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CHAPTER 5

GENETIC DIVERSITY AMONG *Alternaria solani* ISOLATES FROM POTATOES IN SOUTH AFRICA

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Submitted to Plant Disease for publication as:

van der Waals, J.E., Korsten, L. & Slippers, B. 2003. Genetic diversity among *Alternaria solani* isolates from potatoes in South Africa.

ABSTRACT

The genetic diversity between isolates of *Alternaria solani,* the causal agent of early blight of potato, from various potato growing regions in South Africa, was determined using virulence assays, vegetative compatibility tests and random amplified microsatellite (RAMS) primers. The virulence assays showed low virulence levels for the largest part of the population, but failed to otherwise characterise the population diversity. The vegetative compatibility tests revealed 19 different vegetative compatibility groups, indicating a relatively high level of diversity among the isolates. There was little correlation between geographic region and VCG groupings, and some VCGs were even spread over four of the five geographically isolated potato-growing regions in SA that were investigated. Analysis of RAMS profiles revealed 27% genetic diversity among 46 isolates. This value is relatively high for an asexually reproducing fungus, but is similar to diversity values obtained previously by authors studying *A. solani*. Distance analysis of the RAMS profiles also provided no evidence for geographical clustering of isolates. These results from VCG and RAMS profiles indicate that isolates are randomly spread across South Africa. This fact, together with the high diversity of *A. solani* in South Africa, indicates that the fungus has a high potential to adapt to resistant cultivars or fungicides. This information can be used to aid in the breeding and deployment of early blight resistant potato varieties, and also in disease management of early blight in South Africa.

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5.1 INTRODUCTION

The genus *Alternaria* is widespread and of great economic importance as it causes destructive leaf spots, blights (foliar and blossom), blemishes and damage to stored products or seed of numerous hosts (Rotem, 1994). Mycologists around the world have studied this genus with respect to parasitism, host-range, varietal resistance, physiology, taxonomy and control (ibid). *Alternaria solani* Sorauer is one of the best-known and economically important members of this genus and has also been the subject of many such studies (ibid). This species is the causal agent of early blight of potato (*Solanum tuberosum* L.), tomato (*Lycopersicon esculentum* Mill.) and other members of the *Solanum* family. Early blight on potatoes causes major yield losses in most growing regions of the world. Disease symptoms are characteristic dark brown to black lesions with concentric rings, which produce a "target spot" effect.

Genetic analyses of plant pathogen populations are important in understanding epidemiology, host-pathogen co-evolution, resistance management and control methods (McDonald *et al.*, 1989; Leung *et al.*, 1993; Milgroom & Fry, 1997; Morris *et al.*, 2000; Aradhya *et al.*, 2001). Methods commonly used to assess variation in fungal populations include virulence analyses, vegetative compatibility (VC), biochemical and molecular analyses (Leung *et al.*, 1993; Leslie, 1996; Milgroom & Fry, 1997). Of these, molecular techniques such as isozyme analysis and the random amplified polymorphic DNA – Polymerase Chain Reaction (RAPD-PCR) method have been used to study populations of *A. solani* in the USA (Petrunak & Christ, 1992; Weir *et al.*, 1988). Various other authors have provided evidence for the existence of races of *A. solani*, based on morphological, physiological and virulence differences (Bonde 1929; Neergaard, 1945; Henning & Alexander, 1959). Little is, however, known about the population diversity of *A. solani* in South Africa.

The amount and distribution of genetic variation within and between populations in phytopathogenic fungi is of interest in plant pathology as it gives an indication of the potential for the development of pathogenic specialisation and fungicide resistance (Adachi *et al.*, 1993; McDonald & Linde, 2002). It gives an indication of the fitness of the pathogen to circumvent effects of natural or artificial stresses on the population, and thus counteract control measures, such as fungicide applications (McDonald *et al.*, 1989; Adachi *et al.*, 1993; McDonald & Linde, 2002). The objective of this study was thus to determine the levels of genetic variation among *A. solani* isolates causing early blight on potatoes in South Africa. Information on the diversity and spatio-temporal distribution of this species on potato in South Africa can contribute to the development, assessment and accurate deployment in time and space of resistant potato germplasm.

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In this study, three techniques were used to assess the intraspecific diversity of the population of *A. solani* isolates, namely virulence assays, VC and the random amplified microsatellites (RAMS) technique (Zietkiewicz *et al.*, 1994; Hantula *et al.*, 1996). The RAMS technique combines the advantages of RAPDs (no prior knowledge of the organism's sequence is required and a single reaction can produce many polymorphic markers) with those of microsatellite analyses (efficient and accurate for intraspecific studies) (Hantula *et al.*, 1996, 1998). As the chance of finding polymorphisms using RAMS is greater than in other techniques, including RAPDs, it is a promising technique to use for studies of genetic variation (Hantula *et al.*, 1996) and was therefore chosen for this study. Correlation between the RAMS profiles obtained, virulence, vegetative compatibility and geographic origin was also sought.

5.2 MATERIALS AND METHODS

5.2.1 Collection, identification and maintenance of isolates

Early blight infected plants from various potato production regions and cultivars were obtained from potato growers, or collected during field trips from 1999 to 2002. Material was surface sterilised in 1 % active NaOCl for 3 min and then washed twice in sterile, distilled water. Squares with sides of approximately 5 mm were excised from the lesion edges and plated on V8 juice agar or potato dextrose agar (PDA) (Biolab, Midrand, South Africa) and incubated at 25 °C. *Alternaria solani* cultures were identified on the basis of morphological characteristics, spore size and shape. Single spore cultures were made from pure cultures of the fungus. The Plant Protection Research Institute (PPRI) in Pretoria, South Africa, confirmed the identity of selected isolates. These isolates were allocated PPRI numbers. Representative isolates were deposited in the Culture Collection of the Forestry and Agricultural Biotechnology Institute (CMW), University of Pretoria, Pretoria. A description of isolates used in this study can be found in Appendix B.

5.2.2 Virulence assays

Tissue culture plants

Due to the difficulties involved in evaluating the virulence of pathogens (Henning & Alexander, 1959), a novel virulence assay technique using clonal tissue culture plants was designed in an attempt to eliminate most of the host variation that normally causes errors in

such assays. Eight-week-old tissue culture plants from a BP1 clone (moderately susceptible to early blight) (Visser, 1999) were inoculated in test tubes by placing a fully colonised 5 mm diameter V8 juice agar disc, mycelium-side down, on the first fully expanded leaf of each plantlet. The control plantlet received a sterilised, uncolonised block of agar. Twenty isolates were used in the trial, representative of the different areas in South Africa, from which samples were collected. Five plantlets were inoculated per isolate and the experiment was repeated. Plantlets were incubated at 25 °C with a light regime of 12 h near-UV / 12 h dark. After one week, plants were analysed and rated for disease severity, using the following rating scale:

0 No symptoms

1 Slight necrosis

2 Whole leaf necrotic

3 Leaf, petiole and other plant parts necrotic

Detached leaves

Detached leaves of BP1 plants (moderately susceptible to early blight) (Visser, 1999) grown in glasshouses were used in this assay. The same 20 isolates used for the tissue culture plantlet inoculations were used in this study. For each isolate, five fully expanded, unwounded leaves were inoculated and the experiment was repeated. Agar discs were cut from the edge of actively growing *A. solani* cultures using a 5 mm diameter cork borer. One disc was placed mycelium-side down on the adaxial side of each leaf. The leaves were placed in moisture chambers and incubated at 25 °C in a 12 h near-UV / 12 h dark regime. Leaves were rated for disease severity after one week as follows:

0 No symptoms

1 0-30 % of leaf area necrotic and chlorotic

2 31-60 % of leaf area necrotic and chlorotic

3 61-100 % of leaf area necrotic and chlorotic

5.2.3 Vegetative compatibility

Preliminary studies were conducted using different media and different numbers of isolates per Petri dish (90 mm diameter) to optimise the incompatibility reaction. The reactions were most distinct on PDA when six different isolates per Petri dish were incubated in the dark at 25°C for approximately two weeks. Cubes of PDA, with sides of approximately 3 mm, were cut from actively growing cultures and placed approximately 20 mm apart on PDA in the 'dice number six' pattern. Reactions were scored after 14 days. Pairings were repeated to confirm results. Rayner (1991) stressed the need for controlled self-pairings to distinguish

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between true incompatibility and mutual staling or nutrient depletion. Reactions between paired isolates were, therefore, always scored alongside control reactions where isolates were paired against themselves (positive control) and against isolates known to be incompatible from initial trial experiments (negative control). Reactions were scored as compatible or incompatible. Isolates were scored as vegetatively incompatible when they formed a brown line, visible from underneath, at the interface where the mycelia of the two isolates met. Vegetatively compatible isolates produced no detectable interaction zone and the mycelia mixed freely.

5.2.4 Random amplified microsatellites

For preparation of chromosomal DNA, mycelium from actively growing single spore isolate cultures on PDA was used to inoculate liquid malt extract medium (ME) (Biolab, Midrand, South Africa). These cultures were incubated at 25 °C on a shaker for approximately two weeks. The biomass was harvested, freeze-dried and the mycelium ground in a mortar under liquid nitrogen. DNA was extracted using a modification of the method of Raeder & Broda (1985). The chloroform extraction step was repeated several times until the interphase between the aqueous and upper phases was clean from contaminating proteins and cell debris. Precipitation of the nucleic acids was done using 3 M Na Acetate and absolute ethanol. After harvesting the nucleic acids by centrifugation, and washing with 70 % ethanol, the pellet was resuspended in 50 – 100 µl sterile SABAX water. Five µl RnaseA (10 mg/ml) (Roche Diagnostics, Mannheim, Germany) was added to the resuspended sample, which was then placed at 37 °C for approximately 5 h to degrade RNA in the sample.

DNA was amplified by the RAMS technique (Zietkiewicz *et al.*, 1994; Hantula *et al.*, 1996), using primers CAC_5, $GACA_4$ and CA_8 (Table 5.1). Reactions were also done using two primer combinations, namely $GACA_4$–CAC_5 and CA_8–CAC_5. PCR reactions were carried out in total volumes of 20 µl under reaction conditions recommended by the manufacturer of BIOTAQ DNA Polymerase (Bioline, GmbH, Germany). A final concentration of 4 µM was used for all RAMS primers. DNA was amplified in an Eppendorf Mastercycler PCR machine (Eppendorf, Hamburg, Germany) with the following programme:

- Initial denaturation cycle of 95 °C for 2 min,
- 40 cycles of 95 °C for 30 s, 44 °C for 45 s, 72 °C for 1 min,
- 1 cycle of 72 °C for 10 min, and
- A hold at 4 °C.

The PCR products were separated on 2 % agarose gels (Roche Diagnostics, Germany) in TAE buffer (1 mM Tris-acetate, 1 mM EDTA, pH 8.0). Gel electrophoresis was 50 min at

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80 V.cm^{-1}. Gels were stained with ethidium bromide and photographed under ultraviolet illumination. All reactions were repeated at least twice, to confirm results.

Table 5.1. RAMS Primers used to determine genetic diversity among *Alternaria solani* isolates from potatoes in South Africa

Primer	Sequence[1]
CAC-primer	5' DBD $(CAC)_5$
GACA-primer	5' HBDB $(GACA)_4$
CA-primer	5' DB $(CA)_8$

[1] The following designations are used for degenerative sites: H (A, C or T); B (C, G or T) and D (A, G or T).

5.2.5. Statistical analysis

Analysis of results from the virulence assays was performed using non-hierarchical classification (Digby & Kempton, 1987). It is often found that non-hierarchical methods provide a more acceptable classification into fewer major groups. The groupings obtained are also more robust to any aberrant similarities between individual pairs of units (Digby & Kempton, 1987).

Scoring of RAMS amplification products between 280 and 1400 bp was performed visually from photographic prints with no correction for band intensity. Isolates were scored for the presence (1) or absence (0) of a given band, and a binary matrix was constructed. Only reproducible bands with the same molecular weight on a single gel were considered to be identical. Calculation of Nei's genetic diversity (Nei, 1973 in Leung *et al.*, 1993) and construction of a dendrogram was performed using POPGENE Version 1.31 (Yeh *et al.*, 1999). Construction of the dendrogram was based on Nei's genetic distances (Nei, 1978) using the Unweighted Pair – Group Method with Arithmetic mean (UPGMA) cluster analysis. Isolates were also grouped according to geographic origin into six subpopulations, and genetic diversity calculated for each subpopulation

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5.3 RESULTS

5.3.1 Virulence assays

Similar results for all isolates were obtained from both virulence assays. Non-hierarchical grouping of results from virulence assays on detached leaves and tissue culture plants showed that isolates could be divided into two main groups (Table 5.2). Isolates in Group 1 had average virulence ratings of either 0 or 1, while those in Group 2 had average ratings of either 2 or 3. Most isolates fell into Group 1, showing low levels of virulence. Geographical region of isolation had correlation with the relative virulence of isolates, although isolates from the Eastern Free State and North West Province production regions grouped only into Group 1 (low virulence).

Table 5.2. Non-hierarchical grouping of *A. solani* isolates from South Africa into two groups based on virulence on potato tissue culture plants and detached leaves of potatoes. Group 1 is least virulent (average ratings of either 0 or 1) and Group 2 is most virulent (average ratings of either 2 or 3)

	Isolate	
Origin	Group 1	Group 2
Eastern Free State	2, 3, 21	
KwaZulu-Natal	11, 34, 35, 42	26, 41
North West Province	13, 16, 17	
Northern Cape	51, 53, 54, 60	10, 58
Sandveld	64	65

5.3.2 Vegetative compatibility

Forty of the 53 *A. solani* isolates associated with potato early blight included in this study could be divided into six distinct multimember vegetative compatibility groups (VCGs) (Table 5.3). The remaining 13 isolates, however, could not be assigned to any of the six multimember VCGs, and thus formed numerous single-member VCGs.

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Table 5.3. Assignment of *A. solani* isolates from potatoes in South Africa to Vegetative Compatibility Groups

VCG	Isolate	Source
1	1, 2, 3, 4	Reitz, Free State
	5, 12, 14, 16	Lichtenburg, North West Province
	6, 8	Christiana, Northern Cape
	11	Cedara, KwaZulu-Natal
2	13	Lichtenburg, North West Province
	18	Christiana, Northern Cape
	19, 21, 23, 24	Reitz, Free State
	26	Kamberg, KwaZulu-Natal
	30	Lion River, KwaZulu-Natal
	35	Dargle, KwaZulu-Natal
3	37, 41	Cedara, KwaZulu-Natal
	39	Lion River, KwaZulu-Natal
4	51, 53	Christiana, Northern Cape
5	59, 60	Kimberley, Northern Cape
	61, 62, 63, 64, 65	Lambert's Bay, Sandveld
6	71, 72, 74	Roodeplaat, Gauteng
	77, 78, 79, 80, 82	Cedara, KwaZulu-Natal
7	7	Lichtenburg, North West Province
8	10	Uitvlug, Northern Cape
9	17	Lichtenburg, North West Province
10	22	Reitz, Eastern Free State
11	29	Roodeplaat, Gauteng
12	41	Cedara, KwaZulu-Natal
13	42	Cedara, KwaZulu-Natal
14	54	Kimberley, Northern Cape
15	55	Kimberley, Northern Cape
16	58	Kimberley, Northern Cape
17	66	Leipoldtville, Sandveld
18	67	Leipoldtville, Sandveld
19	81	Cedara, KwaZulu-Natal

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5.3.3 Random amplified microsatellites

A total of 47 amplification products were obtained for all RAMS primers and primer combinations. These products ranged in size from 200 bp – 1500 bp. Only fragments between 280 bp and 1400 bp were used for analyses, due to the difficulty in reproducing fragments outside this range. Due to the high number of amplification products, the relationship between loci and alleles was not determined and the variation observed was considered an on / off type polymorphism. Among the 46 isolates examined, 44 unique genotypes were found. The gene diversity for all 46 isolates was 0.27 (Nei, 1973 in Leung *et al.*, 1993). The genetic diversity calculated for each population is presented in Table 5.4. The dendrogram showed no apparent clustering of isolates according to geographic origin (Fig. 5.1).

Table 5.4. Genetic variation within each geographical subpopulation of South African *A. solani* isolates (the number of isolates represented in each population is listed in parenthesis)

Population	Genetic diversity
Eastern Free State (7)	0.1388
Sandveld (6)	0.1625
North West (6)	0.1750
Gauteng (5)	0.2360
KwaZulu Natal (13)	0.2408
Northern Cape (9)	0.2654

5.4 DISCUSSION

This study has shown that the genetic diversity among *A. solani* isolates from South Africa is relatively high for an asexually reproducing fungus. Furthermore, there is no clear clustering of isolates according to geographical origin.

The virulence assays in this study showed a moderate degree of variation in virulence of different isolates. This is indicated by the fact that the largest number of isolates (75%) was not virulent or showed low virulence. Although virulence assays are often used to detect variation in fungal populations, this technique has many disadvantages: It is laborious and time consuming; scoring is subjective and influenced by environmental factors; and genes involved in virulence represent a small fraction of the genes in the pathogen genome, and may be subject to strong selection by the host (Michelmore & Hulbert, 1987; Leung *et al.*, 1993).

In addition, it has been shown for *Alternaria alternata* (Fr.:Fr.) Keissl. that virulence determinants are unstable and virulence may be lost during repeated subculturing (Peever *et al.*, 2002). These factors may have contributed to the low levels of virulence and variation thereof recorded for isolates in this study, and hampered the use of these results to characterize the populations of these fungi.

The amount of genetic variation found in *A. solani* using VC and RAMS markers is relatively high, which is uncommon but not atypical for an asexually reproducing species (Leung *et al.*, 1993; Morris *et al.*, 2000). A possible explanation for the high levels of genetic diversity found among isolates of *A. solani* could be natural chance mutations, combined with the fact that the fungus can produce abundant numbers of spores in a relatively short period of time (Petrunak & Christ, 1992; Leung *et al.*, 1993). High levels of genetic variation are usually due to recombination, which occurs sexually through mating, or asexually through the parasexual cycle. However, it is not known if the parasexual cycle occurs regularly in *Alternaria* and there is no known sexual cycle for *A. solani,* although teleomorphs have been found in other *Alternaria* species (Simmons, 1986; Petrunak & Christ, 1992; Cooke *et al.*, 1998).

Analysis of the virulence data, VC groups and RAMS profiles showed no significant difference of *A. solani* isolates according to geographical origin. In fact, two VC groups were each found in four of the five geographically isolates potato-growing regions in South Africa. Previous studies on *A. solani* populations in the United States (Petrunak & Christ, 1992; Weir *et al.*, 1998) have also shown no geographic clustering of isolates. These results indicate that the various geographical subpopulations are not genetically isolated, which may be due to dissemination of spores by biotic and abiotic factors. However, we consider the distribution of potato tubers and vegetative material over a wide geographic area to be one of the main causes of the even distribution of genetic variation of *A. solani* in South Africa. Early blight is one of the few diseases not specified in the South African Seed Potato Certification Scheme (Republic of South Africa, 1998), thus allowing free movement of early blight infected material throughout the country.

This study is the first to investigate VCG diversity within *A. solani* isolates from potato plants in South Africa. Six multimember and thirteen unique single-member VCGs were identified among the 53 isolates tested. Many researchers have used the natural phenomenon of non-self rejection or VC, which is known to occur in Basidiomycetes and Ascomycetes, as a method of assessing genetic diversity in various fungal species (Michelmore & Hulbert, 1987; Rayner, 1991; Meijer *et al.*, 1994; Worrall, 1997; Tsror (Lakhim) *et al.*, 2001). Although the technique is simple to perform, it is not considered by all to be an ideal genetic marker for determining population structures in fungi. The reasons are that the genetics of VC-loci can be very complicated and because this trait normally lacks the level of

polymorphism needed for an accurate assessment of genetic variability (Leung *et al.*, 1993; Meijer *et al.*, 1994). Nevertheless, this technique was useful in this study to track levels of diversity, as well as geographic distribution of isolates sharing a VCG phenotype. No correlation was noted between VCGs and virulence or RAMS profiles, indicating underlying genetic variation within VCGs.

This study is the first to use RAMS markers to assess genetic diversity of *A. solani* isolates from potato plants. The amount of genetic variation obtained is slightly lower than that found in previous studies using other genetic markers (Petrunak & Christ, 1992; Weir *et al.*, 1998). Petrunak & Christ (1992) studied isozyme variability among 54 *A. solani* isolates from different solanaceous hosts and geographic regions across the USA. Weir *et al.* (1998) used RAPDs to investigate genetic variation among 35 *A. solani* isolates from potato and tomato plants, predominantly from locations in the USA, with the exception of one isolate from South Africa and three from Costa Rica. The fact that the genetic diversity among *A. solani* isolates in this study was found to be lower than in previous studies can possibly be attributed to the fact that the *A. solani* isolates used in this study were only from potatoes and not numerous solanaceous hosts.

The amount and distribution of genetic variation within and among populations of phytopathogenic fungi gives an indication of the fitness of the pathogen to circumvent effects of natural or artificial stresses on the population, and thus counteract control measures, such as fungicide applications (Adachi *et al.*, 1993; Mcdonald *et al.*, 1989; McDonald & Linde, 2002). According to McDonald & Linde (2002), pathogen populations with a high degree of gene flow and high genetic diversity, such as is shown for *A. solani* in this study, pose a great risk of breaking down genetic resistance in the host. This could be one of the reasons no South African potato cultivars are available with complete resistance to early blight (Visser, 1999). McDonald & Linde (2002) suggest that in such cases, breeding efforts should concentrate on quantitative resistance, or the development of cultivar mixtures and multilines that can be used in combination with other control strategies. Resistance screening should include as many isolates from different locations as possible, in order to reflect the genetic diversity present in the pathogen. Understanding the genetic diversity of *A. solani* on potato in South Africa will thus aid in future disease management strategies of early blight in this country. Future studies to improve understanding of the population structure and high levels of genetic variation observed in this species should be conducted using additional molecular markers and more isolates from different hosts and from as many potato-growing locations worldwide as possible.

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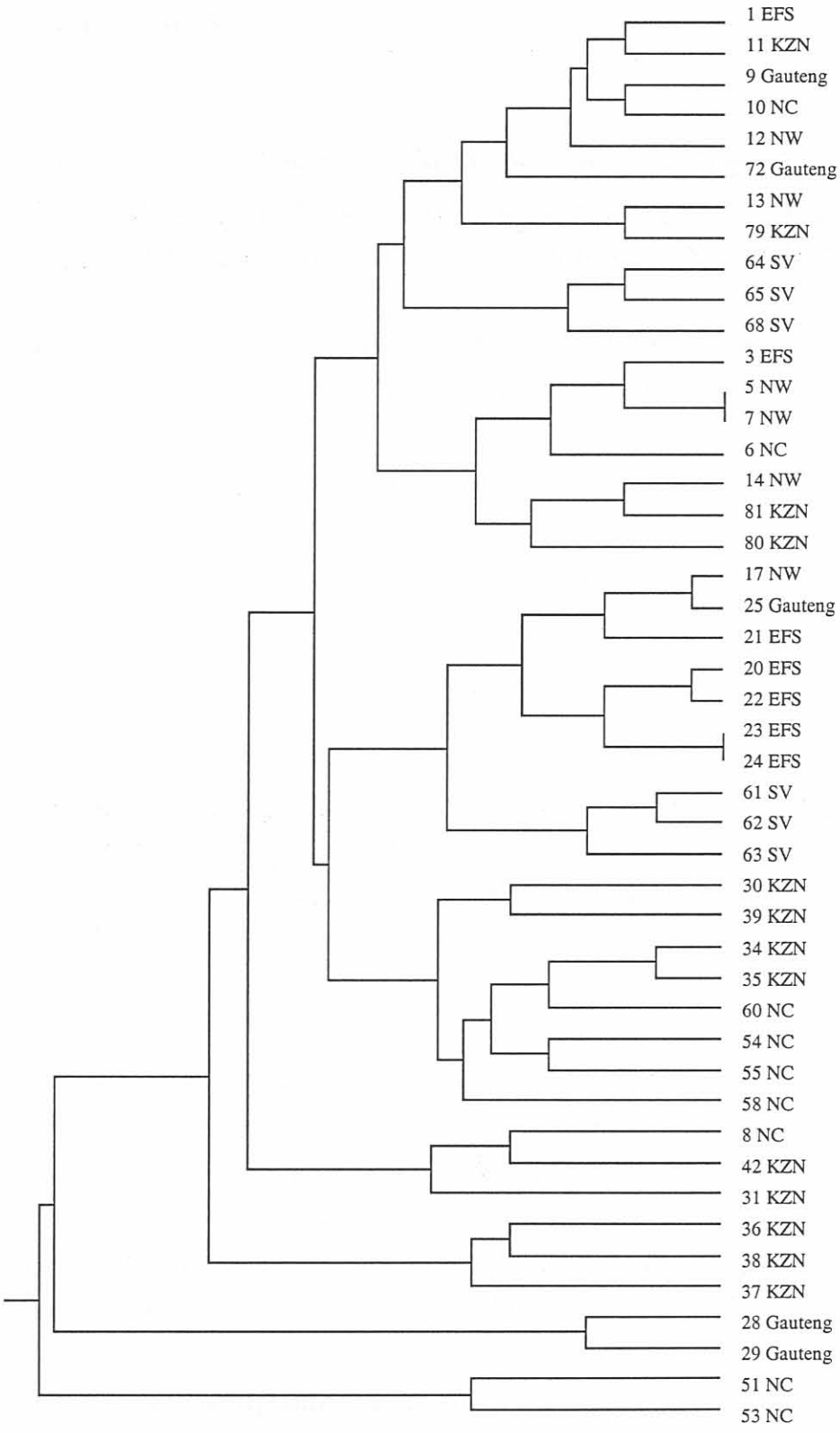

Figure 5.1. Dendrogram of 46 *A. solani* isolates from potatoes in South Africa. UPGMA cluster analysis was based on Nei's (1978) unbiased genetic distances. A description of isolate codes can be found in Appendix B.

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