COMPARISONS OF LEVELS OF GENETIC DIVERSITY AMONG *STREPTOMYCES SCABIES* ISOLATES OF SOUTH AFRICA USING VARIOUS DNA TECHNIQUES

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DECLARATION

I the undersigned hereby declare that the work contained in this thesis is my own work and has not previously in its entirety or in part submitted at any university for a degree.

ABSTRACT

Streptomyces spp. are responsible for a large proportion of the world-wide quality deterioration of potatoes causing a potato tuber disease called common scab. Determining the genetic diversity of the *Streptomyces* spp., especially the main pathogen, *S. scabies*, has been a prerequisite for the ultimate control of common scab. Techniques responsible for the classification and determination of genetic diversity have improved with advances in DNA technology. Analysis of South African (S.A.) *S. scabies* isolates has been focusing on the organisms' morphology, physiology, pathogenicity and melanin production, but the classification of *S. scabies* using DNA techniques has not yet been explored.

In this study various DNA techniques were screened for optimal use in determining the genetic diversity within and among isolates of *S. scabies*. Bacteria had been sampled from the main potato producing regions in S.A. and a few other regions. The techniques explored included RAPDs, AFLPs, RAMS, Rep-PCR, 16S rDNA sequencing and ITS analysis. The first three techniques had to be abandoned due to non-reproducibility between the same isolate extracted on separate occasions and ITS analysis was abandoned due to sequencing difficulties. Of the three Rep-PCR techniques tested (BOX, ERIC and REP), BOX was selected because it produced the clearest and most reproducible results. BOX-PCR and 16S rDNA sequencing were therefore ultimately selected as the methods to analyse the genetic diversity of the *S. scabies* isolates.

Information concerning the pathogenicity of the isolates was supplied by the Vegetable and Ornamental Plant Research Institute of the Agricultural Research Council (VOPI, ARC, Roodeplaat). A brief analysis of the pathogenicity prediction of the isolates in this study was explored with the PCR technique. Presence of the *nec1* gene was previously shown to be an indication of the pathogenicity within the *Streptomyces* spp. group. PCR analysis is based on the amplification of a 0.72kb fragment (*nec1*) in pathogenic isolates which was absent in non-pathogenic isolates. However, in this study the test for pathogenicity lacked specificity and sensitivity and some of the problems experienced included non-reproducibility between PCR reactions and the presence of the pathogenic fragment in the non-pathogenic isolates (as designated by VOPI, ARC). These observations led to the conclusion that this technique is not an ultimate test for pathogenicity of *S. scabies* isolates in a South African context.

The genetic distances and similarity matrices of the Rep-PCR results were calculated using Nei's genetic distance calculation (Nei M, 1975). Clusters from these matrices were constructed using the unweighted pair group average (UPGMA) with the PAUP4 package. The clusters for the 16S rDNA sequences were formed with the Neighbor Joining (NJ) method and the PAUP4 package. The NJ trees do not take small sequencing differences into account, therefore a Parsimony Network had to be constructed.

The trees obtained with the 16S rDNA sequencing techniques grouped most *S. scabies* isolates into one major group with a 100% bootstrap robustness of this group. More genetic diversity was illustrated by the BOX-PCR technique and the isolates were generally grouped according to their different regions of origin. However, the bootstrap values were low, indicating a lack of robustness regarding the BOX-PCR clustering. This was not unexpected as the number of data points employed in the BOX technique is very limited. Both techniques revealed unexpected grouping of a few isolates. Their isolated positions could be attributed to possible misclassification or to the fact that they could be genetically different *S. scabies* isolates. *Streptomyces* spp. (other than *S. scabies*) displayed enough differences to place them in their own distinct groups using both techniques. Comparison of the cluster results obtained in this study did not correlate to the data supplied by the VOPI, ARC (morphology, physiology, pathogenicity and melanin production) which revealed differences between the *S. scabies* isolates within their respective regions.

The lack of diversity displayed by the 16S rDNA technique can be attributed to the fact that only a limited section of the genome is involved making it inappropriate for intra-species genetic diversity analysis. The BOX technique takes various loci within the genome but is still not ideal for a thorough genetic diversity analysis. This study represents the first attempt to determine the genetic diversity of *S. scabies* in S.A. on DNA level.

OPSOMMING

Streptomyces spp. is verantwoordelik vir 'n groot deel van die wêreld afname in aartappel kwaliteit as gevolg van die aartappelknol siekte bruinskurf. Die bepaling van die genetiese diversiteit tussen die *Streptomyces* spp., varal die hoof patogeen in die groep, *S. scabies,* is 'n vooreiste vir die uiteindelike beheer van bruinskurf. Tegnieke verantwoordelik vir die klassifikasie en bepaling van genetiese diversiteit het verbeter met vooruitgang in DNA tegnologie. Analise van Suid Afrika (S.A.) se *S. scabies* isolate konsentreer op die organisme se morfologie, fisiologie, patogenisiteit en malanien produksie, maar die klassifikasie van *S. scabies* met die behulp van DNA tegnieke is nog nie uitgevoer nie.

In hierdie studie is verskeie DNA tegnieke ondersoek vir optimale bepaling van genetiese diversiteit binne en tussen *S. scabies* isolate van S.A. Bakterieë is verkry van die hoof aartappel-produserende areas in S.A. en ook van 'n paar ander areas. Die tegnieke wat in die studie gebruik is, het RAPDs, AFLPs, Rep-PKR, 16S rDNA volgordebepaling en ITS analise ingesluit. Die eersgenoemde drie tegnieke is uitgesluit as gevolg van nie-herhalende resultate tussen dieselfde isolaat geïsoleer op verskillende geleenthede. ITS analise is uitgesluit as gevolg van probleme met volgordebepaling. Rep-PKR en 16S rDNA volgordebepaling is uiteindelik gekies as die mees geskikte metodes vir die analise van genetiese diversiteit tussen *S. scabies* isolate in hierdie studie omdat albei skynbare herhaalbare resultate gelewer het.

Inligting met betrekking tot die patogenisiteit van die isolate is voorsien deur die Groente en Sierplant Instituut van die Landbou Navorsinsraad (VOPI, LNR). 'n Vinnige analise van die patogenisiteits voorspelling van die isolate is uitgevoer met die PKR tegniek. Dit is voorheen aangetoon dat die teenwoordiheid van die *nec1* geen dui op die patogenisiteit in die *Streptomyces sp* groep. PKR analise het 'n 0.72kb fragment (*nec1*) in patogeniese isolate geamplifiseer wat nie teenwoordig was in nie-patogeniese isolate nie. Hierdie toets vir patogenisiteit soos gebruik in hierdie studie was egter onspesifiek en onsensitief en sommige van die probleme wat ondervind is sluit in nie-herhaalbaarheid tussen PKR reaksies en die teenwoordigheid van die patogeniese fragment in die nie-patogeniese isolaat (soos beskryf deur VOPI, LNR). Uit hierdie waarnemings word afgelei dat die tegniek nie 'n geskikte toets vir patogenisiteit van *S. scabies* isolate in 'n S.A. konteks is nie.

Die genetiese afstande en ooreenkomstige matrikse van die Rep-PCR resultate is bereken met die genetiese afstand bepaling van Nei (Nei M, 1975). Groepe is gevorm met die "unweighted pair group average" (UPGMA) en PAUP4 pakket. Die "Neighbor Joining" (NJ) groepe is gevorm met die 16S rDNA volgordebepaling data mbv die PAUP4 pakket. Die NJ groepering neem nie klein volgorde verskille in ag nie en gevolglik moes'n "Parsimony Network" opgestel word.

Die groepering met die 16S rDNA volgordebepaling het meeste van die isolate in een hoof groep geplaas met 'n 100% "bootstrap" waarde. Meer genetiese diversiteit is met die BOX-PCR tegniek gevind en isolate was oor die algemeen gegroepeer volgens van hul oorsprong. Die "bootstrap" waardes vir die BOX tegniek was baie laag. Dit was nie onverwags nie, want die hoeveelheid data punte was beperk met die BOX tegniek. Albei tegnieke het 'n aantal afwykende isolate vertoon. Hul ge-isoleerde posisies kan toegeskryf word aan moontlike misklassifikasies van die isolaat. Die moontlikheid dat daar wel genetiese verkille tussen die isolate is, kan egter nie uitgesluit word nie. *Streptomyces* spp. (uitgesluit *S. scabies*) het genoeg variasie vertoon om hulle in hul eie groepe met die gebruik van beide tegnieke te plaas. Vergelyking tussen die groepe in die studie stem nie ooreen met die data verkry vanaf VOPI, LNR (morfologie, fisiologie, patogenesiteit en melanien produksie) nie wat verskille tussen *S. scabies* isolate binne 'n sekere gebied vertoon.

Die gebrek aan diversiteit soos vertoon deur die 16S rDNA tegniek kan toegeskryf word aan die feit dat slegs 'n beperkte gedeelte van die genoom ondersoek word, wat dit ongeskik vir intra-species genetiese diversiteit analise maak. Die BOX tegniek neem verskeie loci in die genoom in ag, maar is steeds nie ideaal vir deeglike genetiese diversiteit analise nie. Hierdie studie verteenwoordig die eerste poging om die genetiese diversiteit van *S. scabies* in S.A. op DNA vlak te bepaal.

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LIST OF ABBREVIATIONS

%	percentage
&	and
α	alpha
β	beta
γ	gamma
δ	delta
∈	epsilon
µg	microgram
µl	microlitres
µM	micromolar
°C	degrees centigrade
16S rDNA	16S ribosomal deoxyribonucleic acid
AFLP	amplified fragment length polymorphisms
AMOVA	analysis of molecular variance
ARC, VOPI	Agricultural Research Council, Vegetable & Ornamental Plant Research Institute
ATP	Adenosine triphosphate
BOX	BOXIAR
bp	base pair(s)
mCi	millicurie
DAP	diaminopimelic acid
ddH_2O	double distilled deionised water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
E	eastern
E. coli	*Escherichia coli*
eds	editors
EDTA	ethylenediaminetetra-acetic acid
ERIC	enterobacterial repetitive intergenic consensus
EtBr	ethidium bromide
etc.	etcetera
FAME	fatty acid methyl esters

g	gram
href	hypertext reference
hrs	hours
IDT	integrated DNA technologies
i.e.	that is
ISP	International *Streptomyces* Project
ISP2	yeast extract-malt extract agar
ITS	internal transcribed spacer region
kb	kilobase pairs
kg	kilogram
l	liter
LB	"Luria Bertani"
LFRFA	low-frequency restriction fragment analysis
LTD	limited
M	molar
mer	bases
$MgCl_2$	magnesium chloride
min	minute(s)
ml	millilitres
Mol%	molecular percentage
N	northern
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
NJ	Neighbor joining
no	number
nt	nucleotide
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
PE	Perkin Elmer
PAUP	phylogenetic analysis using parsimony
PCR	polymerase chain reaction
pp	page
PYI	paptone-yeast extract iron agar
RAMS	random amplified microsatellite polymorphisms
RAPD	random amplified polymorphic DNA

Rep	repetitive element PCR
REP	repetitive extragenic palindromic
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
s	second(s)
S (16S)	subunit
S.A.	South Africa
S. acidiscabies/ Acid	*Streptomyces acidiscabies*
S. scabies	*Streptomyces scabies*
SDS	sodium dodecyl sulphate
soln	solution
spp.	species
Taq	*Thermus aquaticus*
TBE	tris borate EDTA
TE	tris EDTA
Tm	annealing temperature
Tris	tris[hydroxymethyl]aminomethane
Tris-HCL	tris hydrochloric acid
UPGMA	unweighted pair-group arithmetic averages
US	University of Stellenbosch
U.S.A.	United States of America
UV	ultra-violet
V	volt
v/v	volume per volume
vol	volume
w/w	weight per weight
w/v	weight per volume
XC	xylene cyanol

LIST OF DEFINITIONS

- A haplotype is a unique combination of genetic markers present in a chromosome.

- Bootstrap is based on re-sampling the number of bases over and over again. It operates by drawing random samples with replacement from the original sample, whilst maintaining the original size. Multiple trees are formed and the most likely one is chosen as the correct tree (Weir, 1996).

- Neighbor-Joining method is based on differences. It identifies the closest pairs, or neighbors, of taxonomic units in a way to minimize the total length of a tree. It is based on grouping of the sequences due to unusually high rates of evolution, or some other spurious similarity, but not due to shared ancestry. Only one possible tree is constructed using this method (Weir, 1996; Earnisse, 1998).

- Parsimony is a character-based approach which looks at characters that share an evolutionary origin (out-group). This means that in this study the parsimony approach for tree construction based on sequence results only regards the base changes and results in multiple trees. It follows the shortest possible route and the correct phylogenetic tree is the one that requires the minimum number of nucleotide changes to produce the observed differences between the sequences. It only takes into account the character values observed for each species, rather than working with the distances between sequences (Weir, 1996; Earnisse, 1998).

- UPGMA defines the inter-cluster distance as the average of all the pair-wise distances for members of two clusters (Weir, 1996).

CHAPTER ONE

1 INTRODUCTION

1.1 BACKGROUND

1.1.1 Common scab disease

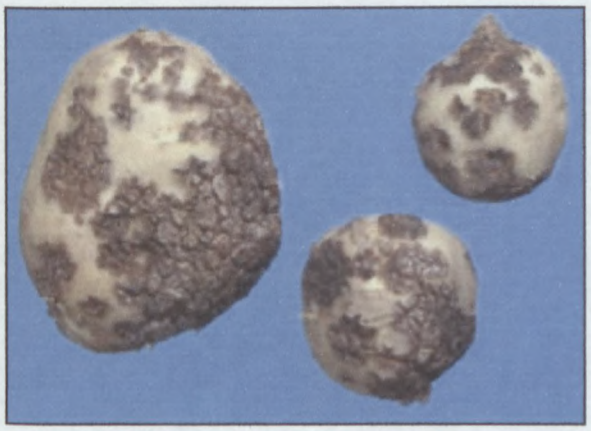
Common scab is a potato disease found in various potato-growing areas in the world. It is considered one of the four most important diseases of potato in North America (Loria *et al.*, 1997) and is also of major importance in South Africa (Gouws and Mienie, 1997). The warm, dry climatic conditions experienced in most production areas in S.A. are extremely favourable for disease development, making it very important to control this disease. The disease is introduced to most potato growing areas on infected seed tubers (href1). Common scab is also found on other crops, such as beetroot, sugar beet, turnips, rutabaga, parsnip, radish, carrot, melon and peanuts. In the latter, the disease is known as "pod wart of peanut" in South Africa. (Goyer and Beaulieu, 1997; Liu *et al.*, 1996; Gouws and Mienie, 1997). The species responsible for these crop diseases belong to a group of bacteria called *Streptomyces* species.

1.1.2 The symptoms of common scab on potatoes

The potato tuber is a modified stem that initially consists of only nodes. Once growth starts, the nodes are separated from each other by the formation of internodes. Lenticels are formed on these internodes through which the *Streptomyces* organisms can infect the tuber. This makes the potato susceptible to infection for the first five weeks of tuber development (Gouws and Mienie, 1997). *Streptomyces* spp. can also infect the tubers through wounds.

The characteristic symptoms of common scab of potato are shallow, raised (common scab), or deep pitted lesions on tubers ranging from localised distribution to complete covering of the tuber surface (Figure 1.1). The lesions first appear as small reddish or brown spots on the developing tuber. These spots may be raised or slightly sunken and wart-like in appearance. As the tuber develops, lesions become circular in outline (href2). This response is due to the action of a toxin or enzyme exuded by the scab organism (Lawrence *et al.*, 1990).

Figure 1.1 *Streptomyces* spp. infection on potatoes (href3).



No potato cultivar is immune to infection by *Streptomyces* spp., but there is considerable variation in cultivar response (Goth *et al.*, 1995). Symptom severity depends on interactions between cultivar resistance, environmental conditions, and the virulence of the species involved while the degree of the scab is determined by the tuber age, time of attack and its subsequent growth (Millard and Burr, 1926). The type of scab is dependent on the infecting *Streptomyces* species. The variety of the potato can therefore modify the severity of scab, but not change its type.

The *Streptomyces* spp. are hydrophobic which explains why they only cause lesions on the potato surface. The potatoes contain moisture which form a protective layer under the skin, not allowing the organisms to penetrate.

The surface lesions on potato tubers caused by the bacteria affect their quality resulting in diminished marketable yields. As high value is placed on external appearance and skin quality of potatoes, blemished tubers are likely to be down graded. Although it has been suggested that it is possible for *S. scabies* to cause root and stolon infection, which can in turn cause a reduction in potato yield (Millard and Burr, 1926), it is generally accepted that common scab does not affect tuber yield, only tuber quality. In the case of groundnuts, the yield and the quality of the nuts are not affected because the symptoms are restricted to the pod tissue (de Klerk *et al.*, 1997). Although the groundnut disease is of minor importance, it is still necessary to be aware of it because any future plantation of potatoes in infected areas can result in common scab disease of the potatoes.

1.1.3 Characteristics of *Streptomyces* species

- The genus *Streptomyces* is classified in the family Streptomycetaceae.
- *Streptomyces* are gram positive, filamentous prokaryotes.
- The organisms are actinomycetes, i.e. bacteria with some of the properties of fungi.

- They produce non-fragmenting substrate mycelium which colonise and penetrate organic matter in the soil.
- Their extensive branching substrate and aerial mycelia contain LL diaminopimelic acid (L-DAP) and glycine (wall chemotype 1) (Becker *et al.*, 1964; Chater, 1989; Wellington *et al.*, 1992; Williams *et al.*, 1983).
- They produce spore chains through fragmentation of aerial hyphae.
- They are nutritionally flexible saprophytes and can aerobically degrade resistant substances (such as pectin, lignin or chitin).
- They produce many antibiotic secondary metabolites and hydrolytic enzymes which can degrade complex carbon, energy and nitrogen sources.
- They possess extensive secondary metabolic pathways, which lead to the production of a wide array of bioactive compounds. These include commercially important substances such as antibiotics (chloramphenicol, neomycin or streptomycin).

1.1.4 *Streptomyces* genome

Streptomyces spp. contain approximately 8Mb DNA that have a very high GC content (69-78%). The chromosomal DNA as well as numerous plasmids are linear in the genus *Streptomyces*. The chromosomes of *Streptomyces* are also genetically unstable, resulting in the huge diversity of species and the production of many different secondary metabolites (Volff and Altenbuchner, 1998; Fischer *et al.*, 1998).

1.1.5 *Streptomyces* scabies

Millard and Burr, 1926, described 11 species of *Streptomyces* that caused scab. Among the *Streptomyces spp.* which can cause scab lesions on potatoes, are *acidiscabies, caviscabies, diastatochromogenes* and the major culprit, *scabies.* The difference between these species justifies their classification into separate groups. Of all the *Streptomyces* spp., *S. scabies* is the most extensively studied.

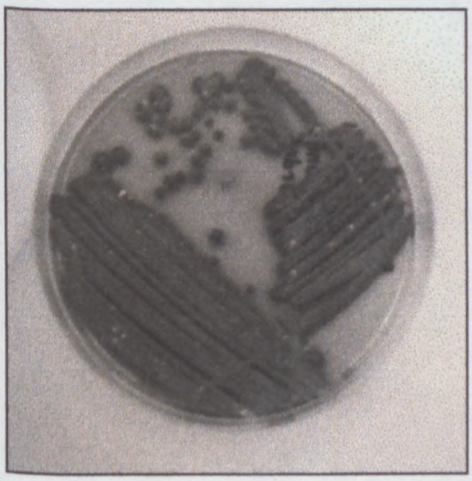
As seen on standard agar, *Streptomyces* spp. grow as a nonseptate, substrate mycelium which give rise to the aerial mycelium. The mycelium undergoes fragmentation to form chains of nonmotile, hydrophobic spores, which each contain one nucleoid. The three to many conidia in each chain are often pigmented and can be smooth, hairy, or spiny in texture. *S. scabies* mycelium is branched and

forms spore chains, which are generally loose, grey, spirals (Figure 1.2). There are 20, or more, smooth, grey spores per chain (Prescot and Harley, 1993).

According to Faucher (1993) *S. scabies* show a high similarity with three other *Streptomyces* species: *S. cyaneus, S.diastaticus*, and *S.microflavus* based on a number of classification criteria such as morphological and physiological characters.

The organism survives as spores or slender strands within crop debris. They can be soil- or tuber-borne. They are adapted to well-drained, near-neutral pH soils in which potatoes and root crop vegetables are commonly grown. High soil moisture inhibits the growth of *S. scabies.* Their spores are spread by water or soil that has been blown by the wind (href1). Their soil dwelling nature and lack of special survival structures suggests that this species has evolved in close association with plants.

Figure 1.2 A *S. scabies* isolate on ISP2 agar after two weeks growth.



1.2 INTEGRATED CONTROL OF COMMON SCAB

There are many methods of controlling this disease (Gouws and Minnie, 1997; Loria *et al.*, 1997) and the following methods are currently employed:

1.2.1 Chemical control

Determination of whether the tuber is from an infected area must be thoroughly investigated. This will determine whether treatment is necessary before plantation into a certain soil. It is recommended that the tubers are placed in a plastic crate and immersed in the product to maintain

constant concentration of the product. Fumigation with PCNB or Bacfume of the soil before tuber plantation is also essential (href2).

1.2.2 Irrigation

It has been suggested that the dividing parenchyma cells of the lenticels during tuber development allows for the transfer of the infection. Antagonistic bacteria move faster through the soil water films than do the *Streptomyces* species. The bacteria colonise the lenticels and provide competition for the other pathogens. Therefore, well-irrigated soil will result in decreased chance of pathogen infection of tubers. Specific monitoring of the variation of tuber growth initiation is essential to obtain the desired success with the irrigation control.

1.2.3 Crop rotation

Streptomyces spp. can survive in the soil for long periods nourished by decomposing plant matter, manure and roots of living plants. They can also infect other fleshy root crops as mentioned at the beginning of this chapter. This means that rotation of potatoes with these crops will not eliminate the presence of *Streptomyces* spp. in the soil. Careful evaluation of the host range is required when using crop rotation as a control mechanism for common scab.

1.2.4 Biological control

Cruciferous species such as cabbage produce volatile compounds which reduce the severity of disease because they are toxic to soilborne pathogens. The release of the volatile compounds is closely associated with the soil temperatures. An increase in temperature results in a greater release of volatile compounds.

Certain *Streptomyces* spp. cause the suppression of common scab by producing antibiotics which are lethal to pathogenic species of *S. scabies* in antibiosis tests. *S. diastatochromogenes* PonSSII and *S. scabies* PonR were identified as suppressive isolates. The success of these species in scab control needs to be investigated so that the correct complement of species can be used in combination without the pathogens developing resistance to them. Environmental conditions also need to be enhanced before the biocontrol can successfully be implemented (Liu *et al.*, 1996).

1.2.5 Soil manuring and pH

Disease development increases with an increase in the pH. Addition of lime which increases the pH will thus produce favourable conditions for pathogen survival (nitrogen, sulphur and phosphorous on the other hand lowers the pH of the soil and inhibit pathogen presence). Certain inorganic compounds such as copper, manganese and sulphur have a direct toxic affect on the pathogen while calcium strengthens plant cell walls giving them resistance to the pathogen (href4).

1.2.6 Resistant cultivars

The ideal long-term control of common scab would be to breed natural disease resistant cultivars. Potato cultivars resistant to common scab have been identified, but they do not have all the desirable characteristics of the more popular cultivars (Gouws and Minnie, 1997). To approach this problem, researchers have to identify markers linked to the resistant gene and incorporate it into the popular cultivars through transformation.

1.3 THE BIOLOGICAL CHEMICALS RESPONSIBLE FOR COMMON SCAB

1.3.1 Thaxtomin A

It has been suggested that many factors are responsible for common scab of potatoes. One of the candidates is an extracellular esterase enzyme which depolimerises suberin, a waxy polyester covering tubers, thus allowing penetrance of the tuber (Beauséjour *et al.*, 1999 and Goyer *et al.*, 1996b). Another factor responsible for pathogenicity is a group of phytotoxins.

It is speculated that certain phytotoxins named Thaxtomins after their founder R. Thaxter in 1890, are involved in the potato scab disease caused by *S. scabies.* Thaxtomin A, a nitroaromatic compound, is the predominant phytotxin produced by *S. scabies* (Doumbou *et al.*, 1998). Disease symptoms caused by *S. scabies* on potato seedlings, (reduction of root and shoot length, radial swelling and tissue chlorosis and necrosis), are identical to the effects of Thaxtomin A on seedlings. Furthermore, Thaxtomin C has been shown to be responsible for common scab of sweet potatoes, *S. ipomoea* (Loria *et al.*, 1997). It is thus suspected that Thaxtomin acts as an important determinant in plant-pathogenicity of *Streptomyces* species. However, Thaxtomin is not the sole determinant of bacterial pathogenicity. Other factors such as phytohormones and hydrolytic enzymes may also be involved (Goyer *et al.*,1998).

The production of Thaxtomin A can be detected as a yellowish halo surrounding the bacterial colonies on oatmeal agar plates. The intermediates of Thaxtomin production are responsible for this yellow colour development (Goyer *et al.*, 1998). Thaxtomin A is produced in media containing plant constituents originating from different species. This suggests that there is a plant compound responsible for Thaxtomin A production that is not limited to potatoes, but other plants as well (Beauséjour *et al.*, 1999). There is a positive correlation between the amount of Thaxtomin A produced and the aggressiveness of a *Streptomyces* isolate, as determined by the percentage affected tuber surface area. However, it is not correlated with the mean number of lesions produced by an isolate. There is an observed 50-fold range in Thaxtomin A production level among *Streptomyces* spp. indicating the diversity in phytotoxin production and the factors influencing the precise level of Thaxtomin A produced by an isolate are very complex (Kinkel *et al.*, 1998).

The variety of the individual isolates able to produce Thaxtomin A, not correlated to any classification methods, suggest a random transfer of these genes. This suggests the unimportance of pathogenicity to the ecology and evolution of the *Streptomyces* spp. (Kinkel *et al.*, 1998).

1.3.2 Genes involved in the pathogenicity of the S. scabies

Having identified Thaxtomins as being the cause of potato common scab (Bukhalid and Loria, 1997), researchers proceeded to identify the genes responsible for Thaxtomin production. This was performed by producing a total genomic library of a pathogenic *S. scabies* isolate and expressing cloned DNA sections of it in *S. lividans*. The *S. lividans* species that displayed necrotic activity similar to that of common scab were isolated and traced back to the *S. scabies* genomic library. Mutational analysis and subcloning was carried out on the fragment to identify a 0.67kb segment, *nec1* gene, responsible for the necrosis of the potato tuber (Bukhalid and Loria, 1997). The *nec1* gene is not directly involved in Thaxtomin A biosynthesis, but is perhaps a virulence factor in plant pathogenic *Streptomyces* species. The gene produces an unidentified extracellular, water-soluble compound that causes necrosis on potato tuber discs. No other function of this gene is known.

The *nec1* gene is linked to a transposase pseudogene (ORFtnp) and there is an intergenic region between the two genes. The predicted sizes of the *nec1*, ORFtnp and intergenic region are 0.72kb, 0.55kb and 0.57kb respectively (Bukhalid *et al.*, 1998). The *nec1* gene's GC content (63%) differs from that of the rest of the genome. This fact and the fact that the three ORFs are linked, suggests that *nec1* may have been mobilised into *S. scabies* through a transposition event mediated by ORFtnp. The ORFtnp displays a high level of identity to a family of transposases whose function is

to mobilise antibiotic resistant genes among clinically important bacteria (Bukhalid *et al.,* 1998). This adds to the speculation that this ORFtnp is responsible for the transposition event. The ORFtnp and the intergenic region are not necrotic in the absence of the *nec1* gene (Bukhalid and Loria, 1997).

Analysis of the hybridisation of the *nec1* clones and ORFtnp clones to various *Streptomyces* spp. genomes (from various regions in the United States, Hungary, Japan and S.A.) was performed (Bukhalid *et al.*, 1998; Bukhalid and Loria, 1997). All pathogenic isolates, except CEK018 from Ceres in South Africa, produced positive hybridisation results. The Southern blot hybridisation patterns for both clones, varied in size within the *S. scabies* genome alone. This suggests that this species is much more genetically diverse and more ancient than other *Streptomyces* spp. that cause potato scab. No record of the gene's presence in other bacterial species has been found.

This genetic element is a useful target for diagnostic PCR (by the occurrence or absence thereof) (Kreuze *et al.*, 1999). Kreuze *et al.* (1999) and Bukhalid *et al.* (1998) revealed a fragment identical in size from all pathogenic isolates. It was suggested that amplification of *nec1* gene could be used to replace the usual long tedious pathogenicity tests.

1.4 CLASSIFICATION OF STREPTOMYCES SPECIES

The major causal agent of common scab identified by Thaxter was a pathogenic strain producing a soluble brown pigment (melanin) and grey spores borne in spiral chains (*Oospora scabies).* The species was renamed *Actinomyces scabies* by Güssow in 1914 and then *Streptomyces scabies* by Waksman and Henrici in1948 (Lambert and Loria, 1989b). In 1961, Waksman erroneously redescribed Thaxter's description of the type strain. This resulted in the false type strain being included in the International *Streptomyces* Project (ISP) and led to the placement of some *S. scabies* isolates in the wrong species groups and some common scab pathogens being erroneously classified as *S. scabies*. Uncertainty of the type strain's true identity prevented it from being listed on the approved list of bacterial names (Goyer *et al.,* 1996b). Lambert and Loria managed to revive the original identity of the *S. scabies* species, as described by Thaxter, and expanded on it (Lambert and Loria , 1989b).

A thorough description of the numerical classification history of the *Streptomyces* spp. is reported by Williams *et al.* (1983). In 1957, Waksman started classifying the *Streptomyces* spp. on the basis of trivial differences in morphological and cultural properties. By 1970, 3000 *Streptomyces* spp. had

been identified. To accommodate the increasing number of *Streptomyces* spp., numerous classification methods had to be devised. Classification using various criteria including sporophore morphology, melanin production, chain morphology, were carried out by various researchers, but no classification consensus was reached. In the early 1960's, a collaborate study with the ISP was carried out on this group of bacteria. Only a small number of traditional tests were used, supplying a very narrow database. Classification research continued during the 1960's and 1970's, but too few major clusters were formed for the large number of *Streptomyces* species. Williams *et al.* (1983) used 139 unit characters to numerically classify the group of bacteria, thus providing the basis for the reduction of the large number of *Streptomyces* species.

However, the above-mentioned classification proved time consuming and sometimes unreliable, leading to the requirement of a more improved classification system (Clarke *et al.*, 1993). Some of these DNA techniques will be discussed in the following section. Since researchers have begun classifying *Streptomyces* spp. by studying their genomes, the quality of the numerical classification results can be assesed by comparing them to the genetical classification results.

Some non-pathogenic isolates are sufficiently related to *S. scabies* for their inclusion in this taxon (Goyer *et al.*,1996b). Most of the classification methods used lack a means of distinguishing pathogenic *S. scabies* isolates from the non-pathogenic *S. scabies* isolates. This could be because the character of pathogenicity was transferred horizontally in the group. It is also unclear whether pathogenicity arose independently more than once in the *Streptomyces* spp., or whether all the *Streptomyces* spp. were originally pathogenic and the trait has been lost multiple times through the history of the organism (Bramwell *et al.*, 1998).

1.4.1 The current classification techniques being employed

Various researchers have contributed to our understanding of the complexity of the genus, but none have resolved the problem of species identification in a satisfactory way. The following bibliography summarises classification techniques carried out on *S. scabies* since the classification reported by Williams *et al.*, (1983) as described in the section above.

- Morphological and physiological tests: Lambert and Loria, 1989a; Lambert and Loria, 1989b; Lorang *et al.*, 1995; Goyer *et al.*, 1996a; Faucher *et al.*, 1992.
- Cellular fatty acid profiles: Paradis *et al.*,1994; Sadowsky, 1996; Ndowora *et al.*,1996; Goyer *et al.*,1996b.
- DNA homologies: Healy and Lambert, 1991.

- 16S ribosomal DNA sequence analysis: Takeuchi *et al.*, 1996.
- Characterisation of inhibitory interactions among isolates: Clark *et al.*, 1998.
- Protein profiles: Paradis *et al.*,1994; and Goyer *et al.*,1996b.
- RAPD profile analysis: Kutchma *et al.*, 1998.
- Restriction fragment length polymorphisms (RFLP's): Doering-Saad *et al.*,1992.
- Polymerase chain reaction (PCR) fingerprinting using repetitive extragenic sequences: Sadowsky *et al.*, 1996; Clark *et al.*, 1998.

These techniques have been applied to many *Streptomyces* spp. groups and are not concerned with the *S. scabies* group alone. Although each method of classification has its advantages and disadvantages, DNA-based methods are emerging as the more reliable, simple and inexpensive way to identify and classify microbes. The use of molecular techniques is thus increasing in popularity among scientists working on the taxonomy of organisms. These techniques, as well as a few others that have not been applied to the *Streptomyces* spp. yet, are discussed below.

1.4.2 Phenotypic characteristics

Initially, scientists were using the species morphology to characterise *Streptomyces* spp. as this provides the basic identification of the bacteria as well as a starting point for researchers trying to characterise them in a more precise manner. Scientists were using a limited number of characters to define species groups which resulted in artificial classification. Williams *et al.* (1983) made use of numerical phenetic techniques (139 characters) to classify 475 Streptomycetaceae species (394 were *Stretomyces* spp.). The use of these phenotypic characters grouped the *Streptomyces* spp. into one major cluster consisting of major, minor and single groups. Phenotypic analysis consists of morphological and physiological classification. The morphology of the bacteria was studied on inorganic salt starch agar using a scanning electron micrograph (Lambert and Loria, 1989a). Physiological analysis includes analysis of the carbon source utilization, nitrogen source utilisation, antibiotic activity, degradative activity and pH sensitivity. These tests were performed by adjusting the agar medium on which the bacteria were propagated (Lambert and Loria, 1989a). The presence of pigment production was tested on tyrosine and peptone yeast extract agar media (Shirling and Gottlieb, 1966).

Lambert and Loria (1989b) used 42 criteria to classify a group of *Streptomyces* spp. based on phenotypic analysis. Twelve *S. scabies* isolates from the United States of America, Canada and Hungary formed a homogenous group displaying the following characteristics:

- Smooth grey spores borne in spiral chains
- Melanin production on PYI medium
- Usage of all eight ISP sugars (Shirling and Gottlieb, 1966) as sole carbon source
- Most *S. scabies* do not degrade xanthine
- They are susceptible to 25µg oleandomycin per ml
  10 IU penicillinG per ml
  20µg streptomycin per ml
  10µg thallous acetate per ml
  5µg crystal violet per ml

Faucher *et al.* (1992) studied a group of *Streptomyces* spp. from Quebec. The group of *Streptomyces* spp. was separated into six classes according to their phenotypic data. *S. scabies* isolates remained homogenous using this classification analysis and they were grouped in class 1. The characteristics of class 1 were the same as those listed above. Goyer *et al.* (1996b) analysed a group of *Streptomyces* spp. in Eastern Canada and *S. scabies* displayed common phenotypic characters. These publications indicate that phenotypic analysis is not ideal for the classification of *S. scabies* isolates. (Appendix A contains a table representing the combined morphological and physiological data obtained from numerous publications).

1.4.3 Fatty acid analysis

It has been suggested that bacteria grown under specific conditions produce a unique set of fatty acids which can be regarded as a means of classifying certain bacteria. Paradis *et al.* (1994) studied 31 *Streptomyces* spp., which were phenotypically related to *S. scabies,* collected from different regions in Eastern Canada and Minnesota. Fatty acids were extracted from them and derivitised to their methyl esters (FAMEs). The FAME profiles of the *Streptomyces* spp. consisted of 12 to 17 fatty acids, most of which were saturated iso- or anteiso acids. Statistical differences in the relative ratios of the two structural isomers enabled a separation of the 31 *S. scabies* isolates from Canada into two groups. The first group had predominantly 16:0 palmitic acid while the second group had two subclusters containing either 15:0 anteiso or 15:0 iso, 15:0 anteiso and 16:0 fatty acids (Goyer *et al.*,1996b).

Pathogenic isolates could not be differentiated from the non-pathogenic isolates, but could be differentiated from suppressive isolates on the basis of fatty acid analysis. This method of classification differed from the DNA-DNA hybridisation method of classification because the latter

was unable to distinguish between pathogenic and suppressive isolates (Paradis *et al.*, 1994; Sadowsky *et al,* 1996 and Ndowora *et al.*,1996).

The groupings formed with fatty acid analysis were compared to those formed with inhibitory reactions, and antibiotic assays (see section 1.4.7). The groupings were similar but not identical (Ndowora *et al.*, 1996).

1.4.4 DNA-DNA hybridization studies

This procedure includes the extraction of DNA from two organisms, the denaturation thereof with alkali and then the re-association of the two DNA populations. Analysis of the re-association results is made possible through the labelling of one organism's DNA by incorporating a radioactively labelled dNTP by the random primer method. The double stranded DNA that is labelled can then be quantified by liquid scintillation counting, providing the DNA-DNA hybridisation values. DNA-DNA homology values exceeding 20% are regarded as a reliable indicator of similarity between the DNA nucleotide sequences (Stackebrandt *et al.*, 1981 and Paradis *et al.*,1994).

According to the DNA-DNA hybridization data, *S. scabies* from Eastern Canada can be divided into three genetic clusters (Goyer *et al.*, 1996b). Pathogenic isolates of *S. scabies* cannot be differentiated from non-pathogenic isolates on the basis of DNA relatedness (Paradis *et al.*,1994) and no correlation between the fatty acid and DNA-DNA hybridisation methods of classification could be identified (Paradis *et al.*, 1994).

The validity of the clusters of isolates using the phenotypic numerical taxonomic studies done by Williams *et al.* (1983) is questionable after analysing the DNA relatedness data (DNA-DNA hybridisation). Although Williams *et al.* (1983) were able to identify several hundred species, the phenotypic characters revealed limited differences between the single *Streptomyces* spp. for use as a classification tool. DNA-DNA hybridisation studies were able to reveal a high diversity among the isolates tested (Doering-saad *et al.*, 1992).

The comparison of DNA-DNA hybridisation data from various *Streptomyces* species expressed more variation in the *S. scabies* hybridisation data. This suggests that the divergence of *S. scabies* occurred earlier than the others (Healy *et al.*, 1999).

1.4.5 Electrophoretic protein profiles

Proteins are extracted from the bacterial cells and run on SDS-polyacrylamide gels to provide whole-cell soluble protein electropherograms (Paradis *et al.*, 1994). Analysis of whole-cell proteins divided the *S. scabies* into two distinct groups in Eastern Canada. The second group was further subdivided into two subgroups. No distinctive patterns were associated with the protein and fatty acid profiles of pathogenic isolates (Paradis *et al.*, 1994 and Goyer *et al.*, 1996b). In contrast to fatty acid analysis, there is a correlation between protein and genetic clusters from DNA-DNA hybridisation tests (Goyer *et al.*, 1996b).

1.4.6 Complementation tests

For complementation tests *Streptomyces* spp. spores are subjected to NTG mutagenesis (N-methyl-N'-nitro-N-nitrosoguanidine). Auxotrophic mutants are selected and co-plated to evaluate prototrophic growth. The rates of prototroph existence in intraspecies (*S. scabies*) and interspecies pairings were 1.11% and 1.43% respectively (Lorang *et al.*, 1995). Prototrophic growth is thought to occur because of the hyphal fusion and complementation between auxotrophic nucleoids in the mycelium. This test could be beneficial to the classification of the *Streptomyces* spp. because it is able to detect genetic relatedness among them. This test demonstrated strong intra-strain complementation and weak inter-strain complementation, indicating that different groups of *Streptomyces* spp. are genetically isolated, including the *S. scabies* group (Goyer *et al.*,1996b). The use of this technique to analyse the classification of the *S. scabies* isolates has not been reported.

1.4.7 Inhibitory interaction

These reactions include the observation of the survival between two isolates growing together. These tests were performed by co-plating two isolates and analysing which isolate survived over the other. The isolates tested according to Clark *et al.* (1998) could be classified into three distinct groups. This suggests that there is significant biological variation within the species *Streptomyces.* Lorang *et al.*, (1995) used criteria from published literature (Lambert and Loria, 1989; Shirling and Gottlieb, 1996 and Pridham and Tresner, 1974), to determine the numerical taxonomic status of a few *Streptomyces* species. The grouping of the bacteria with inhibitory reactions supported this taxonomy. Clark *et al.* (1998) used many *Streptomyces* spp. of which thirteen were *S. scabies*. The *S. scabies* themselves were grouped into different groups.

Antibiotic assays that were carried out by Lorang *et al.* (1995) also supported the suggested numerical taxonomy of the bacteria. *Streptomyces* spp. were grown on agar and then killed with chloroform leaving their antibiotics in the agar. Other *Streptomyces* spp. were plated out onto these plates and tested for growth in the respective antibiotics. Two *S. diastatochromogenes* (PonSS1 and PonsII) and one *S. albogriseolus* (PonSSR) produced antibiotic-like compounds against highly pathogenic isolates of *S. scabies*. These assays grouped the 17 *Streptomyces* spp. (of which thirteen were *S. scabies* from Minnesota) into seven groups. Once again, the *S. scabies* themselves were grouped into different groups, suggesting the variability within the *S. scabies* group.

1.4.8 RAPD analysis (Random amplified polymorphic DNA)

RAPD analysis is based on PCR using a single short (9 to 10-mer) oligonucleotide of arbitrarily chosen sequence to prime DNA synthesis. It synthesises DNA from pairs of sites to which it is matched, or partially matched, resulting in strain specific arrays of DNA products. Amplification only occurs if the two oppositely orientated primers are a short distance away from each other. Low stringency conditions are used (annealing temperature between 35°C-37°C). It is a very sensitive technique because it samples the entire genome at random and many fragments are available for amplification. Slight variation in any of the reaction conditions can significantly alter the banding patterns making reproducibility very difficult. RAPDs allow measurement of genetic diversity and genetic relationships among individuals and populations by analysing the different banding patterns produced on an agarose gel. The attraction to this technique is that no prior sequence knowledge of the genome is required.

RAPD analysis has been performed on the various *Streptomyces* spp. using the d8635 primer (Kutchma *et al.*, 1998). Profiles were produced but no data concerning the *S. scabies* group was published. Mehling *et al.* (1995) attempted RAPD analysis on Actinomycete genomes using arbitrary 12 mer primers, but non-reproducibility was experienced.

1.4.9 Rep-PCR (Repetitive element PCR)

Certain bacteria have multiple copies of intergenic repeated sequences which have highly conserved inverted repeats and are dispersed throughout their genomes. Three unrelated families of repetitive DNA sequences exist:

- BOXA sub-unit of the BOX element of *Streptococcus pneumoniae* (BOX, 154 bp),
- Enterobacterial repetitive intergenic consensus (ERIC, 124 bp) and
- Repetitive extragenic palindromic (REP, 35-40 bp) sequences (Rademaker and de Bruijn, 1997).

Rep-PCR's are detected in a large variety of bacterial genera and can be present in complex clusters, in either orientation, have different locations in different species and are located in non-coding, transcribed regions (Hulton *et al.*, 1991). Their functions are not completely understood, but it has been suggested that they are involved in stabilising mRNA, homologous recombination, chromosome organisation and DNA polymerase binding. Their presence and widespread distribution in both prokaryotic and eukaryotic genomes of microorganisms strongly suggests that they are important to the structure and evolution of genomes (Lupski *et al.*, 1992).

Rep-PCRs are also useful in fingerprinting bacterial genomes and therefore in the taxonomy of bacteria (de Bruijn, 1992 and Lupski *et al.*, 1992). This technique produces fingerprints coupled with a PCR technique. The primers attach outwardly to complementary sequences which are repeated throughout the genomes of certain bacteria. The repetitive element may be present in both orientations. It is conserved in closely related species and distinct in diverse species and genera. The PCR product is comprised of the fragments existing between these elements (Clark *et al.*, 1998). The ERIC and REP-PCR techniques perform amplification on the DNA with primer pairs, whilst the BOX-PCR only makes use of one primer.

The fingerprints produced by Rep-PCR on the *Streptomyces* genome are reproducible over successive generations, despite the fact that their genomes are relatively unstable. Sadowsky *et al.* (1996) divided a group of *Streptomyce* spp. into two groups using BOX-PCR. *S. scabies* isolates from Minnesota were intermingled between the two groups. The average distance between clusters formed using this technique was relatively small. This indicates how closely related the *Streptomyces* spp. are. There is no correlation between the Rep-PCR and numerical taxonomic classification and fatty acid analysis profiles. Spooner *et al.* (1995) used BOX-PCR to analyse *S. scabies* isolates from the US. The fragments from 517bp- 1018bp were selected and all the *S. scabies* fingerprints matched. However this fingerprint differed from *S. scabies* isolates from Washington, North Dakota, Idaho, Montana, Conneticut, West Virginia, Iowa and Maine.

1.4.10 Restriction analysis

Bacterial DNA contains recognition sites for numerous restriction endonucleases, enabling successful digestion. The digestion of DNA provides a unique pattern of fragments when analysed on an agarose gel, providing a potential technique for classifying *S. scabies* bacteria. DNA restriction also provides fragments which can be used in the AFLP procedure.

Hintermann *et al.* (1981) used a set of restriction endonucleases to digest the DNA from *S. glaucescens*. It was concluded that *Bam*H1 furnishes a useful DNA profile for a given strain. It produces patterns that are independent of growth medium, growth temperature and time of harvest. No unique fragment pattern was identified within this *S. glaucescens* group. According to Beyozova and Lechevalier (1993), *Ase*1 digestions of *Streptomyces* spp. are universally suited (both in number and distribution of fragments) for low-frequency restriction fragment analysis (LFRFA). Digestions were performed on 59 *Streptomyces* spp. and 42 isolates formed eight clusters while 17 remained unclustered, but no *S. scabies* isolates were included in this study. The LFRFA patterns were strain specific and placed various *Streptomyces* spp. into different groups which were in good agreement with morphological and physiological groupings. High DNA-DNA hybridisation relatedness between isolates was reflected with high LFRFA similarity. These tests were performed on various *Streptomyces* spp. and did not concentrate solely on the *S. scabies* group. Kutchma *et al.* (1998) reported successful digestion of *Streptomyces* spp. DNA with the *Sma*1 enzyme. Again no *S. scabies* isolates were included in this study. The application of this technique was to test the purity of the isolated chromosomal DNA and not to classify *Streptomyces* spp. Clarke *et al.* (1993) reported successful digestion of a group of *Streptomyces* spp. with six base pair cutters, *Bgl*1, *Eco*R1, *Pst*1 and *Pvu*11. This was in preparation for the RFLP technique and also did not include *S. scabies.*

1.4.11 RFLP analysis (Restriction fragment length polymorphism)

The RFLP procedure entails the digestion of genomic DNA and the electrophoresis thereof on an agarose gel. The fragments are then transferred to a membrane by the Southern blot procedure. A probe is labelled and hybridised to the membrane and a RFLP pattern can be visualized after autoradiography.

Researchers have attempted to use this procedure to classify bacteria. A 7.2kb DNA fragment containing the 16S, 23S and 5S portions of the rRNA operon from *S.coelicolor* IMET 40271, cloned into pUC18 (p64) vector, was used as the probe in the Southern blot reactions. The results

revealed a high degree of diversity among *Streptomyces* spp. and no correlation was observed between RFLP data and numerical classification (Doering-Saad *et al.*, 1992). Once again, a large group of *Streptomyces sp* was used in these studies and not just one species type. Clarke *et al.*, (1993) used RFLP analysis and the rRNA genes to classify 14 *Streptomyces* spp. but not *S. scabies*. Phenetic numerical classification and DNA-DNA hybridisation grouped 13 of these isolates into the same group and the RFLP technique showed considerable variation among the isolates in this group. This makes this method useful for strain identification and verification. (Mehling *et al.*, 1995 and Clarke *et al.*, 1993) but not of much use for taxonomy.

1.4.12 Amplified Fragment length Polymorphisms (AFLP)

AFLP marker technology generates a high level of polymorphism in multiple loci. It is based on the principle of selectively amplifying a small set of restriction fragments from a complex mixture of DNA fragments. These DNA fragments are obtained by digesting DNA with specific restriction endonucleases (Vos *et al.*, 1995; and Maheswaran *et al.*, 1997) and the procedure is described by Vos *et al.* (1995). The banding pattern results can be manipulated by varying the number or the type of selective nucleotides used in the selective amplification procedure. This PCR-based DNA fingerprinting technique generates a large number of polymorphic loci. With a minimum of eight primers, a hierarchical analysis of molecular variance (AMOVA) can quantify and partition levels of variability into between and within form components (Vos *et al.*, 1995). AFLPs can thus provide insights into genetic relatedness. The technique is more time consuming and expensive than RAPDs, but the total number of loci that can be scored is greater and it is a more robust and productive replacement for RAPD technology. The use of restriction site-specific adapters and adapter-specific primers with a variable number of selective nucleotides under stringent amplification conditions ensures the reproducibility of the technique (Maheswaran *et al.*, 1997). To date, no results of AFLP analysis in *Streptomyces* have been reported. Generation of AFLP patterns for different *S. scabies* isolates may be of use in the analysis of their relatedness.

It has been reported that the AFLP patterns using DNA extracted from different organs from the same organism may differ. This has to do with highly methylated repetitive DNA not being able to be cleaved by certain methylation sensitive enzymes (Donini *et al.*, 1997) but this is not likely to be a problem in bacteria.

1.4.13 Random amplified microsattelites (RAMS)

The RAMS technique works on the principle that a degenerate primer attaches to complemenatry microsattelites $(CA)_n$, or any other repeats ($n \geq 3$), at the 5' termini. The non-repetitive nucleotides at the 5' end of the primer anchor the primer to the 5' end of a microsattelite repeat. The primers extend into the flanking regions by 2-4 nucleotide residues. Therefore the amplified DNA is located between the distal ends of two closely placed microsatellites. The multiple bands produced are visualised on an agarose gel, stained with ethidium bromide and viewed under ultra-violet light. This technique has been proven useful as a method to analyse genetic diversity and relatedness between organisms (Zietkiewicz *et al.*, 1994). DNA used in their study was extracted from mammalian blood or tissue samples, rodents, plants (maize, tomatoes) and bacteria (*E. coli*).

However, profiles generated by Zietkiewicz *et al.* (1994) for bacterial DNA were found to be poor Either none or too few fragments were observed. As expected, the higher the complexity of the DNA the more bands will be produced. This requires PCR conditions to be more stringent in order to limit the number of bands.

1.4.14 Ribosomal DNA identification

DNA sequencing is rapidly becoming one of the most important technologies in molecular biology. In the past, sequencing was time consuming and expensive. However, advances in PCR and automated sequencing have allowed these problems to be overcome and the use of DNA sequencing in phylogenetics enables researchers to identify an organism at all taxonomic levels. For phylogenetic analysis of certain organisms, including bacteria, sequencing of slowly evolving protein coding genes is being performed (Hain *et al.*, 1997).

1.4.14.1 The rDNA genes

The genes coding for components of the ribosomal RNA (rRNA) are arranged in the order 16S-23S-5S, known as the RNA operon. These rRNA genes are expressed to produce ribosomal proteins. The RNA operon is found in multiple copies arranged in big clusters in the genome. The copy number of the rRNA gene clusters is thus the same in closely related isolates, but varies among species in the genus *Streptomyces.* The overall GC content of the rRNA gene is much lower compared to the overall GC content of the genome. Specifically, the 16S region has a GC content of 56-59 mol% in comparison to the 72-75 mol% of the rest of the genome, suggesting the transfer of this region into the *S. scabies* genome from another organism (Mehling A *et al.*, 1995). The primary and secondary structures of the 16S, 23S and 5S rRNA revealed conserved and variable regions,

making it a useful area for the taxonomic classification of *Streptomyces* species. The mature rRNAs are predicted to contain 1528, 3120 and 120nt, for the 16S, 23S and 5S rRNAs respectively (Pernodet *et al.*,1989).

1.4.14.1.1 16S rDNA region

The 16S rDNA region is the most variable. It is similar in size and structure to other bacterial 16S rDNAs despite a few regions which contain highly variable sequences (Kim *et al.*, 1993). Three variable regions have been defined in the 16S rDNA region of *Streptomyces* spp., namely α (position 1000), β (position 1133 to 1143) and γ (position 181 to 213) (See Figure 1.3). Sequence comparison of these regions can be used for determining phylogenetic and evolutionary relationships.

Sequencing of the genomes of various *Streptomyces* spp. have already been performed (Takeuchi *et al.*,1996; Witt and Stackebrandt, 1990). Kreuze *et al.* (1999) sequenced the entire 16S rDNA region of various *Streptomyces sp.* from Finland by amplifying two partially overlapping fragments. The levels of similarity among *Streptomyces sp.* examined in this study were more than 95.4%. Three groups were formed based on this data and all the *S. scabies* isolates were found to cluster in one group. Analysis of the *S. scabies* genome alone indicated a limited, intraspecific variability. Bramwell *et al.* (1998) also managed to define three clusters among *Streptomyces sp.* from Europe. Mehling *et al.* (1995) sequenced the complete 16S rDNA regions of some of *Streptomyces sp.* by cloning the amplified 1.5kb fragment into pUC18. The sequences of the 16S rDNA of *Streptomyces* spp. in this study displayed over 95% similarity indicating the high degree of relatedness among them. Hain *et al.* (1997) studied the 16S rDNAs of 30 *S. albidoflavus* isolates. From this group, 27 isolates showed 100% sequence similarity. The 16S rDNAs of other types of *Streptomyces* spp. differed from the *S. albidoflavus* by 1.0% to 1.1%.

Kataoka *et al.* (1997) performed partial sequence analysis of the α region which produced similar tree topologies to the complete 16S rDNA sequence data. Partial sequencing of only the variable region of 16S rDNA is sufficient for deducing phylogenetic relationships of *Streptomyces* spp. at the intra-species level.

Differences accumulated in more conserved regions should not be underestimated (Stackebrandt *et al.*, 1992) and therefore a further two potential variable regions were identified and designated as δ (nt 71 to 102) and ∈ (nt 1.446 to 1.464). Of the five identified variable regions the γ region was found to be the most variable by Kreuze *et al.* (1999) (see Figure 1.3). Takeuchi *et al.* (1996) found

that these 16S rDNA sequences in three isolates of *S. scabies* from geographically different regions (Japan, Hungary and United States) are identical or differ at only one nucleotide position, position 1441 (*E. coli* numbering). These isolates also differed in melanin production.

The differences between the 16S rDNAs are mainly found in the variable regions known to be of restricted significance to phylogenetic relationships (Stackebrandt *et al.*, 1991). This makes species delineation based on the 16S rDNA data not completely successful and require additional taxonomic analysis tools. Analysis of the 16S rDNA sequences is applicable when studying the inter-species genetic diversity. For a finer classification method of analysing isolates (intra-species classification), the ITS region could be more useful (Stackebrandt *et al.*, 1991; Rademaker and de Bruijn, 1997).

Figure 1.3 An example of the sequence composition of the 16S rDNA region from *S. scabies* ATCC 40173^T (Takeuchi *et al.*, 1996)

```
   1 cattcacgga gagtttgatc ctggctcagg acgaacgctg gcggcgtgct taacacatgc
  61 aagtcgaacg atgaaccact tcggtgggga ttagtggcga acgggtgagt aacacgtggg
 121 caatctgccc ttcactctgg gacaagccct ggaaacgggg tctaataccg gatacgacac
 181 tctcgggcat ccgatgagtg tggaaagctc cggcggtgaa ggatgagccc gcggcctatc
 241 agcttgttgg tgaggtaacg gctcaccaag gcgacgacgg gtagccggcc tgagagggcg
 301 accggccaca ctgggactga gacacggccc agactcctac gggaggcagc agtggggaat
 361 attgcacaat gggcgaaagc ctgatgcagc gacgccgcgt gagggatgac ggccttcggg
 421 ttgtaaacct ctttcagcag ggaagaagcg aaagtgacgg tacctgcaga agaagcgccg
 481 gctaactacg tgccagcagc cgcggtaata cgtagggcgc gagcgttgtc cggaattatt
 541 gggcgtaaag agctcgtagg cggtctgtcg cgtcggatgt gaaagcccgg ggcttaaccc
 601 cgggtctgca ttcgatacgg gcagactaga gtgtggtagg ggagatcgga attcctggtg
 661 tagcggtgaa atgcgcagat atcaggagga acaccggtgg cgaaggcgga tctctgggcc
 721 attactgacg ctgaggagcg aaagcgtggg gagcgaacag gattagatac cctggtagtc
 781 cacgccgtaa acggtgggaa ctaggtgttg gcgacattcc acgtcgtcgg tgccgcagct
 841 aacgcattaa gttccccgcc tggggagtac ggccgcaagg ctaaaactca aaggaattga
 901 cgggggcccg cacaagcagc ggagcatgtg gcttaattcg acgcaacgcg aagaacctta
 961 ccaaggcttg acatacaccg gaaacggcca gagatggtcg ccccttgtg gtcggtgtac
1021 aggtggtgca tggctgtcgt cagctcgtgt cgtgagatgt tgggttaagt cccgcaacga
1081 gcgcaaccct tgttctgtgt tgccagcatg cccttcgggg tgatggggac tcacaggaga
1141 ctgccggggt caactcggag gaaggtgggg acgacgtcaa gtcatcatgc cccttatgtc
1201 ttgggctgca cacgtgctac aatggcaggt acaatgagct gcgaagccgt gaggcggagc
1261 gaatctcaaa aagcctgtct cagttcggat tggggtctgc aactcgaccc catgaagtcg
1321 gagttgctag taatcgcaga tcagcattgc tgcggtgaat acgttcccgg gccttgtaca
1381 caccgcccgt cacgtcacga agtcggtaa cacccgaagc cggtggccca acccgtaagg
1441 gagggagctg tcgaaggtgg gactggcgat tgggacgaag tcgtaacaag gtagccgtac
1501 cggaaggtgc ggctggatca cctcctttct
```

■δ ■λ ■α ■β ■ε

1.4.14.1.2 ITS region (Internal Transcribed Spacer)

The rDNA subunits are separated by spacer regions, which differ in length and sequence composition. Like the rDNA subunits, these regions are conserved in bacteria but they display more variation. The ITS region varies in size and composition and classification at a lower taxonomic level is thus more applicable (Jensen *et al.*, 1993). Variability analysis of the ITS region can be performed in three ways: the PCR fragment can be digested and resolved electrophoretically, the region can be used as a probe in Southern blots or the region can be amplified and analysed on a gel for size differences.

Hain *et al.* (1997) analysed the ITS region (16S-23S) of *S. albidoflavus* which displayed identical 16S rDNA sequences. PCR ampification using the ITS region was performed on these bacteria. Sequencing of the products produced unreadable sequence data because of diversity within the ITS region of an individual isolate. Size variation was analysed by running the ITS-PCR products on high-resolution polyacrylamide gels (see Figure 1.4). This could be performed by dye labelling the ITS-PCR fragments and using a software program to determine the fragment size and number. Each band seen in the profiles of each isolate were cloned using a pCR-script cloning kit (Stratagene, La Jolla. California) and subsequently sequenced. Bands which appeared identical on the gel, produced different sequences. Therefore, the ITS regions within and between *S. albidoflavus* isolates are different in their length and or sequence composition (Figure 1.4). The analysis of the ITS region proved useful for the discrimination of *S. albidoflavus* isolates and could be applied to the *S. scabies* species (Hain *et al.*, 1997).

Figure 1.4 Strain diversity as detected by analysing the ITS region. Samples 1 and 2 appear identical on the gels but analysis of the sequences may reveal differences in their composition. Samples 1, 2, 4 and 5 have one band in common, but the sequence composition may be different. The fragments constituting one *S. albidoflavus* isolate have the same fundamental sequence, but certain variable regions making the profiles unique for each isolate.

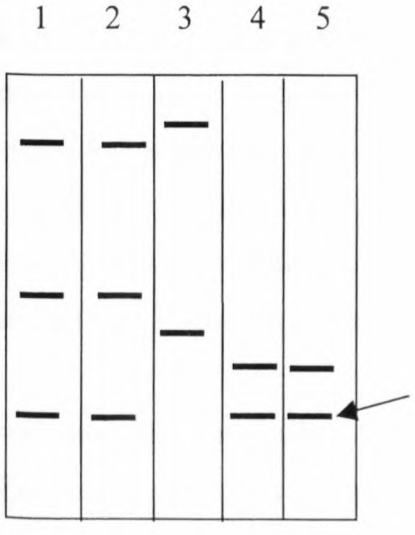


Table 1.1 Summary of the *S. scabies* classification from published data

Morphological Physiological data	Fatty acid profiles	DNA-DNA hybridisation	Protein profiles	Inhibitory reactions	Rep-PCR
Eastern Canada: one group	Canada: two groups	Eastern Canada: three groups	Eastern Canada: two groups	Minnesota: Seventeen *Streptomyces sp.* grouped into seven groups; *S. scabies* dispersed over all groups	Minnesota: *Streptomyces sp.* grouped into two groups; The *S. scabies* dispersed over both groups

1.5 COMPARISON OF THE DNA TECHNIQUES

A disadvantage surrounding the classification techniques already performed on *S. scabies* is inconsistent clustering over techniques evident in Table 1.1. The present study requires a classification technique that takes account of all characteristics of the organism. This can then be used to analyse the genetic diversity of *S. scabies* from different regions in S.A. The genome contains loci which are responsible for all characteristcs of the organism. Analysis of the different loci would be an ideal means of understanding and knowing all of the organism's characteritics. The above-mentioned techniques, such as morphological analysis or 16S rDNA sequences, only analysed a small portion of the genome at one given time.

Molecular techniques such as RAPDs, AFLPs, RAMS and Rep-PCR are more favourable because a wider variety of loci in the genome is sampled. All methods based on PCR are much easier and inexpensive, making them more popular compared to techniques such as sequencing. RAPDs, RAMS and Rep-PCR require no prior knowledge of the genome. However, RAPDs produce profiles that are often not reproducible, posing a major problem in taxonomic or genetic diversity studies. The RAPD-PCR technique can also display two different fragments as the same if they are equally sized. This can lead to ambiguous results making sequencing a more desirable technique as sequencing results are unambiguous. All these implications must be taken into consideration before an ideal data sampling method can be selected for the realistic identification of the *S. scabies* genetic diversity.

1.6 ANALYSIS OF GENETIC VARIABILITY

The analysis of the relatedness between organisms is becoming an increasingly important tool for the study of evolutionary origin, identification of taxonomy and the analysis of genetic diversity between different regions. Taxonomy is concerned with the grouping of organisms into manageable numbers of groups whose members have the same or very similar characters. Taxonomy is essential for the fundamental understanding and knowledge of an organism and, hence, facilitating extensive studies on them, such as the analysis of genetic diversity between isolates found in different regions. It provides knowledge of how an organism evolved. The starting point of taxonomy is to identify similar characters between different organisms and convert the information into specific data. Phylogenetic trees representing the evolutionary pathways can then be constructed. Criteria such as evolutionary processes, constraints and phylogenetic signals must be taken into account (Hillis *et al.*, 1993).

Dendograms depicting the genetic diversity between organisms are constructed using distance matrices. Matrices are commonly constructed from molecular data and converted into clusters using strategies such as UPGMA and Neighbor Joining (Weir, 1996) (see list of definitions pp VII) available in various phylogenetic software packages such as PAUP4 (Swafford, 1998). The choice of strategy must be preceded with the analysis of its applicability to the type of molecular data recorded.

Another approach to the analysis of genetic variability includes Wright's F statistics (Straughan and Lehman, 2000). F_{ST} measures the amount of genetic differentiation among a set of populations or sub-populations of a species and relies on estimates of allele frequencies. Genetic diversity analysis must thus employ DNA techniques which are capable of identifying all alleles at a locus (such as RFLPs or sequence analysis) (Grant and Leslie, 1992).

1.7 AIMS OF THE STUDY

Common scab is an important agricultural disease, which affects the quality of certain crops, such as beetroot, carrots and potatoes. Common scab of potatoes is a worldwide problem and control of this disease is being explored worldwide. The ultimate goal of the studies done on common scab would be to identify cultivars which are naturally resistant to the disease. Many laboratories and greenhouses have proposed screening methods to determine scab resistance in breeding material. However, field evaluation is necessary to determine the true value of resistance to scab. The reason for this is that there are so many variables in nature (scab isolates, soil moisture content, soil's pH)

that can influence the severity of the disease (Gouws and Mienie, 1997). This study focuses on the scab isolate and will ultimately aid researchers in understanding its influence on the scab disease of potatoes. Successful analysis of this isolate requires an understanding of the level of genetic diversity among the different pathogenic isolates. The bacterium species responsible for the presence of the majority of common scab is a species of the *Streptomyces* genus, called *Streptomyces scabies.*

Some research on *S. scabies* has been carried out using the above-mentioned classification techniques (phenotypic analysis, DNA-DNA hybridisation, fatty acids, protein profiles, inhibitory reaction analysis and Rep-PCR). Results suggest that within the species there is more diversity than one would ordinarily expect and suggests the requirement for a more thorough classification investigation of the entire genome at DNA level. Ideally, the relationship between the different functional and taxonomic groupings should eventually be revealed at this level (Beyazova and Lechevalier, 1993).

Tests done on *Streptomyces* spp. to date, such as morphological and fatty acid tests measure only activities coded for by a small portion of the whole genome. This study focuses on DNA molecular techniques (RAPDs, AFLPs, RAMS, Rep-PCR and rDNA analysis) to analyse the genetic diversity of the *S. scabies* genome from different geographical regions in S.A. Most of these techniques sample various genomic regions including the specific region *nec1*. It is speculated that this region is responsible for the production of Thaxtomin A which plays a role in pathogenicity, so amplification of this region could provide a simple assay for pathogenicity polymorphism in *S. scabies* isolates.

Once DNA techniques have refined and numerified statistical analysis can be applied. Pairwise genetic distances are determined using genetic distance formulae followed by cluster analysis using the Neighbor Joining method, the Parsimony Network (PAUP4) or UPGMA ("Statistica", edition 99).

Results of the genetic diversity analysis of *S. scabies* in S.A. will have important implications for the control of the potato scab disease by cultivating potatoes less sensitive, or resistant, to the pathogen or by other biological control measures. Knowledge of whether isolates are similar or genetically diverse in South Africa is important in understanding the response of potato cultivars to scab isolates in various regions. Furthermore, to determine an ideal taxonomy of the genus

The aim of this study was thus to analyse genetic diversity among *S. scabies* isolates from different regions in S.A.

The following targets were set:

- Optimisation of the growth of the bacterium *in vitro*
- DNA extraction
- Application and assesment of the following DNA techniques:
  - RAPDs
  - AFLPs
  - RAMS
  - 16S rDNA sequencing
  - ITS analysis
  - Rep-PCR
- Analysis of the *nec1* region
- Data analysis
  1. Pairwise genetic distances
  2. Cluster analysis

CHAPTER TWO

2 MATERIALS AND METHODS

2.1 MATERIALS

The composition of all buffers and media used in this study are listed in Appendix B. The reagents and some of the apparatus are given in Table 2.1.

Table 2.1 The set conditions and reagents used throughout this study

REAGENTS FOR PCR		
NAME	**COMPANY**	**COMPOSITION**
Biotaq^{TM}DNA polymerase	Bioline,U.K., Ltd.	
10x PCR reaction buffer (Biotaq)	Bioline,U.K., Ltd.	16mM $(NH4)_2SO_4$, 67mM Tris-HCL (pH8.8 at 25°C) 0.01% Tween-20
Stoffel fragment	Applied Biosystems	
Amplitaq Gold	Applied Biosystems	
10x PCR reaction buffer (Amplitaq Gold)	Applied Biosystems	10mM Tris-HCL (pH 8.3), 50mM KCL
dNTPs	Promega Corporation, Madison, WI	10mM of each base from a 100mM stock
REAGENTS FOR ELECTROPHORESIS		
NAME	**COMPANY**	**COMPOSITION**
Ficoll orange G loading buffer		0.1%(w/v) Ficoll, 10mM EDTA (pH7.0)
Xylene Cyanol (XC) loading buffer		0.24% (w/v) Xylene Cyanol FF, 0.24% (w/v) bromophenol blue and 30% (v/v) glycerol in water
Agarose gel 1%- 1.6% (w/v)	FMC Bioproducts, Rockland, ME	1% (w/v) contains 2.5g Agarose in 250ml 1xTBE, 1.5% (w/v) contains 3.75g and 1.6% (w/v) contains 4g Agarose in 250ml 1xTBE. Electrophoresis is performed in a 250ml Biorad gel apparatus
40% (w/v) non-denaturing polyarylamide stock		38.8g polyacrylamide, 1.2g bispolyacrylamide, in a total volume of 100ml. The electrophoresis is performed using a Hoefer® Mighty Small 11 apparatus (Hoefer Parmacia Biotech Inc. USA). 1.5xTBE is used as running buffer.

1kb ladder	Gibco BRL, Life Technologies	3.5µg per lane
100bp ladder	Promega Corporation, Madison, WI	0.65µg per lane
5xTBE		0.45M Tris, 0.45M Boric acid and 0.01M EDTA used as 1xTBE or1.5xTBE
PCR-CYCLERS		
NAME	**MODEL**	
PE PCR cycler	GeneAmp, PCR system 2400, Applied Biosystems	
Eppendorf PCR cycler	Mastercycler gradient, Eppendorf	
Techne PCR cycler	Thermal Cycler PHC-3, Techne	
GEL DOCUMENTATION		
APPARATUS	**COMPANY**	
Ultraviolet transullimunator	UVO Inc. San Gabriel, CA	
GelDoc. system	Multi- Analyst®/PC. Biorad	

2.1.1 Bacterial isolates

Streptomyces spp. were obtained from the Vegetable and Ornamental Plant Research Institute (VOPI, ARC, Roodeplaat). These were sampled from different regions in South Africa and other countries (Table 2.2 and Figure 2.1). Data on the morphology, physiology, melanin and toxin production and the pathogenicity is given in Appendix C. The data that was provided contains all the data that could be obtained for these isolates (R Gouws, VOPI, personal communication).

For control purposes, the following were included:

- A *Streptomyces* spp. from beet (BNT35)
- A *Streptomyces* spp. from peanut (DNKG7)
- A *S. scabies* type strain (ATCC 41973)
- Another *Streptomyces* spp., *S.acidiscabies*, (ATCC 49003)

Figure 2.1 Map of the world indicating the origin of the bacteria used in this study

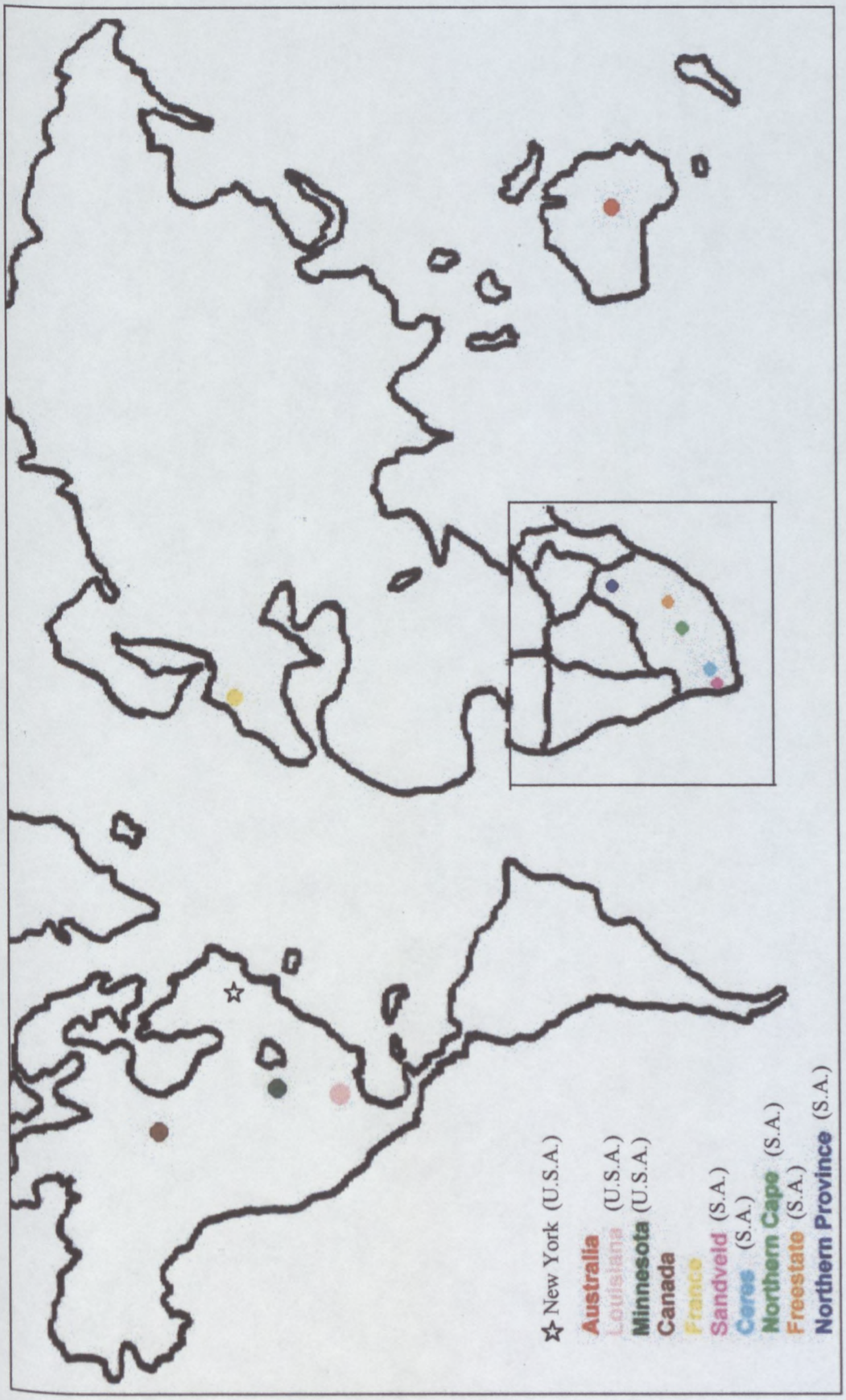
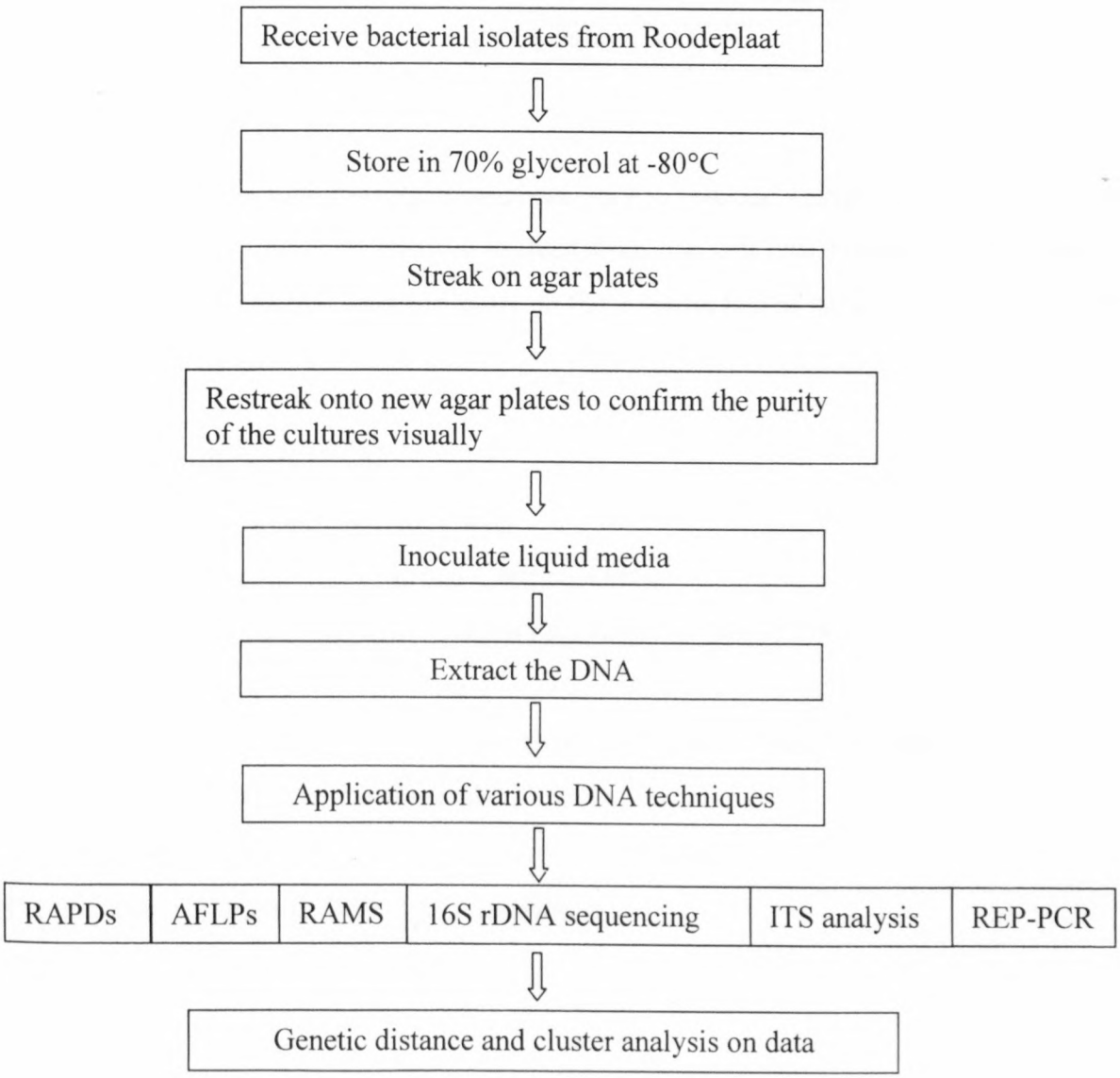


Table 2.2 The number of isolates used and their corresponding localities

REGION	NUMBER OF ISOLATES	NUMBER OF LOCALITIES WITHIN THE REGION		
Sandveld	5	5		
Northern Cape	4	4		
Eastern Freestate	6	6		
Ceres	4	4		
Northern Province	3	3		
Foreign isolates	11	Minnesota 3 France 3	Canada 1 Louisiana 1	Australia 3
TOTAL	33			

2.2 METHODS

Figure 2.2 Summary of procedures followed



2.2.1 Growth of the bacteria

On the arrival of bacterial stocks, various growth parameters (type of media, growth duration, temperatures, agitation speeds and liquid media volumes) were tested as listed in Table 2.3.

Table 2.3 The variables tested during bacterial growth

VARIABLES	CONDITIONS
Liquid media volume	5ml 20ml 30ml 100ml 200ml
Growth shaking speed	100rpm 225rpm 250rpm 300rpm
Growth temperature	26°C 30°C 37°C
Growth time	21hrs 24hrs 42hrs 48hrs 3 days 4 days 5 days
Solid media	Oatmeal Agar
	Inorganic Salt Starch Agar
	ISP2
Liquid media	Trypticase Soy Broth
	Yeast extract-Malt extract Broth
	CRM

Before inoculation into the liquid media, it was necessary to restreak a single bacterial colony onto a new plate to ensure that the cultures were derived from one cell (see Figure 2.3). The bacterial isolates took between one to two weeks to grow on these media before sufficient spores for further inoculations were produced.

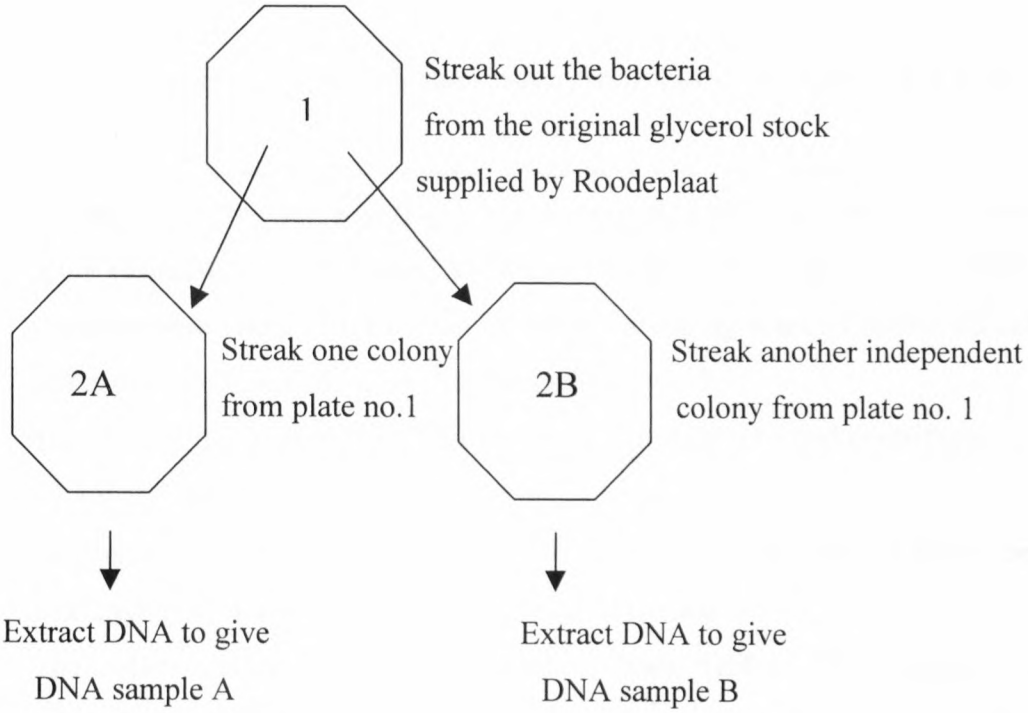
The different solid media tested were:

- Oatmeal Agar (Shirling and Gottlieb, 1966),
- Inorganic Salt Starch Agar (Shirling and Gottlieb, 1966),
- Yeast extract-Malt extract Agar (ISP2, Difco Laborotories- Detroit USA) (see Appendix B).

The liquid media tested for the propagation and preparation of cells for extractions were:

- Trypticase Soy Broth (Fluka, Switzerland),
- Yeast extract-Malt extract Broth (Shirling and Gottlieb, 1966),
- CRM (Pigac and Schrempf, 1995) (see Appendix B).

Figure 2.3 Growth stages followed before DNA extractions were performed. All the DNA techniques performed in this study were carried out on two individual DNA samples (A and B) per isolate.



Shaking the isolates with glass beads to break up the mycelium and inoculation of liquid media with spore suspensions were also tested. Spore suspensions were obtained by scraping the plates and centrifuging them in distilled water. The pellet was resuspended in 60µl of 20% (v/v) glycerol in water. Thirty microlitres were used to inoculate the media.

2.2.2 DNA extractions

Various DNA extraction protocols were tested in order to obtain the best quality DNA:

- A pronase (Boehringer Mannheim), SDS and phenol based protocol (Healy and Lambert , 1991)
- An acetone based protocol (Kutchma *et al.*, 1998)
- A fungal extraction protocol (Raeder and Broda, 1993)
- A yeast extraction protocol (href5)
- A rapid salt based protocol (Aljanabi and Martinez, 1997)
- A modified lysozyme, SDS and phenol protocol (Rao *et al.*, 1987; Pigac and Shrempf, 1995)

Finally total DNA was extracted from 0.5g- 2.0g wet weight mycelium (depending on the rate of growth of the different bacteria) using the following extraction protocol:

2.2.2.1 *Streptomyces* miniprep for total genomic DNA.

The following protocol is a modification taken from Rao *et al.* (1987) and Pigac and Schrempf (1995).

1. Twenty millilitres of CRM (Pigac and Schrempf, 1995) were inoculated with *Streptomyces* spore suspension and incubated at 30°C for 24-48 hrs with agitation at 250rpms.
2. Cultures were checked for contamination by dropping some of the liquid culture onto ISP2 plates the day before the extraction procedure.
3. The total culture was transferred to a 50ml centrifuge tube and centrifuged at 14000rpms for 10min at 4°C.
4. The supernatant was washed once in 3.75ml 10% (v/v) glycerol in water, centrifuged again and used immediately or stored at -20°C until needed.
5. The pellet was resuspended in 2.5ml of **lysis buffer** (15% sucrose, 25mM Tris.HCl (pH 8.0), 25 mM EDTA) plus **lysozyme (5mg/ml)**, and vortexed to disperse the cells. Lysozyme was freshly made.
6. The cells were incubated for 10-15min at 37°C.
7. Twenty-five microlitres of 10mg/ml **proteinase K** was added.
8. The solution was mixed gently while 125µl of **10% SDS** was added. This was IMMEDIATELY incubated at 70°C for 15min.
9. The solution was cooled on ice and 525µl of ice cold **3M sodium acetate** was added. This was mixed well by inverting and then incubated on ice for 15min.
10. The solution was then centrifuged for 10min at RT and the supernatant was transferred to a new 50ml glass Corex™ tube. Precipitation of the DNA was achieved by adding 0.6-0.8 vol of isopropanol.
11. The DNA was spooled out (or centrifuged if it was too fragile to pick up) and redissolved in 500 µl TE.
12. Purification was carried out with an equal vol. of **phenol: chloroform: isoamyl-alcohol** (25:24:1). This was centrifuged at 12,000rpm for 5min.
13. The aqueous layer was transferred to a fresh 2.2ml tube.
14. The protein removal with phenol was repeated.

15. To precipitate the DNA, 0.1 vol of 3 M **NaOAc** and 2.2 vol **EtOH** was added at RT and mixed.
16. The DNA was spooled out (or centrifuged) and redissolved in 500µl of TE.
17. Removal of RNA was achieved by incubation with 2.5µl of **RNase A** (Boerhinger Manheim) (10mg/ml) at 37°C for 30min.
18. One **phenol: chloroform** (1:1) and one (optional) **phenol: chloroform: isoamyl-alcohol** (25:24:1) extraction was carried .
19. Step 15 was repeated. DNA was spooled or centrifuged and washed in **70% EtOH** (RT). The pellet was then dissolved in 50µl TE.

Two DNA extractions were performed on each isolate (A and B in Figure 2.3). These extractions were carried out on different occasions. DNA concentrations were estimated using a Low DNA Mass Ladder (Gibco BRL, Life Technologies) as a marker. The DNA sample (2µl) and ladder (4µl) was loaded onto a 1% agarose gel, together with 10µl Ficoll Orange G (Sambrook *et al.,* 1989), and electrophoresed at 80V for 2hrs. Electrophoresis was carried out in 1xTBE running buffer and staining was achieved by adding 1µg/ml ethidium bromide to the gel (Sambrook *et al.,* 1989). Exact DNA concentrations were found to be of small significance in PCR reactions. Therefore, to accommodate the large flactuations in DNA concentrations, all DNA samples were diluted one in 50 and 5µl of this were used in subsequent PCR reactions.

2.2.3 DNA techniques

2.2.3.1 Amplification of the *nec1* and intergenic region

The *nec1* and intergenic region were selected in this study to test for pathogenicity. Table 2.4 displays the sequences of these primers (Bukhalid *et al.,* 1998) obtained from Integrated DNA Technologies-Inc. (Coralville, Iowa, USA).

Table 2.4 The sequence of the *nec1* (Nf, Nr) and intergenic region (If, Ir) primers

PRIMER	SEQUENCE
Nf	5'- ATGAGCGCGAACGGAAGCCCCGGA -3'
Nr	5' -GCAGGTCGTCACGAAGGATCG -3'
If	5' -GTTGTCTTCGGCGAGGGCGTGCAGG -3'
Ir	5'-AGCGGAAGGATTTGCGACCACAACG -3'

Reactions were carried out in 25µl reaction volumes containing 5µl of the one in 50 diluted DNA stock, 15 pmol of each primer, 1/10xNH_4 buffer, 1mM $MgCl_2$, 200µM of each dNTP, 2.5 units (U) of Biotaq DNA polymerase. The PCR reactions were carried out in a Perkin Elmer cycler with the following PCR profile: denaturing step of 95°C for 10min followed by 30 cycles of 95°C for 1min, 60°C for 1min and 72°C for 2min, and a final extension step of 72°C for 10min.

These PCR reactions were repeated a second time on each bacterial DNA sample to validate the results. Results from these PCR reactions were compared to the pathogenicity data provided by VOPI, ARC (see Appendix C).

To optimise results, Amplitaq Gold was tested and the annealing temperatures were varied from 52°C to 60°C.

2.2.3.2 RAPD analysis

Twenty 10-mer primers from Operon Kit M [Operon ™ Technologies (Alamede, California)], were used to amplify the DNA. Optimisation of the conditions were set by varying the concentrations of the $MgCl_2$ (1mM-4mM), primer (5-50pmol) and DNA, the annealing temperatures (35°C-44°C), the PCR cyclers (Perkin Elmer PCR cycler, Eppendorf PCR cycler, Techne PCR cycler) and different Taq DNA polymerase enzymes (Biotaq™DNA polymerase and Stoffel fragment). Different DNA-extraction protocols were tested (2.2.2) to achieve the best quality DNA able to produce repeatable RAPD profiles.

After optimisation, the final polymerase chain reaction (PCR) amplifications were carried out in 25µl volumes. They contained between 20-120ng DNA, 1.5mM $MgCl_2$, 160µM of each dNTP, 50pmol primer, 1.5units Biotaq™DNA polymerase and the appropriate amount of 1/10x reaction buffer and ddH_2O. Amplification was performed in a PCR thermal cycler programmed for one cycle of 4min at 95°C; followed by 40 cycles of 40s at 94°C, 45s at 44°C and 1.5min at 72°C; and a final extension for 5min at 72°C.

After the PCR amplifications were completed, 10µl of Ficoll-Orange G loading buffer was added to the PCR reaction and 20µl was loaded onto a 1.6% agarose gel prepared in 1xTBE buffer. The gel contained 1µg/ml ethidium bromide. Electrophoresis was performed for 2hrs at 60V. A negative

control containing no template DNA, and a 1kb-ladder used as the molecular marker, were also loaded on the gel. The amplified products were observed on an ultraviolet transullimunator.

PCR products were also analysed on a 16% polyacrylamide gel (using 1.5xTBE). XC loading buffer was used. After completion of electrophoresis, the gels were stained with ethidium bromide (1µg/ml of ethidium bromide in 1.5xTBE buffer) and were observed using the GelDoc. system. A 1kb ladder was used as the molecular length standard.

2.2.3.3 RAMS

The combination of degenerate primers used in the RAMS procedure were:
HBH-$(AC)^N$, DBD$(AC)^5$, D$(GATA)^4$, DBD$(AC)^7$
and B = C, G or T (not A); D= not C and H= not G
For comparison purposes, potato DNA was included in these PCR reactions because previous work proved successful on potatoes (C Lambert, US Genetics department, personal communication). Reactions were carried out in 25µl reaction volumes containing 1µl of the extracted DNA stock, 15pmol of primer, 1/10xNH_4 buffer, 2.5mM $MgCl_2$, 200µM of each dNTP and 1 unit of Biotaq DNA polymerase. The PCR reactions were carried out in a Perkin Elmer cycler with the following PCR profile: denaturing step of 95°C for 10min followed by 37 cycles of 95°C for 30s, 55°C for 45s and 72°C for 2min, and a final extension step of 72°C for 7min.

Ten microlitres of Ficoll Orange G was added to the PCR mixture after completion of the run, and 20µl was loaded onto a 1.6% agarose gel. This was electrophoresed for 4.5 hrs at 80V in 1xTBE. The gel was stained using ethidium bromide and the results were analysed using the GelDoc. system.

2.2.3.4 AFLP analysis

2.2.3.4.1 Restriction digestion of the DNA:

The restriction enzymes tested on the DNA included *Eco*R1, *Pst*1, *Taq*1, *Bam*H1, *Not*1, *Rsa*1, *Hae*111, *Msp*1 (all from Promega) and *Dra*1 (Boerhinger Mannheim). The amount of the DNA to be digested varied from 0.1µg to 1µg and the units of enzyme varied from 5U to 30U. The volume of the buffer was one tenth of the total volume (25µl) and digestions were carried out at 37°C (except *Taq*1 which digests optimally at 65°C) for 4hrs or 16hrs.

2.2.3.4.2 The AFLP procedure:

AFLP reactions were performed using the Life Technologies AFLP™ Analysis system 1 AFLP Starter Primer Kit (Gibco BRL). DNA was digested with *Mse*1 and *Eco*R1 and the procedure was continued as described by Vos *et al.* (1995). The steps followed using this kit involve the digestion of 275ng DNA with *Eco*R1/*Mse*1 enzyme, followed by ligation of double stranded *Mse*1 and *Eco*R1 adaptors. Pre-amplification and selective amplification was subsequently carried out. See Table 2.5 for the combination of primers used in the selective amplification step. The *Eco*R1 primers were fluorescently labelled.

Table 2.5 AFLP primer combinations and their respective 3' nucleotides

Fluorochrome label attached to the *Eco*R1 primers with selective 3' nucleotides	***Eco*R1**	***Mse*1**
Tamra (yellow)	E-ACC	M-CAA
Joe (Green)	E-ACG	M-CAA
Fam (Blue)	E-ACT	M-CAA

The selective PCR products were denatured together with the ABI GENESCAN 500 ROX internal lane size standard for 2 min at 90°C. These were loaded on a 36cm denaturing 5% Long Ranger gel (FMC BioProducts, Rockland, ME) and electrophoresed for 3.5hrs at a limiting parameter of 3000V on an ABI PRISM™ 377 automated genetic analyser. The product size was determined with the ABI GENESCAN 2.1 using the Local Southern method.

AFLPs were also performed, without the use of the kit. DNA (100-200ng) was digested using *Msp*1 (10U) and *Pst*1 (10U) (both supplied by Gibco BRL, Life Technologies). The *Pst* adapter was biotinylated (Gibco BRL). Adaptor ligation with 50pmol *Msp*1 adapter and 5pmol *Pst* adapter was performed using 1.2mM ATP and 0.2µl T4 ligase (stock 7500 weiss U/µl). Dyna beads (Gibco BRL, Life Technologies) were used to select for the biotinylated DNA fragments. *Msp*1 primers (300ng) were labelled with 1µl gamma-^{33}P (dATP) (stock 10mCi) and 0.5µl T4 kinase (stock 7900U/µl) in a total volume of 10µl. Incubation was performed at 37°C for 1 hr and then at 68°C for 10-20 mins. PCR amplification was carried out in 20µl reaction volumes containing 1µl of the ligated reaction mix, 1µl labelled primer, 30ng *Pst* primer, 0.5U Biotaq and 400µM dNTPs. Amplification was performed with two sets of cycles (x 12 and x 23) with Tm's of 65°C and 96°C respectively. Two sets of selective (three base pair extension) primers *Pst*6/*Msp*4 and *Pst*6/*Msp*5 were employed. See Table 2.6 for the corresponding primer sequences.

Table 2.6 Primer combinations including the selective nucleotides (indicated in bold).

PRIMER COMBINATIONS		
1: *Pst*6/ *Msp*4	*Pst* 5' -GAC TGC GTA CAT GCA G -**AG**- 3'	*Msp* 5' -GAT GAG TCC TGA TCG G -**CTC**- 3'
2: *Pst*6/ *Msp*5	*Pst* 5' -GAC TGC GTA CAT GCA G -**AG**- 3'	*Msp* 5' –GAT GAG TCC TGA TCG G -**AGC**- 3'

The amplified samples were loaded onto 6% denaturing polyacrylamide gels and electrophoresed for 90min. Gels were then transferred onto chromatography (Whatman) paper, dried, and exposed to X-ray film (Kodak film, BioMax. Sigma) for one to two days.

2.2.3.5 16S rDNA sequencing

According to Kreuse *et al.* (1999), the 16S rDNA region in various potato pathogenic *Streptomyces* spp. should be about 1.5kb in size. Two isolates from each region in South Africa and one from each region overseas, were used to analyse the 16S rDNA sequencing results.

PCR amplification of the entire region was performed on genomic DNA with primers 1F and 1R (see Table 2.7 and Figure 2.4). The 25µl reaction mixture contained 5pmol of each primer (1F and 1R), 1.5mM $MgCl_2$, 1/10xNH_4 buffer, 5U Biotaq polymerase, 200µM of each dNTP, and 5µl DNA (DNA was diluted one in 50 directly after it was extracted). Amplification was performed with the Perkin Elmer thermal cycler using the following program: denaturation at 95°C for 2.5min, followed by 35 cycles of 95°C for 1min, 55°C for 1min, and 72°C for 2min. A final extension step at 72°C for 10min was included at the end. These PCR products were cleaned with the QIAquick PCR purification kit (Qiagen).

Sequencing reactions were performed with the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (part number 4303152) (Applied Biosystems). Four primers were used for sequencing reactions. These were the 1F and 1R (used in the PCR reaction) and 2F and 2R (corresponding to two regions within the 16S rDNA region, ± 500 bp away from the 1F and 1R primers). See Table 2.7 for the sequence of these four primers and Figure 2.4 for their position in the *Streptomyces* genome.

Table 2.7 Sequences of primers used in the 16S rDNA sequencing reaction

PRIMER	SEQUENCE
1F	5' -CAT TCA CGG AGA GTT TGA TCC- 3'
1R	5' -AGA AAG GAG GTC ATC CAG CC- 3'
2F	5' -ACC AAG GCT TGA CAT ACA CC- 3'
2R	5' -ATT CCG GAC AAC GCT CG- 3'

Figure 2.4 The 16S rDNA region of a *Streptomyces scabies* isolate from Japan (Takeuchi *et al.*, 1996) indicating the position of the four 16S primers.

```
   1 cattcacgga gagtttgatc ctggctcagg acgaacgctg gcggcgtgct taacacatgc
  61 aagtcgaacg atgaaccact tcggtgggga ttagtggcga acgggtgagt aacacgtggg
 121 caatctgccc ttcactctgg gacaagccct ggaaacgggg tctaataccg gatacgacac
 181 tctcgggcat ccgatgagtg tggaaagctc cggcggtgaa ggatgagccc gcggcctatc
 241 agcttgttgg tgaggtaacg gctcaccaag gcgacgacgg gtagccggcc tgagagggcg
 301 accggccaca ctgggactga gacacggccc agactcctac gggaggcagc agtggggaat
 361 attgcacaat gggcgaaagc ctgatgcagc gacgccgcgt gagggatgac ggccttcggg
 421 ttgtaaacct ctttcagcag ggaagaagcg aaagtgacgg tacctgcaga agaagcgccg
 481 gctaactacg tgccagcagc cgcggtaata cgtagggcgc gagcgttgtc cggaattatt
 541 gggcgtaaag agctcgtagg cggtctgtcg cgtcggatgt gaaagcccgg ggcttaaccc
 601 cgggtctgca ttcgatacgg gcagactaga gtgtggtagg ggagatcgga attcctggtg
 661 tagcggtgaa atgcgcagat atcaggagga acaccggtgg cgaaggcgga tctctgggcc
 721 attactgacg ctgaggagcg aaagcgtggg gagcgaacag gattagatac cctggtagtc
 781 cacgccgtaa acggtgggaa ctaggtgttg gcgacattcc acgtcgtcgg tgccgcagct
 841 aacgcattaa gttccccgcc tggggagtac ggccgcaagg ctaaaactca aaggaattga
 901 cgggggcccg cacaagcagc ggagcatgtg gcttaattcg acgcaacgcg aagaacctta
 961 ccaaggcttg acatacaccg gaaacggcca gagatggtcg ccccttgtg gtcggtgtac
1021 aggtggtgca tggctgtcgt cagctcgtgt cgtgagatgt tgggttaagt cccgcaacga
1081 gcgcaaccct tgttctgtgt tgccagcatg cccttcgggg tgatggggac tcacaggaga
1141 ctgccggggt caactcggag gaaggtgggg acgacgtcaa gtcatcatgc cccttatgtc
1201 ttgggctgca cacgtgctac aatggcaggt acaatgagct gcgaagccgt gaggcggagc
1261 gaatctcaaa aagcctgtct cagttcggat tggggtctgc aactcgaccc catgaagtcg
1321 gagttgctag taatcgcaga tcagcattgc tgcggtgaat acgttcccgg gccttgtaca
1381 caccgcccgt cacgtcacga agtcggtaa cacccgaagc cggtggccca acccgtaagg
1441 gagggagctg tcgaaggtgg gactggcgat tgggacgaag tcgtaacaag gtagccgtac
1501 cggaaggtgc ggctggatca cctcctttct
```

■F1 ■R1 ■R2 ■F2

The sequencing reactions were carried out with 3µl of DNA (60ng/µl stock), and 3µl of primer (1.1pmol stock). For improvement of some of the sequences, the concentrations of the DNA and primer used in the sequencing reactions were varied slightly.

The DNA sequences were determined with an ABI PRISM™ 377 automated genetic analyser at the University of Stellenbosch core sequencing facility. DNA alignments were done using Sequence Navigator™ version 1.0.1, supplied by Applied Biosystems.

2.2.3.5.1 Phylogenetic reconstruction from the 16S rDNA sequences

Page vii lists some of the terms and their corresponding definitions which are associated with the following section.

Neighbor Joining trees were constructed for the 16S rDNA region (Weir, 1996) of Region A and B, separately, using the PAUP4 package (Swafford 1998). Trees for the two combined regions were also constructed using the Neighbor Joining method. *E. coli* (Brosius *et al.,* 1978) was used as the out-group. The optimisation of cluster trees tends to give incorrect phylogenies when the number of nucleotides examined is small (Nei *et al.,* 1998). To counter this problem, the validity of the trees were accomplished using bootstrap analysis. A bootstrap value must be greater than 700/1000 before any confidence can be assigned to a particular internal node of a tree (Brown, 1999). The *S. acidiscabies, S. scabies* type strain and the *S. scabies* sequence from Japan (Takeuchi *et al.,* 1996) were used for control and comparitive purposes in tree construction.

For a more detailed analysis of the differences between the sequences of the isolates, a Parsimony Network was constructed using the PAUP4 package (Swafford 1998). The construction of a network was possible using different sized circles to depict the number of identical haplotypes and using cross-hatching between circles to designate the number of base changes between different sequence haplotypes (Matthee and Robinson, 1997). *E. coli* was used as the out-group.

2.2.3.6 ITS fragment amplification

The variable Region between 16S and 23S rDNA sub-units were amplified. The sequences of the two primers (ITS F and ITS R) were obtained from Jensen *et al.* (1993) (Table 2.8).

Table 2.8 Sequence of the primers used for ITS sequencing

PRIMER	SEQUENCE
ITS F	5' -GTC GTA ACA AGG TAI CCG- 3'
ITS R	5' -GAA GTC GTA ACA AGG- 3'

PCR reactions were carried out in 25µl containing 7.5pmol of each ITS primer, 1.5mM $MgCl_2$, 1/10xNH_4 buffer, 2.5U Biotaq polymerase, 200µM of each dNTP and 5µl of a one in 50 DNA dilution stock.

An initial denaturing step at 95°C for 2.5min was followed by 35 amplification cycles, each consisting of 1min at 95°C, 1min at 47°C and 2min at 72°C. A final step at 72°C for 10min completed the PCR program.

The amplification products were separated on a 1.5% agarose gel using 0.5xTBE and on 8% Hoefer® Mighty Small II polyacrylamide gels at 200V for 1 hour at 15°C (using 1.5xTBE).

To separate prominent, consistent double bands (± 300bp), each band was stabbed from the gel using a micropipet tip. This was then added to a PCR reaction as the template. These products were also run on 8% polyacrylamide gels. Further stabbing and PCR amplification of these two separated fragments were repeated another two times in order to eliminate the background (these will be referred to as stab 1/2/3 ITS-PCR). The annealing temperature for these PCR reactions was increased to 58°C.

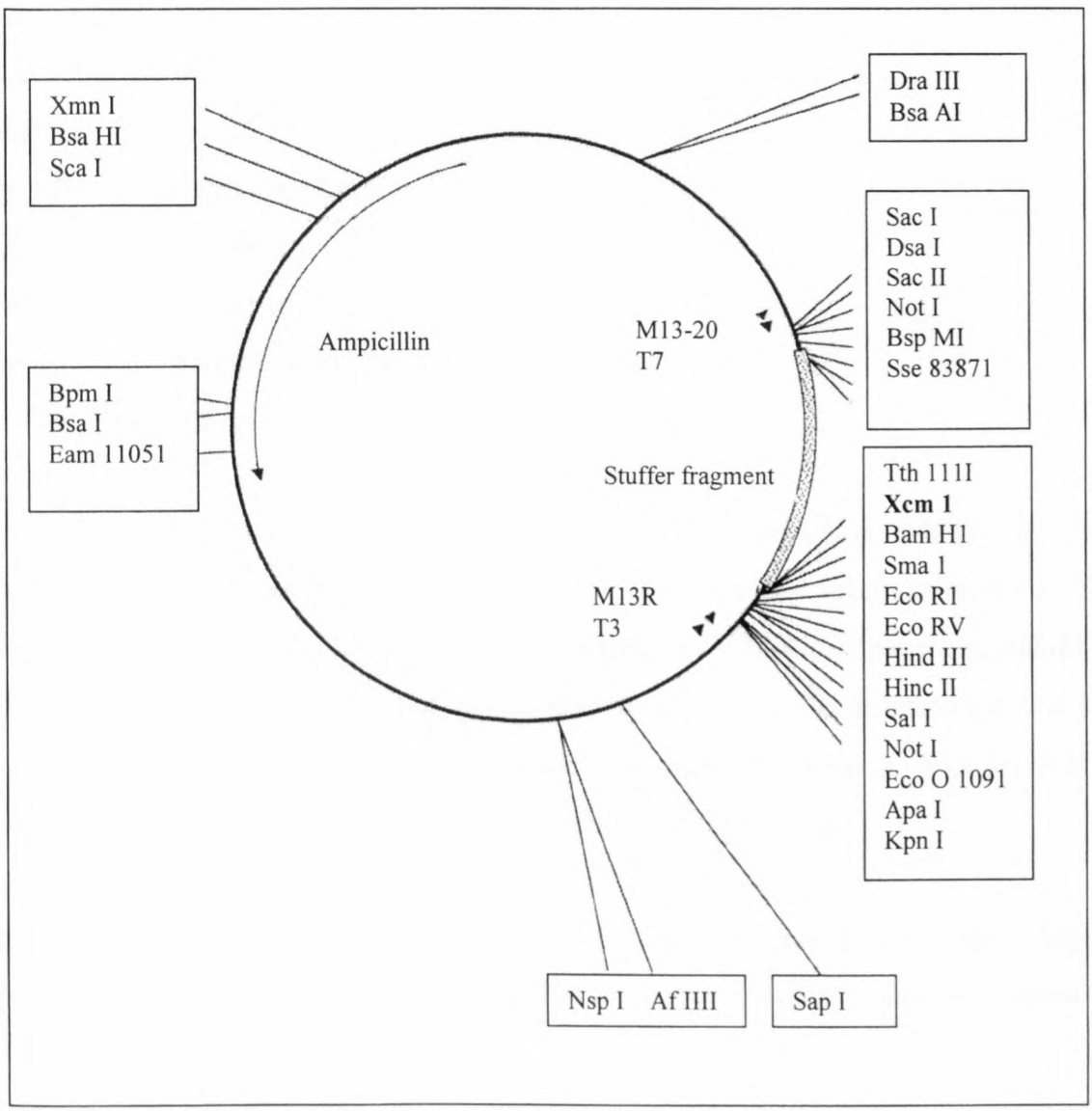
Sequencing of the stab 3 ITS-PCR:
PCR amplification of the last set of stabs was carried out. The products of these PCR reactions (containing the separated double band) were cleaned using the QIAquick PCR purification kit (Qiagen) and subsequently sequenced using 7.5pmol primer and 50ng of DNA.

To eliminate the background present in the sequences of the two bands, cloning of the stab 3 ITS-PCR products in the pT-NOT vector was carried out. The pT-NOT vector is very appropriate because it produces –T overhangs when digested with *Xcm*1. Biotaq amplified PCR products produce complementary–A overhangs.

2.2.3.6.1 Preparation of the pT-NOT vector for the ligation procedure

- The pT-NOT vector (Figure 2.5) was obtained from N Dear (Max-Planck-Institute for Immunobiology at Freiburg, personal communication).
- DH5α *E. coli* bacterial cells (Promega) were transformed with this vector.
- The plasmids were extracted using a modified alkaline lysis plasmid extraction (Xiang *et al.*, 1994).
- The plasmids were digested with *Xcm* 1. Ten units of the enzyme were used to digest 2.4µg of plasmid DNA at 37°C for four hours. Digestions were loaded onto a 1% agarose gel and run at 80V for one hour.
- The 2000bp fragment, ie the vector without the 500bp stuffer fragment, was cut out of the gel and purified using the QIAquick gel extraction kit protocol (Qiagen).

Figure 2.5 The pT-Not vector (N Dear, Max-Planck-Institute for Immunobiology at Freiburg, personal communication).



2.2.3.6.2 Ligation of the vector and the insert

Ligation of the insert and the vector was carried out using a five:one ratio of the insert:vector. The combined insert and vector amount did not exceed 200ng, and 40ng of the vector was used. The ligation reactions were performed with T4 DNA ligase (Promega) at 4°C overnight.

2.2.3.6.3 Transformation of competent cells

The *E. coli* race, DH5α cells, were made competent according to the Calcium Chloride method (Nakata *et al.*, 1997) and transformed with the recombinant pT-NOT vectors using the following methods:

- Use 2µl ligation mix (vector and insert)
- Add 50µl competent cells
- Place on ice for 20mins.
- Incubate at 42°C for 45s.
- Cool on ice for 2mins.
- Add 950µl LB medium
- Shake for one hour (RT)
- Spin the cells down (12000rpms)
- Add 50µl LB medium
- Plate cells onto LB plates, containing ampicillin at a final concentration of 50µg/ml, and incubate overnight at 37°C.

The clones were amplified using M13 primers (pUC M13R, 17mer and pUC M13-40F, 17mer). A single colony was picked with a sterile toothpick and placed in a PCR reaction volume of 25µl. The reaction mix contained 1.5mM $MgCl_2$, 1/10xNH_4 buffer, 0.05U Biotaq polymerase, 200µM of each dNTP and 6.25ng/µl of the forward and reverse M13 primers. An initial denaturing step at 94°C for 5min was followed by 35 amplification cycles, each consisting of 0.5min at 94°C, 45s at 60°C and 1min at 72°C. A final step at 72°C for 7min completed the PCR program.

The plasmids were digested with *Not*1 enzyme (10U) for four hours at 37°C. The products were electrophoresed together with original inserts (for comparison purposes), non-recombinant vector and a 100bp ladder as size indicators.

Sequencing reactions were performed, as described above, using the M13/pUC forward and reverse primers respectively, 300ng of DNA and 30ng of primer per reaction.

2.2.3.7 Rep-PCR:

Three sets of repetitive regions within the genome were regarded in this study: BOXAIR, ERIC and REP (Spooner *et al.*, 1995; Rademaker and de Bruijn, 1997). Primers for amplification of these regions were synthesized by Integrated DNA Technologies-Inc. (Coralville, Iowa, USA). The sequence of the primers are seen in Table 2.9.

Table 2.9 Sequences of the Rep-PCR primers

PRIMER	SEQUENCE
BOX	5'- CTACGGCAAGGCGACGCTGACG- 3'
ERIC 1R	5' -ATG TAA GCT CCT GGG GAT TCA C- 3'
ERIC 2	5' -AAG TAA GTG ACT GGG GTG AGC G- 3'
REP 1R-I	5' -III ICG ICG ICA TCI GGC- 3'
REP 2-I	5' -ICG ICT TAT CIG GCC TAC- 3'

(The I= Iosine).

Analysis was done in duplicate on each isolate using the different colonies (A and B, see Figure 2.3). PCR reactions were carried out in 25µl aliquots containing 2U of *Taq* polymerase, 6mM $MgCl_2$, 1/10xNH_4 buffer, 160µM of each dNTP, 25pmol primer, and 5µl DNA of a one in 50 dilution stock. PCR amplification was performed using the Perkin Elmer thermal cycler programmed for an initial step at 95°C for 10min. This was followed by 37 cycles of denaturation at 95°C for 30s, annealing at 53°C (for BOX)/ 52°C (for ERIC)/ 40°C (for REP) for 45 s and extension at 72°C for 2min. A final extension step at 72°C for 7min was included. BOX-PCR amplifies DNA with one primer and ERIC and REP-PCR make use of primer pairs. After PCR, 8µl of loading buffer (XC loading buffer) were added to each tube and 10µl were loaded onto a 6% polyacrylamide gel and electrophoresed in 1.5x TBE buffer at 15°C, 200V for 45-60 min. The gel was stained with ethidium bromide and photographed using the GelDoc. system. A 100bp ladder (Promega) was used as the molecular size standard.

The Rep-PCR techniques were repeated on each DNA sample to test for reproducibility and performed on duplicates of the same sample obtained from different extraction occasions. This was

to ensure that only the repeatable bands were scored and that the isolate is the true designated isolate and not a contaminant.

2.2.3.7.1 Data analysis of Rep-PCR products analysed on the polyacrylamide gels

Band sharing is a tool used to identify the level of similarity between different individuals exhibiting different banding patterns. The Rep-PCR procedure produced multi-band patterns suitable for this band sharing method of similarity determination.

The DNA profiles generated by Rep-PCR were scored using the Biorad GelDoc. and Microsoft Excel program. The procedure was repeated for all three of the primer sets (BOX, ERIC and REP). Only clear unambiguous bands in repeated PCR reactions were recorded.

The analysis of the Rep-PCR fragments on the Hoefer® Mighty Small polyacrylamide gels was done on the GelDoc. The Molecular Analyst associated with GelDoc. computer program involves the following steps:

1. The image is captured.
2. The lanes and bands are identified using a specific lane finding function on the computer.
3. The top of the lane is identified as the starting point for the measurement of the band sizes.
4. The standard (STD) lane is identified and annotated. (The 100bp ladder from Promega was used on these gels.)
5. GelDoc. measures the distance from the top of each lane to assign a value in bp for each band. This is done in accordance to the values of the STD lane.

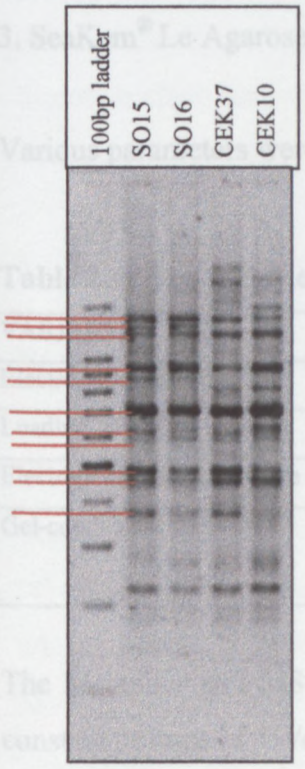
These results were transferred to Excel where a table indicating presence or absence of bands as "1" and "0" was constructed using a program called polymorphism finder (pmfind) (JAN Louw, US and CJ Van Heerden, US Central Sequencing Faculty of the Genetics department, personal communication). Bands had to be scored as present and absent (1 or 0) and matrices had to be produced using pmfind. These scores were then used to calculate a pairwise genetic distance matrix using the formula of Nei (1975). Cluster analysis based on the unweighted pair group average (UPGMA) was used to create cluster trees through "Statistica" (Edition 99) and PAUP4 (Swafford 1998) (see Figure 2.6 for an example of how the procedure was carried out).

Visual analysis of the gels posed a problem because the samples were run on polyacrylamide gels, which can only hold a maximum of ten samples at a time. This meant that a particular band on one gel had to be identified on another gel as being completely identical. This posed a lot of difficulty as

a lot of inter- and intra- gel variation was evident. In an attempt to alleviate these difficulties, the gels were captured on the GelDoc. and analysed using the GelDoc. functions (as indicated above).

Figure 2.6 The steps followed to create a cluster tree (Hillis *et al.*, 1993) by analysing fragments on a gel

1. PCR products are loaded onto a gel and electrophoresed. Red lines indicate the bands scored as seen in step 2.



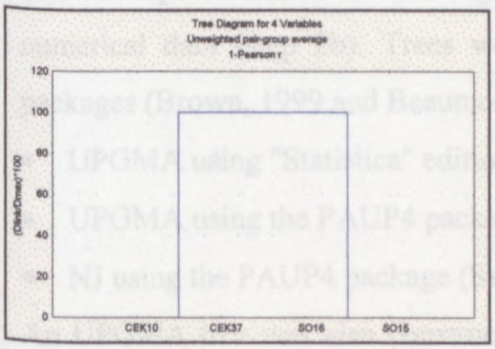
2. Bands are scored as absent (0) or present (1)
a) Using the GelDoc. and Excel or
b) Visual inspection

Band number	SO15	SO16	CEK37	CEK10
1	1	1	1	1
2	1	1	0	0
3	1	1	1	1
4	0	0	0	0
5	1	1	1	1
6	1	1	0	0
7	0	0	0	0
8	0	0	0	0
9	1	1	1	1
10	0	0	0	0
11	0	0	0	0
12	1	1	1	1
13	1	1	1	1
14	0	0	0	0
15	0	0	0	0
16	0	0	0	0
17	0	0	0	0
18	1	1	1	1
19	0	0	0	0
20	1	1	1	1
21	0	0	0	0
22	0	0	0	0
23	0	0	0	0
24	1	1	1	1
25	0	0	0	0
26	0	0	0	0
27	0	0	0	0
28	0	0	0	0

PAUP

"Statistica"

4. Cluster trees are constructed using "Statistica" or PAUP4



3. Similarity matrices (Nei,1975) are produced. This indicates the similarity between isolates by assigning a value of 1 as indicating 100% similarity, and a value 0 indicating complete dissimilarity

	SO15	SO16	CEK37	CEK10
SO15	1.00	1.00	0.90	0.90
SO16	1.00	1.00	0.90	0.90
CEK37	0.90	0.90	1.00	1.00
CEK10	0.90	0.90	1.00	1.00

2.2.3.7.2 Electrophoresis of Rep-PCR products on Metaphor agarose

Ideally, samples should all be run next to each other on one gel to overcoming the problem of inter- and intra- gel variation. A good quality agarose, which displays bands as clearly as the polyacrylamide gels, was necessary. Different types of agarose were tested:

1. MS-8 Agarose (Whitehead Scientific, Hispanagar company, Spain)
2. Metaphor Agarose (FMC BioProducts, Rockland, ME)
3. SeaKem® Le Agarose (FMC BioProducts, Rockland, ME)

Various parameters were tested to obtain the best possible gel (Table 2.10).

Table 2.10 The variables tested during electrophoresis

VARIABLES	CONDITIONS
Electrophoresis buffer	TBE, TAE
Loading buffer	Ficoll Orange G, XC loading buffer
Electrophoresis temperature	RT, 15°C
Gel-comb sizes	3mm wide/ 1.5mm thick, 0.3mm wide/ 1.5mm thick, 4mm wide/ 1.25mm thick

The Metaphor and MS-8 gels were made at a concentration of 1.8%, 4mm thick and run with a constant voltage of 4V/cm and tested with the various parameters mentioned in Table 2.10.

The SeaKem agarose gel was made at a concentration of 1.5% with TAE buffer. The gel was run at 2V/cm for 11 hrs at 15°C using 3mm wide/ 1.5mm thick combs.

2.2.3.7.3 Construction of phylogenetic trees from the Rep-PCR profiles analysed on Metaphor Agarose gels

The same procedure as seen in Figure 2.6 was followed. Molecular data had to be converted to numerical data (step 2b). Trees were constructed using the following strategies and program packages (Brown, 1999 and Beaumont *et al.*, 1998):

- UPGMA using "Statistica" edition 99
- UPGMA using the PAUP4 package (Swafford 1998)
- NJ using the PAUP4 package (Swafford 1998)

An UPGMA tree was also constructed for a combination of the three techniques. The out-group used was *S. acidiscabies*. Bootstrap analysis of 1000 replicas was applied to the trees to determine their validity.

CHAPTER THREE

3 RESULTS AND DISCUSSION

This study focuses on the identification of *Streptomyces scabies* using various DNA techniques. The goal was to produce cluster trees for each DNA technique used in order to deduce the extent of genetic similarity in this group of organisms for comparison with morphological data recorded by other researchers (VOPI, ARC, Appendix C). Access to an easy identification technique and a thorough classification system of *S. scabies* isolates will be a great benefit to researchers working on potato resistance to common scab.

3.1 BACTERIAL GROWTH CONDITIONS

Bacteria commonly exhibit extreme variability in artificial culture and the characters of a species are liable to undergo wide variations. A certain degree of consistency in the culturing is therefore required to obtain true classification and identification systems. In similar studies to this one, researchers therefore attempted to ensure that different isolates of *Actinomyces* from various types of potato scabs were kept at standardised culturing conditions, i.e., growth medium, times, temperatures etc. (Millard and Burr 1926). The optimal growth conditions determined in this study included the growth of the colonies on ISP2 media at 30°C for 48hrs. This was followed by the inoculation of the spores into 20ml CRM which was shaken at 250rpm, 30°C for 48hrs.

The appearance of the bacteria growth in the CRM medium ranged from murky to lumpy and the colour ranged from yellow to a dark brown. There was no consistent appearance within the different groups (isolates from one region), but the appearance of one bacterial isolate between different growth stages remained consistent. The morphology observed correlated with that in Appendix C.

No significant difference between the growth quality of the organism was observed with the use of the glass beads during the shaking process, or inoculation of the liquid media with spore suspensions.

3.2 DNA EXTRACTIONS

The amount of the starting material (mycelium) after centrifugation (after step 4 of the extraction protocol, section 2.2.2.1), varied between 0.5g to 2.0g. The Rao extraction protocol (Pigac and

Schrempf, 1995; Rao *et al.*, 1987) gave the best extraction results and was used routinely. This protocol was chosen as the most successful protocol because the DNA remained intact and yields varied between 550ng to 250000ng. The extensive phenol extraction steps and the spooling of the DNA ensured the purity of the DNA which is beneficial for sensitive PCR reactions. The amount of DNA extracted by other groups was 0.5-5µg of chromosomal DNA/ 50mg wet weight (Kutchma *et al.*, 1998) or 1-2µg DNA / 200-400mg wet weight (Kreuze *et al.*, 1999).

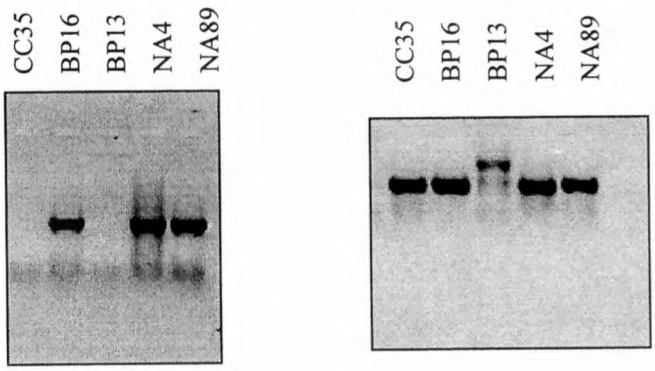
Isolates were supplied from different regions by Roodeplaat (see Table 2.2 in Chapter 2) together with an in detail list concerning their morphology, physiology, melanin production and pathogenicity (Appendix C).

3.3 AMPLIFICATION OF THE *NEC1* AND INTERGENIC REGIONS

It has been speculated that the phytotoxin, Thaxtomin A, is responsible for the pathogenicity of *S. scabies* (Doumbou *et al.*, 1998). Researchers have linked the production of Thaxtomin A to a 0.67kb gene, *nec1*, although this gene is not directly involved in Thaxtomin A synthesis (Bukhalid and Loria, 1997). The *nec1* gene is connected to a transposase pseudogene (ORFtnp) by an intergenic region (Bukhalid *et al.*, 1998). Amplification of the *nec1* and intergenic regions can be applied in PCR reactions as a rapid identification tool of pathogenic *Steptomyces species*. The success of this technique is desired to replace the usual tedious, time-consuming procedures currently used to test for pathogenicity.

In this study the amplification results using the primers described by Bukhalid *et al.* (1998) were not reproducible between subsequent PCR amplification reactions, neither were results consistent with the pathogenicity results from Roodeplaat (Appendix C). PCR reactions were repeated up to three times to ensure that the absence of the fragment was not due to problems within a single PCR reaction. Figure 3.1 show the results of two PCR amplification reactions concerning the same DNA sample performed on different occasions.

Figure 3.1 Duplicate *nec1* amplifications using the NF/NR primers



Although PCR amplification of NA4 and NA89 remained constant in subsequent reactions, the results did not correspond to those obtained from Roodeplaat (Appendix C). The pathogenicity of NA4 is 0, suggesting that no band should be amplified. This is contradicted in the above figures because the fragment is present in both PCR repeats. CC35 has a pathogenicity of 0. Figure 3.1 shows the absence of the fragment in one gel and its presence in the other. BP16 and NA89 are 100% and 75% pathogenic respectively and the fragment is present in both gels as is expected. The pathogenicity of BP13 is unknown and in this study it produces no fragment in one gel and one of a different size in the other gel. This different sized fragment was displayed randomly by some isolates in a few sets of PCR reactions without any consistency. These gels only show the results of five isolates, but similar results were observed with all the other S.A. isolates. Irregular results were also observed with the intergenic region, i.e., the desired fragment was present in non-pathogenic isolates, and visa versa, and amplification results were non-reproducible over successive PCR reactions. Optimisation of this technique was the subject of an honours project performed in 1999 (I Steyn, US Genetics department, personal communication). Inspite of the various parameters tested, PCR results remained inconsistent.

Bukhalid *et al.* (1998) performed Southern blot analysis with the *nec1* clone on 10 pathogenic *S. scabies* isolates and 11 non-pathogenic *Streptomyces sp.* from various regions in the U.S.A., Hungary, Japan and S.A. All reactions produced positive hybridisation resuls except for one Thaxtomin A producing pathogen from Ceres in SA, CEK18. PCR amplification using the corresponding primers complementary to the *nec1* gene was also carried out. The Southern analysis and PCR results employed by Bukhalid *et al.* (1998) had the same outcome. Kreuze *et al.* (1998) PCR amplified the *nec1* and the intergenic regions of 23 pathogenic *S. scabies* isolates. All the pathogenic isolates (determined by conventional pathogenicity tests), except for CEK18, produced a fragment identical in size.

Although not discussed in the above-mentioned studies, PCR amplification of the *nec1* has been reported to pose some problems (RA Bukhalid, Cornell University, personal communication). False positives, in the form of a faint band representing the *nec1* fragment, appeared in non-pathogenic isolates which did not hybridise to the *nec1* clones. The "*nec1*-intergenic region-ORFtnp" area, as well as their respective primers, are fairly GC poor. This could result in amplification occurring irregularly because of poor specific binding. Lowering of the annealing temperature could be a solution to this problem (RA Bukhalid, Cornell University, personal communication). The *nec1* region was lowered to 56°C, but no improvement was observed. Lowering of the Tm for the intergenic region amplification resulted in too much background (results not shown).

No improvement in the PCR reactions was observed when Amplitaq Gold (Applied Biosystems) was used instead of Biotaq (Bioline). Fewer fragments were present and reproducibility was no better than when Biotaq was used (figures not shown). This procedure was an attempt to find a quick and easy way for determining the pathogeneicity of *S. scabies* in S.A. Unfortunately this was not a success and hence the procedure was discarded.

3.4 RAPD ANALYSIS

The RAPD technique is a quick and easy tool for genetic identification and classification. No prior knowledge is required about the DNA sequence. A single 10 mer primer amplifies a set of fragments which can be analysed on a gel to represent a specific fingerprint. Reproducibility is sometimes poor in successive PCR reactions due to contamination and the low annealing temperature used. In this study decamer primers were chosen randomly from the Operon Kit M. Initially, RAPD results were run on agarose gels. Products were electophoresed on polyacrylamide gels as well in an attempt to improve resolution (Figure 3.2 on pg 54)

Difficulty was experienced in obtaining reproducibility of gels and of DNA extracted on different occasions. All results are not shown and Figure 3.2 is a representitive of the results obtained. The variation in the reaction conditions, reagent concentrations and use of the Stoffel fragment (Applied Biosystems) made no improvement to the repeatability or reproducibility of the RAPD procedure.

Severe non-reproducibility was experienced in the following instances:

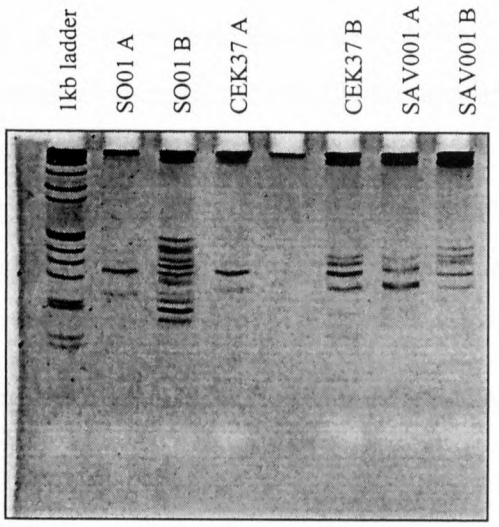
- RAPD-PCR profiles in successive PCR reactions
- RAPD-PCR performed on different clones from the same isolate grown on the same solid agar plate
- RAPD-PCR performed on the same isolate grown and extracted at different times
- RAPD-PCR performed on the same isolate extracted with different and the same extraction protocols.

RAPD errors can be attributed to a number of factors. The outcome of the RAPD profile could be determined by competition for different priming sites. Amplification is possible at many sites, but only a small set of bands are amplified and subsequently observed on the gel. This could result in different banding patterns being observed with each RAPD-PCR repeat. Another reason could be that of poor reproducibility of faint bands in some of the PCR reactions. The presence of faint bands may reflect differences in the specificity of amplification. Sample to sample differences in reaction conditions is another possibility (Halldén *et al.*, 1996; and Skroch and Nienhuis, 1995). It has been observed that with some bacterial species, artifacts occur because of culture condition, culture age and extraction technique (Pooler and Hartung, 1995). Different sources and concentrations of primers and different DNA concentrations also have an effect on reproducibility (Virk *et al.*, 1995).

In an attempt to alleviate these problems, bacteria were grown under constant conditions in the current study. Extraction conditions were also kept as constant as possible. DNA was isolated from one to three different bacterial cultures of the same isolate at different times. Although growth and DNA extraction conditions were kept as constant as possible, the age of bacteria in the glycerol stock was unknown and could also be a contributing factor to the results observed. The age of the cultures at Roodeplaat, before they were received in Stellenbosch, could also have had an influence on the results.

Kutchma *et al.* (1998) and Mehling *et al.* (1995) applied the RAPD technique to a group of *Streptomyces species.* However, no data on *S. scabies* isolates were reported and no investigation into the reproducibility of the RAPD profiles between successive PCR reactions or different extraction occassions was attempted. Jones *et al.* (1997) tested the reproducibility of a number of DNA marker techniques and concluded that the problems experienced with the RAPD technique is that of non-reproducibility. The only solution to these problems would be to perform replicate runs and disregard the non-reproducible bands. However, because of the numerous variable amplification patterns obtained this was not considered in this study.

Figure 3.2 RAPDs carried out with OPM1 using the PE thermal cycler. A and B refer to Figure 2.3

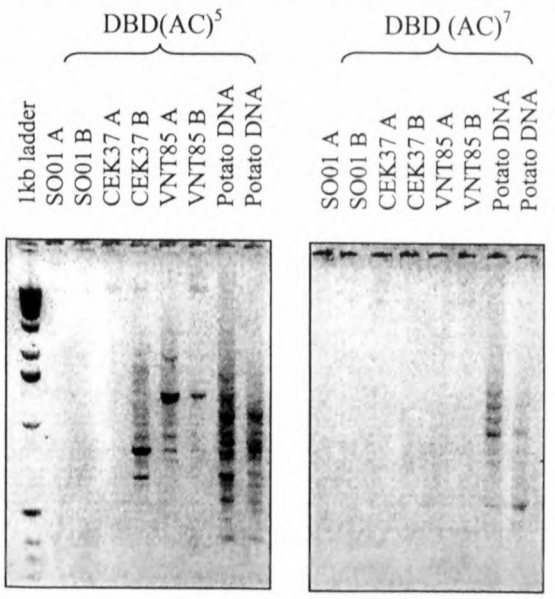


3.5 RAMS

This technique is applicable for the analysis of genetic diversity and relatedness between organisms. It works on the principle of amplifying the regions between microsatellite repeats.

The level of clarity of RAMS profiles in this study on the *S. scabies* isolates was not acceptable. Only a small number of isolates produced fragments (Figure 3.3) and reproducibility from different extractions was poor. The potato genomic DNA used as control did produce acceptable patterns indicating that the fault was probably the *S. scabies* DNA itself and not the primer or PCR reaction. It is not known whether the *S. scabies* genome possesses microsatellite repeats or not. Another factor that might influence the results of the RAMS technique, is low genome complexity (Zietkiewicz *et al.,* 1994). RAMS analysis was not exploited any further because of these inconsistent observations.

Figure 3.3 RAMS profiles produced with two primer sets. A and B refer to Figure 2.3

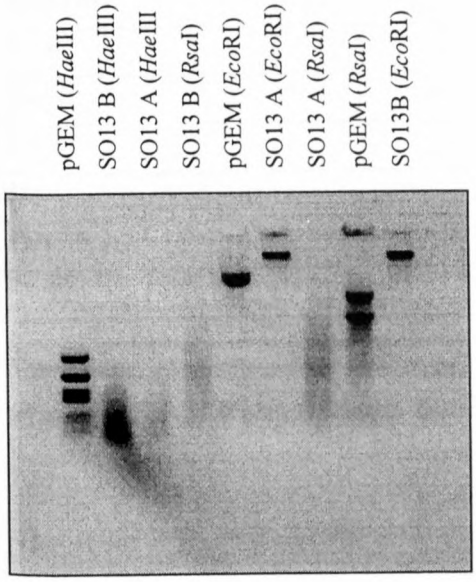


3.6 AFLP ANALYSIS

AFLP analysis is an effective technique which analyses a large number of genetic loci. The profiles generated from this technique are applicable in the study of diversity of organisms.

Optimum digestion results were obtained when 1µg DNA and 30U endonuclease enzyme were incubated at 37°C overnight (16hrs) with the enzymes *Rsa*1 and *Hae*III.

Figure 3.4 Restriction enzyme digests of the bacterial DNA. pGEM was used as control to confirm enzyme activity



*Hae*III and *Rsa*I digested the *S. scabies* DNA successfully but not *Eco*RI (Figure 3.4). The *S. scabies* genome is GC rich which could explain why only the enzymes recognising GC regions digested successfully (Table 3.1).

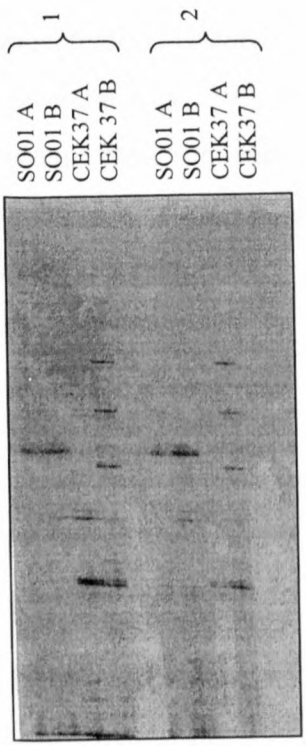
The AFLP procedure using the Gibco AFLP kit with the *Mse*1 and *Eco*R1 endonucleases was thus unsuccessful. Electrophoresis of the ligation products did not produce a clear smear as is normally required for successful AFLP analysis. There were simply too many polymorphic peaks in the two different DNA samples of the same isolate probably ascribable to incomplete digestion.

AFLP analysis was repeated using *Pst*1 and *Msp*1 enzymes both of which recognise GC rich DNA sequences. After optimisation of conditions it was concluded that the three base pair extension primers, Pst6/Msp5, produce the best results, exposing the film for two days. The analysis was then performed on the same *S. scabies* isolate extracted on different occasions. There were too few common bands between them and too many dissimilar bands. AFLP analysis was performed in duplicate on the same DNA sample at the same time and run side by side on the same gel (Figure 3.5). Some reproducibility was evident but non-reproducibility between the different clones of the same isolate discouraged any further use of the procedure.

Table 3.1 Enzymes used to digest the *S. scabies* genome

ENZYME	**RECOGNITION SITE**
*Eco*R1	GAATTC
*Hae*III	GGCC
*Mse*1	TTAA
*Msp*1	CCGG
*Pst*1	CTGCAG
*Rsa*1	GTAC

Figure 3.5 AFLP results using the *Pst*1 and *Msp*1 enzymes. A and B refer to Figure 2.3

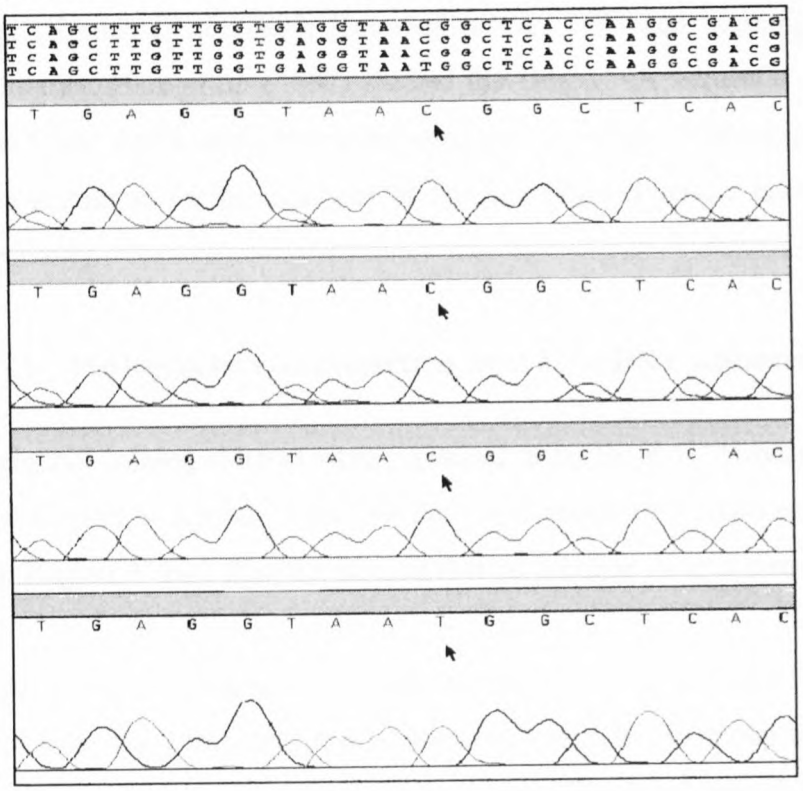


No AFLP analysis on *S. scabies* has been reported to date although the procedure has been succesfully performed on many bacteria and fungi. It was attempted on *E.coli* isolates using *Eco*R1 and *Mse*1 (Zhao *et al.*, 2000) and *Vibrio cholerae* using *Hind*III and *Taq*1 or *Apa*1 and *Taq*1 (Jiang *et al.*, 2000). In a recent publication on plant AFLPs (Donini *et al.*, 1997), it was reported that different patterns are generated for different organs of the plant and could be ascribed to differences in DNA methylation patterns between the different organs. DNA methylation can also vary during the different stages of a plant's growth and development. A way around this problem, in plants, is to use methylation insensitive enzymes. This may apply as well to *S. scabies* and DNA methylation in these bacteria should be further investigated.

3.7 16S rDNA SEQUENCING

DNA sequencing is becoming an important tool in the identification of organisms at all taxonomic levels. The sequencing of the 16S rDNA subunit is being employed by researchers working on the classification of various bacterial species. In a study by Kataoka *et al.* (1997), the partial and total 16S rDNA sequencing results were found to produce similar tree topologies. Furthermore, the genetic distance is more pronounced in the partial sequence tree. The genus *Streptomyces* also contains too many isolates that display two identical 16S rDNA regions making analysis of the complete region impractical and unnecessary. Sequencing was carried out over the first ±500bp (Region A) and over the last ±500bp (Region B) (see Figure 1.3 or 2.4 in Chapter 1 and 2 respectively). Numbers were assigned to each base according to the *E. coli* numbering system (Takeuchi *et al.*, 1996 and Brosius *et al.*, 1978). The 16S rDNA sequencing results were successful because clear sequences were produced and different DNA samples of the same *S. scabies* isolate produced identical sequences. Figure 3.6 provides an example of the signal's uniformity in four *S. scabies* isolates.

Figure 3.6 A section of Region A with one base change in four different isolates of *S. scabies*



The sequences of all the *S. scabies* isolates were either identical or differed by up to a few bases in specific variable regions. The variable regions identified in Region A were the δ region (bp 70-95) and the γ region (bp 180-208). Variable regions identified in Region B were the β region (bp 1131-1152) and the ε region (bp 1458- 1476). These are identical to sequences reported by Kim *et al.* (1993) and Kreuze *et al.* (1999) both of which focused on the relatedness between various *Streptomyces* spp. using the 16S rDNA sequences. A new variable region of 20bp stretching from bp 1263-1283 in Region B was identified in this study. The isolates that differed in this region were VNT85 (Northern Province), SAV022 (Sandveld), CP29 (France), EF92 (Canada), BNT35 (beet isolate) and *S. acidiscabies.*

Sequencing was performed in both directions. Some of the isolates displaying ambiguous sequences were repeated, thus giving some isolates more than two sequences per region. Repeated comparisons of the various sequences available for each isolate were carried out to ensure the accuracy of the results. Only the unambiguous bases identified out of a number of repeated sequencing reactions were taken into account. Eventually a single consensus sequence was constructed for each isolate using the combined sequencing data.

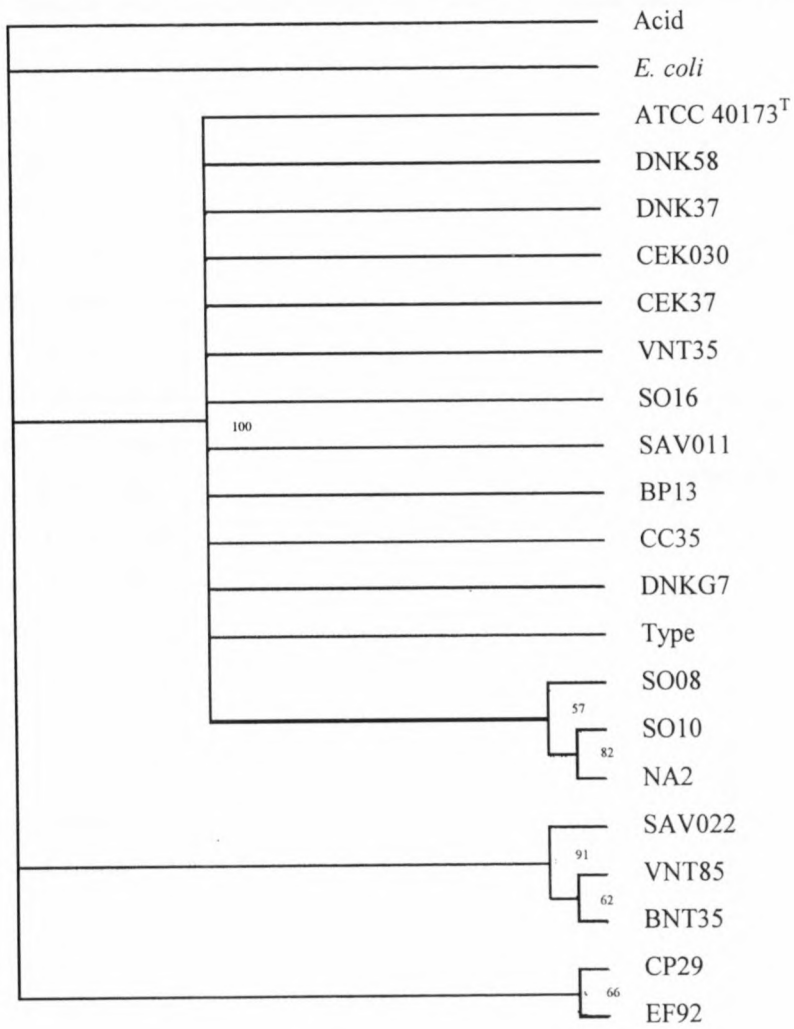
Most 16S rDNA sequencing studies have focussed on differences between various *Streptomyces* spp. (Kreuze *et al.*, 1999; Bramwell *et al.*, 1998 and Mehling *et al.*, 1995) and displayed 95% similarity. Hain *et al.* (1997) studied the 16S rDNA sequence diversity in *S. albidoflavus* isolates and found 100% similarity within this species. These publications hint at the unappropriateness of this technique for the analysis of the *S. scabies* diversity because of their possible intra species similartiy.

3.7.1 Phylogenetic reconstruction from 16S rDNA sequences

Neighbor joining clusters were produced using the PAUP4 package (Swafford 1998) for Region A and Region B separately and for the combination of the two regions. More branching is evident in the Region A trees than the Region B trees because there is more sequence variation in Region A.

Similar grouping between the trees formed for Region A and B was evident. Figure 3.7 shows the tree from the combined data of Region A and B. Bootstrap analysis with 1000 resamplings was applied to the tree to determine the validity of the branching. Bootstrap values should be above 70% before the branching within a tree can be accepted (Brown, 1999). One large major group is evident and two smaller groups are present. The large group has a bootstrap value of 100% indicating complete robustness. One of the small groups contains SAV 022 (Sandveld), VNT85 (Northern Transvaal) and BNT 35 (Beet) and the other small group contains CP 29 (France) and EF 92 (*S. albidoflavus* from Canada). The robustness of these two groups is questionable because their bootstrap values were too low. As expected, the out-group, *E. coli,* and the *S acidiscabies* formed their own separate groups.

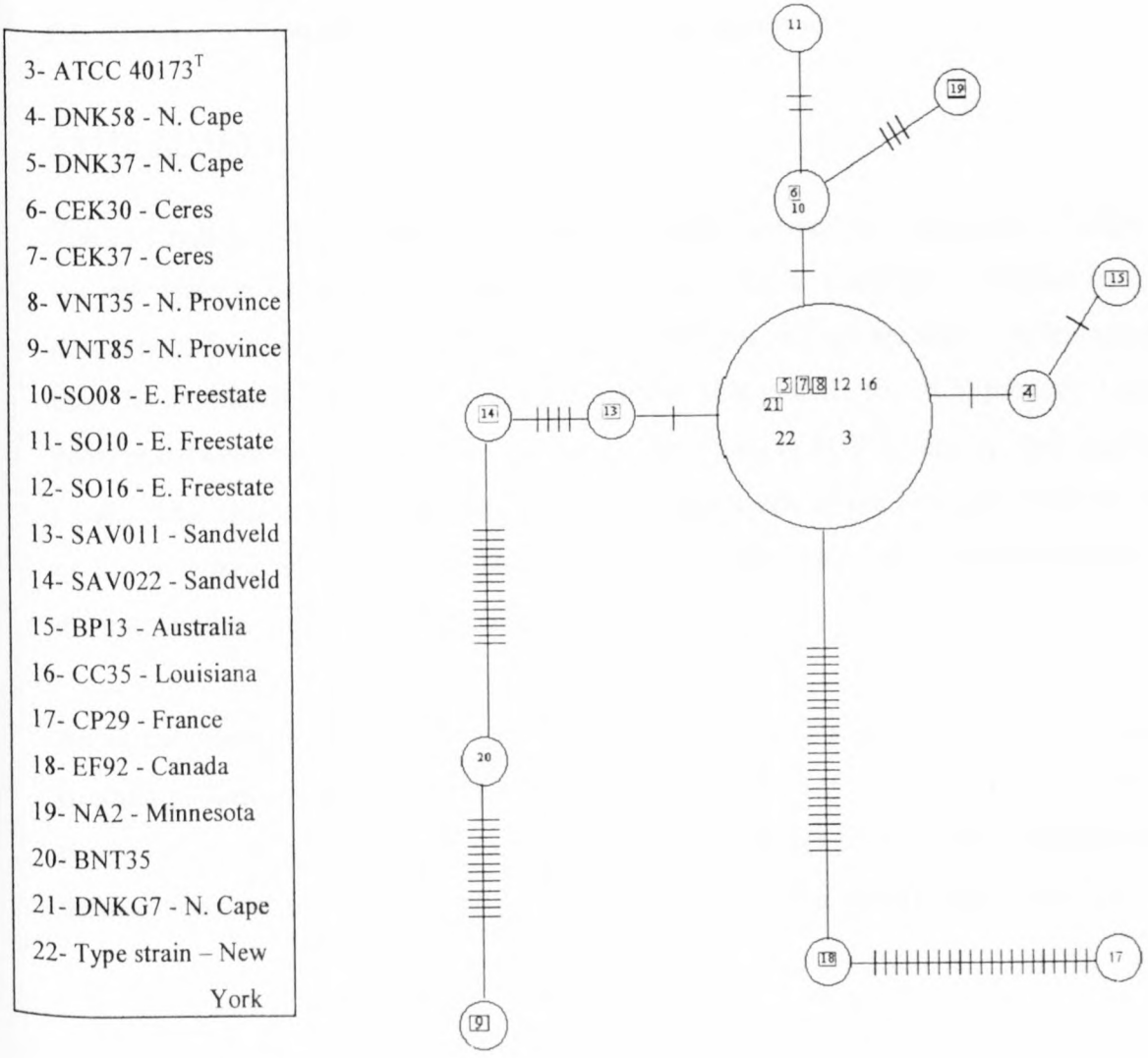
Figure 3.7 NJ tree created by PAUP4 for the combined sequences of Region A and Region B. Bootstrap analysis was applied. Acid is *S. acidiscabies* (ATCC 49003), Type is *S. scabies* (ATCC 41973), ATCC 40173^T is an *S. scabies* isolate obtained from published literature (Takeuchi *et al.*, 1996). The numbers refer to the bootstrap values.



A Parsimony Network was constructed to analyse the sequencing data more thoroughly (Figure 3.8). This technique, otherwise known as the minimum Spanning Network, was developed to improve the resolution of close associations (Matthee and Robinson, 1997). A haplotype contains all the isolates with identical sequences. This method emphasises the distinction between haplotypes (in this case the sequences per isolate) where the number of bases distinguishing them is small.

A grouping similar to the above NJ trees was obtained. Most isolates grouped into one major group or very close to the major group. The anomalous isolates in this network are the same as in Figure 3.7: CP29, EF92, SAV22, BNT35 and VNT85. The parsimony network settles the uncertainty of the robustness of the two small groups evident in Figure 3.7.

Figure 3.8 Parsimony Network created with the combined 16S rDNA sequences of Region A and Region B. The names of the isolates, represented by numbers in the circles, are listed in the box. The size of the circles is proportional to the number of isolates in that particular group. The number of cross-bars between circles represent the number of base changes between each sequence. ATCC 40173^T is an *S. scabies* isolate obtained from published literature (Takeuchi *et al.*, 1996)



No 2 (*S. acidiscabies*) is not included in the Parsimony Network because its position is too far from any of the other isolates. The sequence of the 16S rDNA region of *E. coli* was used as the out-group. Allignment of the 16S rDNA sequences is not available because it requires additional work not suitable for the scope of this study.

Kreuze *et al.* (1998) has reported limited, intraspecific variability between the 16S rDNA sequences of *S. scabies* isolates from Finland. Takeuchi *et al.* (1996) studied the 16S rDNA sequence similarity between three *S. scabies* isolates from different geographical regions (Japan, Hungary and United States) and all were found to be the same except for one nucleotide. Similar results were observed in this study. Most of the isolates had identical sequences while a few others strayed from

this major group. As seen in the parsimony network, most stray isolates differed by only a limited number of base changes. Some isolates, such as EF92 (Canada), BNT35 (beet isolate), VNT85 (Northern Province) and CP29 (France) differed by considerable numbers of bases. This indicates their genetic diversity as compared to other *S. scabies* isolates. Perhaps these stray isolates are in fact other types of *Streptomyces* spp. misclassified as *S. scabies*. Further investigation concerning this misclassification should be explored by plant pathologists.

3.8 ITS ANALYSIS

The ITS region found in bacteria is known to differ in size and composition within and between species. Hain *et al.* (1997) managed to prove that this is a reliable technique in the analysis of diversity of *S. albidoflavus*. The ITS products were run on high-resolution polyacrylamide gels and then the individual bands were then sequenced by first cloning the PCR product. Identical banding profiles between the *S. albidoflavus* isolates were found to be different by studying the sequencing results. This indicates the importance of sequence analysis of the ITS region for the discrimination of the *S. albidoflavus* isolates. The technique was thus expected to be successful in analysing diversity within the *S. scabies* group.

An initial annealing temperature of 47°C was used on *S. scabies* DNA in an attempt to obtain banding patterns as seen with the *S. albidoflavus* (Hain *et al.*, 1997). It is known that ITS regions within one bacterial isolate can vary in composition and length. The PCR products appeared more defined when run on polyacrylamide gels than on the agarose gels (Figure 3.9a and b).

Figure 3.9a The ITS PCR products as seen on an 8% polyacrylamide gel (Annealing temperature of 47°C)

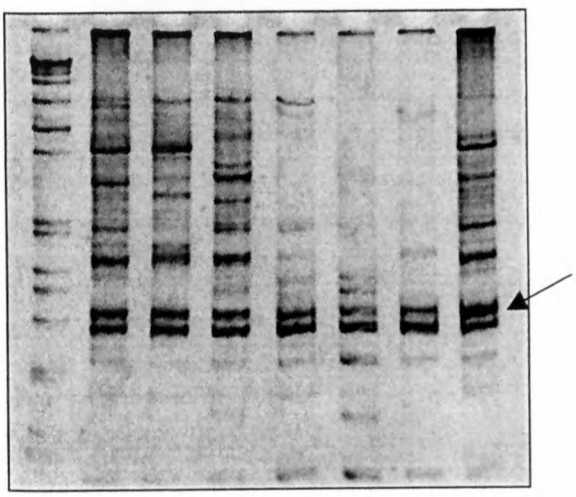
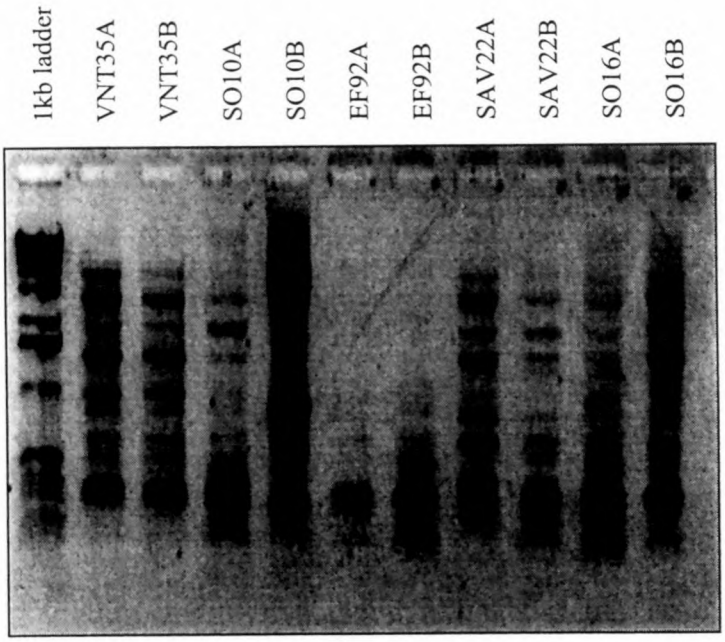


Figure 3.9b ITS-PCR products seen on a 1% Agarose gel (Annealing temperature of 47°C) A and B refer to Figure 2.3



Inconsistency of the band profiles between different samples of the same isolate was evident in some samples, e.g., SO10 A and B in Figure 3.9b. No published reports concerning the analysis of the reproducibility of ITS profiles could be found. A prominent feature present in all the isolates was a double band at the 300bp region indicated with the arrow in Figure 3.9a. With an increase in temperature (to 58°C), the double bands were maintained and the background was decreased (Figure 3.10).

The two bands (1 and 2 in Figure 3.10) were stabbed using a disposable micropipet tip. This was used as the template in a subsequent PCR procedure. Results from these amplifications were visualised on a polyacrylamide gel (Figure 3.11a) and called the stab 1 ITS-PCR products. The stabbing and PCR amplification procedure were repeated to give the stab 2 ITS-PCR products. In an attempt to remove the background completely, as is required for sequencing, the procedure of stabbing and amplification was repeated a third time to provide the stab 3 ITS-PCR products (Figure 3.11b). The background could not be sufficiently elliminated for sequencing therefore the PCR products (1 and 2) had to be cloned into the pT-NOT vector.

Figure 3.10 ITS-PCR results using an annealing temperature of 58°C. (1) and (2) represent the two prominent bands that were always present with the ITS amplification

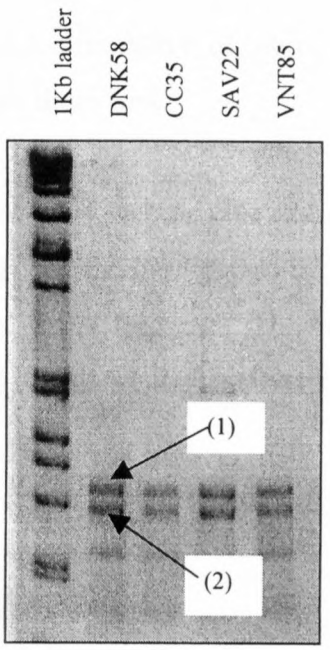
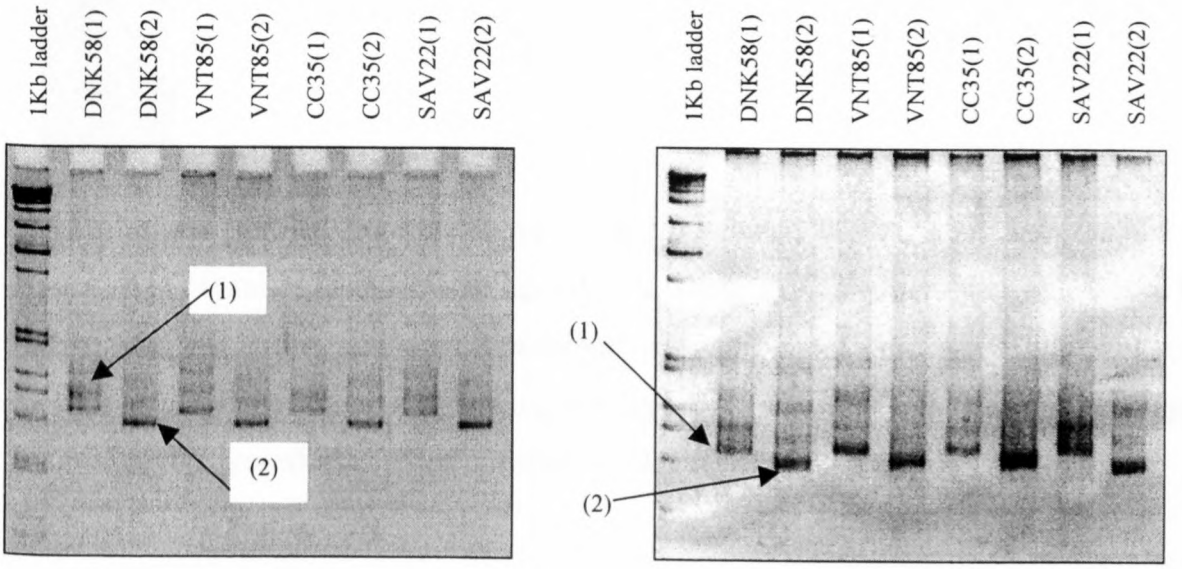


Figure 3.11a Stab 1 (left) and Stab 3 (right) ITS-PCR products. (1) and (2) represent the two bands seen in Figure 3.10

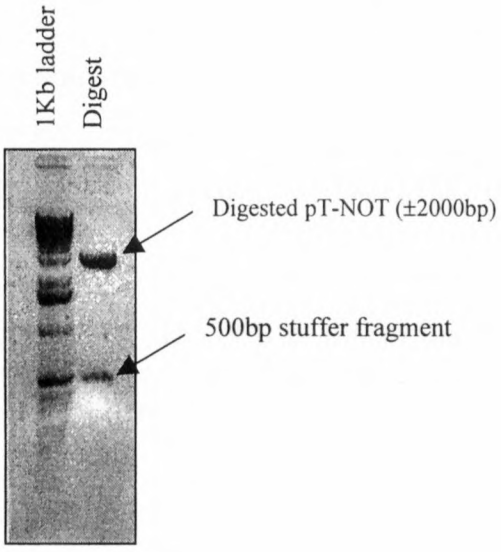


3.8.1 Cloning of the PCR product

In the hope of finding a means of classifying the bacteria using a similar approach as the 16S rDNA sequencing, both of the the consistent bands were sequenced. Successful sequencing requires a complete elimination of the background, therefore the best way to analyse the sequences would be to clone them first.

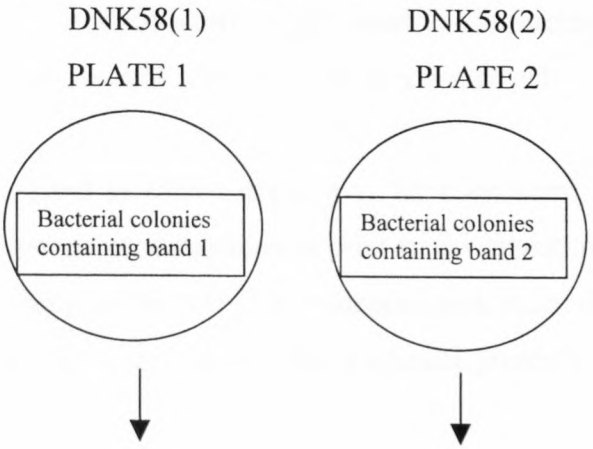
The pT-NOT vector consists of multiple restriction enzyme sites. *Xcm*1 produces T- overhangs appropriate for the ligation of PCR products. Digestion of the vector with this enzyme removes a 500bp stuffer fragment. The vector is 3450bp in size, including the stuffer fragment. Figure 3.12 shows the *Xcm* 1 digested vector, the open vector and the small stuffer fragment.

Figure 3.12 pT-NOT vector digested with the *Xcm* 1 enzyme



The vector was purified from the above gel and the Stab 3 ITS-PCR products (band 1 and 2 in Figure 3.11) of isolate DNK58 were ligated to it. These ligated vectors were introduced back into DH5α cells and propagated on LB medium containing ampicillin. Ampicillin allowed for the survival of only the bacteria containing the ligated vector. The following figure (Figure 3.13) summarises the procedures carried out after the cloning of the bands.

Figure 3.13 Diagramatic representation of the procedures performed on the cloned products

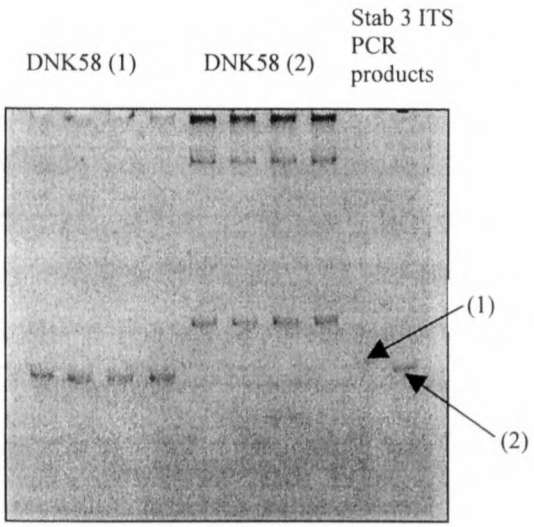


Four clones were isolated from each plate for amplification with the M13 primers (pUC M13R, 17mer and pUC M13-40F, 17mer)

Clones from DNK58(1) were sequenced

The amplification results of band 1 [DNK58 (1)] seen in Figure 3.14 produced a similar sized fragment as was required (see 1 in Figures 3.10- 3.11). The amplification results of band 2 [DNK58(2)] produced two fragments that did not correspond to either of the original fragments 1 or 2.

Figure 3.14 M13 PCR results of the clones. The stab 3 ITS-PCR products are the same products seen in Figure 3.11 (right panel). DNK58 (1) and DNK58 (2) were isolated from plate 1 and 2 respectively in Figure 3.13



Sequencing was performed on the DNK58(1) clones and the Stab 3 ITS-PCR products (Figure 3.11, right panel) The sequencing results indicated the presence of more than one signal suggesting that

products were not completely cleaned of all other DNA sample contaminants. No further analysis of this region was explored due to the difficulty experienced in eliminating the background and the non-reproducibility of the ITS profiles as seen on a polyacrylamide gel.

The presence of the double band in each sample may have occurred as a result of an ancient interspecific hybridisation event or gene duplication prior to the evolutionary radiation (O' Donnell *et al.,* 1998). Perhaps a switching of the two ITS sequences took place during the evolution of the organism without being fixed. This may explain the continual presence of a double band in each ITS-PCR reaction in this study.

Other researchers have also experienced problems with ITS sequencing because of its many base substitutions and instertions or deletions in the ITS region within an individual strain (RA Bukhalid, Cornell University, personal communication). Researchers have been using the analysis of the ITS region for the diversity or evolutionary studies on plants by analysing the ITS sequences (Nickrent *et al.*, 1994) or RFLP analysis of the ITS region (Quijada *et al.,* 1998).

3.9 Rep-PCR

The presence of certain highly conserved inverted repeats that are dispersed throughout the genomes of some bacterial species can be employed as a taxonmic tool for *S. scabies* bacteria. Rep-PCR is a simple technique which requires no prior sequence knowledge of the bacterial genome and works on the principle of analysing PCR products on a gel. This study focussed on the use of three Rep-PCR techniques (BOX, ERIC and REP). The linear combination of fingerprinting data using multiple primer sets improves the robustness of phylogenetic trees.

The best possible Rep-PCR fingerprints were observed on a 6% polyacrylamide gel. Rep-PCR was performed on both A and B DNA (see Figure 2.3 chapter 2). The Rep patterns and numbers of fragments generated differed for each set of primers and the bands produced varied in their intensities. The isolates were subjected to a number of PCR reactions and analysed. The artifacts were minimised by discarding inconsistent amplification data. The BOX-PCR results produced the clearest and most reproducible fragments out of the three Rep techniques. Spooner *et al.* (1995) reported different BOX-PCR results for *S. scabies* isolates from different geographic locations, but similar profiles within one region. Sadowsky *et al.* (1996) showed that the *S. scabies* isolates from Minnesota clustered in different groups. The *S. scabies* isolates analysed in this study produced very similar Rep-PCR fingerprints. Some of the isolates from the same region were identical.

3.9.1 Data analyses

The values assigned to each band by the GelDoc. System (by comparing the band positions to the known band values of the STD) were not as accurate as had initially been anticipated. Inter- and intra-gel variation was prominent. This was evident when repeated PCR products of the same isolate were run on opposite sides of a single gel, or on another gel. Figures 3.15a and 3.15b illustrate this problem.

Figure 3.15a A few BOX-PCR products BP16 run on a polyacrylamide gel. The same colour gel arrow (green and blue) is seen as the same size band using the eye.The red arrow indicates the two bands seen in Figure 3.17 and 3.18

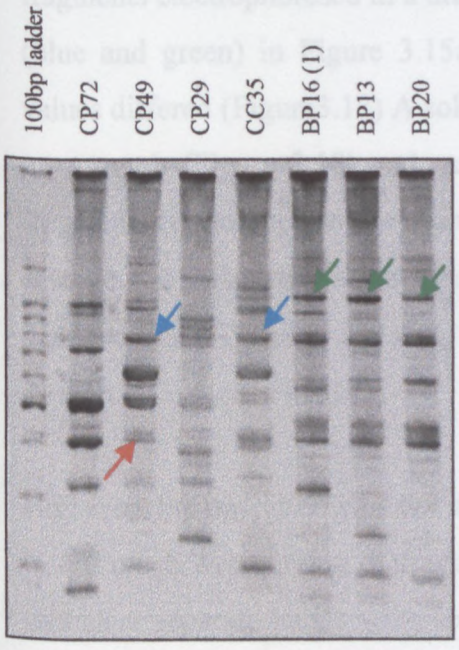
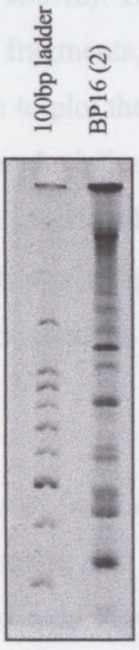


Figure 3.15b The BOX-PCR profile of as seen on a separate polyacrylamide



BP16 was run on two separate gels, each with its own STD, and the GelDoc. calculated the sizes according to the individual gel's STD lanes. The values obtained for the identical fragments between BP16 (1) and BP16 (2) (see Figure 3.15) did not correspond.

Figure 3.16 The fragment sizes of the two BP16 isolates run on two gels as determined using the GelDoc. (1) and (2) indicate the two separate gels seen in Figure 3.15a and b respectively

	BP16 (1)		BP16 (2)
band 1	1270.73	band 1	1424.02
band 2	1075.57	band 2	1205.79
band 3	935.27	band 3	1042.46
band 4	797.15	band 4	813.57
band 5	755.71	band 5	745.4
band 6	587.97	band 6	604.89
band 7	553.3	band 7	578.52
band 8	447.27	band 8	472.02
band 9	404.08	band 9	420.67
band 10	372.24	band 10	328.85

From these results it is evident that the GelDoc. cannot be used to allocate fragment sizes to samples run on different gels. The same difficulty was experienced with visually identical fragments electrophoresed in a different lane of a single gel (not shown). The same coloured arrows (blue and green) in Figure 3.15a indicated visually identical fragments, but their GelDoc. size values differed (Figure3.17) A solution to this problem would be to plot the GelDoc. fragment sizes on a graph (Figure 3.18) and assign an appropriate STD error deviation to each value. All the fragments (no more than one from each isolate) lying within an individual STD error bar could be accepted as being the same size. A 1% STD deviation error was assigned to each value and displayed as STD error bars on the graph following guidelines used in forensic work (National Research Council, 1996).

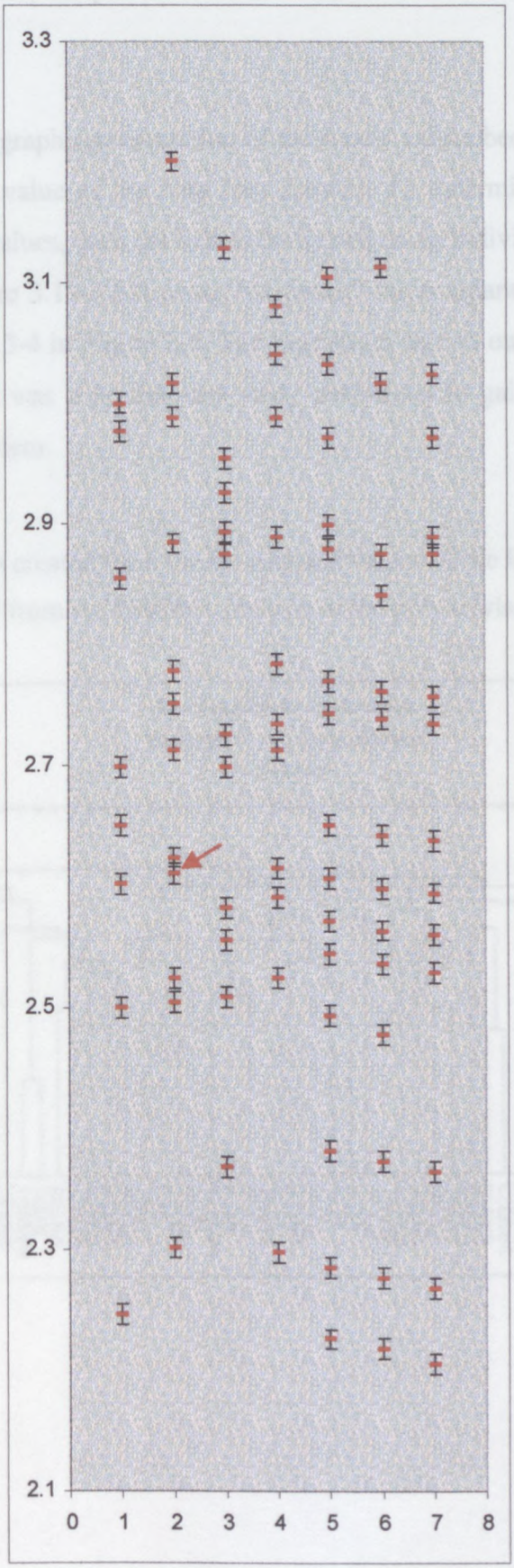
However, the operation was not successful as the large numbers of fragments and isolates resulted in too much overlapping of fragments. Two bands from one isolate also overlapped even though two clear separate bands were seen on the gels (see the red arrow in Figure 3.15a, 3.17 and 3.18 indicating such an example). The whole problem of interpreting GelDoc. fragment sizes with confidence bands should be investigated further.

Figure 3.17 The GelDoc. profile indicating the estimated values of the bands seen on the gel in Figure 3.18

1oobp ladder		CP72		CP49		CP29	
Band1	1500	Band1	1000	Band1	1585.27	Band1	1342.97
Band2	1000	Band2	948.68	Band2	1037.55	Band2	900
Band3	900	Band3	715.75	Band3	974	Band3	842.99
Band4	800	Band4	500	Band4	765.17	Band4	782.39
Band5	700	Band5	447.21	Band5	600	Band5	748.33
Band6	600	Band6	400	Band6	564.62	Band6	591.99
Band7	500	Band7	316.63	Band7	515.43	Band7	500
Band8	400	Band8	176.41	Band8	420.87	Band8	382.42
Band9	300			Band9	408.2	Band9	359.09
Band10	200			Band10	334.17	Band10	322.37
				Band11	319.49	Band11	233.41
				Band12	200		

CC35		BP16		BP13		BP20	
Band1	1202.38	Band1	1270.73	Band1	1294.37	Band1	1056.85
Band2	1055.53	Band2	1075.57	Band2	1037.55	Band2	935.27
Band3	974	Band3	935.27	Band3	748.33	Band3	773.73
Band4	773.73	Band4	797.15	Band4	691.75	Band4	748.33
Band5	607.16	Band5	755.71	Band5	576.18	Band5	570.37
Band6	542.2	Band6	587.97	Band6	547.72	Band6	542.2
Band7	515.43	Band7	553.3	Band7	438.23	Band7	433.81
Band8	412.35	Band8	447.27	Band8	396.42	Band8	352.87
Band9	365.35	Band9	404.08	Band9	365.57	Band9	352.34
Band10	334.17	Band10	372.24	Band10	343.37	Band10	337.79
Band11	198.08	Band11	319.54	Band11	300	Band11	231.16
		Band12	310.98	Band12	235.67	Band12	185.14
		Band13	240.26	Band13	188.74	Band13	160.18
		Band14	192.42	Band14	164.88		
		Band15	168.1				

Figure 3.18 A logarithmic graph plotting the bands seen in Figure 3.15 according to GelDoc. sizes of Figure 3.17. 1% STD error bars were assigned to each band-size value.
(1)-CP72; (2)-CP49; (3)-CP29; (4)-CC35; (5)-BP16; (6)-BP13; (7)-BP20



In another attempt to resolve the problem of the differing size values for the same band, rounding of the data to the nearest five, ten, and 20 was carried out. Graphs were constructed following the same procedure as seen in Figure 2.6, Chapter 2. The size values obtained from the GelDoc. were used for this task.

As expected, the logarithmic graph representation of these new values became more organised with an increase in the rounding value of the data (not shown). To determine which rounding value produces the most accurate values, each set had to be treated as an individual set of data. UPGMA trees were constructed (Figure 3.19-3.22) using "Statistica" for comparative purposes through the same process shown in steps 3-4 in Figure 2.6. The type strain and an out-group were not included in these graphs because this was a preliminary study performed to gain insight into solving the fragment size prediction problem.

Figure 3.19 An UPGMA tree created from the un-rounded values of the BOX-PCR products obtained directly from the GelDoc. analysis of the polyacrylamide gels

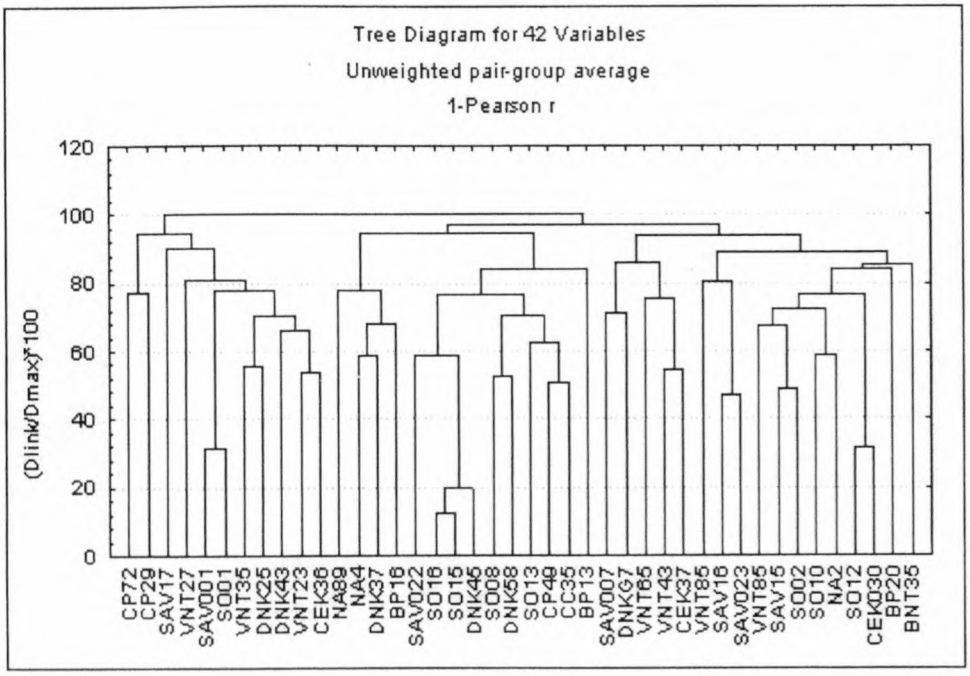


Figure 3.20 An UPGMA tree of the BOX-PCR products created using GelDoc. values rounded to the nearest 5

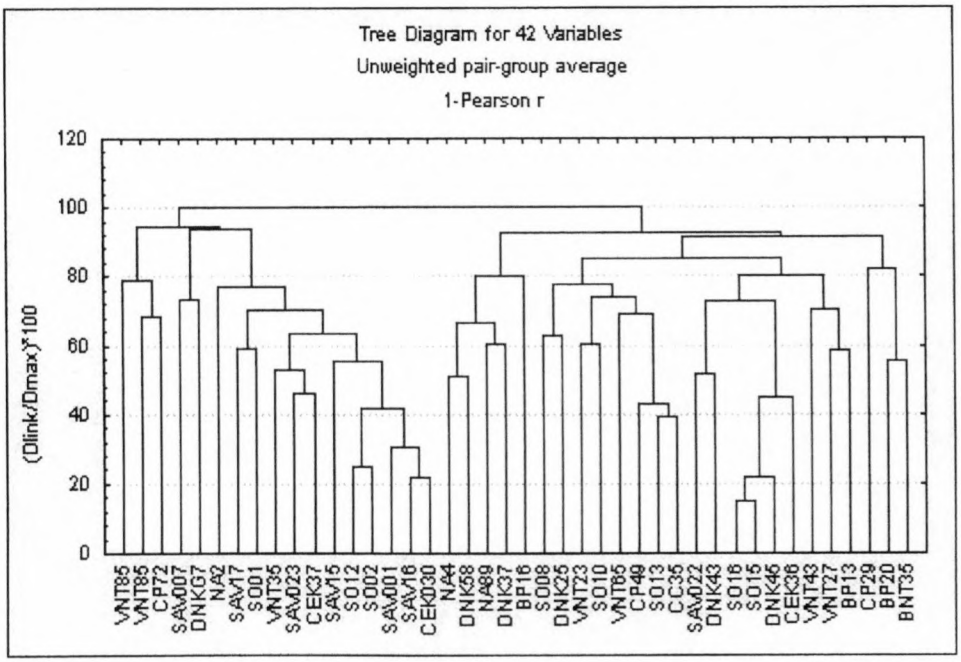


Figure 3.21 An UPGMA tree of the BOX-PCR products created using GelDoc. values rounded to the nearest 10

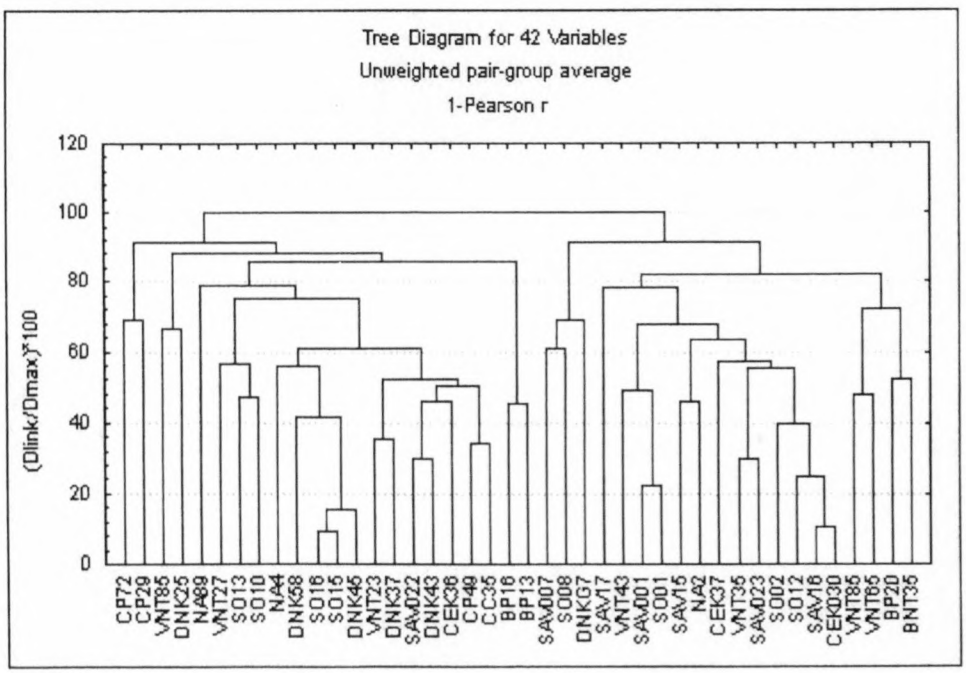
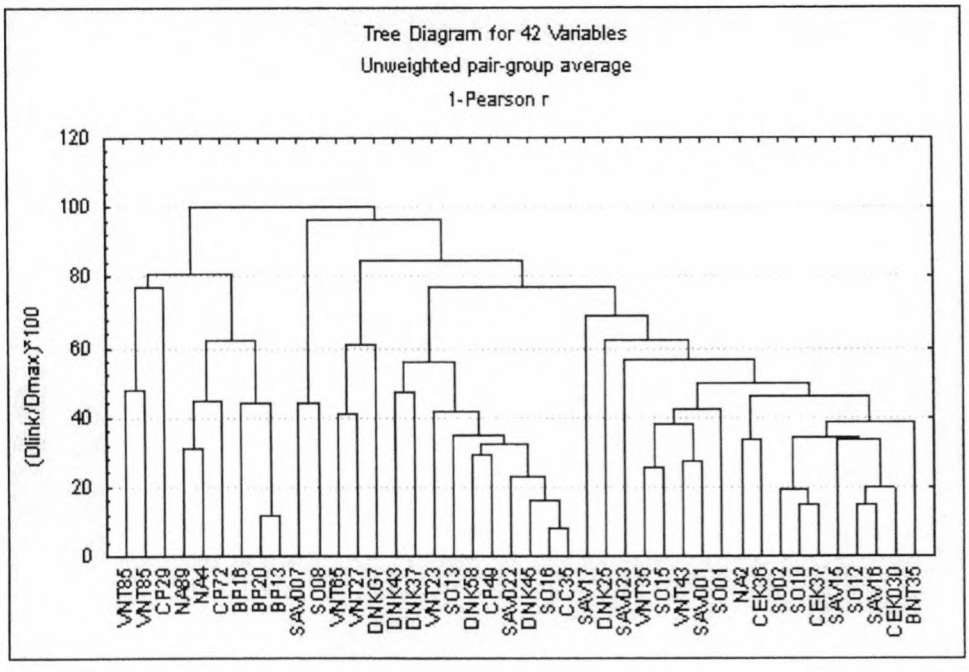


Figure 3.22 An UPGMA tree of the BOX-PCR products created using GelDoc. values rounded to the nearest 20



No grouping consistency was evident between the above four trees. Applying this mathematical model to the analysis of bands on different gels, and to a lesser extent on opposite sides of the same gel, was unsatisfactory. A more complex mathematical model is necessary to resolve this problem, which would be beyond the scope of this study. A subjective way of dealing with this problem would be applicable in analysing the gels. It was decided that under present circumstances, visual analysis would be the most reliable for this type of data. No reported literature has been found that used this mathematical sizing method of gel analysis.

3.9.2 Analysis of PCR products on Metaphor Agarose

The only solution to the problem was to run all the samples next to each other on one gel containing resolution identical to that of polyacrylamide gels. Various agarose gels were tested. Figures 3.23a, b, c and 3.24 display the resolution of different agarose gels.

Figure 3.23a BOX-PCR results analysed on a 6% polyacrylamide gel

Figure 3.23b BOX-PCR results analysed on a 1.8% MS-8 Agarose

Figure 3.23c BOX-PCR results analysed on a 1.5% SeaKem Agarose.

A and B refer to Figure 2.3. The 1kb ladder is not clear in Figure 3.23c. Electrophoresis was repeated to provide the sizes indicated with the arrows

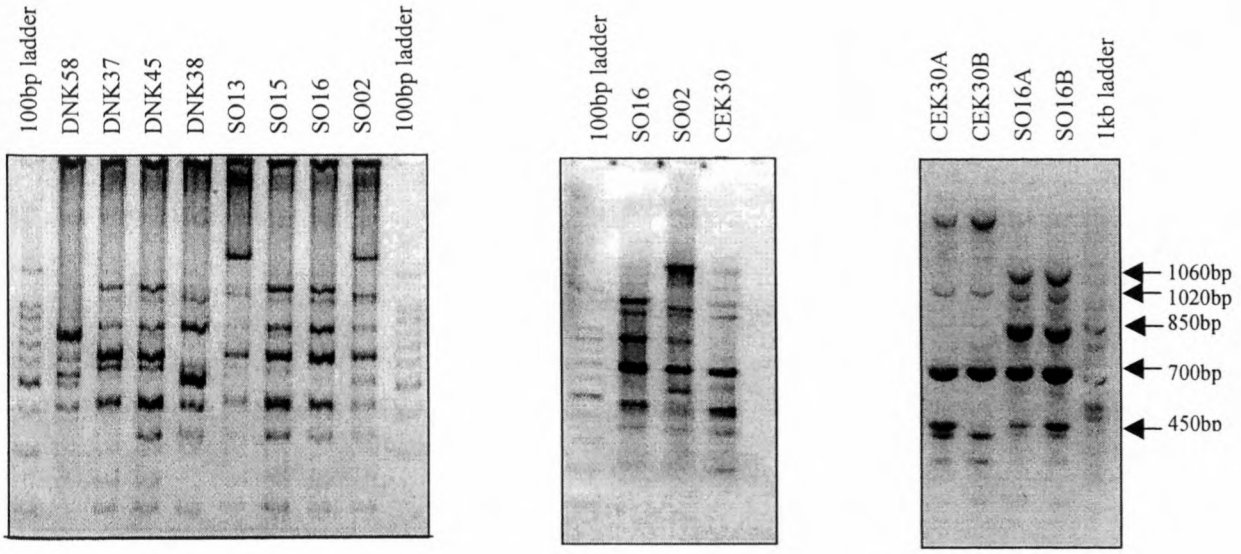
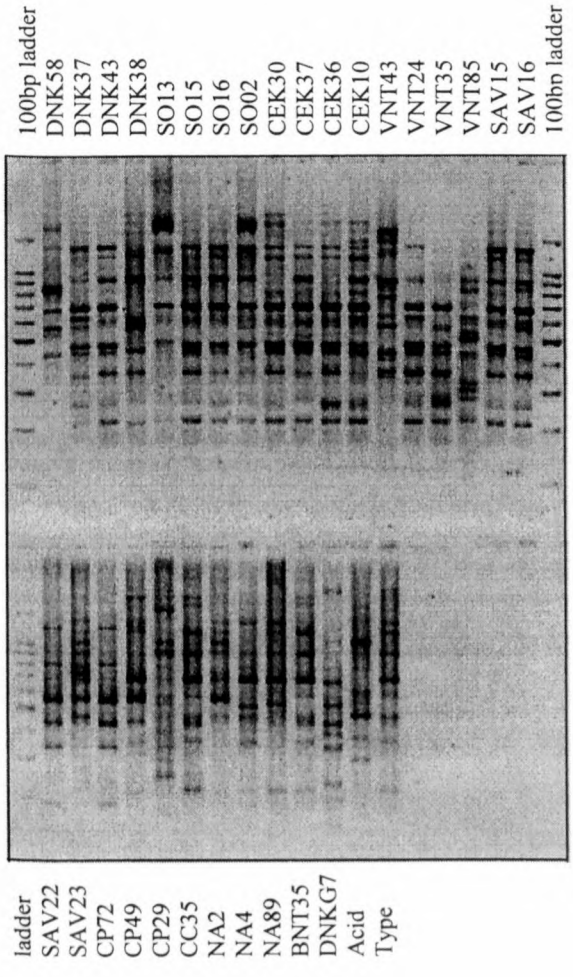


Figure 3.24 BOX-PCR results analysed on 1.8% Metaphor Agarose



Many parameters were involved in getting the gel resolution as clear as possible. Section 2.2.3.7.2 of Chapter 2 indicates the different parameters tested.

The best results were obtained with 1.8% Metaphor Agarose gel (Figure 3.24) run at 4V/cm for 2hrs with 1xTBE buffer, XC loading buffer at room temperature and 5µl of the PCR product being loaded into 4mm wide/ 1mm thick slots. Due to the limited size of the gel apparatus, samples had to be loaded on two different rows within the gel (see Figure 3.24). While visually analysing the standard lanes on these two rows, it became evident that the STD fragments migrate differently depending on the row in which they are loaded. This affects the comparison of the fragments because identical migration is necessary for accurate analysis of the Rep-PCR profiles. A gel tray and gel comb had to be designed to accommodate all the samples next to each other on one row. To account for the large number of samples analysed, the slots were reduced to 1mm thick/ 3mm wide as apposed to the optimum 4mm wide/ 1mm thick. Results were slightly less optimal, but acceptable for the scope of analysis required in this study (see Figure 3.25 for an example of this gel).

The Rep-PCR was repeated to test for reproducibility of the following:

- between successive PCR reactions
- different clones from the same isolate grown on the same solid agar plate
- the same isolate grown and extracted at different times
- the same isolate extracted with different and the same extraction protocols

Reproducibility was acceptable albeit it that a few spurious bands were present. Sadowsky *et al.* (1996) did extensive studies to test for the reproducibility of BOX-PCR on *Streptomyces* spp. (of which 15 were *S. scabies*) and concluded that the BOX-PCR fingerprinting profiles are relatively unique and reproducible despite the fact that the genome of *Streptomyces* spp. are fairly unstable.

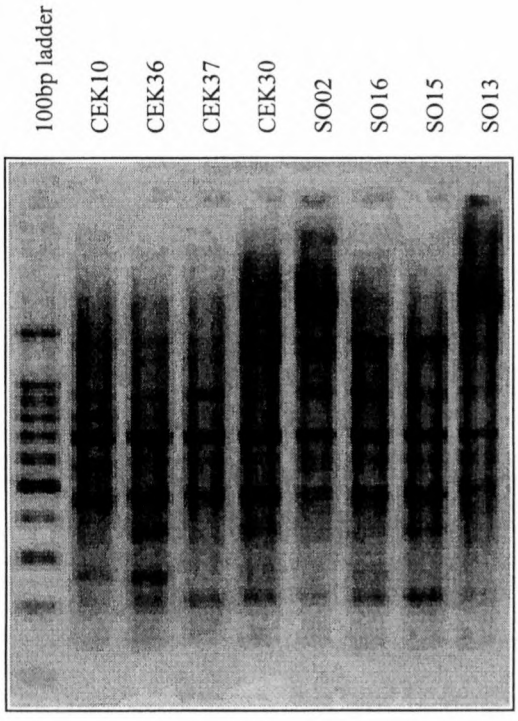
On completion of all the Rep-PCR gels, analyses of the profiles had to be done visually. The best possible means of doing this was to create a mirror image of the Metaphor gels in Excel (Microsoft) after which each band could be numbered, assigning "0" for absent and "1" for present.

The following figures (Figures 3.25- 3.27) display a gel-sample of each Rep-PCR technique and the Excel generated mirror image. The scoring of the Rep-PCR bands (primarily the ERIC and REP profiles) was difficult because a number of bands had to be discarded due to non-reproducibility. Repitition of the PCR and electrophoresis procedure ensured the scoring of only reproducible bands. Still, the identification of the repeatable bands obtained with the ERIC and REP PCRs was

difficult and analyses of more gels than was required for the BOX-PCR, had to be undertaken. The analysis of multiple gels could lead to uncertain results. For this reason, more emphasis should be placed on the clusters formed using BOX-PCR data. The samples producing the suitable and unsuitable patterns were not constant between the three techniques, indicating that the target DNA of the three primer sets were probably different.

Data from the displayed gels (Figures 3.25-3.27) were converted into matrices ("0" indicates absence of band and "1" indicates presence of band). These matrices were converted into similarity data (Nei, 1975) and dendograms depicting the genetic similarity of isolates were constructed. Figures 3.34-3.37 represent the UPGMA clusters (using the PAUP4 package) of the three Rep-PCR techniques and of the combined three techniques. Bootstrap analysis was applied to the UPGMA trees and the values are indicated in Figures 3.28-3.31. Not all the bootstrap values are indicated because the lowest values are considered useless and are eliminated. EF92 (*S. albidoflavus*) was eliminated from the Rep-PCR techniques because only one other type of *Streptomyces* spp. (other than *S. scabies*) is required for comparison purposes and *S. acidiscabies* had already been included. Isolate DNK43 was not included in the REP-PCR dendogram (Figure 3.30) because no fragments were amplified with each REP-PCR replica.

Figure 3.25 A section of a 1.8% Metaphor Agarose gel displaying the BOX-PCR profiles and an Excel generated image constructed from the reproducible fragments of multiple gels



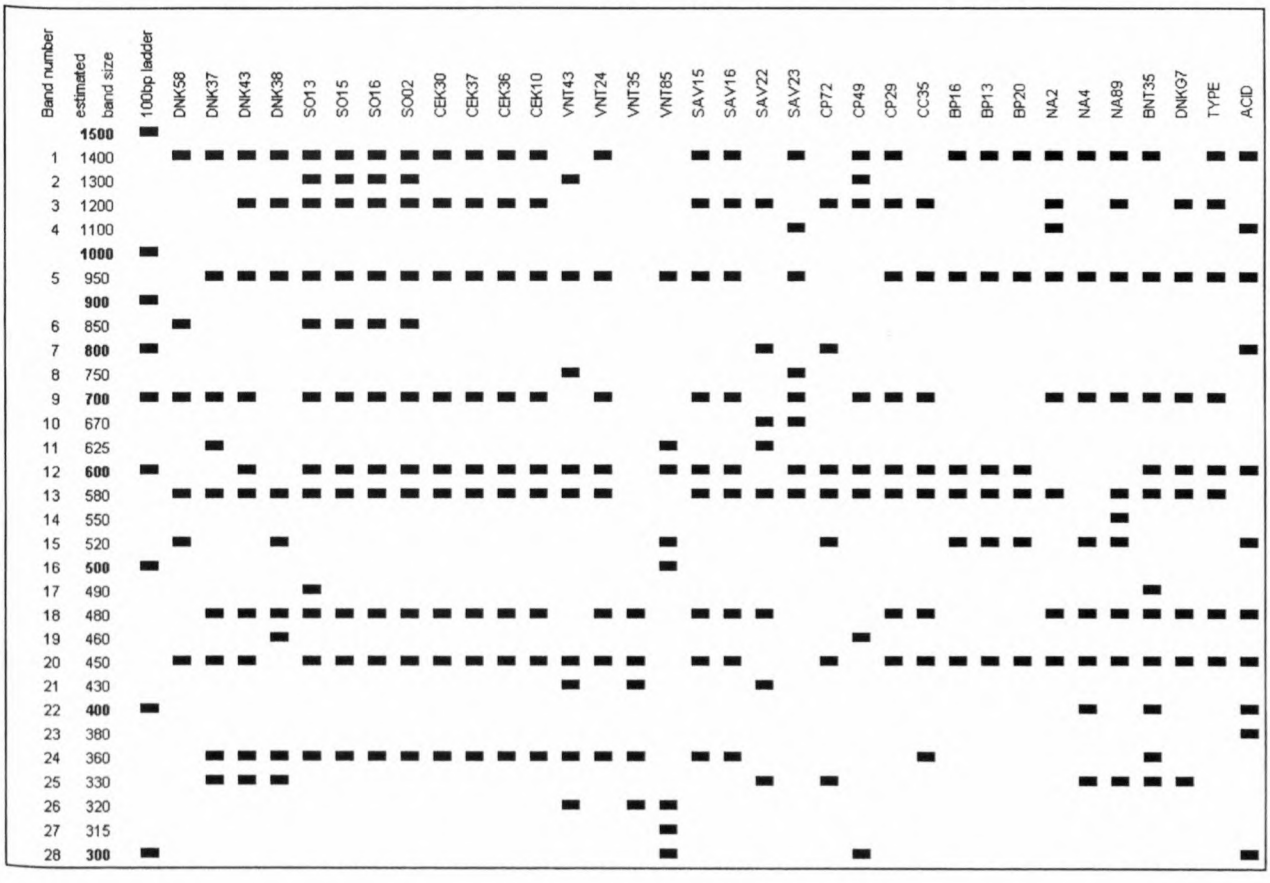
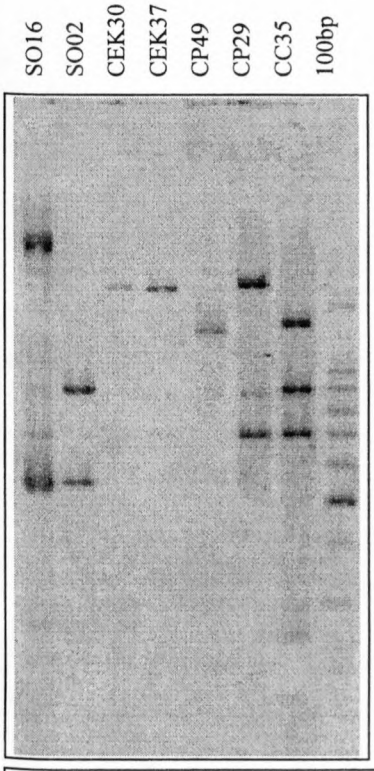


Figure 3.26 A section of a 1.8% Metaphor Agarose gel displaying the ERIC-PCR profiles and an Excel generated image constructed from the reproducible fragments of multiple gels



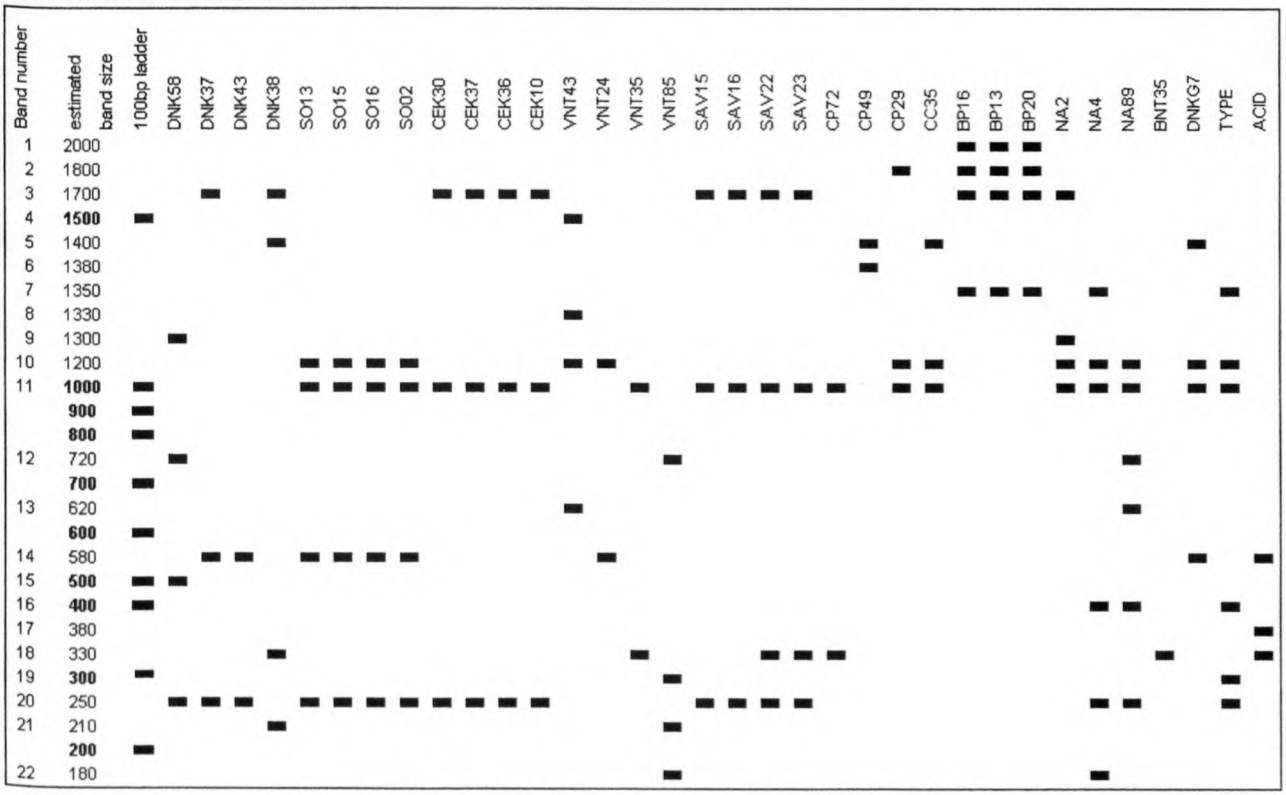
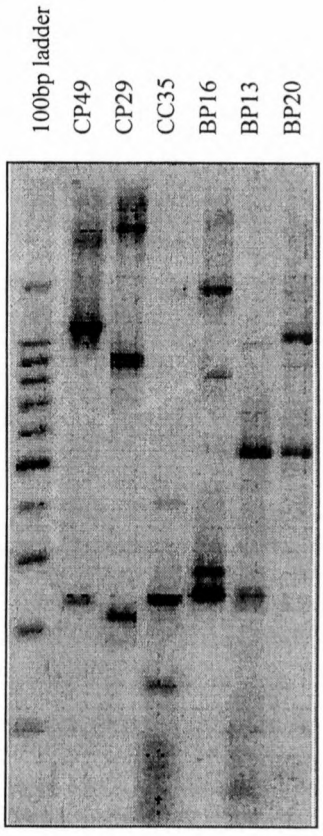


Figure 3.27 A section of a 1.8% Metaphor Agarose gel displaying the REP-PCR profiles and an Excel generated image constructed from the reproducible fragments of multiple gels



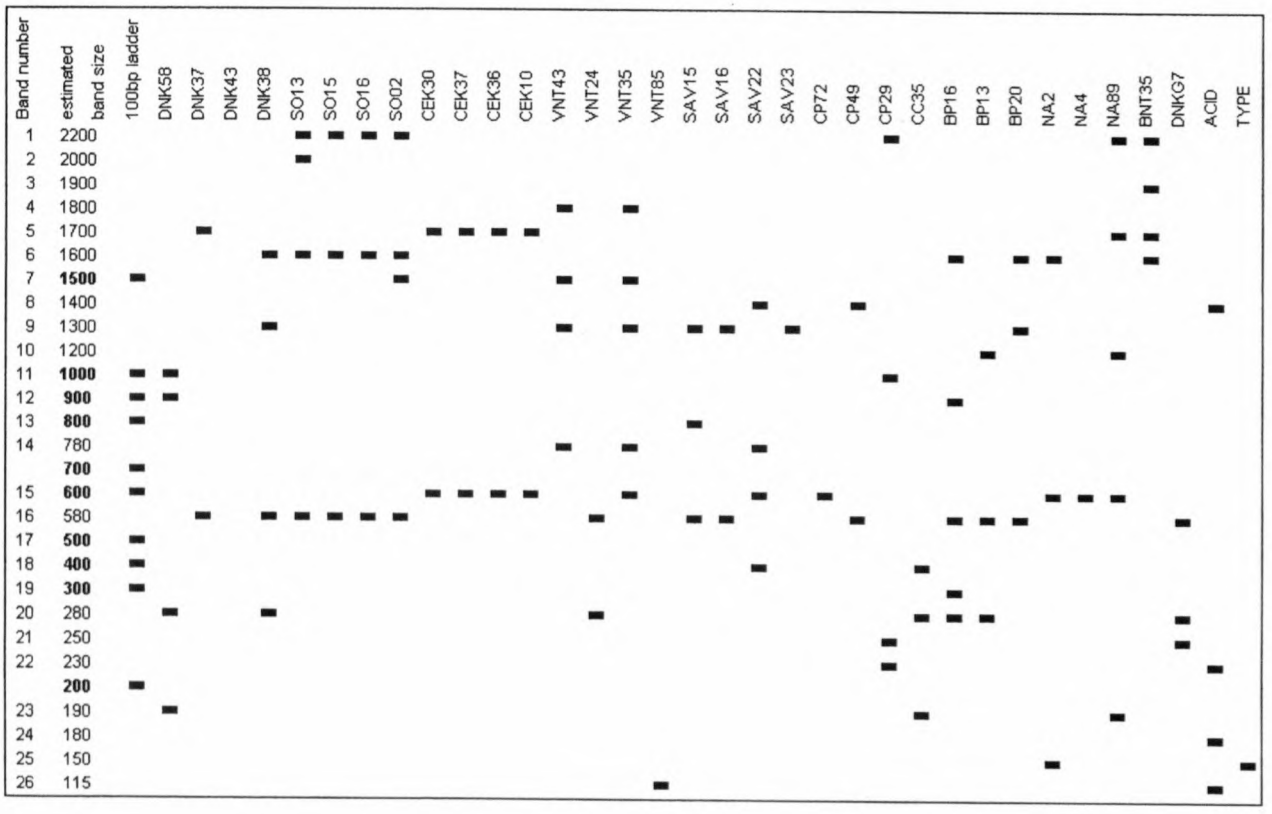


Figure 3.28 BOX UPGMA clusters (PAUP 4)

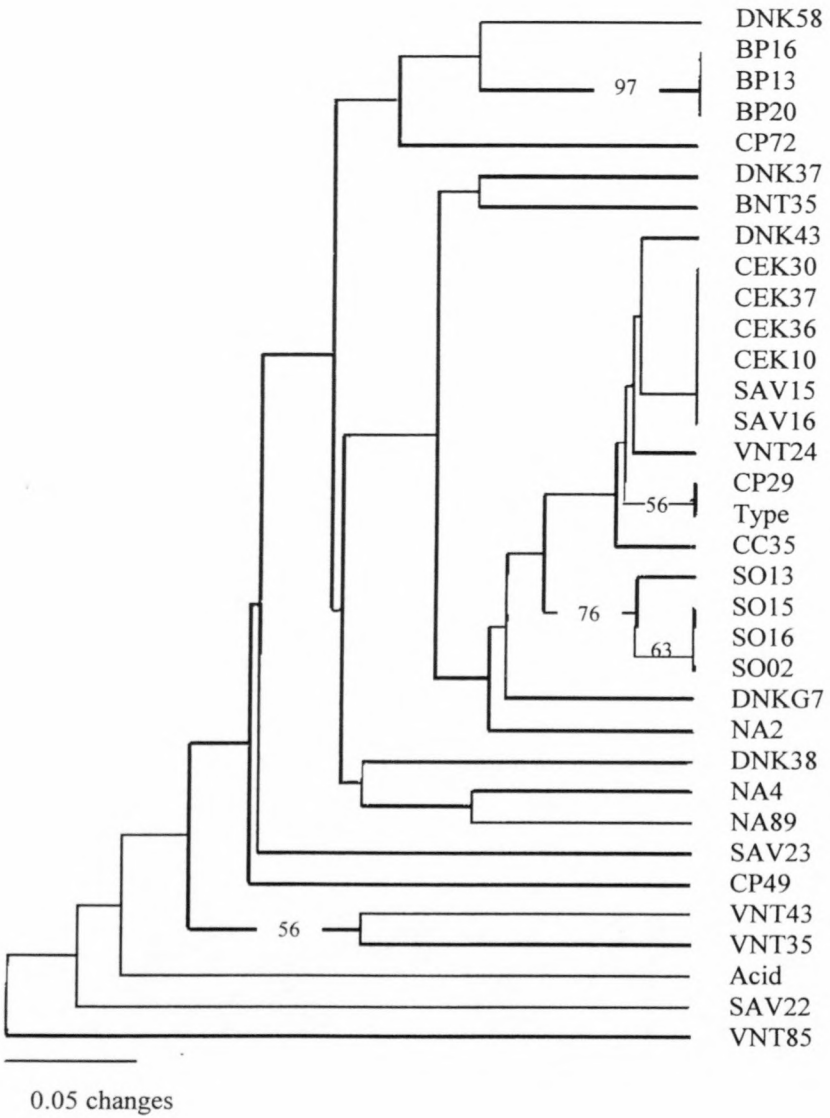
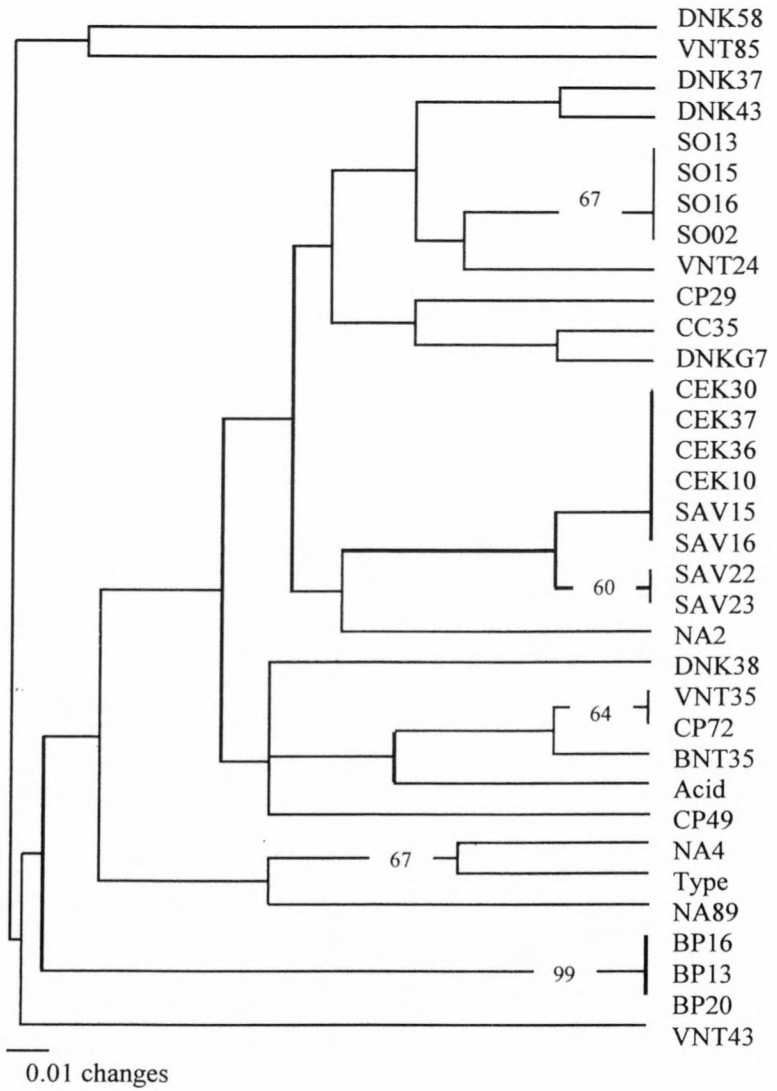


Figure 3.29 ERIC UPGMA clusters (PAUP4)



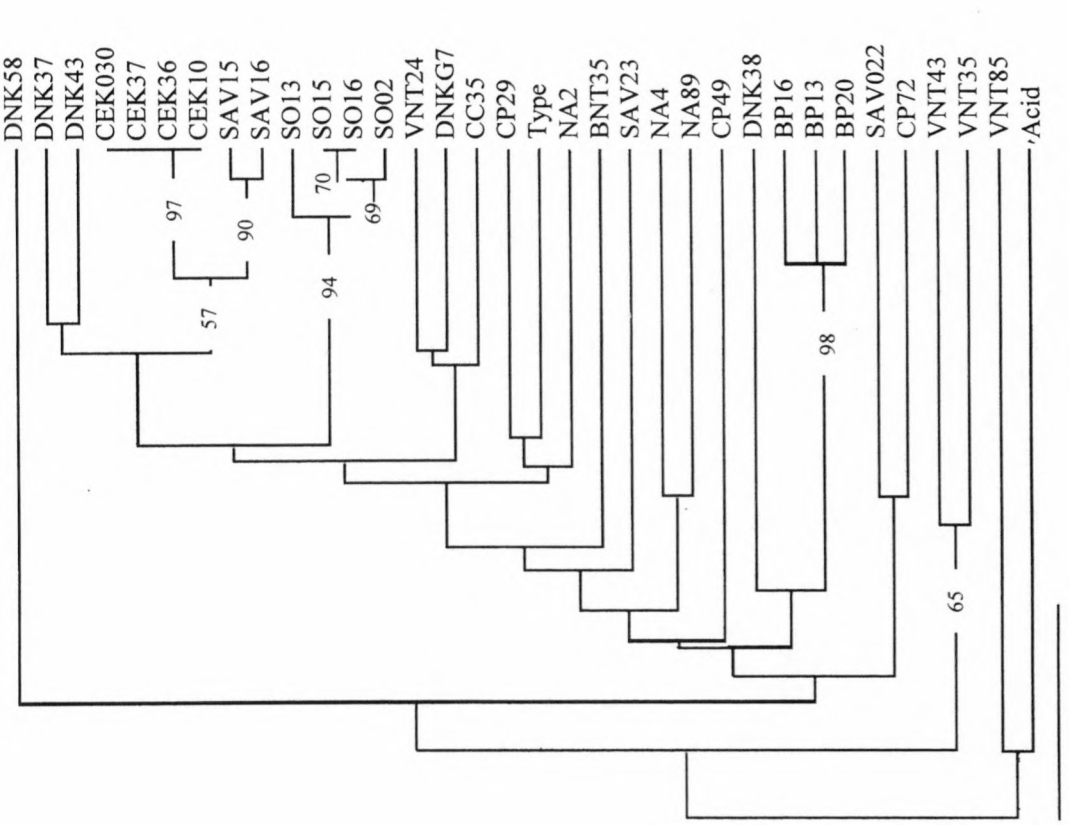


Figure 3.31 UPGMA cluster using combined Rep data

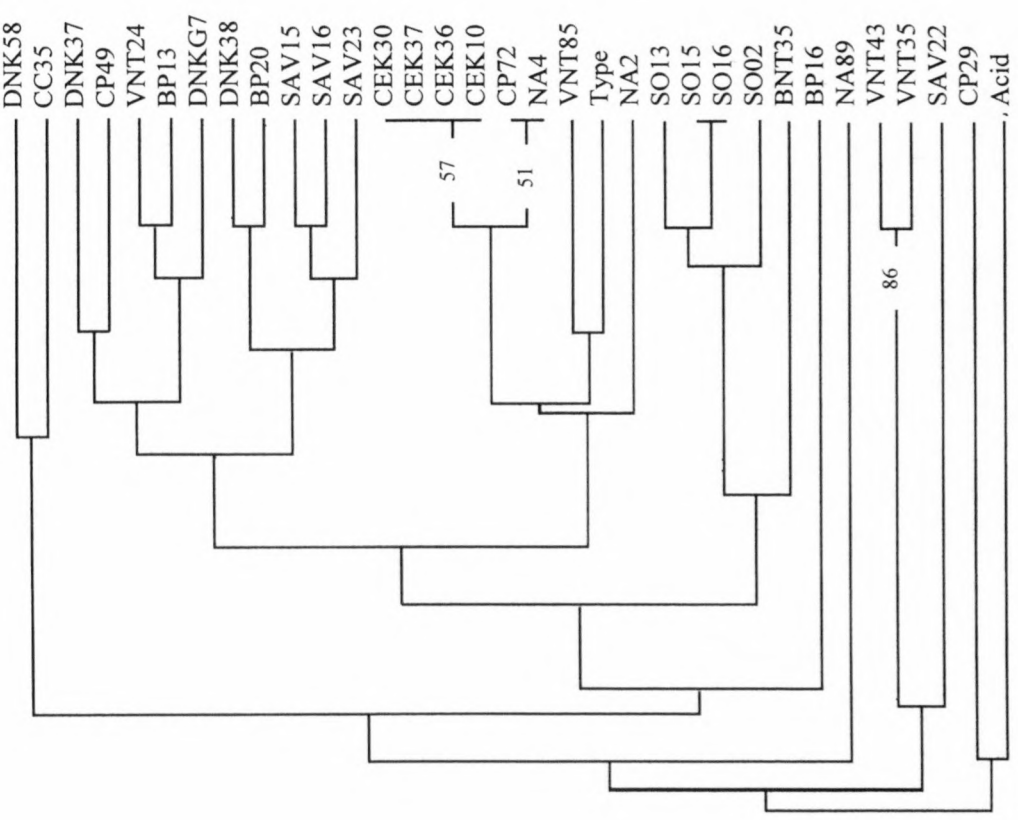


Figure 3.30 REP-UPGMA clusters (PAUP4)

3.9.3 Analysis of the clusters formed with the Rep-PCR technique

UPGMA clusters for each of the three Rep-PCR techniques were constructed using the PAUP4 package as well as "Statistica". Both trees were similar, but not identical. The 16S rDNA clusters were formed with the PAUP4 package, therefore the PAUP4 was used to construct the Rep-PCR trees for consistency purposes. Rep-PCR trees were also analysed using the NJ method with the PAUP4 package (data not shown). Comparison of the NJ and UPGMA trees was also similar and UPGMA clusters with the PAUP4 package were settled on (C Matthee, Zoology department US, personal communication).

Bootstrap analysis with 1000 replicas was applied to the Rep-PCR/UPGMA trees. An overall examination of the dendograms (Figure 3.28-3.31) suggested the existence of genetic diversity between *S. scabies* isolates from different regions. Unlike the high bootstrap values associated with the 16S rDNA dendogram, some of the values for the Rep-PCR clusters were too low to ensure their robustness (lower than 70). The low bootstrap values can be attributed to the fact that the number of characters (Rep-PCR fragments) used in these three techniques was too few. Bootstrap analysis is not appropriate for small character sets (Koji Lum J *et al.*, 2000).

The following groups and their corresponding bootstrap values were observed regarding the four dendograms (Figures3.28-3.31):

- BOX-PCR:
  1)SO13, SO15, SO16, SO02 -76%
  2)BP16, BP13, BP20 -97%
  3)CEK30, CEK37, CEK36, CEK10, SAV15, SAV16 –no value
  4)NA4, NA89 –no value
  5)VNT35, VNT43 –56%
- ERIC-PCR:
  1)BP16, BP13, BP20 –99%
  2)SO13, SO15, SO16, SO02 –67%
  3)CEK30, CEK37, CEK36, CEK10, SAV15, SAV16 –no value
  3a) SAV22, SAV23 –60%
  4)DNK37, DNK43 –no value
- REP-PCR:
  1)VNT43, VNT35 –86%
  2)SAV15, SAV16, SAV23 –no value
  3)CEK30, CEK37, CEK36, CEK10 –57%
  4)SO13, SO15, SO16, SO02 –no value

- Combined Rep-PCR: 1)SO13, SO15, SO16, SO02 –94%
  2)BP16, BP13, BP20 –98%
  3)CEK30, CEK37, CEK36, CEK10 –97%
  4)SAV15, SAV16 –90%
  5)VNT35, VNT43 –65%
  6)DNK37, DNK43 –no value

(No value indicates such a low bootstrap value that it is not considered).

With regard to all four dendograms, the four SO isolates can be accepted as a single group. The BP isolates group together in the BOX, ERIC and combined results. The four CEK (Ceres) isolates appear to group with SAV15 and SAV16 (Sandveld) in both BOX and ERIC dendograms. This is not unexpected, as the two regions are geographically close to each other. The REP dendogram splits the CEKs and the two SAVs into two separate groups. The other SAV isolates (SAV22, SAV23) each remained isolated except for the ERIC dendogram which grouped them adjacent to the others and the REP dendogram which grouped SAV23 with SAV15 and SAV16. VNT43 and VNT35 grouped together in the BOX, REP and combined dendograms while their VNT85 and VNT24 partners each remained separate, indicating more genetic diversity in this group than the others discussed above.

Clark *et al.* (1998) analysed the diversity of 35 *S. ipomoeae* isolates also using the Rep-PCR techniques. The BOX-PCR results grouped all of the *S. ipomoeae* isolates into one group while the ERIC and REP techniques grouped the isolates into different groups. They speculated that the BOX technique amplified conserved regions within the *S. ipomoeae* isolates and ERIC and REP amplified less conserved regions. The different clustering seen with each technique in the current study could thus also be attributed to the target specificity of each Rep primer. Clark *et al.* (1998) included two *S. scabies* isolates in their study and found this group of isolates to possess more diversity than the *S. ipomoeae* species. The REP and BOX profiles were quite dissimilar, as was also seen in our study, and the problems experienced with the ERIC and REP-PCR techniques is confirmed by Clark *et al.* (1998) as they state that inconsistency with the ERIC- and REP-PCR techniques was also experienced by other workers. Inspite of this finding, they have combined the results of the three techniques to provide a more reliable genetic diversity assessment. Although high bootstrap values were also obtained with the combined dendogram in our study, this does not necessarily suggest a more robust indication of diversity analysis, because bootstrap values increase with an increase in the number of data points used. Results obtained using the BOX dendogram were thus chosen as the most conclusive results due to confidence in the BOX-PCR practical procedure.

The BOX dendogram reveals a certain level of grouping within different regions with the following few exceptions:

- the DNK isolates (Northern Cape) did not group together
- two Northern Province isolates (VNT24 and VNT 85) did not group together or with the other VNTs
- two Sandveld isoltes (SAV22 and SAV 23) did not group together or with the other SAVs
- the CP isolates (France) did not group together
- one Minnesota isolate (NA2) did not group together or with the other NAs

These S.A. and overseas isolates could be misclassified or illustrate genetic diversity. BNT35 is the only beet isolate in this study and *S. acidiscabies* is another type of *Streptomyces* spp., validating their isolated positions. Healy and Lambert (1991) states that *S. scabies* and *S. acidiscabies* isolates have a DNA relatedness of only 20%.

Results in this study differ slightly to those of Sadowsky *et al.* (1996) who managed to cluster 15 *S. scabies* isolates from Minnesota into two groups using BOX analysis.

3.10 COMBINED ANALYSIS OF THE CLUSTERS FORMED WITH EACH TECHNIQUE

Analysis of the 16S rDNA derived tree (and Parsimony Network) indicates that most isolates, from South Africa and overseas, grouped in one major cluster or very near to it. The BOX-PCR derived tree indicates grouping within the regions of bacterial origin. This geographical clustering observed with the BOX technique could be explained by short distance distribution of the bacteria as wind or rain borne spores, suggesting that all isolates from one region could be descendants of a single clone. This is supported by the fact that *S. scabies* isolates reproduce asexually (Chater, 1989). The level of geographical differentiation among *S. scabies* isolates could also be due to local adaption to alternative hosts (Peltonen *et al.*, 1996).

Isolates which displayed unexpected positions in both 16S rDNA and BOX data included VNT85, SAV022, CP29 and BNT35. Stray isolates could be genetically different forms of *S. scabies* or they were misclassified. The exclusion of EF92 isolate (Canada) from the major 16S rDNA group is acceptable because it is another type of *Streptomyces* spp, *S. albidoflabvus.* The other stray isolates found with the BOX results (DNK37, CC35, NA2, type strain) that grouped inside the major group of the 16S rDNA dendogram, could be attributed to the fact that the 16S rDNA sequencing technique samples a very limited region within the genome.

Studies of genetic diversity within the *S. scabies* group have revealed high diversity between the isolates. Goyer *et al.* (1996b) divided *S. scabies* isolates from Eastern Canada into two groups using DNA-DNA hybridisation data and fatty-acid analysis. Sadowsky *et al.* (1996) and Doering-saad *et al.* (1992) indicated the lack of correlation between the BOX-PCR and RFLP clusters. These researchers suggested a high level of genetic diversity within the *S. scabies* group of organisms. The results concerning the 16S rDNA sequences do not correspond to the published literature indicating genetic diversity. Perhaps this is a poor technique to use for the analysis of genetic diversity within a species. The analysis of the BOX-PCR profiles of *S. scabies* isolates from different regions reveal more genetic diversity than the 16S rDNA sequencing data.

Comparison of the trees obtained in this study were compared to the data received from Roodeplaat which includes bacterial morphology, physiology, toxicity, origin and pathogenicity (see Appendix C). No correlation is evident with the data from this study and the data from VOPI, ARC because the isolates within one region displayed different morphologies. Within the different regions, pathogenic as well as non-pathogenic isolates were sampled. Lambert and Loria (1989b) and Loria *et al.* (1997) found that the *S. scabies* group is quite homogenous using morphological and physiological characterisation criteria. The data from VOPI, ARC suggests a considerable amount of morphological diversity within the *S. scabies* isolates. This may be attributed to the fact that different morphological tests were carried out by the respective research teams. An explanation for the lack of similarity between *S. scabies* morphology (provided by VOPI, ARC) and their origins may be explained by the fact that a very limited number of genes, responsible for the organism's phenotype, may not be included in the target DNA of the techniques tested in this study and hence not portrayed in the results.

CHAPTER FOUR

4 CONCLUSION

The aim of this study was to analyse the genetic diversity of *S. scabies* isolates from different regions in S.A. and group them according to their similarities. This is best achieved by sampling various loci of the entire bacterial genome. The techniques used in this study include AFLPs, Rep-PCR, RAMS, RAPDs, 16S rDNA sequencing and ITS analysis. The PCR amplification of the *nec1* gene was also investigated as a potential test for pathogenicity. Bacterial growth was optimised and DNA was extracted and subjected to all these techniques.

The amplification of the *nec1* region for determination of pathogenicity, was discarded due to non-reproducible results. This technique is not appropriate for pathogenicity determination of South African *S. scabies* isolates. The use of the gene directly responsible for Thaxtomin A production could provide a more reliable technique for this purpose.

The purpose of this study was to rapidly screen numerous DNA techniques and choose the most promising ones for genetic diversity analysis. Rep-PCR and 16S rDNA sequencing did not require too much otimisation and clear repeatable results were obtained reasonably quickly. These would be desirable methods of choice for further genetic analysis studies. The other techniques displayed non-reproducibility of the same isolate extracted on different occasions or poor sequencing results (ITS sequencing). The difficulty in obtaining repeatable results with certain DNA techniques may be due to a number of reasons. Donini *et al.* (1997) reported that highly methylated repetitive DNA of certain plants are not able to be cleaved by certain methylation sensitive enzymes. This, in turn, would result in non-reproducible AFLP results. Millard and Burr (1926) have stated that cultures undergo many changes during the period of growth and that the final appearance can be totally different to their initial appearance. It can be speculated that such changes could be reflected at the DNA level resulting in different PCR profiles of one isolate at different stages of its life cycle. Clark *et al.* (1998) indicated that prolonged subculturing on artificial media for many years and the fact that *Streptomyces* spp. have relatively unstable genomes can result in genetic changes. Although the conditions were kept as constant as possible, culture age on arrival from Roodeplaat was unknown leading to the inconclusive results experienced. Furthermore, the reasons for non-reproducibility with the RAPD results can be attributed to a number of factors such as competition for priming sites, poor reproducibility of faint bands and sample-to-sample differences between reactions (Halldén *et al.*, 1996 and Skroch and Nienhuis, 1995). No referrals of researchers testing

reproducibility using different DNA extraction techniques on different colonies were reported. It was therefore concluded that techniques can be influenced by a number of factors and should be avoided due to their lack of robustness for routine use.

Both of the sequencing and Rep-PCR techniques had their advantages and disadvantages. 16S rDNA sequencing only samples a limited region of the genome (Clark *et al.,* 1998). Another disadvantage of the 16S rDNA sequencing, which was observed during the course of this study, was that the sequences were too similar between the different *S. scabies* isolates. This is not desirable when trying to establish the genetic differences within the same species group (Rademaker and de Bruijn, 1997). The technique is superior to Rep-PCR because it produces unambiguous results, i.e. some of the Rep-PCR bands could appear visually identical, but not in their sequence composition. The chance of error with 16S rDNA sequencing is therefore less than with the Rep-PCR technique. The advantage of Rep-PCR is that no prior knowledge of the genome is required. Although Rep-PCR only samples a limited number of loci within the genome, the overall number of DNA regions analysed is greater than with the 16S rDNA sequencing technique.

The genetic similarities of the Rep-PCR data were calculated and subsequent NJ/ Parsimony Network (16S rDNA) and UPGMA (Rep-PCR) clusters were formed (PAUP4 package). BOX-PCR is more reliable than ERIC and REP-PCR because results were clearer and more consistent. The conclusion of the genetic diversity between *S. scabies* isolates in this study were thus determined with the two techniques, 16S rDNA sequencing and BOX-PCR analysis.

Comparison of trees constructed from the 16S rDNA technique showed that most of the *S. scabies* isolates grouped into one major group and the others were situated fairly close to it. The BOX-PCR grouped most isolates according to their respective origins and this can be explained by the probability of clonal multiplication. Both techniques displayed some unexpected grouping of a few isolates and could be attributed to possible misclassification or to the fact that they are genetically diverse *S. scabies* isolates. The possibility of misclassification should be followed up by research carried out by plant pathologists.

This study was a first attempt to determine the genetic diversity at DNA level and characterise the genomes of *S. scabies* isolates in S.A. It can be concluded that there is some genetic diversity between the *S. scabies* isolates from different potato producing regions in S.A. This should be noted by researchers working on the control of common scab disease of potatoes in S.A. However, this

study should be followed by a more thorough assessment of genetic diversity and further research is warranted such as:

- A more thorough investigation of the bacteria's genome:
  - A region separating the rDNA operons, known as the intergenic spacer (IGS) can be analysed for differences within the *S. scabies* species.
  - A search for other regions capable of displaying differences can be carried out.
- Consistency of the growth conditions and extractions can be explored to eliminate the non-reproducibility experienced with techniques such as RAPDs and AFLPs.
- An application of a mathematical model to analyse bands on different gels, and to a lesser extent on opposite sides of the same gel should be explored for the ease of Rep-PCR profile analysis.

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APPENDIX A

THE MORPHOLOGICAL AND PHYSIOLOGICAL PROPERTIES OF *S. SCABIES* OBTAINED FROM PUBLISHED LITERATURE.

The morphological and physiological properties were obtained from the following articles:

1. Lambert and Loria (1989b): using the following *S. scabies* isolates: RL-39, RL-42 from New York, RL-70, RL-174 from Maine and RL-151 from Canada
2. Lorang et al. (1995); Lambert and Loria (1989b): using the following *S. scabies* isolates: FLII, Beet, BC, ROY, NC, RB2, RB3, RB3II, RB4, RB5, PonP, PonC, PonR (FLII is from Florida and the rest are from Minnesota)
3. Goyer *et al.* (1996b) and Faucher *at al,* (1992): using the following *S. scabies* isolates: EF-5,-35,-56,-49,-54,-63,-64,-68,-84 (Isolated from Quebec, Canada).

Characteristics	Goyer *et al* (3)	Lorang JM and Lambert DH (1, 2)
Colony color on YME medium	Tan to brown	
Spore mass color on: • Salt starch medium • Oatmeal medium • Glycerol asparagine medium • Yeast malt medium	Grey	Gy Gy (Y) Gy (Y,B) Gy (Br)
Reverse mycelium colour on: • Salt starch medium • Oatmeal medium • Glycerol asparagine medium • Yeast malt medium		Gy (Y) Gy (Y, Br) Gy (Y) Br (Y, Gy)
Spore ornamentation	Smooth	
Spore chain morphology	Spiral	

DAP[1] optical isomer		LL
Melanin production on PYI[2] medium	+	
Melanin on tyrosine agar		+
Production of diffusable pigment	-	
Utilization of carbon sources: • L-Arabinose • D-Fructose • D-Mannitol • Raffinose • Rhamnose • Sucrose • D-Xylose • *meso* inositol • D-Galactose • Salicin • D-Glucose (1% wt/vol)	 + + + V V V V	 + + ± +
Utilization of nitrogen sources: • L-Proline • L-Hydroxyproline • L-Methionine	 + V	 + except for FLII
Degradation of xanthine		-
Hydrolysis of: • Arbutin • Polygalacturonic acid • Xylan	 V V V	
Growth at pH 4.5	-	
Minimum growth pH		5.0

Growth in the presence of: • NaCl (4%) • NaCl (5%) • NaCl (6%) • NaCl (7%) • NaCl (10%) • NaCl (13%)	 V - - -	 + + + except FLII, Roy and Pon R
Growth in the presence of: • Tellurite (10 µg/ml) • Tellurite (100 µg/ml) • Thallium (10 µg/ml) • Thallium (100µg/ml) • Thallous acetate (10 µg/ml) • Thallous acetate (100 µg/ml)	 - -	 + for all except Roy and ponR - for all except RB4 - for all except RB4 -
Growth in the presence of: • Crystal violet (0.5 µg/ml) • Phenol (0.1%)	 V V	 - except RB4 + except FLII, Roy, RB2, PonP/C PonC, and the isolates used in the Acidiscabies article from Lambert and Loria
Growth in the presence of: • Penicillin (10 IU/ml) • Oleandomycin (25 µg/ml) • Oleandomycin (100 µg/ml) • Streptomycin (20 µg/ml)	 + V -	 - for all except RB4 -for all except FLII - -

Gy, grey; Y, yellow; B, blue; Br, brown. Colours in parenthesis indicate secondary hue(s) of predominant colours.

-. negative; +, positive; V, between 20 and 90% of the isolates were positive

(1). PYI- Peptone Yeast Extract Iron, (2). DAP- Diaminopimelic acid

APPENDIX B

COMPOSITION OF BUFFERS AND MEDIA

Trace Salts Solution (TSS)

$FeSO_4.7H_2O$	0.1g
$MnCl_2.4H_2O$	0.1g
$ZnSO_4.7H_2O$	0.1g
Distilled water	100ml

Oatmeal Agar

Oatmeal	20g
Agar	18g

Cook 20g oatmeal in 1litre distilled water for 20 minutes.

Filter through a chiffon fabric

Adjust water volume back to 1 litre

Add 1ml TSS

Set pH at 7.2

Add 18g agar

Sterilize

Inorganic Salt Starch Agar (ISSA)

Solution 1: Make a paste with 10g soluble starch and some distilled water. Adjust volume to 500ml.

Solution 2:

K_2HPO_4 (anhydrous)	1g
$MgSO_4.7H_2O$	1g
NaCl	1g
$(NH_4)_2SO_4$	2g
$CaCO_3$	2g
Distilled water	500ml
TSS	1ml

pH must be 7.0and 7.4

Combine solution 1 and 2

Add 20g agar, sterilize

Yeast Extract-Malt Extract Agar (Isp2 medium)

Bacto-Yeast extract (Difco)	4g
Bacto-Malt extract (Difco)	10g
Bacto-Dextrose (difco)	4g
Distilled water	1l

Set pH at 7.3

Add 20g agar

Sterilize

Trypticase Soy Broth (TSB)

Composition per liter:

Pancreatic digest of casein	17.0g
NaCl	5.0g
Papaic digest of soybean meal	3.0g
K_2HPO_4	2.5g
Glucose	2.5g

pH 7.3

Sterilize

CRM

Glucose	10g
Sucrose	103g
$MgCl_2.6H_2O$	10.12g
TSB	15g
Yeast extract	5g
Distilled water	1l

pH 7.3

Sterilize

APPENDIX C

THE FINAL SET OF ISOLATES FROM VOPI, ARC USED IN THIS STUDY

Isolate no.	origin	YME				Melanin	Toxin	Pathogen-icity
		Spore	Airmyc	Substr	Pigm		Thaxtomin	
CERES								
CEK010 *S. scabies*	Paardekloof	sk	gr201	dbr	-	+	A	0
CEK030A								
CEK36								12.5
CEK37A *S. scabies*	Vredelus	rf	gr201	dbr	-	+	-	0
NORTHERN CAPE								
DNK037 *S.scabies*	Douglas	sl	gr201	br	-	+	A,oA	66
DNK038 *S.scabies*	Douglas	rf	gr201	br	-	+	A	100
DNK043 *S. scabies*	Prieska	spp	gr201	br	-	+	A,oA	100
DNK58								
PEANUTS								
DNKG07 *Streptomyces spp*	N.Cape	rf	gr201	br	-	+	B	50
BEET								
BNT35 *Stretomyces spp*		sl	gr201	br	-	-	-	25
NORTHERN PROVINCE								
VNT024 *S. scabies*	Soutpansberg Nimmersault	rf	gr201	br	-	+	A,B	25
VNT35								
VNT85 *S. scabies*	Pietersburg Hanover	rf	gr201	dbr	-	-	-	0
SANDVELD								
SAV011								
SAV015	Matroosfontein	sl	gr201	dbr	-	+	A,B	62.5

S. scabies								
SAV016								
SAV022								
SAV023 *S. scabies*	Campaynesdrift	sl	gr201	br	-	+	A	25
EASTERN FREESTATE								
SO02 *Streptomy-ces spp*	Bethlehem Anandale	sk	w156	yellow	yellow	+	A	62.5
SO08 *Streptom-yces spp*	Senekal Roodekopjies	sk	w156	yellow	-	+	A	25
SO10 *Streptomy-ces spp*	Reitz Simonsium	sk	w156	yellow	-	+	A	50
SO13								
SO15								
SO16								0
OVERSEAS CULTURES								
NA2 *S.scabies*	Minnesota	sp	gr201	br	-	+	*	0
NA4	Minnesota	sp	gr201	br	-	+	*	0
NA89	Minnesota	sp	gr201	br	-	+	*	75
CP29 *S. scabies*	France	sp	gr201	br	-	+	*	100
CP49	France	sp	gr201	br	-	+	*	100
CP72								
BP13	Australia	sp	gr201	br	-	+	*	*
BP16	Australia	rf	w156	-	-	+	*	100
BP20								
EF92 *S. albidoflavus*	Canada	rf	w156	-	-	-	*	0
CC35 *Streptomy-ces spp*	Louisiana	No sporulation				-	*	0
Type strain ATCC 41973	Ithaca/ New York (Loria R- Cornell University)							

Acidiscabies ATCC 49003 (VOPI, ARC)					

Blank spaces and * represent unavailable information.

ABREVIATION	**DESCRIPTION**
YME	Yeast malt extract
Spore	Spore chain
Airmyc.	Air mycelium
Subst.	Substrate colour
Pigm.	Formation of pigment
sk	Short, spiral
sl	Long, spiral
sp	Spiral
rf	Straight, filamentous
dbr	Dark brown
br	Brown
w156	White (shade 156)
gr201	Grey (shade 201)
bl202	Blue (shade 202)
g197	Green (shade 197)
oA	Ortho-A