Genomic Heterogeneity within Cowpea Bradyrhizobia Isolated from Ghanaian Soils

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Abstract

Bradyrhizobia isolated from nodules of cowpea grown in 20 different Ghanaian soils were characterized on the basis of the 16S rRNA gene. RFLP groups established by four tetrameric restriction endonucleases were used to construct a phylogenetic tree which showed their genetic relatedness. The fast growing isolates produced 9 phylogenetic clusters while the slow growing isolates produced 11. Distribution among the isolates in the 11 clusters of the slow growing isolates was highly unbalanced with one cluster containing more than 50% of the total number of isolates. Diversity assessed using cluster divergence between both the fast and slow growing isolates was high, reaching 80%.

Introduction

Characterization of rhizobial strains naturally associated with the roots of legumes has been recommended as an effective approach to successful management of the legume-*Rhizobium* symbiosis (Richardson *et al.*, 1995; Mpepereki *et al.*, 1997). An array of different methods including host range analysis, serology, antibiotic resistance and biochemical analysis is available for the characterization of rhizobial strains. Currently, however, attention has focused on PCR-based genomic fingerprint methods, which have been shown to be effective for differentiating complex genomes (De Bruijn, 1992; Martinez-Romero, and Caballero-Mellado, 1996; Sessitsch *et al.*, 1997; Laguerre *et al.*, 1994). Indeed DNA sequence analy is of 16S rRNA regions has revealed much greater diversity than previously recognised (Moyer *et al.*, 1996) leading to important revisions in the taxonomy and systematics of the rhizobia.

Although african soils may harbour a large diversity of rhizobial populations, information on diversity is limited. Cowpea rhizobia indigenous to Nigerian soils are probably the only group that has been studied (Fred *et al.*, 1932; Sinclair and Eaglesham, 1984). Assessing the diversity of West African cowpea bradyrhizobia using physiological and biochemical characteristics, (Fred *et al.*, 1932), found some traits common to all or most of the isolates, some related to geographical origin and some colony morphology. The indigenous cowpea rhizobia strains in Zimbabwean soils showed considerable cultural and physiological diversity that included unique types belonging to several as yet undefined species (Mpepereki *et al.*, 1997). Studies on fast growing rhizobia of *Sesbania* and *Acacia* species obtained from soils in Senegal has led to the description of two fast growing species, *Sinorhizobium saheli* and *S. teranga* (Nei and Li, 1979). In Ghana, the diversity of rhizobial populations of different legumes has not been examined. We present here analysis of the genomic diversity of rhizobial strains, isolated from nodules of cowpea plants grown in a wide range of Ghanaian soils.

Materials and methods

Isolation of bradyrhizobia

Bradyrhizobia were isolated from the nodules of cowpea cultivar *Asontem* inoculated with diluted soil samples as described by Somasegaran and Hoben (1994). Soil samples were collected from 20 locations in five ecological zones of Ghana (Guinea savanna, forest savanna transition, semi-deciduous, high rain forest, and coastal savanna). Pre-germinated seeds of cultivar *Asontem* were planted in growth pouches containing a nitrogen-free nutrient solution (Somasegaran and Hoben, 1994). Three days after planting, the root system of each plant was inoculated with 2 ml of 1:10 (soil: water) soil suspension. Plants were grown in the greenhouse for 28 days with mean temperature 30/23 °C (day/night) and natural light of ca.12h photoperiod. One or two nodules from each plant inoculated with a soil suspension were randomly sampled, surface sterilized by immersing in 70% v/v alcohol for 3 min, rinsed with sterilized deionized water, and then placed in 0.1% w/v acidified mercuric chloride solution for 3 min, followed by repeated rinses in sterile water. Each nodule was then crushed in 50µl sterile water and the nodule content was streaked onto yeast extract-mannitol agar plates (Somasegaran and Hoben, 1994) and incubated at 28 °C. Single colonies were selected from isolates that appeared and authenticated by inoculating onto cowpea seedlings; 100 of the authenticated isolates were used for the study.

Sample preparation for DNA amplification

The isolates were grown on YEM agar plates at 28 °C for 24 hr for the fast growers and 48 hr for the slow growers. Cells of each isolate were suspended in 100µl Tris EDTA (TE) (Ausubel *et al.*, 1994) and the optical densities at 600nm of all the samples were adjusted to 2.6 with sterile distilled water. The samples were then deep-frozen for 4 min at –70 °C. Afterwards, the cells were set on ice for 1 min, boiled for 2 min, again set on ice for 1 min and then boiled once more for 2 mins. Finally, the cells were centrifuged for 2 mins at 15 000 rpm and the supernatant used for the PCR assay. The above procedure did not produce optimal DNA for some of the isolates. In these cases, single colonies of the isolates were grown in YEM medium and the cells pelleted by centrifugation at 15 000 rpm. Genomic DNA was then isolated from the pellets using DNeasy Plant Mini Kit (Qiagen) according to the supplier's instruction. DNA concentration was adjusted spectrophotometrically to 100ng/µl.

Polymerase chain reaction (PCR) amplification of the 16S rRNA gene

Primers fD1 (5'-AGAGTTTGATCCTG-GCTCAG-3') and rD1 (5'-AAGGAGGT-GATCCAGCC-3') described by Weisburg *et al.* (1991) were used for PCR amplification. They are derived from conserved regions of the 16S rRNA genes and amplify nearly full-length 16S rRNA genes (Weisburg *et al.*, 1991). PCR amplification was carried out in a total reaction volume of 100µl. DNA was amplified by mixing 100 ng template DNA (pure DNA) or alternatively, 5–8 ul cell extract with 1 x PCR buffer (50 mM KCl; 20 mM Tris.HCl, pH 8.0), 200 µM each of dATP; dCTP; dGTP and dTTP (Boehringer Mannheim), 3 mM $MgCl_2$, 0.2 µM of each primer, and 2 U Taq DNA polymerase (Gibco, BRL). All amplifications were carried out in a Perkin-Elmer thermocycler (GeneAmp PCR System 9600). The temperature profile was as follows: an initial denaturation step at 95 °C for 5 min followed by 35 cycles of 30 sec, denaturation at 94 °C, 1 min annealing at 50 °C and 2 min extension at 72 °C and a final extension step for 4 min at 72 °C.

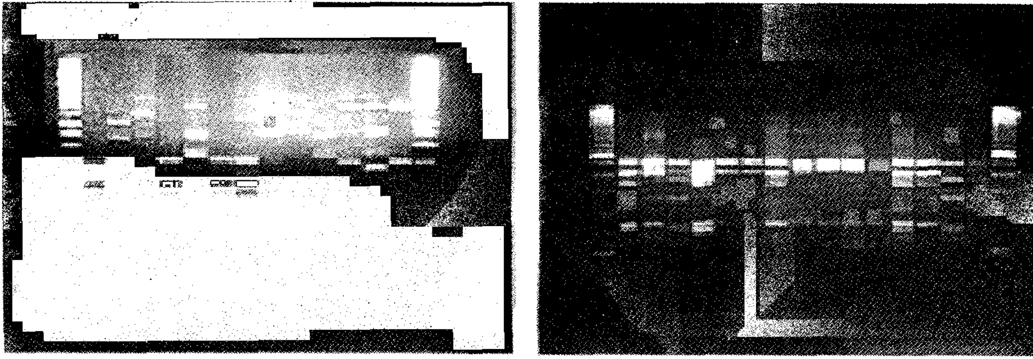

Plate 1. Restriction patterns of PCR-amplified fragment of 16S rRNA genes digested with *HaeIII* (A) or *MspI* (B). The lane assignments (numbers) represent Bradyrhizobium strains. Lane M = molecular marker.

Electrophoresis and imaging

Aliquots (5:l) of the amplified DNA were mixed with loading buffer (2 µl) and analyzed by horizontal agarose gel electrophoresis in 1% w/v agarose gels stained with ethidium bromide. Electrophoresis was carried out at 100 V for 30 min in 1 × Tris-acetate-EDTA (TAE) and the gels were photographed on a UV transilluminator.

Restriction fragment length polymorphism (RFLP) analysis of the PCR-amplified 16S rRNA gene fragment

Aliquots (10µl) of PCR products were digested with the tetrameric restriction endonucleases: *Dde*I, *Hae*III, *Msp*I, and *Rsa*I (Gibco BRL) in a total reaction volume of 20 µl and incubated at 37 °C for 2 hr. The resulting DNA fragments were analyzed by horizontal agarose gel electrophoresis in 2.5% w/v agarose gels stained with ethidium bromide. Molecular weight markers (100 bp) were run in the edge lanes of each gel to enable calculation of the sizes of the resulting restriction fragments. Electrophoresis was carried out at 100 V for 3 hr in 1 × TAE and the gels were photographed on a UV transilluminator. The DNA bands were scored as present or absent. The data obtained was used to determine the pairwise genetic distances among the isolates by the complement of Nei and Li's similarity index (Nei and Li, 1979). The genetic distances were used to cluster the isolates into defined groups.

Results

Gel electrophoresis of the PCR products of the isolates revealed that the amplification reaction produced a common single DNA molecule about 1.5 kilobase long for all the isolates, which corresponded to the expected size of the 16S rRNA genes among bacteria. The PCR products were restricted with enzymes *Dde*I, *Hae*III, *Msp*I, *and Rsa*I (Plate 1). The observed restriction fragments of each type of pattern in the normalised gels are listed in Table 1. From 2 to 11 distinct restriction patterns were detected with each of the four endonucleases, *Dde*I, *Hae*III, *Msp*I, *and Rsa*I (Table 1). The combination of the four restriction digest patterns identified 20 small sub-unit rDNA types that were arbitrarily named A–K (Table 2). The 18 fast growing isolates produced nine distinct RFLP patterns, each of which included between 1 and 5 isolates (Table 2). The ninth digestion pattern of the fast growing isolates designated I, was not included in the relative similarity

TABLE 1
Restriction patterns of cowpea bradyrhizobia isolates revealed by RFLP analysis of PCR-amplified 16s rRNA genes.

Isolates	Restriction pattern[1] of amplified 16s rRNA genes digested with			
	DdeI	Hae III	Msp I	Rsa I
Fast growers				
19	a	a	a	a
20	b	b	b	a
23	c	c	c	b
27	b	b	b	a
29	b	b	b	a
38	d	d	d	c
43	e	c	e	b
44	f	e	f	b
51	b	b	b	a
57	c	c	c	b
59	g	f	g	d
60	b	b	b	a
69	g	f	g	d
79	b	b	b	a
88	a	a	a	a
92	h	g	h	?
98	i	?	i	e
Slow growers				
1	a	b	a	a
2	b	a	b	b
3	a	a	c	b
4	b	c	d	a
5	a	a	c	b
6	a	d	a	c
7	a	f	c	d
8	a	a	e	b
9	a	f	c	d
10	a	b	a	a
11	a	e	f	a
12	a	b	a	a
13	c	a	e	b
14	d	b	d	a
15	a	a	c	b
16	a	a	c	b
17	a	a	a	b
18	a	a	a	b
21	e	e	a	b
22	a	b	a	b
24	a	b	a	a
25	f	f	j	b
26	g	g	c	b
28	h	k	k	b
30	a	b	a	b
31	a	a	a	b
32	i	l	i	d
33	a	a	a	b
34	d	b	d	b
35	a	b	a	b
36	a	b	a	a
37	a	b	a	a

TABLE 1. Contd.

Isolates	Restriction pattern[1] of amplified 16s rRNA genes digested with			
	DdeI	Hae III	Msp I	Rsa I
39	j	j	j	i
40	k	i	g	f
41	k	j	g	f
42	a	a	a	b
45	a	a	a	b
46	g	k	g	c
47	a	a	a	b
48	a	a	a	i
49	a	a	a	b
50	a	a	h	i
52	g	k	g	b
53	a	a	b	a
54	a	b	a	b
55	g	k	g	b
56	g	k	g	c
58	i	l	i	j
61	a	a	a	b
62	a	b	a	b
63	a	a	a	b
64	a	a	a	b
65	a	a	a	b
66	a	a	a	b
67	a	a	a	b
68	d	b	d	d
70	f	f	j	f
71	f	f	j	f
72	a	a	a	b
73	e	e	a	b
74	a	a	a	b
75	a	a	a	a
76	j	j	i	j
77	c	d	a	a
78	c	c	a	a
80	h	k	k	f
81	h	k	k	e
82	c	a	a	a
83	i	l	l	c
84	h	k	k	b
85	a	a	a	b
86	a	a	l	a
87	c	d	a	a
89	h	k	k	c
90	i	l	l	d
91	j	j	j	j
93	j	j	l	j
94	a	b	a	a
95	a	a	a	b
96	e	e	a	b
97	f	f	j	e
99	c	a	a	a
100	f	f	f	e

[1]The different patterns detected with each enzyme among the 100 isolates analyzed are designated by the lower case letters.

TABLE 2
Distribution of cowpea bradyrhizobia isolates among 20 genotypes identified by RFLP analysis of PCR-amplified 16s rRNA genes.

16s rRNA genotype[1]	Isolates
Fast growers	
A	19, 88
B	20, 27, 29, 51, 60, 79
C	23, 57
D	38
E	43
F	44
G	59, 69
H	92
I	98?
Slow growers	
A	1, 3, 5, 6, 7, 8, 9, 10, 11, 12, 15, 16, 17, 18, 22, 24, 30, 31, 35, 37, 42, 45, 46, 49, 50, 53, 61, 62, 63, 64, 65, 66, 67, 74, 75, 85, 86, 94, 95
B	2, 4
C	13, 77, 82, 87, 99
D	14, 34, 68
E	21, 73, 96
F	25, 70, 71, 97, 100
G	26, 46, 52, 55, 56
H	28, 80, 81, 84, 89
I	32, 58, 83, 90
J	39, 76, 91, 93
K	40, 41

[1]The 16s rRNA genotypes lettered A to K represent the species group of bradyrhizobia isolates obtained with the four endonucleases used.

analysis due to irreproducibility in the banding patterns it produced. The slow growing isolates produced 11 distinct RFLP patterns (Table 2). The distribution of the isolates among the slow growing genomic species was highly unbalanced, with one taxon designated A, representing more than half of the total number of the isolates, whilst each of the remaining 10 taxa contained between 1 and 3% of the isolates (Table 2). Diversity among the taxa identified in both the fast and slow growing isolates as revealed by cluster analysis was very high, reaching 80% divergence (Figs. 1 and 2).

Discussion

Knowledge of the diversity of rhizobia indigenous to tropical soils is very sparse. Reports in the literature indicate that strains have been collected piecemeal from various sources and may not have been representative of native populations. Early reports indicated that the microsymbionts of cowpea were typically slow growing bacteria with the characteristics of *Bradyrhizobium* species (Lafay and Burdon, 1998). Later studies however, have suggested fast growers as typical symbionts (Zablotowicz and Focht, 1981; Dakora and Vincent, 1984; Mpepereki *et al.,* 1997). Nevertheless a high

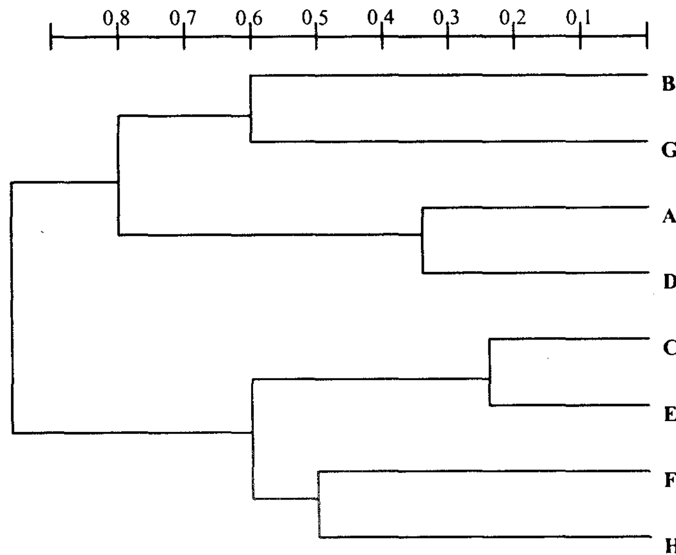

Figure 1. Dendrogram (UPGMA) showing relationship among genomic species of fast-growing cowpea bradyrhizobia isolates as determined by RFLP analysis of the 16s rDNA. The matrix of pairwise genetic distances was used to construct the dendrogram.

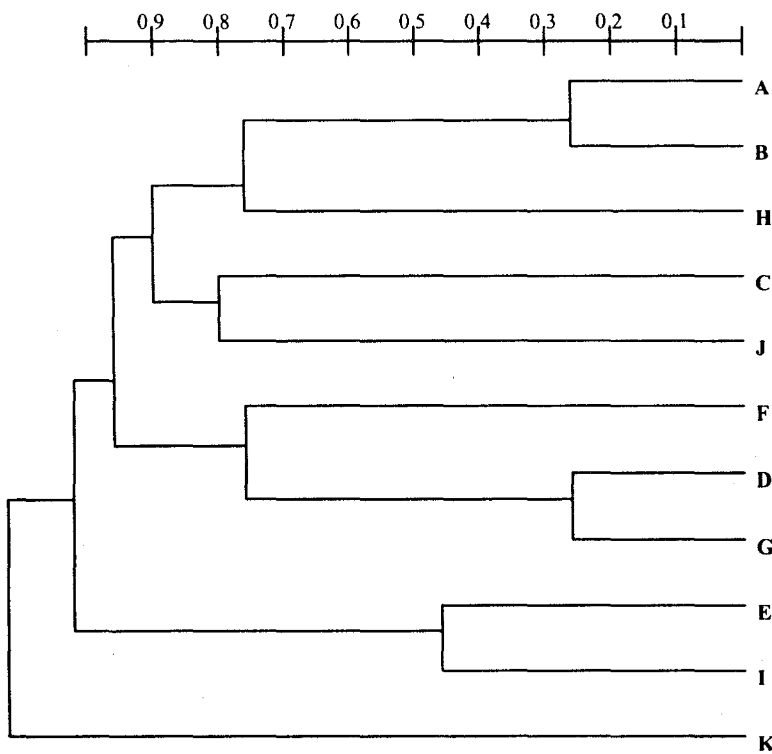

Figure 2. Dendrogram (UPGMA) showing relationship among genomic species of slow-growing cowpea bradyrhizobia isolates as determined by RFLP analysis of the 16s rDNA. The matrix of pairwise genetic distances was used to construct the dendrogram.

predominance of *Bradyrhizobium* species has generally been observed. The various previous descriptions were based solely on growth features and the cross-inoculation concept and thus did not provide precise information about the real nature and structure on the rhizobial population. Considering the broad range of specificities either of rhizobial species towards their host or of the legume species towards their symbionts, molecular identification has become a prerequisite to any study of rhizobial population structure (Laguerre *et al.,* 1994). Indeed, it is fundamental to differentiate between members of the same species to be able to provide some insight on the relationship between the two partners and to infer the factors determining their association. In this study, the 16S rRNA gene of the isolates were analyzed in order to characterize the isolates. Four tetrameric restriction enzymes (*Dde*I, *Hae*III, *Msp*I, and *Rsa*I) were used to determine similarities among the isolates. The choice of the enzymes was based on the results of Laguerre *et al.* (1996) and a recent computer-based study (Mpepereki *et al.,* 1997), which evaluated the efficacy of selected tetrameric restriction enzymes for rDNA-RFLP analysis of rhizobial isolates.

Results obtained using dendrograms constructed from the similarity matrix of the isolates by the method of Nei and Li (1979) indicated that, the diversity of the 100 isolates was high, and provide the first analysis of the genotypic diversity of *Bradyrhizobium* strains nodulating cowpea in Ghanaian soils. The high genotypic diversity revealed by the PCR-RFLP analysis in this study is in good agreement with the great phenotypic diversity revealed on the isolates based on physiological, host range, and serotyping (Fening *et al.,* 2002). However, members of the groupings as delineated by the four methods were not consistent. Thus isolates that had identical rDNA genotypes did not display similar phenotypic characteristics. This illustrates a lack of correlation between phenotypic- and genotypic-based methods for grouping *Bradyrhizobium* strains. Similar observations were made by So *et al.* (1994) and Van Rossum *et al.* (1995). Several reasons may account for this observation. For instance, it was observed that the patterns of serological response to isolates used, as immunogens was not always reciprocal. Also, the indirect ELISA techniques used is dependent on interaction between an antibody and an antigen, which is attached to a solid phase (microtitre plate) by passive adsorption. Results may therefore be biased, depending on the type of microtitre used. During a trial in this study, it was found that ELISA plates type Dynatech and sumilon C gave different results. Furthermore, grouping of isolates is based on a range of absorbance values which may fail to detect widespread differences among the isolates. The cross-inoculation grouping method may also be influenced by the number of test legumes used and also, different rhizobial cell numbers may be required to initiate nodules on different legumes. Age of cell cultures and different batches of PCR reagents were also observed in this study to affect the reproducibility of PCR results. Another possibility might be the level of resolution of each method. For instance, RFLP of 16S rDNA resolves the DNA of the isolates from the genus to the subspecies level. Serogrouping might resolve differently while physiological and metabolic studies might resolve from the family to the strain level. Hence, when the three methods are put together, the results might not correlate nor be consistent. Further studies may therefore be necessary to assess the use or otherwise of these methods for grouping bradyrhizobial isolates.

Conclusion

The genetic diversity of native cowpea bradyrhizobia isolated from soils in different agroecological zones of Ghana has been examined. Data obtained indicate a large genotypic

diversity among the strains which hitherto were assumed to be narrower than other nontropical legumes. This study has provided fundamental information that can pave the way for further characterization and improvement of the cowpea rhizobia–symbiosis through the use of microbial inoculants and other agronomic strategies.

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