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# Numerical taxonomic analysis of cross-inoculation patterns of legumes and Rhizobium

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Summary A survey was made of published results of tests of the capacity of Rhizobium derived from one legume genus to nodulate plants from other genera. The data were derived from more than 14,000 separate cross-inoculation trials involving species from 165 genera of legumes. Numerical taxonomic techniques were applied to 113 of the genera for which results of substantial cross-infection tests were available. The data were examined using mean character difference coefficients re-expressed as total and positive-only similarity coefficients. The resulting similarity matrices were clustered by the unweighted pair-group method using arithmetic averages. Eighteen affinity groups were defined at the 70% similarity level. With few exceptions, the physiological and cultural behavior of the rhizobia was consistent within the defined groups. Two broad categories were suggested in the numerical taxonomic analysis, and their validity is discussed in regard to the geographic, physiological and cultural characteristics of the legumes and their Rhizobium microsymbionts. The taxonomic and agronomic value of this approach and the new groupings are discussed.

## Introduction

Based on early investigations of Rhizobium-legume symbioses that largely focused on agriculturally important crops in temperate climates, the concept of cross-inoculation groups was introduced[6]. Of the groups that were originally described, six were generally considered to be sufficiently unique to have species epithets assigned to them. Recent studies suggest that the rhizobia from Lotus should be placed in a seventh species, *Rhizobium loti*[13]. However, many nodule-forming bacteria have not been studied or grouped.

Cultural, morphological, biochemical and serological methods have been applied to characterize and classify isolates of Rhizobium. Using these methods, a distinction has been made between fast-growing, peritrichously flagellated, acid-producing bacteria (*R. trifolii, R. phaseoli, R. leguminosarum* and *R. meliloti*) and slow-growing, polarly

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flagellated, alkali-producing strains (*R. lupini*, *R. japonicum* and rhizobia nodulating a variety of tropical legumes[3,5,31]. Some of the rhizobia recently have been placed in a new bacterial genus, Bradyrhizobium[17,19]. Exceptions within these categories have been described[18,27]. Application of antigenic and protein analysis has also substantiated differences between these broad groups[25,31], and determination of DNA homologies has revealed wide variation even within a single species[9,11].

The application of computer techniques has allowed for the handling of large data sets. Graham[7] used the results of 100 taxonomic tests on 121 organisms, which included 83 rhizobia, to form groups using the 'nearest neighbor' clustering algorithm. Further investigations included the type of flagella and DNA base composition[22]. In both studies, only two broad groups of rhizobia, fast- and slow-growing, could be characterized. Two similar groups were obtained in a study of 59 bacterial strains, including 21 rhizobia coded for results of 191 tests[23]. Numerical taxonomic techniques have permitted the identification of intrageneric affinity groups[11,33]. Nevertheless, a satisfactory classification scheme encompassing all Rhizobium strains has not emerged.

The lack of a broad, systematic evaluation of nodulation patterns has limited our understanding of the potential for unexplored symbioses involving the many poorly studied legumes and has hindered the development of a practically useful and biologically meaningful classification scheme. Because of its agronomic importance, we have focused on only one trait, the ability of the microsymbiont to nodulate not only its homologous host but also heterologous hosts. It was not our objective to develop a new or definitive classification scheme for Rhizobium. The approach involved summarizing published information on the capacity of Rhizobium strains to nodulate any legume and evaluating by numerical taxonomic techniques whether these data are adequate for taxonomic generalizations.

## Methods

### Original literature

Published reports of cross-infection trials were transcribed into matrices containing a species-versus-species record of the results of the testing. The results of an intergeneric cross in any single study was recorded as successful if nodulation was observed even once, although other Rhizobium isolates from the same legume species or cultivars of the same plant failed to nodulate. For example, if 5 strains of Glycine rhizobia, all isolated independently from nodules on *Glycine max*, were tested in one study for ability to nodulate *Cajanus cajan* and only 1 strain did so successfully, then the relationship between rhizobium from *G. max* and the host *C. cajan* was recorded as positive. While this may bias results toward an anomalous

strain, the potential for successful nodulation was of primary concern to the present evaluation. In the literature, descriptions of nodulation vary greatly, from simple qualitative assessments of nodule abundance and appearance to quantitative assessments. A report of the occurrence of nodulation, however sparse, was presumed to be correct, and the data were recorded as positive. The effectiveness of the symbiosis in fixing nitrogen was not included in the analysis.

Each plant genus, when encountered for the first time, was assigned a code number. Plant species were subcoded according to the genus. Recent changes in botanical taxonomy were included[2]. Only information from primary literature sources was included in the data base, and use was not made of information published in review articles and summaries of cross-inoculation groups.

The methods described in the primary literature were evaluated to maximize the likelihood that the cross-infection data would be valid for use in the data base. An acceptable protocol was one that involved use of sterile pots, bags, tubes or jars containing sterile agar, soil, sand, vermiculite or mixtures which were planted with surface-sterilized seeds and inoculated with a single isolate of Rhizobium. Field trials, tests involving mixed cultures and results from use of genetically altered strains were excluded from this review.

*Data for numerical taxonomy*

Information on cross-infection tests involving 856 individually named plant species was analyzed. Data on the results of cross-inoculation tests were entered according to the direction of the cross; *i.e.*, Rhizobium strain from nodules on plant species A was inoculated onto seeds or roots of legume species B; in this way, the direction of each cross was preserved. Because of the non-reciprocal nature of these Rhizobium-plant symbioses, this was important. Although 14,530 entries describing 3,246 different crosses between different genera were collected, this represented only 23.9% of the total crosses possible among these genera. To accommodate the methodologies developed in numerical taxonomy for far more complete data matrices, rearrangements were made. The number of genera represented by all species was 165, but not all genera contained species that had been tested extensively. Genera which had been tested with rhizobia isolated from nodules derived from fewer than 14 other plant genera were deleted from the final analysis, thereby leaving 113 plant genera in the survey.

If changes in plant taxonomy had been made and involved the reassignment of a species in one genus to an entirely different genus, the change has been made in the survey. These changes are listed in Table 1. In addition, the genus Phaseolus was segregated into 'Phaseolus-beans' containing *P. vulgaris, P. angustifolia(ius)* and *P. coccineus* (same as *P. multiflorus* and *P. multifolia*) and 'Phaseolus-cowpea' containing other *Phaseolus* species. Cassia was likewise segregated into three subgenera[2]. Species in subgenus Lasiorhegma were included in the ananysis; species in subgenera Fistula and Senna were not included because of a lack of information on cross-inoculation.

*Numerical taxonomy*

The analytical methodology of numerical taxonomy may be resolved into two phases: the generation of values of similarity between the taxonomic units (here, plant genera) studied and clustering the resulting similarity matrix. For the purpose of generating similarity values, the set of characters or tests used as a basis for making pair-wise comparisons of all 113 plant genera considered was taken to be the 112 cross-inoculation trials which could be made for each genus with all other genera, plus the one trial which could be made with itself. For the $i^{th}$ genus, the results of cross inoculation trials with the $k^{th}$ genus was numerically expressed as $f_{ik}$, the fraction of all reported cross-inoculation trials between genera i and k that resulted in successful nodulation. The identity of the donor genus in a reported cross was ignored in calculating values of $f_{ik}$. When cross-inoculation trials had not been performed for a particular combination of two genera, that $f_{ik}$ was undefined. For all species, i, $f_{ii}$ was set equal to one.

To calculate values of similarity, $S_{ij}$, between two genera using the $f_{ik}$ values described above, two different similarity coefficients were used: the mean-character similarity (MCS)

Table 1. Taxonomic changes of legume species included in the analysis

| Old name | New name |
|---|---|
| *Astragalus rubyi* | *Oxytropis riparia* |
| *Desmodium gyroides* | *Codariocalyx gyroides* |
| *Dolichos africanus* | *Macrotyloma africanum* |
| *D. lablab* | *Lablab purpureus* |
| *D. uniflorus* | *Macrotyloma uniflorum* |
| *Lotus tetragonolobus* | *Tetragonolobus purpureus* |
| *Phaseolus aconitifolia* | *Vigna aconitifolia* |
| *P. angularis* | *V. angularis* |
| *P. atropurpureus* | *Macroptilium atropurpureum* |
| *P. aureus* | *Vigna radiata* var. *aureus* |
| *P. calcaratus* | *V. umbellata* |
| *P. lathyroides* | *Macroptilium lathyroides* |
| *P. mungo* | *Vigna radiata* var. *mungo* |
| *P. radiata* | *V. radiata* |
| *P. trilobus* | *V. trilobata* |
| *Psoralea acaulis* | *Asphalthium acaulis* |
| *P. esculenta* | *Pediomelum esculentum* |
| *P. onobrychis* | *Orbexilum onobrychis* |
| *Stizolobium deeringianum* | *Mucana deeringianum* |
| *S. utile* | *M. utilis* |
| *S. utilis* | *M. utilis* |
| *Stizolobium* spp. | *Mucuna* spp. |

coefficient described in Sneath and Sokal[26] and a variant of the MCS coefficient here termed the positive-only mean-character similarity (PMCS) coefficient.

Using the MCS coefficient, the similarity value between two genera i and j can be expressed as follows:

$$S_{ij} = \frac{1}{n_{ij}} \sum_{k=1}^{113} 1 - |f_{ik} - f_{jk}|$$

where $n_{ij}$ equals the number of genera, k, for which $f_{ik}$ and $f_{jk}$ were defined. If either $f_{ik}$ or $f_{jk}$ was undefined for a particular value of k, then the quantity $|f_{ik} - f_{jk}|$ was set equal to 1. The PMCS coefficient can be expressed as follows:

$$S_{ij} = \frac{1}{n_{ij}} \sum_{k=1}^{113} 1 - \left( |f_{ik} - f_{jk}| / \max (f_{ik}, f_{jk}) \right)$$

where max $(f_{ik}, f_{jk})$ is the greater of the two fractions. When max $(f_{ik}, f_{jk})$ was equal to zero, the algorithm used set the quantity $|f_{ik} - f_{jk}|/\max (f_{ik}, f_{jk})$ equal to 1. If for a particular combination of genera i and j, the value of $n_{ij}$ was equal to zero, *i.e.*, if the two genera had never been tried against the same test genus, then that value $S_{ij}$ was undefined.

Every genus has associated with it a set of genera, here termed its set of successful partners, with which it has successfully exchanged rhizobia either as donor or recipient. If a large amount of overlap exists between the sets of successful partners for two genera (*i.e.*, 'positive matching'[26]), then both the MCS and PMCS coefficients will assign a high similarity to the two species. Overlap between the sets of unsuccessful partners for two genera is termined 'negative matching'[26]. Although the PMCS coefficient ignores negative matching, the similarity calculated by the MCS coefficient is increased by negative matching. Thus, using the latter coefficient, two genera could be assigned a high similarity despite a complete absence of any overlap in their sets of successful partners.

The clustering algorithm used in this study was the unweighted pairgroup mathematical average (UPGMA) described in Sneath and Sokal[26]. In the UPGMA algorithm used in this study, if the algorithm attempted to average a defined similarity value with an undefined similarity value, the result was set equal to the defined value. The average of two undefined similarity values remained undefined.

The similarity coefficients were calculated using a 6502 assembly language program written by one of the authors (S.S.), and the clustering algorithm was written (by L.M.M.) in interpreted BASIC. Copies of both programs are available upon request.

Groups were defined at the 70 to 85% similarity level using the MCS coefficient. The PMCS coefficient was used to control for groups formed solely on the basis of negative matching.

## Results

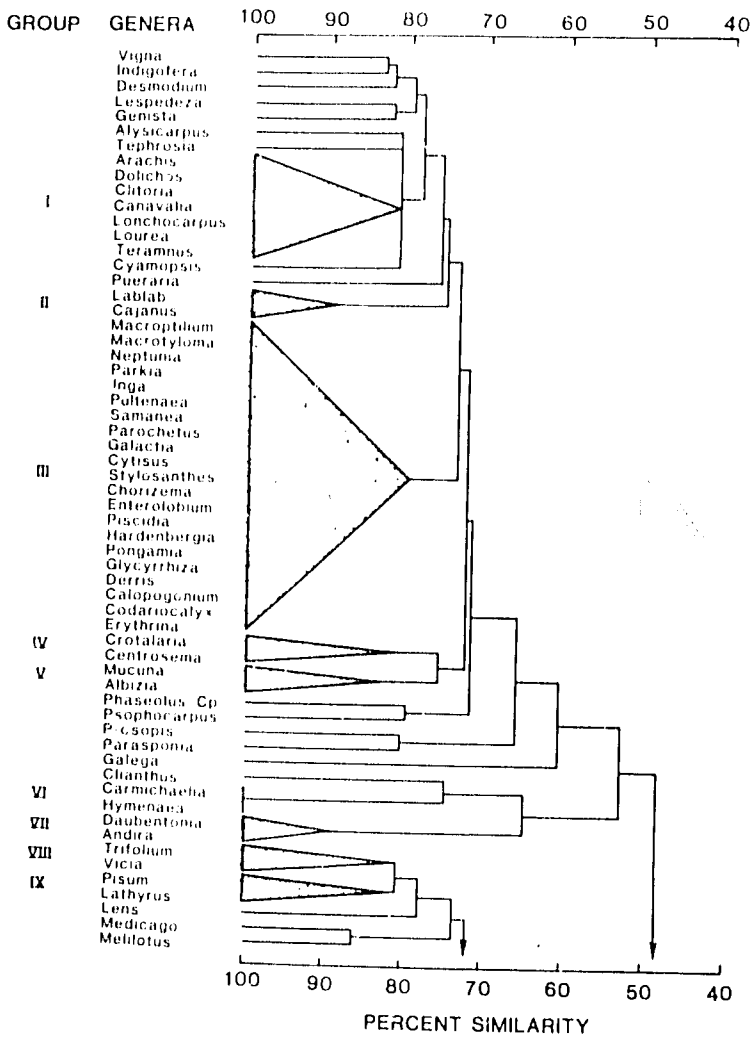
Results of the numerical taxonomic analysis are shown in the simplified dendrogram (Fig. 1). Both the MCS and PMCS coefficients were used in constructing the figure. Initially, groups were defined as consisting of genera clustered at the 70% similarity level by the UPGMA algorithm from the similarity matrix generated using the MCS coefficient. If the genera belonging to a tentative group thus defined were also clustered together when a similarity matrix produced by the PMCS algorithm was used, then that group was regarded as real and is shown as one of 19 numbered groups in the dendrogram. However, if some members of a group generated using the MCS coefficient (*e.g.*, *Alysicarpus*, *Tephrosia* and *Cyamopsis* from group I) clustered with the genera of another group when the PMCS coefficient was used, then those members were regarded as being included in the original MCS group solely on the basis of negative matching (defined previously). Such genera are shown in Fig. 1 attached to one of the 18 numbered groups at the similarity level calculated by the MCS coefficient, but they are not indicated by inclusion within the solid triangle as members of the group.

The 18 groups that were identified by the procedure detailed above contain 75 genera (66% of the total). Group 3, the largest cluster, contained 21 genera. Twelve groups were small and contained 2 or 3 genera in each, and 5 groups were intermediate in size and contained 4 to 7 members.

An attempt was made to cluster the genera at a higher level of similarity, namely 80–85%. This approach was abandoned after studying the results of the PMCS analysis. For example, when group III was examined for clusters defined at the higher level, the group was split into two groups containing 10 and 11 genera defined at the 82 and 83% similarity level (MCS), respectively. Subsequent analysis using the PMCS coefficient indicated that each of these groups split further into 2 groups containing 6 and 4 members and 9 and 3 members.

Thus, if groups were defined at the 80–85% level, 4 groups would be defined from the PMCS results, whereas at the 70% level, a single group is recovered in both the MCS and PMCS analyses. A similar breakdown of clusters was noted in groups XV, XVI, XVII and XVIII when examined at the higher level. Because there is no generally recognized level of similarity to be used for the definition of groups in numerical taxonomy[26] and because use of a stricter level provided no new information in this study, the 70% similarity level was chosen.

Genera recovered in groups illustrated in the dendrogram as well as unclustered genera are listed in Table 2. The number of other genera with which each genus has been tried is noted in column 3; in this context, an intergeneric cross designates the testing for nodulation



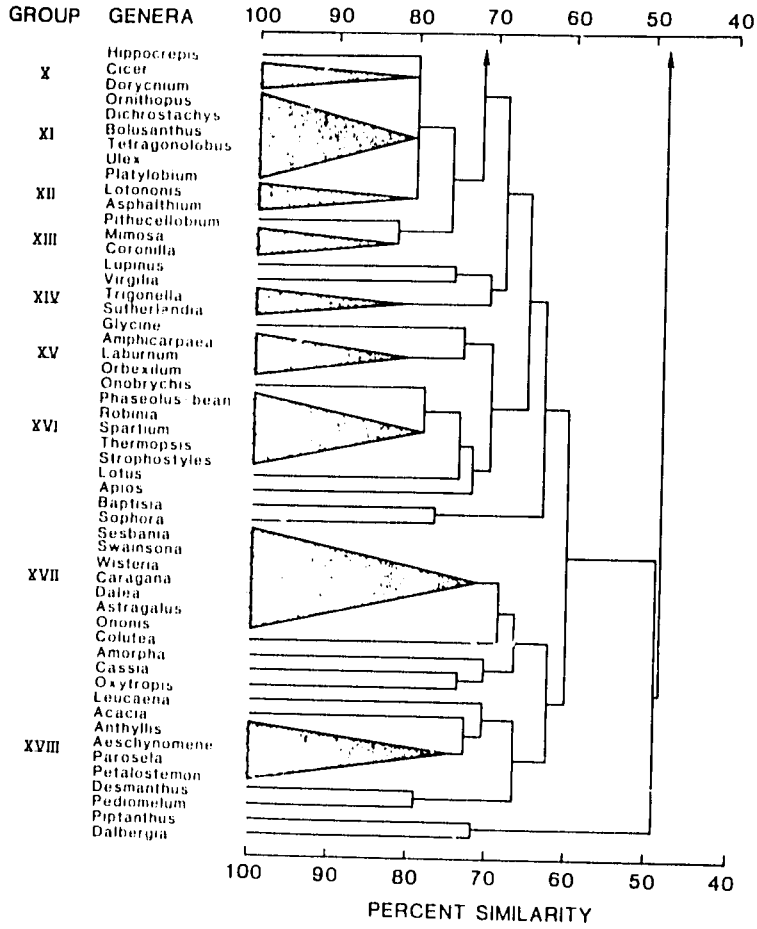



Fig. 1. Dendrogram of relative similarities among legume genera based on cross-infection patterns.

of species of one plant genus by rhizobia derived from nodules present on roots of a different genus; this information provides a picture of the relative abundance or paucity of cross-tests performed with rhizobia from each host genus or using each plant genus as the macrosymbiont for tests with other rhizobia.

Few tests of cross inoculation were conducted with the following genera (the values in parenthesis are the number of legume genera for which cross-infection tests were conducted with the named genus): Abrus (8), Anagyris (1), Aotus (12), Aspalathus (2), Atylosia (3), Bossiaea (12), Brachysema (12), Burtonia (1), Cassia subgenera Fistula and Senna (6), Cratylia (10), Daviesia (12), Delonix (1), Dillwynia (12), Dunbaria (1), Erichsenia (1), Euchilopsis (12), Eutaxia (12), Falcata (1), Flemingia (5), Gastrolobium (12), Gliricidia (1), Gompholobium (12), Goodia (3), Hosackia (6), Hovea (12), Inocarpus (1),

Table 2. Geographic, cultural and physiological characteristics of legume genera and their Rhizobium microsymbionts used in the analysis

| | | | Habitat[b] | | | Growth rate[c] | | pH from carbohydrates[d] | | Reaction in milk | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | pH change | | Serum zone | |
| Group | Genus | No.[a] | Tropical | Subtropical | Temperate | Fast | Slow | Acid | Alkaline | Acid | Alkaline | Formed | Not formed |
| – | Vigna | 135 | 2, 4[e] | 2 | – | – | 1,2,8 | 24(6%)[f] | 1,24(94%) | 32 | 1,2,16 | – | 1,2,16, 32 |
| – | Indigofera | 60 | 2,4 | 2,4 | – | – | 1,2 | – | 1,24 | – | 1,16 | – | 1,16 |
| – | Desmodium | 105 | 4,30 | 2 | – | – | 1,21,29 | – | 1,24 | 32 | 1,3 | 32 | 1,3 |
| – | Lespedeza | 97 | – | 2 | 2,4 | – | 1 | – | 1,24 | – | 1,3,32 | 32 | 1,3 |
| – | Genista | 41 | – | – | 2,3,30 | – | – | – | – | – | – | – | – |
| – | Alysicarpus | 36 | 2,4 | – | – | – | 1 | – | 1,24 | – | 1 | – | 1 |
| – | Tephrosia | 58 | 2,4 | 2,4 | – | – | 1,27 | – | 1,24 | – | 1,3 | 3 | 1 |
| I | Arachis | 72 | 2 | 2,4 | 4,30 | 18 | 23 | 24(25%) | 12,24(75%) | – | 2,8 | 2,8 | – |
| I | Dolichos | 23 | 2,4 | 4 | – | – | – | 24(12%) | 2,24(88%) | – | 2 | – | – |
| I | Clitoria | 63 | 4 | 4 | 2 | – | 23 | – | 24 | – | – | – | – |
| I | Canavalia | 59 | 2 | 2,4 | – | – | 1 | 24(14%) | 1,24(86%) | – | 1 | 1 | – |
| I | Lonchocarpus | 60 | 2,4,30 | – | – | – | 1 | – | 1,2,24 | – | 1 | – | 1 |
| I | Lourea | 31 | 4 | – | – | – | – | – | – | – | – | – | – |
| I | Teramnus | 14 | 2,4 | 2 | – | – | 2 | – | 2,24 | – | 2 | – | – |
| – | Cyamopsis | 45 | 2,4 | 4 | – | – | – | – | – | – | – | – | – |
| – | Pueraria | 48 | 2 | 2,4 | 2 | – | 2,27 | 24(18%) | 2,24(82%) | – | 2 | – | 2 |
| II | Lablab | 73 | 2,4 | – | – | 27,29 | 27 | 27 | – | 27 | – | 27 | – |
| II | Cajanus | 70 | 2,4,30 | 2 | – | – | 27,29 | – | 24 | – | – | – | – |
| III | Macroptilium | 79 | 2 | 2 | – | – | 1,27,29 | – | 1 | – | 1 | – | 1 |
| III | Macrotyloma | 18 | 4 | 4 | – | – | 23,29 | – | – | – | – | – | – |
| III | Neptunia | 16 | 2,4 | 2,4 | – | – | – | 24 | – | – | – | – | – |
| III | Parkia | 15 | 2,4,30 | 2,4 | – | – | 1 | – | 1 | – | 1 | – | 1 |
| III | Inga | 15 | 2,4,30 | 2,4 | – | – | 1,2 | – | 1 | – | 1 | 1 | – |
| III | Pultenaea | 14 | – | 2,4 | – | – | 20 | 24(75%) | 24(25%) | – | 20 | – | 20 |
| III | Samanea | 32 | 2 | – | – | – | 1 | – | 1 | – | 1 | – | 1 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| III | Parochetus | 21 | 4 | 4 | – | – | – | – | – | – | – | – | – |
| III | Galactia | 14 | 4 | 2,4 | – | – | – | – | – | – | – | – | – |
| III | Cytisus | 30 | – | – | 2,4 | – | 1 | – | 1,24 | – | 1 | – | 1 |
| III | Stylosanthes | 48 | 2,4 | 2 | – | – | 1,27,29 | 24(10%) | 1,24(90%) | – | – | – | 1 |
| III | Cherizema | 35 | 2 | 2,4 | – | – | 2,20 | – | – | – | 20 | – | 20 |
| III | Enterolobium | 15 | 2,4 | – | – | – | 1,2 | – | 1 | – | 1 | – | 1 |
| III | Piscidia | 15 | 2,4 | 4 | – | – | 1 | – | 1 | – | 1 | – | 1 |
| III | Hardenbergia | 35 | 2 | 2,4 | – | – | 20 | – | – | – | 20 | – | 20 |
| III | Pongamia | 15 | 2,4 | – | – | – | 1,2 | – | 1,24 | – | 1 | – | 1 |
| III | Glycyrrhiza | 31 | – | 4 | 2,4,30 | – | 2 | – | – | – | 2,3 | 2,3 | – |
| III | Derris | 22 | 2,4,30 | – | – | – | 1,2 | – | 1 | – | 1,2 | – | 1,2 |
| III | Calopogonium | 31 | 2,4 | – | – | – | 2 | 24(12%) | 24(88%) | – | – | – | – |
| III | Codariocalyx | 21 | 2,4 | – | – | – | 2 | – | – | – | – | – | – |
| III | Erythrina | 55 | 2,4,30 | 4,30 | – | – | 1 | – | 1,24 | – | 1 | – | 1 |
| IV | Crotalaria | 114 | 2,4 | 2 | – | – | 2,27,29 | 24(17%) | 24(83%) | – | 2,3,32 | 32 | 2,3 |
| IV | Centrosema | 57 | 2,4 | 2,4 | – | – | 27,29 | – | 24 | – | – | – | – |
| V | Mucuna | 101 | 2,30 | 4 | – | – | 2 | – | 24 | – | – | – | – |
| V | Albizia | 91 | 2,4 | 4 | – | – | 1,20,29 | – | 1,24 | – | 1,2,20,32 | 32 | 1,2,20 |
| – | Phaseolus-C | 58 | 2,4 | 4 | – | – | 1,2,23, 27,29 | 24(7%) | 1,24(93%) | – | 1,2,16 | – | 1,2,16 |
| – | Psophocarpus | 34 | 2,4 | – | – | – | 2 | – | – | – | 2 | – | – |
| – | Prosopis | 40 | 4 | 2,4 | – | – | 2 | – | 2 | – | 2 | – | – |
| – | Parasponia | 29 | – | – | – | – | – | – | – | – | – | – | – |
| – | Galega | 31 | – | 4 | 2,4,30 | 21 | – | 21 | – | – | 21 | – | – |
| – | Clianthus | 43 | 2,4 | 2,4 | – | – | 12 | – | – | – | – | – | – |
| VI | Carmichaelia | 19 | 4 | 2,4 | – | 21 | 12 | – | – | – | – | – | – |
| VI | Hymenaea | 16 | 2,4 | 2 | – | – | 1,2 | – | 1 | – | 1 | – | 1 |
| VII | Daubentonia | 31 | 4 | 4 | – | – | – | – | – | – | – | – | . |
| VII | Andira | 15 | 2,4 | – | – | – | 1 | 1 | 2 | – | 1,2 | 1,2 | – |
| VIII | Trifolium | 121 | – | 2,4 | 2,4 | 2,16, 21,23 | – | 2,24(99%) | – | 2,3 16 | 32 | 2,3 16,32 | – |
| VIII | Vicia | 114 | – | – | 2,4 | 2,21, 23 | – | 2,24 | – | 2 | 3,32 | 2,3,32 | – |

Table 2. Continued

| Group | Genus | No.[a] | Tropical | Subtropical | Temperate | Fast | Slow | Acid | Alkaline | Acid | Alkaline | Formed | Not formed |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IX | Pisum | 91 | – | – | 2,4 | 2,18, 16 | – | 8,24 | – | 8,16 | – | 8,16 | – |
| IX | Lathyrus | 71 | – | 4 | 2 | 2,8 | – | 2,24 | – | – | 3 | 3 | – |
| – | Lens | 107 | – | – | 2,4,30 | 2 | – | 2,24 | – | – | 32 | 32 | – |
| – | Medicago | 118 | – | – | 2,4 | 2,8, 16,21, 23 | – | 8,24(88%) | 24(12%) | 2,3,8 16,32 | – | 2,3 8,16 | 32 |
| – | Melilotus | 53 | – | 4 | 2,4 | 2,16 | – | 2 | – | 2,16 | – | 2,16 | – |
| – | Hippocrepis | 31 | – | – | 4 | – | – | – | – | – | – | – | – |
| X | Cicer | 69 | – | – | 2,4,30 | 2,8 | – | 2,12,30, | – | 8 | – | 8 | – |
| X | Dorycnium | 31 | – | 2,4 | 2,4 | – | – | – | – | – | – | – | – |
| XI | Ornithopus | 75 | 2,4 | 2,4 | – | – | 23 | – | 24 | – | 16 | – | 32 |
| XI | Dichrostachys | 32 | 2,4 | – | – | – | – | – | – | – | – | – | – |
| XI | Bolusanthus | 31 | 4 | 2,4 | – | – | – | – | – | – | – | – | – |
| XI | Tetragonolobus | 32 | – | 4,30 | 2,4 | – | – | – | – | – | – | – | – |
| XI | Ulex | 32 | – | – | 2,4 | – | – | – | – | – | – | – | – |
| XI | Platylobium | 31 | – | – | 2,4 | – | – | – | – | – | – | – | – |
| XII | Lotononis | 30 | 2 | 2,4 | – | – | 2,23 | – | 2,24 | – | – | – | – |
| XII | Asphalthium | 31 | – | 4 | 2,4 | – | – | – | – | – | – | – | – |
| – | Pithecellobium | 32 | 2,4,30 | – | – | – | – | – | 24 | – | – | – | – |
| XIII | Mimosa | 65 | 2,4,30 | 2 | – | 27,29 | 2 | 24(29%),27 | 12,24(71%) | – | 2,27 | 27 | 2 |
| XIII | Coronilla | 48 | 2,4 | 2,4 | 2,4 | 21 | 2 | – | – | – | 2 | – | 2 |
| – | Lupinus | 114 | 2 | 2,4 | 2,4 | 12,21 | 2,8,21 23,29 | 24(20%) | 12,24(80%) | – | 3,8,16 32 | 32 | 3,8,16 |
| – | Virgilia | 31 | 2,4 | 4 | – | – | – | – | – | – | – | – | – |
| XIV | Trigonella | 48 | – | 2,4 | 2,4 | 2,16 | – | 2 | – | 2,16 | – | 2,16 | – |
| XIV | Sutherlandia | 32 | 2 | 2 | – | – | – | – | – | – | – | – | – |
| – | Glycine | 120 | 2,4 | 2,4 | – | 18 | 2,8,21 23,27,29 | – | 2,8,24 | – | 2,8,16 32 | 8 | 16,32 |
| XV | Amphicarpaea | 84 | 2 | 2,4 | 2,4 | – | – | – | – | – | 5,39 | 32 | 3 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| XV | Laburnum | 82 | – | 2,4 | 2,4 | – | 2 | – | – | – | 16,32 | 32 | 16 |
| XV | Orbexilum | 32 | – | – | 2 | – | – | – | – | – | – | – | – |
| – | Onobrychis | 91 | – | 4 | 2,4,30 | – | – | – | – | – | 32 | 32 | – |
| XVI | Phaseolus-bean | 118 | – | – | 2 | 2,8,21 | – | 2,8,24 | 24(7%) | 8,16 | 32 | 8,16,32 | – |
| XVI | Robinia | 88 | – | 4 | 2 | 2 | 21 | 24 | – | – | 2,3,32 | 2,3,32 | – |
| XVI | Spartium | 82 | – | 4 | 2,4 | – | – | – | – | – | 32 | 32 | – |
| XVI | Thermopsis | 82 | – | – | 2,4 | – | – | – | – | – | 32 | 32 | – |
| XVI | Strophostyles | 84 | – | – | 2 | – | – | – | – | – | 3,32 | 3,32 | – |
| – | Lotus | 95 | – | 4 | 2,4,30 | 2,21 | 21 | 24(55%) | 24(45%) | 32 | – | 32 | – |
| – | Apios | 81 | – | – | 2,4 | – | 2 | – | – | – | 3,32 | 32 | 3 |
| – | Baptisia | 83 | – | – | –,4 | – | 2 | – | – | – | 2,3,32 | 32 | 2,3 |
| – | Sophora | 30 | 4 | 2,4 | 2 | 21 | 2,12 | 2 | 24 | – | – | – | – |
| XVII | Sesbania | 95 | 4 | 2,4 | – | 27 | – | 24(95%),27 | 16,24(5%) | 27 | 16,32 | 16,27 | – |
| XVII | Swainsona | 83 | 2,4 | 2,4 | – | – | – | – | – | – | 32 | 32 | – |
| XVII | Wisteria | 92 | – | – | 2,4,30 | – | – | – | – | 32 | – | 32 | – |
| XVII | Caragana | 86 | – | – | 2,4 | 21 | 2 | – | – | – | 32 | 32 | – |
| XVII | Dalea | 87 | 4 | 2,4 | 2,4 | 2 | – | 2,24 | – | – | 32 | 32 | – |
| XVII | Astragalus | 57 | – | 4 | 2,4,30 | 2 | 2 | 24(73%) | 24(27%) | – | 2 | 3 | – |
| XVII | Ononis | 34 | – | – | 2,4 | – | – | – | – | – | – | – | – |
| – | Colutea | 31 | – | 2 | 2,4,30 | – | – | – | – | – | – | – | – |
| – | Amorpha | 92 | – | – | 2,4 | – | – | – | – | – | 3,32 | 3,32 | – |
| – | Cassia[g] | 90 | 2,4 | 2 | 2 | – | 1,27,29 | – | 1,24 | – | 1,3,32 | 32 | 1,3 |
| – | Oxytropis | 82 | – | – | 2,4 | – | 2 | 2 | – | – | 2,3,32 | 3,32 | 2 |
| – | Leucaena | 73 | 2,4 | 2 | – | 27,29 | 2 | 24(94%),27 | 24(6%) | – | 27 | 27 | – |
| – | Acacia | 65 | 2,4,30 | 2,4 | – | 27,29 | 1,2,8 20 | 27 | 1,24 | 27 | 1,2,20 | 27 | 1,2,20 |
| XVIII | Anthyllis | 42 | – | – | 2,4,30 | – | – | 24(66%) | 24(34%) | – | – | – | – |
| XVIII | Aeschynomene | 21 | 2,4 | – | – | – | 2 | 24(20%) | 24(80%) | – | – | – | – |
| XVIII | Parosela | 31 | – | 4 | 4 | – | – | – | – | – | – | – | – |
| XVIII | Petalostemon | 31 | – | – | 2,4 | 2 | – | – | – | – | 2,3 | 3 | – |

Table 2. Continued

| Group | Genus | No.[a] | Tropical | Subtropical | Temperate | Fast | Slow | Acid | Alkaline | Acid | Alkaline | Formed | Not formed |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| – | Desmanthus | 31 | 2,4 | 2,4 | – | – | 2 | – | – | – | 2 | 2 | – |
| – | Pediomelum | 31 | – | – | 2 | 2 | – | 24(92%) | 24(8%) | 2,3 | – | – | 2,3 |
| – | Piptanthus | 31 | – | – | 2,4 | – | – | – | – | – | – | – | – |
| – | Dalbergia | 14 | 2,6 | 2 | – | – | – | – | 24 | – | – | – | – |

[a] Number of tests of cross inoculation among genera.
[b] Principal geographic habitat.
[c] Relative growth rate on agar medium.
[d] pH change on carbohydrate-containing agar media.
[e] The numerals are the references.
[f] Percentage of strains tested by Norris[24] with indicated result. No percentage indicates 100%.
[g] Cassia, subgenus Lasiorhegma.

Isotropis (12), Jacksonia (12), Kennedya (12), Labichea (1), Latrobea (12), Lebeckia (3), Lysiloma (3), Mirbelia (12),Neonotonia (2), Oxylobium (12), Parryella (3), Piptadenia (1), Psoralea (11), Pterocarpus (4), Rafnia (1), Rhynchosia (6), Sarothamnus (1), Schrankia (3), Securigera (1), Smithia (2), Sphaerolobium (12), Stypholobium (10), Templetonia (12), Uraria (5), Viminaria (12), Voandzeia (6) and Zornia (1). These genera were not included in the clustering analysis.

The remaining 113 genera were found to be related at the 48% similarity level using the MCS coefficient. Just above this level, two large groupings were defined: groups I through VII and groups VIII through XVIII, each with associated but unclustered single genera.

Information was compiled from the literature on several characteristics of the legumes and their bacterial symbionts (Table 2). The principal geographic habitat of each genus was determined from information provided by Allen and Allen[2], Capitaine[4] and Tutin[30]. Information on rate of rhizobial growth, the pH change they cause when grown on yeast extract agar containing a suitable carbohydrate and the change the bacteria effect in litmus milk is also included in Table 2.

A summary of this information is presented in Table 3. Although the geographic boundaries for growth and occurrence of individual legume genera are difficult to demarcate, a trend is evident when the principal geographic habitat of each genus is compared with its position in the dendrogram. Forty-four of 52 (85%) legume genera in or among the first 7 groups are found predominantly in tropical or subtropical climates. Five genera are primarily found in temperate and subtrepical regions and only 3 genera grow in all climates. Conversely, of the 60 genera in or adjoining groups VIII to XVIII, 38 (63%) are found predominantly in temperate and subtropical climates. Sixteen genera are mostly tropical, and 6 genera overlap all areas. Although the subtropical regions often contain genera from both tropical and temperate areas and altitude can influence climate and therefore occurrence, the division of the dendrogram into two large groups based on habitat may be valid but is worthy of further exploration.

A common way of comparing growth rates of rhizobia is to measure colony diameter, and often the bacteria are distinguished based on whether the colony diameter is greater or less than 1–1.5 mm in 3 d. Of the 78 plant genera included in this survey for which the growth rates of the rhizobia have been studied, 40 of 44 (91%) in the first 7 groups are slow-growing, and 14 of 47 (41%) in the last 10 groups are fast-growing. Three genera (7%) in the first group produce both fast- and slow-growing rhizobial isolates, as do 11 genera (32%) in the last group.

Table 3. Geographic distribution of the legumes and characteristics of Rhizobium isolate included in the analysis

| Character | Relation to character | Genera in and among groups | | | |
|---|---|---|---|---|---|
| | | I to VII | | VIII to XVIII | |
| | | No. of genera | Percentage of genera | No. of genera | Percentage of genera |
| Plant distribution | Tropical-subtropical | 44 | 85 | 16 | 27 |
| | Temperate-subtropical | 5 | 10 | 38 | 63 |
| | Tropical-temperate | 3 | 6 | 6 | 10 |
| Rhizobium, growth rate[a] | Fast | 1 | 2 | 17 | 41 |
| | Slow | 40 | 91 | 9 | 27 |
| | Fast-slow[b] | 3 | 7 | 11 | 32 |
| Rhizobium, carbohydrate reaction[a] | Acid | 3 | 8 | 11 | 37 |
| | Alkaline | 24 | 63 | 6 | 20 |
| | Acid/alkaline[c] | 11 | 29 | 13 | 43 |
| Rhizobium, in litmus milk[a] | Acid | 1 | 3 | 8 | 21 |
| | Alkaline | 34 | 92 | 25 | 66 |
| | Acid/alkaline[c] | 2 | 5 | 15 | 13 |
| Rhizobium, serum zone[a] | Formed | 6 | 19 | 25 | 64 |
| | Not formed | 20 | 65 | 3 | 8 |
| | Formed/Not formed[d] | 5 | 16 | 11 | 28 |

[a] Data are for the rhizobia isolated from nodules of the designated legume genera.
[b] Both fast- and slow-growing rhizobia were isolated from nodules on plants from the same genus.
[c] Rhizobia producing acidity and alkalinity were isolated from nodules on plants from the same genus.
[d] Rhizobia forming or not forming serum zone in milk were isolated from nodules of plants from the same genus.

Generalizations from the summary of pH changes in carbohydrate-containing media are tenuous because most authors use a wide range of carbohydrates for their testing; there is no one sugar common to all reports, although sucrose is generally used. The results on pH changes were compiled for rhizobia isolated from 68 genera of legumes. In the first group, 63% produced alkali, and only 8% produced acid; the nodules on 29% of the legume genera contained microsymbionts producing both reactions. Of the 30 legume genera in the second group, 37% containing rhizobia that produced primarily acidic products from carbohydrates, 20% produced alkali and 43% bore nodules containing strains generating either acid or alkali.

Of the rhizobia tested, the bacteria derived from 59 of the 75 legume genera produced alkali in litmus milk. Rhizobia from only 9 plant genera produced acid, and 5 of these are found in the pea and alfalfa

cross-inoculation groups of Fred *et al.*[5]. All except Lablab are among the lower group of genera. Rhizobia derived from 7 plant genera are reported as being capable of forming both acidic and alkaline reactions. The formation of a serum zone resulting from casein hydrolysis further illustrates the separation into two broad groups. Of the upper group of plant genera, 65% of the rhizobia tested did not form a serum zone, whereas in the bottom group, 64% hydrolysed casein.

Little attention has been given to physiological characteristics of the microsymbionts nodulating Parasponia[29]. It is a tropical genus, and its cross-inoculation patterns were more similar to those of the tropical than those of the temperate legumes.

Thus, the use of geographical, cultural and biochemical characteristics serves only to divide the rhizobia into two broad categories. In some instances in which rhizobia appear to be exceptions to the general trend in one characteristic, they follow the trend in another. For example, although acid-producing rhizobia from Lablab (group II) and Neptunia (group III) appear to be exceptions to the trend of slow growing, alkali-producing strains predominant among the first 7 groups, the principal geographic habitat of these plants is tropical; thus, in this character, they have some affinity to the other tropical plants with which they cluster. Fewer exceptions to the general trend are found among the top 7 groups, and fewer legumes producing Rhizobium isolates with cultural and physiological characteristics unlike isolates with which they have similar cross-infection patterns have been reported from among these genera. However, the lack of exceptions to the trend of the upper groups is possibly because of a limited amount of study which has been performed on isolates from these predominantly tropical genera. For this reason, more isolates with varying cultural and physiological characteristics are found from among the temperate, agronomically important and well studied legumes of groups VIII to XVIII. From nodules of Lotus, Lupinus, Phaseolus-bean and Acacia, rhizobia with differing biochemical capabilities have been obtained. However, Glycine and Ornithopus are predominantly tropical plants that bear nodules containing rhizobia that are usually slow-growing and alkali-producing, yet they are similar in cross-infection ability to predominantly fast-growing, acid-producing strains.

Of the 18 groups suggested by the computer analysis, only 3 groups contained genera commonly considered as the characteristic genus of the accepted cross-inoculation groups. Rhizobia from Trifolium were found to be 81% similar to Vicia isolates (Group VIII). Pisum isolates clustered tightly with Lathyrus isolates (group IX); and rhizobia from

Phaseolus-bean grouped with Robinia, Spartium, Thermopsis and Strophostyles (group XVI). Medicago was highly similar (86%) to Melilotus in its pattern of cross-infection, but much of this similarity was generated on the basis of negative matching only; for this reason, they were not defined as a group. Lupinus and Glycine remained distinct from all other genera when subjected to either total or positive-only matching. Vigna, which stands out because of its relationship to the so-called 'cowpea miscellaneous cross-inoculation group', also failed to cluster in a group. As in previous examples, although the patterns of cross infection of Indigofera, Desmodium, Lespedeza and Genista were quite similar, defining a group including these genera was not justified because their similarity was largely a result of negative matching in the cross-infection patterns. Phaseolus-cowpea plants and Phaseolus-bean plants had very different cross-inoculation patterns. However, the low affinity (71%) between Phaseolus-cowpea isolates and Vigna isolates was unexpected inasmuch as many Phaseolus species are botanically similar to species of Vigna.

## Discussion

Numerous attempts to classify Rhizobium based on a broad range of cultural, biochemical and genetic characters have been made, but these have only partially helped to unravel the taxonomic confusion. Among the reasons for the only modest progress are the presence of rhizobia with substantially different biochemical or cultural characteristics in nodules on the same plant or from different species within the same plant genus[14,27] and the examination of comparatively few Rhizobium strains. Our study has examined results from cross-inoculation tests involving 165 of the more than 700 legume genera described. In contrast, cultural and biochemical characteristics of rhizobia of a smaller percentage of legume genera and species have been determined, and comparisons by GC ratios, DNA homologies or protein patterns have been made with only few Rhizobium strains.

The symbiotic host range is a trait that reflects important biological, and sometimes agronomic relationships. Results of cross-infection studies conducted by different investigators can be compared, thus allowing for inclusion of a large number of strains in a taxonomic analysis. Nevertheless, the symbiotic host range of Rhizobium isolates from less than 7% of all known legume species have been studied[28]. Our analysis included tests involving more than 1000 legume species or their bacterial symbionts. Except for a few species, such as *Vigna unguiculata, Trifolium repens, Medicago sativa* and several others

of agronomic or horticultural importance, relatively few tests of host specificity for individual legume species have been reported. Grouping the species by genus revealed that 165 legume genera were represented by those species that have been tested. In this way, the amount of information included in the analysis increased to 23.9% of the 15,530 intergeneric crosses that would be possible based on published information on crosses with at least one non-homologous host genus. Further reducing the number of included genera from 165 to the 113 for which crosses with 14 or more non-homologous genera have been evaluated increased the information content to 45.9%. The 52 genera that were excluded contributed little to the analysis because of the paucity of cross-infection tests performed with species of these genera. It should be stressed that, although a comparison of the numerical taxonomic treatment with the data based on host range does not provide sufficient evidence to propose a true classification scheme for the bacteria, it does provide ample evidence of the existence of potentially useful affinities.

Our analysis both supports and contradicts earlier attempts to group rhizobia based on their cross-infection patterns. The close affinity which Pisum, Lathryus, Vicia and Lens exhibit (> 77%) along with Cicer (71%) is consistent with the grouping of Trifolium and Phaseolus-bean. Our analysis supports a high affinity between Trifolium and Pisum (80%) and the identity of a group including Trifolium and Vicia species, but Phaseolus-bean isolates are different in their host range. Regardless of the high affinity among genera thought to be grouped as *R. leguminosarum*, our analysis stops short of placing these together as a 'cross-inoculation group' because much of the similarity is based on negative matching.

The genus Phaseolus has been separated into two groups[2]. Phaseolus-bean includes the temperate type species, *P. vulgaris*, and Phaseolus-cowpea includes many of the tropical species. Our analysis has used this distinction and has shown that the symbiotic pattern of bean species and rhizobia is unrelated to the pattern involving Glycine, Medicago, Lupinus, Trifolium and Pisum, which are genera prominent in the classical cross-inoculation groups, but it does reveal a relatedness of bean species to 4 previously unaffiliated genera, namely, Robinia, Spartium, Thermopsis and Strophostyles. The Phaseolus-cowpea species are found to have slightly over 70% relatedness to the majority of the tropical legumes, but they do not belong within any of the identified groups.

The traditional alfalfa cross-inoculation group contains species of the genera Medicago, Melilotus and Trigonella[6]. By our analysis, Medicago and Melilotus are, in fact, highly similar (86%), but Trigonella

isolates are not (68%). Again, although a high similarity in rhizobial host range is suggested, most published reports show no nodulation when isolates from species of Medicago and Melilotus are tested on most other legume genera, thus precluding the assignment of group status.

An earlier investigation of the relationship between rhizobia of Lupinus, Ornithopus, Lotus and Anthyllis gave little evidence for their designation as a group[15]. In another study of similar isolates involving 37 biochemical tests, all rhizobia except those from Anthyllis were highly similar[12]. Our analysis of the host range and cross-infection patterns of those genera suggests that little, if any, relationship exists to justify their placement in an affinity group.

The use of cross-inoculation groups for taxonomic purposes has been limited because of the relatively few species examined in the assessment, and little attention recently has been given to adding new information. Our analysis of the host ranges of the rhizobia was designed to overcome this limitation. By including a large amount of information, new groupings have been defined, and the validity of some old groups has been verified. Eighteen groups have been identified, and these encompass species from 75 of the 113 genera subjected to numerical taxonomic analysis. The symbiotic promiscuity of rhizobia precludes proposing a taxonomic scheme based solely on cross-inoculation patterns, but the affinities illustrated in the dendrogram provide guidance in the search for new and potentially useful symbioses for agricultural exploitation. The results also show that there is a need for further evaluation of the symbiotic affinities of legumes with their bacterial symbionts.

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