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Comparison of molecular and antibiotic resistance profile methods for the population analysis of *Bradyrhizobium* spp. (TGx) isolates that nodulate the new TGx soybean cultivars in Africa

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Aims: Comparison of molecular and antibiotic resistance profile methods to identify an easy method that can differentiate between strains of introduced *Bradyrhizobium japonicum* and the indigenous *Bradyrhizobium* spp. (TGx) isolates which nodulate the newly developed TGx soybean cultivars in Africa.

Methods and Results: Restriction fragment length polymorphism (RFLP) of 16S rDNA generated by five restriction enzymes, banding patterns in Southern hybridization using *nod* and *nif* genes as probes, and resistance patterns of the isolates to nine antibiotics, were used to group 26 *Bradyrhizobium* spp. (TGx) isolates and four other *Bradyrhizobium* strains. The clusters of isolates obtained from the four grouping methods were all different, although all methods revealed large genetic diversity among the isolates.

Conclusions: Results indicate that the antibiotic resistance profile method is as good as the three molecular methods used in this study for phylogenetic grouping of the *Bradyrhizobium* spp. (TGx) isolates, which may serve as a basis for further characterization of selected isolates from each group.

Significance and Impact of the Study: The antibiotic resistance profile method can be used as a simple means of assessing genetic variability and grouping of a large number of *Bradyrhizobium* spp. (TGx) isolates. Representative isolates from each group can then be selected for further characterization.

INTRODUCTION

Root nodule bacteria belonging to the genus *Bradyrhizobium* consist of several species, among which *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* that nodulate soybean are relatively well characterized. However, the majority of bradyrhizobia that nodulate a wide range of legumes remain mostly uncharacterized and are grouped as *Bradyrhizobium* spp. (misc.). Recently, Abaidoo *et al*. (1999, 2000) described a new group of bradyrhizobia, designated as *Bradyrhizobium* spp. (TGx), which are isolates from the nodules of new soybean genotypes for Africa known as Tropical *Glycine* cross (TGx). These isolates are ineffective on American soybean Clark and are distinct from most *B. japonicum* isolates in their symbiotic phenotype. The majority of these isolates form effective nodules on TGx soybean (Abaidoo *et al*. 2000). *Bradyrhizobium* spp. (TGx) isolates are important for soybean cultivation in Africa because *B. japonicum* or *B. elkanii* populations required for effective nodulation of soybeans are not endemic to African soils. Therefore, plant breeders at the International Institute of Tropical Agriculture (IITA), Nigeria, developed TGx soybean cultivars that are well nodulated by most populations of *Bradyrhizobium* spp. (TGx) present in African soils.

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Since the success of soybean cultivation in Africa depends on effective nodulation by the indigenous *Bradyrhizobium* spp. (TGx) populations, it is necessary to monitor the *Bradyrhizobium* spp. (TGx) populations in different soybean growing areas, and to isolate and characterize superior effective strains from these local populations and use them as inoculant strains in areas where their numbers are not large enough in the soils for effective nodulation of TGx soybean. This will involve taking a large number of isolates from effective nodules of TGx soybean in Africa and determining whether they are related to other well characterized *B. japonicum* or *B. elkanii* strains. It is also important that bradyrhizobia populations in soybean breeding sites are characterized in order to establish that such populations do not contain *B. japonicum* strains from earlier introductions. Only a limited number of *Bradyrhizobium* spp. (TGx) isolates have so far been characterized, and the genetic characterization of isolates from many parts of Africa has not yet started. It is necessary to be able to distinguish between these species of *Bradyrhizobium* in order to carry out correctly the symbiotic screening part of the breeding and selection programme. Indeed, *B. japonicum* is not present in most African soils, but most of the effective strains isolated from nodules of TGx soybean were *B. japonicum* (Abaidoo *et al*. 1999). In this same study, it was found that significant *B. japonicum* populations were more frequent at research stations than in farmers' fields. The primary aim of the present study was to identify an easy method for differentiating between strains of introduced *B. japonicum* and the indigenous *Bradyrhizobium* spp. (TGx). The only known method of distinguishing *Bradyrhizobium* spp. (TGx) from *B. japonicum* is the lengthy procedure of inoculating isolates onto North American soybeans and African TGx soybeans. It is especially important to establish that *B. japonicum* is not present at sites where the TGx soybean genotypes are selected for nodulation. The presence of *B. japonicum* at such sites eliminates or reduces the selection pressure for effective nodulation by *Bradyrhizobium* spp. (TGx). Another goal of the present study was to determine genetic diversity among *Bradyrizobium* spp. (TGx) populations and to measure their relatedness to other reference *Bradyrhizobium* strains.

In recent years, several molecular methods have been developed to determine genetic diversity, phylogenetic relationships and strain identity among bacterial isolates. These methods include restriction fragment length polymorphism (RFLP), Southern hybridization, analysis of variable intergenic spacer regions, randomly amplified polymorphic DNA (RAPD) analysis, detection of insertion sequences, PCR amplification of repetitive sequences, and sequencing of the 16S rRNA gene and other genes. These methods provide many of the fine details needed for sharp differentiation of closely-related strains, but they require expensive equipment and reagents. Among various molecular methods, 16S rRNA gene sequencing is the most commonly used method for phylogenetic cluster analysis. Other traditional methods of phylogenetic studies and strain differentiation include analysis of morphological characters (Eaglesham *et al*. 1987; Fuhrmann 1990), nutritional requirements (Graham and Parker 1969), biochemical characters (Noel and Brill 1980), serological characters (Bohlool and Schmidt 1980), fatty acid composition (Kuykendall *et al*. 1988), phage typing (Kowalski *et al*. 1974), hydrogenase phenotype and induction of rhizobitoxine chlorosis (Fuhrmann 1990). Also, in several studies involving genetic diversity in bradyrhizobia, intrinsic antibiotic resistance patterns were used as the distinguishing characters (Kuykendall *et al*. 1988; Mueller *et al*. 1988; Date and Hurse 1991). In the present study, a comparison was made of two molecular methods, RFLP analysis of the 16S rRNA gene and Southern hybridization analysis using *nod* and *nif* genes as probes, and a physiological method involving antibiotic resistance patterns, to differentiate and establish phylogenetic relationships among the *Bradyrhizobium* spp. (TGx) isolates obtained from nodules of the newly developed TGx soybean in Africa. By comparing these methods, it should be possible to determine whether the antibiotic profiling method can be as effective as a molecular method in differentiating between introduced *B. japonicum* and the indigenous *Bradyrhizobium* spp. TGx populations.

MATERIALS AND METHODS

Bradyrhizobium strains

Bradyrhizobium japonicum, *B. elkanii*, *Bradyrhizobium* sp. (misc.) and *Bradyrhizobium* spp. (TGx) isolates used in this study are shown in Table 1. The 26 *Bradyrhizobium* spp. (TGx) isolates were a random sample from a collection of 258 isolates obtained from the nodules of TGx soybeans inoculated with soils from 65 locations in nine countries in Africa.

Bacterial media and growth conditions

All *Bradyrhizobium* strains were grown on yeast extract-mannitol agar (YMA) medium with bromothymol blue indicator (Somasegaran and Hoben 1994) at 28°C.

Genomic DNA preparation

Genomic DNA from the *Bradyrhizobium* strains was extracted by the method of Heath *et al*. (1986).

Table 1 Original host legumes and geographical origin of *Bradyrhizobium* strains

Strains/isolate	Host legume	Geographic origin
Bradyrhizobium spp. (TGx)		
UG-10	*Glycine max* (TGx)	Namul, Uganda
TG-1, TG-5	*Glycine max* (TGx)	Kara, Togo
TG-9	*Glycine max* (TGx)	Sotouboua, Togo
GH-1	*Glycine max* (TGx)	Kamasi, Ghana
GH-2	*Glycine max* (TGx)	Nyankpala, Ghana
CA-1, CA-7	*Glycine max* (TGx)	Dschang, Cameroon
CA-4, CA-8	*Glycine max* (TGx)	Bambui, Cameroon
BN-3	*Glycine max* (TGx)	Ina, Benin
NI-1, NI-2, NI-29, NI-32, NI-43	*Glycine max* (TGx)	Zaria, Nigeria
NI-17	*Glycine max* (TGx)	Abuja, Nigeria
NI-10, NI-19, NI-21, NI-39, NI-40, NI-56	*Glycine max* (TGx)	Abeokuta, Nigeria
NI-24, NI-23,	*Glycine max* (TGx)	Zonkwa, Nigeria
NI-52	*Glycine max* (TGx)	Jos, Nigeria
Bradyrhizobium sp. (misc.)		
TAL309	*Macrotyloma africanum*	Zimbabwe
Bradyrhizobium japonicum		
USDA110	*Glycine max*	FL, USA
USDA59	*Glycine max*	NC, USA
Bradyrhizobium elkanii		
USDA 74	*Glycine max*	CA, USA

RFLP analysis of PCR-amplified 16S rRNA gene fragment

A 1·5 kb 16S rRNA gene fragment, corresponding to positions 27–1525 of the *Escherichia coli* 16S rRNA gene sequence, was amplified from the *Bradyrhizobium* isolates by PCR using primers and reaction conditions as previously described (Abaidoo *et al*. 2000). Portions (15 μl) of the amplification product were digested in a final volume of 20 μl with each of five restriction enzymes (*Rsa*I, *Hin*fI, *Msp*I, *Cfo*I and *Hae*III). 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 using averages (UPGMA) algorithm (Sneath and Sokal 1973); a dendrogram was generated from the matrix using NTSYS-Pc software (Exeter Software Co., New York, USA).

Southern hybridization

Genomic DNA (approximately 4 μg) was digested with a restriction enzyme, electrophoresed on a 1·0% agarose gel and transferred to a nylon membrane. For Southern hybridizations, DNA probes were labelled by random priming using the digoxigenin labelling and detection kit from Boehringer Mannheim (Indianapolis, USA). The probes were prepared from 0·5–3·0 μg DNA using the random priming technique according to the manufacturer's protocol. Hybridizations were done at 42°C in a buffer containing 5× SSC, 0·02% SDS, 2% blocking reagent and 50% formamide (1× SSC is 0·15 mol l^{-1} NaCl, 15 mmol l^{-1} sodium citrate) for 12 h, and washed at 60°C with 2× SSC, 0·1% SDS for 30 min. Chemiluminescent alkaline phosphatase substrate Lumi-Phos 530 (Boehringer Mannheim) was used to detect the signal by exposure to X-ray film.

Comparison of sequence divergence in and around *nod* and *nif* genes

Bradyrhizobium strains were compared with *B. japonicum* strain USDA110 on the basis of the presence or absence of *Eco*RI and *Pst*I restriction fragments of the genomic DNAs hybridized with *nolA*, *nodS*, *nodIJ* and *nodDYABC*, and

nifHD gene probes. The hybridizing bands were scored for intensity on a 0–3 scale, where 0 = no band, 1 = weak band, 2 = moderate intensity band and 3 = strong band, with intensity similar to that of USDA110. The fraction of restriction fragments conserved for all pairwise comparisons of all strains, and the expected proportion of nucleotide substitutions in and around the probed nod gene sequences, were derived using equation 6b of Upholt (1977). A similarity matrix based on the presence or absence of a hybridized band was generated using the SIMQUAL (Similarity for Qualitative data) (Sokal and Sneath 1963) subroutine of NTSYS-pc (Exeter Software Co., New York, USA). The SAHN (Sequential Agglomerative Hierarchical and Nested) (Sneath and Sokal 1973) and Treeplot subroutines were used to determine the genetic relationships between *Bradyrhizobium* isolates and *B. japonicum* strain USDA110, based on the proportion of sequence divergence.

Antibiotic resistance

The agar diffusion technique (Antoun *et al.* 1982) was used to measure the resistance of bradyrhizobia isolates to nine antibiotics. Antibiotic-impregnated discs were made by transferring 20 μl volumes of antibiotic solutions, containing the desired antibiotic concentration levels, onto 6 mm sterile blank paper discs (BBL, Becton and Dickinson & Co., Cockeysville, MD, USA). The antibiotic discs were allowed to dry in a laminar flow cabinet in the dark and stored at –20°C until used. Approximately 10^7 cells in a 100 μl volume were spread uniformly onto YMA agar plates in duplicate. Plate cultures were incubated for 6–8 h before antibiotic discs were placed on them. Plates were then incubated at 28°C for 10 days. Zones of inhibition of bacterial growth were measured on the 7th and 10th day. Measurements were made at four different points on each disc and average radii were recorded. Minimum inhibitory concentrations were chosen as the antibiotic levels that produced clear zones of inhibition on the 7th day and maintained the clarity of the zone on the 10th day. Minimum inhibitory concentrations for the nine antibiotics were determined by preliminary experiments using a few isolates. Based on minimum and maximum radii of zones of inhibition, the isolates were classified into four phenotypes: highly resistant, < 5 mm; resistant, 5–10 mm; susceptible, 11–20 mm; and highly susceptible, > 20 mm. Thus, the relationships between isolates with regard to their response to the minimum inhibitory antibiotic concentrations were analysed by developing a matrix of the isolates and their antibiotic resistance coefficients. These were then converted into similarity coefficients using the SIMINT subroutine of the NTSYS-pc. Isolates were grouped by unweighted paired group mean average (UPGMA) clustering (Sneath and Sokal 1973) using the SAHN subroutine of the NTSYS-pc.

RESULTS

Clustering on the basis of 16S rDNA-RFLP

PCR amplification using the 16S rRNA gene-specific primers produced a 1·5 kb DNA fragment from the *Bradyrhizobium* spp. (TGx) isolates and other reference strains, as expected. This 1·5 kb 16S rDNA fragment was digested with a number of restriction enzymes to establish RFLP patterns. Banding patterns of the DNA fragments obtained by digesting the 1·5 kb fragment separately with five restriction enzymes (*Rsa*I, *Hin*fI, *Msp*I, *Cfo*I and *Hae*III), and separating them on an agarose gel, could distinguish most of the *Bradyrhizobium* spp. (TGx) isolates from the reference strains and established 16S rDNA RFLP groups among these isolates. Clustering of the *Bradyrhizobium* spp. (TGx) and other reference strains by the UPGMA method differentiated them at the 70% similarity level into group 1 and group 2 (Fig. 1a), which further separated into clusters I–IV at approximately 85% similarity levels. Group 2 contained 18 isolates while the remaining eight isolates clustered with the reference 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. Three isolates in group 1 clustered with the two *B. japonicum* reference strains USDA110 and USDA59, while five isolates clustered with the *B. elkanii* strain USDA74.

Clustering on the basis of hybridization patterns with *nod* genes

Four different *nod* gene probes were used to determine the relatedness of *Bradyrhizobium* spp. (TGx) isolates to the reference strains and the extent of diversity among the *Bradyrhizobium* spp. (TGx) isolates. The *nodDYABC* probe from *B. japonicum* USDA110 hybridized with 1–3 *Eco*RI and 1–4 *Pst*I fragments in the isolates. The sizes of the hybridizing fragments ranged from 0·7 to 16·7 kb for *Eco*RI-digested DNA, and from 0·5 to 15·6 kb for *Pst*I-digested DNA (Tables 2 and 3). The banding patterns showed that most *Bradyrhizobium* spp. (TGx) isolates produced different hybridization bands with reference to *B. japonicum* strain USDA110. Similarly, *B. japonicum* USDA59 and *B. elkanii* USDA74 did not produce the same hybridization profiles as the homologous strain USDA110, indicating that sequences around the *nodDYABC* gene region are also not highly conserved in all *Bradyrhizobium* strains. The *nodIJ* gene probe produced hybridizing bands of between 1·6 and 16·7 kb for the

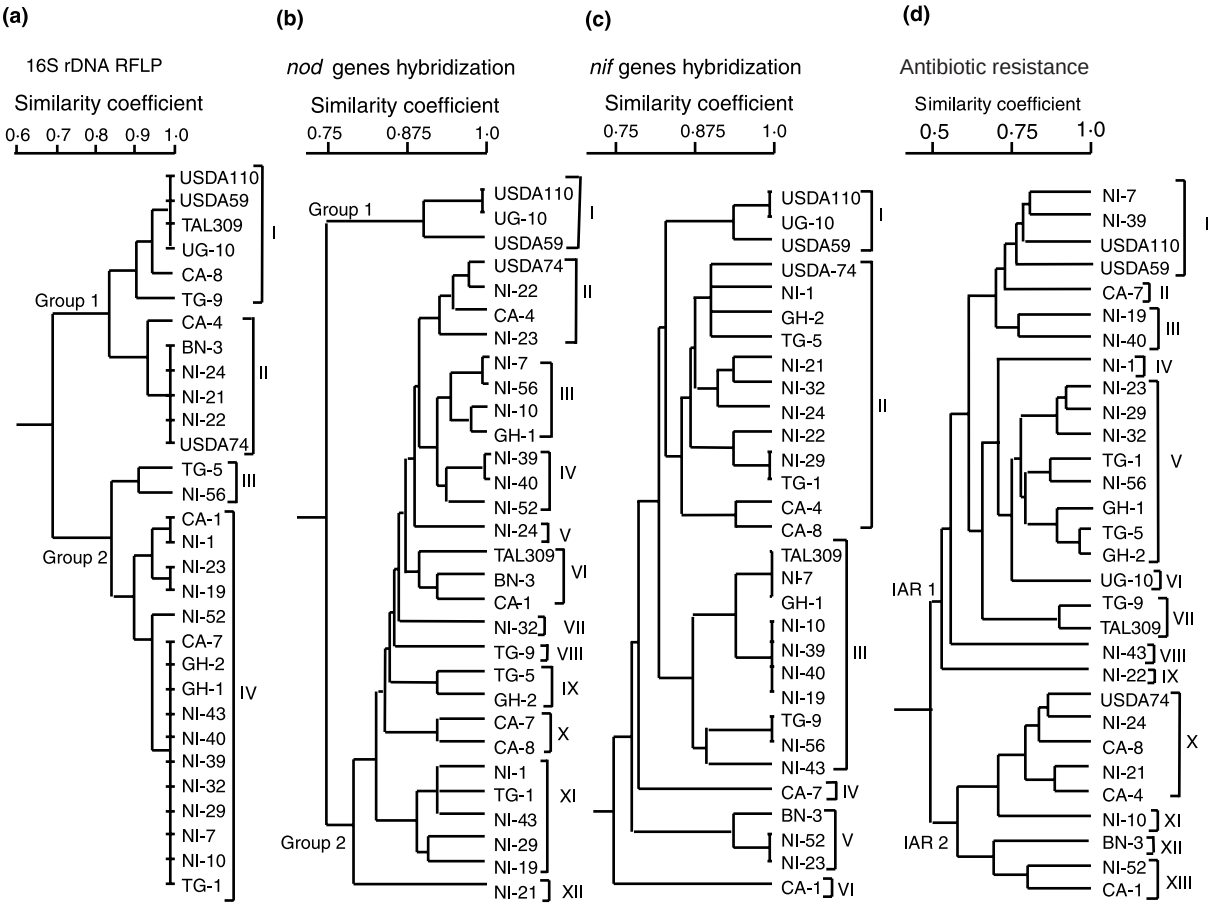

Fig. 1 Phylogenetic relationships among various *Bradyrhizobium* spp. (Tgx) isolates and reference strains as determined by four different methods: (a) 16S-RFLP; (b) *nod* gene hybridization; (c) *nif* gene hybridization; (d) antibiotic resistance. Various clusters are shown with brackets on the right-hand side of each dendrogram

*Eco*RI-digested DNA (Table 2), and between 1·1 and 7·3 kb for the *Pst*I-digested DNA (Table 3). Genomic DNA fragments that hybridized with the *nodS* gene probe ranged from 4·1 to 16·7 kb for *Eco*RI-digested DNA and from 2·1 to 15·6 kb for *Pst*I-digested DNA. Similarly, hybridization with the *nolA* probe to *Eco*RI-digested genomic DNA revealed that *nolA* is located in fragments of sizes ranging from 2·7 to 15·8 kb for the *Eco*RI-digested DNA, and 1·8 to 15·6 kb for the *Pst*I-digested DNA of different isolates. The *nolA* probe hybridized with a 6·1 and a 5·4 kb hybridizing band in USDA110 digested with *Eco*RI and *Pst*I, respectively. These results indicate that DNA sequences around this gene are highly variable in the *Bradyrhizobium* spp. (TGx) isolates.

Clustering the isolates based on simple matching coefficients (presence or absence of the different *Eco*RI and *Pst*I restriction fragments) resulted in two major groups at the 75% similarity level (Fig. 1b). Group 1 comprised one isolate and the *B. japonicum* reference strains, while group 2 included the rest of the isolates, *Bradyrhizobium* sp. (misc.) strain TAL309 and the *B. elkanii* reference strain. Group 2 isolates showed wide diversity and were classified into 11 clusters at the 90% similarity level.

Clustering on the basis of hybridization patterns with *nif* genes

The *nifHD* probe from *Sinorhizobium meliloti* hybridized with different *Eco*RI fragments of the isolates which ranged from 2·2 to 14·7 kb. The isolates were separated into six clusters at the 85% similarity level of association (Fig. 1c), with only one (UG-10) of the isolates showing common hybridization bands and clustering with *B. japonicum* USDA110 and USDA59. *Bradyrhizobium elkanii* strain USDA74 grouped with 11 isolates in cluster II, while *Bradyrhizobium* sp. (misc.) strain TAL309 grouped with nine isolates in cluster III. Clusters IV, V and VI contained *Bradyrhizobium* spp. (TGx) isolates that did not compare with any of the reference strains on the base sequence in and around the *nifHD* gene region.

Table 2 Distribution of *Eco*RI fragments of different sizes (kb) around the nodulation gene regions in 26 *Bradyrhizobium* spp. (TGx) isolates

nolA		*nodS*		*nodIJ*		*nodYABC*	
Size	Number*	Size	Number	Size	Number	Size	Number
15·8	4	16·7	3	16·7	3	16·7	2
15·3	3	15·3	3	15·3	3	15·8	3
13·9	3	13·3	2	13·9	3	15·1	7
13·3	1	12·2	1	13·3	2	13·9	4
10·4	2	10·3†	2	12·2	1	13·3	2
6·4	1	8·8	2	10·3†	2	12·2	1
6·1†	2	6·6	2	8·8	3	10·4	1
4·7	1	6·1	1	6·6	2	8·5†	4
3·5	1	4·3	1	6·1	1	6·8	2
3·1	2	4·1	5	4·3	1	6·2†	5
2·9	5			4·1	5	5·9	1
2·7	1			3·6	1	5·0	4
				3·1	6	4·7	2
				1·8	1	4·2	8
				1·6	2	3·9	3
						2·5	1
						1·6	3
						0·7	1

*Number of isolates producing a fragment.
†*Bradyrhizobium japonicum* USDA 110 fragment size.

Table 3 Distribution of *Pst*I fragments of different sizes (kb) around the nodulation gene regions in 26 *Bradyrhizobium* spp. (TGx) isolates

nolA		*nodS*		*nodIJ*		*nodYABC*	
Size	Number*	Size	Number	Size	Number	Size	Number
15·6	1	15·6	3	7·3	4	15·6	2
6·5	6	7·3	2	6·4	1	13·1	1
5·6	5	6·4	3	5·6	1	10·7	1
5·4†	3	5·6	2	5·1†	5	8·3	2
4·9	2	5·1†	5	4·1	1	7·3	3
4·5	2	4·5	1	3·5	2	6·5	12
4·1	4	4·1	1	3·3	2	5·6†	9
3·0	2	3·5	8	3·1	5	4·9†	5
1·8	3	3·3	1	2·8	6	4·5	2
		2·8	8	2·6	2	3·7	3
		2·1	12	2·1	15	2·6	1
				1·6	5	2·1	4
				1·5	2	1·6†	3
				1·3	21	1·2	1
				1·1	5	0·5†	4

*Number of isolates producing a fragment.
†*Bradyrhizobium japonicum* USDA 110 fragment size.

Clustering on the basis of antibiotic resistance patterns

The various *Bradyrhizobium* spp. (TGx) isolates and the reference strains responded to the antibiotic concentrations differently. Nearly 50% of the isolates were susceptible to carbenicillin (400 μg disc^{-1}), kanamycin (100 μg disc^{-1}) and nalidixic acid (600 μg disc^{-1}), while only 4% were susceptible to gentamycin (100 μg disc^{-1}). Forty-two percent of isolates were highly resistant to rifampicin (40 μg disc^{-1}), while 81% were highly resistant to erythromycin (60 μg disc^{-1}). A phenogram developed from similarity coefficients between the isolates showed that the isolates formed two intrinsic antibiotic resistance (IAR) groups (IAR

1 and IAR 2) that clustered at the 50% similarity level (Fig. 1d). At the 75% similarity level, IAR 1 separated into nine clusters and IAR 2 separated into four clusters. The *B. japonicum* reference strains grouped with isolates NI-7 and NI-39 in cluster I, indicating that these isolates may be related to *B. japonicum*, while the isolates in cluster X that grouped with USDA74 may be related to *B. elkanii*. Clusters I, III, V and X can be further divided into subclusters at the 80% similarity level. All isolates appeared to be distinct from each other with respect to antibiotic resistance patterns. Thus, the antibiotic resistance profiles could not distinguish the *Bradyrhizobium* spp. TGx isolates from the *B. japonicum*, *B. elkanii* or *Bradyrhyzobium* sp. (misc.) strains.

DISCUSSION

Twenty-six randomly selected isolates from a large collection of *Bradyrhizobium* spp. (TGx) isolates collected from six countries across a wide geographical range in Africa were used for a comparative analysis of genetic and phylogenetic relatedness and diversity using different molecular and physiological parameters as the measure of diversity. Although the isolates of *Bradyrhizobium* spp. (TGx) are distinct from *B. japonicum* or *B. elkanii* in their inability to effectively nodulate American soybean Clark, all four analyses indicate that they are heterogeneous, with some members showing relatedness to *B. japonicum*, some to *B. elkanii* and some to the *Bradyrhizobium* sp. (misc.) reference strain.

Comparison revealed that the groups and clusters of isolates were all different with different methods. None of the methods could separate the *Bradyrhizobium* spp. TGx isolates as distinct groups different from *B. japonicum*, *B. elkanii* or *Bradyrhyzobium* sp. (misc.) strain TAL309. The cultivar specificity of these isolates was not revealed by the 16S RFLP generated by restriction enzymes, or *nod* or *nif* gene hybridization patterns. The isolates were distributed throughout the phylogenetic clusters, and the same isolates did not show a consistent grouping pattern with the strains of *B. japonicum*, *B. elkanii* or *Bradyrhyzobium* sp. (misc.) using the different methods. It is possible that a finer molecular method, such as partial sequencing of a gene segment from each of these strains, will be required to distinguish these TGx isolates from other related *Bradyrhizobium* species. Although none of the methods used could clearly distinguish them from *B. japonicum* or *Bradyrhyzobium* sp. (misc.) strains, all four methods revealed large diversity within TGx bradyrhizobia. Thus, the antibiotic resistance profile method is neither better nor worse than the three molecular methods used in this study. All four methods appear suitable for determining genetic diversity among the isolates and their relatedness to other known *Bradyrhizobium* strains.

Restriction enzyme site polymorphism was identified within the 16S rRNA gene of the isolates. Different restriction patterns were observed with the six different tetrameric enzymes used. Although the 16S rDNA RFLP method appeared to be good for determining the phylogenetic relationship among isolates, this method did not distinguish many isolates within a cluster from each other. Southern hybridization results using both *nod* and *nif* genes as probes revealed that *Bradyrhizobium* spp. (TGx) isolates were divergent from *B. japonicum* strain USDA110. Sadowsky *et al.* (1990) used $nodD_2$ and *nodDYABC* probes to differentiate between nodulation-restricted and nodulation-non-restricted strains of *B. japonicum*. Extensive sequence divergence around the *nodDYABC* genes of *B. japonicum* strains from Thailand have been reported from Japan (Yokoyama *et al.* 1996). Such variation might have resulted from a series of nucleotide substitutions during the evolutionary process. Only one isolate, UG-10, showed similarity in banding patterns with USDA110, suggesting that it may be a *B. japonicum* strain. Based on the patterns of the hybridizing bands, the *Bradyrhizobium* spp. (TGx) isolates have extensive sequence differences around *nodS* and *nodU* genes relative to *B. japonicum* strain USDA110 (data not shown). Similar results were obtained by Göttfert *et al.* (1990), who used a 1·2 kb *Nsi*I-*nodSU* fragment to probe *Eco*RI restriction fragments of genomic DNA of four *B. japonicum* and three *Bradyrhizobium* spp. strains.

Resistance patterns of the isolates to nine antibiotics were studied to provide phenotypic data for differentiating the *Bradyrhizobium* spp. (TGx) isolates from each other and to determine diversity among the isolates. The concentrations used to characterize the strains were higher than those used by Date and Hurse (1991), who used a similar approach to characterize indigenous rhizobial populations nodulating *Desmodium intortum* and *Macroptilium atropurpureum*. Many of the *Bradyrhizobium* spp. (TGx) grew back into the zones of growth inhibition after 7 days. This phenomenon was common with the lower concentrations of nalidixic acid, erythromycin and carbenicillin. Therefore, the concentrations of these antibiotics were increased gradually until a zone of inhibition remained clear until the 10th day of incubation. Cluster analysis of antibiotic resistance revealed that the responses to erythromycin were most dissimilar (58%), followed by rifampicin (50%), among the nine antibiotics (Fig. 2). Carbenenicillin and kanamycin were least effective in grouping and distinguishing the isolates into different clusters. All nine antibiotics were necessary to obtain resistance profiles of the isolates that could distinguish them from each other and group them into 13 clusters. The grouping of the *Bradyrhizobium* spp. (TGx) isolates based on antibiotic resistance profiles indicated that the isolates comprised two major groups. Using the *B. japonicum*

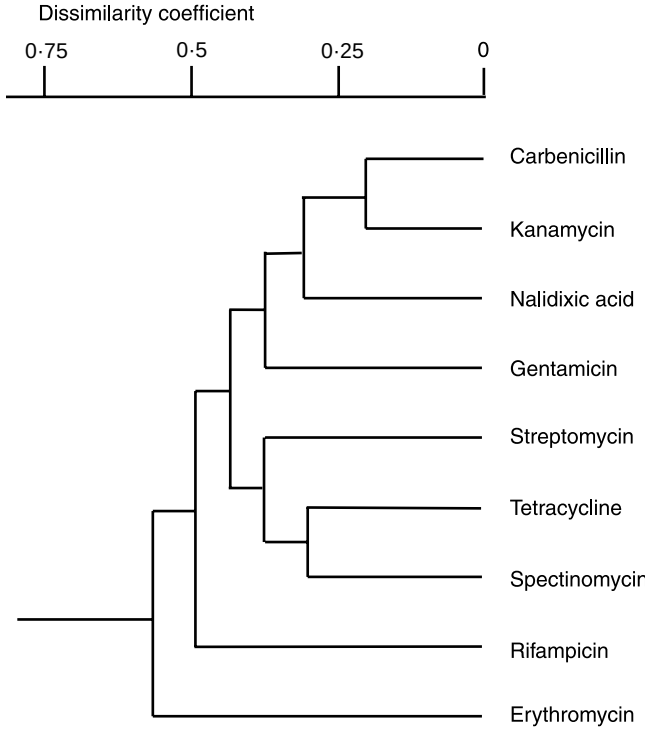

Fig. 2 Dendrogram derived for comparative effects of antibiotics on *Bradyrhizobium* spp. (Tgx) isolates and reference strains

strains as indicators, IAR 1 and IAR 2 correspond to DNA homology groups I and II described by Hollis *et al.* (1981). IAR 1 contains *B. japonicum* USDA110 and USDA59, while IAR 2 contains *B. elkanii* USDA74. Previously, Kuykendall *et al.* (1988) showed that DNA homology groups I and II have definite antibiotic resistance patterns.

In the present study, variations in one or a few attributes of the *Bradyrhizobium* populations are considered as the measure of overall variations in the whole organism. Thus, any single method considers the variations within certain attributes of the population and excludes all other variables in the organism. This may be the reason why different grouping and clustering patterns were obtained from the different methods. The results may be more reliable when a number of parameters are used instead of just one or two. The grouping due to *nod* gene hybridization in Fig. 1 is based on several probes, while the grouping due to *nif* gene hybridization is based on only one probe. Similarly, for the antibiotic resistance method, nine antibiotics were used to determine the resistance profiles of the isolates. When this method is applied to the study of several hundred isolates, it is likely that some of the isolates will appear to be similar. Those isolates that appear similar may be distinguished by using additional antibiotics, or by considering other morphological or physiological attributes such colony morphology, ability to grow at low pH, tolerance to high temperature etc. Molecular methods such as 16S rDNA RFLP and Southern hybridization require sophisticated laboratory facilities and expensive chemicals and enzymes. Such facilities are not generally available in laboratories in many parts of Africa. The antibiotic resistance method, on the other hand, does not require sophisticated laboratory facilities and expensive supplies, and is simple and easy to perform. Therefore, this appears to be a suitable method for assessment of genetic variability and grouping of the large number of *Bradyrhizobium* spp. (TGx) isolates obtained from the nodules of TGx soybeans in Africa. Representative isolates from each group can then be selected on the basis of nodulation and nitrogen fixing potential in TGx soybeans, and the phylogenetic relationship of the superior isolates selected with other bradyrhizobia may be established by sequencing a segment of a gene such as the 16S rRNA gene.

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