



Ghent University, Belgium
Faculty of Sciences
Department of Biochemistry, Physiology and Microbiology
Laboratory of Microbiology

**Molecular study of the microbial community in pasture soil under
Acacia tortilis subsp. *raddiana* and *Balanites aegyptiaca* in
North Senegal**



Moudjahidou Demba Diallo

Thesis submitted to obtain the degree of Doctor (PhD) in Sciences (Biochemistry)
Specialisation in Microbiology

Promotor: Professor Dr. Monique Gillis

Directeur de thèse: Dr. Philippe de Lajudie
Co-directeur de thèse : Dr. Marc Neyra





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Cover photo by author: *Acacia tortilis* subsp. *raddiana* (center), *Balanites aegyptiaca* (right).

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"Life is like a box of chocolates, you never know what you're going to get", Forrest Gump said in the movie with the same name; and it is indeed so. It's so wonderfully addictive and full of new surprises, both pleasant and less pleasant ones. The chocolate might look so nice, but upon eating we find we don't like the filling or vice versa, when the last one remaining doesn't seem to be your taste but you 'sacrifice' yourself to eat it (before somebody else can!) and you find it was the best chocolate in the box. Chocolates are always a nice present (let this be a hint!), the person who gives them is eager for you to open the box and let them go round for everybody to taste. We have learned to share; the only thing you can do is hope that your favorite one will still be there when the box comes back to you. The nicest thing of all is when there are two layers of them, what a surprise when you find that out! That means double pleasure! Imagine lying awake at night, a mosquito buzzing around your ears, your tummy nagging for some food and then... then you remember there are some chocolates on the first shelf on your left in the basement. Chocolates are definitely something to get out of bed for; no need for lights even, just follow the instinct!!!!!!

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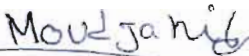
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Bacterial community structure in semi-arid soil (Senegal) under *Acacia tortilis* subsp. *raddiana* as revealed by PCR-DGGE analysis.

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Phylogenetic Analysis of the Partial Bacterial 16S rDNA Sequences of Tropical Grass pasture Soil under *Acacia tortilis* subsp. *raddiana* in Senegal.

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Chapter 1

Introduction and outline of the thesis

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1- General

Up to the last 2 decades, characterization and classification of micro-organisms was largely based on phenotypic and biochemical characterization of pure cultures (Bergey's Manual of Systematic Bacteriology, 1st edition, 1984). For the identification, differential phenotypic tests were used and many of these conventional methods were also appropriate for the detection and enumeration of specific bacteria. Growth on a selective medium containing defined nitrogen and carbon or other energy sources played an important role in these methods. Although these methods were in general sufficiently precise to distinguish certain groups of bacteria, they did not provide stable classifications because solely a limited part of the genome was used and also because the methods did not allow to estimate phylogenetic relationships among the diverse groups of bacteria. The introduction of genomic methods (in the sixties) was a milestone in the bacterial taxonomy because these techniques allowed for the first time to indirectly compare the bacterial genomes. The determination of the mean % G+C and the % of DNA:DNA hybridizations gave the first indications of heterogeneity within the established genera and species. The discovery of conserved parts in the ribosomal RNA, to be used for characterization, definitely opened the study towards a phylogenetically based bacterial classification (Woese et al. 1985; De Ley, 1992). The introduction of several molecular techniques with varying discriminatory powers further led to the development of a multidisciplinary characterization and classification in which phenotypic, chemotaxonomic and genomic data have been integrated.

2- Ribosomal RNA sequences

The two subunits (30S and 50S) of the prokaryotic ribosome contain, besides approximately 50 proteins, three ribonucleic acids: the 5S rRNA (120 nucleotides), the 16S rRNA (1540 nucleotides) and the 23S rRNA (2900 nucleotides). These rRNAs are encoded by an operon, which was first characterized in *Escherichia coli* and contains a tRNA^{glu} gene in the intergenic sequence between the 16S and 23S rRNA genes (Brosius et al. 1981). Later it was demonstrated that *E. coli* contains seven copies of the operon located at different genomic positions (Wagner, 1994). This operon organization is generally found in all prokaryotes although there are variations in number and identity of the tRNAs and also the sequential order of the rRNAs gene. Most organisms carry more than one copy of the operon in their genome and generally they are supposed to have all the same sequence. However, in some organisms the presence of two different 16S rDNA sequences has been demonstrated. These differences

vary from only a few bases as in *Mycobacterium gordonae* (Kirschner and Bottger, 1992), to up to 74 bases in *Haloarcula marismortui* (Mylvaganam and Dennis, 1992). In the first proposed secondary structure model of 16S rRNA (Woese et al. 1983), a number of helical elements (± 50) and the main functional domains were described. In 1991, Hubbard and Hearst presented models of the three-dimensional structure of the 16S rRNA. Already in 1987, Woese was able to identify the functionally most important and thereby most conserved regions in the 16S rRNA by comparative sequence analysis of various 16S and 18S rRNAs sequences (Woese, 1987) and by using the cataloguing technique. Due to the fact that rRNA consists of alternating conserved and more variable regions it is uniquely suited as a molecular chronometer. Results of cataloguing and DNA:rRNA hybridisations introduced several changes in the former bacterial classification. Cataloguing and sequencing of some rDNAs allowed to determine the phylogenetic relationships between diverse lineages of bacteria and to estimate the separate position of the *Archaeobacteria* (Woese et al. 1990) leading to the universal tree of life. DNA:rRNA hybridisations allowed to study the relationships within the *Proteobacteria* leading to diverse reclassifications e.g. within the former genus *Pseudomonas* (De Ley, 1992). Sequencing of more 16S rDNAs confirmed the former results but remained a laborious and time-consuming technique. The introduction of the reverse transcriptase sequencing (Laue et al. 1985) resulted in an exponentially growing number of 16S rDNA sequences further revolutionising the bacterial classification. Since the development of the Polymerase Chain Reaction (PCR; Saiki et al. 1988), and new automated sequencing techniques, rDNA sequences can directly be obtained from lysed cell cultures (Bottger, 1989), resulting in another exponential increase in known 16S rDNA and 23S rDNA sequences in recent years.

Comparative analysis of the sequences of 16S and 23S rDNAs by taxonomists has led to the construction of phylogenetic trees, illustrating the evolutionary relationships between organisms (Gutell et al. 1994) and providing new insights in the development of cellular life (Olsen et al. 1994). Based on the phylogenetic trees obtained, Woese et al. (1990) (Figure 1.1)

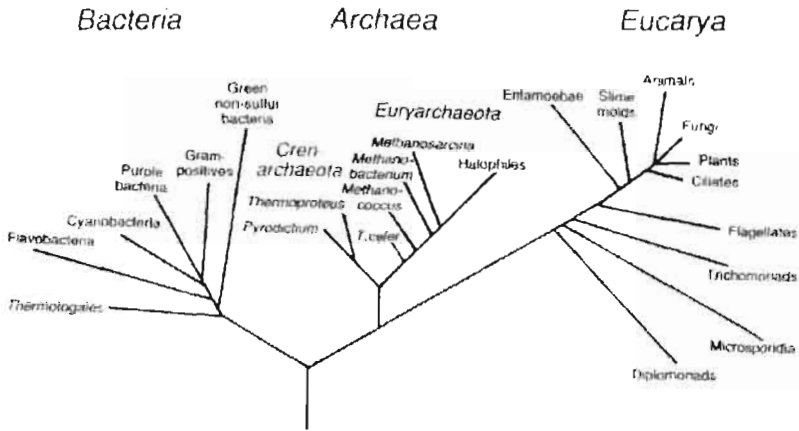


Figure 1.1 Universal phylogenetic tree in rooted, showing the three domains, *Archaea*, *Bacteria* and *Eucarya* (Woese et al. 1990)

postulated the existence of three domains namely the *Eucarya*, containing all former eukaryotes, the *Archaea*, previously known as *Archaeobacteria* and consisting of the *Euryarchaeota* and the *Crenarchaeota*, and the *Bacteria* containing the former *Eubacteria* divided in 23 phyla (Ludwig and Klent, 2001) (see Figure. 1.2). From now on we will use the nomenclature of the phyla and lineages as proposed by Garrity and Holt. (2001) and we do not use for the not yet validated names (e.g *Alphaproteobacteria*) quotation marks because we suppose that these names will soon be validated.

Ribonucleic acid sequence comparison resulted not only in completely new insights into microbial evolution and classification, but has also opened the perspective to study non culturable bacteria. In fact, comparing of the many 16S and 23S rDNA and ITS sequences available allowed designing custom specific primers (for each domain, lineage, genus and species).

This was in first instance used to characterize endophytic bacteria (Amann et al. 1997) but could later be used to study mixed biotopes. The application of direct 16S rDNA amplification, cloning and sequencing from natural habitats became indeed a standard

technique and the first studies indicated that most micro-organisms present in the environment have not yet been characterized (Ward et al. 1992).

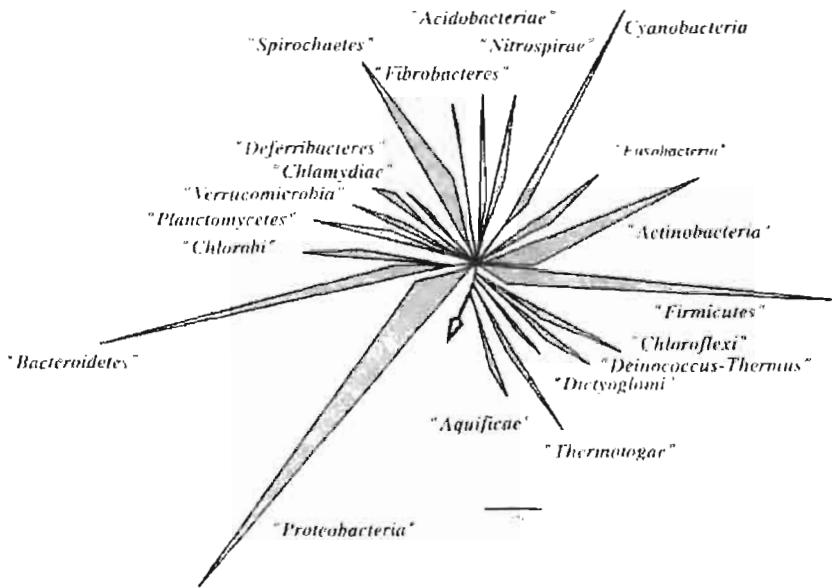


Figure 1.2 16S rRNA based tree showing 20 major bacterial phyla (Ludwig and Klenk, 2001)

3- Techniques in molecular environmental studies

Traditionally, microbiologists have attempted to recover as many different types of organisms from an environmental sample as possible. Depending on the aims of the study, organisms were selected either on the basis of physiological properties (photosynthesis, N_2 -fixation, sulphate or sulphur reduction, anaerobic respiration, H_2 production etc.), formation of products (antibiotics, metabolites), or morphological characters. Isolation of micro-organisms from a given biotope usually resulted in the recovery of a large number of strains to be identified by the current techniques. Several new groups have been described. From up to now unculturable organisms, 16S rDNA has been amplified, sequenced and compared with sequences from databases leading to the introduction of many new sequences for which in some cases the status *Candidatus* has been introduced (Murray and Stackebrandt, 1995; Vandekerckhove et al. 2002).

3-1- Hybridisation of DNA extracted from whole communities against that obtained from individual species, or other communities, allows the determination of the presence or absence of defined taxa, and the temporal and spatial distribution of defined and undefined community members, respectively (Sayler and Layton, 1990). The homogeneity of the microbiota of three different open ocean samples (Lee and Fuhrman, 1990), and the differences in microbial populations between coastal water and the open ocean have been studied by DNA:DNA hybridisation.

3-2- Analysis of the reassociation kinetics of total DNA indicated the presence of approximately 4000 different prokaryotic genomes in 1 g of soil (Torsvik et al. 1990). If such a result, indeed, reflects the extent of biodiversity, and each genome represents an individual species, the number of species in a single soil sample of 1 g is of the same order as the number of all described prokaryotic species.

3-3- Probing. A specific sequence can also be detected by the use of taxon-specific diagnostic oligonucleotide probes. This approach has in first instance been used to verify the molecular identity of symbionts within their hosts and to determine the presence and relative abundance of organisms in the environment, either of a particular organism or of a group of organisms. For endosymbiotic bacteria (characterized by their 16S rDNA) rDNA based probes had to be used, mostly for *in situ* hybridisations (Amann and Schleifer, 2001). Also for the determination of the relative abundance of microorganisms in environmental samples rDNA based probes were frequently used and often in dot blot or slot blot hybridisations (Stahl et al. 1988; Giovannoni et al. 1990; DeLong, 1992; Devereux et al. 1992; Liesack and Stackebrandt, 1992). Precise quantification of a given micro-organism by rDNA based probes (in DNA:DNA or DNA:rRNA hybridisations) cannot be achieved, since different bacteria contain different numbers of *rm* operons, and probably different numbers of ribosomes. Other factors negatively affecting quantitative measurements are: masking of low-abundance homologous rRNAs by large amounts of heterologous rRNA, binding of probes to their targets with different efficiencies (since not all sites in rRNA are equally accessible to probes) etc. Refinement of the technique e.g. by using sophisticated fluorescent labelled probes allowed more precise results (Zarda et al. 1991; Kessler, 1992; Hahn et al. 1993). To detect whole cells directly in their natural environment (Yu and Gorovsky, 1986; DeLong et al. 1989; Hicks et al. 1992; Manz et al. 1992) different probes can be labelled with different fluorescent dyes allowing to visualize

numerous targets in a single experiment (Ried et al. 1992, Amann and Schleifer, 2001). Other probes based on other part of the genome have been used in several studies (Ref)

3-4- Cloning and sequencing

The main breakthrough in the determination of genomic variation within natural samples came with the introduction of the PCR technique, cloning strategies and the use of DNA that was isolated either from harvested cells (Giovannoni et al. 1990) or from the autochthonous population in the natural matrix (Liesack and Stackebrandt, 1992). Cloning of rDNA or crDNA is needed to obtain individual sequences from the bulk rDNA amplificate. Identification of the clones can be done by hybridisation with specific probes or sequencing of part of the molecule.

The combined application of cloning and oligonucleotide probing facilitated the recognition of clones in clone libraries (Britschgi and Giovannoni, 1991; Liesack and Stackebrandt, 1992).

Sequence information obtained from nucleic acids isolated from an environmental sample allowed detection of a significantly broader range of diversity than achieved by isolation procedures alone. Three observations are very important:

1. In an environment that contains a phylogenetically highly diverse population, few of the clone sequences obtained are identical to those of isolates obtained by culturing. Although reference sequences are not available for all described species, this finding was a confirmation of the assumption stated above that only a fraction of prokaryotic species is culturable. This finding emphasizes the previously documented bias of culture conditions that may select for a portion of the microbiota that is not predominant. The data also showed that the as yet not cultured bacteria were not necessarily closely related to the culturables. Several new lineages containing almost solely uncultured sequences have been found e.g. the *Acidobacteria*, the TM7 lineage and other new lineages (Hugenholtz et al. 1998; Hugenholtz et al. 2001)

2. Often phylogenetic analysis revealed groups of related sequences, suggesting the presence of several members of the same, previously unknown, taxon. Although the sequence differences were sometimes small and comparable to those observed in culturable members of a species or a genus (Fuhrman et al. 1993; Stackebrandt et al. 1993), larger differences have also been detected indicating relationships on a lower level.

3. If a sequence from an environmental sample is shown to be a member of a taxon of culturable organisms, attempts to cultivate those organisms often have failed.

4- Molecular identification and detection methods based on ribosomal DNA sequences in soil

The classical biological approach for the description of an ecosystem was to first characterize the community structure by identification and enumeration of the species present, and then to assign roles to species or groups. This strategy, typically employed by ecosystem and population investigators, has not always been practical for the microbial ecologist. For reasons explained above, cultured based methodology is inadequate to serve the need of microbial ecologists seeking to describe the diversity of bacterial communities in environmental samples.

4-1- Culture-independent assessments of microbial communities

A number of methods have been developed that exploit the 16S rDNA divergence among taxa to examine microbial community structure. Initially, rRNA and rDNA were considered to be equally useful, in their suitability as templates for sequence analysis, and for subsequent determination of the biodiversity. However, the introduction of PCR-mediated amplification of rRNA genes and the recognition that reverse transcriptase may not faithfully transcribe the rRNA, resulted in a shift towards the use of DNA. These culture-independent methods utilize in first instance the polymerase chain reaction (PCR) to amplify rDNA sequences according to the primers used. The use of denaturing gradient gel electrophoresis (DGGE) for the separation of PCR-amplified environmental 16S rDNA marked an important milestone (Muyzer et al. 1993; Palus et al 1996), especially when combined with high-resolution gelelectrophoretic techniques (eventually in a two-dimensional system), probing with taxon-specific oligonucleotides, and sequence analysis of the separated DNA fragments. Community analyses based on PCR have number of steps that may introduce biases, starting with the DNA extraction. Bacterial cell wall structure varies among groups, some being more easily disrupted than others. In addition, environmental factors require special consideration for both sample and DNA extraction. Inhibition of PCR by environmental compounds (as e.g. humic substances from soil) has been reviewed by Wilson et al. (1997) and optimizing the extraction procedure may be required for each different group. Niemi et al. (2001) demonstrated that soil bacterial community profiles differed depending on the DNA extraction and purification

methods utilized. Methods that include mechanical lysis using a bead beater were found to yield the most consistent results (Harmsen et al. 1995; Zoetendal et al. 1998).

Despite these caveats, PCR-based community analysis methods are commonly used because of the ease to analyse many samples and the ability to tailor the analysis for particular organisms or taxa of interest through the use of universal or group specific primers (Bruns, 1999; Henckel et al. 1999; Robleto et al. 1998). In addition to DGGE, a number of community "fingerprint" methods are commonly used to assess differences in populations. Such techniques are ribosomal intergenic spacer analysis (RISA) (Borneman and Triplett, 1997; Ranjard et al. 2000; Robleto et al. 1998), and temperature gradient gel electrophoresis (TGGE) (Heuer and Smalla, 1997; Smalla et al. 2001) of an rDNA fragment, single-strand-conformation polymorphism (Schmalenberger et al. 2001) of rDNA, ITS-restriction fragment length polymorphism (ITS-RFLP) (Cho and Tiedje, 2000), or amplified ribosomal DNA restriction analysis (ARDRA) (Massol-Deya et al. 1995) yield complex community profiles that do not directly offer phylogenetic information but do allow analysis and comparison of community composition. Differences in electrophoretic profiles between samples reflect differences in community composition and abundance of individual microbial populations in a community. Phylogenetic information about particular community member's maybe obtained by isolation and sequence analysis of bands of interest. A number of approaches have been developed to improve the detection and resolution of fragment analysis, including automated ribosomal intergenic spacer analysis (ARISA) (Fisher and Triplett, 1999; Ranjard and Richaume, 2001), length heterogeneity PCR (LH-PCR) (Robleto et al. 1998; Suzuki and Giovannoni, 1996), and terminal restriction fragment length polymorphism (T-RFLP) (Kitts, 2001; Liu et al. 2001). These methods utilize a fluorescent labelled oligonucleotide primer for the PCR amplification and an automated system such as Applied Biosystems capillary or classical gel electrophoresis for separation and detection of PCR fragments. Automation of the procedure increases sample throughput and allows the rapid analysis of the bacterial community structure. The high resolution offered by automated electrophoresis instruments and the high sensitivity of fluorescence gel electrophoresis detection increase the number of peaks detected compared to methods that use standard electrophoresis and detection. In addition, the band intensity can be measured more precisely by fluorescence detection methods, which allow more accurate comparison of community profiles. When fluorescence fragment analysis techniques are used, information on the relative abundance of individual

fragments (presumed to represent different bacterial taxa) is collected. These data can be used to calculate the diversity, richness and evenness of the community using ecological indexes such as Shannon-Weaver diversity index, Sorenson's similarity index, or the Bray-Curtis similarity index (Magurran, 1988).

Of the fragment analysis methods listed above, only T-RFLP can offer some phylogenetic information without further sequencing of the fragments. Fragment lengths obtained from T-RFLP analysis of a microbial community may be compared to the expected terminal restriction fragment length obtained from analysis of known 16S rRNA gene sequences (Marsh et al. 2000). In practice, however, the complexity of T-RFLP profiles obtained from environmental samples can hinder phylogenetic assignment of individual fragments (Marsh, 1999; Liu et al. 1997; Kitts, 2001).

When phylogenetic information is desired, researchers employ the more laborious strategy of constructing a clone library from the amplified rDNA (Bahr et al. 1996; Bowman et al. 2000a; Dojka et al. 2000; Hiorns et al. 1997) and to sequence statistically significant numbers of clones. This approach allows identifying the phylogenetic clusters to which dominant community members belong. Clone libraries are also useful for refining molecular fingerprinting techniques (Kitts, 2001; McSpadden et al. 2000). Approaches utilizing DNA microarrays are being developed to study clone libraries (Small et al. 2001).

While correlating the distribution of PCR-amplified rDNA with the distribution of species is limited because of the presence of multiple rRNA operons in bacteria (Farrelly et al. 1995, Rainey et al. 1996) and PCR and cloning biases (Suzuki and Giovannoni, 1996), these molecular methods can indeed reveal the presence of micro-organisms that remain intractable to traditional cultivation techniques. Fernández et al. (1999) reported that the effects of PCR and cloning biases are minimized when relative changes are studied in the same ecosystem and when replicate community profiles are produced.

Mostly, solely the rDNA's and the ITS regions are used although 5S rRNA has also been used in combination with the pool of transfer RNA's (Höfle et al. 1999).

4-2- Early analyses of microbial diversity in soils.

In the first assessments of soil by culture-independent means, *Proteobacteria* were found to dominate 16S rDNA clone libraries using template DNA from Queensland soil (Liesack and Stackebrandt, 1992). A more diverse population was found in a Japanese soybean field (Ueda et al. 1995) however based on a few clones. In another study, three bacterial divisions were

represented in nearly 60% of the 16S rDNA of a Wisconsin pasture soil (Borneman et al. 1996). In contrast to these agricultural soils, analysis of 16S rDNA clones from a Siberian tundra soil showed that over 60% of the clones belong to the *Proteobacteria* and 16% to the *Fibrobacteres* (Zhou et al. 1997)

The publication of this early work encouraged analyses of soil microbial diversity under a wide variety of conditions. For review papers we refer to Amann (2000); DeLong and Pace (2001); Hughes et al. (2001); Johnsen et al. (2001); Øvreås, (2000); and Pace (2000).

5- Other genes and techniques used.

When specific traits or functional characteristics are under investigation, phylogenetic markers other than the rRNA genes can be used to characterize microbial communities.

Marsh (1999) summarized several bacterial housekeeping genes as potential phylogenetic markers. These include genes for heat shock proteins, glutamine synthetase, ATPases, and topoisomerases (Marsh, 1999).

For soil microorganisms, functions associated with nitrogen metabolism have been widely used for community analysis. The phylogenetic markers used in these studies include a structural gene for nitrogenase (*nifH*) [studies reviewed by (Nicholson et al. 1998; Poly et al. 2001a, b)]. *NifH* gene has been largely studied by PCR-based culture-independent approaches providing a more complete picture of the diazotrophic community than culture-based approaches. Various techniques, such as PCR cloning (Zehr et al. 1998), denaturing gradient gel electrophoresis (Piceno and Lovell, 2000; Lovell et al. 2001; Bagwell et al. 2002), PCR-restriction fragment length polymorphism (RFLP), and fluorescent labelled terminal (FLT)-RFLP (Chelius and Lepo, 1999; Noda et al. 1999; Ohkuma et al. 1999; Shaffer et al. 2000; Widmer et al. 1999), have been used to analyse the composition of the *nifH* gene pools in various environments. The results show that *nifH* is present in diverse environments: forest soil (Shaffer et al. 2000; Widmer et al. 1999; Rösch et al. 2002), the rhizosphere of native wetland species, such as *Spartina* (Chelius and Lepo, 1999; Piceno and Lovell, 2000) or of crop species, such as rice (Ueda et al. 1995), aquatic (Braun et al. 1999; Zehr et al. 1998) and polar (Olson et al. 1998) cyanobacteria, and the bacteria found in termite guts (Noda et al. 1999, Ohkuma et al. 1999; Ohkuma et al. 1996). A large number of unknown sequences, which correspond to diverse unidentified bacteria, have been described. Some *nifH* genes may be characteristic for an ecological niche (Chelius and Lepo, 1999; Shaffer et al. 2000). Shaffer et

al. (2000) evoked the possible relationship between the habitats of soil nitrogen-fixing bacteria and the structure of *nifH* gene pools.

Other phylogenetic markers like nitrous oxide reductase (*nosZ*) (Scala and Kerkhof, 1999, 2000) and nitrite reductase genes (*nirK* and *nirS*) (Braker et al. 2000) were used as well. Genes involved in methane oxidation (*pmoA*, *mmoB*, and *nxoF*) have also been used to characterize soil microbial communities (Henckel et al. 1999). When a particular function is restricted to specific bacterial taxa, 16S rRNA gene sequences may be used to differentiate these community members.

Methods that examine physiological or metabolic characteristics of microbial communities are alternatives to PCR-based approaches.

Fatty acid methyl ester (FAME) profiles and phospholipid fatty acid analysis have been used extensively to characterize the composition of soil microbial communities [(Ibekwe and Kennedy, 1998; Ritchie et al. 2000) and references therein].

Careful morphological analysis of bacterial cells can provide powerful information on the diversity, microbial abundance, and two-dimensional spatial distribution of microbial community members. A computer-aided system has been developed by the Center for Microbial Ecology at Michigan State University to assist in such assessments. CMEIAS (Center for Microbial Ecology Image Analysis System) is a semi-automated analysis tool that uses digital-image processing and pattern-recognition techniques in conjunction with microscopy to gather size and shape measurements of digital images of micro-organisms to classify them into their appropriate morphotype, allowing culture-independent quantitative analysis of the diversity and distribution of complex microbial communities (Liesack and Stackebrandt, 1992). This tool holds much promise for automating a tedious but important evaluation of microbial communities.

6- Methods to assess community function in soil.

As microbial ecology involves the study of both the structure and function of an ecosystem, meaningful assessments of microbial communities must consider not only the abundance and distribution of species but also the functional diversity and redundancy present in a microbial community. Gaston (1996) has described functional diversity as the number of distinct processes (functions) that can potentially be performed by a community, whereas functional redundancy is measured as the number of different species within the functional groups present in a community. The diversity of metabolic functions possessed by microbial

communities is often examined using BIOLOG substrate assays (Garland and Mills, 1991; Heuer and Smalla, 1997a; Insam, 1997; Smalla et al. 1998; Pace, 2000), which assess the ability of the community as a whole to oxidase selected carbon substrates.

An alternative technique assesses community response (measured as CO₂ respiration) after the addition of selected carbon substrates directly to the soil environment (Degens, 1997).

To gain a better insight in the microbial processes within an ecosystem, it is essential to study functional diversity in combination with taxonomic diversity. Recent studies have attempted to characterize the portion of the microbial community that responds to nutrient availability by comparing community fingerprints after incubation in individual BIOLOG wells (Smalla et al. 1998) or by isolating DNA from microbial community populations that responded to nutrient addition by uptake and incorporation of a thymidine nucleotide analogue, bromodeoxyuridine (BrdU) (Borneman, 1999). Molecular fingerprint analysis of the responsive portion of the microbial community (as defined by BrdU labelling) was also used to assess the functional redundancy of bacterial communities along a vegetation gradient (Yin et al. 2000).

A DNA microarray technique for the simultaneous identification of ecological function and phylogenetic affiliation of microbial populations has been recently developed (Bertilsson and Polz, 2001).

7- Change in uncultured bacterial soil communities with disturbance.

7-1- Heavy metals.

In soil treated (Sandaa et al. 2001) with sludge containing either high or low amounts of heavy metals it was found that heavy metal treatment more than doubled *Alphaproteobacteria* members while the abundance of the *Cytophaga-Flavobacterium* division declined by more than two thirds. In another study, Sandaa et al. (1999) showed that the number of prokaryotic genomes per gram of wet weight of soil declined eightfold following many years of heavy metal treatment and that solely the % of *Alphaproteobacteria* more than doubled while the proportion of all other group decreased. Addition of Hg (II) to a silt loam caused an increase in abundance of two RISA bands (Ranjard et al. 2000). These bands were excised, sequenced, and identified as having originated from a *Clostridium*-like Gram-positive organism and a *Ralstonia*-like *Betaproteobacterium*.

7-2- Addition of pollutants to soil.

Various papers report on the changes in the diversity of the microbial population in function of the addition of pollutants as pentachlorophenol resulting in an increase of

Sphingomonas members (Beaulieu et al. 2000), methane provoking an enrichment of methylotrophic *Alphaproteobacteria* (Jensen et al. 1998, Øvreås and Torsvik, 1998), and of chlorinated benzoates resulting in a general decline of the bacterial diversity (Ramirez-Saad et al. 2000).

7-3- Pesticide treatment.

El Fantroussi et al. (1999) examined the effect of three phenyl urea herbicides on microbial communities in soils over an 11-year period. All three herbicides significantly decreased the number of culturable heterotrophic bacteria. BIOLOG GN fingerprint analysis also showed that the treated communities differed significantly compared to the control. A striking result of this work is the apparent decline of uncultured *Acidobacteria* upon treatment with any of the three herbicides. Uncultured *Acidobacteria* are commonly found in culture-independent analyses of soils. It is not clear whether the decline is caused directly by the herbicides or as a consequence of the changes in the macroflora community resulting from herbicide use. Treatment of soil (Yang et al. 2000) with the fungicide triadimefon caused a decline in organic carbon and soil microbial biomass but no decline in microbial DNA diversity as measured with RAPD (Random amplified polymorphic DNA) This can be explained by the common contradiction that although fungi can comprise a large proportion of soil biomass, fungal DNA concentrations in soil are low.

Xia et al. (1995) evaluated microbial community response to the experimental application of 2,4-dichlorophenoxyacetic acid (2,4-D) and did not find changes in community structure in three soils. Two culture-dependent studies on the effects of herbicides on soil bacterial diversity present conflicting results. Nicholson and Hirsch (1998) showed an increase in culturable bacterial populations in soils treated with glyphosate and suggest that the increased crop yield resulting from the herbicide treatment might have contributed to higher bacterial numbers. In contrast, Busse et al. (2001) found lower bacterial numbers in a pine plantation treated with glyphosate compared to the untreated control.

Fumigants are widely used in high-value crops for the control of eukaryotic soil-borne pests such as fungal pathogens, nematodes, and weeds. Ibekwe et al. (2001) studied their effect on soil prokaryotic communities from a culture-independent perspective. Of four fumigants used, methyl bromide caused the greatest and longest-lasting impact on soil bacterial diversity. Chloropicrin had virtually no impact.

7-4- Agricultural management.

Through the use of ribosomal intergenic space analysis (RISA), deforestation in Amazonia was shown to have a profound, qualitative impact on soil bacterial diversity (Borneman and Triplett, 1997). Another analysis of community changes in tropical soils with deforestation was done by Nüsslein and Tiedje (1998). The G + C content of the pasture soil DNA was significantly higher than that of the forest soil DNA. Whereas the *Fibrobacters* were dominant in the forest soil, the *Betaproteobacteria* and *Alphaproteobacteria* dominated the pasture soil.

Improved and unimproved Scottish grasslands differing in fertilizer regimes and plant cover were assessed for microbial diversity using 16S rDNA clone libraries (McCaig et al. 1999). Both pastures were dominated by *Alphaproteobacteria* (about 40% of the total clones) followed by the *Actinomycetes* (13.3% of the total). Indices of diversity including the Shannon-Weaver index as well as evenness and dominance measurements were similar between the two pastures.

Grasslands in the Netherlands out of agricultural production over a period of 30 years were examined for changes in microbial diversity (Felske et al. 2000). The multiple competitive RT-PCR procedure used, did not have sufficient resolution to distinguish those pastures currently in agricultural production from those out of production. Through a culture-independent analysis of soils collected from the Kellogg Biological Station's Long Term Ecological Research project of Michigan State University, Buckley and Schmidt (2001) found that the microbial diversity of cultivated fields differed little from each other regardless of the specific agricultural management regime. However, the bacterial diversity of the managed soils was significantly different from soils of nearby fields that had never been cultivated. This is an excellent site for such analyses because the Long Term Ecological Research sites have long term data on the temporal and spatial variability of a wide range of physical, chemical and biological characteristics of the experimental location.

8- Outline of the thesis.

This thesis is part of a large-scale research project (Minimising competition in dry land agroforestry. INCO-DC ERBIC18CT98322) that investigates the competition between trees and crops in West Africa.

In the pastoral-forestry zone of Northern Senegal, the availability of water and nitrate are limiting factors for the growing of crops. Soil bacteria play an important role in the exchange

of energy and nutrient. in the decomposition of organic material and in the development of the soil, where they promote humidity and fertility.

Nitrogen is essential for all life on this planet. but most of it is in the air, making up about 78% of the earth's atmosphere. Three processes dominate the nitrogen cycle: denitrification, nitrogen fixation and nitrification (Figure 1.3). Nitrogen can only be absorbed by plants in the form of ammonium and nitrate. Nitrate originates of abiotical (lightening) and biotical (nitrification) (Figure 1.3) processes. In the grasslands from Senegale the presence of the nitrificating bacteria is rather low (Richards. 1987) and the nitrate sources are in the soil only available at a depth of 5-25 meter, so that plants are mainly dependent on ammonium as source of nitrogen.

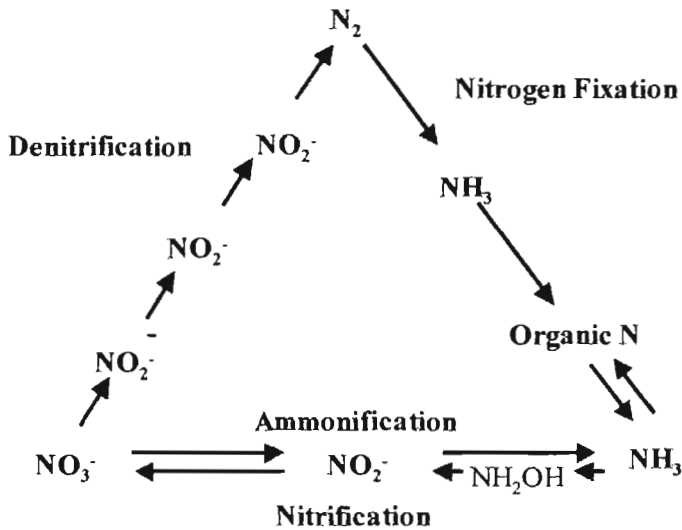


Figure 1.3 The nitrogen cycle. The three primary processes and primary intermediates are indicated (Apr, 2000)

Bacteria are the only organisms capable of nitrogen fixation: mostly they are free-living soil organisms, but some plants (*Leguminosae*, *Parasponia*, *actinorhizal plant*) have developed a symbiosis with bacteria which infect their roots and stems, fix nitrogen in the formed nodules and which obtain in return nutrients from the plant. The most important symbiotic nitrogen fixing bacteria are the “rhizobia” constituting several lineages in the *Alphaproteobacteria* (see Figure 1.4).

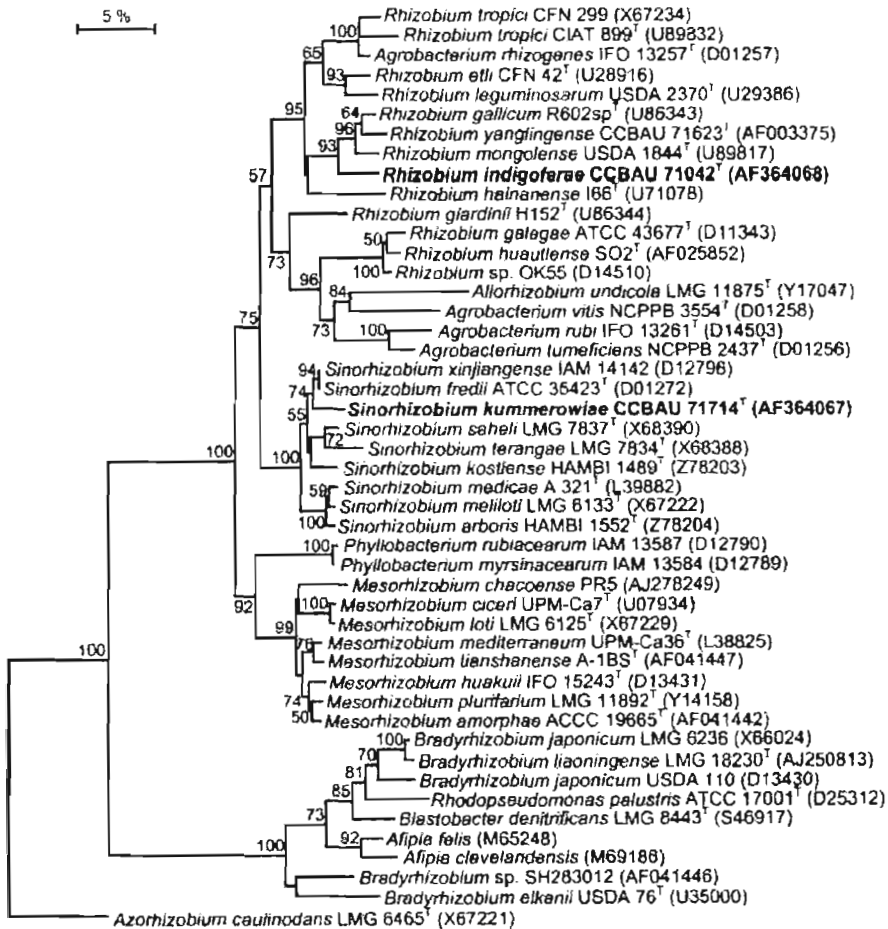


Figure 1.4 Phylogram of 16S rRNA gene sequences showing the phylogenetic relationships of rhizobia (Wei et al. 2002)

Recently symbiotic bacteria belonging to the *Betaproteobacteria* have been described (Moulin et al. 2001). The presence of free living and symbiotic nitrogen fixers in the soil is essential.

In Senegal, a semi-arid region, crops are grown under and in the neighbourhood of trees and wood-like vegetation, because the soil-fertility on those places is often higher than in other areas, which are further removed from trees. Only limited information is available on the impact of trees on microbial populations and their dynamics in tropical semi-arid regions of Senegal.

This project aimed to study the competition between trees and agricultural crops, and how this competition can be minimised to improve the productivity and product diversity. As such, minimising the competition should improve the income and ensure food of the local people.

The competition was studied in different tree-crops combinations and the aims were:

- * To investigate the yield of the agricultural crops
- * To study the soil-fertility (analysis of P, N, K, C and organic matter) in function of the season.
- * To examine the root system of the trees and to study to what extent they are in competition with the root system of the crops.
- * To define the amount of nitrate in the groundwater.
- * To study the bacterial diversity in the soil was the main objective of our participation in this project and of this thesis; as discussed above the choice to use molecular methods on total extracted DNA was obvious.**

The microbial part of this project concerned a collaboration between Dr. M. Neyra (co-directeur these for IRD) from the Laboratoire de Microbiologie des Sols Tropicaux, IRD, in Dakar, Senegal, the Laboratory of Microbiology at the University of Ghent and Dr. P. de Lajudie from the Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM) in Montpellier (France) who was my IRD supervisor (Directeur de thèse from IRD). The work was performed in Dakar and Ghent.

In first instance we wanted to study the total bacterial population on different distances from the stem of a legume tree (*Acacia tortilis* subsp. *raddiana*) and on different depths in soil of Souilène (IRD's Biological Research Station in North Senegal) on samples from the dry and the rainy season.

A second objective was to estimate the number and diversity of the N₂-fixing bacteria in the bacterial community. To determine the effect of the host plant we studied the composition

of the symbiotic nitrogen-fixing bacteria we studied soil samples under *A. tortilis* subsp. *raddiana* and *Balanites aegyptiaca* a non legume tree naturally growing in this biotope.

To obtain an overall view of the bacterial diversity we used in first instance PCR-DGGE of a variable fragment (V3 region) of the 16S rDNA, in combination with sequencing of the excised intense bands (**Chapter 2**). Then we constructed two clone libraries (one from the rainy and one from the dry season). The libraries were screened for groups and representatives of various OTUs were partially sequenced (**Chapter 3**). In **Chapter 4**, we compared ARDRA (Amplified 16S rDNA restriction analysis) and DGGE (Denaturing gradient gel electrophoresis) to screen one of our 16S rDNA clone libraries. The results were compared with a set of partial 16S rDNA sequences obtained for representative clones from the clusters obtained with both techniques. This strategy allowed us to compare and evaluate the discriminatory power of both screening methods.

To study the composition of nitrogen-fixing bacteria in these populations we used in **Chapter 5** *nifH* gene specific primers to monitor the DGGE changes of the *nifH* gene pool from soil samples collected under *Acacia tortilis* subsp. *raddiana* (legume tree) and *Balanites aegyptiaca* (non-legume tree). Intense bands were excised and sequenced.

The results of the various Chapters will be analysed with the appropriate software to distinguish groups of results and to test the significance of the groups found.

Chapter 2

Bacterial community structure in semi-arid soil (Senegal) under *Acacia tortilis* subsp. *raddiana* as revealed by PCR-DGGE analysis

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ABSTRACT

Bacterial community structure was studied under *Acacia tortilis* subsp. *raddiana*, a legume tree naturally growing in the dry part of Senegal (West Africa), using denaturing gradient gel electrophoresis (DGGE) and sequencing of excised 16S ribosomal DNA fragments. Samples were taken along transects from the stem up to 10 m distance from, at depths of 0-0.25 m and 0.25-0.50 m. Sampling was done in the dry (June 25th 1999) and rainy seasons (August 28th 1999). Overall DGGE profiles showed that the bacterial community was characterized by few (5-6) abundant phylotypes, present in every sample, and a larger number of less prevalent phylotypes. Cluster analysis and principal component analysis (PCA) demonstrated that the bacterial community differed according to the season. Differences as a function of the depth or distance from the tree were less pronounced. Sequence analysis of prominent bands revealed that the most abundant taxa were members of the genera *Bacillus* and *Acinetobacter*, both bacterial genera commonly found in soil habitats. Phylotypes belonging to the *Alpha*, and *Betaproteobacteria*, the *Cytophaga-Flavobacter-Bacteroides* group, the genus *Streptomyces*, and the TM7 lineage were also detected.

INTRODUCTION

In the semi-arid region of West Africa, soil fertility under the canopy of leguminous trees is frequently superior to that in open grassland areas (Deans et al. 1999). This is exploited by farmers using tree fallows and the spaced-tree parkland system in the Sahel (Le Houérou, 1989). The promotion of low-input agriculture over the recent decades has sparked a great interest in soil microorganisms, that are able to increase soil fertility or to stimulate plant nutrition and/or health. Several studies in different parts of the world have indicated that the structural and functional diversity of rhizosphere microbial communities is affected by plants (Piceno and Lovell, 2000; Lovell et al. 2000; Smalla et al. 2001; Gomes et al. 2001; Duineveld et al. 2001). However to date, only limited information is available on the impact of trees on microbial populations and their dynamics in tropical semi-arid regions of West Africa and particularly in the Northern pastoral-forestry zone of Senegal. *Acacia tortilis* subsp. *raddiana*, often called the “umbrella thorn” for its distinctive spreading crown, is one of the most widespread trees in seasonally dry areas of Africa and the Middle East. *Acacia* is the largest mimosoid genus and includes nearly 1200 species (Founoune et al. 2002). They are abundant in savanas and arid regions, and are valuable for their symbiotic bacteria fixing atmospheric N₂. Some of them prevent wind and rain erosion, control sand dunes and are important sources of wood and fodder for browsing livestock. Additionally, bound nitrogen is returned to the soil by the roots and by the natural loss of leaves, resulting in humus enrichment, which is advantageous for the fertility of the soil and its physical properties. Symbiotic and other microorganisms play an important role in this process.

Surveys of soil bacteria by PCR amplification of their 16S rRNA genes followed by cloning and sequencing have shown that soil bacterial communities are extremely diverse and may contain large numbers of new taxa. Some of these taxa may be dominantly uncultured bacteria (Hugenholtz et al. 1998; Smit et al. 2001). Most sequences detected by such methods belong to the *Proteobacteria*, the Gram-positive bacteria or the *Acidobacteria* division (Barns et al. 1999; Dunbar et al. 2001; McCaig et al. 2001). The composition of the populations differs according to the soil, the season, the depth of sampling and the plants grown on the sampling sites (Gomes et al. 2001; Smit et al. 2001; McCaig et al. 2001; Zhou et al. 2002; Kuske et al. 2002). Various studies indicate that the composition of the bacterial population in the rhizosphere is different from that of rhizoplane, soils and interspaces (Gomes et al. 2001; Duineveld et al. 2001; Smit et al. 2001; Dunbar et al. 2001; McCaig et al. 2001; Kuske et al. 2002; Felske and Akkermans 1998; Normander et al. 2000). It has also been demonstrated that the surface soil shows a high diversity in its bacterial

population and a lack of dominant groups whereas subsurface communities have a lower diversity and a greater dominance of a few groups (Zhou et al. 2002). Due to the complexity of soil communities and the effort required for this type of analysis, cloning and sequencing have been restricted to the analysis of a limited number of samples in the environment. Denaturing gradient gel electrophoresis on the V3 region of the 16S rDNA and sequencing of the excised bands is an alternative method to study microbial community structure in numerous samples (Muyzer, 1999) and can also be used to study changes in the bacterial populations. Whether the soil-borne bacterial community changes in response to different environmental conditions along a gradient away from the stem to outside the canopy remains largely unknown. Information on the changes in the diversity and composition of the soil bacterial population could be important in understanding the ecosystem and in predicting plant productivity in the Northern semi-pastoral forestry areas of Senegal.

To obtain baseline knowledge on the microbial diversity and population dynamics associated with *Acacia tortilis* subsp. *raddiana* trees grown in the pastoral forestry zone in Senegal, we used denaturing gradient gel electrophoresis (DGGE) of PCR-amplified V3 16S rDNA fragments in combination with sequencing of excised bands (Muyzer et al. 1993). The aim was to characterize the bacterial structure and the diversity, and to detect possible shifts in the bacterial community as a function of the sampling date, soil depth or the distance from the tree. The soil under *Acacia tortilis* subsp. *raddiana* was therefore sampled in the dry and the rainy seasons along transects (up to 10 m) from the stem and at 2 depths (surface and subsurface).

MATERIALS AND METHODS

Study site and samples collection

The study was carried out in Souilène (IRD's Biological Research Station in North Senegal), in the grassland part of the Ferlo Region near the village of Windou Thiengoly and Mbeulekhe, situated between 15°40'-16° N, 15°40' W. The soil of the region consists of degraded red/brown ferruginous sand, which extends to a depth of at least 30 m, and is characterized by low organic matter content, and poor water and chemical retention properties. Annual long-term (1981-1993) rainfall, which occurs in a single rainy season between July and October, was on average 297 mm for the period 1981-1993 but more recently has decreased to about 150 mm. A comprehensive description of the area is given

by Le Houérou (1989) and floristic details of the district were reported by Mieke et al. (1990). Soil cores (2.5 by 25 cm) were taken under *Acacia tortilis* subsp. *raddiana* along a transect from 1 to 10 m from the stem at 1 m intervals, beneath the canopy up to the open field. Samples were taken at depths of 0-0.25 m, where fine tree roots were reported as being most heavily concentrated, and 0.25-0.50 m. Samples in which some fine root fragments were found were treated as the other bulk soil samples. The tree roots extended more than 9 m on each side of the stem. Sampling was done at the end of the dry season (June 25th 1999) and in the middle (50 days from the start) of the rainy season (August 28th 1999). Samples of the cores were stored in a plastic bag at -20°C.

DNA extraction

Total DNA was extracted from 1.5 g of soil by a modification of the procedure of Porteous et al. (Porteous et al. 1997). Soil (1.5 g) was mixed with 1.5 ml Na₂HPO₄ (0.1 M) and washed by shaking for 30 min at room temperature. After centrifugation at 7000 g (10 min at 4°C), the supernatant was removed. The pellet was resuspended in 500 µl of lysis buffer [2% CTAB (Cetyltrimethylammonium bromide); 0.15 M NaCl; 0.1 M Na₂EDTA (pH8); 1% PVPP (PolyVinyl PolyPyrrolidone)], and 7.5 mg lysozyme (Sigma) was added. Samples were incubated overnight at 37°C. Twenty-five µl of proteinase K (20 mg.ml⁻¹, Boehringer, Mannheim) was added and the tubes were incubated at 50°C for 40 min. The temperature was increased to 65°C for 20 min and 300µl extraction buffer (0.2 M NaCl; 0.1 M Tris-HCl pH 8; 2% SDS) was added. Then, the mixture was incubated at 65°C for another 10 min. After addition of 350 µl of 5 M NaCl the samples were cooled on ice for 15 min. The supernatant was collected after centrifugation (7000 g, 10 min, 4°C) and transferred into 2 ml centrifuge tubes. To precipitate the crude DNA, 75 µl of 5 M potassium acetate (Kac) and 250 µl 40% polyethylene glycol 8000 (PEG) were added and the mixture was incubated at -80°C for 1h. The pellet, obtained by centrifugation (13000 g for 15 min at 4°C) was resuspended in 900 µl 2 x CTAB (2 % CTAB; 1.4 M NaCl; 0.1 M Na₂EDTA) and incubated for 15 min at 68°C. After addition of 900 µl of chloroform the solution was gently mixed and centrifuged at 13000 g for 10 min at room temperature. The DNA was precipitated by addition of 1 ml of isopropanol and incubated for at least 15 min at 20°C. The pellet (13000 g for 15 min at 4°C) was dissolved in 450 µl 2.5 M ammonium acetate (NH₄OAc) and subsequently, the DNA was precipitated by the addition of 1000 µl 95% ethanol and then incubated for at least 15 min at -20°C. The pellet of DNA was obtained by centrifugation at 13000 g for 15 min at 4°C and resuspended in 200µl sterile water (Sigma). For each soil sample we performed two independent 1.5 g soil preparations

of which the purified DNA-samples were pooled and stored in one single vial. A 100 μ l aliquot of the crude extract was further purified using the Wizard[®] DNA CleanUp kit (Promega, Madison, WI USA).

PCR amplification of 16S rDNA

Five μ l (ca 100 ng) of the purified DNA was amplified in a Genius temperature cycler. The PCR mixture contained: 5 μ l of template DNA, 0.5 μ M of each of the appropriate primers, 200 μ M of each deoxynucleoside triphosphate, 5 μ l of 10 x PCR buffer (100 mM Tris-HCl (pH 9); 500 mM KCl; 15 mM MgCl₂, 20 ng of bovine serum albumine and 2.5 U of Taq DNA polymerase (Ampli-Taq Perkin Elmer). Each mixture was adjusted to a final volume of 50 μ l with sterile water (Sigma). The primers are F357GC(5'CGCCCGCCGCGCCCGCGCCCGGCCCCGCCGCCGCCGCCCTACGG GAGGCAGCAG-3') and R518 (5'-ATTACCGCGGCTGCTGG-3'). Both primers designed by Muyzer et al. (1993) are used to amplify the 16S rDNA region corresponding to position 341 to 534 in *E. coli*. Primer F357GC that contains a GC-rich clamp is specific for most *Bacteria* and R518 is specific for most *Bacteria*, *Archaea*, and *Eucarya* (Van Hannen et al. 1999). In order to improve the specificity of the amplification and to reduce the formation of spurious by-products, a "touchdown" PCR (Muyzer et al. 1993; Don et al. 1991) was performed starting with 5 minutes at 94 °C, followed by 20 cycles of denaturation at 94°C for 1 min, annealing at 65°C (the temperature was decreased by 0.5°C every cycle until the touchdown temperature of 56°C was reached) for 1 min, and primer extension at 72°C for 1 min. Five additional cycles were carried out at an annealing temperature of 55°C. The tubes were then incubated for 10 min at 72°C. The presence of PCR products and their concentration were determined by analysing 5 μ l of PCR-product on a 2% agarose gel. A molecular weight marker (Smartladder-Eurogentec, SA Belgium) was included.

DGGE analysis

The DGGE technique was carried out using the D-Code System from Bio-Rad Laboratories. The PCR products were loaded onto 8% (w/v) polyacrylamide gels of 1 mm thickness, in 1 x TAE buffer [20 mM Tris-acetate with pH 7,4; 10mM acetate; 0,5mM disodium EDTA]. The denaturing gradient contained 35% to 70% denaturants (100% denaturant corresponded to 7 M urea and 40% (vol/vol) deionized formamide). The total lane intensity was normalised between the samples on 400 ng of DNA. Electrophoresis was performed at a constant voltage of 75 V for 16 h and at a constant temperature of 60°C.

After electrophoresis, the gels were stained for 1 h in 1X TAE containing ethidium bromide (0.5 mg.l⁻¹). The bands were visualized on a UV transillumination table equipped with a digital CCD camera.

As standards, we used a mixture of DNA from 9 clones (Van der Gucht et al. 2001). On each gel, three standard lanes were analysed in parallel to the samples, to facilitate comparison between gels.

Sequencing of excised DGGE bands

Bands were excised from DGGE gels with a surgical scalpel, placed in sterile vials and 0.5 g of zirconium beads (0.1 mm diameter) were added. To elute the DNA, 500µl of TE buffer (10mM Tris, pH7.6, 1mM EDTA) was added and the tube was shaken with a Mini Bead-beater (BioSpec Products, USA) for 2 min at 5000 rpm with intermittent cooling on ice. The DNA was incubated overnight at 4°C. Five µl of the supernatant was used as template DNA in a PCR with the primers F357 (no GC clamp) and R518plus as described above. The PCR products were purified by using a QIAquick PCR Purification Kit (Qiagen Inc, Germany), according to the manufacturer's instructions. Sequencing was performed with an automated sequencer (ABI-Prim377, Applied Biosystems, USA) using the appropriate sequencing Kit (PE-Biosystems) and the primer StefITex (5'-GCGTTCTTCATCGTTGCGAG-3') (Van Der Gucht et al. 2001).

The sequences (122 to 181 bp long) were compared against GenBank and EMBL sequences with BLAST (Altschul et al. 1997).

Data analysis

DGGE profiles were analysed using the software program BioNumerics 2.5 (Applied Maths BVBA, Kortrijk, Belgium). The DGGE banding patterns were converted to a binary matrix. Using presence-absence data, a pairwise similarity of the banding-patterns of the different samples was calculated using the Dice coefficient $S_d = 2j/(a+b)$ where j is the number of bands common to both samples, a and b are the total number of bands in sample A and B respectively. This number was then multiplied by 100 to obtain the percentage similarity. We also used the Pearson (product-moment) correlation coefficient, by which the place and the density of the bands are evaluated. A value of 0 indicates that the profiles are completely different, while a value of 100 indicates that they are identical. Using these pairwise similarity values, a UPGMA cluster analysis was performed to determine whether the samples reveal a non-random pattern, or whether they cluster according to the season and sampling place. Bootstrap values were calculated for each dichotomy.

Principal Components Analysis (PCA) was used to investigate the variation in the DGGE banding patterns. PCA allowed ordering of samples and taxa (i.e. bands) along axes (principal components) on the basis of the banding patterns alone

RESULTS

DGGE analysis

All (39) soil DGGE patterns consisted (Figure 2.1) of a few strong bands and a large number of less intense bands, indicating that the 16S rDNA fragments of only a few organisms dominated. A total of 58 different band positions could be identified. The number of bands for the individual samples, varied from 11 to 29, with the highest number found in the samples from the rainy season at a depth of 0.25-0.5 m (mean value 24 versus 20 for samples taken in the rainy season at a depth of 0-0.25 m, and versus 20 for the samples taken in the dry season). Eight bands, (appearing at positions 10, 13, 14, 15, 18, 33, 34, 36) which were also the most dominant bands, were present in all of the samples examined. Less intense bands at positions 1 to 9 were almost solely present in the samples from the rainy season. In addition, two band positions (17 and 28) seem to be specific for the dry season. At position 17, a band was present in 17 out of 20 samples of the dry season and absent in all samples of the rainy season; at position 28 a band was present in all dry season samples but only in 6 out of 19 samples from the rainy season. In the lower part of the profiles some bands were only present in samples from near the stem or in samples further away from the stem (band positions 52 and 54). Other bands showed no clear pattern of presence or absence.

Numerical analysis of the DGGE patterns with two similarity coefficients showed distinct differences between soil samples taken in different seasons (Figure 2.2). Results of the cluster analysis, based on the Dice correlation coefficient, showed visually three separate clusters (Figure 2.2A) with a percentage of similarity above 66. Cluster 1 groups all the samples taken in the rainy season at a depth of 0-0.25 m (except the sample taken at 8 m from the tree, which clusters separately probably because of the limited number (11) of bands revealed). Cluster 2 contains all the samples taken in the dry season and cluster 3 contains the samples taken in the rainy season at a depth of 0.25-0.5 m.

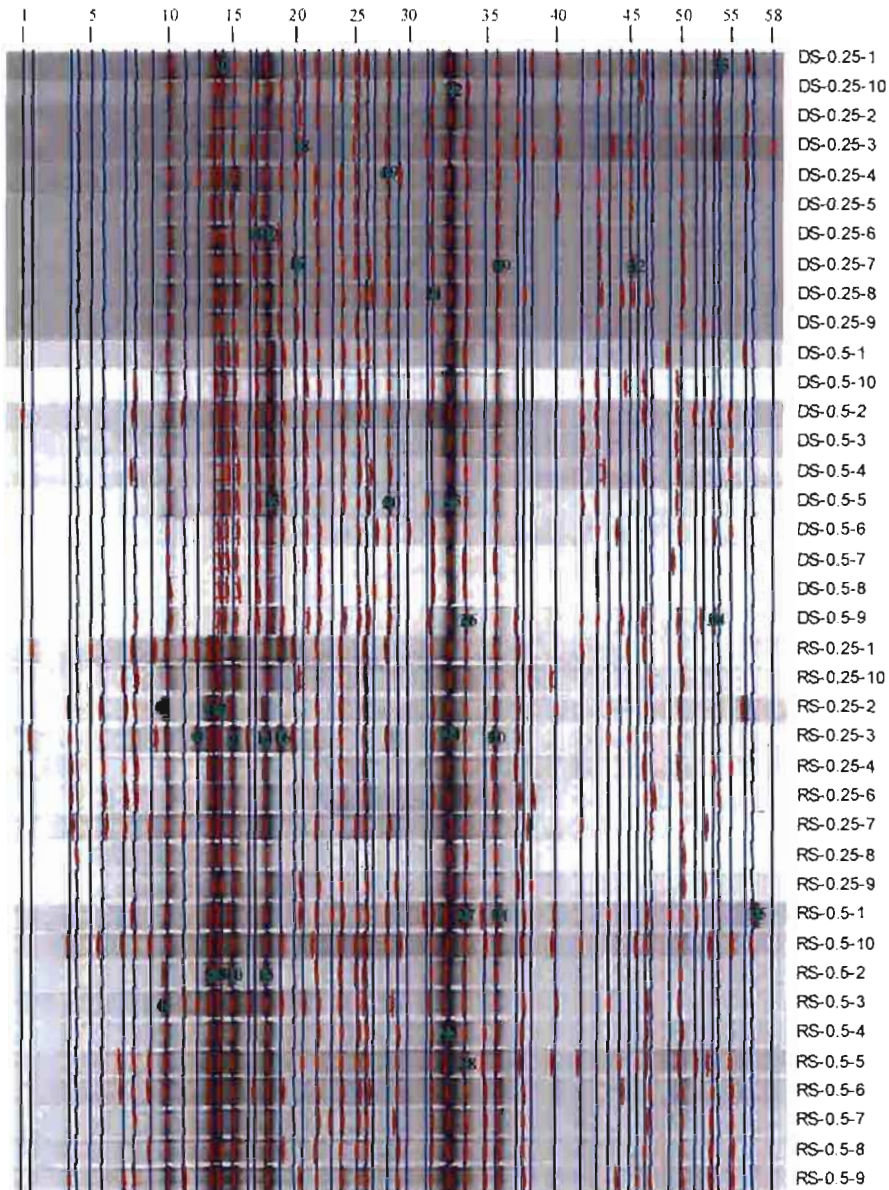


Figure 2.1 DGGE banding patterns from soil samples taken under *Acacia tortilis* subsp. *raddiana* in the rainy season (RS) and in the dry season (DS) at a depth of 0-0.25 m and 0.25-0.50 m along a transect of 1 to 10 m distance from the stem of the tree. The DGGE profiles were analysed using the software program BioNumerics 2.5 (Applied Maths BVBA, Kortrijk, Belgium). Number 1 to 58 indicate the different band positions. Numbered dots refer to the excised bands (see Table 1). The DGGE profiles were ranged numerically by the computer program.

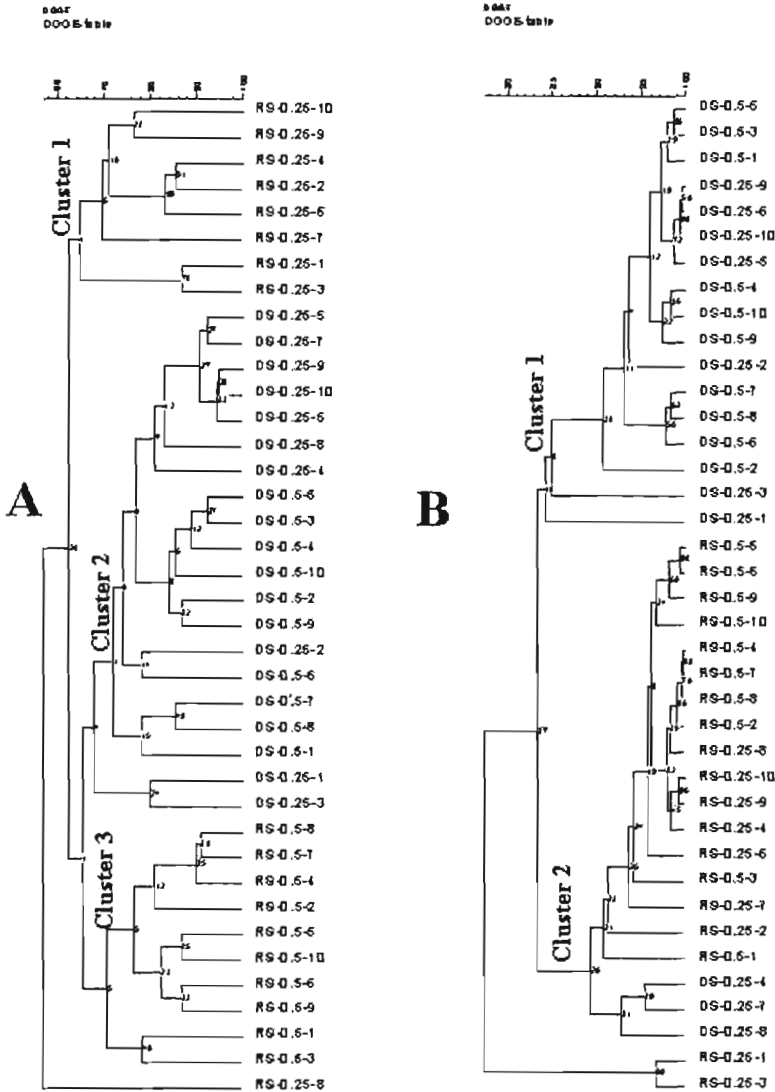


Figure 2.2 Dendrograms obtained by UPGMA clustering of DGGE patterns from Souilène (North of Senegal) soil samples taken under *Acacia tortilis* subsp. *raddiana*. The similarity is expressed as a percentage value of Dice correlation coefficient (A) and Pearson correlation coefficient (B); bootstrap values are shown for each dichotomy in the cluster. AR= *Acacia tortilis* subsp. *raddiana*, RS= Rainy season, DS= Dry season, 0-0.25 (à.25-0.50)-x = depth at xm distance from the tree.

Cluster analysis, based on the Pearson correlation coefficient which takes into account the presence and the intensity of the bands (Figure 2.2B), showed that two samples taken during the rainy season, at a depth of 0-0.25 m. and at 1 and 3 m distance from the stem, are the most divergent. The presence of a more intense band at position 19 is probably at the origin of this divergence. The remaining samples are visually divided over two clusters (above a similarity percentage of 83): one (cluster 2) representing most of the samples taken in the rainy season and three samples from the dry season, and the second one (cluster 1) containing most of the samples taken in the dry season. Samples DS 0.25-4, 0.25-7 and 0.25-8 cluster with the rainy season samples probably because of a more intense band at position 15. The DGGE profiles of different samples taken along the transect at a depth of 0.25-0.5 m were very similar, in the dry season (similarity > 90%) as well as in the rainy season (similarity > 90%) (Figure 2.2).

Sequence analysis of DGGE bands

From the DGGE fingerprints, 35 bands were selected for excision from the DGGE-gels, followed by amplification, purification, DGGE and sequence analysis (Figure 2.1). In some cases two bands were found in DGGE; solely bands corresponding to the original position were sequenced. Representatives of the most intense bands in the profiles (appearing at positions 18 and 33), present in all the samples, were among the sequenced bands (respectively B12, B13, B14, B15 and B22, B23, B24 and B25). The position of the 35 bands, the results of the sequence analysis and their tentative identification (according to a BLAST analysis) are shown in Table 2.1.

The sequence of the bands appearing at position 33 showed 99-100% similarity to a *Bacillus* sp., isolated from a hot-spring microbial mat and described as closely related to *Anoxybacillus thermiflavus*. Also the sequence of bands B29, B30, B31 (at position 36), and B26, B28 (at position 34) showed a high similarity (98%) with *Anoxybacillus thermiflavus*. The sequences of B12, B13, B14 and B15 (position 18) were most similar to that of *Acinetobacter hvoffii* (closest match at 98%). Four of five other dominant bands in the rainy season (appearing at position 10, 13, 14 and 15) were also related to *Acinetobacter* sp. B4 and B5 (position 13) showed 100% similarity to *Acinetobacter schindleri*, B9 and B10 (position 15) 98%.

Table 2.1. Results of a BLAST analysis on the sequences of the 35 excised and sequenced bands. The band positions and the localisation of the bands in the DGGE profiles are given in Figure 1.

Band position on gel	Band n°	Closest match in NCBI search	Bacterial group	Homology range (%)	Accession number
10	B1, B2	Uncultured isolate	<i>Gammaproteobacteria</i>	98-100%	AB002659
12	B3	Elbe River snow isolate Iso32	<i>Gammaproteobacteria</i>	97%	AF150691
13	B4, B5	<i>Acinetobacter schindleri</i>	<i>Gammaproteobacteria</i>	100%	AJ275040
14	B6, B7	<i>Acinetobacter</i> sp. BC111	<i>Gammaproteobacteria</i>	99%	AF189693
	B8	<i>Acinetobacter</i> sp. BC111	<i>Gammaproteobacteria</i>	100%	AF189693
15	B9, B10	<i>Acinetobacter schindleri</i>	<i>Gammaproteobacteria</i>	97-98%	AJ275040
17	B11	<i>Zoogloea</i> sp. GOBU3	<i>Betaproteobacteria</i>	97%	AF321021
18	B12, B13, B14, B15	<i>Acinetobacter hwoffii</i>	<i>Gammaproteobacteria</i>	98%	Z93441
19	B16	<i>Flavobacterium psychrophilum</i>	<i>Cytophaga/Flexibacter/Bacteroides</i> group	96%	AY034478
20	B17	<i>Brevundimonas alba</i>	<i>Alphaproteobacteria</i>	98%	AF296688
21	B18	<i>Bacillus</i> sp. Jsbs2	Endospore forming Gram positive bacteria	99%	AY039821
28	B19	Uncultured soil bacterium TM71.H21	<i>Bacteria</i> , TM7 lineage	93%	AF269009
	B20	<i>Comamonas denitrificans</i>	<i>Betaproteobacteria</i>	94%	AF233876
32	B21	<i>Sphingomonas alaskensis</i>	<i>Alphaproteobacteria</i>	99%	AF378796
33	B22, B23	<i>Bacillus</i> sp. OS-ac-18	Endospore forming Gram positive bacteria Low GC	99%	U46747
	B24, B25	<i>Bacillus</i> sp. OS-ac-18	Endospore forming Gram positive bacteria Low GC	100%	U46747
34	B26	<i>Bacillus niacini</i>	Endospore forming Gram positive bacteria Low GC	97%	AB021194
	B27	Uncultured earthworm cast bacterium	Endospore forming Gram positive bacteria Low GC	92%	AY037670
	B28	<i>Bacillus</i> sp OS-ac-18	Endospore forming Gram positive bacteria Low GC	100%	U26932
36	B29, B30, B31	<i>Anoxybacillus flavithermus</i>	Endospore forming Gram positive bacteria Low GC	98%	Z26932
45	B32	<i>Arthrobacter</i> sp	<i>Streptomyces</i>	99%	AF388034
53	B33	<i>Streptomyces</i> sp. VIT E-99-1336 (B329)	<i>Streptomyces</i>	99%	X95969
54	B34	<i>Streptomyces lividans</i>	<i>Bacteria</i>	100%	AF429400
57	B35	Uncultured soil bacterium C0224	<i>Bacteria</i>	98%	AF218637

B6, B7 and B8 had as nearest relative (99-100% similarity) an *Acinetobacter* sp. BC111, isolated from activated sludge (Barberio et al. 2001). B1 and B2 (position 10) were closely

related to an uncultured *Gammaproteobacterium*, isolated from the deep sea floor (Takami et al. 2001). These bands were also present in the dry season but less intense. From the two band positions (17 and 28) specific for the dry season; the B11 sequence had 97 % similarity with a *Zoogloea* sp. while B19 and B20, occurring at similar positions, represented different phylotypes: B19 had 93 % of similarity with *Comamonas denitrificans*, a representative of the *Betaproteobacteria* and B20 had 93% of similarity with a representative of the TM7 lineage. The similar banding position of these two phylotypes was verified by a separate DGGE analysis.

PCA-analysis

The first two axes of a principal components analysis (PCA) on the samples revealed a clear separation according to the season, with the samples taken in the dry season situated on the left side of the diagram and the rainy season samples on the right side (Figure 2.3). The first and second principal components, PC1 and PC2, explained respectively 44.1 and 12.3 % of the variance of the data. Rainy season samples, on the left side of the diagram, are characterized by very intense bands situated at position 13, 14, and 15, having highest similarity with the sequences of members of the genus *Acinetobacter* e.g. *A. schindleri* related organisms. Band positions 12 and 19 are separated from the main group of positions within the rainy season samples. The most characteristic band (B16 at position 19) of the rainy season samples, taken at a depth of 0-0.25 m, at 1 and 3 m distance from the tree has a sequence that is most similar with the sequence representing a member of the *Cytophaga-Flexibacter-Bacteroides* group, a *Flavobacterium psychrophilum*-like organism. Dry season samples, on the right side of the diagram are characterized by the presence and intensity of bands situated at position 17 and 28, representing respectively members of the genus *Zoogloea* and *Comamonas*, and bands at position 18 and 33, the most intense bands in the profiles, representing respectively members of the genera *Acinetobacter* and *Bacillus*.

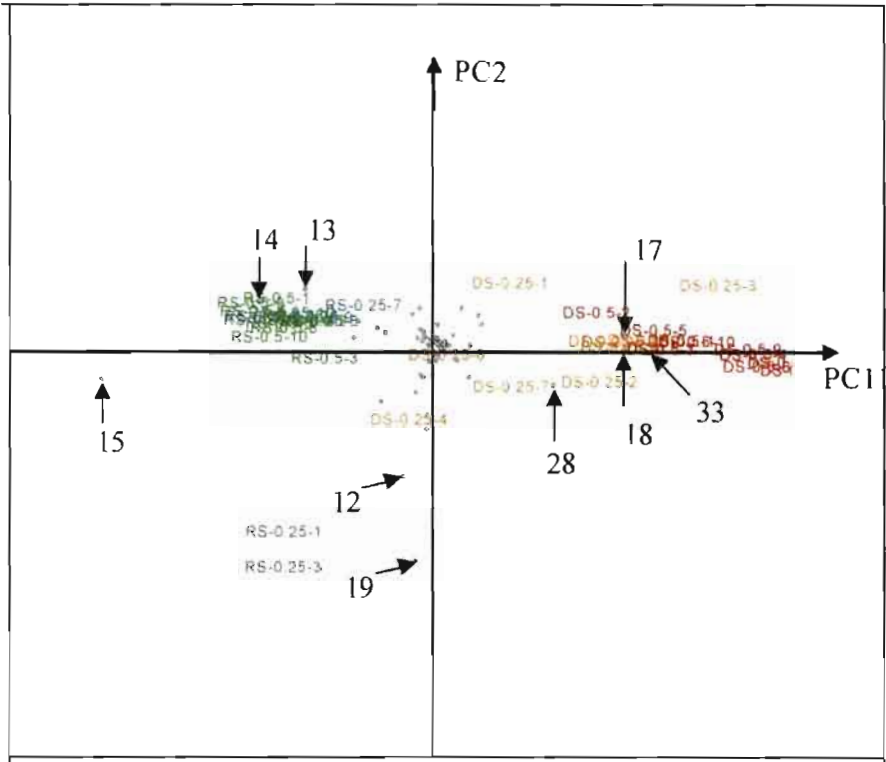


Figure 2.3 PCA biplot of the DGGE banding patterns from Souilène (North of Senegal) soil samples taken under *Acacia tortilis* subsp. *raddiana*. All samples studied were included (for details on the samples see Figure 1). Arrows indicate bands, which are characteristic for the groups found.

DISCUSSION

DGGE analysis combined with limited sequencing of several of the bands found as used in the present study does not allow a complete characterization of the bacterial community. Yet, the technique gives an idea of the relative most abundant bacterial groups in the soil and of differences between samples.

In Souilène soil we found by PCR-DGGE a rather low diversity within the relative most abundant groups (only 58 bands were detected) when compared to e.g. what was found in temperate grassland soils (McCaig et al. 2001; Gomes et al. 2001). A possible explanation for these differences in DGGE patterns between temperate and tropical soil might be found in the amount of rainfall and the temperature.

The DGGE patterns from the different samples have a similar aspect: an average number of bands for each sample with a few dominant bands of which the strongest are situated on the same position in all lanes (Figure 2.1). This indicates already a relatively

large, uniform and dominant population, corresponding to a more competitive population than found in most biological communities (Zhou et al. 2002). Our DGGE profiles correspond with those described for low carbon-subsurface sites as described by Zhou et al. (2002) who proposed that the dominant structure is the result of competitive interactions. In such a pattern, some species can be eliminated by competitive exclusion while a few others that are better adapted to the conditions, can dominate.

The cluster analysis of our DGGE banding patterns and the PCA-analysis provided evidence that bacterial communities differ according to the sampling date. The results obtained in the cluster analysis using the Pearson correlation coefficient corresponded better with the results of the PCA analysis than the results of the cluster analysis obtained with the Dice coefficient. This could be expected since the Pearson coefficient takes into account the presence and the intensity of the bands. The dendrogram obtained with the Pearson coefficient is also supported by slightly higher bootstrap values (Figure 2.2) than the dendrogram obtained with the Dice coefficient. The differences between the samples taken in the two seasons are more important than the differences observed between the samples taken at different depths (0-0.25 versus 0.25-0.5 m) or the observed spatial shifts. Spatial shifts seemed to be more obvious in the samples taken at a depth of 0-0.25 m, where most of the roots are found, than at a depth of 0.25-0.5 m. The fact that almost no spatial shifts of the abundant bacterial 16S rDNA molecules were observed, suggests that the presence and activity of the dominant soil bacteria remains stable. The same results were also found by Felske and Akkermans (1998).

Interpretation of our results of the cluster and PCA analyses demand cautious. As indicated by the sequence analysis of bands occurring at similar positions in lanes, these sequences might affiliate to different phylogenetic clusters, e.g. B19 and B20 at position 28 (the closest matches are respectively a member of the *Betaproteobacteria* the TM7-lineage). These biases are of general significance in the analysis and therefore it is of major importance to combine cluster and/or PCA analysis with sequence analysis of representative bands.

The taxa that we were able to characterize by sequencing the most prominent DGGE bands are similar to apparently common and widespread soil taxa (Smalla et al. 2001; Gomes et al. 2001; Duineveld et al. 2001; Smit et al. 2001; Felske and Akkermans 1998; Normander et al. 2000; El-Beltagy et al. 1994; Ludwig et al. 1997). The majority of the analysed bands belong to the genera *Bacillus*, *Anoxybacillus* (10) and *Acinetobacter* (11). For the bands corresponding with *Anoxybacillus thermiflavus* three bands are found on three different bandpositions in the gel due to sequence heterogeneities of the 16S rDNA

operon. Most bands corresponding to representatives of the genus *Acinetobacter* were less intense in the dry season samples. This seasonal difference was not observed in the bands related to representatives of the genus *Bacillus*. A possible explanation for this difference in intensity between *Acinetobacter* and *Bacillus* populations might be that the spores of the latter genus are more resistant to extreme conditions. Amongst the less intense bands, sequences were found corresponding to various members of other *Proteobacteria*, *Streptomyces*, a representative of the *Cytophaga-Flavobacterium-Bacteroides* lineage and one sequence having 93% of similarity with a representative of group 3 of division TM7 containing only environmental 16S rDNA sequences (Hugenholtz et al. 2001). The TM7 division is described as a division level phylogenetic lineage in the *Bacteria*; several morphotypes have been found using the FISH-technique (Hugenholtz et al. 2001; Dojka et al. 2000). When compared to populations described in other papers using DGGE and/or 16S rDNA cloning (Smalla et al. 2001; Gomes et al. 2001; Duineveld et al. 2001; Smit et al. 2001; Felske and Akkermans 1998; Normander et al. 2000; El-Beltagy et al. 1994; Ludwig et al. 1997), the composition of our samples corresponds with their data except that we found no member of the *Acidobacterium-Holophaga* group. It has been suggested that the ratio between *Proteobacteria* and sequences belonging to this group might be indicative of the trophic level of the soil (Smit et al. 2001). This hypothesis, not universally accepted (McCaig et al. 2001) and based on temperate-cultivated soils, would suggest that our soil has a high trophic level. However, since this is the first study in tropical semi-arid soil a more thorough analysis, including physico-chemical analysis of soil samples and clone library construction, is needed before drawing general conclusions.

Another striking result was that no sequence shows a high similarity with rhizobia-related organisms, even though the sampling was done under *Acacia tortilis* subsp. *raddiana*, a legume tree that can establish a symbiotic relation with species of *Mesorhizobium* and *Sinorhizobium*. de Lajudie et al. (1998) isolated and described *Mesorhizobium plurifarium* from root nodules taken under several *Acacia tortilis* subsp. *raddiana* trees present on the sampling site. The reason that we did not detect any member of the *Acidobacterium-Holophaga* group in our sequence analysis and no rhizobia-related organism might be that their abundance in soil is very low compared to the other community members. The detection limit of PCR-amplified 16S rRNA gene fragments in DGGE analysis has been estimated to be 1% of the total amplified 16S rDNA (Muyzer et al. 1993). Another possible reason is biased PCR reactions due to selective amplification (Cottrell and Kirchman, 2000), or biased DNA composition due to selective isolation. It might also be that we did not pick any of the *Acidobacteria* bands for the sequence analysis.

Evidence that these taxa are present in our samples is given elsewhere (Demba Diallo et al. 2003b). We assume that, despite these shortcomings, the taxa we detected on our gels, and in particular the most intense bands represented the great majority of the bacteria present in the soil.

In conclusion, our results show that the bacterial community in Souilène soil under *Acacia tortilis* subsp. *raddiana* is dominated by members of the genera *Bacillus* and *Acinetobacter* and a limited number of members of *Alpha*- and *Betaproteobacteria*, the CFB-group, the genus *Streptomyces* and the TM7-lineage. No representative of the *Acidobacterium-Holophaga* group, of major importance in similar ecosystems, was detected. On the one hand, the soil is characterized by a dominant structure consisting of a few relatively large, stable, uniform populations of the most abundant taxa. On the other hand, our data suggest that bacterial communities under *Acacia tortilis* subsp. *raddiana* might differ according to the season, although the design of our study (sampling only one transect in the rainy season and one in the dry season) does not allow us to generalize.

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Chapter 3

Phylogenetic Analysis of Partial Bacterial 16S rDNA Sequences of Tropical Grass Pasture Soil under *Acacia tortilis* subsp. *raddiana* in Senegal

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ABSTRACT

We used direct recovery of bacterial 16S rRNA gene sequences to investigate the bacterial diversity under *Acacia tortilis* subsp. *raddiana*, a legume tree naturally growing in the dry land part of Senegal (West Africa). Microbial DNA was purified directly from soil samples and subjected to PCR with primers specific for bacterial 16S rRNA gene sequences. 16S rDNA clone libraries were constructed from two soil samples taken at two dates, i.e. June 25th 1999 (dry season) and August 28th 1999 (rainy season) at depths of 0.25-0.50 m and at 3 m distance from the stem. The 16S rDNA of 117 clones was partially sequenced. Phylogenetic analysis of these sequences revealed extensive diversity (100 phylotypes). Comparative sequence analysis of these clones identified members of the *Gammaproteobacteria* (35% of the phylotypes) as the most important group, followed by the *Firmicutes* division with 24%. *Alphaproteobacteria*, *Betaproteobacteria*, *Acidobacteria* and *Actinobacteria* were found to be less represented. Our data suggest that bacterial communities under *Acacia tortilis* subsp. *raddiana* might differ according to the season. The relative compositions of the populations is different in both samples: the *Acidobacteria* are present in a much higher percentage in the dry season than in the rainy season sample while the inverse effect is observed for the members of the other groups. Within the *Gammaproteobacteria* we found a shift between the dry season and the rainy season from pseudomonads to *Acinetobacter* and *Escherichia* related organisms.

INTRODUCTION

In the semi-arid region of West Africa, soil fertility under the canopy of leguminous trees is frequently superior to that in open grassland areas (Deans et al. 1999) and this beneficial effect is exploited by the farmers (Le Houérou, 1989). Legume trees play an important role in reforestation and maintenance of soil fertility (Ba et al. 2002). Vegetation is one environmental factor thought to be a major determinant of the composition of the soil microbial community since it provides the primary resource for heterotrophic growth. Bacteria are known to have a high abundance in the soil (up to 10^9 cells per gram of soil) (Ranjard et al. 2000) and a high diversity (a minimum of 4000-7000 different bacterial genomes per gram of soil) (Torsvik et al. 1990). They represent a functionally diverse group of organisms known to play crucial roles in decomposition, food chains, and biogeochemical cycling. Different micro-organisms might be expected to grow in soil under *Acacia tortilis* subsp. *raddiana*, often called the "umbrella thorn" for its distinctive spreading crown. Some acacia trees (around 10 species) are native to the Sahelian and Saharan areas of the African continent, and are valuable for their symbiotic bacteria fixing atmospheric N_2 . Some of them prevent wind and rain erosion, control sand dunes and are important sources of wood and fodder for browsing livestock. Additionally, bound nitrogen is returned to the soil by the roots and by the natural loss of leaves, resulting in humus enrichment, which is advantageous for the fertility of the soil and its physical properties. Except for our previous study in which these populations were studied by DGGE and sequencing of some abundant bands (Demba Diallo et al. 2003a), to our knowledge no other data exist on the composition of soil communities under this tree in the dry land part of Senegal. Nowadays it is established that more than 90% of the micro-organisms existing in nature are refractory to selective enrichment cultures (Ward et al. 1990). To overcome these drawbacks, interest is currently focused on the use of molecular biological techniques, given their powerful capacity to allow the analysis of bacterial communities in their natural habitats. In this context, since 1990 (Ward et al. 1990), analysis of the 16S rRNA molecule or its corresponding gene (16S rDNA) has been by far the most widely used approach in the last decade.

The aim of the present work was to study bacterial diversity associated with *Acacia tortilis* subsp. *raddiana* and to compare the bacterial communities from the same soil taken at 2 different sampling dates (rainy and dry season) in the Northern pastoral-forestry zone of Senegal. To obtain phylogenetic information, we used the classical cloning-sequencing approach on DNA directly extracted from soil. Universal *Bacteria* primers were used to

amplify cloned 16S rDNA. The 16S rDNA of the various different clones was partially sequenced and the sequences analysed.

MATERIAL AND METHODS

Study site and sample collection

The study was carried out in Souilène (IRD's Biological Research Station in North Senegal), in the grassland part of the Ferlo Region near the village of Windou Thiengoly and Mbeulekhe, situated between 15°40'-16° N, 15°40' W. The soil of the region consists of degraded red/brown ferruginous sand, which extends to a depth of at least 30 m (Deans et al. 1999), and is characterized by a low organic matter content, and poor water and chemical retention properties. Consisting almost completely of quartz it has a very low cation exchange capacity. Annual long-term rainfall, which occurs in a single rainy season between July and October, was on average 297 mm for the period 1981-1993 but more recently, it has decreased to about 150 mm. A comprehensive description of the area is given by Le Houérou (1989), and floristic details of the district were reported by Miehe (1990). Two soil cores (2.5 by 25 cm) were taken under *Acacia tortilis* subsp. *raddiana* at depths of 0.25-0.50 m beneath the canopy at a distance of three meters from the stem. Sampling was done at the end of the dry season (June 25th 1999) and in the middle of the rainy season (August 28th, 1999). These samples were selected, after examination of the banding patterns of various soil samples analyzed by DGGE (Demba Diallo et al. 2003a), to capture the samples with the most bands.

DNA extraction and PCR amplification of 16S rDNA

Bacterial community DNA was extracted from soil samples by a direct-lysis method previously described (Demba Diallo et al. 2003a). The extracted DNA was subjected to PCR amplification with the bacteria-specific primers: F27 (5'-AGAGTTTATCMTGGCTCAG-3', (Lane et al. 1991) and R1492 (5'-GRTACCTTGTTACGACTT-3'). Numbering refers to the *Escherichia coli* 16S rRNA gene position corresponding to the 3' end of the primers. PCR amplification was performed by means of a Genius temperature cycler (Biometra, Göttingen, Germany) in 50 µl reactions containing approximately 100 ng of purified DNA, 10 mM Tris/HCl pH 8.3, 15 mM MgCl₂, 500 mM KCl, 0.1% gelatine, 10 µl µg⁻¹ BSA, 1U µl⁻¹ of Expand high-fidelity DNA polymerase (Boehringer, Mannheim, Germany); and 200 µM of each deoxyribonucleotide. The temperature and cycling conditions were as follows. First,

preheating at 94°C for 5 min; then 25 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and a final incubation at 72°C for 5 min. The presence of PCR products and their concentration were checked by electrophoresis of 5 µl product on a 2% agarose gel, stained with ethidium bromide. A molecular weight marker (Smart ladder-Eurogentec, SA Belgium) was included.

Clone library construction

To generate nearly full-length 16S rDNA clones, the PCR product was ligated into the pGEM-T vector (Promega, Madison, Wis. USA) and the ligation reaction was used to transform competent *Escherichia coli* strain JM109. Recombinant colonies were selected on Luria-Bertani agar plates containing 20 µg ml⁻¹ X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and 100 µg ml⁻¹ ampicillin. Plates were incubated overnight at 37°C. The presence of inserts was determined by direct PCR on a sample from white (positive) bacterial colonies, using primers flanking the cloning sites on the vector.

DGGE analysis

To screen the clones, the V3 region of the 16S rDNA of each clone was amplified by using 1 µl of each clone culture directly as a DNA template. The primers used are: F357GC (5'-CGCCCCGCGCGCCCCGCGCCCGGCCCGCCCCGCCCCCCTACGGGAGGCAGCAG-3') and R518 (5'-ATTACCGCGGCTGCTGG-3'). The PCR amplification procedure was performed as described by Van der Gucht et al. (2001).

The DGGE technique was performed using the D-Code System from Bio-Rad Laboratories. PCR products were loaded onto 8% (w/v) polyacrylamide gels, 1 mm thick, in 1 × TAE buffer [20 mM Tris-acetate pH 7.4, 10 mM acetate, 0.5 mM disodium EDTA]. The denaturing gradient contained 35% to 70% denaturant (100% denaturant corresponded to 7 M urea and 40% (vol/vol) deionized formamide). Electrophoresis was done at a constant voltage of 75 V for 16 h. The temperature was set to 60°C. After electrophoresis, gels were incubated for 1 hour in TAE containing ethidium bromide (0.5 mg l⁻¹). The bands were visualized on a UV transillumination table equipped with a digital CCD camera.

As standard, we used a mixture of DNA from 9 clones (Van der Gucht et al. 2001). On every gel, three standard lanes were included in parallel with the samples in order to compare the

patterns formed in different gels. This procedure was semi-automated using the software package BioNumerics 2.5 (Applied Maths BVBA, Kortrijk, Belgium).

The similarity between the profiles was calculated using the Dice correlation coefficient and a similarity matrix was constructed. The data were clustered by UPGMA.

Phylogenetic analysis

Representatives of the 16S rDNA clones were partially sequenced; the sequence of 500 base pair long fragment was determined with an automated sequencer (ABI-Prism377) combined with a sequencing kit (PE-Biosystems) and the primer R519 (5'-GTATTACCGCGGCTGCTG-3')

A GenBank BLAST search (Altschul et al. 1997) was performed for each of our sequences in order to sort out the closest relatives. The selected sequences were aligned automatically with BioNumerics 2.5 and manually corrected. Phylogenetic reconstructions were performed separately for the *Alphaproteobacteria*, the *Betaproteobacteria*, the *Gammaproteobacteria*, the *Firmicutes* and for the *Acidobacteria*. A final alignment of 413 bases was imported into BioNumerics 2.5. Distances, including deletions and insertions were calculated according to Jukes and Cantor (1969), whereupon the overall neighbour-joining (NJ) phylogenetic dendrogram was inferred, rooted and bootstrapped 500 times (Hillis and Bull. 1993). Identical sequences were considered as a single phylotype.

Rarefaction analysis

Rarefaction curves (Heck et al. 1975) were produced by using a web-based program written by C.J. Krebs and J. Brzustowski (University of Alberta, Edmonton, Canada) available online at <http://www.biology.ualberta.ca/jbrzusto/rarefact.php>.

Nucleotide sequences accession numbers.

The sequences obtained in this study have been deposited in the GenBank database under accession no: AY344844 to AY344960.

RESULTS

Clone library construction and screening of the library

16S rRNA gene clone libraries were constructed from DNA extracted from 2 Senegalese grassland soil samples (Demba Diallo et al. 2003a). Nearly full-length 16S rRNA gene clones were obtained by PCR amplification with primers near the ends of the gene and cloning into a TA cloning vector.

Felske et al. (1998), and Bano and al. (2002) showed that clone screening by DGGE or TGGE is a convenient and efficient way to detect and retrieve the most abundant 16S rRNA sequences.

One hundred and forty-nine 16S rDNA clones were collected from the dry season sample and screened by DGGE. Ten clones delivered a single band corresponding to the place of the *E. coli* band and four contained three bands. From the remaining clones, 48 representing various band classes containing in total 120 clones, were selected for sequencing. One hundred and forty-eight clones were isolated from the rainy season sample. Four contained three DGGE bands, and 10 contained a single band corresponding with *E. coli*. After further screening, 68 clones were selected for sequencing.

The representative clones were partially sequenced producing on average 450 bp long quality sequences. After a BLAST analysis, phylogenetic reconstructions were made on an alignment of 413 bases.

Comparative sequence analysis and coverage of libraries

All the sequences appeared to cluster in bacterial phyla and classes, which are generally found in soil: *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* as well as *Firmicutes*, *Acidobacteria* and *Actinobacteria*. Only one clone (ARDS52) was found to belong to the *Actinobacteria* showing 96% similarity with *Streptomyces diastatochromogenes*. Forty-seven and 53 phylotypes were identified in samples from the dry and the rainy season respectively.

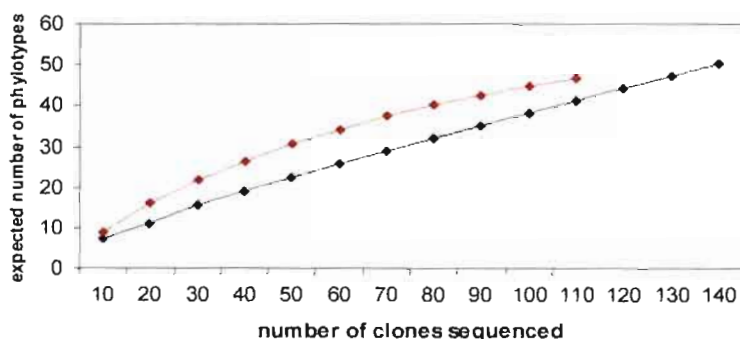


Figure 3. 1. Rarefaction curves generated for 16S rRNA genes in clones libraries from samples collected in the dry season (DS) and rainy season (RS).

As indicated by the coverage analysis (Figure 3.1) the number of clones screened and sequenced in this study was not enough to cover the full bacterial diversity.

Gammaproteobacteria clones

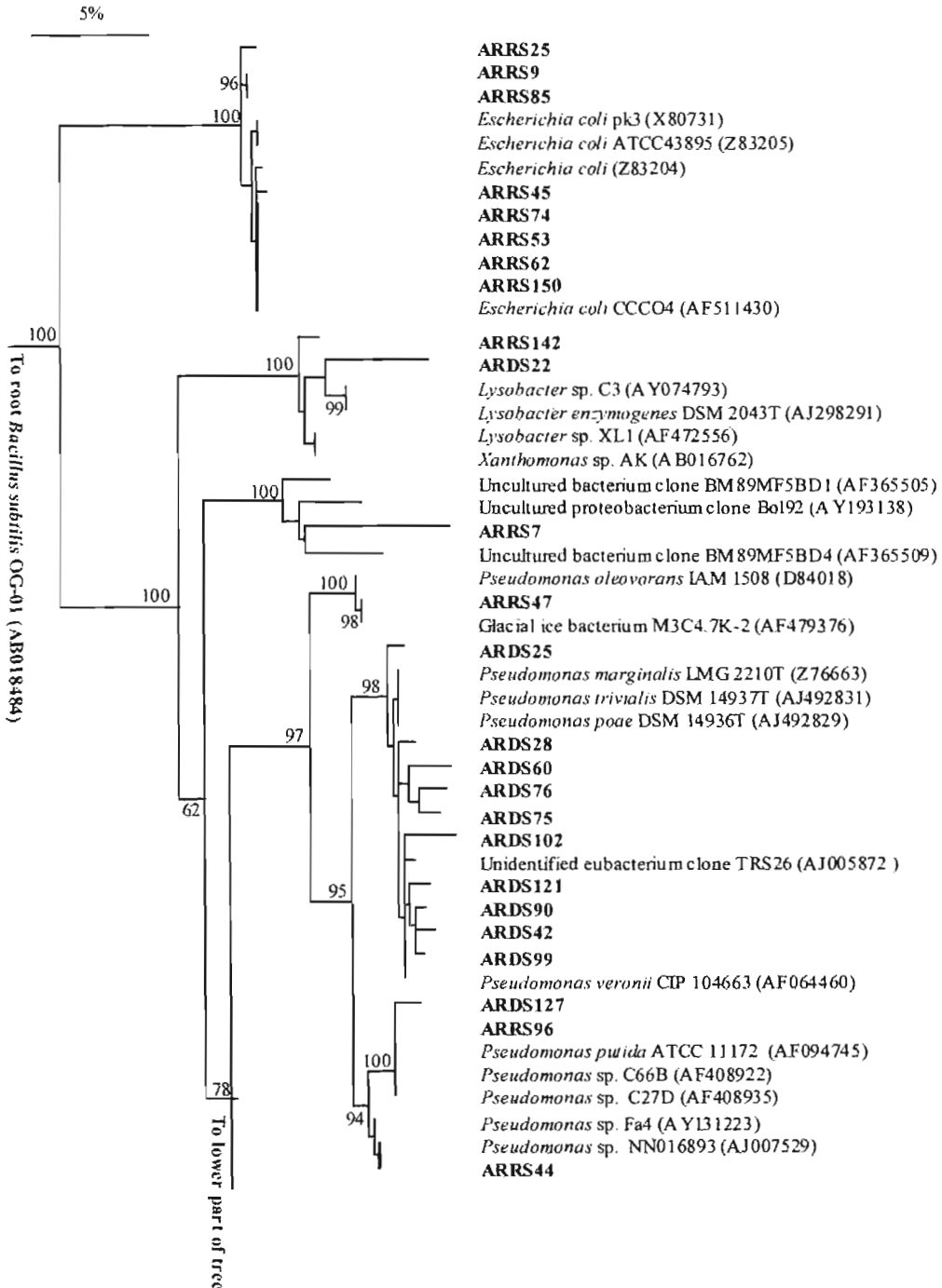
The largest number (35) of phylotypes (35% of the 100 phylotypes and representing 108 clones) was found to belong in the *Gammaproteobacteria* (Figure 3.2). Within this lineage our phylotypes belonged mostly in the *Acinetobacter*, the *Pseudomonas* and the *Escherichia* clusters. Two phylotypes (ARDS22, ARRS142) clustered with *Lysobacter* and one with two uncultured bacteria occupying a separate position. *Lysobacter* contains non-fruiting gliding bacteria with a high % G+C; they have been isolated from soil and water and can lyse all kinds of micro-organisms (Christensen et al. 1978).

Fifteen phylotypes from both seasons clustered in the *Acinetobacter* group (supported by a 100% bootstrap value). *Acinetobacter* consists of aerobic, oxidase-negative, penicillin-resistant rods that are common soil and water inhabitants (Baumann, 1968; Bouvet and Grimont, 1986; Nemeč et al. 2001; Ibrahim et al. 1997). Their nutritional properties and ubiquitous occurrence in soil and water suggest that they may be very important agents in the aerobic mineralisation of organic matter in nature (Baumann, 1968). Amongst the phylotypes of the *Acinetobacter* cluster, 4 [ARRS110 (= ARRS40, ARRS49, ARRS105), ARRS32 (=ARRS124), ARDS92 and ARRS41] representing 36 clones had the highest sequence similarity with a subcluster containing *Acinetobacter hwoffii*, a soil bacterium (Kim et al. 2002). Another 4 phylotypes (ARRS48, ARRS125, ARRS28 and ARRS39, representing 7 clones) were related to the subcluster containing the type strain of *Acinetobacter johnsonii* (DSM 6963^T). Clones ARDS48 and ARDS8 constitute another *Acinetobacter* subcluster together with *Acinetobacter* sp. BC187 isolated from activated sludge of a treatment plant collecting wastes enriched in ethoxylated nonylphenols (Barberio et al. 2001) and more closely related to *A. calcoaceticus*. Phylotype ARDS100 has *A. schindleri* (Nemeč et al. 2001) as its closest relative. Phylotypes ARRS20 (= ARRS 60) belongs to a separate subcluster containing also ARRS70 and ARDS125 which has an aberrant sequence. Their closest relative is *Acinetobacter* sp. Hop10 (Barberio and Fani, 1998).

Thirteen phylotypes (mostly from the dry season) cluster in the *Pseudomonas* group, supported by a 97% of bootstrap value. The authentic pseudomonads contain many species isolated from soil (Vermeiren et al. 1999; Cilia et al. 1996; Anzai et al. 1997; Anzai et al. 2000). Phylotype ARRS47 clusters together with the type strain of *Pseudomonas oleovorans* (IAM1508^T). Ten phylotypes (ARDS25, ARDS28, ARDS60, ARDS76, ARDS75, ARDS102, ARDS121, ARDS90, ARDS42 and ARDS99 comprising 22 clones) fall in a subcluster containing *P. marginalis*, *P. veronii*, '*P. trivialis*' and '*P. poae*'. The latter two species have recently been created for strains isolated from the phyllosphere of grasses (Behrendt et al. 2003). Phylotypes ARDS127 and ARRS96 grouped together with *P. putida* and a *Pseudomonas* isolate NZ17, a

pathogen of *Agaricus bisporus* (Godfrey et al. 2001), while phylotype ARRS44 clustered with *Pseudomonas* sp. NNO16893 (Johnsen et al. 1999) and Fa20 (Krimm et al. unpublished, 2002).

Four phlotypes, all from the rainy season [ARRS45, ARRS9 (= ARRS85), ARRS62 = (ARRS150, ARRS74, ARRS53) and ARRS25]] grouped with *Escherichia coli* strain PK3 and *E. coli* ATCC 43895 (Cilia et al. 1996; Kahn, 1968). All the clones related to the *Escherichia* group were from the rainy season sample while 12 clones out of 14 related to the *Pseudomonas* group were from the dry season sample.



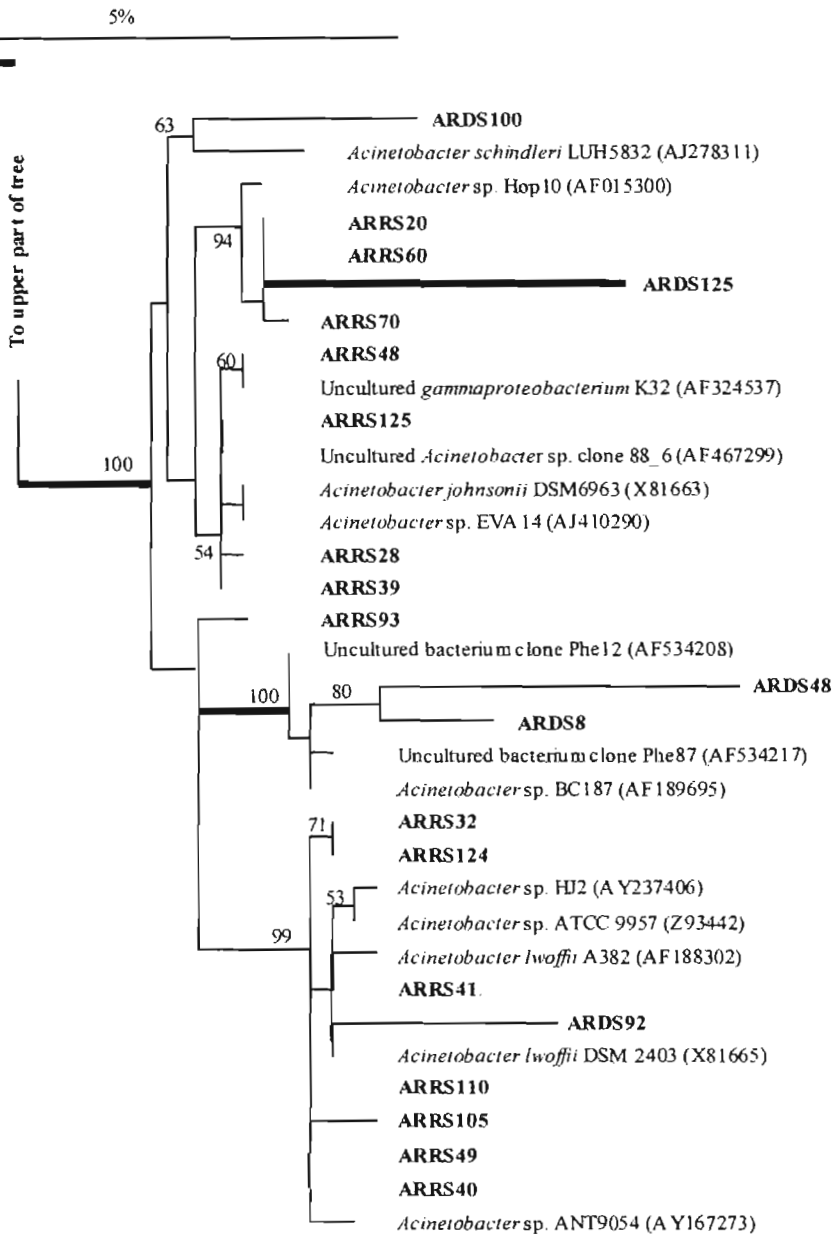


Figure 3.2 Neighbor-joining tree depicting the phylogenetic relationships among the *Gammaproteobacteria* sequence in this study. Species or strain names are preceded by their GenBank accession numbers. Clones detected in this study are given in boldface. Bootstrap values above 50 are shown, representing the percentage support for cluster out of 500. AR= *Acacia tortilis* subsp. *raddiana*, RS= Rainy season, DS= Dry season. The scale gives genetic distances. Bold lines corresponding to a separate scale are used to reduce long branches.

***Firmicutes* clones**

The second largest series with 24% phylotypes representing 64 clones belong to the *Firmicutes* (Figure 3.3); several clusters and subclusters were found. One phylotype [ARRS100 (= ARRS65 and ARRS121), representing 18 clones] grouped with the formerly called '*Bacillus flavothermus*' now reclassified as *Anoxybacillus thermiflavus* (Rainey, et al. 1994a; Pikuta et al. 2000), while phylotype ARRS6 clustered more remotely from the other *Firmicutes* relates phylotypes. Phylotypes ARDS81, ARDS79 and ARRS55 (together 8 clones) occupied separate positions. Three phylotypes (ARRS113, ARRS126 and ARRS42) clustered with both species of *Ammoniphilus* (Zaitsev et al. 1998) obligately oxalotrophic ammonium-dependent organisms isolated from the rhizosphere of sorrel (*Rumex acetosa*). Within the *Paenibacillus* cluster we found 2 subclusters one with *P. turicensis* (Bosshard et al. 2002) and another one containing the potato endophytic bacteria described as an uncultured *Paenibacillus* sp. (Reiter et al. 2002). Phylotype ARRS33, clustered in the *P. turicensis* subcluster while phylotype ARRS132 (= ARRS143) were more closely related to potato bacterium B9 (Reiter et al. 2002). Several clones were found in the *Bacillus* cluster: phylotypes ARDS51 and ARRS3 were related to the "*Bacillus litoralis*" sublineage (Gilvanova unpublished. 2001), two phylotypes (ARDS93, ARDS62) were related to *Bacillus methanolicus* (Arfman et al. 1992), and ARRS94 and ARRS77 grouped with *Bacillus licheniformis*. Two phylotypes (ARRS123 and ARDS67) form a group affiliated with *Bacillus* sp. (strain MKO3) isolated and described by Suzuki et al. (2002), and, interestingly, also with bacteria from very cold habitats, like the glacial ice bacterium G500K-19; phylotype ARRS83 belongs in the same subcluster and has a higher sequence similarity with *Bacillus* sp. LMG19415 (Gurtner et al. 2000). ARDS4 and ARRS19 formed a distinct lineage encompassing also *Bacillus firmus* and two clones of uncultured bacteria (335-1 and 511-1) described by Valinsky et al. (2002). Three other phylotypes (ARDS149, ARRS109 and ARDS64) constitute another subcluster containing *Bacillus niacini*, a nicotinate-metabolizing mesophile isolated from soil (Nagel and Agneesen, 1991).

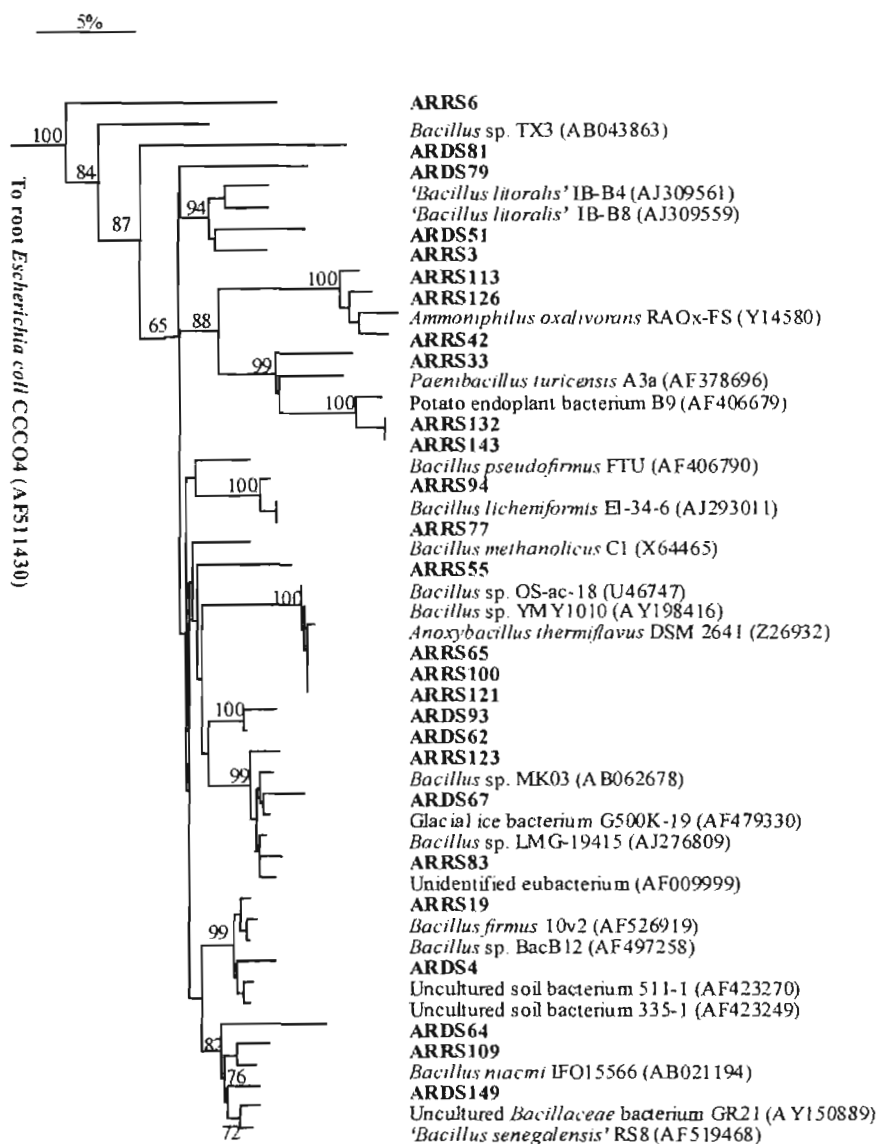


Figure 3.3 Neighbor-joining tree depicting the phylogenetic relationships among the *Firmicutes* sequences in this study. Species or strain names are preceded by their GenBank accession numbers. Clones detected in this study are given in boldface. Bootstrap values above 50 are shown, representing the percentage support for cluster out of 500. AR= *Acacia tortilis* subsp. *raddiana*, RS= Rainy season, DS= Dry season. The scale gives genetic distances.

Betaproteobacteria clones

Fourteen phylotypes (14% representing 27 clones) belong to the *Betaproteobacteria* (Figure 3.4). Two phylotypes [ARRS26, (= ARRS4) and ARRS2, representing 11 clones] grouped together in a cluster containing *Massilia timonae* isolated from human patients (Lindquist et al. 2003). Three dry season phylotypes (ARDS96, ARDS108 and ARDS14) grouped with “*Pseudomonas mephitica*” belonging to the *Janthinobacterium lividum* cluster (Anzai et al. 2000), *Duganella zoogloeoides* and *Zoogloea* sp. BAL15. *Zoogloea* sp. BAL15 is the closest relative of ARDS14, ARDS108 and ARDS96. The sequence of *Zoogloea* sp. BAL15 has more similarity with that of *Duganella zoogloeoides* indicating that the former species is probably not a genuine *Zoogloea* member because *Zoogloea ramigera* IAM12137^T belongs in the same cluster but on another branch also containing phylotype ARDS127 and *Betaproteobacterium* MC10 that is capable of catabolising biodegradable polymers (Suyama et al. 1998). Three phylotypes [ARRS46, ARDS36 and ARRS66 (= ARRS114)] were found in a cluster containing the sequences of two unidentified bacterial clones oxSCC-6 and oxSCC-26 isolated from flooded paddy soil (Lüdemann et al. 2000). Three phylotypes [ARDS84, ARRS51 and ARRS27 (= ARRS18)] clustered within *Acidovorax* with the highest similarity to a strain (B2/74) isolated from the pupa of *Wohlfahrtia magnifica*. Phylotypes ARRS101 occupies a separate position. One sequence, ARRS29, clustered with *Comamonas*.

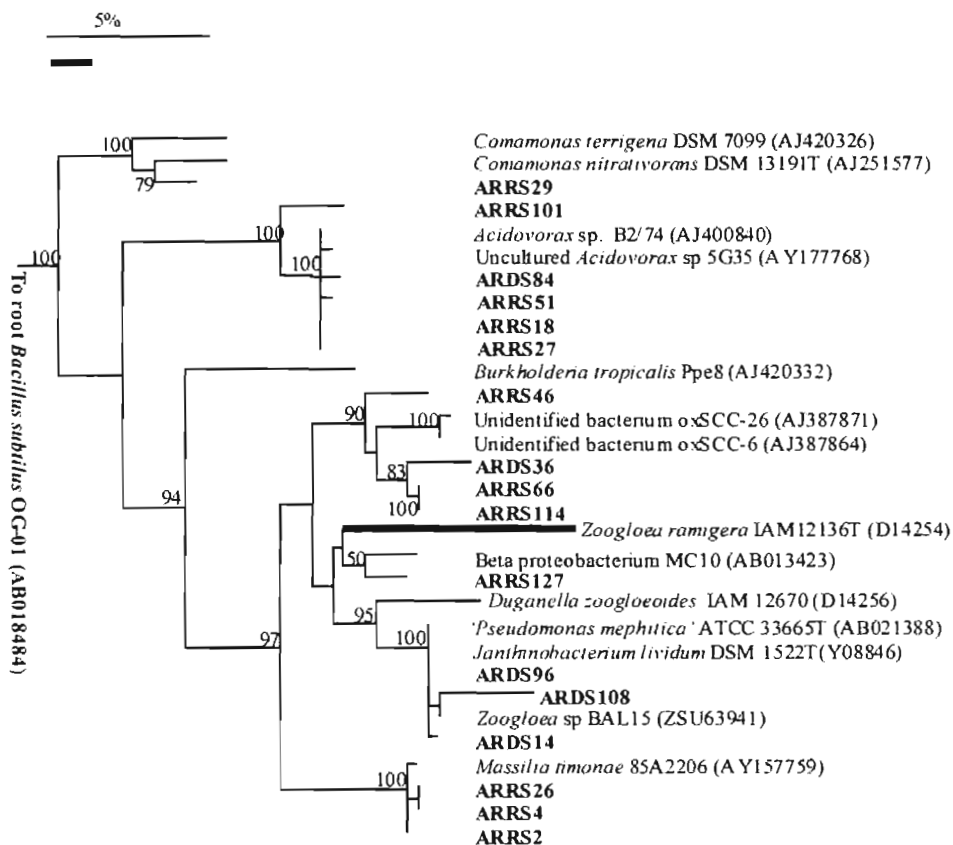


Figure 3.4 Neighbour-joining tree depicting the phylogenetic relationships among the *Betaproteobacteria* sequences in this study. Species or strain names are preceded by their GenBank accession numbers. Clones detected in this study are given in boldface. Bootstrap values above 50 are shown, representing the percentagesupport for cluster out of 500. AR= *Acacia tortilis* subsp. *raddiana*, RS= Rainy season. DS= Dry season. The scale gives genetic distances. Bold lines corresponding to a separate scale are used to reduce long branches

***Alphaproteobacteria* clones**

Eleven phylotypes (11% representing 29 clones) belong to the *Alphaproteobacteria* (Figure 3.5) of which 4 clustered with members of the rhizobia: two sequences (ARRS139, ARRS10) clustered with *Mesorhizobium* sp. WG detected in a bioreactor and characterized by Costa et al. (2000). Phylotype ARDS107 occupied a separate position close to the *Mesorhizobium* cluster; sequence ARRS102 had the highest sequence similarity with *Sinorhizobium* sp. C5 (Chen et al. 2000) a halotolerant rhizobia isolated from root nodules of *Canavalia rosea* from seaside areas and clustered with *Sinorhizobium meliloti* including *S. meliloti* ORS1044 isolated from *Acacia tortilis* subsp. *raddiana* nodules (Ba et al. 2002). Four phylotypes belonged to the *Sphingomonas* cluster: sequence ARRS82 grouped closely to *Sphingobium yanoikuyae* strain B10 isolated from soil (Takeuchi et al. 2001), while sequence ARRS57 was closely related to *Sphingomonas* sp. SLA181-A1 isolated from Lake Vostok accretion ice (Christner et al. 2001, unpublished). The phylotypes ARRS63 and ARDS47 had the highest similarity with members of *Caulobacter leidyi* and *Caulobacter subvibrioides*, now reclassified in *Sphingomonas* (Abraham et al. 1999) Sequence ARRS67 was very similar to *Brevundimonas* sp. FWC 43 and *Mycoplana bullata* a species that has to be transferred to *Brevundimonas* (Abraham et al. 1999). Phylotype ARRS17 has a separate position.

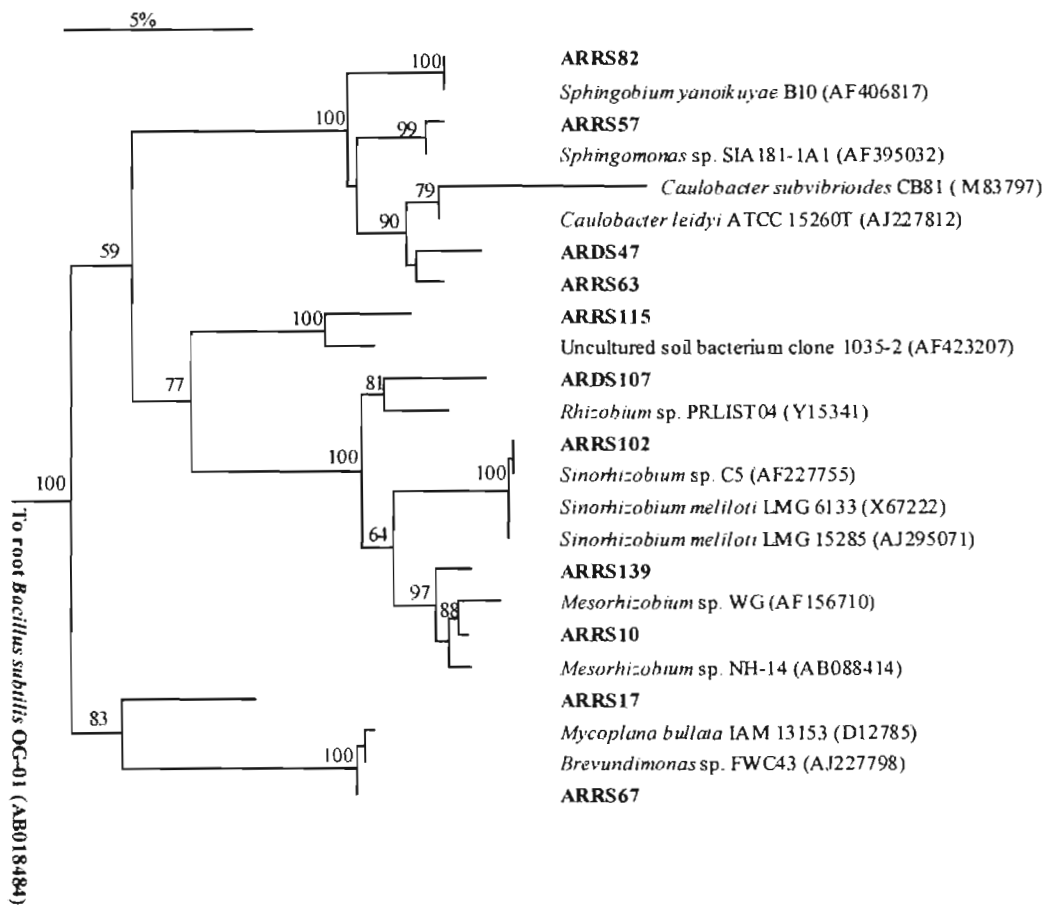


Figure 3.5 Neighbor-joining tree depicting the phylogenetic relationships among the *Alphaproteobacteria* sequences in this study. Species or strain names are preceded by their GenBank accession numbers. Clones detected in this study are given in boldface. Bootstrap values above 50 are shown, representing the percentage support for cluster out of 500. AR= *Acacia tortilis* subsp. *raddiana*, RS= Rainy season, DS= Dry season. The scale gives genetic distances.

Finally, an unidentified soil bacterial clone 1035-2 retrieved from soil by Valinsky et al. (2002) proved to be the closest relative of phylotype ARRS115.

***Acidobacteria* clones**

Fifteen phylotypes (15%) were most closely related to environmental clones of uncultivated bacteria obtained in other studies and belonging in the *Acidobacteria* phylum (Figure 3.6) (Ludwig et al. 1997). Except one (ARRS99 having the same phylotype as ARSD39) they all are from the dry season sample. The sequences found in this phylum are very heterogeneous. Based on our partial sequences we found 5 clusters: a first one contains phylotypes ARDS3 and ARDS1 only distantly related to an uncultured *Acidobacterium* clone W2a-4C detected in rocky mountain alpine soil (Lipson and Schmidt, 2003, unpublished). Within the second cluster we found 2 phylotypes [ARDS17 (= ARDS12) and (ARDS112)]. Phylotype ARDS112 was more closely related to an uncultured soil bacterium clone 513-2 (Valinsky et al. 2002) and to an uncultured soil bacterium Ac74 cluster (Sessitsch et al. 2001). A third cluster contains phylotype ARDS88 closely related to a British Columbia forest soil clone NM8. 79WL (Axelrood et al. 2002).

A fourth cluster contains phylotype ARDS122 closely related to uncultured bacterium Br-z33. A fifth cluster contained three subclusters: in a first one we found phylotype ARDS45 containing the sequence of uncultured bacteria OF20 and Ac37 (Sessitsch et al. 2001), three phylotypes (ARDS7, ARDS2 and ARDS105) constitute a separate subcluster and ARDS99 (= ARDS39) group in a third subcluster together with an uncultured soil bacterium 576-2 detected by Valinsky et al. (2002). Phylotypes ARDS146, ARDS6 and ARDS103 occupy separate positions.

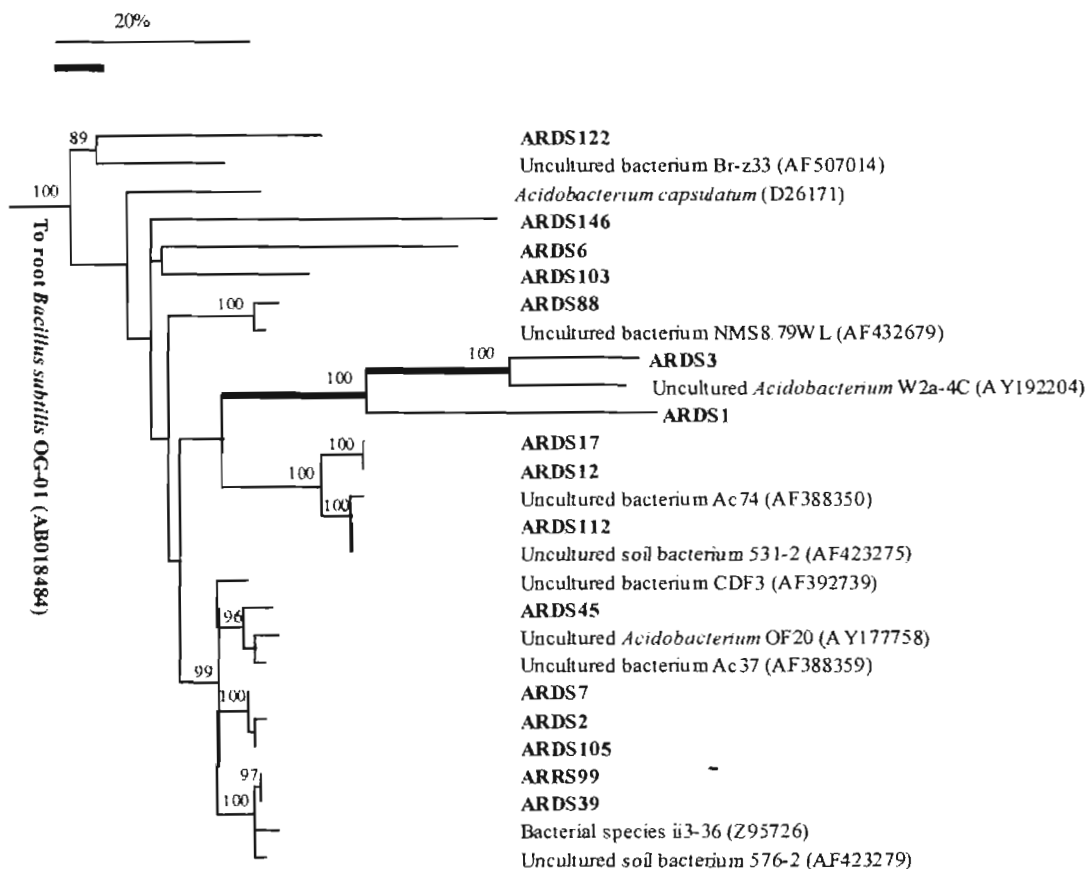


Figure 3.6 Neighbour-joining tree depicting the phylogenetic relationships among the *Acidobacterias* equences in thisstudy. Species or strain names are preceded by their GenBank accession numbers. Clones detected in this study are givenin boldface. Bootstrap values above 50 are shown, representing the percentage support for cluster out of 500. AR= *Acacia tortilis* subsp. *raddiana*. RS= Rainy season, DS= Dry season. The scale indicates genetic distances. Bold lines correspondingto a separate scale are used to reduce long branches.

DISCUSSION

This work is part of a comprehensive study on the microbial diversity in tropical semi-arid soil under *Acacia tortilis* subsp. *raddiana* in the Northern pastoral-forestry zone of Senegal using culture-independent molecular approaches. In a previous study we assessed the bacterial community structure in various soil samples from a transect under this tree by DGGE analysis of the 16S rDNA V3 region combined with sequencing of the excised dominant bands (Demba Diallo et al. 2003a). In the present paper, we phylogenetically analysed the 16S rDNA clone libraries of two samples from a single place from this transect but from different dates (one in each season). The sampling site (3 m from the stem and at a depth of 0.25 to 0.50m) was chosen for its highest diversity as found by DGGE (Demba Diallo et al. 2003a). Our first intention was to study the diversity of the bacterial communities and not to fully characterize the diverse members of the bacterial population; consequently we preferred to only partially sequence the various 16S rDNA clones. After screening by DGGE and sequencing we obtained 100 different phylotypes. Most of them (35%) were identified as members of the *Gammaproteobacteria*, 24% belonged to the *Firmicutes*, 14% to the *Betaproteobacteria*, 15% to the *Acidobacteria*, 11% to the *Alphaproteobacteria* and only one (1%) to the *Actinobacteria*.

As we only analysed one sample in the rainy season and one sample in the dry season, caution is needed interpreting the results. The differences detected in this study are not based on replicates and therefore have no significant values. We also did not characterize the full bacterial diversity present in tropical soil under *Acacia* trees but only offer a first glimpse of this bacterial diversity as is shown by the low coverage of our clone libraries (see Figure 3.1).

Comparison with previous results

In our previous study, where we sequenced the most prominent bands of the DGGE profiles, we found (Demba Diallo et al. 2003a) that members of *Bacillus* and *Acinetobacter*, represent the most intense bands in all the samples studied. In both 16S rDNA clone libraries members of the *Bacilli* represent the second largest group after the *Gammaproteobacteria* with almost half of them being representatives of the genus *Acinetobacter*. We found 55 and 70 *Acinetobacter* related clones in the rainy and dry season respectively for 36 (rainy season) and 16 (dry season) clones related to the *bacilli*. It was to be expected that we found a much higher diversity in the 16S rDNA clones than in our previous work in which we only sequenced the most intense bands of the DGGE profiles.

The diverse groups of bacteria found in our former study were confirmed by the results of the current study except that we did not detect 16S rDNA clone sequences corresponding to *Arthrobacter* members nor to representatives of the TM7 lineage (Hugenholtz et al. 2001) and to the *Bacteroidetes* phylum although a representative of this lineage was found in many

samples in both seasons and as a rather intense band present in part of the rainy season samples. A possible explanation for this might be that the *Bacteroidetes* cluster is under-represented in our clone libraries. Kirchman (2002) showed in his review on the ecology of *Bacteroidetes* in aquatic environments that 16S rDNA clone library construction is often biased against the *Bacteroidetes* cluster.

On the other hand solely by using the 16S rDNA clone library approach we found members of the *Acidobacteria* phylum, typical for many soil bacterial populations (Kuske et al. 1997; Dunbar et al. 1999; Sessitsch et al. 2001; Dunbar et al. 2002). The 16S rDNA clone library approach revealed many more members of the *Alphaproteobacteria* amongst which 4 clones belonging to *Mesorhizobium* and *Sinorhizobium*. As *A. tortilis* subsp. *raddiana* can be nodulated by *Mesorhizobium plurifarum* and members of *Sinorhizobium*, these organisms are presumed to be present in this soil. A possible explanation for the absence of representatives of *Mesorhizobium* and *Sinorhizobium* in our DGGE study might be their low abundance in comparison with other organisms. Also in our 16S rDNA clone libraries their presence is low (only four clones) which confirms our DGGE results unless PCR or cloning bias can also be invoked to explain the low coverage of these sequences (Cottrell and Kirchman, 2000)

Comparison with similar studies in the literature

In other studies characterizing the soil microbial diversity by culture-independent methods most clones found were also members of the described bacterial lineages but with varying percentages of participation depending on the soil, sample origin and plant growth. Felske et al. (1998) and Duineveld et al. (2001) described members of *Bacillus* as the most predominant in grassland soil in Drente and in a loamy sand soil from Ede (the Netherlands). Also, Smalla et al. (2001) studying the bulk and rhizospheric soil under three different plant species showed that *Bacillus megaterium* and *Arthrobacter* sp. prevailed in temperate loamy sand soil in Germany. *Arthrobacter* spp. were also found as dominant populations in the TGGE molecular fingerprints of 16S rDNA fragments amplified from the rhizosphere of maize grown in tropical soil (Gomez et al. 2001). In other studies using comparable techniques on other tropical or subtropical but forest soils, *Alphaproteobacteria* are described as most abundant sequences (as high as 40%) in Scottish grassland soils (McCaig et al. 2001), in soil from a Hawaiian rainforest (Nüsslein and Tiedje, 1998) and in subtropical acid forested soil in Australia (Stackebrandt et al. 1993) in which the bacteria customly identified by culturing in acid soils could not be detected in the 16S rDNA clone library approach. In a forest soil of Central Java (Indonesia) Krave et al. (2002) also found a strong dominance of *Alphaproteobacteria* (especially members of the *Rhizobium-Agrobacterium* group) and *Actinobacteria*. However, in other studies (Borneman et al. 1996; Nüsslein and Tiedje, 1999;

Kuske et al. 1997) *Alphaproteobacteria* clones accounted for maximally 3% in a library obtained from Wisconsin soil and were absent, in a clone library from another Hawaiian rainforest sample and pinyon-juniper woodland soil. The composition of our samples is different since we detected mostly *Gammaproteobacteria* followed by the *Firmicutes*. Representatives of the *Alpha-* and *Betaproteobacteria* were less abundant. The reasons for this can be that the semi-arid tropical soil may contain other dominant bacterial populations than temperate soil or other sub or tropical forest soils and that the populations are influenced by growth of the tree. Another explanation can be that we extracted and/or amplified only a selection of the abundant DNA from the soil or that our experimental conditions were different from those used in the other studies. Smit et al. (2001) who studied the bacterial community in a wheat field in temperate soil (The Netherlands), found e.g. no Gram-positive bacteria by using molecular techniques, while these organisms dominated in the cultured isolates.

Comparison between the samples and the season

Figure 3.7 shows the percentages of the clones belonging to the various bacterial divisions in both seasons. Based on the number of different phlotypes found, 39% of the clones represent a unique phlyotype in both samples. For *Betaproteobacteria*, the *Gammaproteobacteria* and the *Firmicutes* more clones are present in the rainy season sample than in the dry season sample. These high percentages may be linked to the rainy season, when more nutrients become available for micro-organisms. This indicates that various micro-organisms could profit from these nutrients.

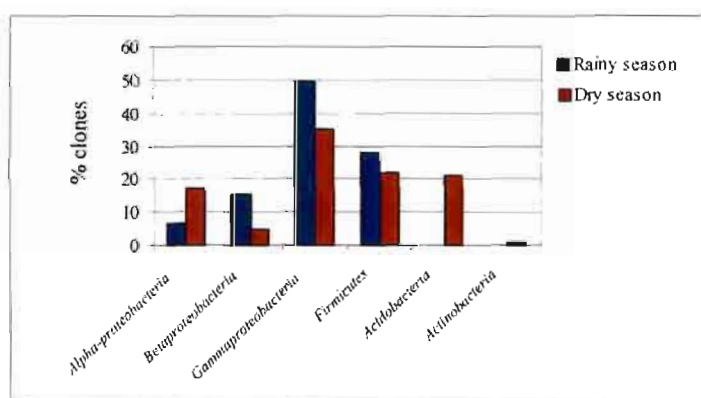


Figure 3.7 Distribution of 16S rDNA clones derived from the rainy season and the dry season within the bacterial phyla.

In contrast, *Acidobacteria* and *Alphaproteobacteria* are more represented in the dry season than in the rainy season. The vast majority of *Acidobacteria* have not yet been cultured and their ecological functions in soils remain unknown (Kuske et al. 2002). They have been detected in a wide variety of environments worldwide (Barns et al. 1999; Hugenholtz et al. 1998; Kuske et al. 1997) and Dunbar et al. (1999) suggested that they might be abundant in arid soils. We identified more than 20% of *Acidobacteria* in the 16S rDNA clone library of the sample taken in the dry season, although we did not detect them amongst the most intensive bands in our DGGE gels. A possible explanation can be that they are that diverse that they are spread over various weaker bands on the DGGE gels from the dry season samples since the presence of the different representatives is rather low. Smit et al. (2001) suggest that the ratio between the percentages of *Proteobacteria* and the *Acidobacterium* division can be indicative of the nutrient status of the soil ecosystem. We found an index of 0.73 for the dry season samples when calculated as $\% \text{ Proteobacteria} / \text{sum of } \% \text{ Proteobacteria and } \% \text{ Acidobacteria}$; in the rainy season the index is 1. The relatively low proportion of *Acidobacteria* in this poor soil could be due to plant roots having a preference for *Gammaproteobacteria* or to the detriment of Gram-positive bacteria and *Acidobacteria* (Marilley and Aragno, 1999). *Actinobacteria* were very rare, representing less than 1% of our clones.

Distribution of clones in the different phyla

If we take a closer look within the divisions we observe a remarkable shift from the dry to the rainy season within the *Gammaproteobacteria*. The members of the *Escherichia* group are exclusively found in the rainy season, the majority of the pseudomonads are almost all from the dry season sample (65 versus 11) while the representatives of the *Acinetobacter* group changed from 30% in the dry season to 60% in the rainy season. The same phenomenon was also found in our previous study where the DGGE bands corresponding to representatives of the genus *Acinetobacter* were less strong in the dry season samples.

In conclusion, our results show that a large proportion, approximately 53% of the sequences from tropical semi-arid soil of the Northern pastoral-forestry zone of Senegal belong to the *Proteobacteria* division, which members are omnipresent in soil worldwide (Valinsky et al. 2002; Suyama et al. 1998; Desmarais et al. 2002; Johnsen et al. 1999). Approximately 24% of our clones belonged to the *Firmicutes*.

Furthermore, our data suggest that bacterial communities under *Acacia tortilis* subsp. *raddiana* might differ according to the season.

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Chapter 4

Comparison of amplified 16S rDNA restriction analysis (ARDRA) and denaturing gradient gel electrophoresis (DGGE) for screening of a bacterial 16S rDNA clone library.

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Keywords: 16S rDNA, clone library, DGGE, ARDRA

ABSTRACT

A complex microbial population originating from tropical pasture soil in North Senegal was studied by constructing a 16S rDNA clone library. One hundred and fifty clones were obtained and they were screened by DGGE (denaturing gradient gel electrophoresis) of the V3 region and by ARDRA (Amplified rDNA restriction analysis) of 16S rDNA. The discriminating power of both screening methods was determined by comparing the composition of the clusters with a set of available partial sequences of the 16S rDNA of the clones. Clone screening by DGGE revealed 31 OTUs (Operational Taxonomic Unit) while the ARDRA technique detected 48 OTUs. The clusters obtained after ARDRA screening corresponded quite well with the sequencing results while the clusters obtained with DGGE contained members with quite different sequences indicating that the discriminatory power of the DGGE technique, in the conditions used, was lower than that of ARDRA.

INTRODUCTION

Molecular PCR-based techniques for bacterial identification, especially those including the 16S rDNA gene sequencing, have become important to study bacterial community composition in environmental samples (Giovannoni et al. 1990. Muyzer et al. 1993). A 16S rDNA clone library is a useful tool to assess the diversity of a bacterial community. To limit the number of clones to be sequenced a clone library can be screened for identical clones. A number of community "fingerprint" methods are available to screen a clone library: restriction fragment length polymorphism (RFLP) (Bowman et al. 2000b), terminal restriction fragment length polymorphism (TRFLP) (Moeseneder et al. 2001), denaturing and temperature gradient gel electrophoresis (DGGE and TGGE) (Muyzer et al. 1993; Heuer and Smalla. 1997b; Felske et al. 1997,1998), and amplified ribosomal DNA restriction analysis (ARDRA) (Massol-Deya et al. 1995; Watts et al. 2001). The techniques RFLP, TRFLP and ARDRA are based on the digestion of the PCR-product with one or more restriction enzymes, generating fragments with different lengths, depending on the DNA sequence and on the specificity of the enzyme. In the DGGE analysis, separation is dependent not on the size but on the melting behaviour of the PCR-product. Additionally, bands of interest can be excised and used for subsequent sequencing reactions. In the present study we compare ARDRA and DGGE analyses to screen a 16S rDNA clone library from a soil sample. We also made a DGGE-fingerprint of the total bacterial community. The results were compared with a set of partial 16S rDNA sequences obtained for representative clones from the clusters obtained with both techniques. This strategy allowed us to compare and evaluate the discriminatory power of both screening methods.

MATERIALS AND METHODS

Study site and soil sample collection

The soil sample, used to construct the 16S rDNA clone library is one of the various samples studied to evaluate bacterial communities in a Senegalese pasture soil under two tree species. The study was carried out in Souilène (IRD's Biological Research Station in North Senegal), in the grassland part of the Ferlo Region near the village of Windou Thiengoly and Mbeulekhe, situated between 15°40'-16° N, 15°40' W. Many DGGE profiles of the amplified V3 region of the 16S rDNA were compared for series of soil samples taken in the dry and in the rainy season. After examination of the profiles (Demba Diallo et al. 2003a) the sample

from the rainy season with the highest number of bands was selected to construct the 16S rDNA clone library. It is a sample taken between 2.5 by 25 cm deep under *Acacia tortilis* subsp. *raddiana* at a distance of three meters from the stem.

DNA extraction and PCR amplification of 16S rDNA

Bacterial DNA was extracted by a direct-lysis method described previously (Demba Diallo et al. 2003a). The extracted DNA was subjected to PCR amplification with the bacteria-specific primers: F27 (5'-AGAGTTTATCMTGGCTCAG-3', Lane et al. 1991), and R1492 (5'-GRTACCTTGTTACGACTT-3'). Numbering refers to the *Escherichia coli* 16S rRNA gene position corresponding (Brosius et al. 1978) to the 3' end of the primers. PCR amplification was performed by means of a Genius temperature cycler (Biometra, Göttingen, Germany) in 50 μ l reactions containing approximately 100 ng of purified DNA, 10 mM Tris/HCl pH 8.3, 15 mM MgCl₂, 500 mM KCl, 0.1% gelatine, 10 μ l μ g⁻¹ BSA (Bovine Serum Albumine), 1 U μ l⁻¹ of Expand high-fidelity DNA polymerase (Boehringer, Mannheim) and 200 μ M of each deoxyribonucleotide. The temperature and cycling conditions were as follows. First, preheating at 94°C for 5 min; then 25 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and a final incubation at 72°C for 5 min. The presence of PCR products and their concentration were determined by electrophoresis of 5 μ l product on a 2% agarose gel, stained with ethidium bromide. A molecular weight marker (Smartladder-Eurogentec, SA Belgium) was included.

Clone library construction

To generate nearly full-length 16S rDNA clones, the PCR product was ligated into the pGEM-T vector (Promega, Madison, Wis.) and the ligation reaction was used to transform competent *Escherichia coli* strain JM109. Recombinant colonies were selected on Luria-Bertani agar plates containing 20 μ g ml⁻¹ X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), 0.5 mM IPTG (isopropyl- β -D-thiogalactopyranoside), and 100 μ g ml⁻¹ ampicillin. Plates were incubated overnight at 37°C. The presence of inserts was determined by direct PCR on a sample from white (positive) bacterial colonies, using primers flanking the cloning sites on the vector.

DGGE analysis

To screen the clones, the V3 region of the 16S rDNA of each clone was amplified by using 1 μ l of each clone culture directly as a DNA template. The primers used are: F357GC (5'-CGCCCGCCGCGCCCCGCGCCCGCCCCGCCCCGCCCCCTACGGGAGGCAG

CAG-3') and R518 (5'-ATTACCGCGGCTGCTGG-3'). PCR amplification was as described by Van der Gucht et al. (2001).

DGGE was performed using the D-Code System from Bio-Rad Laboratories. PCR products were loaded onto 8% (w/v) polyacrylamide gels, 1 mm thick, in 1 × TAE buffer [20 mM Tris-acetate pH 7.4, 10 mM acetate, 0.5 mM disodium EDTA]. The denaturing gradient contained 35% to 70% denaturant [100% denaturant corresponded to 7 M urea and 40% (vol/vol) deionized formamid]. Electrophoresis was done at a constant voltage of 75 V for 16 h. The temperature was set to 60°C. After electrophoresis, gels were incubated for 1 hour in TAE containing ethidium bromide (0.5 mg l⁻¹). The bands were visualized on a UV transillumination table equipped with a digital CCD camera.

As standard, we used a mixture of DNA from 9 clones (Van der Gucht et al. 2001). On every gel, three standard lanes were included in parallel with the samples in order to compare the patterns formed in different gels. This procedure was semi-automated using the software package BioNumerics 2.5 (Applied Maths BVBA, Kortrijk, Belgium).

The similarity between the profiles was calculated using the Dice correlation coefficient and a similarity matrix was constructed. The data were clustered by UPGMA.

ARDRA analysis

The clones were also screened by ARDRA. The 16S rDNA amplicons were purified using the Quantum Prep plasmid miniprep kit (Bio-Rad) for quick cleaning and purification of plasmid DNA. Purified amplicons were digested with three different restriction enzymes (*Hae III*, *Rsa I* and *Hha I*) (Gibco BRL, Life Technologies). The enzymes were selected using Webcutter 2.0 (Max Heiman, Yale University, USA) aiming to use a tetracutter, to have a low cost technique and to obtain profiles with a sufficient stability. Ten microliter of the amplicons was digested with 10 U of restriction endonuclease. The digestions were carried out in a total volume of 20 µl according to the manufacturer's recommended buffer and incubation conditions, for 2 h at 37°C. Reaction products were loaded onto 8% (w/v) polyacrylamide gels in 1 × TAE buffer. Electrophoresis was done at a constant voltage of 300 V for 110 minutes. The temperature was set to 18°C. Staining and visualisation was as described.

As molecular weight marker we used Marker VIII (Roche) in three lanes per gel, whereupon patterns were compared using the Dice correlation coefficient with UPGMA in BioNumerics 2.5 (Applied Maths BVBA, Kortrijk, Belgium).

RESULTS AND DISCUSSION

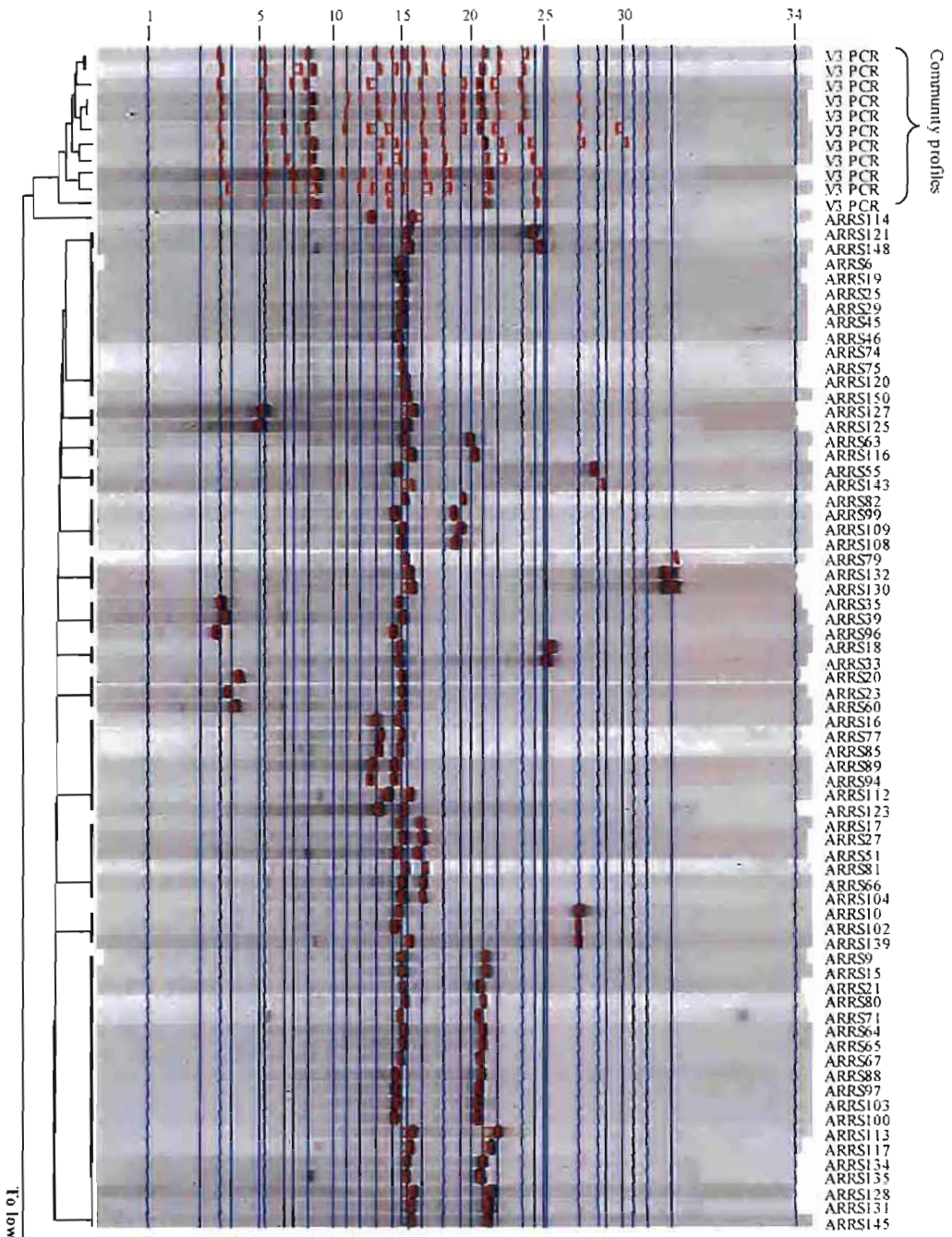
Clone library construction

A 16S rRNA gene clone library was constructed from DNA extracted from a Senegalese grassland soil sample (Demba Diallo et al. 2003b). One hundred and fifty clones were selected for further study. They were numbered ARRS1 to ARRS150

Screening with DGGE

The majority of the clones have two bands in their DGGE profile: one band corresponding to the V3 region of *E. coli* 16S rDNA which, always migrates at the same position in the middle of the gel (band class 15), and a band with the V3 region of the clone (Figure 4.1.) which position varies according to its sequence. Some clones have three bands (not shown) possibly because competent *E. coli* cells have taken up a second vector during transformation, or because during the picking up of a transformant colony part of a neighbour colony was also taken. These clones, ARRS52, ARRS137, ARRS147 and ARRS149 are not further considered in this work.

There are also 10 clones (ARRS6, ARRS19, ARRS25, ARRS29, ARRS45, ARRS46, ARRS74, ARRS75, ARRS120, ARRS150) with only one band (Figure 4.1) at the *E. coli* position. Several reasons could explain this observation. First, it is possible that there was no insert, secondly it is also possible that the *E. coli* V3 region may be identical to the V3 region of the insert or the V3 region sequence of the insert may differ from the *E. coli* V3 sequence but has similar denaturation characteristics resulting in the same position on the gel.



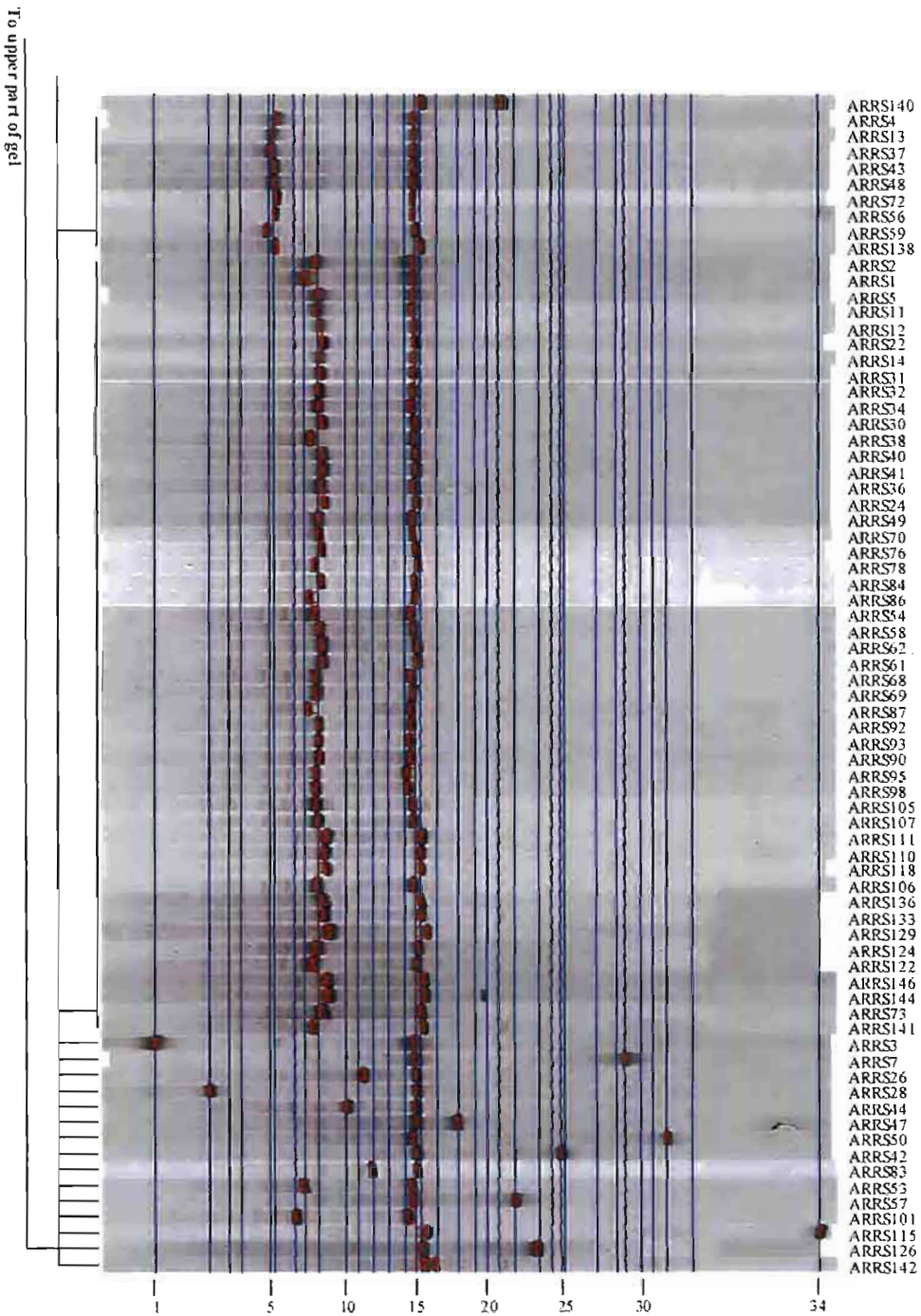


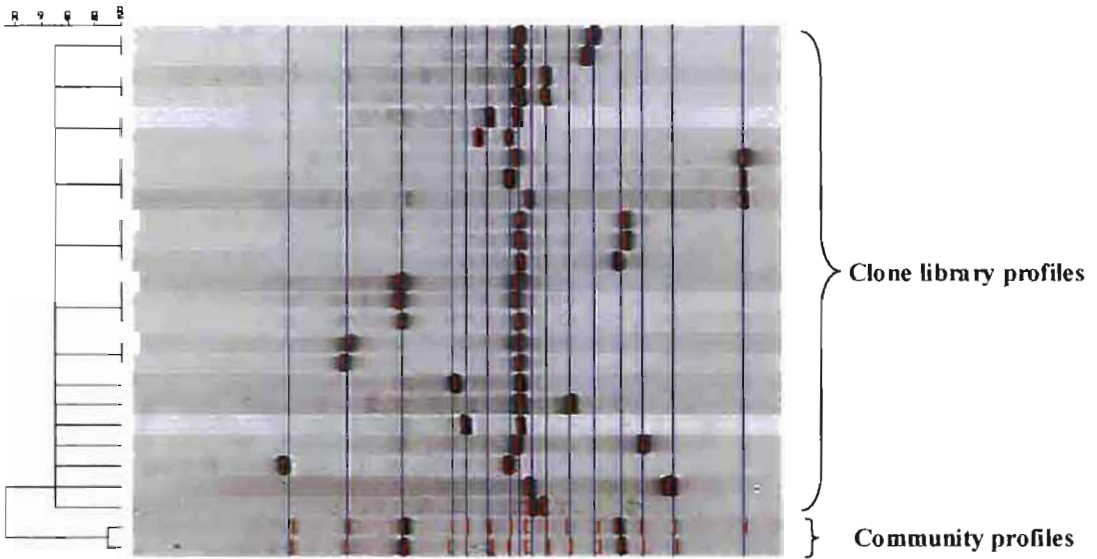
Figure 4.1 Dendrogram obtained by UPGMA clustering of DGGE patterns from a Souliène (North of Senegal) soil sample taken under *Acacia tortilis* subsp. *raddiana*. The similarity is expressed as a percentage value of the Dice correlation coefficient. AR= *Acacia tortilis* subsp. *raddiana*, RS= Rainy season. The vertical lines on the gel image show the bands that are recognized as separate OTUs.

Screening of the clones by DGGE resulted in 34 different band classes (Figure 4.1; Table 4.1; column, "screening DGGE") of which e.g. band class 9 contains the band corresponding with the highest number of clones. Clustering of all bands according to their band class was performed after that the position of each band was visually checked with original gels and manually corrected. The results are represented in Figure 1. Even though the gels were poured using a pump, small differences between gels cannot be avoided, as can be seen from the position of the marker bands and the *E. coli* bands. This is illustrated in Figure 4. 1 for clones ARRS113 and ARS143 (band class 21 and 29). The bands of forty-nine clones belong to band class 9 corresponding with about 1/3 of the clone library. Other band classes contain 2 to 9 clones (see Figure 4.1). There are 15 band positions represented by a single clone.

Comparison with the DGGE profile of the community

Comparison between the DGGE profile of the soil sample and the bands of the clones shows that almost all intense bands (15) can be found back in the clone library (Figure 4.2) while 19 more band classes were contained in the library. This means that we have not only screened for the most abundant species of the bacterial community but also for species that could not be detected by DGGE of total DNA of the same sample. In some cases it is difficult to decide if a band belongs to a specific band class or a very close one. Especially in the region of the very intensive *E. coli* band it is possible that the latter masks a slighter band. A narrower denaturation gradient could be used to separate such bands: we did not do that because many of the clones were already partially sequenced (Demba Diallo et al. 2003) and we compared the composition of our DGGE clusters with these sequences.

The two most intense bands (Figure 4.2) in the community profile correspond to both largest clusters of clones found by DGGE containing about 33% and 14% of the studied clones respectively (band class 9 and 21). A similar result was also found in the study of Felske et al. (1998). However, as long as various members of the clusters have not been identified we cannot claim that all members of each cluster will have the same sequence. In fact only sequencing of the excised bands or of the 16S rDNA of the clone can give a decisive answer because it is always possible that the band identification was not fine enough. We have also to take into account that sequences that differ considerably can have the same denaturation properties (Demba Diallo et al. 2003a; Nicol et al. 2003).



Screening with ARDRA

One hundred and forty eight clones of the library were also screened by ARDRA. Cleavage with three restriction endonucleases, *Hae III*, *Hha I* and *Rsa I*, resulted in a variety of different 16S rDNA fragment patterns (Figure 4.3).

When we make the sum of the molecular weights of the 16S rDNA fragments obtained in one profile, we may expect to come to approximately 1500 bp. As shown in Figure 4.3 that is indeed what we found after digestion with *Hae III* and *Rsa I* (Figure 4.3). This means that these restriction enzymes cut specifically.

With *Hha I* (Figure 4.3) the sum of the fragment lengths is in most cases bigger than the molecular weight of the 16S rDNA indicating that this restriction enzyme cuts aspecifically in some clones. Therefore the patterns obtained with this enzyme were not used in the final analysis. The analysis of the combined results obtained with *HaeIII* and *HhaI* using the Dice coefficient and UPGMA is shown in Figure 4.4.

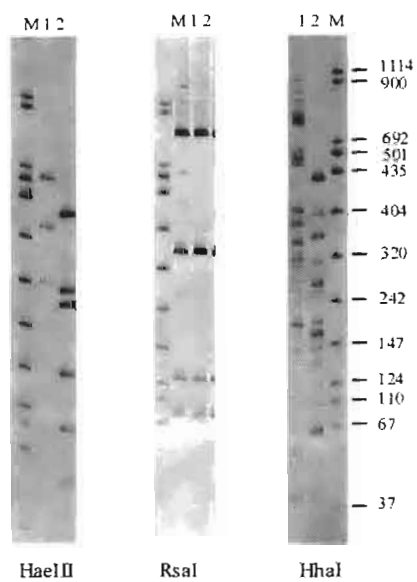
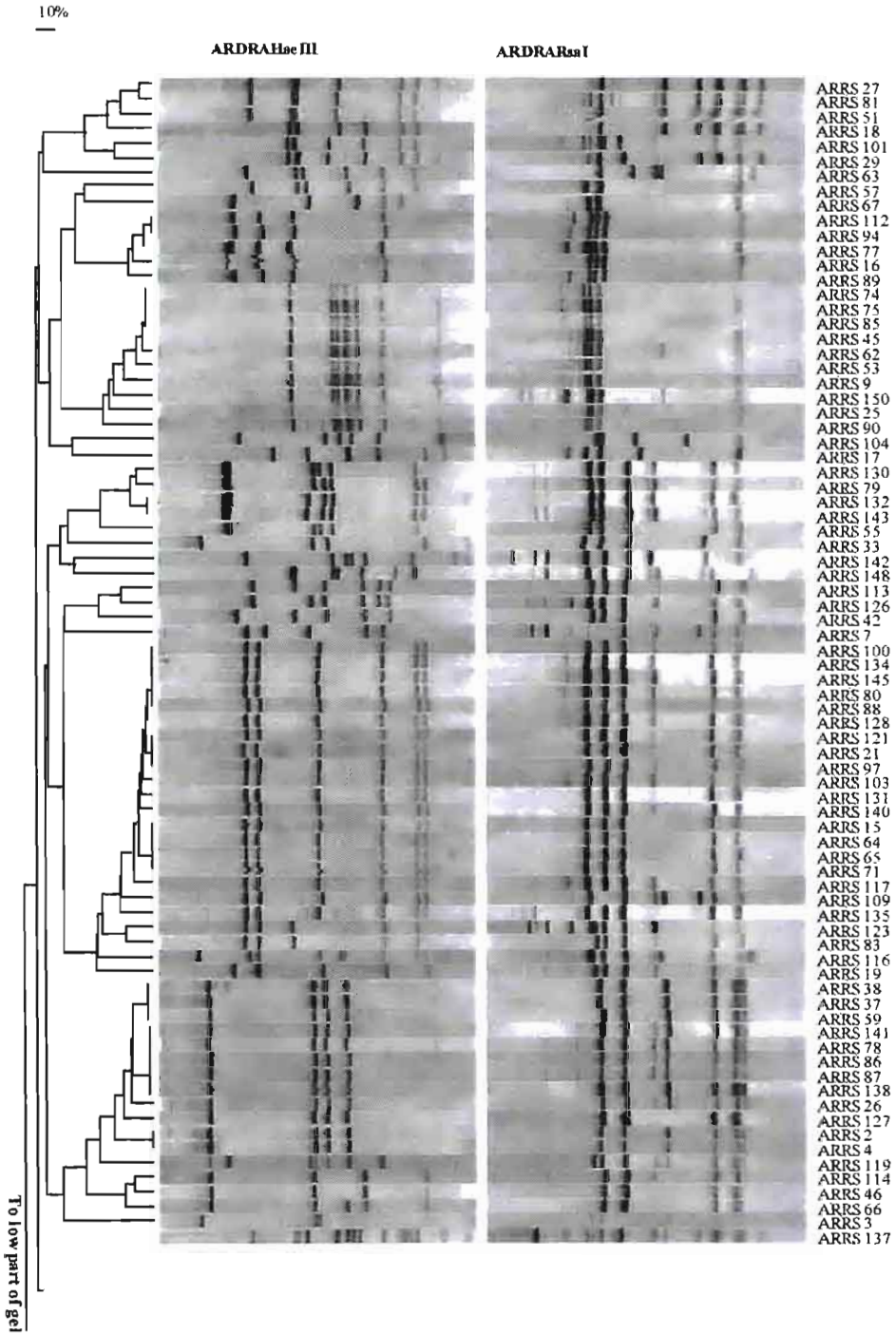


Figure 4.3 ARDRA profiles obtained for 2 clones (lane 1 and 2) after restriction with *HaeII*, *RsaI* and *HhaI*. M = marker VIII (Roche).



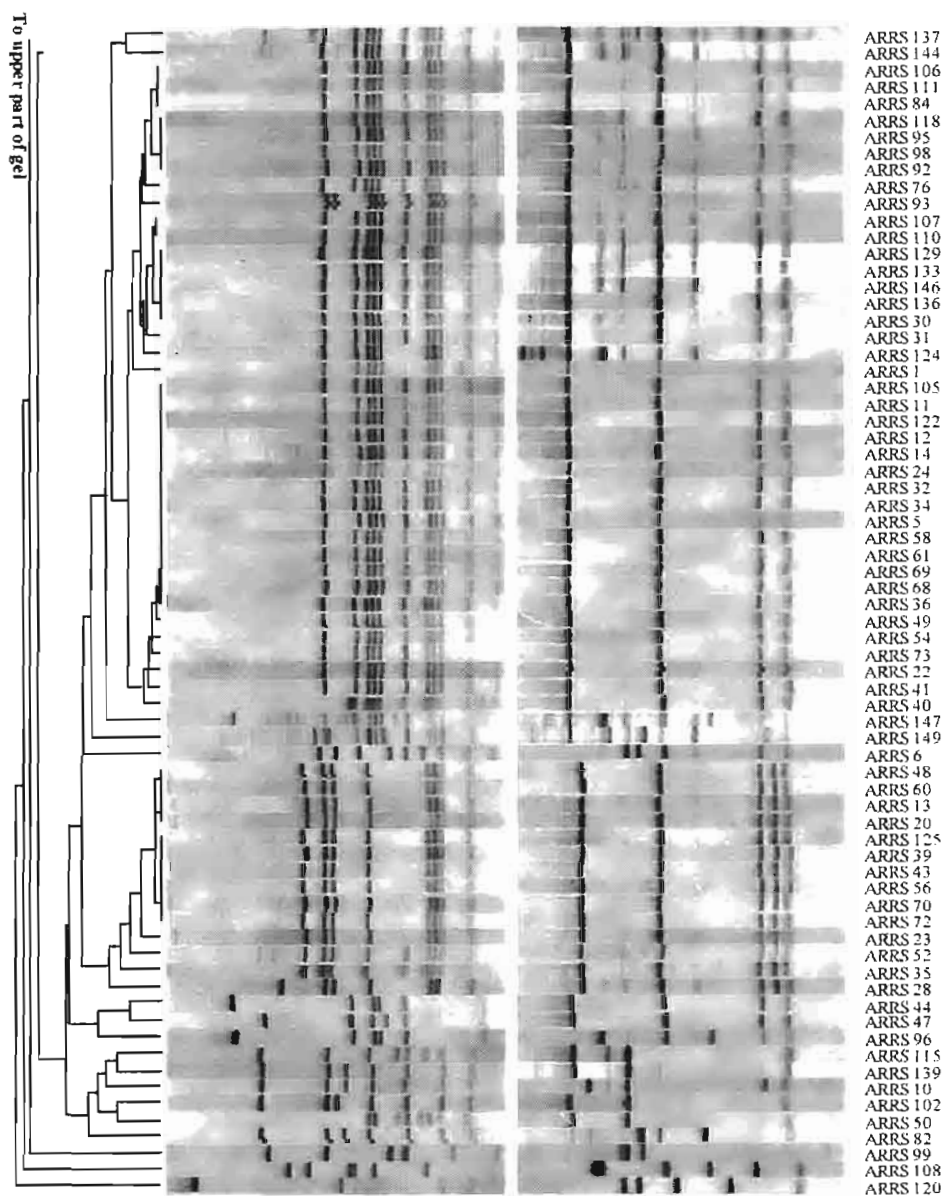


Figure 4.4 Dendrogram obtained after UPGMA clustering using the Dice similarity coefficient. Also shown are the digitalized ARDRA gel lane with profiles obtained after *Rsa I* and *HaeIII* digestion and processed in BioNumerics 2.5.

Using the 100% similarity level, we can distinguish 100 different OTUs. To avoid that some weaker bands were not registered by the automated analysis we visually extra checked each profile and the clusters, and we traced out 48 different fingerprint types or OTUs (Table 4.1). Within each of these OTUs the similarity of the profiles was minimally 90%. The clusters and the many OTUs containing a single clone can be found in Table 4.1.

Table 4.1 Results of total clones screened. Different OTUs found were placed in frame. OTUs belonging to a same division were placed in a white frame. DGGE OTUs that run over different division were represented in a specific colour.

Category	Bandclass	Screening DGGE	Screening ARDRA	Sequencing	Most related genus and/or species according to the a blast search and a phylogenetic analysis (Demba Diallo et al. 2003b)
<i>Alphaproteobacteria</i>	27	ARRS10	ARRS10	ARRS10	<i>Mesorhizobium</i> sp
		ARRS102	ARRS102	ARRS102	<i>Sinorhizobium</i> sp. C5 (AF227755)
		ARRS139	ARRS139	ARRS139	<i>Mesorhizobium</i>
	34	ARRS115	ARRS115	ARRS115	Uncultured soil bacterium (AF423207)
	17	ARRS17	ARRS17	ARRS17	<i>Caulobacter</i> sp (AJ227767)
	21	ARRS57	ARRS57	ARRS57	<i>Sphingomonas</i> sp. (AF395032)
	20	ARRS63	ARRS63	ARRS63	<i>Caulobacter</i>
	21	ARRS67	ARRS67	ARRS67	<i>Brevundimonas</i> sp. FWC43 (AJ227798)
	19	ARRS82	ARRS82	ARRS82	<i>Sphingomonas yanoikuyae</i> (AF 406817)
<i>Betaproteobacteria</i>	7	ARRS101	ARRS101	ARRS101	<i>Acidovorax</i>
	17	ARRS51	ARRS51	ARRS51	<i>Acidovorax</i>
		ARRS27	ARRS27	ARRS27	
		ARRS81	ARRS81		
		ARRS104*	ARRS104		
	26	ARRS18	ARRS18	ARRS18	
	15	ARRS29	ARRS29	ARRS29	<i>Comamonas</i>
		ARRS46	ARRS46	ARRS46	Unidentified bacterium o5SCC-26 (AJ387871)
	17	ARRS127	ARRS127	ARRS127	<i>Betaproteobacterium</i> MIC10 (AB013423)
		ARRS66	ARRS66	ARRS66	Unidentified <i>Betaproteobacteria</i>
	17	ARRS114	ARRS114	ARRS114	
		ARRS26	ARRS26	ARRS26	<i>Massilia umonae</i> 85A2206 (AY157759)
	9	ARRS38	ARRS38		
ARRS78		ARRS78			
ARRS86		ARRS86			
ARRS87		ARRS87			
ARRS141		ARRS141			
6	ARRS2	ARRS2	ARRS2		
	ARRS4	ARRS4	ARRS4		
	ARRS37	ARRS37			
	ARRS59	ARRS59			
<i>Gammaproteobacteria</i>	5	ARRS48	ARRS48	ARRS48	<i>Acinetobacter</i>
		ARRS43	ARRS43		
		ARRS56	ARRS56		
		ARRS13	ARRS13		
	5	ARRS72	ARRS72		
ARRS125		ARRS125	ARRS125		
4	ARRS20	ARRS20	ARRS20		
	ARRS23	ARRS23			
	ARRS60	ARRS60	ARRS60		
9	ARRS32	ARRS32	ARRS32	<i>Acinetobacter lwoffii</i>	
	ARRS31	ARRS31			
	ARRS30	ARRS30			
	ARRS12	ARRS12			
	ARRS36	ARRS36			
	ARRS34	ARRS34			
ARRS61	ARRS61				

	ARRS14	ARRS14		
	ARRS73	ARRS73		
	ARRS69	ARRS69		
	ARRS110	ARRS110	ARRS110	
	ARRS111	ARRS111		
	ARRS76	ARRS76		
	ARRS106	ARRS106		
	ARRS136	ARRS136		
	ARRS98	ARRS98		
	ARRS24	ARRS24		
	ARRS22	ARRS22		
	ARRS146	ARRS146		
	ARRS133	ARRS133		
	ARRS129	ARRS129		
	ARRS122	ARRS122		
	ARRS118	ARRS118		
	ARRS5	ARRS5		
	ARRS95	ARRS95		
	ARRS92	ARRS92		
	ARRS107	ARRS107		
	ARRS1	ARRS1		
	ARRS84	ARRS84		
	ARRS144	ARRS144		
	ARRS105	ARRS105	ARRS105	
	ARRS41	ARRS41	ARRS41	
	ARRS11	ARRS11		
	ARRS58	ARRS58		
	ARRS68	ARRS68		
	ARRS54	ARRS54		
	ARRS49	ARRS49	ARRS49	
	ARRS124	ARRS124	ARRS124	
	ARRS40	ARRS40	ARRS40	
	ARRS70	ARRS70	ARRS70	<i>Acinetobacter</i>
	ARRS93	ARRS93	ARRS93	
2	ARRS28	ARRS28	ARRS28	<i>Acinetobacter</i> sp. (AJ410290)
3	ARRS39	ARRS39	ARRS39	
	ARRS35	ARRS35		
	ARRS96	ARRS96	ARRS96	<i>Pseudomonas putida</i> (AF094745)
13	ARRS85	ARRS85	ARRS85	<i>Escherichia coli</i>
15	ARRS74	ARRS74	ARRS74	
	ARRS75	ARRS75		
	ARRS45	ARRS45	ARRS45	
8	ARRS53	ARRS53	ARRS53	
21	ARRS9	ARRS9	ARRS9	
9	ARRS62	ARRS62	ARRS62	
15	ARRS150	ARRS150	ARRS150	
	ARRS25	ARRS25	ARRS25	
	ARRS7	ARRS7	ARRS7	Unidentified <i>Gammaproteobacterium</i> (AF365509)
29				
10	ARRS44	ARRS44	ARRS44	<i>Pseudomonas</i> sp. (AJ007529)
18	ARRS47	ARRS47	ARRS47	<i>Pseudomonas oleovorans</i> (D84018)
17	ARRS142	ARRS142	ARRS142	<i>Lysobacter</i>
25	ARRS42	ARRS42	ARRS42	<i>Ammoniplutis oxalivorana</i> (Y14580)
	ARRS113	ARRS113	ARRS113	
	ARRS126	ARRS126	ARRS126	
	ARRS55	ARRS55	ARRS55	<i>Bacillus</i>
<i>Firmicutes</i>				

	21	ARRS65 ARRS64 ARRS15 ARRS100 ARRS117 ARRS71 ARRS128 ARRS131 ARRS135 ARRS134 ARRS145 ARRS80 ARRS140 ARRS103 ARRS21 ARRS97 ARRS88	ARRS65 ARRS64 ARRS15 ARRS100 ARRS117 ARRS71 ARRS128 ARRS131 ARRS135 ARRS134 ARRS145 ARRS80 ARRS140 ARRS103 ARRS21 ARRS97 ARRS88	ARRS65 ARRS100	<i>Anoxybacillus flavithermus</i> DSM2641(Z26932)	
	24	ARRS121	ARRS121	ARRS121		
		13	ARRS77 ARRS16 ARRS89 ARRS112 ARRS94 ARRS123	ARRS77 ARRS16 ARRS89 ARRS112 ARRS94 ARRS123	ARRS77 ARRS94 ARRS123	<i>Bacillus licheniformis</i> (AY30335)
		19	ARRS109 ARRS108*	ARRS109 ARRS108	ARRS109	<i>Bacillus nacin</i> (AB021194)
		1	ARRS3	ARRS3	ARRS3	<i>Bacillus litoralis</i> IB-B4 (AF326365)
		12	ARRS83	ARRS83	ARRS83	<i>Bacillus</i> sp. LMG-19415 (AJ276809)
		15	ARRS6 ARRS19	ARRS6 ARRS19	ARRS6 ARRS19	<i>Clostridium thermocapriac</i> (L09167) <i>Bacillus firmus</i> 10v2 (AF526919)
	26	ARRS33	ARRS33	ARRS33	<i>Paenibacillus nurensis</i> (AF378699)	
		33	ARRS132 ARRS79 ARRS130	ARRS132 ARRS79 ARRS130	ARRS132	Potato endoplant bact (AF406679)
		28	ARRS143	ARRS143	ARRS143	
		19	ARRS99	ARRS99	ARRS99	Uncultured bacterium
	<i>Acidobacterium</i>	32	ARRS50*	ARRS50		

* No similarity in the Blast

Comparing DGGE and ARDRA (Table 4.1)

In a previous work (Demba Diallo et al. 2003b) we compared the results of partial sequencing of various clones selected on the basis of OTUs obtained by DGGE for a soil sample taken in the dry season and for the sample taken in the rainy season used in this study. Therefore several clones for each cluster were sequenced. For the rainy season we could select more clones for sequencing because we also had the ARDRA results at our disposition. The number of clones sequenced is primarily based on the number of ARDRA OTUs because ARDRA has a higher discriminative power than DGGE. Further included in the sequencing were clones that had the same ARDRA profile but occupied different band classes in DGGE (e.g. ARRS26, ARRS2 and ARRS4). Within OTUs representing more than one clone, a various number of clones were sequenced. The results are assembled in Table 4.1 in which the band classes and the corresponding clones in DGGE and the clusters found in ARDRA are given together with the results of a BLAST search (Altschul et al. 1990) to identify the most similar sequence. Results of the phylogenetic analysis presented in our former paper (Demba Diallo et al. 2003b) have also been integrated in the Table 4.1. For the sake of clarity the ARDRA clusters are bordered by a rectangle while the clusters in DGGE are indicated by using different colors and by their corresponding band class. The reasons why clones from the same ARDRA cluster can give different bands in DGGE can be that the restriction enzymes used in ARDRA have not cut in the V3 region yielding exactly the same fragments despite a sequence difference in this V3 region. In total the partial sequences of 72 clones from the rainy season sample were available. The sequences of three clones ARRS50, ARRS108 and ARRS104 did not match any sequence from the database suggesting that they may represent unknown bacteria, endemic to the Senegalese grasslands, or alternatively they might be chimeras. However this possibility could be excluded after using the program CHECK CHIMERA of RPD (Ribosomal Database Project).

Our results in Table 4.1 clearly demonstrate that clusters delineated by ARDRA correspond quite well with the sequencing results, in that different ARDRA OTUs have the highest sequence similarity with sequences from different lineages in the different phyla and classes of bacteria. Within the *Alphaproteobacteria* each ARDRA OTU corresponds with a member of a different lineage. Within the *Betaproteobacteria* the correspondence between the various ARDRA clusters and the sequencing results is also high. Exceptionally 2 separate OTUs e.g. ARRS18 and a clone ARRS27 have identical partial sequences. Analogous conclusions can be drawn for the *Gammaproteobacteria* and the *Firmicutes*. On the contrary bands appearing at the same position in DGGE and thus representing the same OTU cannot

always be identified as belonging to the same phylogenetic lineage. Eleven of the 31 OTUs represent more than one species and are sometimes that different that they can be identified as belonging to different phylogenetic classes. As an example, we found that the cluster corresponding with band class 9 (49 clones) contains subclusters corresponding with sequences having the highest sequence similarity with members of the *Betaproteobacteria*: *Massilia timonae* and members of the *Gammaproteobacteria*: *Acinetobacter lwoffii*, *Acinetobacter sp.* and a not cultivated bacterium respectively. Band class 6 contains clones showing the highest similarity with members of the *Betaproteobacteria* and members of the *Gammaproteobacteria* respectively. Band class 21 contains one band phylogenetically belonging in the *Alphaproteobacteria*, another one belonging in the *Gammaproteobacteria* and the majority having the highest sequence similarity with *Anoxybacillus flavithermus* within the *Firmicutes*. For the group of clones generating only one band we found the highest similarity with *E. coli* (ARRS74, ARRS75 and ARRS45, ARRS85, ARRS9, ARRS150) but also with 2 members of the *Betaproteobacteria* (ARRS29 and ARRS46) and with 2 members of the *Firmicutes* (ARRS6 and ARRS19). As suggested before this can be provoked by a masking effect or by having bands with different sequences but with an analogous denaturation behavior in DGGE. A possible explanation for the lower discriminative power of DGGE in general can be that we defined the band classes too broadly or that the denaturation gradient was too ample.

On the other hand we also found that a single group can be represented by more than one OTU in DGGE. We found this e.g for ARRS85 and ARRS9 (band class 13 and 21 respectively) belonging to the same phylotype but generating bands in a different band class. A possible explanation could be that different copies of the rDNA are available in these genomes.

We can conclude that for this soil sample DGGE is a low discriminatory technique (with the gradient applied), which is illustrated by the number of 31 different OTUs found, while the diversity of the clone library screened by ARDRA gave 48 OTUs. As mentioned before the ARDRA based OTUs correspond largely to the identification found by phylogenetic analysis of the sequences (40 OTUs). This means that ARDRA does even have a slightly higher discriminative power than partial sequence analysis itself, probably because in ARDRA the full 16S rDNA is analyzed whereas in the sequencing solely the 500 first bases are included. ARDRA is thus very suitable to screen a 16S rDNA clone library.

To investigate whether we have screened enough clones to estimate the diversity of the clone library we used rarefaction analysis (Eilers et al. 2000). If the rarefaction relation has the shape of a saturation curve enough clones have been screened.

From the DGGE rarefaction analysis it is clear that we might not have screened enough clones. Likewise, ARDRA curve reveals even better that our clone library was possibly not large enough to estimate the bacterial diversity of the Senegalese soil.

Conclusion

A first aim of this study was to evaluate screening of a 16S rDNA clone library by DGGE of the V3 region and by ARDRA using two restriction enzymes. Therefore the compositions of the clusters obtained with both techniques were compared with the sequencing results described before (Demba Diallo et al. 2003b). A comparison between both screening methods showed that ARDRA is finer than DGGE and gave a better picture of the diversity in the clone library, as was confirmed by sequencing. Bacterial diversity estimated on the basis of clusters delineated solely by DGGE is lower.

Chapter 5

PCR-DGGE analysis of the N₂-fixing bacterial diversity in soil under *Acacia tortilis* subsp. *raddiana* and *Balanites aegyptiaca* in the dryland part of Senegal

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Keywords: Grass pasture, *Acacia tortilis* subsp. *raddiana*, *Balanites aegyptiaca*, diazotrophs bacteria, Tropical soil, PCR-DGGE analysis, *nifH* gene.

ABSTRACT

Denaturing gradient gel electrophoresis (DGGE) of amplified *nifH* gene fragment used to study the diazotrophic community of soil samples under *Acacia tortilis* subsp. *raddiana* (legume tree) and *Balanites aegyptiaca* (non-legume tree), two dominant plant species grown naturally in the dryland part of Senegal. Samples were taken along transects from the stem to 10 m distance from it, at depths of 0-0.25 m and 0.25-0.50 m. Sampling was done in the dry season (June 25th 1999) and in the rainy season (August 28th 1999). The community structure and diversity of the bacterial groups from the different samples was further analysed using different techniques, such as statistical analysis and diversity index evaluation of the band patterns. Diazotrophic diversity was lower under *Balanites aegyptiaca* than under *Acacia tortilis* subsp. *raddiana*. Multidimensional scaling (MDS) analysis and ANOSIM tests showed a significant effect of the tree on the diazotroph assemblages. SIMPER analysis showed that the electrophoretic bands responsible for the dissimilarity are a member of the *Rhizobium* cluster, which is characteristic for the samples taken under *Acacia tortilis* subsp. *raddiana* and a member of *Mesorhizobium* and *Bradyrhizobium* for the samples taken under *Balanites aegyptiaca*. Forty-six major bands were sequenced, yielding 35 different *nifH* sequences, which were used in phylogenetic reconstructions. Most sequences were affiliated with the *Alpha- Betaproteobacteria* and *Gammaproteobacteria*. Five *nifH* sequences were identical to those of *Pseudomonas stutzeri*, one sequence showed 100% similarity with that of *Azotobacter vinelandii*. Four bands were affiliated with the *Cyanobacteria* and a single one with the *Firmicutes*. For both trees there were also clear differences between the samples taken in the dry and the rainy season. Overall, for the samples taken under *Acacia tortilis* subsp. *raddiana* a significant difference was observed between the two sampling depths.

INTRODUCTION

Nitrogen is the limiting nutrient for crop growth in most developing countries. Exploitation of biological N₂ fixation offers a unique opportunity to harness nitrogen from the air. The promotion of low-input agriculture over the last decades has sparked a great interest in soil microorganisms, able to increase soil fertility or to stimulate plant nutrition and/or health. Knowledge of the diazotrophic community structure and population dynamics responsible for the nitrogen fixation is important for agricultural applications as well as for understanding the ecosystem processes (Bormann et al. 1980; Reinhold-Hurek and Hurek, 1998). Several studies attempting to study the functionally active diazotrophs are based on classical isolation techniques, although for most ecosystems, it is known that the majority of bacteria cannot be cultivated yet (Pace, 1997). Evidence is accumulating to support the notion that a change in the species composition and dominance of the plant community may be an important determinant of biological properties of grassland soil. Recent studies in the field (Wardle et al. 1999) and in the laboratory (Wardle et Nicholson, 1996; Bardgett and Shine, 1999) have shown that individual grassland plant species can greatly influence the size and activity of the microbial biomass, and that these effects are often closely related to various plant ecophysiological traits (Wardle et al. 1998). Such findings support the emerging view in ecology that individual plant species effects are important determinants of ecosystem properties, of organic matter decomposition and nutrient recycling (Bardgett et al. 1999).

The Northern pastoral-forestry zone of Senegal is a mixture of grassland and shrub steppe (37%), largely dominated by Sahelian species, such as *Acacia tortilis* subsp. *raddiana* (legume tree) and *Balanites aegyptiaca* (non-legume tree). Soil fertility under the canopy of *Acacia tortilis* subsp. *raddiana* trees is frequently superior to that in open grassland areas (Deans et al. 1999). This is exploited by farmers using tree fallows and the spaced-tree parkland system in the Sahel (Le Houérou, 1989). However to date, only limited information is available on the diazotrophic bacterial community structure, the impact of the tree on the community and the bacterial dynamics in this zone. Nitrogen fixation is performed by phylogenetically diverse groups of prokaryotic organisms belonging to the *Bacteria* and the *Archaea*. (Young, 1992). Different growth requirements resulting from the different physiological properties of these prokaryotic organisms preclude their simultaneous cultivation (Knowles and Laserna, 1994). Because some of these micro-organisms are as yet unculturable (Roszak and Colwell, 1987), a general culturing approach for evaluating nitrogen-fixing populations will be incomplete, even though many important contributions to the characterization of nitrogen-fixing prokaryotes have been based on traditional culturing techniques (Knowles and Laserna, 1994). Numerous

researchers have employed various PCR primers to amplify a segment of *nifH*, the structural gene encoding the nitrogen iron protein, (Bagwell et al. 1998; Ben-Porath and Zehr, 1994; Haukka et al. 1998; Zehr and McReynolds, 1989; Olson et al. 1999) from various environmental samples, including marine plankton (Braun et al. 1999; Zehr et al. 1998), termite hindguts (Lin and Brown, 1989; Ohkuma et al., 1996), microbial mats and aggregates (Olson et al. 1998, Zehr et al. 1998) and terrestrial soils (Rosado et al. 1998; Widmer et al., 1999; Poly et al. 2001a and b; Rösch et al. 2002). These studies yielded a diverse array of *nifH* sequences representing many, often unknown, lineages of diazotrophic members of the *Bacteria* and the *Archaea*. PCR amplification of *nifH* segments followed by their separation through denaturing gradient gel electrophoresis (DGGE), has been used to examine the complexity and stability of the diazotrophic assemblage in the *Spartina alterniflora* rhizosphere (Piceno et al. 2000), to study its diversity (Lovell et al. 2000, 2001) and to investigate the diazotrophic diversity in oligotrophic tropical seagrass bed communities (Bagwell et al. 2002).

In this study, *nifH* sequence diversity was examined for soil samples collected under *Acacia tortilis* subsp. *raddiana* and *Balanites aegyptiaca*. Denaturing gradient gel electrophoresis (DGGE), which separates amplified sequences on the basis of their melting points, was used to profile the recoverable *nifH* sequence diversity under the two different trees, on two different sampling dates and on two different sampling depths. Several intensive bands were excised and sequenced to determine their phylogenetic position.

The objectives of this work were (i) to compare the diazotrophic assemblages from soil samples taken under two different trees growing in the pastoral forestry zone in Senegal. (ii) to detect possible shifts in the diazotrophic bacterial community in function of sampling date, depth or distance from the tree.

MATERIALS AND METHODS

Study site and sampling procedure

The study was carried out in Souilène (IRD's Biological Research Station in North Senegal), in the grassland part of the Ferlo Region near the village of Windou Thiengoly and Mbeulekhe, situated between 15°40'-16° N, 15°40' W. The soil of the region consists of degraded red/brown ferruginous sand, which extends to a depth of at least 30 m (Dean et al. 1999) and which is characterized by a low organic matter content, and poor water and chemical retention properties. Annual long-term (1981-1993) rainfall, which occurs in a single rainy

season between July and October, was on average 297 mm for the period 1981-1993 but more recently, has decreased to about 150 mm. A comprehensive description of the area is given by Le Houérou (1989) and floristic details of the district were reported by Miehe (1990). Soil cores (2.5 by 25 cm) were taken under *Acacia tortilis* subsp. *raddiana* and *Balanites aegyptiaca* along a transect from 1 to 10 m from the stem at 1 m intervals, beneath the canopy up to the open field. Samples were taken at depths of 0-0.25 m, where fine tree roots were most heavily concentrated under *Acacia tortilis* subsp. *raddiana*, and 0.25-0.50 m. The tree roots extended more than 9 m on each side of the stem. Sampling was done at the end of the dry season (June 25th 1999) and in the middle of the rainy season (August 28th, 1999). Samples of the cores were stored in a plastic bag at -20°C prior to DNA extraction.

Soil, physical and chemical analysis

Ten soil samples of each 25 g collected along a transect under *Acacia tortilis* subsp. *raddiana* and *Balanites aegyptiaca*, at 0-0.25 m depth in each season were pooled according to the tree and the season. Two hundred and fifty gram of soil of each category was analysed in the Department of Geology and Pedology at the University of Gent (Belgium): grain size analysis was performed after destruction of organic matter by wet sieving on a 63- μ m sieve. The coarse residue was, after drying, separated by sieving. The fine fractions (silt equilibration period. Total carbon (Walkley-Black), total nitrogen (Kjeldahl), exchangeable basic cations and CEC (M NH₄OAc, pH 8.2) were determined through the procedures outlined by Black et al. (1965). Total P was determined colorimetrically, and NH₄-N and NO₃-N in a H₂O extract using ionchromatography.

DNA extraction and PCR amplification

Bacterial community DNA was extracted from soil samples by a direct-lysis method described previously (Demba Diallo et al. 2003a). Following the extraction, the DNA was purified on a Wizard column (Promega, Madison, WI USA) as recommended by the manufacturer. The *nifH* DNA sequences from divergent nitrogen-fixing micro-organisms were amplified using a nested PCR to increase the sensitivity. The primers used are listed in Table 5: 1 and were originally developed by Poly et al. (2001a) and Simonet et al. (1991). The first PCR was performed with the forward primer FGPH19 and the reverse primer PolR. The forward and reverse primers are 24 \times and 8 \times degenerate, respectively. The amplification product is 429 bp. The second PCR was performed with the forward primer PolF containing the GC clamp and the reverse primer AQER. The forward and reverse primers are 24 \times and 2 \times degenerate respectively. The amplification product is approximately 320 bp including the GC-clamp sequence.

table equipped with a digital CCD camera. As standards, we used a mixture of DNA from 9 clones (Van der Gucht et al. 2001) and two *nifH*/DGGE bands. On each gel, three standard lanes were analysed in parallel to the samples. Since these bands always should be formed at the same denaturant concentration in the gel, their position was used to compare the patterns formed in different gels. This procedure was semi-automatized using the software package BioNumerics 2.5. (Applied Maths BVBA, Kortrijk, Belgium). Since previous studies have shown that DGGE bands intensity is stable and reproducible, we decided to use the relative intensity in our analyses because it provides extra information (Schauer et al. 2000). The BioNumerics software measures an optical density profile through each lane (corresponding to a single sample), identifies bands position, and calculates the percent contribution of the intensity of each band to the total intensity of the lane. This procedure yielded a matrix with the relative intensity of each band in all samples.

Statistical analysis

Primer v.5 statistical software was used for multivariate tests. Multivariate procedures were carried out on $\text{Log}(x + 1)$ transformed data.

Separation of diazotroph species groups according to the tree, season and depth was examined by MDS (multi-dimensional scaling) ordination plots based on Bray-Curtis similarities (10 restarts, 999 iterations each).

ANOSIM (analysis of similarity) tests using 999 permutations were run to test for statistically significant differences in diazotroph assemblages. The contribution of each species to the average Bray-Curtis dissimilarity between pairs of groups was computed using the similarity percentages procedure (SIMPER, Clarke. 1993).

DGGE banding data were used to estimate the diversity indices by treating each band as an individual OTU and using the number of bands as an indicator of richness. The Shannon diversity index (Shannon and Weaver, 1963) and the Simpson's index (Simpson, 1949) were calculated from the number of bands present and the relative intensities of bands in each lane.

Sequencing of excised *nifH*/DGGE bands and phylogenetic analysis

Forty six bands were excised from DGGE gels with a surgical knife and placed into sterile Eppendorf vials. To elute the DNA, 50 μl of TE (10 mM Tris, pH7.6, 1mM EDTA), was added. The DNA was incubated at 4°C overnight. Three μl of the supernatant was used as template DNA in a PCR with the primers PolIF (without GC clamp) and AQER as described above. The PCR products were purified by using a QIAquick PCR Purification Kit (Qiagen Inc, Germany), according to the manufacturer's instructions. Sequencing was performed with

the automated sequencer (ABI-Prim 377, Applied Biosystems, USA) using the ABI-Prism sequencing Kit (PE-Biosystems) and the primer AQER.

Environmental *nifH* sequences from NCBI GenBank database were selected on the basis of *nifH* sequence similarity, these sequences as well as sequences from relevant known, formally described diazotrophs were utilized for phylogenetic reconstructions. The *nifH* sequence segments corresponding to the primers were removed from all sequences prior to phylogenetic analysis. Sequences were aligned with BioNumerics 2.5 and then corrected by manual inspection. A phylogenetic tree was constructed by using the neighbor-joining method (Saitou and Nei, 1987). The *nifH* sequence for *Klebsiella pneumoniae* (GenBank accession number K00044) was used as the outgroup taxon for all phylogenetic reconstructions. The tree was bootstrapped 500 times to provide a confident estimate for its topology.

Nucleotide accession numbers: all sequences obtained in this study have been submitted to the GenBank database under accession numbers no xxxxxxxxx to xxxxxxxxx

RESULTS

Comparison of the physicochemical characteristics of the soils

Physical, chemical, and biological characteristics of soil sample sites are given in Table 5.2. All analysed samples have a sandy texture and are almost completely composed out of quartz (no other minerals could be detected by XRD). The dominance of quartz explains the extremely low cation exchange capacity (CEC) and the very low amounts of exchangeable basic cations. These mineralogically poor sandy materials have very low O. C contents. The soil samples taken under *Acacia tortilis* subsp. *raddiana* have higher total nitrogen contents, as well as higher NO₃-N contents compared to the soil samples under *Balanites aegyptiaca*.

Table 5.2. Physical, chemical, and biological characteristics of soil samples.

Field code	Texture			pH		CEC cmol.kg ⁻¹	Exchangeable cations				N-tot mg ⁻¹	NH ₄ -N mg ⁻¹	NO ₃ -N mg ⁻¹	P-tot mg ⁻¹	OC	
	< 2 µm	2- 63 µm	63- 2000 µm	pH H ₂ O (1:2.5)	pH KCl (1:2.5)		Ca cmol.kg ⁻¹	Mg cmol.kg ⁻¹	Na cmol.kg ⁻¹	K cmol.kg ⁻¹					%	C/N
ARDS 0- 0.25 m	3	4	93	7.7	7.2	1.6	1.7	0.4	<0.1	0.2	158.1	1.2	26.2	88.6	0.2	15.18
ARRS - 0.25 m				8.1	7.8	1.7	2.0	0.5	<0.1	0.3	187.8	1.0	20.9	95.1	0.3	16.51
BDS 0- 0.25 m	4	6	90	8.0	6.8	1.6	1.0	0.2	<0.1	0.1	103.5	1.0	2.9	31.5	0.2	19.32
BRS 0- 0.25 m				8.1	6.8	2.1	1.2	0.4	<0.1	0.1	120.4	0.7	1.9	83.0	0.2	14.95

CEC: ammonium acetate pH 8.2

OC: Walkley and Black

Comparison of DGGE profiles and biodiversity indices

A circa 320 bp long fragment from the *nifH* gene was amplified using primers with GC-clamps and the resulting products were separated on DGGE gels. The DGGE profiles showed various banding patterns in the different soil samples (Figure 5.1).

A total of 76 different band positions could be identified.

Richness was assessed by determining the number of DGGE bands (the mean values were 11.5 for samples taken under *Acacia tortilis* subsp. *raddiana* and 8.5 for samples taken under *Balanites aegyptiaca*) and this difference was significant ($P = 0.00065$).

The Shannon diversity index (2.2 for *Acacia tortilis* subsp. *raddiana*, 1.85 for *Balanites aegyptiaca*), and Simpson's index (0.87 for *Acacia tortilis* subsp. *raddiana*, 0.815 for *Balanites aegyptiaca*) also differ significantly ($P = 0.00023$ for the Shannon diversity index, and $P = 0.001$ for the Simpson's index) for the two groups of samples being significantly lower for the samples taken under *Balanites aegyptiaca*.

For the samples taken under *Acacia tortilis* subsp. *raddiana* the dry season samples had significantly higher richness values (13.4 versus 9.4; $p = 0.00056$) and higher values of the Shannon and Simpson index (2.4 versus 2; $p = 0.00073$; 0.90 versus 0.85, $p = 0.00162$) than the samples taken in the rainy season. Comparing the two depths of sampling we found significantly lower values for the surface samples (richness 10.9 versus 13.9, $P = 0.02507$; Shannon index, 2.11 versus 2.42, $p = 0.02720$; Simpson's index, 0.86 versus 0.95, $p = 0.04742$)

No comparisons were statistically significant for the samples taken under *Balanites aegyptiaca*.

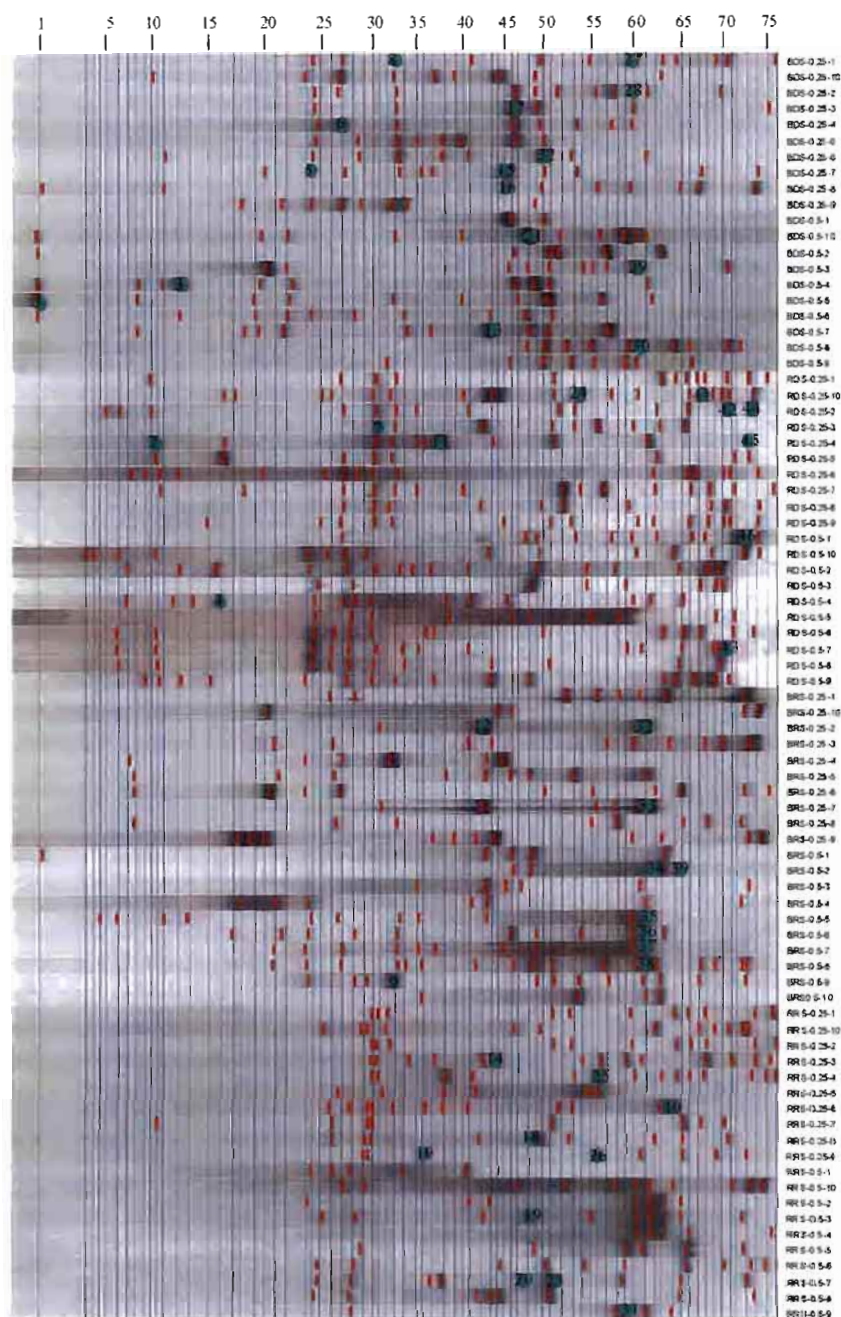


Figure 5.1 DGGE banding patterns from soil samples taken under *Acacia tortilis* subsp. *raddiana* (R) and *Balanites aegyptiaca* (B), RS= Rainy season, DS= Dry season, 0.25 (0.50)-x = depth at xm distance from the tree. The DGGE profiles were analysed using the software program BioNumerics 2.5 (Applied Maths BVBA, Kortrijk, Belgium). Number 1 to 76 indicate the different band positions. Numbered dots refer to the excised bands (see Table 5.3). The DGGE profiles were ranged numerically by the computer program.

Table 5.3. Results of a BLAST analysis on the sequences of the 46 *nifH*/DGGE excised and sequenced bands. The band positions and the localisation of the bands in the DGGE profiles are given in Figure 5.1

Band position DGGE gel	Band n°	Closest match in NCBI search	Bacterial group	Homology(%)	Accession no
1	BDS1	<i>Lyngbya lagerheimii</i>	<i>Lyngbya</i>	85%	L15550
11	RDS2	Uncultured eubacterium stream13	<i>Bacteria</i>	96%	AF049034
13	BDS3	marine stromatolite eubacterium HB Z05	<i>Bacteria</i>	86%	AF227937
16	RDS4	<i>Plectonema boryanum</i> (IU 594)	<i>Cyanobacteria</i>	83%	L15552
24	BDS5	<i>Bradyrhizobium</i> sp. MAFF 210318	<i>Alphaproteobacteria</i>	91%	AB079620
27	BDS6	<i>Bradyrhizobium</i> sp. 125c	<i>Alphaproteobacteria</i>	96%	AF484631
30	RDS7	<i>Rhizobium</i> sp. NGR234	<i>Alphaproteobacteria</i>	91%	AE000105
33	BDS8	<i>Bradyrhizobium</i> sp. 125c	<i>Alphaproteobacteria</i>	98%	AF484631
	BRS9	<i>Bradyrhizobium</i> sp. 125c	<i>Alphaproteobacteria</i>	98%	AF484631
36	RRS10	<i>Faenibacillus macerans</i>	<i>Firmicutes</i>	84%	AJ224427
37	RDS11	<i>Plectonema boryanum</i> (IU 594)	<i>Cyanobacteria</i>	83%	L15552
42	BRS12	<i>Mesorhizobium</i> sp. LMG 11892	<i>Alphaproteobacteria</i>	94%	AF484654
43	BDS13	Uncultured bacterium clone CB907H9	<i>Alphaproteobacteria</i>	92%	AY223997
44	RRS14	Uncultured bacterium clone KqT2A	<i>Betaproteobacteria</i>	92%	AY231519
45	BDS15	Uncultured bacterium clone SH1	<i>Bacteria</i>	93%	AF414665
	BDS16	Uncultured bacterium clone SH1	<i>Bacteria</i>	97%	AF414665
46	BDS17	<i>Bradyrhizobium</i> sp. 125c	<i>Alphaproteobacteria</i>	97%	AF484631
48	RRS18	<i>Azoarcus</i> sp BH72	<i>Betaproteobacteria</i>	92%	AF200742
	RRS19	<i>Azoarcus</i> sp BH72	<i>Betaproteobacteria</i>	93%	AF200742
	RRS20	<i>Azoarcus</i> sp BH72	<i>Betaproteobacteria</i>	92%	AF200742
49	BDS21	<i>Azotobacter vinelandii</i>	<i>Gammaproteobacteria</i>	100%	M20568
50	BDS22	uncultured alphaproteobacterium nifH51	<i>Alphaproteobacteria</i>	92%	AY159593
51	RRS23	<i>Azovibrio restrictus</i>	<i>Betaproteobacteria</i>	96%	U97119
54	RDS24	uncultured bacterium clone NIS7-2	<i>Alphaproteobacteria</i>	90%	AF389803
56	RRS25	uncultured temperate bacterium VF003	<i>Betaproteobacteria</i>	91%	AF315432
	RRS26	uncultured temperate bacterium VF003	<i>Betaproteobacteria</i>	92%	AF315432
60	BDS27	<i>Pseudomonas stutzeri</i> A15	<i>Gammaproteobacteria</i>	100%	AJ297529
	RRS28	<i>Pseudomonas stutzeri</i> A15	<i>Gammaproteobacteria</i>	100%	AJ297529
	BDS29	<i>Pseudomonas stutzeri</i> A15	<i>Gammaproteobacteria</i>	100%	AJ297529
	BDS30	<i>Pseudomonas stutzeri</i> A15	<i>Gammaproteobacteria</i>	100%	AJ297529
	BDS31	<i>Pseudomonas stutzeri</i> A15	<i>Gammaproteobacteria</i>	100%	AJ297529
61	BRS32	<i>Sinorhizobium</i> sp. GR-06	<i>Alphaproteobacteria</i>	91%	AF506515
	BRS33	<i>Sinorhizobium</i> sp. GR-06	<i>Alphaproteobacteria</i>	91%	AF506515
	BRS34	<i>Sinorhizobium</i> sp. GR-06	<i>Alphaproteobacteria</i>	92%	AF506515
	BRS35	<i>Sinorhizobium</i> sp. GR-06	<i>Alphaproteobacteria</i>	92%	AF506515
	BRS36	<i>Sinorhizobium</i> sp. GR-06	<i>Alphaproteobacteria</i>	90%	AF506515
	BRS37	<i>Sinorhizobium</i> sp. GR-06	<i>Alphaproteobacteria</i>	89%	AF506515
	BRS38	<i>Sinorhizobium</i> sp. GR-06	<i>Alphaproteobacteria</i>	90%	AF506515
64	BRS39	uncultured bacterium NR1614	<i>Betaproteobacteria</i>	87%	AF035498
	RRS40	uncultured bacterium clone (I-HA9	<i>Betaproteobacteria</i>	92%	AY196448
67	RDS41	uncultured bacterium cloneCB907H9	<i>Betaproteobacteria</i>	93%	AY224034
71	RDS42	uncultured bacterium clone CB914H5	<i>Betaproteobacteria</i>	90%	AY223997
	RDS43	uncultured bacterium clone CB914H5	<i>Betaproteobacteria</i>	93%	AY224034
	RDS44	uncultured bacterium clone CB916H1	<i>Betaproteobacteria</i>	94%	AY224034
	RDS45	uncultured bacterium clone CB916H1	<i>Betaproteobacteria</i>	93%	AY224034
	RDS46	uncultured bacterium clone CB916H1	<i>Betaproteobacteria</i>	93%	AY224034

Direct sequence analysis of DGGE bands

In total 46 intense bands were reamplified, 16 and 9 bands obtained from soil under *Balanites aegyptiaca* in the dry season and in the rainy season respectively, and 11 and 10 from soil under *Acacia tortilis* subsp. *raddiana* in the dry season and in the rainy season respectively. The excised 321-bp *nifH* (+GC clamp) gene segments could be sequenced in one single step and the results showed that the target sequence of the *nifH* gene had been amplified in all cases. The results of a GenBank search are given in Table 5.3. Hundred % similarity was found for 6 major bands: with *Pseudomonas stutzeri* A15 for 4 bands from samples in the dry season under *Balanites aegyptiaca* and 1 sample from the rainy season under *Acacia tortilis* subsp. *raddiana*, and with *Azotobacter vinelandii* for one band under *Balanites aegyptiaca*. The other results showed 4 and 36 bands with respectively between 97 % and 100 % and between 83 and 94% similarity with known data. Sequences generated from DGGE bands were further subjected to cluster analysis. Simple homology searching is not always sufficient to place a given sequence within a taxonomic group, especially when the fragments are short, approximately 320 bp. A phylogenetic approach also establishes whether the banding patterns of DGGE reflect the presence/absence of certain phylogroups in relation to the tree. The results of this analysis (on 218 common bp) are shown in Figure 5.2.

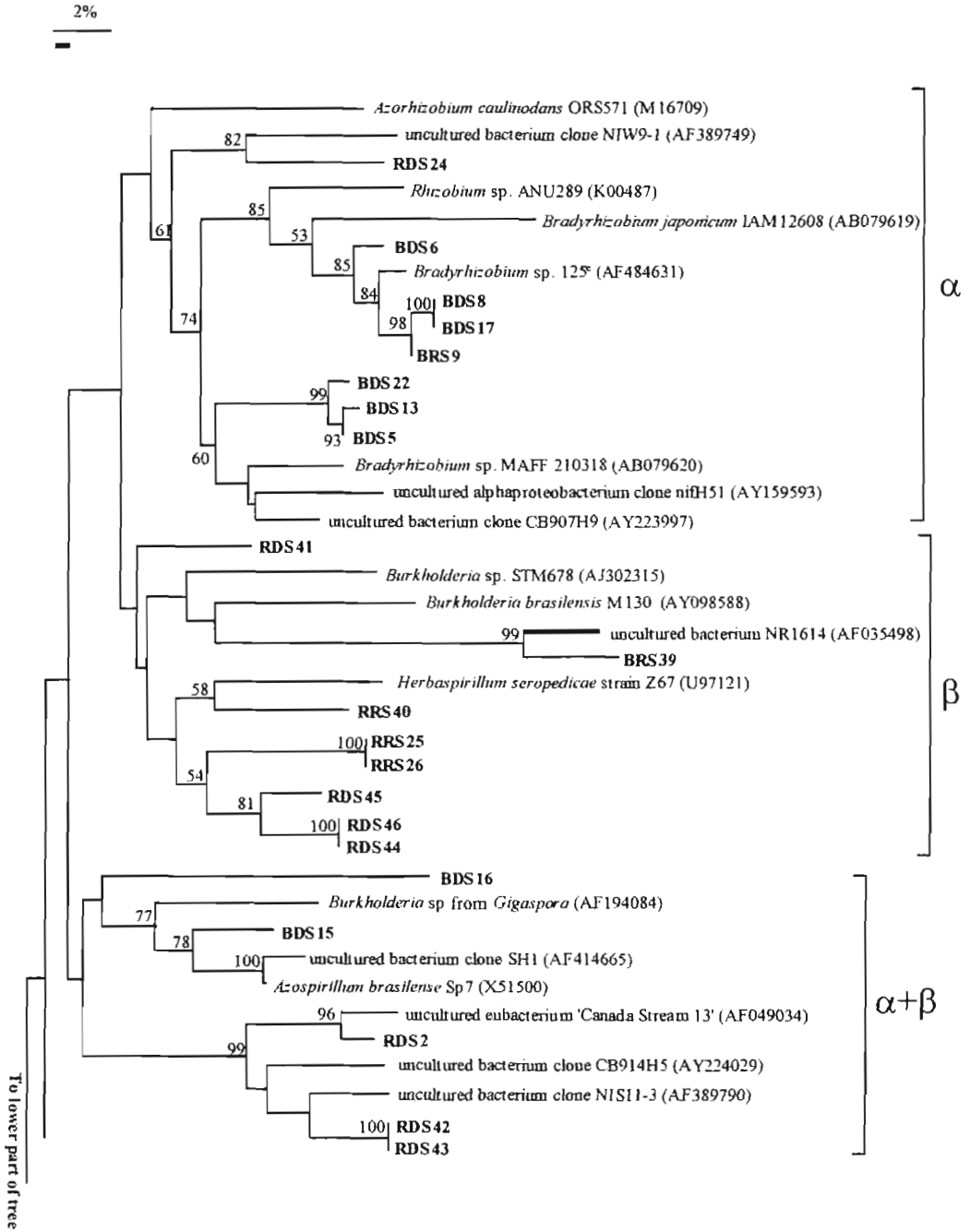
Most of the sequences cluster in the *Proteobacteria*. Only 4 sequences group within the *Cyanobacteria* and a single one with the *Firmicutes*. Within the *Proteobacteria* the *nifH* relationships (Figure 5.2) of members of the various classes were as described in various other studies (Lovell et al. 2001; Bagwell et al. 2002): the members belonging to the *Gammaproteobacteria* constitute a clearly separate cluster while the *nifH* sequences of some members of the *Alphaproteobacteria* and the *Betaproteobacteria* are more intertwined e. g. *Burkholderia*, *Azospirillum*. Many sequences cluster with those of uncultured bacteria (Figure 5.2). We distinguish 35 *nifH* phylotypes of which 28 were single sequences.

Six of our sequences representing 2 band positions belong in the cluster of the *Gammaproteobacteria*. One (BDS21) had 100% similarity with the *nifH* sequence of *Azotobacter vinelandii* (Jacobson et al. 1989). The other 5 (BDS27, RRS28, RRS29, BDS30, BDS31) sequences all on band position 60 (Figure 5.1) constitute a single phylotype and show to be 100 % similar with the corresponding sequence of *Pseudomonas stutzeri* A15 (Table 5.3, Figure 5.2) a nitrogen-fixing organism isolated from the rice rhizosphere (Vermeiren et al. 1999). According to this part of the *nifH* sequence this strain clusters together with another *Pseudomonas stutzeri* CMT.9A isolated from the rhizosphere of *Sorghum* (Krotzky and Werner, 1987).

The sequences of bands RRS14, RRS18, RRS19, RRS20 at bandposition 44 and 48 cluster with *Azoarcus*; RRS18, RRS19 and RRS20 belong to a single phylotype. Band RRS23 (bandposition 51) clustered with *Azovibrio restrictus* S5b2. The genus *Azoarcus* was originally proposed to include various nitrogen-fixing *Betaproteobacteria* occurring in large numbers in roots of Kallar grass (Reinhold-Hurek et al. 1993), a pioneer plant grown on flooded, salt-affected, low-fertility soils in Pakistan. These strains can also infect rice under laboratory conditions (Hurek et al. 1994). Later the taxonomic structure of this genus was reassessed and three new genera *Azovibrio*, *Azospira* and *Azonexus* were proposed (Reinhold-Hurek and Hurek, 2000). Members of the former *Azoarcus* genus have not been isolated from soil as yet (Reinhold-Hurek and Hurek, 2000).

Most of our sequences cluster with the corresponding ones of various members of the *Aphaproteobacteria*. The sequences of 9 bands BRS32, BRS33, BRS34, BRS35, BRS36, BRS37 and BRS38 at position 61, BRS12 at position 42, and RDS7 at position 30 clustered together within a large cluster containing the *nifH* sequences of several members of *Rhizobium*, *Mesorhizobium* and *Sinorhizobium*. Seven sequences had similarities from 89 to 92% with *Sinorhizobium* sp. GR-06 isolated from *Phaseolus vulgaris* (Laguerre et al. 2001) but did not (except BRS34 and BRS35) cluster in the same phylotype; band RDS7 (91% similarity with *Rhizobium* sp. NGR234) clustered among these 7 sequences. These 8 bands constitute a separate cluster. The % of similarity between the 218 base pairs of RDS7 and BRS34 was 97.7. A single band (BRS12) seems to be distinct but related to *Mesorhizobium* sp. LMG 11892 (Jaftha and Steyn, unpublished).

Sequences of bands BDS6, BDS8, BDS9, BDS17 (respectively at position 27, 33, 46) subclustered together with *Bradyrhizobium japonicum* IAM12608 (Cantera et al. 2002), *Bradyrhizobium* sp. 125e, *Rhizobium* sp. ANU 289 (isolated from *Parasponia*); BDS8 and BDS17 belong to the same phylotype (Figure 5.2). BDS5, BDS13 and BDS22 (respectively at position 24, 43 and 50) are quite similar and subcluster together. Band RDS24 was related (90% similarity) to an uncultured bacterial clone isolated from the rhizosphere of the smooth cordgrass, *Spartina alterniflora* (Lovell et al. 2000).



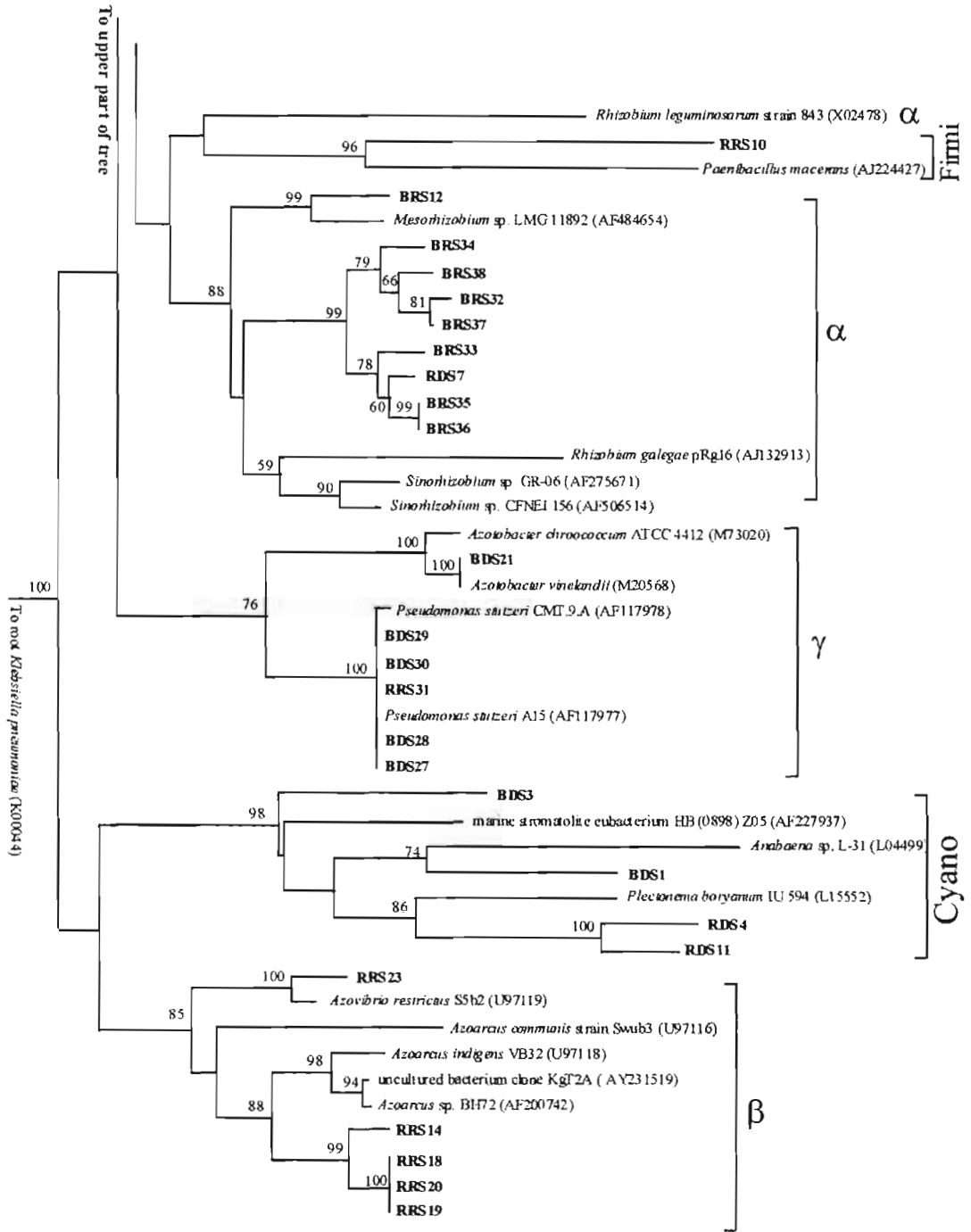


Figure 5.2. Neighbor-joining tree depicting the phylogenetic relationships among the *NifH* sequences in this study. Species or strain names are preceded by their GenBank accession numbers. DGGE bands detected in this study are given in boldface. Bootstrap values above 50 are shown, representing the percentage support for cluster out of 500. R= *Acacia tortilis* subsp. *raddiana*, B= *Balanites aegyptiaca*, RS = Rainy season, DS = Dry season. The scale indicates genetic distances. Boldline corresponding to a separate scale is used to reduce long

nifH sequences of bands RRS25 and RRS26 (position 53) form a single phylotype. BRS39, RDS40 (at position 64), and sequences of RDS44, RDS45, RDS46 (at position 73) fell into a large *Betaproteobacteria* cluster containing the *nifH* sequences of several members of *Herbaspirillum seropedicae*, members of *Burkholderia* and uncultured bacteria with a % of similarity ranging from 91 to 94. RDS46 and RDS44 belong to the same phylotype. Band RDS41 (at position 67) that is distinct, can also be considered as member of the *Betaproteobacteria* cluster.

The sequence of BDS15 appearing at position 45 is for 93% similar to that of an uncultured bacterial clone SH1 and fell into a cluster containing *Burkholderia* sp. (*Gigaspora margarita* endosymbiont) and *Azospirillum brasilense* Sp7 (de Zamaroczy et al. 1989); also BDS16 occupying the same band position, as BDS15 could be member of that cluster. Bands RDS42 and RDS43 (at position 71) form a phylotype, together with RDS2 they cluster with sequences from uncultured organisms. Only one band RRS10 matched with *Paenibacillus macerans* LMD24.3 belonging to the *Firmicutes* (Rosado et al. 1998).

We did not detect any putative sequences from *nifH* belonging to *Archaea*, *Frankia*, or *Vibrio*.

Comparison of the diazotroph species composition under *Acacia tortilis* subsp. *raddiana* and *Balanites aegyptiaca*

Multi-dimensional scaling (MDS) analysis – ANOSIM and SIMPER

The best configuration following MDS ordination using Bray-Curtis dissimilarities for the samples is presented in Figure 5. 3. Figure 5. 3a shows the samples taken in the dry season; Figure 5.3b shows the samples taken in the rainy season. From both (Figure 5.3a and b) it can be seen that the data can nominally be assigned to two distinct groupings. These groupings reflect the different trees under which the samples were taken. This separation suggests that the *nifH* gene pool in the bacterial community under these two trees is quite different.

While it may be suggested from the MDS plots that the *nifH* gene pool in the bacterial community under *Acacia tortilis* subsp. *raddiana* and *Balanites aegyptiaca* is different, this is based solely on a visual inspection of the plot.

ANOSIM analysis of the data revealed a significant difference between trees and this in both seasons (global R of 0.377 and 0.179 was calculated for respectively the dry season data and rainy season data, significance level 0.1%).

The most important elements responsible for the high percentage of dissimilarity (87%) between the samples taken under *Balanites aegyptiaca* and those taken under *Acacia*

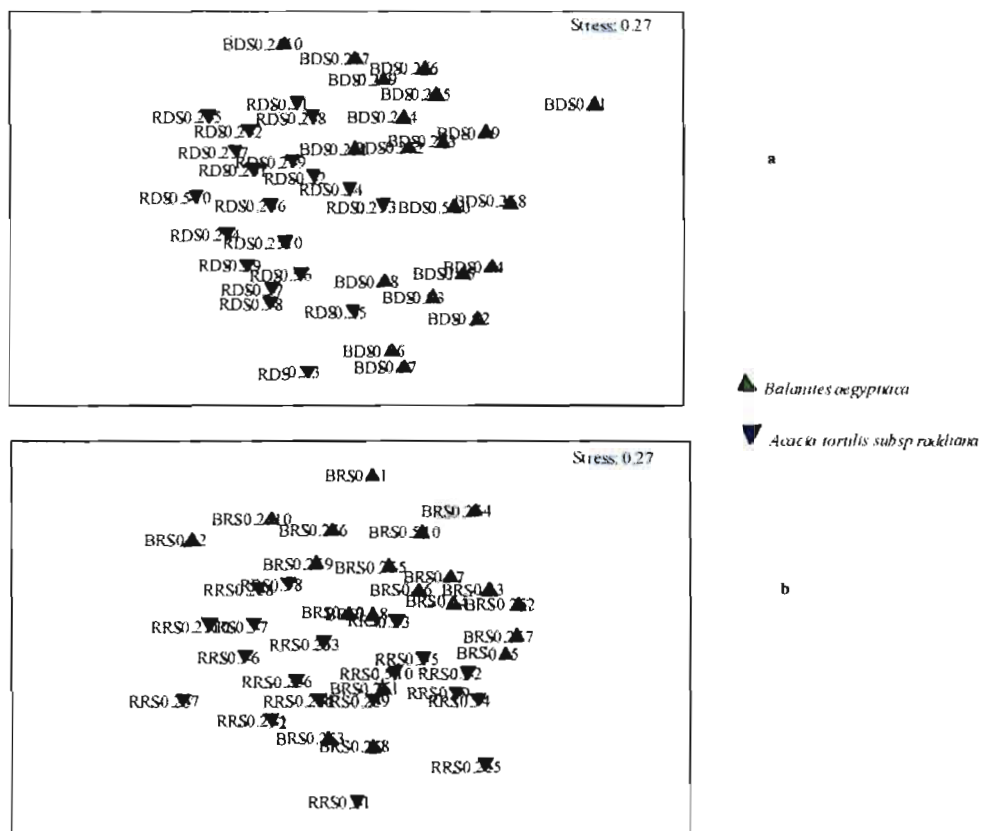


Figure 5.3. MDS ordination of Bray-Curtis similarity matrix of population abundance data [Log (x + 1) transformation] DGGE patterns from Souilène (North of Senegal) soil samples taken under *Acacia tortilis subsp. raddiana* (R) and *Balanites aegyptiaca* (B), RS= Rainy season, DS= Dry season, 0.25 (0.50)-x = depth at xm distance from the tree, (a) = for the factor level (*B. aegyptiaca* and *A. tortilis subsp. raddiana* in the dry season) (b) = for the factor level (*B. aegyptiaca* and *A. tortilis subsp. raddiana* in the rainy season).

tortilis subsp. *raddiana* are the high abundance of bands on bandposition 30 (of which one band RDS7 had the highest similarity with *Rhizobium* sp. NGR234) in the samples taken under *Acacia tortilis* subsp. *raddiana* and this in both seasons (SIMPER analysis), and the abundance of bands on bandpositions 27 and 33 (of which BDS6 and BDS8 and BDS9 have the highest similarity with *Bradyrhizobium* sp. 125e) in the samples under *Balanites aegyptiaca*. On top of that we also found in the rainy season samples, a higher abundance of bands on bandposition 42 (having the highest similarity with the *nifH* fragment of *Mesorhizobium* sp.) and of bands on bandposition 61 (having the highest similarity with sequences forming a separate subcluster in the rhizobia) under *Balanites aegyptiaca* than under *Acacia tortilis* subsp. *raddiana*.

Community structure under *Acacia tortilis* subsp. *raddiana*

Focusing our analysis on the samples taken under *Acacia tortilis* subsp. *raddiana* the MDS plots suggest a clear grouping according to the season and a clear grouping according to the depth (see Figure 5.4a and 4b).

An ANOSIM analysis confirmed significant differences between the samples taken in the dry and the rainy season (global R: 0.179, significance level 0.3%) and between the samples taken on the surface and the deeper layer (global R: 0.311, significance level 0.1%).

The major elements responsible for the dissimilarity between the samples taken in the dry and rainy season are the higher abundance of bands on position 61 and on position 60 in the rainy season.

The major elements responsible for the dissimilarity between the surface samples and the samples taken at 0.5 m are higher abundance of bands on bandposition 30, in the surface layers and lower abundance of bands on position 61 and 60.

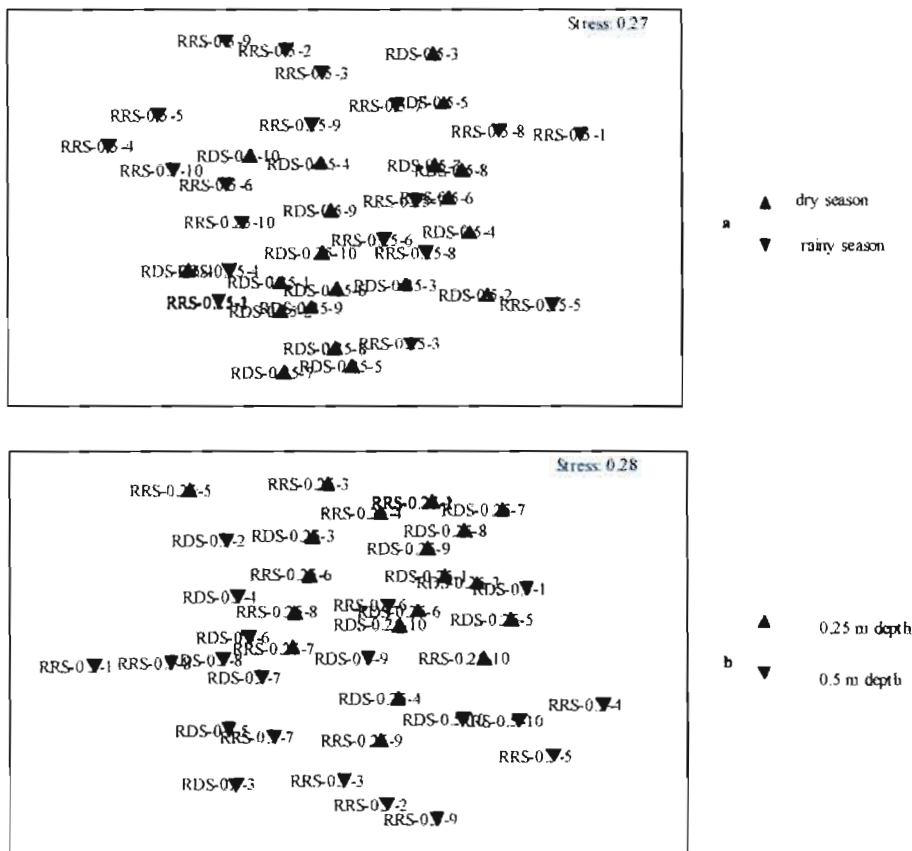


Figure 5.4 MDS ordination of Bray-Curtis similarity matrix of population abundance data [Log (x + 1) transformation] DGGE patterns from Souilène (North of Senegal) soil samples taken under *Acacia tortilis* subsp. *raddiana* (R), RS= Rainy season, DS= Dry season, 0.25 (0.50)-x = depth at xm distance from the tree, (a) = for the factor level (dry and rainy season), (b)= for the factor level (0. 25 m and 0. 50 m depth)

Community structure under *Balanites aegyptiaca*

Focusing our analysis on the samples taken under *Balanites aegyptiaca* the MDS plots suggest more or less distinct groupings according to the season while the samples taken at two different depths show no clear clustering (see Figure 5.5a and b). An ANOSIM analysis confirmed significant differences for the samples according to the season (global R: 0.274, significance level 0.1%) while differences according to the depth were not significant.

The major elements responsible for the dissimilarity between the samples taken in the dry and rainy season are higher abundance of bands in bandposition 61 and 42 (in the rainy season) and a lower abundance of bands on position 46.

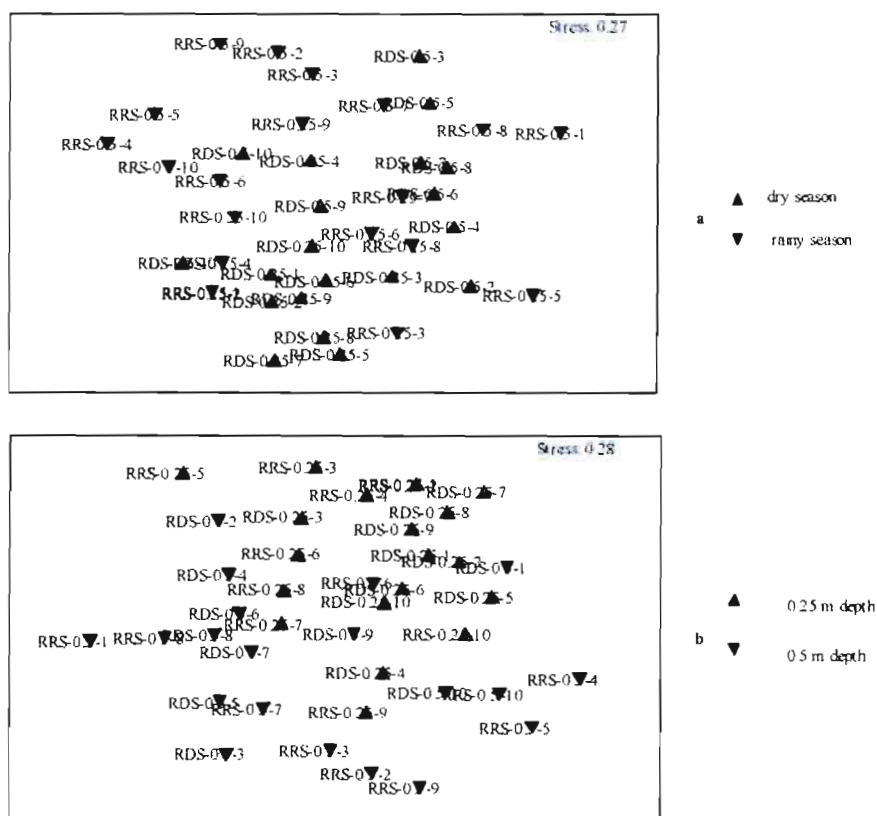


Figure 5.5 MDS ordination of Bray-Curtis similarity matrix of population abundance data [$\text{Log}(x + 1)$ transformation] DGGE patterns from Souilène (North of Senegal) soil samples taken under *Balanites aegyptiaca* (B), RS= Rainy season, DS= Dry season. 0.25 (0.50)-x = depth at xm distance from the tree, (a) = for the factor level (dry and rainy season), (b) = for the factor level (0.25 m and 0.50 m depth).

DISCUSSION

Nested PCR

We used PCR/DGGE on the *nifH* gene pool to investigate the genetic diversity of the diazotrophic communities associated with two different tree species naturally growing in the dryland part of North Senegal. All *nifH*/DGGE analyses were based on amplification products resulting from a nested PCR, since direct amplification with the primers PolF and AQER (Poly et al. 2001a) was unsuccessful. A possible explanation might be that the number of nitrogen fixers is quite low when compared to the other community members present in our soil samples. In previous studies (Demba Diallo et al. 2003a and b) in which we investigated the overall biodiversity in Souilène soil under *Acacia tortilis* subsp. *raddiana* using respectively a DGGE/16S rDNA approach and a 16S rDNA clone library we only identified nitrogen-fixing bacteria in the 16S rDNA clone library (namely 4% of the clones). This corresponds with reported values of approximately 5 % in upper soil layers in forest soil in Germany (Mergel et al. 2001) and in a Douglas fir forest in Oregon (Widmer et al. 1999). Although they are highly adapted to the soil physical and chemical conditions, diazotrophic bacteria are rarely dominant populations in terrestrial ecosystems (Tate, 1995).

DGGE analysis

Visually comparing the 80 *nifH*/DGGE profiles showed that very few similarities among the profiles could be detected (Figure 5.1) suggesting that the *nifH* pool in the dryland part of North Senegal is variable in relation to depth, distance from the tree, and season.

A computer assisted analysis (results not shown) confirmed the heterogeneity among the profiles, solely the profiles of RRS0.25-1 and RRS0.25-2 being identical as well by using the Dice as the Pearson similarity coefficient. On the contrary Bagwell et al. (2002) who studied the molecular diversity of diazotrophs in oligotrophic tropical seagrass bed communities found that all DGGE profiles from these sites showed the same prominent bands. Also in other studies it was shown that the diazotrophic community remains quite stable in function of time (Poly et al. 2001b; Shaffer et al. 2000). However Poly et al. (2001b) also found that the composition of the diazotrophic community varies among microenvironments in pasture soil. We could not evaluate this aspect because all our soil samples had the same sandy texture. By using 16S rRNA DGGE on the same soil samples taken under *Acacia tortilis* subsp. *raddiana* we showed (Demba Diallo et al. 2003a) that the profiles of the bacterial community soil samples were very similar suggesting stable communities. The heterogeneity observed within

the *nifH*/DGGE profiles might be an indication of a relatively high level of species richness of the soil nitrogen-fixing bacterial community in the samples studied.

Richness values and two diversity indices (Shannon and Simpson) indicated that the diazotrophic population recovered by DGGE is consistently less diverse under *Balanites aegyptiaca* than under *Acacia tortilis* subsp. *raddiana*. These differences might be due to the higher nitrogen content, as well as higher NO₃-N contents in the soil samples under *Acacia tortilis* subsp. *raddiana*.

Phylogenetic analysis

Although the *nifH* genes are quite conserved, mainly at the amino acid sequence level (Young et al. 1992, Ohkuma et al. 1996), several reports have shown that there is a sufficient variation at the DNA sequence level to distinguish similarity groups that may represent taxonomic clusters. Therefore we sequenced 46 intense bands (approximately 320 bases) from various samples and analysed the results by a NCBI-Blast search (Table 5.1) and by a phylogenetic analysis using a 218 long common part (Figure 5.2).

The phylogenetic dendrogram we obtained is conform with phylogenetic trees described in several other studies (Lovell et al. 2000; Lovell et al. 2001; Rösch et al. 2002; Poly et al. 2001b; Widmer et al. 1999; Bagwell et al. 2001). Within the *Gammaproteobacteria* we found *Azotobacter vinelandii* and *Pseudomonas stutzeri* A15, detected in many soils and of which the *nifH* genes have been compared in several studies (Lovell et al. 2000; Eardly et al. 1992; Hurek et al. 1997; Bagwell et al. 2002). Within the *Alpha*- and *Betaproteobacteria* the % of similarity between our sequences and sequences from the database vary between 83 to 98%. Lateral transfer of *nifH* gene may explain the appearance of sequences from *Beta* and *Alphaproteobacteria* in the same *nifH* cluster (Hurek et al. 1997; Lovell et al. 2000; Bagwell et al. 2002).

The percentages of members of the *Alphaproteobacteria* in the *nifH* gene pool vary with the soil studied: in the rhizosphere of *Spartina alterniflora*, the overwhelming majority of *nifH* sequences (Lovell et al. 2001) were affiliated with sequences from presumptive *Gammaproteobacteria* while the *Alphaproteobacteria* were poorly represented; in forest soil close to Cologne (Germany) most *nifH* sequences belonged in the *Proteobacteria* with the highest number in the *Alphaproteobacteria* (Rösch et al. 2002) and a smaller number in the *Betaproteobacteria*.

Statistical analysis

The results of the statistical analysis showed that there are significant differences in the diazotrophic community under *Acacia tortilis* subsp. *raddiana* and *Balanites aegyptiaca*

(Figure 5.3). The differences mainly concerned relative abundance of bands clustering with symbiotic nitrogen-fixing bacteria. Especially the high abundance of a sequence affiliated with a *Rhizobium* sp. (band position 30) in the samples under *Acacia tortilis* subsp. *raddiana* was striking. Our results show that the diversity of N₂ fixing bacteria is higher under *Acacia tortilis* subsp. *raddiana* than under *Balanites aegyptiaca* indicating a strong influence of the tree on the *nifH* pool. This hypothesis is supported by Bardgett et al. (1999) who suggested that the abundance and activity of soil microorganisms are more regulated by the dominant plants species than by the physical or chemical properties of the soil. We assume however that in our study the species constituting the dominant grass population under both trees will also have influenced the diazotrophic population. Poly et al. (2001b) did not support the influence of the plant species and suggested that soil management seemed to be the major parameter influencing differences in the *nifH* gene pool by controlling inorganic nitrogen content and that its variation and plants effect was negatively correlated to the *nifH* gene pool.

Several other studies (Riffkin et al. 1999; Giller et al. 1998) suggested that the soil physicochemical properties could influence the diazotrophic activity. Our results (Table 5.2) showed that soil samples taken under *Acacia tortilis* subsp. *raddiana* have higher total nitrogen contents, as well as higher NO₃N contents when compared to the soil samples under *Balanites aegyptiaca*. The difference in *nifH* gene pool structure may also be related to the root architecture of both trees and to the quality and quantity of compounds lost by rhizodeposition and by deposition of leafs on the ground. Indeed, *Acacia tortilis* subsp. *raddiana* root system comprised a plagiotropic tap root (at a depth of 30 cm from the soil surface) bearing abundant lateral roots along its length, while, *Balanites aegyptiaca* comprised an orthotropically tap root and produced lateral ramification, but they are less abundant and less voluminous than those of *Acacia tortilis* subsp. *raddiana* and were mainly located in the central region of the tap root with only a few branches located close to the soil surface (Vancura, 1964; Klein et al. 1988).

Our results also provide evidence that the communities were different in function of the season. Differences between both season's concern varying abundances of bands clustering with the symbiotic nitrogen-fixing bacteria, of which we found a higher abundance in the rainy season. For *Acacia tortilis* subsp. *raddiana* we also found a higher abundance of *Pseudomonas stutzeri* and almost no bands in the lower region in the rainy season. From this lower part, 4 bands of band classes 1, 11, 13 and 16 were sequenced: they correspond with members of the *Cyanobacteria* and of uncultured bacteria with an uncertain affiliation (Table 5.3). In function of the depth we only found significant differences for *Acacia tortilis* subsp. *raddiana* (Figure 5.4, Figure 5.5), a difference which might be explained by the root structure of this tree (see

above). Differences between both depths were provoked by a higher abundance of symbiotic nitrogen-fixers in the surface layers, and a lower abundance of symbiotic nitrogen-fixers and *P. stutzeri* in the lower layers.

The use of DNA banding patterns for determination of community similarities proved to be a powerful technique for resolving the different communities, but also was shown to be considered with caution. On the one hand we detected different sequences in bands from the same band position indicating that the separation of the various fragments was not optimal or that some bands were that intense that they masked others. On the other hand bands at different positions (band positions 27, 33, 46) as shown here for bands having a high sequence similarity with that of *Bradyrhizobium* sp. 125e, may represent very similar species.

Conclusion

This study clearly shows that it is possible to obtain a view on diazotrophic communities. The integration of the fingerprinting data with the statistical tools can be used to enable distinction between diazotrophic communities from different sampling spots.

Tree species have been shown to have an effect on the structure of the diazotrophic community, as well as the season. For the community under *Acacia tortilis* subsp. *raddiana* we also found an effect of the sampling depth.

Sequence analysis was limited to 46 bands and revealed that most belonged to the *Alpha-Beta* and *Gammaproteobacteria*.

Acknowledgments

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Chapter 6

General discussion and future perspectives

Three years ago, when we started this work, very little was known about global microbial and diazotrophic community structure in the tropical semi-arid regions of West Africa more particularly in the Northern pastoral-forestry zone of Senegal. This thesis is a first attempt to provide an overall view of the abundant bacterial population, and possible shifts in the bacterial community in function of the sampling date, the depth and the distance from the stem under two of the most dominating trees species growing naturally in this zone (*Acacia tortilis* subsp. *raddiana* and *Balanites aegyptiaca*).

Because the results of our work have been presented in 4 papers, part of the discussion is integrated in each of the papers and in this chapter we will consequently solely discuss the strategy used, its possible weaknesses and the obvious perspectives.

In the first paper, the aim was the investigation of the total microbial diversity of soil samples under *Acacia tortilis* subsp. *raddiana*. For reasons explained in the introduction we focused from the beginning on the use of molecular methods because we wanted to study in first instance the total bacterial population. One of the aims was also to isolate and characterize diazotrophic bacteria. Because of shortness of available time we could not start the isolation procedure. Nevertheless it would have been interesting to compare our results on total DNA, with the results obtained by isolation and characterisation of the bacteria.

Our first challenge was to obtain pure DNA without any interfering material for the PCR. As humic acids are known as inhibitors of the PCR and are often encountered in soils and sediments, we have developed and optimised a protocol for total DNA extraction and purification that could be used for direct lysis, taking into account that this tropical soil is poor (Demba Diallo et al. 2003a) in organic matter.

Efficient lysis of all cells in an ecosystem is necessary to obtain a reliable picture of the microbial community structure. Efficient cell lysis may be hampered by the variations in cell envelope composition of the microorganisms to be extracted. Hence, a protocol suitable for one species may not work for another one. Therefore we combined in our technique chemical and enzymatic lysis with slight mechanical lysis. Gram-negative bacteria can be lysed chemically by treatment with detergents, such as sodium dodecyl sulphate (SDS) while for the disruption of the cell envelope of the Gram-positive bacteria prior treatment with enzymes such as lysozyme before the use of detergents is needed. Because it remains very difficult to conceive chemical and/or enzymatic based lysis methods for complex communities (Johnson et al. 1991), a procedure which includes mechanical lysis by beat beating is mostly preferred (Akkermans et al. 1998). A disadvantage of this mechanical cell lysis is that nucleic acids can

be extremely sheared increasing the risk of formation of chimeric structures during PCR (Kopczynski et al. 1994).

To evaluate the dominant bacterial population we used in first instance denaturing gradient gel electrophoresis (DGGE) on the variable V3 region of the 16S rDNA, directly amplified from the bulk DNA. Therefore the conditions to obtain reliable profiles had to be standardised for these soil samples. We used a 35 to 70 % (Muyzer, 1993) denaturing gradient to detect as many bands as possible in each sample. The reproducibility of the profiles from one soil sample was checked in the starting period of this work: we repeated the technique on several samples from soil under *Acacia tortilis* subsp. *raddiana* and visually checked the profiles obtained on the number of bands and their position. For each repetition both parameters were visually identical proving that the standardisation of our procedure was optimal. It would however have been more complete if we should have also disposed of the % of similarity between the DGGE profiles obtained from various repetitions on the same sample.

The profiles from the various samples were analysed using the BioNumerics 2.5 software and Principal Component Analysis. The profiles all had a few strong bands and a large number of less intense bands. The fifty-eight identified band positions indicated a relatively low diversity corresponding with results from other tropical soils (Gomez et al. 2001). Overall DGGE profiles showed that a few dominant bands were present in all samples. Numerical analysis of all DGGE patterns showed a clear difference between samples taken in both seasons but few differences according to the depth and distance from the stem of the tree. Only when using the Dice coefficient we found the samples from the rainy season and from 0.5 m depth clustering together. Our conclusion is that there is a spatial homogeneity in the numerically dominant bacterial populations in the single transect we used for the sampling.

To characterise some of the taxa, 35 prominent bands have been excised and sequenced and the results analysed by a BLAST analysis. The results showed that prominent bacteria in the samples studied, are members of the *Firmicutes* (*Bacillus*, *Anoxybacillus*) and the *Acinetobacter* cluster in the *Gammaproteobacteria*.

From the combination of the results obtained by both techniques (16S rDNA/DGGE/sequencing) we made the following observations:

- 1 Two different band positions (33 and 36) contained bands having 100% sequence similarity with a *Bacillus* species (see Table 2.1) suggesting that identical sequences can arrive on two different band positions. However the sequences had not the same length making it possible that the corresponding bands contain slightly different part and consequently occupy different band positions. More efforts are needed to obtain sequences of the same maximum

length. Multiple operons with small sequence divergences have been described in several genera and can be responsible for mixed sequences in one band or for several bands for a single organism. We have no indications of more than one sequence in the bands we sequenced. Including control profiles with the relevant pure cultures could have been useful.

2 Two bands on the same band position (28 in Table 2.1) were found to belong to the *Betaproteobacteria* and the TM7 lineage respectively. Theoretically it is possible that 2 bands with different sequences can have the same denaturing profile in DGGE (Graeme et al. 2003). However the reason can also be that in the conditions used, that the separation of the fragments was not optimal or that some intense bands masked some less intensive ones. Therefore more bands of the same position should have been sequenced to decide whether some positions did indeed contain more than one rather different sequence. Bands that deviated slightly from some band positions would have been good candidates. Cloning of PCR products of re-amplified DNA contained in excised bands and sequencing of several clones could help to analyse the composition of some bands. A few publications describe the possible heterogeneous composition of a single DGGE band (Bagwell et al. 2002; Lovell et al. 2000).

Despite these observations we can conclude that the combination of DGGE and sequencing is minimally required to correctly interpret the DGGE profiles. This strategy allows us to obtain a view of the dominant population in the samples studied. The discriminatory power of the DGGE technique needs however further study.

Moreover it would have been very interesting to have disposed of the sequences of the less intensive bands, surely because some of these were unique for some samples. However we were not able to obtain enough pure DNA to be sequenced. More efforts to refine the extraction and purification techniques are therefore required.

By using 16SrDNA/DGGE/sequencing we did not detect rhizobia in our soil samples, which is a striking observation because the roots of this tree are nodulated by members of *Sinorhizobium* and *Mesorhizobium*. In fact de Lajudie et al. (1998) isolated and described *Mesorhizobium plurifarum* strains from root nodules from the roots of several *Acacia tortilis* subsp. *raddiana* trees present on the sampling site. A weak point is that we did our sampling in a single transect in function of the distance from the stem of the tree and in function of the depth. The transect was chosen randomly without special attention to the neighbourhood of fine roots and/or nodules. Our hypothesis is that the detection limit of PCR-amplified 16S rRNA gene fragments in DGGE analysis of 1% of the total amplified 16S rDNA is beneath the detection rate of rhizobia (Muyzer et al. 1993). However further (Chapter 5) studies estimated that the symbiotic nitrogen-fixing population is approximately 4% of the total detectable

population in the soil studied. Another possible reason is biased PCR reactions due to selective amplification (Cottrell and Kirchman, 2000). It is also possible that we did not pick any of these (less intense bands) bands for the sequence analysis confirming that the rhizobia are not amongst the most abundant bands in the profiles.

Another weak point is that we did not study replica's of the samples and that we used solely a single transect for each tree. We did study a transect of a second *Acacia tortilis* subsp. *raddiana* tree on the same field by PCR-DGGE and the results confirmed the results of Chapter 2. Missing are also control soil samples from the open grassland (without crops or trees).

To further analyse the population under *Acacia tortilis* subsp. *raddiana* we constructed a 16S rDNA clone library from a sample from the rainy and a sample from the dry season (Chapter 3). The aim was to screen each library by DGGE and consequently to sequence representative clones from the various OTUs found. The purpose was to compare the results of both libraries amongst each other but also with the results described in Chapter 2.

Within both clone libraries we found obviously a larger (when compared with the bands sequenced in the first approach) biodiversity, containing 100 phylotypes. The percentages of clones belonging to the various bacterial phyla differed amongst the seasons, *Acidobacteria* and *Alphaproteobacteria* being more present in the dry than in the rainy season. Within the *Gammaproteobacteria* we observed a shift in function of the season. We do not have an explanation for this phenomenon. With this technique we did find sequences (4 clones) corresponding with members of the rhizobia.

Taking into account that we found (in Chapter 2) examples of two bands belonging to two different lineage's on the same band position and that screening by DGGE is time consuming we wanted to evaluate DGGE as screening technique of a clone library. Therefore we screened (Chapter 4) one of both 16S rDNA clone libraries (rainy season) with DGGE and with 16S ARDRA (amplified ribosomal DNA restriction analysis) to evaluate the discriminatory power of both screening techniques and to evaluate the efforts needed for both. To us ARDRA has the extra advantage that the digitised profiles can be stocked in a database to be compared at any time with profiles of new clones.

Our results clearly indicate that 16S ARDRA is a much finer technique for screening for groups than DGGE. The definition of the band positions may be at the origin of the lower resolution and this may be correlated with the denaturing gradient used. However using other gradients to differentiate bands on a single band position is a laborious technique and the use of

a clone library of reamplified DNA from one band is more indicated to analyse the variation within one DGGE band.

We have to conclude that the estimated diversity of the most abundant organisms in soil as estimated by 16SrDNA/DGGE/clone library (Chapter 2 and 3) may be too low and that in the conditions used, the DGGE screening technique is not optimal to screen a 16S rDNA clone library.

In Chapter 5 we described the study of the abundant nitrogen-fixing population in soil samples under *Acacia tortilis* subsp. *raddiana* and *Balanites aegyptiaca* by using a *nifH*-DGGE approach combined with sequencing of representative bands from the different band classes. We also determined the chemical composition of the soil samples used.

Most of the sequences belong in the *Proteobacteria*. Hundred % of similarity was only found with the corresponding fragments from *Pseudomonas stutzeri* A15 and *Azotobacter vinelandii* both well known free-living diazotrophs in various soils. Most of the band sequences cluster in the *Alphaproteobacteria* in which, symbiotic nitrogen-fixing clusters contained several of our sequences. The overall diversity of diazotrophs is the highest under *Acacia tortilis* subsp. *raddiana*. This may be correlated with the higher content of NO₃ and N found in the soil under *Acacia tortilis* subsp. *raddiana* but more samples need to be examined to confirm this conclusion. This shows in any way that dominant trees present may influence the abundant *nifH* gene population. The heterogeneity of the symbiotic nitrogen fixers is comparable under both trees. The nitrogen-fixing communities were also different in both seasons for each tree and for *Acacia tortilis* subsp. *raddiana* also differences according to the depth were found. Statistical analyses revealed the bands responsible for these differences.

When evaluating our *nifH*/DGGE/sequencing approach the following observations need attention:

* As mentioned before for the comparable approach in Chapters 2 and 3, bands from a single band position contained some diversity in their common *nifH* fragment with a % of similarity with the sequence of the same organism between 89 and 92% (Table 5.3). As suggested above other techniques should be used to study the variation within abundant bands e.g. by constructing a clone library of the whole *nifH* pool or of single bands.

* Also in the *nifH*/DGGE profiles we found that bands from two different band positions may have 100% of similarities in the sequence of the common compared fragments. This can be provoked by sequence differences outside the common part used for comparison but this needs also to be confirmed in further investigations. Not enough attention was given to obtain *nifH* sequences of exactly the same (maximum) length as used in the DGGE gel. This is a

prerequisite to compare directly both groups of results and to make more reliable phylogenetic reconstructions.

Both observations lead to the conclusion that the *nifH* /DGGE/sequencing combination may underestimate the abundant population of nitrogen-fixing bacteria. Nevertheless the technique allowed us to obtain a preliminary view of the diversity of the most abundant groups.

It is evident that the use of PCR-based fingerprinting techniques is useful in answering ecological questions. Although the use of these techniques is still in development, their application has already been shown to be a powerful tool for determining the structure of microbial communities in different environments and monitoring changes in microbial communities without cultivation procedures.

In summary the following perspectives can be presented:

- * To be able to make general conclusions about the soil under both trees the sampling procedure needs improvement: replicas are needed, as are samples of more transects, control soil samples and more intensive sampling for seasonal evaluation. A single sample for each season is not representative to complete a study on seasonal shifts. Our results only allow to compare two isolated sampling dates. More transects should have been included. To decrease the number of samples, pooling of samples from different transects would have been advisable.
- * It would have been very interesting to do more efforts to sequence also the weaker bands from our DGGE gels because these bands were not contained in all samples and may fulfil a special role. The extraction and purification techniques need to be refined for these less intense bands.
- * The possible heterogeneity of single DGGE bands needs further study.
- * We did not have time enough to compare in detail the results obtained with other denaturing gradients; we tried out various gradients merely, to obtain the optimal gradient for the best overall band separation for all samples.
- * The chemical composition of the soil could only be determined on a pooled sample containing the diverse samples from a single transect under each tree. Replication is advised.
- * Originally it was the purpose to also study deeper soil samples. We obtained some results but not enough to be evaluated. One of the problems was that we did not obtain enough DNA from these deeper samples. Here again the extraction procedure needs to be adjusted.
- * More attention should be given to the dominating vegetation under the trees because these can also strongly influence the bacterial population (Bardgett et al. 1999).

In conclusion: by succeeding to give an overall view of the most dominant bacterial populations in the soil studied we created the start material for further experiments to evaluate

more in detail the bacterial community structure and their functional diversity. One of the questions to be solved remains whether the symbiotic nitrogen-fixing bacteria do have an influence on the improvement of the soil under *Acacia tortilis* subsp. *raddiana* or that the improvement is rather linked to the effect of the deep roots which play a role in the recycling of plant nutrients.

Summary

This thesis is part of a large-scale research project (Minimising competition in dry land agroforestry, INCO-DC ERBIC18CT98322) that investigates the competition between crops and trees in the dry land parts of West Africa. Crops are grown in the vicinity of naturally growing trees in the open grassland and two tree species have been chosen to be studied: *Acacia tortilis* subsp. *raddiana* (a legume tree) and *Balanites aegyptiaca* (a non-legume tree).

Our partnership in this project was to study the bacterial diversity in the soil on the study site.

The microbial part of this project concerned a collaboration between Dr. M. Neyra (co-director of this thesis for IRD) from the Laboratoire de Microbiologie des Sols Tropicaux, IRD, in Dakar, Senegal, the Laboratory of Microbiology at the University of Ghent and with Dr. P. de Lajudie from the Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM) in Montpellier (France) who was my IRD supervisor (Directeur de thèse). The work was performed in Dakar and Ghent.

During the start and study period (Chapter 1) we came to the conclusion that a molecular study of the soil samples has to be preferred on isolation and culturing methods to obtain an overview of the total detectable bacterial population in the experimental field. Nevertheless one of our aims remained to do also isolation, characterize the isolates and to compare the results obtained by both approaches

In first instance the sampling was done in Souilène in Senegal and we have chosen several *Acacia tortilis* subsp. *raddiana* trees and one *Balanites aegyptiaca* tree, two naturally growing trees in that region. Both trees species are used in agroforestry.

In order to limit the number of samples to be included we took samples under 2 *Acacia tortilis* subsp. *raddiana* trees and one *Balanites aegyptiaca* tree. We took samples along a single transect from the stem to approximately 10 m from it; sampling was done on two depths. In the overall project a deeper sampling was planned but unfortunately it was not possible to go further than to a depth of 12 m.

The DNA extraction protocol was optimised to guarantee that a maximum of different taxa in the soil could be lysed. The procedure was standardized.

In the second part of our work we did the first experiments to analyse samples from soil under a single *Acacia tortilis* subsp. *raddiana* by 16S rDNA/DGGE. Various conditions and the reproducibility were tested and an optimised protocol was delineated. The aim was to obtain in first instance a global view on the total bacterial population in our samples, to estimate the % of diazotrophs and to register changes in function of place and time. This part is described in Chapter 2.

All profiles consisted of a few strong bands and a large number of less intense bands; some of the intense bands were found in all samples while some other bands were typical for specific groups of samples. Numerical analyses of the profiles showed that there were distinct differences between the samples taken on two dates one in the dry and one in the rainy season. After we excised 35 of the most prominent bands from the profiles, the DNA was purified and sequenced. The sequences were analysed with a GenBlast search and the clusters to which these sequences belonged were identified. The most prominent bacteria in the soil samples were members of the *Bacillae* and of *Acinetobacter* from the *Gammaproteobacteria*.

In a third part of the work (Chapter 3) we decided to complete the former study by constructing two clone libraries one from a sample from the dry and one from a sample from the rainy season respectively.

We screened the profiles in each season for the one with the highest number of bands and the corresponding soil sample was chosen for making the clone library. Both libraries were screened by DGGE and representatives of each group found were partially sequenced (the first 500 bases). The sequences were analysed by a GenBlast search and by a phylogenetic reconstruction. The results of both approaches were analysed. A larger biodiversity was found than in the PCR/DGGE/sequencing approach. Members of the *Gammaproteobacteria* were the most dominant, followed by the *Firmicutes*, the *Alphaproteobacteria*, the *Betaproteobacteria*, the *Acidobacteria* and the *Actinobacteria*. The relative compositions of both samples are different: the *Acidobacteria* are in a much higher percentage present in the dry season than in the rainy season, while the inverse effect is found for the members of the other groups. Within the *Gammaproteobacteria* we found a shift between the dry season and the rainy season from the pseudomonads to *Acinetobacter* and *Escherichia* related organisms. A coverage analysis showed that the number of clones (135 in each sample) screened was still not big enough to cover the full clone diversity.

Because we observed that in some cases the screening for groups in the library by using DGGE was not completely satisfying we decided to analyse one of our 16S rDNA clone library also by 16S ARDRA. The results are presented in Chapter 4. One hundred and fifty clones were analysed and screened by both methods (DGGE and 16S ARDRA). The results were compared with the sequence results from Chapter 3. The clusters obtained by ARDRA corresponded quite well with the sequencing results while the clusters obtained by DGGE were delineated less fine. Our conclusion is that screening of a 16S rDNA clone library with ARDRA is superior on screening with DGGE. The conclusion is also that the results obtained

with DGGE and sequencing of representative bands underestimate the most abundant bacterial populations.

Contrary to what we found in the 16SrDNA/DGGE/sequencing approach, the clone library approach revealed a few symbiotic nitrogen-fixing bacteria in our samples.

In a last part we wanted to determine the abundant nitrogen-fixing population in soil samples under *Acacia tortilis* subsp. *raddiana* and compare these populations with these found under a non legume tree *Balanites aegyptiaca*. Therefore we wanted to use a *nifH* fragment wherefore the primers and the PCR conditions were analysed and optimised. We needed a nested PCR to amplify *nifH* genes from our soil samples. This work is described in Chapter 5. To analyse the community structure and the diversity of the bacterial groups we used statistical analysis and diversity index evaluation of the band profiles. The diazotrophic diversity was lower under *Balanites aegyptiaca* than under *Acacia tortilis* subsp. *raddiana*. The differences were confirmed with multidimensional scaling analysis and ANOSIM tests. By SIMPER test we showed which element were responsible for the differences found. Additionally we excised several prominent bands on various positions, sequenced these and performed phylogenetic reconstructions. Most sequences are affiliated to the *Proteobacteria*. We found 4% of clones belonging to the rhizobia. Hundred % of similarity was only found with the corresponding *nifH* sequence of *Pseudomonas stutzeri* and *Azotobacter vinelandii*. We found differences in the populations under each tree according to the season. Only under *Acacia tortilis* subsp. *raddiana* a difference was found in the composition of the populations according to the depth. The chemical composition of the soils was different under both trees the one under *Acacia tortilis* subsp. *raddiana* having a much higher nitrogen content than the one under *Balanites aegyptiaca*.

We concluded that it is possible with the techniques used to obtain a view on the most abundant members of the diazotrophic communities. Tree species seem to have an effect on these populations as well as the season. Only for *Acacia tortilis* subsp. *raddiana* we found an effect of the sampling depth.

In Chapter 6 we evaluated our overall strategy and focused on the shortcomings of some of the techniques used and the issues for further study.

Nederlandse samenvatting van de thesis

Dit onderzoek maakt deel uit van een grootschalig project (Minimising competition in dry land agroforestry, INCO-DC ERBIC18CT98322) met de bedoeling de competitie tussen gewassen en bomen te bestuderen in de droge gebieden van West-Afrika. De plaatselijke bevolking teelt er gewassen in de open plaatsen tussen de plaatselijke struiken en bomen. Onze deelname in dit project bestond erin de microbiële diversiteit in de grond te bestuderen op de gekozen studieplaats. Twee onderzoeksplaatsen werden gekozen, één onder *Acacia tortilis* subsp. *raddiana* (een boom behorende tot de Leguminosae) en één onder *Balanites aegyptiaca* (een boom die niet tot de Leguminosae behoort). Beide boomsoorten maken daar deel uit van de natuurlijke begroeiing.

Het microbiële luik van dit onderzoek omvatte een samenwerking tussen Dr. M. Neyra [co-Directeur van deze thesis voor IRD (Institut de Recherche pour le Développement)] van het Laboratoire de Microbiologie des Sols Tropicaux, IRD, in Dakar (Senegal), het laboratorium voor Microbiologie aan deze universiteit en Dr. P. De Lajudie van het Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM) in Montpellier (Fr). In het kader van mijn doctoraatsbeurs van IRD was Dr. P. de Lajudie mijn supervisor (Directeur de thèse). Deze drie onderzoeksgroepen werken reeds verscheidene jaren samen om de diversiteit van allerhande isolaten afkomstig uit wortel- en stengelnodules van een grote variatie van planten uit allerhande landen en vegetaties te karakteriseren.

Gedurende een eerste studieperiode werden in een literatuur onderzoek de verschillende technieken, die beschikbaar zijn om de microbiële diversiteit in grondstalen te onderzoeken, met elkaar vergeleken en kwamen wij tot het besluit dat moleculaire methoden die het totale DNA in deze stalen kon onderzoeken het meest voor de hand liggend waren. Het werk werd in Gent en in Dakar uitgevoerd en het was de bedoeling om ons en op DGGE en op de isolatie van bacteriën uit de grondstalen toe te leggen. Uiteindelijk hebben we ons (wegens tijdsgebrek) moeten beperken tot moleculaire methoden uitgaand van totaal geëxtraheerd DNA uit de diverse grondstalen.

In eerste instantie werden de stalen verzameld in het biologisch station in Souilène in Noord-Senegal. We namen stalen onder twee *Acacia tortilis* subsp. *raddiana* bomen en onder een *Balanites aegyptiaca* boom. De stalen werden genomen over één transect per boom op twee verschillende diepten. Het transect liep van de stam tot 10 m ervan weg.

In het project was er ook voorzien dat er een boring zou gebeuren tot op 30 m om diepestalen te bekomen. Het bleek niet mogelijk te zijn om dieper dan 12 m te boren. Diepestalen tot 12 m werden ook ingesloten.

Het protocol voor de extractie van totaal DNA werd geoptimaliseerd om een maximum van diverse bacteriën te kunnen lyseren. De procedure werd gestandaardiseerd en gebruikt op de verschillende stalen.

In een tweede deel werden de voorbereidende experimenten gestart om 16SrDNA/DGGE uit te voeren op stalen genomen onder *Acacia tortilis* subsp. *raddiana*. Verschillende omstandigheden en de reproduceerbaarheid van de techniek werden grondig uitgetest en een geoptimaliseerd protocol werd vastgelegd. Het was onze bedoeling om eerst een algemeen beeld te verkrijgen van de totale bacteriële populatie in onze stalen en om daarin het % van stikstof-fixeerders te kunnen schatten. De resultaten werden beschreven in Hoofdstuk 2.

We bekwamen DGGE profielen bestaande uit een beperkt aantal sterke banden en een groter aantal lichtere banden. Sommige van de sterke banden kwamen voor in alle stalen terwijl andere typisch voorkwamen in bepaalde groepen van stalen. De numerieke analyse van de profielen toonde aan dat er een duidelijk verschil was tussen de profielen, bekomen in het regenseizoen en deze bekomen in het droge seizoen.

Vijfendertig van de meest sterke banden uit de verschillende profielen werden uitgesneden, gezuiverd en gesequeneerd. De bekomen sequenties werden onderworpen aan een GenBlast onderzoek, om de clusters waartoe deze sequenties behoorden te identificeren. De meest voorkomende sequenties behoorden tot de *Firmicutes* en tot het *Acinetobacter* cluster in de *Gammaproteobacteria*.

In een derde deel van dit werk werd de vorige studie vervolledigd door twee clone libraries te maken van het 16S rDNA (voor een staal uit respectievelijk het regen- en het droge seizoen).

Beide bibliotheken werden gescreend voor groepen door DGGE en representatieve banden van de diverse groepen werden partiëel gesequeneerd (eerst 500 bp). De sequenties werden terug aan een GenBlast onderzoek onderworpen en fylogenetische reconstructies werden gemaakt. De bekomen resultaten werden geanalyseerd en vergeleken met de resultaten bekomen in Hoofdstuk 2. De resultaten toonden een grotere biodiversiteit dan deze gevonden met 16SrDNA/DGGE/sequencing. Organismen behorende tot de *Gammaproteobacteria* kwamen het meest voor, gevolgd door leden van de *Firmicutes*, de *Alphaproteobacteria*, de *Betaproteobacteria*, de *Acidobacteria* en de *Actinobacteria*. De samenstelling was verschillend in beide seizoenen: de *Acidobacteria* komen in een hoger percentage voor in het droge seizoen dan in het regenseizoen, terwijl het omgekeerde zich voordoet voor leden van de andere

groepen. Een statistische test toonde dat het aantal clones dat wij onderzochten toch nog niet groot genoeg was om de gehele bacteriële diversiteit te omvatten.

Omdat wij waarnamen dat het screenen voor groepen d.m.v. DGGE niet voldoende fijn leek te zijn, werd voor één van de bibliotheken ook een screening d.m.v. 16S ARDRA uitgevoerd (Hoofdstuk 4). Honderd en vijftig clones werden ingesloten. De resultaten toonden duidelijk dat de resultaten bekomen door screenen met 16S ARDRA veel beter overeenkwamen met de sequentieresultaten dan deze bekomen met DGGE screening. Hieruit moeten we ook besluiten dat de resultaten bekomen in Hoofdstuk 2 de bacteriële diversiteit onderschatten.

Onderzoek van de clone library toonde aan dat er een beperkt aantal clones overeenkwamen met leden van de rhizobia. Waarschijnlijk waren deze organismen in een te klein aantal aanwezig in onze stalen om ze als band via DGGE te detecteren ofwel hebben we de relevante banden niet gesequeneerd.

In een laatste deel werd specifiek onderzoek verricht naar de stikstof-fixeersers in onze stalen door de *nifH* populatie te onderzoeken door gebruik te maken van DGGE, gecombineerd met het sequencen van uitgesneden sterke banden. Hierbij werden stalen van onder *Acacia tortilis* subsp. *raddiana* en *Balanites aegyptiaca* ingesloten; de stalen waren afkomstig van één transect per boom en twee dieptes en werden éénmalig genomen in het droge en het regenseizoen. De PCR condities werden uitgetest en geoptimaliseerd (nested PCR was nodig). Dit werk wordt beschreven in Hoofdstuk 5. Om de bekomen resultaten te analyseren hebben wij gebruik gemaakt van statistische methodes. Het besluit was dat de diversiteit van de diazotrofe bacteriën verschillend was onder beide bomen en dat de diversiteit groter was in de grond onder *Acacia tortilis* subsp. *raddiana* dan in de grond onder *Balanites aegyptiaca*. De stikstof fixerende populaties verschilden per boom ook in beide seizoenen. Alleen voor *Acacia tortilis* subsp. *raddiana* werd er ook een verschil gevonden tussen de populaties afkomstig van stalen uit verschillende diepten.

In Hoofdstuk 6 hebben wij de algemene resultaten besproken en een aantal perspectieven voor bijkomende studies voorgesteld. Ook werden een aantal zwakke punten over de gevolgde strategie en technieken besproken.

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