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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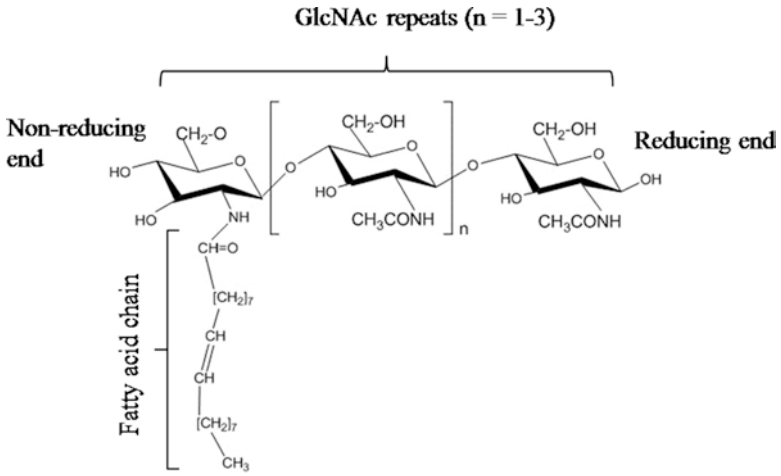


Fig. 5.1 Basic structure of LCOs

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

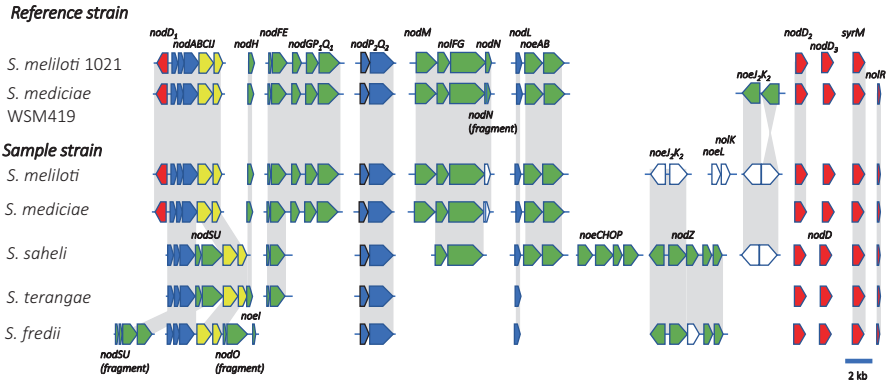


Fig. 5.2 Gene organisation and correlation of Nod factor biosynthetic genes in some *Sinorhizobium* species (Sugawara et al. 2013). Blue arrows indicate the genes encoding enzymes for Nod factor synthesis commonly detected in all tested *Sinorhizobium* strains. Yellow arrows indicate the genes involved 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

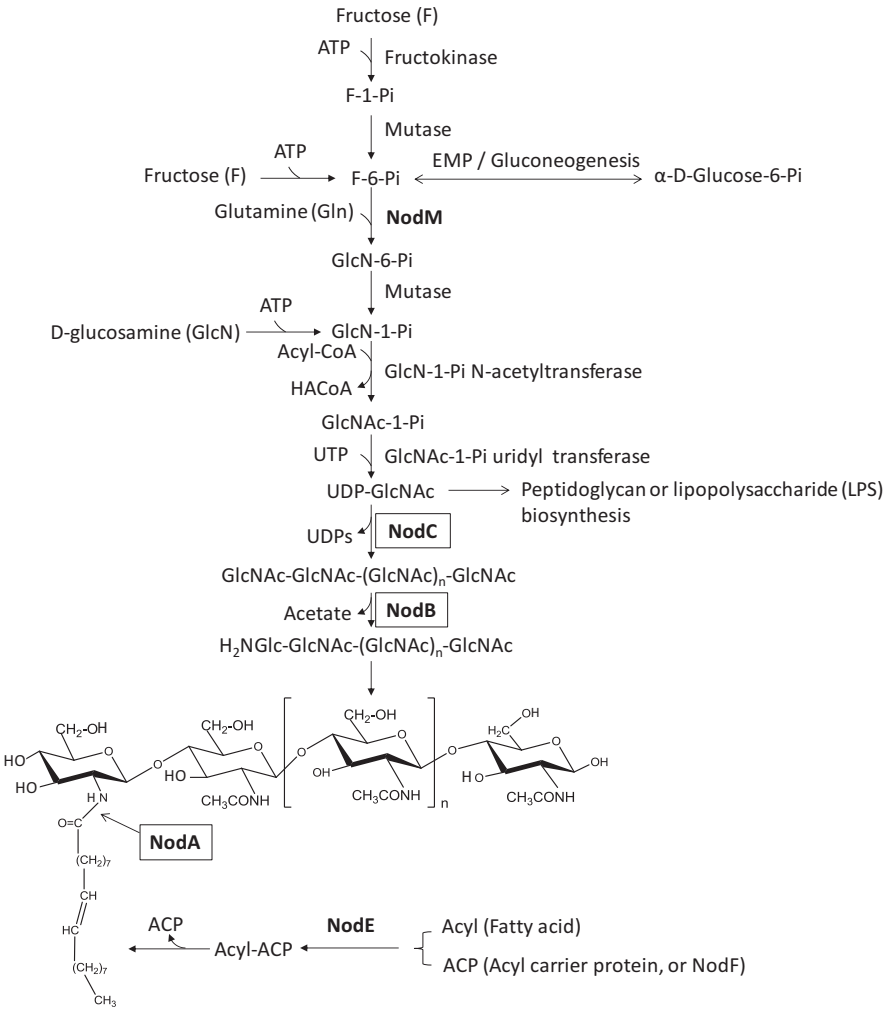


Fig. 5.3 The central pathway of basic LCO biosynthesis and the enzymes involved (Liu et al. 2018a)

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).

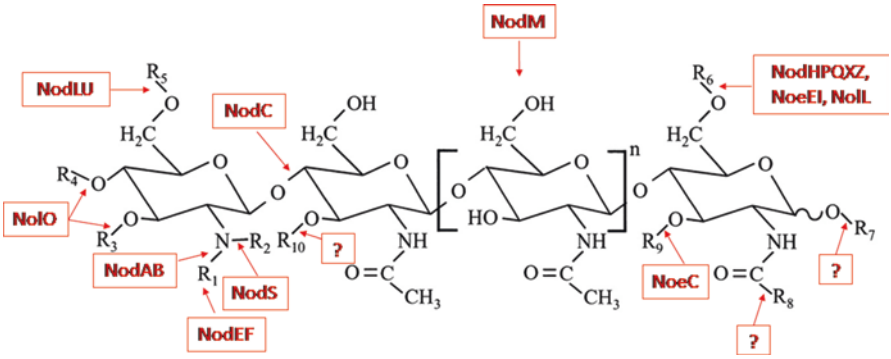


Fig. 5.4 Various substitutions (R_1–R_{10}) and enzymes (boxed) responsible for the synthesis and modification of LCOs produced by rhizobia (Revised based on D'Haeze and Holsters (2002)). R_1 = fatty acid acyl chain; R_2 = methyl (CH_3-) or hydrogen (H-); R_3 = H- or carbamoyl (NH_2CO-); R_4 = H-, NH_2CO- 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:

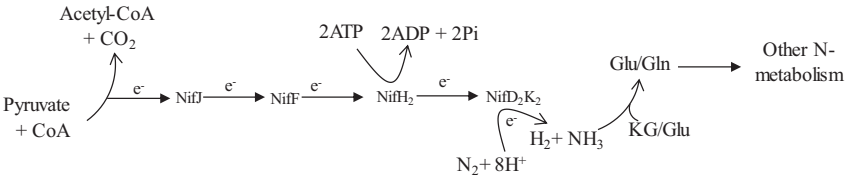


Fig. 5.5 Reaction and molecular mechanism of biological nitrogen fixation (Revised based on Kneip et al. (2007)). (**a**) General reaction of molecular nitrogen fixation. (**b**) Schematic structure and operation of the nitrogenase enzyme complex and subsequent metabolism of nitrogen. Functions of enzymes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

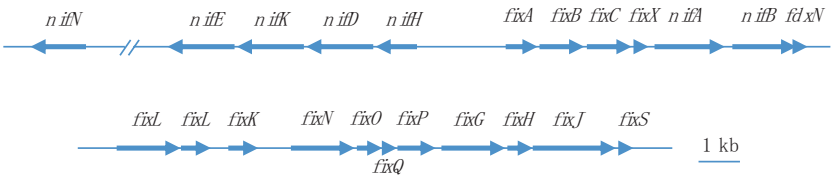
Different rhizobia use different secretion systems – type III, type IV and type VI – to transport effector proteins into host cells (Nelson and Sadowsky 2015). These secretion systems have an effect on rhizobial host specificity and the nodule number on legumes (Nelson and Sadowsky 2015). Abolition of 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*)

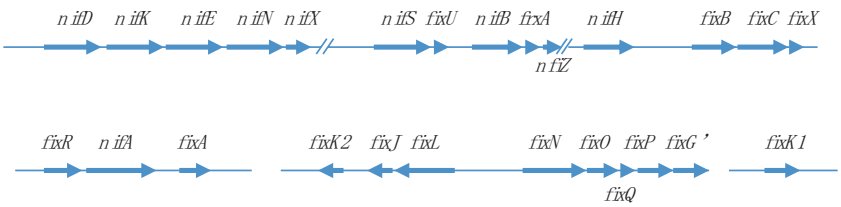


Fig. 5.6 Organisation of *nif* and *fix* gene clusters in *S. meliloti* (**a**) and *B. japonicum* (now *B. diazoefficiens*) (**b**) (Fischer 1994)

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.

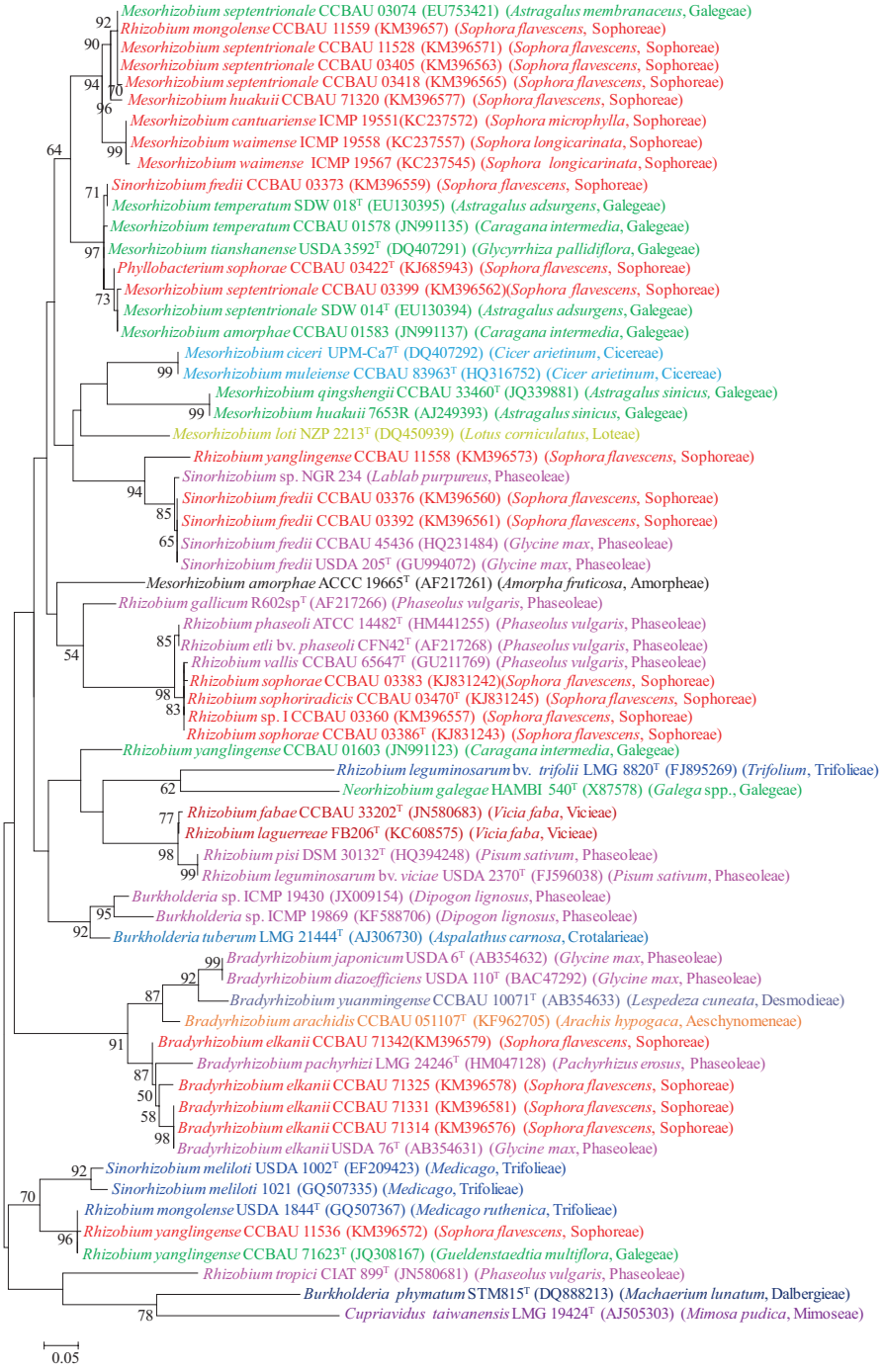


Fig. 5.7 Maximum likelihood phylogenetic tree based on *nodC* genes (Revised from Jiao et al. (2015a)). The model T92+G+I was used to construct the tree. Bar, 5% nucleotide substitution per site. Strains isolated from same tribe of plants were printed in same colour

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/*

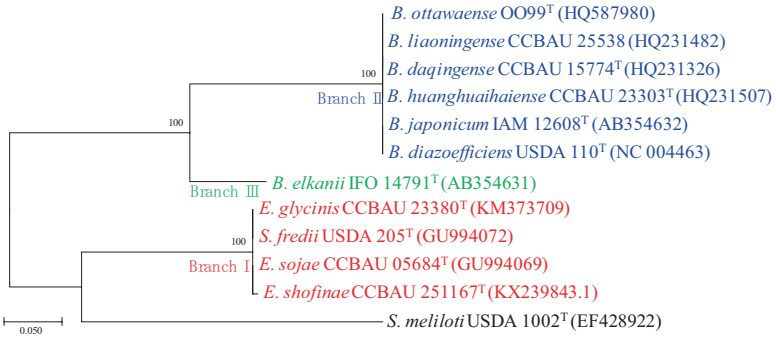


Fig. 5.8 Maximum likelihood phylogenetic tree based on *nodC* genes of soybean rhizobia. The tree was constructed based on the Kimura two-parameter model using Mega 7 software. Bar, 5% nucleotide substitution per site. *Sinorhizobium* (*S.*) *meliloti* USDA 1002^T was used as an outgroup. T in superscript,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

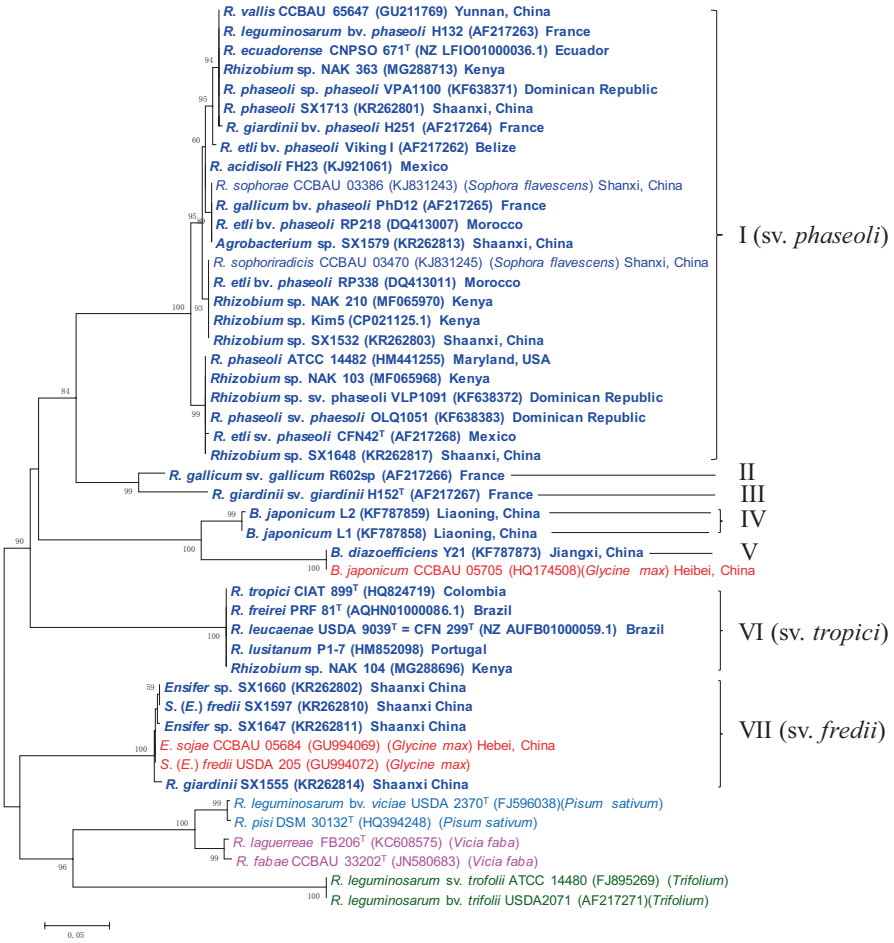


Fig. 5.9 Neighbour-joining (NJ) phylogenetic tree based on *nodC* genes of rhizobia mainly isolated from root nodules of common bean (*Phaseolus vulgaris*). Rhizobia isolated from common bean are shown in blue and boldface. Rhizobia from plants other than common bean are printed in other colours. T in 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.

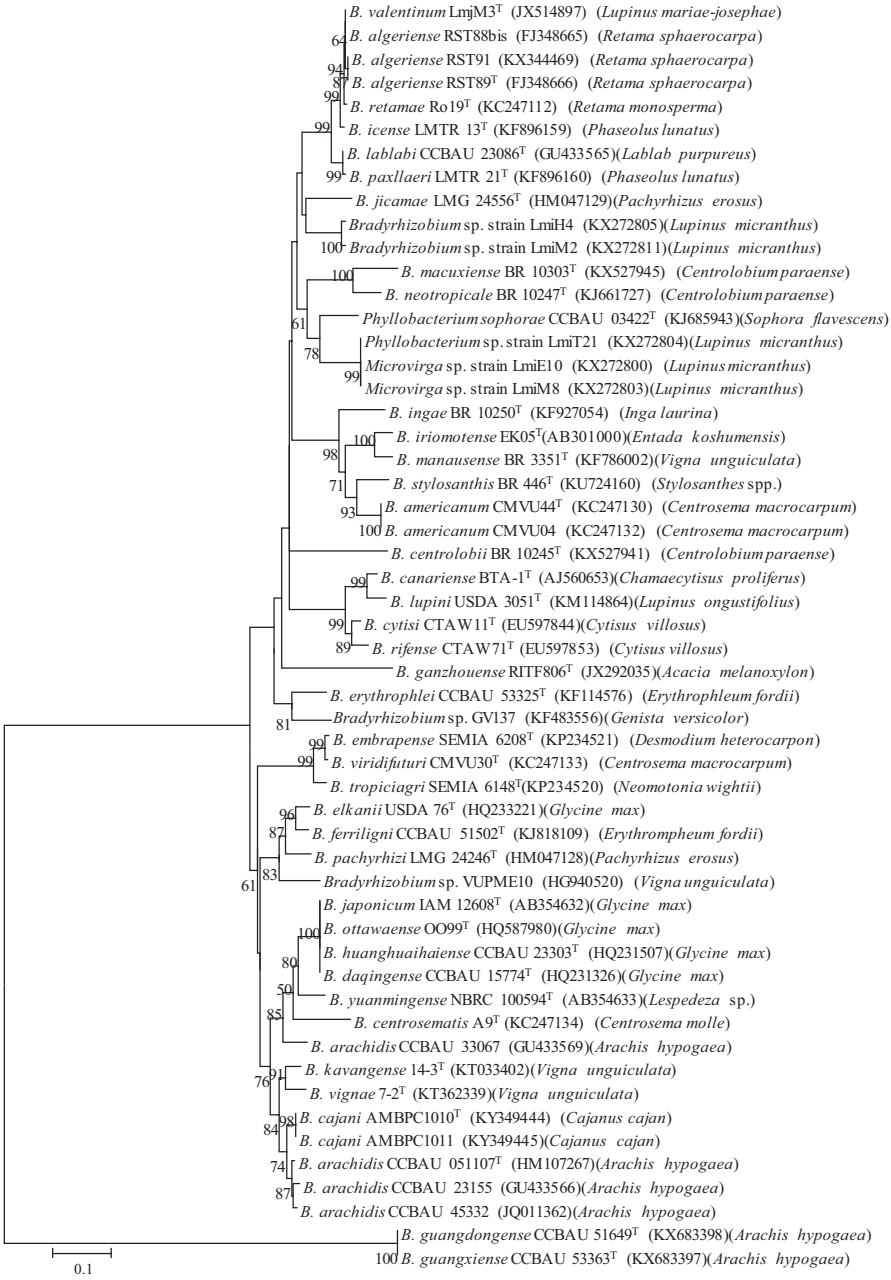


Fig. 5.10 NJ phylogenetic tree based on *nodC* genes of bradyrhizobia. T in superscript, type strain. Bootstraps over 50 are shown at each branch node. Bar, 1% nucleotide substitution per site. GenBank accession No. and rhizobial host plants were shown after the strain numbers

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