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# Taxonomy of the Rhizobia: Current Perspectives

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### Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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## ABSTRACT

The classification of rhizobia has been gone through a substantial change in recent years due to the addition of several new genera and species to this important bacterial group. Recent studies have shown the existence of a great diversity among nitrogen-fixing bacteria isolated from different legumes. Currently, more than 98 species belonging to 14 genera of  $\alpha$ - and  $\beta$ - *proteobacteria* have been described as rhizobia. The genera *Rhizobium*, *Mezorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Bradyrhizobium*, *Phyllobacterium*, *Microvirga*, *Azorhizobium*, *Ochrhobactrum*, *Methylobacterium*, *Devosia*, *Shinella* (Class of  $\alpha$ -*proteobacteria*), *Burkholderia*, *Cupriavidus* (formerly *Ralstonia*) (Class of  $\beta$ -*proteobacteria*) and some  $\gamma$ -*proteobacteria*, form the set of the bacteria known as legume's symbionts. There is certainly much to discover, since only 23% of known legumes were identified specifically for symbiotic relationship up to date. The discovery of new symbionts associated with legumes is necessary to gain deep insight into the taxonomy of the rhizobia. A literature review of the currently recognized classification of rhizobia is presented in this paper.

**Keywords:** *Rhizobia; Legume; Proteobacteria; Taxonomy; Classification.*

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## 1. INTRODUCTION

Rhizobia are soil bacteria able to form nodules and establish a symbiosis with the roots or the stems of leguminous plants. During the symbiotic process, rhizobia reduce atmospheric nitrogen into a form directly assimilated by plants (ammonium).

The ability of rhizobia to fix nitrogen reduced significantly the use of chemical fertilizers in agriculture. In fact, nearly 90% of the nitrogen plant needs are well satisfied and the soil is enriched with nitrogen that will be used by subsequent crops, with no need to add chemical fertilizers. In general, this symbiosis has several advantages including improved agricultural productivity, maintenance and restoration of soil fertility, economy of expensive fertilizers and limitation of groundwater's pollution by nitrates playing therefore a significant ecological and economical function.

The study of the rhizobial diversity is a valuable biological resource and attempts to find bacterial strains with interesting features to maximize the agricultural productivity [1]. Such studies allow us not only to find new strains of rhizobia but they also support research efforts to select efficient combinations of rhizobium-legume association.

Because of their ecological and economical importance, the diversity and taxonomy of these microorganisms have been extensively studied over the last twenty years. From one genus including four species in 1981, the classification now includes at least 14 genera comprising more than 98 species and this number continues to increase [2].

Zakhia et al. proposed the term BNL (Bacteria Nodulating Legumes) to avoid confusion between the general term of rhizobium and the genus name. Currently, all BNL described so far belong to the *Proteobacteria* class. The majority of them belong to the genera of the  $\alpha$ -*Proteobacteria* class, in the genera of *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium* and *Ensifer* (formerly *Sinorhizobium*). In addition, new types were found in the  $\alpha$ -*Proteobacteria* class, namely *Methylobacterium*, *Devosia*, *Microvirga*, *Ochrobactrum*, *Phyllobacterium* and *Shinella* [3].

The BNL were also found in the  $\beta$ -*Proteobacteria* class, such as *Burkholderia* and *Cupriavidus* (formerly *Ralstonia*) [4]. Finally, several studies reported the presence of  $\gamma$ -*Proteobacteria* class in black locust by the *Pseudomonas* sp. [5].

More than 19.700 legume species grow around the world but only a few microsymbionts have been studied [6]. The increase in advanced study of legume's new species in different geographical regions opens new perspectives to isolate and to characterize more rhizobial species.

## 2. PHYLOGENY OF RHIZOBIA

The study of evolutionary relationships between organisms using molecular data (DNA, rRNA or protein sequences) is known as molecular phylogenetics [1]. Thereafter, the concept of theoretical phylogenetic reconstruction was proposed, which views macromolecules as documents of evolutionary history that may therefore help to reconstruct phylogenies [7]. Therefore, the phylogeny can be defined as the evolutionary history revealing the relationships which exists between organism's groups with a common ancestor located at a higher taxonomic level [8].

A phylogenetic tree is a diagram composed of nodes and branches, where nodes are connecting with adjacent branches. Branches represent taxonomic units that could be species, populations or individuals. Relationships between taxonomic units are defined by branches in terms of descent and ancestry; this branching pattern is known as the tree topology. Following a cladistic approach only monophyletic groups derived from a common ancestor, should form taxonomic units such as genera, tribes, families, species or subspecies. Monophyly, paraphyly or polyphyly may be inferred from phylogenetic analyses. Monophyletic and paraphyletic groups have a single evolutionary origin. Polyphyletic groups are the result of convergent evolution and the main characteristic used to define the group is absent in the most recent common ancestor and consist of a hodgepodge of unrelated forms [7].

### **3. POLYPHASIC TAXONOMY**

Originally based on phenotypic criteria, the application of polyphasic terminology has been reviewed by the revolution of different molecular techniques mainly the Polymerase Chain Reaction (PCR) [9]. Thus, the standard principles were proposed in 1991, for the description of new genus or species on the basis of numerical analysis which is the synthesis of the results of different phenotypic and molecular tests [10]. Therefore, polyphasic taxonomy takes into account all available phenotypic and genotypic data and integrates them in a consensus type of classification, framed in a general phylogeny derived from 16S rRNA sequence analysis. This new definition of the polyphasic approach allowed a proper systematic study of major bacterial groups, an assessment of taxonomic resolution of different techniques of diversity study [11] and an establishment of comparable databases between different applied microbiology laboratories [12].

### **4. GENOTYPIC AND PHYLOGENY CLASSIFICATION METHODS**

During the last years, many methods for bacterial characterisation have been developed at each information level and computer facilities are becoming every day more available for numerical analysis of data. Each method used in taxonomy has (i) its own discriminating power varying from the individual or species levels to the genus, family and higher levels, and (ii) its field of application, dependent on the addressed question, the particular conditions, the number and the type of strains. The level of discrimination of a method may vary depending on the studied bacterial taxon [18].

The most useful methods for identifying bacterial species include: sequencing of the 16S rRNA gene, restriction fragment length polymorphism (RFLP) typing, multilocus sequence analyses of different protein-coding housekeeping genes (MLSA), whole-genome sequence analysis, Fourier transformed infrared spectroscopy (FTIR) and pyrolysis mass spectrometry for analysis of cellular components. However, the housekeeping gene's sequencing, the DNA profiling or DNA microarrays are preferred. There are also some powerful PCR-based techniques like REP- and ERIC-PCR available for bacterial taxonomy and their discriminatory power is higher than serological, RFLP and multilocus enzyme electrophoresis (MLEE) techniques [11].

Sequencing of ribosomal 16S rDNA genes have been widely used for rhizobial genetic variability evaluations. In recent years, the phylogenetic position of several species has been defined only on the basis of 16S rDNA sequences. However, it has been shown that the resolving power of this technique is limited in the studies of strains or closely related species

whose divergence is very recent. It is now well established that two organisms that have less than 97% homology of 16S rDNA sequence belong to two different species. Furthermore, housekeeping gene's sequencing is successfully used to distinguish between different rhizobial species.

RFLP frequently used for rhizobia classification and genetic diversity studies is generally coupled with southern blot or with PCR (ARDRA or PCR-RFLP) which corresponds to an RFLP of the 16S rDNA coupled with PCR. The amplified DNA is submitted to restriction enzyme digestion then revealed by electrophoresis. The number of fragments obtained and their migration enables to evaluate and analyse their apparent polymorphism.

The REP-PCR technique is advantageous both for strain's diversity or identification studies, due to the stability and reproducibility of results linked to similarity between electrophoretic profiles of amplified fragments whatever is their origin (from a cell grown in petri-dish, a preculture or even nodular extract). While the MLEE technique is limited by the necessity to obtain sufficient quantity of each one of the analysed strains to test their enzymatic activity and also by the low number of existing enzymes. However, it was largely used to study genetic diversity between several rhizobial species (*R. leguminosarum*, *R. etli*, *R. tropici*, *R. sulae* and some *Sinorhizobium* species). It was also successfully used to distinguish between two closely related species *R. huautlense* and *R. galegae* [38].

Nowadays, use of 16S rRNA sequence is the main tool in the study of microbial phylogeny. However, it has limitations to differentiate among close species and for this purpose several metabolic genes (housekeeping) have been proposed for species identifying in several groups of bacteria [8].

Two genes (*recA* and *atpD*) were firstly analyzed, currently sequenced and usefully used to differentiate between rhizobial species for which 16S rRNA genes were nearly identical. Then, new techniques such as MLSA (Multilocus sequence analysis) and MLST (Multilocus sequence typing) based on the analysis of several housekeeping genes have been applied in phylogenetic analyses and identification of concrete groups of rhizobia [8].

Besides these housekeeping genes qualified "core genes", other ones involved in the legume symbiosis and called "auxiliary" or "accessory" genes are commonly included in rhizobial species description and in some MLST analysis [8]. The most studied symbiotic genes of rhizobia are *nodD*, *NodA*, *nodC* and *nifH*, however, they are generally encoded by plasmids. Therefore, the symbiotic genes are not useful in rhizobial taxonomy due to their ability to be transferred from plasmids in the islands, in nature from bacteria to plants or to other bacteria. Nevertheless, the analysis of these symbiotic genes is generally useful to identify non-*Rhizobium* species able to nodulate legumes and conduct studies of biogeography endosymbionts pulses [8].

Other most commonly used molecular techniques are: FT-IR technique (Fourier-transform infrared spectroscopy), which allows to study the diversity of bacteria at an intraspecific level [13], the technique of typing by PCR target (target PCR fingerprinting) [14] and especially the technique of DNA chips commonly known as DNA microarrays. In this last technique, the conception of the chips permits to detect simultaneously thousands of sequences or even to cover the entire genome of an organism [15]. By the same technique, Rüberg et al. have analyzed the entire genome of *Sinorhizobium meliloti* [16].

Other new methods are potential alternatives to DNA-based ones for rapid and reliable characterization of bacteria. The most commonly used methods are Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) which has been suggested as a fast and reliable method for bacterial identification, based on the characteristic protein profiles for each microorganism. Using this technology it has been estimated that up to 99% of strains tested are correctly identified when comparing with commercial phenotypic identification panels or *rrs* gene sequencing [17].

All these techniques can reveal more information about the diversity of rhizobia and can therefore provide more data available for their phylogenetic study.

Thus, modern bacterial taxonomy is based on the integration of all phenotypic, genotypic and phylogenetic data for a more stable classification [11, 18]. Hence, a minimum standard has been proposed by Graham et al. [10] for the description of root- and stem-nodulating bacteria, who suggested using a combination of traditional morphological and culture characteristics, symbiotic properties, DNA fingerprinting methods, 16S rRNA gene sequencing and DNA hybridization.

The following paragraphs will discuss the current classification of rhizobia with this new approach.

## 5. CURRENT CLASSIFICATION OF RHIZOBIA

The first classifications of rhizobia were based on cross-inoculation tests between rhizobia and their host plants. The host plant was not the only criteria taken into account for the classification of rhizobia for which species were classified into two groups: the fast growing strains and the slow-growing strains, based on their generation time and their growth's rate on culture medium [19].

However, discordant observations between the notion of bacterial growth speed and the host range showed a lot of doubt on the validity of this classification. This makes place to comparative methods such as the serology, the coefficient of Chargaff, RNA/DNA or DNA/DNA hybridization, analysis of plasmids, etc [20].

This period marked the beginning of a new taxonomy studies based on the results from different phenotypic and biochemical analysis for the identification of symbiotic bacteria [20]. Since then, the isolation of rhizobia from an increasing number of plant species in the world and their characterization by modern polyphasic taxonomy has led to the description of other new genera and species.

On the basis of the 16S ribosomal DNA sequence, the currently described legume's symbionts belong to three main distinct phylogenetic subclasses:  $\alpha$ ,  $\beta$  and  $\gamma$ -*Proteobacteria* (Fig.1). More than 98 species grouped in 11 genera belonging to the subclass  $\alpha$ -*Proteobacteria* and 2 genera belonging to the order of Burkholderiales in subclass  $\beta$ -*Proteobacteria* [18, 16, 21,22], and finally, one genus belonging to the order Pseudomonales in the subclass  $\gamma$ -*Proteobacteria* [23].

This high number of species able to nodulate legumes (Table 1) was unexpected only two decades ago and the previsions are that it will be increased in the future.

Table 1. Current classification of rhizobia

Genus species	Isolation source	References
<b>Class: Alphaproteobacteria</b>		
<b>Order: Rhizobiales</b>		
<b>Family: Rhizobiaceae</b>		
<b>Genus: Rhizobium</b>		
<i>R. leguminosarum</i>		[24]
<i>symbiovar viciae</i>	<i>Pisum, Viciae, Lens, Lathyrus</i>	[24,25]
<i>symbiovar trifolii</i>	<i>Trifolium pratense</i>	[29,25]
<i>symbiovar phaseoli</i>	<i>Phaseolus vulgaris</i>	[24,25]
<i>R. galegae</i>	<i>Galega, Leucaena</i>	[26,27]
<i>symbiovar officinalis</i>	<i>Galega orientalis</i>	[27]
<i>symbiovar orientalis</i>	<i>Galega officinalis</i>	[27]
<i>R. tropici</i>	<i>Phaseolus, Medicago, Macroptilium</i>	[28]
<i>R. leucaenae</i>		[29]
<i>R. tropici</i>		[28]
<i>R. endophyticum</i>	<i>Phaseolus vulgaris</i>	[30]
<i>R. phaseoli</i>	<i>Phaseolus</i>	[30]
<i>R. fabae</i>	<i>Vicia faba</i>	[31]
<i>R. etli</i>	<i>Phaseolus,</i>	[32]
<i>symbiovar mimosae</i>	<i>Mimosa affinis</i>	[33]
<i>symbiovar phaseoli</i>	<i>Phaseolus</i>	[34]
<i>R. undicola</i>	<i>Neptunia natans</i>	[35]
<i>R. gallicum</i>	<i>Phaseolus vulgaris</i>	[36]
<i>symbiovar phaseoli</i>	<i>Phaseolus vulgaris</i>	[36]
<i>symbiovar gallicum</i>	<i>Phaseolus vulgaris</i>	[36]
<i>R. giardinii</i>	<i>Phaseolus vulgaris</i>	[36]
<i>symbiovar phaseoli</i>	<i>Phaseolus</i>	[36]
<i>symbiovar giardinii</i>	<i>Phaseolus vulgaris</i>	[36]
<i>R. hainanensis</i>	<i>Desmodium sinuatum, Centrosema, etc.</i>	[37]
<i>R. huautlense</i>	<i>Sesbania herbacea</i>	[38]
<i>R. mongolense</i>	<i>Medicago ruthenica, Phaseolus</i>	[39]
<i>R. yanglingense</i>	<i>Amphicarpaea</i>	[40]
<i>R. larrymoorei</i>	<i>Ficus benjamina</i>	[41]
<i>R. indigoferae</i>	<i>Indigofera spp.</i>	[42]
<i>R. sullae</i>	<i>Hedysarum</i>	[43]
<i>R. loessense</i>	<i>Astragalus, Lespedeza</i>	[44]
<i>R. cellulosityticum</i>	<i>Populus alba</i>	[45]
<i>R. miluonense</i>	<i>Lespedeza</i>	[46]
<i>R. multihospitium</i>	Multiple legume species	[47]
<i>R. oryzae</i>	<i>Oryza alta</i>	[48]
<i>R. pisi</i>	<i>Pisum sativum</i>	[49]
<i>R. mesosinicum</i>	<i>Albizia, Kummerowia Dalbergia</i>	[50]
<i>R. alamii</i>	<i>Arabidopsis thaliana</i>	[51]
<i>R. alkalisoli</i>	<i>Caragana intermedia</i>	[52]
<i>R. tibeticum</i>	<i>Trigonella archiducis-nicolai</i>	[53]
<i>R. tubonense</i>	<i>Oxytropis glabra</i>	[54]
<i>R. halophytocola</i>	Coastal dune plant	[53]
<i>R. radiobacter</i>	*	[55]

**Continued Table 1** .....

<i>R. rhizogenes</i>	*	[55]
<i>R. rubi</i>	*	[55]
<i>R. vitis</i>	*	[55]
<i>R. nepotum</i>	*	[56]
<b>Genus: Ensifer</b>		
<i>E. meliloti</i>	<i>Medicago, Melilotus, Trigonella</i>	[57]
<i>E. fredii</i>		
<i>symbiovar fredii</i>	<i>Glycine, Vigna, Cajanus</i>	[58]
<i>symbiovar siensis</i>	<i>Glycine</i>	[37]
<i>E. sahelense</i>	<i>Acacia, Prosopis, Neptunia, Leucaena</i>	[59]
<i>E. teranga</i>	Different host plants	[59]
<i>symbiovar acaciae</i>	<i>Acacia</i>	[55]
<i>symbiovar sesbania</i>	<i>Sesbania</i>	[60]
<i>E. medicae</i>	<i>Medicago truncatula, Melilotus</i>	[61]
<i>E. arboris</i>	<i>Acacia, Prosopis</i>	[62]
<i>E. kostiense</i>	<i>Acacia, Prosopis</i>	[62]
<i>E. xingianense</i> (Formerly: <i>Sinorhizobium xingianense</i> )	<i>Glycine max</i>	[63]
<i>E. adhaerens</i>	*	[64]
<i>E. kummerowiae</i>	<i>Kummerowia stipulaceae</i>	[42]
<i>E. americanum</i>	<i>Acacia</i>	[67]
<i>E. mexicanus</i>	<i>Acacia angustissima</i>	[68]
<i>E. numidicus</i>	<i>Medicago sativa</i>	[69]
<b>Genus: Shinella</b>		
<i>S. kummerowiae</i>	<i>Kummerowia stipulacea</i>	[50]
<b>Family: Phyllobacteriaceae</b>		
<b>Genus: Mesorhizobium</b>		
<i>M. loti</i>	<i>Lotus, Cicer, Anthyllis, Astragalus, etc.</i>	[70]
<i>M. huakuii</i>	<i>Astragalus sinicus</i>	[71]
<i>M. ciceri</i>	<i>Cicer arietinum</i>	[72]
<i>M. tianshanense</i>	<i>Glycyrrhiza pallidiflora</i>	[130]
<i>M. mediterraneum</i>	<i>Cicer arietinum</i>	[73]
<i>M. plurifarum</i>	<i>Acacia, Chamaecrista, Leucaena,</i> <i>Prosopis,</i>	[74]
<i>M. amorphae</i>	<i>Amorpha fruticosa</i>	[75]
<i>M. chacoense</i>	<i>Prosopis alba</i>	[76]
<i>M. septentrionale</i>	<i>Astragalus adsurgens</i>	[77]
<i>M. temperatum</i>	<i>Astragalus adsurgens</i>	[77]
<i>M. thiogangeticum</i>	*	[77]
<i>M. albiziae</i>	<i>Albizia kalkora</i>	[65]
<i>M. caraganae</i>	<i>Caragana spp.</i>	[66]
<i>M. gobiense</i>	Wild legumes	[47]
<i>M. tarimense</i>	Wild legumes	[47]
<i>M. australicum</i>	<i>Biserrula pelecinus</i>	[78]
<i>M. opportunistum</i>	<i>Biserrula pelecinus</i>	[78]
<i>M. metallidurans</i>	<i>Anthyllis vulneraria</i>	[79]
<i>M. alhagi</i>	<i>Alhagi</i>	[80]
<i>M. camelthorni</i>	<i>Alhagi sparsifolia.</i>	[80]
<i>M. abyssinicae</i>	Different agroforestry legume trees	[81]

**Continued Table 1** .....

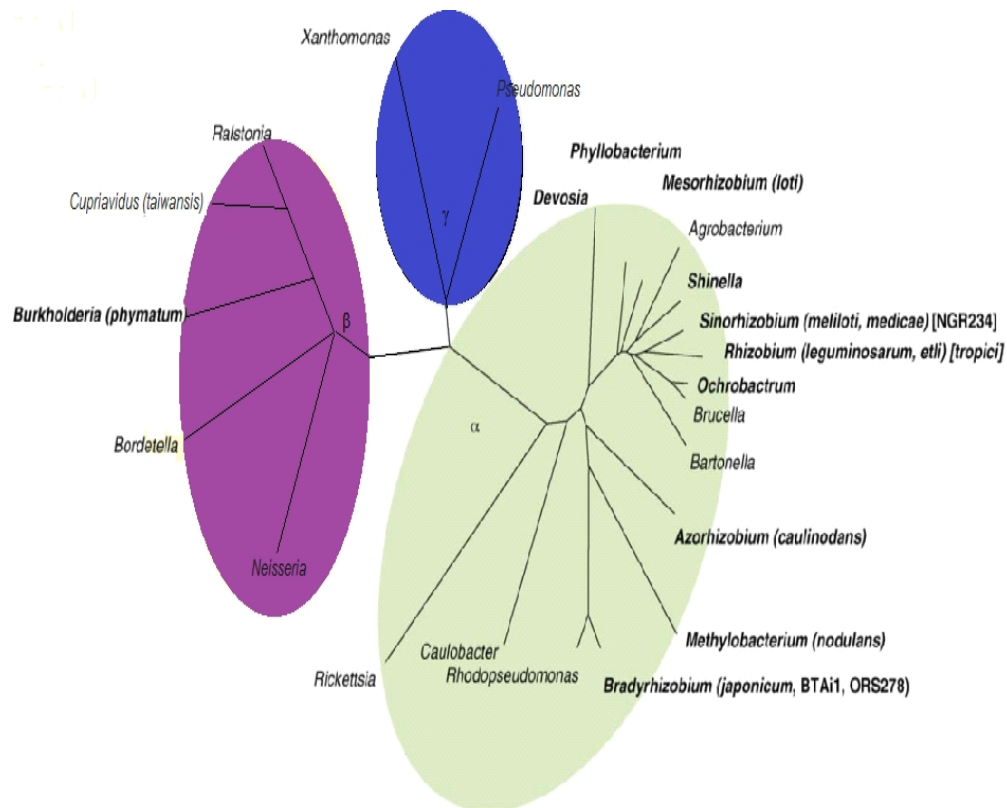
<i>M. muleiense</i>	<i>Cicer arietinum</i>	[82]
<i>M. hawassense</i>	Different agroforestry legume trees	[81]
<i>M. qingshengii</i>	<i>Astragalus sinicus</i>	[83]
<i>M. robiniae</i>	<i>Robinia pseudoacacia</i>	[84]
<i>M. shonense</i>	Different agroforestry legume trees	[71]
<i>M. shangrilense</i>	<i>Caragana</i> species	[85]
<i>M. silamurunense</i>	<i>Astragalus</i> species	[86]
<i>M. tamadayense</i>	<i>Anagyris latifolia</i> , <i>Lotus berthelotii</i>	[87]
<b>Genus: Phyllobacterium</b>		
<i>P. trifolii</i>	<i>Trifolium pratense</i>	[88]
<b>Family: Methylobacteriaceae</b>		
<b>Genus: Methylobacterium</b>		
<i>M. nodulans</i>	<i>Crotalaria</i> spp.	[89]
<b>Genus: Microvirga</b>		
<i>M. lupini</i>	<i>Lupinus</i> sp.	[90]
<i>M. lotoonidisi</i>	Different legume host	[90]
<i>M. zambiensis</i>	Different legume host	[90]
<b>Family: Brucellaceae</b>		
<b>Genus: Ochrobactrum</b>		
<i>Ochrobactrum cytisi</i>	<i>Cytisus</i>	[91]
<i>Ochrobactrum lupini</i>	<i>Lupinus albus</i>	[92]
<b>Family: Hyphomicrobiaceae</b>		
<b>Genus: Azorhizobium</b>		
<i>A. caulinodans</i>	<i>Sesbania rostrata</i>	[93]
<i>A. dobereinereae</i>	<i>Sesbania virgata</i>	[94]
<i>A. oxalatiphilum</i>		[95]
<b>Genus: Devosia</b>		
<i>Devosia neptuniae</i>	<i>Neptunia natans</i>	[[96]
<b>Family: Bradyrhizobiaceae</b>		
<b>Genus: Bradyrhizobium</b>		
<i>B. japonicum</i>	<i>Glycine max</i> , <i>Glycine soja</i>	[25,97]
<i>B. elkanii</i>	<i>Glycine max</i>	[98]
<i>B. liaoningense</i>	<i>Glycine max</i>	[99]
<i>B. yuanmingense</i>	<i>Lespedeza</i>	[100]
<i>B. betae</i>	<i>Betae vulgaris</i>	[101]
<i>B. canariense</i>	<i>Genisteae et Loteae</i>	[102]
<i>B. iriomotense</i>	<i>Entada koshunensis</i>	[103]
<i>B. jicamae</i>	<i>Pachyrhizus erosus</i>	[109]
<i>B. lablabi</i>	<i>Lablab purpureus</i>	[103]
<i>B. huanghuaihaiense</i>	<i>Glycine max</i>	[104]
<i>B. cytisi</i>	<i>Cytisus villosus</i>	[105]
<i>B. daqingense</i>	<i>Glycine max</i>	[106]
<i>B. denitrificans</i>	<i>Aeschynomene</i>	[107]
<i>B. oligotrophicum</i>		[108]
<i>B. pachyrhizi</i>	<i>Pachyrhizus erosus</i>	[109]
<b>Class: Beta Proeobacteria</b>		
<b>Order: Burkholderiales</b>		
<b>Family: Burkholderiaceae</b>		
<b>Genus: Burkholderia</b>		[110]



**Continued Table 1** .....

<i>B. caribensis</i>	Vertisol microaggregates	[111]
<i>B. cepacia</i>	<i>Alysicarpus glumaceus</i>	[4]
<i>B. tuberosum</i>	<i>Aspalatus carnosus</i>	[112]
<i>B. phymatum</i>	<i>Machaerium lunatum</i>	[112]
<i>B. nodosa</i>	<i>Mimosa bimucronata, Mimosa scabrella</i>	[113]
<i>B. sabiae</i>	<i>Mimosa caesalpinifolia</i>	[114]
<i>B. mimosarum</i>	<i>Mimosa</i> spp.	[115]
<i>B. rhizoxinica</i>	<i>Rhizopus microsporus</i>	[116]
<i>B. diazotrophica</i>	<i>Mimosa</i> spp.	[117]
<i>B. endofungorum</i>	<i>Rhizopus microsporus</i>	[116]
<i>B. helea</i>	<i>Eleocharis dulcis</i>	[118]
<i>B. symbiotica</i>	<i>Mimosa</i> spp.	[119]
<b>Genus: Cupriavidus</b>	<i>Aspalatus carnosus</i>	
<i>C. taiwanensis</i>	<i>Mimosa</i> sp.	[120]
<b>Class: Gamma-Proteobacteria</b>		
<b>Order: Pseudomonadales</b>		
<b>Family: Pseudomonaceae</b>		
<i>Pseudomonas</i> sp.	<i>Robinia pseudoacacia</i>	[5]

\*Species with no described nodulation ability included in traditionally considered rhizobial genera.



**Fig. 1. Simplified phylogenetic tree of *Proteobacteria* based on the 16S rRNA gene sequences. The rhizobial genera are shown in bold-face [121].**

## 5.1 Alpha-subclass of *Proteobacteria*

### 5.1.1 *Rhizobium* / *Ensifer* / *Shinella* branch

This branch is subdivided into three sub-branches corresponding to the genera *Rhizobium* [24], *Ensifer* (formerly *Sinorhizobium*) [37,59] and *Shinella* [50]. These genera are grouped with:

- Bacteria ameliorating plant growth (PGPR: Plant growth-promoting rhizobacteria), such as *Azospirillum*.
- Human and animal pathogens, including *Brucella*, *Ochrobactrum*, *Bartonella* or soil bacteria as *Mycoplana*.

The first sub-branch corresponding to genus *Rhizobium*, defines a group of thirty four species, from various hosts and includes *R. leguminosarum*, *R. tropici* [28], *R. etli* [32], *R. gallicum* [36], *R. mongolense* [39], *R. undicola* [35] and also other species previously named *Agrobacterium*. The members of this genus are short Gram-negative rods, of a 0.5 to 0.9 µm width and a 1.2 to 3 µm length, often with an uncolored region due to the presence of polymer β-hydroxybutyrate (PHB). These bacteria do not form endospores but are motile with polar or peritrichous flagella (4 to 6), aerobic, chemo-organotrophs. They become pleomorphic under adverse conditions [122].

There are some species present in this genus that have not been observed to form nodules and therefore do not fit the functional definition of rhizobia. These include the species formerly known as *Agrobacterium* (e.g. *R. larrymoorei*, *R. rubi*, and *R. vitis*) [55]. However, recent evidence showed that some *Agrobacterium* members are able to nodulate leguminous plants. For example some *R. radiobacter* strains could nodulate *Phaseolus vulgaris*, *Campylotropis* spp., *Cassia* spp. [123] and *Wisteria sinensis* [124]. Both nodules and tumours were formed on *Phaseolus vulgaris* by *R. rhizogenes* strains containing a Sym plasmid [125].

In 2001, Young et al. have revised taxonomic data and showed that the discriminatory phenotypic characteristics to distinguish between these types are not convincing, and that the phylogenetic relationships between genera deduced from the comparative analysis of 16S rDNA sequences differ depending on the chosen algorithm and more especially on the chosen sequences [55]. Therefore, despite phenotypic differences, these authors suggested to combine the three genera *Rhizobium*, *Agrobacterium* and *Allorhizobium* in a single genus: *Rhizobium*, including all species of *Agrobacterium* and *Allorhizobium* as new combinations: *R. radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*.

The second sub-branch corresponds to genus *Ensifer* (formerly *Sinorhizobium*) and includes the species *E. fredii* [37,58], *E. meliloti* [57], *E. teranga* and *E. sahelense* [59,126], *E. medicae* [61], *E. kostense* and *E. arboris* [62], *E. xingianense* [63], *E. adhaerens* [64], *E. kummerowiae* [42], *E. americanum* [67], *E. mexicanus* and *E. numidicus* [68]. Rods of this genus are 0.5 to 0.9 µm wide and 1.2 to 3 µm length, motile by polar flagellum or some peritrichous flagella, Gram-negative, aerobic accumulating PHB. Optimal growth of this genus is at 25-30°C (10-35°C) and at pH 6-8 (5-10.5) and tolerates 10 g/l of NaCl [127].

*Sinorhizobium* have been previously separated from *Rhizobium* and *Bradyrhizobium* and proposed as a new genus of the fast-growing soybean rhizobia. This classification was based not only on phenotypic characteristics (numerical taxonomy, serological analysis data,

composition of extracellular gum, bacteriophage typing data and soluble protein pattern) but also on genotypic ones like GC content and DNA-DNA hybridization data. Then, the species of the genus *Sinorhizobium* were transferred to the genus *Ensifer* since the latter is the synonym that takes the priority [128].

The third sub-branch corresponds to genus *Shinella* and includes one species: *S. kummerowiae* [50], a symbiotic bacterium nodulating *Kummerowia stipulacea*. Cells of this strain are Gram-negative, strictly aerobic, non-spore-forming, motile short rods [129].

### **5.1.2 Mesorhizobium branch**

A new genus proposed in 1997 to include the species of intermediate growth named *Mesorhizobium* [130] was included in the family *Phyllobacteriaceae* including also the former genus *Phyllobacterium* (with one specie: *Phyllobacterium trifolii* isolated from *Trifolium pratense* nodules) [88].

The branch of *Mesorhizobium* contains almost thirty species among which we quote: *M. loti* [70], *M. huakuii* [71], *M. ciceri* [72], *M. mediterraneum* [73], *M. tianshanense* [130], *M. plurifarum* [74], *M. amorphae* [75], *M. muleiense* [82], *M. qingshengii* [83], *M. robiniae* [84] and *M. tamadayense* [87]. They are phylogenetically related and distinct from the large phylogenetic group that includes *Rhizobium*, *Agrobacterium* and *Sinorhizobium* [130]. They are characterized by a growth rate intermediate between the fast and slow-growing rhizobia. They are Gram-negative rods, motile by polar or subpolar flagellum, aerobic, accumulating PHB. They assimilate glucose, rhamnose and sucrose with acid production [127].

### **5.1.3 Azorhizobium branch**

This family regroups only one genus and three species of symbiotic bacterium: *Azorhizobium caulinodans*, *A. dodereinereae* and *A. oxalatophilum*. They are short rods very similar to *Rhizobium*, with polar and peritrichous ciliation but produce an alkaline reaction on glucose as *Bradyrhizobium*. Only this sugar is used. Strains also degrade fatty acids, organic acid and alcohols and are the only ones to use alcohols. Their growth is intermediate between the two previous genus, with a Generation time = 7-9 h. No strain is denitrifying. They grow up to 43°C. They have only the dihydrolase arginine and lysine decarboxylase. They do not use mannitol, conventional substrate of the other two genera, but lactate gives good growth yields. They seem more sensitive to antibiotics [127]. In addition, it doesn't only form nodules on root parts but also on aerial parts of *Sesbania rostrata* [93]. Another species *Azorhizobium johanna* was proposed [96]. However, this species is characterized by a low level of DNA/DNA hybridization compared to the typical strain of this genus.

The analysis of the 16S rRNA sequences showed that the typical strain is mixed with species of the genus *Xanthobacter* and *Aquabacters*. The combination of these last two genera with *Azorhizobium* was investigated without being suggested because of the great dissimilarity of phenotypic traits [131,132].

### **5.1.4 Bradyrhizobium branch**

This genus has been defined to include all slow-growing rhizobia [25] with a Generation time = 10-12h. These are rods with a single polar or subpolar flagellum. Their colonies do not exceed 1 mm diameter in YMA medium [19]. These symbiotic bacteria use many sugars and organic acids but prefer pentoses, with production of polysaccharidic mucus.

Some species can grow as chimiolithotrophic in the presence of H<sub>2</sub>, CO<sub>2</sub> and low content in O<sub>2</sub> due to the presence of an hydrogenase. Some strains can fix N<sub>2</sub> in vitro. It's more resistant to antibiotics than *Rhizobium* [127].

This branch has included, for a long time, a single species: *Bradyrhizobium japonicum* that regrouped all strains nodulating the soybean (*Glycine max*). Very heterogeneous, it was then divided into three groups (I, Ia and II), based on homologies obtained by DNA/DNA hybridization [133]. A new species: *Bradyrhizobium elkanii* was created in the Group II, which differs from the species *Bradyrhizobium japonicum* [94]. For other very slow growing strains, isolated from nodules of soybean (*Glycine max* and *Glycine soja*) in China, species *Bradyrhizobium liaoningense* has been proposed [99]. Recently, *B. huanghuaihaiense* was defined as a novel species to regroup rhizobial strains isolated from *Glycine max* L. nodules collected in different sites of the Northern (Huang-Huai-Hai) Plain of China [104]. These strains formed effective nodules also with *Glycine soja* and *Vigna unguiculata* in cross-nodulation tests. Despite they harbour the same nod C and nif H symbiotic genes compared to *Bradyrhizobium japonicum*, *Bradyrhizobium liaoningense* and '*Bradyrhizobium daqingense*' reference strains, they present some differences in cellular fatty acids content and phenotypic characters.

The *Bradyrhizobium* genus includes strains for which the taxonomic position is not clearly defined at the species level. The discrepancy between *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* is clear. It is more important than the divergence between *Bradyrhizobium* and non-symbiotic species as: *Afipia* (animal bacterial pathogens), *Nitrobacter* (nitrifying bacteria in the soil), *Rhodopseudomonas palustris* (photosynthetic bacteria) and *Blastobacter denitrificans* [110].

In recent years, the characterization of different strains of *Bradyrhizobium* by comparing several molecular techniques revealed the existence of 11 different genotypes, three of which correspond to known strains, while 8 genotypes are very distinct.

A new species *B. yuanmingense* was isolated from the genus *Lespedeza* [100,134]. In the same year, a strain of *Bradyrhizobium* nodulating a wild plant of the genus *Phaseolus* was reported [135]. Other recognized species of this group are *Bradyrhizobium betae* from the roots of *Beta vulgaris* afflicted with tumor-like deformations [101] and *Bradyrhizobium canariense* from genistoid legumes from the Canary Islands [102]. Many other slow-growing rhizobia were isolated from other legume hosts and are commonly referred as *Bradyrhizobium* sp., followed by the name of the host legume: *B. lablabi* was isolated from *Lablab purpureus* [131], *B. cytisi* has been isolated from *Cytisus villosus* [105] and *B. pachyrhizi* was isolated from effective nodules of *Pachyrhizus erosus* [109].

Based on ITS sequence data, the photosynthetic bradyrhizobia isolated from stem-nodules of *Aeschynomene*, form a distinct group closely related to *Blastobacter denitrificans* [110]. As a result of a comprehensive study of both groups, van Berkum et al. recently proposed to transfer *Blastobacter denitrificans* to *Bradyrhizobium* and unite the species with the isolates from *Aeschynomene indica* as *Bradyrhizobium denitrificans* [107].

Recently, new species namely *B. cytisi* from *Cytisus villosus* [105], *B. daqingense* from *Glycine max* [106], *B. pachyrhizi* from *Pachyrhizus erosus* [109] and *B. oligotrophicum* [108] were reported.

### **5.1.5 Methylobacterium branch**

The *Methylobacterium* genus [136] has included a single symbiotic species: *M. nodulans* that regrouped all strains nodulating the *Crotalaria* [89]. Bacteria of this genus have the ability to grow on single-carbon compounds such as formate, formaldehyde, methylamine and methanol as only source of carbon and energy. These are chemo-organotrophic and optional methylotrophic, able to use multi-carbon compounds for growth. The bacteria of this genus are rodshaped (0.8 - 1.0 x 1.0 to 3.0  $\mu\text{m}$ ), most frequently isolated and occasionally in rosette. They are often branched or pleomorphic, especially in cultures in late stationary phase. They are strictly aerobic bacteria, Gram-negative, oxydase and catalase positive and have a typical pink pigmentation due to the presence of carotenoids [137]. *Methylobacterium* strains are commonly called "pink-pigmented facultative Methylophs" (PPFM). However, at least one case, *M. nodulans* is not pigmented [104]. They are mesophilic and have optimal temperatures between 25 and 30°C. All these strains are motile with a single polar flagellum. Their cells often contain inclusions of poly- $\beta$ -hydroxybutyrate and sometimes inclusions of polyphosphates [138].

## **5.2 Beta-subclass of *Proteobacteria***

### **5.2.1 Burkholderia branch**

The *Burkholderia* [139] genus has been proposed by Yabuuchi et al. in 1992. Its definition was based primarily on genomic considerations and on cellular lipid's composition. Regarding the main phenotypic characteristics, they are right bacilli, Gram negative, usually accumulating granules of poly-beta-hydroxybutyrate, motile with one or more polar flagella (*Burkholderia mallei* is however lacking flagella and stationary), strictly aerobic, catalase positive, oxidase variable depending on the species, can grow using as sole carbon source glucose, glycerol, inositol, galactose, sorbitol, mannose (unlike *Ralstonia sp.* that do not use this sugar) and mannitol. The different species of the genus can be differentiated by the results of auxanograms (137 substrates were tested in the study of Gillis et al.) [140].

This genus contains 39 species including rhizospheric bacteria, human pathogenic bacteria, as well as nitrogen fixing bacteria as *Burkholderia vietnamiensis* and *Burkholderia kururiensis*. Nevertheless, the genus also includes important species to the ecology of environment. In particular *Burkholderia xenovorans* (formerly known as *Pseudomonas cepacia*, then *Burkholderia cepacia* and *Burkholderia fungorum*) was studied for its faculty to deteriorate the organochlorine links in pesticides and polychlorobiphenyl (PCB) [141].

Following the discoveries of Moulin et al. and Chen et al. [4,115], showing the ability of bacteria from the subclass  $\beta$ -*Proteobacteria* (*Burkholderia* and *Ralstonia*) to induce nodulation in legumes, bacteria of the subclass  $\gamma$ -*Proteobacteria* (*Pseudomonas sp.*) was also isolated from legume nodules [23]. Based on these findings, it is not excluded to find other bacteria able to nodulate legumes, even outside the *Proteobacteria* phylum.

## **5.3 Other nitrogen-fixing legume symbionts**

Recently, several isolates from legume nodules have been reported to be able to fix nitrogen but were phylogenetically located outside the traditional groups of rhizobia in the  $\alpha$ -*Proteobacteria*. New lines that contain nitrogen-fixing legume symbionts include *Devosia*,

*Ochrobactrum* and *Microvirga* in the  $\alpha$ -Proteobacteria and *Cupriavidus* in the  $\beta$ -Proteobacteria.

In the  $\alpha$ -Proteobacteria, *Devosia neptuniae* was proposed for strains from *Neptunia natans* from India [96]. *Ochrobactrum lupini* and *Ochrobactrum cytisi* were described for nodule isolates from *Lupinus Albus* [92] and from *Cytisus* [91] respectively. *Microvirga lupini* was described for nodule isolates from *Lupinus* sp., *Microvirga lotononidis* and *Microvirga zambiensis* were described for nodule isolates from different legume host [90].

In 2001, the nodulation of *Mimosa* by *Ralstonia taiwanensis* or *Wautersia taiwanensis* was published [21]. This species was firstly isolated from nodules of *Mimosa pudica* and *Mimosa diplotricha*. In this case the strain was erroneously classified as *Ralstonia* and it has been later named *Cupriavidus taiwanensis* [120]. Nowadays, *Cupriavidus* is a  $\beta$ -Proteobacteria, belonging to the family *Burkholderiaceae* of *Burkholderiales* order. Several species of this genus were isolated from soil and human clinical specimens but *C. taiwanensis* was the only specie able to form effective nodules and to fix atmospheric nitrogen in legumes.

## 6. CONCLUSION

From one genus and 4 species in 1981, the rhizobia taxonomic studies have currently led to a total of 14 genera and 98 recognized species. This is in constant progress due to various studies in the world. Progress in taxonomy is also due to increasing numbers of effective techniques available in the characterization of bacteria, more accessible to laboratories.

So far only 23% of the total number of legumes species (between 16 500 and 19 700) were characterized for their microsymbionts. The majority (88%) of the studied legumes proved to be able of forming nodules. Specifically, tropical rhizobia are still poorly documented compared to what can be expected based on recent data, which show a great diversity in China, Brazil, Senegal, Sudan and Morocco.

These findings suggest that several other new groups of symbiotic leguminous plant's bacteria (genera, species) may emerge in the future.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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