Invited Review

Changing concepts in the systematics of bacterial nitrogen-fixing legume symbionts

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As of February 2003, bacteria that form nitrogen-fixing symbiotic associations with legumes have been confirmed in 44 species of 12 genera. Phylogenies of these taxa containing legume symbionts based on the comparative analysis of 16S rDNA sequences show that they are not clustered in one lineage but are distributed in the classes *Alphaproteobacteria* and *Betaproteobacteria*, and dispersed over the following nine monophyletic groups, being intermingled with other taxa that do not contain legume symbionts (shown in parentheses below): Group 1, which comprises *Rhizobium* and *Allorhizobium* species containing legume symbionts (intermingled with *Agrobacterium* and *Blastobacter* species, which are nonsymbionts); Group 2, *Sinorhizobium* and *Ensifer* species (with unclassified nonsymbionts); Group 3, *Mesorhizobium* species (with nonsymbiotic *Aminobacter* and *Pseudaminobacter* species); Group 4, *Bradyrhizobium* species and *Blastobacter denitrificans* (with nonsymbiotic *Agromonas*, *Nitrobacter*, *Afipia*, and *Rhodopseudomonas* species); Group 5, “*Methylobacterium nodulans*” (with nonsymbiotic *Methylobacterium* species); Group 6, *Azorhizobium* species (with nonsymbiotic *Xanthobacter* and *Aquabacter* species); Group 7, “*Devosia neptuniae*” (with nonsymbiotic *Devosia* species and unclassified nonsymbionts); Group 8, symbiotic *Burkholderia* strains (with nonsymbiotic *Burkholderia* species); and Group 9, *Ralstonia taiwanensis* (with nonsymbiotic *Ralstonia* species). For Groups 5, 8, and 9, the present classification, in which “each monophyletic group comprises one genus wherein legume symbionts and nonsymbionts are intermingled with each other,” is considered to be retained as is because they are clearly separated from other genera at high bootstrap values and have already been sufficiently characterized based on polyphasic taxonomy. As for the remaining six monophyletic groups, on the other hand, there are currently three options for emending their current classification (definitions and circumscriptions) at the generic level: A) the current classification shall be retained as is; B) all the genera within each monophyletic group shall be amalgamated into one single genus in conformity with the results of phylogenetic analysis; or C) each subordinate lineage in each monophyletic group shall be proposed as a genus. It is considered that research and discussions will be continuously conducted for emending the classification of these monophyletic groups based chiefly on Options B and C as preferable candidates.

Key Words—evolution; legume symbiont; molecular phylogeny; polyphasic taxonomy; rhizobia; systematics

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Introduction

To date, diazotrophy, the biological ability to reduce atmospheric nitrogen to ammonia for use as a nutrition source for growth, has not been confirmed in *Eucarya*,

Fig. 1

and is considered as a character shared exclusively by *Bacteria* and *Archaea*. Of these, bacteria that can fix nitrogen in a free-living state and utilize the fixed nitrogen mainly for their own growth are called free-living diazotrophs. Well-known examples are aerobic *Azotobacter* species and obligately anaerobic *Clostridium* species, both of which live in soil. Various other bacte-

ria with diversified types of physiology, such as facultative anaerobes, autotrophs, heterotrophs, and phototrophs, are also known to be classified as free-living diazotrophs. They became extensively distributed worldwide by adapting themselves to different environmental conditions, such as soils and waters (Eady, 1991). In addition, some bacteria, such as *Klebsiella*,

Azospirillum, *Gluconacetobacter*, *Azoarcus*, and *Herbaspirillum* species, colonize the rhizosphere, rhizoplane, phyllosphere, and intercellular spaces of plants, where they conduct plant-associative nitrogen fixation.

On the other hand, some specialized bacteria, which form symbiotic associations with specific hosts, fix nitrogen by forming special structures in hosts and/or the bacteria themselves, and supply the fixed nitrogen to their hosts, are called symbiotic diazotrophs. Important symbiotic associations occur between nodulating bacteria and members of the family *Leguminosae* (legumes), as well as between the actinomycete *Frankia* and the actinorhizal plants, such as *Alnus*, *Myrica*, *Ceanothus*, *Elaeagnus*, and *Casuarina*. In terms of efficiency and the amount of nitrogen fixed, the contribution of symbiotic diazotrophs in ecosystems is immense. In particular, as legumes are of agricultural importance, research has sought vigorously to better understand the features of nodulating bacteria which form symbiotic associations with legumes (hereafter the term legume symbionts will be used when referring to this type of bacteria) for improving the efficiency in agricultural production (Eady, 1991; Spaink et al., 1998).

At the early stage of bacterial taxonomy, all legume symbionts were classified into the single genus *Rhizobium* (Fig. 1), based on their unique feature of conducting nitrogen fixation by forming symbiotic associations with legumes. In addition, the intrageneric classification of the genus *Rhizobium* at the species level

was based primarily on the types of host plants (host specificity) infected by legume symbionts (Jordan and Allen, 1974). However, as inoculation studies using larger numbers of isolates from more diverse sources in wider ranges of legume plants showed a significant number of exceptions, host specificity gradually lost its reliability as a taxonomic criterion. Meanwhile, various genome-encoded phenotypic characters (physiological and biochemical characters) and genotypic characters (G+C content, DNA-DNA similarity, and DNA-rRNA similarity) became new taxonomic criteria, and consequently the classification of legume symbionts underwent major emendations at both the generic and species levels (Fig. 1) (Jordan, 1984). Subsequently, successive research on bacterial taxonomy (systematics) further improved our understanding, and chemotaxonomic markers (e.g., isoprenoid quinones and cellular fatty acids) and molecular phylogenetic data (16S ribosomal RNA gene (16S rDNA) sequences and protein-coding gene sequences) became positively used as taxonomic criteria for classifying legume symbionts. Consequently, incompatibilities between the earlier phenotypic classification and recent phylogenetic classification surfaced (van Berkum and Eardly, 1998), and some taxa are now facing a pressing need for further emendations (Kuykendall et al., 2003b; Willems et al., 2003; Young et al., 2001). In addition, rapidly completing genome analyses of legume symbionts are providing results that may affect classification in the future (Freiberg et al., 1997; Galibert et al., 2001; Goodner et al., 2001; Jumas-Bilak et al., 1998; Kaneko et al.,

Fig. 1. Changes in the classification of legume symbionts.

Figure 1 shows a schematic diagram of the changes in the classification of legume symbionts at the generic level from the past, through the stage in around 1984, when the first edition of Bergey's Manual of Systematic Bacteriology (BMSB) was published (as shown by a horizontal broken line), up to the present as compiled in Volume 2 of the second edition of BMSB (BMSB (2003)). The data for the present in this diagram are based primarily on the descriptions appearing in BMSB (2003); many other data published from 2001 until February 2003 are also used as supplementary sources of information. This schematic diagram does not represent the phylogenetic relationships among bacterial species. Such relationships, including legume symbionts, and presumed changes in the classification toward the future are shown on the phylogenetic tree in Fig. 2. The figures placed before the generic names are the years when the genus concerned was validly published. The figures in parentheses placed after the generic names indicate the number of "species which contain legume symbionts" described in the respective genera at each point of time. The vertical dotted lines show the period when no legume symbionts were described within the respective genera: legume symbionts were described for the first time in 2001 for the genera *Methylobacterium*, *Burkholderia*, and *Ralstonia* (Chen et al., 2001; Moulin et al., 2001; Sy et al., 2001); in 2002 for the genera *Blastobacter* and *Devosia* (Rivas et al., 2002; van Berkum and Eardly, 2002); and in 2003 for the genus *Ensifer* (Willems et al., 2003). As for the species containing legume symbionts which have not been validly published yet but are considered quite likely to be formally accepted as new species only if they are described following the formal procedures stipulated in the Bacteriological Code, their number is shown in parentheses with a question mark, i.e., *Devosia* (1?), *Methylobacterium* (1?), and *Burkholderia* (2?).

2000, 2002; Moriguchi et al., 2001; Suzuki et al., 2000; Wood et al., 2001).

The first edition of Bergey's Manual of Systematic Bacteriology (BMSB), which is a reference guide for bacterial systematics, was published in five volumes between 1984 and 1989 (Fig. 1). Chapters on legume symbionts and their relatives were included in Volume 1 (Jordan, 1984; Kersters and De Ley, 1984). However, in the face of the rapid progress of bacterial systematics as described so far, many researchers took authorship in compiling the recent changes into the second edition of BMSB, with chapters on legume symbionts in Volume 2. Based on the information in this volume and the latest research results reported so far, the changes in the classification of legume symbionts from the past to the present and into the future will be discussed in the following chapters.

1. Distribution of Legume Symbionts on a 16S rDNA-Based Phylogenetic Tree

Modern bacterial taxonomy (systematics) has enabled the construction of a hierarchical classification at the generic level and at higher taxonomic levels mainly on the basis of the results obtained by phylogenetic

analysis using 16S rDNA sequences. Species circumscriptions are determined mainly on the basis of the results of DNA-DNA hybridization analysis, which provides a quantitative evaluation of the inter- and intra-species relatedness. Furthermore, the appropriateness of each taxon is validated by confirming the cut-off points on the inter-taxon boundaries by incorporating various phenotypic, genotypic, and chemotaxonomic profiles in the hierarchical structure based on molecular criteria established by the above techniques. This series of techniques is called a polyphasic taxonomic approach (Vandamme et al., 1996), with which a 16S rDNA tree that is the basis for constructing the classification is evaluated and validated multidimensionally by examining various characteristics, and is considered to be the standard approach in modern bacterial systematics (Boone et al., 2001; Stackebrandt et al., 2002).

As the first step in this review, a 16S rDNA tree was constructed using all the known bacterial species containing legume symbionts, as well as selected representatives of their close relatives, to clarify the phylogenetic distribution of legume symbionts (Fig. 2). This showed that these legume symbionts do not form a single monophyletic group, but are dispersed over nine separate and unrelated monophyletic groups (Groups

Fig. 2. Phylogenetic relationships among legume symbionts and their relatives inferred on the basis of the 16S rDNA sequence divergence.

Figure 2 shows a neighbor-joining tree constructed on the basis of the distances estimated by the maximum-likelihood method with the rate heterogeneous HKY model (gamma shape parameter=0.21; number of rate categories=9). This tree was rooted using *Betaproteobacteria* as an outgroup. Horizontal branch lengths are proportional to the estimated number of nucleotide substitutions. Nine major monophyletic groups which contain legume symbionts are shown in order, and each group number (serial number) corresponds to Groups 1 to 9 in the text and Table 1. The figures above or beside the internal branches indicated by thick lines are bootstrap values (as percentages), as determined for 1,000 resamplings, which show the degree of unity of each monophyletic group (Groups 1 to 9). The bacterial species shown by their validly published names, which are either adopted in Volume 2 of the second edition of BMSB (BMSB (2003)) or validly published between 2001 and February 2003, were analyzed using the 16S rDNA data of their type strains. Those which have not been validly published are shown in quotation marks (" "). The names of the species which include legume symbionts are highlighted. Of these, the symbol ● is placed for *Allorhizobium undicola*, *Ensifer adhaerens* and *Blastobacter denitrificans* for the convenience of readers (see text).

a. The respective genera are listed for their respective monophyletic groups (Groups 1 to 9). The genera confirmed to belong to the respective monophyletic groups based on the results of a more comprehensive phylogenetic analysis conducted separately (Sawada, H., unpubl.) are shown in parentheses. The names of the genera which contain legume symbionts are highlighted.

b. With the further progress of research based on polyphasic taxonomy in the future, the classification will be emended, and the treatment of the monophyletic groups containing legume symbionts as taxa is considered likely to undergo amendments. Supposedly, Groups 5, 8, and 9, which correspond to the genera *Methylobacterium*, *Burkholderia*, and *Ralstonia*, respectively, will be treated as separate genera, as they are at present. Regarding Groups 1 and 2, it has been proposed that the entirety of each monophyletic group should be considered as a genus, and named as the genera *Rhizobium* and *Sinorhizobium*, respectively (Willems et al., 2003; Young et al., 2001). The same may apply to the remaining four monophyletic groups; it is thought that Groups 3, 4, 6, and 7 may be treated as four separate genera in the future.

Fig. 2

1 to 9) with different phylogenetic positions. A monophyletic group is the assemblage of all the descendants derived from a single common ancestor, and is a unit treated as one independent taxon in systematics. The classification of legume symbionts will be discussed here in terms of the identified monophyletic groups, focusing on how it has been and will be established: the process and background (past), the current situation (present), and the outstanding taxonomic problems and presumed directions of their emendations (future). The "present" state herein (Fig. 1) is based on the descriptions shown in Volume 2 of the second edition of BMSB (2003) (hereafter referred to as BMSB (2003)), as well as data reported from 2001 until February 2003 used as supplementary sources of information.

2. Classification of Legume Symbionts in Each Monophyletic Group

2.1. Monophyletic Group 1 (*Rhizobium* and *Allorhizobium* species)

Species of the genera *Rhizobium* and *Allorhizobium*, each of which includes legume symbionts, constitute Group 1, together with various nonsymbionts (species of the genus *Agrobacterium*, etc.) (Fig. 2). This subchapter will focus on the genera *Rhizobium* and *Allorhizobium*.

2.1.1. Past

At the early stage of bacterial taxonomy, legume symbionts were classified based primarily on their symbiotic ability and host-specificity (Jordan and Allen, 1974). As more detailed genotypic and genome-encoded phenotypic information was gathered, their classification was progressively amended (Jordan, 1982). The first edition of BMSB (1984) adopted a classification that divided the legume symbionts, previously allocated to the single genus *Rhizobium*, into two genera. Species comprising legume symbionts which grow fast and produce acid on a yeast extract-mannitol agar (YMA) medium (fast growers) were retained in the genus *Rhizobium*, and those which grow slowly and produce alkali were classified into the genus *Bradyrhizobium* (Fig. 1) (Jordan, 1984). Both of these genera were defined to consist of species that include legume symbionts, and in 1984 only three species, i.e., *R. leguminosarum*, *R. meliloti*, and *R. loti*, were included in the genus *Rhizobium* based on their distinguishing phenotypic and genotypic characters.

However, with the progress of polyphasic taxonomy, studies of isolates from diverse origins have provided the characterization of numerous *Rhizobium* species (Fig. 1, Table 1). Some species were transferred from the genus *Rhizobium* to other genera because they were found to be genotypically and phylogenetically different from typical *Rhizobium* species: *R. fredii* and *R. meliloti* were transferred to the genus *Sinorhizobium* (Group 2), whereas *R. ciceri*, *R. huakuii*, *R. loti*, *R. mediterraneum*, and *R. tianshanense* were transferred to the genus *Mesorhizobium* (Group 3) (Fig. 1, Table 1).

Incidentally, the nitrogen-fixing symbionts that were isolated from the root nodules of *Neptunia natans* in Senegal, West Africa, were confirmed to be phylogenetically more closely related to *Agrobacterium vitis*, which is the causal agent of crown galls on grapevine, rather than to *R. leguminosarum*, which is the type species of the genus *Rhizobium* (indicated by ● in Group 1 in Fig. 2). This finding, together with uncertainties considered to exist in the relationships between *Rhizobium* species and *Agrobacterium* species expressed at that time, led to a conclusion that these isolates should be kept separate from *Rhizobium* species at the generic level, and described as a novel genus and species, for which the name *Allorhizobium undicola* was proposed in 1998 (de Lajudie et al., 1998) (Fig. 1, Table 1).

2.1.2. Present

In BMSB (2003), the genera *Rhizobium* and *Allorhizobium* are both described as members constituting the family *Rhizobiaceae* of the order *Rhizobiales* of the class *Alphaproteobacteria* (Fig. 1, Table 1). The genus *Rhizobium* is defined therein as "a group of species which have either peritrichous or peritrichous/subpolar flagella, grow fast and produce acid on a YMA medium, and include strains that possess the ability of symbiotic nitrogen fixation," and thirteen species are described in the genus *Rhizobium*: *R. etli*, *R. galegae*, *R. gallicum*, *R. giardinii*, *R. hainanense*, *R. huautlense*, *R. indigoferae*, *R. leguminosarum*, *R. lupini*, *R. mongolense*, *R. sullae*, *R. tropici*, and *R. yanglingense* (Kuykendall et al., 2003b; Squartini et al., 2002; Tan et al., 2001; Wei et al., 2002). Of these, *R. etli*, *R. gallicum*, *R. giardinii*, *R. leguminosarum*, and *R. tropici* are subdivided into several varieties or subgroups based on the differences in their host specificity and other features (Table 1). In addition, there is an opinion that *R. lupini* should be transferred to the genus

Table 1. Legume symbionts reported so far^a and their host plants.

Monophyletic group ^b	Present name ^c	Former name(s) ^d	Host plants with which each species is associated
1	Class Alphaproteobacteria		
	Order Rhizobiales		
	Family Rhizobiaceae		
	Genus Rhizobium		
	<i>Rhizobium etli</i>	<i>Rhizobium leguminosarum</i> bv. <i>phaseoli</i> type I	<i>Phaseolus vulgaris</i> <i>Mimosa affinis</i>
	biovar <i>phaseoli</i>		<i>Phaseolus vulgaris</i>
	biovar <i>mimosae</i>		<i>Mimosa affinis</i>
	<i>Rhizobium galegae</i>	<i>Rhizobium leguminosarum</i>	<i>Galega orientalis</i> , <i>Galega officinalis</i>
	<i>Rhizobium gallicum</i>		<i>Leucaena leucocephala</i> , <i>Macroptilium atropurpureum</i> , <i>Onobrychis viciifolia</i> , <i>Phaseolus</i> spp.
	biovar <i>gallicum</i>		<i>Phaseolus</i> spp.
	biovar <i>phaseoli</i>		<i>Phaseolus</i> spp.
	<i>Rhizobium giardinii</i>		<i>Phaseolus</i> spp., <i>Leucaena leucocephala</i> , <i>Macroptilium atropurpureum</i>
	biovar <i>giardinii</i>		<i>Phaseolus</i> spp.
	biovar <i>phaseoli</i>		<i>Acacia sinicus</i> , <i>Arachis hypogaea</i> , <i>Centrosema pubescens</i> , <i>Desmodium gyroides</i> (= <i>Codariocalyx gyroides</i>), <i>D. sinuatum</i> , <i>D. triquetrum</i> (= <i>Pteroloma triquetrum</i>), <i>D. heterophyllum</i> , <i>Macroptilium lathyroides</i> , <i>Stylosanthes guianensis</i> , <i>Tephrosia candida</i> , <i>Uraria crinita</i> , <i>Zornia diphylla</i>
	<i>Rhizobium hainanense</i>		<i>Sesbania herbacea</i> , <i>S. rostrata</i> , <i>Leucaena leucocephala</i>
	<i>Rhizobium huautlense</i>		<i>Indigofera amblyantha</i> , <i>I. carlesii</i> , <i>I. potanini</i>
	<i>Rhizobium indigoferae</i>		
	<i>Rhizobium leguminosarum</i>		
	biovar <i>trifolii</i>	<i>Rhizobium trifolii</i> *	<i>Trifolium</i> spp.
	biovar <i>phaseoli</i>	<i>Rhizobium phaseoli</i>	<i>Phaseolus vulgaris</i> , <i>P. angustifolius</i> , <i>P. multiflorus</i>
	biovar <i>viceae</i>		<i>Lathyrus</i> spp., <i>Lens</i> spp., <i>Pisum</i> spp., <i>Vicia</i> spp.
	<i>Rhizobium lupini</i> ^e		<i>Lupinus</i> spp., <i>Ornithopus</i> spp.
	<i>Rhizobium mongolense</i>		<i>Medicago ruthenica</i> , <i>Phaseolus vulgaris</i>
	<i>Rhizobium sullae</i>	" <i>Rhizobium hedysari</i> "	<i>Hedysarum coronarium</i>
	<i>Rhizobium tropici</i>	<i>Rhizobium phaseoli</i> , <i>R. leguminosarum</i> bv. <i>phaseoli</i> type II	
	Type A		<i>Phaseolus vulgaris</i> , <i>Leucaena</i> spp., <i>Amorpha fruticosa</i>
	Type B		<i>Phaseolus vulgaris</i> , <i>Leucaena</i> spp.
	<i>Rhizobium yanglingense</i>		<i>Amphicarpaea trisperma</i> , <i>Coronilla varia</i> , <i>Gueldenstaedtia multiflora</i>
	Genus Allorhizobium		
	<i>Allorhizobium undicola</i> (<i>Rhizobium undicola</i>) ^f		<i>Medicago sativa</i> , <i>Neptunia natans</i> , <i>Acacia senegal</i> , <i>Acacia seyal</i> , <i>Acacia tortilis</i> subsp. <i>raddiana</i> , <i>Lotus arabicus</i> , <i>Faidherbia albida</i> (= <i>Acacia albida</i>)

Table 1. (Continued).

Monophyletic group ^b	Present name ^c	Former name(s) ^d	Host plants with which each species is associated
2	Family Rhizobiaceae		
	Genus Sinorhizobium		
	<i>Sinorhizobium arboris</i>		<i>Acacia senegal</i> , <i>Prosopis chilensis</i>
	<i>Sinorhizobium fredii</i>	<i>Rhizobium fredii</i> *	<i>Cajanus cajan</i> , <i>Glycine max</i> cv. Peking, <i>Glycine soja</i> , <i>Vigna unguiculata</i> , <i>Medicago sativa</i>
	chemovar <i>fredii</i>		
	chemovar <i>siensis</i>		
	<i>Sinorhizobium kostiense</i>		<i>Acacia senegal</i> , <i>Prosopis chilensis</i>
	<i>Sinorhizobium kummerowiae</i>		<i>Kummerowia stipulacea</i>
	<i>Sinorhizobium medicae</i>	<i>Sinorhizobium meliloti</i>	<i>Medicago orbicularis</i> , <i>M. polymorpha</i> , <i>M. rugosa</i> , <i>M. truncatula</i>
	<i>Sinorhizobium meliloti</i> subgroup A	<i>Rhizobium meliloti</i> *	<i>Melilotus</i> spp., <i>Medicago</i> spp., <i>Trigonella</i> spp.
	subgroup B		
	<i>Sinorhizobium morelense</i>		<i>Leucaena leucocephala</i>
	<i>Sinorhizobium saheli</i>		<i>Sesbania cannabina</i> , <i>S. grandiflora</i> , <i>S. rostrata</i> , <i>S. pachycarpa</i> , <i>Acacia seyal</i> , <i>Leucaena leucocephala</i> , <i>Neptunia natans</i>
	<i>Sinorhizobium teranga</i>		
	biovar <i>sesbaniae</i>		<i>Sesbania grandiflora</i> , <i>S. pubescens</i> , <i>S. rostrata</i>
	biovar <i>acaciae</i>		<i>Acacia senegal</i> , <i>A. tortilis</i> subsp. <i>raddiana</i> , <i>A. nilotica</i> , <i>Leucaena leucocephala</i>
	<i>Sinorhizobium xinjiangense</i>		<i>Glycine max</i>
	Genus Ensifer		
	<i>Ensifer adhaerens</i>		<i>Sesbania grandiflora</i> , <i>Leucaena leucocephala</i> , <i>Pithecellobium dulce</i> , <i>Medicago sativa</i>
	(<i>Sinorhizobium adhaerens</i>) ^g		
3	Family Phyllobacteriaceae		
	Genus Mesorhizobium		
	<i>Mesorhizobium amorphae</i>		<i>Amorpha fruticosa</i>
	<i>Mesorhizobium chacoense</i>		<i>Prosopis alba</i>
	<i>Mesorhizobium ciceri</i>	<i>Rhizobium ciceri</i> *	<i>Cicer arietinum</i>
	<i>Mesorhizobium huakuii</i>	<i>Rhizobium huakuii</i> *	<i>Astragalus sinicus</i>
	<i>Mesorhizobium loti</i>	<i>Rhizobium loti</i> *	<i>Lotus corniculatus</i> , <i>L. tenuis</i> , <i>L. japonicum</i> , <i>L. krylovii</i> , <i>L. filicalius</i> , <i>L. schoelleri</i> , <i>Anthyllis</i> spp., <i>Lupinus</i> spp.
	<i>Mesorhizobium mediterraneum</i>	<i>Rhizobium mediterraneum</i> *	<i>Cicer arietinum</i>
	<i>Mesorhizobium plurifarium</i>		<i>Acacia senegal</i> , <i>A. seyal</i> , <i>A. tortilis</i> subsp. <i>raddiana</i> , <i>Leucaena leucocephala</i> , <i>L. diversifolia</i> , <i>L. pulvurulenta</i> , <i>Prosopis juliflora</i> , <i>Chamaecrista ensiformis</i>
	<i>Mesorhizobium tianshanense</i>	<i>Rhizobium tianshanense</i> *	<i>Glycyrrhiza pallidiflora</i> , <i>Glycyrrhiza uralensis</i> , <i>Sophora alopecuroides</i> , <i>Glycine max</i> , <i>Swainsonia salsula</i> , <i>Halimodendron holodendron</i> , <i>Caragana polourensis</i>

Table 1. (Continued).

Monophyletic group ^b	Present name ^c	Former name(s) ^d	Host plants with which each species is associated
4	Family Bradyrhizobiaceae		
	Genus Bradyrhizobium		
	<i>Bradyrhizobium elkanii</i>	<i>Bradyrhizobium japonicum</i> DNA homology group II	<i>Glycine</i> spp., <i>Vigna</i> spp., <i>Macroptilium atropurpureum</i>
	<i>Bradyrhizobium japonicum</i> <i>Bradyrhizobium liaoningense</i> <i>Bradyrhizobium yuanmingense</i> "Bradyrhizobium lupini" ? ^e	<i>Rhizobium japonicum</i> *	<i>Glycine</i> spp., <i>Macroptilium atropurpureum</i> <i>Glycine max</i> , <i>G. soja</i> <i>Lespedeza cuneata</i> <i>Lupinus</i> spp.
	Genus Blastobacter		
	<i>Blastobacter denitrificans</i>		<i>Aeschynomene indica</i>
	Genus "Photorhizobium" ?^h		
	"Photorhizobium thompsonianum" ? ^h		<i>Aeschynomene indica</i>
5	Family Methylobacteriaceae		
	Genus Methylobacterium		
	"Methylobacterium nodulans" ⁱ		<i>Crotalaria podocarpa</i> , <i>C. perrottetii</i> , <i>C. glaucoides</i>
6	Family Hyphomicrobiaceae		
	Genus Azorhizobium		
	<i>Azorhizobium caulinodans</i>		<i>Sesbania rostrata</i>
7	Family Hyphomicrobiaceae		
	Genus Devosia		
	"Devosia neptuniae" ⁱ	"Rhizobium neptunii"	<i>Neptunia natans</i>
8	Class Betaproteobacteria		
	Order Burkholderiales		
	Family Burkholderiaceae		
	Genus Burkholderia		
	<i>Burkholderia</i> sp. STM678 ("Burkholderia tuberum") ^j		<i>Aspalathus carnosa</i>
	<i>Burkholderia</i> sp. STM815 ("Burkholderia phymatum") ^j		<i>Machaerium lunatum</i>
9	Family Burkholderiaceae		
	Genus Ralstonia		
	<i>Ralstonia taiwanensis</i>		<i>Mimosa pudica</i> , <i>M. diplotricha</i>

^a Taxa which have been confirmed to contain nitrogen-fixing bacteria forming symbiotic associations with legumes (as of February 2003) are shown here.

^b The serial numbers of the nine monophyletic groups including legume symbionts shown here are the same as those in Fig. 2.

^c The scientific names shown here are those adopted in the second edition of BMSB (2003), to which those validly published as new species from 2001 until February 2003 and other unclassified bacteria which are considered to deserve new species are added. The names which have not been validly published yet are shown in quotation marks (" ").

^d Synonyms of the present scientific names are indicated by an asterisk (*).

^e Although there is an opinion that *Rhizobium lupini* should be transferred to the genus *Bradyrhizobium*, this has not been formally proposed yet (van Berkum and Eardly, 1998).

^f It has been proposed to amalgamate the genera *Allorhizobium*, *Rhizobium*, and *Agrobacterium* into one genus that should be named the genus *Rhizobium*, and change *Allorhizobium undicola* to *Rhizobium undicola* (Young et al., 2001).

^g It has been proposed to amalgamate the genera *Ensifer* and *Sinorhizobium* into one genus that should be named the genus *Sinorhizobium*, and change *Ensifer adhaerens* to *Sinorhizobium adhaerens* (Willems et al., 2003).

^h There is an opinion that *Bradyrhizobium* strains which have the abilities of both symbiotic nitrogen fixation and photosynthesis (the so-called photosynthetic bradyrhizobia) should be made independent at the generic level and named the genus "*Photorhizobium*," although this has not been validly published and thus has not been formally accepted yet (van Berkum and Eardly, 2002).

ⁱ It is considered quite likely that these strains will be accepted as new species, only if they are validly published following the formal procedures stipulated in the Bacteriological Code together with sufficient data.

^j Recently, the names "*Burkholderia tuberum*" and "*Burkholderia phymatum*" were proposed for strains STM678 and STM815, respectively (Vandamme et al. (2002) *Syst. Appl. Microbiol.*, **25**, 507–512).

Bradyrhizobium as “*Bradyrhizobium lupini*,” although this has not been validly published yet (van Berkum and Eardly, 1998).

The genus *Allorhizobium* is treated in BMSB (2003) as a separate genus independent from the genus *Rhizobium* in conformity with the original description (de Lajudie et al., 1998). However, the descriptions of these two genera are considerably similar to each other (Kuykendall and Dazzo, 2003). *All. undicola* is the only species described as the member comprising this genus (Fig. 1, Table 1).

2.1.3. Future

There are several taxonomic problems with the genera *Rhizobium* and *Allorhizobium*, and it is considered likely that their classification will have to undergo major amendments in the future. The most vital problem is that there is no consistency between the results of phylogenetic analyses and the current definitions and circumscriptions of these genera. Specifically, in Group 1 on the 16S rDNA phylogenetic tree, thirteen *Rhizobium* species and *All. undicola* are intermingled together with some other bacteria (*Agrobacterium* species, etc.), and they are never neatly clustered into the respective genera they are supposed to belong to (Fig. 2) (de Lajudie et al., 1999; Sawada, 2003a,b; Sawada et al., 1993; Willems and Collins, 1993; Yanagi and Yamasato, 1993; Young et al., 2001).

Symbiotic strains of the *Rhizobium* species possess a Sym plasmid which harbors genes involved in nodulation and nitrogen fixation (Kuykendall et al., 2003b; Spaink et al., 1998). Meanwhile, *Agrobacterium* is a genus defined on the basis of phytopathogenicity (pathogenic ability to cause crown gall and hairy root diseases to plants) (Kerstens and De Ley, 1984; Young et al., 2003), and the major genes involved in phytopathogenicity are carried by either a Ti (tumor-inducing) or Ri (root-inducing) plasmid (Goodner et al., 2001; Moriguchi et al., 2001; Spaink et al., 1998; Suzuki et al., 2000; Wood et al., 2001). These three types of symbiotic and phytopathogenic plasmids are phylogenetically very closely related to one another, and are considered to have originated from a common ancestor (Freiberg et al., 1997; Moriguchi et al., 2001). Many of them are transmissible plasmids (although Ti and Ri plasmids are considered to be conjugative, some Sym plasmids are known to be mobilizable by other conjugative elements), and possess the ability to transfer among the members of Group 1 (Spaink et al., 1998). Furthermore, it has been confirmed that the

genes located on a plasmid, which has been transferred into another member of Group 1, express and function normally in the transconjugant. Thus, *Agrobacterium* strains, into which a Sym plasmid has been transferred, acquire the ability to form root nodules and fix nitrogen symbiotically (Brom et al., 1988; Martinez et al., 1987; Novikova and Safronova, 1992), whereas the *Rhizobium* strains, which have obtained a Ti plasmid, incite galls on plants (Hooykaas et al., 1977). Therefore, it is presumed that certain dynamic changes and interactions due to the loss and/or transfer of plasmids have been actually occurring in the process of evolution among the members of Group 1, i.e., the loss of a plasmid from one strain results in the loss of the symbiotic ability or phytopathogenicity, and the transfer of a plasmid results in the gain of such characters for the transconjugant (Kuykendall et al., 2003b; Laguerre et al., 2001; de Lajudie et al., 1999; Segovia et al., 1991; Suominen et al., 2001). This is considered to be the reason for the strains with these different types of plasmids to be randomly intermingled as members of Group 1.

At the time when the classification of these genera was established, the understanding of how the above-mentioned genetic and ecological factors would affect classification was not sufficient. Accordingly, the ability of symbiotic nitrogen fixation and phytopathogenicity were considered to be primary characters in anthropocentric terms, and were regarded as primary taxonomic criteria for conducting classification at the generic level. As a result, the strains harboring particular plasmids became selected artificially from Group 1 and classified into the respective genera, thereby causing the confusion that the members of the three genera are randomly intermingled on the phylogenetic tree (Fig. 2) (Kuykendall et al., 2003b; de Lajudie et al., 1999; Sawada, 2003b; Sawada et al., 1993; Young et al., 2001, 2003). In other words, these three genera included in Group 1 were established on the basis of an artificial classification which does not conform to the natural classification and systematics which BMSB (2003) follows.

To decide the direction of the emendation of the classification of Group 1 at the generic level in the future, the following three options are considered to be available at present (Farrand et al., 2003; Gaunt et al., 2001; Kuykendall et al., 2003b; Sawada, 2003a,b; Young et al., 2001, 2003):

A) The current artificial classification

within Group 1 shall be retained as is because this system has hitherto been considered to excel in practicability and expediency.

B) All the genera within Group 1 shall be amalgamated into one single genus in conformity with the results of phylogenetic analysis.

C) Each subordinate lineage in Group 1 (e.g., IG I and IG II (abbreviations for Intra-generic Clusters I and II) as shown in Fig. 2) shall be proposed as a genus.

Regarding Option A, the problem has been pointed out that there are chances for artificially defined new genera to be born one after another as long as the current artificial classification is accepted. Specifically, it has gradually been clarified that Group 1 comprises not only legume symbionts and phytopathogenic bacteria, but also various "environmental microbes," such as *Blastobacter* species (*Bla. aggregatus* and *Bla. capsulatus*) which are aquatic budding bacteria isolated from fresh water habitats, arsenite-oxidizing bacteria (NT-26 and "*Agrobacterium albertimagni*"), and a denitrifying bacterium (*Pseudomonas* sp. G-179), some of which are shown in Fig. 2 (Bedzyk et al., 1999; Effendi et al., 2000; Hugenholtz et al., 1994; Santini et al., 2000; Sawada, 2003b). With the present artificial classification, "species which exclusively comprise strains with neither the ability of symbiotic nitrogen fixation nor phytopathogenicity" are not accepted as new formal members of the genera *Rhizobium*, *Allorhizobium*, or *Agrobacterium*. Accordingly, it is considered likely that new artificial genera will be established for describing the species that do not conform to any of the criteria of these three currently described genera. In addition, the taxonomic position of such non-conforming species belonging to new artificial genera will be variable at the generic level because the other genera of Group 1 are defined based on the symbiotic ability or phytopathogenicity encoded on the transmissible plasmids, and members of such non-conforming species may gain the conforming characters through the acquisition of the transmissible plasmids, which will eventually result in making the taxonomy unstable. Thus, Option A is considered to aggravate the confusion in both the classification and identification of members belonging to Group 1.

Option C has the following problems (Gaunt et al., 2001; de Lajudie et al., 1999; Sawada, 2003b): 1) It was confirmed that the bootstrap probability, which shows the unity of a lineage, was low for both the IG I

and II lineages (Fig. 2). 2) The phylogenetic position of species near the root of each lineage is not necessarily stable, and some species were observed to move between these two lineages depending on the analytical conditions. These tendencies have also been recognized in the analyses using *atpD* and *recA* (Gaunt et al., 2001). Furthermore, 3) no discontinuity has been found in phenotypic characters between IG I and IG II, so these lineages have not been differentiated based on their phenotypic characters. These analytical results suggest that neither IG I nor IG II meets the requirements for being recognized as a taxonomic or evolutionary unit; thus it is inappropriate under the present circumstances to define IG I and IG II as two separate genera (Sawada, 2003b). Continued studies are required for carefully examining the taxonomic positioning of such subordinate lineages as IG I and IG II in Group 1.

Option B is considered to be most preferable under the present circumstances, based on the following reasons: 1) All the known species belonging to the genera *Agrobacterium*, *Allorhizobium*, and *Rhizobium* can be clustered into Group 1 (although the value of the bootstrap probability, which shows the unity of Group 1, is 72% as shown in Fig. 2, some other analyses based on the maximum likelihood method (Sawada, 2003b; Young et al., 2001) and analyses using *atpD* and *recA* as markers (Gaunt et al., 2001) have provided higher bootstrap probability values). 2) Species of these three genera share many common features in regard to phenotypic and chemotaxonomic characters, and there is discontinuity of such characters on the boundary with the *Sinorhizobium* species that are their close relatives (Kuykendall and Dazzo, 2003; Kuykendall et al., 2003a, b; Tighe et al., 2000; Yokota et al., 1993; Young et al., 2001, 2003). 3) Environmental microbes which have not been confirmed to possess either the ability of symbiotic nitrogen fixation or phytopathogenicity (various soil- or water-borne unclassified bacteria including the so-called xenobiotic-degrading bacteria) are also included in Group 1; however, if Group 1 is described as one single genus named *Rhizobium*, they can be described as "new formal species of the genus *Rhizobium*" by slightly emending the generic definitions whenever necessary (Sawada, 2003b).

As has been discussed so far, Option B is considered to be appropriate from the viewpoints of systematics and practical expediency, so the authors and their colleagues have proposed to amalgamate the

genera *Agrobacterium*, *Allorhizobium*, and *Rhizobium* into one single genus called *Rhizobium* as the first step for future emendations (Young et al., 2001). It must be noted that this proposal is not binding, but is one of the candidates for straightening up the classification of Group 1. In future studies, the adequacy of these three options (Options A to C) needs to be carefully examined, based also on polyphasic taxonomy, using not only 16S rDNA but also other markers with higher resolution, in due consideration of practical expediency of the generic classification and identification.

2.2. Monophyletic Group 2 (*Sinorhizobium* and *Ensifer* species)

Group 2 is a sister group to Group 1, and the phylogenetic positions of these two groups are very closely related to each other (Fig. 2). Species of the genus *Sinorhizobium*, each of which includes legume symbionts, constitute Group 2, together with *Ensifer adhaerens*, the one and only species of the genus *Ensifer*, which has been found to include legume symbionts just recently. This sub-chapter will focus on these two genera which constitute Group 2.

2.2.1. Past

Rhizobium fredii, a fast grower isolated from *Glycine soja* and *G. max* cv. Peking in the People's Republic of China, was described as a new species of the genus *Rhizobium* in 1984 (Scholla and Elkan, 1984). However, the results of numerical taxonomy using various phenotypic characters revealed that *R. fredii* was different from other *Rhizobium* species, and on the basis of this finding a new genus named *Sinorhizobium* was established based on *Sinorhizobium fredii* in 1988 (Fig. 1) (Chen et al., 1988). Subsequently, it was suggested that this generic division was not justified because *R. fredii* appeared to be closely related to *R. meliloti*, based on a comparative analysis of partial 16S rDNA sequences (Jarvis et al., 1992). Nevertheless, further studies based on polyphasic taxonomy revealed that *R. fredii* and *R. meliloti*, as well as the unclassified symbionts of *Sesbania* and *Acacia* (later named *S. teranga* and *S. sahelii*), were in a phylogenetic clade separate from other typical *Rhizobium* species, leading to a proposal that these four species (*S. fredii*, *S. meliloti*, *S. teranga*, and *S. sahelii*) should be classified into the genus *Sinorhizobium* and kept separate from the genus *Rhizobium* at the generic level (de Lajudie et al., 1994).

Ensifer adhaerens was originally described as a predatory soil-borne bacterium that attacked other bacteria (Casida, 1982); however, this species has recently been shown to include legume symbionts based on the fact that some strains with symbiotic nitrogen-fixing capacity isolated from the root nodules of legumes were identified to be *E. adhaerens* (Willems et al., 2003).

2.2.2. Present

In BMSB (2003), the genera *Sinorhizobium* and *Ensifer* are described as members constituting the family *Rhizobiaceae* of the order *Rhizobiales* of the class *Alphaproteobacteria*, together with the genera *Rhizobium*, *Allorhizobium*, and other genera (Fig. 1, Table 1). The genus *Sinorhizobium* is defined therein as "a group of species which grow fast and produce acid on a YMA medium, and include strains that have the ability of performing symbiotic nitrogen fixation," which is basically identical to that of the genus *Rhizobium*. There are now ten species (*S. arboris*, *S. fredii*, *S. kostiense*, *S. kummerowiae*, *S. medicae*, *S. meliloti*, *S. morelense*, *S. sahelii*, *S. teranga*, and *S. xinjiangense*) described in this genus (Kuykendall et al., 2003a; Wang et al., 2002; Wei et al., 2002). Of these, *S. fredii*, *S. meliloti*, and *S. teranga* are subdivided into several varieties or subgroups depending on the differences in their host specificity and/or various phenotypic characters (Table 1). (Recently, "*Sinorhizobium americanus*," a new *Sinorhizobium* species nodulating native *Acacia* spp. in Mexico, was proposed (Toledo et al. (2003) *System. Appl. Microbiol.*, **26**, 54–64).)

Group 2, which contains the genus *Sinorhizobium*, is in a sister group relationship with Group 1 discussed in Sub-Chapter 2.1. (Fig. 2), and these two groups share many similar phenotypic characters. The question as to whether or not a generic boundary should be established between these two groups has been a controversial issue since the genus *Sinorhizobium* was first proposed. With the progress of research, however, it has gradually been clarified that these two groups are separate monophyletic groups, based on phylogenetic analyses using various markers (Gaunt et al., 2001; de Lajudie et al., 1994; Sawada, 2003b; Turner and Young, 2000; Young et al., 2001) and that they also differ in details of fatty acid composition (Tighe et al., 2000). Thus, Groups 1 and 2 are considered to represent separate genera at present (Kuykendall et al., 2003a).

E. adhaerens is the only valid member constituting

the genus *Ensifer*, which is described as “a predatory soil-borne bacterium that is capable of adhering to and lysing other bacteria” (Casida, 1982). It has already been confirmed that a nonsymbiotic *E. adhaerens* strain gains the ability of forming nitrogen-fixing nodules when the Sym plasmid of *R. tropici* is transferred into it (Rogel et al., 2001). Furthermore, it has recently been confirmed that several strains, which were isolated from root nodules and taxonomically identified as *E. adhaerens*, are capable of performing symbiotic nitrogen fixation (Willems et al., 2003). Thus, *E. adhaerens* is now recognized as a species containing legume symbionts (Fig. 1, Table 2).

2.2.3. Future

Recently, Willems et al. (2003) proposed that the genera *Sinorhizobium* and *Ensifer* should be amalgamated, based on the following findings: *E. adhaerens* is included in the same monophyletic group as *Sinorhizobium* species on the 16S rDNA tree (indicated by ● in Group 2 in Fig. 2) (Wang et al., 2002; Willems et al., 2003); the same result is obtained by constructing a *recA* tree (Willems et al., 2003); nonsymbiotic *E. adhaerens* strains are able to form nitrogen-fixing nodules upon acquisition of a Sym plasmid (Rogel et al., 2001); and legume symbionts identified as *E. adhaerens* have been isolated from natural environments (Willems et al., 2003). Willems et al. (2003) have submitted a Request for an Opinion to the Judicial Commission of the International Committee on Systematics of Prokaryotes to propose that the generic name *Sinorhizobium* should be preserved over the older name *Ensifer* to avoid confusion and *E. adhaerens* should be replaced with *S. adhaerens*, because the genus *Sinorhizobium* contains many important legume symbionts and its research has much more advanced than that of the genus *Ensifer*.

Meanwhile, it has been reported that Group 2 contains various unclassified soil-borne bacteria including xenobiotic-degrading bacteria and an isolate from the gut of termite, in addition to *Sinorhizobium* species and *E. adhaerens* (Song et al., 2000; Willems et al., 2003) (these nonsymbionts are not shown in Fig. 2). This suggests that Group 2 is richer in diversity, containing both legume symbionts and nonsymbionts, than has previously been thought. As with *Rhizobium* and *Al-lorhizobium* species in Group 1, this is considered attributable to the fact that many of the genes of *Sinorhizobium* species involved in symbiotic nitrogen fixation exist on a transmissible Sym plasmid (Galibert et al.,

2001; Spaink et al., 1998) and that the transfer and/or loss of such plasmids have been actually occurring during the process of evolution (Haukka et al., 1998; Laguerre et al., 2001). Thus, further research needs to be conducted for examining the classification of Group 2 at the generic level on the basis of the three options (Options A to C) discussed in the sub-chapter on Group 1, in consideration of the proposal of Willems et al. (2003).

2.3. Monophyletic Group 3 (*Mesorhizobium* species)

Group 3 contains species of the genus *Mesorhizobium*, each of which includes legume symbionts, together with nonsymbionts, such as species of the genera *Aminobacter* and *Pseudaminobacter* (Fig. 2).

2.3.1. Past

In the early 1990's, legume symbionts were classified into four genera: fast growers into the genera *Rhizobium*, *Sinorhizobium*, and *Azorhizobium*, and slow growers into the genus *Bradyrhizobium* (Fig. 1). However, as phylogenetic inferences based on 16S rDNA comparative analysis showed that five *Rhizobium* species, i.e., *R. loti*, *R. huakuii*, *R. ciceri*, *R. mediterraneum*, and *R. tianshanense*, were clustered into one monophyletic group at a position far from other fast growers, these species became considered to be an independent “*R. loti* group” that should be separated from other fast growers (Young and Haukka, 1996). Subsequently, the members of this group were found to have specific phenotypic characters, such as being slower in growth on a YMA medium than the typical fast growers, and producing acid unlike slow growers, and based on these findings a formal proposal was presented in 1997 that these members should be transferred to the new genus *Mesorhizobium* as *M. loti*, *M. huakuii*, *M. ciceri*, *M. mediterraneum*, and *M. tianshanense* (Fig. 1, Table 1) (Jarvis et al., 1997). The word “meso” prefixed to the generic name means “intermediate,” which thereby denotes that the phylogenetic position and the growth rate of the members of this genus are between those of fast growers and slow growers. Thereafter, more isolates belonging to this genus were found, and described as *M. amorphae*, *M. chacoense*, and *M. plurifarium*.

2.3.2. Present

In BMSB (2003), the genus *Mesorhizobium* is described as a member constituting the family *Phyllobacteriaceae* of the order *Rhizobiales* of the class *Al-*

phaproteobacteria (Fig. 1, Table 1). A total of eight species (*M. amorphae*, *M. chacoense*, *M. ciceri*, *M. huakuii*, *M. loti*, *M. mediterraneum*, *M. plurifarum*, and *M. tianshanense*) have been described in this genus (Chen et al., 2003).

As a wider range of strains of various origins have been examined, it has become increasingly clear that among the *Mesorhizobium* strains are those which do not comply with some of the characters considered to be specific to this genus when it was first described. Although the *Mesorhizobium* species were originally distinguished by their intermediate growth rate on a YMA medium, some members have been shown to grow as fast as fast growers or as slowly as slow growers (van Berkum and Eardly, 1998; Chen et al., 2003). In addition, although the genes of *Mesorhizobium* species involved in nodulation and nitrogen fixation used to be considered to exist on the chromosome (Jarvis et al., 1997; Kaneko et al., 2000; Martinez et al., 1990), they have been found on a megaplasmid in some strains of *M. amorphae* and *M. huakuii* (Wang et al., 1999; Zou et al., 1997). Thus, it has gradually been clarified that *Mesorhizobium* strains are not homogeneous but are richly variable in regard to these characters that used to be considered to characterize this genus.

From the phylogenetic viewpoint, it has been confirmed that *Mesorhizobium* species are invariably clustered into a monophyletic group that is independent from other legume symbionts irrespective of the markers used for analysis including 16S rDNA and protein-coding genes (Fig. 2) (Gaunt et al., 2001; Turner and Young, 2000; Young et al., 2001). In addition, a fatty acid composition pattern particular to *Mesorhizobium* species has been identified (Tighe et al., 2000). Furthermore, the number of copies of rRNA operons on the chromosome is three for fast growers (*Rhizobium*, *Sinorhizobium*, and *Azorhizobium* species) (Kündig et al., 1995) and two for *M. loti* (Kaneko et al., 2000), which is considered to be a distinctive character of *Mesorhizobium* species.

2.3.3. Future

The same outstanding taxonomic problem of the *Rhizobium*, *Allorhizobium*, *Sinorhizobium*, and *Ensifer* species described before applies to the *Mesorhizobium* species. Specifically, Group 3 comprises not only *Mesorhizobium* species but also several other bacteria. Although all the legume symbionts contained in Group 3 are *Mesorhizobium* species, various nonsym-

bionts are also intermingled with them, including xenobiotic-degrading bacteria and other environmental microbes (*Aminobacter* species, *Pseudaminobacter* species, and numerous other unclassified bacteria (IMB-1, Iso26, etc.)) (some of these nonsymbionts are shown in Fig. 2).

As mentioned before, although the symbiotic genes of some strains belonging to *M. amorphae* and *M. huakuii* are located on the plasmid, such genes of many other *Mesorhizobium* strains are considered to be present within a symbiosis island on the chromosome (Jarvis et al., 1997; Kaneko et al., 2000; Martinez et al., 1990). However, the chromosomal symbiosis island is also transmissible and capable of conducting horizontal transfer between strains in soil and under laboratory conditions (Sullivan and Ronson, 1998; Sullivan et al., 1995). The molecular mechanism of the horizontal transfer of the symbiosis island has gradually been clarified, i.e., the symbiosis island contains conjugative transfer genes (a *trb* operon and *tra* genes) and a P4-type integrase gene, and lacks plasmid replication genes, suggesting that this island is a site-specific conjugative transposon, which is inserted into the phenylalanine tRNA gene on the recipient's chromosome after having conjugatively transferred from the donor (Kaneko et al., 2000; Sullivan and Ronson, 1998; Sullivan et al., 2002). Accordingly, it is likely that the reason why the *Mesorhizobium* species and nonsymbionts are intermingled in Group 3 is because this mobile symbiosis island has frequently been transferred to other members or lost from the chromosome during the process of evolution (Finan, 2002; Sullivan et al., 2002). Thus, it is considered that the definitions and circumscriptions of the genus *Mesorhizobium* need to be examined as with the case of Groups 1 and 2. Specifically, based on Options A to C discussed in the sub-chapter on Group 1, comparative studies should be zealously conducted, based on the viewpoint of polyphasic taxonomy, for properly classifying *Mesorhizobium* species, environmental microbes that are their close relatives, and species of the genus *Phyllobacterium* that is in a sister relationship with Group 3 (Fig. 2).

2.4. Monophyletic Group 4 (*Bradyrhizobium* and *Blastobacter* species)

Species of the genus *Bradyrhizobium*, each of which includes legume symbionts, *Blastobacter denitrificans*, which has been confirmed to contain symbiotic nitro-

gen-fixing strains just recently, and various nonsymbionts, are intermingled in Group 4 (Fig. 2). This subchapter will focus on the genera *Bradyrhizobium* and *Blastobacter*.

2.4.1. Past

As mentioned in the sub-chapter on Group 1, legume symbionts were originally all classified into the single genus *Rhizobium*, but were reclassified at the generic level based on the differences in their phenotypic and genotypic characters, i.e., slow growers, which grow slowly and produce alkali on a YMA medium, were separated from fast growers and made independent as the genus *Bradyrhizobium* (Fig. 1) (Jordan, 1982, 1984). At the time when the first edition of BMSB (1984) was published, *B. japonicum* was the only species formally described as the member constituting this genus (Jordan, 1984). However, as more isolates from various origins have been examined, this genus has gradually been revealed to be rich in serological and genotypical diversity. In particular, soybean-originating *Bradyrhizobium* strains were found to be classifiable into several groups based on their DNA-DNA similarity (Hollis et al., 1981), and one of these groups (Group II) was described as a new species (*B. elkanii*) (Kuykendall et al., 1992) (Table 1). Recently, there have been successive reports on new species (*B. liaoningense* and *B. yuanmingense*) belonging to this genus (Xu et al., 1995; Yao et al., 2002).

The genus *Blastobacter* is an artificial genus established by amalgamating aquatic bud- and cluster-forming bacteria in a single taxon (Hirsch and Müller, 1985). In 2002, *Bla. denitrificans*, which is one of the *Blastobacter* species, was found to be very closely related to *Bradyrhizobium* species in terms of evolution (indicated by ● in Group 4 in Fig. 2), and to contain a strain that is capable of conducting symbiotic nitrogen fixation by forming root nodules on *Aeschynomene indica* (van Berkum and Eardly, 2002).

2.4.2. Present

In BMSB (2003), the genera *Bradyrhizobium* and *Blastobacter* are both described as members constituting the family *Bradyrhizobiaceae* of the order *Rhizobiales* of the class *Alphaproteobacteria* (Fig. 1, Table 1). The genus *Bradyrhizobium* is defined therein as "a group of species which have either a polar or subpolar flagellum, grow slowly and produce alkali on a YMA medium, and include strains which form root nodules on legumes," and four species (*B. japonicum*, *B. elka-*

nii, *B. liaoningense*, and *B. yuanmingense*) are described to constitute this genus (Kuykendall, 2003a; Yao et al., 2002). The characters reported to characterize the chromosome of *Bradyrhizobium* species are that most of the genes involved in nodulation and nitrogen fixation are clustered within the symbiosis island on the chromosome (Göttfert et al., 2001; Kaneko et al., 2002), and that only one or two rRNA operons are present on their chromosome (Kaneko et al., 2002; Kündig et al., 1995).

Meanwhile, the genus *Blastobacter* is an artificial and polyphyletic genus defined to comprise aquatic bud- and cluster-forming bacteria (van Berkum and Eardly, 2002; Hirsch and Müller, 1985; Hugenholtz et al., 1994). At present, four species (*Bla. denitrificans*, *Bla. aggregatus*, *Bla. capsulatus*, and *Bla. henrici*) are described as members constituting this genus. Of these, *Bla. denitrificans* is the only species that belongs to Group 4 (Fig. 2), and its type strain has the ability of symbiotic nitrogen fixation (van Berkum and Eardly, 2002).

2.4.3. Future

There are various outstanding problems in the descriptions of *Bradyrhizobium* species. Specifically, the results of phylogenetic analyses based on 16S rDNA and ITS regions and analyses based on DNA-DNA similarity have revealed that *B. japonicum* strains are too diverse to be grouped into one species (van Berkum and Fuhrmann, 2000; Willems et al., 2001a, b; Young and Haukka, 1996). The genus *Bradyrhizobium* also contains many unclassified wild strains that are considered to deserve classification as new *Bradyrhizobium* species; thus, it is an urgent necessity to analyze these strains and proceed with their description at the species level appropriately (Kuykendall, 2003a; Willems et al., 2001b; Young and Haukka, 1996). Incidentally, when the taxonomic position is uncertain, the common practice for expressing wild strains is to follow the generic name with the scientific name of their host plants in parentheses (Kuykendall, 2003a), e.g., *Bradyrhizobium* sp. (*Acacia*).

The problem with the current species definitions and circumscriptions also applies to *Bla. denitrificans*. Phylogenetically, *Bla. denitrificans* is very closely related to *Agromonas oligotrophica*, which is a free-living diazotroph, and to some photosynthetic *Bradyrhizobium* strains which induce stem nodules on *Aeschynomene* spp. (ORS278 is shown as their representative in Fig. 2), and they are located almost at the same position

on the phylogenetic tree (Fig. 2) (Molouba et al., 1999; Willems et al., 2001a). Because 16S rDNA differences among these strains are 0.7% or lower, it has been suggested that these strains should be amalgamated into one species; thus, it is necessary to proceed with taxonomic studies of these strains by accumulating more data of their DNA-DNA similarity (van Berkum and Eardly, 2002; Willems et al., 2001a).

It has also been suggested that the photosynthetic *Bradyrhizobium* strains should be allocated to a new genus "*Photorhizobium*" and distinguished from other *Bradyrhizobium* species at the generic level (Eaglesham et al., 1990). However, the genus "*Photorhizobium*" has not been validly published yet because whether or not the photosynthetic *Bradyrhizobium* strains can constitute a separate genus is still in question due to the fact that they belong to the same monophyletic group as other *Bradyrhizobium* species (Fig. 2) (van Berkum and Eardly, 2002; Willems et al., 2001b). Meanwhile, the feasibility of dividing these strains into several groups at the species level has also been pointed out because these strains have been shown to be genotypically heterogeneous based on the results of examining the DNA-DNA similarity of numerous photosynthetic *Bradyrhizobium* strains with diverse origins (Willems et al., 2001b). Thus, the photosynthetic *Bradyrhizobium* strains need to be carefully examined together with other members of Group 4 based on the polyphasic taxonomic approach for properly determining their taxonomic position at the generic and species levels.

As with the case of Groups 1 to 3 discussed before, the classification of Group 4 is clearly in need of careful investigation at the generic level because studies show that several genera with different physiological and ecological characters are intermingled (Kamagata et al., 1997; Saito et al., 1998; Song et al., 2000; Willems et al., 2001a). Specifically, nonsymbionts, such as *Agromonas* spp. (free-living diazotrophs), *Nitrobacter* spp. (nitrifying soil bacteria), *Afipia* spp. (mammalian pathogens), *Rhodopseudomonas* spp. (phototrophic bacteria), and other environmental microbes whose taxonomic positions have not been established (halobenzoate-degrading denitrifying bacteria, 2,4-D-degrading bacteria, etc.), are included in Group 4 together with such legume symbionts as *Bradyrhizobium* strains (including those treated as "*Photorhizobium*" strains) and *Bla. denitrificans*. Only representative strains of these nonsymbionts are

shown in Fig. 2.

These strains share the common basic character that they are all oligotrophic bacteria and grow slowly on laboratory media, and are confirmed to be grouped into one lineage on the phylogenetic tree (Fig. 2) (Minamisawa and Mitsui, 2000; Saito et al., 1998). In addition, it has been pointed out that, as with *Mesorhizobium* species, the chromosomal symbiosis island of *Bradyrhizobium* species may be transmissible (Göttfert et al., 2001). Genome analyses showed recently that this symbiosis island is a mobile element which is horizontally transferred and then integrated into the val-tRNA gene on the recipient's chromosome by the mediation of conjugative transfer genes and an integrase gene (Kaneko et al., 2002). Phylogenetic analyses suggested that *nifD* of *Bradyrhizobium* species has expanded distribution by conducting horizontal transfer in the process of evolution (Parker et al., 2002). Among the nonsymbionts included in Group 4 are strains with imperfect chromosomal symbiosis islands where some symbiosis-related genes are missing (Saito et al., 1998). These findings have led to the inference that the chromosomal symbiosis island has been transferred among these closely related strains, but that some strains have lost some symbiotic genes in the course of time, resulting in the irregular and sporadic presence of legume symbionts in Group 4 (Minamisawa and Mitsui, 2000; Parker et al., 2002). Thus, it is considered that the definitions and circumscriptions of the genera *Bradyrhizobium* and *Blastobacter* need to be scrutinized and emended in the future based on polyphasic taxonomy in consideration of the three options (Options A to C) as with Groups 1 to 3 (van Berkum and Eardly, 1998, 2002; Willems et al., 2001a).

2.5. Monophyletic Group 5 (*Methylobacterium* species)

Recently, it has been shown that some *Methylobacterium* strains, which constitute Group 5, perform nitrogen fixation in symbiotic associations with legumes (Fig. 2).

2.5.1. Past

In 2001, the symbiotic diazotrophs isolated from the root nodules of *Crotalaria podocarpa*, *C. perrottetii*, and *C. glaucoidea* in Senegal were analyzed on the basis of their phenotypic and phylogenetic characters, and identified to be *Methylobacterium* strains (Fig. 2, Table 1) (Sy et al., 2001). As none of the known

species of the genus *Methylobacterium* have 16S rDNA similarity higher than 95% with these isolates, these isolates are considered to deserve classification as a new species of the genus *Methylobacterium*, perhaps named "*Methylobacterium nodulans*" (Sy et al., 2001); however, this species name has not been validly published yet.

2.5.2. Present

In BMSB (2003), the genus *Methylobacterium* is described as a member constituting the family *Methylobacteriaceae* of the order *Rhizobiales* of the class *Alphaproteobacteria* (Fig. 1, Table 1). Fourteen species described in this genus are all facultatively methylotrophic bacteria. None of these known species have been shown to possess the ability of conducting symbiotic nitrogen fixation, so "*Met. nodulans*" is the only known symbiotic member of this genus. Nevertheless, the name "*Met. nodulans*" has not been validly published according to the International Code of Nomenclature of Bacteria (Bacteriological Code).

The facts that no *Methylobacterium* species other than "*Met. nodulans*" have been confirmed to have the ability of symbiotic nitrogen fixation and that the structures of NodA of "*Met. nodulans*" and *Bradyrhizobium* species are similar, have led to the inference that "*Met. nodulans*" gained this ability by obtaining symbiotic genes which were horizontally transferred from *Bradyrhizobium* species (Sy et al., 2001).

2.5.3. Future

Unlike Groups 1 to 4 discussed so far, there seems to be no particular problem with the classification of Group 5 at the generic level. In other words, the definitions and circumscriptions of the genus *Methylobacterium* are widely accepted because of the following facts: 1) the members of the genus *Methylobacterium* are confirmed to form a separate and compact monophyletic group with a high bootstrap value (Fig. 2); 2) the monophyletic group is comprised only of *Methylobacterium* species; and 3) there are several phenotypic characters specific to this genus.

"*Met. nodulans*" is indubitably a member of the genus *Methylobacterium*, based on the results of phylogenetic analyses, as well as its phenotypic characters such as its being facultatively methylotrophic (Sy et al., 2001). Thus, the only outstanding issue regarding Group 5 is to validly publish "*Met. nodulans*" as a new species by following the formal procedures. There seems to be no problem for "*Met. nodulans*" to be accepted as a formal member of the genus *Methylobac-*

terium without emending the definitions of this genus, if only this new species name is validly published along with necessary data, including those on DNA-DNA similarity.

2.6. Monophyletic Group 6 (*Azorhizobium* species)

Azorhizobium caulinodans, which includes stem-nodule bacteria, and species of the genera *Xanthobacter* and *Aquabacter*, which are nonsymbionts, are intermingled in Group 6 (Fig. 2).

2.6.1. Past

The symbiotic diazotrophs isolated from the stem-nodules of *Sesbania rostrata* in Senegal were confirmed to have phenotypic and genotypic characters that are greatly different from those of other known legume symbionts, so they were described as a new species of a new genus named *Azorhizobium caulinodans* in 1988 (Fig. 1, Table 1) (Dreyfus et al., 1988).

2.6.2. Present

In BMSB (2003), the genus *Azorhizobium* is described as a member constituting the family *Hyphomicrobiaceae* of the order *Rhizobiales* of the class *Alphaproteobacteria* (Fig. 1, Table 1), and characterized as "having peritrichous flagella, fixing atmospheric nitrogen under microaerobic conditions, utilizing lactate, being fast in growth but unable to utilize mannitol unlike other legume symbionts, and forming stem- and root-nodules on *Sesbania rostrata*" (Kuykendall, 2003b). *Azo. caulinodans* is the only species described to constitute this genus.

2.6.3. Future

Although *Azo. caulinodans* is the only species described in this genus at present, there are several other isolates (genomic species 2) which are considered to deserve classification as a new species of this genus based on DNA-DNA similarity (Rinaudo et al., 1991).

In addition to *Azorhizobium* species, *Xanthobacter* and *Aquabacter* species, which are nonsymbionts, are also intermingled in Group 6 (Fig. 2). Although there have been reports that these three genera are phylogenetically closely related to one another (Dreyfus et al., 1988; Rainey and Wiegand, 1996), opinions about their treatment have differed. Dreyfus et al. (1988) proposed that the genus *Azorhizobium* should be made independent and differentiated from the other two genera at the generic level on the ground of the difference in their phenotypic characters including the ability of

symbiotic nitrogen fixation, while Rainey and Wiegel (1996) suggested that these three genera could be amalgamated into one genus called *Xanthobacter*. The revision of classification of Group 6 at the generic level can only be determined by further studies based on polyphasic taxonomy in consideration of Options A to C, exactly as with the cases of Groups 1 to 4 (Rainey and Wiegel, 1996).

2.7. Monophyletic Group 7 (*Devosia* species)

Legume symbionts closely related to *Devosia riboflavina* were isolated just recently, and have been classified as members constituting the genus *Devosia* (Fig. 2).

2.7.1. Past

Several strains isolated from the root nodules of *Neptunia natans* in India, which are fast growers with the ability of symbiotic nitrogen fixation, were considered to be members of the genus *Devosia* because a phylogenetic analysis showed that they were more closely related to *D. riboflavina* than any other known bacterial species (Fig. 2, Table 1) (Rivas et al., 2002). However, given that their 16S rDNA similarity with *D. riboflavina* is relatively low (95.9%) and that they are the first legume symbionts ever found to belong to the genus *Devosia*, it is considered that these strains represent a new species of the genus *Devosia*, perhaps to be named "*D. neptuniae*" (Rivas et al., 2002), although this has not been validly published yet.

2.7.2. Present

In BMSB (2003), the genus *Devosia* is described as a member constituting the family *Hyphomicrobiaceae* of the order *Rhizobiales* of the class *Alphaproteobacteria* (Fig. 1, Table 1). *D. riboflavina*, which is a soil-borne bacterium, is the only species formally described in this genus, and its characters so far identified include its possession of long-chain 3-hydroxy fatty acids (Nakagawa et al., 1996). Unlike other *Devosia* strains, however, "*D. neptuniae*" has been confirmed to have the ability of symbiotic nitrogen fixation. Incidentally, preparations for validly publishing "*D. neptuniae*" as a new species are currently underway by Rivas et al. (Rivas et al., 2002).

Because *nodD* and *nifH* of "*D. neptuniae*" are present on a plasmid and because their sequences are very similar to those of *R. tropici*, it is inferred that these symbiotic genes were transferred from *R. tropici* or its relatives to "*D. neptuniae*" by the mediation of the transmissible plasmid (Rivas et al., 2002).

2.7.3. Future

Currently, "*D. neptuniae*" is considered to be a member of the genus *Devosia* merely based on the results of a phylogenetic analysis (Rivas et al., 2002). However, as Rivas et al. are continuously analyzing the fatty acid composition and various other characters based on polyphasic taxonomy (Rivas et al., 2002), "*D. neptuniae*" will be validly published as a new species of this genus when sufficient data that support formal publication of the species epithet is available. (Recently, Rivas et al. described the detailed features of "*D. neptuniae*" (Rivas et al. (2003) *System. Appl. Microbiol.*, **26**, 47–53).)

Although *D. riboflavina* is the only species formally described in this genus at present (Nakagawa et al., 1996), Group 7 contains various unclassified bacteria isolated from soils and the travertine of hot springs (strains BSV03, Bici23, and SM1E02 are shown as examples in Fig. 2), in addition to *D. riboflavina* and "*D. neptuniae*." Thus, *Devosia* species, together with these unclassified environmental microbes, should be further examined on the basis of polyphasic taxonomy to refine the definitions and circumscriptions of the genus *Devosia* and to establish the species that represent the genus.

2.8. Monophyletic Group 8 (*Burkholderia* species)

Recently, it has been reported that legume symbionts are included in the genus *Burkholderia*, which constitute Group 8 (Fig. 2).

2.8.1. Past

The results of phylogenetic analysis have shown that symbiotic diazotrophs (strains STM678 and STM815) isolated from the root nodules of *Aspalathus carnosa* and *Machaerium lunatum*, which are tropical legumes, in the Republic of South Africa and French Guiana, respectively, constitute one independent monophyletic group together with *Burkholderia* species (Fig. 2) (Moulin et al., 2001). However, the fact that the 16S rDNA similarity between these strains and any of the known *Burkholderia* species is less than 97% suggests that these strains may deserve classification as new species of the genus *Burkholderia* (Moulin et al., 2001).

2.8.2. Present

In BMSB (2003), the genus *Burkholderia* is described as a member constituting the family *Burkholderiaceae* of the order *Burkholderiales* of the class *Betaproteobacteria* (Fig. 1, Table 1). The genus *Burk-*

holderia contains diverse species with different physiological and ecological properties, which were isolated from soils, plants, animals, and humans. Twenty-four species have been described in this genus. However, none of the known species have been confirmed to have the ability of symbiotic nitrogen fixation, and only two *Burkholderia* strains, i.e., STM678 and STM815, were confirmed to have this ability at present (Moulin et al., 2001).

Based on the fact that high *nodAB* similarity is observed between these strains and legume symbionts of the class *Alphaproteobacteria*, it is inferred that the symbiotic genes have horizontally transferred among strains, even crossing the boundary between these classes (Moulin et al., 2001).

2.8.3. Future

As with the genus *Methylobacterium* of Group 5, no problem has hitherto been pointed out regarding the classification of Group 8 at the generic level. In other words, the definitions and circumscriptions of the genus *Burkholderia* are well established because of the following facts: 1) the members of the genus *Burkholderia* are confirmed to form a discrete and compact monophyletic group with a high bootstrap value (Fig. 2); 2) the monophyletic group is comprised only of *Burkholderia* species; and 3) the circumscription of this genus comprises chemotaxonomic and phenotypic characters specific to this genus (Gillis et al., 1995; Yabuuchi et al., 1992, 1995).

A phylogenetic analysis has suggested that strains STM678 and STM815 are members of the genus *Burkholderia* (Moulin et al., 2001). However, these strains have only been analyzed phylogenetically, but have not undergone taxonomic examinations, nor have their phenotypic characters other than the ability of symbiotic nitrogen fixation been clarified. Thus, to describe these strains as new species, it is considered necessary to accumulate more data by analyzing more isolates based on various viewpoints of polyphasic taxonomy. There seems to be no problem for these strains to be accepted as formal members of the genus *Burkholderia* without emending the definitions of this genus, if only new species names of these strains are validly published along with necessary data following the formal procedures. (Recently, the names "*Burkholderia tuberum*" and "*Burkholderia phymatum*" were proposed for strains STM678 and STM815, respectively (Vandamme et al. (2002) *System. Appl. Microbiol.*, **25**, 507–512).)

2.9. Monophyletic Group 9 (*Ralstonia* species)

Legume symbionts were also found among the *Ralstonia* strains (Fig. 2), and these isolates were validly published as a new species of this genus.

2.9.1. Past

Symbiotic diazotrophs isolated from the root nodules of *Mimosa pudica* and *M. diplotricha* in Taiwan were analyzed based on polyphasic taxonomy, which revealed that they have the basic characters common to *Ralstonia* species and that they are included in a monophyletic group on the phylogenetic tree, which corresponds to the genus *Ralstonia* (Fig. 2) (Chen et al., 2001). Furthermore, as these isolates were found to differ from other known *Ralstonia* species in DNA-DNA similarity and phenotypic characters, they were confirmed to deserve classification as a new species of the genus *Ralstonia*, which was validly published under the name of *Ralstonia taiwanensis* (Chen et al., 2001).

2.9.2. Present

In BMSB (2003), the genus *Ralstonia* is described as a member constituting the family *Burkholderiaceae* of the order *Burkholderiales* of the class *Betaproteobacteria* (Fig. 1, Table 1). As with the genus *Burkholderia* discussed before, the genus *Ralstonia* contains various members with diversified physiological and ecological properties isolated from soils, waters, plants, and clinical specimens, and eleven species, including *Ral. taiwanensis*, are currently described as members of this genus. Among all these known *Ralstonia* species, however, *Ral. taiwanensis* is the only species confirmed to have the ability of symbiotic nitrogen fixation (Chen et al., 2001).

2.9.3. Future

As with the genera *Methylobacterium* and *Burkholderia*, which correspond to Groups 5 and 8, respectively, no problem has hitherto been pointed out regarding the classification of Group 9 at the generic level. In other words, the definitions and circumscriptions of the genus *Ralstonia* are widely accepted because Group 9 is comprised only of *Ralstonia* species which present a high bootstrap value (Fig. 2), and because chemotaxonomic and phenotypic characters specific to this genus have been detected (Yabuuchi et al., 1995).

It has been agreed from the initial stage of defining the genus *Ralstonia* that this genus comprises various species with diversified physiological and ecological properties. Furthermore, *Ral. taiwanensis* has suffi-

ciently been characterized based on polyphasic taxonomy, and its description as a *Ralstonia* species has been acknowledged as a valid publication. Thus, there seems to be no outstanding problem regarding the taxonomic treatment of *Ral. taiwanensis* at the species level.

Given that *Ral. taiwanensis* also contains strains isolated from clinical specimens in the United States (Chen et al., 2001), and that *Ral. taiwanensis* is the only species that has the ability of symbiotic nitrogen fixation among all the *Ralstonia* species, it is considered quite interesting to elucidate the process in which *Ral. taiwanensis* gained this ability. It is thus expected that continued research will be conducted for analyzing this species, focusing on its genomic and genotypic properties.

3. Conclusions

Regarding classification at the species level, it is considered unlikely that the definitions and circumscriptions of the currently described species that contain legume symbionts will undergo major emendations, except for *B. japonicum* and a few other species, because most species have already been carefully defined based on the analytical results of DNA-DNA similarity. However, the number of the genera of legumes, whose bacterial partners have already been taxonomically investigated and described, accounts for less than 10% of all the legume genera, about 750 in total (Moulin et al., 2001). Accordingly, if the remaining legumes are investigated, more strains with different characters may be isolated. Some of them may deserve classification as new species, which may belong to new groups other than the nine monophyletic groups discussed in this review. It is thus important to investigate diverse legume symbionts more extensively, proceed with their species descriptions, and study the origin and evolution of the ability of symbiotic nitrogen fixation of legume symbionts.

Regarding classification at the generic level, the future direction of Groups 5, 8, and 9 differs from that of the remaining six monophyletic groups. Legume symbionts isolated recently and identified to belong to Groups 5 (the genus *Methylobacterium*), 8 (the genus *Burkholderia*), and 9 (the genus *Ralstonia*) can be considered as new types of legume symbionts (Fig. 1). Despite the outstanding problem at the species level that these new legume symbionts belonging to the

genera *Methylobacterium* and *Burkholderia* must be validly published as new species by going through the formal procedures, these three monophyletic groups have no taxonomic problem at the generic level. This is because the genera *Methylobacterium*, *Burkholderia*, and *Ralstonia* have carefully been analyzed on the basis of polyphasic taxonomy, and have therefore been sufficiently characterized on the basis of various properties and confirmed to be clearly separated as monophyletic groups at high bootstrap values (Fig. 2) (Gillis et al., 1995; Yabuuchi et al., 1992, 1995). Regarding these three monophyletic groups, the present classification that "each monophyletic group shall be treated as one genus" will be retained as is toward the future. In addition, it has been agreed from the initial stage of defining these three genera that they are constituted of various members with diversified physiological and ecological properties. Thus, the newly isolated legume symbionts of the genera *Methylobacterium* and *Burkholderia* awaiting valid publications will be accepted as formal members of the respective genera they belong to without emending the generic definitions, only if they are properly described following the formal procedures stipulated in the Bacteriological Code, as with the exemplary case of *Ralstonia taiwanensis* (Chen et al., 2001).

Meanwhile, the legume symbionts contained in Groups 1, 2, 3, 4, 6, and 7, except for "*Devosia neptuniae*," *Blastobacter denitrificans*, and *Ensifer adhaerens*, are "long-standing legume symbionts" which have been studied for decades (Fig. 1), and a considerable amount of data and knowledge on the plant-microbe interactions have been accumulated (Spaink et al., 1998). However, as discussed in the previous sub-chapters, there is an outstanding problem regarding the classification at the generic level, which is common to all the genera belonging to these monophyletic groups. Specifically, although most of the symbiotic genes of these legume symbionts are known to be present on mobile genetic elements (transmissible plasmids or conjugative transposon-like symbiosis islands), there exist numerous closely related environmental microbes in soil without such symbiosis-related mobile genetic elements. For the purpose of implementing environmental bioremediation, screening of soil-borne bacteria has been widely conducted all over the globe and various xenobiotic-degrading bacteria have been isolated. It has gradually been clarified that such nonsymbiotic xenobiotic-degrading bacteria also

belong to the monophyletic groups which contain legume symbionts (only some of them are shown in Fig. 2). The respective genera, which the long-standing legume symbionts belong to, are defined as "a group of species which contains legume symbionts." Accordingly, due to the restriction of this generic definition, these genera are unable to accept the species that exclusively comprise nonsymbionts as their formal members. Thus, if the current classification is retained as is toward the future, it is considered likely that new artificial genera will be established for describing such species, resulting in further congestion of members within the respective monophyletic groups where even more genera will be randomly intermingled with one another. In addition, if nonsymbionts belonging to such new artificial genera acquire mobile symbiotic genes, they may gain the ability of symbiotic nitrogen fixation and as a result their taxonomic position at the generic level will change, which will invite the serious problem that taxonomy will become quite unstable. Thus, it is considered likely that continued use of the current classification in the future will result in aggravating confusion in the classification and identification of legume symbionts and xenobiotic-degrading bacteria.

Regarding Groups 1, 2, 3, 4, 6, and 7, research and discussions of the classification at the generic level need to be vigorously conducted toward the future based chiefly on Options B and C as preferable candidates out of the three options discussed in the subchapter on Group 1. Considering the fact that the taxonomic position of environmental microbes is variable depending on the transfer of mobile symbiotic genes, the classification needs to be made more flexible for describing such changeable members. To achieve such flexibility, the classification of the genera *Methylobacterium*, *Burkholderia*, and *Ralstonia*, which already accept the new legume symbionts, may be a good example.

Nevertheless, it is vitally essential to proceed with research by giving due considerations to practical expediency to avoid confusion among rhizo-microbiologists who are users of the classification. It is also essential to pursue scientific exactitude as much as possible. At present, a hierarchical classification is constructed based on polyphasic taxonomy focusing on the 16S rDNA sequence and DNA-DNA similarity; however, problems have been pointed out such that the 16S rDNA sequence and DNA-DNA similarity are defective as markers for inferring the course of evolu-

tion and that the amount of information they provide is insufficient. It is considered that the so-called multilocus sequence typing will be most required some day in the future for obtaining the basic data for taxonomy, which is to clarify the course of evolution of the fundamental backbone of the genome (basic genome) by phylogenetically analyzing several housekeeping genes under stabilizing selection that are present on the basic genome (Stackebrandt et al., 2002; Turner et al., 2002). From the standpoint of pursuing scientific exactitude in the genomic era, the idealistic way may be to complete the classification by incorporating the information of how the mobile genetic elements (accessory genome) have been transferred and lost during the process of evolution of the basic genome. Thus, taxonomists must continue working on straightening up the classification toward the future in consideration of both scientific exactitude and practical expediency.

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