GENETIC DIVERSITY OF NATURALLY-OCCURRING

RHIZOBIA NODULATING PIGEONPEA

By

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PREFACE

This study was conducted to provide information regarding the naturally-occurring rhizobia capable of nodulating pigeonpea. It also provided information on the use of two selected rhizobial strains to fix atmospheric nitrogen for the plant. The specific aims of this research were to characterize (a) the persistence of inoculant strains of pigeonpea rhizobia after the initial inoculation, and (b) the genetic diversity of naturally-occurring rhizobia capable of nodulating pigeonpea in Oklahoma soils. REP (Repetitive Extragenic Palindromic sequences) PCR (Polymerase Chain Reaction) amplifications were used to follow the fate of the introduced rhizobial strains. This technique plus 16S, 23S IGS (intergenic region) RFLP's (Restriction Fragment Length Palindromic) sequences, 16S rRNA gene amplification RFLP's (Restriction Fragment Length Palindromic) sequences and partial sequencing of 16S genes were used to characterize the gentic diversity of the indigenous rhizobial populations.

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Chapter 1

Introduction

The tropical legume pigeonpea, *Cajanus cajan* Millispecies, is widely used in Asia and the Caribbean for human consumption and as an intercrop with cereals, pulses or oilseeds (82). Pigeonpea has the potential to contribute positively to agricultural systems typical of the Southern Great Plains if included in a winter wheat-legume cropping system. Pigeonpea planted during the normally fallow summer months can help protect soils from erosion, contribute to soil nitrogen pools, and produce forage for livestock or high protein seed for animal feed or human consumption. Furthermore, crop rotation with pigeonpea may help improve the soil structure by promoting the efficient use of water and fertilizer by the main crop and may also help break disease cycles (36).

Pigeonpea is a promiscuously nodulated legume that can form effective and ineffective nitrogen fixing nodules with many species of rhizobia (8). Specific strains of slow-growing *Bradyrhizobium* can form effective nodules on pigeonpea (81). Some fast-growing species of *Rhizobium* can also nodulate pigeonpea (3), but the symbiotic associations are often ineffective (i. e. creating nodules with little nitrogenase activity) (58). When no other significant source of nitrogen is available, nitrogen fixation by effective strains of rhizobia could provide in part for the nitrogen requirements of the plants.

Frequently, populations of naturally-occurring rhizobia are ineffective at biological nitrogen fixation (48). Seed inoculation with selected effective strains of rhizobia is intended to promote nitrogen fixation thus reducing the need for fertilizer nitrogen. (91).

Nodulation and nitrogen fixation in pigeonpea have been studied in nodulation trials. Unfortunately, depending on the natural levels of available nitrogen in the soil, competition by the naturally-occurring rhizobia, and varied soil and climatic conditions, different results on nodulation, nitrogen fixation, and crop yield have been reported. In other words, these studies were performed in a variety of conditions that do not allow for

generalizations about the ability of selected inoculants to nodulate and fix nitrogen effectively (7, 11, 81).

The introduction of pigeonpea into the wheat cropping system of the Southern Great Plains region requires the evaluation of potential inoculant strains of rhizobia. It is necessary to identify effective strains of rhizobia that effectively nodulate pigeonpea under widely-varying field conditions. Selected strains need to be evaluated as to their abilities to competitively nodulate pigeonpeas and to persist under local field conditions. To meet these goals, the symbiotic effectiveness of selected strains of rhizobia--*Bradyrhizobium* sp. (*Cajanus*) TAL1127 and TAL1132 were evaluated under field conditions. These field trials with pigeonpea cultivar ICPL-87 were performed in collaboration with Dr. J. Bidlack of the University of Central Oklahoma and Dr. S. Rao of the United States Department of Agriculture Agricultural Research Station (USDA/ARS Grazinglands Research Laboratories), El Reno, Oklahoma. Field experiments were conducted over a four-year period to evaluate the competitiveness and persistence of strains TAL1127 and TAL1132. **R**epetitive **E**xtragenic **P**alindromic – polymerase chain reaction (REP PCR) was chosen to identify the isolates of rhizobia obtained from the respective inoculant plots either as inoculants strains or indigenous rhizobial isolates. Restriction analysis of the 16S/23S ribosomal intergenic region was used to verify the REP PCR results. In addition, the genetic diversity of indigenous strains of rhizobia obtained from pigeonpea from the uninoculated plots was assessed using additional molecular approaches. REP PCR, restriction analysis of the 16S/23S ribosomal intergenic region, and the 16S ribosomal DNA sequences were used to characterize the isolates from the uninoculated plots. Partial sequencing of the 16S ribosomal DNA (rDNA) locus was used to establish the phylogenetic relationships of the unknown rhizobial strains.

Chapter 2

Literature Review

Pigeonpea

Pigeonpea, *Cajanus cajan* Millispecies. L., is an attractive plant species for the winter-wheat based agricultural system found in the U. S. Southern Great Plains region. Pigeonpea can produce seed and plant material for livestock forage and feed. Pigeonpea can also provide cover for the ground and protection from erosion, hence improving soil structure when planted after the spring wheat is harvested. Pigeonpea can be used as a green-manure and replace in part fertilizer nitrogen. Pigeonpea can contribute to the sustainability of these agroecosystems. For these reasons, and those discussed below pigeonpea is a legume of great benefit to the winter-wheat based agronomic practices.

Pigeonpea is useful because of high nutritional value of the grain and its potential for nitrogen fixation (37). It is widely used in Asian, African, Latin American, and Caribbean countries, with India producing over 96% of the world's supply of pigeonpea (79). It grows well in tropical and subtropical environments. This legume has the capacity to produce more seed than other crops on a limited residual moisture supply and is widely adapted to different climates and soils (79). Pigeonpea seeds are nutritionally valuable, containing 18-20% dry weight protein. They are also a good source of nutrients such as calcium, phosphorus, niacin and choline (73).

Pigeonpea is used as a green-manure crop to provide incorporation of nitrogen-rich organic matter into the soil. As it is a shrubby grain legume, it provides food production with fast growth and high biomass productivity (7). In Australia, pigeonpea is sometimes grown for green manure rotated with pineapples or bananas (56). Pigeonpea may be grown as forage in pure stands and grazed or cut for handfeeding. It is grown as a protein supplement for feeding during periods of low pasture quality. Also, some countries have

used the pigeonpea seeds as grain in animal feeds with studies in poultry and pig nutrition giving positive results (56).

Unfortunately, pigeonpea requires large quantities of nitrogen that can be derived from biological nitrogen fixation by rhizobia. It is believed that seed production of pigeonpea can be increased by inoculating its seeds with effective strains of rhizobia (51).

Rhizobia--the microsymbionts

Rhizobia are soil-borne bacteria with the ability to form a symbiotic association with legumes: the legume benefits from the atmospheric nitrogen fixed by the bacterial symbiont; the rhizobia receive shelter and nutrients from the plant (47).

Both the fast-growing rhizobia, *Rhizobium* sp. and the slow-growing rhizobia *Bradyrhizobium* sp. have been found to nodulate pigeonpea (3). In many physiological, phenotypic and serological aspects the slow-growers differ from the highly characterized, fast-growing *Rhizobium* species. These genera vary for example in their flagellation patterns, preferred carbon and nitrogen sources and localization of their *sym* (symbiotic) genes. The symbiotic genes are plasmid encoded in the fast-growing rhizobia (25). Slow growers have been demonstrated not to oxidize disaccharides as do *Rhizobium* spp. (3).

Most rhizobial species are traditionally defined by indicating the species of legumes that the rhizobia can nodulate (95). Most legumes can be nodulated by one or a few species of rhizobia. However, there are some legumes that are referred to as "promiscuous" because they can be nodulated by several species of rhizobia.

Rhizobial Inoculation for Legumes

An important step in the rhizobial inoculation of legumes is selection and testing of strains of rhizobia in the soil environment (20). There are many biological and environmental factors that affect the introduction of a selected strain of rhizobia into a new soil environment, thus the importance of testing different strains. In most cases, it is

preferable to use a single strain of inoculant where climatic conditions are constant and there a is history of indigenous rhizobia present in the soil (66).

A desirable rhizobial inoculant must i) compete for infection and nodulation sites in the presence of indigenous rhizobia, and ii) fix nitrogen with a specific host legume over a range of environmental conditions, including in the presence of soil nitrogen (42). In addition inoculant strains should colonize the soil when the legume is not present (86).

Ecology of Rhizobia

Rhizobia can live as normal components of the soil microbial community when the host legume is absent from the plant community (86), though populations of rhizobia decrease in number when the host plant is absent and the rhizobia must survive saprophytically (42).

One major hurdle in the introduction of new rhizobial strains into soil is the establishment of a new rhizobial population when indigenous, well-adapted rhizobia are already present in the soil. The naturalized bacteria are usually better saprophytic competitors (91).

The origin of the rhizobial populations found in soil could be either indigenous or introduced with subsequent naturalization. If new populations are introduced into soils that contain indigenous rhizobia that form nodules on the introduced host plant, but do not fix nitrogen no benefits from inoculation will occur. The introduced rhizobial strains can be reduced in numbers or eliminated if ineffective rhizobia can outcompete the introduced effective strains of rhizobia (65). While living as saprophytes, the inoculant strains are forced to compete with other microorganisms for limiting resources such as nutrients, moisture. For example, when the soil is low in nutrients, the rhizobial populations face the problem of having to "share" the nutrient sources (9). The supply of nutrients in soil is found in localized spaces of organic matter in the soil (42). Even in low nutrient

concentration in the soil, populations of rhizobia may persist as it has been demonstrated that adding carbon sources to the soil raises the number of rhizobia recovered (25, 101).

Factors Affecting the Persistence of Rhizobia

There are many factors that can affect the persistence of rhizobial populations in soil during their initial proliferation in the soil, colonization, nodulation and saprophytic survival. This may lead to a decline in the population or to its elimination it from the soil altogether.

I. Abiotic Factors

Factors like temperature, soil moisture, and pH can influence rhizobia in many ways. The persistence of inoculant strains can be affected adversely by physical and chemical stresses such as drought, acidity, salinity, alkalinity, and high temperatures (26). Changes in the soil pH affect the populations of rhizobia that may nodulate a specific legume host (33). Also, the infection process can be affected by low pH for some strains of rhizobia (65). Slow growers have been demonstrated to be more resistant to lower pH in soil than *Rhizobium* sp. It has been suggested that acidity may alter the soil environment in favor of survival of some rhizobial species over others. Low pH can affect the nodulation process in such a way that certain strains have a better chance at the infection of the roots than others when that bacterium-plant interaction is benefited by a lower pH (17, 65). The infection step of the nodulation process seems to be the most sensitive phase to environmental stresses, such as acidity and salinity, the latter needing further studies (65). These stresses are very important factors in determining which strain of rhizobia will nodulate the host plant. Nodulation by a second strain seems to be reduced or limited by the earliest infection (20).

Temperature effects have been shown to be dependent upon the amount of inoculum, time of exposure to high temperatures, and to the strains studied (18).

Unfortunately, most work related to the effect of temperature has assessed mainly high temperatures and the results varied depending on soil type. Some studies simply showed that the higher temperatures yielded lower numbers of recovered rhizobia (65). Effects of high temperatures can be correlated to delayed nodule initiation, deformed nodules and changes in nitrogen fixation rates (32). The pigeonpea-*Rhizobium* symbiosis has been described to be adversely affected by high temperature leading to poor development of nodules (80). Dry heat may accelerate death of the rhizobial population in areas of hot, dry summers (65).

Water availability can also be an important factor in the survival of rhizobia in the soil environment. It has been reported that most rhizobia die when desiccated (19, 65). Also deformation of the root hairs can occur during a drought promoting problems in nodulation (18).

Fungicides, herbicides, and plant protectants can also reduce the rhizobial populations in soil or affect the nodulation process (89). Some herbicides do not prevent rhizobial infection of the root hairs, but delay the process of nodulation resulting in reduction of total nitrogen fixed by the symbiont (25).

II. Presence or Absence of Host Plant and Effect of Nitrogen Concentration in the Soil

The populations of rhizobia in soil are influenced by the presence of a plant host. Population sizes of rhizobia may decrease when the host plant is not present, but this has not been demonstrated to be a permanent change. Studies of *Bradyrhizobium japonicum* in French soils showed that the strains are still present up to 13 years later without significant phenotypic changes even when the host plant was not present (16, 60).

The reduction of rhizobial populations may be either because not all plant roots do provide an advantageous environment for the bacteria or because some strains of rhizobia are poor soil saprophytes.

The presence of a host plant is no guarantee for nodulation. When the soil levels of available nitrogen are adequate for the plant there is no advantage for the plant to form nodules with nitrogen fixing bacteria. In such a scenario, there may be no nodules formed by inoculants even though the selected strain is capable of nodulating the host plant under nitrogen-limited test soil conditions (91). High levels of mineral nitrogen in the soil can significantly decrease infection of root hairs, nodule formation and nitrogen fixation (89). By testing pigeonpea rhizobia with adequate mineral nitrogen concentrations in soil, small inoculation responses were obtained in such a way that a minimal amount of nodules were formed if any (67).

III. Biotic factors

There are many biological factors that can affect the survival of rhizobia in the soil community and the chances for the bacteria to persist in the soil. These factors can include antagonistic interactions such as competition, amensalism, predation, parasitism and lysis, and beneficial associations such as commensalism and symbiosis, and neutral interactions (18).

Competition for nodulation is a very interesting phenomenon about which little is understood (52). Introduced *Bradyrhizobium* sp. must compete with the indigenous rhizobia that are already well adapted to the soil environment (106). The competition may be for resources in the soil and/or for the host plant. The limited nutrient availability in some types of soil can allow the adapted strains of bacteria to be the best competitors. If there is a chance for competition on the same nutrient sources, the rhizobia would have to be able to survive saprophytically by quickly using readily available substrates before other microorganisms in order proliferate in the soil (16).This is difficult because indigenous rhizobia can more quickly take advantage of readily available substrates in the vicinity of plant roots, root exudates and debris since they are generally capable of colonizing the rhizosphere of both leguminous and non-leguminous plants (55).

Naturally occurring rhizobia have adapted to variations in the environment and they can usually proliferate faster than introduced rhizobia (70). Quick proliferation helps in the process of competing for a host plant once one is available (31). Seed inoculants can be placed strategically to form nodules as the root develops before the introduced can be outcompeted by the indigenous rhizobia.

Survival of introduced rhizobia in diverse environments during the saprophytic phase can vary widely which renders difficult the maintenance of the inoculant population in the soil (42). Free-living rhizobia must compete with each other for infection sites to improve the chances of their continuity in the soil. After prompt colonization by the inoculant, there is a better chance for adaptation to the competitive soil environment that can lead to persistence (86).

It has also been purported that the competition can be a function of the soil characteristics for many rhizobial strains (26). Selected inoculant strains are usually chosen for their long term survival characteristics mainly based on data from sterile soils (8, 65). These studies help characterize possible inoculants, but are limited in their practical application in the fields. Nonsterile soil studies like this one can provide more information on what happens to the introduced organisms when better adapted rhizobia are present (8).

It has been reported that indigenous strains can outcompete the inoculant strains even though the inoculant strains were proven more effective after infection and nodulation in laboratory experiments (2). Inoculant strains tend to be outcompeted by indigenous rhizobia when the introduced rhizobia do not express good survival characteristics immediately upon introduction (70). Indigenous rhizobia may be highly competitive because they have adapted well to the local environmental conditions, even if those conditions seem unfavorable to other microorganisms (53). Indigenous rhizobia are better adapted to their own soil environment and can dominate nodule occupancy in many legume introduction cases (14). An introduced strain can be outcompeted so severely that its

numbers in the soil cannot be detected. This can happen even when the numbers of introduced rhizobia are very high (83).

Interactions with predatory protozoa and bacteriolytic bacteria such as *Bdellovibrio* has been proven deleterious towards rhizobia (65, 68). Other soil microorganisms like bacteria, fungi, and actinomycetes can inhibit or stimulate rhizobia. Even rhizobia can produce antibiotics that can suppress effective nodulation by chosen strains (76, 77). Slow growers seem less susceptible to the exudates from other microorganisms than fast growers for reasons not clearly understood. Also, rhizobial numbers can decline due to bacteriophages, but this only applies to the rhizobia that succumb to infection by phages (65, 89).

Inoculation studies

I. Stability of inoculant

Variation in effectiveness has been observed in some strains of *Rhizobium* over time (97). There has been little offered as explanation of what could have caused these apparent changes from the time the bacterial strains were stored and cultured in lab to the time they were introduced to plant roots. It may be part of the adaptation process that happens to an introduced rhizobium in the soil when compared to the parental lab strains response (57). It is possible that it is a phenotypic drift caused by the selection process and it may not be of major importance, as long as the rate of nitrogen fixation remains comparable. Changes in the characteristics of introduced rhizobia can be indicators of adaptation to their new environment (15). The inoculant strain variability after this study in an introduced legume such as pigeonpea can be helpful to obtain this type of information; however four years of study may not be enough time to study a population that has reached its equilibrium in the new environment. There is possibly going to be a selection process in the soil environment towards the most fit organisms. This selection process may generate

heterogeneity due to mutations or genotypic variation through the process of recombination (22).

II. Adaptation

Populations of naturally occurring rhizobia and their persistence through time in soil in the absence of host plants can be used as a measure of adaptation. After the initial soil, colonization there may not be a sufficient numbers of introduced rhizobia left in the soil to form nodules once the host plant is replanted after the winter-wheat season. This may be due to poor survival of rhizobia that are not well adapted to the prevailing soil conditions (78). For example, it is desirable to release a large number of rhizobia upon nodule senescence and decay for a better chance for survival into the next season as the introduced rhizobia still has to compete in the soil (17). It is desirable for the inoculant strain to adapt to the new environment so its survival possibilities are greater through the years thus reducing the need for reinoculation (8). Only a few strains can compete for the nodulation process in the soil once the host plant is present because not every legume can be nodulated by every rhizobium present in the soil (1). This may not be the case for introduced pigeonpea as it is a promiscuous legume, but this can be addressed by this study. The rhizobial strains that have persisted and adapted through changes in the soil climate have a better chance at survival and nodulation.

This scenario was observed with the widely studied *Bradyrhizobium japonicum* serocluster 123, a soybean symbiont. *Bradyrhizobium japonicum* serocluster 123 appears to be widely distributed throughout the soybean-growing region of the upper Midwest of the United States. The strain may have been introduced at some point with imported soybeans. When attempts were made to inoculate soybeans with selected strains of serocluster 110 (which can fix nitrogen at a higher level than serocluster 123), the already predominating serocluster 123 was found to outcompete the inoculant strain (29). *Bradyrhizobium japonicum* serocluster 110 was not a good competitor against serocluster 123 and thus did not improve crop yield.

Unfortunately, the process of adaptation can lead to changes in the inoculant strains (39), such as loss of nitrogen fixation effectiveness and/or changes in carbohydrate utilization patterns. This phenomenon has been observed not only in the field but also in laboratory strains stored on solid media (38). A change was observed in *Lotononis* rhizobia in which there was slightly lower nitrogen fixation reported over a period of 12 years from the recovered strains as compared with the parental lab strains tested. However, this lower nitrogen fixation rates did not seem significant as the bacteria recovered were confirmed to be the original inoculant and nitrogen was still being fixed, but at a slightly lower rate (23). The effectiveness of rhizobia in the process of nodulation can also change over time (23). The occurrence of spontaneous mutations has been suggested to explain this phenomenon (5). These mutations may be the result of the process of adaptation to a new environment or to obtain advantage over indigenous strains (32, 75). In long term studies of ten to twelve years it has been shown that equilibrium may take more than four years (the length of this study) to be achieved in the new environment and that the changes in rhizobial populations may be due to adaptational changes through time (23).

Promiscuity of host plant

Promiscuously nodulating legumes such as pigeonpea have been reported to have a larger number of ineffective nodules as there is seemingly no discrimination between effective and ineffective rhizobial strains. For example, the fast growers *R. tropici*, *R. etli*, and other *Rhizobium* sp. have been shown to produce ineffective nodules in pigeonpea (35). It appears that the fastest and fittest rhizobia will take over the nodulation process because the prompt symbionts usually limit the success of other rhizobia that could nodulate the same host (12). The low nitrogen fixation levels obtained from these nodules is of great concern and urges study into the use of a possible competitive effective strain for successful crop yield (13). It would be of interest to study how a novel inoculant strain interacts with indigenous nodulating bacteria as this process has been studied with only a

few rhizobial species of commonly used legumes (90). The outcome would depend on the ability of the introduced rhizobia to persist in soil that may already have indigenous rhizobia capable of colonizing, surviving and nodulating the exotic legume.

Phylogenetics and diversity of rhizobia

Rhizobial characterization was previously based on interaction with a plant host, creating a taxonomic challenge (28). This made the identification and characterization of rhizobia even more difficult in the case of promiscuously nodulated legumes such as pigeonpea.

Conventional methods of characterization

Many methods have been developed to characterize natural rhizobia. Conventional methods such as serology, antibiotic sensitivity patterns and biochemical utilization tests are intended to be used with specific rhizobial strains that can react accordingly (54).

Serology has been one of the most common methods for *Rhizobium* sp. differentiation. This method is limited to the strains reacting to specific antisera (46). Although this technique can be useful, there is no conclusive evidence that indicates each individual serotype represent distinct strains of bacteria (8).

Also available are antibiotic sensitivity pattern studies (6), and biochemical utilization studies which are very specific to certain *Rhizobium* sp. Unfortunately, they are not very effective for bradyrhizobia because there is a limited number of distinctive antibiotic markers, and due to their long incubation time, biochemical tests are difficult to execute and interpret (54). Another very specific method that applies to *Rhizobium* and some *Bradyrhizobium japonicum* strains is phage typing; however, its use is limited to the strains that can be infected with phage (74). Although these methods are useful for a selected group of rhizobia, they are tedious to use in classifying isolates that are possibly heterogeneous.

Molecular techniques of characterization

There has been wide use of molecular biology techniques to characterize strains of rhizobia. Some of them include restriction enzyme analysis of genomic DNA and Southern hybridization probing with nodulation and nitrogen fixation genes (71). The technique of multilocus allozyme electrophoresis also allows the study the genetic relationship among bacterial strains (88).

There are many techniques that can be used for the purpose of studying genetic diversity and relatedness among bacteria. Newer molecular biology techniques permit the classification of genetically related strains in a short time (44). Methods that are strain and/or species specific can yield information about the genetic diversity and genetic relationships among isolates (34). In population studies of *Rhizobium* sp., it has been necessary to use identification methods at the molecular level to provide information at the evolutionary level (24). The use of the polymerase chain reaction (PCR) with selected primers allows for fast genetic fingerprinting of rhizobia. This can be done using strain-, species- or genus- specific primers. For example, the repetitive extragenic palindromic sequences (REP) are often used to genotypically fingerprint bacterial isolates, including rhizobia (21, 59).

The use of repetitive extragenic palindromic (REP) sequences with PCR provides a way of differentiating bacterial strains in many cases (41). The REP sequence is a highly conserved inverted repeat present in one or more adjacent copies throughout the chromosome (41). These REP sequences are widely distributed in Gram negative soil bacteria and have been shown to produce banding patterns in agarose gels that are strain specific (21). A good example of this is the case of *B. japonicum* serocluster 123 in which the strains have been phylogenetically classified and clustered with REP PCR (41). In studies of indigenous soil populations of *Rhizobium leguminosarum bv. trifolii*, Leung et al. (45) found that the REP PCR clustering results were consistent with the results obtained

by multilocus enzymatic electrophoresis (MLEE) analysis. However, REP PCR has been demonstrated to be faster and more sensitive than MLEE analysis (59).

Besides REP PCR, other types of genetic analysis can be used. One of these is restriction fragment length polymorphisms of specific PCR products (RFLP-PCR), such as 16S-23S rRNA PCR. This technique reveals polymorphisms due to the presence of conserved and variable intergenic sequences (IGS) regions (61). It uses a region that has been known to exhibit variations in length and sequence at species level. Some studies show that the IGS can be used to detect genetic differences at intrageneric and intraspecific level (64). These differences depend in the type of tRNA found in the spacer regions (40). Ideally, this method helps in grouping population samples based on analysis of PCR products digested with several restriction enzymes to provide band patterns in gel electrophoresis that can be grouped based on similar banding patterns. Nour et al. (62) used such RFLP analysis of the IGS between the 16S and 23S rDNAs to differentiate chickpea rhizobia.

Sixteen-S PCR is a new trend in genotypic studies. The 16S rRNA molecule is considered important in establishing phylogenetic relationships among bacteria (99). The sequencing of genes for the 16S or small subunit of ribosomal RNA (SSU of rRNA) has become an essential part of the description of a new bacterial species. Molecular sequences have provided reliable data to reconstruct phylogenies and to redefine bacterial species (103).

Many researchers assert that the 16S gene is long and variable enough to permit reliable conclusions, especially for comparison of close relatives (105). Variable regions cannot be used to phylogenetically cluster species for all organisms (102). Observations based on 16S analysis vary depending in the organisms studied (87). Some researchers argue that 16S rRNA sequencing is not sufficient to recognize recently divergent species (30). Some argue that when 1-2-bp differences are observed in the sequences there is close genetic relatedness between species (50). Partial sequence determination of 16S rDNA is a

helpful approach for the identification of rhizobia (63), but only complete sequencing allows a totally reliable comparison (87).

The comparisons of sequences of 16S rRNA genes has permitted differentiation between *Rhizobium* sp., and has started a new taxonomic system (43). Based on 16S sequencing, *Rhizobium* sp. (fast-growing rhizobia) is in a group closely related group to *Agrobacterium* sp. However, *Bradyrhizobium* sp. (slow-growing rhizobia) and other slow growers are more closely related to *Rhodopseudomonas palustris* than to *Rhizobium* (28). This cluster of slow growers also includes a known pathogen, *Afipia felis,* a causative agent of cat-scratch disease, adding to the phenotypic variety of this group (104). It has also been suggested that a photosynthetic stem-nodulating bacterium (strain BTAi) is a very close relative of *B. japonicum* and *Rhodopseudomonas.* This group includes other slow-growing rhizobia that are within the alpha-2 subdivision of proteobacteria (100). This cluster is closer to the rhizobial genus *Azorhizobium* than to a *Rhizobium.* (104). Overall, these data indicate that slow-growing rhizobia forms parts of heterogeneous cluster distant from the *Rhizobium* sp. cluster. Interestingly, this cluster is heterogeneous, but includes organisms that have some metabolic traits like autotrophy. Rhizobia survives saprophytically without its legume and while in this state, it is autotrophic.

By doing amplified ribosomal DNA restriction analysis on 1.5 to 1.6 Kb of 16S rDNA PCR products (ARDRA), different patterns are detected. The information obtained with ARDRA is a reliable tool to identify bacterial species (93), but some other study must be correlated to it (such as partial 16S rDNA sequencing or DNA-DNA hybridizations). Using a second molecular typing technique is commonly urged in an attempt to confirm other typing data as accurate (27, 44).

Using phenotypic data alone we cannot construct adequate evolutionary trees due to the instability of characteristics that are variable in culture. The bradyrhizobia cluster formed based on 16S rRNA data is very close (85), not allowing for distinction of genus.

This genetic method still provides information on the relation among a diverse group of samples.

Few studies have been published in which an exotic introduced legume and its symbiotic partner are followed over time. Such a study could provide needed information about introduced exotic bacteria and their introduced exotic legume to a new environment that has an existing rhizobial population. By using REP PCR, 16S, 23S IGS PCR, nodulation trials, examining 16S RFLPs and sequences I have characterized this group of rhizobia and established phylogenetic relationships for sample groups from four years.

Chapter 3

Materials and methods

Bacterial Strains, Pigeonpea variety and growth conditions

The bacterial strains used as inoculants on pigeonpea seeds were *Bradyrhizobium* sp. (Cajanus) TAL1127 and *Bradyrhizobium* sp. (Cajanus) TAL1132 provided by NifTAL (Nitrogen fixation in Tropical Agricultural Legumes, Hawaii). The other strains used were isolated from root nodules on *Cajanus cajan Millispecies* L cv. ICP87 plants growing in areas with no prior history of inoculation with rhizobia. The pigeonpea seeds used were ICP87 provided by Dr. Rao at USDA/ARS Grazinglands Research Laboratories in El Reno, Oklahoma. Strains of rhizobia were grown and maintained on AG media (L^{-1}): 1 gr arabinose, 1 gr sodium gluconate, [HM (13g Hepes, 11 g MES) buffer to pH 6.6, .0067g $FeCl_3$, .0018 g $MgSO_4$, .013 g $CaCl_2$, .24 g, Na_2SO_4, .32g NaCl, .125g Na_2HPO_4, to pH 6.6] (71) at 28°C.

Sampling Procedures

This study was conducted in experimental plots of the USDA/ARS Grazinglands Research Laboratories in El Reno, Oklahoma. Four treatments were applied to the plot soil in triplicate in a randomized complete block design. The treatments consisted of i) pigeonpea seeds inoculated with *Bradyrhizobium sp.* (Cajanus) TAL1127, ii) pigeonpea seeds with *Bradyrhizobium* sp. (Cajanus) TAL1132, iii) uninoculated pigeonpea seeds, and iv) fallow soil (see Figure 1). Seeds were individually inoculated with the selected inoculant strains in a preparation of peat-based inoculant preparation provided by NifTAL. Inoculated seeds were sown in late May, 1993. Uninoculated seeds were sown in May 1993, 1994, 1995 and 1996.

Nodules were recovered from the roots of 12 to 20 randomly chosen plants for each treatment for each of four years from 1993-1996. Samples were bagged and kept on ice

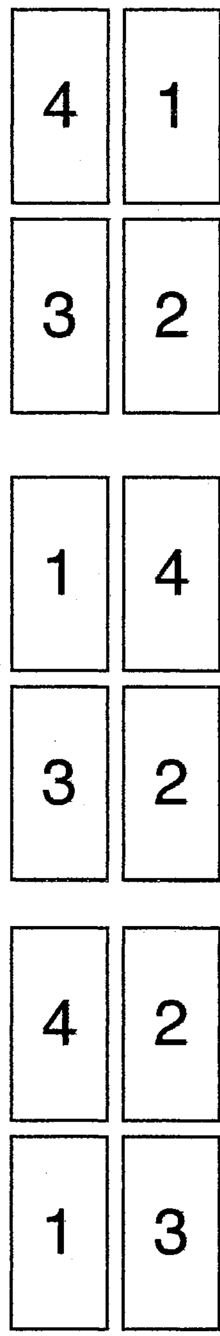

Figure 1. Experimental plots randomized block design.

1. TAL1127; 2. TAL1132; 3. Uninoculated plots; 4. Fallow soil.

until brought to the laboratory. The nodules were surface sterilized by immersion in 95% ethanol for 30 seconds, 25% bleach for 5 minutes and six consecutive washes with sterile distilled water. The surface-sterilized nodules were crushed in sterile microtiter plates and the nodule contents streaked on AG plates. Isolated colonies of bacteria which resemble rhizobia were further restreaked onto AG plates to obtain a pure culture. Each isolate was maintained in AG agar slants kept at 4°C as working stocks and 15% glycerol stocks were prepared and kept at –80 °C for long-term storage.

Chemicals, Enzymes and Media Additives

Bacteriological agar and media additives were obtained from Difco Laboratories, (Detroit, MI). Chemical additives for the media and buffers were obtained from Sigma Chemical Corporation, (St. Louis, MO), and Fluka Chemical Corporation, (Ronkonkoma, NY). *Taq* DNA polymerase in buffer B was obtained from Promega Corporation, (Madison, WI). Restriction enzymes were purchased from Gibco BRL Laboratories and Boerhinger Mannheim, (Indianapolis, IN) and were used as described by the manufacturer. SeaKem Agarose and Metaphor agarose were obtained from FMC Bioproducts, (Rockland, ME).

DNA Extraction

Genomic DNA extraction was performed by a modified standard protocol (4). Each isolate was grown on 5 ml of AG broth shaking for 5-7 days at 28°C. A total of 3 ml of culture was harvested by centrifugation in a microcentrifuge tube at 14,000 rpm. The cell pellet was washed in TE (10 mM Tris HCl,(pH 8.0), 1 mM EDTA) with 1% Sarkosyl and 1M sodium chloride then again in TE to remove extracellular polysaccharide material. The cell suspension was centrifuged to recover the washed cells. The cell pellet was resuspended in 100 μl of 25% (w/v) sucrose in TE, then 20 μl of lysozyme (5 mg/ml in 0.25 M Tris pH 8.0) and 40 μl of 250 mM EDTA were added. Cell suspensions were

incubated at 37 °C for 10 minutes. Two hundred µl of water were added to the suspensions, and thoroughly vortexed. Ten µl of 20% (w/v) SDS and 10 µl of RNase (10 mg RNase/ml) were added and incubated at 37 °C for 15 minutes. Five microliters of pronase (10 mg /ml) were added and the suspension was incubated at 37 °C for 30 minutes. Four hundred µl TE were added followed by mixing by gently inverting the tubes several times. Several phenol:chlorophorm:isoamyl alcohol extractions with long centrifugation times (15 minutes) were performed to optimize DNA purification. A final chloroform:isoamyl alcohol extraction was performed. One-tenth volume of 3M sodium acetate (pH 5.5) and 1 volume of isopropanol were mixed in to precipitate the DNA, followed by centrifugation for 30 minutes at 14,000 rpm. The DNA pellet was washed with 70% and 95% ethanol and dried in a laminar flow hood for 10-15 minutes The DNA pellet was dissolved in 50-100 µl of TE and stored at 4 °C.

Oligonucleotide Primers

The oligonucleotide primers used in this study are described in Table 1.The REP PCR primers, REP1R-1 and REP2-I were synthesized by Genosys Biotechnologies, Inc. (The Woodlands, TX). The primers used for other types of PCR were synthesized by the Oklahoma State University Department of Biochemistry Core Facility (OSU DBCF) (Stillwater, OK).

Primers FGPS 6-63 and FGPL 132′-38 (62) were used to amplify the 16S, 23S IGS region of the rRNA gene. The FGPS 6-63 is derived from a conserved sequences in the 3' part of the 16S rDNA gene and the reverse primer, FGPL 132′-38 corresponds to conserved sequence near the 5' end of the 23S rDNA gene. These primers were synthesized at the Oklahoma State University Department of Biochemistry Core Facility.

To amplify the 16S rDNA gene, primers fD1 and rD1 were used (98). These primers were synthesized without the cloning sequences at the Oklahoma State University Department of Biochemistry Core Facility.

REP primers	REP 1R-1 [5'-IIIICGICGICATCIGGC-3']
	REP 2-1 [5'-ICGICTTATGIGGCCTAC-3']
16S, 23S IGS primers	FGPS 6-63 [5'-GGAGAGTTAGATCTTGGCTCAG-3']
	FGPL 132'-38 [5'-CCGGGTTTCCCCATTCGG-3']
16S primers	fD1 [5'-AGAGTTTGATCCTGGCTCAG-3']
	rD1 [5'-AAGGAGGTGATCCAGCC-3']
16S partial sequencing primers	Y1 [5'-TGGCTCAGAACGAACGCTGGCGGC-3']
	Y2 [5'-CCCACTGCTGCCTCCCGTAGGAGT-3']

Table 1. Primers used in the project

Primers Y1 and Y2 were used to amplify and sequence portions of the 16S rDNA genes (105). These primers amplify the sequences corresponding to nucleotides 20-43 to 61-338 in the *E. coli* 16S rRNA sequence. These primers were synthesized at the Oklahoma State University Department of Biochemistry Core Facility.

REP (Repetitive Extragenic Palindromic) PCR Fingerprinting

Each isolate was characterized by PCR fingerprinting with REP primers, REP1R-1 and REP2-I. The PCR reactions were carried out as described (94). Each reaction included 1X Gistchier buffer (16.6 mM $(NH_4)_2 SO_4$; 67 mM Tris HCl, pH 8.8; 6.7 mM $MgCl_2$; 6.7 μM EDTA; 30 mM β-mercaptoethanol; 4 μg bovine serum albumin (Boerhinger Mannheim, Indianapolis, IN), 10% dimethylsulfoxide (v/v) (Fluka Chemical Corp., Ronkonkoma, NY), 50 picomoles each of the REP primers, 1.25 mM of each of four deoxynucleotides (Boerhinger Mannheim, Indianapolis, IN), 2 U of *Taq* DNA polymerase in buffer B (Promega Corporation) and approximately 50 ng of DNA. Each PCR reaction mix was covered with 25 μl of mineral oil (Sigma Chemical Corp, St. Louis, MO). Reaction mixes without DNA were used as negative controls. A Perkin Elmer Thermal Cycler (Perkin Elmer Cetus) was used to carry out the amplification reactions as follows in a total volume of 25 μl: 1 cycle at 94 °C for 1 minutes, 30 cycles at 94 °C for 1 minutes, 40 °C for 1 minutes, and 65 °C for 8 minutes followed by a cycle at 65 °C for 16 minutes. The reactions were held at 4 °C until analyzed. After the amplification cycles, 8-10 μl of the REP PCR products were separated on 1.5% (w/v) agarose gels (12 x 14 cm) in TBE (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA) for 4.5 h at 75 V. The gels were stained with a ethidium bromide solution (0.5 μg/ml) and photographed under UV illumination by using Polaroid type 665 film and Polaroid type 55 film for negatives. Identical and similar fingerprints were compared by electrophoresis. To verify reproducibility of results, PCR was repeated for each sample at least six times.

16S and 16S, 23S IGS PCR Amplification and Restriction Fragment Length Polymorphisms Analysis

The oligonucleotides used were FGPS 6-63 and FGPL 132-38' and the amplification was carried out according to Nour et al. (62). The reaction mixture of a total of 50 µl contained gelatin buffer (10 µM Tris HCl (pH 8.2); 1.5 mM $MgCl_2$; 50 mM KCl; 0.01% (w/v) gelatin), 20 µM each of dATP, dTTP, dGTP, and dCTP, 0.1 µM of each primer, 2.5 U of *Taq* DNA polymerase in buffer B and DNA. Twenty-five µl of mineral oil were overlayered on top of each reaction tube. Reaction mixes without DNA were used as negative controls. Amplifications were carried out in a Perkin Elmer Thermal Cycler as follows: 1 cycle of 3 minutes at 95 °C, 35 cycles of 1 minute at 95 °C, 1 minutes at 55 °C, and 2 minutes at 72 °C followed by a cycle of 3 minutes at 72 °C. The samples were kept at 4 °C until further analyzed. The PCR products (5 µl) were run on 1% agarose gels (8 x 6 cm) in TBE at 75 V for 2 h. The gels were stained with ethidium bromide (0.5 µg/ml) and photographed under UV illumination by using Polaroid type 665 film.

Restriction enzyme analyses of PCR products were performed in a total volume of 20 µl that contained 10 µl of PCR products, with 5 U of one of the following restriction endonucleases: *Rsa* I, *Hpa* II, and *Hae* III. The reaction conditions were those specified by the manufacturers. The restriction fragments (8-10 µl) were separated by agarose gel electrophoresis in a 2.5% (w/v) Metaphor agarose gels (12 x 14 cm) in TBE buffer for 4.5 h at 75 V. The gels were stained with ethidium bromide (0.5 µg/ml) and photographed under UV illumination by using Polaroid type 665 film.

16S rDNA Amplification and Restriction Analysis

The 16S rRNA genes of the isolates obtained from the uninoculated plots were amplified and analyzed. The PCR reactions were carried out as described by Weisburg et al. (98) with minor modifications. The primers used were fD1 and rD1. Each reaction included in a total volume of 100 µl: gelatin buffer (10 µM Tris HCl (pH 8.2); 1.5 mM

$MgCl_2$; 50 mM KCl; 0.01% (w/v) gelatin), 20 μM each of the dNTPs, 0.1 μM of each primer, 2.5 U of *Taq* DNA polymerase in buffer B and 50 ng of template DNA (in 1-2 μl). Twenty-five μl of mineral oil were overlayered on top of each reaction. Reaction mixes without DNA were used as negative controls. Amplifications were carried out in a Perkin Elmer Thermal Cycler as follows: 1 cycle of 3 minutes at 95 °C, 35 cycles of 2 minutes at 95 °C, 30 seconds at 42 °C, and 4 minutes at 72 °C followed by 1 cycle of 20 minutes at 72 °C. The samples were kept at 4 °C until further analysis. The PCR products (5 μl) were run on 1% agarose gels (8 x 6 cm) at 75 V for 2 h. Gels were stained with ethidium bromide (0.5 μg/ml) and photographed under UV illumination by using Polaroid type 655 film.

Restriction enzyme analyses of PCR products were done in a total volume of 20 μl that contained 10 μl of PCR products with 5 U of one of the following restriction endonucleases: *Rsa* I, *Hpa* II, and *Hae* III. The reaction conditions were those specified by the manufacturers. The restricted fragments were separated by agarose gel electrophoresis in 2.5% (w/v) Metaphor agarose (12 x 14 cm) for 4.5 h at 75 V. The gels were stained with ethidium bromide (5 μg/ml) and photographed under illumination by using Polaroid type 665 film.

16S rDNA Amplification and Sequencing

The sequences of segments of approximately 264-bp of the 16S rRNA genes of isolates obtained from the uninoculated plot were analyzed after being amplified. The PCR reactions were carried out as described by Young et al. (105) with minor modifications. The primers used were Y1 and Y2. Each reaction included in a total volume of 100 μl: gelatin buffer (10 μM Tris HCl (pH 8.2); 1.5 mM $MgCl_2$; 50 mM KCl; 0.01% (w/v) gelatin), 200 μM each of dATP, dCTP, dGTP, and dTTP, 500 nM of each primer, 1 U of *Taq* DNA polymerase in buffer B, and approximately 20 ng of DNA(in 1 μl). 25 μl of mineral oil were overlayered on top of each reaction. Reaction mixes without DNA were

used as negative controls. Amplifications were carried out in a Perkin Elmer Thermal Cycler as follows: 30 cycles of 1 minutes at 94 °C, 30 seconds at 65 °C, 2.5 minutes at 72 °C. The samples were kept at 4 °C until further analyzed. The PCR products (5 µl) were run on 1% agarose gels (8 x 6 cm) in TBE at 75 V for 2 h. Gels were stained with ethidium bromide (0.5 µg/ml) and photographed under UV illumination by using Polaroid type 665 film. The entire volume of PCR products were purified by using the QIAquick PCR purification kit from QIAgen corp. (Valencia, CA) as specified in user's handbook of July 1997. Purified PCR products (2-3 µl) were electrophoresed on 1% agarose gels (8 x 6 cm) in TBE buffer at 75 V for 2 h. Gels were stained with ethidium bromide (5 µg/ml) and photographed under UV illumination by using Polaroid type 665 film. Purified products were sequenced at the OSU DBCF using a model 370A DNA sequencer (Applied Biosystems, Inc., Foster City, CA) and an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Applied Biosystems, Inc., Foster City, CA).

Data Analysis

The REP PCR fingerprints were compared to each other. Similarities among fingerprints were based on the presence or absence of bands and a similarity matrix was computed based on Dice's coefficient, $2n / n_x + n_y$, where n is the number of bands common to two isolates and n_x and n_y are the total number of bands in isolate x and isolate y (84). Dendograms were generated using the unweighted pair group method with arithmetic average algorithm in SAHN (Sequential, Agglomerative, Hierarchical, and Nested) of NTSYS-pc (ver 1.8; Exeter Software, Setauket, NY). The same analysis was used for the 16S, 23S IGS PCR RFLPs and 16S rRNA gene RFLPs.

Partial 16S rRNA gene sequence data were aligned by using the BCM Search Launcher : Multiple sequential alignments using the Clustal W 1.7 alignment method (92) programs and were compared with published sequence data at the Ribosomal Database

Project –Michigan State University Center for Microbial Ecology with the similarity rank program and the option of searching the entire small subunit prokaryotic database (49). The Clustal X windows interface program was used to create phylograms by the Neighbor Joining method (72). Additional 16S rRNA sequence data was accessed from the Gene Bank database.

Nodulation Trials

Pigeonpea seeds were inoculated with putative rhizobia isolated from the uninoculated plots to confirm their ability to nodulate aseptically grown pigeonpea. Seeds were surface sterilized as follows: 95% ethanol for 30 seconds, bleach for 5 minutes, and 5 sterile distilled water rinse washes of 3 minutes each. The sterile seeds were held overnight at 4 °C on 1% water agar plates in resealable zipper seal storage bags. The plates with seeds were kept in the dark for 1-3 days to allow seeds to germinate. The germinated pigeonpea seeds were transferred aseptically to Pyrex culture flat bottom, rimless tubes (25 mm x 150 mm) with between 20-25 ml of Jensen's seedling agar (0.6 g $CaCl_2$, 0.2 g $MgSO_4$, 0.1 g NaCl, 0.02 g ferric citrate, 0.34 g K_2HPO_4, KH_2PO_4, 1 ml trace elements mixture (1.43 g H_3BO_3 , 1.02 g $MnSO_4$, 0.22 g $ZnSO_4$, 0.08 g $CuSO_4$, 0.1 g $CoCl_2$, 0.05 g $Na_2 MoO_4$ per 1 L distilled water), 15 g agar in 1 L distilled water to pH 6.8. The seedlings were inoculated with 1 ml of a cell suspension in 0.85% saline. Aluminum foil was used to cover the bottom of the tubes. The plant tubes were kept under a plant light with 16 hours of light, and watered with sterile distilled water as needed. Plant roots were monitored for the presence of nodules.

Chapter 4

Competitiveness and Persistence of introduced *Bradyrhizobium* sp. (Cajanus) TAL1127 and TAL1132 on pigeonpea

Inoculant strains of rhizobia for a new species of legume should be able to compete for nodulation and persist in the soil for several years. The inoculant strain should be able to colonize, compete and survive saprophytically even when other rhizobia are present in the soil. Thus, it is important to determine potential inoculant strains for pigeonpea that can compete with indigenous rhizobia for nodulation in the inoculation year and persist in the soil to nodulate and fix nitrogen with the host legume in subsequent years. In this study, I performed a field trial with pigeonpea seeds inoculated with *Bradyrhizobium* sp. (Cajanus) TAL1127 and TAL1132 in the initial year to determine the competitiveness of the potential inoculant strains. Uninoculated seeds of pigeonpeas were sown in subsequent years to determine the persistence and ability of the inoculant strains to nodulate pigeonpeas.

To determine the competitiveness of the inoculant strains, it is necessary to be able to unequivocally identify the strains. Many different techniques including serology, antibiotic resistance, and whole-cell protein patterns have been used in the past to identify rhizobial strains (46). Newer molecular techniques, including restriction fragment length polymorphism analysis and polymerase chain reaction, have been used (41, 96). Amplification with Repetitive Extragenic Palindromic sequence primers (94) can provide a quick genetic fingerprinting approach that is sensitive at the strain level. This technique can be used to follow the fate of introduced rhizobia in experimental plots. In addition the 16S,23S IGS PCR analysis can be used to confirm REP-PCR results.

Initially, the REP-PCR profiles of *Bradyrhizobium* sp. (Cajanus) TAL1127 and TAL1132 were compared to each other. The REP-PCR profiles of TAL1127 and TAL1132 are distinctly different (Figure 2). The REP-PCR profiles of each strain contained circa

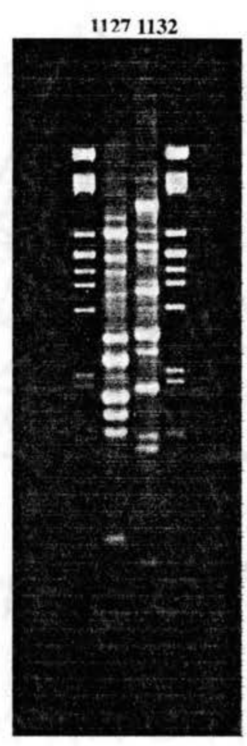

Figure 2. Inoculant strains REP-PCR fingerprints. Leftmost and rightmost lanes contain *Pst* I-digested phage lambda DNA.

seven robust bands that were reproducible. The differences in the REP-PCR patterns make the identification of the inoculant strains unequivocal.

To determine if either TAL1127 or TAL1132 or both are competitive nodulators of pigeonpeas, seeds of pigeonpea were inoculated individually with a peat-based inoculant of either strain. The inoculated seeds were then sown into replicated plots. Mature plants were removed and nodules collected from the root systems of the plants. Isolations of rhizobia were made from the nodules and the isolates identified initially using REP-PCR. In the inoculation year, 100% of the isolates from nodules collected from plants grown in plots sown with TAL1127-inoculated seeds were indistinguishable from TAL1127 based on REP-PCR (see Table 2). Fifty, 66 and 75% of the isolates collected from nodules collected from plants growing in replicates 1, 2, and 3, respectively, sown with seed inoculated with TAL1132 were indistinguishable from TAL1132 based on REP-PCR. The balance of the nodules collected from the TAL1132-inoculated replicates contained isolates that were indistinguishable from TAL1127. No REP PCR patterns from the uninoculated plots had TAL1127 or TAL1132 REP patterns. The cross-contamination of the plots sown with seeds inoculated with TAL1132 with seeds inoculated with TAL1127 may be due to one or more of the following: (i) incomplete sterilization of the seed drill between first sowing seeds inoculated with TAL1127 and then seeds inoculated with TAL1132, (ii) incomplete removal of TAL1127 seeds from the seed drill before sowing seed inoculated with TAL1132 or (iii) seeds inoculated with TAL1127 were dragged to replicates to be sown with TAL1132-inoculated seeds during planting.

To determine if either or both inoculant strains TAL1127 and TAL1132 were persistent in the soil, uninoculated seeds of pigeonpeas were sown into the plots in 1994, 1995 and 1996. Table 2 summarizes the percentage of isolates obtained from the plots sown with inoculated seeds for 1993 and with uninoculated seeds of pigeonpea after the 1993 inoculation in 1994, 1995 and 1996. Isolates of rhizobia were obtained from nodules on plants growing in the sown replicates and identified using REP-PCR. Isolates

1993[a]	Repetition #1	Repetition #2	Repetition #3
Inoculant Strain	%age of isolates recovered identical to inoculant strains		
1127	100	100	100
1132	50	66	75
1994[b]	Repetition #1	Repetition #2	Repetition #3
Inoculant Strain	%age of isolates recovered identical to inoculant strains		
1127	100	***	71
1132	0	40	***
1995[c]	Repetition #1	Repetition #2	Repetition #3
Inoculant Strain	%age of isolates recovered identical to inoculant strains		
1127	53	12	10
1132	0	0	0
1996[d]	Repetition #1	Repetition #2	Repetition #3
Inoculant Strain	%age of isolates recovered identical to inoculant strains		
1127	68	25	95
1132	44	44	0

TABLE 2. Recovery of inoculant strains summary

[a]the remainder %age for 1993 was identical to TAL1127
[b]the remainder %age for 1994 was not similar to the inoculant strains
[c]the remainder %age for 1995 was not similar to the inoculant strains
[d]the remainder %age for 1996 was not similar to the inoculant strains

indistinguishable from TAL1127 were obtained from nodules of uninoculated seed sown into each of the 3 replicates in 1994, 1995, and 1996. Isolates indistinguishable from TAL1132 were obtained from plants sown in a single replicate in 1994, 0 of 3 replicates in 1995 and two of three replicates in 1996.

Pigeonpea have not been sown in this area of the southern Great Plains region (Dr. Srinivas Rao, personal communication). Therefore, an indigenous population of rhizobia capable of nodulating pigeonpea may had not been characterized in the soil. There is evidence that a diverse population of rhizobia capable of nodulating pigeonpea under the prevailing conditions is present in the soil as REP PCR patterns other than the TAL1127 and TAL1132 patterns were found in the uninoculated plots (see Chapter 5). Inoculant strains TAL1127 and TAL1132 were competitive and capable of outcompeting the indigenous strains of rhizobia in the inoculation year. Strains indistinguishable from either TAL1127 or TAL1132 were isolated from each of the nodules. Furthermore, isolates indistinguishable from TAL1127 and TAL1132 were isolated from uninoculated seeds sown into the previously inoculated plots in subsequent years. Seemingly TAL1127 and TAL1132 persisted in the soil for three years following the inoculation year and could still be present in the soil. Future studies should be conducted to determine if soil populations TAL1127 and TAL1132 are still symbiotically competent, i.e., capable of effectively nodulating pigeonpea. Potentially ineffective at nitrogen fixation populations of either strain could present a problem in the future if new more effective strains were to be used as inoculant strains. Adapted, ineffective soil populations of inoculant strains could be strong competitors and outcompete new, effective inoculant strains, but we don't know for sure. This could result in a situation similar to that of the soybean-growing region of upper Midwestern United States. The indigenous *Bradyrhizobium japonicum* serogroup 123 is a strong competitor for nodulation agronomically important cultivars of soybeans used in this region (71, 74). Isolates of serogroup 123 are generally ineffective at nitrogen fixation on these same cultivars soybeans (32, 106). Because of the strong competitive abilities of

serogroup 123, inoculation with effective strains of *B. japonicum* failed to result in increased yields of soybeans.

Based on this study, *Bradyrhizobium* sp. TAL1127 could be recommended as the best nodulator if it seems necessary to choose between the two inoculants tested. This strain could be used as an inoculant in very similar soils and locations. There is always a chance that this inoculant strain will not work as well in different soil because the indigenous rhizobial populations could be different from the one described for this study. For this reason it is imperative to perform inoculation studies with inoculants that have been tested only under laboratory conditions.

Chapter 5

Genotypic diversity of naturally occurring rhizobia in the introduced legume, *Cajanus cajan* Millisp., pigeonpea

Data for the uninoculated plots in 1994 was not available because unfortunately, rabbits and deer ate the pigeonpea plants in the experimental plots.

Previous studies on genetic diversity of indigenous rhizobia for other legumes have characterized heterogeneous populations. The outcome was depending on the geographical areas of study and the soil characteristics. In studies of soybean bradyrhizobia in North Carolina, separate experimental plot locations differed significantly in diversity (69). These types of studies allowed understanding of the population structure of the rhizobia nodulating the legume of interest.

Pigeonpea is a good legume to introduce and try in the United States for its many possible uses. This legume is a promiscuous plant that has been previously described to be nodulated by slow- and fast-growers (3, 51). Its introduction into Oklahoma soils allows for nodulation by indigenous rhizobia if there are any that could nodulate pigeonpea utilizing pigeonpea as a host trap. The selected inoculant strains would have to compete with any possible indigenous "pigeonpea" rhizobia, hence the importance of studying the indigenous rhizobial population. There is also a chance of finding new pigeonpea rhizobia with desirable characteristics such as efficient nitrogen fixation and competition for further studies.

The genetic diversity information is valuable for pigeonpea because it is a promiscuous legume. This information can help determine the types of rhizobia that nodulate it by providing more data concerning its host range. This study brings information about pigeonpea ecology and identify possible rhizobial competitors for the legume for the inoculant rhizobial strains (10). In previous pigeonpea studies, indigenous rhizobia have

nodulated the host plant in low numbers with variations in the effectiveness in the nitrogen fixation process (26).

The plants in the uninoculated plots were sampled and rhizobia were obtained from the nodules collected. These rhizobia were analyzed to study genotypic diversity. The use of REP PCR and 16S, 23S PCR allow for distinctions at species level . This analysis allows for subgrouping of strains to a higher degree when compared with 16S rDNA sequencing and 16S ARDRA. REP PCR allows for comparisons at species level of strain depending on the organisms. (21, 41).

The study of the intergenic spacer region (IGS) of 16S, 23S rDNA allows clustering of the samples and helps group strains identified on the basis of REP PCR. Studies have shown that the 16S, 23S rDNA IGS regions are conserved among a wide range of bacterial species, the technique can be used to cluster related strains; but as there can be insertion sequences in some strains IGS results have to be carefully analyzed (44).

REP PCR analysis

The goal for the sample set number for each year was 36 isolates, or 12 isolates/repetition. The genomic DNA isolated from the samples was used as a template for PCR with REP primers. The inoculant strains TAL1127 and TAL1132 were included in the analysis to establish comparisons with indigenous rhizobia. The letters representing the REP-types across the years are not equivalent, e. g. REP-type A in the 1993 sample set is not necessarily the same as REP-type A in the 1995 sample set because it proved difficult to compare diversity among the years of sampling.

I.1993 isolates

The isolates collected in1993 were a homogeneous set of slow-growers with REP types that were similar to each other (Fig. 3a, 3b). There were 25 different REP-patterns including the inoculant strains REP types. Isolates with similar REP-patterns based on a core set of shared bands were grouped together and labeled with the same letter. There were two main groups: A and B. REP group A contained nine REP-types that shared most

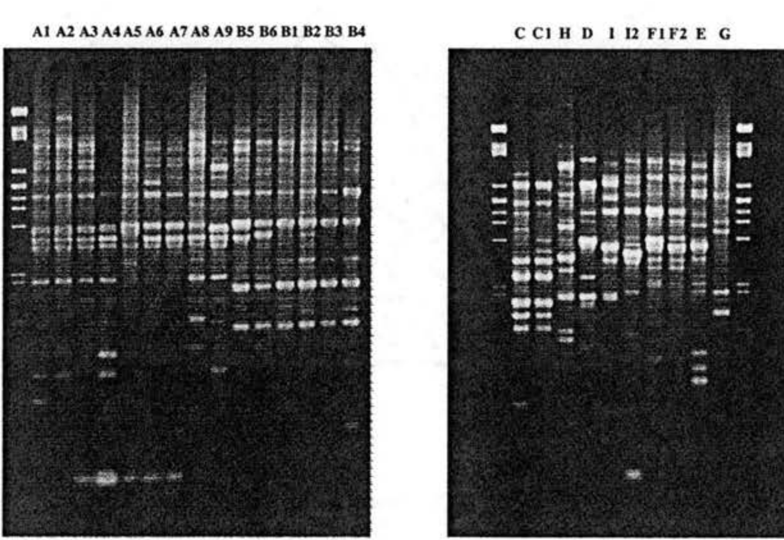

Figure 3a and 3b. REP-PCR fingerprints of uninoculated plot isolates collected in 1993. Letters indicate REP pattern types. Leftmost lane contains *Pst* I-digested phage lambda DNA.

of their bands, but with an average similarity coefficient of .50. Group B contained six REP types with very similar patterns clustered closely at a coefficient of 0.7. There were also REP groups C-I with distinctive REP patterns (see Figures 3a and 3b).

The dendogram presented in Figure 4 summarizes the estimated average similarities, derived from UPGMA. It shows two main branches that separate group A from group B. Types E and G are the furthest away from groups A and B.

II.1995 sample set

The isolates collected in 1995 had complex REP patterns with multiple product bands. This group was very heterogeneous due to the complexity of the fingerprints obtained as shown in Figures 5a and 5b.The heterogeneity may be due to the presence of both fast- and slow-growing rhizobia in the sample set. There were 35 REP-patterns labeled A-AB including the inoculant strains types. Clusters of samples in REP groups were not easily formed. There were only 3 small main groups: A, S and Y. Group A contained pattern types A_0-A_3 sharing many bands among each other with a coefficient of 0.7. Group S includes S_1-S_4 with many shared bands with a coefficient of 0.82. Group Y had patterns Y_1-Y_3 being very similar among each other with a coefficient of 0.86 for Y_1 and Y_2. These groups included very distinct patterns with estimated average similarities from UPGMA summarized in the dendogram presented in Figure 6. There are many branches in the tree because of the differences among the strains REP patterns.

III.1996 sample set

The isolates collected in 1996 did not include as many different REP-patterns as the 1995 sample set. There were 25 REP-patterns including the inoculant strains types (see Fig. 7a and 7b). Some of the REP patterns are very similar to REP patterns in the 1993 sample set possibly because this 1996 sample group includes mainly slow-growers. There were two main groups A and C. Group A (similar to TAL1127 with a coefficient of .94 from A1) included A_1-A_3 with many repeats each. Group C contained REP types C-C_2 with a coefficient of 0.65 from C to C1 and C2. The dendogram presented in Figure 8

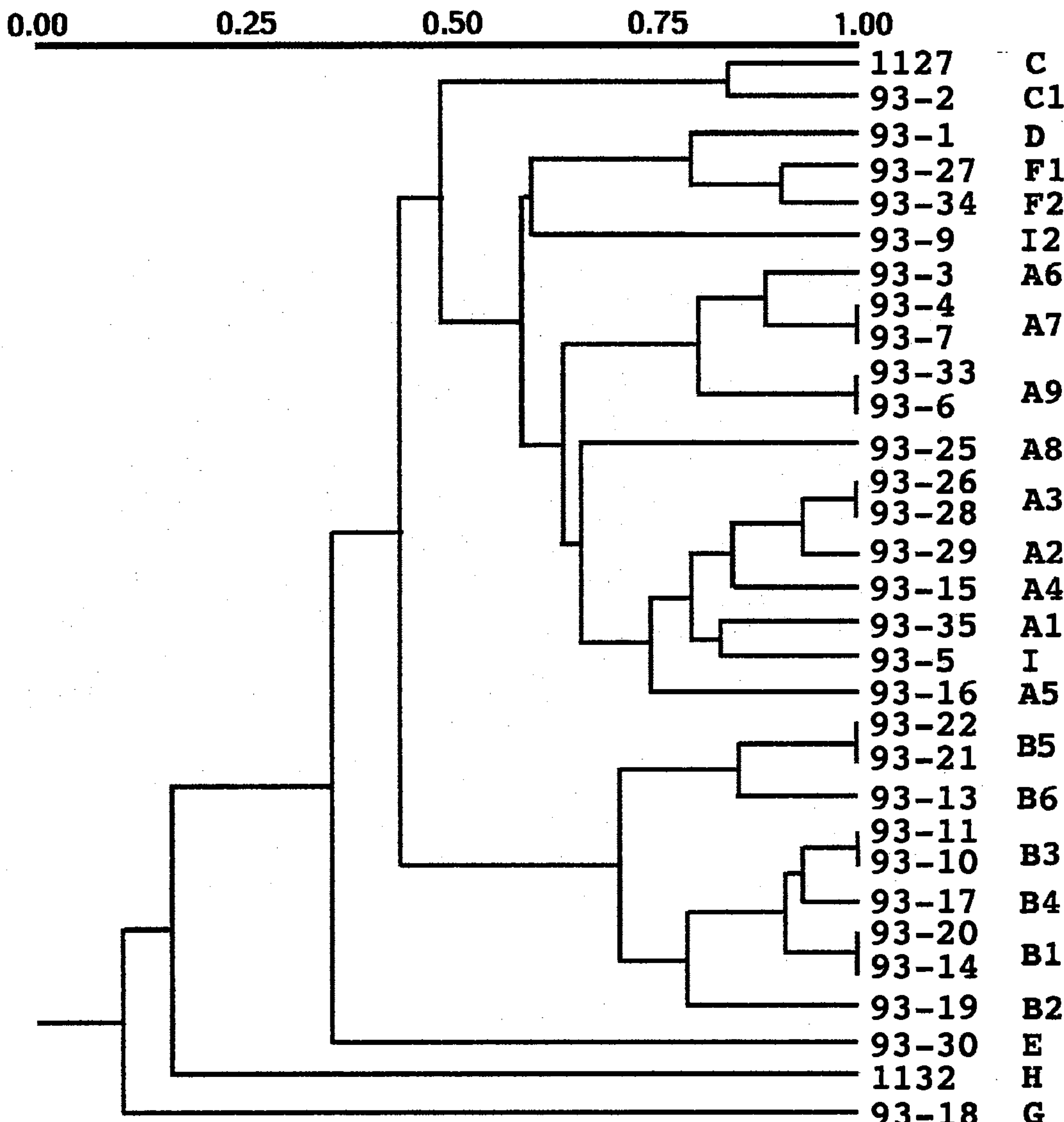

Figure 4. Dendogram from 1993 uninoculated plots REP-PCR fingerprinting data. Letters indicate REP pattern types.

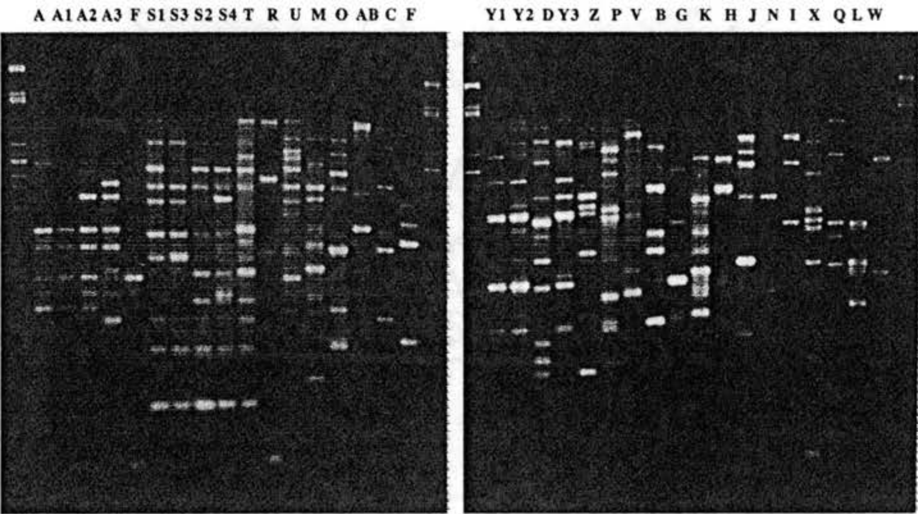

Figure 5a and 5b. REP-PCR fingerprints of uninoculated plot isolates collected in 1995. Leftmost and rightmost lanes contain *Pst* I-digested phage lambda DNA.

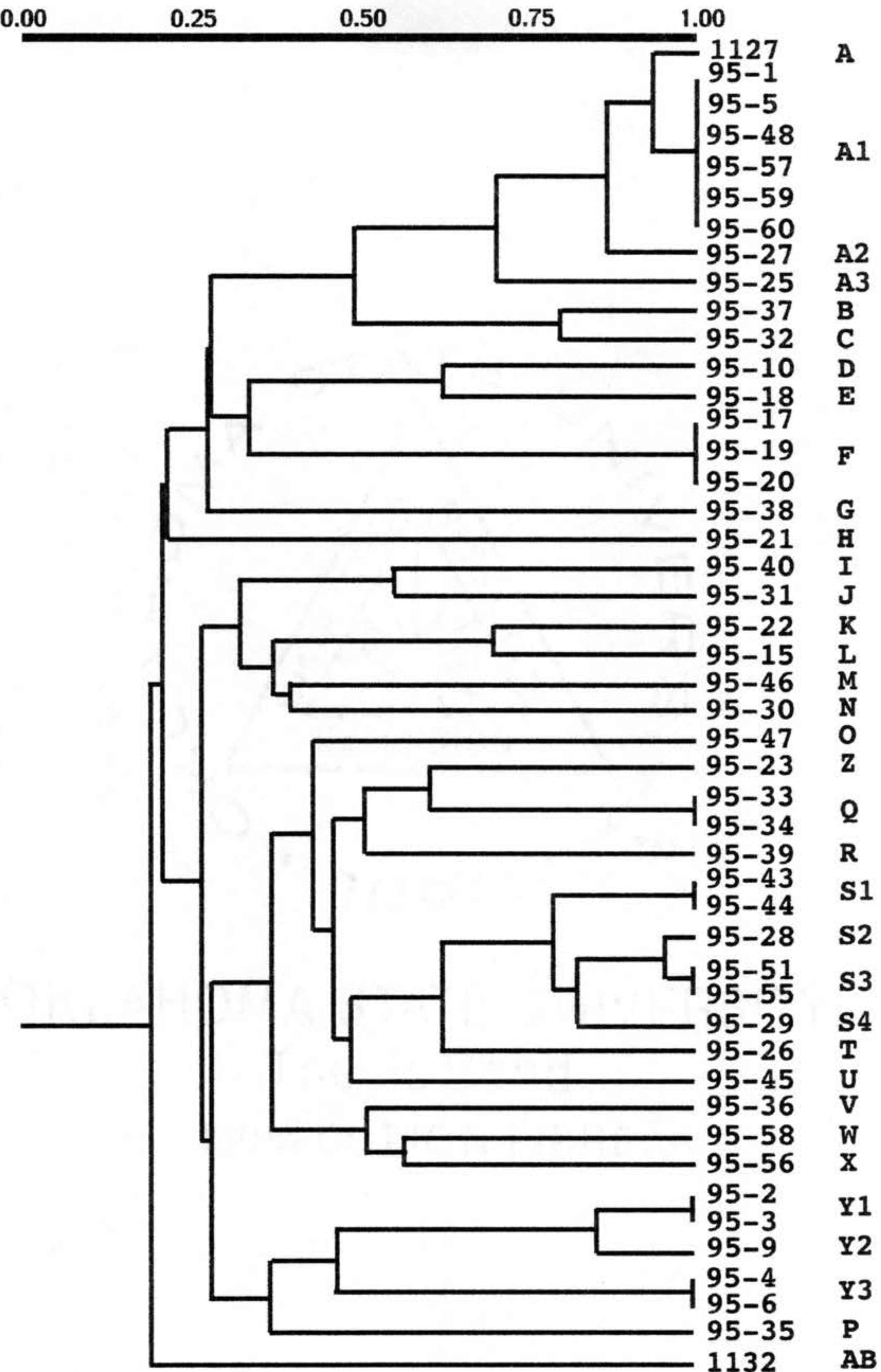

Figure 6. Dendogram from 1995 uninoculated plots REP-PCR fingerprinting data. Letters indicate REP pattern types.

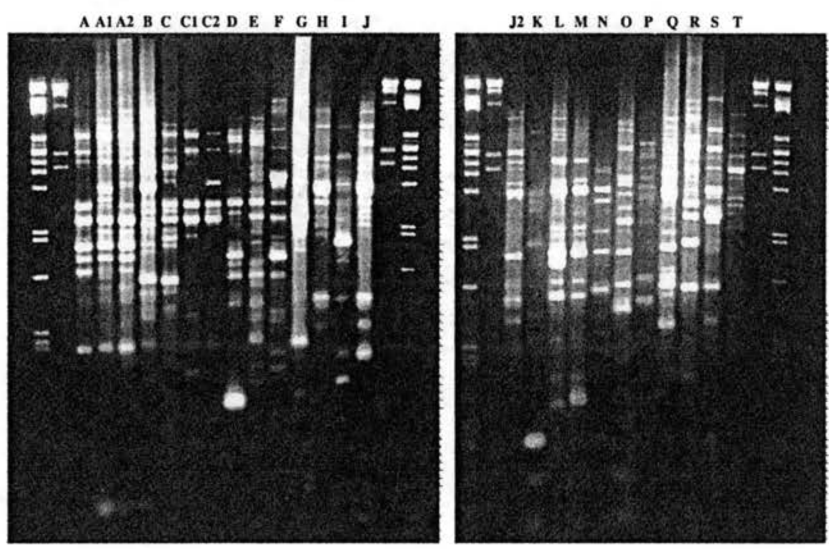

Figure 7a and 7b. REP-PCR fingerprints of uninoculated plot isolates collected in 1996. Leftmost and rightmost lanes contain *Pst* I-digested phage lambda DNA.

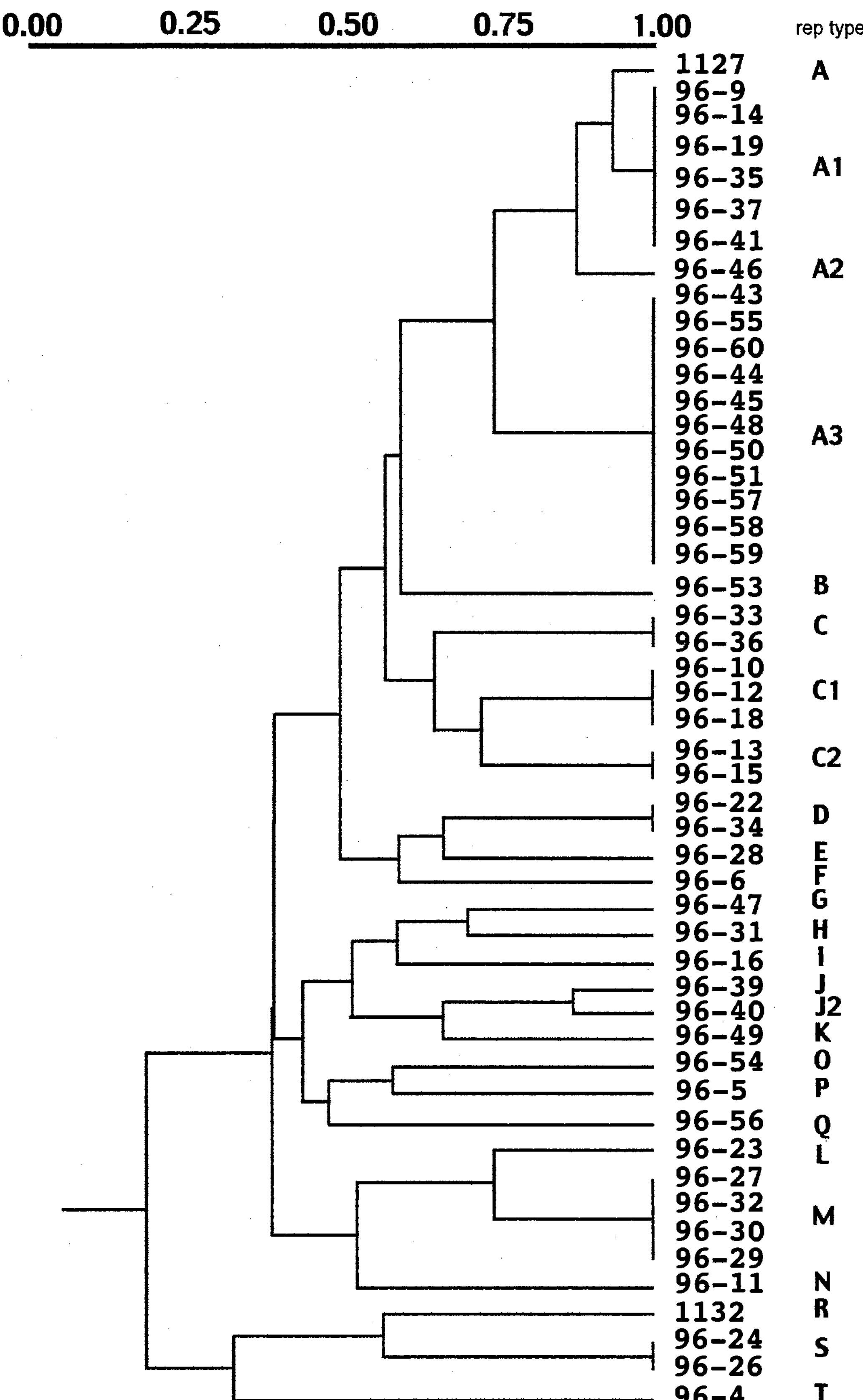

Figure 8. Dendogram from 1996 uninoculated plots REP-PCR fingerprinting data. Letters indicate REP pattern types.

summarizes the estimated similarities derived from UPGMA for all the 1996 samples. The groups close to TAL1127 REP type are possibly slow-growing rhizobia similar to the inoculant. TAL1127 identical REP prints may be due to its movement in the soil naturally or mechanically.

Based on REP PCR analysis, there is a diverse population of rhizobial isolates that nodulated pigeonpea through the four years of this project. These rhizobia show dominant populations at the start of the project, becoming more heterogeneous in the following years. Four years do not allow enough time for equilibrium of the ecological system to be reached. Every possible nodulator seemed to have tried to nodulate the "new" legume without having set dominant strains throughout the four years of this study.

16S, 23S IGS PCR -Restriction Length Polymorphism Analysis

Most samples produced a single band of 16S, 23S IGS PCR amplifications products and a few produced an additional band (data not shown). The sizes of the amplification bands were homogeneous ranging from 1.0-1.2-Kb. By using three different 4-base pair cutting restriction endonucleases distinct polymorphisms were obtained.

I.1993 sample set

The isolates collected in 1993 showed 6 restriction patterns for *Rsa*I, 8 for *Hpa*II and 10 for *Hae* III. *Hae* III was the most discriminating enzyme for this group. The patterns for each enzyme included many common bands (see Figure 9a and 9b). The previously similar REP groups were clustered together or shared very similar restriction patterns. There were no unique restriction patterns for any of the enzymes utilized that could indicate a distinct isolate. The dendogram summarizes these data in Figure 10 showing clusters formed among isolates of the same REP group, and adding on other REP types indicating that these strains were closely related. The capital letters indicate the REP types assigned previously. These results (summarized in Table 3) further indicate a very homogeneous, closely related group of samples for 1993 with a coefficient of .73.

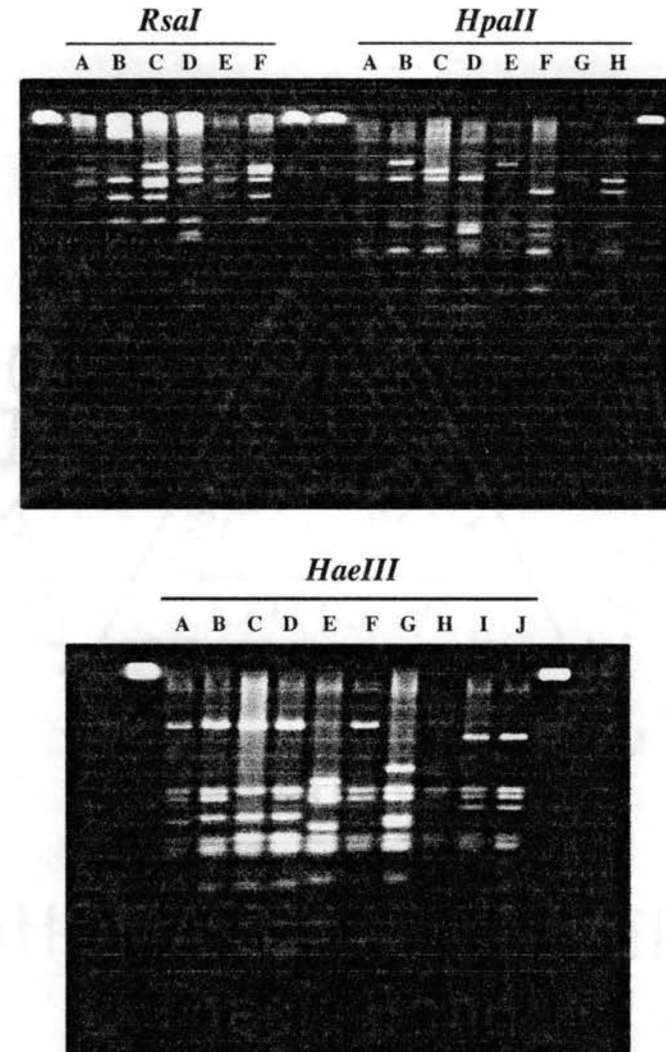

Figure 9a and 9b. 16S, 23S IGS unique polymorphisms in digests of uninoculated plot isolates collected in 1993. Letters represent unique restriction patterns.

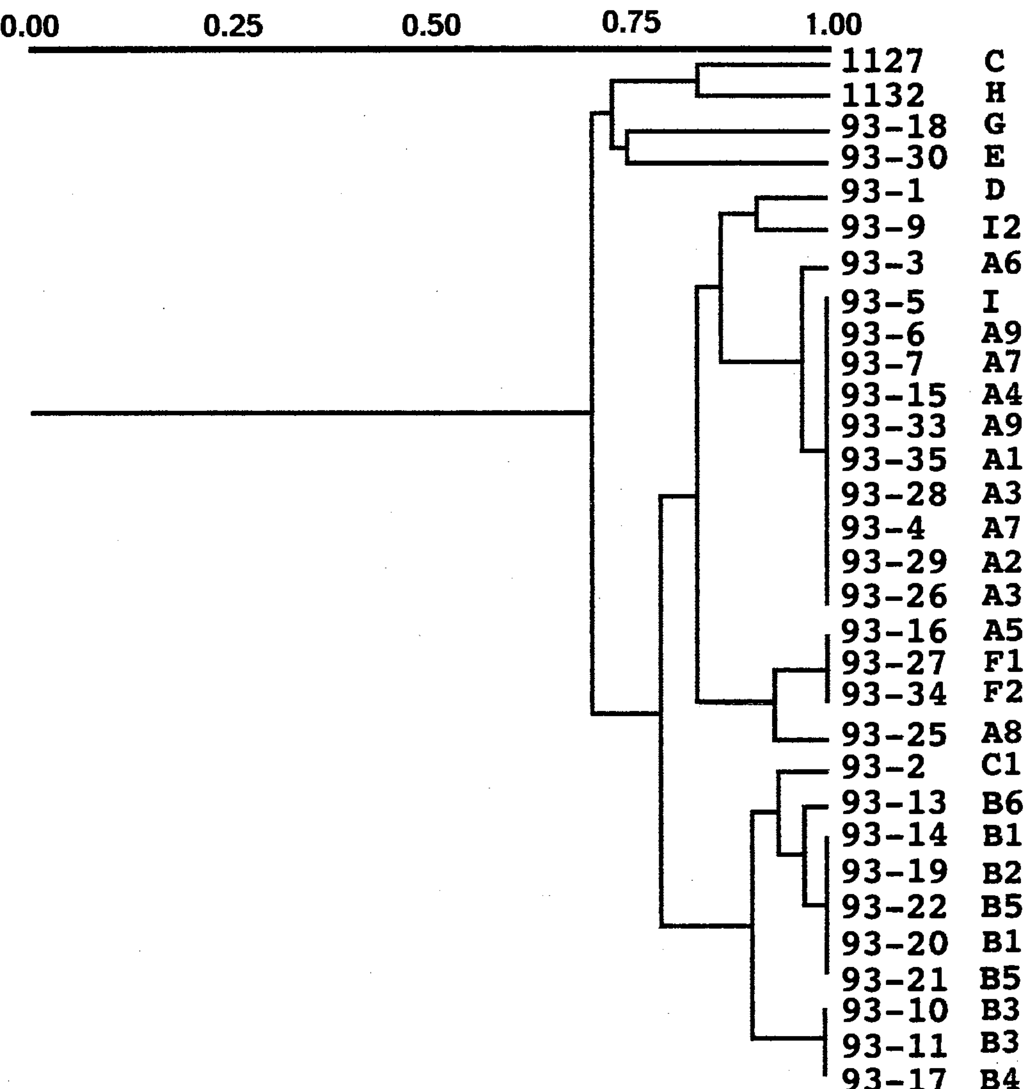

Figure 10. Dendogram from 1993 uninoculated plots 16S, 23S IGS polymorphisms.

Letters represent REP pattern types.

REP type	16S/23S IGS RFLP type		
A1	RsaI B	HpaII B	HaeIII A
A2	B	B	A
A3	B	B	A
A4	B	B	A
A5	C	C	D
A6	B	B	B
A7	B	B	A
A8	C	B	C
A9	B	B	A
I	B	B	A
I2	A	C	D
B5	F	H	J
B6	F	G	I
B1	F	G	I
B2	F	G	I
B3	F	B	I
B4	F	B	I
C1	C	E	F
C2	F	G	H
D	A	A	A
E	A	C	J
F1	C	C	A
F2	C	C	A
G	D	D	E
H	E	F	G

TABLE 3. 1993 16, 23S IGS (intergenic spacer region) PCR (polymerase chain reaction) RFLP (Restriction Fragment Length Polymorphisms) analysis

REP group B clustered closer together with a coefficient of .92. REP group A clustered closer together with a coefficient of .85.

II.1995 sample set

The 1995 sample group, with very heterogeneous REP patterns includes many restriction patterns for each enzyme used, this further confirms the heterogeneity of the sample set. The analysis of this set proved very complex and difficult. The main groups described based on REP PCR analysis remained close based on these analyses, pointing to a closer relation among samples.

The results are summarized in the dendogram in Figure 11 and in Table 4, which contains all the restriction pattern types for each enzyme for each REP type assigned. Isolates of REP groups A and Y were grouped closer together based on IGS analysis with a coefficient of .77. *Rsa* I-produced patterns A-X, *Hpa* II-produced patterns A-ZZ, and *Hae* III-produced patterns A-Y. There were many uncommon restriction patterns for each enzyme used.

III.1996 sample set

The 1996 sample group had closer clusters and the previously described REP groups were clustered together with groups A, B, C at a coefficient of 0.75. *Rsa*I-produced patterns A-Q, *Hpa*II-produced patterns A-P and *Hae*III-produced A-O. Polymorphisms for each enzyme included many common bands. Results are summarized in the dendogram in Figure 12 and in Table 5 which contains all restrictions pattern types for each enzyme. These results show IGS clusters composed of several REP types.

In this study, the genetic diversity of the collected pigeonpea nodulators was analyzed. The majorities of the REP patterns were distinct, and could be used to distinguish among strains as it distinguished among the inoculant strains. It was determined that during the first sampling year, a group of slow-growing rhizobia nodulated the newly introduced legume. These slow-growing rhizobia were the ones who competed best in the indigenous rhizobial populations. The prompt nodulators are usually the ones who dominate the

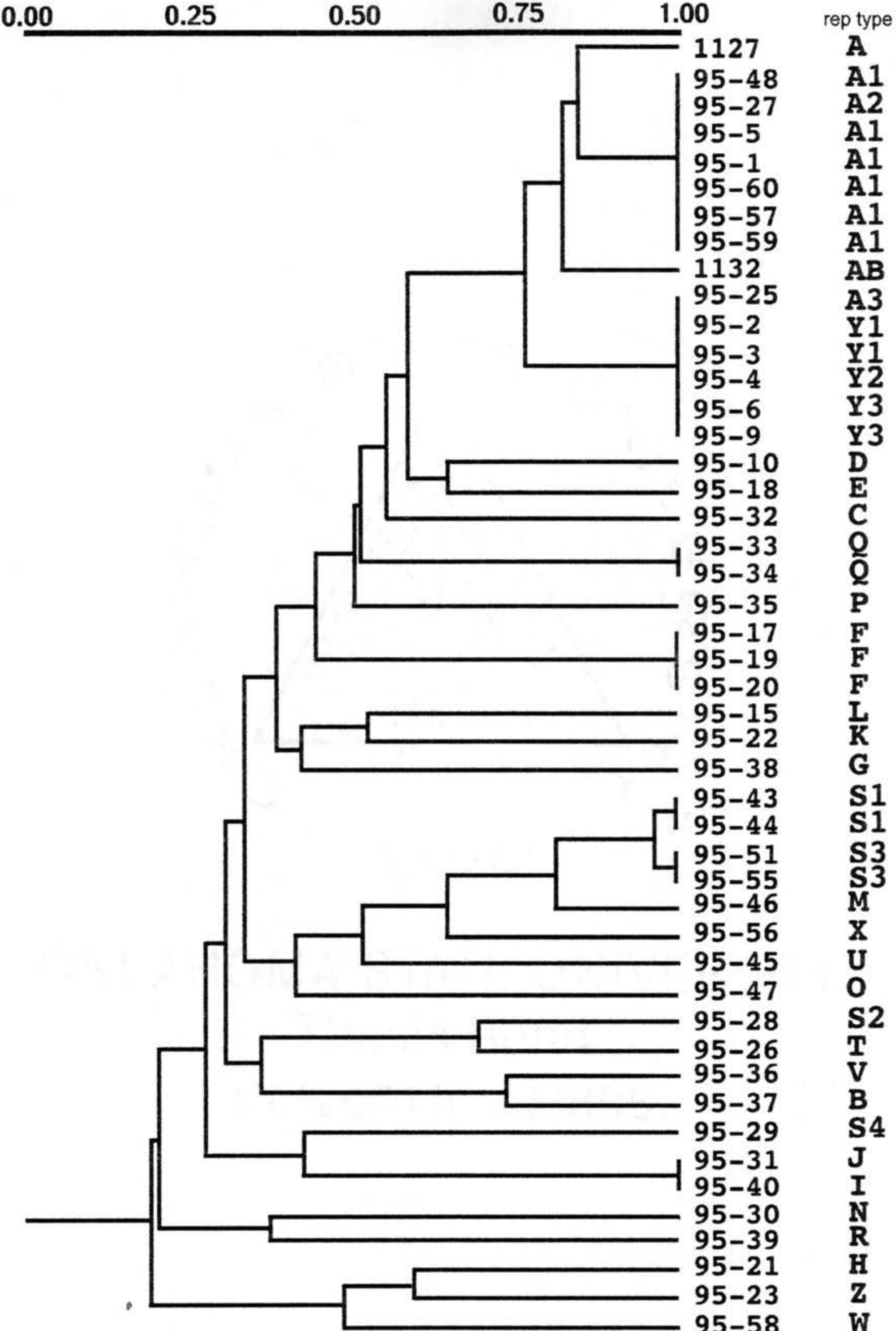

Figure 11. Dendogram from 1995 uninoculated plots 16S, 23S IGS polymorphisms. Letters represent REP pattern types.

REP type	16S, 23S IGS RFLP type		
A	RsaI B	HpaII C	HaeIII C
A1, A2	A	A	A
A3	F	B	B
B	U	M	S
C	Q	F	Q
D	G	D	D
E	H	J	F
F	H	I	H
G	T	T	Y
H	I	K	J
I, J	P	Q	P
K	J	L	I
L	E	G	G
M	V	X	T
N	O	R	O
O	K	Y	V
P	N	K	N
Q	R	R	R
R	X	U	M
S1	V	V	T
S2	N	O	L
S3	V	V	V
S4	N	O	N
T	M	E	L
U	D	W	U
V	W	H	X
W	L	N	T
X	V	AA	T
Y, Y2, Y3	F	B	B
Z	S	S	K
AB	C	AB	E

Table 4. 1995 16S, 23S IGS (intergenic spacer region) PCR (polymerase chain reaction) RFLP (restriction fragment length polymorphisms) analysis

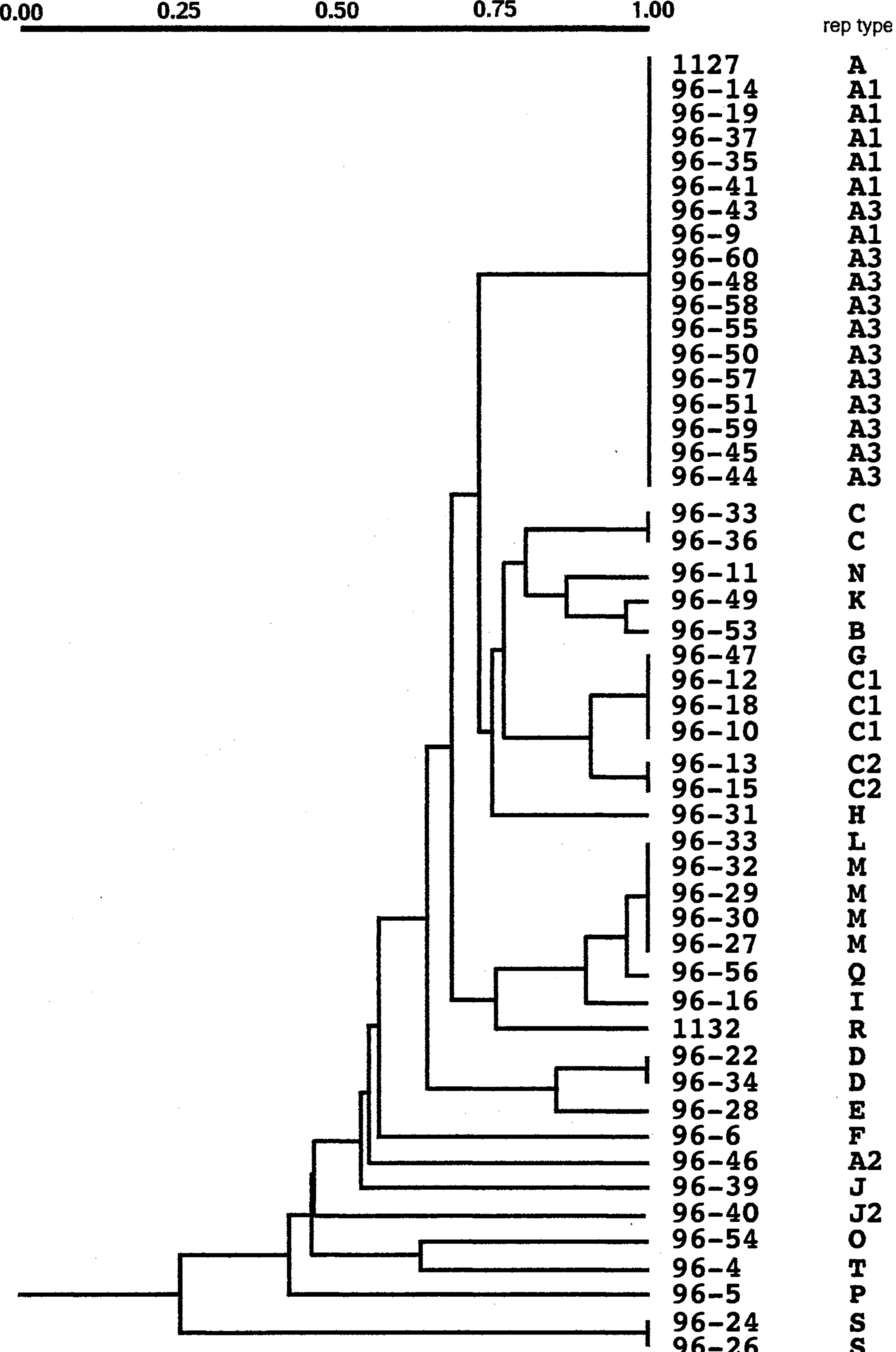

Figure 12. Dendogram from 1996 uninoculated plots 16S, 23S IGS polymorphisms. Letters represent REP pattern types.

REP type	16S/23S IGS RFLP type		
A1	RsaI A	HpaIIA	HaeIII A
A2	L	B	I
A3	A	A	A
B	E	D	B
C	B	B	A
C1	F	E	B
C2	G	E	B
D	J	I	F
E	J	J	G
F	K	I	H
G	F	E	B
H	C	F	C
I	I	G	D
J	M	K	J
J2	N	L	K
K	D	D	B
L	H	B	D
M	H	B	D
N	C	C	A
O	O	M	L
P	P	O	N
Q	H	B	E
R	H	H	E
S	Q	P	O
T	P	N	M

TABLE 5. 1996 16, 23S IGS (intergenic spacer region) PCR (polymerase chain reaction) RFLP (Restriction Fragment Length Polymorphisms) analysis

nodulation process. In 1993, two main REP groups (A and B) dominated the nodulation process and perhaps promptly took over it. These strains of slow-growing rhizobia were found to be related based on 16S, 23S IGS RFLP analysis. For the 1995 year other rhizobia had the chance to compete for the legume. This diverse population was potentially composed of slow and fast growers maybe due to a shift in the population. As equilibrium had not been reached, any possible pigeonpea rhizobia could nodulate pigeonpea--whether fast or slow-growing.

It is clear that there was a diverse population in the experimental plots including both fast and slow growers (based on growth phenotype). This indigenous "pigeonpea" rhizobial population was composed of many strains that can potentially compete with introduced rhizobia. The 1996 samples included more slow growers than 1995 probably because they competed better and tried to nodulate a legume for which no rhizobial equilibrium had been reached. It seemed that slow-growing rhizobia could dominate the nodulation process like in previous pigeonpea studies. Changes in the environment and or shifts in the population can explain the variety in the populations through the years.

The indigenous rhizobial population in these plots was capable of forming nodules in pigeonpea. Mainly slow-growing rhizobia nodulated pigeonpea, but some fast-growing rhizobia achieved to form nodules too, especially in the 1995 sample set. the rhizobial population in the experimental plots consisted of a heterogeneous population that included closely-related slow-growing rhizobia and also a group of fast-growing rhizobia. Pigeonpea was nodulated by many different strains in the experimental plots even though there had been no prior history of pigeonpea cultivation in this site. These molecular techniques of REP PCR and 16S, 23S IGS PCR RFLP made the genotypic analysis faster and easier.

Chapter 6

Phylogenetic analysis of indigenous pigeonpea rhizobia with ARDRA and partial 16S rDNA sequencing

Amplified rDNA restriction analysis (ARDRA) is a technique that helps to group strains at a taxonomic resolution of genus or species, depending on the organism under study (96). To consistently achieve a resolution of species full-length sequencing must be used. Partial sequencing can help distinguish heterogeneous samples that include *Bradyrhizobium* sp., and *Rhizobium* sp., but does not reveal variation within species (104). The analysis of rDNA can help group and classify the samples collected through the four sampling years of this study. It can help distinguish what type of fast or slow growers were present in the experimental soil. This analysis will also provide a better idea of diversity among the potential competitors for nodulation.

The use of nodulation assays will help verify that the isolates analyzed can in fact form nodules on pigeonpea.

ARDRA

The 16S rDNA was amplified with rD1 and fD1 universal primers. The product obtained averaged 1.5 Kb in size. The products were digested with the restriction enzymes *Rsa*I, *Hpa*II, and *Hae*III.

The inoculant strains *Bradyrhizobium* sp. (Cajanus) TAL1127 and TAL1132 had identical restriction patterns indicating that this method does not necessarily discern among these rhizobial strains as predicted from literature.

I.1993 ARDRA

The isolates collected in1993 appeared to be a very homogeneous group of slow-growing bacteria as there was close resemblance to the inoculant restriction patterns. There were only two types of 16S restriction patterns for each one of the enzymes used. These types include the inoculant strains types (see Table 6 and Figure 13).

HpaII RsaI HaeIII

A B A B A B

Figure 13. 16S unique polymorphisms in digests of uninoculated plot samples collected in 1993. Letters represents unique restriction digest patterns for each enzyme.

REP type	16S RFLP type		
A1	HpaII A	RsaI A	HaeIII A
A2	A	A	A
A3	A	A	B
A4	A	A	A
A5	A	A	A
A6	A	A	A
A7	A	A	A
A8	A	A	A
A9	A	A	A
I	A	A	A
I2	A	A	B
B5	A	A	A
B6	A	A	A
B1	A	A	A
B2	A	A	A
B3	A	A	A
B4	A	A	A
C1	A	A	A
C2	B	B	A
D	A	A	A
E	A	A	B
F1	A	A	A
F2	A	A	A
G	A	A	A
H	B	B	A

TABLE 6. 1993 16S rDNA PCR (polymerase chain reaction) RFLP (Restriction Fragment Length Polymorphisms) analysis

In the 1993 sample set there were two main groups of closely related genotypes. These groups shared restriction pattern bands with the inoculant strains, but the inoculants remained clustered in a close branch in the dendogram produced from this data (see Figure 14). This dendogram illustrates the two main groups of indigenous rhizobia found and their relatedness to the inoculant strains branch. The two main groups had a similarity coefficient of 0.8. These data showed that the clusters formed based on 16S, 23S IGS RFLP analysis were closely related. They were bradyrhizobia of closely related species. This analysis compared with the REP PCR data for this sample set indicated close relatedness among the strains.

II.1995 ARDRA

In the 1995 sampling year, the differences found previously with REP PCR can be simplified into smaller groups. The samples get clustered closer than with REP PCR and 16S, 23S IGS PCR RFLP analysis to separate fast- and slow-growers. From the three enzymes used *Hpa*II seems to be the most discriminatory giving the most polymorphisms. For *Rsa*I there were A-P restriction patterns, for *Hpa*II there were A-N patterns and for *Hae*III there were A-N patterns. The patterns for each enzyme were similar to each other (see Figures 15 a-c) but more complex than in the 1993 sample set. This was further confirmation of the heterogeneity of this sample set when compared with the 1993 results as there were far more restriction digest types for this sample set (see Table 7) than for the 1993 sampling year. The dendogram in Figure 16 shows that the slow-grower branch was separated from the fast-grower branch by a coefficient of 0.6. ARDRA allowed for better subgrouping among such heterogeneous sample groups. The presence of fast-growing rhizobia may add to the differences as *Bradyrhizobium* and *Rhizobium* are clustered separately based on 16S data. The dendogram in Figure 16 illustrated the grouping of samples into larger clusters allowing us to see that those very diverse REP types were related based on 16S rDNA data. There is a major branch including TAL1127 and TAL1132 that includes slow growers similar to *Bradyrhizobium* sp. This group extends

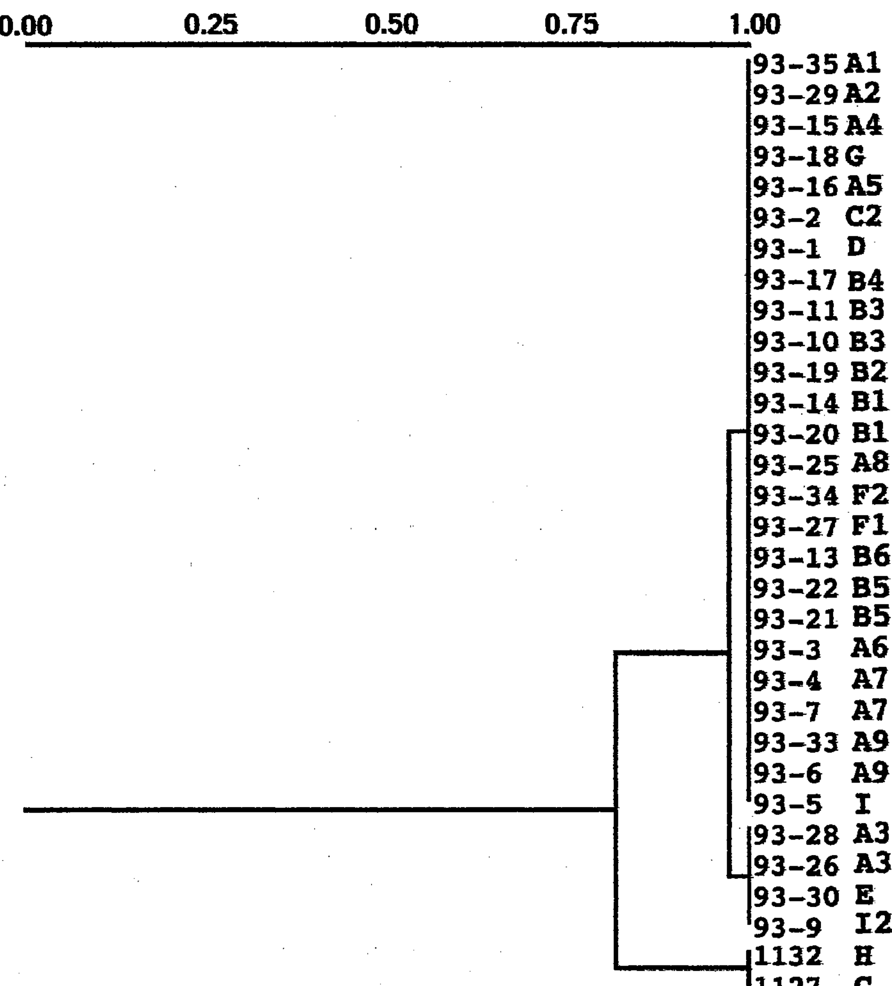

Figure 14. Dendogram from 1993 uninoculated plots ARDRA. Letters represent REP pattern types.

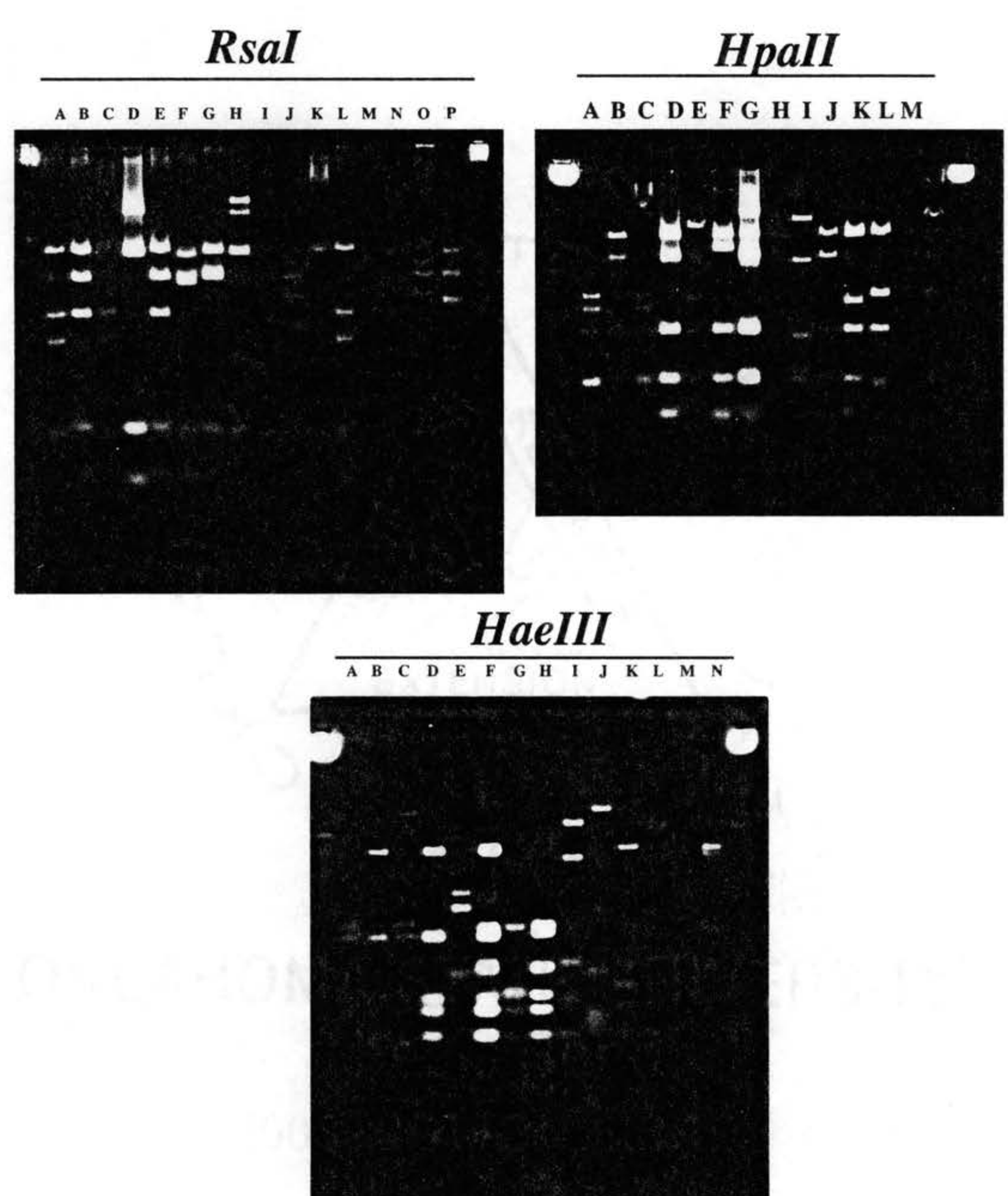

Figure 15a, 15b and 15c. 16S unique polymorphisms in digests of uninoculated plot samples collected in 1995. Letters represents unique restriction digest patterns for each enzyme.

REP type	16S RFLP type		
A, AB	RsaI A	HaeIII A	HpaII A
A1	A	B	A
A2	L	B	A
A3	N	B	B
B	B	B	B
C	E	B	B
D	K	J	K
E	B	B	B
F	C	C	C
G	H	F	G
H	J	I	I
I, J	F	E	E
K	K	J	J
L	O	N	N
M	D	D	D
N	J	H	F
O	K	K	K
P	O	M	D
Q	D	B	A
R	P	M	M
S1, S3	D	D	D
S2	D	D	J
S4	M	D	D
T	D	G	D
U	D	D	D
V	E	B	B
W	D	D	D
X	B	B	B
Y, Y2, Y3	B	B	L
Z	I	L	H

TABLE 7. 1995 16S rDNA PCR (polymerase chain reaction) RFLP (Restriction Fragment Length Polymorphisms) analysis

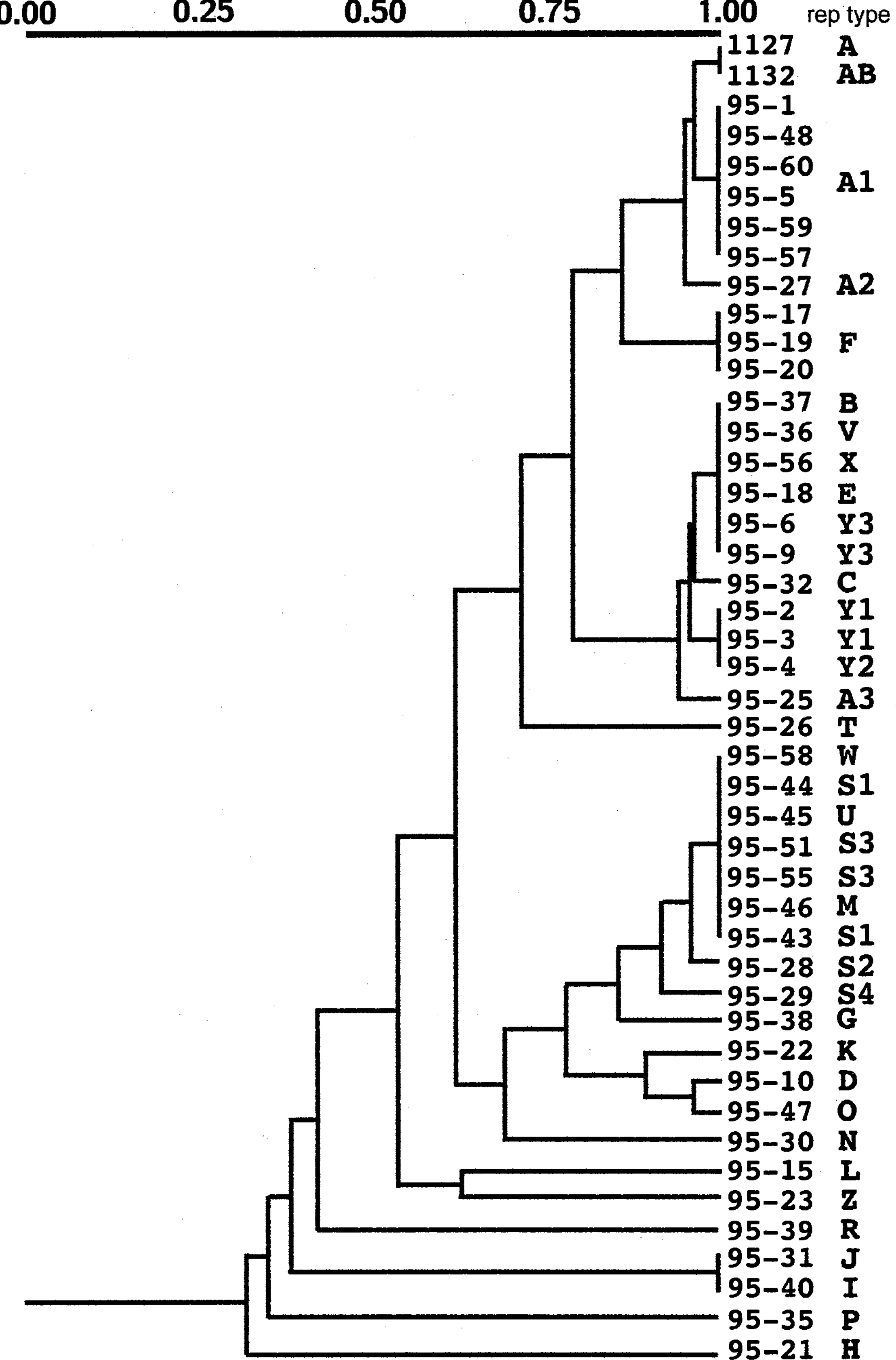

Figure 16. Dendogram from 1995 uninoculated plots ARDRA. Letters represent REP pattern types.

from REP type A through A3 in the upper half of the dendogram and includes closely related slow growers. The fast growers clustered together, separately from slow growers as expected for 16S analysis.

III.1996 ARDRA

The 1996 16S RFLP analysis included a big group of slow growers and a smaller group of fast growers. The most discriminatory enzyme was again *Hpa*II. For *Rsa*I there were A-N restriction patterns with most of them sharing many bands (see Figure 17a). For *Hpa*II there were A-L patterns with most patterns resembling each other (see Figure 17b). For *Hae*III there were A-M patterns with many similarities (see Figure 17c). The restriction type data are summarized in Table 8.

The 1996 16S ARDRA dendogram (see Figure 18) illustrates the diversity of this sampling group including a big group of closely related samples based on restriction data and a few very different samples that are separated in lower branches. Most of the slow growers were clustered together separate from other fast growers by a coefficient of 0.75. It is shown that REP types A, R, A1, A3, and A3 are closely related. These types were characterized as closely related based on 16S, 23S IGS RFLP analysis, but 16S ARDRA illustrated that these REP types were closely related to more REP types, grouping all slow groups together as expected for 16S analysis.

The 1996 samples include more slow growers than 1995 probably because they competed better and tried to nodulate a legume for which no rhizobial equilibrium had been reached. Changes in the environment and or shifts in the population can explain the variety in the populations through the years.

16S Partial Sequencing

Samples with different REP types were analyzed by partial 16S rDNA sequencing. The partial sequencing of an approximately 264-bp 16S DNA fragment helped in classifying samples being analyzed. This partial sequencing did not differentiate at the

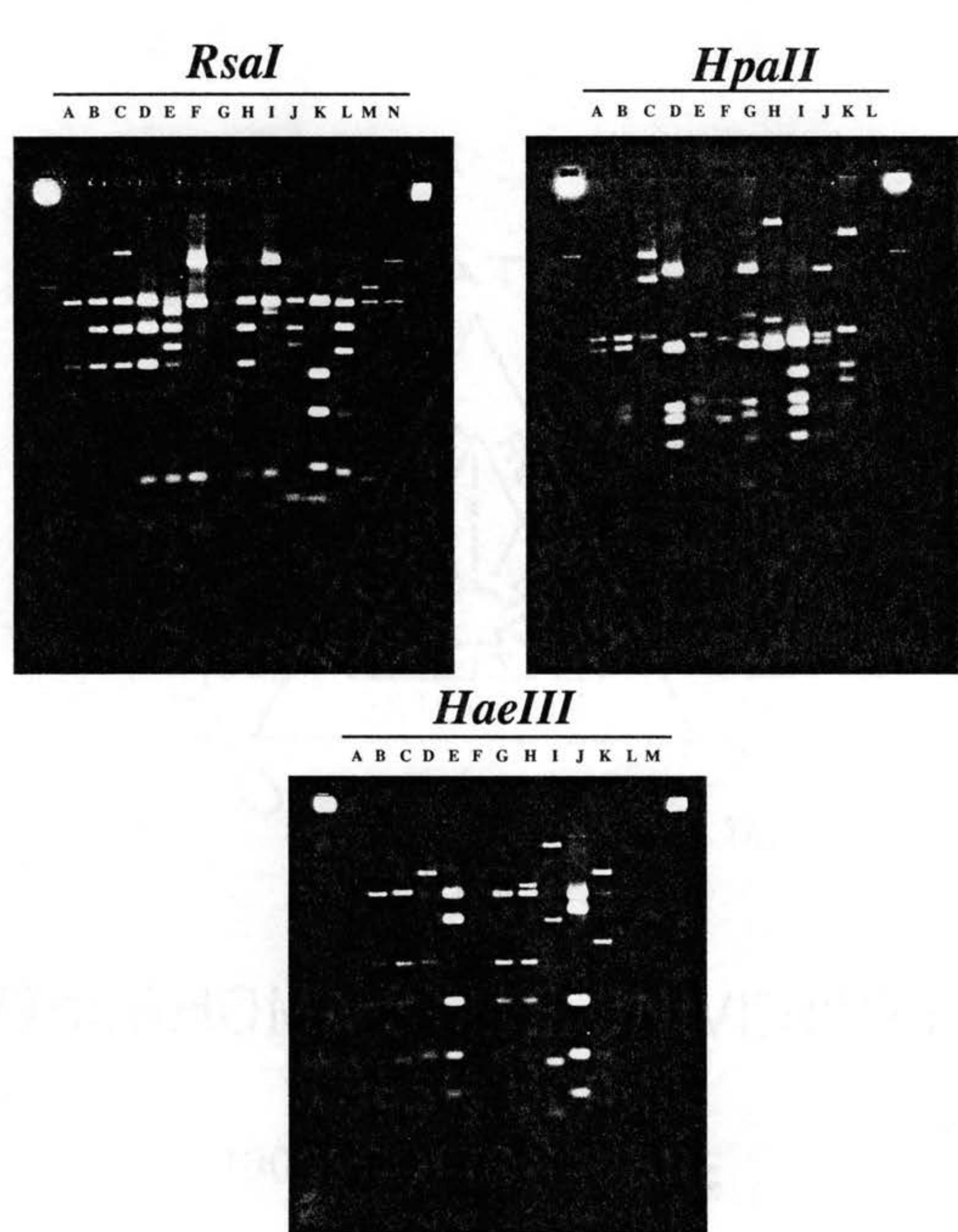

Figure 17a, 17b and 17c. 16S unique polymorphisms in digests of uninoculated plot samples collected in 1995. Letters represents unique restriction digest patterns for each enzyme.

REP type	16S RFLP type		
A1	RsaI A	HpaIIA	HaeIII A
A2	K	F	C
A3	B	A	A
B	J	E	A
C	B	B	A
C1	D	D	A
C2	E	D	A
D	F	D	A
E	N	I	F
F	L	G	D
G	G	D	A
H	I	C	B
I	D	D	A
J	M	H	E
J2	R	M	D
K	J	E	A
L	C	B	A
M	A	C	A
N	H	D	A
O	O	J	G
P	Q	L	H
Q	C	B	A
R	A	A	A
S	S	N	J
T	P	K	H

TABLE 8. 1996 16S rDNA PCR (polymerase chain reaction) RFLP (Restriction Fragment Length Polymorphisms) analysis

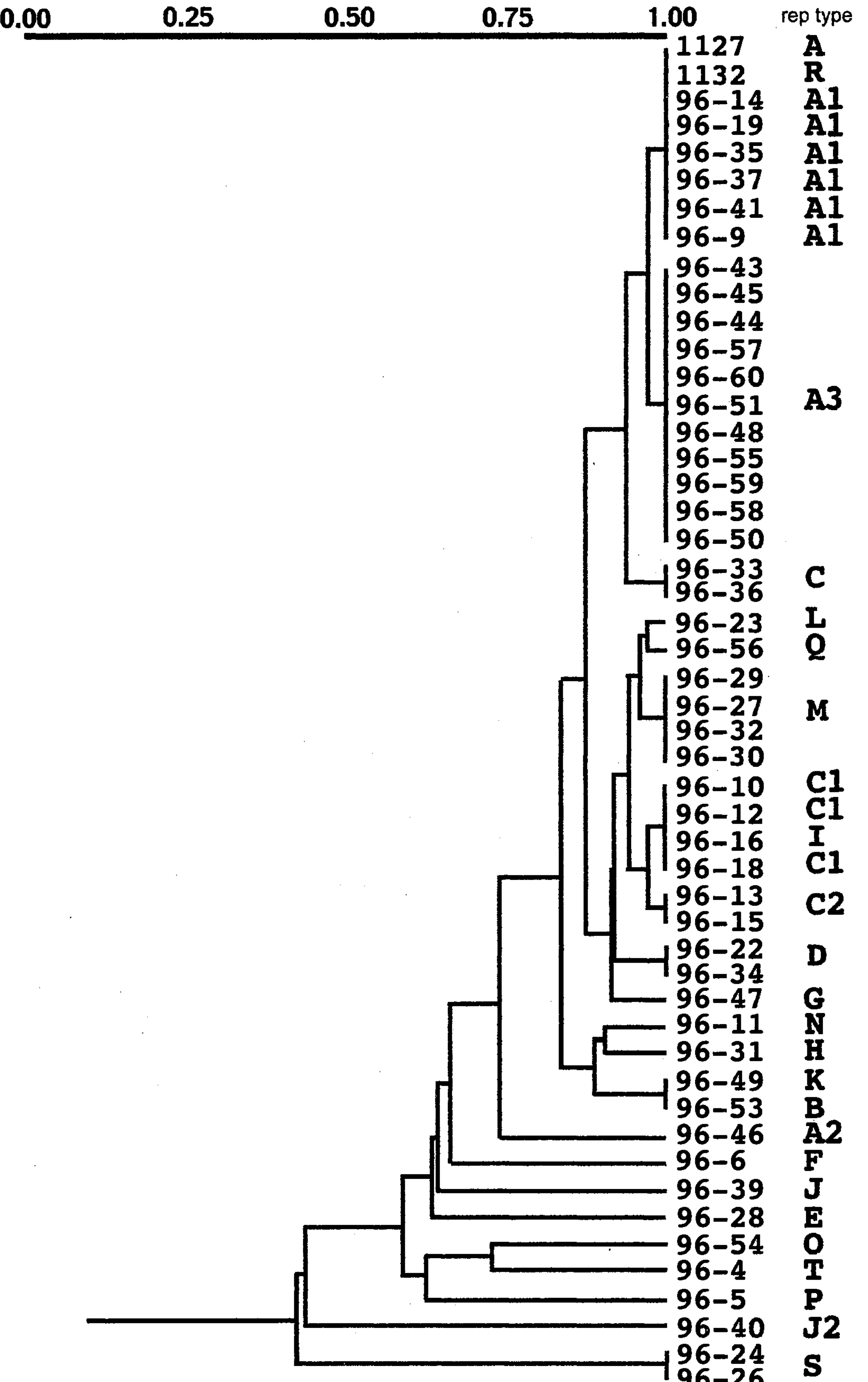

Figure 18. Dendogram from 1993 uninoculated plots ARDRA. Letters represent REP pattern types.

strain or species level, necessarily, but it did help in identifying genus. Inoculant strains were included to establish comparisons.

Based on 16S RFLP analysis of the 1993 samples, along with partial sequence analysis, it was confirmed that there were two groups of closely related slow-growing rhizobia. When compared to sequences available at the Ribosomal Database Project website, the recovered indigenous strains were found to be closely similar to the species included in Table 9. For the samples in 1993, *Bradyrhizobium lupini* (slow-growing rhizobia nodulator of lupines) and *Bradyrhizobium* sp. (LMG9520; slow-growing rhizobia nodulator of *Acacia*) were the most similar sequences including the inoculant strains (Table 10).The alignment of these sequences indicated that there were only minor differences among the samples (see Figure 19). There were only a few different sequences in addition to the two inoculant strains sequences. There were four types of sequences identified in 1993 (see Table 10): isolates 93-1 and 93-27 had type 1; isolate 93-18 had type 2; isolates 93-22, 93-10, 93-13, 93-2, 93-9, 93-16, 93-25, 93-3, 93-5, 93-15, 93-26, 93-13 and 93-30 had identical sequences to inoculant strain TAL1127 for type 3 and type 4 included inoculant strain TAL1132. It seemed that only REP types D and F had different partial sequences from the inoculant strains.

Phylograms indicate closer groups in vertical lines and relatedness among those in horizontal lines. From the sequence alignment data a phylogram was created. This phylogram includes comparisons with the strains found to be similar to sequences deposited at the Ribosomal Database Project (Michigan State University) to establish the relatedness of the indigenous rhizobial strains to these similar strains. *E. coli* was included as an outgroup and also because the primers used to amplify the partial sequence were created from *E. coli*.

The phylogram in Figure 20 for the 1993 distinct sequences illustrates that TAL1127, TAL1132, isolates 93-1 and 93-18 are closely related as expected from the

GenBank ascession numbers	Genus and species
M55494	Rhizobium leguminosarum
M11223	Agrobacterium tumefasciens
M55485	*Bradyrhizobium japonicum* 110
M55242	*Rhizobium meliloti* A
L20867	*Afipia felis*
M55233	*Rhizobium tropici* CIAT899
X13695	*Brucella abortus*
M55491	*Azorhizobium caulidonans*
M55496	*Rhodopseudomonas palustris*
M55492	*Bradyrhizobium japonicum BTAi 1*
X63824	*Rhizobium (leucaena)* TAL1145
M55497	*Rhodospirillum rubrum*
U07934	*Rhizobium cicer*
L20781	*Bradyrhizobium elkani*

TABLE 9. List of GenBank strains used to create phylogram with indigenous rhizobia

Sequence Type	Isolate ID	REP type	Sequence similarity in Ribosomal Database Project	Nodulator in assays (y/n)
	35, 29, (4,7), (6,33), (14,20), 19, 17, 34	A1, A2, A7, A9, B1, B2, B4, F2	sequence not possible	test not possible
2	(26,28), 15, 3, 25, 5, 9, 3, (21,22), 2, 30	A3, A4, A6, A8, I, I2, B6, B5, C2, E	*B. lupini* 1.00 similarity	test not possible
2	16, 1127, (10,11)	A5, C, B3,	*B. lupini* 1.00 similarity	Y
1	1, 27	D, F1	*B. lupini* .954 similarity	test not possible
3	18	G	*Bradyrhizobium sp.* (LMG9520).981 similarity	test not possible
4	1132	H	*B. lupini* .977 similarity	Y

TABLE 10. 1993 similarity ranks with the RDP (Ribosomal Database Project) and nodulation trials results summary
(parenthesis indicates samples with identical REP type)

CLUSTAL X (1.64b) multiple sequence alignment

```
B.lupini                AACACATGCAAGTCGAGCGGGCGTAGCAATACG-----TC----------
1127                    AACACATGCAAGTCGAGCGGGCGTAGCAATACG-----TC----------
1132                    AACACATGCAAGTCGAGC-----TAGCAATACG-----TC----------
93-34                   AACACATGCAAGTCGAGCGGGCGTAGCAATACG-----TC----------
93-18                   AACACATGCAAGTCGAGCGGGCATAGCAATATG-----TC----------
Bradyrhizobiumsp.LMG9520AACACAUGCAAGUCGAGCGGGCAUAGCAAUAUG-----UC----------
E.coli                  AACACATGCAAGTCGAACGGTAACAGGAAGAAGCTTGCTCTTTGCTGACG
                        ****** ***** *** *      ** ** * *      *

B.lupini                AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
1127                    AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
1132                    AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
93-1                    AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
93-18                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
Bradyrhizobiumsp.LMG9520AGCGGCAGACGGGUGAGUAACGCGUGGGAACGUACCUUUUGGUUCGGAAC
E.coli                  AGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGAT
                        ** *** ****** *** ** *   *****    **    **   ** *

B.lupini                AACACAGGGAAACTTGTGCTAATACCGGATAAGCCCTTACGGGGAAAGA-
1127                    AACACAGGGAAACTTGTGCTAATACCGGATAAGCCCTTACGGGGAAAGA-
113234                  AACACAGGGAAACTTGTGCTAATACCGGATAAGCCCTTACGGGGAAAGA-
93-1                    AACCCAGGGAAACTTGGGCTAATACCGGATAAGCCCTTACGGGGAAAGA-
93-18                   AACTGAGGGAAACTTCAGCTAATACCGGATAAGCCCTTACGGGGAAAGA-
Bradyrhizobiumsp.LMG9520AACUGAGGGAAACUUCAGCUAAUACCGGAUAAGCCCUUACGGGGAAAGA-
E.coli                  AACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAG
                        ***    ******    ** ** **** * **   *  * *   *****

B.lupini                -------TT------TATCGCCGAAAGATCGGCCCGCGTCTGATTAGCTA
1127                    -------TT------TATCGCCGAAAGATCGGCCCGCGTCTGATTAGCTA
1132                    -------TT------TATCGCCGAAAGATCGGCCCGCGTCTGATTAGCTA
93-1                    -------TT------TATCGCCGAAAGATCGGCCCGCGTCTGATTAGCTA
93-18                   -------TT------TATCGC-GAAAGATCGGCCCGCGTCTGATTAGCTA
Bradyrhizobiumsp.LMG9520-------UU------UAUCGCCGAAAGAUCGGCCCGCGUCUGAUUAGCUA
E.coli                  GGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTA
                                           **     **   ****      **  *** *

B.lupini                GTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTGAGA
1127                    GTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTGAGA
1132                    GTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTGAGA
93-1                    GTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTGAGA
93-18                   GTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTGAGA
Bradyrhizobiumsp.LMG9520GUUGGUGAGGUAAUGGCUCACCAAGGCGACGAUCAGUAGCUGGUCUGAGA
E.coli                  GTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGA
                        *  ** * ** ** *** **** ********* *   *** ** * ****

B.lupini                GGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
1127                    GGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
1132                    GGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
93-1                    GGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
93-18                   GGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
Bradyrhizobiumsp.LMG9520GGAUGAUCAGCCACAUUGGGACUGAGACACGGCCCAAACUCCUACGG
E.coli                  GGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG
                        *** ** ********  ** ** ********* *** ** ** ****
```

Figure 19. Clustal X alignment of the unique sequences in isolates collected in 1993 and the Ribosomal Database Project similar sequences

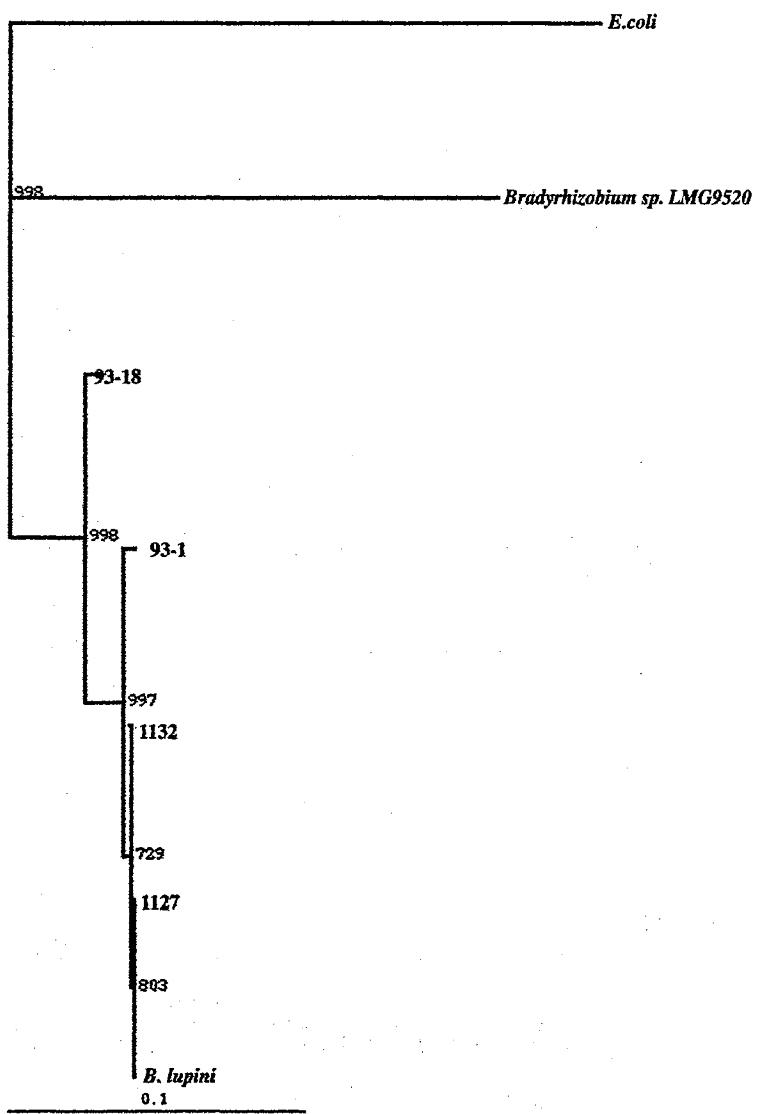

Figure 20. Phylogram created with N-J method through ClustalX and viewed through Viewtree 1.0 from 1993 unique sequences and their Ribosomal Database Project similar sequences.

previous ARDRA. These data further clarified that the 1993 isolates are closely related slow-growing rhizobia.

The 1995 data set included both fast- and slow-growing rhizobia. Sequencing data confirmed that most slow-growers clustered together and so did the fast-growers as seen previously in the dendogram created by ARDRA. The alignment of these sequences indicated the overall differences among the slow- and fast-growers based on partial sequencing. Consensus asterisks indicated conserved regions (see Figure 21). There were 17 types of sequences found that include (see Table 11): 1) 95-31, 95-40, 2) 95-26, 3) 95-30, 4) 95-29, 95-58, 95-55, 95-51, 95-43, 95-44, 5) 95-43, 95-44, 6) 95-58, 7) 95-22, 8) 95-47, 9) 95--10, 10) TAL1127, 95-18, 95-2, 95-4, 95-3, 95-17, 95-19, 95-20, 11) TAL1132, 12) 95-9, 95- 6, 13) 95-36, 14) 95-39, 95-37, 95-40, 95-48, 95-5, 95-1, 15) 95-33, 95-34, 16) 95-25, 17) 95-32, 95-37.

The phylogram for the 1995 sequencing data included the unique sequence types found and the selected similar sample set accessed from RDP for comparisons. The shape of the phylogram for the slow growers (see Figure 22) remains constant as the main differences from the 1993 sequence data came from the presence of fast-growing rhizobia.

The isolates in the slow-growing rhizobia branch for the 1995 sequence data were found to be similar to *Blastobacter denitrificans* (budding bacteria), *Bradyrhizobium lupini* (nodulator of lupines), *Bradyrhizobium* sp. (LMG9520; nodulator of *Acacia*), *B. japonicum* (IAM12608; nodulator of soybean) and *Photorhizobium thompsonianum str.* BTA*i* (photosynthetic stem-nodulating bacteria from *Aeschynomene*). The isolates in the fast-growing rhizobia branch were found to be similar to *Rhizobium* sp. OK-55 (nodulator of *Sesbania aculeata*), *R. leguminosarum* (nodulator of bean), *A. caulidonans* (nodulator of *Sesbania rostrata*), *R. etli* and *A. amazonense* when compared to sequences available at the Ribosomal Database Project.

The sequencing data for the 1996 sample group confirmed that it contained both fast- and slow-growing rhizobia. The slow-growing rhizobia dominated the nodulation

```
CLUSTAL X (1.64b) multiple sequence alignment
95-29                   --AACACATGCAAGTCGAGCG----CC-------CGCAGGGG--------
95-43                   ACTACGTGAGGCAGCAGTGGG----CC-------GCAAGGGG--------
95-22                   --AACACATGCAAGTCGAGCG----CCC-----CGCAAGGGG--------
95-38                   --AACACATGCAAGTCGAGCG----CCC------GCAAGGGG--------
95-10                   --AACACATGCGAGACGAGCG----CCC-----CGCAAGGGG--------
95-47                   --AACACATGCAAGTCGAGCG----CCC-----CGCA-GGGG--------
Bradyrhizobiumsp.LMG9520--AACACAUGCAAGUCGAGCG----GGCA---UAGCAAUAUG------UC
B.japonicumIAM12608     --AACACAUGCAAGUCGAGCG----GGCG---UAGCAAUACG------UC
B.denitrificansLMG8443  --AACACAUGCAAGUCGAGCG----GGCG---UAGCAAUACG------UC
P.thompsonianumstr.BTAi --AACACAUGCAAGUCGAGCG----GGCG---UAGCAAUACG------UC
R.etlistr.CFN-244.      --------------------------------------------------
R.leguminosarum         --AACACAUGCAAGUCGAGCG----CCC-----CGCAAGGGG--------
Rhizobiumsp.str.OK-55.  --AACACAUGCAAGUCGAGCG----CCC-----CGCAAGGGG--------
A.amazonense            --AACACAUGCAAGUCGAACG----A-------AGGCUUCGG------CC
Az.caulinodansstr       --AACACAUGCAAGUCGAGCG----CCC-----AGCAAUGGG--------
95-9                    --AACACATGCGAGTCGAGCG----GGCG---TAGCAATACG------TC
95-5                    ---ATACATGCGGCACGAGCG----GGCG---TAGCAATACG------TC
B.lupini                --AACACATGCAAGTCGAGCG----GGCG---TAGCAATACG------TC
1132                    --AACACATGCAAGTCGAGC------------TAGCAATACG------TC
1127                    --AACACATGCAAGTCGAGCG----GGCG---TAGCAATACG------TC
95-36                   --AACACATGCAAGTCGAGCG----GGCA---TAGCAATATG------TC
95-32                   --AACACATGCAAGTCGAGCG----GGCG---TAGCAATACG------TC
95-25                   --AACACATGCAAGTCGAGCG----GGCG---TAGCAATACG------TC
95-33                   --AACACATGCAAGTCGAGCG----GGCG---TAACAATACG------TC
95-30                   --AACACATGCAAGTCGAGCG----C-------ATCCTTGGG------GT
95-26                   --AACACATGCAAGTCGAACG----CTC-----CGCAAGGGG--------
95-31                   ---ACACATGCAAGTCGAACGGCAGCACG--GGAGCAATCC---TGGTGG
Burkholderiasp.         --UACACAUGCAAGUCGAACGGCAGCACG--GGAGUAAUCC---UGGUGU
E.coli                  --AACACATGCAAGTCGAACGGTAACAGGAAGAAGCTTGCTCTTTGCTGA


95-29                   --AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCCTTTACTACGGA
95-43                   --AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCCTTTACTACGGA
95-22                   --AGCGGCAGACGGGTGAGTAACGCGTGGGAATCTACCCTTGACTACGGA
95-38                   --AGCGGCAGACGGGTGAGTAACGCGTGGGAATGTACCCTCTACTACGGA
95-10                   --AGCGGCAGACGGGTGAGTAACGCGTGGGAATCTACCCATCCCTACGGA
95-47                   --AGCGGCAGACGGGTGAGTAACGCGTGGGAATCTACCCATCCCTACGGA
Bradyrhizobiumsp.LMG9520--AGCGGCAGACGGGUGAGUAACGCGUGGGAACGUACCUUUUGGUUCGGA
B.japonicumIAM12608     --AGCGGCAGACGGGUGAGUAACGCGUGGGAACGUACCUUUUGGUUCGGA
B.denitrificansLMG8443  --AGCGGCAGACGGGUGAGUAACGCGUGGGAACGUACCUUUUGGUUCGGA
P.thompsonianumstr.BTAi --AGCGGCAGACGGGUGAGUAACGCGUGGGAACGUACCUUUUGGUUCGGA
R.etlistr.CFN-244.      -------CAGACGGGUGAGUAACGCGUGGGAACGUACCCUUUACUACGGA
R.leguminosarum         --AGCGGCAGACGGGUGAGUAACGCGUGGGAAUCUACCCUUGACUACGGA
Rhizobiumsp.str.OK-55.  --AGCGGCAGACGGGUGAGUAACGCGUGGGAAUCUACCCAUCCCUACGGA
A.amazonense            UUAGUGGCGCACGGGUGAGUAACGCGUGGGAACCUGCCCUUUGGUUCGGA
Az.caulinodansstr       --AGCGGCAGACGGGUGAGUAACGCGUGGGGAUGUGCCCAAUGGUGCGGA
95-9                    --AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGA
95-5                    --AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGA
B.lupini                --AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGA
1132                    --AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGA
1127                    --AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGA
95-36                   --AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGA
95-32                   --AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGA
95-25                   --AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGA
95-33                   --AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGA
95-30                   G-AGCGGCAGACGGGTGAGTAACGCGTGGGGATGTGCCCAGAGGTGGGGA
95-26                   --AGTGGCGCACGGGTGAGTAACACGTGGGAACCTACCTTCTGGTACGGA
95-31                   CGAGTGGCGAACGGGTGAGTAATACATCGGAACGTGTCCTGTAGTGGGGG
Burkholderiasp.         CGAGUGGCGAACGGGUGAGUAAUACAUCGGAACGUGUCCUGUAGUGGGGG
E.coli                  CGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGG
                               *  ***** *** **      ** *     *         **
```

Figure 21 . Clustal X alignment of the unique sequences in isolatescollected in 1995 and the RDP similar sequences

```
95-29                   ATAACGCAGGG-AAACTTGTGCTAATACCGTATGTGCCCTTCGG------
95-43                   ATAACGCAGGG-AAACTTGTGCTAATACCGTATGTGCCCTTCGG------
95-22                   ATAACGCAGGG-AAACTTGTGCTAATACCGTATGTGTCCTTCGG------
95-38                   ATAACGCAGGG-AAACTTGTGCTAATACCGTATGTGTCCTTTGG------
95-10                   ACAACTCCGGG-AAACTGGAGCTAATACCGTATACGCCCTTCGG------
95-47                   ACAACTCCGGGGAAACTGGAGCTAATACCGTATACGCCCTTCGG------
Bradyrhizobiumsp.LMG9520ACAACUGAGGG-AAACUUCAGCUAAUACCGGAUAAGCCCUUACG------
B.japonicumIAM12608     ACAACACAGGG-AAACUUGUGCUAAUACCGGAUAAGCCCUUACG------
B.denitrificansLMG8443  ACAACACAGGG-AAACUUGUGCUAAUACCGGAUAAGCCCUUACG------
P.thompsonianumstr.BTAi ACAACACAGGG-AAACUUGUGCUAAUACCGGAUAAGCCCUUACG------
R.etlistr.CFN-244.      AUAACGCAGGG-AAACUUGUGCUAAUACCGUAUGUGCCCUUCGG------
R.leguminosarum         AUAACGCAGGG-AAACUUGUGCUAAUACCGUAUGUGUCCUUCGG------
Rhizobiumsp.str.OK-55.  ACAACUCCGGG-AAACUGGAGCUAAUACCGUAUACGCCCUUCGG------
A.amazonense            AUAACUCCGGG-AAACUGGAGCUAAUACCGGAUGAGCCUGAUGGUUGUGG
Az.caulinodansstr       AUAACCCAGGG-AAACUUGGAUUAAUACCGCAUGUGCCCUUCG---G---
95-9                    ACAACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG------
95-5                    ACAACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG------
B.lupini                ACAACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG------
1132                    ACAACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG------
1127                    ACAACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG------
95-36                   ACAACTGAGGG-AAA-TTCAGCTAATACCGGATAAGCCCTTACG------
95-32                   ACAACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG------
95-25                   ACAACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG------
95-33                   ACAACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG------
95-30                   ATAACTCAGGG-AAACTTGAGCTAATACCGCATGAGCCCTTCGG------
95-26                   ACAACCAAGGG-AAACTTTGGCTAATACCGTATACGACCTCCGG------
95-31                   ATAGCCCGGCG-AAAGCCGGATTAATACCGCATACGCTCTGCGGAGGAAA
Burkholderiasp.         AUAGCCCGGCG-AAAGCCGGAUUAAUACCGCAUACGAUCUGUGGAUGAAA
E.coli                  ATAACTACTGG-AAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAA
                        * * *     * ***        ** **** *

95-29                   ----------GGGA-AAGATTTATCGGTAAGGGATCGGCCCGCGTTGGATT
95-43                   ----------GGGA-AAGATTTATCGGTAAGGGATCGGCCCGCGTTGGATT
95-22                   ----------GAGA-AAGATTTATCGGTCAAGGATGAGCCCGCGTTGGATT
95-38                   ----------GGGA-AAGATTTATCGGTAGGGGATGAGCCCGCGTTGGATT
95-10                   ----------GGGA-AAGATTTATCGGGGATGGATGAGCCCGCGTTGGATT
95-47                   ----------GGGA-AAGATTTATCGGGGATGGATGAGCCCGCGTTGGATT
Bradyrhizobiumsp.LMG9520----------GGGA-AAGAUUUAUCGCCGAAAGAUCGGCCCGCGUCUGAUU
B.japonicumIAM12608     ----------GGGA-AAGAUUUAUCGCCGAAAGAUCGGCCCGCGUCUGAUU
B.denitrificansLMG8443  ----------GGGA-AAGAUUUAUCGCCGAAAGAUCGGCCCGCGUCUGAUU
P.thompsonianumstr.BTAi ----------GGGA-AAGAUUUAUCGCCGAAAGAUCGGCCCGCGUCUGAUU
R.etlistr.CFN-244.      ----------GGGA-AAGAUUUAUCGGUAAGGGAUCGGCCCGCGUUGGAUU
R.leguminosarum         ----------GAGA-AAGAUUUAUCGGUCAAGGAUGAGCCCGCGUUGGAUU
Rhizobiumsp.str.OK-55.  ----------GGGA-AAGAUUUAUCGGGGAUGGAUGAGCCCGCGUUGGAUU
A.amazonense            AGACUGUCAGGGA-AAGAUUUAUCGCCGAAGGAGGGGCCCGCGUCCGAUU
Az.caulinodansstr       ----------GGGA-AAGAUUUAUCGCCAUUGGAUCAACCCGCGUCUGAUU
95-9                    ----------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATT
95-5                    ----------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATT
B.lupini                ----------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATT
1132                    ----------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATT
1127                    ----------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATT
95-36                   ----------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATT
95-32                   ----------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATT
95-25                   ----------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATT
95-33                   ----------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATT
95-30                   ----------GGGA-AAGATTTATCGCCTTTGGATCAACCCGCGTCAGATT
95-26                   ----------GTGA-AAGATTTATCGCCGGAAGAGGGGCCCGCGTCCGATT
95-31                   GCGGGGGATCCTTCGGGACCTCGCGCTACAGGGGCGGCCGATGGCAGATT
Burkholderiasp.         GCGGGGGAUCUU--AGGACCUCGCGCUACAGGGGCGGCCGAUGGCAGAUU
E.coli                  GAGGGGGACCTT--CGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATT
                                        *       *      *      **      **
```

Figure 21 cont. Clustal X alignment of the unique sequences in isolatescollected in 1995 and the RDP similar sequence

```
95-29                     AGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTC
95-43                     AGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTC
95-22                     AGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTC
95-38                     AGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTC
95-10                     AGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTC
95-47                     AGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCG---ATCCATAGCTGGTC
Bradyrhizobiumsp.LMG9520AGCUAGUUGGUGAGGUAAUGGCUCACCAAGGCGACGAUCAGUAGCUGGUC
B.japonicumIAM12608       AGCUAGUUGGUGAGGUAAUGGCUCACCAAGGCGACGAUCAGUAGCUGGUC
B.denitrificansLMG8443    AGCUAGUUGGUAGGGUAAUGGCCUACCAAGGCGACGAUCAGUAGCUGGUC
P.thompsonianumstr.BTAi AGCUAGUUGGUAGGGUAAUGGCCUACCAAGGCGACGAUCAGUAGCUGGUC
R.etlistr.CFN-244.        AGCUAGUUGGUGGGGUAAAGGCCUACCAAGGCGACGAUCCAUAGCUGGUC
R.leguminosarum           AGCUAGUUGGUGGGGUAAAGGCCUACCAAGGCGACGAUCCAUAGCUGGUC
Rhizobiumsp.str.OK-55.    AGCUAGUUGGUGGGGUAAAGGCCUACCAAGGCGACGAUCCAUAGCUGGUC
A.amazonense              AGGUAGUUGGUGAGGUAACGGCUCACCAAGCCGACGAUCGGUAGCUGGUC
Az.caulinodansstr         AGCUAGUUGGUGAGGUAAAGGCUCACCAAGGCGACGAUCAGUAGCUGGUC
95-9                      AGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTC
95-5                      AGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTC
B.lupini                  AGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTC
1132                      AGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTC
1127                      AGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTC
95-36                     AGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTC
95-32                     AGCTAGTTGGTAGGGTAATGGCCTACCAAGGCGACGA-CAGTAGCTGGTC
95-25                     AGCTAGTTGGTAGGGTAATGGCCTACCAAGGCGACGATCAGTAGCTGGTC
95-33                     AGCTAGTTGGTCGGGTAATGGCCTACCAAGGCGACGATCAGTAGCTGGTC
95-30                     AGCTAGTTGGTAGGGTAATGGCCTACCAAGGCGACGATCTGTAGCTGGTC
95-26                     AGGTAGTTGGTGGGGTAACGGCTCACCAAGCCGACGATCGGTAGCTGGTC
95-31                     AGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCTGGTC
Burkholderiasp.           AGCUAGUUGGUGGGGUAAAGGCCUACCAAGGCGACGAUCUGUAGCUGGUC
E.coli                    AGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTC
                          **  **  **   ** ** ***  *** ** **   * *   *** ** *

95-29                     TGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC
95-43                     TGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC
95-22                     TGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC
95-38                     TGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC
95-10                     TGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC
95-47                     TGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC
Bradyrhizobiumsp.LMG9520UGAGAGGAUGAUCAGCCACAUUGGGACUGAGACACGGCCCAAACUCCUAC
B.japonicumIAM12608       UGAGAGGAUGAUCAGCCACAUUGGGACUGAGACACGGCCCAAACUCCUAC
B.denitrificansLMG8443    UGAGAGGAUGAUCAGCCACAUUGGGACUGAGACACGGCCCAAACUCCUAC
P.thompsonianumstr.BTAi UGAGAGGAUGAUCAGCCACAUUGGGACUGAGACACGGCCCAAACUCCUAC
R.etlistr.CFN-244.        UGAGAGGAUGAUCAGCCACAUUGGGACUGAGACACGG-------------
R.leguminosarum           UGAGAGGAUGAUCAGCCACAUUGGGACUGAGACACGGCCCAAACUCCUAC
Rhizobiumsp.str.OK-55.    UGAGAGGAUGAUCAGCCACAUUGGGACUGAGACACGGCCCAAACUCCUAC
A.amazonense              UGAGAGGAUGAUCAGCCACACUGGGACUGAGACACGGCCCAGACUCCUAC
Az.caulinodansstr         UGAGAGGAUGAUCAGCCACACUGGGACUGAGACACGGCCCAGACUCCUAC
95-9                      TGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC
95-5                      TGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC
B.lupini                  TGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC
1132                      TGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC
1127                      TGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC
95-36                     TGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC
95-32                     TGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC
95-25                     TGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC
95-33                     TGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC
95-30                     TGAGAGGATGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
95-26                     TGAGAGGATGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
95-31                     TGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
Burkholderiasp.           UGAGAGGACGACCAGCCACACUGGGACUGAGACACGGCCCAGACUCCUAC
E.coli                    TGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTAC
                           ******* ** ********  ** ** *********
```

Figure 21 cont. Clustal X alignment of the unique sequences in isolates collected in 1995 and the RDP similar sequences

```
95-29                        GG
95-43                        GG
95-22                        GG
95-38                        GG
95-10                        GG
95-47                        GG
Bradyrhizobiumsp.LMG9520GG
B.japonicumIAM12608          GG
B.denitrificansLMG8443       GG
P.thompsonianumstr.BTAi      GG
R.etlistr.CFN-244.           --
R.leguminosarum              GG
Rhizobiumsp.str.OK-55.       GG
A.amazonense                 GG
Az.caulinodansstr            GG
95-9                         GG
95-5                         GG
B.lupini                     GG
1132                         GG
1127                         GG
95-36                        GG
95-32                        GG
95-25                        GG
95-33                        GG
95-30                        GG
95-26                        GG
95-31                        GG
Burkholderiasp.              GG
E.coli                       GG
```

Figure 21 cont. Clustal X alignment of the unique sequences in isolates collected in 1995 and the Ribosomal Database Project similar sequences

Sequence Type	Sample ID	REP type	Sequence similarity in Ribosomal Database Project	Nodulator in assays (y/n)
10	1127	A	*B. lupini* 1.00 similarity	Y
4	95-1, -5, -48, -57, -59, -60	A1	*B. japonicum* (IAM12608) .963 similarity	test not possible
	95-27	A2	sequence not possible	Y
16	95-25	A3	*P. thompsonianum str.* BTAi 1.00 similarity	Y
17	95-37	B	*P. thompsonianum str.* BTAi.977 similarity	Y
17	95-32	C	*P. thompsonianum str.* BTAi.977 similarity	test not possible
9	95-10	D	*Rhizobium sp.* OK-55 .965 similarity	Y
10	95-18, (-2, -3), -4, (-17, -18, -20)	E, Y1, Y2, F	*B. lupini* 1.00 similarity	Y
6	95-38	G	*R. leguminosarum* (LMG8820) .876 similarity	Y
	95-21	H	sequence not possible	Y
1	95-40, -31	I	*Burkholderia sp.* .880 similarity	Y
7	95-22	K	*R. leguminosarum* (LMG8820) 1.00 similarity	Y
	95-15	L	sequence not possible	Y
	95-46	M	sequence not possible	Y
3	95-30	N	*A. caulidonans* (LMG6465) .695 similarity	test not possible
8	95-47	O	*Rhizobium sp.* OK-55 .992 similarity	test not possible
	95-35	P	sequence not possible	test not possible
15	95-34	Q	*B.denitrificans* .958 similarity	test not possible
	95-39	R	sequence not possible	Y
5	95-43, -44	S1	*R. etli str.* CFN-244 .954 similarity	test not possible
4	(95-51, -55), -29, -58	S3, S4, W	*R. etli str.* CFN-244 1.00 similarity	Y
2	95-26	T	*Azospirillum amazonense str.* Y1 .631 similarity	Y
	95-45	U	sequence not possible	Y
13	95-36	V	*Bradyrhizobium sp.* (9520) .981 similarity	Y
	95-56	X	sequence not possible	Y
12	95-6, -9	Y3	*B. lupini* .976 similarity	Y
	95-23	Z	sequence not possible	Y
11	1132	AB	*B. lupini* .977 similarity	Y
	95-28	S2	sequence not possible	test not possible

TABLE 11. 1995 similarity ranks with the RDP (Ribosomal Database Project) and nodulation trials results summary (parenthesis indicates samples with identical REP type)

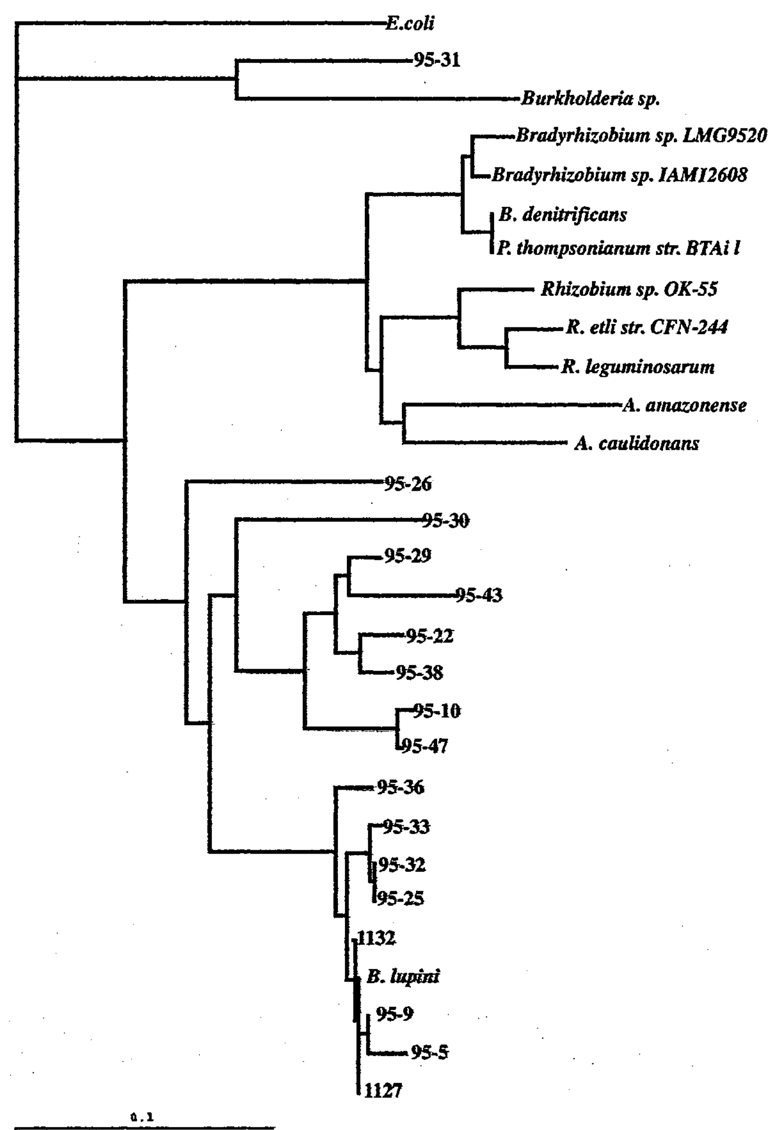

Figure 22. Phylogram created with N-J method through ClustalX and viewed through Viewtree 1.0 from 1995 unique sequences and their Ribosomal Database Project similar sequences.

process that sampling year. The alignment of these sequences indicates the differences among fast- and slow-growing rhizobia based on these 264-270 bp. Any area without consensus is inferred by the absence of asterisks (see Figure 23). There were 11 types of sequences found that include (see Table 12): 1) 96-12, 96-18, 96-13, 96-15, 96-47, 96-10, 2) TAL1127, 96-14, 96-19, 96-9, 96-41, 96-35, 96-16, 96-29, 96-30, 96-27, 3) 96-53, 96-11, 96-56, 96-31, 96-49, 4) 96-23, 5) 96-24, 96-36, 96-43, 96-55, 96-60, 96-44, 96-45, 96-48, 96-50, 96-51, 96-57, 96-58, 96-59, 96-26, 96-33, 6) TAL1132, 7) 96-34, 96-22, 8) 96-54, 9) 96-5, 10) 96-4, 11) 96-6.

The clustering in Figure 24 illustrates that there were fast and slow growers in the 1996 sequence data. Isolates 96-4, 96-5, and 96-6, and 96-54 are clustered separate from fast- and slow-growing rhizobia.

Isolates in the slow-growing rhizobia branch were found to be similar to *B. lupini* (nodulator of lupines), *B. japonicum* (nodulator of soybeans), *Bradyrhizobium* sp. (9520) and *Photorhizobium thompsonianum str.* BTAi (photosynthetic stem-nodulating bacteria from *Aeschynomene*) based on comparisons with RDP. Isolates in the fast-growing rhizobia branch were found to be similar to *R. leguminosarum* (nodulator of bean), *Azospirillum amazonense* str. Y1, or *A. caulidonans* (nodulator of *Sesbania rostrata*) based on comparisons with RDP.

The phylogram in Figure 25 demonstrated the relationship among the similar sequences accessed from RDP and the unique sequences from the 1993, 1995 and 1996 sequencing data. Slow-growers were clustered together in the lower branch while fast-growers were clustered together in the higher branch. The alignment of these sequences used to create this phylogram can be seen in Figure 26.

The alignment of the partial sequences, the creation of phylograms and the similarity by analysis with the RDP database provided a complete comparison among the samples. These analyses allowed determination of relatedness among sample sets and genus identification of rhizobia were potentially recovered from pigeonpea nodules. Partial

```
CLUSTAL X (1.64b) multiple sequence alignment


1132                    AACACATGCAAGTCGAGC-----TAGCAATACGTC---------------
1127                    AACACATGCAAGTCGAGCGGGCGTAGCAATACGTC---------------
B.lupini                AACACATGCAAGTCGAGCGGGCGTAGCAATACGTC---------------
96-12                   AACACATGCAAGTCGAGCGGGCGTAGCAATACGTC---------------
96-24                   AACACATGCGAGTCGAGCGGGCGTAGCAATACGTC---------------
96-23                   AACACATGCGAGTCGAGCGGGCGTAGCAATACGTC---------------
96-53                   AACACATGCAAGTCGAGCGGGCGTAGCAATACGTC---------------
96-34                   AACACATGCAAGTCGAGCGGGCATAGCAATATGTC---------------
E.coli                  AACACATGCAAGTCGAACGGTAACAGGAAGAAGCTTGCTCTTTGCTGACG
96-4                    AACACATGCAAGTCGAACGC--ACCGCAAGGTG-----------------
96-6                    AACACATGCAAGTCGAGCGG---CATCCTTCGGGGT-------------G
96-5                    AACACATGCAAGTCGAACGCT--CCGCAAGGGG-----------------
96-54                   AACACATGCAAGTCGAGCGCC--CCGCAAGGGG-----------------
P.thompsonianumstr.BTAi AACACAUGCAAGUCGAGCGGGCGUAGCAAUACGUC---------------
B.japonicumIAM12608     AACACAUGCAAGUCGAGCGGGCGUAGCAAUACGUC---------------
Bradyrhizobiumsp.LMG9520AACACAUGCAAGUCGAGCGGGCAUAGCAAUAUGUC---------------
T.novellusIAM12100.     AACACAUGCAAGUCGAACGCA--CCGCAA--GGUG---------------
Az.caulinodansstr       AACACAUGCAAGUCGAGCGCC--CAGCAA--UGGG---------------
Rh.leguminosarumIAM12609AACACAUGCAAGUCGAGCGCC--CCGCAA--GGGG---------------
A.amazonense            AACACAUGCAAGUCGAACGAAGGCUUCGG--CCUU---------------
                        ****** ** ** *** *


1132                    AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
1127                    AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
B.lupini                AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
96-12                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
96-24                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
96-23                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
96-53                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
96-34                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
E.coli                  AGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGAT
96-4                    AGTGGCAGACGGGTGAGTAACACGTGGGGATCTGCCCAATGGTACGGAAT
96-6                    AGCGGCAGACGGGTGAGTAACGCGTGGGGATGTGCCCAGAGGTGGGGAAT
96-5                    AGTGGCGCACGGGTGAGTAACACGTGGGAACCTACCTTCTGGTACGGAAC
96-54                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCCTTTACTACGGAAT
P.thompsonianumstr.BTAi AGCGGCAGACGGGUGAGUAACGCGUGGGAACGUACCUUUUGGUUCGGAAC
B.japonicumIAM12608     AGCGGCAGACGGGUGAGUAACGCGUGGGAACGUACCUUUUGGUUCGGAAC
Bradyrhizobiumsp.LMG9520AGCGGCAGACGGGUGAGUAACGCGUGGGAACGUACCUUUUGGUUCGGAAC
T.novellusIAM12100.     AGUGGCAGACGGGUGAGUAACACGUGGGGAUCUGCCCAAUGGUACGGAAU
Az.caulinodansstr       AGCGGCAGACGGGUGAGUAACGCGUGGGGAUGUGCCCAAUGGUGCGGAAU
Rh.leguminosarumIAM1260 AGCGGCAGACGGGUGAGUAACGCGUGGGAACGUACCCUUUACUACGGAAU
A.amazonense            AGUGGCGCACGGGUGAGUAACGCGUGGGAACCUGCCCUUUGGUUCGGAAU
                        ** ***  ***** *** **     *** *    **         ** *
```

Figure 23. Clustal X alignment of the unique sequences in isolates collected in 1996 and the Ribosomal Database Project similar sequences

```
1132                    AACACAGGGAAACTTGTGCTAATACCGGATAAGCCCTTACG---------
1127                    AACACAGGGAAACTTGTGCTAATACCGGATAAGCCCTTACG---------
B.lupini                AACACAGGGAAACTTGTGCTAATACCGGATAAGCCCTTACG---------
96-12                   AACACAGGGAAACTTGTGCTAATACCGGATAAGCCCTTACG---------
96-24                   AACACAGGGAAACTTGTGCTAATACCGGATAAGCCCTTACG---------
96-23                   AACACAGGGAAACTTGTGCTAATACCGGATAAGCCCTTACG---------
96-53                   AACCCAGGGAAACTTGGGCTAATACCGGATAAGCCCTTACG---------
96-34                   AACTGAGGGAAACTTCAGCTAATACCGGATAAGCCCTTACG---------
E.coli                  AACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAG---------
96-4                    AGCTCCGGGAAACTGGAATTAATACCGTATGTGCCCGCAAG---------
96-6                    AACTCAGGGAAACTTGAGCTAATACCGCATGAGCCCTTCGG---------
96-5                    AACCAAGGGAAACTTTGGCTAATACCGTATACGACCTCCGG---------
96-54                   AACGCAGGGAAACTTGTGCTAATACCGTATGTGCCCTTTGG---------
P.thompsonianumstr.BTAi AACACAGGGAAACUUGUGCUAAUACCGGAUAAGCCCUUACG---------
B.japonicumIAM12608     AACACAGGGAAACUUGUGCUAAUACCGGAUAAGCCCUUACG---------
Bradyrhizobiumsp.LMG9520AACUGAGGGAAACUUCAGCUAAUACCGGAUAAGCCCUUACG---------
T.novellusIAM12100.     AGCUCCGGGAAACUGGGAUUAAUACCGUAUGUGCCCGCAAG---------
Az.caulinodansstr       AACCCAGGGAAACUUGGAUUAAUACCGCAUGUGCCCUUCGG---------
Rh.leguminosarumIAM12609AACGCAGGGAAACUUGUGCUAAUACCGUAUGUGCCCUUUGG---------
A.amazonense            AACUCCGGGAAACUGGAGCUAAUACCGGAUGAGCCUGAUGGUUGUGGAGA
                        * *    ******       ** **** *           *
```

```
1132                    ------GGGAAAGA--------TT------TATCGCCGAAAGATCGGCCC
1127                    ------GGGAAAGA--------TT------TATCGCCGAAAGATCGGCCC
B.lupini                ------GGGAAAGA--------TT------TATCGCCGAAAGATCGGCCC
96-12                   ------GGGAAAGA--------TT------TATCGCCGAAAGATCGGCCC
96-24                   ------GGGAAAGA--------TT------TATCGCCGAAAGATCGGCCC
96-23                   ------GGGAAAGA--------TT------TATCGCCGAAAGATCGGCCC
96-53                   ------GGGAAAGA--------TT------TATCGCCGAAAGATCGGCCC
96-34                   ------GGGAAAGA--------TT------TATCGCCGAAAGATCGGCCC
E.coli                  ------ACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCC
96-4                    ------GGGAAAGA--------TT------TATCGCCATTGGATGAACCC
96-6                    ------GGGAAAGA--------TT------TATCGCCTTTGGATCAACCC
96-5                    ------GTGAAAGA--------TT------TATCGCCGGAAGAGGGGCCC
96-54                   ------GGGAAAGA--------TT------TATCGGTAAAGGATCGGCCC
P.thompsonianumstr.BTAi ------GGGAAAGA--------UU------UAUCGCCGAAAGAUCGGCCC
B.japonicumIAM12608     ------GGGAAAGA--------UU------UAUCGCCGAAAGAUCGGCCC
Bradyrhizobiumsp.LMG9520------GGGAAAGA--------UU------UAUCGCCGAAAGAUCGGCCC
T.novellusIAM12100.     ------GGGAAAGA--------UU------UAUCGCCAUUGGAUGAACCC
Az.caulinodansstr       ------GGGAAAGA--------UU------UAUCGCCAUUGGAUCAACCC
Rh.leguminosarumIAM12609------GGGAAAGA--------UU------UAUCGGUAAAGGAUCGGCCC
A.amazonense            CUGUCAGGGAAAGA--------UU------UAUCGCCGAAGGAGGGGCCC
                                 *****                    *      **    ***
```

Figure 23 cont. Clustal X alignment of the unique sequences in isolates collected in 1996 and the Ribosomal Database Project similar sequence

```
1132                    GCGTCTGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCA
1127                    GCGTCTGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCA
B.lupini                GCGTCTGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCA
96-12                   GCGTCTGATTAGCTAGTTGGTAGGGTAATGGCCTACCAAGGCGACGATCA
96-24                   GCGTCTGATTAGCTAGTTGGTAGGGTAATGGCCTACCAAGGCGACGATCA
96-23                   GCGTCTGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCA
96-53                   GCGTCTGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCA
96-34                   GCGTCTGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCA
E.coli                  AGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCC
96-4                    GCGTCGGATTAGCTAGTTGGTGTGGTAAAGGCGCACCAAGGCGACGATCC
96-6                    GCGTCAGATTAGCTAGTTGGTAGGGTAATGGCCTACCAAGGCGACGATCT
96-5                    GCGTCCGATTAGGTAGTTGGTGGGGTAACGGCTCACCAAGCCGACGATCG
96-54                   GCGTTGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCC
P.thompsonianumstr.BTAi GCGUCUGAUUAGCUAGUUGGUAGGGUAAUGGCCUACCAAGGCGACGAUCA
B.japonicumIAM12608     GCGUCUGAUUAGCUAGUUGGUGAGGUAAUGGCUCACCAAGGCGACGAUCA
Bradyrhizobiumsp.LMG9520GCGUCUGAUUAGCUAGUUGGUGAGGUAAUGGCUCACCAAGGCGACGAUCA
T.novellusIAM12100.     GCGUCGGAUUAGCUAGUUGGUGUGGUAAAGGCGCACCAAGGCGACGAUCC
Az.caulinodansstr       GCGUCUGAUUAGCUAGUUGGUGAGGUAAAGGCUCACCAAGGCGACGAUCA
Rh.leguminosarumIAM12609GCGUUGGAUUAGCUAGUUGGUGGGGUAAAGGCCUACCAAGGCGACGAUCC
A.amazonense            GCGUCCGAUUAGGUAGUUGGUGAGGUAACGGCUCACCAAGCCGACGAUCG
                              **  **  **  **   ** ** ***  *** ** ****** *

1132                    GTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCC
1127                    GTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCC
B.lupini                GTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCC
96-12                   GTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCC
96-24                   GTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCC
96-23                   GTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCC
96-53                   GTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCC
96-34                   GTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCC
E.coli                  CTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCC
96-4                    GTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCC
96-6                    GTAGCTGGTCTGAGAGGATGACCAGCCACACTGGGACTGAGACACGGCCC
96-5                    GTAGCTGGTCTGAGAGGATGACCAGCCACACTGGGACTGAGACACGGCCC
96-54                   ATAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCC
P.thompsonianumstr.BTAi GUAGCUGGUCUGAGAGGAUGAUCAGCCACAUUGGGACUGAGACACGGCCC
B.japonicumIAM12608     GUAGCUGGUCUGAGAGGAUGAUCAGCCACAUUGGGACUGAGACACGGCCC
Bradyrhizobiumsp.LMG9520GUAGCUGGUCUGAGAGGAUGAUCAGCCACAUUGGGACUGAGACACGGCCC
T.novellusIAM12100      GUAGCUGGUCUGAGAGGAUGAUCAGCCACACUGGGACUCAGACACGGCCC
Az.caulinodansstr       GUAGCUGGUCUGAGAGGAUGAUCAGCCACACUGGGACUGAGACACGGCCC
Rh.leguminosarumIAM1260 AUAGCUGGUCUGAGAGGAUGAUCAGCCACAUUGGGACUGAGACACGGCCC
A.amazonense            GUAGCUGGUCUGAGAGGAUGAUCAGCCACACUGGGACUGAGACACGGCCC
                          *** ** * ******* ** ********  ** **  ******** **
```

Figure 23 cont. Clustal X alignment of the unique sequences in isolates collected in 1996 and the Ribosomal Database Project similar sequences

```
1132                        AAACTCCTACGG
1127                        AAACTCCTACGG
B.lupini                    AAACTCCTACGG
96-12                       AAACTCCTACGG
96-24                       AAACTCCTACGG
96-23                       AAACTCCTACGG
96-53                       AAACTCCTACGG
96-34                       AAACTCCTACGG
E.coli                      AGACTCCTACGG
96-4                        AGACTCCTACGG
96-6                        AGACTCCTACGG
96-5                        AGACTCCTACGG
96-54                       AAACTCCTACGG
P.thompsonianumstr.BTAi     AAACUCCUACGG
B.japonicumIAM12608         AAACUCCUACGG
Bradyrhizobiumsp.LMG9520AAACUCCUACGG
T.novellusIAM12100.         AGACUCCUACGG
Az.caulinodansstr           AGACUCCUACGG
Rh.leguminosarumIAM12609AAACUCCUACGG
A.amazonense                AGACUCCUACGG
                              ** ** ****
```

Figure 23 cont. Clustal X alignment of the unique sequences in isolates collected in 1996 and the Ribosomal Database Project similar sequences

Sequence type	Sample ID	REP type	Sequence similarity in Ribosomal Database Project	Nodulator in lab trials (y/n)
2	1127, (96-9, -14, -19, -35, -37, -41), (-27, -29, -30, -32)	A, A1, M	*B. lupini* 1.00 similarity	Y
	96-46	A2	sequence not possible	test not possible
5	(96-43, -44, -45, -48, -50, -51, -55, -57, -58, -59, -60), (-33, -36), (-24, -26)	A3, C, S	*Photorhizobium thompsonianum str.* BTAi .977 similarity	Y
3	(96-49, -53), -56, -11	B, Q, N	*B. japonicum* (IAM12608) .954 similarity	test not possible
1	(96-10, -12, 18)	C1	*P. thompsonianum str.* BTAi 1.00 similarity	Y
1	(96-13, -15), -47	C2, G	*P. thompsonianum str.* BTAi 1.00 similarity	test not possible
7	(96-22, -34)	D	*Bradyrhizobium sp.* (9520) 1.00 similarity	Y
	96-28	E	sequence not possible	Y
11	96-6	F	*Azorhizobium caulidonans* .650 similarity	Y
3	96-9, 31	H, K	*B. japonicum* (IAM12608) .954 similarity	Y
2	96, 16	I	*B. lupini* 1.00 similarity	test not possible
	96-39	J	sequence not possible	Y
	96-40	J2	sequence not possible	Y
4	96-23	L	*B. japonicum* (IAM12608) .977 similarity	Y
8	96-54	O	*R. leguminosarum* (IAM12609) 1.00 similarity	Y
9	96-5	P	*Azospirillum amazonense* str Y1.632 similarity	test not possible
6	1132	R	*B. lupini* .977 similarity	Y
10	96-4	T	*T. novellus (IAM12100)* .949 similarity	Y

TABLE 12. 1996 similarity ranks with the RDP (Ribosomal Database Project) and nodulation trials results summary
(parenthesis indicates samples with identical REP type)

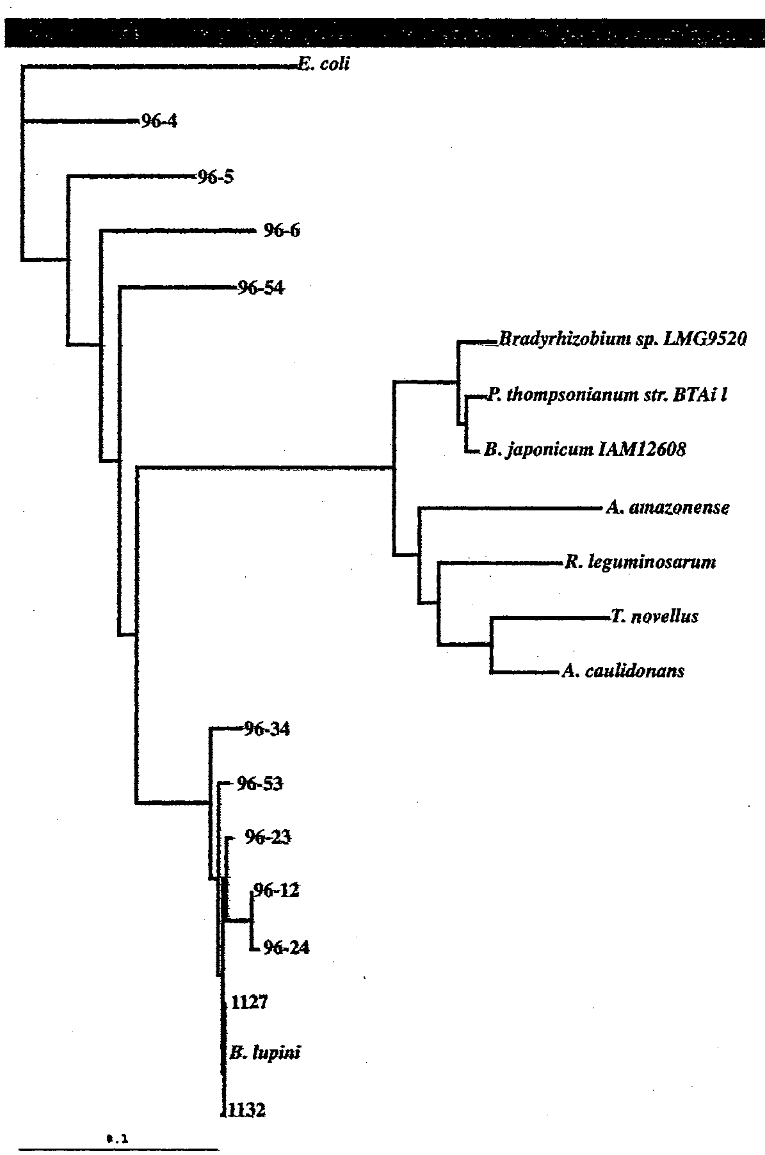

Figure 24. Phylogram created with N-J method through ClustalX and viewed through Viewtree 1.0 from 1996 unique sequences and their Ribosomal Database Project similar sequences.

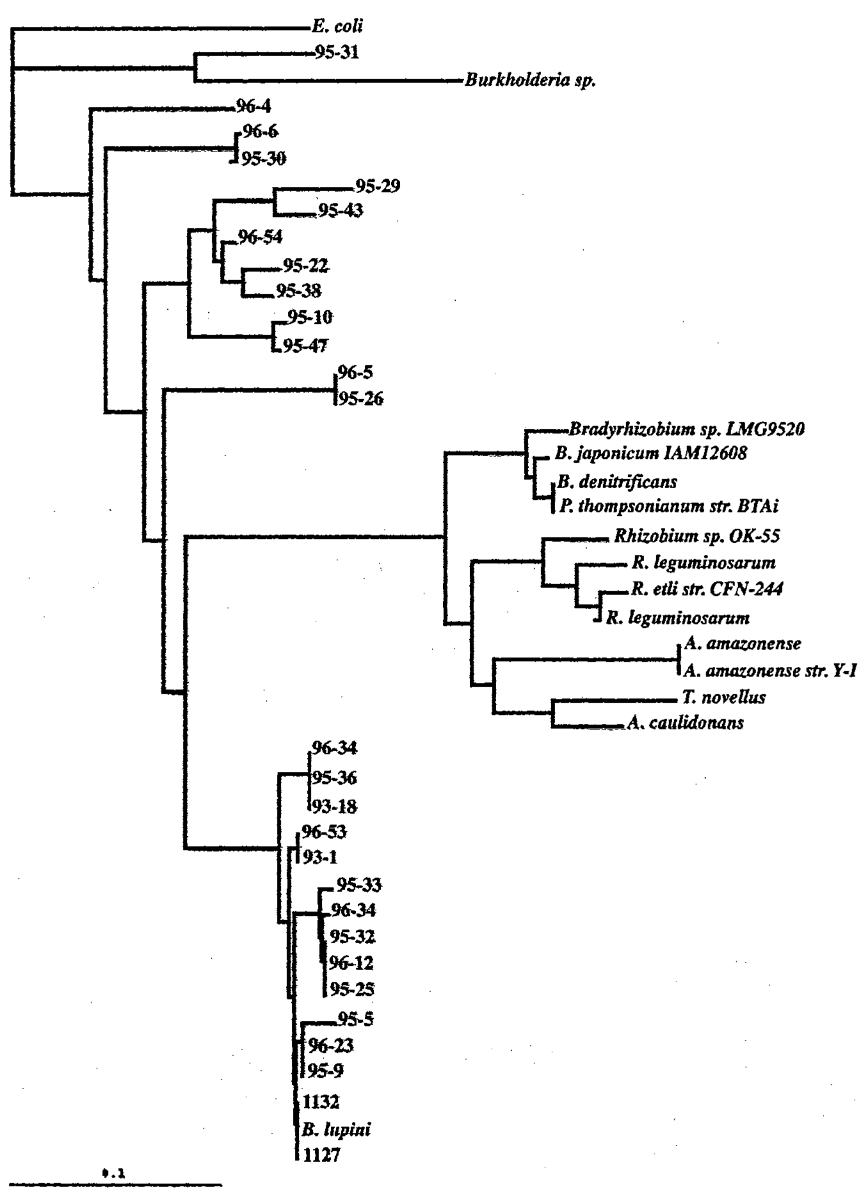

Figure 25. Phylogram created with N-J method through ClustalX and viewed through Viewtree 1.0 from all unique sequences and their Ribosomal Database Project similar sequences.

CLUSTAL X (1.64b) multiple sequence alignment

```
B.denitrificansLMG8443 AACACAUGCAAGUCGAGCGGGCGUA-------GCAAUA--------CGUC
P.thompsonianumstr.BTAiAACACAUGCAAGUCGAGCGGGCGUA-------GCAAUA--------CGUC
B.japonicumIAM12608    AACACAUGCAAGUCGAGCGGGCGUA-------GCAAUA--------CGUC
BradyrhizobiumspLMG9520AACACAUGCAAGUCGAGCGGGCAUA-------GCAAUA--------UGUC
R.etlistr.CFN-244.     --------------------------------------------------
R.leguminosarumIAM12609AACACAUGCAAGUCGAGCGCC--CC-------GCAA----------GGGG
R.leguminosarum        AACACAUGCAAGUCGAGCGCC--CC-------GCAA----------GGGG
Rhizobiumsp.str.OK-55. AACACAUGCAAGUCGAGCGCC--CC-------GCAA----------GGGG
Az.amazonensestr.Y-1DSMAACACAUGCAAGUCGAACGAAGGCU-------UCGG----------CCUU
A.amazonense           AACACAUGCAAGUCGAACGAAGGCU-------UCGG----------CCUU
T.novellusIAM12100.    AACACAUGCAAGUCGAACGCA--CC-------GCAA----------GGUG
Az.caulinodansstr      AACACAUGCAAGUCGAGCGCC--CA-------GCAA----------UGGG
1132                   AACACATGCAAGTCGAGC-----TA-------GCAATA--------CGTC
1127                   AACACATGCAAGTCGAGCGGGCGTA-------GCAATA--------CGTC
B.lupini               AACACATGCAAGTCGAGCGGGCGTA-------GCAATA--------CGTC
96-23                  AACACATGCGAGTCGAGCGGGCGTA-------GCAATA--------CGTC
95-5                   -ATACATGCGGCACGAGCGGGCGTA-------GCAATA--------CGTC
95-9                   AACACATGCGAGTCGAGCGGGCGTA-------GCAATA--------CGTC
96-12                  AACACATGCAAGTCGAGCGGGCGTA-------GCAATA--------CGTC
95-32                  AACACATGCAAGTCGAGCGGGCGTA-------GCAATA--------CGTC
95-25                  AACACATGCAAGTCGAGCGGGCGTA-------GCAATA--------CGTC
96-24                  AACACATGCGAGTCGAGCGGGCGTA-------GCAATA--------CGTC
95-33                  AACACATGCAAGTCGAGCGGGCGTA-------ACAATA--------CGTC
96-53                  AACACATGCAAGTCGAGCGGGCGTA-------GCAATA--------CGTC
93-1                   AACACATGCAAGTCGAGCGGGCGTA-------GCAATA--------CGTC
96-34                  AACACATGCAAGTCGAGCGGGCATA-------GCAATA--------TGTC
95-36                  AACACATGCAAGTCGAGCGGGCATA-------GCAATA--------TGTC
93-18                  AACACATGCAAGTCGAGCGGGCATA-------GCAATA--------TGTC
95-29                  --AACACATGCAAGTCGAGCG--CC-------CGCA----------GGGG
95-43                  ACTACGTGAGGCAGCAGTGGG--CC-------GCAA----------GGGG
96-54                  AACACATGCAAGTCGAGCGCC--CC-------GCAA----------GGGG
95-22                  AACACATGCAAGTCGAGCGCC--CC-------GCAA----------GGGG
95-38                  AACACATGCAAGTCGAGCGCC--C--------GCAA----------GGGG
95-10                  AACACATGCGAGACGAGCGCC--CC-------GCAA----------GGGG
95-47                  AACACATGCAAGTCGAGCGCC--CC-------GCA-----------GGGG
96-5                   AACACATGCAAGTCGAACGCT--CC-------GCAA----------GGGG
95-26                  AACACATGCAAGTCGAACGCT--CC-------GCAA----------GGGG
96-6                   AACACATGCAAGTCGAGCGGCATCC-------TTC-GG--------GGTG
95-30                  AACACATGCAAGTCGAGCG-CATCC-------TT--GG--------GGTG
96-4                   AACACATGCAAGTCGAACGCA--CC-------GCAA----------GGTG
95-31                  -ACACATGCAAGTCGAACGGCAGCACG--GGAGCAATCC---TGGTGGCG
Burkholderiasp.        UACACAUGCAAGUCGAACGGCAGCACG--GGAGUAAUCC---UGGUGUCG
E.coli                 AACACATGCAAGTCGAACGGTAACAGGAAGAAGCTTGCTCTTTGCTGACG
```

Figure 26. Clustal X alignment of all the unique sequences collected and the Ribosomal Database Project similar sequences

```
B.denitrificansLMG8443  AGCGGCAGACGGGUGAGUAACGCGUGGGAACGUACCUUUUGGUUCGGAAC
P.thompsonianumstr.BTAi AGCGGCAGACGGGUGAGUAACGCGUGGGAACGUACCUUUUGGUUCGGAAC
B.japonicumIAM12608     AGCGGCAGACGGGUGAGUAACGCGUGGGAACGUACCUUUUGGUUCGGAAC
BradyrhizobiumspLMG9520 AGCGGCAGACGGGUGAGUAACGCGUGGGAACGUACCUUUUGGUUCGGAAC
R.etlistr.CFN-244.      -----CAGACGGGUGAGUAACGCGUGGGAACGUACCCUUUACUACGGAAU
Rh.leguminosarumIAM12609AGCGGCAGACGGGUGAGUAACGCGUGGGAACGUACCCUUUACUACGGAAU
R.leguminosarum         AGCGGCAGACGGGUGAGUAACGCGUGGGAAUCUACCCUUGACUACGGAAU
Rhizobiumsp.str.OK-55.  AGCGGCAGACGGGUGAGUAACGCGUGGGAAUCUACCCAUCCCUACGGAAC
Az.amazonensestr.Y-1DSM AGUGGCGCACGGGUGAGUAACGCGUGGGAACCUGCCCUUUGGUUCGGAAU
A.amazonense            AGUGGCGCACGGGUGAGUAACGCGUGGGAACCUGCCCUUUGGUUCGGAAU
T.novellusIAM12100.     AGUGGCAGACGGGUGAGUAACACGUGGGGAUCUGCCCAAUGGUACGGAAU
Az.caulinodansstr       AGCGGCAGACGGGUGAGUAACGCGUGGGGAUGUGCCCAAUGGUGCGGAAU
1132                    AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
1127                    AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
B.lupini                AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
96-23                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
95-5                    AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
95-9                    AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
96-12                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
95-32                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
95-25                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
96-24                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
95-33                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
96-53                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
93-1                    AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
96-34                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
95-36                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
93-18                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCTTTTGGTTCGGAAC
95-29                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCCTTTACTACGGAAT
95-43                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCCTTTACTACGGAAT
96-54                   AGCGGCAGACGGGTGAGTAACGCGTGGGAACGTACCCTTTACTACGGAAT
95-22                   AGCGGCAGACGGGTGAGTAACGCGTGGGAATCTACCCTTGACTACGGAAT
95-38                   AGCGGCAGACGGGTGAGTAACGCGTGGGAATGTACCCTCTACTACGGAAT
95-10                   AGCGGCAGACGGGTGAGTAACGCGTGGGAATCTACCCATCCCTACGGAAC
95-47                   AGCGGCAGACGGGTGAGTAACGCGTGGGAATCTACCCATCCCTACGGAAC
96-5                    AGTGGCGCACGGGTGAGTAACACGTGGGAACCTACCTTCTGGTACGGAAC
95-26                   AGTGGCGCACGGGTGAGTAACACGTGGGAACCTACCTTCTGGTACGGAAC
96-6                    AGCGGCAGACGGGTGAGTAACGCGTGGGGATGTGCCCAGAGGTGGGGAAT
95-30                   AGCGGCAGACGGGTGAGTAACGCGTGGGGATGTGCCCAGAGGTGGGGAAT
96-4                    AGTGGCAGACGGGTGAGTAACACGTGGGGATCTGCCCAATGGTACGGAAT
95-31                   AGTGGCGAACGGGTGAGTAATACATCGGAACGTGTCCTGTAGTGGGGGAT
Burkholderiasp.         AGUGGCGAACGGGUGAGUAAUACAUCGGAACGUGUCCUGUAGUGGGGGAU
E.coli                  AGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGAT
                            *   ***** *** **      ** *     *         ** *
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Figure 26 cont. Clustal X alignment of all the unique sequences collected and the Ribosomal Database Project similar sequences

```
B.denitrificansLMG8443  AACACAGGG-AAACUUGUGCUAAUACCGGAUAAGCCCUUACG--------
P.thompsonianumstr.BTAi AACACAGGG-AAACUUGUGCUAAUACCGGAUAAGCCCUUACG--------
B.japonicumIAM12608     AACACAGGG-AAACUUGUGCUAAUACCGGAUAAGCCCUUACG--------
Bradyrhizobiumsp.LMG9520AACUGAGGG-AAACUUCAGCUAAUACCGGAUAAGCCCUUACG--------
R.etlistr.CFN-244.      AACGCAGGG-AAACUUGUGCUAAUACCGUAUGUGCCCUUCGG--------
Rh.leguminosarumIAM12609AACGCAGGG-AAACUUGUGCUAAUACCGUAUGUGCCCUUUGG--------
R.leguminosarum         AACGCAGGG-AAACUUGUGCUAAUACCGUAUGUGUCCUUCGG--------
Rhizobiumsp.str.OK-55.  AACUCCGGG-AAACUGGAGCUAAUACCGUAUACGCCCUUCGG--------
Az.amazonensestr.Y-1DSM AACUCCGGG-AAACUGGAGCUAAUACCGGAUGAGCCUGAUGGUUGUGGAG
A.amazonense            AACUCCGGG-AAACUGGAGCUAAUACCGGAUGAGCCUGAUGGUUGUGGAG
T.novellusIAM12100.     AGCUCCGGG-AAACUGGGAUUAAUACCGUAUGUGCCCGCAA---G-----
Az.caulinodansstr       AACCCAGGG-AAACUUGGAUUAAUACCGCAUGUGCCCUUCG---G-----
1132                    AACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG--------
1127                    AACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG--------
B.lupini                AACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG--------
96-23                   AACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG--------
95-5                    AACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG--------
95-9                    AACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG--------
96-12                   AACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG--------
95-32                   AACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG--------
95-25                   AACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG--------
96-24                   AACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG--------
95-33                   AACACAGGG-AAACTTGTGCTAATACCGGATAAGCCCTTACG--------
96-53                   AACCCAGGG-AAACTTGGGCTAATACCGGATAAGCCCTTACG--------
93-1                    AACCCAGGG-AAACTTGGGCTAATACCGGATAAGCCCTTACG--------
96-34                   AACTGAGGG-AAACTTCAGCTAATACCGGATAAGCCCTTACG--------
95-36                   AACTGAGGG-AAA-TTCAGCTAATACCGGATAAGCCCTTACG--------
93-18                   AACTGAGGG-AAACTTCAGCTAATACCGGATAAGCCCTTACG--------
95-29                   AACGCAGGG-AAACTTGTGCTAATACCGTATGTGCCCTTCGG--------
95-43                   AACGCAGGG-AAACTTGTGCTAATACCGTATGTGCCCTTCGG--------
96-54                   AACGCAGGG-AAACTTGTGCTAATACCGTATGTGCCCTTTGG--------
95-22                   AACGCAGGG-AAACTTGTGCTAATACCGTATGTGTCCTTCGG--------
95-38                   AACGCAGGG-AAACTTGTGCTAATACCGTATGTGTCCTTTGG--------
95-10                   AACTCCGGG-AAACTGGAGCTAATACCGTATACGCCCTTCGG--------
95-47                   AACTCCGGGGAAACTGGAGCTAATACCGTATACGCCCTTCGG--------
96-5                    AACCAAGGG-AAACTTTGGCTAATACCGTATACGACCTCCGG--------
95-26                   AACCAAGGG-AAACTTTGGCTAATACCGTATACGACCTCCGG--------
96-6                    AACTCAGGG-AAACTTGAGCTAATACCGCATGAGCCCTTCGG--------
95-30                   AACTCAGGG-AAACTTGAGCTAATACCGCATGAGCCCTTCGG--------
96-4                    AGCTCCGGG-AAACTGGAATTAATACCGTATGTGCCCGCAAG--------
95-31                   AGCCCGGCG-AAAGCCGGATTAATACCGCATACGCTCTGCGGAGGAAAGC
Burkholderiasp.         AGCCCGGCG-AAAGCCGGAUUAAUACCGCAUACGAUCUGUGGAUGAAAGC
E.coli                  AACTACTGG-AAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGA
                        * *     * ***        ** **** *
```

Figure 26 cont. Clustal X alignment of all the unique sequences collected and the Ribosomal Database Project similar sequence

```
B.denitrificansLMG8443   -------GGGA-AAGAUUUAUCGCCGAAAGAUCGGCCCGCGUCUGAUUAG
P.thompsonianumstr.BTAi  -------GGGA-AAGAUUUAUCGCCGAAAGAUCGGCCCGCGUCUGAUUAG
B.japonicumIAM12608      -------GGGA-AAGAUUUAUCGCCGAAAGAUCGGCCCGCGUCUGAUUAG
Bradyrhizobiumsp.LMG9520-------GGGA-AAGAUUUAUCGCCGAAAGAUCGGCCCGCGUCUGAUUAG
R.etlistr.CFN-244.       -------GGGA-AAGAUUUAUCGGUAAGGGAUCGGCCCGCGUUGGAUUAG
Rh.leguminosarumIAM12609-------GGGA-AAGAUUUAUCGGUAAAGGAUCGGCCCGCGUUGGAUUAG
R.leguminosarum          -------GAGA-AAGAUUUAUCGGUCAAGGAUGAGCCCGCGUUGGAUUAG
Rhizobiumsp.str.OK-55.   -------GGGA-AAGAUUUAUCGGGGAUGGAUGAGCCCGCGUUGGAUUAG
Az.amazonensestr.Y-1DSM  ACUGUCAGGGA-AAGAUUUAUCGCCGAAGGAGGGGCCCGCGUCCGAUUAG
A.amazonense             ACUGUCAGGGA-AAGAUUUAUCGCCGAAGGAGGGGCCCGCGUCCGAUUAG
T.novellusIAM12100.      -------GGGA-AAGAUUUAUCGCCAUUGGAUGAACCCGCGUCGGAUUAG
Az.caulinodansstr        -------GGGA-AAGAUUUAUCGCCAUUGGAUCAACCCGCGUCUGAUUAG
1132                     -------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATTAG
1127                     -------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATTAG
B.lupini                 -------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATTAG
96-23                    -------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATTAG
95-5                     -------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATTAG
95-9                     -------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATTAG
96-12                    -------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATTAG
95-32                    -------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATTAG
95-25                    -------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATTAG
96-24                    -------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATTAG
95-33                    -------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATTAG
96-53                    -------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATTAG
93-1                     -------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATTAG
96-34                    -------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATTAG
95-36                    -------GGGA-AAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATTAG
93-18                    -------GGGA-AAGATTTATCGC-GAAAGATCGGCCCGCGTCTGATTAG
95-29                    -------GGGA-AAGATTTATCGGTAAGGGATCGGCCCGCGTTGGATTAG
95-43                    -------GGGA-AAGATTTATCGGTAAGGGATCGGCCCGCGTTGGATTAG
96-54                    -------GGGA-AAGATTTATCGGTAAAGGATCGGCCCGCGTTGGATTAG
95-22                    -------GAGA-AAGATTTATCGGTCAAGGATGAGCCCGCGTTGGATTAG
95-38                    -------GGGA-AAGATTTATCGGTAGGGGATGAGCCCGCGTTGGATTAG
95-10                    -------GGGA-AAGATTTATCGGGGATGGATGAGCCCGCGTTGGATTAG
95-47                    -------GGGA-AAGATTTATCGGGGATGGATGAGCCCGCGTTGGATTAG
96-5                     -------GTGA-AAGATTTATCGCCGGAAGAGGGGCCCGCGTCCGATTAG
95-26                    -------GTGA-AAGATTTATCGCCGGAAGAGGGGCCCGCGTCCGATTAG
96-6                     -------GGGA-AAGATTTATCGCCTTTGGATCAACCCGCGTCAGATTAG
95-30                    -------GGGA-AAGATTTATCGCCTTTGGATCAACCCGCGTCAGATTAG
96-4                     -------GGGA-AAGATTTATCGCCATTGGATGAACCCGCGTCGGATTAG
95-31                    GGGGGATCCTTCGGGACCTCGCGCTACAGGGGCGGCCGATGGCAGATTAG
Burkholderiasp.          GGGGGAUCUU--AGGACCUCGCGCUACAGGGGCGGCCGAUGGCAGAUUAG
E.coli                   GGGGGACCTT--CGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAG
                                       *       *      *     **       **  **
```

Figure 26 cont. Clustal X alignment of all the unique sequences collected and the Ribosomal Database Project similar sequences

```
B.denitrificansLMG8443   CUAGUUGGUAGGGUAAUGGCCUACCAAGGCGACGAUCAGUAGCUGGUCUG
P.thompsonianumstr.BTAi  CUAGUUGGUAGGGUAAUGGCCUACCAAGGCGACGAUCAGUAGCUGGUCUG
B.japonicumIAM12608      CUAGUUGGUGAGGUAAUGGCUCACCAAGGCGACGAUCAGUAGCUGGUCUG
Bradyrhizobiumsp.LMG9520CUAGUUGGUGAGGUAAUGGCUCACCAAGGCGACGAUCAGUAGCUGGUCUG
R.etlistr.CFN-244.       CUAGUUGGUGGGGUAAAGGCCUACCAAGGCGACGAUCCAUAGCUGGUCUG
Rh.leguminosarumIAM12609CUAGUUGGUGGGGUAAAGGCCUACCAAGGCGACGAUCCAUAGCUGGUCUG
R.leguminosarum          CUAGUUGGUGGGGUAAAGGCCUACCAAGGCGACGAUCCAUAGCUGGUCUG
Rhizobiumsp.str.OK-55.   CUAGUUGGUGGGGUAAAGGCCUACCAAGGCGACGAUCCAUAGCUGGUCUG
Az.amazonensestr.Y-1DSM  GUAGUUGGUGAGGUAACGGCUCACCAAGCCGACGAUCGGUAGCUGGUCUG
A.amazonense             GUAGUUGGUGAGGUAACGGCUCACCAAGCCGACGAUCGGUAGCUGGUCUG
T.novellusIAM12100.      CUAGUUGGUGUGGUAAAGGCGCACCAAGGCGACGAUCCGUAGCUGGUCUG
Az.caulinodansstr        CUAGUUGGUGAGGUAAAGGCUCACCAAGGCGACGAUCAGUAGCUGGUCUG
1132                     CTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTG
1127                     CTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTG
B.lupini                 CTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTG
96-23                    CTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTG
95-5                     CTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTG
95-9                     CTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTG
96-12                    CTAGTTGGTAGGGTAATGGCCTACCAAGGCGACGATCAGTAGCTGGTCTG
95-32                    CTAGTTGGTAGGGTAATGGCCTACCAAGGCGACGA-CAGTAGCTGGTCTG
95-25                    CTAGTTGGTAGGGTAATGGCCTACCAAGGCGACGATCAGTAGCTGGTCTG
96-24                    CTAGTTGGTAGGGTAATGGCCTACCAAGGCGACGATCAGTAGCTGGTCTG
95-33                    CTAGTTGGTCGGGTAATGGCCTACCAAGGCGACGATCAGTAGCTGGTCTG
96-53                    CTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTG
93-1                     CTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTG
96-34                    CTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTG
95-36                    CTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTG
93-18                    CTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCAGTAGCTGGTCTG
95-29                    CTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTG
95-43                    CTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTG
96-54                    CTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTG
95-22                    CTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTG
95-38                    CTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTG
95-10                    CTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTG
95-47                    CTAGTTGGTGGGGTAAAGGCCTACCAAGGCG---ATCCATAGCTGGTCTG
96-5                     GTAGTTGGTGGGGTAACGGCTCACCAAGCCGACGATCGGTAGCTGGTCTG
95-26                    GTAGTTGGTGGGGTAACGGCTCACCAAGCCGACGATCGGTAGCTGGTCTG
96-6                     CTAGTTGGTAGGGTAATGGCCTACCAAGGCGACGATCTGTAGCTGGTCTG
95-30                    CTAGTTGGTAGGGTAATGGCCTACCAAGGCGACGATCTGTAGCTGGTCTG
96-4                     CTAGTTGGTGTGGTAAAGGCGCACCAAGGCGACGATCCGTAGCTGGTCTG
95-31                    CTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCTGGTCTG
Burkholderiasp.          CUAGUUGGUGGGGUAAAGGCCUACCAAGGCGACGAUCUGUAGCUGGUCUG
E.coli                   CTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTG
                           **  **   ** ** ***  *** ** **   * *   ***. ** * *
```

Figure 26 cont. Clustal X alignment of all the unique sequences collected and the Ribosomal Database Project similar sequences

```
B.denitrificansLMG8443  AGAGGAUGAUCAGCCACAUUGGGACUGAGACACGGCCCAAACUCCUACGG
P.thompsonianumstr.BTAi AGAGGAUGAUCAGCCACAUUGGGACUGAGACACGGCCCAAACUCCUACGG
B.japonicumIAM12608     AGAGGAUGAUCAGCCACAUUGGGACUGAGACACGGCCCAAACUCCUACGG
Bradyrhizobiumsp.LMG9520AGAGGAUGAUCAGCCACAUUGGGACUGAGACACGGCCCAAACUCCUACGG
R.etlistr.CFN-244.      AGAGGAUGAUCAGCCACAUUGGGACUGAGACACGG---------------
Rh.leguminosarumIAM12609AGAGGAUGAUCAGCCACAUUGGGACUGAGACACGGCCCAAACUCCUACGG
R.leguminosarum         AGAGGAUGAUCAGCCACAUUGGGACUGAGACACGGCCCAAACUCCUACGG
Rhizobiumsp.str.OK-55.  AGAGGAUGAUCAGCCACAUUGGGACUGAGACACGGCCCAAACUCCUACGG
Az.amazonensestr.Y-1DSM AGAGGAUGAUCAGCCACACUGGGACUGAGACACGGCCCAGACUCCUACGG
A.amazonense            AGAGGAUGAUCAGCCACACUGGGACUGAGACACGGCCCAGACUCCUACGG
T.novellusIAM12100.     AGAGGAUGAUCAGCCACACUGGGACUCAGACACGGCCCAGACUCCUACGG
Az.caulinodansstr       AGAGGAUGAUCAGCCACACUGGGACUGAGACACGGCCCAGACUCCUACGG
1132                    AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
1127                    AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
B.lupini                AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
96-23                   AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
95-5                    AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
95-9                    AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
96-12                   AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
95-32                   AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
95-25                   AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
96-24                   AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
95-33                   AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
96-53                   AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
93-1                    AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
96-34                   AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
95-36                   AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
93-18                   AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
95-29                   AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
95-43                   AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
96-54                   AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
95-22                   AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
95-38                   AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
95-10                   AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
95-47                   AGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGG
96-5                    AGAGGATGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
95-26                   AGAGGATGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
96-6                    AGAGGATGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
95-30                   AGAGGATGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
96-4                    AGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
95-31                   AGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
Burkholderiasp.         AGAGGACGACCAGCCACACUGGGACUGAGACACGGCCCAGACUCCUACGG
E.coli                  AGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG
                        ****** ** ********  ** **  ********
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Figure 26 cont. Clustal X alignment of all the unique sequences collected and the Ribosomal Database Project similar sequences

sequencing does not allow for a total identification, but strongly suggested the closest relatives of an isolate.

Nodulation Trials

Nodulation data allowed for the assessment that the isolates recovered were the true nodulators of the legume (and not simply contaminants). It also helped in clarifying whether an organism whose partial 16S sequence could not be determined were in fact a "pigeonpea" rhizobia.

For the 1993 sample set, nodulation data was only available for isolates 10 and 16 because these were the only viable isolates after four years in cold storage which can be deleterious for rhizobia. Both isolates nodulated pigeonpea under laboratory (greenhouse) conditions. Both isolates belong to the larger cluster of identical partial sequence in 1993 (see Table 10).

In the 1995 data set the nodulation data confirmed that the isolates sequenced and determined to be slow and fast-growing rhizobia that nodulate pigeonpeas. For those where sequence data was not available it confirmed their status as "pigeonpea" rhizobia, including isolates C2-7, C2-1 and C3-6 (see Table 11).

In the 1996 data set, isolates were also confirmed as pigeonpea rhizobia including those without partial sequencing data, isolates 3-2:8, 3-2:19 and 3-2:20 (see Table12). Nodulation data allows for a more complete analysis and clarification of the organisms previously analyzed and classified as rhizobia. Only a handful of isolates lacked partial sequence and nodulation data because cells were not viable after freezer storage, but the analysis was completed for all the other organisms.

The available data on pigeonpea nodulation patterns is not complete. The data provided by this project suggested that other rhizobia known to nodulate leguminous trees, and other legumes can nodulate pigeonpea and were present in Oklahoma soils. Phylogenetic data obtained indicated that both fast- and slow-growing rhizobia can be

found in these experimental plots. It was previously reported that both fast and slow growers could nodulate pigeonpea but the legume host range had not been clearly defined (3, 51). With these new data the pigeonpea host range was described for the experimental plot soil.

Chapter 7

Conclusion

There is a chance of not recovering inoculant strains after their introduction into the soil and interaction with other soil organisms. The inoculant strains *Bradyrhizobium* sp. (Cajanus) TAL1127 and TAL1132 were persistent through four years after their initial introduction into Oklahoma soils. These inoculant strains are suitable for introduction with pigeonpea seeds as competitive inoculants. The recovery of the inoculant strains demonstrates that these strains were able to survive and persist saprophytically in the experimental soils.

Many rhizobial strains were found to nodulate the introduced pigeonpea in the experimental plots. The potential for competition with introduced inoculant strains was demonstrated, but TAL1127 and TAL1132 seemed to be able to compete effectively with them. The inoculant strain TAL1127 seemed to be superior to the inoculant strain TAL1132 as an inoculant, because it was recovered through the years in higher numbers than TAL1132. It was a good prompt nodulator that did not get outcompeted by the indigenous rhizobial population. In conclusion, inoculant strains TAL1127 and TAL1132 have the ability to form nodules in introduced pigeonpea and can persist in the soil throughout at least four years.

Much was also learned from the genetic diversity of the indigenous "pigeonpea" rhizobia. As described previously pigeonpea is promiscuously nodulated by both slow and fast-growing rhizobia. In this study, the diversity of the group of rhizobia nodulating pigeonpea changed from year to year. This diversity allowed for the collection and analysis of additional "pigeonpea" rhizobia. The comparisons of partial sequences, ARDRA, and confirmation of nodulators in nodulation assays allowed for a rapid identification of pigeonpea rhizobia.

REP PCR allowed for the description of a heterogeneous group of pigeonpea rhizobial strains in Oklahoma soils. These rhizobial strains had the opportunity to outcompete the inoculant strains, but the inoculants persisted through the four years of sampling. Based on REP PCR there were several pigeonpea rhizobial strains capable of nodulating pigeonpea.

The 16S, 23S IGS RFLP analysis indicated that these strains characterized by REP PCR were in fact closely related especially the slow-growers. The use of both REP PCR and 16S, 23S IGS RFLP analysis allowed to characterize a group of strains of rhizobia and to study their genetic relationships. Based on REP PCR analysis I could define the diversity of the indigenous pigeonpea rhizobia and look at study relationships to strains level, but clustered them to species level by using the 16S, 23S IGS RFLP analysis. These techniques did not allow for direct phylogenetic analysis, so ARDRA analysis and 16S partial sequencing was utilized also.

16S RFLP analysis allowed for the grouping of organisms based on their relatedness that split them into fast- and slow-growing rhizobia. By partial sequencing of the isolates it was possible to identify relatedness to close relatives and their place phylogenetically. This information can enrich the data already available about what kinds of rhizobia can nodulate pigeonpea. With 16S analysis there was also a confirmation of isolation of true rhizobia instead of contaminants.

Four years of sampling to identify diversity are not enough to demonstrate equilibrium in the population after the introduction of microorganisms. There could still be other pigeonpea rhizobia in the experimental plots that were not trapped due to competition from other rhizobia. The four years of the study did allow for a genotypic analysis of the diversity among the indigenous pigeonpea rhizobia. There is a chance that by going back to the experimental plots and using pigeonpea seeds as a trap, we could see if the introduced inoculant strains are still present in the soil. Additionally, the indigenous slow-growing

rhizobial population may be the reason why the inoculant TAL1132 was not as prevalent as TAL1127; i.e. it could not compete sufficiently.

If a farmer in Oklahoma desired to use pigeonpea as an intercrop plant to protect the soil from erosion I would recommend TAL1127 as the inoculant of choice because it was able to compete better than the second inoculant strain tested (TAL1132). Tests should still be performed to confirm that there is not already sufficient nitrogen available in the soil and that the inoculant strain can persist in the new soil conditions.

Thus, the techniques used for this study were proven powerful and allowed for a thorough study of genetic diversity. These techniques also allowed for the description of new pigeonpea rhizobia.

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