IDENTIFICATION AND DIFFERENTIATION OF *FUSARIUM* SPECIES USING SELECTED MOLECULAR METHODS

By

Milcah Dhoro

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CONTENTS

TABLES

FIGURES

ABSTRACT

The taxonomy of *Fusarium* at the species level is based on morphological characteristics which include hyphae, conidia and microconidia. These features require expertise in taxonomy for an accurate and reliable diagnosis which is crucial as it aids in disease management and genetic diversity studies. This study aimed to develop alternative and/ or complementary taxonomic tools through the use of molecular based techniques. Initially, 113 morphologically identified *Fusarium* isolates were selected for polymerase chain reaction (PCR) amplification with a set of six inter-simple sequence repeat (ISSR) primers and four universally-primed (UP-PCR) primers to establish identifiable differences and similarities. Genetic variation was also assessed by amplification and sequencing of internally transcribed spacer (ITS) regions of rDNA from 13 isolates using *Fusarium* specific primers. ISSR amplifications employing four primers and UP-PCR analysis using one primer pair revealed scorable polymorphisms among an average of 86 isolates per primer. Some of the isolates did not yield amplifiable DNA with the selected primers used. The four ISSR primers yielded a total of 500 polymorphic bands and the UP-PCR primer pair revealed 126 polymorphic bands. Genetic similarities among the isolates were calculated using Jaccard's coefficient while cluster analysis was used to generate dendrograms showing genetic relationships. The isolates were grouped according to similarity levels.

Results obtained indicated a high degree of genetic variability in the genus *Fusarium*. High intraspecies diversity was observed in *Fusarium oxysporum* and *Fusarium solani* isolates. Some unexpected genetic similarities were observed among the isolates indicating non-agreement between morphological and molecular identification of the isolates. This suggests the need to use species-specific primers in further analyzing the revealed genetic relationships. Sequencing of the amplified ITS regions among the 13 *Fusarium* species revealed two groups with 85% genetic similarity. The ITS regions in the two groups showed a relative similarity ranging from 87 to 100%. A 100% genetic similarity was noted between two *F. oxysporum* isolates which indicated an agreement between morphological and molecular identification. Another 100% genetic similarity was noted among three species, *F. moniliforme*, *F. pallidoroseum* and *F. lateritium* suggesting that species designation can be unreliable if based on morphological data alone. Based on the overall results, the use of molecular methods constitutes an important complement of the morphological criteria needed to allow fungi to be more easily identified

CHAPTER 1

1.0 INTRODUCTION

The fungus *Fusarium* (Ascomycotina: Eumycota) comprises cosmopolitan parasitic species to humans, agricultural crops and forest trees. In Zimbabwe, *Fusarium* species have been recorded as parasitizing over 100 agricultural crops of importance including tobacco (Masuka *et al.*, 2003). In relation to tobacco several species that include *F. solani* and *F. oxysporum* have been recorded as causing sore shin and wilts, respectively (Gordon and Martyn, 1997). On the basis of the number of different economic plant species involved, the wilt diseases caused by *Fusarium* are by far the most numerous.

Fusarium wilt diseases have been reported on crops such as tomato, crucifers, peas, watermelons and bananas. Worldwide, *Fusarium* species annually cause damage worth billions of dollars (Synder and Hansen, 1989). Members of this genus also produce a range of toxic compounds that contaminate food and food products that can adversely affect both livestock and humans.

Fusarium species were traditionally classified in the Deuteromycotina/ Fungi Imperfecti although affinities to Ascomycotina have been established. Within the Hyphomycetes, the genus *Fusarium* is among the most diverse and important pathologically (Synder and Hansen, 1989). There is no universally accepted species delineation concept in *Fusarium*, and, as a consequence, taxonomists disagree on the number of species in the genus.

The taxonomy of *Fusarium* at the species level is based on conventional methods. These include physical macroscopic description of colonies on appropriate media (based on colony growth, texture, colour and pigment) and microscopic description of hyphae, phialides, conidiogenous cells, conidia and microconidia. Hyphae are units of structure of most fungi. Conidiogenous cells are hyphal cells from which a nonmotile asexual spore (conidia) is formed. Phialides are general types of conidiogenous cells open at one end (Alexopoulos *et al.,* 1996). Macroscopic characteristics are variable depending on incubation time and temperature. Microscopic characteristics require expertise in taxonomy for an accurate and reliable diagnosis (Nelson *et al.,* 1981). For such an important genus, the accurate identification of species is crucial, as an aid in disease management and genetic diversity studies.

The need for alternative and/ or complementary taxonomic techniques for the rapid and accurate identification of *Fusarium* species is therefore high, especially in the tropics and developing countries where the species are abundant. These techniques, which include monoclononal antibody development techniques and DNA fingerprinting, provide a realistic opportunity (Bruns *et al.*, 1991). The use of DNA markers in fungal diagnostics and molecular taxonomy is now well established (Williams *et al.,* 1995).

This study, therefore, sought to employ a robust method for the identification of *Fusarium* species collected over a period of three years (2003 - 2005), at one of Zimbabwe's biggest Plant Diagnostic Clinics based at Kutsaga Research Station, Tobacco Research Board.

2.0 LITERATURE REVIEW

2.1 The Genus *Fusarium*

Fusarium is a filamentous fungus widely distributed on plants and in the soil. It is found in the normal mycoflora of commodities, such as rice, bean, soybean, and other crops (Pitt *et al.,* 1994). While most species are more common at tropical and subtropical areas, some inhabit in soil in cold climates. *Fusarium* is a genus of the Hyphomycetes, formerly classified in the Deuteromycetes or Fungi Imperfecti. Within the genus the following 16 sections have been recognized: Eupionnotes, Macroconia, Spirarioides, Submicrocera, Pseudomicrocera, Arachnites, Sporotrichiella, Roseum, Arthrosporiella, Gibbosum, Discolor, Lateritium, Liseola, Elegans, Martiella and Ventricosum. Diverse literature has accumulated on the classification of *Fusarium* species but a system developed by Wollenweber and Reinking (1935), appears to be the most widely accepted (Nelson *et al.,* 1981).

Approximately 1000 *Fusarium* species had been described by 1900, based largely on examination of fruiting structures (sporodochia) on plant material. This large number of species was reduced by Wollenweber and Reinking, (1935) to 65 species, 55 varieties and 22 forms, in 16 sections, and all taxonomic systems proposed since then have been based on this system (Burgess *et al.,* 1994). Gerlach and Nirenberg (1982) recognized over 90 species in *Fusarium*. They proposed delimitation and further emphasized the importance of analyzing variation in a large number of cultures from a wide range of substrates and geographic sources (Burgess *et al.,* 1994).

2.1.1 Species concept

The ways in which systematists define species are as numerous and controversial as are the methods of building phylogenetic trees; but there seems to be some consensus on three basic concepts (Wiley, 1981; Mishra and Donoghue, 1982; Otte and Endler, 1989). A morphological species concept is based on observed similarities of isolates and these are distinguished from other groups of isolates based on discontinuities in the characters (Alexopoulos *et al.,* 1996). Conventional taxonomists regarded this as the working or operational concept since the majority of species, including *Fusarium*, were named based on this model

2.1.2 Biological species concept

The biological species concept defines a species as a natural population or populations of individuals that are potentially interbreeding and are isolated reproductively from other populations (De Queiroz, 2007). This concept is ideal and is readily applicable to animals and can be used for fungi. Species have also been defined as groups of individuals having a shared genealogical relationship determined by phylogenetic analysis (Alexopoulos *et al.,* 1996). This is known as the phylogenetic species concept and the method has found wide and complementary application to the morphological species concept.

2.1.3 Macroscopic and microscopic features

The genetic structure of *Fusarium* species is variable and the morphology of the species is influenced by environmental factors. For many of the species, specific conditions are required for optimal morphology manifestations and the tendency to mutate causes difficulties in identification. Most *Fusarium* species grow rapidly on Sabouraud dextrose agar at 25°C and produce woolly to cottony, flat, spreading colonies.

The only slow-growing species is *Fusarium dimerum*. The colour of the colony may be white, cream, tan, salmon, cinnamon, yellow, red, violet, pink or purple (Fig 1); and on the reverse, it may be colorless, tan, red, dark purple, or brown (Kontoyiannis *et al.*, 2000).

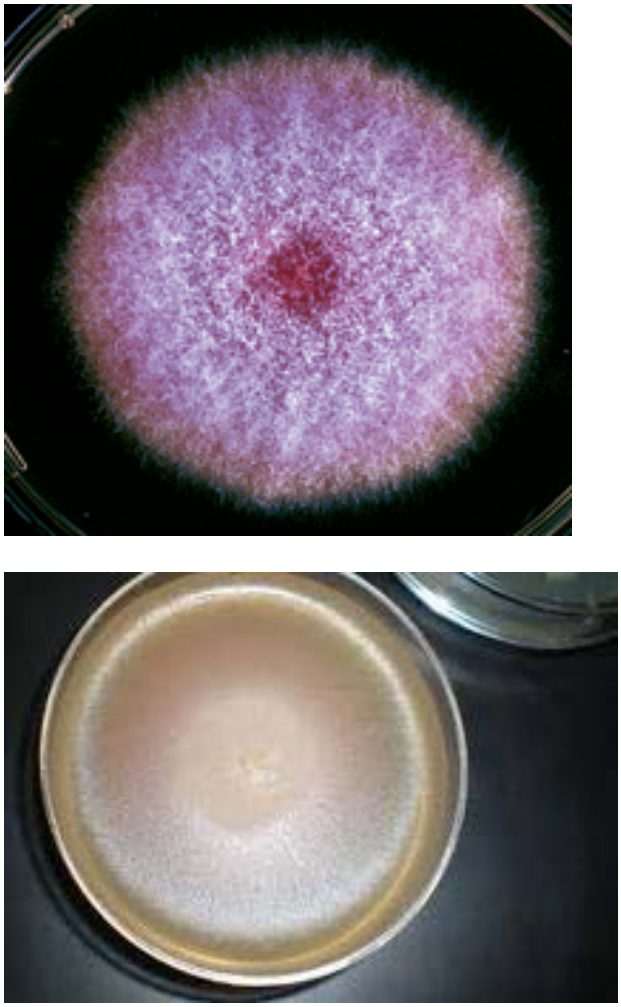

Fig 1. Two types of colonies produced by *Fusarium*. From De Hoog *et al*., 2000.

Sclerotia are masses of hyphae that remain dormant under unfavorable growth conditions; they may be observed macroscopically in some species and are usually dark blue in colour (Arora, 1986). In addition some species have a sporodochium, which is a cushion-like mat of hyphae bearing conidiophores over its surface. This is usually absent in culture. When present, it may be observed in cream to tan or orange color, except for *Fusarium solani*, which gives rise to blue-green or blue sporodochia (Boonk *et al.*, 1998).

Fusarium species produce hyaline macroconidia (3-8 x 11-70 µm) which are septate (Fig 2), and generally have a foot-shaped or notched base to the basal cell. These can be observed microscopically in different species. However, species such as *Fusarium poae* have globose to ellipsoid cells while all other characteristics are similar to those in other *Fusarium* species.

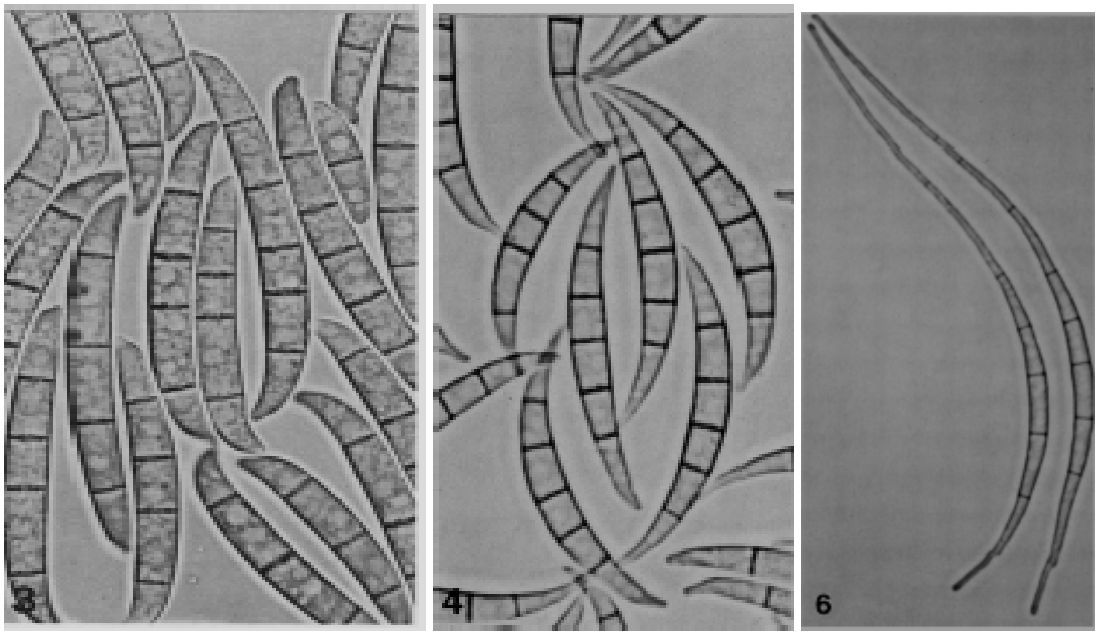

Fig 2. Hyaline septate macroconidia of *Fusarium solani* (3), *Fusarium equiseti* (4), *Fusarium longipeps* (6) .From Nelson *et al*., 1994 .

Macroconidia are produced from phialides on unbranched or branched conidiophores (Boonk *et al.*, 1998). They are two or more celled, thick-walled, smooth, and cylindrical or sickle shaped with pointed distal ends (Fig 2). Microconidia and chlamydospores (Fig 3), which are resting structures produced by hyphae and conidia, may be present or absent. The perithecial states (teleomorph or sexual state) are known for some species and belong to the Hypocreales in the Ascomycotina (Burgess *et al,* 1994).

Microconidia (2-4 x4-8 μm), are formed on long or short simple conidiophores. They are 1-celled (occasionally 2- or 3-celled), smooth, hyaline, ovoid to cylindrical, and are arranged in balls. Chlamydospores, when present, are sparse and grow in pairs, clumps or chains (Fig 3). They can be thick-walled, hyaline, intercalary or terminal. Phialides are cylindrical structures, with a small collarate and maybe solitary or produced as a component of a complex branching system. Monophialides and polyphialides (in heads or in chains) may also be observed (Fig 4).

Macroscopic and microscopic features such as length and shape of the macroconidia, the number, shape and arrangement of microconidia, and presence or absence of chlamydospores are key features for the differentiation of *Fusarium* species.

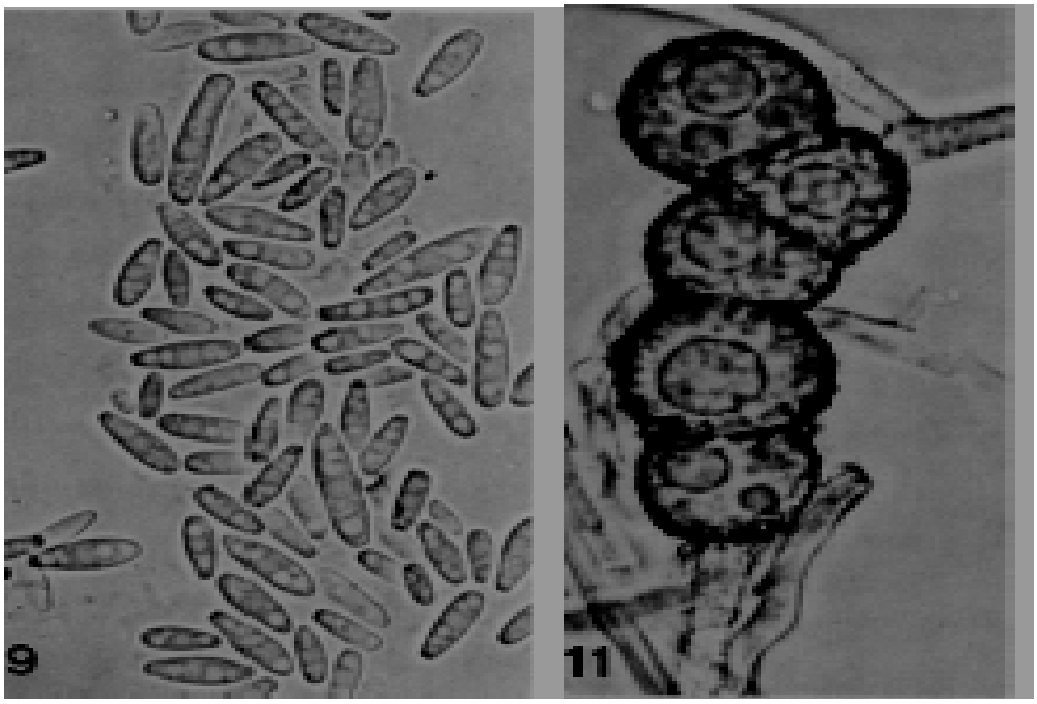

Fig 3. Two types of *Fusarium* spores. *Fusarium moniliforme* microconidia (9) and *Fusarium equiseti* chlamydospores (11). Pictures from Nelson *et al*., 1994.

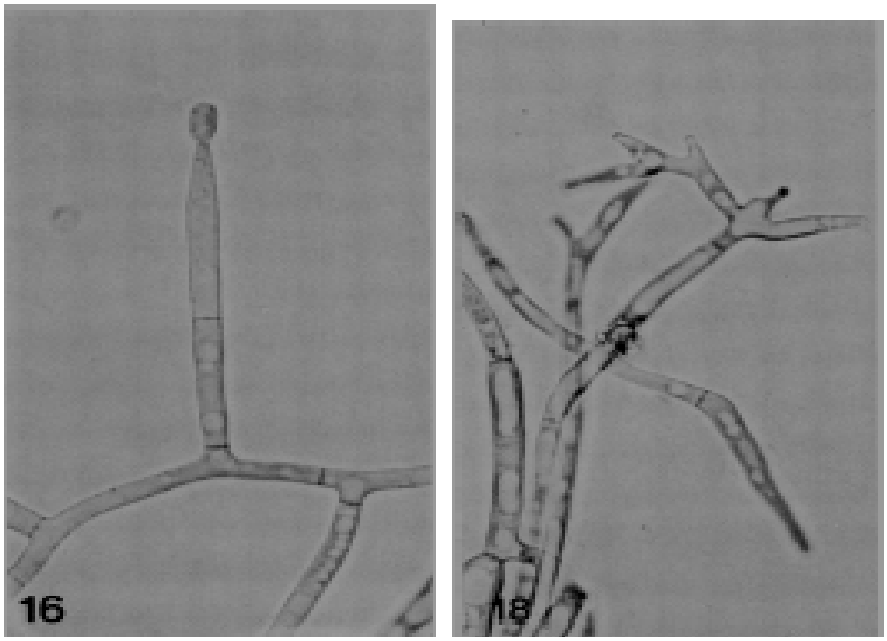

Fig 4. Monophialides and polyphialides of *Fusarium* species. *Fusarium moniliforme* (16), *Fusarium subglutinans* (18). From Nelson *et al*., 1994.

2.1.4 Isolation and ecology

Fusarium species can be found in soil, water and on seeds, roots and leaves of most plants. Several selective media have been developed for the isolation, growth and sporulation of *Fusarium* species, including Selective Fusarium Agar (SFA), Dichloran Chloramphenicol Peptone Agar (DCPA), Spezieller Nahrstoffarmer Agar (SNA) and Modified Potato Dextrose Agar (MPDA). The isolation of *Fusarium s*pecies from plants is affected by the nature of the source material, method of surface sterilization, plating procedures, medium and incubation conditions (Burgess *et al.,* 1994).

The choice of medium depends largely on the nature of the tissue involved in the isolation exercise. Selective media are normally used for the isolation of *Fusarium* species from diseased crown or root samples. There are several other techniques for recovering *Fusarium* species, directly or indirectly, from plant samples, which do not involve plating tissue segments on agar media. Some species produce sporodochia on the surface of the diseased tissue. Macroconidia can be taken from these sites and used to prepare a conidial suspension, which is plated on Water Agar containing antibiotics. Germinated single conidia are later taken to initiate pure cultures for identification of *Fusarium* species (Burgess *et al.,* 1994).

2.1.5 Problems with conventional *Fusarium* species identification

For taxonomic purposes robust nomenclatural and classification schemes, which ideally should reflect natural relationships, have been developed; but taxonomists do not attach the same

weight to the criteria available (Webster, 1980). As a consequence, different species concepts (morphological, biological, phylogenetic) have arisen (Alexopoulos *et al.,* 1996).

Morphological characteristics are fundamental for the identification and taxonomic assignment of *Fusarium*. *Fusarium* species however, exhibit considerable morphological and physiological variability largely attributed to genetic plasticity and to the ability of *Fusarium* species to vary in response to changes in the environment (Burgess *et al.,* 1994). It is, therefore, essential that standard culturing procedures be used in taxonomic studies to minimize variation caused by environmental changes.

Some of the most pathologically important species of *Fusarium* such as *F. oxysporum* do not produce a perfect or sexual state (characterized by presence of sexual structures). Such species are for convenience accommodated in the order Hyphomycetes. *Gibberella fujikuroi,* is a perfect state but is thought to be a species complex that encompasses many *Fusarium* species (Nirenberg and O'Donnell, 1998; O'Donnell and Cigelnik 1998; Steenkamp *et al.*, 1999). The taxonomy of species in this complex has been subject to much controversy (Leslie, 1995) due to a lack of consensus on the morphological species concept for species characterization (Gerlach and Nirenberg, 1982).

In an attempt to resolve this problem, the biological species concept was introduced and eight species have been identified in the complex. These biological species were designated as mating populations A to H (Britz *et al.*, 2002). Fungal species in this complex can also be classified through the application of the phylogenetic species concept and more than 40

different species have been identified (O'Donnell and Cigelnik, 1998). However, the inconsistency between the biological and phylogenetic species concepts introduces serious complications for classification of the fungi. Since many of the *Fusarium* species are still identified on the basis of the morphological species concept, the taxonomic problems associated with *Fusarium* identification are further compounded (Nelson *et al.,* 1994).

2.1.6 Economic importance of *Fusarium*

As well as being common plant pathogens, *Fusarium* species are causative agents of superficial and systemic infections in humans (Mayayo *et al*., 1999). There are species which are highly mycotoxigenic, producing a range of toxins affecting wildlife, livestock and humans (Marasas *et al.,* 1984). The genus has a wide distribution and some of its species occur in all major geographic regions of the world (Burgess, 1994).

Dietary exposure to fusarial toxins causes irreversible tissue damage through biochemical mechanisms that produce pro-oxidative, pro-inflammatory, carcinogenic and/or immune-suppressive effects at a cellular level (Baumrucker and Prieschl, 2002). Some toxigenic species have furthermore been implicated as causative agents of life-threatening opportunistic infections in immune-suppressed humans. Mortalities ranged between 50–80% in these cases (Nelson *et al*., 1994).

Several species produce airborne conidia and are common colonizers of stems, leaves and floral parts of plants (Burgess, 1994). Consequently farming practices, such as conservation tillage are likely to increase the level of inoculum of *Fusarium* species (Summerbell *et al.,*

1989). *Fusarium oxysporum* is one of the most variable species within the genus. It includes populations that cause vascular wilt diseases (Burgess, 1994) and populations that cause root, crown, tuber, corn, and bulb rots (Nelson *et al.,* 1981). The serious wilts, such as tobacco wilts and Panama disease of bananas caused by *F. oxysporum*, are among the most devastating plant diseases in the world.

Fusarium head blight (scab) has recently re-emerged as a devastating disease of wheat and barley throughout the world (Windels, 2000). Undesirable effects of the disease include reduction of grain yield and quality, mycotoxin poisoning in livestock fed with contaminated cereals and mycotoxin carry-over to food products (Chelkowski, 1998). Species such as *F. moniliforme*, *F. graminearum, F. avenaceum* and *F.culmorum* are serious pathogens of Gramineae causing pokkah-boeng of sugarcane, bakanae disease of rice as well as, pre- and post-emergence blight of cereals. In addition, *F. graminearum* is a major fungal pathogen of cultivated cereals responsible for billions of dollars in agriculture losses. Strains of *F. solani* are also of worldwide occurrences, causing root rots and cankers of hardwood trees (Munkvold and Desjardins, 1997).

2.2 Molecular Methods for Identification of *Fusarium*

Molecular biology has offered a number of insights into the detection and enumeration of fungal pathogens and information on identifying unknown species from their DNA sequences (Paminondas and Paplomatas, 2004). In recent years, there has been vast progress in the development of molecular biological tools and technologies (Beckmann, 1988). Each technique can be used as a tool to study variation amongst fungal isolates, and hence provide

important information on genetic relationships, taxonomy, population structure and epidemiology associated with fungi (Cooley, 1991).

The amplification of DNA sequences through the polymerase chain reaction (PCR) has found widespread application in the diagnosis and detection of fungi (Cooley, 1991; Bruns *et al.,* 1991; Reischl and Lohmann, 1997; Alexandrakis *et al.*, 1998; Louis *et al.*, 2000). Unlike fungal culture approaches, PCR does not require the presence of viable organisms for implementation and may be performed with very small quantities of biological material (Abd-Elsalam *et al.,* 2003).

2.2.1 Isozyme technology

Isozymes are varied forms of enzymes, which found extensive application in plant, animal and insect systematics and were later adapted for use in phytopathogenic fungi taxonomy (Bonde *et al.*, 1993). Isozymes usually have similar, if not identical, enzymatic properties but slightly different amino acid sequences (Bonde *et al.,* 1993). Only those isozymes with amino acid compositions of different net charge, or those isozymes characterized by large differences in the molecular shape of an enzyme can be differentiated by electrophoresis.

Isozyme variation analysis was initially applied to fungi whose morphological characteristics exhibited high levels of variation, usually with overlaps between species. Although they could provide adequate levels of polymorphic loci in a number of cases, their use in the study of fungal plant pathogens has been limited because they are subject to environmental influences

that could cause polymorphisms that did not reflect evolutionary events (Bonde *et al.,* 1993). The greatest disadvantage of isozyme analysis is that relatively large quantities of the material must be present, in order to extract sufficient enzymes for detection, compared with that required for immunological or polymerase chain reaction (PCR) techniques.

2.2.2 DNA probes

With the advent of techniques for the isolation, purification, cloning and hybridization of DNA from various organisms, DNA probes were among the first molecular markers applied in the detection, identification and phylogenetic analysis of fungal pathogens (Manicon *et al.,* 1987; Rollo *et al.,* 1987). Species-specific DNA probes generated from cloned random DNA fragments derived from genomic DNA that was digested with specific restriction endonucleases had a number of advantages over classical approaches (Goodwin *et al.,* 1989).

Due to high specificity of probes, a pure culture of the target organism is not necessary. Furthermore, DNA can be extracted from any form of mycelia without the fungus having to produce spores and sclerotia. Since repetitive sequences found in fungal genomes were usually preferred, DNA probes improved the speed, sensitivity and objectivity of the detection and identification of fungi as compared with conventional methods (Paminondas and Paplomatas, 2004).

2.2.3 Restriction fragment length polymorphisms (RFLPs)

Analysis of restriction fragment length polymorphisms (RFLPs) has been extensively used in the detection and characterization of fungi (Goodwin *et al.,* 1990). RFLPs are based on specific differences in the sequence of amplified DNA that results in fragments of different sizes when the DNA is digested by restriction enzymes (Beckmann and Soller, 1983, 1986). Restriction fragments are separated according to their size by electrophoresis and subsequently transferred to nylon membrane filters by capillary forces and immobilized by ultraviolet crosslinking.

Labeled DNA probes are hybridized to the membrane bound DNA fragments and specific bands are visualized by appropriate methods (Paminondas and Paplomatas, 2004). Clonal chromosomal DNA probes were applied to detect various *Phytophthora* species in soil and host tissue (Goodwin *et al.,* 1989). Mitochondrial DNA (mtDNA) probes were subsequently developed because of the nature of mitochondrial genome; it is found in high copy numbers and produces simpler restriction fragment patterns. Such probes were used to differentiate between *Phytophthora spp* that display overlapping of morphological characteristics (Paminondas and Paplomatas, 2004).

This technology has also been applied to distinguishing special forms of the soil-borne fungal pathogen *Fusarium oxysporum*, the causative agent of vascular wilt of a large number of plant species. The genetic relationships of isolates from the taxa traditionally grouped in *Fusarium* sections *Fusarium* (*Discolor*) and *Roseum* have been studied using the RFLP technique, by Southern hybridisation with random genomic and mitochondrial DNA probes originating from *Fusarium* species. Strong genetic relationships have been observed among such isolates (Benyon *et al*., 2000). While RFLPs will continue to be used widely, they have limited use in

routine diagnostic applications. This method is not technically easy, as it requires high quality genomic DNA preparations (Cooley, 1991). Due to technical difficulties associated with mitochondrial RFLP, they were considered not useful for diagnostic purposes (Steenkamp *et al.,* 1999).

Baayen and co-workers (2001) diagnosed *F.oxysporum* and *F. redolens* using RFLP patterns of their internal transcribed spacer regions; but this technique could not differentiate *F. redolens* from the closely related *F. hostae*. Most studies have found RFLPs valuable for taxonomy study purposes only at or below the species level and have used them as additional investigating tools to test classifications already derived from other characterisations such as morphology or host preference (Cooley, 1991; Semagn *et al.,*2006).

2.2.4 Randomly amplified polymorphic DNA (RAPD)

The randomly amplified polymorphic DNA (RAPD) assay involves amplification of genomic DNA at target sites, which are distributed throughout the genome. Using short universal primer sequences, essentially any genomic DNA can be amplified. The technique is especially useful for comparing closely related genomes at the subspecies and pathotype level, and has been used extensively in defining fungal populations at species, intraspecies, race and strain levels because it is simple and requires only small amounts of genomic DNA (Baleiras *et al.,* 1995). RAPDs have the advantage that no sequence information about the target DNA is required (Semagn *et al.,* 2006).

The analysis of DNA from different species results in very divergent electrophoretic patterns. Polymorphic fragments are the result of variation in the number of appropriate primer matching sites of the different DNA (Caetano *et al.,* 1991; Welsh and McClelland, 1991). RAPDs have been tested for their efficacy in differentiating *F. subglutinans* isolates; but due to the low repeatability of RAPD data, the technique is not considered useful for diagnostic purposes (Steenkamp *et al.,* 1999). The non-reproducibility of RAPD patterns due to annealing difficulties of the random primers used, has led to the development of other PCR fingerprinting technology variants (Semagn *et al.,* 2006).

2.2.5 Universally primed-polymerase chain reaction (UP-PCR)

Universally primed PCR (UP-PCR) is a PCR fingerprinting method, which involves specific amplification of target DNA sequences using species-specific primers (Paminondas and Paplomatas, 2004). The UP-PCR technique is similar to the randomly amplified polymorphic DNA (RAPD) technique, but longer primers (approximately 16 to 21 nucleotides) with unique designs are used. The reactions are carried out at relatively high annealing temperatures and result in highly reproducible amplification products from single organisms (Abdelsalam *et al.,* 2002).

UP-PCR has been applied successfully in the identification of differences between populations of fungal pathogens, including *Fusarium* species (Abdelsalam *et al.,* 2002; Bulat *et al.,* 1995). This method has demonstrated its applicability in different aspects of mycology. These applications involve analysis of genome structures, the identification of species, analysis of population and species diversity, demonstrating genetic relatedness at intra - and inter-species level, and identification of UP-PCR markers at different taxonomic levels (strain, group and /

or species) (Lubeck, 2004; Yli-Mattila *et al.*, 2004; Abdelsalam *et al.,* 2002). UP-PCR is an effective tool for the rapid intraspecific typing of strains at the molecular level and for the study of *F. oxysporum* populations (Bulat *et al*., 1995). According to Lübeck *et al.,* (1999), UP primer L45 generates fingerprints with a relatively large number of bands.

2.2.6 Inter simple sequence repeat (ISSR) markers

Inter-simple sequence repeat (ISSR) markers are identified by PCR amplification of DNA using a single primer composed of a microsatellite sequence that may be anchored at the 3'or 5'end by 2 to 4 arbitrary and often degenerate nucleotides (Ziekiewicz *et al.*, 1994; Meyer *et al.,* 1993; Gupta *et al.,* 1994; Wu *et al.,* 1994). The ISSR technology is based on the amplification of regions (100-3000 bp) between inversely oriented closely spaced microsatellites (Fig 5) by single primers (25-30 bp) consisting of several simple sequence repeats (Morgante *et al.,* 2002).

These primers anneal to simple-sequence repeats (microsatellites) that are abundant throughout the eukaryotic genome (Tautz and Renz, 1984; Kijas *et al.*, 1995) and evolve rapidly (Levinson and Gutman, 1987). Prior knowledge of DNA sequence of the genome to be analysed is not required for primer design (Joshi *et al.,* 2000). ISSR markers rapidly reveal high polymorphic fingerprints and shed light on genetic variability. Two unrelated species are likely to have different numbers of microsatellites at a given locus (Bornet *et al.,* 2002).

The major advantage of this method is that it does not require a time-consuming and expensive step of genomic library construction and it is possible to study ISSR abundance and

distribution in genomes. The bands produced by an ISSR primer with a given microsatellite repeat, should reflect the relative frequency of that motif in a given genome (Morgante and Olivieri, 1993). Although ISSRs are mostly random-type markers, they are thought to be highly useful for genetic diversity and phylogenetic studies (Morgante *et al.,* 2002).

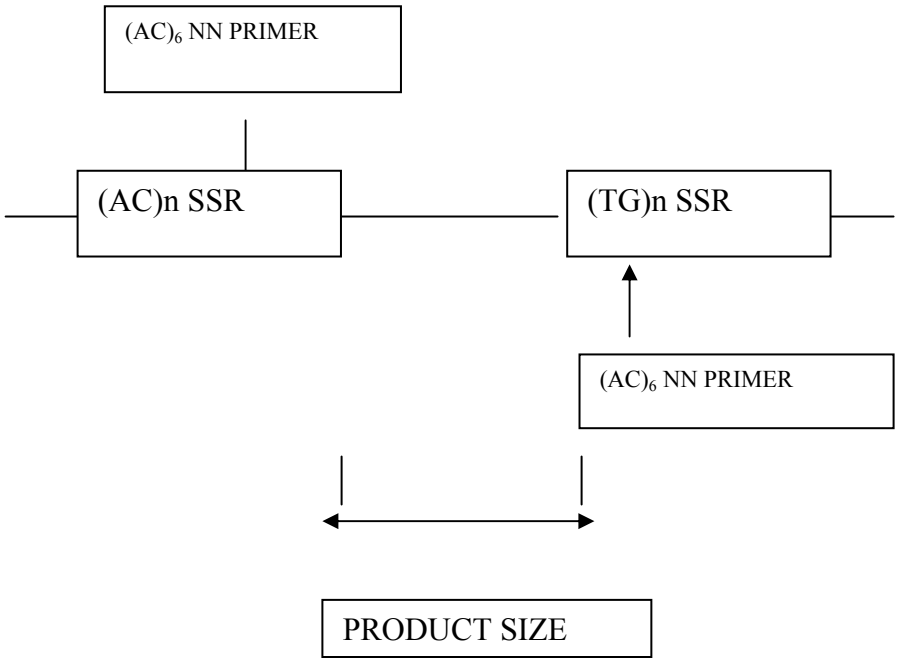

Fig 5. Schematic diagram showing ISSR amplification. ISSR primers $(AC)_6$ NN, will anneal to the SSR (microsatellites (AC)n and (TG)n) enabling the amplification of the region between them.

2.2.7 Sequence analysis of the rDNA internal transcribed spacer (ITS) region

The ribosomal RNA (rRNA) genes in ribosomal DNA possess characteristics that are suitable for the detection of pathogens at the species level (O'Donell, 1992). These rDNA sequences are highly stable and exhibit a mosaic of conserved and diverse regions within the genome (Hibbett, 1992). The rDNA sequences also occur in multiple copies with up to 200 copies per

haploid genome (Bruns *et al.*, 1991) arranged in tandem repeats. Each repeat consists of the 18S small subunit (SSU), the 5.8S, and the 28S large subunit (LSU) genes (Fig 6).

A rRNA gene also includes variable regions such as the internal transcribed spacers (ITS) that are formed between the subunits and intergenic spacers (IGS) found between the cluster repeats that facilitate discrimination between closely related species of a fungal genus (Hennequin *et al.,* 1999). The more conserved 18S and 28S regions tend to combine the more closely related species into the same taxonomic group, but ITS regions tend to split groups into distinct species (O'Donell and Gray, 1995).

The rDNA has been utilized by many investigators for species determination in a wide variety of yeasts and fungi (Rehner and Uecker, 1994; Zhang *et al.,* 1998; Farr *et al.,* 2002). The most successful methods of species determination have employed PCR amplification of target sequences within the gene cluster. These methods rely on the conserved nature of rDNA such that isolates from the same species maintain the same sequence, whereas the more phylogenetically diverse the species is, the greater is the difference in the sequences of rDNA.

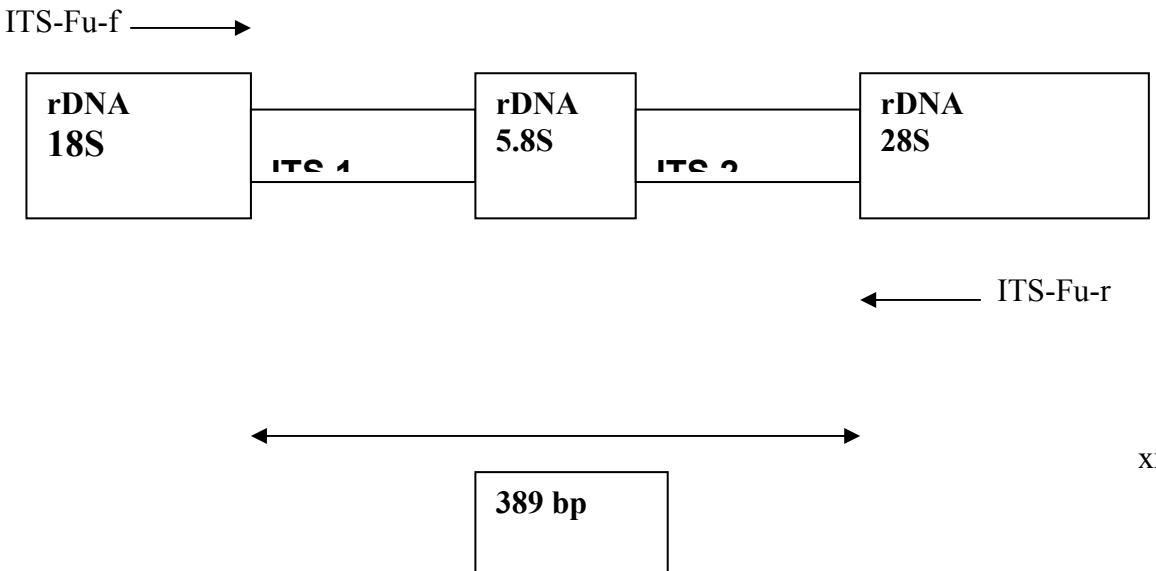

Fig 6. Schematic diagram of the fungal rDNA gene cluster. Primers ITS-Fu-f and ITS-Fu-r have been used to amplify the ITS region (ITS1, 5.8S and ITS2) from *Fusarium* species.

Variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions because of their high degree of variation, compared to the 18S and 28S rRNA coding regions of rDNA (Carbone and Kohn, 1993; Berthier *et al.,* 1996).

Identical ITS sequences do not automatically mean that the fungi are conspecific but that they are closely related. Differences in the nucleotide sequence composition of the variable ITS regions have been successfully employed to design specific primer sets that amplify DNA selectively among and within species of plant pathogens (Nazar *et al.*, 1991; Moukhamedov *et al.*, 1994; Schilling *et al.*, 1996; Moricca *et al.*, 1998).

ITS primers 1 and 4 have been used to amplify the entire 5.8S rRNA gene, both ITS regions I and II, and a portion of the 18S small-subunit rRNA gene. This region was sequenced with the

M13 forward and reverse sequencing primers to develop a genus-specific PCR assay for the rapid identification of *Fusarium* (Abd-Elsalam *et al.,* 2003).

Two taxon-selective primers for quick identification of members of the *Fusarium* genus have been developed (Fig 6). These primers, ITS-Fu-f and ITS-Fu-r were designed by comparing the aligned sequences of ITS regions of a range of *Fusarium* species (Abd-Elsalam *et al.*, 2003). The primers showed good specificity for the genus *Fusarium*, and an approximately 389-bp product was amplified exclusively (Bryan *et al*., 1995).

Specific primers have also been designed to amplify *F.oxysporum* f.sp *vasinfectum* DNA in cotton, but not the isolates of any other mycoflora associated with cotton (Moricca *et al.,* 1998). In soybean roots and soil, *F. solani* f.sp *glycines* was detected using a mitochondrial SSU rRNA gene primer set (Paminondas and Paplomatas, 2004). PCR-based identification and detection of various fungal pathogens exploiting the ITS region specificity has been reported for *Rosellinia necatrix, Cylindrocarpon destructans* and *Cylindrocladium floradanum* and *Gaeumannomyces graminis var tritici* (Keller *et al.*, 1995).

Sequence analysis of ITS regions has been employed to study intrageneric relationships within *Pythium* (Matsumoto *et al.,* 1999) and *Phytophthora (*Lee and Taylor, 1992; Crawford *et al.,* 1996; Cooke and Duncan, 1997; Forster *et al.,* 2000). In contrast to the ITS sequences, IGS has been less popular as a region for primer designing due to the high evolutionary pressure acting on the sequences.

3.0 AIM OF THE PROJECT

The broad aim of this study was to investigate the efficiency of two molecular based techniques in identifying and differentiating *Fusarium* species. The specific objectives of this work were to:

1. Distinguish morphologically identified *Fusarium* species from each other,
2. Examine genetic relatedness of the *Fusarium* species, and
3. Assess genetic diversity among the isolates from within species.

CHAPTER 2

4.0 MATERIALS AND METHODS

4.1 Fungal Isolates

A total of 113 morphologically identified isolates (Table 1) comprising different *Fusarium* species isolated from tobacco, cotton, groundnuts, coffee, onions, pea seed and paprika, were initially used in the study. Isolates were obtained from the *Fusarium* collection in Plant Pathology, Crop Protection Division at Kutsaga. Five reference isolates from the Centre for Agriculture Biosciences International (CABI), United Kingdom, formerly International Mycological Institute (IMI), were included in the ITS analysis. In addition, one unrelated control isolate, *Rhizoctonia solani,* was included to test the specificity of the ITS primers. All isolates were cultured for 7 to 14 days at 25°C on Potato Dextrose agar (PDA). A plant pathologist in the Division confirmed the isolates to be *Fusarium* after observing colony characteristics and microscopic features.

Table 1. Fungal species used in the study.

Species of *Fusarium*	**Isolate**	**Source**	**Date last subcultured**
F. oxysporum	029	Kutsaga	29/ 04/ 03
	031	Kutsaga	29/ 04/ 03
	054	Kutsaga	18/ 06/ 03
	074	Burma valley	29/ 04/ 03
	079	Burma valley	29/ 04/ 03
	081	Burma valley	29/ 04/ 03
	085	Burma valley	29/ 04/ 03
	087	Burma valley	29/ 04/ 03
	088	Burma valley	05/ 05/ 03
	089	Burma valley	29/ 04/ 03

	091	Banket	29/ 04/ 03
	093	Banket	29/ 04/ 03
	094	Banket	29/ 04/ 03
	095	Banket	29/ 04/ 03
	096	Banket	05/ 05/ 03
	099	Banket	05/ 05/ 03
	100	Banket	05/ 05/ 03
	102	Banket	05/ 05/ 03
	103	Banket	05/ 05/ 03
	104	Banket	05/ 05/ 03
	105	Banket	05/ 05/ 03
	106	Karoi	05/ 05/ 03
	108	Karoi	05/ 05/ 03
	112	Kadoma	05/ 05/ 03
	120	Kutsaga	05/ 05/ 03
	121	Kutsaga	05/ 05/ 03
	122	Kutsaga	05/ 05/ 03
	123	Kutsaga	05/ 05/ 03
	124	Kutsaga	05/ 05/ 03
	125	Kutsaga	05/ 05/ 03
	127	Kadoma	05/ 05/ 03
	139	Kutsaga	05/ 05/ 03
	140	Kutsaga	05/ 05/ 03
	141	Kutsaga	05/ 05/ 03
	142	Kutsaga	05/ 05/ 03
	143	Kutsaga	05/ 05/ 03
	219	Kutsaga	05/ 05/ 03
	237	Kutsaga	15/ 05/ 03
	241	Kutsaga	15/ 05/ 03
	257	Karoi	15/ 05/ 03
	263	Burma valley	15/ 05/ 03
	264	Burma valley	15/ 05/ 03
	266	Burma valley	20/ 05/ 03
	268	Burma valley	15/ 05/ 03
	276	Bindura	15/ 05/ 03
	277	Bindura	15/ 05/ 03
	279	Tengwe	15/ 05/ 03
	283	Centenary	15/ 05/ 03
	284	Mutare	15/ 05/ 03

	285	Mutare	15/ 05/ 03
	286	Mutare	18/ 06/ 03
	287	Mutare	15/ 05/ 03
	290	Kutsaga	15/ 05/ 03
	291	Kutsaga	15/ 05/ 03
	295	Trelawney	15/ 05/ 03
	296	Trelawney	15/ 05/ 03
	297	Trelawney	15/ 05/ 03
	298	Kadoma	15/ 05/ 03
	299	Kadoma	15/ 05/ 03
	301	Kadoma	15/ 05/ 03
	302	Ruwa	15/ 05/ 03
	303	Belvedere	15/ 05/ 03
	304	Belvedere	15/ 05/ 03
	305	Ruwa	30/ 05/ 03
	306	Makonde	30/ 05/ 03
	307	Mutare	30/ 05/ 03
	312	Mutare	30/ 05/ 03
	313	**Mutare**	30/ 05/ 03
	319	Harare	30/ 05/ 03
	325	Harare	30/ 05/ 03
	326	Harare	30/ 05/ 03
	327	Marondera	16/ 05/ 03
	337	Harare	23/ 09/ 05
	338	Harare	23/09/ 05
Fusarium solani	008	Kutsaga	29/ 04/ 03
	013	Kutsaga	29/ 04/ 03
	021	Kutsaga	29/ 04/ 03
	027	Kutsaga	29/ 04/ 03
	030	Kutsaga	29/ 04/ 03
	032	Kutsaga	29/ 04/ 03
	050	Kadoma	18/ 06/ 03
	261	Karoi	15/ 05/ 03
	262	Karoi	15/ 05/ 03
	265	Burma valley	18/ 06/ 03
	272	Burma valley	15/ 05/ 03
	293	Harare	15/ 05/ 03
	294	Harare	15/ 05/ 03

Fusarium equiseti	126	Kutsaga	05/ 05/ 03
	133	Kadoma	05/ 05/ 03
	134	Kutsaga	05/ 05/ 03
	316	Enterprise	30/ 05/ 03
	317	Enterprise	18/ 06/ 03
Fusarium moniliforme	131	Kutsaga	05/ 05/ 03
	132	Kutsaga	05/ 05/ 03
	258	Karoi	15/ 05/ 03
	318	Mutare	18/ 06/ 03
	320	Harare	30/ 05/ 03
Fusarium poae	329	Harare	10/ 12/ 03
	331	Harare	10/ 12/ 03
Fusarium species	282	Centenary	15/ 05/ 03
	308	Mutare	30/ 05/ 03
	309	Mutare	30/ 05/ 03
	311	Mutare	18/ 06/ 03
	321	Mutare	30/ 05/ 03
	322	Enterprise	18/ 06/ 03
	323	Enterprise	18/ 06/ 03
	324	Enterprise	18/ 06/ 03
	332	Guruve	06/ 02/ 04
	334	Glendale	17/ 05/ 04
Fusarium graminearum	259	Kutsaga	15/ 05/ 03
Fusarium pallidoroseum	136	Kutsaga	05/ 05/ 03
Fusarium lateritium	273	Burma valley	18/ 06/ 03
F. moniliforme var *subglutinans*	128	Kadoma	05/ 05/ 03
F. moniliforme var *verticilloides*	129	Kutsaga	05/ 05/ 03

The reference isolates from CABI used in this study were *F. oxysporum, F.solani, F. poae, F. moniliforme var subglutinans* and *F.moniliforme var intermedium*.

4.2 DNA Isolation

DNA was extracted using the large-scale extraction protocol as described by Carling *et al.,* (1999). Flasks containing 100 ml Czapecks media were inoculated with all conidial

suspensions of *Fusarium* isolates from the PDA plates and incubated at 25 °C for 10 days. The mycelia were harvested by filtration, washed with distilled water and dried overnight at 40 °C. For total genomic DNA extraction, all the dried mycelia were ground using a mortar and pestle into a fine powder and suspended in 25 ml of extraction buffer (50 mM Tris-HCl, 100 mM sodium EDTA, 150 mM sodium chloride, 0.5 g sodium dodecyl sulphate and 5 ml toluene). The centrifuge tubes were placed in a shaker incubator and centrifuged at 150 rpm for 72 hr at 37 °C for maximum cell lysis. The tubes were subsequently centrifuged at 1509.3 g in a benchtop centrifuge (Beckman Coulter) at room temperature for 15 min and the supernatant mixed with an equal volume of phenol: chloroform: pentanol (25:24:1).

DNA was precipitated by adding 2.5 volumes of cold 100% ethanol and incubating for 10 minutes at -20 °C. After centrifuging at room temperature for 15 min at 28 341.3 g, the DNA pellet was re-suspended in 5 ml of 1M Tris-EDTA (pH 8.0) + 10 µl RNAse (50 µg/ml) to degrade RNA and 10 µl Proteinase K (100 µg/ml) to digest the proteins. DNA samples were further purified by shaking in an equal volume of phenol: chloroform: pentanol (25:24:1) and centrifuged at 16 770 g at room temperature for 10 mins. The supernatant was shaken with an equal volume of chloroform:pentanol (24:1) and centrifuged at 16 770 g at room temperature for 10 mins. The DNA was precipitated with 0.54 x volume cold isopropanol. The tubes were centrifuged at 16 770 g for 10 min and DNA pellets were rinsed with 70% ethanol, air-dried, re-suspended in 200 µl 1 M Tris-EDTA buffer (pH 8.0) and stored at -80 ºC until use.

4.3 Determination of DNA yield and purity

After extraction, the DNA samples were first run on 2% agarose gels in 0.5 M Tris borate EDTA (TBE) buffer (pH 8) at room temperature at a constant voltage of 100 V to determine the quality. The DNA concentration and purity were further determined by measuring optical density (OD) at 260 nm and 280 nm using a spectrophotometer. Briefly, 10 µl of DNA sample was diluted in 990 µl distilled water (1:100) dilution. The OD of the solution was measured at 260 nm and 280 nm alternately. DNA concentration was calculated using the following formula:

DNA conc (µg/ ml) = A_{260} X 50X dilution factor

DNA purity was determined by calculating the ratio OD_{260}/ OD_{280}. Samples with values of between 1.8 – 2.0 were considered for the PCR analyses.

4.4 PCR Amplification

4.4.1 Amplification and sequencing of the ITS region

Fusarium isolates from eight different species (*F. moniliforme* 131, *F. equiseti* 133, *F. pallidoroseum* 136, *F. graminearum* 259, *F. solani* 261, *F. lateritium* 273, *F. oxysporum* 277 and *F. poae* 331), including the five reference isolates were analyzed. Amplification of the ITS region was done using the *Fusarium* specific primers ITS-Fu-f and ITS-Fu-r (Table 2). The PCR reaction mixture contained 5 µl of 10 x PCR buffer (100 mM Tris-Cl, 500 mM KCl, pH 8.3), 5 µl of 0.1 mg/ml bovine serum albumin (BSA), 4 µl of 0.2 mM each of the four deoxynucleoside triphosphates (dATP, dTTP, dGTP, dCTP), 6 µl of 3 mM $MgCl_2$, 5 µl of 1 µM each primer, 1 µl (5 units) of *Taq polymerase* (Inqaba Biotech), 1 µl of 100 ng/µl DNA and 17µl of PCR water to bring to a total volume of 50 µl.

The thermal cycling profile using a Perkin Elmer 2400 thermocycler was denaturation for 2 min at 94 ºC followed by 30 cycles at 94 ºC for 1 min, 54 ºC annealing for 30 s, and 72 ºC extension for 1 min, followed by a final extension at 72 ºC for 7 min. The entire PCR product was resolved on 2% agarose gels in 0.5 M TBE buffer pH 8.0, at room temperature at a constant voltage of 100 V. The product was visualised by staining with ethidium bromide (0.5µg/ml) and photographed under ultraviolet light using an ultraviolet transilluminator. After gel electrophoresis, the PCR products were purified using a High Pure Purification Kit (Roche Diagnostics, Germany).

The kit contains three different buffers, high pure filter tubes and collection tubes. A total of 400 µl of binding buffer was mixed with PCR solution after amplification and transferred into a high pure tube to allow DNA binding to the filter. After 30s of centrifugation at 28 341.3 g, bound DNA was washed twice with 200 µl wash buffer. The filter tube, now containing purified DNA, was then transferred into a clean 1.5 ml microcentrifuge tube where 200 µl of elution buffer was added to the upper reservoir and another 30s of centrifugation transferred the DNA directly into the microcentrifuge tube. Purified products were sent to Inqaba Biotechnical Industries (Pretoria, South Africa) for sequencing, using cycle sequencing based on the Fred Sanger method.

4.4.2 ISSR analysis

To identify primers that generated informative arrays of PCR products, the eight *Fusarium* isolates belonging to the species numbered 131, 133, 136, 259, 261, 273, 277 and 331 (Table

1) were used in the initial analyses. After the best primers were selected, all 113 isolates belonging to different species were analyzed to determine the degree of genetic diversity among and within collections of the *Fusarium* species. The reference isolates were also included in the analyses. For the analysis, six oligonucleotides were tested (Table 2). For primers SSR 1, SSR 17, SSR 7 and IS 36 the PCR reactions contained 2 µl PCR buffer, 1 µl of 0.5 mg/ml BSA, 2 µl of 4% DMSO, 1.5 µl of 2 mM $MgCl_2$, 1.5 µl of 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 2 µl of 1 µM primer, 1 µl of 5units *Taq Polymerase* and 1 µl of 100 ng/µl DNA in a total volume of 20 µl, with the rest of the volume being water. The thermal cycling profile using Perkin Elmer 2400 thermocycler was denaturation for 2 min at 94 ºC, followed by 38 cycles at 94 ºC for 25 s, 48 ºC annealing for 25 s, and 72 ºC extension for 1 min, followed by a final extension at 72 ºC for 7 min. in all PCRs, water was included as a non template control (NTC).

For primers 17898B and 17899B, the PCR reactions contained 1 µl of 100 ng/µl DNA, 5 µl of 10 x PCR buffer, 3 µl of 25 mM $MgCl_2$, 4 µl of 2.5 mM dNTPs, 4 µl of 10 mM primer, 0.5 µl 2.5units *Taq polymerase* in a total volume of 50 µl. The thermal cycling profile was 94 ºC for 2 min followed by 35 cycles at 94 ºC for 30 s, 42 ºC annealing for 1 min, 72 ºC for 1 min 30 s, followed by a final extension at 72 ºC for 5 min. In each case, the entire PCR product was resolved on 2% agarose gels in 0.5 M TBE buffer (pH 8) at room temperature at a constant voltage of 100 V. The product was visualised by staining with ethidium bromide (0.5 µg/ml) and photographed under ultraviolet light.

Table 2 Primers used in this study

Primer name	Nucleotide sequence of primer	Annealing T°C
SSR1	$(ACGT)_4$	48
SSR7	$GC(AC)_5$	38
SSR17	$CSC(GA)_6$	48
IS36	$(AG)_6TC$	54
17898B	$(CA)_6GT$	42
17899B	$(CA)_6GG$	44
L15	GAGGGTGGCGGTTCT	55
L45	GTAAAACGACGGCCAGT	55
ITS-Fu-f	CAACTCCCAAACCCCTGTGA	54
ITS-Fu-r	GCGACGATTACCAGTAACGA	54
AS15	GGCTAAGCGGTCGTTAC	52
AA2M2	CTGCGACCCAGAGCGG	52

4.4.3 Universally primed PCR amplification.

Universally primed PCR was done using two primer sets L15/L45 and AS15/AA2M2 (Table 2) in pairwise combinations. The PCR was performed in a 25 µl volume containing 2µl of 10 x PCR buffer, 2µl of 0.35 mM dNTPs, 2µl of 2.5 mM $MgCl_2$, 2µl of 0.5 µM primer, 2.5 µl of 100 ng/µl DNA, 0.5 µl (2.5 U) of *Taq polymerase* and 14µl PCR water. A Perkin Elmer 9700 thermal cycler was used. PCR conditions were as follows initial denaturation at 94 ºC for 5 min followed by 29 cycles at 94 ºC for 50 s, 50 ºC annealing for 1 min, 72 ºC for 1 min , followed by a final extension at 72 ºC for 5 min. For primers AS15/AA2M2, the thermal cycling profile

was initial denaturation at 94 ºC for 3 min followed by 29 cycles of 92 ºC for 50 s, 52 ºC annealing for 1min 10sec, 69 ºC for 1min and a final extension at 69 ºC for 3 min.

4.5 Data Analysis

Computer analysis of ISSR and UP-PCR data was performed utilising the band patterns obtained from agarose gel electrophoresis employing manual digitalisation to a two-discrete-character matrix (0 and 1 for absence and presence of bands, respectively). Comparison of band profiles for the products of each of the primers was based on presence (1) or absence (0) of bands that migrated to the same position in the gel. Bands of the same size reproducible in at least three experiments with the same primer at different times were scored as identical. From the ISSR and UP-PCR data, similarity matrices based on Jaccard's coefficient (measure of similarity) were calculated and these were used to construct dendrograms using the nearest neighbour method of analysis. This is a technique for measuring the extent to which a particular pattern is clustered, random or uniform.

CHAPTER 3

5.0 RESULTS

5.1 Amplification and Sequencing of the ITS Region

Agarose gel electrophoresis profile of DNA extracted from seven isolates is shown in Fig 7. Amplification of the ITS region in the 13 *Fusarium* species using ITS-fu-f and ITS-fu-r primers successfully amplified an approximately 389 bp product in all 13 isolates. The gel electrophoresis patterns of purified products sent for sequencing are shown in Fig 8.

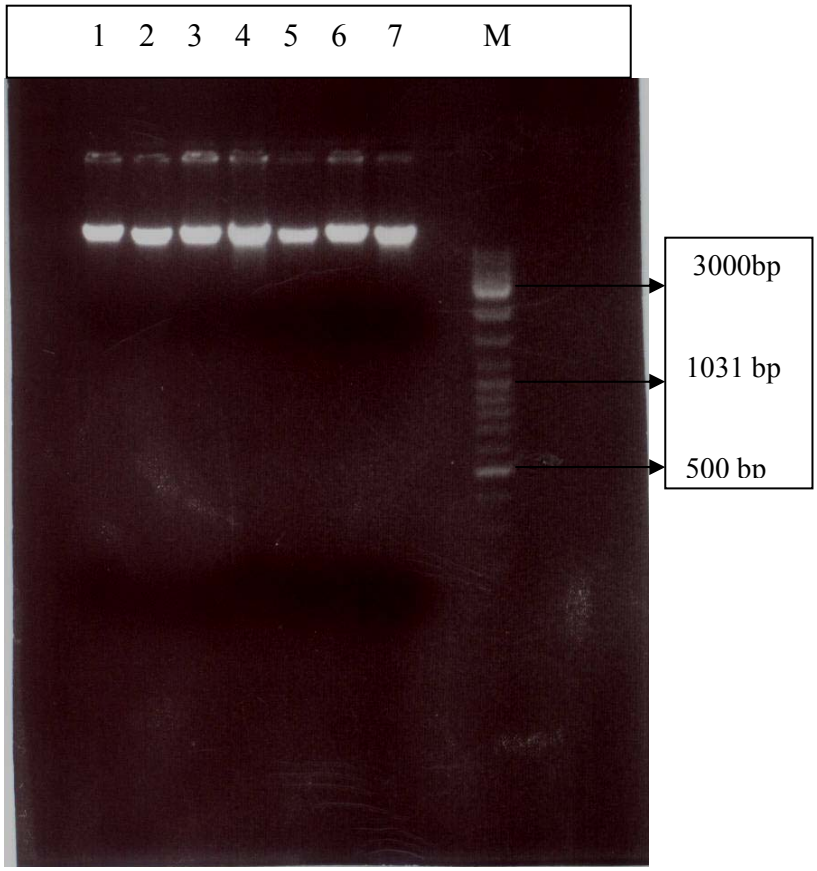

Fig 7. A representative ethidium bromide stained gel electrophoresis pattern of DNA extracted from 7 *Fusarium* isolates using the large scale extraction protocol. Lanes 1 – 7 isolates F237, F268, F291, F299, F302, F312, F313, respectively, lane M molecular weight marker (DNA ladder mix).

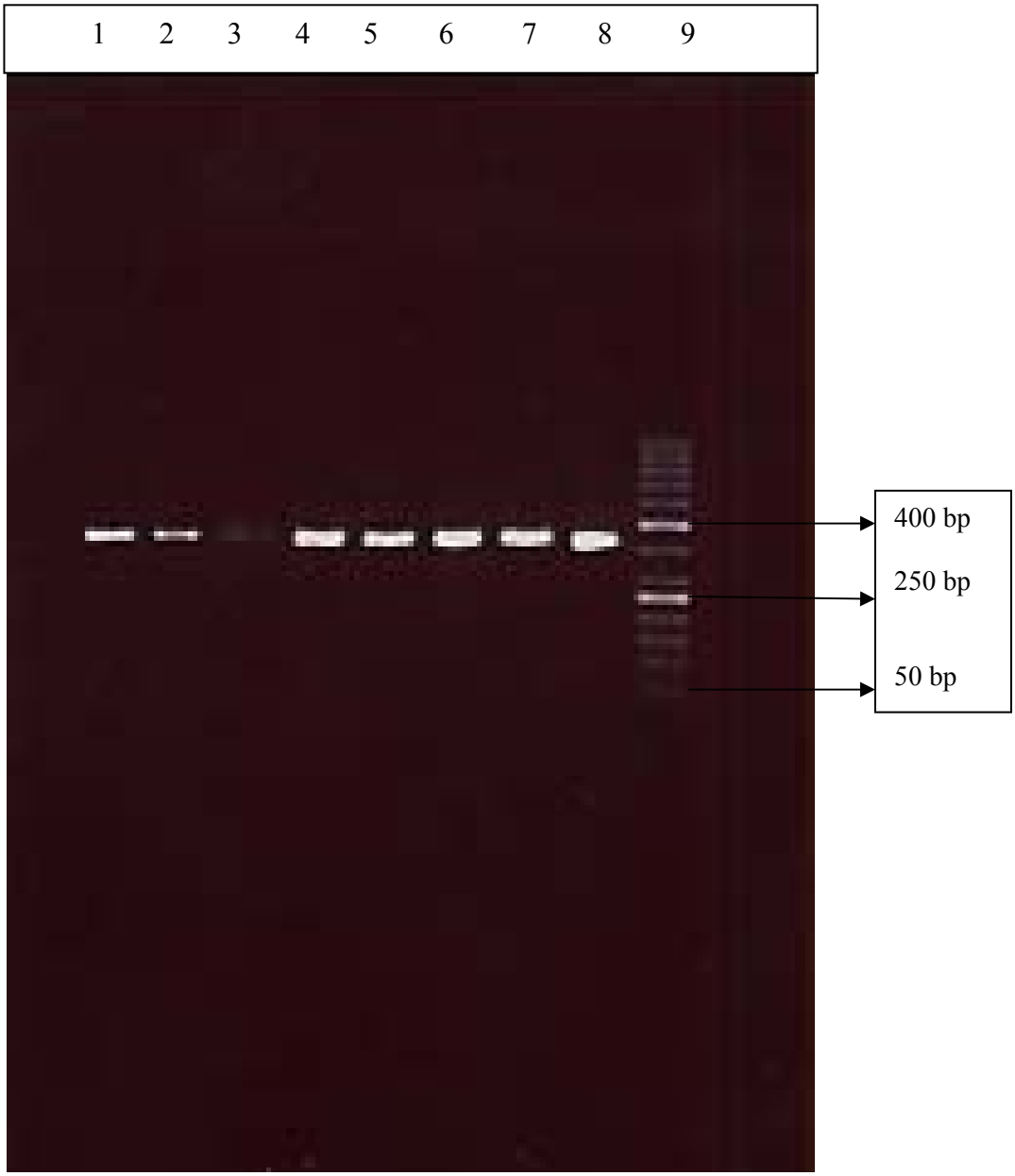

Fig 8. A representative ethidium bromide stained gel electrophoresis pattern showing purified 389bp ITS products that were subsequently sequenced. Lane 1 *F. oxysporum*, lane 2 *F. solani*, lane 3 *Rhizoctonia solani*, lane 4 *F. poae*, lane 5 *F. lateritium*, lane 6 *F. moniliforme*, lane 7 *F. graminearum*, lane 8 *F. pallidoroseum*, lane 9 molecular weight marker (100 bp DNA ladder).

Using the computer programme DNAMAN (Version 4.3, Lynnon Biosoft), multiple alignments of the nucleotide sequences of the 13 species were performed and their homology tree was constructed (Fig 9).The 13 species were initially split into two groups based on ITS region similarities. The results show that the ITS regions were relatively similar with a range of 87 - 100% within the two groups. The larger group comprised eight species, *F. equiseti, F. moniliforme var subglutinans, F. oxysporum* (CABI isolate), *F. oxysporum, F. poae* (CABI isolate)*, F. solani* (CABI isolate)*, F.solani* and *F. moniliforme var intermedium* (CABI isolate) with similarities ranging from 95 to 100%. All the CABI reference isolates were clustered into this group.

This group of eight species was further split into five subgroups. *F. solani* (CABI)*, F. solani* and *F. moniliforme var intemedium* (CABI) were placed into three distinct groups with similarities ranging from 95 to 98%. *F. poae* had an ITS region that was highly homologous to that of the two *F. oxysporum* species by 99%. These three species were clustered into the fourth subgroup. The two *F. oxysporum* species had 100% similarity in their ITS region. The fifth subgroup was composed of *F. equiseti* and *F. moniliforme* var *subglutinans* with a high ITS region similarity of 99%.

The smaller group contained the other five species with an ITS region similarity of 93%. This group was further split into two subgroups. One subgroup contained *F. moniliforme*, *F. pallidoroseum* and *F. lateritium* with an unexpected 100% similarity in the ITS region. In the other subgroup, *F. poae* and *F. graminearum* had a high similarity of 98%.

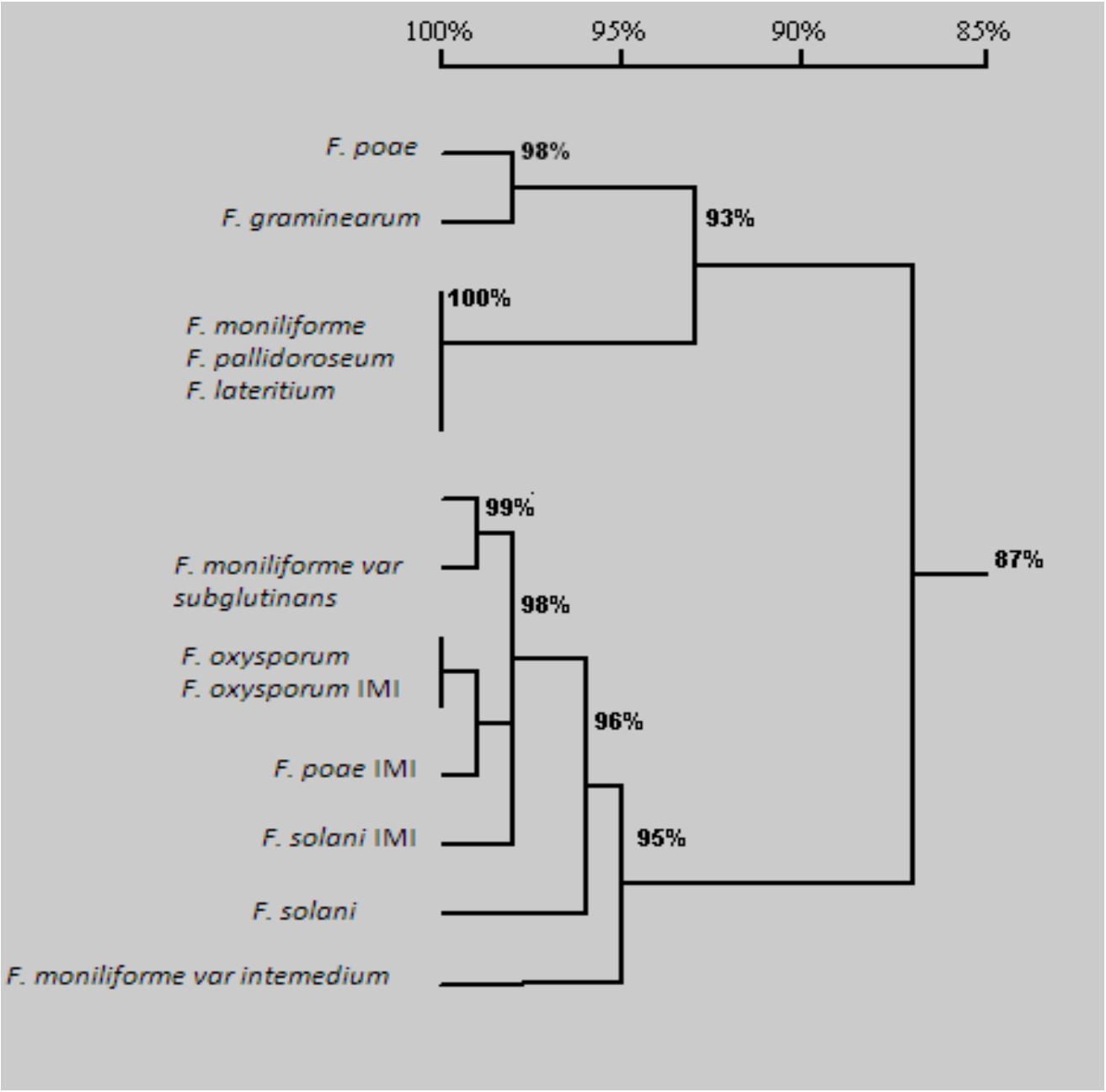

Fig 9. Homology tree showing ITS region percentage similarities among the 13 *Fusarium* species.

5.2 Inter-Simple Sequence Repeat Analysis

Of the six primers used (Table 2), four primers, IS 36, SSR 1, SSR 7 and SSR 17 gave reproducible banding profiles for most of the isolates tested. Out of the 113 isolates analysed, an average of 84 isolates per primer gave reproducible polymorphic bands and analyses done were based on these results. These primers yielded a total of 500 polymorphic bands. IS 36, SSR 7 and SSR 17 generated more polymorphic bands than SSR 1.

Primer IS 36 yielded 140 polymorphic bands across 82 isolates. Among the 82 isolates, 47 were *F. oxysporum* including the CABI isolate, 14 *F. solani* including the CABI isolate, 7 *F. spp*, 5 *F. equiseti,* 2 *F. poae,* 1 *F. moniliforme,* 1 *F.moniliforme var verticilloides,* 1 *F. moniliforme var subglutinans,* 1 *F. graminearum,* 1 *F.lateritium,* 1 *F.pallidoroseum* and the *Rhizoctonia solani* control isolate. The electrophoresis gel banding profiles of the IS 36 primed products for the 14 *F. solani* isolates and 5 *F.equiseti* isolates are shown in Fig 10.

Several isolates had similar banding profiles such as those in lanes 6 and 7 (isolates 032 and 050), lanes 8 and 9 (isolates 261 and 262), lanes 15 and 17 (isolates 126 and 134). Some of the polymorphic bands appeared more than once across the different isolates. These bands are marked by the arrows in Fig 10.

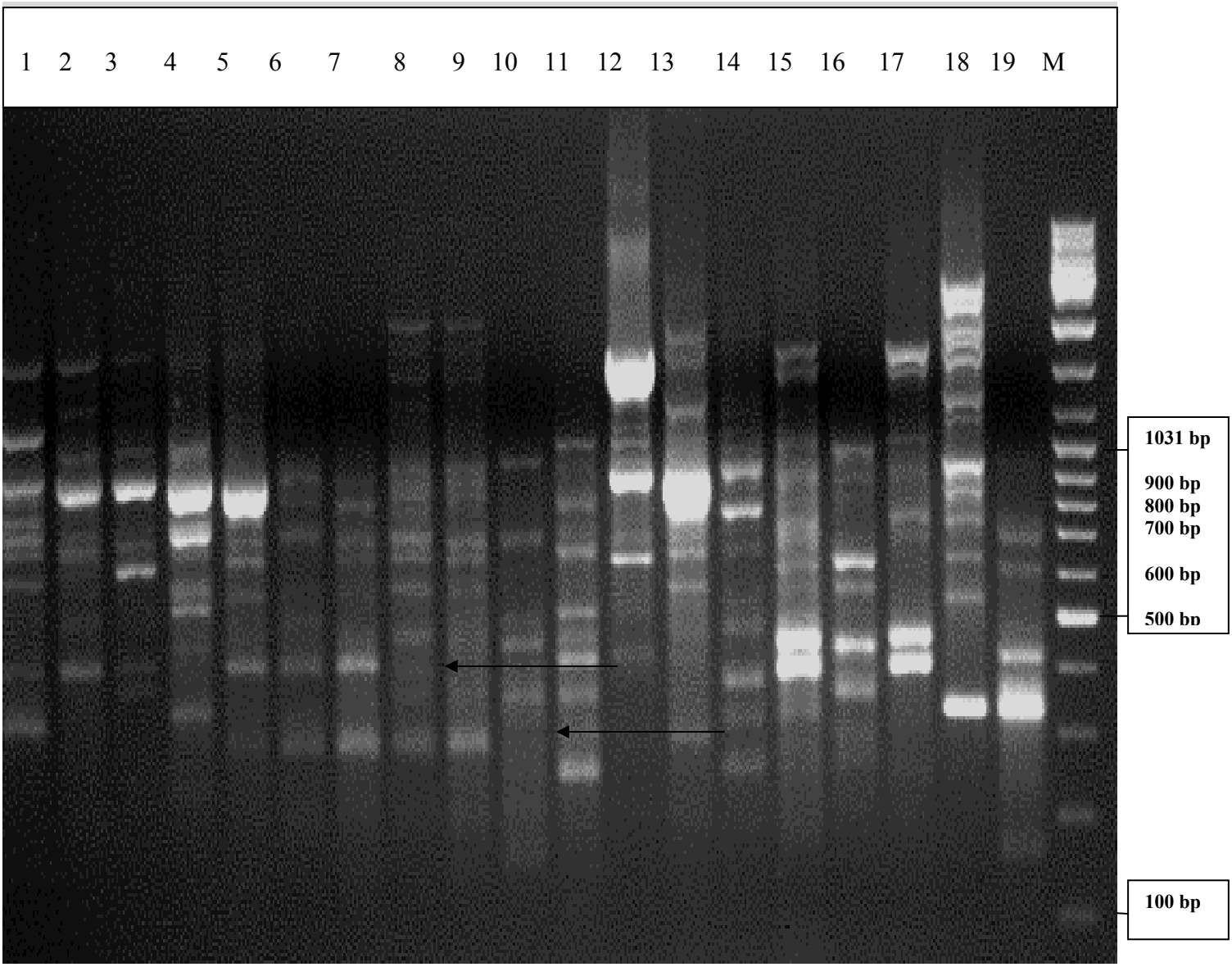

Fig 10. A representative ethidium bromide stained gel ISSR profile of the PCR products generated using primer IS 36. Lanes 1-13 *F.solani* isolates, lane 14 *F. solani* (CABI isolate), lanes 15-19 *F.equiseti* isolates, M, molecular-weight marker (DNA ladder mix).

Analysis of all the ISSR data revealed a high genetic diversity among the 84 *Fusarium* isolates. Figure 11a shows the dendrogram that was constructed using the nearest neighbour cluster analysis. The genetic similarities (x-axis) are expressed as percentages from 0 – 100%. The isolates were separated and placed into distinct groups based on their genetic relatedness with the closely related isolates having the highest genetic similarities. This primer initially split all 84 isolates into two groups at 4.4% genetic similarity (Fig 11a, I and II). The smaller group contained six *F. oxysporum* isolates and the larger group comprised the other 78 isolates, which were further split into seven subgroups.

Figures 11 b – e are enlarged sections of the dendrogram indicating how the isolates were further split into subgroups. This primer revealed a high intraspecies diversity among the *F. oxysporum* isolates as these were clustered into all the subgroups. Wide genetic similarity ranges were also seen among the *F. oxysporum* isolates. The dendrogram in Fig 11b shows 21 *Fusarium* isolates that were split into a further two subgroups A and B at a genetic similarity of 10%.

In subgroup A were 9 *F. oxysporum* isolates with a genetic similarity range of 20% – 84.6%. Subgroup B had 7 *F.species* isolates with genetic similarities ranging from 25% to 77%, 2 *F. poae* isolates that were 50% genetically similar, *F. lateritium, F. pallidoroseum* and *F. graminearum* isolates. There was an unexpected high genetic similarity of 80% between *F. lateritium* and *F. pallidoroseum*. The high intraspecies diversity observed in *F. oxysporum* isolates is shown in Fig 11c.

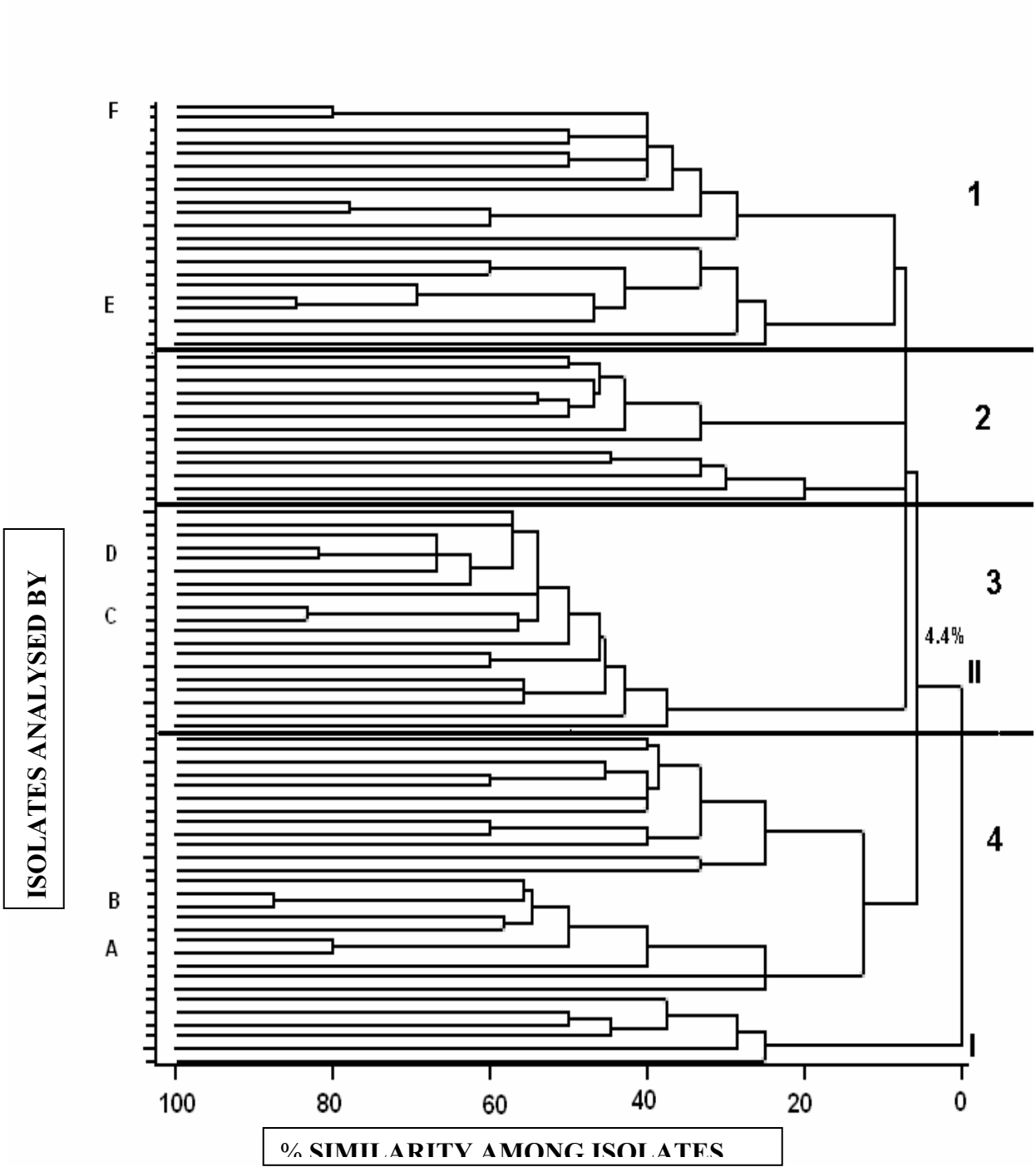

Fig 11a. Dendrogram showing clustering of isolates based on genetic similarities in PCR reactions using primer IS36. The two main groups formed are shown under I and II. Isolates with highest genetic similarities (more than 80%), are indicated by the letters A, B, C, D, E, F.

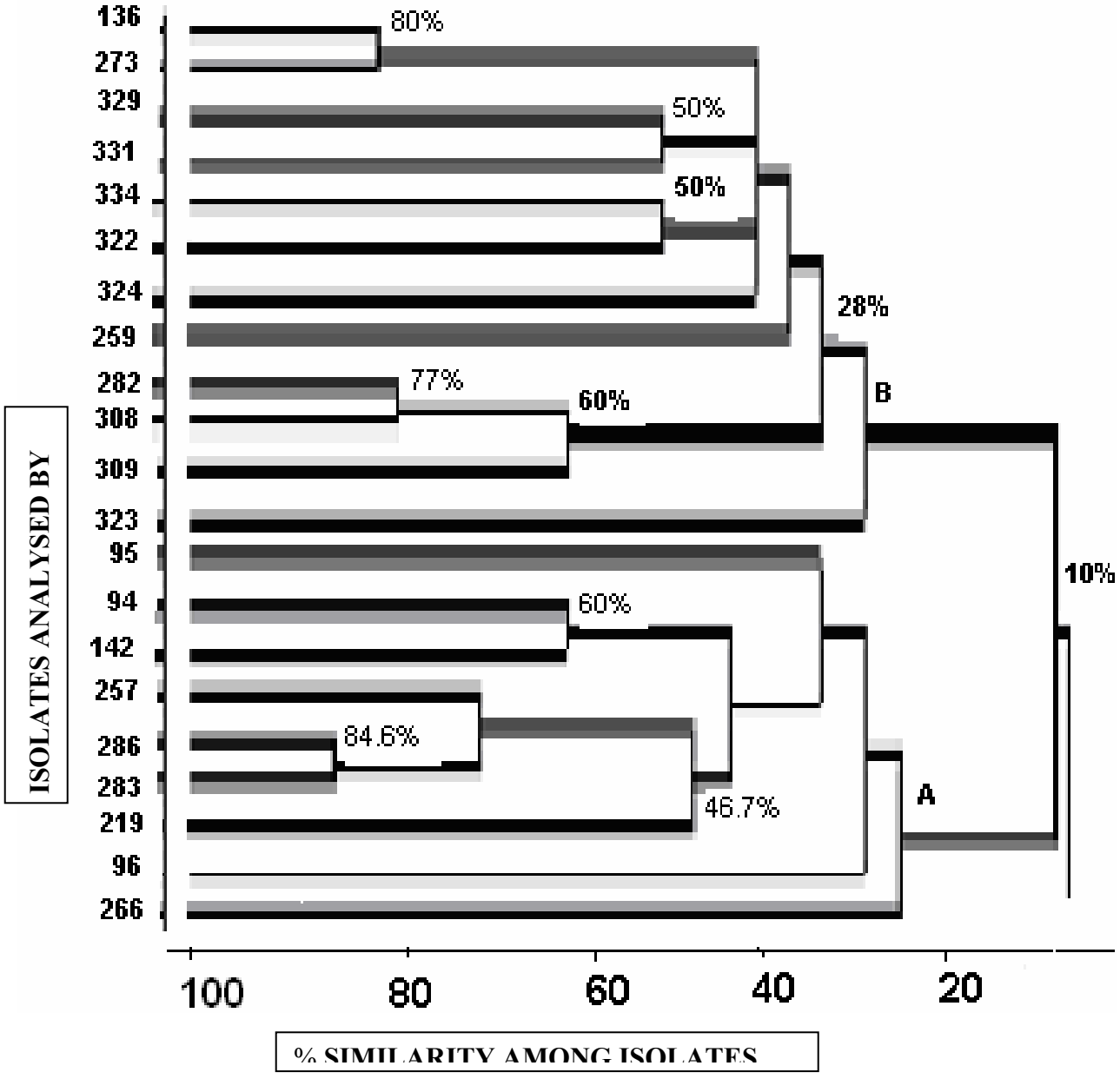

Fig 11b. Section 1 of dendrogram in Fig 11a showing clustering of 21 *Fusarium* isolates. Nine *F. oxysporum* isolates were clustered into subgroup A. Subgroup B contained seven *F.spp*, two *F. poae* (329 and 331), *F. pallidoroseum* (136), *F. lateritium* (273) and *F. graminearum* (259).

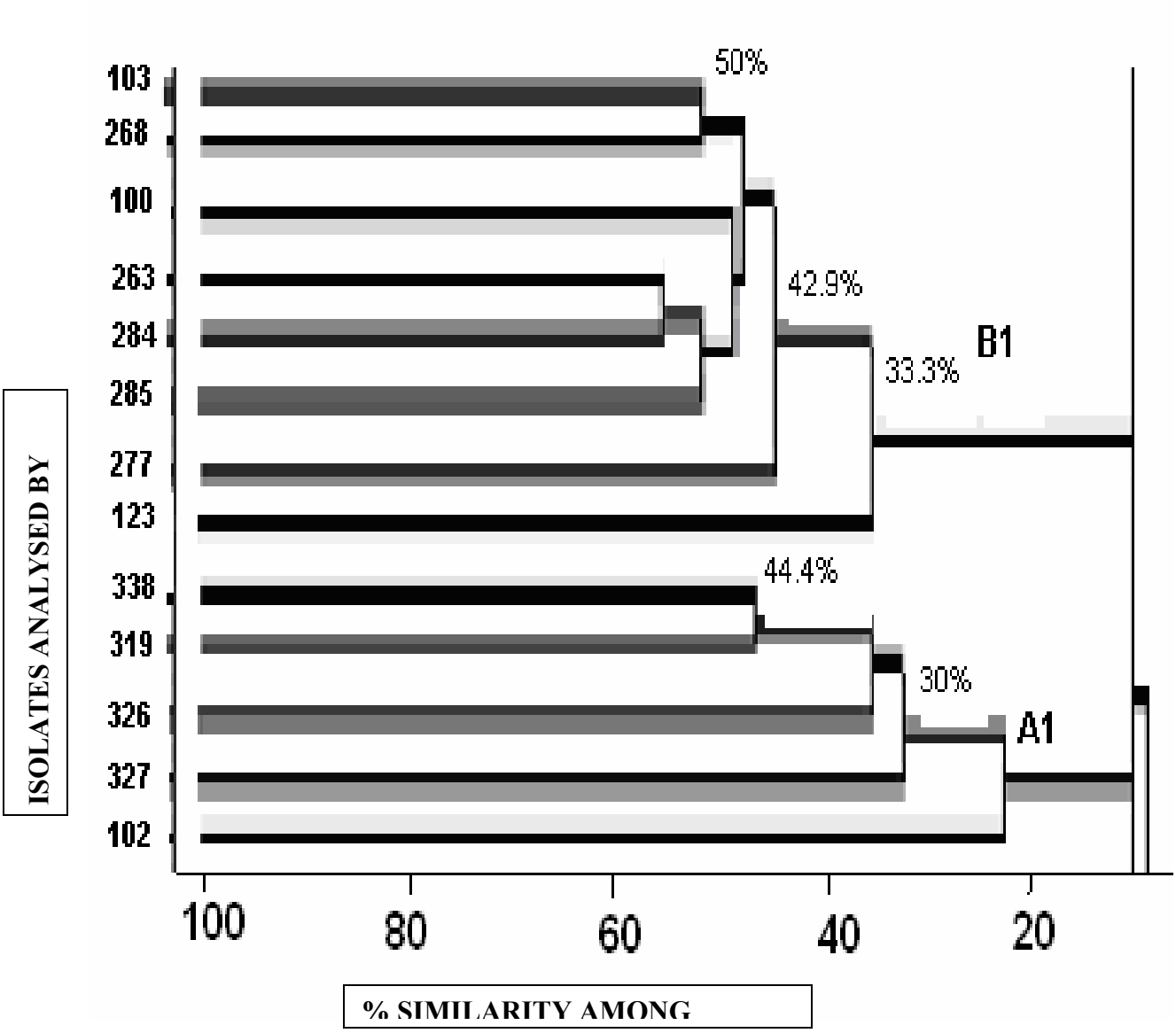

Fig 11c. Expanded section 2 of Fig 11a showing clustering of 13 *Fusarium oxysporum* isolates into two subgroups A1 and B1. The two groups separated at 10% genetic similarity, with most isolates being 50% similar.

The 13 *F. oxysporum* isolates were split into two subgroups at 10% genetic similarity. Subgroup A1 had five isolates with a genetic similarity range of 22% - 44.4%. Group B1 contained eight isolates with a genetic similarity range of 33.3% - 50%. The highest genetic similarity observed in this subgroup was 59% between isolates 284 and 263.

All *F.solani* and *F.equiseti* isolates were clustered into one subgroup shown in Fig 11d. Genetic similarities in this subgroup ranged from 37.5% to 83.3%. Two *F.solani* isolates 27 and 265, and one *F. equiseti* isolate 133 had a genetic similarity of 55.7%. The *F. solani* CABI isolate had a similarity of 60% with one of the *F. solani* isolates 272. *F. solani* isolates 261 and 262 had a high genetic similarity of 83.3%. Similarly *F. equiseti* isolates 126 and 134 had a high genetic similarity of 81.1%. This subgroup revealed a close genetic relationship between *F. solani* and *F. equiseti* isolates.

The remaining 24 *F. oxysporum* isolates were grouped with three *F moniliforme* isolates and the *R. solani* control isolate Fig 11e. Six of the *F. oxysporum* isolates were placed in one cluster with a genetic similarity range of 25% - 50%. The CABI *F. oxysporum* isolate was clustered with nine *F. oxysporum* isolates with genetic similarities ranging from 25% to 87.5%. Unexpected similarities were observed, where *R. solani* was related to *F. moniliforme var subglutinan*s by 40%. One of the *F. oxysporum* isolates, 337, and *F. moniliforme var subglutinans* had a genetic similarity of 60%. The remaining *F. oxysporum* isolates had a narrow genetic similarity range of 33.3% - 40%.

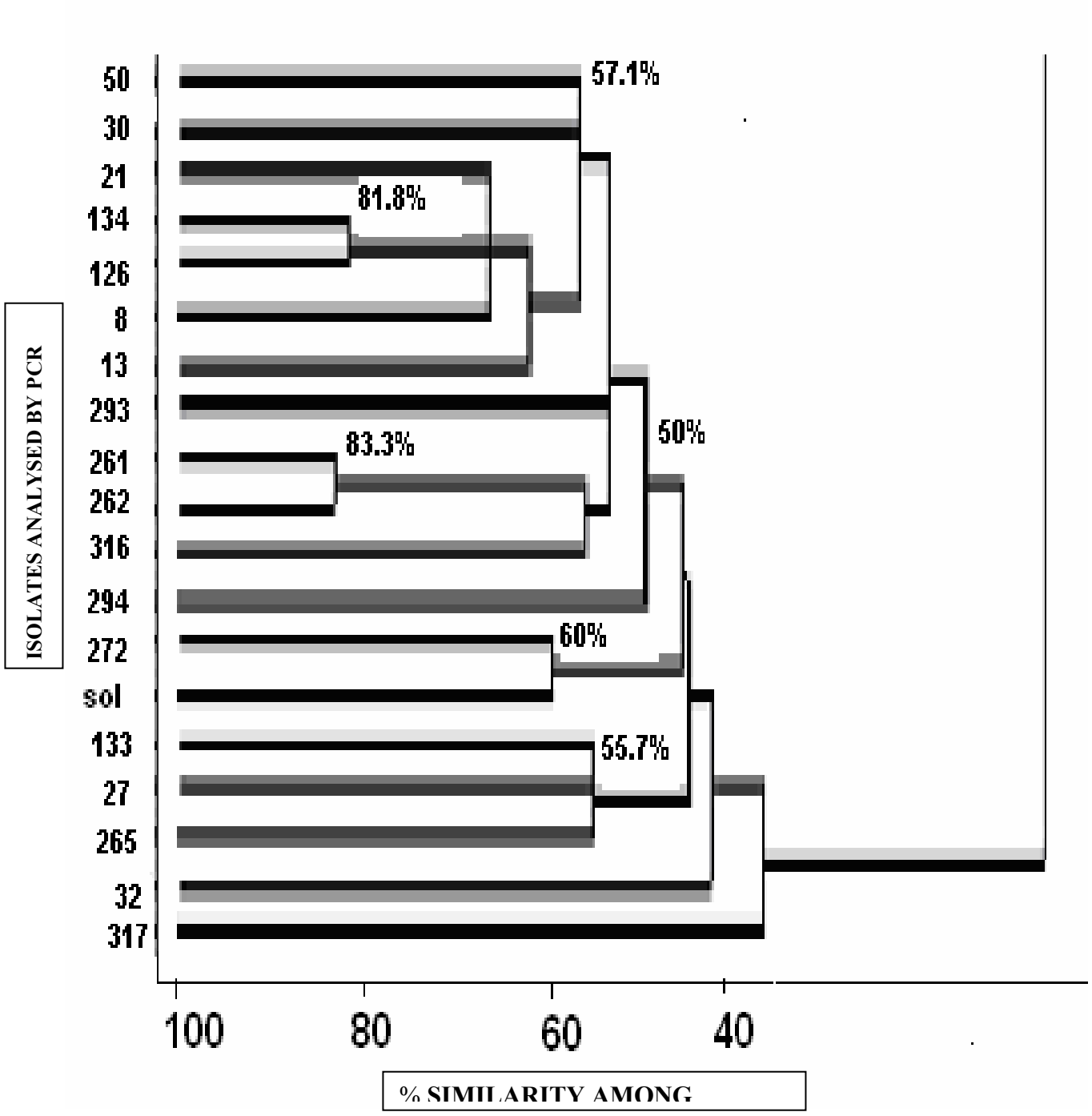

Fig 11d. Expanded section 3 of Fig 11a showing the groups formed among 14 *F. solani* and 5 *F. equiseti* isolates. The *F. solani* (CABI isolate) was grouped with another *F. solani* isolate (272) at 60% similarity.

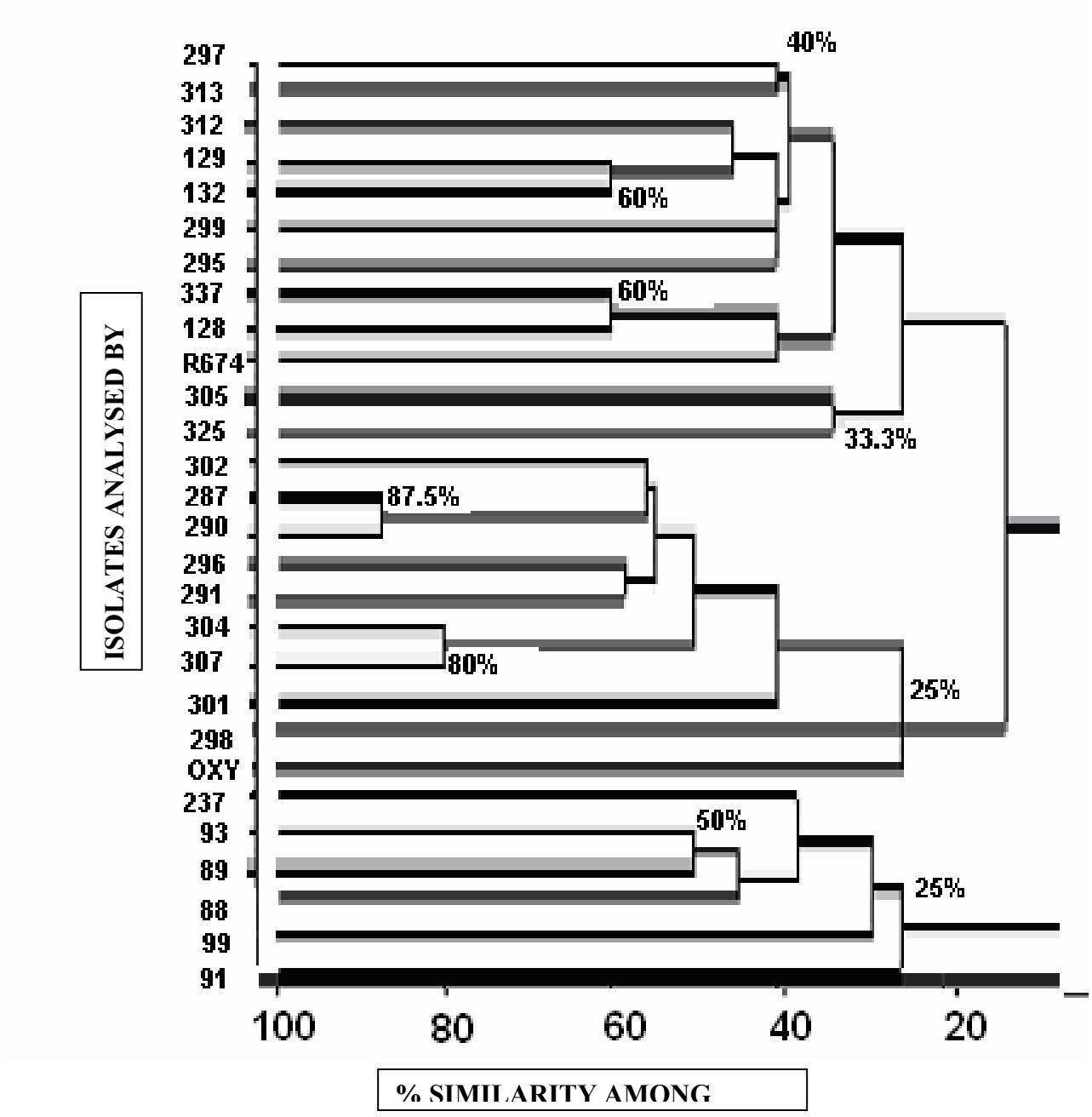

Fig 11e. Expanded Section 4 of Fig 11a showing subgroups of 24 *F. oxysporum* isolates and 3 *F. moniliforme* isolates (129, 132,128). *R. solani* control (R674) was grouped with *F. moniliforme* isolate at 40% genetic similarity.

Primer SSR 1 yielded 98 polymorphic bands in 84 isolates of which 44 were *F. oxysporum* including the IMI control, 14 *F. solani* including the CABI isolate, 9 *F. species*, 5 *F. equiseti*, 4 *F. moniliforme,* 2 *F. poae*, 1 *F. moniliforme* var *verticilloides,* 1 *F. moniliforme* var *subglutinans*, 1 *F. lateritium*, 1 *F. pallidoroseum* and 1 *F. graminearum* and *R.solani*. Some of the banding patterns generated by this primer are shown in Fig 12.

Similarly as with primer IS 36, most of the polymorphic bands were common across the isolates (indicated by the arrows). The *F. solani* isolates in lanes 5- 9 shared two distinct bands also shown by the arrows. Four *F. solani* isolates 13 and 21, in lanes 2 and 3; 261 and 262 in lanes 8 and 9, had the same banding patterns. Two *F. equiseti* isolates in lanes 15 and 17 had a similar banding pattern with slight differences in the band sizes.

After cluster analysis, the 84 isolates were separated into three distinct groups at 6.7% genetic similarity (Fig 13a). Subgroups formed by this primer are shown clearly in Figures 13b – d. The first one shown in Fig 13b had 25 *F. oxysporum* isolates, one *F. species*, *F. lateritium* and *R. solani*. Six of the *F. oxysporum* isolates, (284, 285, 302, 305, 307 and 312) were 100% similar. A wide genetic similarity range of 25% - 83.8% was observed among the other 19 *F. oxysporum* isolates. A surprising similarity of 50% was observed between *R. solani* and *F. oxysporum* isolate 290. The CABI *F. oxysporum* isolate was in the same cluster with two *F. oxysporum* isolates 287 and 313.

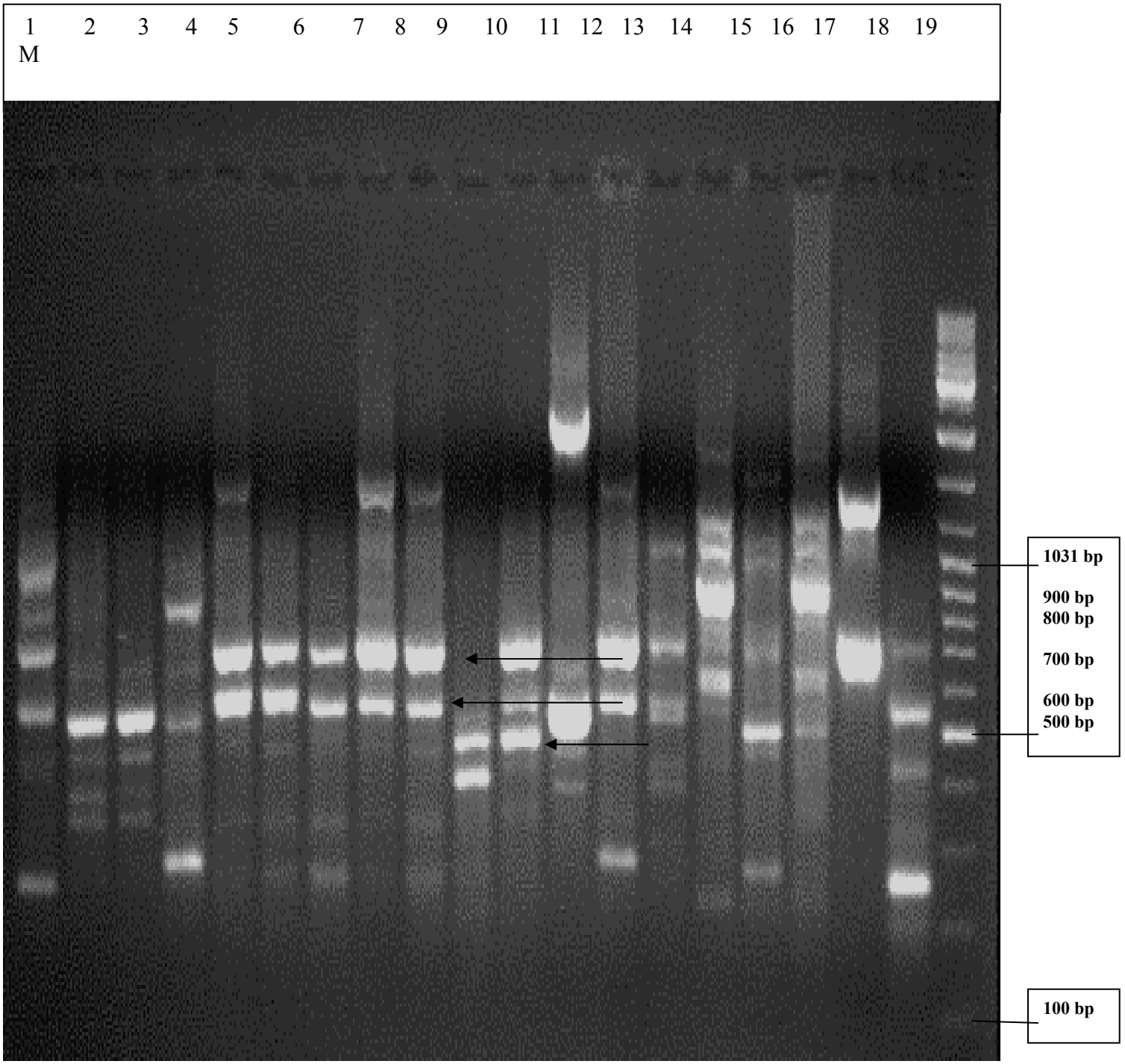

Fig 12. An ISSR profile of the PCR products generated by primer SSR 1. Lanes 1-13 *F.solani* isolates, lane 14 *F.solani* (CABI isolate), lanes 15-19 *F.equiseti* isolates, M, molecular-weight marker (DNA ladder mix). Polymorphic bands common across the isolates are indicated by the arrows.

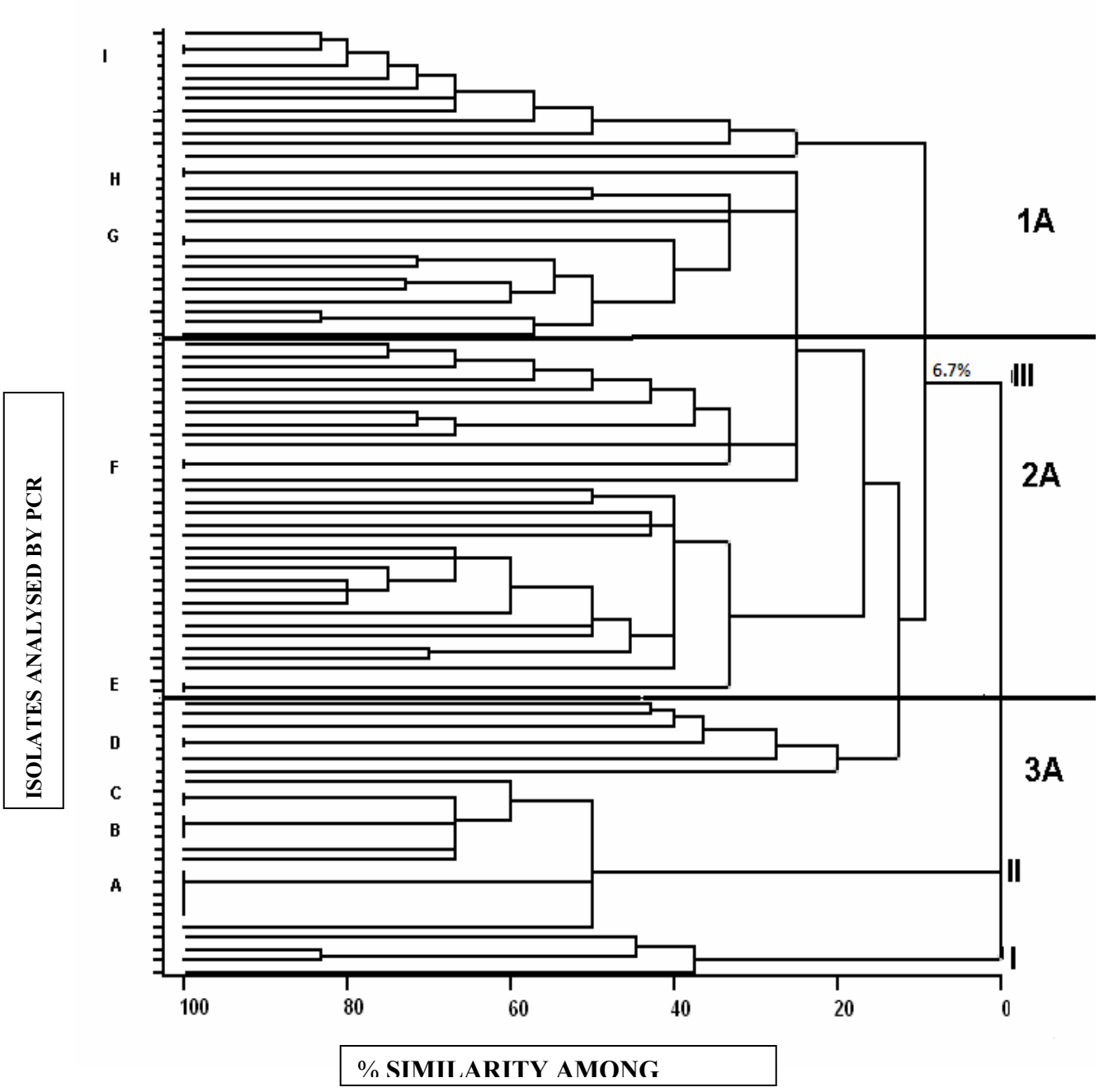

Fig 13a. Dendrogram generated from ISSR data of primer SSR1. **I, II** and **III** showing the three main groups formed at 6.7% genetic similarity. The letters A-I along the vertical axis indicate isolates that were 100% similar.

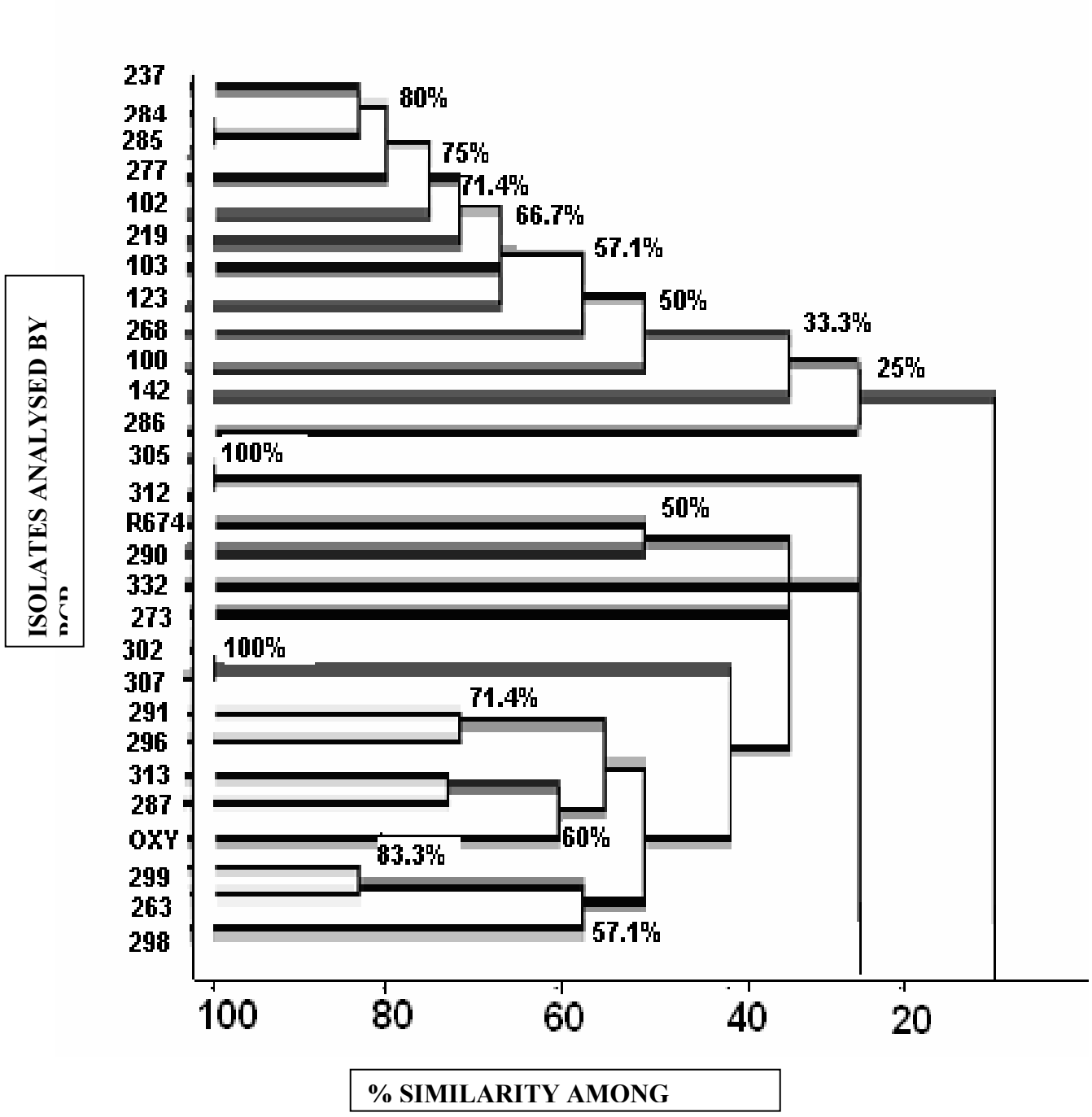

Fig 13b. Expanded section 1A of Fig 13a showing subgroups of 25 *F. oxysporum* isolates, 1 *F. species* isolate (332), 1 *F. lateritium* isolate (273) and the *R. solani* control (R674). The *F. oxysporum* CABI isolate (OXY) was 60% similar to *F. oxysporum* isolate 313. The *R. solani* isolate was grouped with an *F. oxysporum* isolate at 50% genetic similarity.

As with primer IS36, all *F. solani* and *F. equiseti* isolates were clustered into one subgroup (Fig 13c). The CABI *F.solani* isolate was closely linked to the two *F. equiseti* 317 and 133 by a similarity of 42.9%. Two *F. solani* isolates (13 and 21) and two *F. poae* isolates (329 and 331) were 100% similar. Other isolates in this subgroup included eight *F. species* with a genetic similarity range of 57.1% - 73%. There was one *F. oxysporum* isolate in this subgroup (301) which was linked to the *F. graminearum* isolate (259) at 25% similarity.

The last subgroup formed by this primer is shown in Fig 13d. In this subgroup, 18 *F. oxysporum* isolates were clustered with six *F. moniliforme* isolates and one *F. species*. *F.oxysporum* isolates74, 79, 81, 87, 88, 91, 93, 94, 95 and 96 were 100% similar and the remaining five *F. oxysporum* isolates had a similarity range of 37.5% - 66.7%. *F. moniliforme* isolate 132 and *F. moniliforme var verticilloides* isolate 129 had 100% genetic similarity.

Primer SSR 7 yielded 113 polymorphic bands in 86 isolates. The banding profiles generated by PCR products from 17 *F. oxysporum* isolates are shown in Figure 14. The arrows point to polymorphic bands that were more frequent. Similar banding patterns were observed for PCR products from isolates 289 and 299 in lanes 5 and 6 respectively. It can be noted that isolates 291, 296 and 319 in lanes 1, 3 and 15 respectively had no amplification products primed by primer SSR7. This could be an indication of no priming sites for this primer.

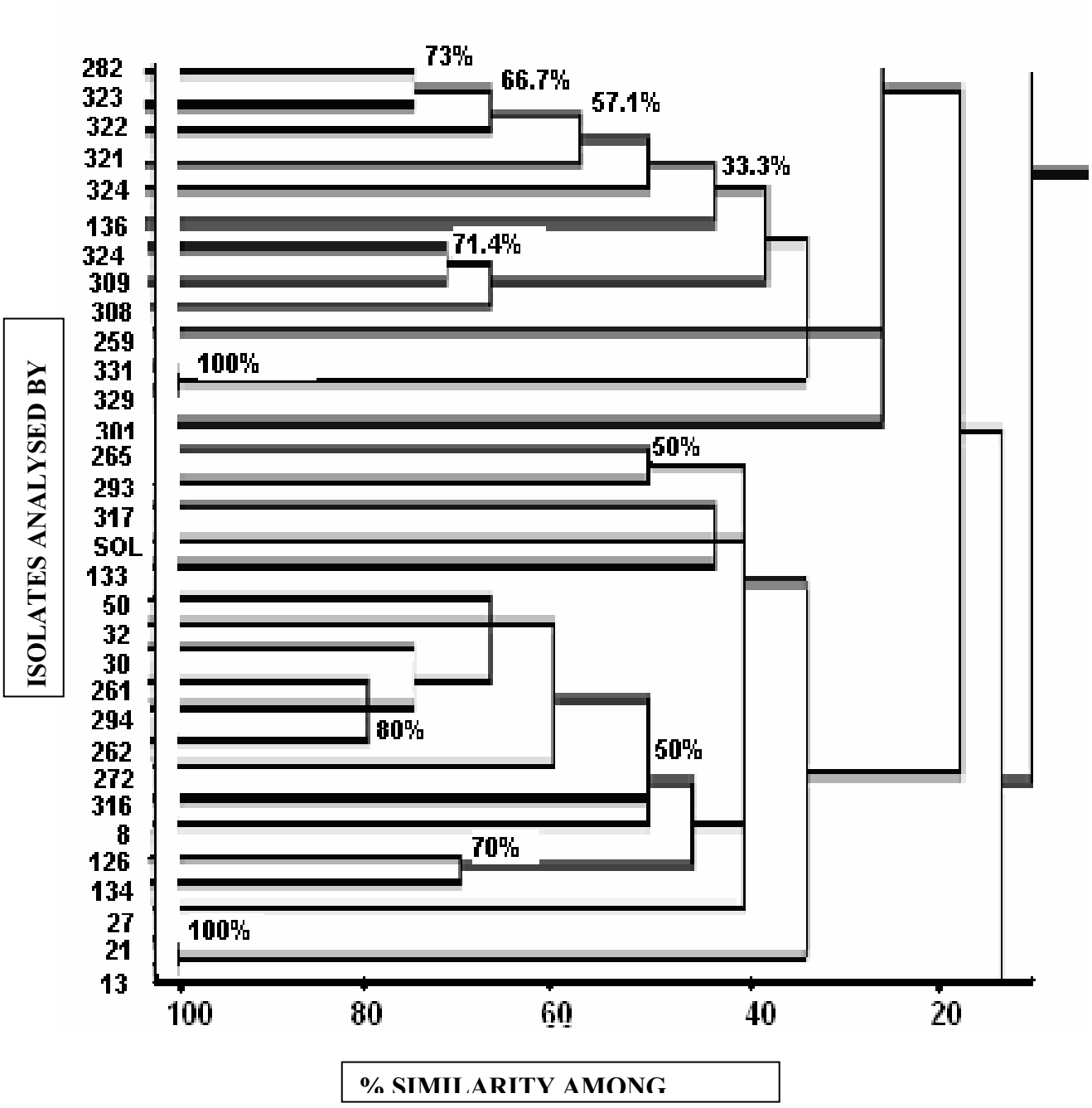

Fig 13c. Expanded section 2A of Fig 13a showing clustering of 8 *F. species* isolates, 14 *F. solani* isolates, 5 *F.equiseti* isolates (134, 126, 316, 133 and 317) , 2 *F. poae* isolates (331 and 329) with 100% genetic similarity, *F. graminearum* isolate (259), *F. pallidoroseum* isolate (136) and 1 *F. oxysporum* isolate (301).

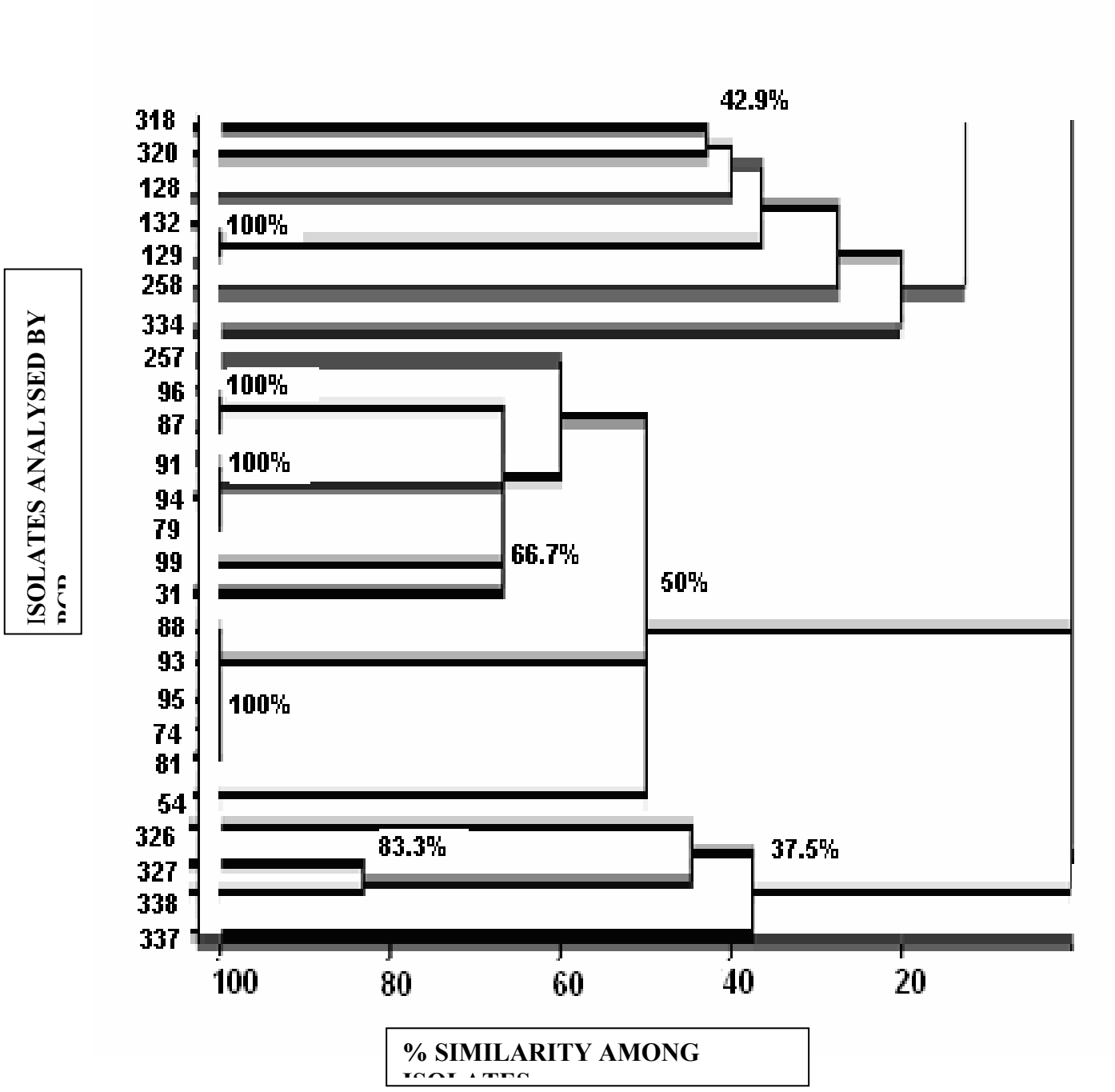

Fig 13d. Expanded section 3A of Fig 13a showing subgroups of 18 *F. oxysporum* isolates, 6 *F. moniliforme* isolates and 1 *F. species* with 12 *F. oxysporum* isolates 100% genetically similar. The *F. species* isolate (334) was grouped with the *F. moniliforme* isolates.

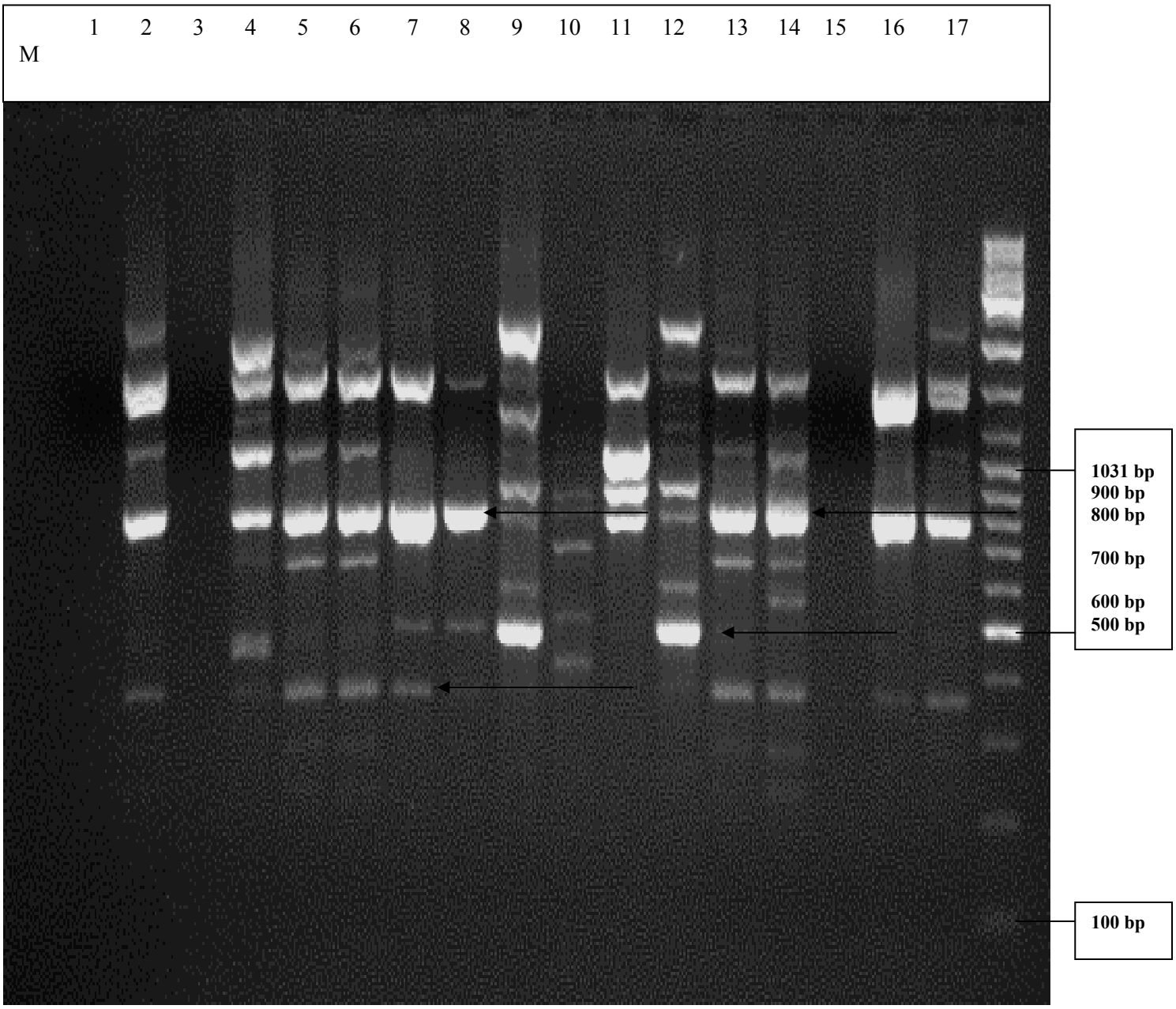

Fig 14. An ISSR profile of the PCR products generated by primer SSR7. Lanes 1-17 *F. oxysporum* isolates, M, molecular weight marker (DNA ladder mix). Isolates in lanes 5 and 6 show similar banding profiles. Polymorphic bands more frequent among isolates are shown by the arrows.

After cluster analysis of the combined ISSR data for primer SSR 7, the 86 isolates were split into five groups at 6.7% genetic similarity (Fig 15a). The high intraspecies diversity observed among the *F. oxysporum* isolates using primers IS 36 and SSR 1, was also evident with this primer.

Figures 15b – e are enlarged sections of the dendrogram showing the different subgroups that were formed. In Fig 15b, the high intraspecies diversity among the *F. oxysporum* isolates was observed with genetic similarity ranges of 62.5% - 100% among 16 *F. oxysporum* isolates. Eight of the isolates (29, 31, 81, 85, 88, 93, 94 and 95) were 100% similar.

In another subgroup, Fig 15c, 14 *F. oxysporum* isolates were related to seven *F. moniliforme* isolates with genetic similarities ranging from 25% to 87.5%. One *F. moniliforme* isolate 132 and *F. moniliforme var verticilloides* isolate129 had a high genetic similarity of 80%. This result was also seen with primer SSR1. All 14 *F. solani* isolates were placed in one subgroup (Fig 15d) together with five *F. equiseti* isolates and 10 *F. species*. Most of the *F. solani* isolates were similar by 50% except for two isolates 30 and 32 that were 100% similar. The CABI *F. solani* isolate was also placed in this subgroup. The last subgroup (Fig 15e) comprised 20 *F. oxysporum* isolates including the CABI isolate. Genetic similarities in this group ranged from 6.7% to 85.7%. This wide range revealed the high intraspecies diversity among the *F. oxysporum* isolates.

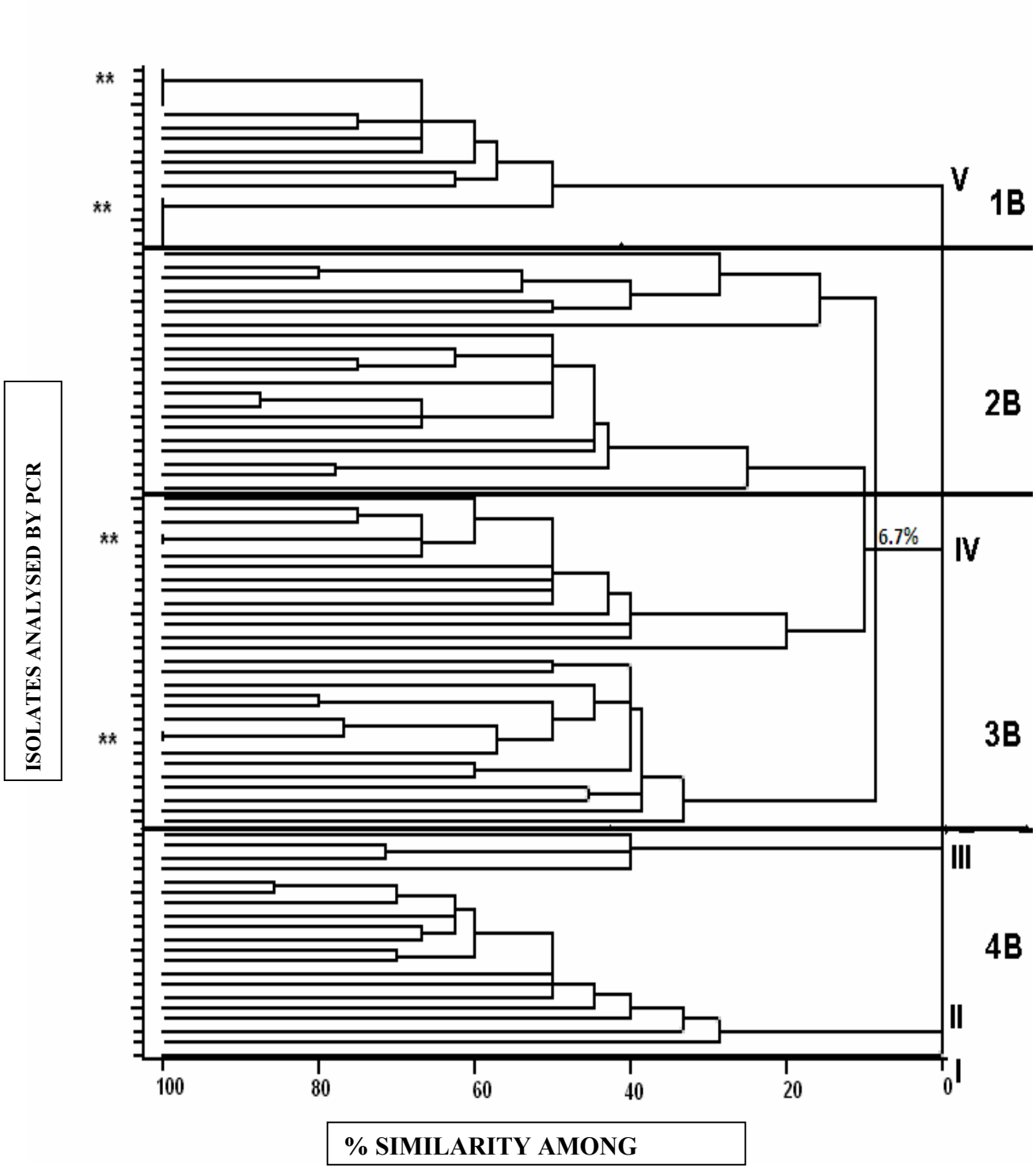

Fig 15a. Dendrogram generated from ISSR data of PCR products generated by primer SSR7. Five distinct groups I, II, III, IV and V were formed at 6.7% genetic similarity. The asterisks (along the vertical axis) indicate isolates that were 100% genetically similar.

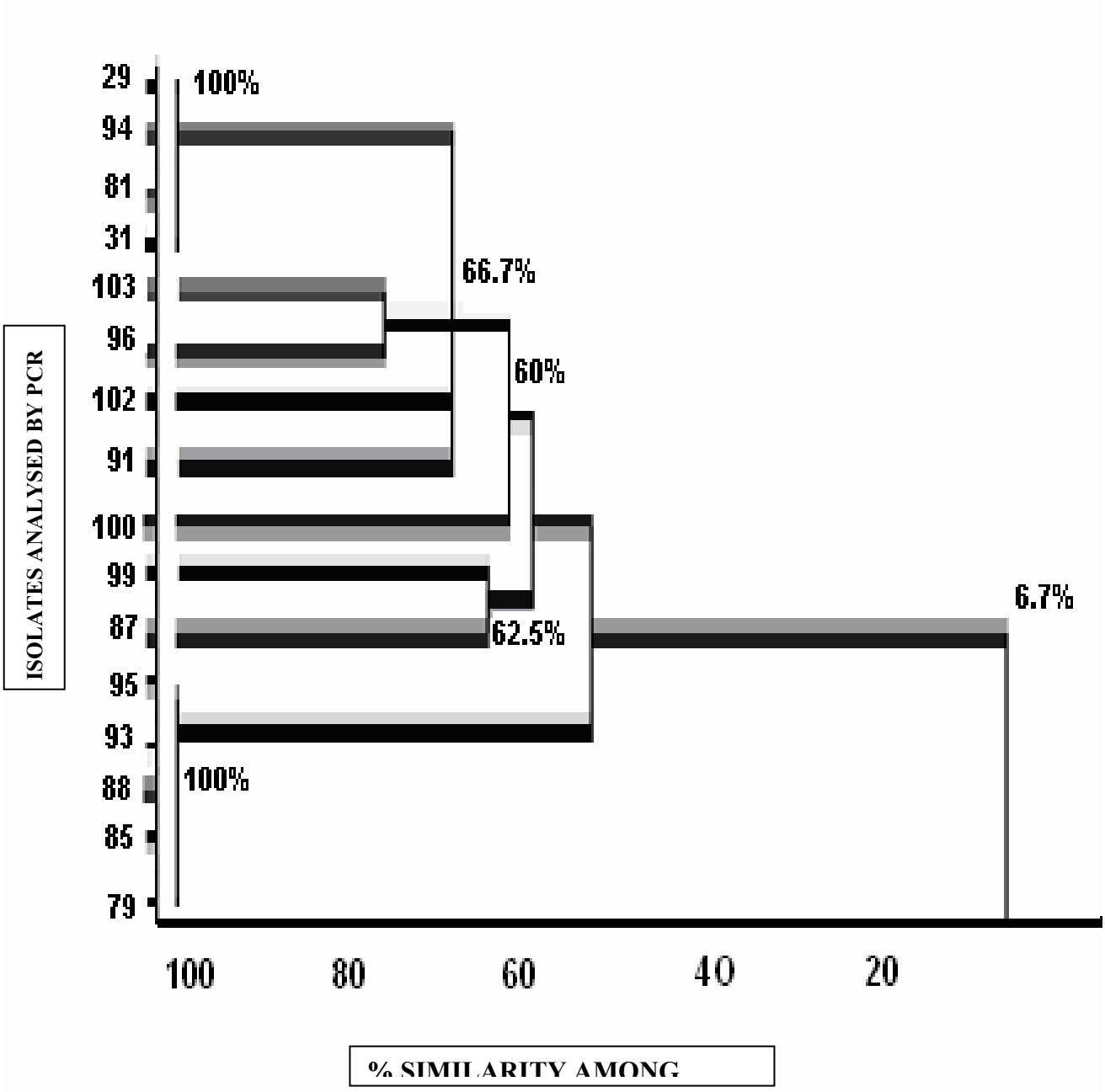

Fig 15b. Expanded section 1B of Fig 15a showing a group of 16 *F. oxysporum* isolates with a genetic similarity range of 62.5% - 100%.

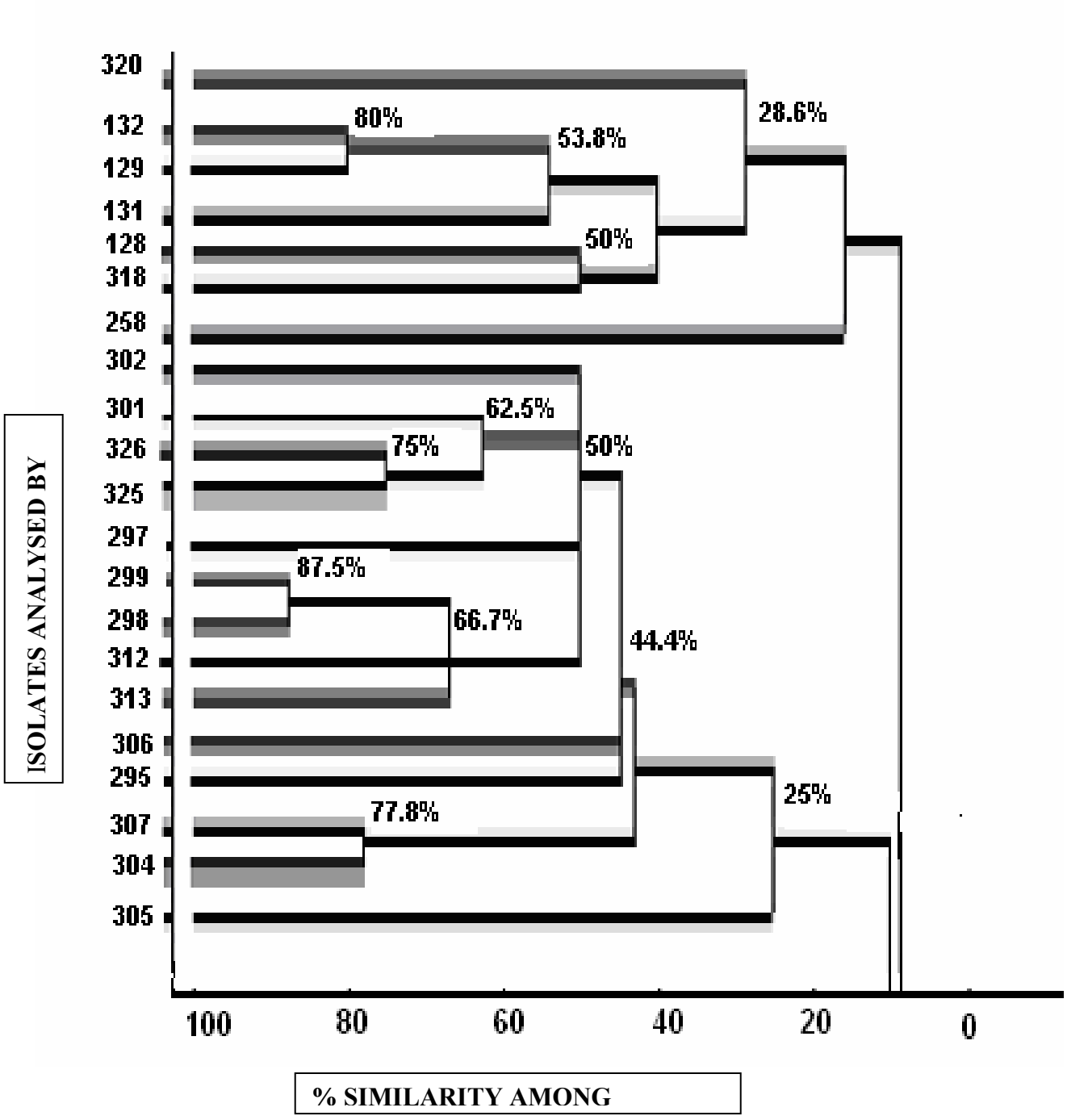

Fig 15c. Expanded section 2B of Fig 15a showing subgroups of 14 *F. oxysporum* isolates and 7 *F. moniliforme* isolates.

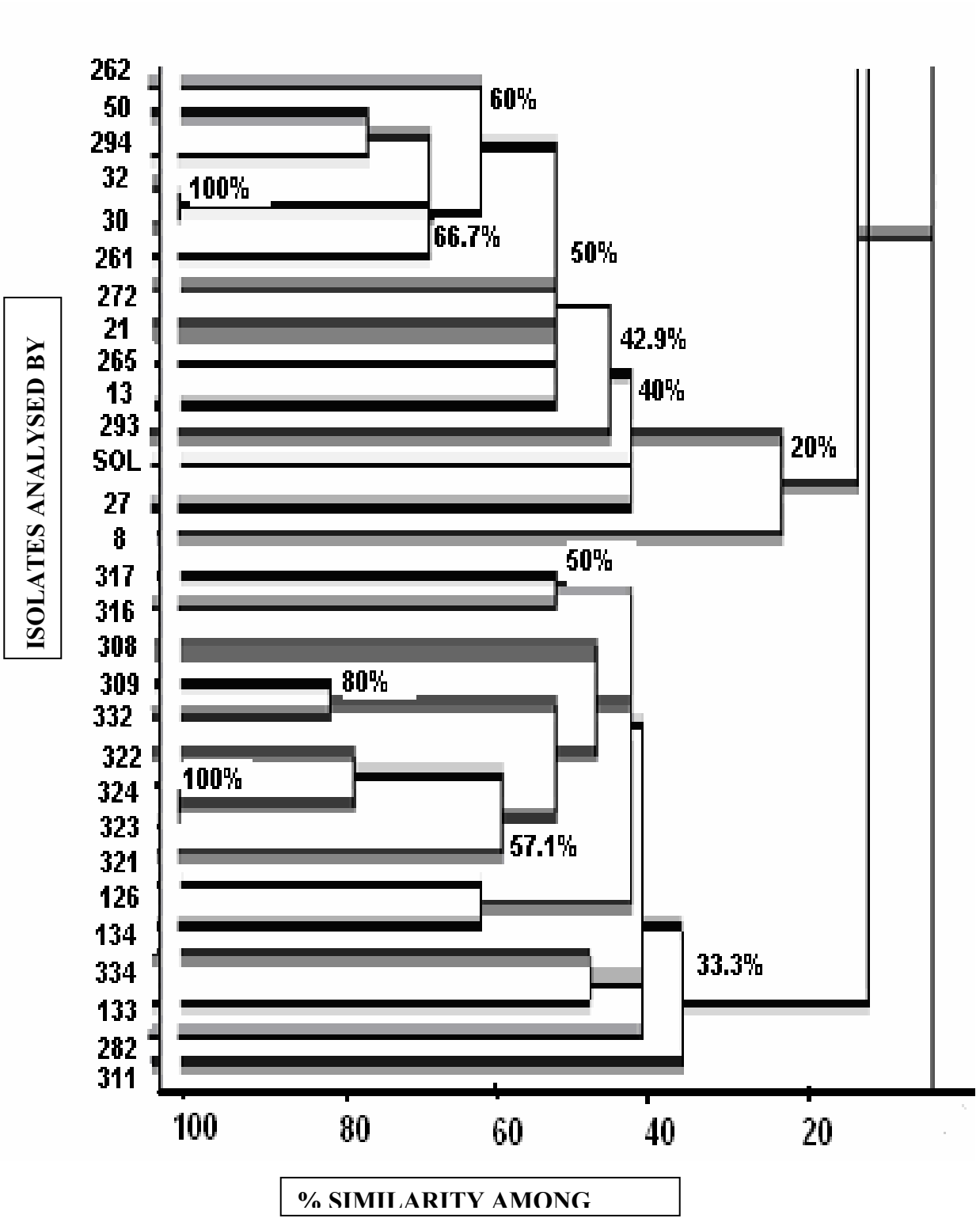
ISOLATES ANALYSED BY
262
50
294
32
30
261
272
21
265
13
293
SOL
27
8
317
316
308
309
332
322
324
323
321
126
134
334
133
282
311
60%
100%
66.7%
50%
42.9%
40%
20%
50%
80%
100%
57.1%
33.3%
100
80
60
40
20
% SIMILARITY AMONG

Fig 15d. Expanded section 3B of Fig 15a showing 14 *F. solani* isolates, 5 *F. equiseti* isolates and 10 *F.spp* isolates. Most *F. solani* isolates had 50% genetic similarity but two isolates 32 and 30 had 100% genetic similarity.

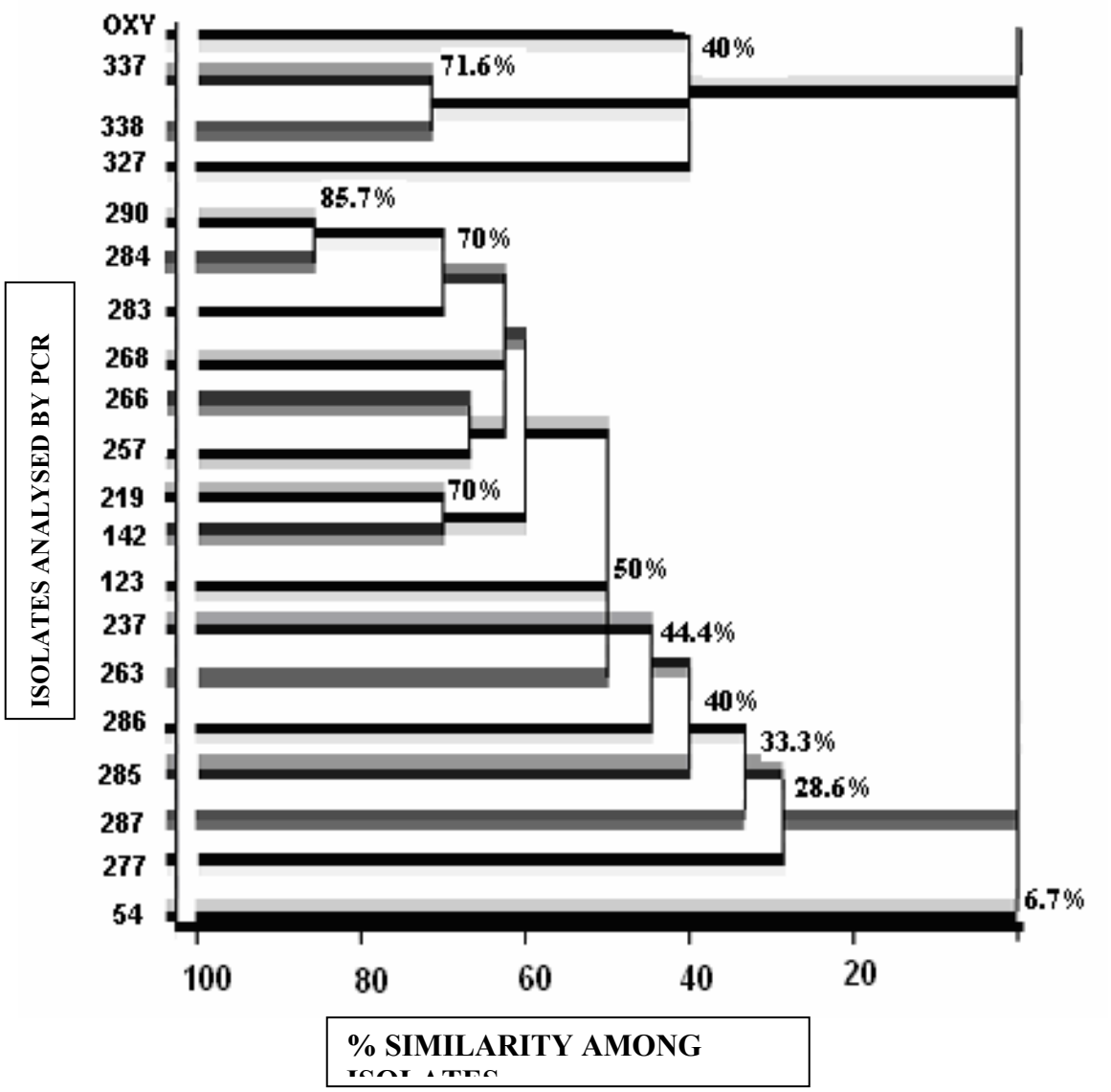

Fig 15e. Expanded section 4B of Fig 15a showing subgroups of 20 *F. oxysporum* isolates including the CABI isolate (OXY). The genetic similarities among the isolates ranged from 6.7% to 85.7%.

Primer SSR 17 gave the highest number of polymorphic bands (Fig 16) giving a total of 149 bands across 84 isolates. Several isolates (lanes 4, 5, 12 and 13) had similar banding patterns. Among the 84 isolates, 47 were *F. oxysporum*, 14 *F. solani*, 10 *F. spp*, 5 *F. equiseti*, 5 *F. moniliforme*, 1 *F. moniliforme* var *verticilloides*, 1 *F. moniliforme* var *subglutinans* and the *R. solani* isolate.

After cluster analysis, primer SSR 17 divided the 84 isolates into two groups at 6.7% genetic similarity (Fig 17a). The smaller group contained 17 *F. oxysporum* isolates with genetic similarities ranging from 25 to 100%. Two isolates in this group had 100% genetic similarity. The larger group was further divided into three subgroups. Figures 17b – e show the different subgroups into which the 84 isolates were placed. The clustering of 14 *F. oxysporum* isolates and 7 *F. moniliforme* isolates is shown in Fig 17b. The same clustering of isolates was observed with primer SSR 7 although the genetic similarities among the isolates were different for the two primers. Similarities in this case ranged from 20% to 69.2%. The CABI *F. oxysporum* isolate was linked to another *F. oxysporum* isolate though the genetic similarity was very low (20%).

The strong genetic relationship between *F. solani* isolates and *F. equiseti* isolates was also revealed by primer SSR 7. In Fig 17c, all 14 *F. solani* isolates were placed in a subgroup with four *F. equiseti* isolates and one *F. species*. Two *F. solani* isolates (32 and 263) had a genetic similarity of 100%. An unexpected high genetic similarity of 73.3% was noted between *F. equiseti* isolate 126 and the CABI *F. solani* isolate. Similarities for the other *F. solani* isolates ranged from 25% to 80%.

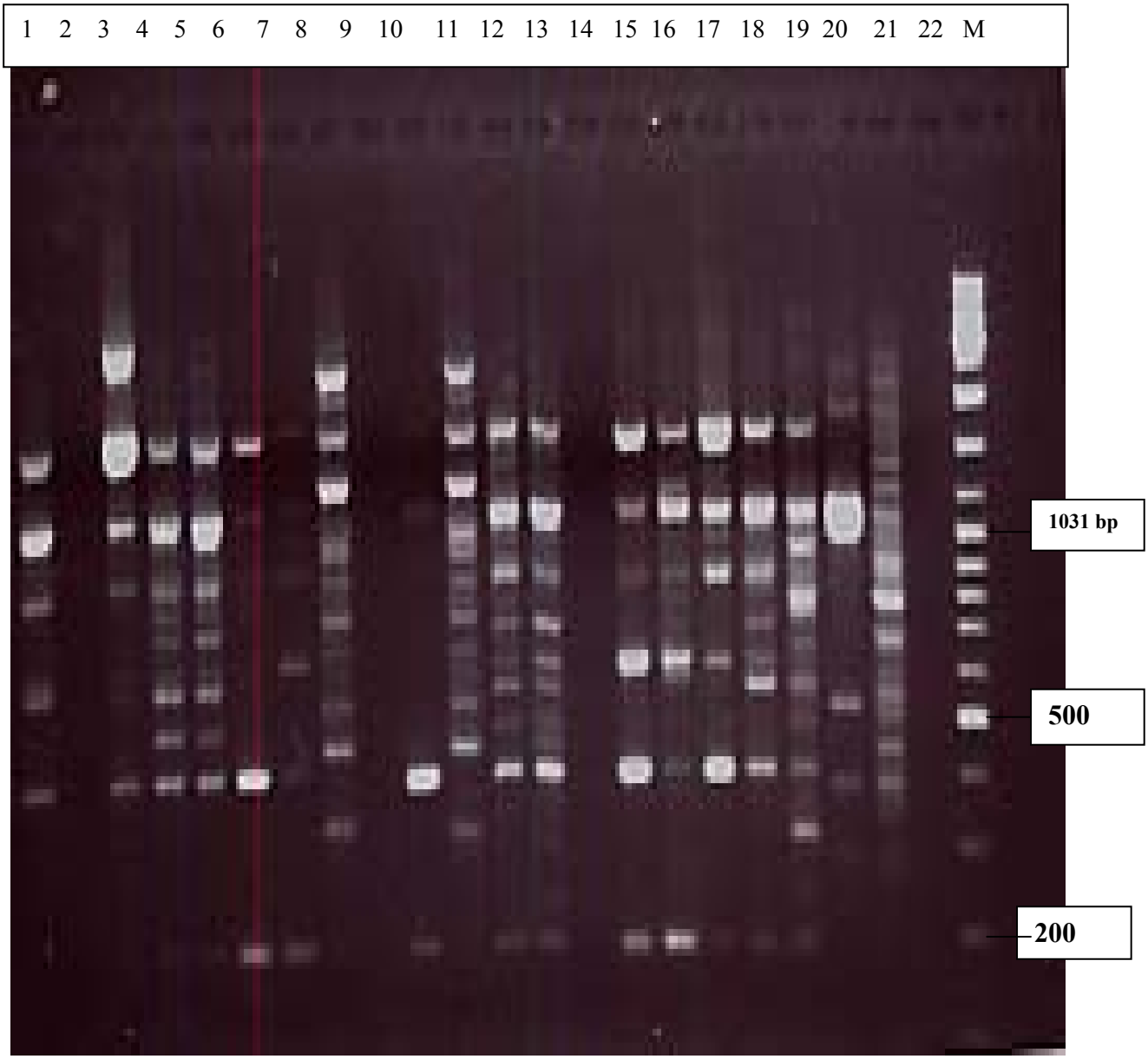

Fig 16. Gel electrophoresis pattern of a representative ISSR profile generated by primer SSR 17. Lanes 1-20 *F.oxysporum* isolates, lane 21 *R. solani* isolate, lane 22 NTC, M molecular marker (DNA ladder mix).

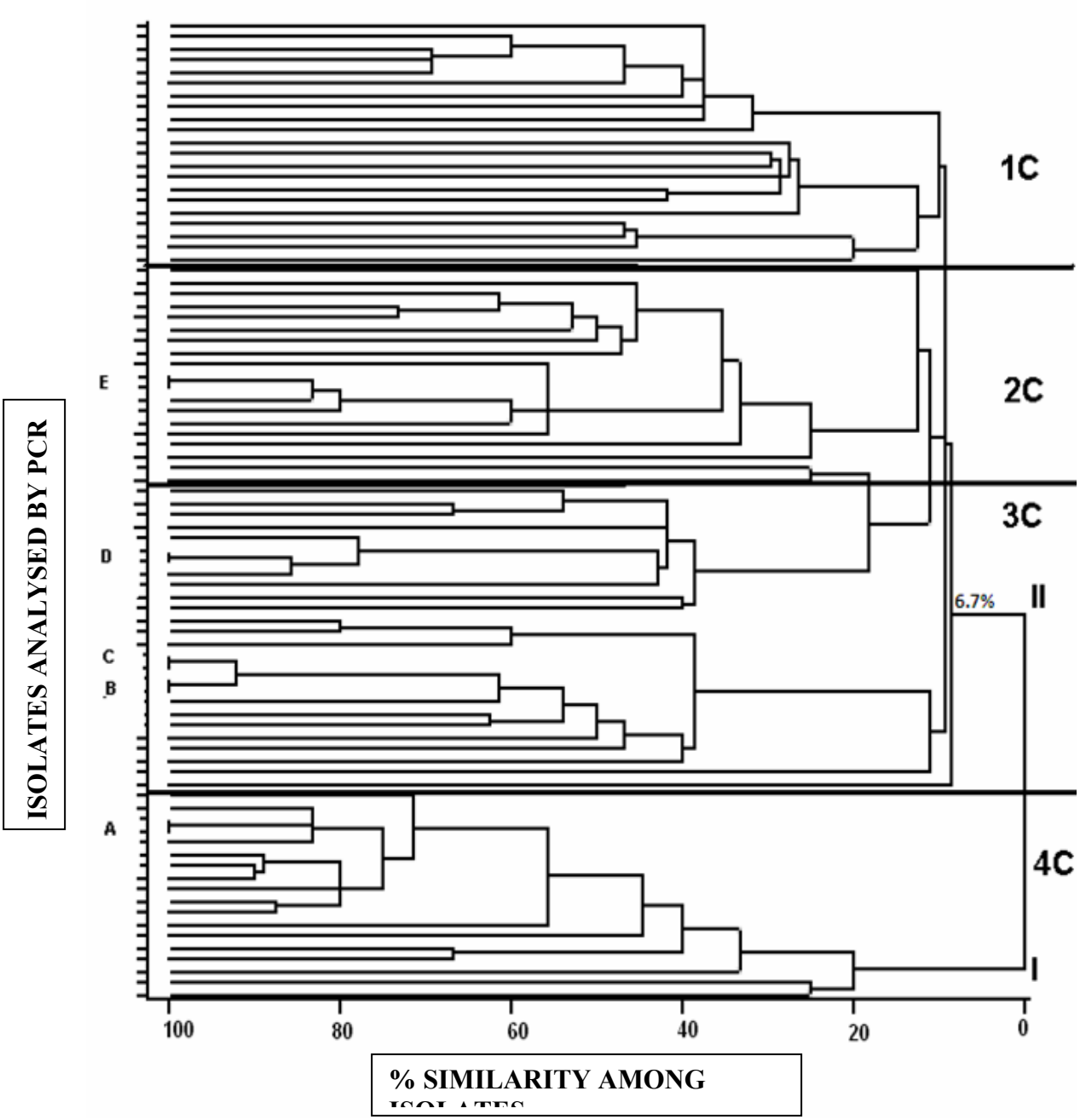

Fig 17a. Dendrogram generated from ISSR data of primer SSR 17. The letters A - E show 100% similarity between isolates. I and II indicate the two groups that were formed containing 6.7% similarity.

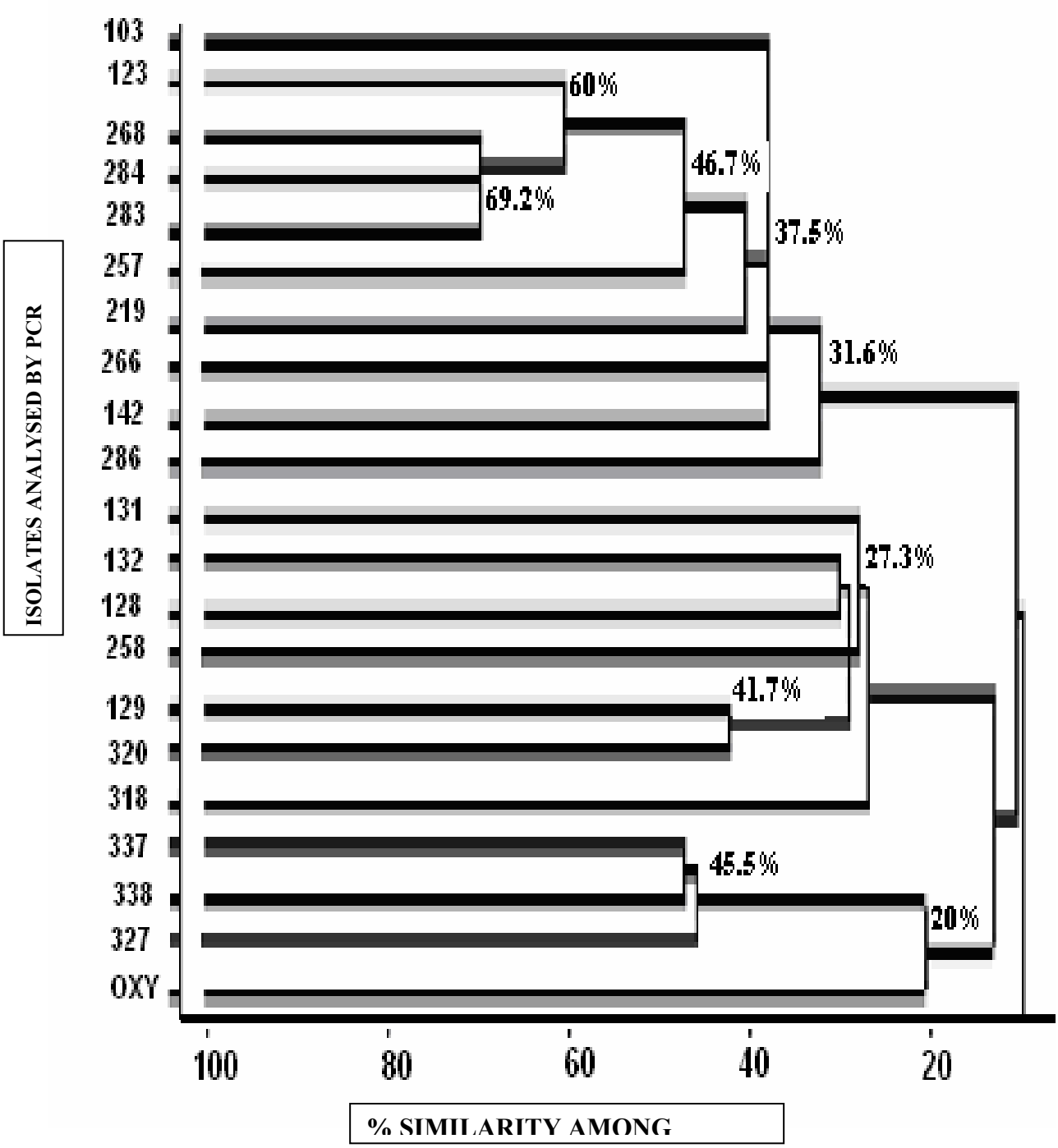

Fig 17b. Expanded section 1C of Fig 17a showing subgroups of 14 *F. oxysporum* isolates and 7 *F. moniliforme* isolates. All *F. moniliforme* isolates were placed into one subgroup and *F. oxysporum* CABI isolate (OXY) was grouped with the other *F. oxysporum* isolates.

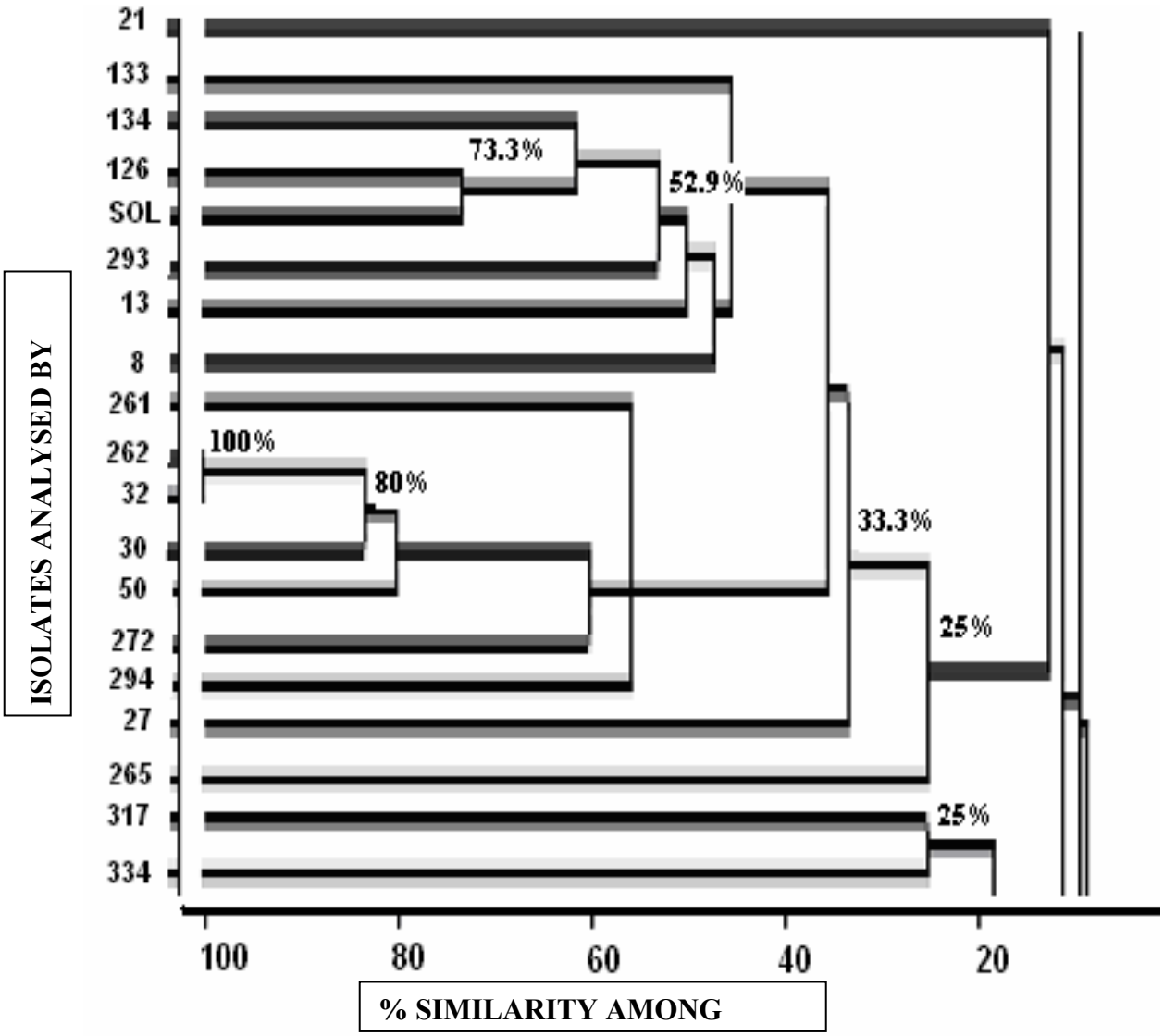

Fig 17c. Expanded section 2C of Fig 17a showing clustering of 14 *F. solani* isolates, 4 *F. equiseti* isolates and 1 *F. species* isolate (334). 2 *F. solani* isolates had 100% genetic similarity.

The wide genetic similarity ranges observed with IS 36, SSR 1 and SSR 7 were seen with primer SSR 17 as well. In Fig 17d, 15 *F.oxysporum* isolates were grouped with nine *F. species,* one *F. equiseti* isolate and *R. solani* control isolate. In this group, four *F. oxysporum* isolates (298, 299,312 and 313) were 100% similar. Two *F. species* isolates (323 and 324) also had 100% genetic similarity.

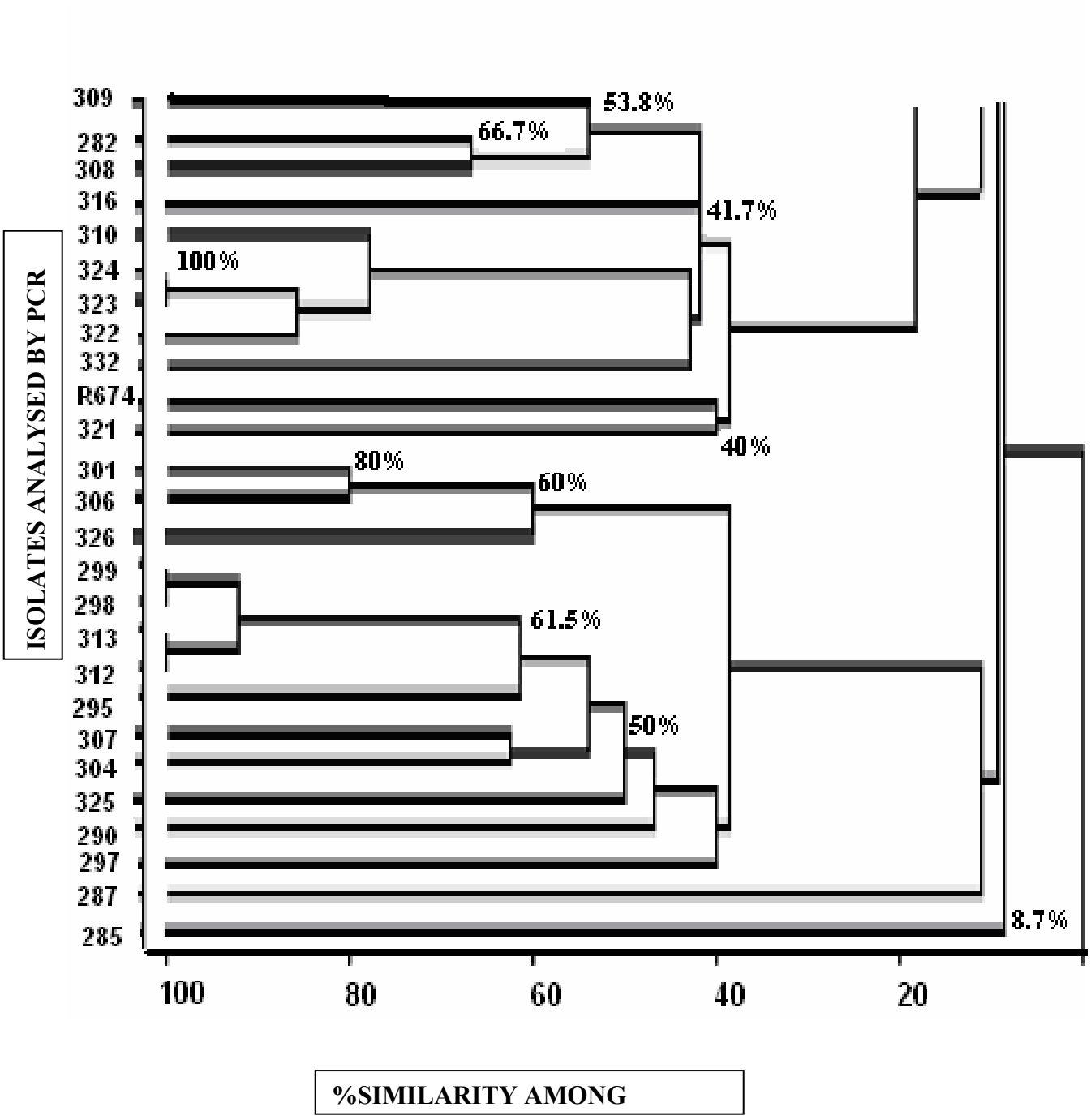

Fig 17d. Expanded section 3C of Fig 17a showing subgroups of 15 *F. oxysporum* isolates, 9 *F. species,* 1 *F. equiseti* isolate (316) and *R. solani* control (R674). Two *F. species* isolates (324 and 323) had 100% genetic similarity.

The last subgroup comprising 18 *F. oxysporum* isolates is shown in Fig 17e. Two isolates in this group (78 and 89) were 100% similar. The genetic similarities among the other isolates ranged from 25% to 87.5%.

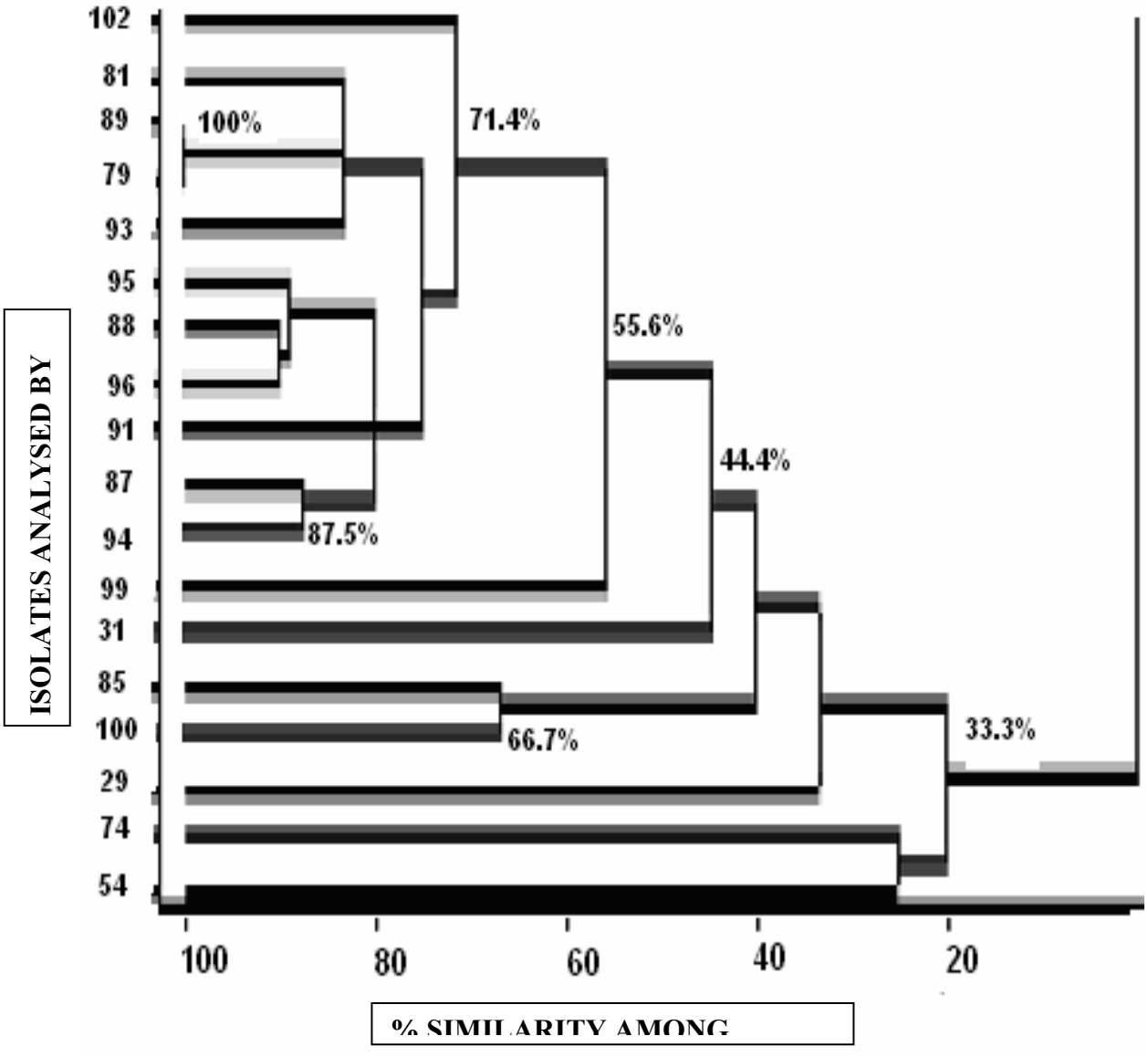

Fig 17e. Expanded section 4C of Fig 17a showing subgroups of 18 *F. oxysporum* isolates. Two isolates (79 and 89) had 100% genetic similarity. Genetic similarities among the other isolates ranged from 25% to 87.5%.

5.3 UP-PCR analysis

The primer pair L15/ L45 generated 126 reproducible bands in 72 isolates. A representative UP-PCR banding profile is shown in Figure 18. Among the 72 isolates, 40 were *F. oxysporum*, 14 *F. solani*, 8 *F. spp*, 4 *F. equiseti*, 3 *F. moniliforme*, 2 *F. poae* and 1 *F. moniliforme* var *subglutinans.* Results obtained with this primer pair were comparable to results obtained with the ISSR primers. Several polymorphic bands were common among the isolates (indicated by arrows) (Fig 18). This primer pair generated the least number of scorable bands from the isolates tested, indicating few priming sites for the primer pair used.

After cluster analysis of the UP-PCR data, the dendrogram in Fig 19a was constructed. This L15/L45 primer pair gave six distinct groups at 6.7% genetic similarity. Several subgroups were formed using this primer pair (Fig 19b – e). The intraspecies diversity among *F. oxysporum* isolates was also indicated using this primer pair. The first subgroup shown in Fig 19b had 13 *F. solani* isolates, seven *F. species* isolates and one *F. moniliforme var subglutinans*. Genetic similarities among the *F.solani* isolates ranged from 20% to 66.7%. The CABI *F. solani* isolate was not placed in this subgroup as was the case with the ISSR primers. There was a relatively wide genetic similarity range of 42.9 % - 66.7% among the *F. species* isolates.

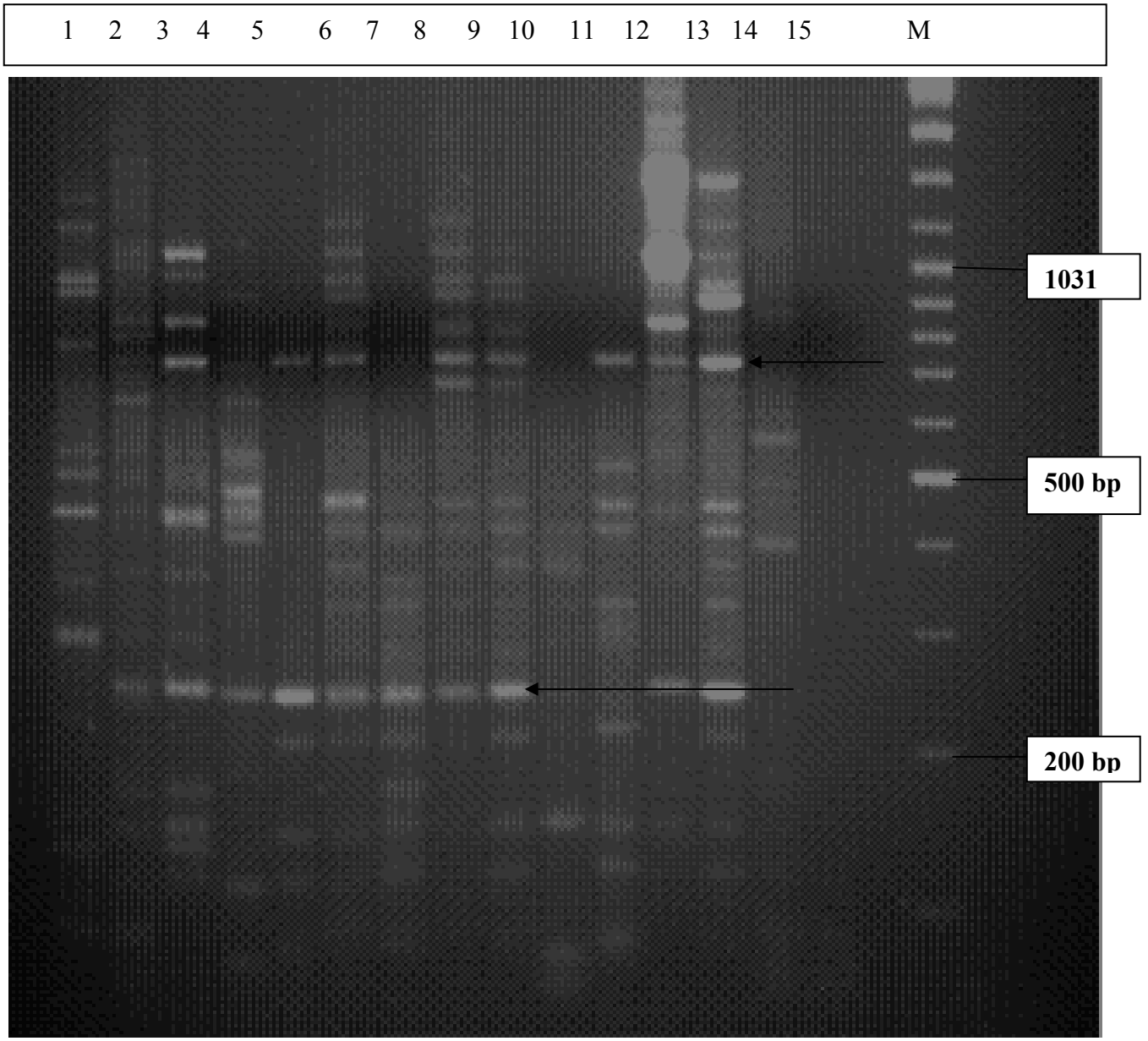

Fig 18. A representative ISSR profile of PCR products generated by primer combination L15 and L45. Lanes 1-13 *F. solani* isolates, lane 14 *F.solani* (CABI), lane 15 NTC, lane M, DNA ladder mix. The polymorphic bands marked by arrows were identified as common.

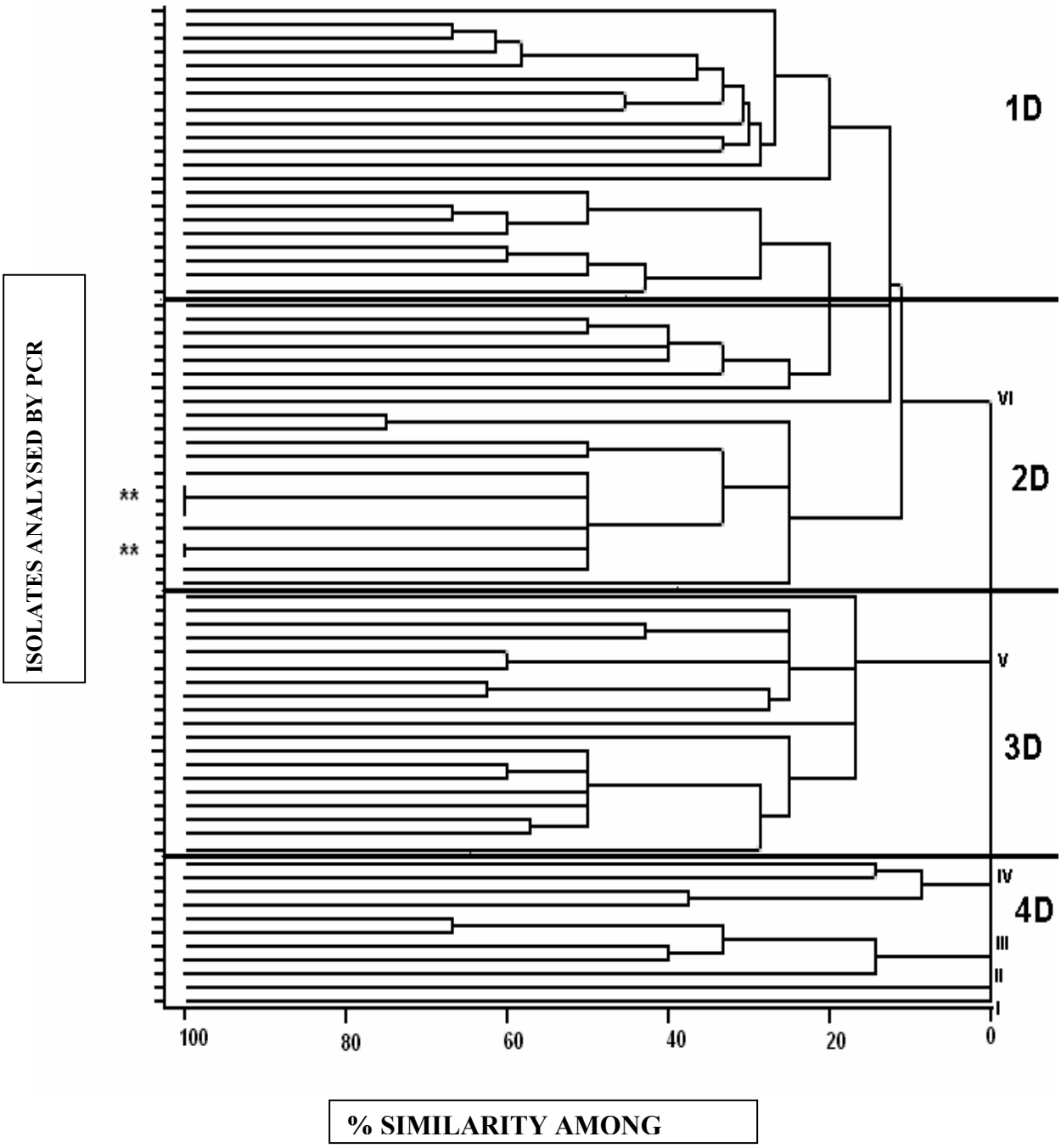

Fig 19a. Dendrogram generated from UP-PCR data of primer pair L15/L45 showing six groups formed at 6.7% similarity. The asterisks indicate *F. oxysporum* isolates that had genetic similarities of 100%.

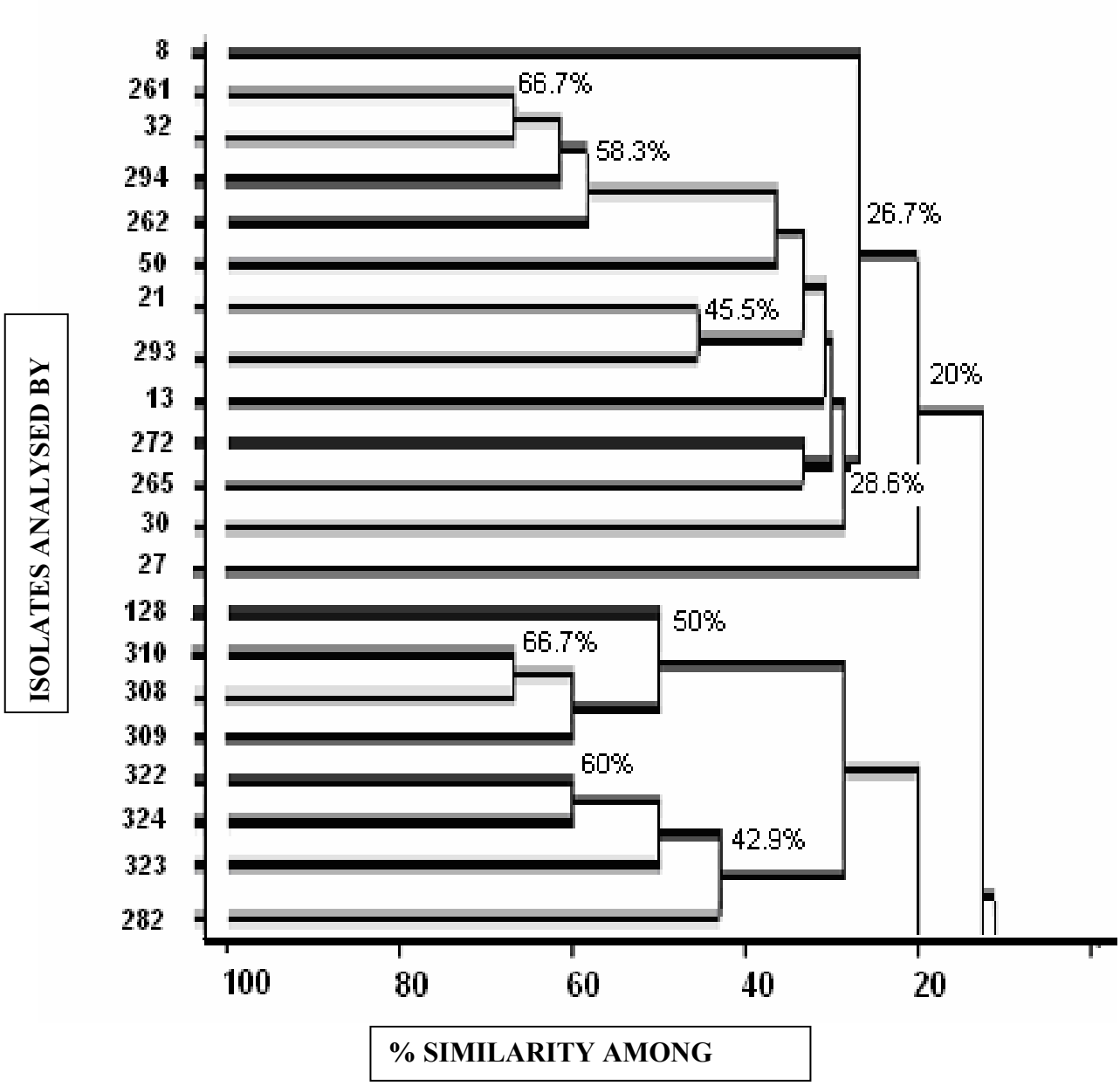

Fig 19b. Expanded section 1D of Fig 19a showing subgroups of 13 *F. solani* isolates, 7 *F. species* isolates and 1 *F. moniliforme* var *subglutinans* isolate (128).

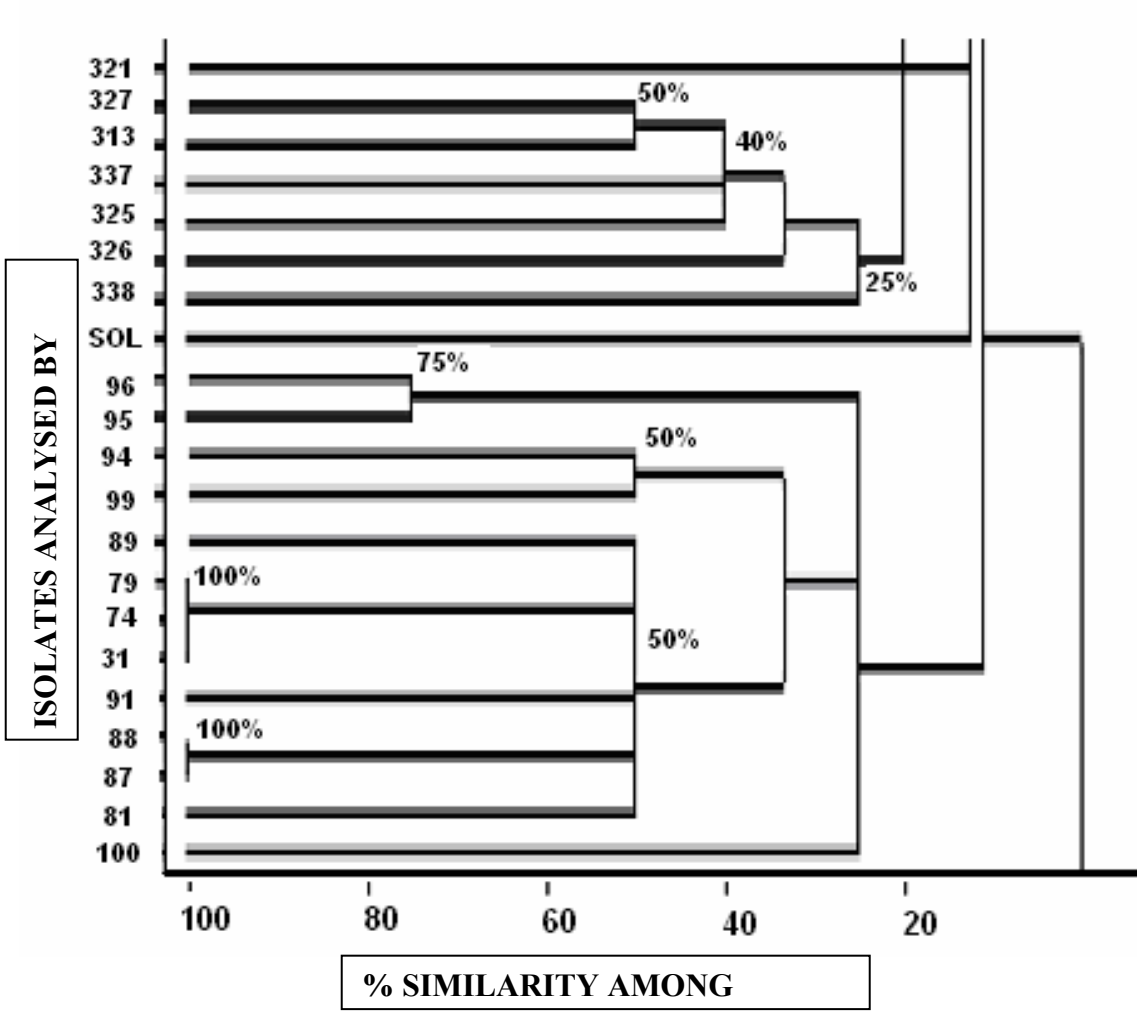

Fig 19c. Expanded section 2D of Fig 19a showing clustering of 19 *F. oxysporum* isolates, 1 *F. species* isolate (321) and *F. solani* CABI isolate (SOL). Most of the *F. oxysporum* isolates had 50% genetic similarity.

Similarly as with ISSR primers 1, 7 and 17, this primer pair also resulted in 100% genetic similarity among some *F. oxysporum* isolates (Fig 19c). In this subgroup, 19 *F. oxysporum* isolates were related to one *F. species* isolate and the CABI *F. solani* isolate but the genetic similarity was low (12.5%). Five *F.oxysporum* isolates (31, 74, 79, 87 and 88) were 100% similar. Most of the other *F. oxysporum* isolates in this subgroup were similar by 50%.

The clustering of the subgroup comprising 19 *F. oxysporum* isolates is indicated in Fig 19d. Genetic similarities among these isolates ranged from 16.7% to 62.5%. This wide range was also observed with the ISSR primer products.

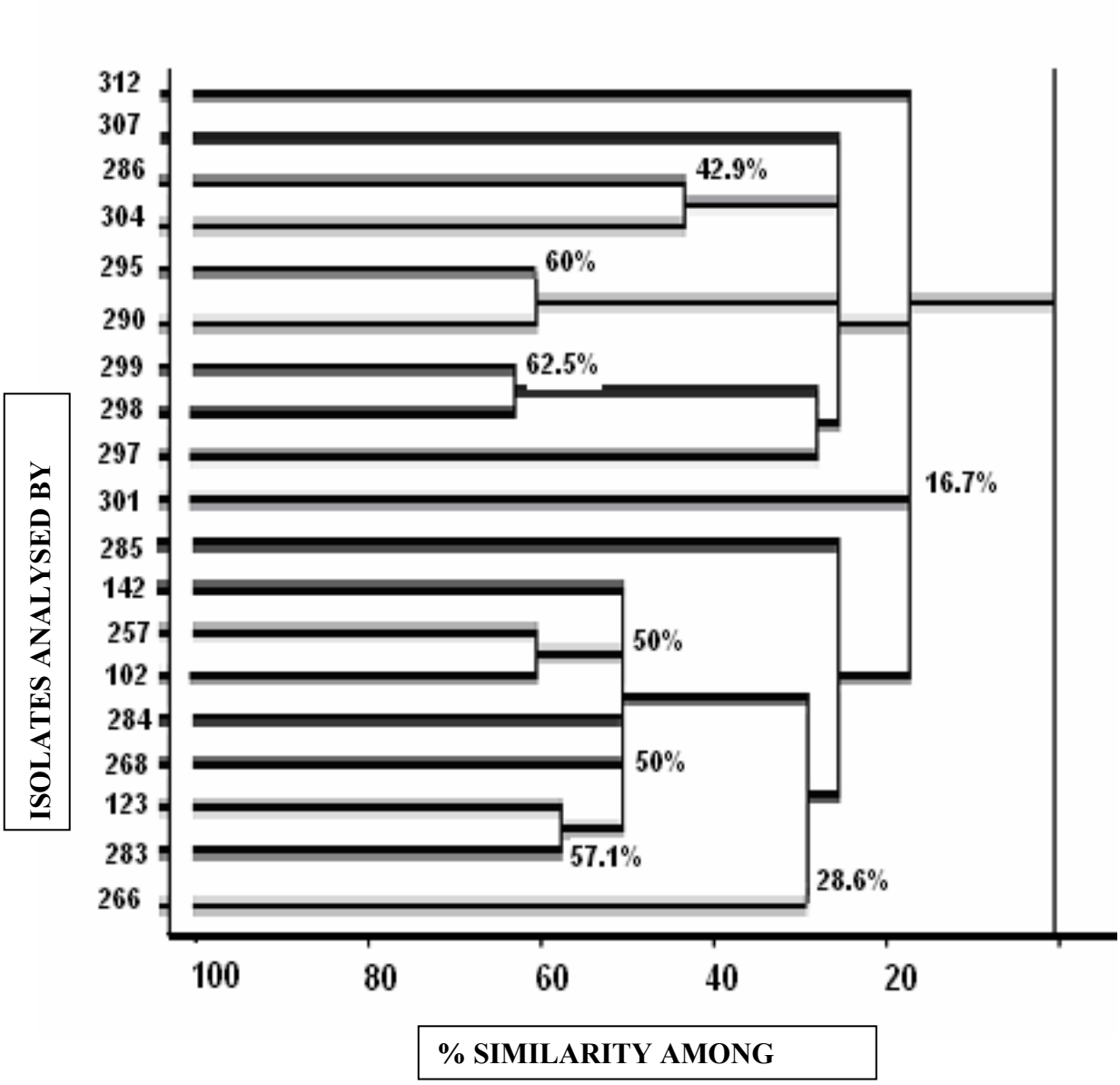

Fig 19d. Expanded section 3D of Fig 19a showing clustering of 19 *F. oxysporum* isolates. Genetic similarities ranged from 16.7% to 62.5%.

Fig 19e shows the last subgroup in which four *F. equiseti* isolates were clustered with two *F. poae* isolates, three *F. moniliforme* isolates and two *F. oxysporum* isolates. The two *F. poae* isolates had a genetic similarity of 66.7% whilst the *F. equiseti* isolates had low genetic similarities ranging from 14.3% to 37.5%.

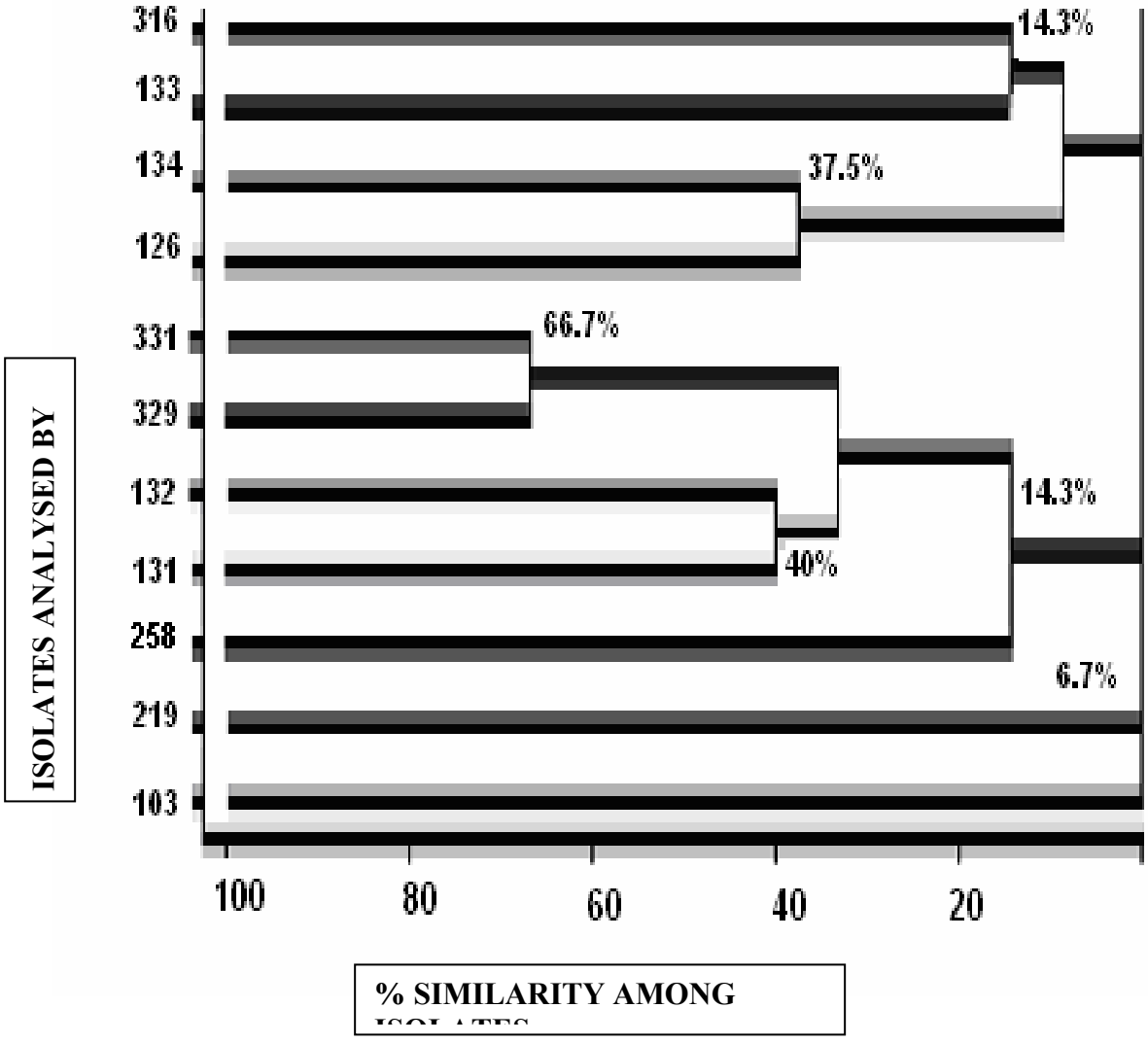

Fig 19e. Expanded section 4D of Fig 19a showing groups of 4 *F. equiseti* isolates, 2 *F. poae* isolates (329 and 331), 3 *F. moniliforme* isolates and 2 *F. oxysporum* isolates (103 and 219).

CHAPTER 4

6.0 DISCUSSION

The current fungal taxonomic systems have used macroconidia and microconidia in the asexual stages to identify fungal species. However, the plasticity and intergradation of the phenotypic traits has presented difficulties in identifying the filamentous fungi (Duggal *et al.,* 1997). Most genetic diversity studies of the genus *Fusarium* have sought, as an initial goal, more stable and reliable markers for delineation of the species and subspecific rankings. These studies have used approaches such as isozymes, DNA fingerprinting, RFLPs, RAPDs and DNA sequence analysis as the basis for comparison of individual isolates (Kistler, 1997). Two molecular based techniques were employed in this work to achieve a similar goal.

Analyses of rDNA sequences constitute an important complement of the morphological criteria needed to allow fungi to be more easily identified (Guarro *et al.,* 1999). The nucleotide sequence analysis of the rDNA region of a genome has been widely taken to have phylogenetic significance, and is, therefore, used in taxonomy and the study of phylogenetic relationships. In order to accurately identify the genus as *Fusarium*, species-specific primers, ITS-F-f and ITS-F-r, were used to amplify the ITS region of the rDNA.

In this study, the sequencing of the 389bp ITS fragment revealed 10 different sequence classes among the 13 species analyzed. The 100% genetic similarity among the three species *F. moniliforme*, *F. pallidoroseum* and *F. lateritium* (Fig 9) suggests that species designation based on morphological data alone could be unreliable. This result was further supported by the ISSR

analysis of these species using primer IS 36. A surprisingly high genetic similarity of 80% was observed between *F. pallidoroseum* and *F. lateritium*. Similar work done by Young-Mi *et al.*, 2000, where sequences of the internal transcribed spacers of the ribosomal DNA among 13 species from *Fusarium* sections Elegans, Liseola and Dlaminia were compared, also revealed discordant results. Consequently, further analysis of these three species is required before a definitive conclusion can be made on their genetic relationship.

The 100% similarity between the two *F. oxysporum* isolates (Fig 9) suggests that they are from the same species and have the same ITS sequence. This result could be an indication of an agreement between morphological and molecular identification of this species. A previous report by Bruns *et al.,* (1991), showed that the section Elegans *(F. oxysporum*) was genetically very closely related to the section Liseola (*F. moniliforme*). This finding is in agreement with results obtained in this work where the two species had a genetic similarity of 98%.

In this study, the ISSRs exposed a significant number of polymorphisms, which gave an indication of species diversity in the genus. Molecular marker analysis can be useful for determining the finer details of the relationship between genotypes and phenotypes. The efficiency of a molecular marker technique depends upon its polymorphism level in the set of accessions tested. ISSR techniques have a high degree of reproducibility, possibly due to the use of longer primers of 16–25 base pairs.

Usually, ISSR primers based on di-nucleotide repeats reveal high polymorphism (Blair *et al*. 1999; Joshi *et al*., 2000; Nagaoka and Ogihara 1997) and this was confirmed in this study.

Although definitive placement of some species remains uncertain due to no amplification in some isolates, these initial results demonstrated the diversity of *Fusarium,* hence this technique can be used in genetic diversity and phylogenetic studies.

In the present analysis, selected ISSRs revealed a significant number of polymorphisms, which can distinguish some of the species from each other. In most cases, the four ISSR primers used revealed a good agreement between morphological and molecular identification of the species. Isolates belonging to the same species were clustered into the same groups, despite the low genetic similarities in some cases. All four primers grouped *F. solani* isolates and *F. equiseti* isolates into the same subgroup. This result suggests a close relationship between these two species which can further be studied using species-specific primers and rDNA sequencing. The different subgroups formed by each primer were indicative of inter-species diversity in this genus. Primers IS 36, SSR 17 and SSR 1 grouped the *Rhizoctonia solani* isolate with the *Fusarium* isolates with genetic similaritites of 40% and 50%, respectively. This result indicates that genomes of the two species have several similar priming sites for the ISSR primers used. The use of species-specific primers will be necessary in further analyzing this genetic relatedness.

Some workers have employed ISSRs to study the genetic structure of various *Fusarium* species. Similarly in the present study, each of the four ISSR primers revealed a high intraspecies diversity among the *F. oxysporum* isolates which have been reported to have considerable variation in morphological features. Several investigators recognized true variants within the species with respect to host plant species specialization and more than 70

physiologic strains that are indistinguishable from saprophytic strains of the same species, but show different physiological properties in their ability to parasitise specific hosts (Kistler, 1997). This characteristic may be used to explain how the several groups of *F.oxysporum* isolates were formed by the ISSR primers. The varying similarity ranges within this species could also be a result of isolates that share a host range. It is also possible that some of these isolates are not *F. oxysporum*. There is, therefore, a need to gather more molecular data in order to classify these isolates further.

According to the report by Kistler (1997), genetic relationships based on rDNA sequences suggest *F. oxysporum* to be a species complex consisting of at least five phylogenetically distinct species. Work done by Glass and Donaldson (1995), revealed a greater RFLP diversity for *F. oxysporum* than for isolates of other *Fusarium* species; cluster analysis placed the isolates into several distinct groups. Similarly Paavanen *et al.,* (1998), distinguished 27 *F. oxysporum* isolates from each other by RAPD-PCR analysis, and clustered them into seven groups. The high degree of genetic diversity observed among these isolates could also be a result of cryptic speciation in the species. This possibility was observed in *F. subglutinans* isolates that were subdivided into more than one phylogentic lineage, suggesting that the existing populations of *F. subglutinans* were in the process of undergoing divergence and each of the resulting lineages were undergoing separation into distinct taxa (Steenkamp *et al.,* 1999).

The different levels of genetic similarities observed among all the analysed isolates were expected since this genus is large and diverse and the species have a broad latitude for genetic variation. Leissner and co-workers (1997), used AFLP fingerprinting to study 18 different *F.*

graminearum strains, 15 of which showed a high degree of similarity in the banding patterns. O'Neill and co-workers (1998), found less than 70% similarity between *F. udum* and *F. oxysporum* strains pathogenic to coca, cowpea and tomato. Woo and co-workers (1996) also found that some pathogenetically diverse isolates of *F. oxysporum* f.sp *phaseoli* had very similar banding patterns. The similar banding profiles observed between some isolates in this study would be an indication of isolates belonging to the same forma speciales.

Several discordant groupings were observed among the isolates with primers IS36, SSR1 and SSR17. Primer IS 36 revealed a high genetic similarity (80%) between *F. lateritium* and *F. pallidoroseum*. This result was unexpected as the two isolates belong to different sections of the genus according to the morphological identification. Similarly with primer SSR1, *F. poae, F. graminearum, F. pallidoroseum* and *F. species* isolates were placed in the same cluster. In this case, this result would suggest that these *Fusarium* species are closely related. However, the use of more species specific primers and rDNA sequencing will probably reveal a wide genetic relatedness between the isolates.

UP-PCR is an effective tool for rapid intraspecific typing of strains at the molecular genetic level (Bulat *et al*., 1995). According to Lubeck and co-workers (1999), UP primer L45 generates fingerprints with a relatively large number of bands. In this work, one primer combination was used L15/ L45, which produced 126 polymorphic bands across the 72 isolates. The results obtained are in agreement with the ISSR based phylogeny. The high intraspecies diversity among the *F. oxysporum* isolates was also revealed using this primer pair. Six distinct groups were formed using this primer pair. This revealed a high degree of

interspecies diversity among all isolates analysed. These findings confirm that UP-PCR is also a reliable method of differentiating *Fusarium* species.

7.0 CONCLUSION AND RECOMMENDATIONS

Literature shows that it has been problematic in many cases to determine the phylogenetic relationships of fungi by morphological methods alone. Morphological species identification is strongly biased by human perception and severely limited when one applies it to organisms without clear morphological characters to discern (Redecker, 2002). This work is a preliminary study of *Fusarium* species currently in the Plant Clinic collection at the Tobacco Research Board. Many workers globally have used DNA based markers extensively to evaluate genetic diversity in this important fungus.

All the two molecular techniques used in this present study, gave an indication of genetic diversity within *Fusarium*. For the ISSR and UP-PCR techniques, further analysis is required using more species-specific primers to complement results obtained in this work.

This study revealed that the use of molecular methods constitutes an important complement to the morphological criteria needed to allow fungi to be more easily identified. Whilst this technology gives useful information, there is need to use many more ISSR and UP-PCR primers to get more reliable data. Other molecular marker techniques like amplified fragment length polymorphism (AFLP), can be used for this genetic assessment. The sequencing of rDNA sequences using the *Fusarium* specific primers is more reliable as a diagnostic technique as well as revealing genetic relatedness of *Fusarium* isolates.

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