# *Bradyrhizobium* spp. (TGx) isolates nodulating the new soybean cultivars in Africa are diverse and distinct from bradyrhizobia that nodulate North American soybeans

Robert C. Abaidoo,[1]† Harold H. Keyser,[1] Paul W. Singleton[1] and Dulal Borthakur[2]

Author for correspondence: Dulal Borthakur. Tel: +1 808 956 6600. Fax: +1 808 956 3542. e-mail: dulal@hawaii.edu

[1] University Hawaii NifTAL Project, 1000 Holomua Avenue, Paia, HI 96779, USA

[2] Department of Plant Molecular Physiology, University of Hawaii, Honolulu, HI 96822, USA

**The newly developed cultivars of soybean in Africa, known as Tropical *Glycine* cross (TGx), are nodulated by bradyrhizobia indigenous to African soils, here designated *Bradyrhizobium* spp. (TGx). Isolates of *Bradyrhizobium* spp. (TGx) obtained from nodules of TGx soybeans that were inoculated with soils from 65 locations in six African countries were characterized and grouped into 11 phylogenetic clusters on the basis of RFLP of the 16S rRNA gene. Five restriction enzymes (*Rsa*I, *Hin*fI, *Msp*I, *Cfo*I and *Hae*III) established RFLP groups within these *Bradyrhizobium* spp. (TGx) isolates, which were used to construct a phylogenetic tree showing their genetic relationship with other *Bradyrhizobium* species. RFLP analysis indicated that *Bradyrhizobium* spp. (TGx) is a heterogeneous group with some isolates related to *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* strains and some to *Bradyrhizobium* spp. (misc.) reference strains isolated from a variety of tropical legumes. The heterogeneity within the large phylogenetic clusters was further examined through analysis of randomly amplified polymorphic DNA (RAPD) using GC-rich PCR primers. The RAPD analysis showed additional heterogeneity in the *Bradyrhizobium* spp. (TGx) phylogenetic clusters, which was not revealed by separations based on RFLP analysis. The *Bradyrhizobium* spp. (TGx) isolates were classified into effective and ineffective types based on their symbiotic performance on TGx soybean. The isolates were randomly distributed throughout the phylogenetic clusters regardless of their symbiotic effectiveness on TGx soybean.**

**Keywords:** *Bradyrhizobium* spp. (TGx), TGx soybean, symbiosis, RFLP, randomly amplified polymorphic DNA (RAPD)

## INTRODUCTION

*Bradyrhizobium japonicum* populations required for effective nodulation of soybeans are not endemic to African soils (Hadley & Hymowitz, 1973). To avoid the need to inoculate soybean with *B. japonicum*, soybean breeders at the International Institute of Tropical Agriculture (IITA), Nigeria, developed new soybean genotypes for Africa, known as Tropical *Glycine* cross (TGx), which nodulate with *Bradyrhizobium* spp. populations indigenous to African soils (Pulver *et al*., 1985). TGx soybean genotypes in some locations, however, are reported to develop nitrogen deficiency symptoms (Okereke & Eaglesham, 1992; Pal & Norman, 1987), suggesting that the TGx soybean may require inoculation with bradyrhizobia at some locations if high soybean yields are to be obtained. To better understand the degree to which bradyrhizobia populations in Africa nodulate TGx soybean, it is essential to characterize these *Bradyrhizobium* spp. populations and determine their genetic relatedness with *B. japonicum* and other *Bradyrhizobium* species. The following definitions of

†**Present address:** Department of Biological Sciences, University of Science and Technology, Kumasi, Ghana.

**Abbreviation:** RAPD, randomly amplified polymorphic DNA.



Downloaded from www.microbiologyresearch.org by
IP: 54.70.40.11
On: Wed, 06 Mar 2019 19:29:00

the different bradyrhizobia groups are used in this paper: *Bradyrhizobium* spp. (misc.) are isolates from tropical legumes belonging to the 'cowpea cross-inoculation group' of promiscuous legumes; *Bradyrhizobium* spp. (TGx) are isolates from the nodules of TGx soybean genotypes which are ineffective on *Glycine max* cultivar Clark (see nodulation and nitrogen fixation assay in Methods). Those isolates from the nodules of TGx soybean genotypes which are effective on Clark, and are therefore similar to *B. japonicum* or *Bradyrhizobium elkanii* strains in their symbiotic phenotypes, are not considered as *Bradyrhizobium* spp. (TGx) and are excluded from this study.

The genetic characteristics of *Bradyrhizobium* spp. (TGx) that nodulate TGx soybean genotypes have not been determined. Available information, however, indicates that *Bradyrhizobium* spp. in general do not nodulate North American soybean genotypes (Leonard, 1923; Sears & Carroll, 1926). Other reports suggest that the bradyrhizobia which nodulate TGx soybeans are unique populations within the *Bradyrhizobium* spp. (Bromfield & Roughley, 1980; Pulver *et al*., 1985; Roughley *et al*., 1980). Bromfield & Roughley (1980) established that the bradyrhizobia populations nodulating the local soybean cultivars grown in Nigeria were not strains of *B. japonicum*. The present investigation was undertaken to determine the extent of genetic diversity within the *Bradyrhizobium* spp. (TGx) populations and their relatedness to *B. japonicum* and other *Bradyrhizobium* spp. We characterized isolates of *Bradyrhizobium* spp. (TGx) obtained from nodules of TGx soybeans that were inoculated with soils from 65 locations in six countries in Africa, and grouped them into several phylogenetic clusters on the basis of RFLP of the 16S rRNA gene. The heterogeneity within the large phylogenetic clusters was further examined using the random primer PCR method.

## METHODS

**Isolation of *Bradyrhizobium* spp. (TGx) isolates.** *Bradyrhizobium* spp. (TGx) isolates were isolated from the nodules of *Glycine max* cultivar TGx 1456-2E inoculated with diluted soil samples as described by Somasegaran & Hoben (1994). Soil samples were collected from 65 locations in 14 geographic regions within six African countries. Pregerminated seeds of cultivar TGx 1456-2E were planted in growth pouches containing a nitrogen-free nutrient solution (Somasegaran & Hoben, 1994). Five days after planting, the root system of each plant was inoculated with 2 ml of a 1:10 (soil:water) soil suspension. Plants were grown under 1000 W sodium lamps providing approximately 1200 μE photosynthetically active radiation. Five uninoculated control plants did not nodulate. One or two nodules from each plant inoculated with a soil suspension were randomly sampled, surface-sterilized by immersing in 2·6% sodium hypochlorate, rinsed with sterile deionized water and then placed in 0·01% acidified $HgCl_2$ solution, followed by repeated rinses in sterile deionized water. Nodules were then crushed in 50 μl sterile water and the nodule content was streaked onto yeast extract-mannitol agar (YMA) with either Congo red or bromothymol blue indicators (Somasegaran & Hoben, 1994). Individual isolates were established from single colonies and used to inoculate the same TGx soybean line, 1456-2E, and the American soybean cultivar Clark. A few isolates which were effective on Clark and therefore not considered as *Bradyrhizobium* spp. (TGx) were excluded from this study. *Bradyrhizobium* spp. (TGx) isolates and the geographic regions from where the soil samples were collected are listed in Table 1.

***Bradyrhizobium* and *Rhizobium* reference strains.** Reference strains included 15 strains from the University of Hawaii NifTAL Center (TAL prefix), nine strains from the USDA Beltsville National *Rhizobium* Collection (USDA prefix), and a few other strains belonging to several *Bradyrhizobium* and *Rhizobium* species, which are listed in Table 1.

**Genomic DNA preparation.** Genomic DNA was isolated from stationary phase bacterial cultures grown in arabinose-gluconate (AG) broth medium (Sadowsky *et al*., 1987). Cells were washed in 1·0 M NaCl and DNA was extracted by the method of Heath *et al*. (1986).

**PCR amplification of the 16S rRNA gene.** The 16S rRNA gene fragment was amplified by PCR using two primers from the conserved region. The forward primer, 5′-AGA GTT TGA TCC TGG CTC AG-3′, corresponds to positions 27–46 of the *Escherichia coli* 16S rRNA gene sequence, and the reverse primer, 5′-ACG CCG ACC TAG TGG AGG AA-3′, corresponds to positions 1525–1506 of the *E. coli* sequence (Lane, 1991). The reactions were carried out in 50 μl volumes containing 80–100 ng genomic DNA, 1·25 U *Taq* polymerase in 20 mM Tris (pH 8), 2·5 mM $MgCl_2$, 200 μM each dATP, dGTP, dCTP and dTTP, and 0·4 μM each of the forward and reverse primers. The mixture was subjected to 25 PCR cycles in a thermocycler (GeneAmp PCR System 2400; Perkin-Elmer). The temperature regimes were as follows: pre-PCR denaturation, 94 °C for 5 min; PCR denaturation, 94 °C for 30 s; primer annealing, 58 °C for 30 s; primer extension, 72 °C for 30 s; and final extension, 72 °C for 7 min. Ten microlitre portions of the product were separated electrophoretically on a 1·5% agarose gel at approximately 80 mV to establish successful separation.

**RFLP analysis of the PCR-amplified 16S rRNA gene fragment.** Fifteen microlitre portions of the amplification product were digested in a final volume of 20 μl, with each of seven restriction enzymes (*Rsa*I, *Hin*fI, *Msp*I, *Cfo*I, *Hae*III, *Alu*I and *Taq*I). The DNA fragments were separated electrophoretically on a 2·5% agarose gel at 80–85 mV for approximately 3 h. Restriction patterns were normalized by comparing with selected reference strains which were included at regular intervals on the gel. RFLP groups were established based on the restriction fragment patterns generated by the different enzymes. Relationships between strains were established by using data from restriction enzymes that adequately differentiated strains. A binary scoring system (1 for the presence of a band and 0 for the absence) was used to generate an input matrix, which was analysed using the unweighted pair group method with averages (UPGMA) algorithm (Sneath & Sokal, 1973), and a dendrogram was generated from the matrix using NTSYS-PC software (Exeter Software).

**Differentiation of *Bradyrhizobium* spp. (TGx) isolates within RFLP clusters by randomly amplified polymorphic DNA (RAPD) analysis.** Heterogeneity within the 16S rRNA gene RFLP clusters was examined by arbitrarily primed PCR amplification of genomic DNA fragments using one GC-only and two GC-rich primers. The GC-only primer, 5′-GCCCCGCCGCC-3′, was used previously by Mathis &



Downloaded from www.microbiologyresearch.org by
IP: 54.70.40.11
On: Wed, 06 Mar 2019 19:29:00

***Table 1.*** Original host legume and the geographic origin of *Bradyrhizobium*, *Sinorhizobium* and *Rhizobium* strains

| Strain/isolate | Host legume | Geographic origin |
|---|---|---|
| ***Bradyrhizobium* spp. (TGx)** | | |
| NI-1, NI-2, NI-3, NI-20, NI-27, NI-28, NI-29, NI-30, NI-31, NI-32, NI-43, NI-47, NI-48, NI-49 | *Glycine max* | Zaria, Nigeria |
| NI-6, NI-14, NI-16, NI-23, NI-24, NI-41, NI-42, NI-45, NI-50, NI-51, NI-53, NI-54, NI-57 | *Glycine max* | Zonkwa, Nigeria |
| NI-8, NI-17, NI-35, NI-36 | *Glycine max* | Abuja, Nigeria |
| NI-9, NI-10, NI-11, NI-12, NI-13, NI-19, NI-21, NI-25, NI-26, NI-39, NI-40, NI-56 | *Glycine max* | Abeokuta, Nigeria |
| NI-52 | *Glycine max* | Jos, Nigeria |
| UG-4, UG-5, UG-6, UG-9 | *Glycine max* | Kaban, Uganda |
| UG-3, UG-7, UG-8, UG-10 | *Glycine max* | Namul, Uganda |
| CA-1, CA-7 | *Glycine max* | Dschang, Cameroon |
| CA-4, CA-5, CA-6, CA-7, CA-8 | *Glycine max* | Bambui, Cameroon |
| TG-1, TG-2, TG-4, TG-5, TG-6, TG-10, TG-46 | *Glycine max* | Kara, Togo |
| TG-3, TG-7, TG-8, TG-9, TG-11, TG-12 | *Glycine max* | Sotouboua, Togo |
| BN-1, BN-2, BN-3, BN-4 | *Glycine max* | Ina, Benin |
| GH-1, GH-3, GH-7 | *Glycine max* | Kumasi, Ghana |
| GH-2, GH-8 | *Glycine max* | Nyankpala, Ghana |
| ***Bradyrhizobium* spp. (misc.)** | | |
| GU-120, GU-121, GU-122, GU-123 | *Vigna unguiculata* | Barrigada, Guam |
| NC-3, NC-9, NC-11, NC-57, TAL 173 | *Vigna unguiculata* | Ibadan, Nigeria |
| TAL 16 | *Arachis glabrata* | FL, USA |
| TAL 36, TAL 191 | *Cajanus cajan* | Nigeria |
| TAL 309 | *Macrotyloma africanum* | Zimbabwe |
| TAL 363 | *Albizia lebbek* | Burma |
| TAL 574 | *Mucuna deeringianum* | Zimbabwe |
| TAL 800 | *Vigna unguiculata* | Colombia |
| TAL 894 | *Vigna* sp. | Philippines |
| TAL 1039 | *Cajanus cajan* | Kenya |
| TAL 1069 | *Vigna ungularis* | Taiwan |
| TAL 1446 | *Acacia auriculiformis* | HI, USA |
| TAL 1881 | *Acacia auriculiformis* | Côte-d'Ivoire |
| USDA 3764 | *Pueraria phaseoloides* var. javanica | Brazil |
| ***Bradyrhizobium japonicum*** | | |
| USDA 110 | *Glycine max* | FL, USA |
| USDA 31 | *Glycine max* | WI, USA |
| USDA 59 | *Glycine max* | NC, USA |
| J-1 | *Glycine max* | Ibadan, Nigeria |
| ***Bradyrhizobium elkanii*** | | |
| USDA 38 | *Glycine max* | Japan |
| USDA 74, USDA $76^{T}$ | *Glycine max* | CA, USA |
| ***Sinorhizobium fredii*** | | |
| USDA 191, USDA 193 | *Glycine max* | China |
| ***Rhizobium leguminosarum* bv. trifolii** | | |
| SU7 | *Trifolium* sp. | Australia |
| ***Rhizobium etli*** | | |
| TAL 182 | *Phaseolus vulgaris* | HI, USA |
| ***Rhizobium tropici*** | | |
| CIAT $899^{T}$ | *Phaseolus vulgaris* | Columbia |
| ***Rhizobium* sp.** | | |
| NGR 234 | *Lablab purpureus* | Papua New Guinea |
| TAL 1145 | *Leucaena diversifolia* | Australia |



Downloaded from www.microbiologyresearch.org by
IP: 54.70.40.11
On: Wed, 06 Mar 2019 19:29:00

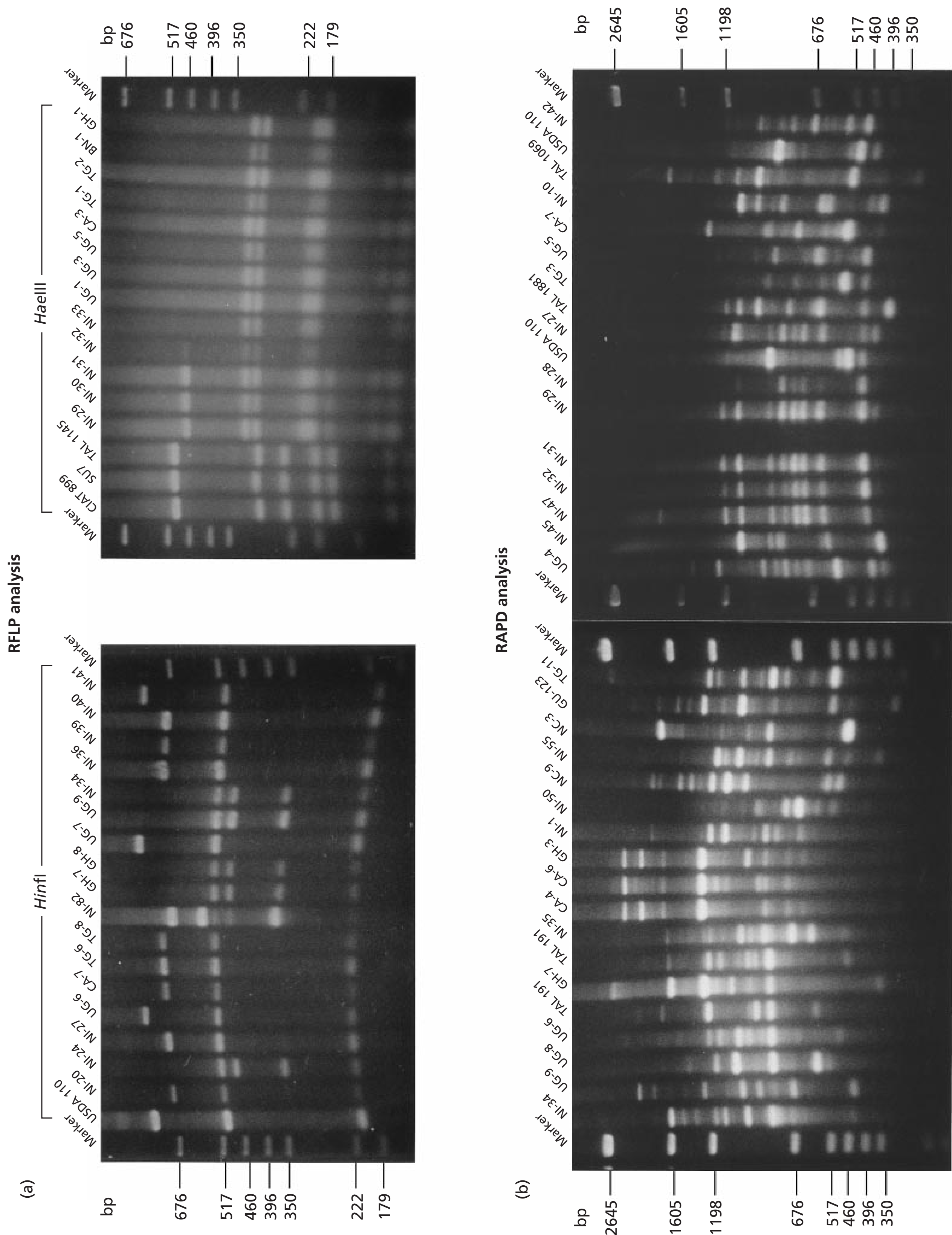


***Fig. 1.*** Photographs showing examples of RFLP and RAPD analyses of some *Bradyrhizobium* spp. (TGx) isolates and reference strains. (a) RFLP analysis of 1·5 kb PCR-amplified 16S rDNA digested with *Hin*fI and *Hae*III. The fragments were separated on a 1·5% agarose gel. (b) RAPD analysis showing RAPD fragments generated by PCR using a GC-only primer. These fragments were separated on a 2% agarose gel.



Downloaded from www.microbiologyresearch.org by
IP: 54.70.40.11
On: Wed, 06 Mar 2019 19:29:00

***Table 2.*** Distribution of 16S rDNA restriction enzyme polymorphic groups among 22 isolates of *Bradyrhizobium* spp. (misc.) and 81 isolates of *Bradyrhizobium* spp. (TGx)

| Restriction enzyme | Recognition sequence (5′–3′) | RFLP group | Proportion (%) in: | |
|---|---|---|---|---|
| | | | ***Bradyrhizobium* spp. (misc.)** | ***Bradyrhizobium* spp. (TGx)** |
| *Rsa*I | GA′T C | Rsa-a | 78·3 | 46·0 |
| | | Rsa-b | 21·7 | 54·0 |
| *Hinf*I | G′ANTC | Hinf-a | 47·8 | 24·1 |
| | | Hinf-b | 34·8 | 25·3 |
| | | Hinf-c | 17·4 | 47·1 |
| | | Hinf-d | 0·0 | 3·4 |
| *Msp*I | C′CGG | Msp-a | 69·6 | 42·5 |
| | | Msp-b | 30·4 | 57·5 |
| *Cfo*I | GCG′C | Cfo-a | 65·2 | 42·5 |
| | | Cfo-b | 34·8 | 29·9 |
| *Hae*III | GG′CC | Hae-a | 100·0 | 93·1 |
| | | Hae-b | 0·0 | 6·9 |

McMillin (1996) and the two GC-rich primers, 5′-GAC GAC GAC GAC GAC-3′ and 5′-TGC GCC GAA TTA TGC GG-3′, were used by Van Rossum *et al.* (1995) to differentiate *Bradyrhizobium* isolates. The PCR reactions were carried out as for the 16S rRNA gene, except that the primer annealing temperature was 40 °C, the primer concentration was 1·0 µM and the number of cycles was 40. PCR products were separated electrophoretically on a 2% agarose gel at 80 mV for 3·5 h, and comparison of banding patterns of *Bradyrhizobium* strains was done as described above. Similarity between members of the RFLP group was measured by visual assessment of the presence and absence of DNA fragments within the range of approximately 200–1900 bp. The results for the three primers were combined in a binary data matrix. These were converted into similarity coefficients, and dendrograms were generated from the matrices as described above.

**Nodulation and nitrogen fixation assay.** Seeds of soybean line TGx 1456-2E, obtained from IITA, Nigeria, or North American variety Clark (NifTAL Project) were surface-sterilized, pregerminated in sterile horticultural vermiculite, then planted in 1 litre plastic pots containing vermiculite. The rooting medium was wetted with nitrogen-free nutrient solution as described previously (Singleton & Tavares, 1986). Three days after transplanting the seedlings were inoculated with $10^7$ bradyrhizobia from freshly grown cultures diluted with sterile plant nutrient solution. Three replicates were used for each treatment with each pot containing two seedlings. Uninoculated controls were used to check for cross-contamination. Plants were placed in a completely randomized block design. At harvest the uninoculated control plants were free of root nodules. Plants were harvested 28 d after inoculation and shoots were dried to constant weight at 70 °C. Total shoot dry weight was used to measure symbiotic effectiveness of the isolates. Data from a concurrent growth experiment with 54 treatments involving both nitrogen-fixing and non-fixing plants in the greenhouse were analysed for nitrogen (data not shown). Correlation values between dry matter and total nitrogen for genotype 1456-2E and Clark were 0·90 and 0·89, respectively. Thus the use of plant dry weight produced in this nitrogen-free growth system is a valid proxy for nitrogen fixation. Isolates were classified as effective if shoot dry weight was not statistically different ($P < 0{\cdot}05$) from dry weight produced by *B. japonicum* strain USDA 110 but was significantly different from the uninoculated control. Shoot dry weight of ineffective isolates was not significantly different to the uninoculated control but was significantly less than the dry weight produced by USDA 110.

## RESULTS

### RFLP analysis of the 16S rRNA gene of *Bradyrhizobium* spp. (TGx) isolates

A 1·5 kb DNA fragment was obtained by PCR amplification of the 16S rRNA gene (16S rDNA) of all *Bradyrhizobium* spp. (TGx) isolates and other reference strains. RFLP was identified within the 16S rDNA from the *Bradyrhizobium* spp. (TGx) isolates and strains of various *Bradyrhizobium* and *Rhizobium* species. Examples of RFLP of *Bradyrhizobium* spp. (TGx) and some reference strains, produced by 16S rDNA digested with *Hinf*I and *Hae*III, are shown in Fig. 1. Only the enzymes *Alu*I and *Taq*I clearly differentiated the *Rhizobium* strains from the *Bradyrhizobium* strains, but these two enzymes did not establish different RFLP groups for the *Bradyrhizobium* spp. (misc.) and *Bradyrhizobium* spp. (TGx) isolates (data not shown). *Taq*I produced a similar pattern for all the bradyrhizobia used but two different patterns for the *Rhizobium* strains. Five restriction enzymes (*Rsa*I, *Hinf*I, *Msp*I, *Cfo*I and *Hae*III) established 16S rDNA RFLP groups within the *Bradyrhizobium* spp. (TGx) isolates (Table 2). Various restriction patterns were observed at different frequencies within the *Bradyrhizobium* spp. (misc.) and *Bradyrhizobium* spp. (TGx) groups using these five enzymes.



Downloaded from www.microbiologyresearch.org by
IP: 54.70.40.11
On: Wed, 06 Mar 2019 19:29:00

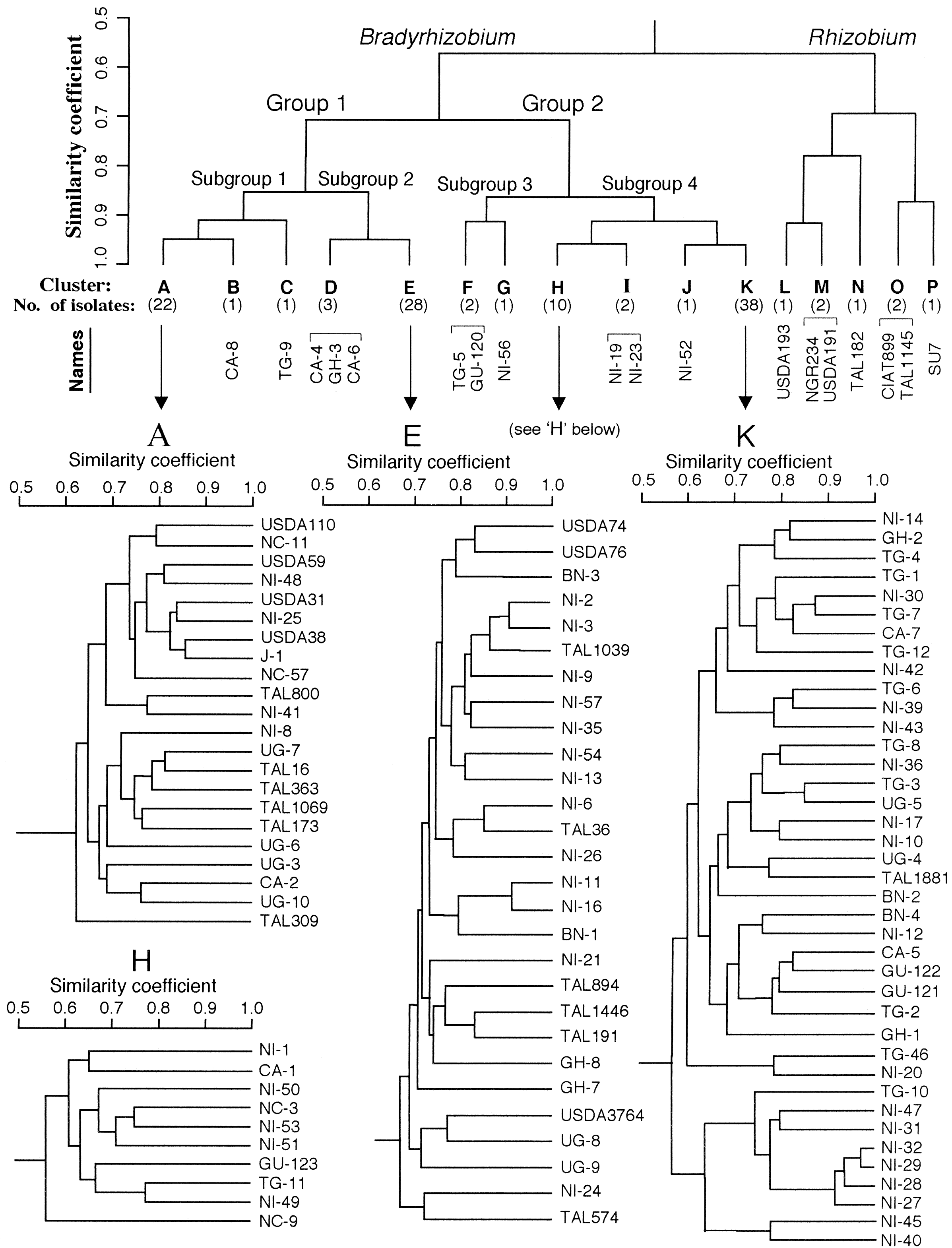


***Fig. 2.*** Phylogenetic relationship among various *Bradyrhizobium* spp. (TGx) isolates and reference strains. The upper horizontal dendrogram showing relatedness among isolates in various clusters was derived from 16S rDNA RFLP analysis. RFLP clusters A, E, H and K were separated further by RAPD analysis using three random primers. The relationships among various isolates within these four clusters, as derived from RAPD analysis, are shown by separate vertical dendrograms.



Downloaded from www.microbiologyresearch.org by
IP: 54.70.40.11
On: Wed, 06 Mar 2019 19:29:00

### Clustering of *Bradyrhizobium* spp. (TGx) isolates on the basis of 16S rDNA RFLP patterns

Clustering of the *Bradyrhizobium* spp. (TGx) and other reference strains by UPGMA differentiated these root nodule bacteria into two major groups (*Bradyrhizobium* and *Rhizobium*) at the 56% similarity level (Fig. 2). The major group *Bradyrhizobium* separated at the 70% similarity level into group 1 and group 2, which further separated into subgroups 1–4 at approximately 85% similarity levels. Group 2 contained 57% of the *Bradyrhizobium* spp. (TGx) isolates while the remaining 43% of the *Bradyrhizobium* spp. (TGx) isolates clustered with the *B. japonicum* and *B. elkanii* strains in group 1. Thus the isolates in group 2 appear genetically distinct from *B. japonicum* and *B. elkanii* strains that nodulate North American soybean. Within group 1, 25 isolates are in subgroup 2, with two *B. elkanii* and four *Bradyrhizobium* spp. (misc.) strains, while only 12 isolates clustered in subgroup 1, with the *B. japonicum* strains. *Bradyrhizobium* isolates in group 1 separated into five clusters (A, B, C, D and E) at the 95% similarity level. Clusters A and E contained 37% of the *Bradyrhizobium* spp. (TGx) isolates which may be phylogenetically closer to *B. japonicum* and *B. elkanii*. The 10 *Bradyrhizobium* spp. (TGx) isolates in cluster A show genetic relatedness to *B. japonicum*, whereas the 22 *Bradyrhizobium* spp. (TGx) isolates in cluster E show similarities with *B. elkanii*.

The two subgroups of bradyrhizobia in group 2 separated into six clusters (F, G, H, I, J and K). These clusters in group 2 are distinct from those in group 1 and did not contain any strains of *B. japonicum* or *B. elkanii*. Cluster K comprised 37 *Bradyrhizobium* spp. (TGx) isolates, TAL 1881 (isolated from *Acacia auriculiformis*) and two *Bradyrhizobium* spp. (misc.) isolates from the island of Guam. Similarly, cluster H, containing nine *Bradyrhizobium* spp. (TGx) isolates and one *Bradyrhizobium* spp. from Nigeria, is the second largest cluster in group 2. Thus clusters K and H comprise 52% of the *Bradyrhizobium* spp. (TGx) isolates in this study, which represent a diverse group of isolates distinct from the *B. japonicum* and *B. elkanii* strains that nodulate North American soybean cultivars.

### Differentiation of *Bradyrhizobium* spp. (TGx) isolates within RFLP clusters by RAPD analysis

The four major RFLP clusters of *Bradyrhizobium* spp. (TGx) isolates based on 16S rDNA RFLP analysis were further analysed for RAPD with three random primers. PCR amplification of genomic DNA of these isolates using any one of these primers resulted in the synthesis of multiple bands of variable sizes. Examples of such randomly amplified multiple bands from some *Bradyrhizobium* spp. (TGx) and reference strains using one of the primers are shown in Fig. 1. The banding patterns generated separately by the three primers were combined and dendrograms were constructed for the isolates in the major clusters A, E, H and K (Fig. 2). The RAPD analysis revealed the latent diversity that exists among the *Bradyrhizobium* spp. (TGx) isolates within each of these phylogenetic clusters. This method separated *B. japonicum* USDA 110 from *Bradyrhizobium* sp. (misc) strain NC-11 at an 80% similarity level. It also differentiated several *Bradyrhizobium* spp. (TGx) isolates from a few *Bradyrhizobium* spp. (misc.) strains at 77–80% similarity levels. On the basis of this analysis, the phylogenetic clusters A and E consist of six and 17 groups, respectively, at the 80 % similarity level. Similarly, the RAPD banding patterns obtained for clusters H and K indicate the existence of 11 and 24 groups, respectively. The existence of distinct groups within cluster K at lower similarity level (<80%) indicates a higher degree of heterogeneity in this cluster than within other clusters (Fig. 2).

***Table 3.*** Percentages of effective *Bradyrhizobium* spp. (TGx) isolates in various phylogenetic clusters and geographic groups

| Class | No. of isolates | Effective (%) |
|---|---|---|
| **Phylogenetic cluster** | | |
| A | 9 | 66·7 |
| E | 19 | 52·6 |
| H | 7 | 100·0 |
| K | 36 | 75·0 |
| Group I | 33 | 57·6 |
| Group II | 48 | 79·2 |
| **Geographic group** | | |
| Nigeria | 44 | 75·0 |
| Uganda | 8 | 62·5 |
| Cameroon | 7 | 42·9 |
| Togo and Benin | 17 | 76·5 |
| Ghana | 5 | 60·0 |
| **All isolates** | 81 | 70·3 |

### Symbiotic types within the phylogenetic clusters and the geographic groups

*Bradyrhizobium* spp. (TGx) isolates were classified into effective and ineffective types based on their symbiotic performance on TGx soybean; 70·3% of the isolates in the *Bradyrhizobium* spp. (TGx) collection were effective. The symbiotically effective and ineffective isolates were unevenly distributed in various phylogenetic clusters and geographic groups (Table 3). Group 2 contained higher percentages of effective isolates than group 1. Among the phylogenetic clusters, all cluster H isolates were effective while one-third of the isolates in cluster A were ineffective. In the largest cluster, K, one-quarter of the isolates were ineffective, whereas in cluster E nearly half of the isolates were ineffective. Nigeria, Togo and Benin contained a relatively higher percentage of effective isolates, whereas Cameroon contained a relatively higher proportion of ineffective isolates.



Downloaded from www.microbiologyresearch.org by
IP: 54.70.40.11
On: Wed, 06 Mar 2019 19:29:00

## DISCUSSION

The *Bradyrhizobium* spp. (TGx) isolates used in this study were collected from six countries across a wide geographic range in Africa. These isolates are ineffective on the American soybean Clark and are therefore distinct from most *B. japonicum* isolates in their symbiotic phenotype. Although they were isolated from the nodules of TGx soybean, all isolates were not effective for nitrogen fixation on TGx soybeans; 30% of the isolates formed ineffective nodules on the TGx soybean cultivars. However, the ineffective isolates did not differ from the effective ones with respect to the number and size of nodules they formed on TGx soybean. On the other hand, these isolates formed no nodules or only a few ineffective nodules on North American soybean Clark. Thus the North American commercial soybean restricts nodulation by isolates of *Bradyrhizobium* spp. (TGx). This is analogous to certain North American soybean genotypes identified by Keyser & Cregan (1987), which restricted the nodulation by *B. japonicum* serogroup 123 isolates. Another example of soybean cultivar specificity has been observed with most *Sinorhizobium fredii* strains, which form effective nodules on Asian soybean cultivar Peking, but induce the formation of ineffective nodules on North American commercial cultivars (Keyser *et al*., 1982).

Although the isolates of *Bradyrhizobium* spp. (TGx) are distinct from *B. japonicum* or *B. elkanii* in their inability to effectively nodulate American soybean Clark, PCR-RFLP analysis indicates that *Bradyrhizobium* spp. (TGx) is a heterogeneous group with some members showing relatedness to *B. japonicum* and *B. elkanii* strains and some to *Bradyrhizobium* spp. (misc.) reference strains isolated from a wide range of legume hosts. The separation of *Rhizobium* and *Bradyrhizobium* strains into known taxonomic groups demonstrates the usefulness of the PCR-RFLP method as a taxonomic tool. The majority of the *Bradyrhizobium* spp. (TGx) isolates in this study belong to group 2 and are phylogenetically very distinct from *B. japonicum* and *B. elkanii* strains that nodulate North American soybeans. The isolates of group 2 separate into two distinct subgroups and six clusters, thus showing diversity among themselves. The isolates in group 2 came from all six African countries from where the isolates were obtained. Eighty per cent of these isolates form effective nodules on TGx soybean.

Isolates in cluster A show phylogenetic relatedness to *B. japonicum* reference strains, therefore, it is not surprising that a relatively greater proportion (70·3%) of these isolates belongs to the effective symbiotic type. Similarly, cluster E shows phylogenetic relatedness with the *B. elkanii* reference strains and contains isolates from all six countries. Nine out of 19 isolates in this cluster were effective. *B. elkanii* strain USDA 38 did not cluster with two other *B. elkanii* strains, USDA 74 and USDA 76$^T$, but grouped with the *B. japonicum* strains USDA 110 and J-1 in cluster A. This suggests that the 16S rDNA RFLP method cannot distinguish all *B. japonicum* strains from all *B. elkanii* strains. Moreover, there may be diversity in the 16S rDNA sequence among *B. elkanii* strains of different geographic origin. Strains USDA 74 and USDA 76$^T$ originated in California while USDA 38 came from Japan. Both clusters A and E include several *Bradyrhizobium* spp. (misc.) isolates obtained from the nodules of different legumes in several countries. The clustering of *B. japonicum* and *B. elkanii* strains with *Bradyrhizobium* spp. (misc.) strains is not peculiar to this study. Young *et al*. (1991), using partial 16S rRNA sequence data, found limited or no base differences between *B. japonicum* strains USDA 110, USDA 76$^T$, USDA 3407 and *Bradyrhizobium* sp. (*Lotus*) strain NZP 2257. Similarly, the PCR-RFLP analysis carried out by Laguerre *et al*. (1994) could not separate *B. japonicum* strain USDA 110 from *Bradyrhizobium* sp. (*Lupinus*) and *Bradyrhizobium* sp. (*Macrotyloma*) strain TAL 309. Recently, Barrera *et al*. (1997) determined the 16S rRNA sequences of 41 *Bradyrhizobium* isolates from the nodules of *Lupinus* grown in Mexico and found that the lupine isolates were highly related to the *B. japonicum* type strain.

The PCR-RFLP analysis reported in our study separated *Rhizobium* and *Bradyrhizobium* genera at a similarity level of 56%. This level of similarity is comparable to the similarity coefficient of 0·53 obtained by Hennecke *et al*. (1985), where they compared the 16S rRNA gene sequences of *Bradyrhizobium* and *Rhizobium* strains. The procedure we used also separated *Rhizobium etli* strain TAL 182 from *Rhizobium* sp. NGR 234 and *S. fredii* strain USDA 193 at a similarity level of 77%. It separated *Rhizobium leguminosarum* bv. trifolii strain SU7 from *Rhizobium tropici* CIAT 899$^T$ and *Rhizobium* sp. TAL 1145 at an 85% similarity level. Similarly, this method separated the *B. japonicum* strains USDA 110 and J-1 from *B. elkanii* strains USDA 74 and USDA 76$^T$ at an 85% similarity level. Considering that at a 77 % similarity level *R. etli* separated from *S. fredii* and at an 85% similarity level *R. leguminosarum* bv. trifolii differentiated from *R. tropici* and *B. japonicum* from *B. elkanii*, the separation of bradyrhizobia into group 1 and group 2 at the 70% similarity level indicates that many more species taxa exist within the genus *Bradyrhizobium*. *Bradyrhizobium* spp. (TGx) isolates in group 2 may constitute a taxonomic group of significant genetic distance from *B. japonicum*, *B. elkanii* and other *Bradyrhizobium* spp. (misc.) populations.

The RAPD analysis using GC-rich random primers showed additional heterogeneity in the *Bradyrhizobium* spp. (TGx) phylogenetic clusters, which was not revealed by separations based on 16S rDNA RFLP analysis. The procedure also adequately served as a means of fingerprinting various *Bradyrhizobium* spp. (TGx) isolates. This method amplified multiple DNA bands from different regions of the bacterial genome and thus produced different banding patterns for



Downloaded from www.microbiologyresearch.org by
IP: 54.70.40.11
On: Wed, 06 Mar 2019 19:29:00

individual isolates. RAPD has not been used extensively for bacterial taxonomic studies because its results are sensitive to reaction conditions. Welsh & MacClelland (1990) showed that primer annealing temperature, template concentrations, primer length and sequence, and concentration of salts altered banding patterns. In our study, these parameters were carefully optimized to obtain reproducible results. The RAPD analyses for all isolates were repeated at least twice and similar results were obtained. The RAPD analysis showed that the isolates within clusters A, E, H and K, which were found to be homogeneous from 16S rDNA RFLP analysis, showed heterogeneity in banding patterns, and comprised several RAPD subclusters. When the RAPD analysis was applied to isolates from any two 16S rDNA RFLP groups together, the isolates were not separated into two distinct groups similar to the 16S rDNA RFLP clusters; instead, the isolates from the two RFLP groups were randomly distributed in different RAPD groups. Thus the RAPD analysis is a suitable method to screen internal diversity inside each phylogenetic group defined by 16S rDNA RFLP analysis; however, its use to make phylogenetic clusters within a large number of diverse isolates results in clusters which may be different from those based on RFLP analysis.

Our analyses demonstrate that the *Bradyrhizobium* spp. (TGx) populations that nodulate TGx soybean are highly diverse. Some of these isolates are closely related to *B. japonicum* and *B. elkanii* while others are related to *Bradyrhizobium* spp. that nodulate a wide range of tropical legume species. RFLP groups of *Bradyrhizobium* spp. (TGx) or their genetic relatedness to *B. japonicum* did not correspond to geographic origin or symbiotic effectiveness on TGx soybean. RAPD analysis within RFLP groups revealed further diversity within the *Bradyrhizobium* spp. (TGx) isolates. The genes controlling the cultivar specificity of *Bradyrhizobium* spp. (TGx) have not been identified and characterized. The cultivar specificity of these isolates is apparently not revealed by 16S RFLP generated by the restriction enzymes used in this study.

## ACKNOWLEDGEMENTS

The work was supported in part by the US Agency for International Development grant DAN-4177-A-00-6035-00 (NifTal Project) and the International Institute of Tropical Agriculture (IITA). We are grateful to F. Kumaga and P. Woomer for providing us with soil samples from Ghana and East African countries, respectively, and Kathy MacGlashan for technical assistance. Journal series number 4418 of the College of Tropical Agriculture and Human Resources, University of Hawaii, Honolulu.

## REFERENCES

**Barrera, L. L., Trujillo, M. E., Goodfellow, M., Garcia, F. J., Hernandez-Lucas, I., Davila, G., van Berkum, P. & Martinez-Romero, E. (1997).** Biodiversity of bradyrhizobia nodulating *Lupinus* spp. *Int J Syst Bacteriol* **47**, 1086–1091.

**Bromfield, E. S. P. & Roughley, R. J. (1980).** Characterization of rhizobia isolated from nodules of locally-adapted *Glycine max* grown in Nigeria. *Ann Appl Biol* **95**, 185–190.

**Hadley, H. H. & Hymowitz, T. (1973).** Speciation and cytogenetics. In *Soybeans: Improvement, Production, and Uses*, pp. 97–116. Edited by B. E. Caldwell. Madison, WI: American Society of Agronomy.

**Heath, L. S., Sloan, G. L. & Heath, H. E. (1986).** A simple and generally applicable procedure for releasing DNA from bacterial cells. *Appl Environ Microbiol* **51**, 1426–1434.

**Hennecke, H., Kaluza, K., Thönyl, B., Fuhrmann, M., Ludwig, W. & Stackebrandt, E. (1985).** Concurrent evolution of nitrogenase genes and 16S rRNA in *Rhizobium* species and other nitrogen fixing bacteria. *Arch Microbiol* **142**, 342–348.

**Keyser, H. H. & Cregan, P. B. (1987).** Nodulation and competition for nodulation of selected soybean genotypes among *Bradyrhizobium japonicum* serogroup 123 isolates. *Appl Environ Microbiol* **53**, 2631–2635.

**Keyser, H. H., Bohlool, B. B., Hu, T. S. & Weber, D. F. (1982).** Fast-growing rhizobia isolated from root nodules of soybean. *Science* **215**, 1631–1632.

**Laguerre, G., Allard, M., Revoy, F. & Amarger, N. (1994).** Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. *Appl Environ Microbiol* **60**, 56–63.

**Lane, D. J. (1991).** 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. London: Wiley.

**Leonard, L. T. (1923).** Nodule production kinship between the soybean and cowpea rhizobia. *Soil Sci* **15**, 277–283.

**Mathis, J. N. & McMillin, D. E. (1996).** Detection of genetic variation in *Bradyrhizobium japonicum* USDA 110 variants using DNA fingerprints generated with GC rich arbitrary PCR primers. *Plant Soil* **186**, 81–85.

**Okereke, G. U. & Eaglesham, A. R. J. (1992).** Nodulation and nitrogen fixation by 79 'promiscuous' soybean genotypes in a soil in east Nigeria. *Agronomie Africaine* **5**, 123–136.

**Pal, U. L. & Norman, J. C. (1987).** Differential response of soybean cultivars promiscuous to native rhizobia to split application of fertilizer N in Nigerian savanna. *J Agron Crop Sci* **158**, 340–345.

**Pulver, E. L., Kueneman, E. A. & Ranga-Rao, V. (1985).** Identification of promiscuous nodulating soybean efficient in $N_2$ fixation. *Crop Sci* **25**, 1065–1070.

**Roughley, R. J., Bromfield, E. S. P., Pulver, E. L. & Day, J. M. (1980).** Competition between species of *Rhizobium* for nodulation of *Glycine max*. *Soil Biol Biochem* **12**, 467–470.

**Sadowsky, M. J., Tully, R. E., Cregan, P. B. & Keyser, H. H. (1987).** Genetic diversity in *Bradyrhizobium japonicum* serogroup 123 and its relation to genotype-specific nodulation of soybeans. *Appl Environ Microbiol* **53**, 2624–2630.

**Sears, O. H. & Carroll, W. R. (1926).** Cross inoculation with cowpea and soybean nodule bacteria. *Soil Sci* **24**, 413–419.

**Singleton, P. W. & Tavares, J. W. (1986).** Inoculation response of legumes in relation to the number and effectiveness of indigenous *Rhizobium* populations. *Appl Environ Microbiol* **51**, 1013–1018.

**Sneath, P. H. A. & Sokal, R. R. (1973).** *Numerical Taxonomy*. San Francisco: Freeman.

**Somasegaran, P. & Hoben, H. J. (1994).** *Handbook for Rhizobia: Methods in Legume-Rhizobium Technology*, p. 450. New York: Springer.



Downloaded from www.microbiologyresearch.org by
IP: 54.70.40.11
On: Wed, 06 Mar 2019 19:29:00

**Van Rossum, D., Schuurmans, F. P., Gillis, M., Muyotcha, A., van Verseveld, H. W., Stouthamer, A. H. & Boogerd, F. C. (1995).** Genetic and phenetic analysis of *Bradyrhizobium* strains nodulating peanut (*Arachis hypogea* L.) roots. *Appl Environ Microbiol* **61**, 1599–1609.

**Welsh, J. M. & MacClelland, M. (1990).** Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res* **18**, 7213–7218.

**Young, J. P. W., Downer, H. L. & Eardly, B. D. (1991).** Phylogeny of the phototropic *Rhizobium* strain BTAil by polymerase chain reaction sequencing of a 16S rRNA gene segment. *J Bacteriol* **173**, 2271–2277.



Downloaded from www.microbiologyresearch.org by
IP: 54.70.40.11
On: Wed, 06 Mar 2019 19:29:00