

## Numerical Taxonomy and DNA Relatedness of Tropical Rhizobia Isolated from Hainan Province, China

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**A total of 63 strains of rhizobia isolated from Hainan Province, a tropical region of the People's Republic of China, and 27 representative strains belonging to the genera *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium* were compared by performing numerical taxonomy, DNA hybridization, and DNA base composition analysis to determine the relationships among these rhizobia. The results indicated that the strains isolated from Hainan Province fell into two distinct phena, the slowly growing rhizobia and the fast-growing rhizobia. The slowly growing rhizobia, which formed three subphenae that seemed to be three subspecies, are *Bradyrhizobium japonicum* strains. The fast-growing strains belong to the genus *Rhizobium* and might be further divided into three specific groups. Sometimes both slowly growing rhizobia and fast-growing rhizobia were isolated from host plants belonging to the same genus or species or even from the same nodule. There was no correlation between hosts and the distribution of rhizobia in the subphenae. Isolates obtained from members of the same host genus or species fell into different groups or subgroups.**

The root nodule bacteria of tropical leguminous plants have been investigated by workers in many laboratories (1, 5–8, 10, 23, 25), but taxonomic studies of these bacteria have been sparse and have been limited to a few leguminous symbionts (16, 19, 24, 27, 28). Dreyfus and Dommergues (6) classified 13 *Acacia* species into three groups on the basis of effective nodulation with fast-growing and slowly growing tropical strains of rhizobia. The first group formed nodules effectively with slow growers; the second group formed effective nodules with fast growers; and the third group formed effective nodules with both fast and slow growers. Zhang et al. (27) obtained 12 clusters by using numerical taxonomy to study 97 strains isolated from root nodules of *Acacia senegal*, *Prosopis chilensis*, and other legumes. Thus, it is interesting to study the taxonomy of rhizobia isolated from a wide range of tropical legumes.

In this work 63 rhizobial strains were isolated from various legumes, including trees, herbs, and vines, growing in different geographic regions of Hainan Province of the People's Republic of China, a tropical region south of latitude 20° N. These strains were compared with previously described species of rhizobia by performing numerical taxonomy, DNA composition, and DNA-DNA hybridization analysis.

### MATERIALS AND METHODS

**Bacterial strains.** A total of 90 strains were used in this study (Table 1); 27 of these strains were type and representative strains of *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium* species, and 63 strains were isolated from root nodules collected in different geographic regions of Hainan Province. The purity of the cultures was assured by using single-colony isolates and checking colony morphology, bacterial morphology, and the Gram stain reaction. The identity of each strain was checked by performing a plant infection test with the original host plant.

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**Characteristics analyzed.** The following characteristics were analyzed: (i) utilization as sole carbon sources of L-alanine (0.1%), ammonium tartrate (0.1%), amygdalin (0.1%), D-arabinose (0.1%), D-arabitol (0.1%), D-arginine (0.1%), DL-arginine (0.1%), DL-asparagine (0.1%), DL-aspartic acid (0.1%), calcium gluconate (0.1%), calcium malonate (0.1%), D-(+)-cellobiose (0.1%), iso-cytosine (0.1%), dextrin (0.1%), 2,4-dimethoxybenzoate (0.1%), dulcitol (0.1%), meso-erythritol (0.1%), ferulic acid (0.1%), D-fucose (0.1%), D-galactose (0.1%), glucose (0.1%), D-glutamic acid (0.1%), L-glutamic acid (0.1%), DL-glutamine (0.1%), L-glutamine (0.1%), glycyl-DL-valine (0.1%), L-histidine (0.1%), m-hydroxybenzoic acid (0.1%), inositol (0.1%), lactose (0.1%), D-lyxose (0.1%), malic acid (0.1%), maltose (0.1%), D-(+)-melezitose (0.1%), D-methionine (0.1%), DL-ornithine monohydrobromide (0.1%), DL-proline (0.1%), raffinose (0.1%), L-(+)-rhamnose (0.1%), D-ribose (0.1%), salicin (0.1%), saliginin (0.1%), L-serine (0.1%), sodium benzoate (0.1%), sodium D-gluconate (0.1%), sodium hippurate (0.1%), sodium oxalate (0.1%), sodium salicylate (0.1%), sorbose (0.1%), sucrose (0.1%), syriagic acid (0.1%), D-(–)-tagatose (0.1%), tannic acid (0.1%), L-threonine (0.1%), D-tryptophan (0.1%), L-tryptophan (0.1%), D-tyrosine (0.1%), uric acid (0.1%), D-valine (0.1%), valeric acid (0.1%), and D-xylose (0.1%); (ii) utilization as sole nitrogen sources of D-arginine (0.1%), DL-arginine hydrochloride (0.1%), DL-aspartic acid (0.1%), D-cysteine (0.1%), DL-cysteine hydrochloride (0.1%), iso-cytosine (0.1%), 6-furfuryl aminopurine (0.1%), D-glutamic acid (0.1%), L-glutamic acid (0.1%), DL-glutamine (0.1%), glycine (0.1%), glycyl-DL-valine (0.1%), L-histidine (0.1%), D-histidine monohydrochloride (0.1%), D-methionine (0.1%), L-ornithine monohydrobromide (0.1%), DL-proline (0.1%), D-serine (0.1%), DL-serine (0.1%), D-tryptophan (0.1%), L-tryptophan (0.1%), L-valine (0.1%), and DL-valine (0.1%); (iii) resistance to the antibiotics chloramphenicol (5, 10, 25, 50, and 200  $\mu\text{g} \cdot \text{ml}^{-1}$ ), chlortetracycline (5, 25, and 50  $\mu\text{g} \cdot \text{ml}^{-1}$ ), erythromycin (25 and 125  $\mu\text{g} \cdot \text{ml}^{-1}$ ), gentamicin (5, 25, 50, and 100 U  $\cdot \text{ml}^{-1}$ ), kanamycin sulfate (5, 20, 50, 100, and 500 U  $\cdot \text{ml}^{-1}$ ), lincomycin (500  $\mu\text{g} \cdot \text{ml}^{-1}$ ), neomycin (25 and 75  $\mu\text{g} \cdot \text{ml}^{-1}$ ), penicillin GK (5, 20, 40, 100, and 500 U  $\cdot \text{ml}^{-1}$ ), qianglimycin (1, 10, 25, and 100  $\mu\text{g} \cdot \text{ml}^{-1}$ ), streptomycin (1, 5, 20, and 120  $\mu\text{g} \cdot \text{ml}^{-1}$ ),

TABLE 1. Origins and host plants of the bacterial strains examined

| Strain  | CCBAU no. <sup>a</sup> | Host plant                     | Origin <sup>b</sup> | Source <sup>a</sup> |
|---|------------------------|--------------------------------|---------------------|---------------------|
| <i>R. meliloti</i>  |                        |                                |                     |                     |
| USDA 1002 <sup>T</sup>  |                        |                                | United States       | USDA                |
| 102F28  |                        | <i>Medicago sativa</i>         |                     | UCD                 |
| H1  |                        | <i>Melilotus albus</i>         | Heilongjiang        | CCBAU               |
| <i>R. leguminosarum</i>   |                        |                                |                     |                     |
| USDA 2370 <sup>T</sup> ( <i>R. leguminosarum</i> biovar viceae) |                        |                                | United States       | USDA                |
| 127K17 ( <i>R. leguminosarum</i> biovar phaseoli)               |                        |                                | United States       | USDA                |
| 162X68 ( <i>R. leguminosarum</i> biovar trifolii)               |                        |                                | United States       | USDA                |
| <i>R. loti</i> ATCC 33669 <sup>T</sup>                          |                        |                                | Canada              | Canada              |
| <i>R. huakuii</i>   |                        |                                |                     |                     |
| A106  |                        | <i>Astragalus sinicus</i>      | Hubei               | HAU                 |
| PL-52   |                        | <i>Astragalus sinicus</i>      | Hubei               | HAU                 |
| 103 <sup>T</sup>  |                        | <i>Astragalus sinicus</i>      | Nanjing             | NAU                 |
| <i>R. galegae</i>   |                        |                                |                     |                     |
| HAMBI 540 <sup>T</sup>  |                        |                                | Finland             | HAMBI               |
| HAMBI 503   |                        |                                | United States       | HAMBI               |
| HAMBI 1185  |                        |                                | United Kingdom      | HAMBI               |
| <i>R. fredii</i>  |                        |                                |                     |                     |
| USDA 205 <sup>T</sup>   |                        | <i>Glycine soja</i>            | Henan               | USDA                |
| 2408  |                        | <i>Glycine soja</i>            | Liaoning            | SFRI                |
| USDA 194  |                        | <i>Glycine soja</i>            | Henan               | USDA                |
| <i>Rhizobium</i> sp.  |                        |                                |                     |                     |
| CCBAU 105   |                        | <i>Glycine max</i>             | Xinjiang            | CCBAU               |
| CCBAU 108   |                        | <i>Glycine max</i>             | Xinjiang            | CCBAU               |
| CCBAU 109   |                        | <i>Glycine max</i>             | Xinjiang            | CCBAU               |
| CCBAU 110   |                        | <i>Glycine max</i>             | Xinjiang            | CCBAU               |
| <i>R. tropici</i> CIAT 899 <sup>T</sup>                         |                        | <i>Phaseolus vulgaris</i>      |                     | CIAT                |
| <i>B. japonicum</i>   |                        |                                |                     |                     |
| USDA 6 <sup>T</sup>   |                        | <i>Glycine max</i>             | United States       | USDA                |
| USDA 110  |                        | <i>Glycine max</i>             |                     | USDA                |
| B15   |                        | <i>Glycine max</i>             | Liaoning            | IOC                 |
| <i>Agrobacterium tumefaciens</i>                                |                        |                                |                     |                     |
| B6S3  |                        |                                |                     | CCBAU               |
| C58   |                        |                                |                     | CCBAU               |
| <i>Agrobacterium radiobacter</i> 1.150                          |                        |                                |                     | CCBAU               |
| Strains isolated in this study                                  |                        |                                |                     |                     |
| A-2-Bs  |                        | <i>Lotus</i> sp.               | Xinjiang            | CCBAU               |
| B-6-1   |                        | <i>Lotus</i> sp.               | Xinjiang            | CCBAU               |
| I12   | 57002                  | <i>Centrosema pubescens</i>    | Hainan              | CCBAU               |
| I17   | 57517                  | <i>Centrosema pubescens</i>    | Hainan              | CCBAU               |
| I28   | 57520                  | <i>Centrosema pubescens</i>    | Hainan              | CCBAU               |
| H17   | 57502                  | <i>Centrosema pubescens</i>    | Hainan              | CCBAU               |
| H68   | 57507                  | <i>Centrosema pubescens</i>    | Hainan              | CCBAU               |
| H112  | 57509                  | <i>Centrosema pubescens</i>    | Hainan              | CCBAU               |
| I7  | 57514                  | <i>Crotalaria anagyroides</i>  | Hainan              | CCBAU               |
| I14   | 57515                  | <i>Crotalaria anagyroides</i>  | Hainan              | CCBAU               |
| I25   | 57519                  | <i>Crotalaria juncea</i>       | Hainan              | CCBAU               |
| I61   | 57522                  | <i>Crotalaria chinensis</i>    | Hainan              | CCBAU               |
| I70   | 57523                  | <i>Crotalaria hainanensis</i>  | Hainan              | CCBAU               |
| I79   | 57524                  | <i>Crotalaria linifolia</i>    | Hainan              | CCBAU               |
| S49   | 57532                  | <i>Crotalaria mucronata</i>    | Hainan              | CCBAU               |
| I80   | 57525                  | <i>Dalbergia obtusifolia</i>   | Hainan              | CCBAU               |
| H54   | 57505                  | <i>Dalbergia odorifera</i>     | Hainan              | CCBAU               |
| H101  | 57508                  | <i>Dalbergia balansae</i>      | Hainan              | CCBAU               |
| I15   | 57516                  | <i>Desmodium heterocarpon</i>  | Hainan              | CCBAU               |
| I20   | 57003                  | <i>Desmodium gyroides</i>      | Hainan              | CCBAU               |
| I32   | 57008                  | <i>Desmodium triquetrum</i>    | Hainan              | CCBAU               |
| I88   | 57527                  | <i>Desmodium biarticulatum</i> | Hainan              | CCBAU               |
| I94   | 57531                  | <i>Desmodium heterocarpon</i>  | Hainan              | CCBAU               |
| H22   | 57503                  | <i>Desmodium triangulare</i>   | Hainan              | CCBAU               |
| H121  | 57535                  | <i>Desmodium intortum</i>      | Hainan              | CCBAU               |
| I23   | 57518                  | <i>Desmodium intortum</i>      | Hainan              | CCBAU               |
| I30   | 57007                  | <i>Desmodium biandum</i>       | Hainan              | CCBAU               |
| I66   | 57015                  | <i>Desmodium sinuatum</i>      | Hainan              | CCBAU               |
| H50   | 57504                  | <i>Desmodium triangulare</i>   | Hainan              | CCBAU               |
| H62   | 57506                  | <i>Desmodium</i> sp.           | Hainan              | CCBAU               |
| H123  | 57511                  | <i>Desmodium triquetrum</i>    | Hainan              | CCBAU               |
| H33   | 57021                  | <i>Desmodium triquetrum</i>    | Hainan              | CCBAU               |

Continued on following page

TABLE 1—Continued

| Strain | CCBAU no. <sup>a</sup> | Host plant                        | Origin <sup>b</sup> | Source <sup>a</sup> |
|--------|------------------------|-----------------------------------|---------------------|---------------------|
| H14    | 57019                  | <i>Desmodium heterophyllum</i>    | Hainan              | CCBAU               |
| I86    | 57526                  | <i>Indigofera litoralis</i>       | Hainan              | CCBAU               |
| I40    | 57521                  | <i>Indigofera suffruticosa</i>    | Hainan              | CCBAU               |
| I3     | 57512                  | <i>Macroptilium atropurpureum</i> | Hainan              | CCBAU               |
| I27    | 57006                  | <i>Macroptilium lathyroides</i>   | Hainan              | CCBAU               |
| H24    | 57536                  | <i>Pachyrhizus erosus</i>         | Hainan              | CCBAU               |
| H31    | 57020                  | <i>Pueraria phaseoloides</i>      | Hainan              | CCBAU               |
| H122   | 57510                  | <i>Pueraria phaseoloides</i>      | Hainan              | CCBAU               |
| S74    | 57533                  | <i>Pueraria phaseoloides</i>      | Hainan              | CCBAU               |
| S88    | 57534                  | <i>Pueraria phaseoloides</i>      | Hainan              | CCBAU               |
| I5     | 57513                  | <i>Vigna sinensis</i>             | Hainan              | CCBAU               |
| I2     | 57001                  | <i>Stylosanthes guyanensis</i>    | Hainan              | CCBAU               |
| S68    | 57026                  | <i>Stylosanthes hamata</i>        | Hainan              | CCBAU               |
| S71    | 57027                  | <i>Stylosanthes guyanensis</i>    | Hainan              | CCBAU               |
| I64    | 57013                  | <i>Tephrosia candida</i>          | Hainan              | CCBAU               |
| S25    | 57022                  | <i>Tephrosia candida</i>          | Hainan              | CCBAU               |
| S93    | 57028                  | <i>Tephrosia purpurea</i>         | Hainan              | CCBAU               |
| S38    | 57023                  | <i>Leucaena diversifolia</i>      | Hainan              | CCBAU               |
| S43    | 57024                  | <i>Leucaena leucocephala</i>      | Hainan              | CCBAU               |
| H3     | 57016                  | <i>Leucaena</i> sp.               | Hainan              | CCBAU               |
| H7     | 57017                  | <i>Leucaena</i> sp.               | Hainan              | CCBAU               |
| H8     | 57018                  | <i>Leucaena leucocephala</i>      | Hainan              | CCBAU               |
| I54    | 57012                  | <i>Acacia sinuata</i>             | Hainan              | CCBAU               |
| S62    | 57025                  | <i>Cassia mimosoides</i>          | Hainan              | CCBAU               |
| I65    | 57014                  | <i>Arachis hypogaea</i>           | Hainan              | CCBAU               |
| I22    | 57004                  | <i>Sesbania cannabina</i>         | Hainan              | CCBAU               |
| I24    | 57005                  | <i>Calopogonium mucunoides</i>    | Hainan              | CCBAU               |
| I33    | 57009                  | <i>Zornia diphylla</i>            | Hainan              | CCBAU               |
| I36    | 57010                  | <i>Uraria crinita</i>             | Hainan              | CCBAU               |
| I39    | 57011                  | <i>Uraria lagopodioides</i>       | Hainan              | CCBAU               |
| I91    | 57528                  | <i>Alysicarpus vaginalis</i>      | Hainan              | CCBAU               |
| I92    | 57529                  | <i>Alysicarpus vaginalis</i>      | Hainan              | CCBAU               |
| I93    | 57530                  | <i>Alysicarpus vaginalis</i>      | Hainan              | CCBAU               |

<sup>a</sup> CCBAU, Culture Collection of Beijing Agricultural University, Beijing, People's Republic of China; HAU, Huazhong Agricultural University, Wuhan, People's Republic of China; HAMBI, Department of Microbiology, University of Helsinki, Helsinki, Finland; IOC, Institute of Oil Crops, Chinese Academy of Agricultural Science, Wuhan, People's Republic of China; NAU, Nanjing Agricultural University, Nanjing, People's Republic of China; SFRI, Soil and Fertilizer Research Institute, Chinese Academy of Agricultural Science, Beijing, People's Republic of China; UCD, University of California, Davis; USDA, United States Department of Agriculture, Beltsville, Md.

<sup>b</sup> All locations in the People's Republic of China unless indicated otherwise.

terramycin (5, 10, 30, and 100  $\mu\text{g} \cdot \text{ml}^{-1}$ ), tetracycline hydrochloride (5, 20; 40, and 100  $\mu\text{g} \cdot \text{ml}^{-1}$ ), and maidimycin (20, 40, and 100  $\mu\text{g} \cdot \text{ml}^{-1}$ ); (iv) resistance to the dyes acridine orange (0.1 and 0.05%), bromocresol purple (0.1%), bromophenol blue (0.1%), bromothymol blue (0.1 and 0.05%), Giemsa stain (0.1%), methyl green (0.1%), methyl red (0.1%), methylene blue (0.1%), methyl violet (0.1%), Nile blue (0.1%), phenol red (0.1%), chrysoidine (0.1%), gentian violet (0.05%), rose bengal (0.08%), neutral red (0.20%), and Congo red (0.20%); (v) tolerance to the chemicals bismuth sulfate (0.5, 1.0, and 2.0%), potassium tellurite (0.01, 0.02, and 0.05%), sodium desoxycholate (0.025, 0.05, 0.1, 0.2, and 0.5%), and sodium nitrite (0.025, 0.05, and 0.1%); (vi) growth on yeast extract-mannitol agar supplemented with NaCl at concentrations of 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0%; (vii) growth at pH 4.5, 5.5, 9.0; (viii) growth at 4, 37, 41, and 46°C; (ix) reactions in litmus milk; (x) reduction of methylene blue, nitrite, and Nile blue; (xi) acid or alkali production on yeast extract-mannitol agar; and (xii) production of urease, catalase, and cytochrome oxidase.

Most of the biological tests were conducted by using plates inoculated with a multipoint inoculator (12); about  $10^5$

cells per point was used as the inoculum. Other tests were conducted in broth. Unless indicated otherwise, the cultures were incubated at 28°C. Growth of the fast-growing and slowly growing strains was observed after incubation for 3 to 5 and 7 to 10 days, respectively. Carbohydrate utilization and organic acid utilization were determined on the medium of White (26), which contained the trace elements of medium Cs7 (17). Utilization of sole nitrogen sources was examined by using the same medium, except that  $\text{NaNO}_3$  was replaced with various nitrogen sources at a concentration of 0.1% (wt/vol). Resistance to antibiotics and dyes, tolerance of NaCl at various concentrations and the pH range for growth were examined by using yeast extract-mannitol agar (11) and the method of Thompson and Skerman (22). Nitrate reduction was tested by using a modification of the method of Pohlman (18), as described previously (2).

**Computer analysis.** The coding of characteristics, calculating similarity with the simple matching coefficient, determining the central strain of each phenotypic group, and clustering by the unweighted average linkage method were all performed by the methods of Sneath and Sokal (21). The analysis was performed at the Computer Research Labora-

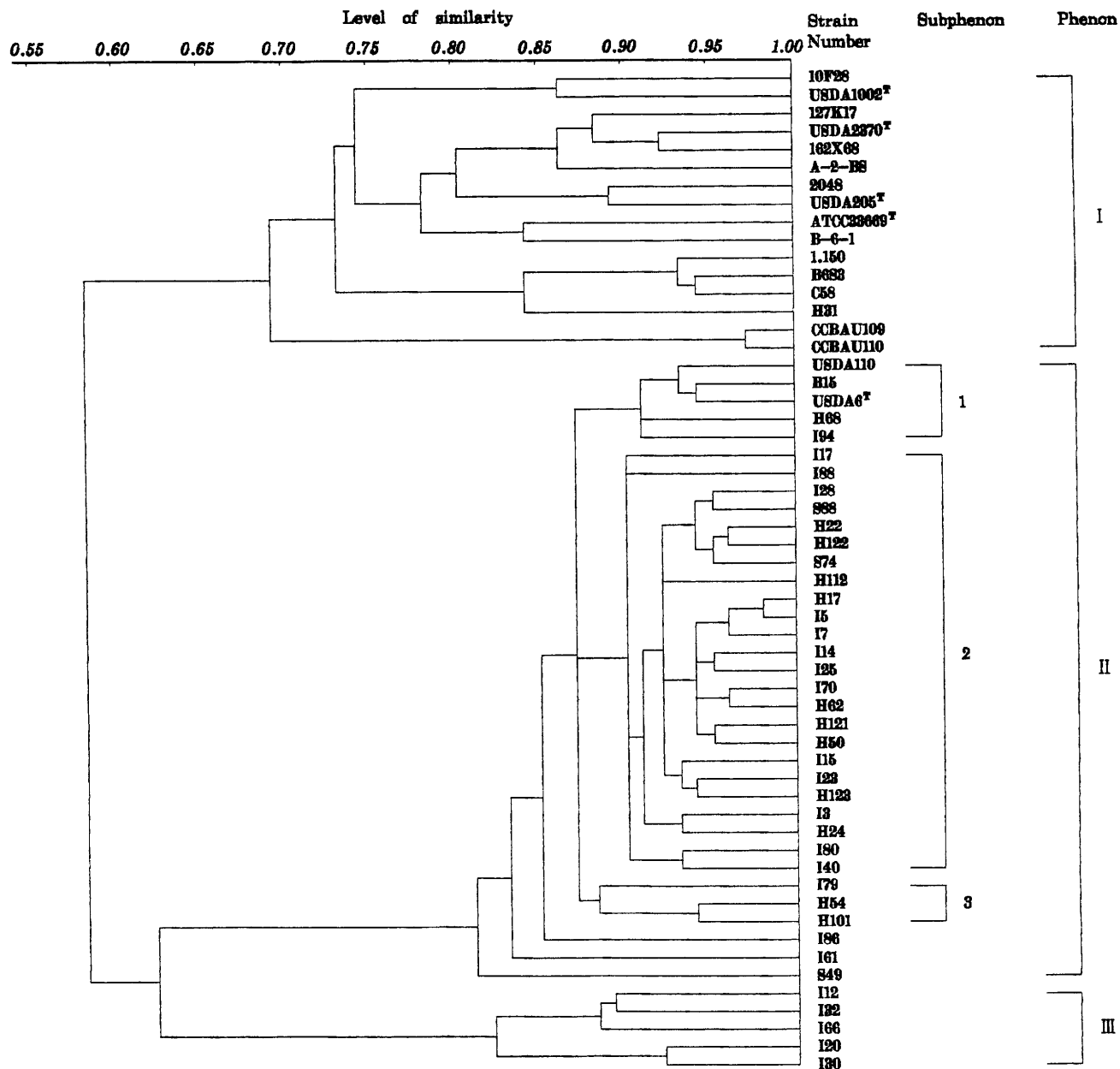


FIG. 1. Phenetic dendrogram showing the relationships among slowly growing rhizobia isolated from Hainan Province and reference strains.

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**Estimation of DNA base compositions and levels of DNA-DNA hybridization.** DNAs were isolated and purified by Marmur's method, with some modifications (14). The average G+C content of the DNA of each strain was measured by the thermal denaturation method (15), and the equation of De Ley (3) was used. *Escherichia coli* K-12 was used as the standard. The levels of DNA homology were determined by the initial renaturation rate method (4).

**RESULTS AND DISCUSSION**

**Numerical analysis of slowly growing rhizobia obtained from Hainan Province.** The numerical taxonomic analysis

was conducted in two steps. First, 32 slowly growing strains and 6 fast-growing strains isolated from Hainan Province were compared with 18 reference strains, including the type strains of previously described species belonging to the genera *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium*, by performing a numerical analysis of 193 phenotypic features. The resulting dendrogram is shown in Fig. 1.

Figure 1 shows that all of the strains examined clustered into three phenons at a level of similarity of 62%.

Phenon I, whose members clustered at a level of similarity of 69%, contained 13 representatives of *Rhizobium* and *Agrobacterium* species. Two strains isolated from *Lotus* sp. (strains A-2-BS and B-6-1 from Xinjiang Region) and one strain isolated from *Pueraria phaseoloides* (strain H31 from Hainan Province) also fell into this group.

TABLE 2. Distinctive features of the genus *Rhizobium* and the slowly growing and fast-growing rhizobia isolated from Hainan Province

| Characteristic   | <i>Rhizobium</i> | Slowly growing rhizobia | Fast-growing rhizobia |
|--|------------------|-------------------------|-----------------------|
| Utilization of the following compounds as sole carbon sources:   |                  |                         |                       |
| Amygdalin  | d <sup>a</sup>   | -                       | +                     |
| Dulcitol   | d                | -                       | +                     |
| Lactose  | +                | -                       | +                     |
| Sodium oxalate   | d                | -                       | +                     |
| Sucrose  | +                | -                       | +                     |
| Salicin  | d                | -                       | +                     |
| Uric acid  | d                | -                       | +                     |
| Dextrin  | +                | -                       | +                     |
| D-Arginine   | d                | -                       | +                     |
| DL-Arginine  | d                | -                       | +                     |
| D-Glutamic acid  | -                | d                       | +                     |
| L-Histidine  | d                | -                       | +                     |
| DL-Ornithine monohydrobromide                                    | d                | -                       | +                     |
| D-Tryptophan   | d                | -                       | +                     |
| L-Threonine  | -                | +                       | d                     |
| DL-Asparagine  | d                | -                       | +                     |
| D-Tyrosine   | d                | -                       | +                     |
| Utilization of the following compounds as sole nitrogen sources: |                  |                         |                       |
| DL-Cysteine hydrochloride  | -                | d                       | +                     |
| Histidine monohydrochloride                                      | d                | -                       | +                     |
| D-Tryptophan   | -                | d                       | +                     |
| Resistance to antibiotics  |                  |                         |                       |
| Chlortetracycline (25 µg · ml <sup>-1</sup> )                    | -                | +                       | +                     |
| Neomycin (25 µg · ml <sup>-1</sup> )                             | -                | +                       | d                     |
| Qianglimycin (1 µg · ml <sup>-1</sup> )                          | -                | +                       | +                     |
| Qianglimycin (10 µg · ml <sup>-1</sup> )                         | -                | +                       | d                     |
| Qianglimycin (25 µg · ml <sup>-1</sup> )                         | -                | +                       | d                     |
| Streptomycin (20 µg · ml <sup>-1</sup> )                         | -                | d                       | +                     |
| Terramycin (10 µg · ml <sup>-1</sup> )                           | -                | +                       | d                     |
| Terramycin (30 µg · ml <sup>-1</sup> )                           | -                | +                       | d                     |
| Tetracycline HCl (5 µg · ml <sup>-1</sup> )                      | -                | +                       | +                     |
| Tetracycline HCl (20 µg · ml <sup>-1</sup> )                     | -                | +                       | +                     |
| Tetracycline HCl (40 µg · ml <sup>-1</sup> )                     | -                | +                       | d                     |
| Resistance to dyes   |                  |                         |                       |
| Bromothymol blue (0.1%)  | d                | -                       | +                     |
| Bromothymol blue (0.05%)   | d                | -                       | +                     |
| Chrysoidine (0.1%)   | d                | -                       | +                     |
| Growth at:   |                  |                         |                       |
| pH 4.5   | -                | -                       | +                     |
| pH 9.0   | d                | -                       | +                     |
| Methylene blue reduction   | +                | -                       | +                     |
| Litmus milk alkali production                                    | d                | +                       | -                     |
| Litmus milk peptonization  | d                | -                       | +                     |
| Acid production  | +                | -                       | +                     |
| Alkali production  | -                | +                       | -                     |

<sup>a</sup> +, at least 95% of the strains are positive; d, less than 95% but more than 5% of the strains are positive; -, less than 5% of the strains are positive.

The members of phenon II, which contained 32 strains of slowly growing rhizobia obtained from Hainan Province and three representative strains of *Bradyrhizobium japonicum*, clustered at a level of similarity of 81%. In addition, at a level of similarity of 88%, phenon II was divided into three subphenon. Subphenon 1, whose members clustered at a level of similarity of 91%, was composed of three representative strains of *B. japonicum* and two strains isolated from *Centrosema pubescens* and *Desmodium heterocarpon*. Subphenon 2 formed at a level of similarity of 90% and contained 24 strains isolated from members of nine host plant genera,

including the genera *Centrosema* (strains I17, I28, H112, and H17 from *Centrosema pubescens*), *Desmodium* (strain I88 from *Desmodium biarticulatum*, strains H22 and H50 from *Desmodium triangulare*, strain H62 from *Desmodium* sp., strains H121 and I23 from *Desmodium intortum*, strain I15 from *Desmodium heterocarpon*, strain H123 from *Desmodium triquetrum*), *Pueraria* (strains H122 and S74 from *Pueraria phaseoloides*), *Vigna* (strain I5 from *Vigna sinensis*), *Crotalaria* (strains I7 and I14 from *Crotalaria anagyroides*, strain I25 from *Crotalaria juncea*, strain I70 from *Crotalaria hainanensis*), *Macroptilium* (strain I3 from *Macroptilium atropurpureum* cv. siratro), *Paehyrhizus* (strain H24 from *Pachyrhizus erosus*), *Dalbergia* (strain I80 from *Dalbergia obtusifolia*), and *Indigofera* (strain I40 from *Indigofera suffruticosa*). Strain H22 was the central strain of phenon II. Subphenon 3 was composed of three strains isolated from the genera *Crotalaria* (strain I79 from *Crotalaria linifolia*) and *Dalbergia* (strain H54 from *Dalbergia odorifera*, strain H101 from *Dalbergia balansae*). These strains were linked at a similarity level of 88%. Three strains (strain I86 from *Indigofera litoralis*, strain I61 from *Crotalaria chinensis*, strain S49 from *Crotalaria mucronata*) did not form a subcluster; these strains joined the three subphenon at levels of similarity of 85, 83, and 81%, respectively.

The slowly growing rhizobia isolated from Hainan Province (phenon II, including three subphenon), clustered with the representative strains of *B. japonicum* at a high similarity level (81%) and were distinctly separated from the other two subphenon. On the basis of the view of Sneath (20) that the level of similarity of strains belonging to the same species is about 80%, it seemed that the slowly growing rhizobia isolated from Hainan Province are *B. japonicum* strains and that the three subphenon are three subspecies of *B. japonicum*.

Five strains were isolated from *Centrosema pubescens*; one of these strains (H68) fell into subphenon 1, and the other four strains (I17, I28, H112, H17) fell into subphenon 2. Nine strains were isolated from *Desmodium* spp.; one of these strains (I94) fell into subphenon 1, and the other 8 strains (I88, H22, H62, H121, H50, I15, I23, H123) fell into subphenon 2. Seven strains were isolated from *Crotalaria* spp.; four of these strains (I7, I14, I25, I70) fell into subphenon 2, one strain (I79) fell into subphenon 3, and two strains (I61, S49) did not fall into any subphenon. These results show that slowly growing rhizobia may be members of different clusters even though they were isolated from members of the same host plant genus. Thus, the clustering of the slowly growing rhizobia was not closely related to the genera of their host plants. This indicated that the host specificity of the slowly growing rhizobia is very weak. Meanwhile, the high level of similarity in phenon II indicated that all of the slowly growing rhizobia are closely related to each other.

Phenon III contained five fast-growing rhizobia isolated from Hainan Province, and its members clustered at a level of similarity of 82%. These organisms were isolated from members of the genera *Centrosema* (strain I12 from *Centrosema pubescens*) and *Desmodium* (strain I32 from *Desmodium triquetrum*, strain I66 from *Desmodium sinuatum*, strain I20 from *Desmodium gyroides*, strain I30 from *Desmodium biandum*). These fast-growing rhizobia are distinctly different from either the slowly growing rhizobia or the previously described fast-growing species shown in Fig. 1. Figure 1 also shows that nine slowly growing rhizobia isolated from *Desmodium* spp. (strain I94 from *Desmodium heterocarpon* var. *stigosum*, strain I88 from *Desmodium biarticulatum*, strains H22 and H50 from *Desmodium triangulare*, strain H62 from *Desmodium* sp., strains H121 and

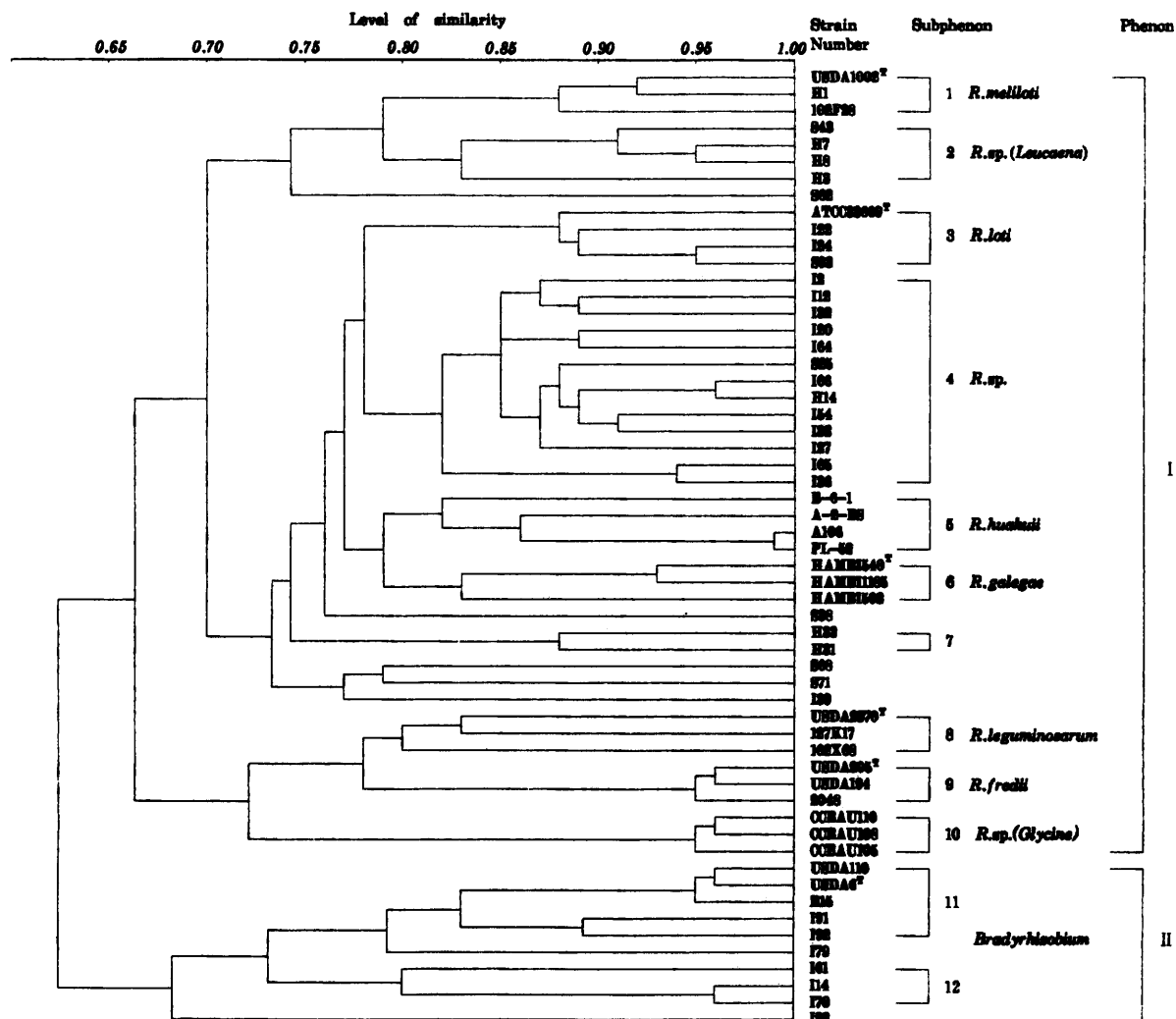


FIG. 2. Phenetic dendrogram showing the relationships among fast-growing rhizobia isolated from Hainan Province and reference strains.

I23 from *Desmodium intortum*; strain I15 from *Desmodium heterocarpon*, strain H123, from *Desmodium triquetrum*) fell into phenon II, while four fast-growing rhizobia isolated from members of the same genus of host plants (strain I32 from *Desmodium triquetrum*, strain I66, from *Desmodium sinuatum*, strain I20 from *Desmodium gyroides*, strain I30 from *Desmodium biantum*) fell into phenon III. These results indicate that slowly growing and fast-growing rhizobia may fall into different phenon that are distantly related to each other even though they were isolated from members of the same genus of host plants.

The differential characteristics of the three phenon are shown in Table 2. The data show that the fast-growing Hainan Province rhizobia could utilize a wider range of carbon and nitrogen sources than previously recognized *Rhizobium* and *Bradyrhizobium* strains could. These organisms were also more resistant than *Rhizobium* strains and more susceptible than *Bradyrhizobium* strains to the antibiotics used in this study, and they could grow at either pH 4.5 or pH 9.0.

**Numerical analysis of fast-growing rhizobia from Hainan Province.** Since the fast-growing Hainan Province rhizobia

are very different from previously described *Rhizobium* strains, we increased the number of fast growers to 27 isolates obtained from a wide range of leguminous plants growing in Hainan Province and conducted a second numerical analysis with 23 representative strains belonging to previously recognized *Rhizobium* species, including *Rhizobium huakuii* and *Rhizobium galegae* (2, 13). A total of 143 phenotypic characteristics were used. The results are shown in the dendrogram in Fig. 2.

Figure 2 shows that all of the strains examined clustered into two phenon at a similarity level of 66%. Phenon I included 27 strains of fast-growing rhizobia obtained from Hainan Province and 18 representative strains of *Rhizobium* species. A total of 10 subphenon were formed at a level of similarity of 80%. Subphenon 1, 5, 6, 8, 9, and 10 contained the known *Rhizobium meliloti*, *R. huakuii*, *R. galegae*, *Rhizobium leguminosarum*, *Rhizobium fredii*, and *Rhizobium* sp. strains, respectively. Subphenon 2 contained four strains isolated from *Leucaena* spp. Subphenon 3 contained the type culture of *Rhizobium loti* and three strains obtained from *Sesbania cannabina*, *Calopogonium mucunoides*, and *Tephrosia purpurea*. Subphenon 4 contained 13 strains iso-

TABLE 3. Levels of DNA relatedness between fast-growing rhizobia isolated from Hainan Province and belonging to subphenon 2<sup>a</sup>

| Strain | % Homology with strain: |     |     |     |
|--------|-------------------------|-----|-----|-----|
|        | S43                     | H7  | H8  | H3  |
| S43    | 100                     |     |     |     |
| H7     | 86                      | 100 |     |     |
| H8     | 73                      | 78  | 100 |     |
| H3     | 71                      | 84  | 73  | 100 |

<sup>a</sup> See Fig. 2.

lated from 12 species belonging to nine genera of host plants (*Stylosanthes guyanensis*, *Centrosema pubescens*, *Desmodium triquetrum*, *Desmodium gyroides*, *Desmodium sinuatum*, *Desmodium heterophyllum*, *Tephrosia candida*, *Acacia sinuata*, *Zornia diphylla*, *Macroptilium lathyroides*, *Arachis hypogaea*, and *Uraria crinita*). Subphenon 7 contained two strains isolated from *Desmodium triquetrum* and *Pueraria phaseoloides*.

Phenon II contained three strains of *B. japonicum* and seven slowly growing rhizobia obtained from Hainan Province. Two subphena were formed at a level of similarity of 80%. Subphenon 11 contained three representative strains of *B. japonicum* and two strains isolated from *Alysicarous vaginalis*; three strains isolated from *Crotalaria chinensis*, *Crotalaria anagyroides*, and *Crotalaria hainanensis* made up subphenon 12.

Five fast-growing rhizobia (S62, S38, S68, S71, I39) and two slowly growing rhizobia (I79, I93) did not fall into any subphenon at a similarity level of 80%.

**DNA relatedness of fast-growing rhizobia isolated from Hainan Province.** The levels of DNA homology and the DNA base compositions of representative strains of rhizobia belonging to each subphenon isolated from Hainan Province, as well as type strains of previously recognized *Rhizobium* and *Bradyrhizobium* species, were determined by the spectrophotometric method (3). The results showed that the levels of DNA relatedness between strains in subphenon 2 ranged from 71 to 86% (Table 3). The levels of DNA relatedness for four strains in subphenon 3, including *R. loti* ATCC 33669<sup>T</sup> (T = type strain), ranged from 78 to 91% (Table 4). And the levels of DNA relatedness for five strains in subphenon 4 ranged from 75 to 93% (Table 5). Strains that exhibited more than 70% DNA-DNA relatedness were members of a specific group, and the levels of DNA homology between the groups were less than 30%. The levels of DNA homology between most representative strains of the three subphena and eight type strains of previously described *Rhizobium* and *Bradyrhizobium* species ranged from 0 to 46%; the only exception was the 82% level of homology

TABLE 4. Levels of DNA relatedness between fast-growing rhizobia isolated from Hainan Province and belonging to subphenon 3<sup>a</sup>

| Strain | % Homology with strain: |     |     |                         |
|--------|-------------------------|-----|-----|-------------------------|
|        | I22                     | S93 | I24 | ATCC 33669 <sup>T</sup> |
| I22    | 100                     |     |     |                         |
| S93    | 78                      | 100 |     |                         |
| I24    | 91                      | 82  | 100 | 82                      |

<sup>a</sup> See Fig. 2.

TABLE 5. Levels of DNA relatedness between fast-growing rhizobia isolated from Hainan Province and belonging to subphenon 4<sup>a</sup>

| Strain | % Homology with strain: |     |     |     |     |
|--------|-------------------------|-----|-----|-----|-----|
|        | I12                     | I20 | I36 | I54 | I66 |
| I12    | 100                     |     |     |     |     |
| I20    | 82                      | 100 |     |     |     |
| I36    | 85                      | 92  | 100 |     |     |
| I54    | 78                      | 78  | 93  | 100 |     |
| I66    | 77                      | 89  | 75  | 80  | 100 |

<sup>a</sup> See Fig. 2.

observed between strains ATCC 33669<sup>T</sup> and I24, which were in the same subphenon, subphenon 3 (Table 6). The results described above indicated that strains isolated from *Sesbania cannabina*, *Calopogonium mucunoides*, and *Tephrosia purpurea* should be placed in *R. loti*. Subphenon 2 contained four strains isolated from *Leucaena* spp. These organisms constituted a distinct group that was different from *Rhizobium tropici* (16). And subphenon 4 was also an independent group different from previously recognized *Rhizobium* species.

The DNA G+C contents of representative strains of subphena 2 through 4, as well as the type strains of *R. leguminosarum* biovar viceae and *R. loti*, ranged from 59 to 64 mol%; these values were within the range expected for the genus *Rhizobium* (11).

According to the minimal standards for description of new genera and species of root- and stem-nodulating bacteria proposed by the Subcommittee on the Taxonomy of *Rhizobium* and *Agrobacterium* (9), subphenon 4 might be a new *Rhizobium* species on the basis of the results of the numerical taxonomy and DNA relatedness analysis. In order to determine its phylogenetic position, DNA-rRNA hybridization or 16S rRNA sequence analysis will be required.

From the data described above, it seemed that the slowly growing rhizobia isolated from various legumes growing in a tropical region were very closely related but the fast-growing rhizobia were highly diverse and that the classification of these organisms is not closely related to the identities of the host plants.

TABLE 6. Levels of DNA relatedness between representative strains belonging to subphena 2 through 4 and type strains of *Rhizobium* and *Bradyrhizobium* species<sup>a</sup>

| Strain                  | % Homology with strain: |     |     |
|-------------------------|-------------------------|-----|-----|
|                         | I66                     | H7  | I24 |
| USDA 1002 <sup>T</sup>  | 0                       | 0   | 0   |
| USDA 2370 <sup>T</sup>  | 46                      | 2   | 0   |
| ATCC 33669 <sup>T</sup> | 0                       | 0   | 82  |
| CCBAU 110               | 8                       | 32  | 0   |
| USDA 205 <sup>T</sup>   | 0                       | 18  | 0   |
| CCBAU 2609              | 0                       | 40  | 35  |
| HAMBI 540 <sup>T</sup>  | 0                       | 0   | 0   |
| CIAT 899 <sup>T</sup>   | 11                      | 0   |     |
| USDA 6 <sup>T</sup>     | 10                      | 0   | 0   |
| I66                     | 100                     | 29  | 24  |
| H7                      |                         | 100 | 8   |
| I24                     |                         |     | 100 |

<sup>a</sup> See Fig. 2.

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