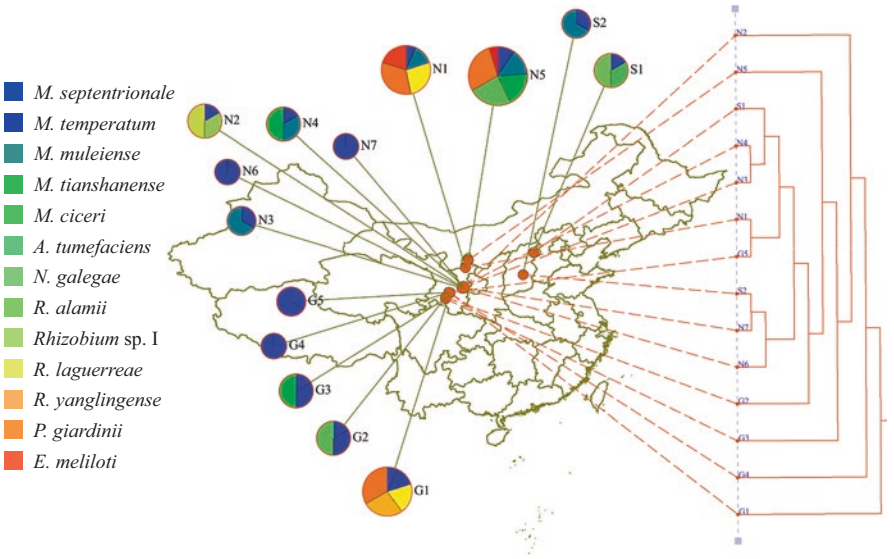

Fig. 8.1 Distribution and ratio of different *Astragalus* rhizobia-associated genospecies in different sampling sites of China (Yan et al. 2016a). The pie charts show the ratio of each genospecies placed beside the map. Sampling sites S1 and S2 from Shanxi Province, N1–N7 from Ningxia Province, G1–G5 from Gansu Province. The geographic information for these sampling sites are shown in detail in reference (Yan et al. 2016a)

Among the different mesorhizobia, *M. septentrionale*, *M. temperatum* and *M. ciceri* were the major rhizobial species isolated from *A. mongholicus*, while *M. tianshanense* was the major microsymbiont of *A. membranaceus*. The majority of rhizobia of *A. adsurgens* growing in Liaoning Province belong to *M. temperatum* and *M. septentrionale* (Gao et al. 2001, 2004).

8.3.3 Conclusion and Perspective Remarks

Though the largest genus of family Leguminosae, no more than 200 species (about 8%) were surveyed for the nodulation and rhizobial diversity in detail. The overwhelming microsymbionts of *Astragalus* spp. belong to *Mesorhizobium*, followed by *Rhizobium* and *Sinorhizobium/Ensifer*. Endophytes were often isolated from root nodules of *Astragalus*, but the nodulation ability of these endophytes has not been clearly demonstrated. The importance of these *Mesorhizobium* species cannot be overemphasised for they are symbiotic nitrogen-fixing bacteria associated with a wide variety of legumes, including the currently described *Astragalus*, the important crop species like chickpea, the sand-fixing *Caragana* and others (Laranjo et al. 2014). Resources, genomes and evolution should be further investigated to understand their distinct characteristics and to develop more effective mesorhizobia inoculants for *Astragalus* and other plants.

8.4 Biogeography of Rhizobia Associated with Faba Bean

Faba bean (*Vicia faba* L.) is cultivated either in winter or spring season mainly in China and Europe (Duc et al. 2010). *Rhizobium leguminosarum* sv. *viciae* (Mutch and Young 2004), *R. fabae* (Tian et al. 2008), *R. laguerreae* (Saïdi et al. 2014) and *R. anhuiense* (Zhang et al. 2015) have been isolated from root nodules of faba bean in various soils around the world. Of these species, only the symbiovar (sv.) *viciae* with *nodC* gene sequence highly similar (>95%) to FJ596038 (GenBank No.) could nodulate faba bean, reflecting the high selection by the host plant on the symbiosis genes of rhizobia (Saïdi et al. 2014; Zhang et al. 2015) and the bacterial adaptation to host plant (Xiong et al. 2017).

R. laguerreae is the predominant microsymbiont of faba bean in the northwest of China (Xiong et al. 2017), Europe, Africa and America (Saïdi et al. 2014). Only very few of *Rhizobium* spp. I and II were found in nodules of faba bean grown in northwest of China (Xiong et al. 2017). In the eastern regions of China, three rhizobial species including *R. anhuiense*, *R. laguerreae* and *R. fabae*/*R. pisi* were found there (Xiong et al. 2017; Zhang et al. 2015). In the southwest of China, six rhizobial species associated with faba bean were found, with *R. laguerreae* as the first dominant microsymbiont, followed by *R. anhuiense*, *R.* spp. I and II and a minor component of *R. leguminosarum* (Xiong et al. 2017). Soil pH and salt content are correlated positively to the abundance of *R. laguerreae*, whereas they are negatively correlated to the distribution of *R. anhuiense* (Xiong et al. 2017). However, until now the experiments performed could not confirm the direct role of these two soil factors in the contrasting distribution patterns of *R. laguerreae* and *R. anhuiense* (Xiong et al. 2017).

In the Bejaia region of Algeria, North Africa, faba bean was nodulated by *R. leguminosarum* sv. viciae, *R. laguerreae* and two new genospecies in the genus *Rhizobium* (Belhadi et al. 2018).

In studies of faba bean-nodulating rhizobia in Panxi, Sichuan Province, southwestern China (a region not surveyed by Xiong et al. (2017)), the authors found *R. anhuiense* was the predominant (41 of 65 isolates) species, followed by *R. fabae*, *R. vallis*, *R. sophorae*, *Agrobacterium radiobacter* (no *nodC* gene amplified) and four unknown species in the genera *Rhizobium* and *Agrobacterium* (Chen et al. 2018).

8.5 Biogeography of Rhizobia Associated with Peanut

Most of the peanut rhizobia belong to the slow-growing genus *Bradyrhizobium*, with *B. arachidis*, *B. guangdongense*, *B. guangxiense*, *B. lablabi*, *B. yuanmingense*, *B. iriomotense*, *B. japonicum* and *B.* spp. as the representatives.

The type strain CCBU 051107^T of the species *B. arachidis* was isolated from root nodule of peanut grown in Hebei Province of China (Wang et al. 2013), and this species represented 22 isolates from peanut and one isolate from *Lablab purpureus* grown in different regions of China (Wang et al. 2013). *B. guangdongense* and *B. guangxiense* were isolated respectively from Guangdong and Guangxi Provinces, southeast China (Li et al. 2015). Strain CCBAU 23160, isolated from nodules of peanut grown in Anhui Province, central China, was identified as *B. lablabi* (Chang et al. 2011). Bacterial isolates of peanut grown in Ghana (Africa) were found to be closely related to *B. yuanmingense* (Osei et al. 2018). This species and *B. iriomotense*, *B. japonicum* and *Bradyrhizobium* sp. were found to be predominant peanut microsymbionts in Córdoba, Argentina (Muñoz et al. 2011; Taurian et al. 2006).

Besides the bradyrhizobia, some fast-growing bacteria were isolated from root nodules of peanut from Argentina, Morocco, Ghana and South Africa (El-Akhal et al. 2009; Jaiswal et al. 2017; Osei et al. 2018; Taurian et al. 2006). Fast-growing effective rhizobia from peanut nodules have not yet been isolated from China.

8.6 Biogeography of Rhizobia Associated with *Sophora*

8.6.1 Nodulation of Sophora *spp.*

Sophora, a genus of about 50 species of shrubs or tree in tribe Sophoreae of pea family Fabaceae (Sprent 2009). The species are native to southeast Europe, Asia, Australasia, various Pacific islands, western South America, the western United States, Florida and Puerto Rico. The generic name is derived from *sophera*, an Arabic name for a pea-flowered tree. A mix of nodulation- and non-nodulation genera was found in tribe Sophoreae. Total of 19 species in the genus *Sophora* are known to have nodulation, while the other species appears to be unable to nodulate (Sprent 2009). Most species in tribe Sophoreae have the 50 kb inversion of the chloroplast genome, similar to the presence in most taxa of the subfamily Papilionoideae, while four small groups in tribe Sophoreae lack this inversion (Doyle et al. 1996). Whether there is a positive correlation between the existence of this 50 kb inversion and the own of nodulation is unclear.

Reports of nodulated species in the genus *Sophora* include *S. alopecuroides*, *S. angustifolia* = *S. flavescens*, *S. chathamica*, *S. chrysophylla*, *S. davidii*, *S. howinsula*, *S. inhambanensis*, *S. longicarinata*, *S. microphylla*, *S. mollis*, *S. moorcroftiana*, *S. prostrata*, *S. tetraptera*, *S. tomentosa* and *S. velutina*, as well as *S. arizonica* var. *formosa, S. gypsophila* and *S. secundiflora*, which have been transferred to the genus *Dermatophyllum* (Gandhi et al. 2011). Most of the rhizobia were isolated from *S. alopecuroides*, *S. flavescens*, *S. microphylla*, *S. prostrata* and *S. tomentosa*, and the rhizobial diversity from these plants had been studied extensively.

8.6.2 *General Diversity of Rhizobia Associated with* Sophora *spp.*

Diversity of nodule bacteria isolated from the legume genus *Sophora* has been reviewed in Chap. 7 of this book. In summary, six genera of bacteria, including *Bradyrhizobium*, *Rhizobium*, *Sinorhizobium*, *Phyllobacterium* and *Agrobacterium*, were isolated from nodules of *Sophora* spp. in different places, mainly in China and New Zealand. The predominant rhizobia of *Sophora* spp. belong to the genera of *Mesorhizobium* and *Rhizobium*.

8.6.3 *Biogeography of Rhizobia Associated with* Sophora flavescens

The biogeography of rhizobial isolates obtained from nodules of *Sophora flavescens* in different regions of China were studied extensively (Jiao et al. 2015a). Therefore, in this subsection, we discuss them in detail.

A total of 266 pure isolates, confirmed to be nitrogen-fixing symbionts, were isolated from root nodules of *S. flavescens* grown in three ecoregions of China: Shanxi Province (ecoregion I), Shaanxi Province (ecoregion II) and Liaoning Province (ecoregion II). The soil pH values of the three ecoregions were 7.26–7.95 (slightly alkaline), 5.44–6.68 (acidic) and 8.36–8.59 (alkaline), respectively. In addition, ecoregion I had a much higher concentration of available potassium (AK) than the other two ecoregions. Ecoregion III had total nitrogen content (TN), organic matter (OM), available nitrogen (AN), available phosphorus (AP), available potassium (AK) and total salt (TS) lower than those of the other two ecoregions (Table 8.3).

Of the 266 rhizobia, 17 genospecies in 5 genera were identified based on the multilocus sequence analysis (MLSA) of 3 housekeeping genes (*atpD-glnII-recA*) (Table 8.4): *Sinorhizobium fredii*, *Mesorhizobium septentrionale*, *Bradyrhizobium elkanii*, *Rhizobium yanglingense*, *Mesorhizobium* sp. I, *R. sophorae*, *R. sophoriradicis*, *M. huakuii* and *Rhizobium* sp. I were the major groups; *Phyllobacterium sophorae*, *Bradyrhizobium* sp. I, *Mesorhizobium* sp. II, *R. mongolense*, *R. lusitanum*, and *Rhizobium* spp. II, III and V were the minor groups. *Sinorhizobium fredii* was the most widely spread major genospecies (including 46 isolates) occurring on 8 sampling sites. The minor groups were only found in one to three sampling sites.

The highest diversity (Shannon-Weiner index, H') was found at site S18 (1.68), followed by that at site S5 (1.58) and site S17 (1.22) (Table 8.4). Seven genospecies were found at each of above three sites. The lowest H' values (0) were found at sites S2, S9, S10, S14 and S15 because only one genospecies was isolated there. The other sampling sites had H' values ranging from 0.97 to 0.30. The values of Simpson's index (D) varied between 0.78 and 0 at 18 sampling sites and were well

Table 8.3 Geographic information, climate and soil characteristics of the 18 sampling sites (Jiao et al. 2015a)

Geographic origin and sampling sites (S1~S18)	Altitude (m)	GPS		Rainfall (mm)[a]	Soil characteristics[b]							Ecoregions [c]
		Longitude	Latitude		TN	OM	AN	AP	AK	TS	pH	
Shanxi Province												
S1: Zhenxin town, Changzhi	1224	113°03′08″E	36°02′24″N	411	1.18	16.7	70.0	5.9	436	0.75	7.95	I
S2: Zhenxin town, Changzhi	1224	113°03′10″E	36°02′27″N	411	1.06	15.4	53.4	56.7	138	0.70	7.52	I
S3: Zhenxin town, Changzhi	1224	113°03′09″E	36°02′30″N	411	0.82	16.3	42.0	19.9	122	0.83	7.76	I
S4: Wugu Mt., Changzhi	1041	113°03′02″E	36°02′24″N	411	1.43	30.4	44.3	25.8	170	1.27	7.26	I
S5: Niusi town, Qinxian	993	112°03′30″E	36°54′36″N	606	0.89	11.4	58.5	14.8	273	0.85	7.75	I
S6: Niusi town, Qinxian	993	112°03′36″E	36°54′35″N	606	0.94	10.3	44.5	25.4	390	0.86	7.78	I
S7: Hanbei town, Wuxiang	1225	113°11′56″E	36°45′40″N	560	1.01	23.0	103.0	33.4	398	0.91	7.76	I
S8: Hanbei town, Wuxiang	1225	113°11′55″E	36°45′25″N	560	1.02	28.4	90.4	10.9	322	0.90	7.81	I
S9: Hanbei town, Wuxiang	1225	113°11′57″E	36°45′32″N	560	0.90	17.7	50.9	20.8	470	0.73	7.91	I
S10: Hanbei town, Wuxiang	1225	113°11′49″E	36°45′35″N	560	1.19	23.8	52.2	7.3	316	0.67	7.84	I
S11: Hanbei town, Wuxiang	1225	113°11′50″E	36°45′31″N	560	1.06	20.9	57.3	16.7	562	0.80	7.91	I
Shaanxi Province												
S12: Maping town, Luonan	1066	110°04′10″E	34°12′06″N	700	1.27	18.4	52.7	23.1	93	0.41	5.80	II
S13: Maping town, Luonan	1066	110°04′03″E	34°12′11″N	700	1.33	18.0	123.0	34.8	80	0.43	5.44	II
S14: Maping town, Luonan	1050	110°03′59″E	34°12′06″N	700	1.43	20.2	89.3	25.5	110	0.45	5.57	II
S15: Youfang town, Luonan	1109	110°02′44″E	34°13′14″N	700	1.06	13.0	83.5	18.9	65	0.53	6.68	II
Liaoning Province												
S16: Heishui, Jianping	554	119°29′48″E	42°03′33″N	500	0.74	8.18	49.8	9.0	86	0.47	8.36	III
S17: Changlong, Jianping	526	119°23′16″E	41°58′41″N	500	0.47	5.6	36.6	1.6	40	0.64	8.59	III
S18: Yangshuling, Jianping	690	119°45′58″E	41°50′36″N	500	0.73	9.29	49.2	7.3	81	0.55	8.46	III

[a]Rainfall, mean annual rainfall (mm)

[b]*TN* total nitrogen content (g/kg), *OM* organic matter (g/kg), *AN* available nitrogen (mg/kg), *AP* available phosphorus (mg/kg), *AK* available potassium (K) (mg/kg), *TS* total salt (g/kg), *pH* soil pH

[c]I, ecoregion I with clay loam, semihumid, alkaline soil; II, ecoregion II with sandy loam, moist, acid soil; III, ecoregion III with sandy loam, semi-arid land, highly alkaline soil

Table 8.4 Representative genospecies identified based on MLSA similarities and their distribution in each sampling site and ecoregion (Jiao et al. 2015a)

Representative isolate (CCBAU no.)[a]	17 Genospecies[b]	Numbers of isolates in different ecoregions and sites																		
		Ecoregion I (105)											Ecoregion II (81)				Ecoregion III (83)			
		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	Total
03470	*R. sophoriradicis*	2	2		12														4	20
03386	*R. sophorae*					3	2					11					1	5	3	**25**
03360	*Rhizobium* sp. I	3			2	2		2	1			2								**12**
03429	*Rhizobium* sp. II					1														**1**
03419	*Rhizobium* sp. III					1												2		**3**
11560	*Rhizobium* sp. IV																	1	1	**2**
71316	*R. lusitanum*													2						**2**
11559	*R. mongolense*																1	2	1	**4**
11536	*R. yanglingense*																27	1	1	**29**
03373	*S. fredii*			5	20			3	6	2	8	1							1	**46**
71358	*Mesorhizobium* sp. I												1			28				**29**
71303	*Mesorhizobium* sp. II												2							**2**
71320	*M. huakuii*												11	8						**19**
03405	*M. septentrionale*			1		9	1											24	6	**41**
71325	*B. elkanii*													6	23					**29**
03416	*Bradyrhizobium* sp. I					1														**1**
03422	*P. sophorae*					2												2		**4**
Total		**5**	**2**	**6**	**34**	**19**	**3**	**5**	**7**	**2**	**8**	**14**	**14**	**16**	**23**	**28**	**29**	**37**	**17**	**269**

(continued)

Diversity indexes	Shannon-Wiener index (H')	0.67	0.00	0.45	0.85	1.58	0.64	0.67	0.41	0.00	0.00	0.66	0.66	0.97	0.00	0.00	0.30	1.22	1.68	/
	Simpson index (D)	0.48	0.00	0.28	0.53	0.72	0.44	0.48	0.24	0.00	0.00	0.36	0.36	0.59	0.00	0.00	0.13	0.55	0.78	/
	Pielou index (J)	0.97	Null	0.65	0.77	0.81	0.92	0.97	0.59	Null	Null	0.60	0.60	0.89	Null	Null	0.27	0.63	0.86	/

Shannon-Wiener index (H') is the diversity considering the species richness in a community. Simpson index (D) shows the species dominance in a community. Pielou index (J) shows the species evenness in a community

Nodules collected in S1–S11 were from Shanxi Province; S12 to S15 were from Shaanxi Province; S16 to S18 were from Liaoning Province

[a]These three isolates (03429 (from site S5), 03419 (from site S5), 71,316 (from site S13)) could not nodulate *Sophora flavescens* in the nodulation tests in laboratory condition, and the *nodC* gene could not be amplified

[b]*R.*, *Rhizobium*; *M.*, *Mesorhizobium*; *B.*, *Bradyrhizobium*; *S.*, *Sinorhizobium*; *P.*, *Phyllobacterium*

/ no data

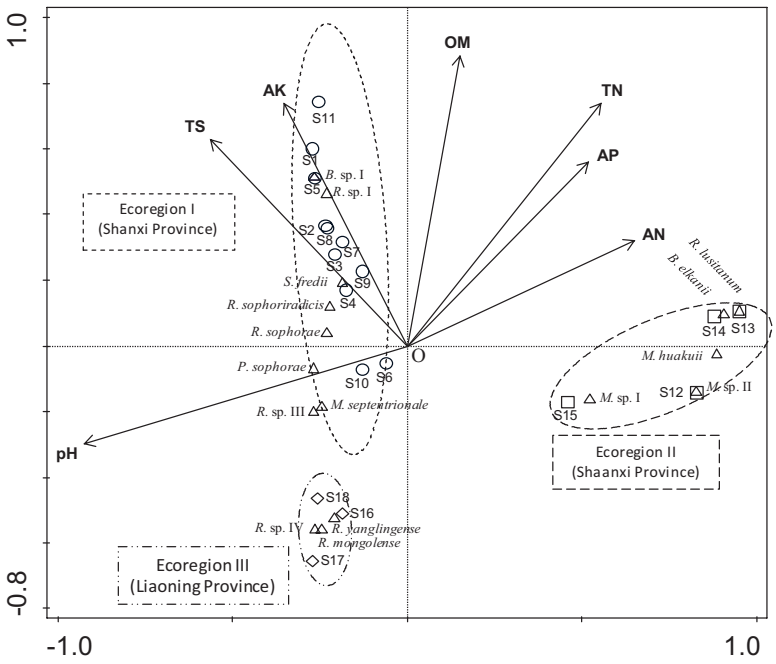

Fig. 8.2 Biplot showing the relationship between the 17 genospecies and soil factors in 3 ecoregions (Jiao et al. 2015a) drawn using CANOCO software. Abbreviation: *AN* available nitrogen, *AP* available phosphorus, *AK* available potassium, *OM* organic materials, *TS* total salt. The arrows and triangles present soil factors and genospecies, respectively. The circles (○), squares (□) and diamonds (◇) present sampling sites in three ecoregions I, II, and III, respectively. The triangles (△) present the distribution of genospecies. The longer the arrow is, the greater the influence of that specific soil factor on the distribution of the certain genospecies; the smaller the angle between the arrow and the line (not drawn) linking the triangle and origin point (O), the closer the relationship between the soil factor and the specific rhizobial genospecies

consistent with the H' values. Evenness Pielou index (J) varied from 0.97 in the case of sites S1 and S7 to 0.27 in the case of site S16. These results demonstrated that the diversity and genospecies composition of the rhizobial community associated with *S. flavescens* varies at different sampling sites.

According to the correlation analysis (biplot) shown in Fig. 8.2, the soil pH, total salt (TS) and available potassium (AK) had an obviously positive correlation with the distribution of genospecies isolated from ecoregion I but a negative correlation with the distribution of genospecies isolated from ecoregion II and III. Soil pH is one of the most important soil factors related to the distribution of these rhizobia ($p = 0.0020$) (Fig. 8.2 and Table 8.3). *Rhizobium yanglingense*, *R. mongolense* and *Rhizobium* sp. IV were solely found in alkaline soil in ecoregion III, while *Rhizobium lusitanum*, *Mesorhizobium* spp. I and II, *M. huakuii* and *Bradyrhizobium elkanii* were only found in acidic soil in ecoregion II.

8.6.4 *Biogeography of Rhizobia Associated with* Sophora alopecuroides

In this subsection, we summarise the biogeography of nodule isolates of *Sophora alopecuroides* in different regions of China (Zhao et al. 2010).

A total of 75 isolates were isolated from root nodules of wild *Sophora alopecuroides* grown in different regions of China's Loess Plateau. Nine genomic species were identified, including *Mesorhizobium alhagi* (34 isolates) and *M. gobiense* (28 isolates) as the main groups, as well as *Agrobacterium tumefaciens* (2 isolates), *M. amorphae* (1 isolate), *Phyllobacterium trifolii* (2 isolates), *Rhizobium giardinii* (1 isolate), *R. indigoferae* (3 isolates), *Sinorhizobium fredii* (3 isolates) and *S. meliloti* (1 isolate) as the minor groups according to the 16S rRNA and *recA* gene sequence comparison. The existence of symbiotic *A. tumefaciens* strains in root nodules of *S. alopecuroides* was first described, and these agrobacteria had symbiosis genes (*nodA* and *nifH*) whose sequences were highly similar (93% and 97%, respectively) to those in *M. alhagi*, indicating potential lateral gene transfer among these strains.

The wide distribution of the predominant species, *M. gobiense* and *M. alhagi*, can be found in many sampling sites of the surveyed regions in Ningxia Autonomous Region (including sampling sites of Qingtongxia, Daba Town and Zhongwei), Gansu Province (including sampling sites of Guazhou, Minqin, Gaotai, Yumen, Zhangye) and Shaanxi Province (including sampling sites of Dingbian). These minor groups had a scattered distribution in Gansu Province and Ningxia Autonomous Region, but not in Shaanxi Province.

In addition, Han et al. identified three strains isolated from *S. alopecuroides* growing in Xinjiang Province as *Bradyrhizobium elkanii* (two strains) and *Rhizobium multihospitium* (one strain) (Han et al. 2008a, b).

8.6.5 *Biogeography of Rhizobia Associated with Other Sophora Species in New Zealand and Other Places*

Nodulation of six species of the genus *Sophora* in New Zealand, including *S. godleyi*, *S. microphylla*, *S. longicarinata*, *S. prostrata*, *S. tetraptera* and *S. chathamica*, has been surveyed, and most of the rhizobia belong to *Mesorhizobium* but with highly diverse species (Nguyen et al. 2017; Tan et al. 2015).

Until now, eight *Mesorhizobium* species have been isolated from New Zealand, including *M. ciceri*, *M. calcicola*, *M. waitakense*, *M. sophorae*, *M. newzealandense*, *M. kowhaii*, *M. waimense* and unknown *M.* sp. ICMP 14430. Only one species, *Rhizobium leguminosarum*, was isolated from nodules of *S. chathamica* in New Zealand. The diversity of the rhizobia and their host range are discussed in detail in Chap. 7.

A few rhizobial strains isolated from nodules of *S. tomentosa* growing in Rio de Janeiro, Brazil were identified as three species: *Sinorhizobium adhaerens*, *Sinorhizobium mexicanum* and *Sinorhizobium chiapanecum* (Toma et al. 2017).

8.7 Biogeography of Rhizobia Associated with *Phaseolus vulgaris*

Common beans (*Phaseolus vulgaris*) are mainly distributed in three geographic regions, one in Mesoamerica (Mexico, Central America and Colombia); another in the southern Andes, and the third in Ecuador and northern Peru (Aguilar et al. 2004). This crop had been exported to the rest of the world, and it is currently an important crop worldwide (Gepts 1990; Gepts and Bliss 1988).

The common bean is a promiscuous legume nodulated by several species predominantly in the genus *Rhizobium* including species of *R. etli* sv. *phaseoli* (Aguilar et al. 2004), *R. leguminosarum* sv. *phaseoli* (Amarger et al. 1994; García-Fraile et al. 2010; Mulas et al. 2011), *R. lusitanum* (Valverde et al. 2006), *R. gallicum* and *R. giardinii* (Amarger et al. 1997), *R. phaseoli* (Ramírez-Bahena et al. 2008), *R. tropici* (Amarger et al. 1994; Martinez-Romero et al. 1991), *R. leucaenae* (Ribeiro et al. 2012), *R. paranaense* (Dall'Agnol et al. 2014), *R. vallis* (Wang et al. 2011) and *R. sophoriadicis* (Jiao et al. 2015b; Ormeño-Orrillo et al. 2018). These species are distributed in different places around the world. In addition, some nodulating isolates are classified in the bacterial genera *Agrobacterium* (Wang et al. 2016), *Bradyrhizobium* (Cao et al. 2014) and *Ensifer* (Wang et al. 2016), and another, non-nodulating, species, *Phyllobacterium endophyticum* (Flores-Félix et al. 2012) was reported to be isolated from root nodules of common beans growing in different soils of China, Mexico and Spain.

The species *R. etli* is the predominant symbiotic rhizobium of both wild and cultivated common bean in Mexico (Silva et al. 2003), Colombia (Eardly et al. 1995), the southern Andes (Amarger 2001) and elsewhere in the Americas (Aguilar et al. 2004). It also has been isolated from Spain (Rodriguez-Navarro et al. 2000), France (Laguerre et al. 1993), Austria (Sessitsch et al. 1997), Tunisia (Mhamdi et al. 1999) and Indonesia (Amarger 2001).

At least six *Rhizobium* genospecies, most belonging to *R. phaseoli* and novel *Rhizobium* species, can nodulate common bean grown in Kenya (Mwenda et al. 2018). Infrequently, isolates belonging to *R. paranaense*, *R. leucaenae*, *R. sophoriradicis* and *R. aegyptiacum* sv. *phaseoli* were also found in Kenya (Mwenda et al. 2018).

Two species, *R. leguminosarum* sv. *phaseoli* and *R. tropici,* were found to be predominant at four different sites in France (Amarger et al. 1994). In another study, the authors established two novel species, *R. gallicum* and *R. giardinii* (Amarger et al. 1997), both nodulating common bean growing in France.

Several strains (total 22) effectively nodulating common bean in several soils from the region of Arcos de Valdevez, in the northwest of Portugal, were identified as *R. lusitanum* (Valverde et al. 2006), phylogenetically close to *Rhizobium rhizogenes* and *R. tropici.* The type strain, ATCC 14482^T (=DSM 30137^T), was isolated from effective nodules of *P. vulgaris* and also nodulates *Trifolium repens*, but not *Pisum sativum* (Ramírez-Bahena et al. 2008).

R. leucaenae was isolated from common bean grown in Mexico and Brazil, and this species was split from type A of the well-known species *R. tropici (*Ribeiro et al. 2012*)*. Another species, *R. paranaense*, also split from *R. tropici*, was distributed more broadly in Brazil and was an effective N_2-fixing symbiont of common bean (Dall'Agnol et al. 2014).

The type strain of *R. vallis* CCBAU 65647^T was isolated from effective nodules of *P. vulgaris* growing in Yunnan Province of China (Wang et al. 2011). *R. sophoriradicis* was previously isolated from the promiscuous legume *Sophora flavescens* growing in China and had highly a similar *nodC* gene to those of common bean rhizobia (Jiao et al. 2015b). This species was also found in nodules of common bean growing on the coast of Peru (Ormeño-Orrillo et al. 2018), suggesting it has a wider distribution.

In a systematical survey of the genetic diversity and geographic distribution of common bean rhizobia in Shaanxi Province, China (Wang et al. 2016), the authors found genus *Rhizobium* was the most dominant group, in which *Rhizobium* sp. II was the major group, followed by *R. phaseoli* and *Rhizobium* spp. I, IV and III. They also found bacteria belonging to *Agrobacterium*, *Ensifer*, *Bradyrhizobium*, *Pararhizobium* and *Ochrobactrum*. Soil pH, available nitrogen and potassium were the three most important factors determining the distribution of rhizobial species in Shaanxi Province, China (Wang et al. 2016).

8.8 Biogeography of Rhizobial Populations Associated with Alfalfa

Alfalfa (*Medicago sativa* L.) is an important leguminous forage crop, and it highly selects *Sinorhizobium meliloti* (syn. *Ensifer meliloti*) as its predominant rhizobial partner to form nitrogen-fixing nodules either in farmlands or natural ecosystems in the Yarlung Zangbo River valley of the Tibetan Plateau (Wang et al. 2018). Of the 581 alfalfa nodule isolates, most of them (473 isolates) belonged to *ropB*-I type, followed by *ropB*-II type (95 isolates), *ropB*-IV type (10 isolates) and *ropB*-III (1 isolate). All these four *rpoB* haplotypes were classified as the species *S. meliloti*, suggesting strict selection by its host plants. In rhizosphere soils of alfalfa, the *rpoB*-I type was also detected as the predominant population. A significant differentiation of the total rhizobial community between farmlands and natural ecosystems was demonstrated. Moreover, soil conditions, especially pH and nitrogen content, were revealed as important factors shaping the observed beta-diversity of rhizobial community.

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Chapter 9
Environmental Determinants of Biogeography of Rhizobia

Abstract In this chapter, the effects of soil factors and agricultural practices on the biogeography of rhizobia are discussed. Soil properties, such as pH level, significantly affect the distribution of soybean rhizobia especially in China. Further, land use and crop management have a distinct influence on the distribution of soybean rhizobia. For the rhizobia associated with medicinal legume *Astragalus* spp., distribution of these nodule isolates is negatively related to the application of fertilisers. Geographic separation for a long time prevents the gene exchange and recombination of rhizobia.

9.1 Soil Factors Affecting the Distribution of Rhizobia

9.1.1 Soil Factors Affecting the Distribution of Soybean Rhizobia

The distribution of soybean rhizobia is clearly affected by soil factors, especially the soil pH. Species in the genus *Bradyrhizobium* are widely distributed around the world but mainly found in acid soils, while these species belonging to *Sinorhizobium* (syn. *Ensifer*) are distributed in Asia mainly in alkaline soils (Zhang et al. 2011). No *Sinorhizobium* symbionts of soybean, but only bradyrhizobia, were found in the USA (Shiro et al. 2013). This is clearly related to the acidic to slightly alkaline pH (5.18–7.68) in the American soils. Both *B. japonicum* and *B. elkanii* were isolated from nine field soils in the USA. Isolates related to *B. japonicum* Bj123, *B. japonicum* Bj6 and *B. elkanii* Be46 were found, respectively, mainly in northern, middle and southern regions, significantly related to latitude ($r^2 = 0.815$) (Shiro et al. 2013).

Besides the soil pH, the concentration of available Fe (AFe) was found to determine the distribution of *Bradyrhizobium* and *Sinorhizobium* in China (Yang et al. 2018). The higher concentration of AFe (54·1–155 mg kg^{-1}), corresponding to acid soil, favoured the distribution of *Bradyrhizobium* species. Soils with lower AFe con-

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tent (8.75–8.94 mg kg^{-1}), corresponding to alkaline pH (7.59–7.60), were occupied by *S. fredii*, while in soils with intermediate AFe content (61.9 mg kg^{-1}) and neutral soil (pH 6.98), both *Sinorhizobium* and *Bradyrhizobium* species were found.

In another study in Hebei Province, north of China by Li et al. (2011), they found that *B. japonicum* and *B. elkanii* strains were found only in neutral to slightly alkaline soils, whereas *B. yuanmingense*, *B. liaoningense*-related strains and five strains within the genus *Sinorhizobium* were found predominantly in alkaline-saline soils. The pH and available phosphorus in soils had the greatest influence on the soybean rhizobia distributed in Hebei Province of China. Especially, most isolates (total 91 of 215) within *S. fredii* and three isolates belonging to the *B. liaoningense*-related group were adapted to saline-alkali soils with high concentration of available phosphorus and organic material. In addition, strains belonging to *Sinorhizobium* spp. I and II, *S. americanum*-related, *S. sojae* and *B. yuanmingense*, could adapt to saline-alkali soils with a high concentration of available potassium. Only two isolates belonging to *S. fredii*, but most bradyrhizobia (27 isolates classified as *B. japonicum*, and 16 isolates clustered into *B. elkanii*) preferred soils with relative low pH and low concentration of available potassium and salt.

The soil pH-dependent characteristic distribution of the two genera of *Bradyrhizobium* and *Sinorhizobium* associated with soybean was supported by the comparative genomes of these rhizobia (Tian et al. 2012). Several gene clusters known to be involved in osmoprotection and adaptation to alkaline pH were found specifically in the *Sinorhizobium* core genome (Tian et al. 2012). The genome of *Bradyrhizobium* contained lipid and secondary metabolism-related genes, consistent with the biogeographic patterns and wider adaptation in acid soils but not in alkaline soils (Tian et al. 2012). The soil pH can be used as a gold marker to select applicable rhizobial species in *Bradyrhizobium* or *Sinorhizobium* when considering the inoculation of soybean in different soils of China.

The geographic distributions of the two genera, *Bradyrhizobium* and *Sinorhizobium*, were not significantly affected by the different soybean varieties (Yang et al. 2018).

9.1.2 *Soil Factors Affecting the Distribution of* Astragalus *Rhizobia*

The geographic distribution of *Astragalus* rhizobia has been discussed in detail in Chap. 8 of this book. Here we only focus on the factors in soil affecting the distribution of rhizobia associated with medicinal legumes, *Astragalus mongholicus*, *Astragalus membranaceus* and their closer relative *Hedysarum polybotrys*, based on the work of Yan et al. (2016).

The fields used for growing the above medicinal legumes are of two kinds: with fertiliser applied and without fertiliser. Clearly, the components of these two kinds of soils are different, and therefore they influence the bacterial communities and

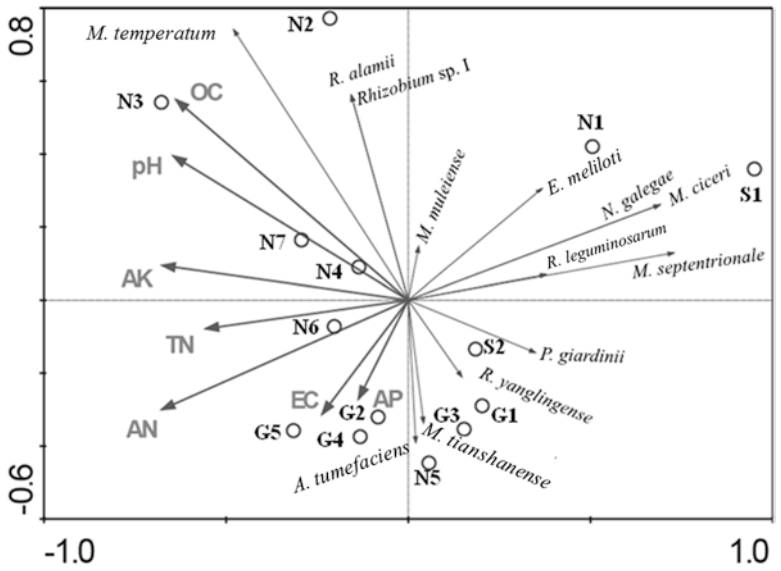

Fig. 9.1 Correspondence analysis among the soil factors, sampling sites and distribution of *Astragalus*-rhizobia-associated genospecies (Yan et al. 2016). Abbreviations for soil factors are as follow: *TN* total nitrogen, *OC* organic carbon, *AN* available nitrogen, *AP* available phosphorus, *AK* available potassium, and *EC* electrical conductivity. Nodule bacteria were isolated from in Ningxia (N1–N7), Gansu (G1–G5) and Shanxi (S1 and S2) provinces, respectively (Yan et al. 2016)

populations inhabiting the nodules of these medicinal legumes. In our survey of the soil properties of the fields used for the growth of the medicinal legumes, we found the fields at some sites (N7, N4, N6, G5, G4, G2, N5, G3, S2) were supplied by farmers with higher concentration of nitrogen, phosphate and potassium fertilisers (AN, TN, AP, AK), and the distribution of nodule isolates there was negatively related to these fertilisers (Fig. 9.1) (Yan et al. 2016). The species *M. temperatum* was distributed more widely than other species, suggesting its environmental adaptation and higher competitive abilities than other species. The species *M. ciceri* was only found in nodules of Site S1, whose soil was very barren, with no fertilisers used there. The *M. ciceri* identified here should be reclassified as *M. cantuariense* based on *recA* sequence similarity (99%) (De Meyer et al. 2015). Site S1 was also occupied by the species *M. septentrionale*, even more predominant than *M. ciceri* (*M. cantuariense*). The higher content of organic carbon (OC) in Sites N2 and N3 favoured the wide distribution of *M. temperatum*. The species *M. tianshanense* was distributed mainly at Site N5, where the OC content was relatively lower than at other sampling sites (Fig. 9.1).

In contrast, *R. yanglingense* and *P. giardinii* were also found in nodules of *Astragalus* species, but they tended to exist in soils with low OC and alkaline pH values. The pH was positively correlated with the distribution of *M. temperatum*, *M. muleiense*, *R. alamii* and *Rhizobium* sp. I in nodules collected from sampling site

N2 but was negatively correlated with the distribution of other genospecies. Electrical conductivity (EC) and available phosphorus (AP) were negatively correlated with the distribution of *M. muleiense* but positively correlated with the distribution of *A. tumefaciens* and *M. tianshanense* (Yan et al. 2016).

It is suggested that, since these medicinal legumes have N_2-fixing ability by associating with rhizobia, the usage of fertilisers, especially nitrogen fertiliser, should be restricted or limited to the lowest amount, so that symbiotic nitrogen fixation can be made functional by inoculating effective rhizobial inoculants. In considering the selection of effective rhizobia, species in the genus *Mesorhizobium*, especially the two species *M. temperatum* and *M. septentrionale*, should be considered first. Even though *M. temperatum* was predominant in nodules where these medicinal legumes were grown in nitrogen-rich fields (Yan et al. 2016), the nitrogen-fixing activity may be inhibited because of the higher nitrogen content in the soils.

9.2 Effects of Agricultural Practices on Distribution of Rhizobia

9.2.1 Effect of Land Use and Crop Management on the Distribution of Soybean Rhizobia

The long-term effects of land use and crop management on the distribution of soybean rhizobia was extensively studied by Yan et al. (2014). In their studies, seven different treatments were set up, including grassland, bare land and different rotations of soybean, maize and wheat with different fertilization and tillage (Yan et al. 2014). Because of the acid pH of the soils (5.68–6.30), all the soybean rhizobia in the experimental fields (Hailun) in Heilongjiang Province, northeast China, were *Bradyrhizobium* but included different species (Yan et al. 2014).

Four species, *B. japonicum* and three potential novel *Bradyrhizobium* spp. (I, II and III) were found in the experimental fields (Yan et al. 2014). *Bradyrhizobium* sp. III was later identified as *B. ottawaense* (Yan et al. 2017; Yu et al. 2014). Grassland maintained a higher diversity of soybean bradyrhizobia than the other cultivation systems. All the four species could be trapped using soybean from the grassland, whereas from the other treatments only two or three species were found. Of the four species, *B.* sp. I and III were predominant in the experimental fields, accounting for 90.7% of the total isolates, followed by *B. japonicum* (7.9%) and *B.* sp. II (1.4%).

The predominant species in grassland was *B.* sp. I (42.5%), followed by *B.* sp. III (35.0%), *B. japonicum* (12.5%) and *B.* sp. II (10.0%). The significantly increased organic carbon, available phosphorus contents and the pH level because of long-term natural restoration in grassland may contribute to the high diversity of bradyrhizobia compared to other treatments. In particular, *B.* sp. II was the only species that was isolated from the grassland but could not be found in other

treatments. Though the abundance of *B*. sp. II was lower, it would be worth studying the reasons for the emergence of this species, its competitiveness with other species and symbiotic nitrogen-fixing efficiency.

In the bare land treatment, where the grasses were manually eliminated periodically, *Bradyrhizobium* sp. III (77.5%) was the dominant species, followed by *B.* sp. I (15.0%) and *B. japonicum* (7.5%). The composition of the soybean rhizobia was similar to that in the cropland treatment (rotation among maize/soybean/wheat and no fertiliser supplied since 1985) and similar to the treatment with rotation of the above crops but with chemical fertiliser since 1990. However the usage of chemical fertiliser in the rotation of the three above crops changed the ratio of the three rhizobial species, with *B.* sp. I as the dominant species (65.0%), followed by *B. japonicum* (25.0%) and *B*. sp. III (10%).

Curiously, only two rhizobial species, *B.* sp. I and III, were found in all the three monocropped treatments of soybean, maize or wheat. In the monocropped soybean treatment, *B*. sp. I was the dominant species, while in the monocropped maize/wheat treatment, *B.* sp. II was the main rhizobial species. Clearly, the legume and cereals changed the long-term ratio of the rhizobial species in soil. The symbiotic nitrogen-fixing efficiency and adaptation of these two species should be evaluated in the future.

The relationships between soil environmental factors and soybean rhizobial genospecies were also analysed. The organic carbon (OC) content and pH had a strong positive correlation with the existence of *B.* sp. I, *B. japonicum* and *B.* sp. II and strong negative correlation with the distribution of *B.* sp. III. This was due to the increase of grass in the soil in the grassland treatment. The available potassium (AK) and phosphorus (AP) contents had slight effects on the distribution of soybean rhizobia, especially in fields with no chemical fertiliser supply, such as cropland and grassland treatments. The soil bulk density was positively correlated with the distribution of *B.* sp. III but negatively correlated with *B.* spp. I, II, *B. japonicum* and the population size (abundance) of soybean rhizobia in the soil.

In conclusion, the population, communities and abundance of rhizobia were determined not only by the host legume cultivation history but also by fertiliser application and crop management.

9.2.2 *Distribution of Bradyrhizobia Associated with* Kummerowia *spp. Grown in Urban and Rural Areas*

Kummerowia stipulacea (Korean clover) and *Kummerowia striata* (Japanese clover) are small legumes widely distributed in rural and urban areas (Ji et al. 2018; Lin et al. 2007; Park et al. 1999). Most rhizobia associated with *Kummerowia* spp. were classified into the slow-growing genus *Bradyrhizobium* (Lin et al. 2007), though some fast growers were isolated from the nodules of these plants (Liu et al. 2012; Wei et al. 2002; Yao et al. 2012).

Kummerowia-associating bradyrhizobia isolated from rural (130 isolates) and urban (153 isolates) areas, respectively, were compared and analysed to explore the distribution and gene exchange among these strains (Ji et al. 2018).

Based on the analysis of housekeeping genes, all the strains isolated from urban areas were clustered into one large branch, containing three genospecies, while the other strains from rural areas were very diverse and they separated clearly from the urban strains (Ji et al. 2018). Similarly, the analyses of symbiosis genes (*nodC* and *nifH*) were consistent with results of housekeeping genes (Ji et al. 2018). The phylogenetic consistency between the housekeeping genes and the symbiosis genes suggests the coevolution of these two kinds of gene. In addition, it also reveals that geographic segregation has occurred between the strains in urban and rural areas.

The rate of nonsynonymous substitutions of the strains from rural areas was higher than in those from urban areas for the housekeeping genes, suggesting gene flow and genetic exchange among these strains. In addition, nonsynonymous substitution rates were higher among strains from southern China than those from northern China. However the opposite was true for the symbiosis genes, where synonymous substitution rates were higher.

In conclusion, the results confirm that the diversity of *Kummerowia*-associating rhizobia has been drastically reduced in urban environments. Gene exchange and recombination were frequent within the rural or urban areas separately but seldom occurred between the two ecoregions. Furthermore, a single evolutionary lineage formed gradually for the *Bradyrhizobium* species of cultivated *Kummerowia* because of urban segregation (Ji et al. 2018).

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Part V
Agricultural Applications of Rhizobia and Other PGPR

Chapter 10
Usage of Rhizobial Inoculants in Agriculture

10.1 Introduction

The element nitrogen (N) is highly abundant in the atmosphere of the Earth, making up 80% of it, but exists there as nitrogen gas (N_2), which is not directly usable for most organisms, including plants and humans. Living organisms prefer reduced nitrogen, ammonium (NH_4^+), or oxidised nitrogen, nitrate (NO_3^-), for incorporation in their proteins, DNA, RNA, ATP and various nitrogen-containing metabolites. Only certain prokaryotes, either free-living or symbiotic with plants and animals, can perform biological nitrogen fixation (BNF). Rhizobia are one kind of important soil bacteria, infecting and nodulating legumes by forming functional nodules for symbiotic nitrogen fixation (SNF). Approximately 300 million hectares of legumes are grown around the world, and they can fix about 60 teragrams (Tg) (6×10^7 metric tonnes) of N each year by symbiosis with various rhizobia (Kinzig and Socolow 1994; Sadowsky et al. 2013).

The industrial production of nitrogenous fertiliser via the Haber-Bosch process is close to the level of natural BNF. However, the negative consequences of the overuse of fertiliser additions are alarming because they lead to pollution of soil, water and air and increase the greenhouse effect.

10.2 Brief History: Present and Future of Rhizobial Inoculant Application

The commercial application of rhizobial inoculants in agriculture dates back to the end of the nineteenth century (Nobbe and Hiltner 1896), right after the first pure rhizobial cultures were obtained and the function of nitrogen-fixing root nodules was confirmed (Brockwell and Bottomley 1995). Before the rapid worldwide

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growth of chemical N fertiliser in the late 1940s, most crops were mainly dependent on biological N fixation or the organic nitrogen from human and animals. Through rotation, the main Australian crop, wheat, still receives about 70% of its N requirement from the mineralisation of legume residues and soil organic matter and only 30% from chemical fertiliser (Angus 2001). The amount of N_2 fixed by rhizobia associating with legumes varies greatly (up to 450 Kg N/ha) among host legume species and rhizobial strains (Rai 2006). Therefore, it is essential to select the best strains adapted to the soils and the legume varieties (Gyogluu et al. 2018) so that the symbiosis is able to achieve maximum nitrogen-fixing efficiency. Additionally, the maintenance of cell viability (Berninger et al. 2018), agricultural practices and the usage of trace elements are also important to the efficiency of symbiotic N_2 fixation. Proper inoculation of legumes is a significant measure for improved crop productivity and soil fertility.

In the USA, commercial production of legume inoculants commenced in 1895 (Nobbe and Hiltner 1896). Various carriers, such as peat, coal, clays, lignite, polyacrylamide gel, cellulose gels, fluid gels, etc., were used to entrap *B. japonicum* around the coat of soybean seeds (Brockwell and Bottomley 1995). Williams (Williams 1984) and Smith (Smith 1992) have provided detailed guides to the production, quality control and proper use of legume inoculants (Brockwell and Bottomley 1995). Despite strict control, contamination cannot be avoided completely, and some contaminant bacteria were even able to inhibit the growth of *Rhizobium leguminosarum* bv. *trifolii* or *Sinorhizobium meliloti* (Olsen et al. 1995). Now, some companies still produce effective rhizobial inoculants in the USA. The ABM company produces America's Best Inoculant® brand bradyrhizobia used to inoculate soybeans, peanuts and peas/lentils in a wide variety of soils. Other inoculant producers and trademarks include Monsanto BioAg Alliance (Optimize®), BASF (Vault®), ABM (Excalibre™), MycoGold™, XiteBio Technologies (XiteBio® and SoyRhizo®). In addition to live bradyrhizobial cells, enhancers, biostimulants or other microbes may be added into the soybean inoculants to boost the increase of nodule numbers, healthier plant growth and better yield. The total consumption of rhizobia-containing biofertiliser in the USA was 87842.1 t in 2017 (Wookroof 2018). The popular genera of rhizobia used are *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium* and *Allorhizobium* (Wookroof 2018). The shelf life of such carrier-enriched biofertilisers in powder form is limited to 6–12 months (Wookroof 2018). *Bradyrhizobium japonicum* (now reclassified as *B. diazoefficiens*) strain USDA 110 has been used in commercial inoculants in the USA and in other countries and is now being broadly evaluated in Africa, so far with excellent results (http://www.n2africa.org/).

Currently, three countries, Argentina, Brazil and the USA, are the main soybean producers at the global scale, comprising 16, 32 and 33%, respectively, of the estimated global soybean production (Torres et al. 2018). In Brazil, SNF can meet up to 80% of the nitrogen (N) requirement of soybean (Hungria et al. 2006). In another report, SNF was estimated to provide 0–98% N uptake to soybean, depending on

rhizobial activity (Torres et al. 2018). In Brazil, reinoculation to soybean is reinforced every year even the soils contain high populations of *Bradyrhizobium* (Hungria et al. 2006).

Australia may be one of the best countries that promotes the application of rhizobial inoculation in agriculture and pasture. Australian farmers sow inoculated legume seed in about 2.5 million hectares, equivalent to 50% of the area sown to legumes (Drew et al. 2012). The total amount of nitrogen fixed by agricultural legumes is estimated at 2.7 million tonnes annually, with a nominal value for the industry of close to $4 billion annually (Drew et al. 2012; Herridge 2011). Strong research teams, strict quality control and management, habits to use inoculants every year and reduction of N fertilisers are the important aspects of successful application of rhizobial inoculants in Australia (Drew et al. 2012; Deaker et al. 2016).

The use of rhizobial inoculants in China is not popular. The unavailability and instability of inoculants, differences in soil properties, the wide usage of chemical nitrogen fertiliser (Chen et al. 2018) and the diminishing soybean cultivated area are the important reasons that inoculants are not widely used in China. With the implementation of agricultural policies, including the reduction of fertiliser input, enhancing nutrient use efficiency with zero increase in chemical fertiliser input until 2020 (Jiao et al. 2018), adjustment of planting structure and the application of environmentally friendly biofertiliser in agriculture, a new era of the usage of rhizobial inoculant is coming.

10.3 Strategy to Screen for Effective Rhizobia

Effective rhizobia must be those that can nodulate the host legumes well by forming functional and healthy N_2-fixing nodules with red or pink cross-sections indicating the presence of the pigment leghaemoglobin, adapted to local soils, and with high nodule occupation because they are strongly competitive against the indigenous soil rhizobia. The plants inoculated with effective rhizobia should be healthy with dark green leaves and higher plant height than the uninoculated ones. Genetic characteristics of the effective rhizobia include the phylogenic position based on 16S rRNA or housekeeping genes and the presence of *nod* and *nif* gene types matching the host plants. Some suggested effective rhizobia will be discussed in the next section.

Effective rhizobia can be obtained by collecting healthy and functional nodules of the relevant legumes. Then, the rhizobia are isolated from the nodules using standard methods described in the appendix section of this book. The efficiency of the rhizobia can be evaluated by inoculating them onto surface-sterilised leguminous seeds grown in vermiculite or perlite in Leonard jar assemblies (Leonard 1943; Trung and Yoshida 1983), irrigated with an N-free nutrient solution. Effective rhizobia will promote the nodulation and the growth of the plants well. Then the selected rhizobia are inoculated on legumes grown in relevant soils in pots or in fields to

determine the competitiveness and adaptation. Lastly, the best performing strains are selected. Alternatively, effective strains can be selected from preserved resources using the above two-step screening strategy, i.e. firstly in sterilised vermiculite and then in soils (Jia et al. 2008; Ji et al. 2017).

10.4 Suggested Rhizobia Used for Inoculants for Legumes

Only effective rhizobia matched to their legumes should be used in the inoculation process. In addition, the rhizobia used should be adapted to the soil environment. The rhizobial species recommended to inoculate various legumes are listed in Table 10.1.

Table 10.1 Recommended rhizobial species to inoculate corresponding legumes

Host plant	Recommended rhizobial species	Reference
Soybean (*Glycine max*)	*Sinorhizobium fredii*, *Bradyrhizobium elkanii*, *B. japonicum*, *B. diazoefficiens*, *B. ottawaense*, etc.	Yang et al. (2018b)
Chickpea (*Cicer arietinum*)	*Mesorhizobium ciceri*, *M. mediterraneum*, *M. muleiense*, *M. wenxiniae*	Zhang et al. (2018)
Peanut (*Arachis hypogaea*)	*B. arachidis* and other *B.* spp.	Wang et al. (2013a), Li et al. (2015)
Common bean (*Phaseolus vulgaris*)	*Rhizobium tropici*, *R. freirei* and other *R.* spp.	Dall'Agnol et al. (2013)
Faba bean (*Vicia faba*)	*R. anhuiense*, *R. laguerreae*, *R. fabae*, *R. leguminosarum* sv. *viciae*	Xiong et al. (2017), Belhadi et al. (2018)
Mung bean (*Vigna radiata*)	*B. elkanii*, *B. yuanmingense*, *B. japonicum* and *B. liaoningense* spp.	Zhang et al. (2008), Yang et al. (2008)
Clover (*Trifolium* spp.)	*R. leguminosarum* sv. *trifolii* and other *R.* spp. sv. *trifolii*	Zhang et al. (2016)
Alfalfa (*Medicago sativa*)	*S. meliloti*	Wang et al. (2018)
Chinese milk vetch (*Astragalus sinicus*)	*M. huakuii*, *M. qingshengii*	Zheng et al. (2013)
Astragalus mongholicus, *A. membranaceus*, *A. adsurgens*	*M. septentrionale*, *M. temperatum*	Gao et al. (2004), Yan et al. (2016)
Caragana spp.	*M. caraganae*, *M. septentrionale*, *M. temperatum*, *R. yanglingense*	Li et al. (2012b), Lu et al. (2009)
Amorpha fruticosa	*M. amorphae*	Wang et al. (1999)
Liquorice (*Glycyrrhiza uralensis*)	*M. tianshanense*	Mousavi et al. (2016), Li et al. (2012a)
Sophora flavescens	*S. fredii*, *M. septentrionale*, *R. yanglingense*	Jiao et al. (2015)
Vicia sativa	*R.* spp. sv. *viciae*	Xu et al. (2018)

For the soybean, two genera of rhizobia, *Sinorhizobium* and *Bradyrhizobium*, should be considered firstly according to the soil pH or Fe content. For high pH alkaline soil, *Sinorhizobium fredii* should be chosen, while for low pH acid soil, *Bradyrhizobium* spp. should be chosen (Yang et al. 2018b; Albareda et al. 2009b). Soybean cultivar can also influence the nodule occupation ratio by the two genera of rhizobia even in the same soil. Alkaline pH (7.8) favoured *S. fredii* SMH12 to occupy nodules with American soybean cultivars, while *B. diazoefficiens* USD A110 was more competitive in soils with a moderately acid pH (6.6). By contrast, the Asiatic cultivar Peking was almost exclusively nodulated by *S. fredii* SMH12 in both types of soils (Albareda et al. 2009b). In another study, in the near-neutral soil (pH6.98) of Feicheng, China, *B. elkanii* and *B. diazoefficiens* were always more dominant than *S.* (*E.*) *fredii* for the three soybean varieties grown in the greenhouse, while the nodule occupancy rate of *S. fredii* was higher than that of *B. elkanii* in the field when *S. fredii* was used as inoculant (Yang et al. 2018b). Because of the faster growth rate, the time saved in the fermentation process, and the greater competitiveness in neutral and alkaline soils, it is recommended to use *S. fredii* as inoculant for soybean in these soils.

For the chickpea, at least four novel species in the genus *Mesorhizobium* have been found around the world, including *M. muleiense* and *M. wenxiniae* that were only found in China (Zhang et al. 2018), and the other two species, *M. ciceri* and *M. mediterraneum*, that have not been found in China. In competition studies, the two exotic chickpea mesorhizobia, *M. mediterraneum* and *M. ciceri,* were more competitive than the resident species *M. muleiense* in sterilised vermiculite or soils sampled from Xinjiang where the *M. muleiense* was isolated. However, in non-sterilised soils, *M. muleiense* was the absolutely predominant nodule occupier in chickpea nodules (Zhang et al. 2014). Therefore, the mesorhizobial species inoculated on chickpea should be chosen carefully, firstly, according to the symbiosis gene type (see Chap. 5 of this book), and secondly, depending on the specific species of *Mesorhizobium*. In uncultivated soils, or if chickpea has not been grown previously in cultivated soils, inoculation is absolutely required (De Meyer et al. 2018).

For peanut, bradyrhizobia should be chosen rather than other genera, though some fast-growing bacteria have been found in root nodules. Peanut does not select strictly and specifically on the bradyrhizobial species, but these bradyrhizobia do not nodulate soybean. Peanut bradyrhizobia can, however, be used to inoculate mung bean and *Lablab purpureus* (Wang et al. 2013a).

For alfalfa and *Amorpha fruticosa*, two highly specific legumes, it is recommended to choose *S. meliloti* and *M. amorphae*, respectively, as their effective microsymbionts.

For common bean, faba bean and clover, the most effective rhizobia are in the genus *Rhizobium* (Table 10.1). Though belonging to the *R. leguminosarum* complex, the symbiosis gene types of these rhizobia are different from each other, and they do not cross-nodulate. Therefore, specific symbiosis gene types should be considered. On the other hand, rhizobia from *Vicia sativa* and *V. faba* nodulate both hosts.

For legumes in *Astragalus* and *Caragana*, the predominant microsymbionts are in the genus *Mesorhizobium*. Some species in *Rhizobium* could nodulate some species in *Astragalus* and *Caragana* and can be used as inoculants.

The selection of other rhizobia used for inoculation of legumes is covered in other chapters of this book.

10.5 Inoculant Production and Application

In the production of inoculants, selected rhizobia are cultured at a smaller scale in flasks or at a larger scale in fermenters to the late exponential stage, and then the culture is used directly or mixed with solid carriers. Two formulations of rhizobial inoculants, liquid and solid (granular or powder), are generally used to inoculate legumes in agriculture (Denton et al. 2017). The primary product of rhizobial inoculant is usually fermented liquid, and it can be applied directly to leguminous seeds or the seed furrow, after checking the purity and viable cell counting. Solid inoculants have various formulations: peat granules, bentonite clay granules, attapulgite clay and freeze-dried powders (Deaker et al. 2016).

Under laboratory or greenhouse conditions, 1 mL liquid inoculant with cell density up to 10^8 CFU per mL can be poured directly over one surface-sterilised seed. To promote symbiotic nitrogen fixation, microelements (Bajandi et al. 2011) and Jensen nitrogen-free plant mineral nutrients should be used (Vincent 1970). In the field, the liquid inoculant can be added to the surface of seed supplied with appropriate adhesive, such as 1.5–2% carboxymethylcellulose (CMC). If the liquid inoculant is sprayed directly into the seed row, CMC is not necessary. Liquid inoculant can be absorbed in sterilised peat to create the peat slurry formulation. The application of this peat slurry on seeds of faba bean and lupin always provided the best nodulation, grain yield and N_2 fixation (Denton et al. 2017).

Solid-based inoculants can be used to coat the seed through the help of adhesive solution or sprinkled into the seed row directly.

Cautions must be taken to avoid drought, high and low temperature, combinations with chemical fertiliser, bactericide and herbicide, chlorinated water, acidic or alkaline water and various wastes, as these factors or conditions will kill the live rhizobia.

10.6 Intercropping and Crop Rotation Between Legumes and Other Measures to Enhance Nitrogen Fixation

Interspecific interactions can be antagonistic, mutualistic or competitive. However, the interactions between legumes and other crops are often mutualistic, and the beneficial interactions usually happen underground through the interactions among

roots and microbes. In intercropping systems, maize can enhance the nodulation, N_2 fixation and productivity of faba bean by root interactions and the participation of beneficial symbiotic rhizobia (Li et al. 2016). Root exudates from maize increase root hair deformation and nodulation in faba bean and double exudation of signalling flavonoids for rhizobia and up-regulate expression of a gene involved in flavonoid synthesis and genes mediating nodulation and auxin responses (Li et al. 2016). Rhizobial inoculation increased the yield of soybean in both monocropping and intercropping with maize (Yang et al. 2018a), perhaps by increasing the diversity of soybean root endophytes (Zhang et al. 2011b). Moreover, lower N input also increased rhizobial N fixation and N use efficiency without decreasing grain yield in the maize-soybean relay intercropping system (Yong et al. 2018). The land equivalent ratio (LER) values under intercropping between soybean and maize were 1.5 times higher than those under monocropping, reflecting the advantage of the intercropping systems (Yang et al. 2018a). In mixed legume/grass pastures, nitrogen (N) fixed by legumes can be transferred partially to associated grasses. The estimated amounts of N fixed from atmospheric N_2 in legume/grass pastures throughout the world range from 13 to 682 kg N ha^{-1} $year^{-1}$ (Ledgard and Steele 1992). The amount of N transferred "below ground", predominantly through decomposition of legume roots and nodules, has been estimated at 3–102 kg N ha^{-1} yr^{-1} or 2–26% of SNF (Ledgard and Steele 1992). Uptake of soil N by grasses and the transfer of N to grass can reduce the inhibitory effect of soil N on symbiotic nitrogen fixation (SNF) of legumes, therefore enhancing the SNF. The intercropping system of alfalfa with corn rye provided higher forage production performance than that in rotated and monocropped alfalfa systems, respectively (Zhang et al. 2015). Intercropping of alfalfa and Siberian wild rye (*Elymus sibiricus* L.) and the rhizobial inoculation of alfalfa showed major effects on root microbial activities as well as community structure (Sun et al. 2009). Interestingly, intercropping of ryegrass (*Lolium perenne* L.) and alfalfa grown in metal-contaminated soil not only increases biomass of both plants but also reduces the absorption of heavy metals (Cui et al. 2018). In summary, to increase SNF in mixed systems, selection or breeding of applicable legumes and grasses, rhizobial inoculation and minimisation of negative factors, such as nutrient limitations, soil moisture, soil acidity and pests and disease, will be essential measures (Ledgard and Steele 1992).

In rotation systems of sustainable agriculture, components from the organic residues and root nodules of legumes can be decomposed, and the nutrients are assimilated by the subsequent crops, reducing the additional fertiliser inputs and improving the subsequent crop yield and quality, as well as benefitting the environment (Zeng et al. 2016). Yield increases by crops following legumes are often equivalent to applications of between 30 and 80 kg fertiliser N ha^{-1} (Jensen and Haahr 1990; McDonagh et al. 1993; Peoples and Craswell 1992; Peoples et al. 1995). Soybean is estimated to fix on average 79 kg N ha^{-1}, and 24% "below ground" N is left to the next crop (Salvagiotti et al. 2008). The N fixed by red clover (*Trifolium pratense* L.), white clover (*T. repens* L.) and alfalfa (*Medicago sativa* L.) is estimated to be up to

373, 545 and 350 kg N ha^{-1} $year^{-1}$, respectively (Carlsson and Huss-Danell 2003). The benefits from these N_2-fixing legumes are highest in the rotated cereal crops (Preissel et al. 2015).

10.7 Inoculation of Soybean, Peanut, Alfalfa, Medicinal Legumes and Chickpea

10.7.1 Soybean Inoculation

Soybeans contain 37–45% protein content by weight, so a 3600 kg ha^{-1} crop requires 136 kg of nitrogen (Beuerlein 2008). SNF can meet up to 84% of soybean nitrogen (N) requirement (Hungria et al. 2006); therefore, it is very important to fully utilise the natural nitrogen factory, the root nodule, rather than the extra chemical fertiliser added to the soil by humans. In addition, even 5 mM nitrate in the medium (Fujikake et al. 2011) or 0.17% N level in soil (Nyaguthii 2017) can inhibit the nodulation and nitrogen fixation of soybean. The addition of 50 kg N/ha ammonium nitrate to soybean at the beginning of flowering resulted in a decrease of nodulation in *S. fredii* strain SMH12 inoculated plots (Albareda et al. 2009a). Furthermore, both the uninoculated and N-fertilised control (200 kg N/ha) produced seed yields lower than those obtained with inoculated and non-fertilised treatments (Albareda et al. 2009a).

Soybean can establish symbiosis with different rhizobial species in the genera *Bradyrhizobium* and *Sinorhizobium* (syn. *Ensifer*) in different soils (Yang et al. 2018b; Zhang et al. 2011a). Clearly, the biogeographic characteristics of soybean rhizobia determine the selection and application of appropriate species belonging to these two genera used in inoculation (Yang et al. 2018b; Albareda et al. 2009b). In neutral soil pH, the inoculation of strains belonging to either of the two genera can increase the nodule occupation ratio (Yang et al. 2018b). Inoculation of soybean with selected rhizobia according to geographic regions and soil conditions increased the nodule number, fresh weight, occupation ratio, seed protein content and soybean yields (Yang et al. 2018b; Khaitov 2018).

Rhizobial inoculation together with magnesium (Mg) application in Mg-deficient acidic soils substantially increased shoot and root biomass, nodulation and N-fixation in soybean, but plants displayed toxicity in higher Mg concentration (Khaitov 2018). Determination of Mg level and the supplement of appropriate Mg content to soils will be a positive measure to increase soybean yield and quality in Mg-deficient acidic soils (Khaitov 2018).

10.7.2 Peanut Inoculation

The predominant peanut or groundnut (*Arachis hypogaea*) rhizobia belong to the slow-growing genus *Bradyrhizobium*, though fast growers (such as *Rhizobium* sp. NGR234) were reported but were ineffective symbionts (Boogerd and van Rossum 1997; Stanley and Cervantes 1991; Wong et al. 1988). Therefore, the selection of peanut inoculants should consider strains in *Bradyrhizobium* first. Although the crack entry infection pattern is characterised by a lack of strong specificity (Boogerd and van Rossum 1997) for rhizobial symbiosis genes, peanut still selects specific bradyrhizobia as its microsymbionts.

Early studies showed that inoculation of peanut at 8–12 cm depth was quite effective, resulting in the best peanut yields and quality (Schiffmann and Alper 1968). Similarly, inoculation with the more effective, competitive siderophore-producing strain NC 92 increased yields of certain cultivars in India when the inoculant was used as a liquid slurry below the seed (Nambiar 1985). To meet the P requirement in nitrogen fixation, a combination of higher inorganic P (82 kg ha^{-1}) and lower N (27 kg ha^{-1}) fertilisers, and rhizobial inoculation, led to the highest growth and yield of peanut in Malaysia (Hasan and Sahid 2016). If the bradyrhizobia in soils are more effective than the inoculated ones, it is suggested to take better measures to improve nodulation and nitrogen fixation by utilising the indigenous strains (Bogino et al. 2006). The co-inoculation of *Bradyrhizobium* sp. and plant growth-promoting rhizobacteria (PGPR) not only promoted the growth of peanut grown under sandy loam soil conditions but also enhanced its productivity greatly (Badawi et al. 2011). For the combined application of PGPR with rhizobia, the reader can refer to the next section of this book.

10.7.3 Alfalfa Inoculation

Throughout the world, the predominant microsymbiont of alfalfa (*Medicago sativa*) belongs to *Sinorhizobium meliloti* (Wang et al. 2018; Stajkovic-Srbinovic et al. 2012; Mnasri et al. 2009; Talebi et al. 2008). However, effective strains should be selected according to the alfalfa cultivars and environments (Jia et al. 2008, 2013; Provorov et al. 2016). By using the two-step strategy, first in vermiculite, and then in field, more effective and competitive strains were selected (Jia et al. 2008), and the selected strains improved the growth by 32–156% in all the three alfalfa cultivars tested (Jia et al. 2013).

Because alfalfa seed is small, it is better to coat it using rhizobial inoculant. Results from studies by Wigley et al. showed that the coated seed had the highest nodule occupancy with 45% and 47% of nodules containing *S. meliloti* strain RRI128 at the Ashley Dene and Lincoln University field sites, respectively (Wigley et al. 2015).

Application of a lower level of chemical nitrogen fertiliser (50 kg ha^{-1}) combined with rhizobial inoculation of alfalfa improved the maximum nodule numbers, nodule fresh weight, nitrogen fixation and grass yield compared with the uninoculated treatments (Ma et al. 2013). Application of nitrogen fertiliser above 50 kg ha^{-1} inhibited nodulation and nitrogen fixation significantly (Ma et al. 2013).

Potential improvements in nodulation and plant growth by combining of PGPR with rhizobial inoculation have also been reported frequently (Younesi et al. 2013b). In addition, co-inoculation of *S. meliloti* and *Pseudomonas fluorescens (*Younesi et al. 2013a*)*, *Halomonas maura* (Martinez et al. 2015) or arbuscular mycorrhizal fungi (Ashrafi et al. 2014) moderated the negative effects of salinity on measured plant growth characteristics.

10.7.4 Medicinal Legume Inoculation

Many nitrogen-fixing legumes, such as *Glycyrrhiza uralensis* (Mousavi et al. 2016; Li et al. 2012a), *Astragalus membranaceus*, *Astragalus mongholicus* (Yan et al. 2016), *Sophora flavescens* (Jiao et al. 2015), *Sophora alopecuroides (*Zhao et al. 2010*)*, *Psoralea corylifolia* (Wang et al. 2013b), *Crotalaria* spp. (Irmer et al. 2015), *Caragana* spp. (Lu et al. 2009) and so on, are used as medicines. By inoculating these legumes with appropriate rhizobia, the yield of the active constituents of these plants may be improved.

Pyrrolizidine alkaloids (PAs) are among the defence compounds against herbivores produced by *Crotalaria*, and the biosynthesis of PAs was demonstrated to be restricted to nodulated plants (Irmer et al. 2015). The nodules contain the first specific enzyme of the PA biosynthesis, and they are the source from which the PAs are transported to the above ground parts of the plant to defend against herbivores (Irmer et al. 2015). In addition, 5 mM nitrate inhibited nodulation, but exogenously applied nitrate had no direct impact on the biosynthesis of PAs (Irmer et al. 2015).

Psoralea corylifolia is a psoralen-producing medicinal legume. Co-inoculation with *R. leguminosarum* PCC2 and *S.* (*E.*) *meliloti* PCC7 increased the psoralen content (46%) in the seeds (Prabha et al. 2013).

The relationship between the production of medicinal compounds and the symbiotic nitrogen fixation in other legumes should be considered and studied further. Some candidate genes involved in the biosynthesis of isoflavonoids and quinolizidine alkaloids in *Sophora flavescens* had been proposed by using RNA-Seq analysis (Jiao et al. 2015), while the effects of symbiotic nitrogen fixation on the biosynthesis of these compounds, oxymatrine and matrine, has not been explored yet.

10.7.5 Chickpea Inoculation

Chickpea (*Cicer arietinum* L.) is a major food legume widely grown around the world, especially in India. Inoculation of chickpea with *Mesorhizobium* species provides positive effects on the growth attributes, symbiotic parameters, yield and yield components, nutrient uptake and quality in chickpea (Zorawar and Guriqbal 2018).

Results from studies by Rudresh et al. in 2005 showed that the combined inoculation of *Mesorhizobium ciceri* strain IC 2091 (Nour et al. 1994; Jarvis et al. 1997), phosphate-solubilising *Bacillus megaterium* subsp. *phospaticum* strain-PB and a biocontrol fungus *Trichoderma* spp. had positive effects on the growth, nutrient uptake and yield of chickpea under glasshouse and field conditions (Rudresh et al. 2005). The plant height (3.3%), number of branches per plant (23.3%) and biomass per plant (144%) of inoculated chickpea were higher than those of the uninoculated control (Rudresh et al. 2005). Other studies have shown that rhizobial inoculation increases plant, shoot dry weight, chlorophyll content and number of branches in chickpea (Elkoca et al. 2008; Giri 2010; Singh et al. 2014a).

In the case of agronomic traits that contribute to chickpea yield, the values of plant height, number of primary and secondary branches, number of pods per plant and number of grains per plant were highest in the treatment of combined N fertiliser supply (100 kg urea/ha-1) and rhizobial inoculation (Namvar et al. 2013).

The role of rhizobial inoculation on chickpea production and environmental factors affecting the symbiosis was recently reviewed extensively by Singh and Singh (Zorawar and Guriqbal 2018). Because of the strict selection on the rhizobial symbiosis genes by the chickpea plant, the selection of mesorhizobia used for chickpea inoculation should be considered carefully. The specificity between chickpea and mesorhizobia is discussed in other chapters in this book.

10.8 Usage of Microelements and Biostimulants to Enhance Symbiotic Nitrogen Fixation

Biological nitrogen fixation is not only influenced by the rhizobia and legumes themselves, but mineral microelements (Mo, B, Fe, Zn, etc.) and biostimulants (Nod factors, fulvic acid) are also important factors affecting nodulation and symbiotic nitrogen fixation (SNF). Microelements are micronutrients that are essential for plant growth, but the amounts required are much smaller than those of the macroelements such as nitrogen (N), phosphorus (P), sulphur (S) and potassium (K) (Hänsch and Mendel 2009). Different plants need certain micronutrients for their healthy growth, and the most important ones for legumes include molybdenum (Mo), boron (B), iron (Fe) and zinc (Zn). The Nod factors are secreted by rhizobia when induced by legume flavonoids, and they can stimulate the legumes to form root nodules (see Chap. 5 of this book). Another nodulation enhancer, the

water-soluble fulvic acid, was found recently by Gao et al. (Gao et al. 2015). This section will focus on the functions of the microelements molybdenum, boron and fulvic acid in nodulation and SNF. Other micronutrients are discussed in the review by Weisany et al. (Weisany et al. 2013).

10.8.1 Molybdenum (Mo)

Among the microelements essential for plant growth, the amount of molybdenum (Mo) required by plants is lowest (Zimmer and Mendel 1999). However, Mo is a key constituent of nitrogenase (Armiadi 2009) in all N_2-fixing prokaryotes (Shah et al. 1984), so plants associating with N_2 fixers have a relatively high Mo element requirement, particularly in the root nodules (Hille 1999; Marschner 2012). Mo uptake has been shown to occur in *Bradyrhizobium japonicum* free-living cells and in bacteroids of soybean nodules (Delgado et al. 2006). The *modABC* gene cluster encoding a high-affinity molybdate ABC-type transporter in *B. japonicum* has been characterised (Delgado et al. 2006). In soybean, gene ID Glymao4g07690.1 (homologous to MOT1 in *Arabidopsis*) is responsible for the efficient uptake and translocation of molybdate (Kastoori Ramamurthy et al. 2014). LjMOT1, a high-affinity molybdate transporter from *Lotus japonicus*, is essential for molybdate uptake, but not for delivery to the nodules (Duan et al. 2017). In *Medicago truncatula*, the molybdate transporter MtMOT1.2 is responsible for molybdate supply to root nodules (Gil-Díez et al. 2019), and MOT1.3 is a plasma membrane molybdenum transporter required for nitrogenase activity in root nodules (Tejada-Jimenez et al. 2017). The average abundance of Mo in the Earth's crust is only about 15 μmol kg^{-1} (Pope et al. 1980), and much of the Mo in natural environments is insoluble, so a specific high-affinity Mo transporter is important for rhizobia and legumes (Maier and Graham 1988).

Because Mo frequently limits nitrogen fixation under natural conditions (Shah et al. 1984), availability of Mo in soils can significantly influence nodulation and biological nitrogen fixation, thus determining the legume yield (Fageria et al. 2015; Brikics et al. 2004; Jongruaysup et al. 1993) and the use efficiency of N fertiliser (Biscaro et al. 2011).

Mo is particularly in short supply in many acidic soils (Barron et al. 2009; Armiadi 2009), where the oxoanion molybdate (MoO_4^{2-}) can interact with iron oxides to drastically reduce Mo bioavailability. Besides the scarcity of Mo in acidic soil, Yang et al. (Yang et al. 2018b) found the alkaline soils in Jining of China also lacked Mo, as well as iron (Fe), which may be due to the easier uptake by soybeans at higher soil pH values (Zimmer and Mendel 1999). Khan et al. (Khan et al. 2014) suggested that a supplement of 0.5 kg ha^{-1} Mo and 2 kg ha^{-1} Fe is needed for getting maximum yield, nodulation and nitrogen fixation by chickpea genotypes under the prevailing conditions. A much lower supplement of Mo (80 g ha^{-1}) still increased the grain yield observed by Araújo et al. (Araújo et al. 2009). In another study, Alam et al. (Alam et al. 2015) stated that application of 0.63 mg Mo kg^{-1} to soil would be

the optimum dose to maximise the biomass yield of hairy vetch (*Vicia villosa* Roth) under their conditions. Foliar-applied molybdenum on common beans resulted in an 81% increase in nodule molybdenum levels (Brodrick and Giller 1991). Application of 1kg of Mo/ha along with rhizobial inoculum to soybean in a drip irrigation system produced the most (51) nodules per plant and the highest nodule weight (199 mg/plant) and plant dry weight (72 g/plant) (Jabbar et al. 2013). In addition, this treatment also produced significantly the highest yield (29.46 g/plant) compared to the control which produced only 9.15 g/plant (Jabbar et al. 2013).

If the Mo content is lower than 0.1 mg/kg in soils, a supplement is necessary to enhance nitrogen fixation. On the other hand, excessive Mo doses (1.0 mg kg^{-1}) will lead to the deterioration of nodule structure and, hence, reduced enzymatic activity in plants (Alam et al. 2015). In brief, soil pH (below 6), Mo and Fe content and precipitation should be considered to determine the supplement dose of Mo to soils in order to enhance nitrogen fixation.

10.8.2 Boron (B)

Boron (B) is another essential microelement required for the normal growth of plants (Sommer and Sorokin 1928). Deficiency of boron and some other essential elements can cause a disturbance in the regulation of growth and development of pea (*Pisum sativum*), leading to short, thick and stunted roots (Sommer and Sorokin 1928). The meristematic region of the root tips of *P. sativum* grown without boron becomes abnormal (Sommer and Sorokin 1928).

The absence of boron in pea resulted in a decrease of the number of nodules and nitrogenase activity (Bolaños et al. 1994). Boron-deficient nodules display dramatic changes in cell walls and in both peribacteroid and infection thread membranes, indicating that boron is an indispensable element for normal development and functionality of nodules (Bolaños et al. 1994). Misshapen vascular development in ineffective nodules of *Vicia faba* under B deficiency also supported the importance of B in SNF (Brenchley and Thornton 1925). Root nodules of B-deficient soybeans were damaged and showed lower N_2-fixing activities (Yamagishi and Yamamoto 1994). Seed production of B-deficient soybeans was seriously depressed (Yamagishi and Yamamoto 1994). More recent studies indicate that B is also essential for bacteroid maturation (Bolaños et al. 2001) and for early signalling interaction between plant and rhizobium (Redondo-Nieto et al. 2001). Further studies showed that the development of the symbiosis also depends on a certain concentration of calcium (Ca^{2+}) as well as B (Redondo-Nieto et al. 2003). Cell wall elasticity decreased in the B-deficient treatment, and this abnormality, with disorganised pectin polysaccharides and without associated structural proteins, cannot be prevented by extra addition of Ca (Redondo-Nieto et al. 2003).

Besides, B also has effects on rhizobial polysaccharide production. B deficiency led to a 65–80% reduction in the amount of exopolysaccharide (EPS) and to modi-

fications of lipopolysaccharide (LPS), resulting in the decreased nodulation and nitrogen fixation (Abreu et al. 2012).

Groundnut crop Var GPBD-4 responds positively in respect of growth, yield, quality and uptake of nutrients to the application of borax at 2.5 kg ha^{-1} + sodium molybdate at 1.5 kg ha^{-1} + borax foliar spray at 0.2% + sodium molybdate foliar spray at 0.05%. (Ghanti 2012). At concentrations of 29–88 μg B L^{-1}, nitrogen fixation and nodule weight of soybean were markedly enhanced (Yamagishi and Yamamoto 1994). However, at high concentration of B (1300 μg L^{-1}), soybean plants were damaged, followed by a decrease in N_2 fixation (Yamagishi and Yamamoto 1994). In another study, foliar boron application increased seed protein by 13.7% and oleic acid by 30.9% compared to the control soybean plants (Bellaloui et al. 2010).

10.8.3 Fulvic Acid (FA)

Fulvic acid (FA) is a natural component of humus and can be derived from degraded lignite products by *Penicillium*. FA was found to enhance the cell density of *Bradyrhizobium liaoningense* strain CCBAU05525, as well as the nodule number, nodule fresh weight and nitrogenase activity of the inoculated soybean plants (Gao et al. 2015). FA is a complex mixture that includes compounds similar to flavonoids such as genistein, which is secreted by soybean roots, and FA can induce stronger *nod* gene expression than genistein in strain CCBAU05525 (Gao et al. 2015).

10.9 Combination of Rhizobia with PGPR

Co-inoculation of beneficial plant growth-promoting rhizobacteria (PGPR) with rhizobia cannot only improve legume growth and yield but can also be cost-effective and cost-efficient. A range of PGPR microbes, including *Azospirillum* (Tchebotar et al. 1998), *Azotobacter* (Dashadi et al. 2011), *Bacillus* (Mishra et al. 2009; Rugheim et al. 2017; Alagawadi and Gaur 1988), *Pseudomonas* (Fox et al. 2011; Alagawadi and Gaur 1988), *Serratia* (Shahzad et al. 2014), *Paenibacillus* (Figueiredo et al. 2008), etc., can be combined with rhizobium as co-inoculants for plants.

Here, we focus on the combination of rhizobia and *Azospirillum* and their influence on the inoculated plants. The combined inoculation of white clover with *R. leguminosarum* bv. *trifolii* and *Azospirillum lipoferum* enhanced the number and acetylene reduction activity of root nodules by 2–3 times from 20 days after inoculation (DAI) (Tchebotar et al. 1998). Co-inoculation of *A. brasilense* Az39 and *B. japonicum* E109, both producing the three phytohormones indole-3-acetic acid

(IAA), gibberellic acid (GA3) and zeatin (Z), promoted seed germination and early seedling growth in corn (*Zea mays* L.) and soybean (*Glycine max* L.) (Cassan et al. 2009). *Azospirillum-Rhizobium* co-inoculation increased the amount of fixed nitrogen and the yield of genotype DOR364 of common bean (*Phaseolus vulgaris* L.) across all sites of Cuba, while they had a negative effect on the yield and nitrogen fixation when inoculated on genotype BAT477 of common bean (Remans et al. 2008). Therefore, the importance of genotype × inoculum interactions should be emphasised in agricultural application of these inoculants.

For other applications of combined inoculation of rhizobia and PGPR microbes, we refer the reader to a detailed review article (Gopalakrishnan et al. 2015).

10.10 Rhizobial Activity Beyond Nitrogen Fixation

The main function of rhizobia in agriculture is SNF. In addition, rhizobia also play roles in producing various compounds that are beneficial to the legumes and other plants directly and indirectly. In rotation or intercropping systems, the N fixed by the legume-rhizobium symbiosis can benefit the cereal crops, such as maize, rice, wheat and sorghum, with a relative yield increase of 11–353 % (Peoples and Crasswell 1992).

10.10.1 Denitrification

Genes involved in denitrification have been identified in several rhizobial species, including *Rhizobium sullae*, *R. etli*, *Sinorhizobium meliloti* and *Bradyrhizobium japonicum* (Bedmar et al. 2005), under free-living or symbiotic conditions (Delgado et al. 2007). The species *B. japonicum* and *Allorhizobium caulinodans*, in the free-living state, can reduce NO_3^- to N_2 when cultured anaerobically with nitrate as the terminal electron acceptor (Smith and Smith 1986; Breitenbeck and Bremner 1989). Bacteroids inside the root nodules also express the denitrification pathway, independent of soil aeration (Smith and Smith 1986). Emission of NOx from legume nodules contributes to greenhouse gases in the atmosphere. Though the rate of denitrification activity is not much higher than that in other soil microbes, the vast area of cultivated legumes around the world makes a highly significant contribution of free-living and symbiotic rhizobia to global denitrification (Delgado et al. 2007). For rhizobia themselves, the ability to denitrify may promote survival of free-living rhizobia in soils subject to waterlogging and the alternative use of nitrate or nitrite as electron acceptors to maintain nodule integrity in the symbiotic state (Breitenbeck and Bremner 1989).

10.10.2 Phosphate Solubilisers

Phosphorus (P), followed by nitrogen (N), is the most important nutrient limiting crop yield. Soils are often abundant in insoluble P, but deficient in soluble phosphates essential for growth of most plants and microorganisms (Singh et al. 2014b). Some bacteria and fungi have the ability to solubilise insoluble P to available forms absorbed easily by plants. The potential of rock phosphate solubilisation by strains of *Bradyrhizobium*, *Rhizobium*, *Mesorhizobium* and *Sinorhizobium* isolated from nodules of various plants (*Vicia*, *Phaseolus*, *Medicago*, *Lotus*, *Cicer arietinum* and *Aeschynomene aspera*) was systematically evaluated by Halder et al. (Halder et al. 1990). Results showed that almost all the test strains could effectively solubilise rock phosphate and reduce the pH level of the medium (Halder et al. 1990). Among the strains, *R. leguminosarum* sv. *viciae* BICC635, producing 2-ketogluconic acid as the primary agent for dissolving rock phosphate in the culture medium, was the most effective solubiliser. Rhizobia isolated from root nodules of *Chamaecrista absus* (formerly *Cassia absus*), *Vigna trilobata* (Kumar and Ram 2014), *Lens culinaris* (Mulissa and Fassil 2011) and *Sesbania sesban* were also confirmed to have the ability to solubilise tricalcium phosphate (Sridevi and Mallaiah 2009).

In a plant growth-promoting test, an isolate RASH6 from root nodules of chickpea not only could solubilise insoluble phosphate by producing citric, succinic and gluconic acids but also could promote chickpea growth significantly. Nodulation and nitrogenase were also enhanced by inoculating this strain, RASH6 (Singh et al. 2014b). Rhizobia having N_2-fixing ability and P-solubilising capability will be of huge value for sustainable agriculture in the future (Singh et al. 2014b).

10.10.3 IAA Production

Indole-3-acetic acid, abbreviated as IAA, is the foremost plant hormone that enhances plant growth and development. IAA formation in rhizobia takes place via the indole-3-pyruvic acid (Minamisawa et al. 1996) or indole-3-acetic aldehyde pathway (Spaepen et al. 2007) or the tryptamine (TAM) pathway (Torres et al. 2018). Many rhizobia can produce IAA, and some salient species include *R. tropici* CIAT899 (from *Phaseolus vulgaris*) (Figueiredo et al. 2008), *Rhizobium* sp. (from *Desmodium gangeticum*) (Bhattacharyya and Basu 1997), *Rhizobium* sp. (from *Vigna trilobata*) (Kumar and Ram 2012), *Bradyrhizobium japonicum* (from soybean) (Torres et al. 2018; Minamisawa and Fukai 1991), *B. elkanii* (from soybean) (Minamisawa et al. 1996), *R. leguminosarum* bv. *trifolii* (from *Trifolium*) (Garg et al. 2015), *Mesorhizobium loti* (from *Lotus*) (Sarkar and Laha 2013) and *R. phaseoli* (from *P. vulgaris*) (Atzorn et al. 1988). Attention should be paid that IAA production is significantly inhibited by ammonium (NH_4^+) (Imada et al. 2016), and the latter also inhibits nodulation and SNF (Laws and Graves 2005; Moudiongui

and Rinaudo 1987; Kamberger 1977). Other environmental stress factors (acidic pH, osmotic and matrix stress and carbon limitation) also influence the biosynthesis of IAA (Spaepen et al. 2007). In addition to IAA, some strains of *R. phaseoli* secreted gibberellins (Atzorn et al. 1988). Some symbiotic strains of *R. leguminosarum* sv. *viciae* showed plant growth-promoting characteristics such as inorganic phosphate solubilisation as well as IAA production (Mulissa and Fassil 2011). The IAA had a special function in enhancing the resistance to stress via an increase of PHB content, which is used as a carbon and energy source during long-term starvation (Imperlini et al. 2009).

IAA can also promote the uptake of nutrients (Etesami et al. 2009). In a greenhouse study, wheat growth and uptake of N, P and K nutrients were enhanced by inoculating with IAA-producing rhizobia, *R. leguminosarum* sv. *phaseoli* and sv. *viciae*, with supplements of L-tryptophan and Ag^{+} ion (Etesami et al. 2009).

It should be noticed that high concentrations of IAA might be harmful to plant growth. For example, *R. leguminosarum* sv. *trifolii* strain TV-13 produced 171.1 μg/ml IAA in media supplied with tryptophan, but it caused damage to lettuce seedlings (Schlindwein et al. 2008). *Bradyrhizobium* sp. isolates produced between 1.2 and 3.3 μg/ml of IAA and improved seedling vigour (Schlindwein et al. 2008). Lower concentrations of IAA (1 mM) on *B. japonicum* caused differences in expression of genes involved in heat, cold, oxidative, osmotic and desiccation stress and EPS biosynthesis, but had no significant influence on the nodulation ability on soybean (Donati et al. 2013).

10.10.4 Siderophore Formation

Water-soluble siderophores secreted by some bacteria can efficiently sequester insoluble Fe^{3+}, making the iron available for themselves and plants (Gopalakrishnan et al. 2015). Rhizobial species, such as *S. meliloti*, *R. tropici*, *R. leguminosarum* (bvs. *viciae*, *trifolii* and *phaseoli*) and *Bradyrhizobium* sp., are known to produce siderophores (Antoun et al. 1998). About 25% of all the strains of rhizobia and bradyrhizobia stimulated radish growth (20% or more increase) in greenhouse conditions, and among them, a siderophore-producing strain of *B. japonicum*, Tal 629, significantly increased the dry matter yield of radish (Antoun et al. 1998). Rhizobial isolate RASH6, isolated from chickpea, promoted growth of plants by producing a siderophore, IAA, ammonia and HCN; this isolate could also nodulate and fix nitrogen on chickpea (Singh et al. 2014b).

Other beneficial functions, such as production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Ma et al. 2004; Duan et al. 2009), abscisic acid (ABA) (Boiero et al. 2007), biocontrol abilities (Ozkoc and Deliveli 2001), inducing plant resistance (Mishra et al. 2006) and other indirect roles in plant growth promotion, are discussed in some detailed review articles (Gopalakrishnan et al. 2015; Lugtenberg and Kamilova 2009).

10.11 Concluding Remarks and Perspectives

The applications of rhizobial inoculants to legumes not only can increase the nodulation but also enhance the SNF, therefore promoting the growth of legumes and yields. The other functions of rhizobia, such as producing IAA and solubilisation of the phosphate, are also beneficial to legumes. Attentions should be paid that rhizobia are kinds of Gram-negative and non-spore-forming bacteria and their resistance to drought, desiccation and high temperature is relatively weaker than that of the spore-forming *Bacillus*. In addition, the usage of chemical nitrogen fertiliser, which can inhibit the nodulation and SNF, should be reduced to minimum quantity. And supplement with microelements to inoculants will enhance the SNF.

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Part VI
Technology and Methods

Chapter 11
Working on the Taxonomy, Biodiversity, Ecology and Evolution of Rhizobia

11.1 Sampling Soils and Nodules and Isolation of Rhizobia

11.1.1 Recognition of Legumes

Through the world, about 770 genera covering more than 19,500 species have been recognised in the family Leguminosae or Fabaceae, distributed in diverse habitats (LPWG 2017). Traditionally, the legumes were divided into three subfamilies, Caesalpinioideae, Mimosoideae and Papilionoideae, according to their flower morphology, and these are also treated as three families [Caesalpiniaceae, Mimosaceae and Papilionaceae (Fabaceae)] in some publications. However, based upon molecular studies, especially the extensive phylogenetic analysis of plastid *matK* genes, it was found that "the classification of legumes into three subfamilies is not natural because the monophyletic Mimosoideae and Papilionoideae are nested within a paraphyletic Caesalpinioideae" (LPWG 2017). Combining with morphological data, a new system has been proposed, with six subfamilies within the Leguminosae: Caesalpinioideae, Cercidoideae, Detarioideae, Dialioideae, Duparquetioideae and Papilionoideae (LPWG 2017). In addition to its importance for the legume taxonomic community, this change is also relevant for rhizobial studies that address the coevolution of rhizobia with their hosts, since all the known nodulating legumes are included in Papilionoideae and the revised Caesalpinioideae (as Mimosoideae-Caesalpinieae-Cassieae or MCC clade). The other four subfamilies recircumscribed from the former Caesalpinioideae contain the non-nodulating members (Sprent et al. 2017).

For sampling the nodules of wild plants in the field, the recognition of legumes is the first step. In relation to the great diversity of legumes, leguminous plants can be annual herbaceous (*Glycine soja*, *Mimosa affinis*), biennial herbaceous (*Hedysarum coronarium, Melilotus officinalis*), perennial herbaceous (*Medicago sativa*, *Arachis pintoi*), climbing plants (*Lablab purpureus*, *Pueraria lobata*), shrubs

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(*Acacia farnesiana*, *Caragana microphylla*) and trees (*Sesbania sesban*, *Robinia pseudoacacia*), which present dramatic variation in morphology (Table 11.1).

In fields, legume plants are mainly recognised by the morphology of their leaves, flowers and/or fruits. As listed in Table 11.1, the leaves of leguminous plants can be unifoliolate (like *Cercis chinensis* and *Bauhinia alba*) or compound leaves that could be bifoliolate (*Zornia latifolia*), trifoliolate (*Glycine max* and *Trifolium repens*), tetrafoliolate (*Arachis hypogaea*), palmate (*Lupinus polyphyllus*) and pinnate (bipinnate, paripinnate, imparipinnate) (*Mimosa affinis* and *Albizia julibrissin*). Some species have a leaf tendril or short point. The base of the leaf stalk has a pulvinus, a structure responsible for movement of the leaf or leaflet. Leaf stipules are common in many legumes.

The morphology of flowers is also an important feature for recognition of legumes (Table 11.1) (LPWG 2017). The flowers of nodulating legumes usually have zygomorphic (bilaterally symmetrical) or papilionate flowers (*Glycine max*) but occasionally actinomorphic (radially symmetrical) flowers (*Erythrophleum fordii*). Plants in the mimosoid clade have flowers in bundles with many stamens much longer than the petals, which make the inflorescence appears as a single downy globe.

11.1.2 Collection of Nodules, Seeds and Soil in the Field and Plant Trapping of Rhizobia

For rhizobial isolation, the root and/or nodules can be obtained from the field plants or from trapping plants in the laboratory. In general, nodules are easily obtained from herbaceous legumes or tree seedlings of the same year in the field. In the field, it is better to maintain the live root system with nodules together with the surrounding soil in plastic bags (water can be added to keep the humidity if necessary), which should be transported to the laboratory within a few days, depending on the temperature. An alternative way is to dehydrate several nodules in vials (5–10 ml volume) half filled with silica gel that can be stored at room temperature for months. These dry nodules can be rehydrated at 4 °C overnight and then used for rhizobial isolation (Vincent 1970). Based on our experience, isolation efficiency is much greater for fresh nodules than dehydrated-rehydrated nodules, whereas the dehydrated nodules may be used to estimate the nodule occupancy of rhizobial strains/species in the field by PCR amplification-sequencing of the marker gene.

For the perennial legumes, including herbaceous (*Glycyrrhiza*), liana (*Pueraria*) and trees (*Leucaena*), root nodules are difficult to find in most cases. Therefore, the seeds and soils around the root zone can be collected and used for trapping rhizobia by growing seedlings in the laboratory (Wang et al. 2002) as follows:

Table 11.1 Characteristics of the nodulating subfamilies in Leguminosae. Characters highlighted by bold letters are particularly valuable for recognition of the members in the subfamilies (Simplified from LPWG 2017)

	Subfamily	
Character	Caesalpinioideae	Papilionoideae
Habit	Trees, shrubs, lianas, suffruticose or functionally herbaceous, unarmed or commonly armed with prickles or spines	Usually unarmed trees, shrubs, lianas, herbs or twining vines with tendrils
Specialised extrafloral nectaries	Often present on the petiole and/or on the primary and secondary rachises, usually between pinnae or leaflet pairs, sometimes on stipules or bracts	Lacking on petiole and leaf rachis; occasionally present on stipules, stipels, bracts or swollen and nectar-secreting peduncles or sepals
Stipules	Lateral, free or absent	Lateral, free or absent, very rarely interpetiolar
Leaves	Commonly bipinnate, otherwise pinnate and then mostly paripinnate, rarely imparipinnate or bifoliolate, modified into phyllodes or lacking	Mostly pari- or imparipinnate or palmately compound, commonly unifoliolate, trifoliolate, rarely bifoliolate or tetrafoliolate
Leaflets and pinnae	Mostly opposite, rarely alternate	Opposite or alternate, sometimes modified into tendrils, rarely in phyllodes
Inflorescence	Globose, spikes, panicles, racemes or flowers in fascicles	Mostly racemes, pseudoracemes or panicles, less often cymes, spicate or capitate, or flowers solitary
Bracteoles	Small or absent	Mostly small, rarely large, valvate, enveloping the bud
Flowers	Usually bisexual, rarely unisexual or bisexual flowers combined with unisexual and/or sterile flowers in heteromorphic inflorescences; radially, less frequently bilaterally symmetrical, sometimes papilionate or asymmetric	Bisexual, rarely unisexual, usually bilaterally symmetrical, usually papilionate, rarely asymmetrical, radially symmetrical or nearly so
Hypanthium	Lacking or cupular, rarely tubular	Present or absent
Sepals	(3–)5(−6), free or fused, or sepal whorl lacking	(3–)5, united at least at the base, sometimes entire and splitting into irregular lobes or lobes dimorphic and some petaloid
Petals	3–)5(−6), free or fused, or petal whorl lacking, valvate or imbricate, then adaxial petal innermost	Usually (0–)5(−6), rarely 1 (standard) petal and 4 absent, imbricate, the adaxial petal outermost, in radially symmetrical flowered species, corolla with 5 small or undifferentiated petals, less often only 1 (standard) petal is present or all petals absent

(continued)

Table 11.1 (continued)

	Subfamily	
Character	Caesalpinioideae	Papilionoideae
Stamens	Diplostemonous or haplostemonous, sometimes reduced to 3, 4 or 5, frequently many (100+), sometimes heteromorphic, some or all sometimes modified or staminodial	Usually 10, rarely 9 or many
Stamen fusion	Filaments free or connate	Filaments usually connate into a sheath or tube, uppermost filament wholly or partly free, sometimes all filaments free
Anthers	Uniform or heteromorphic, basifixed or dorsifixed, often with a stipitate or sessile apical gland, dehiscing via longitudinal slits or apical or basal poricidal slits or pores	Uniform or dimorphic, basifixed or dorsifixed, dehiscing via longitudinal slits
Pollen	Monads, tricolporate or porate tetrads, bitetrads or polyads, sculpture pattern never striate	Monads, mostly 3-colporate, 3-colpate or 3-porate
Gynoecium	Usually 1-carpellate, rarely polycarpellate, stipitate or sessile, stipe free	Usually 1-carpellate, rarely 2-carpellate, stipitate or sessile, stipe free
Ovules	Ovary 1–many-ovulate	Ovary 1–many-ovulate
Fruits	Commonly thinvalved, 1–many-seeded pod, dehiscent along one or both sutures, also often a lomentum, a craspedium or thick and woody and then indehiscent or explosively dehiscent, often curved or spirally coiled	Dehiscent pods along one or both sutures, or indehiscent, or loments, samaras or drupes
Embryo	Straight	Usually curved, rarely straight
Vestured pits in 2° xylem	Present	Present
Root nodules	Variably present and indeterminate	Usually present, either indeterminate or determinate
Chromosome counts	2*n* mostly 24, 26, 28 (but 14, 16, 52, 54, 56 also reported)	2*n* mostly 16, 18, 20, 22 (but 12, 14, 24, 26, 28, 30, 32, 38, 40, 48, 64, 84 also reported)
Chemistry	Non-protein amino acids frequently reported; coumarins, cyanogenic glucosides, phenylethylamine, tryptamines and β-carboline alkaloids also reported	Isoflavonoids, prenylated flavonoids, indolizidine or quinolizidine alkaloids reported. Non-protein amino acids widespread, some exclusively found in the subfamily (e.g. canavanine)

1. Scribing the seeds on sandpaper to break the seed coat if a wax layer exists. This is always necessary for seed of wild soybean (*Glycine soja*), *Acacia* spp., *Leucaena leucocephala* and *Pueraria* spp.
2. Surface sterilisation of the seeds by immersing them in 90% (v/v) ethanol for 30 sec. Followed by immersing in 1% (w/v) sodium hypochlorite for 5 min and washing 6 times with sterile distilled water.
3. Putting the surface-sterilised seeds separately on agar-water plates (0.7% agar in distilled water); turning the plates with seeds upside down; and incubating them in the dark at 28 °C after the seeds have attached to the medium. The germination of seeds should be checked daily.
4. The germinated seedlings with around 0.5 cm of root are transferred into Leonard jars (Vincent 1970) filled with sterilised vermiculite moisturised with N-free plant nutrient solution and the seeds covered with 1 g of the soil. The 1000× stock N-free plant nutrient solution (pH 6.5) contains in g L^{-1}: KSO_4, 87.14; KH_2PO_4, 68.04; $CaCl_2{\cdot}2H_2O$, 147.01; $MgSO_4{\cdot}7H_2O$, 123.24; EDTA, 372.24; $FeSO_4{\cdot}7H_2O$, 278.02; $ZnSO_4{\cdot}7H_2O$, 0.143; $CuSO_4{\cdot}5H_2O$, 0.030; $MnSO_4{\cdot}H_2O$, 0.845; H_3BO_3, 1.855; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.099; $Co(NO_3)_2$, 0.003; and $NiSO_4$, 0.026 (Vincent 1970). Alternatively, seedlings of trapping plants can be grown directly in the sampled soils and moistured with distilled water.
5. Checking the nodules after growing the seedlings for 1–2 months under greenhouse conditions. Then the nodules will be used for isolation of rhizobia as mentioned subsequently.

This procedure is also used for nodulation tests of the isolates on their host of origin according to Koch's postulates (Vincent 1970) and on different legumes to test their host range as suggested (Graham et al. 1991) or to determine the symbiovar (Andrews and Andrews 2017). In some of the recent studies, the symbiovar has been deduced from *nod* gene sequence analyses, as reported previously (Laguerre et al. 2001).

In addition, the seeds are also necessary for confirming the nodulation ability after the rhizobia are isolated and purified, while the soils can be used for soil characterisation that provides data for biogeographic analysis (Tian et al. 2007).

11.1.3 Isolation of Rhizobia from Nodules and Preservation of Isolates

For both the fresh nodules and the dehydrated nodules, the general isolation protocol is:

– Separating the nodules from roots and washing them with sterile water in a vial.
– Immersing the cleaned nodules in 95% (v/v) alcohol for 30s, followed by immersing the nodules in 1% sodium hypochlorite for 5 min and finally washing six times with sterile water.

– Crushing each nodule in a small drop (10 μL) of sterile water with forceps directly on a plate of PY medium (peptone of casein 5 g; yeast extract 3 g; $CaCl_2$ 0.6 g; agar 18 g; distilled water 1 L; pH 7.0–7.2) and separating the rhizobia by cross-striking the nodule fluid on the plate.
– Checking the plates after incubating 2–15 days at 28 °C and picking up a single colony representing the dominant bacteria on the plate for further purification by repeatedly striking on other plates until all the colonies show the same colony morphology. It should be mentioned here that the single colonies for some rhizobial species, like those in the genus *Bradyrhizobium*, may occur after 2 weeks of incubation at a size smaller than 1 mm in diameter.
– The purified isolates should be stored at −70 °C in PY broth supplied with 30% of glycerol or stored by lyophilization.

Traditionally, yeast extract-mannitol agar (YMA) medium (Vincent 1970: yeast extract, 3 g; mannitol, 10 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; agar, 18 g; in 1 L of distilled water; pH 7.0–7.2) is used for isolation of rhizobia. However, this medium can be replaced by PY medium, which supports growth of a wider arrange of rhizobia.

11.1.4 Nitrogenase Activity Determination for Nodules

The effectiveness of nodules obtained in the fields or by plant trapping can be determined qualitatively and quantitatively. For qualitative determination, the red colour inside nodule and the growth (height of plants, dark green leaves) of the host plant can be used as evidence for effective (nitrogen-fixing) nodules (Fig. 11.1). For quantitative determination, the gas chromatograph assay of acetylene reduction into ethylene by the nitrogenase was the most popular method (Dilworth 1966), and some modification has been reported to make it more effective (David et al. 1980).

Fig. 11.1 Nitrogen-fixing effectiveness of nodules of colorín (*Erythrina coralloides*). (**a**) Short and yellowish seedling of colorín without nodules. (**b**) Strong and green seedling of colorín with effective nodules. (**c**) Root nodules of colorín seedling. (Photographed by ET Wang)

In the field, nitrogenase activity can be determined by putting the clean nodules (about 0.5 g) into a 5 mL-syringe and aerating it with 0.5 ml acetylene and 4.5 ml of air. After incubation at environmental temperature for at least 1 h, 0.5 ml of the gas is injected into a pre-vacuumed (sucking out 0.5 mL air) 5-ml vial sealed with a rubber plug. Then the vial is further sealed with Parafilm to prevent the escape of gas. The gas samples stored in vials will be used for a gas chromatograph assay according to David et al. (1980). The nitrogenase activity (NA) will be calculated with the formula NA (nmol of C_2H_4 mg^{-1} h^{-1}) = C_2H_4 (nmol) / [nodule weight (mg) × reaction time (h)].

11.1.5 Soil Characterisation

In general, the soil characters with major effects on rhizobial populations are pH, salinity and contents of organic materials, phosphorus and nitrogen (Han et al. 2009; Zhang et al. 2011). The contents of contaminants, like heavy metals, in contaminated soils should also be considered (Stan et al. 2011). These analyses can be performed by commercial services.

11.2 Phenotypic Characterisation

In this chapter, phenotypic characterisation refers to the determination of biophysical and biochemical traits of rhizobia. During the dacades of 1960s–1990s, a wide range of phenotypic features were used for numerical taxonomy of rhizobia, while the number of strains and number of characters were increased from tens (Graham 1964) to hundreds (Gao et al. 1994) according to the development of the characterisation techniques and of the computer technology. The phenotypic features commonly used in the characterisation involved (i) utilisation as sole carbon sources (at 0.1% final concentration) of sugars, alcohols, organic acids, amino acids, polysaccharides and so on; (ii) utilisation as sole nitrogen sources of amino acids and other nitrogen compounds at the final concentration of 0.1%; (iii) resistance to antibiotics, dyes, heavy metals, etc. at varied concentrations from 5 to 200 units (or μg ml^{-1}); (iv) biochemical tests such as catalase, peroxidase, urease, reduction of nitrate, reduction of methyl blue, etc.; (v) growth in medium with different values of pH and salinity and growth at different temperature; and (vi) motility/flagellation and cellular observation. Currently, most of the basic biochemical characteristics can be estimated by using the commercial kits, such as API-20NE test strips (bioMérieux) and Biolog GN2 microplates according to the manufactures' instruction. However, the classical methods are still useful in many cases.

The utilisation of sole carbon and nitrogen sources can be performed on plates of basal medium, such as White's medium (1972) supplied with trace elements of

medium Cs7 (Pagan et al. 1975), by inoculating with a multipoint inoculator (Josey et al. 1979). The composition of White's medium (White 1972) is $NaNO_3$, 2.5 g; KH_2PO_4, 1.0 g; K_2HPO_4, 2.0 g; $MgSO_4{\cdot}7H_2O$, 0.3 g; $CaCl_2$, 0.1 g; NaCl, 0.1 g; $FeCl_3$, 0.01 g; bacteriological agar, 18 g; vitamin solutions A, B and C, 2.5 ml of each; and distilled water, 900 ml. The vitamin solutions are (A) biotin 20 pg and vitamin B12 40 pg in 100 ml of distilled water; (B) 10 mg for each of thiamine-HCl, nicotinic acid, calcium pantothenate and p-amino-benzoic acid in 100 ml of distilled water; and (C) folic acid 2.0 mg solubilised in 100 ml of 1 mM NaOH. After autoclaving at 105 °C for 10 min or filter-sterilising (Millipore 0.22 μm filter), these stock solutions can be stored at 4 °C. The prepared medium is distributed in 250 ml flasks at aliquot of 90 ml for sterilisation at 121 °C for 15 min, and then 10 ml of filter-sterilised carbon source (1%, w/v, solution) is added to the cooled basal medium (about 50 °C). Then complete medium is poured into five plates. To improve the growth of some rhizobia, 1 ml of a stock solution of trace elements in medium Cs7 can be added, which contains $MnSO_4{\cdot}4H_2O$ 87 mg, H_2BO_4 50 mg, $ZnSO_4{\cdot}7H_2O$ 10 mg, KI 10 mg, $CuSO_4{\cdot}5H_2O$ 2 mg, $NaMoO_4{\cdot}2H_2O$ 1 mg and $CoCl_2{\cdot}6H_2O$ 1 mg, in 100 ml distilled water (Pagan et al. 1975).

In the earlier studies, the prepared plates were inoculated in duplicate with a multipoint inoculator with 62 pins (Lighthart 1968), and the inoculants were a suspension of washed cells at a concentration of OD_{600} about 0.8 that offered approximately 10^5 cells per pin (Gao et al. 1994). For inoculating, the multipoint inoculator is immersed first in the cell suspension and then stamped softly on the surface of the medium in the plate. Growth is observed after 3–10 days of incubation (depending on the growth rate of the tested rhizobia). Visibly more growth than that on control plates (without addition of carbon source) is considered as positive.

Resistance to antibiotics, morphology and growth at different pH/temperatures can be examined on YMA or on PY agar by using the multipoint inoculator. For resistance to antibiotics, two strategies have been used. One is to spread the strain on the surface of the medium in the plate and place agar diffusion discs (Difco) containing different antibiotics (e.g. ampicillin 10 pg, bacitracin10 U, cefoperazone 75 pg, etc.) on the surface. After incubation for 3–10 days (depending on the growth rate), the presence of an inhibition ring around a disc indicates that the strain is sensitive to the corresponding antibiotic (van Berkum et al. 1998). The other strategy is to add the filter-sterilised antibiotic solution at different concentrations into the melted medium to prepare the plates and then to inoculate the strains with a multipoint inoculator. The concentrations for each antibiotic can be varied, such as 5, 25 and 50 pg ml^{-1} for chlortetracycline but 25 and 125 pg ml^{-1} for erythromycin (Gao et al. 1994), which are determined according to the references. This method can also be applied for resistance determination for other chemicals (metals, dyes, etc.) and NaCl (1–10% with interval of 0.5% or 1%, w/v).

For determination of pH range for growth, pH 4 to pH 12 can be used; this may be modified according to the pH of the soil where the rhizobia were isolated. In most of the earlier studies, the pH of the medium was adjusted with 1 M HCl and NaOH after autoclaving, which can avoid the destruction by highly acid or alkaline conditions of the nutrients in the medium. With this method, the pH may change

during growth, so buffer solutions are now used to adjust the pH: pH 4.0 and 5.0 with 0.1 M citric acid/1 M Na_3-citrate; 6.0 through 10.0 with 0.05 M NaH_2PO_4/0.05 M $Na_3PO_4.12H_2O$; pH 11.0 with 0.05 M NaH_2PO_4/0.1 M NaOH; and pH 12.0 with 0.2 M KCl/0.2 M NaOH. The pH of the medium should be checked and measured after autoclaving.

The Biolog system, such as Biolog GEN III microplates (Biolog, Inc., Hayward, CA, USA), has been widely used for determining the utilisation of different carbon sources, while the API 20NE kit (bioMérieux) has been used to examine biochemical activities (Kuzmanović et al. 2018; McInroy et al. 1999).

11.3 Chemical Characterisation

The cellular fatty acid methyl ester profile (Tighe et al. 2000), respiratory quinone composition and polar lipids (Tindall et al. 2010) have been suggested and applied in rhizobial taxonomy, in which the fatty acid patterns are commonly used and the latter are rarely reported.

11.3.1 Cellular Fatty Acid Patterns

For analysis of cellular fatty acid composition, the rhizobial strains should be incubated on plates of a medium adequate for their growth. In most cases, YMA or TY media can be used, but other media are also needed if the strain does not grow in these media. Since the medium composition and incubation conditions affect the cellular fatty acid patterns, reference strains for closely related species are always required and cultured in parallel to make the results comparable. In this analysis, the strains are incubated on plates for 3–10 days according to the growth rate of the strains. Then the biomass is collected and fatty acids are extracted for conversion into methyl esters. Finally, the fatty acid pattern is determined by gas chromatography with the Microbial Identification System (Sherlock version 6.1, TSBA40 method) (Kuzmanović et al. 2018). In practice, the use of a commercial service is always preferred, and only the live or acetone-dried cells are needed in this case.

11.3.2 Composition of Respiratory Quinones

The respiratory quinones have been characterised for rhizobia in several studies (Choma and Komaniecka 2003; Jiao et al. 2015; Wang et al. 2013). Since the predominant respiratory quinone is ubiquinone-10 (Q-10) for bacteria in the order *Rhizobiales*, this analysis does not offer valuable information to differentiate or define rhizobial species. This analysis can be performed by using reversed-phase

HPLC (Komagata and Suzuki 1987). In general, attention should be paid to the following points (Tindall et al. 2010):

– For extraction of quinones from cell material, extreme pH, strong light and highly oxidising conditions should be avoided (Minnikin et al. 1984).
– Some respiratory lipoquinone classes, like ubiquinones, rhodoquinones, menaquinones, *Sulfolobus-Caldariella* quinones, menathioquinones and demethyl-, monomethyl- and dimethylmenaquinones, can be identified with TLC pre-scanning.
– The simple quinone mixtures can be separated and identified by using reverse phase TLC (Collins 1994; Tindall 2010).
– For identification of unknown quinones, UV-visible spectroscopy and HPLC systems can be used, while the full structural identification may require a combination of MS and NMR (Collins 1994).

11.3.3 Composition of Polar Lipids

Although this analysis is not universally applied in the species definition of rhizobia, the following polar lipids have been reported in some rhizobial strains: phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, ornithine-containing lipids, phosphatidyl-*N*-dimethylethanolamine and cardiolipin. A recommended procedure (Nguyen and Kim 2017) is:

1. Mix 0.25–0.3 g of wet cells cultured at 28 °C in YM or PY broth at the late exponential phase with 75 ml of chloroform/methanol (2:1, v/v) by stirring overnight.
2. Remove the cell debris by centrifugation or filtration with paper.
3. Eliminate the solvents by evaporating at <37 °C, and resuspend the precipitate in 6.5 ml of chloroform/methanol/0.3% aqueous NaCl (6:10:3, v/v/v), followed by adding 2.0 ml chloroform and 2.0 ml 0.3% aqueous NaCl. After mixing for 15 min, the chloroform and aqueous phases are separated by centrifugation, and the chloroform phase is evaporated again. As noted by Nguyen and Kim (2017), if a white and viscous appearance occurs in the chloroform phase after centrifugation, it should be transferred to a new glass tube and mixed with an additional 3 mL chloroform. After vortexing for 60 s, it is centrifuged again.
4. Step 3 should be repeated once again to purify the extract of polar lipids, and the final extract is resuspended in 300 μl chloroform/methanol (2:1, v/v). This extract will be stored at −20 °C.
5. Aliquots (15 μl) of the extract are loaded for two-dimensional thin-layer chromatography (TLC) by using chloroform/methanol/water (14:6:1, v/v/v) at first and chloroform/methanol/glacial acetic acid (13:5:2, v/v/v) at second.
6. To visualise the TLC patterns, the spray reagents molybdenum blue (Dittmer and Lester 1964) for phospholipids, ninhydrin (0.4% in n-butyl alcohol saturated with distilled water, w/v; developed at 100 °C for 5 min) for aminolipids and

anisaldehyde reagent [95% ethanol/concentrated sulphuric acid/anisaldehyde/glacial acetic acid (54:3:3:0.6, v/v), developed at 110 °C for 4–6 min] for glycolipids are used. The spots are identified by comparing them with the standard mixtures of polar lipids or with the chromatogram of a defined reference strain (Choma and Komaniecka 2003).

11.4 Genomic Analyses

In contrast to the taxonomy of plants and animals, the simple morphology of bacteria makes their classification mainly based on a combination of analyses (Stackebrandt et al. 2002; Vandamme et al. 1996). Among these criteria, DNA-based analyses have played key roles to define the taxa, such as BOX-PCR for strain identification (Schneider and de Bruijn 1996), DNA-DNA hybridisation (DDH) (Wayne et al. 1987), sequence analyses of 16S rRNA genes, multilocus sequence analysis (MLSA) of housekeeping genes (Martens et al. 2008) and genome sequence similarity (Wang et al. 2016) for species, genus and family, etc.

11.4.1 Extraction of Genomic DNA

For genome-based analysis, DNA is extracted in a mini-preparation as template for gene extraction and genome sequencing or on a large scale (mg) for DNA hybridization.

1. Cell lysate as DNA template:
   - Suspend ¼ bacteriological loop of a fresh rhizobial colony into 100 μl of 0.1% Tween 20 (polyoxyethylene sorbitan monolaurate).
   - Incubate the suspensions at 95 °C for 10 min to lyse the cells, and cool the lysate immediately on ice.
   - Use 5 μl of the lysate samples in 120 μl PCR mixture as template DNA.
2. Extraction of genomic DNA with CTAB (hexadecylytrimethyl ammonium bromide) (Wilson 1997) for PCR or for genome sequencing:
   - Incubate the strain in 5 ml of PY broth at 28 °C with shaking (180 rpm) up to the middle exponential phase (about 0.5–0.8 of OD_{600}: overnight for fast-growing rhizobia or 24 h for bradyrhizobial strains).
   - Collect the cells by centrifuging 1.5 ml of the culture in a microcentrifuge for 1 min at 12,000 rpm and resuspending the pellet in 567 μl of 1 × TE buffer (10 mM Tris-HCl, 1 mM EDTA·Na_2, pH 8.0 at 25 °C). After vortexing with 30 μl of 10% SDS and 3 μl of 20 mg/ml proteinase K, incubate the mixture for 1 h. at 37 °C, and mix it by inversion every 10 min until the solution becomes viscous.

- Add 100 µl of 5 M NaCl and vortex for 30 s to mix thoroughly. Then add 80 µl of CTAB/NaCl solution (dissolve 4.1 g NaCl and 10 g CTAB in 80 ml water at 65 °C with stirring, finally, adjust the volume to 100 ml with water) and vortex for 30 s.
- After incubating 10 min at 65 °C, add equal volume (0.7–0.8 ml) of chloroform/isoamyl alcohol (24:1 v/v) and vortex for 30 s, and centrifuge for 5 min at 12,000 rpm in a microcentrifuge.
- Remove the aqueous, viscous supernatant (without touching the white sediment in the interface) to a clean Eppendorf tube. Add an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and vortex for 30 s, and then centrifuge at 12,000 rpm for 5 min.
- Transfer the supernatant to a clean Eppendorf tube, and add 0.6 volume of precooled (−20 °C) isopropanol. Shake the tube up and down until the white DNA precipitate occ.
- Centrifuge the tube at 12,000 rpm for 5 min to precipitate the DNA.
- After discarding the liquid, add 70% ethanol (pre cooled at −20 °C) and vortex 30 s. Then centrifuge 5 min and discard the liquid carefully.
- Invert the tube on an absorbent paper for 10 min to dry the pellet, and finally dissolve the DNA in 50–100 µl TE buffer. The extracts can be stored at −20 °C.

11.4.2 DNA Fingerprinting by REP-PCR (BOX-A1R, ERIC)

Targeted to the DNA sequences located between the specific interspersed repeated sequences in bacterial genomes, the patterns of PCR-amplified repetitive DNA elements in bacteria (REP-PCR) are used to define strains (Schneider and de Bruijn 1996). These repeated sequences are termed BOX, REP and ERIC elements (Versalovic et al. 1994). BOX elements consist of conserved multiple sequences universal in bacterial genomes, including the rhizobia (Cardoso et al. 2017; Gao et al. 2001; Menna et al. 2009). REP sequences are 38 bp palindromic units with a stem-loop (5 bp) structure, while ERIC sequences are 126 bp, possessing a cEntral conserved palindromic structure. Both REP and ERIC sequences are found in all the Gram-negative enteric bacteria and in other bacteria, such as the rhizobia (Gao et al. 2001; Hameed et al. 2016; Kundu and Dudeja 2008). Among these three types of rep-elements, BOX is used most commonly.

For BOX-PCR, the primer Box-A1R (5'-CTA CGG CAA GGC GAC GCT GAC G-3′) is used, and the PCR protocol is initial denaturation at 95 °C for 7 min followed by 30 cycles of 94 °C 1 min, 53 °C 1 min, and 65 °C 8 min and then the final extension at 65 °C for 16 min. In a reaction mixture of 25 µl, add 0.5 µl of template DNA, 0.5 µM of the primer, 50 µM of each deoxynucleoside triphosphate and 0.5 U of TaqDNA polymerase (Ecogen, Barcelona, Spain). The amplifications are performed in duplicate, and the PCR products are separated and visualised by

electrophoresis of 5 μL amplicons in a 2% (w/v) agarose gel in TE buffer (Sambrook and Russell 2001). An aliquot of 0.5 μL of the 100 bp DNA ladder is included in each electrophoresis to standardise the patterns, and only the bands occurring in both replicates are considered for typing the isolates by calculating the Pearson correlation coefficient and grouping with the average linkage (UPGMA) method (Gao et al. 2001). Isolates sharing the same PCR patterns are identified as clones of the same strain (Schneider and de Bruijn 1996).

For other REP-PCR, the primers REP1R-I (5'-III ICG ICG ICA TCI GGC-3′) and REP2-I (5'-ICG ICT TAT CIG GCC TAC-3′) for REP-PCR and ERIC1R (5′-ATG TAA GCT CCT GGG GAT TCAC-3′) and ERIC2 (5′-AAG TAA GTG ACT GGG GTG AGCG-3′) for ERIC-PCR are used (Versalovic et al. 1994). The protocol of REC-PCR is 95 °C for 6 min as initial denaturation and 30 cycles at 94 °C for 1 min, 40 °C for 1 min and 65 °C for 8 min, with the final extension at 65 °C for 16 min (for REP-PCR). The protocol for ERIC-PCR is the same as for BOX-PCR.

11.4.3 16S rRNA Gene for Genus Definition

The 16S rRNA genes can be amplified and sequenced by the same primer pairs. For rhizobia, the commonly used primers are fD1 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and rD1 (5′-AAG GAG GTG ATC CAG CC-3′) (Weisburg et al. 1991), which correspond to the positions 7–26 and 1541–1525 in the *Escherichia coli* 16S rRNA gene. Another commonly used primer pair is P1 (5′-AGA GTT TGA TCC TGG CTC AGA ACG AAC GCT-3′) and P6 (5′-TAC GGC TAC CTT GTT ACG ACT TCA CCCC-3′), corresponding to the positions 8–37 and 1479–1506 in *E. coli* 16S rRNA (Tan et al. 1997). The PCR protocol is 95 °C for 3 min to initially denature the DNA; then 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min; and, finally, an extension at 72 °C for 3 min. The reaction mixture of 25 μL contains 2.5 μL of 10 × buffer (20 mmol Tris-HCl, pH = 8.4), 1 μL of 50 mmol $MgCl_2$, 1 μL (100 μmol) of each dNTPs, 2.5 μL of each primer (1 μmol), 5 μL of genomic DNA, 1 μL (1 U) of Taq DNA polymerase and 6.5 μL of sterile Milli-Q water.

The amplified 16S rRNA can be sequenced commercially, and the sequences are aligned with related ones extracted from the database (GenBank) using Clustal X software (Thompson et al. 1997). Phylogenetic trees can be reconstructed with the methods of neighbour-joining (Saitou and Nei 1987), maximum likelihood (Chor and Tuller 2005) and maximum parsimony (*Felsenstein* 1978) combined with an adequate model (Sullivan and Joyce 2005) in the PHYLIP package. Bootstrap analysis using 1000 replications should be applied to estimate the topological stability. In general, 95% and 97% similarities are used as thresholds for genus and species, respectively. However, the grouping results of well-defined species should be considered as a reference.

11.4.4 Multilocus Sequence Analysis (MLSA) for Species/ Genus Definition

For MLSA of rhizobia, more than ten genes have been recommended, including *atpD* (ATP synthase F1, beta subunit), *dnaK* (chaperone protein), *gap* (glyceraldehyde-3-phosphate dehydrogenase), *gltA* (citrate synthase), *glnA* (glutamine synthetase), *gyrB* (DNA gyrase B subunit), *pnp* (polyribonucleotide nucleotidyltransferase), *recA* (homologous DNA repair protein), *rpoB* (RNA polymerase, beta subunit), *thrC* (threonine synthase) (Martens et al. 2008), *glnII* (glutamine synthetase), *SMc00019* (conserved hypothetical protein), *thrA* (homoserine dehydrogenase) and *truA* (RNA pseudouridine synthase A) (Zhang et al. 2012). The corresponding primers and PCR conditions are summarised in Table 11.2.

To estimate the phylogenetic relationships of the strains, the acquired sequences and the related sequences extracted from the database (GenBank) are aligned, and phylogenetic trees are reconstructed for the single genes and for the concatenated sequences as described for the 16S rRNA gene. In practice, subsets with 3 or 4 genes can be used in MLSA for genospecies definition, such as the combinations of *SMc00019-thrA-truA* (Zhang et al. 2012), *gyrB-gltA-recA-thrC* (Martens et al. 2008) and *recA-glnII-atpD* (Zhang et al. 2011) which have been used for successfully differentiating diverse rhizobial species. Depending on the rhizobial group, the threshold for species can vary between 95% and 97% (Martens et al. 2007, 2008; Zhang et al. 2011, 2012). In MLSA, the phylogenetic relationships among the rhizobial strains also can be estimated from the sequence divergence or average nucleotide identity (ANI), for example, 2.7% sequence divergence was detected at the intraspecies level based upon seven housekeeping genes which corresponds to an ANI value of 97.3% (Martens et al. 2008).

11.4.5 Phylogenetic Analysis of Symbiosis Genes

The symbiosis genes in rhizobia refer to the nodulation (*nod*, *nol*, *nor*) genes and the nitrogen fixation (*nif*, *fix*) genes. The phylogenetic analysis of these genes has been used for determining the symbiovar of rhizobial strains (Laguerre et al. 2001; Saïdi et al. 2014; Zhang et al. 2011). The commonly used genes are *nifH* (dinitrogenase reductase), *nodC* (N- acetylglucosaminyltransferase) and/or *nodA* (acyltransferase). The primers and PCR conditions for amplification and sequencing of the *nifH*, *nodC* and *nodA* are summarised in Table 11.3.

The *nodC* gene can be amplified in a 100-µl PCR mixture containing 25 pmol of each primer, 0.1 µg of DNA template and 1 U of DNA polymerase, in 1× buffer supplied by the manufacturer. The *nifH* gene can be amplified in 100 µL reaction mixture containing 1 µg of template DNA, 1× buffer (10 mM tris-HCl pH 8.3), 1·5 mM $MgCl_2$, 20 µM (each) dNTP and 0·1 µM of each primer. PCR products can be visualised by electrophoresis of 10 µL aliquot of the PCR products in 1.2% (w/v)

Table 11.2 Primers and PCR conditions for MLSA from Martens et al. (2007, 2008), Stepkowski et al. (2005) and Zhang et al. (2012)

Gene	Primer	Sequence of primer (5′-3′)	Position*	PCR condition#
glnA	glnA144F	GTCATGTTCGACGGYTCYTCG	144–164	2× (2′ 94 °C, 2′ 62 °C, 1′ 72 °C), 32× (30s 94 °C, 1′ 61 °C, 1′ 72 °C)
	glnA1142R	TGGAKCTTGTTCTTGATGCCG	1162–1142	
	glnA572F	GGACATGCGYTCYGARATGC	572–591	
	glnA572R	GCATYTCRGARCGCATGTCC	591–572	
gltA	gltA428F	CSGCCTTCTAYCAYGACTC	428–446	3× (2′ 94 °C, 2′ 53 °C, 1′ 72 °C), 30× (30s 94 °C, 1′ 53 °C, 1′ 72 °C),
	gltA1111R	GGGAAGCCSAKCGCCTTCAG	1130–1111	
	gltA1089R	ATRCCSGARTAGAAGTCG	1106–1089	
	gltA667R	GCRTGCAGGATGAANAT	683–667	
thrC	thrC1231R	GGRAATTTDGCCGGRTGSGC	1250–1231	3× (2′ 94 °C, 2′ 55 °C, 1′ 72 °C), 30× (30s 94 °C, 1′ 55 °C, 1′ 72 °C)
	thrC577F	GGCAMKTTCGACGAYTGCCAG	577–597	
	thrC766F	GGCAATTTCGGCGAYAT	766–782	
	thrC766R	ATRTCGCCGAAATTGCC	782–766	
	thrC925R	GASGARAYCTGGATRTCCAT	944–925	
dnaK	TsdnaK4	GTACATGGCCTCGCCGAGCTTCA	1057–1075	35× (60s 94 °C, 60s 55°C 40s 72 °C)
	TsdnaK2	GGCAA- GGAGCCGCAYAAGG	1794–1772	
	dnaK1466Fd	AAGGARCANCAGATCCGCATCCA	1466–1488	35× (1′ 94 °C, 1′ 64 °C, 40s 72 °C)
	dnaK1777Rd	TASATSGCCTSRCCRAGCTTCAT	1777–1799	
glnA	glnA144f	GTCATGTTCGACGGYTCYTCG		2× (2′ 94 °C, 2′ 62 °C, 1′ 72 °C), 32× (30 s 94 °C, 1′ 61 °C, 1′ 72 °C)
	glnA1142R	TGGAKCTTGTTCTTGATGCCG		
recA	TSrecAf	CAACTGCMYTGCGTATCGTCGAAGG	8–32#	35× (45 s 95 °C, 30 s 58 °C, 1.5′ 72 °C
	TSrecAr	CGGATCTGGTTGATGAAGATCACCATG	620–594#	
atpD	atpD352F	GGCCGCATCATSAACGTCATC	352–372*	3× (2′ 94 °C, 2′ 64 °C, 1′ 72 °C), 30× (30′′ 94 °C, 1′ 64 °C, 1′ 72 °C)
	atpD871R	AGAGCCGACACTTCMGARCC	890–871*	
	TSatpDf	TCTGGTCCGYGGCCAGGAAG	189–208#	35× (45 s 95 °C, 30 s 58 °C, 1.5′ 72 °C)
	TSatpDr	CGACACTTCCGARCCSGCCTG	804–784#	

(continued)

Table 11.2 (continued)

Gene	Primer	Sequence of primer (5′-3′)	Position*	PCR condition#
glnII	TSglnIIf	AAGCTCGAGTACATCTGGCTCGACGG	13–38#	35× (45 s 95 °C, 30 s 58 °C, 1.5′ 72 °C)
	TSglnIIr	SGAGCCGTTCCAGTCGGTGTCG	681–660#	
gyrB	gyrB343F	TTCGACCAGAAYTCCTAYAAGG	343–364*	5× (2′ 94 °C, 2′ 58 °C, 1′ 72 °C), 28× (30′′ 94 °C, 1′ 58 °C, 1′ 72 °C)
	gyrB1043R	AGCTTGTCCTTSGTCTGCG	1061–1043*	
	gyrB846F	CACCAACAACATYCCSCAGC	846–865*	
	gyrB846R	GCTGSGGRATGTTGTTGGTG	865–846*	
rpoB	**rpoB83F**	CCTSATCGAGGTTCACAGAAGGC	83–103*	3× (2′ 94 °C, 2′ 58 °C, 1′ 72 °C), 30× (30′′ 94 °C, 1′ 58 °C, 1′ 72 °C)
	rpoB1061R	AGCGTGTTGCGGATATAGGCG	1081–1061*	
	rpoB456F	TCGTYTCGCAGATGCACCG	456–475*	
	rpoB458R	GAACGGTGCATCTGCGARACG	478–458*	
Gap	**gap109F**	TCGGNCCGGTYGARACCAAYGC	109–130*	3× (2′ 94 °C, 2′ 57 °C, 1′ 72 °C), 30× (30′′ 94 °C, 1′ 57 °C, 1′ 72 °C)
	gap940R	CCCCAYTCRTCGTCGTACC	958–940*	
	gap528F	ATGACSACGATCCACKCCTA	528–547*	
	gap528R	TAGGMGTGATCGTSGTCAT	547–528*	
Pnp	pnp913F	AAGRTCGTKCGCTGGAAC	913–930*	3× (2′ 94 °C, 2′ 50 °C, 1′ 72 °C), 30× (30′′ 94 °C, 1′ 50 °C, 1′ 72 °C)
	pnp1473R	ACCTTGAAGTCCATRTCG	1490–1473*	
SMc00019	SMc00019B-F	CATTCVKCSGARGGVGCSATGGGYATC		30× (45 s 94 °C, 1′ 66 °C, 1′ 72 °C)
	SMc00019B-R	GCGTGBCCBGCSKCGTTSGAVAGCAT		
thrA	thrAB-R	TGCTTCGTCGARYTGATGG		13× (45 s 94 °C, 1′ 61 °C to 48 °C with −1 °C/cycle, 1′ 72 °C), 22× (45 s 94 °C, 1′ 48 °C, 1′ 72 °C)
	thrAB-F	ACRCCCATCACCTGYGCRATC		
truA	truAB-F	CGCTACAAGCTCAYYATCGA		10× (45 s 94 °C, 1′ 60 °C to 50 °C with −1 °C/cycle, 1′ 72 °C), 25× (45 s 94 °C, 1′ 50 °C, 1′ 72 °C)
	truAB-R	CCSACCATSGAGCGBACCTG		

Note: N = A, G, C or T; R = A or G; Y=C or T; M = A or C; S = G or C; K = G or T; W = A or T; V = A, C or G; D = A, G or T; H = A, C or T; B=C, G or T

*, #, positions in the corresponding sequence of *Ensifer meliloti* 1021 and *Bradyrhizobium japonicum* USDA 110, respectively

Table 11.3 Primers and PCR conditions for symbiosis gene sequencing reported by Martens et al. (2007, 2008), Stepkowski et al. (2005) and Zhang et al. (2012)

Gene	Primer	Sequence of primer (5′-3′)	Size of fragment	PCR condition
nifH	nifH-univ(F)	GCIWTITAYGGNAARGGNGG	780 (of 890) bp	2′ 95 °C; 35× (45 s 95 °C, 30s 53 °C, 2′ 72 °C); 7′ 72 °C (Gnat et al. 2015)
	nifH-univ(R)	GCRTAIABNGCCATCATYTC		
	NifH1	CGTTTTACGGCAAGGGCGG	780 bp	
	NifH2	TCCTCCAGCTCCTCCATGGT		
	TSnodH1	VTKGAGYAACGGTGARYTGCTCA	567 bp	
	TSnodH2	GCGAAGTGAWSCCGCAACTC		
nodC	nodCF4	AYGTHGTYGAYGACGGATC	930 (of 1300) bp	3′ 95 °C; 35× (1′ 94 °C, 1′ 55 °C, 2′ 72 °C); 10′ 72 °C (Perret and Broughton 1998)
	nodC1	CGYGACAGCCANTCKCTATTG		
	NodCFu	AYGTHGTYGAYGACGGITC	890–930 bp	
	NodCI	CGYGACAGCCANTCKCTATTG		
nodA	nodA-1F	TGCRGTGGAARNTRNNCTGGGAAA	660 bp	2′ 93 °C, 35× (45 s 93 °C, 45 s 62 °C, 2′ 72 °C), 5′ 72 °C (Haukka et al. 1998)
	nodA-2R	GGNCCGTCRTCRAAWGTCARGTA		

Note: nucleotide codes as in Table 16.1

agarose, as mentioned above for BOX-PCR. Acquired sequences can be analysed as discussed above for 16S rRNA genes.

11.4.6 Genome Analysis for Species/Genus Definition

Genome sequence can be realised via a commercial service, such as the genome sequencing companies Novogene (Beijing), Macrogen Inc. (Seoul, Korea) and so on. Although a lot of information can be obtained from the genome sequence, the most useful information for diversity and taxonomy of rhizobia extracted from the genome data is the average nucleotide identity (ANI).

ANI has been widely used to compare prokaryotic genome sequences for classifying or identifying their taxonomic affiliation at different taxonomic levels, such as species, genus, family and so on. The finding that an ANI value of around 95% corresponds to the 70% DDH threshold for species (Goris et al. 2007) indicates that an ANI of 95–96% is a suitable threshold for species delineation in most bacterial groups, including rhizobia. For calculating the ANI value of two prokaryotic genome sequences, software is available, including the Ezbiocloud ANI calculator (Yoon et al. 2017; http://www.ezbiocloud.net/tools/ani), the Kostas lab ANI calculator (Rodriguez-R and Konstantinidis 2014; http://enve-omics.ce.gatech.edu/ani/) and ANItools (Han et al. 2016; http://ani.mypathogen.cn/).

11.5 Ecological Analyses

11.5.1 Diversity Estimation

In the analysis of community structure and species richness of rhizobia, genospecies defined based upon MLSA are used, while the genotypes defined by genomic fingerprints (BOX-PCR) can be used in the estimation of genetic diversity. The alpha and beta diversity can be estimated by calculating the diversity index, species richness and evenness in the sampling sites (Hill et al. 2003) with the Vegan package (version 1.17–4; http://ftp.ctex.org/mirrors/CRAN/) in the R statistical language (version 2.12.0; http://www.r-project.org/):

- Shannon-Wiener (H') index for diversity: $H' = -\sum p_i \ln p_i$, where p_i is the proportion of strains in the ith OTU (genospecies or genotype) estimated as n_i/N (=the number of isolates in the ith OTU/the total number of the studied isolates). This index considers both the number of OTUs and the relative abundance of each OTU. In this case, the greater the H´ value, the greater of diversity in the site or community.
- Simpson (D) index for diversity: $D = \sum[n_i(n_i\text{-}1)/N(N\text{-}1)]$, where n_i is the number of strains in the ith OTU (genospecies or genotype) and N is the total number of the studied strains. Similar to H', the D index also considers both the number of OTUs and the relative abundance of each OTU.
- Pielou (J) index for species evenness: $J = H'/H'_{max}$, where H' is the value of the Shannon diversity index and H'_{max} (=ln$S$, S is the total number of species) is the maximum possible value of H' (if every OTU were in the same proportion). This index varies between 0 and 1 and reflects the ratio of observed diversity and the theoretical maximum diversity in the sampling site.
- Chao1, a nonparametric estimate of species richness: $S^* = S + (a^2/2b)$, where S is the observed number of species; a is the number of OTUs recorded only once, while b is the number of OTUs recorded twice. This index reflects the efficiency of revealing all the species in the sampling site.

11.5.2 Analysis of Biogeography

The biogeographic patterns and correlations between the rhizobial distribution and environmental factors can be estimated by correspondence analysis (CA) and principal component analysis (PCA) (Mhadhbi et al. 2007). For this analysis, the best subset of environmental variables is selected with the program Bioenv (Clarke and Ainsworth 1993) to ensure that the scaled environmental variables present the maximum (rank) correlation with community dissimilarities in biodiversity. The CA can be realised with the Correspondence 1.0 program in the SPSS 12.0 package, in which the OTUs of rhizobia and the sampling sites are treated as two factors. An

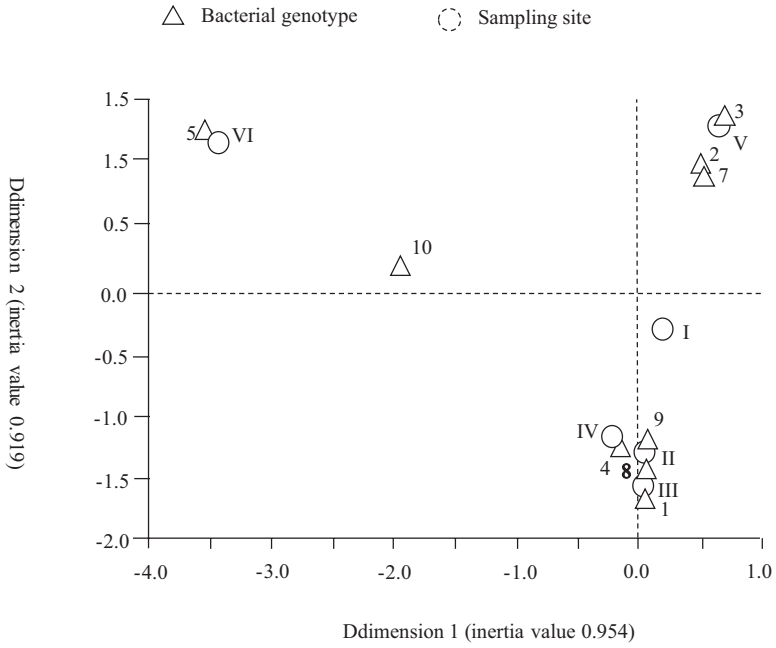

Fig. 11.2 Diplot model showing the results of correlation analysis with the Correspondence 1.0 program in SPSS 12.0 package between genotypes (○) and geographic origins (Δ) of the rhizobia. The clusters formed by genotypes and geographic sites demonstrated a biogeographic pattern of the analysed rhizobia (Pearson coefficient was 0.476, $p = 0.01$). The inertia values >0.9 for both dimensions indicated the two dimensions were closely related to the two factors (genotype and sampling sites). (Deduced from Han et al. 2009)

example is that in Han et al. (2009) (Fig. 11.2). In Fig. 11.2, the nine rhizobial IGS genotypes and the five sampling sites formed three groups, which indicated a clear biogeographic pattern of rhizobia.

PCA can be applied to get a clear idea of the associations among the various parameters. Mhadhbi et al. (2007) performed a PCA to analyse the plant performance (such as the number and fresh weight of nodules, dry weight of shoot and root, nitrogen fixation) of rhizobial inoculant. They found that most of the combinations involving *M. ciceri* 835 and CMG6 and *M. mediterraneum* C11 formed two groups located on the positive sides of the PC1 and PC2, which are characterized by high levels of shoot biomass, nitrogen-fixing capacity and CAT activity.

Redundancy analysis (RDA) (Rao 1964) as a variant of the canonical version of principal component analysis (PCA) was used to examine the relationships between various soil factors (available N, P and K and soil pH) and the rhizobial genospecies detected in different sampling sites (Fig. 11.3) (Zhang et al. 2011). In the diplot, the longer the arrow is, the greater the influence it has; the smaller the angle is between two arrows, the closer their relationship.

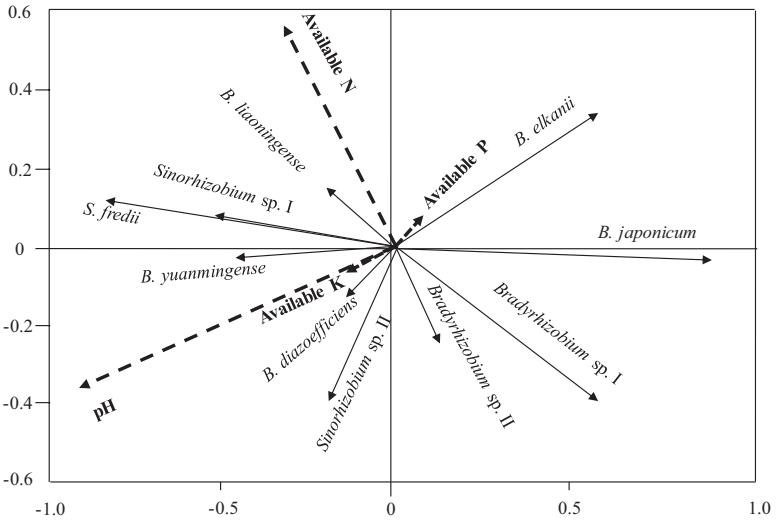

Fig. 11.3 Diplot of RDA showing the effects of soil factors on the distribution of soybean rhizobia estimated by canonical correspondence analysis. The length of the arrow of soil factor is positively related to its impacts on rhizobial community. The direction and angle of the rhizobial group with the soil factor reflect its responds to the soil factor. (Deduced from Zhang et al. 2011)

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