Chapter 6
Rhizobia: Culture Collections, Identification, and Methods of Preservation

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Abstract Across the globe nitrogen is limiting primary productivity. Although fertilizer nitrogen could supplement soil nitrogen depletion, efforts should be to augment soil biological nitrogen fixation mediated by microbes. This could be addressed by conservation and sustainable use of rhizobia that can fix nitrogen in soil in association with legumes. Rhizobia commonly occur in soils but often fail to produce effective nodulation either because their population in soil is low or those present cannot effectively nodulate the particular legume. Rhizobia present in various ecosystems are considerably diverse; at present 130 species within 15 genera are reported. Rhizobial inoculation is almost always needed when certain new leguminous crops are introduced to new areas or when ineffective and incompatible rhizobia are present in the soils. For this, depository of authentic microbial gene pool is a must. Culture collection centers act as repository of valuable microbial strains. In the changing global scenario, these centers are becoming Biological Resource Centres (BRCs), carry out research, enhance value of strains, and control access to dangerous microorganisms. For adoption and popularizing use of rhizobium-legume technology, their isolation, identification, preservation, and deposition in biological banks are imminent. Moreover, there is a need to identify the compatible rhizobium-legume symbioses for sustainable agriculture.

6.1 Introduction

The major challenge of modern agriculture is to achieve a food production level sufficient to feed the rapidly increasing global population. Current world population of 7.6 billion is projected to reach 8.6 billion by 2030, 9.8 billion in 2050, and 11.2 billion in 2100 (Anonymous 2017). Half of this population will be shared by nine countries: India, Nigeria, Congo, Pakistan, Ethiopia, Tanzania, the United States,

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Uganda, and Indonesia. Hence, India will be the most populated country. The other challenges for increasing food production are the sharp decline in cultivable land area and the increased deterioration of soil health and environmental quality as a result of intense agriculture practices, particularly since the green revolution of the 1960–1970s (Tilman et al. 2001; Trewavas 2001). High agriculture productivity will put immense pressure on soil nitrogen (N) and phosphorous (P) demands. Human N requirements are fulfilled directly or indirectly from plants. Plants acquire N from (a) soil, through commercial fertilizers, manure, and/or mineralization of organic matter; (b) from the atmosphere through biological N_2 fixation; and (c) minor contributions from other biological processes. However, since the green revolution, management of N inputs into agricultural system has become a contentious issue. It is already known that cultivation of legumes can enhance N acquisition and use. However with the goal of enhanced grain production, increased awareness of organic production, health issues, importance of biodiversity, and maintenance of soil fertility, the role of legumes in the sustainable management of N, as well as of P, is reaffirmed. It has been estimated that properly managed alfalfa (*Medicago sativa* L.) in rotation with corn (*Zea mays* L.) will reduce fertilizer inputs by up to 25%. Further legume green manures can replace more than 100 kg N ha^{-1} for the subsequent crop (Peterson and Russelle 1991). The use of legumes accompanied with good agronomic practices could save up to 20 TgN $year^{-1}$. Legumes can be used for reclaiming acid soils low in P and N, by the incorporation of residues, resulting in higher soil organic matter content and increased P and N availability. Legumes with low harvest index also improve P and N sustainability in low technified agriculture. Intercropping of pigeon pea (*Cajanus cajan*) with cereals is a proven management strategy for increasing N and P. Thus, in addition to providing an immediate source of dietary N, incorporation of pigeon pea residues after seed harvest increases the availability of P and N, contributing to agriculture sustainability. In another example, the development of rhizobial inoculants for low fertility acidic soils of the Brazilian Cerrado has allowed Brazil to become a leading exporter of soybeans.

In India research on legume crops is being undertaken through All India Coordinated Research Projects (AICRP) of Indian Council of Agricultural Research (ICAR). There are five AICRPs undertaking research on pigeon pea, arid legumes, groundnut, soybean, and MULLaRP [mung bean (*Vigna radiata*), urad bean (*Vigna mungo*), lentil (*Lens culinaris*), lathyrus (*Lathyrus sativus*), rajma (*Phaseolus vulgaris*), pea (*Pisum sativum*)]. Under the aegis of AICRPs, there is large gene pool of rhizobia specific to chickpea, lentil, and other legumes from various agroclimatic regions in India which have been screened for high N_2-fixing efficacy and various plant growth-promoting attributes (www.aicrpchickpea.res.in; www.icar.org.in/content/aicrps-network-projects). However, there are no records to prove the submission of these rhizobial isolates in culture collection centers. Similarly Centre for Research on Bacteria and Archaea (CRBA) operational at the Department of Microbiology, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, India, as part of the Ministry of Environment and Forests funded All India Coordinated Project on Taxonomy during 2000–2012 have characterized and identified rhizobia from nodules of tree (*Dalbergia sissoo*), medicinal

(*Mucuna pruriens*), pulse legumes (*Lens culinaris*, *Cicer arietinum*), and spice legume (*Trigonella foenum-graecum*). A few rhizobia nodulating *Dalbergia sissoo* have been deposited at the culture collection center at IMTECH, Chandigarh, India (Sahgal et al. 2004). The development of high-quality rhizobial inoculants is not possible unless the authentic rhizobial strains are available for advanced research, efficacy trial in fields, and for industrial applications. Thus ex situ conservation of microbial wealth is essential under Convention on Biological Diversity (CBD). Hence, establishment of culture collection centers is imminent (Smith 2003).

6.2 Global Directory of Culture Collection

The culture collection centers around the globe are registered under the World Federation of Culture Collection (WFCC) founded in 1963 and based at University of Queensland, Australia. In 1972, it published the first printed volume of world directory of culture collection centers of microorganisms. WFCC has been relocated in 1986 to RIKEN, Saitama, Japan, and again in 1999 to the National Institute of Genetics, Japan (Smith 2003; Sly 2010). All the microbial resource centers registered under WFCC are networked under WFCC-MIRCEN World Data Centre for Microorganisms (WDCM) (www.wdcm.org). WDCM constitutes the *World Directory of Culture Collections* (sixth version, 2014) and has various constituent databases such as CCINFO http://www.wfcc.info/ccinfo/ and STRAIN http://www.wfcc.info/ccinfo/search/strain_search/. Culture Collections Information Worldwide (CCINFO) is the directory of all registered culture collection in world, whereas STRAIN is the database that includes the list of holdings from registered culture collections. CCINFO allows the search through culture collection centers, whereas STRAIN allows the search through name and type of strain. Nowadays, there are 728 culture collection centers registered globally. They hold 29,63,164 microbial cultures, of which 12,20,838 represent bacteria, 8,14,206 fungi 38,002 viruses and 32,128 cell lines (www.wfcc.info/ccinfo/).

6.3 Global Catalogue of Microorganisms

The global catalogue of microorganisms (GCM) is the database of microbial culture deposits in the collection centers worldwide (gcm.wfcc.info). The main objective of the GCM is to develop a data bank of information on biological holdings of registered culture collection centers (s). It provides the description of microbial strains and a friendly user system to manage and disseminate the information. Besides, it also provides the information of strains to scientific and industrial communities for their usage. All WFCC members are required to publish online or a printed catalogue of microbial holdings with them. However, as per recent statistics, only 1/6 of the total collection centers registered under CCINFO have

published their online catalogue. Nonpublication of an online catalogue by all WFCC members greatly hinders the visibility and accessibility of strains. Only 112 culture collections representing 43 countries or regions have participation in the GCM (gcm.wfcc.info). From these, only 77 collection centers have rhizobial culture deposits (Fig. 6.1).

6.3.1 Rhizobial Culture Collections: Past to Present

There is increased interest in *Rhizobium* research because of its symbiotic biological nitrogen fixation (BNF). The research on rhizobia and BNF is almost 125 years old. During this period numerous rhizobial strains have been identified and tested for nitrogen fixing and plant growth-promoting potential. In spite of being researched since long, rhizobium/legume technology is not widely used as yet. The major constraint has been availability of well-characterized and identified authentic cultures. Here comes the importance and role of culture collection centers. They are depository of authentic rhizobial strains available for advanced research and application, as patent deposits and as microorganisms cited in research publications for confirmation of results.

The status and progress of rhizobial culture collection centers have been reviewed by Freire and Kolling (1986). The first rhizobial culture collection was established as early as 1889. Five decades later O.N. Allen established the earliest and largest rhizobial culture repository at Department of Bacteriology, University of Wisconsin, Australia (Johnson and Allen 1952). Thereafter several other collection centers were established in Australia. They are Commonwealth Scientific and Industrial Research Organization (CSIRO) Cunningham Laboratory, Brisbane (https://www.csiro.au/en/Publications), and Australian Inoculant Research and Control Service, New South Wales Department of Agriculture, Melbourne (http://www.dpi.nsw.gov.au/). Several large rhizobial culture collections are present in South America mainly in Brazil, Uruguay, and Argentina. In Europe, the largest collections of rhizobium are in Belgium, Bulgaria, Czechoslovakia, Poland, and the United Kingdom. In Asia, China has the largest repository of rhizobia at Beijing Agriculture University. The first edition of the World Catalogue of Rhizobium Culture Collection (1973) listed 3000 strains from 59 collection centers spread over 29 countries, whereas its second edition (1983) listed the same number of strains from 64 repositories in 38 countries. The online printed catalogue is available for 112 collection centers, of which only 77 centers have rhizobial strain deposits (gcm.wfcc.info). Till today 15 genera representing rhizobia have been described. They are *Aminobacter*, *Azorhizobium*, *Bradyrhizobium*, *Burkholderia*, *Cupriavidus*, *Devosia*, *Ensifer* (earlier *Sinorhizobium*) *Mesorhizobium*, *Methylobacterium*, *Microvirga*, *Neorhizobium*, *Ochrobactrum*, *Phyllobacterium*, *Rhizobium*, and *Shinella* (Weir 2016). Within 15 genera, a total of 130 species of rhizobia have been identified (Table 6.1). World over all rhizobial culture collection centers have several strains that have only genus designation. They are not yet identified at the species level. The majority of culture collections, of the 77 with online published catalogue, have deposits designated as *Bradyrhizobium* sp.,

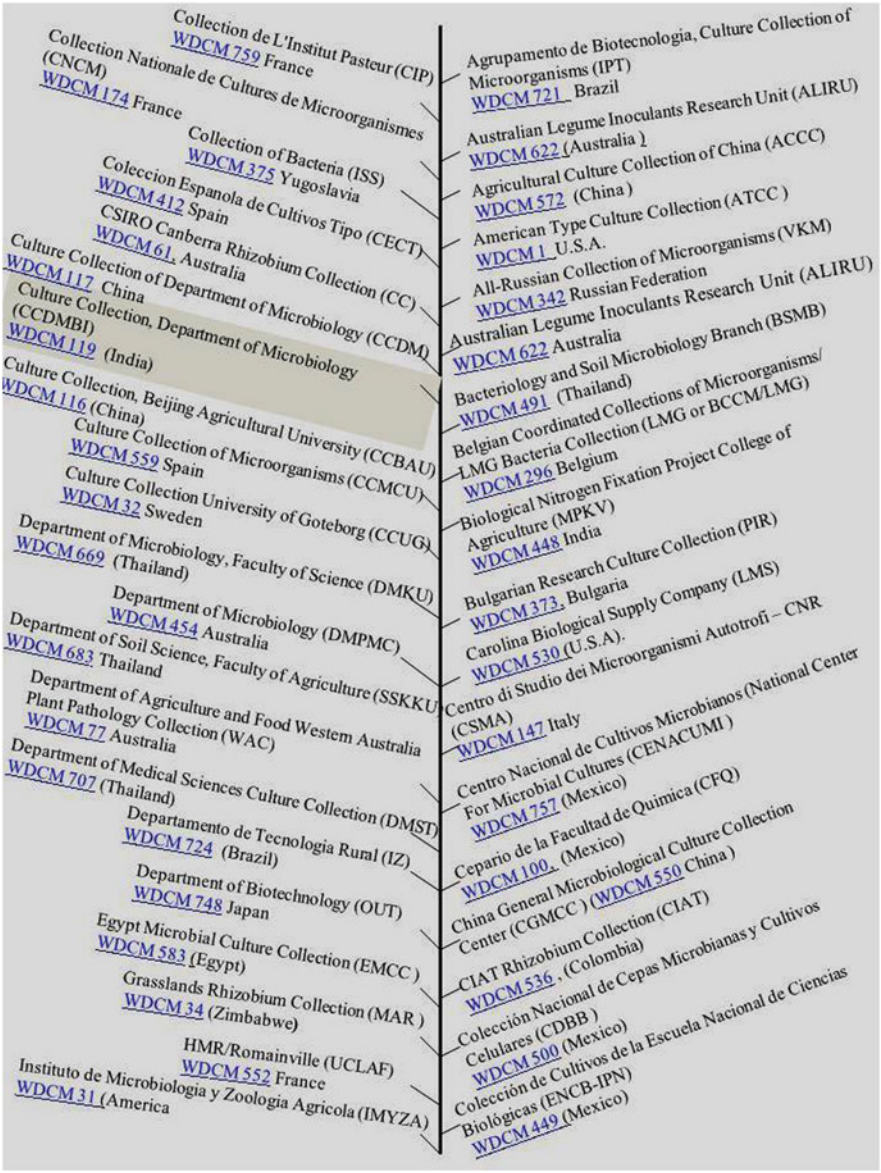

Fig. 6.1 The list of rhizobial culture collections that have participation in global catalogue of microorganisms and published online printed catalogue

Mesorhizobium sp., and *Rhizobium* sp. They have not been assigned species designation. The strains have been assigned to genus on the basis of alignment of their 16S rDNA region with that of completely identified strains. To identify species of these strains, a set of housekeeping genes (two to five) and symbiotic genes (*nod*, *nif*, and *fix*) must be sequenced and analyzed. The sequence analysis of these genes is used to infer

Fig. 6.1 (continued)

the phylogenetic relationship. In these culture collection centers, several strains are still designated by their former names, such as *R. japonicum*, *R. meliloti*, *R. phaseoli*, *R. trifolii*, *R. fredii*, *R. loti*, *and R. viceae*, although they have been reassigned to different groups.

Table 6.1 Genera of nodulating bacteria along with species described within each genus

Genus	Species within the genus
Aminobacter	This genus contains only one species *Aminobacter anthyllidis*
Azorhizobium	This genus currently contains two species. *Azorhizobium caulinodans, A. doebereinerae*
Bradyrhizobium	This genus contains 10 species. *Bradyrhizobium canariense, B. cytisi, B. denitrificans, B. elkanii, B. iriomotense, B. japonicum, B. jicamae, B. liaoningense, B. pachyrhizi, B. yuanmingense.* They are slow growing bacteria
Devosia	This genus contains only one species. *Devosiane ptuniae*, fast growing rhizobia
Mesorhizobium	This genus contains 31 species. *Mesorhizobium abyssinicae, M. albiziae, M. alhagi, M. amorphae, M. australicum, M. camelthorni, M. caraganae, M. chacoense, M. ciceri, M. erdmanii, M. gobiense, M. hawassense, M. huakuii, M. jarvis*ii, *M. loti, M. mediterraneum, M. metallidurans, M. muleiense, M. opportunistum, M. plurifarium, M. qingshengii, M. robiniae, M. sangaii, M. shangrilense, M. shonense, M. silamurunense, M. septentrionale, M. tamadayense, M. tarimense, M. temperatum and M. tianshanense*
Methylobacterium	This genus contains only one species *Methylobacterium nodulan.* Colonies 0.5–1 mm diameter after 4–7 days incubation
Microvirga	This genus contains only three species. *Microvirga lupine, M.lotononidis* and *M. zambiensis*
Neorhizobium	This genus contains only one species *Neorhizobium galegae*
Ochrobactrum	This genus contains two species. *Ochrobactrum cytisi* and *O. lupine*
Phyllobacterium	This genus contains three species. *Phyllobacterium trifolii, P. ifriqiyense* and *P. leguminum*
Rhizobium	This genus contains 49 species namely *Rhizobium alamii, R. alkalisoli, R. azibense, R.calliandrae, R.cauense, R. cellulosilyticum, R.daejeonense, R.endophyticum, R. etli, R. fabae, R. freirei, R. galegae, R. gallicum, R. giardinii, R. grahamii, R. hainanense, R. halophytocola, R. herbae, R. huautlense, R. indigoferae, R.jaguaris, R. laguerreae, R. leguminosarum, R. leucaenae, R. loessense, R. lusitanum, R.mayense, R. mesoamericanum, R.mesosinicum, R. miluonense, R.mongolense, R. multihospitium, R. oryzae, R. paranaense, R. petrolearium, R. phaseoli, R. pisi, R. tibeticum, R. sophorae, R. sophoriradicis, R. sphaerophysae, R.sullae, R. taibaishanense, R. tropici, R. tubonense, R. undicola, R. vallis, R. vignae* and *R. yanglingense.* They are fast growing bacteria
Ensifer (Sinorhizobium)	This genus contains 17 species. *Ensifer abri, E. americanum, E. arboris, E. fredii, E.garamanticus, E.indiaense, E. kostiensis, E. kummerowiae, E. medicae, E. meliloti, E. mexicanus, E. morelense', E. adhaerens, E. numidicus, E.saheli, sojae* and *E. terangae*
Shinella	This genus contains one species. *Shinella kummerowiae*
Burkholderia	This genus contains seven species. *Burkholderia caribensis, B. cepacia, B. mimosarum, B. nodosa, B. phymatum, B. sabiae* and *B. tuberum*
Cupriavidus	This genus contains only one species. *Cupriavidus taiwanensis*

6.3.2 MIRCENs' Culture Collection

In 1974 the concept of the Microbial Resource Centers (MIRCENs) was coined by the Microbiology Panel of UNEP/UNESCO/ICRO with specific objectives like (1) world network of regional and interregional cooperating laboratories, (2) efforts for conservation of microorganisms specifically *Rhizobium*, (3) technology development for strengthening rural economics, and (4) trained manpower. All five MIRCENs with BNF as the main aim have been established during 1977–1982. These are the (1) University of Nairobi, Kenya (1977); (2) Porto Alegre, Brazil (1978), jointly at the Department of Soils, University of Rio Grande do Sul, and Institute of Agronomic Research, and State Department of Agriculture; (3) NifTAL at the University of Hawaii (1981); (4) Cell Culture and Nitrogen Fixation Laboratory, USDA, Beltsville, USA (1981); and (5) at the Centre National de Recherches Agronomiques de Bambey, Senegal (1982). All rhizobia MIRCENs hold 4000 strains. The NifTAL MIRCEN has about 2000 strains from 50 countries and 283 legume species. Of these 41 strains are recommended for economically important legumes and 55 have been characterized for lesser known potential legumes. FEPAGRO/UFRGE-MIRCEN has a collection of 700 strains of which 150 are *Bradyrhizobium japonicum*. About 106 strains from this collection have proven high N_2 fixation efficiency for 50 legume species. The USDA-Beltsville MIRCEN has around 1000 strains.

6.3.2.1 NifTAL MIRCEN

Nitrogen fixation of tropical agricultural legume (NifTAL) was established at the University of Hawaii under United States Agency for International Development (USAID) contract to promote use of legume-rhizobia technology for increasing food production in developing countries. It has a collection of 1774 rhizobial strains for legume cultivated at both high and low elevation in tropics for grains, forage, fodder, firewood, green manure as cover crop, erosion control, and shade-providing trees. The rhizobium germplasm resource at NifTAL is a member of WFCC. It contains complete information for rhizobial strains tested as inoculant for 18 agriculturally important tropical legumes that were part of the International Network of Legume Inoculation Trials (INLIT) (NifTAL catalogue of strains 1994).

6.3.3 Rhizobial Repositories in India

6.3.3.1 Indian Type Culture Collection

It is an oldest culture collection established at the Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi in 1936. Indian Type

Culture Collection (ITCC) is a member of the WFCC and registered as WDCM 430. The main objectives of ITCC are to act as a repository, to supply authentic fungal/bacterial cultures as well as identification and provide related services to farmers and scientists working in research institutions/universities and industries. It has 3800 bacterial and fungal deposits.

6.3.3.2 National Microbial Resource Centre

The Department of Biotechnology (DBT), Government of India, funded national facility affiliated to National Centre for Cell Science (NCCS), Pune, India. It is a member of WFCC and is registered as WDCM 930. It was recognized by the World Intellectual Property Organization (WIPO), Geneva, Switzerland as an International Depository Authority (IDA) in April 2011. The total fungal and bacterial deposits including rhizobia at National Microbial Resource Centre (*NMRC*) (formerly MCC), Pune, is 15338 and 149314, respectively.

6.3.3.3 The International Crops Research Institute for the Semiarid Tropics

It is an international organization established in 1972 at Patancheru, India, by the Ford and the Rockefeller foundations with two regional hubs, Nairobi, Kenya, and Bamako, Mali. International Crops Research Institute for the Semiarid Tropics (ICRISAT's) research agenda is achieved through CGIAR Research Program on Grain Legumes. The rhizobial strains deposited in the collection are associated to various drought-tolerant legumes such as chickpea (*Cicer arietinum* L.), groundnut (*Arachis hypogaea* L.), and pigeon pea (*Cajanus cajan* (L.) Millsp.) The total deposits in this collection include 819 strains, of which 259 are of chickpea, 150 of groundnut, and 410 of pigeon pea collected from 6 countries including Niger, Nigeria, Zimbabwe, Malawi, Ethiopia, and Mozambique.

6.3.3.4 National Agriculturally Important Microbial Culture Collection

NAIMCC was established in the year 2004 by the National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau, Uttar Pradesh India. It is recognized as one of national repositories of India by the National Biodiversity Authority of India and has storage facility of 10,000 agriculturally important microorganisms (AIMs). The AIMs comprising of fungi, bacteria, actinomycetes, and cyanobacteria are being preserved at National Agriculturally Important Microbial Culture Collection (NAIMCC). The accessioned microorganisms at NAIMCC are 6327 including fungi (3809), bacteria and actinomycetes (2327), and cyanobacteria (228). It has published a *Catalogue of Microbial Cultures: Supplement-2014* giving detailed information on microbial deposits.

6.4 Authentication

Authentication is the ability of rhizobial isolates to form a nodule on a legume and fix nitrogen (effectiveness). This is done through in vivo plant infection assays. The various methods for authentication of rhizobia are described and compiled in a recent manual (Howeison and Dilworth 2016). The effective nodulation of legume with a rhizobial strain is a result of genetic compatibility between both partners. This also gives clue to the host range of a specific strain. Rhizobia host range is often the main criterion for the release of strain in the field. Nodulation of a legume with rhizobia may either be effective or noneffective (Terpolilli et al. 2008).

6.5 Identification of Rhizobia

Rhizobia are Gram-negative bacteria present in soil. They are distinguished from other prokaryotes by the ability to elicit nodules on roots and stems of leguminous plants and fixing atmospheric nitrogen. Initially one species of legume root-nodulating bacterium, *Rhizobium leguminosarum*, was known (Frank 1889). Nearly five decades later, it was observed that rhizobia considerably differed in the range of host plants; they could nodulate effectively and thus emerged the concept of cross-inoculation group in rhizobia. Hence *R. leguminosarum* (symbiont of peas and vetches), *R. trifolii* (clover rhizobium), *R. phaseoli* (bean rhizobium), *R. lupini* (host plant lupin), *R. japonicum* (nodulated soybean in Japan), and *R. meliloti* (sweet clover) were identified and described (Fred et al. 1932). Another 50 years later, Jordan proposed that growth rate and biochemical properties along with host range should be the basis of taxonomy (Jordan 1982). Hence second rhizobial genus, *Bradyrhizobium*, was described, and *R. leguminosarum*, *R. trifolii*, and *R. phaseoli* were amalgamated into single species *R. leguminosarum* with three different "biovars" denoting their symbiotic preferences. Since then emerged the concept of polyphasic taxonomy which is currently followed for identification of rhizobia. Consequently, till date 15 genera (13 in α-proteobacteria and 2 in β-proteobacteria) of rhizobia with 120 species have been described (Table 6.1). Since authentication of rhizobia for nodulation and nitrogen fixation on legume plant is the key step prior to their identification. Thus the concept of symbiovar (earlier biovar) is still useful.

6.5.1 Polyphasic Taxonomy of Rhizobia

In the 1990s the concept of defining prokaryotic species using the combination of phenotypic, biochemical, and genotypic methods, collectively known as polyphasic taxonomic approach, emerged. Almost a decade later in 2002, the International Committee on Systematics of Prokaryotes (ISCP) defined prokaryotic species as a

collection of strains that have phenotypic and/or morphological similarity; genome similarity of >70% in DNA–DNA hybridization; G+C content similarity, as indicated by melting temp of DNA within 5 °C; and less than 3% divergence of 16S rRNA sequence (Stackebrandt et al. 2002). The description of any novel prokaryotic species must be first published in the *International Journal of Systematic and Evolutionary Microbiology* (http://ijs.sgmjournals.org/). For rhizobia, polyphasic taxonomy has been used by several workers (Graham et al. 1991; Vandamme et al. 1996) and is overseen by the ICSP Subcommittee on the taxonomy of *Rhizobium* and *Agrobacterium* (Lindström and Young 2011). Under this scheme, the species designation to rhizobia is based on the combination of morphological, biochemical, physiological, and genetic fingerprinting methods, along with host range for nodulation. The genotypic fingerprinting is performed using several techniques, viz., restriction fragment length polymorphism (RFLP), pulse-field gel electrophoresis (PFGE), PCR-fingerprinting, and gene sequencing. The sequence analysis of 16S rRNA gene has been used in phylogeny and taxonomy of rhizobia, but this does not reflect the symbiotic features including legume host range. Moreover, there are multiple copies of the 16S rRNA gene, with up to 5% intragenomic differences (Kampfer and Glaeser 2012); the 16S rRNA gene sequence is highly conserved and has ability to resolve closely related strains but only up to genus level, and it is also vulnerable to horizontal gene transfer (Willems et al. 2001; Gevers et al. 2005). Stackebrandt and co-workers (2002) have proposed the16S–23S rRNA ITS region and genes located in the core genome (housekeeping) as alternative phylogenetic markers. According to them, at least five housekeeping genes are necessary for reliable taxonomy. Although housekeeping genes have evolved at a rate faster than the 16S rRNA, they are considerably conserved to retain genetic information. The housekeeping genes frequently used in taxonomic characterization are *dnaK*, *dnaJ*, *glnA*, *gyrB*, *gltA*, *glnII*, *recA*, *rpoA*, *rpoB*, and *atpD* (Menna et al. 2009; Menna and Hungria 2011; Azevedo et al. 2015). There are several criteria for selecting housekeeping genes for MLSA such as that they should be (1) present in single copies in a genome; (2) distributed throughout the genome, spaced by distance of at least 100 kb between two genes; (3) with nucleotide length sufficient for sequencing; (4) carry information sufficient for the analysis; and (5) be broadly distributed among taxa. The combination of genes for use in taxonomic analysis is also important and should be carefully selected. The increased use of MLSA with rhizobia suggests that it may soon be an accepted tool to define new species. It is further proposed that 94–96% identity for multilocus sequences may replace the 70% homology in DNA–DNA hybridization. In case of rhizobia, analysis of nodulation (*nod*) and nitrogen-fixing (*nif*, *fix*) genes can provide the information on evolution of the symbioses and its host range for nodulation.

Since whole genome sequences for bacterial species are available, average nucleotide identity (ANI) of the whole genome has also been increasingly used for the identification of novel species (Konstantinidis and Tiedje 2005a, b, Ormeño-Orrillo et al. 2015). For the genome comparison, 94% identity of the shared genes between two strains would be equivalent to the 70% level of DNA–DNA hybridization. This

criterion might be reevaluated as more rhizobial genomes are sequenced and available for analysis.

6.5.2 Criteria for Description of Rhizobia

Taxonomy includes three elements: characterization, classification, and nomenclature. The characterization of one strain is a key step in prokaryotic systematic and precedes classification and nomenclature. Over the past century, characterization methodologies have considerably improved. Presently, both modern and traditional methods are used for assigning a strain to a taxon. The scheme of currently available methods for characterization of the strain comprehensively has been outlined by Tindall and co-workers (2010). The scheme of tests for rhizobial species demarcation is as follows:

1. *Number of strains used to describe a new rhizobial species*. The new strain descriptions are preferentially based on a minimum of at least three different isolates as revealed by IGS-PCR RFLP, rep-PCR, and AFLP fingerprints or sequence data. Sampling should be made from different ecological settings, and more than 12 isolates should preferentially represent each ecological setting. Hence, *new species descriptions based on a single isolate are strongly discouraged!*
2. *Number and type of molecular markers. The high-resolution molecular typing method appropriate to reveal diversity within species (i.e., rep-PCR or AFLP genomic fingerprints) combined with MLSA of at least three protein-coding loci should be used.* For rhizobia it *would be appropriate to generate full-length 16S rDNA sequences for a few carefully selected strains, along with the partial sequencing of three protein-coding loci (e.g. glnII, recA, and rpoB) and at least one symbiotic (nifH, nodA, and nodC) locus.*
3. *Group of phenotypic tests*. New isolates should be screened for host range in vivo which gives insights on biovars and symbiotic varieties. Other commonly used and potentially relevant attributes are pH and temperature growth range, salt tolerance, growth on different C and N sources, as well as antibiotic resistance profiling and fatty acid methyl ester analysis (FAME). *The selection of phenotypic and chemotaxonomic tests is based on the niches from where isolate(s) was recovered.*
4. *DNA–DNA hybridization vs MLSA. For delineation of species, a thorough MLSA must be combined with key phenotypic tests. They should further be supported by full-length 16S rDNA sequencing of the type strain and two other strains, along with DNA–DNA hybridization data. DNA–DNA hybridization has been increasingly replaced by MLSA and genomic comparisons (ANI).*

6.5.3 *Classification of Rhizobia on Plant Host Range for Nodulation*

Rhizobia are characterized by inherent ability to nodulate legume plants effectively. Another characteristic property of rhizobia is host specificity. At genetic level host specificity is determined by nodulation (e.g., *nod*, *rhi*) and nitrogen fixation (*fix*, *nif*) genes that form accessory genome and/or present on plasmids. The stable taxonomy is based on "core" or housekeeping genes. Hence, bacteria of same species can have different host specificity. The host specificity or symbiotic preferences in rhizobia were denoted through biovar description (Jordan 1984). In subsequent years several biovars have been described within rhizobia (Table 6.2). Biovars can be differentiated on the basis of different biochemical and physiological properties. Considering the polyphasic taxonomy, in rhizobia the biovar must be supported with sequence data of symbiotic genes. Thus the use of "symbiovar" is more appropriate to reflect symbiotic preferences of a particular rhizobium species (Rogel et al. 2011).

6.6 The Need to Preserve Cultures

Preservation of the cultures in a genetically stable form is highly important, as experimentation with strains of rhizobia can last for many decades. It has been more than 100 years since the first bacteria from the nodules were isolated, and several decades have elapsed since the symbiosis was scientifically understood, yet very few rhizobial cultures are available for exploitation. The probable reason is that strains were commonly stored on agar. Agar slope-borne cultures have a relatively short shelf life. For this reason, methods for long-term preservation of valuable cultures are required to ensure survival over long period of inattention.

6.7 Methods of Preservation

The main goal of any method of preservation is to maintain the purity and viability of the culture for the longest duration possible. In addition, the method should be easy to implement, of low cost, and easily accessible (Romeiro 2001). The requirements for short-term storage after the strain has been isolated from a nodule and passed through the purification process and long-term storage to preserve the integrity of the strain after authentication are different. A short-term system is based on agar or glycerol storage (with strains labelled with a temporary code) and long-term preservation (freeze-drying or lyophilization) after authentication (Table 6.3). The strains may lose desirable properties during storage or after repeated subculture. Hence, storage methods must be such that minimize the opportunity for variation or mutation besides keeping cultures viable for long duration. Several methods are

Table 6.2 Description of different biovars within rhizobial species and their legume host

Biovars	Rhizobial species	Legume host	References
acaciae	*S. terangae* *S. sahelense* *S. meliloti*	*Acacia* *Acacia* *A. tortilis*	Lortet et al. (1996) Haukka et al. (1998) Ba et al. (2002)
acaciellae	*S. chiapanecum* *S. mexicanum*	*Acaciella angustissima* *A. angustissima*	Rogel et al. (2011)
biserrulae	*M. opportunistum*	*Biserrula pelecinus*	Nandasena et al. (2007)
ciceri	*M. amorphae* *M. tianshanense* *M. ciceri* *M. mediterraneum* *S. meliloti*	*Cicer arietinum* *C. arietinum* *C. arietinum* *C. arietinum* *C. arietinum*	Rivas et al. (2007) Rivas et al. (2007) Nandasena et al. (2007) Nour et al. (1995) Maatallah et al. (2002)
gallicum	*R. gallicum* *R. giardinii*	*Phaseolus vulgaris, Leucaena leucocephala* *P. vulgaris, L. leucocephala*	Amarger et al. (1997) Amarger et al. (1997)
genistearum	*B. japonicum*	*Genisteae, Loteae*	Vinuesa et al. (2005)
giardinii	*R. giardinii*	*P. vulgaris, L. leucocephala*	Amarger et al. (1997)
glycinearum	*B. japonicum*	*Glycine*	Vinuesa et al. (2005)
lancerottense	*S. meliloti*	*Lotus lancerottense*	Leon-Barrios et al. (2009)
medicaginis	*S. meliloti*	*Medicago laciniata*	Villegas et al. (2006)
mediterranense	*S. fredii* *S. meliloti*	*P. vulgaris* *P. vulgaris*	Mnasri et al. (2007) Mnasri et al. (2007)
meliloti	*S. meliloti*	*Medicago sativa,* *M. truncatula*	Villegas et al. (2006) Mnasri et al. (2007)
mimosae	*R. etli*	*P. vulgaris, L. leucocephala, Mimosa affinis*	Mnasri et al. (2007) Wang et al. (1999)
officinalis	*R. galegae*	*Galega officinalis*	Radeva et al. (2001)
orientalis	*R. galegae*	*Galega orientalis*	Quispel (1988)
orientale	*R. mongolense, Rhizobium* spp.	*Medicago ruthenica,* *P. vulgaris*	Silva et al. (2005) Amarger et al. (1997)
phaseoli	*R. gallicum P* *R. giardini* *R. leguminosarum* *R. etli* *R. phaseoli*	*P. vulgaris* *P. vulgaris* *P. vulgaris* *P. vulgaris* *P. vulgaris*	Amarger et al. (1997) Amarger et al. (1997) Jordan (1984) Segovia et al. (1993) Ramírez-Bahena et al. (2008)

(continued)

Table 6.2 (continued)

Biovars	Rhizobial species	Legume host	References
sesbaniae	*S. terangae* *S. sahelense* *Agrobacterium* sp.	*Sesbania* *Sesbania* *Sesbania*	Lortet et al. (1996) Lortet et al. (1996) Cummings et al. (2009)
trifolii	*R. leguminosarum* *R. leguminosarum*	*Trifolium* *Vicia sativa*	Jordan (1984) Jordan (1984)
viciae	*R. fabae* *R. pisi*	*Viciafaba* *V. sativa*	Tian et al. (2008) Ramírez-Bahena et al. (2008)

Table 6.3 Cell viability of various methods for preserving rhizobia

Methods	Characteristics and conditions	Period of viability
Agar slopes	YEMA, stored at 5–7 °C Low cost and transfer is simple	1 year
Agar slopes covered with mineral oil or paraffin wax	As above. In addition the agar slopes were covered with sterilized mineral or paraffin oil	2 years
Porcelain beads	Suspension of rhizobium cells is poured on sterilised porcelain beads, air dried and kept in a tube with dehydrated silica. Stored at 5–7 °C	2 years
Soil, peat or clay	The material is finely ground, corrected for chemical properties and sterilized. The culture to be stored is inoculated into the measured amount of soil, peat or clay. Stored at 5–7 °C	2–4 years
Freezing	Storage in temperatures ranging from −70 to −190 °C in deep freeze or liquid nitrogen. Viability depends on the culture medium; freezing speed, freezing temperature, type of cryoprotectant used	Months to several years
Lyophilization	Viability depends on the physiological state of the culture, cell concentration, and medium and lyophilization rate; can be kept at room temperature for decades but little information available	Several decades

(Source: modified from Hungria et al. 2016)

available for the preservation of bacterial cultures such as immersing in mineral oil, ordinary freezing, drying, in situ preservation in soil, in sterile distilled water (SDW), glycerol, synthetic polymers, liquid nitrogen, and lyophilization (van Elsas 2001; Campos et al. 2004; Denardin and Freire 2000; Fernandes Júnior et al. 2009). Carboxymethyl cellulose (CMC) and glycerol are commonly used for preserving bacterial cultures. Carboxymethyl cellulose (CMC) is cellulose-derived ester and a highly hygroscopic and viscous polymer, nontoxic to humans (Sanz et al. 2005). Glycerol is a cryoprotectant commonly used as osmoregulator in freezing processes (Campos et al. 2004).

6.7.1 Storage on Agar Slopes

1. Select preferred growth medium (yeast extract mannitol agar or tryptone yeast agar). Heat to dissolve the agar and ensure it is thoroughly mixed.
2. Dispense mixture with a screw-capped bottle or test tube to fill 33% of the volume (i.e., 10 into 30 ml McCartney bottle or 1 into 3 ml plastic vial).
3. Place the bottles and tubes into autoclavable basket. After autoclaving place the bottles and tubes at an angle of 45–60 °C until set.
4. Take a loopful of culture, and streak across the surface of agar slope, allow growing until visible.
5. Store at 5–7 °C.
6. Alternatively cover the agar slope with sterile paraffin or mineral oil to decrease the rate of desiccation.

6.7.2 Storage on Porcelain Beads

1. The desiccated silica gel is placed in the autoclavable vial occupying up to 30% of the volume. The vial is covered with cotton wool, topped with cleansed porcelain beads.
2. The vial is screw capped on loosely then autoclaved.
3. The culture is grown in selected liquid medium to visible turbidity. Alternatively, culture suspension is prepared by washing from solid medium with diluents.
4. Transfer the sterilized beads aseptically to broth culture in the tubes and re-plug. Allow the beads to soak for 1–2 h. The beads impregnated with rhizobia culture are placed into storage tubes aseptically; replace and tighten the caps; store at 5–7 °C. The tubes are observed for moisture. If silica gel becomes pink or colorless, either too much moisture was absorbed during transfer or there is an improper seal permitting entry of moisture.
5. For reviving a culture, one bead is removed and dropped into a yeast mannitol broth medium. This is allowed to grow till visible turbidity is observed; a loopful is then streaked on YM agar with bromothymol blue (BTB) or congo red (CR) indicator dye to check purity.

6.7.3 Long-Term Preservation

(a) *Lyophilization*

This method allows removal of moisture from the culture sample without concurrent change in their physiological, biochemical, or genetic properties. It is based on the principle of removing moisture from culture under strong vacuum; the evaporation of moisture results in drop of the temperature. Although cultures are desiccated, cells in sufficient numbers survive. Freeze-

drying of cultures is carried out in two stages, primary stage where 90–95% of the water is removed and secondary stage where additional 4% of water is removed; retention of 1% of water is essential for survival of bacteria. This is achieved by suspending the cells in medium that will not permit complete removal of water. For example, a 50–50 mixture of 10% w/v peptone with 10% w/v Na-glutamate or 5% w/v peptone with 10% w/v sucrose is used. Cultures are then sealed in glass ampoules under very low vacuum. Various cryoprotectants such as dimethyl sulfoxide, glycerol, albumin, skim milk, and peptone (Hubálek 2003; Day and Stacey 2007) are used.

(i) *Preparation of Ampoules*

1. Glass ampoules, dimensions 4 mm × 50 mm open at one end, are made of high-quality glass.
2. A small cotton wool swab is placed at the bottom of the tube with the help of inoculating loop. Then the labels are placed into ampoules above the cotton wool, plugged with non-absorbent cotton wool and autoclaved at 121 °C for 30 min.

(ii) *Preparation of Cultures*

1. The rhizobial strains are grown in the preferred agar medium to check for purity.
2. Dispense 2 ml of the lyophilization mixture (e.g., 1:1 mixture of 50% peptone and 10% Na-glutamate) into screw-capped vials and autoclave.
3. A loopful of the rhizobial strain is added into the cooled lyophilization mixture, and lid replaced and vortexed. Alternatively, take 1 ml of a broth suspension of rhizobia and mix well into the lyophilization medium.
4. The suspension (0.1 ml) is transferred to the ampoule using a sterile pasture pipette. Usually per strain 10 ampoules are prepared. If less than 0.1 ml culture suspension is added, then primary drying process can be omitted.
5. The cotton wool plug is pushed halfway down the ampoule, below the constriction point, to close the vial and protect the culture during the lyophilization process.

(iii) *Primary Drying Process*

1. Turn on the lyophilizer, introduce the ampoules, and follow the manufacturer's instructions. The first drying stage (90–95% of dehydration) usually takes about 90 to 120 min in most lyophilizers.

(iv) *Ampoule Constriction*

1. After the culture is lyophilized, ampoules are constricted in the middle above the inserted plug by gently turning ampoules while holding the center point over a flame, allowing the glass to melt.

2. During the process ampoules are rolled. Consequently glass flows into the middle of the ampoule. At this point ampoule is removed from the flame while continuing to gently stretch the middle section. The ampoules are not sealed during this process.

(v) *Secondary Drying*

1. Constricted ampoules are applied to a manifold by pushing over into rubber sleeve so that they are held tightly.
2. The vacuum is started again, and drying is continued for approximately 1 h until a vacuum reaches 6.7 pascals. Drying over a desiccant, for example, phosphorus pentoxide, is done for the removal of remaining moisture. It is noteworthy that phosphorus pentoxide is poisonous.
3. Once the vacuum has been achieved, ampoules may be sealed by holding a flame to the constricted part of the ampoule. After the narrow neck has melted, ampoule is held at the end and twisted, so that it is separated from the top half of the ampoule.
4. The ampoules should retain a vacuum. The vacuum within sealed ampoule is checked using a high-frequency spark tester. If correct level of vacuum is present, a faint glow like a neon light appears inside ampoule.
5. Viability of cell should be ascertained in at least one ampoule per culture per batch immediately

(vi) *Recovery from Lyophilized Ampoules*

1. Break the ampoule by hitting the glass above the cotton plug with a steel file, heat the score mark, and gently bend the glass allowing it to crack.
2. The cotton from ampoule is removed with sterile forceps, and 0.1–0.3 ml growth medium or lyophilizing mixture is added with a micropipette.
3. Homogenize and transfer a drop or two to a Petri plate containing growth medium. Spread and incubate at optimum temperature for growth.

(b) *Cryopreservation*

It is the process of maintenance of living organisms at low temperature (−80 °C or below) so that they survive thawing. Generally at −80 °C temperature, the viability of cultures decreases with time. For long-term preservation, bacteria should be stored at temperature below −140 °C (OECD 2007). For preservation, rhizobia are grown in glycerol-peptone medium (Gerhardt et al. 1981).

(i) *Preparation of Glycerol Stock*
Method 1

1. The pure culture is inoculated into liquid glucose-peptone broth till log phase is achieved.

2. 150 µl of sterile 80% (v/v) glycerol is added to the cryotube. The final volume is made up to 1 ml by adding broth culture and mixed thoroughly.
3. The temperature of the tube is decreased at a rate of 1.0 °C per minute till it reaches −50 °C and then stored at the final temperature.

Method 2

1. The glucose-peptone medium without indicator dyes is mixed with 80% v/v glycerol in the ratio 85:15 (final concentration = 12% glycerol) and autoclaved.
2. The above mixture is dispensed into sterile cryotubes (1–2 ml).
3. Add loopful of culture from solid agar after confirming purity to the cryotube, and vortex to suspend the culture.
4. Alternatively the cryotubes can be dipped into liquid nitrogen allowing a rapid drop of temperature.

Method 3

1. The glycerol cell suspensions may also be added to vials containing small sterile glass beads. The excess liquid is removed; the beads get coated with cell suspensions. For revival, individual coated beads are removed and inoculated into the standard medium.

Method 4

1. The glucose-peptone medium without indicator dyes is mixed with 100% glycerol in the ratio1:1.
2. The loopful of culture is inoculated into glucose-peptone broth (10 ml) in a test tube and grown till log phase is attained.
3. Now the mixture of glucose-peptone broth and glycerol, 4.5 ml in 10 ml of log phase culture broth is added and thoroughly mixed. The mixture is transferred to cryotubes in aliquots of 1 ml each. The cryotubes were labelled properly and stored at −80 °C

(c) *Revival of Preserved Cultures*

The preserved cultures should be checked every 5 years.

1. The frozen cultures should be kept on ice, if they are to be returned to cold storage or deep freezer.
2. A loopful of frozen culture is transferred on to agar plate with the help of warm sterile loop.
3. Once thawed, the culture is streaked on to the plate. The purity is checked for which the cultures should be streaked till single colonies appear.
4. The growth of rhizobial cultures streaked from frozen stocks may be slower and required longer incubation period than those streaked out from routine slopes or plates. There can be loss of nod character during storage/frequent transfers.

6.8 Conclusion and Future Scenario

There is network of 728 culture collection centers registered under WFCC-MIRCEN and coordinated by WDCM and of these 77 have published online catalogues. The Convention on Biological Diversity and Budapest Treaty has been adopted globally. The use and peruse of rhizobium-legume technology is well established. Although advent and advancement of sequencing techniques have led to increased number of rhizobial strains being described and identified, till date 130 rhizobial species have been described. However, for rhizobia plant infection, tests are very useful and relevant. Presently, hundreds of rhizobial strains have been recovered, explored, exploited, and preserved. But still 90% of the legumes spread across the globe remain to be explored for their microbial partner. More and more microbial repositories should be established around the world especially in regions that are hotspots of biodiversity.

Biogeographically India has characteristic elements from all three realms—Afrotropical, Indo-Malayan, and Paleo-Arctic realms. Therefore it harbors 2 of the 18 hotspots of biodiversity spread across the globe. These include floristically rich areas of Northeast India, the Western Ghats, northwest Himalayas, and the Andaman and Nicobar Islands. Considering rich biodiversity, very few culture collection centers are established across India, and only six have published online catalogues.

The exploration of legumes for their microbial partner, its characterization, description, and conservation should be taken up exhaustively and systematically so that we do not lose out rich biodiversity. This will further help in strict implementation of the Convention on Biological Diversity.

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References

Amarger N, Macheret V, Laguerre G (1997) *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. Int J Syst Bacteriol 47:996–1006

Anonymous (2017) India to overtake China's population in 7 yrs. A UN report published in Times of India

Australian Inoculant Research and Control Service, New South Wales Department of Agriculture Melbourne. http://www.dpi.nsw.gov.au/

Azevedo H, Lopes FM, Silla PR, Hungria M (2015) A database for the taxonomic and phylogenetic identification of the genus *Bradyrhizobium* using multilocus sequence analysis. BMC Genomics 16:S10

Ba S, Willems A, de Lajudie P, Roche P, Jeder H, Quatrini P, Neyra M, Ferro M, Prome JC, Gillis M, Boivin-Masson C, Lorquin J (2002) Symbiotic and taxonomic diversity of rhizobia isolated from *Acacia tortilis* subsp. Raddiana in Africa. Syst Appl Microbiol 25:130–145

Campos AK, de Andrade Mota M, de Araújo JV, Cecon PR (2004) Predatory activity, radial growth and sporulation of fungi predating *Monacrosporium* nematode submitted to cryopreservation. Sci Rural 34:465–469

Commonwealth Scientific and Industrial Research Organization's (CSIRO) Cunningham Laboratory, Brisbane. https://www.csiro.au/en/Publications

Cummings SP, Gyaneshwar P, Vinuesa P, Farruggia FT, Andrews M, Humphry D, Elliott GN, Nelson A, Orr C, Pettitt D, Shah GR, Santos SR, Krishnan HB, Odee D, Moreira FMS, Sprent JI, Young JPW, James EK (2009) Nodulation of *Sesbania* species by *Rhizobium (Agrobacterium)* strain IRBG74 and other rhizobia. Environ Microbiol 11:2510–2525

Day JG, Stacey GN (eds) (2007) Cryopreservation and freeze-drying protocols, 2nd edn. Methods in molecular biology series 368. Humana, Totowa

Denardin ND, Freire JRJ (2000) Assessment of polymers for the formulation of legume inoculants. World J Microbiol Biotechnol 16:215–217

Fernandes Júnior PI, Rohr TG, de Oliveira PJ, Xavier GR, Rumjanek NG (2009) Polymers as carriers for rhizobia inoculant formulations. Pesq Agropec Bras Brasília 44:1184–1190

Frank B (1889) Über die Pilzsymbiose der Leguminosen. Ber Dtsch Bot Ges. 7:332–346

Fred EB, Baldwin IL, McCoy E (1932) Root nodule bacteria and leguminous plants. University of Wisconsin. Digitized19 Feb 2008

Freire JRJ, Kolling I (1986) World culture collections of Rhizobium. World J Microbiol Biotechnol 2:231–235

Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Phillips GB (eds) (1981) Manual of methods for general bacteriology. American Society of Microbiology, Washington

Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, Feil EJ, Stackebrandt E, de Peer YV, Vandamme P, Thompson FL, Swings J (2005) Re-evaluating prokaryotic species. Nat Rev Microbiol 3:733–739

Graham PH, Sadowsky MJ, Kersters HH, Barnet YM, Bradley RS, Cooper JE, De Ley DJ, Jarvis BDW, Roslycky EB, Strijdom BW, Young JPW (1991) Proposed minimal standards for the description of new genera and species of root-and stem-nodulating bacteria. Int J Syst Bacteriol 41:582–587

Haukka K, Lindstrom K, Young JPW (1998) Three phylogenetic groups of nodA and nifH genes in S*inorhizobium* and *Mesorhizobium* isolates from leguminous trees growing in Africa and Latin America. Appl Environ Microbiol 64:419–426

Howeison JG, Dilworth MJ (2016) Working with rhizobia. Australian Centre for International Agricultural research, Canberra

Hubálek Z (2003) Protectants used in the cryopreservation of microorganisms. Cryobiology 46:205–229

Hungria M, Ardley J, O'Hara GW, Howeison JG (2016) Preservation of rhizobia. In: Howeison JG and Dilworth MJ (eds) Working with rhizobia. Australian Centre for International Agricultural Research, Canberra, pp 61–72

Johnson MD, Allen ON (1952) Cultural reactions of rhizobia with special reference to strains isolated from *Sesbania* species. Ant Leewenhoek 18:1–12

Jordan DC (1982) Notes: transfer of Rhizobium japonicum Buchanan 1980 to Bradyrhizobium gen. Nov., a genus of slow-growing, root nodule bacteria from leguminous plants. Int J Syst Bacteriol 32(1):136–139

Jordan DC (1984) Family III. Rhizobiaceae Conn (1938). In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 1. Williams and Wilkins, Baltimore, pp 234–254

Kampfer P, Glaeser SP (2012) Prokaryotic taxonomy in the sequencing era the polyphasic approach revisited. Environ Microbiol 14:291–317

Konstantinidis K, Tiedje JM (2005a) Genomic insights that advance the species definition for prokaryotes. Proc Natl Acad Sci USA 102:2567–2572

Konstantinidis KT, Tiedje JM (2005b) Towards a genome-based taxonomy for prokaryotes. J Bacteriol 187:6258–6264

Leon-Barrios M, Lorite MJ, Donate-Correa J, Sanjuan J (2009) *Ensifer meliloti* bv. lancerottense establishes nitrogen-fixing symbiosis with Lotus endemic to the Canary Islands and shows distinctive symbiotic genotypes and host range. Syst Appl Microbiol 32:413–420

Lindström K, Young JP (2011) International Committee on Systematics of Prokaryotes Subcommittee on the taxonomy of *Agrobacterium* and *Rhizobium*: minutes of the meeting, 7 September 2010, Geneva, Switzerland. Int J Syst Evol Microbiol 61:3089–3093

Lortet G, Mear N Lorquin J, Dreyfus B, de Lajudie P, Rosenberg C, Boivin C (1996) Nod factor thin-layer chromatography profiling as a tool to characterize symbiotic specificity of rhizobial strains: application to *Sinorhizobium saheli*, *S. teranga*, and *Rhizobium sp*. strains isolated from Acacia and *Sesbania*. Mol Plant–Microbe Interact 9:736–747

Maatallah J, Berraho E, Munoz S, Sanjuan J, Lluch C (2002) Phenotypic and molecular characterization of chickpea rhizobia isolated from different areas of Morocco. J Appl Microbiol 93:531–540

Menna P, Hungria M (2011) Phylogeny of nodulation and nitrogen fixation genes in *Bradyrhizobium*: supporting evidence for the theory of monophyletic origin, and spread and maintenance by both horizontal and vertical transfer. Int J Syst Evol Microbiol 61:3052–3067

Menna P, Barcellos FG, Hungria M (2009) Phylogeny and taxonomy of a diverse collection of *Bradyrhizobium* strains based on multilocus sequence analysis of the 16S rRNA gene, ITS region and glnII, recA, atpD and dnaK genes. Int J Syst Evol Microbiol 59:2934–2950

Mnasri B, Mrabet M, Laguerre G, Aouani ME, Mhamdi R (2007) Salt-tolerant rhizobia isolated from a Tunisian oasis that are highly effective for symbiotic N2-fixation with *Phaseolus vulgaris* constitute a novel biovar (bv. mediterranense) of *Sinorhizobium meliloti*. Arch Microbiol 187:79–85

Nandasena KG, O'Hara GW, Tiwari RP, Sezmis E, Howieson JG (2007) In situ lateral transfer of symbiosis islands results in rapid evolution of diverse competitive strains of mesorhizobia suboptimal in symbiotic nitrogen fixation on the pasture legume *Biserrula pelecinus L*. Environ Microbiol 9:2496–2511

Nour SM, Cleyet Marel JC, Normand P, Fernandez MP (1995) Genomic heterogeneity of strains nodulating chickpeas (*Cicer arietinum L*.) and description of *Rhizobium mediterraneum* sp. nov. Int J Syst Bacteriol 45:640–648

OECD (2007) Promoting pro-poor growth: policy guidance for donors. OECD Publishing. https://doi.org/10.1787/9789264024786-en

Ormeño-Orrillo E, Servín-Garcidueñas LE, Rogel MA, González V, Peralta H, Mora J, Martínez-Romero J, Martínez-Romero E (2015) Taxonomy of *Rhizobia* and *Agrobacteria* from the Rhizobiaceae family in light of genomics. Syst Appl Microbiol 38:287–291

Peterson TA, Russelle MP (1991) *Alfalfa* and the nitrogen cycle in the Corn Belt. J Soil Water Conserv 46:229–235

Quispel A (1988) Hellriegel and Wilfarth's discovery of (symbiotic) nitrogen fixation one hundred years ago. In: Bothe H, de Bruijn FJ, Newton WE (eds) Nitrogen fixation: one hundred years after. Gustav Fisher, Stuttgart, pp 3–12

Radeva G, Jurgens G, Niemi M, Nick G, Suominen L, Lindstrom K (2001) Description of two biovars in the *Rhizobium galegae* species: *biovar orientalis* and *biovar officinalis*. Syst Appl Microbiol 24:192–205

Ramírez-Bahena MH, García Fraile P, Peix A, Valverde A, Rivas R, Igual JM, Mateos PF, Martínez Molina E, Velázquez E (2008) Revision of the taxonomic status of the species *Rhizobium leguminosarum* (Frank 1879) Frank 1889AL, *Rhizobium phaseoli* Dangeard 1926AL and *Rhizobium trifolii* Dangeard 1926AL. *R. trifolii* is a later synonym of *R. leguminosarum*. Reclassification of the strain *R. leguminosarum* DSM 30132 (=NCIMB 11478) as *Rhizobium pisi* sp. nov. Int J Syst Evol Microbiol 58:2484–2490

Rivas R, Laranjo M, Mateos PF, Oliveira S, Martínez-Molina E, Velázquez E (2007) Strains of *Mesorhizobium amorphae* and *Mesorhizobium tianshanense*, carrying symbiotic genes of common chickpea endosymbiotic species, constitute a novel biovar (*ciceri*) capable of nodulating *Cicer arietinum*. Lett Appl Microbiol 44:412–418

Rogel MA, Ormeño-Orrillo E, Martinez Romero E (2011) Symbiovars in rhizobia reflects bacterial adaptation to legumes. Syst. Appl Microbiol 34:96–104. https://doi.org/10.1016/j.syapm.2010.11.015

Romeiro RS (2001) Methods in plant bacteriology. UFV, Viçosa, p 279

Sahgal M, Sharma A, Johri BN, Prakash A (2004) Selection of growth promotory rhizobia for *Dalbergia sissoo* from diverse soil ecosystems of India. Symbiosis 36:83–96

Sanz T, Fernández MA, Salvador A, Muñhoz J, Fiszman SM (2005) Thermogelation properties of methylcellulose (MC) and their effect on a batter formula. Food Hydrocoll 19:141–147

Segovia L, Young JPW, Martinez-Romero E (1993) Reclassification of American *Rhizobium leguminosarum* biovar *phaseoli* type I strains as *Rhizobium etli* sp. nov. Int J Syst Bacteriol 43:374–377

Silva C, Vinuesa P, Eguiarte LE, Souza V, Martinez-Romero E (2005) Evolutionary genetics and biogeographic structure of *Rhizobium gallicum* sensu lato, a widely distributed bacterial symbiont of diverse legumes. Mol Ecol 14:4033–4050

Sly LI (2010) Biodiversity and the role of microbial resource centres. In: Brown AG (ed) Proceedings of conference "biodiversity and world food security: nourishing the planet and its people". Conducted by the Crawford Fund for International Agricultural Research, Parliament House, Canberra, Australia, 30 August–1 September, 2010, pp 60–67

Smith D (2003) Culture collections over the world. Int Microbiol 6:95–100

Stackebrandt E, Frederiksen W, Garrity GM, Grimont PAD, Kampfer P, Maiden X, Nesme MCJ, Rossello-Mora R, Swings J, Truper HG, Vauterin L, Ward AC, Whitman WB (2002) Report of the adhoc committee for the re-evaluation of the species definition in bacteriology. Int J Syst Evol Microbiol 52:1043–1047

Terpolilli J, O'Hara GW, Tiwari RP, Dilworth MJ, Howieson JG (2008) The model legume *Medicago truncatula* A17 is poorly matched for N2 fixation with the sequenced microsymbiont *Sinorhizobium meliloti* 1021. New Phytol 179:62–66

Tian CF, Wang ET, Wu LJ, Han TX, Chen WF, Gu CT, Gu JG, Chen WX (2008) *Rhizobium fabae* sp. nov., a bacterium that nodulates *Vicia faba*. Int J Syst Evol Microbiol 58:2871–2875

Tilman D, Reich PB, Knops J, Wedin D, Mielke T, Lehman C (2001) Diversity and productivity in a long-term grassland experiment. Sci 294:843–845

Tindall BJ, Rosselló-Móra R, Busse H-J, Ludwig W, Kämpfer P (2010) Notes on the characterization of prokaryote strains for taxonomic purposes. Int J Syst Evol Microbiol 60:249–266

Trewavas A (2001) Urban myths of organic farming. Nature 410:409–410

Van Elsas JD (2001) Effects of ecological factors on the survival and physiology of *Ralstonia solanacearum* bv.2 in irrigation water. Can J Microbiol 47:842–854

Vandamme P, Pot B, Gillis M, De Vos P, Kersters K, Swings J (1996) Polyphasic taxonomy; a consensus approach to bacterial systematics. Microbiol Mol Biol Rev 60:407–438

Villegas MC, Rome S, Maure L, Domergue O, Gardan L, Bailly X, Cleyet- Marel JC, Brunel B (2006) Nitrogen-fixing *sinorhizobia* with *Medicago laciniata* constitute a novel biovar (bv. *medicaginis*) of *S. meliloti*. Syst Appl Microbiol 29:526–538

Vinuesa P, Leon-Barrios M, Silva C, Willems A, Jarabo-Lorenzo A, Perez-Galdona R, Werner D, Martinez-Romero E (2005) *Bradyrhizobium canariense* sp. nov., an acid-tolerant endosymbiont that nodulates endemic genistoid legumes (Papilionoideae: Genisteae) from the Canary Islands, along with *Bradyrhizobium japonicum* bv. *genistearum*, *Bradyrhizobium* genospecies alpha and *Bradyrhizobium* genospecies beta. Int J Syst Evol Microbiol 55:569–575

Wang ET, Rogel MA, Garcia-De los Santos A, Martinez-Romero J, Cevallos MA, Martinez-Romero E (1999) *Rhizobium etli* bv. *mimosae*, a novel biovar isolated from *Mimosa affinis*. Int J Syst Bacteriol 49:1479–1491

Weir BS (2016) The current taxonomy of rhizobia. NZ Rhizobia website. https://www.rhizobia.co.nz/taxonomy/rhizobia. Last updated: 10 Jan 2016

Willems A, Coopman R, Gillis M (2001) Comparison of sequence analysis of 16S–23S rDNA spacer regions, AFLP analysis and DNA–DNA hybridizations in *Bradyrhizobium*. Int J Syst Evol Microbiol 51:623–632

World Directory of Culture Collections, sixth version (2014) www.wfcc.info/ccinfo/index.php/home/statistics/#m2