Plant and Soil **204:** 127–134, 1998.

Detection of *Bradyrhizobium* spp. and *B. japonicum* in Thailand by primer-based technology and direct DNA extraction

N. Teaumroong* and N. Boonkerd
School of Biotechnology, Suranaree University of Technology, Nakhon-Ratchasima, 30000 Thailand

Key words: Arachis hypogaea, Glycine max, PCR, *Vigna radiata*

Abstract

Total chromosomal DNAs from 20 *Bradyrhizobium* spp. strains (10 strains isolated from *Vigna radiata* and 10 from *Arachis hypogaea*) and 18 *B. japonicum* strains isolated from *Glycine max* were extracted. These DNAs served as templates for REP, ERIC and RAPD primers in PCR analyses. The patterns of the resulting PCR products were analyzed and highly specific for each strain, especially when grouped together with their antibiotic-resistance profiles. A method for extracting DNA directly from soil was developed. Recovery was approximately 30 μg DNA g^{-1} soil and the procedure yielded DNA suitable for PCR amplification.

Introduction

Legumes play a major role in sustainable management of arid areas. Rhizobia have been extensively used in agricultural systems for enhancing the ability of legumes to fix atmospheric nitrogen (Elkan, 1992). One of the impediments to greater nitrogen fixation efficiency is the inability of superior rhizobia used as inoculants to form the majority of nodules under field conditions. Competition from indigenous strains usually limits the performance of the inoculant strains (Keyser and Cregan, 1987). It is therefore important to distinguish inoculant strains from other indigenous rhizobia and to identify the strain responsible for nodulation.

Many methods to identify rhizobia exist but recently new approaches to evaluate the diversity of indigeneous rhizobia using the polymerase chain reaction (PCR) have been developed. PCR can be performed rapidly with strain-, species- or genus-specific primers that generate fingerprints characteristic of each strain. DNA primers corresponding to repetitive palindromic (REP), enterobacteria-repetitive intergenic-consensus (ERIC) coupled with PCR can be used to fingerprint the genomes of rhizobia (De Bruijn, 1993). In addition, short primers of arbitrary nucleotide sequences, RAPD (random amplified polymorphic DNA), have been used since it is simple to detect polymorphisms that allow rapid identification and isolation of chromosome-specific DNA fragments (e.g. Judd et al., 1993; Nick and Lindstrom, 1994; Trevors and Elsas, 1989; Versalovic et al., 1991; Williams et al., 1993).

To fingerprint rhizobia using primer-based techniques, DNA was extracted directly from soil since it overcomes limitations imposed by conventional methods such as the need to culture cells (Sayler and Layton, 1990). We also compared conventional techniques such as antibiotic-resistance profiles with the PCR data.

Materials and methods

Bacterial strains

Twenty *Bradyrhizobium* spp. strains isolated from *Vigna radiata* (L.) *R. Wilezek* and *Arachis hypogaea* L. as well as 18 *B. japonicum* strains isolated from *Glycine max* (L.) *Merr.* are listed in Table 1. TY (Tryptone Yeast-extract: Tryptone 5.0 g, Yeast extract 3.0 g, and $CaCl_2$–2 H_2O 0.1 g per deionized water 1 *l*) was used for cultivation (Beringer, 1974).

* FAX No: 66-44-216-310. E-mail: neung@ccs.sut.ac.th
Plant and Soil is the original source of publication of this article. It is recommended that this article is cited as: *Plant and Soil* **204**: 127–134, 1998.

Table 1. Bradyrhizobium spp. and *B. japonicum* strains used in this study

Host plant of origin	Strain
Vigna radiata	*Bradyrhizobium* spp. TAL 209, USDA 3267, TAL 306, TAL 305, TAL 301, THA 302, TAL 442, THA 304, TAL 425 and TAL 441
Arachis hypogaea	*Bradyrhizobium* spp. E-7-1, E-17-1, 280 A, NE-36-19, N-22-18, M-47-12, M-43-10, NE-41-15, M-50-2 and 22-2A
Glycine max	*B. japonicum* TAL 102 (USDA 110), TAL 432, THA 2, TAL 379, TAL 944, TAL 212, TAL 211, USDA 8-T, TAL 220, USDA 94, USDA 35, USDA 117, TAL 377, THA 7, THA 5 and TAL 216

Isolation of genomic DNA

Bacterial cells were pelleted in 0.1% lauroylsarcosine in TE buffer by centrifugation at 13,000 *g* for 2 min. Cells were resuspended in 60 μl of 25% sucrose in TE buffer, 700 μl of TE buffer and 80 μl of 1.0% lauroylsarcosine prior to incubation with 20 μl of 2.0 mg/ml lysozyme for 30 min at 37 °C. Cell lysates were extracted twice with saturated phenol and once with chloroform: isoamyl alcohol (24:1). The aqueous phase was precipitated with two volumes of cold 99% ethanol. DNA pellets were dried under vacuum before dissolution in TE buffer and stored at 4 °C.

Oligonucleotide primers and PCR conditions

Oligonucleotide PCR primers and their sequences are listed in Table 2. 50 ng of template DNA was used per reaction. PCR reactions contained 50 pmol each of two opposing primers, 1.25 m*M* each of the four deoxynucleoside triphosphate, 2.5 U of Ampli Taq DNA polymerase (Takara Biomedical, Japan) and distilled water to final volume of 100 μl. Each tube was overlaid with 40 μl of mineral oil to avoid evaporation. The PCR conditions for the different primers are shown in Table 3.

Antibiotic resistance

The antibiotics and the concentration used in this study are listed in Table 4. All preparations were filter-sterilized using 0.45 μm membrane filters. Antibiotics were added to molten agar after sterilization.

Direct extraction of DNA from soil

Aliquots (1.0 g) of soil were mixed with 2.5 mL of 0.12 *M* phosphate buffer (pH 8.0) on an end-over-end shaker for 15 min at room temperature. The slurry was pelleted by centrifugation at 9,300 *g* for 10 min. The pellet was washed again with the same buffer. Disintegration of bacterial cell walls and extraction of humic substances from soil was performed with end-over-end shaking for 15 min in a final concentration of 0.2 *N* NaOH and 1.0% Brij 58. After centrifugation (2 times at 13,000 *g* for 20 min), the dark-brown supernatant was discarded. The pellet was extracted three times with 2.5 mL of 0.12 *M* phosphate buffer (pH 8.0) followed by centrifugation at 13,000 *g* for 5 min. Supernatants from all three extractions were collected in one tube which contained 15 mg of lysozyme/ml and incubated at 37 °C for 2 h. Subsequently, 2.0 μL of 10% SDS was added followed by incubation at 50 °C for 1 h. Three cycles of freezing and thawing (-80 °C and 70 °C) were conducted to break bacterial cells. After freezing and thawing, the solution was extracted twice with phenol-chloroform. DNAs from the aqueous phase were precipitated with cold isopropanol; the pellet was washed again with cold 70% ethanol and dried under vacuum. To further purify DNA obtained this way, we used the method for extraction of

Table 2. Oligonucleotide sequences of PCR primers

Primer	Oligonucleotide sequence
RAPD	5′ - G G A A G T C G C C - 3′
REP IR-I	3′ - C G G I C T A C I G C G C I I I I - 5′
REP 2-I	5′ - I C G I C T T A T C I G G C C T A C - 3′
ERIC IR	3′ - C A C T T A G G G G T C C T C G A A T G T A - 5′
ERIC 2	5′ - A A G T A A G T G A C T G G G G T G A G C G - 3′

Abbreviations: A, adenine; T, thymine; G, guanine; C, cytosine; I, inosine.

Table 3. The PCR conditions for the different primers

Primer	Danaturation	Annealing	Primer extension	No. of cycles
RAPD	94 °C, 1 min	36 °C, 1 min	72 °C, 2 min	45
REP	95 °C, 6 min	–	–	1
	94 °C, 1 min	40 °C, 1 min	65 °C, 8 min	30
	–	–	65°C, 16 min	1
ERIC	95 °C, 7 min	-	-	1
	94 °C, 1 min	52 °C, 1 min	65 °C, 8 min	30
	–	–	65 °C, 16 min	1

DNA from Low-Melting-Agarose by QN (N-cetyl-N, N, N-trimethylammonium- bromide) (Langridge et al., 1980).

Results and discussion

REP, ERIC and RAPD classification of some Bradyrhizobium *spp. and* B. japonicum

Total genomic DNAs used as DNA templates for PCR using REP, ERIC and RAPD primers. PCR products were separated on 1.5% agarose gels and stained with ethidium bromide. To control the reproducibility of the system, REP, ERIC and RAPD PCRs were repeated three times; the generated patterns were reproducible (Figure 1). Each strain that had a PCR product of a given size was scored positive at that location in the matrix, while strains not having a product of that size were scored negative. Data were analyzed using the Primer Version 3.1 B programme and used to generate the dendrogram representing the products from each primer.

One set of primers detected differences in the genome of strains that was not seen when other sets of primers were used. To maximize the specificity of the REP, ERIC and RAPD derived PCR patterns, we generated a combined matrix of the product patterns from all three primers. The dendrogram generated from the combined matrix is shown in Figures 2–4.

The 10 tested *Bradyrhizobium* spp. that were isolated from *V. radiata* (Figure 2) can be divided into two main clusters. Strains TAL 425 and TAL 306 are genetically more similar than the other strains. As shown by Kuykendall et al. (1988), however, differentiation based on multiple resistance markers provides a rapid and efficient method for distinguishing between strains of *Bradyrhizobium* spp. with distinct genetic and phenotypic backgrounds. Accordingly we combined the results of antibiotic resistance profiles with the dendrogram. The only difference between TAL 425 and TAL 306 is the sensitivity to chloramphenicol. Similar results were also found in the dendrogram of *Bradyrhizobium* spp. isolated from *A. hypogaea* (Figure 3); strains E-7-1 and E-17-1 were genotypically similar but different in erythromycin resistance. With the 18 strains of *B. japonicum*, the dendrogram showed that strains USDA 117 and THA 5 are genotypically similar but had the same antibiotic profiles, suggesting that the data obtained from both genotypic and phenotypic analyses are necessary to distinguish strains of bradyrhizobia.

Table 4. Type and concentration of antibiotics used in the study

Antibiotic	Concentration (μg/mL)
Carbenicillin (Car)	500
Chloramphenicol (Chl)	500
Erythromycin (Ery)	250
Nalidixic acid (Nal)	50
Streptomycin (Str)	100
Tetracyline (Tet)	100
Trimethoprim (Tmp)	50

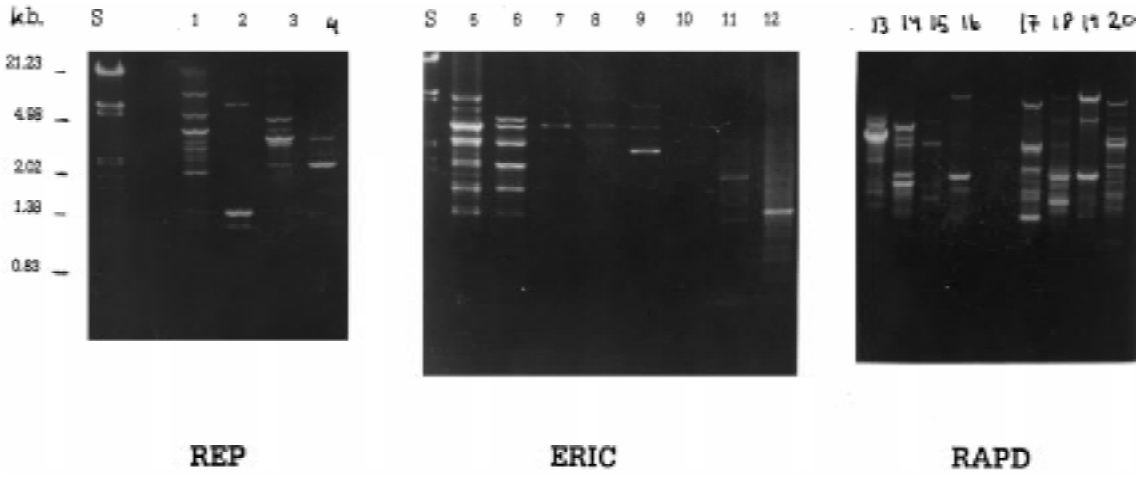

Figure 1. REP-, ERIC- and RAPD-PCR fingerprint patterns of genomic DNA from some *Bradyrhizobium* spp. and *B. japonicum* strains isolated from *Arachis hypogaea, Vigna radiata* and *Glycine max.* Lanes: S, 560-bp DNA ladder size standard; 1-4 show the REP PCR pattern of chromosomal DNA from *Bradyrhizobium* spp. (isolated from *Vigna radiata*) strains TAL 305, THA 301, TAL 209 and USDA 3267, respectively. Lanes 5-12 show the ERIC patterns of chromosomal DNA from *Bradyrhizobium* spp. (isolated from *Arachis hypogaea*) strains E-7-1, 280 A, E-17-1, NE-36-19, N-22-18, M-47-12, M-43-12 and M-43-10, respectively. Lanes 13–20 show the RAPD pattern of chromosomal DNA from *B. japonicum* (isolated from *Glycine max*) strains USDA 8-0, USDA 94, USDA 35, USDA 117, TAL 377, THA 7, THA 5 and TAL 216, respectively.

Direct extraction of DNA from soil

In soil microbial ecology, there is a need to develop methods to analyze microbial communities. Many microbes that are part of these communities are hard to cultivate and therefore difficult to detect or classify. Perhaps primers for REP-, ERIC- and RAPD-PCR will facilitate analysis, since these methods do not require microbial cultivation and yield results with very small amounts of DNA. Direct extraction of DNA from soil is widely used for these purposes (e.g. Hilger and Myrold, 1991; Sayler and Layton, 1990; Trevors and Elsas, 1989). Thus, sandy-loam was collected from a cassava field in Nakhon-Ratchasima, Thailand. It had a pH (H_2O) of 6.3, organic substance = 6.1%, nitrogen = 0.42% (Kjel), total carbon = 4.83%, and water (105 °C from air-dried sample) of 2.2%. After phenol extraction the DNA solution still contained humic substances (brown color), which could not be removed and gave a bright blue-green fluorescent band under UV. This band migrated ahead of DNA in electrophoresis gels (data not shown). To avoid the inhibitory effect of humic acids on the activity of Taq polymerase (Picard et al., 1992), the DNA was purified by extraction from low-melting-agarose. Approximately 30 μg DNA was recovered per g soil. This represents a recovery of 30–35% assuming a mean DNA content per bacterial cell of 6 fg (Ingraham et al., 1983), and a DNA content of *E.coli* cell of 9 fg (McCoy and Olson, 1995). This recovery was intermediate between that estimated for direct extraction using

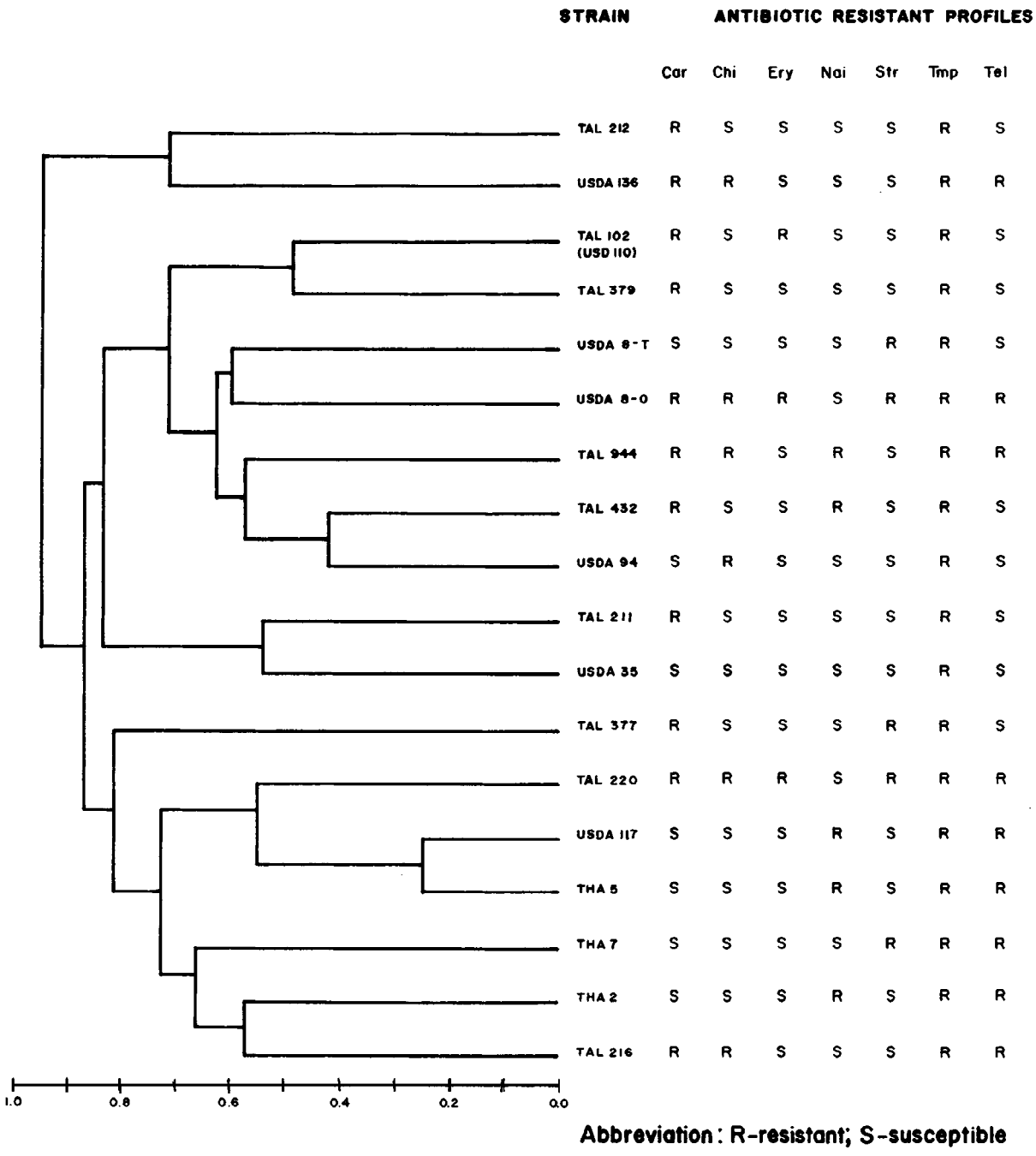

Figure 2. Dendrogram of *B. japonicum* isolated from *Glycine max* and their antibiotic profiles.

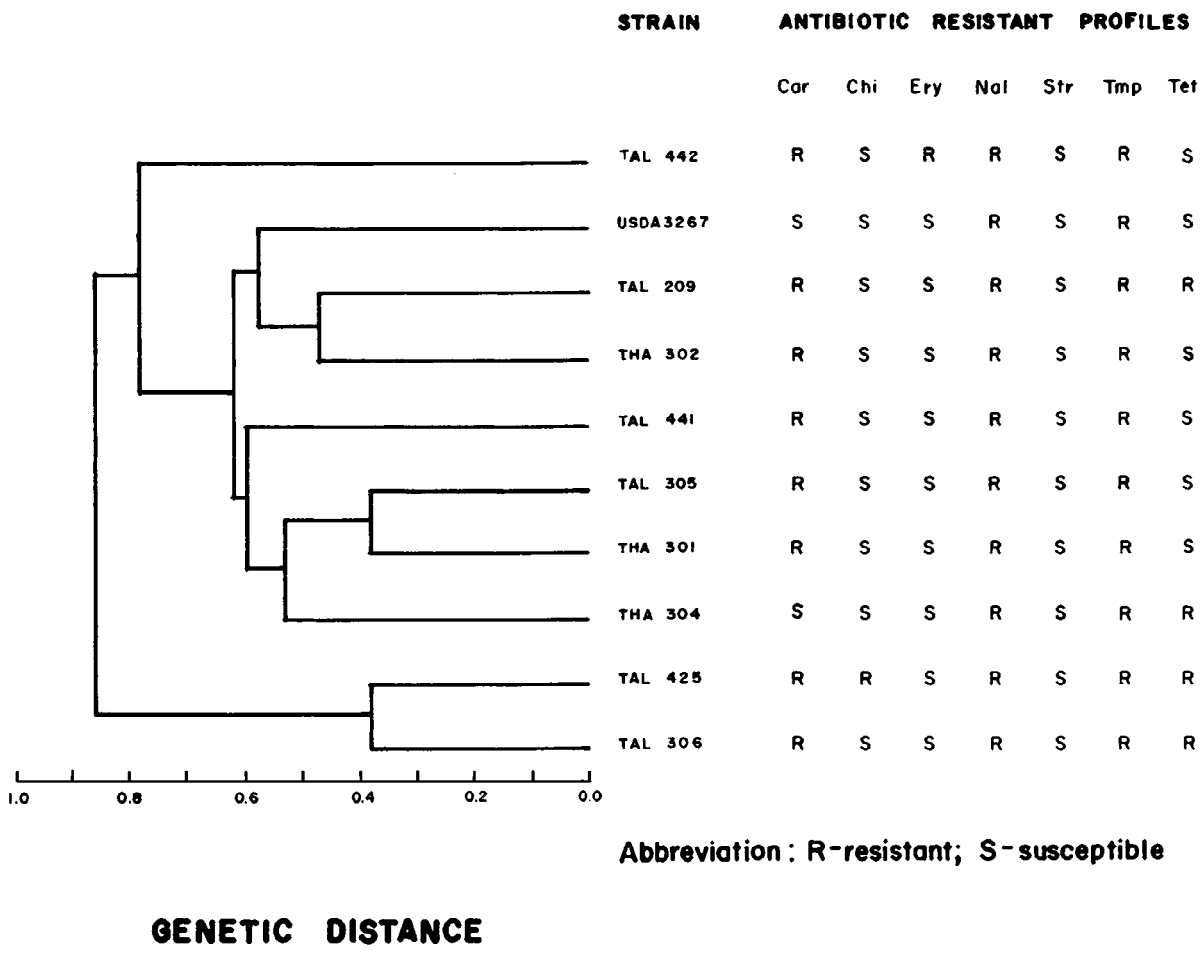

Figure 3. Dendrogram of *Bradyrhizobium* spp. isolated from *Vigna radiata* and their antibiotic profiles.

lysozyme (10%) (Hilger and Myrold, 1991), and the direct lysis method (22%) (Holben et al., 1992). Holben et al (1992) and Jacobsen and Rasmussen (1992) developed procedures to purify microbial DNA from soil, which give an average yield of purified DNA of 0.88 μg of soil.

Sensitivity of PCR amplification in DNA extracted from soil

When purified DNAs from soil samples were used as the DNA template, the PCR products generated from REP, ERIC and RAPD primers were not reproducible between experiments (data not shown). Perhaps soil heterogenity and complexity which affects the establishment and population dynamics of soil bacteria played a role. Use of autoclaved soil followed by the DNA extraction procedure, coupled with the addition of pure chromosomal DNA corresponding to $\geq 10^2$ cells of *Bradyrhizobium* spp. strain TAL 305, E-7-1 and from *B. japonicum* strain TAL 432 routinely gave PCR-products of the expected size in agarose gels. Such bands could not be obtained using lower template DNA concentrations. This shows that our methodology yields purified DNA that can be amplified using PCR.

Acknowledgements

This research was supported in part by the International Atomic Energy Agency. We thank Ms P Changpo and Ms P Prukhett for helping to prepare the manuscript.

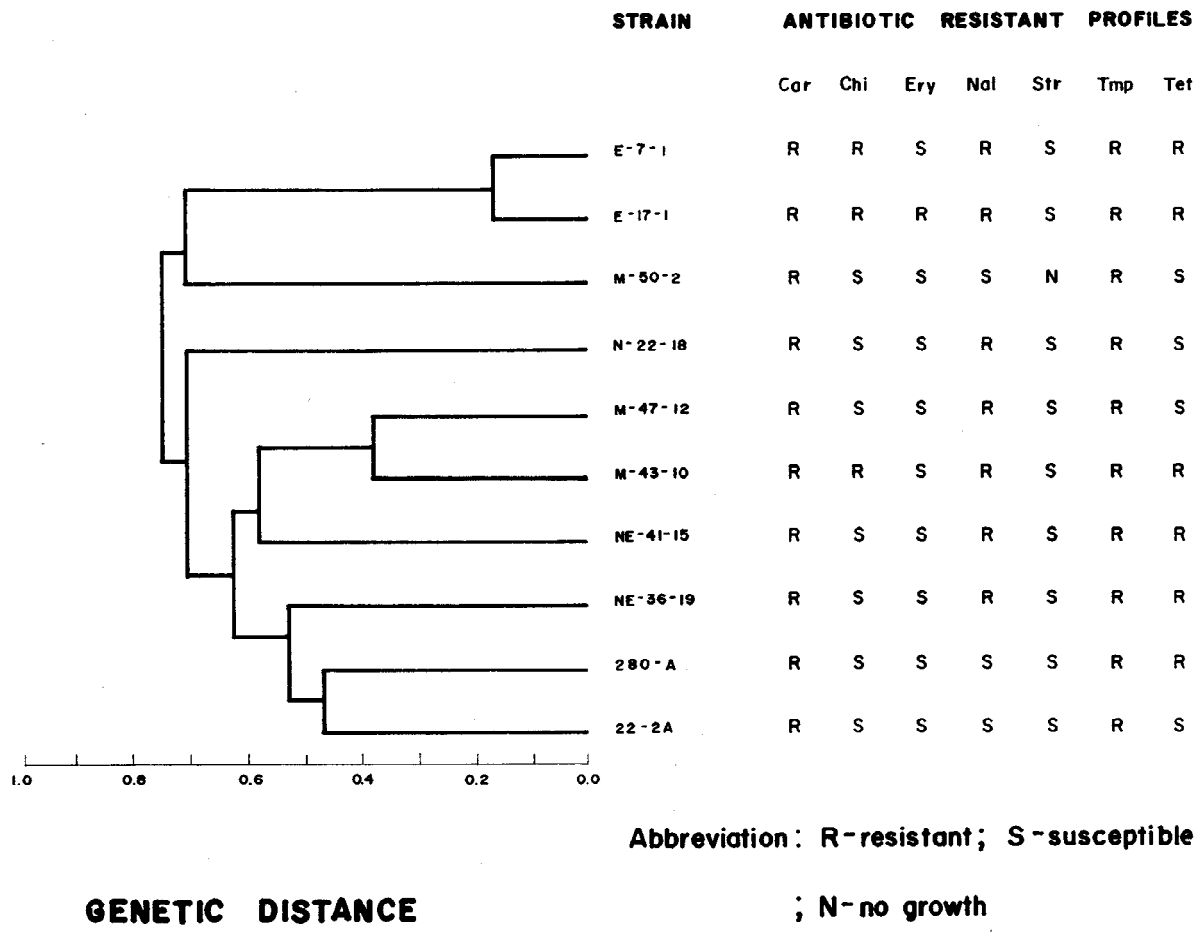

Figure 4. Dendrogram of *Bradyrhizobium* spp. isolated from *Arachis hypogaea* and their antibiotic profiles.

References

Beringer J E 1974 R-factor transfer in *Rhizobium leguminosarum*. J. Gen Microbiol. 84, 188–198.

Bruijn De F J 1993 Use of Repetitive (Repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. 58, 2180–2187.

Elkan G H 1992 Taxonomy of the Rhizobia. Can. J. Microbiol. 38, 446–450.

Hilger A B and Myrold D D 1991 Method for extraction of *Frankia* DNA from soil. Agric. Ecosystems. Environ. 34, 107–113.

Holben W E, Schroeter B M, Calabrese V G M, Olsen R H, Kukor J K, Biederbeck V O, Smith A E and Tiedje J M 1992 Gene probe analysis of soil microbial populations selected by amendment with 2,4-dichlorophenoxyacetic acid. Appl. Environ. Microbiol. 58, 3941–3948.

Holben W E, Jansson J K, Chelm B K and Tiedje J M 1988 DNA probe method for detection of specific microorganism in the soil bacterial community. Appl. Environ. Microbiol. 54, 703–711.

Ingraham J L, Maaloe O and Neidhardt F C 1983 Growth of the Bacterial Cell. Sinauer Associates, Inc., Sunderland, MA. pp 1–48.

Jacobsen S C and Rasmussen O F 1992 Development and application of a new method to extract bacterial DNA from soil based on separation of bacterial from soil with cation-exchange resin. Appl. Environ. Microbiol. 58, 2458–2462.

Judd K A, Schneider G M, Sadowsky M J and de Bruijn F J 1993 Use of repetitive sequences and the polymerase chain reaction technique to classify genetically related *Bradyrhizobium japonicum* serocluster 123 strains. Appl. Environ. Microbiol. 59, 1702–1708.

Keyser H H and 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.

Kuykendall L D, Roy M A, O'Neill J J and Devine T E 1988 Fatty acids, antibiotic resistance and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. Int. J. Syst. Bacterial. 38, 358–361.

Langridge L, Langridge P and Bergquist L 1980 Extraction of nucleic acids from agarose gels. Anal. Biochem. 103, 264–271.

McCoy W F and Olson B 1995 Fluorometric determination of DNA concentration in municipal drinking water. Appl. Environ. Microbiol. 49, 811–817.

Nick G and Lindstrom K 1994 Use of repetitive sequences and the polymerase chain reaction to fingerprint the genomic DNA of *Rhizobium galegae* strains and to identify the DNA obtained by sonicating the liquid cultures and root nodules. System. Appl. Microbiol. 17, 265–273.

Picard C, Ponsonnet C, Paget E, Nesme X and Simonet P 1992 Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. Appl. Environ. Microbiol. 58, 2717–2722.

Sayler S G and Layton A C 1990 Environmental application of nucleic acid hybridization. Annu. Rev. Microbiol. 44, 625–648.

Trevors J T and van Elsas J D 1989 A review of selected methods in environmental microbial genetics. Can. J. Microbiol. 35, 895–901.

Versalovic J, Koeuth T and Lupski J R 1991 Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucl. Acids Res. 19, 6823—6831.

Williams G K J, Kubelik A N, Livak K J, Rafalski J A and Tingey S V 1993 DNA polymorphisms amplified by arbitrary primers are useful as genetic marker. Nucl. Acids Res. 18, 6531–6535.