Rostaniha, Vol. 4, 2003

A REVISION OF PLANT-ASSOCIATED *VERTICILLIUM* SPECIES

R. ZARE

Department of Botany, Plant Pests & Diseases Research Institute

Received 17.11.2002 Accepted 15.07.2003

Abstract

Molecular approaches were used to re-evaluate the morphological criteria used to identify plant-associated *Verticillium* species. ITS-RFLPs divided the 31 studied strains of seven *Verticillium* species, (including the type species *V. luteo-album*) into four clusters. Cluster one comprised strains of the type species, *V. luteo-album*, cluster two *V. albo-atrum*, *V. dahliae*, *V. nubilum* and *V. tricorpus*; cluster three comprised strains of *V. theobromae*, and cluster four comprised strains of *V. nigrescens*. β-tubulin gene RFLPs offered a higher degree of resolution, distinguishing all seven species from each other. The highest degree of resolution was obtained from mitochondrial DNA-RFLPs that divided strains of *V. theobromae* and *V. nigrescens* into infraspecific groups. The β-tubulin gene digested by *Hae* III offers a reliable way to separate the two economically important and controversial species *V. albo-atrum* and *V. dahliae*.

Key words: *Verticillium*, Molecular studies, taxonomy

Introduction

NEES (1816) introduced the genus *Verticillium* for a saprotrophic fungus that he named *V. tenerum*. The genus is featured by having usually 1-celled, hyaline conidia (NEES 1816), verticillate conidiophores with aculeate phialides inserted in a mesotonous to acrotonous position (GAMS 1971). Among the numerous synonyms cited for the type species by HUGHES (1951, 1958), ISAAC (1967), and DOMSCH *et al.* (1980), *Sporotrichum luteo-album* Link 1809, is the oldest name sanctioned by FRIES (1832). Subramanian correctly combined this in *Verticillium* (*V. luteo-album*). The type species, *V. luteo-album* was claimed to be the anamorph of a *Nectria* (PETHYBRIDGE 1919) and was reported as a fungal hyperparasite (HUGHES 1951, TSUNEDA *et al.* 1976). The genus is traditionally classified as an anamorph of pyrenomycetes, a class that accommodates fungi with flask-shaped, mostly ostiolate perithecia that produce asci from a single basal hymenium (MÜLLER & VON ARX 1973). *Verticillium* comprises a heterogeneous assemblage of taxa which are included according to relatively simple and poorly defined characters (JUN *et al.* 1991). It is generally considered a highly polyphyletic form-genus containing distantly related fungi, with affiliations to several ascomycete families and even orders (GAMS 1971, MÜLLER & VON ARX 1973, GAMS & VAN ZAAYEN 1982, ZARE *et al.* 2000). Based on molecular and morphological studies (GAMS & ZARE 2001, GAMS & ZARE 2002, SUNG *et al.* 2001, ZARE & GAMS 2001 a, b, ZARE *et al.* 2001) a large number of entomogenous, fungicolous and nematophagous species previously classified in *Verticillium sensu lato* are now re-classified in *Lecanicillium*, *Pochonia*, *Haptocillium* and *Simplicillium*. Species of *Verticillium* associated with plants (section *Nigrescentia* W. Gams in GAMS & VAN ZAAYEN 1982) were shown to be affiliated with the Phyllachoralean genus *Glomerella* (MESSNER *et al.* 1996) while members of section *Prostrata* are anamorphs of Clavicipitalean genera, *Cordyceps* and *Torrubiella*.

Verticillium section *Nigrescentia* comprises *Verticillium albo-atrum* Reinke & Berthold, *V. dahliae* Klebahn, *V. nigrescens* Pethybridge, *V. nubilum* Pethybridge, *V. theobromae* (Turconi) E. Mason & S. Hughes, *V. tricorpus* I. Isaac and *V. longisporum* (Stark) Karapapa, Bainbridge & Heale. The differentiation between species in this section, using morphological criteria alone, is not difficult, but it usually involves working with cultures for some weeks until resting structures develop. Members of this section have a tendency to lose the expression of diagnostic characters on prolonged maintenance in culture or when kept on inappropriate media (DOMSCH *et al.* 1980). All members in this group produce black or brownish black pigment. However, after repeated transfer and prolonged preservation, especially on rich media, some strains may lose the ability of pigment production becoming 'albino'.

The best known species of *Verticillium* section *Nigrescentia* are *V. albo-atrum* Reink & Berthold and *V. dahliae* Klebahn, about which a large quantity of phytopathological literature have been published (ISAAC 1949, 1953, 1967, JENSEN 1965, SKADOW 1969, SCHNATHORST 1973, PEGG 1974, DOMSCH *et al.* 1980, JOAQUIM & ROWE 1990, OKOLI *et al.* 1994, KOIKE *et al.* 1995, WAKATABE *et al.* 1997). Over 300 agriculturally important plants are susceptible to devastating losses due to the most common species, *V. albo-atrum* and *V. dahliae*. Important crops at risk are cotton, tomato, hops, alfalfa, potato and olive (HAWKSWORTH & TALBOYS 1970a, 1970b, DOMSCH *et al.* 1980, NAZAR *et al.* 1991). These two species are the most important plant pathogens within the genus, causing diseases in a wide range of economically important crops worldwide, particularly in temperate and cool temperate regions (ISAAC 1967). Host-specific isolates of *V. dahliae* are indistinguishable on the basis of morphological characters (MESSNER *et al.* 1996). The two species have been regarded as controversial for a long time and there has been confusion in the application of the names *V. dahliae*

and *V. albo-atrum*, particularly in the USA where both species were merged, whilst in Europe the two species were mostly kept apart (WILLIAMS *et al.* 1992). Several attempts were made to separate *V. albo-atrum* and *V. dahliae* and other *Verticillium* species at the species level, using molecular approaches (CARDER & BARBARA 1991, NAZAR *et al.* 1991, WILLIAMS *et al.* 1992, CARDER *et al.* 1993, LI *et al.* 1994, KARAPAPA *et al.* 1997).

ISAAC & KEYWORTH (1948) provided evidence that *V. albo-atrum*, *V. dahliae* and *V. nigrescens* are three distinct species that can be distinguished on the basis of morphological, physiological and phytopathological criteria. *Verticillium dahliae* never produces structures resembling the dark resting mycelium of *V. albo-atrum* while the latter does not produce microsclerotia (also ISAAC 1949). Studies by MORTON *et al.* (1995) using sub-repeat sequences in the RNA intergenic regions and sequences of the internal transcribed spacers (ITS) showed considerable differences between haploid and diploid strains of *V. dahliae* (the latter known as *V. dahliae* var. *longisporum* Stark) and the authors suggested that the differences may be great enough to put the respective isolates into two separate species. Subsequently the diploid isolates were described as a separate species, *V. longisporum* (Stark) Karapapa, Bainbridge & Heale (KARAPAPA *et al.* 1997). This species is reported as a heterodiploid of *V. dahliae* and *V. albo-atrum* differing from the former in conidial size which are nearly double the size of those in *V. dahliae* and from the latter in the production of microsclerotia. This species, which was not included in the present study, was originally isolated from horse-radish, but is now mainly found to attack rapeseed.

Verticillium tricorpus is known to produce wilt in tomato (ISAAC 1953) and is commonly isolated from potato, where it causes no harm. This species is readily distinguished from *V. albo-atrum* and *V. dahliae* by its production of a yellow, diffusing pigment on OA and PDA and the production of three types of

resting structures (chlamydospores, microsclerotia and resting mycelium). However, some strains may lack or lose the ability to produce any of these features, causing difficulties in identification.

Verticillium nigrescens and *V. nubilum* are distinguished from each other on the basis of chlamydospore size which is larger in the latter (ISAAC 1949). The two species are saprotrophs or weak pathogens found in soil and sometimes on stored seeds (WILLIAMS *et al.* 1992). *Verticillium theobromae* differs from *V. albo-atrum* in the absence of a torulose resting mycelium, the different colour of the colony reverse, and the rather smaller conidia (HAWKSWORTH & HOLLIDAY 1970).

The goal of the present study was to integrate morphological criteria with information derived from DNA from multiple loci to distinguish the plant-associated species of *Verticillium* focussing on *V. albo-atrum* and *V. dahliae*. This study presents a review based on data derived from RFLPs of the ITS (internal transcribed spacer) region, the β-tubulin gene and mitochondrial DNA in addition to morphology. Eukaryotic β-tubulin sequences are highly conserved (YAN & DICKMAN 1996) and the majority of fungi such as *Saccharomyces cerevisiae*, *Candida albicans* and *Neurospora crassa* are reported to contain a single highly conserved gene for β-tubulin (BUHR & DICKMAN 1993). Sequence data from β-tubulin gene introns and exons have been used to resolve evolutionary relationships among several species of *Stanjemonium* (GAMS *et al.* 1998) and *Fusarium* species (AOKI & O'DONNELL 1999).

Materials and Methods

1. Morphological studies: Fungal strains examined in this study are listed in Table 1. All strains were examined morphologically to confirm their identification and purity. Colony growth rate and features (shape, colour, pigmentation, etc.) were recorded on PDA (Potato-Dextrose Agar; Oxoid Ltd, Hampshire, England). The growth rate and

colony features of each taxon were determined on PDA in plastic Petri dishes after 10 days incubated at 24±1°C in the dark. Conidiophore structure and branching, phialides, conidia, and resting structures were examined on PCA (Potato-Carrot Agar) incubated at 24±1°C in the dark. Slide preparations were made in lactic acid-cotton blue and structures were measured from freshly prepared slides under the light microscope.

2. Molecular studies: A range of DNA characters including RFLPs of the ITS regions, β-tubulin gene and mtDNA were used to characterize the fungal strains. Glucose-Yeast Medium (GYM, MUGNAI *et al.* 1989) was used to produce mycelium for DNA extraction. Mycelium was macerated from the surface of a 10-20 day old PDA plate and inoculated into a 28 ml Universal bottle containing 10 ml GYM. Bottles were incubated in an orbital shaking incubator at 25°C/150 rpm for 48-72 h before transfer to 250 ml screw cap conical flasks containing 60 ml GYM. Flasks were incubated on the shaking incubator (25°C/100 rpm) for a further 3-6 days (depending on fungal growth rates). Cultures were harvested by vacuum filtration onto Whatman No. 3 filter paper, rinsed with sterile distilled H_2O and placed in sterile Petri dishes. Harvested cultures were stored at -20°C and then lyophilized. Biomass was prepared for extraction by grinding up the freeze-dried material in an alcohol sterilized mortar and pestle. Total DNA was extracted using the method of RAEDER & BRODA (1985). Approximately 50-100 ng DNA samples were subjected to PCR amplification using primers ITS1F (GARDES & BRUNS 1993) and ITS4 (WHITE *et al.* 1990). The DNA concentration was tested against standard Lambda Phage DNA (*Escherichia coli* host strain W3110, SIGMA). Conditions for the amplification of the ITS region were the same as those used by ZARE *et al.* (1999).

Twelve restriction enzymes were screened to select those that showed the greatest level of polymorphism for the ITS region. These were *Bam*H I, *Sal* I, *Pst* I,

*Eco*R I, *Eco*R V, *Hind* III, *Msp* I, *Xba* I, *Alu* I, *Hae* III, *Hinf* I and *Dra* I (Promega, UK). Three restriction enzymes, *Hae* III (site GG:CC), *Hinf* I (site G:ANTC), and *Msp* I (site C:CGG), gave the greatest numbers of polymorphic bands and were subsequently used to generate ITS-RFLPs for all strains. Aliquots of 3-5 μl of amplified ITS-PCR products were digested for between 4 h and overnight with 20 units of the selected restriction enzymes together with 2 μl 10× buffer (as supplied by the manufacturer) made up to a total volume of 20 μl with sterile HPLC-grade H_2O. The restriction fragments were separated by horizontal electrophoresis in 1× TBE buffer in 3% NuSieve 3:1 agarose (FMC BioProducts, Rockland, USA). The electrophoresis was carried out in 16 (l) × 12 (w) × 0.5 (h) cm gels for about 4 hours at a constant voltage of 5 V cm^{-1} (Conset Flowgen E455 Microcomputer Electrophoresis Power Supply). Five μl of 100-bp size markers (100 ng/μl, Gibco BRL) were loaded into two wells on each side of the gels.

A region of the β-tubulin gene containing exons and an intron was selected for PCR amplification using primers Bt1a and Bt1b (GLASS & DONALDSON 1995). PCR reactions were performed in a total volume of 50 μl, containing 50-100 ng of genomic DNA, 50 mM KCl, 75 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2$ SO_4, 100 μM of each dATP, dCTP, dGTP, dTTP, 30 pmol of each primer, 2 mM $MgCl_2$, 1 unit *Tth* DNA polymerase, and the total volume was adjusted to 50 μl by adding HPLC grade H_2O. PCR reactions were overlaid by a drop of mineral oil. The PCR was performed in an MJ Research thermal cycler programmed at 5 min at 94°C for hot start and then followed by 35 cycles of 94°C (1 min), 60°C (1 min), 72°C (1 min) and a final extension of 72°C (5 min). Seven restriction enzymes including *Msp* I, *Alu* I, *Hae* III, *Hinf* I, *Dra* I, *Cfo* I and *Alu* I (Promega, UK) were screened and four of them, *Hae* III (site GG:CC), *Hinf* I (site G:ANTC), *Alu* I (site AG:CT) and *Cfo* I (site GCG:C) gave the greatest number of polymorphic bands and were used to generate β-tubulin gene RFLPs. Aliquots of 5-8 μl (approximately 1 μg

DNA) of β-tubulin PCR product was digested with the selected restriction enzymes and the condition for incubation and separation of bands by electrophoresis was as for ITS-RFLPs.

Mitochondrial DNA restriction fragment length polymorphisms (mtDNA-RFLPs) were obtained by using a feature of fungal DNA, which appears to be almost universal. The four-base restriction sites consisting of purely cytosine and guanine are found much less frequently in fungal mitochondrial DNA than in the chromosomal DNA (BRUNS *et al.* 1988, TYPAS *et al.* 1992, BUDDIE *et al.* 1998, KOUVELIS *et al.* 1999). The method used by KOUVELIS *et al.* (1999) that involves electrophoresis of *Hae* III digested total DNA, was followed here to generate mtDNA RFLPs.

Analysis of banding patterns was carried out using Jaccard's similarity coefficient with UPGMA (unweighted pair-group method using arithmetic averages) and furthest neighbour clustering through the package MVSP version 3.11a (multivariate statistical program; Kovack Computing, Anglesey, UK). Binary codes were used to score the bands for presence (1) and absence (0). Separate and combined analyses were performed using different combinations of the RFLP data. In all cases a single accession (of each species) is used to represent strains with identical banding patterns. Dendrograms resulting from UPGMA and Furthest neighbour clustering methods were very similar and the UPGMA results only are shown. Principal component analysis using covariance of data was also performed using MVSP to further explore the pattern of variation. The first three components are plotted.

Results

1. Morphological studies: *Verticillium luteo-album* is distinct from all other species studied here because of its brick-red colonies and the lack of resting structures. *Verticillium albo-atrum* and *V. dahliae* are the closest species in this genus and the main distinguishing feature between them is the presence of microsclerotia in the

latter and dark-brown resting mycelium in the former. *Verticillium tricorpus* is clearly distinguished from all other *Verticillium* species by the orange-yellow colour of the young mycelium (margin of the colony) and the production of chlamydospores and microsclerotia in addition to the dark resting mycelium (HAWKSWORTH 1970a,b, ISAAC 1953), however, any of these features can be absent in some isolates, rendering identification difficult. *Verticillium nigrescens* and *V. nubilum* are close morphological relatives, but can be separated by the larger chlamydospores in the latter. *Verticillium theobromae* shares with *V. albo-atrum* the dark resting mycelium. The two species can be differentiated by their colony colour, which is greyish brown in reverse (after 1-2 weeks) in the former and entirely black in the latter. Besides, individual cells of the resting mycelium in *V. albo-atrum* are frequently torulose, a feature which does not occur in *V. theobromae*.

Key to the plant-associated *Verticillium* species

1 Microsclerotia present, dark resting mycelium or brown chlamydospores usually absent.....2

1′ Microsclerotia absent, dark resting mycelium or brown chlamydospores usually present.....4

2 Microsclerotia elongate to almost spherical, mostly 60-85 µm diam; cells distinctly swollen and globular; colonies often with yellow diffusing pigment on PDA; dark resting mycelium and brown chlamydospores present or absent ....*V. tricorpus*

2′ Microsclerotia elongate to irregularly spherical, very variable in size 15-50 (-100) µm diam.; cells often elongate; colonies always without yellow diffusing pigment on PDA; dark resting mycelium and chlamydospores absent.....3

3 Conidia measuring 2.5-8 × 1.4-3.2 µm.....*V. dahliae*

3′ Conidia measuring 5-12.5 × 1.6-3.4 µm..... *V. longisporum*

4 Dark resting mycelium present, brown chlamydospores absent.....5

4′ Dark resting mycelium absent; brown chlamydospores present.....6

5 Dark mycelium torulose, colony reverse often black.....*V. albo-atrum*

5′ Dark mycelium non-torulose, colony reverse often greyish brown..*V. theobromae*

6 Chlamydospores 8.5-17 μm diam, often forming chains; conidia sometimes septate.....*V. nubilum*

6′ Chlamydospores 5.5-8 (-10) μm diam, simple or in pairs; conidia non-septate.....*V. nigrescens*

2. Molecular studies: (a) ITS RFLPs: No polymorphism was detected within *V. luteo-album* which was consistently distinct from sect. *Nigrescentia*. The total size of the amplified ITS regions (including the 5.8S gene) was estimated to be 600-bp. Twenty seven strains of the six plant-associated *Verticillium* species (Table 1) were divided into two groups based on the size of undigested PCR products. PCR amplification from all strains of *V. albo-atrum*, *V. dahliae*, *V. tricorpus* and *V. nubilum* resulted in a 580-bp product; and from all strains of *V. theobromae* and *V. nigrescens*, a 600-bp product. Digestion of the ITS products using the three restriction enzymes resulted in identical banding patterns of the first four species while the last two species *V. theobromae* and *V. nigrescens* had distinct patterns (Figs 1, 2a, b).

(b) Mitochondrial DNA RFLPs: Of the four strains of *V. luteo-album*, three showed one of two different mtDNA-RFLP banding patterns with no common bands while the remaining one did not produce bands. Twenty-seven strains from six species of section *Nigrescentia* were included in the study to elucidate the relationship between these taxa and other sections of *Verticillium*. Pattern 3 (Fig. 3) represents the profile obtained from all strains of *V. albo-atrum* and pattern 4 (Fig. 3) represents those from all strains of *V. dahliae*. Of three bands in *V. albo-atrum*, which were longer than 2-kbp, two were common to both species. The five strains of *V. nigrescens* were divided into four subgroups, one of them being very similar to *V. dahliae* and *V. albo-atrum*. Pattern 5 (Fig. 3) which represents strain IMI 118380 has four (out of five) bands in common with *V. dahliae* and two with *V. albo-atrum*. Two strains (IMI 295728 and IMI 112791) of the species had unique profiles (Fig. 3, patterns 6 and 8) while two other strains (IMI 044575 and IMI 326064) had a 4-kbp fragment in common with *V. albo-atrum*, *V. dahliae* and strains IMI 118380 and IMI 295728

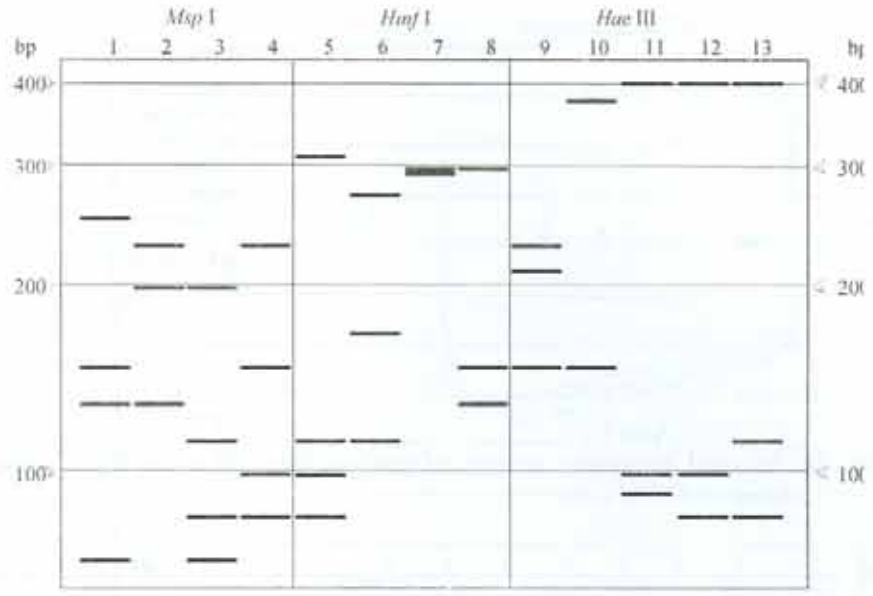

Fig. 1. Schematic representation of ITS-RFLP patterns of sections *Verticillium* and *Nigrescentia*. 1, 5, 9. *V. luteo-album*; 2, 6, 10. *V. albo-atrum*; 3, 7, 11. *V. theobromae*; 4, 8, 12. *V. nigrescens* 1; 4, 8, 13. *V. nigrescens* 2. (*V. dahliae*, *V. tricorpus* and *V. nubilum* had identical patterns to *V. albo- atrum*).

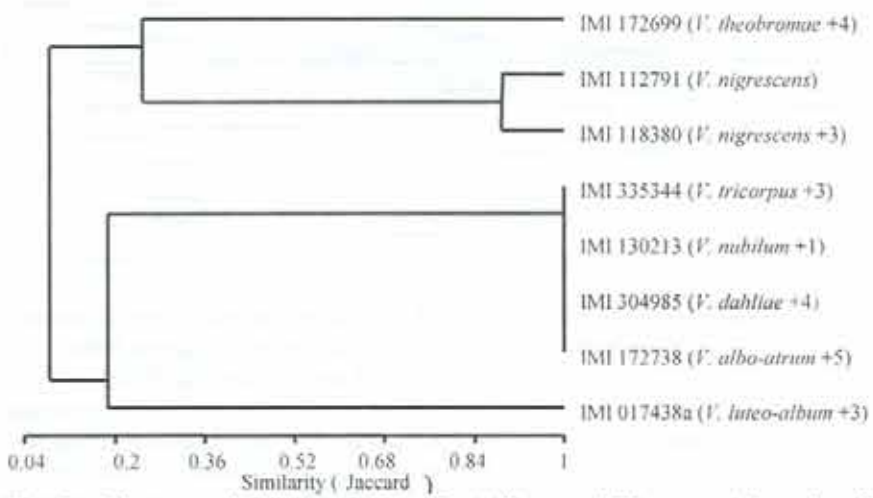

Fig. 2a. Cluster analysis of sections *Verticillium* and *Nigrescentia* based on ITS-RFLPs using Jaccard's Coefficient with UPGMA method.

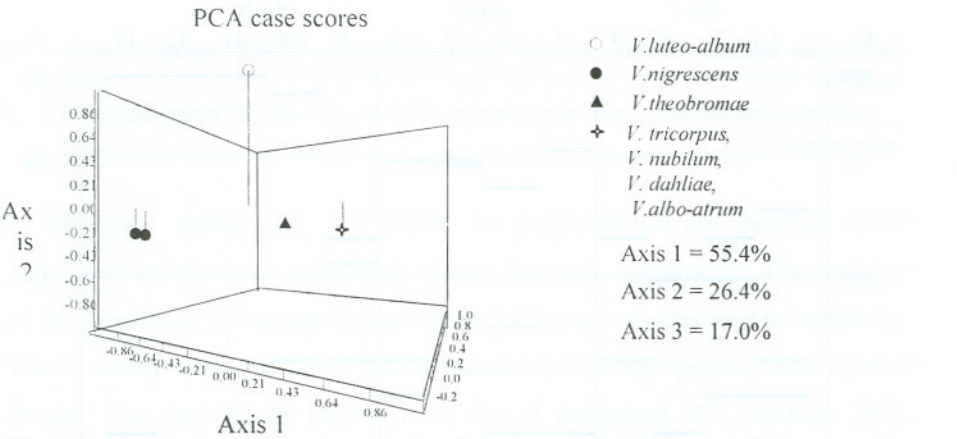

Fig. 2b. Principal component analysis of sections *Verticillium* and *Nigrescentia* based on ITS-RFLPs.

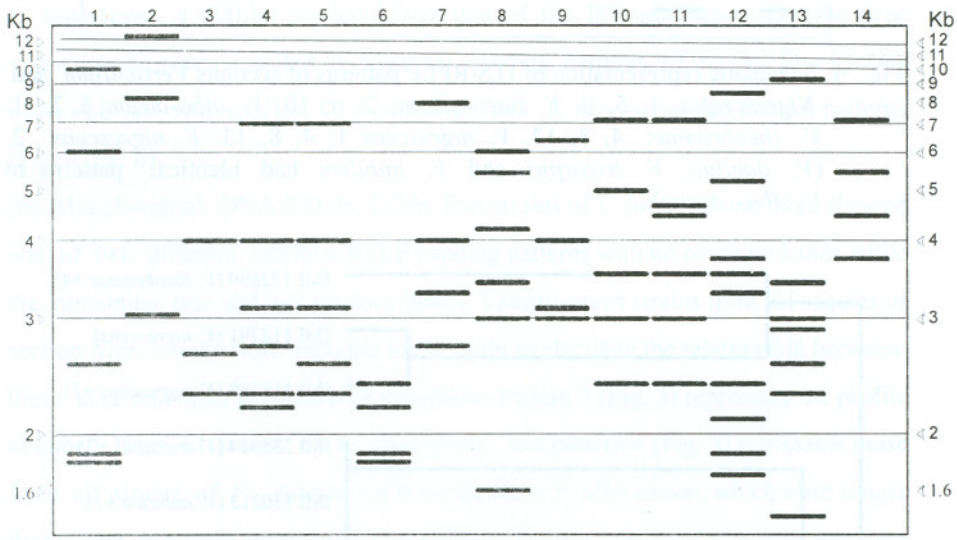

Fig. 3. Schematic representation of mtDNA-RFLPs of sections *Verticillium* and *Nigrescentia* resulting from *Hae* III digestion of total genomic DNA. 1, 2. *Verticillium luteo-album* (IMI 017438a Lane 1, IMI 182719 Lane 2); 3. *V. albo-atrum* (all strains); 4. *V. dahliae* (all strains); 5, 6. *V. nigrescens* (IMI 118380 Lane 5, IMI 295728 Lane 6); 7. *V. nigrescens* (IMI 044575, IMI 326064); 8. *V. nigrescens* (IMI 112791); 9. *V. nubilum* (IMI 278734, IMI 130213); 10-12. *V. theobromae* (IMI 172699 Lane 10, IMI 225818 Lane 11, IMI031432a Lane 12); 13. *V. theobromae* (IMI 073524, IMI 084424); 14. *V. tricorpus* (all strains).

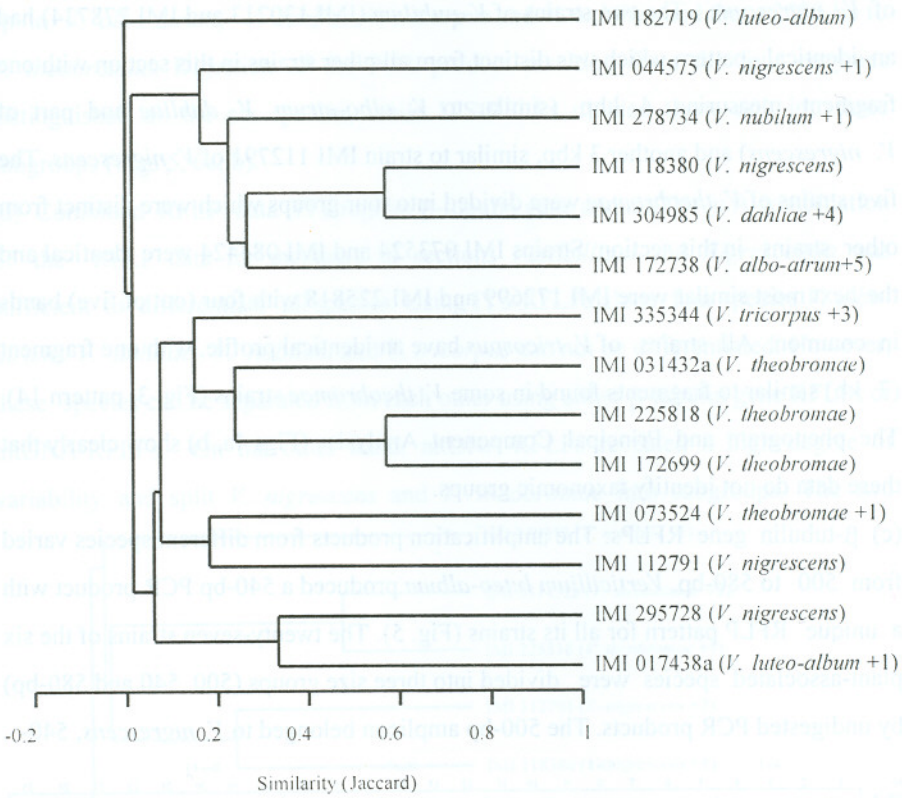

Fig. 4a. Cluster analysis of sections *Verticillium* and *Nigrescentia* based on mtDNA-RFLPs using Jaccard's Coefficient with UPGMA method.

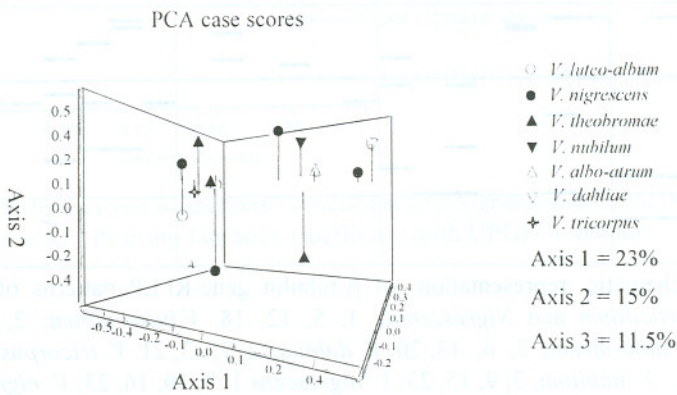

Fig. 4b. Principle component analysis of sections *Verticillium* and *Nigrescentia* based on mtDNA-RFLPs.

of *V. nigrescens*. The two strains of *V. nubilum* (IMI 130213 and IMI 278734) had an identical pattern which was distinct from all other strains in this section with one fragment measuring 4 kbp (similar to *V. albo-atrum*, *V. dahliae* and part of *V. nigrescens*) and another 3 kbp, similar to strain IMI 112791 of *V. nigrescens*. The five strains of *V. theobromae* were divided into four groups which were distinct from other strains in this section. Strains IMI 073524 and IMI 084424 were identical and the next most similar were IMI 172699 and IMI 225818 with four (out of five) bands in common. All strains of *V. tricorpus* have an identical profile, with one fragment (7 kb) similar to fragments found in some *V. theobromae* strains (Fig. 3, pattern 14). The phenogram and Principal Component Analysis (Figs 4a, b) show clearly that these data do not identify taxonomic groups.

(c) β-tubulin gene RFLPs: The amplification products from different species varied from 500 to 580-bp. *Verticillium luteo-album* produced a 540-bp PCR product with a unique RFLP pattern for all its strains (Fig. 5). The twenty-seven strains of the six plant-associated species were divided into three size groups (500, 540 and 580-bp) by undigested PCR products. The 500-bp amplicon belonged to *V. nigrescens*, 540-

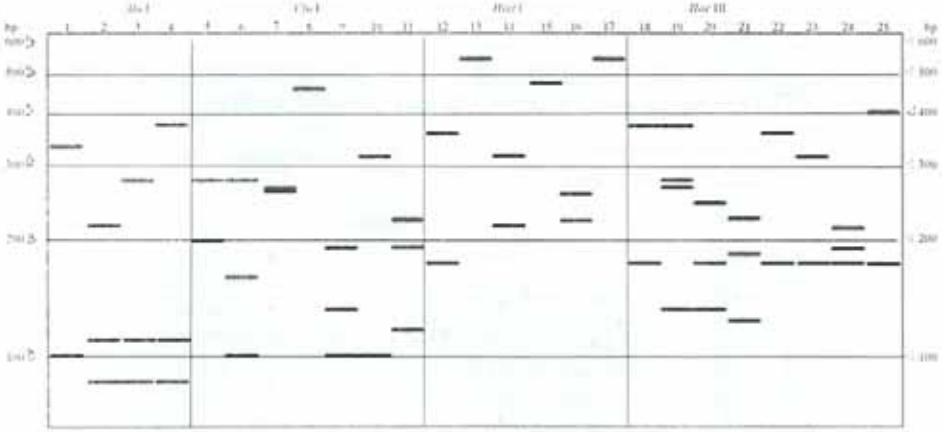

Fig. 5. Schematic representation of β-tubulin gene-RFLP patterns of sections *Verticillium* and *Nigrescentia*. 1, 5, 12, 18. *V.luteo-album*; 2, 6, 13, 19. *V. albo-atrum*; 2, 6, 13, 20. *V. dahliae*; 2, 7, 13, 21. *V. tricorpus*; 2, 8, 14, 22. *V. nubilum*; 3, 9, 15, 23. *V. nigrescens* 1; 3, 10, 16, 23. *V. nigrescens* 2; 4, 11, 17, 24. *V. theobromae* 1; 4, 11, 17, 25. *V. theobromae* 2.

bp to *V. albo-atrum*, *V. dahliae*, *V. tricorpus* and *V. nubilum* and that of 580-bp to *V. theobromae*. Digestion of the PCR product resulting from the four enzymes distinguished all the six species, while ITS-RFLPs divided them only into three subgroups (Figs 5, 6a, b).

(d) Combined RFLP data (ITS region, β-tubulin gene and mtDNA): A combination of the RFLP data for members of sections *Verticillium* and *Nigrescentia* was sufficient to differentiate all species. Using ITS-RFLPs alone, four species *V. albo-atrum*, *V. dahliae*, *V. nubilum* and *V. tricorpus* can not be differentiated. However, these species can be separated from each other using either β-tubulin gene RFLPs or mtDNA-RFLPs. On the other hand, mtDNA-RFLPs revealed a high degree of variability and split *V. nigrescens* and *V. theobromae* into subgroups. The two

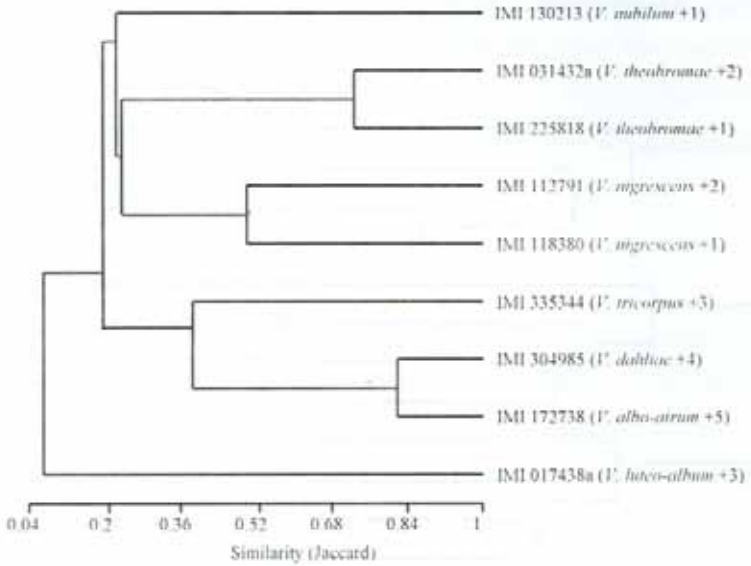

Fig. 6a. Cluster analysis of sections *Verticillium* and *Nigrescentia* based on β-tubulin gene RFLPs using Jaccard's Coefficient with UPGMA method.

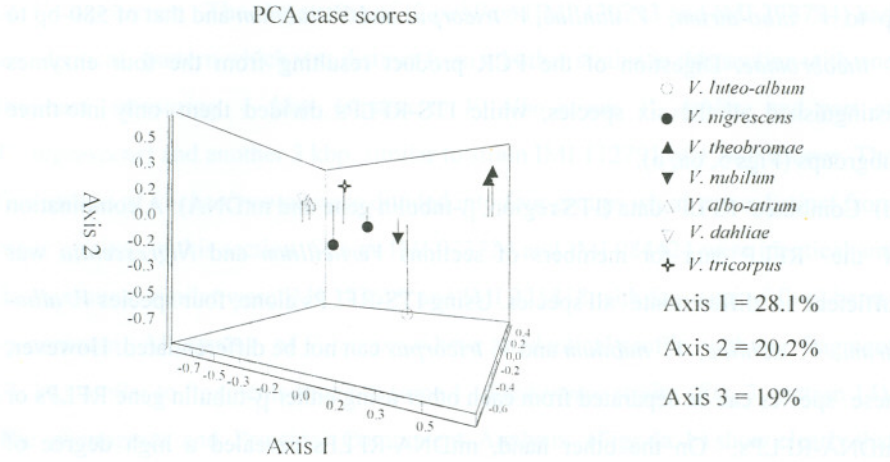

Fig. 6b. Principle component analysis of sections *Verticillium* and *Nigrescentia* based on β-tubulin gene RFLPs.

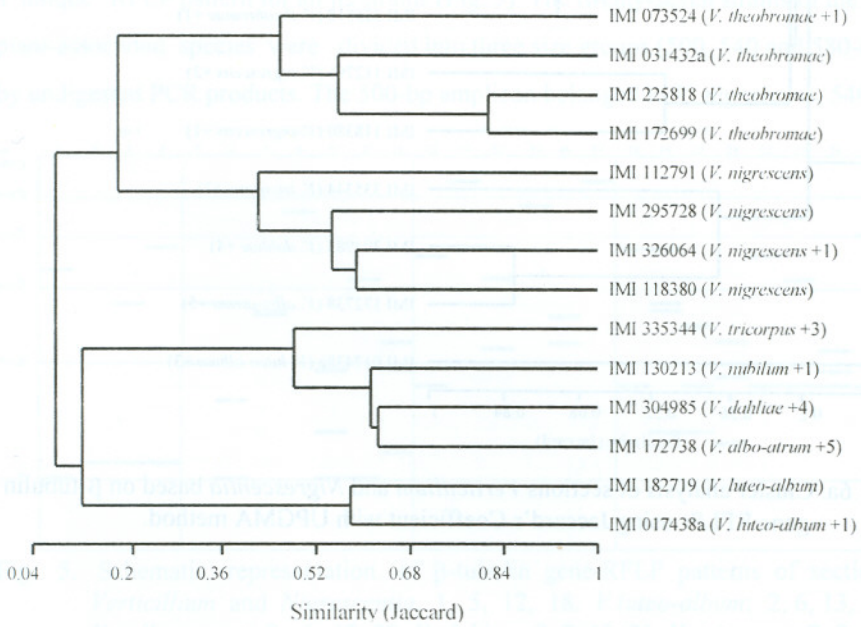

Fig. 7a. Cluster analysis of sections *Verticillium* and *Nigrescentia* based on combined RFLPs of ITS region, mtDNA and β-tubulin gene using Jaccard's Coefficient with UPGMA method.

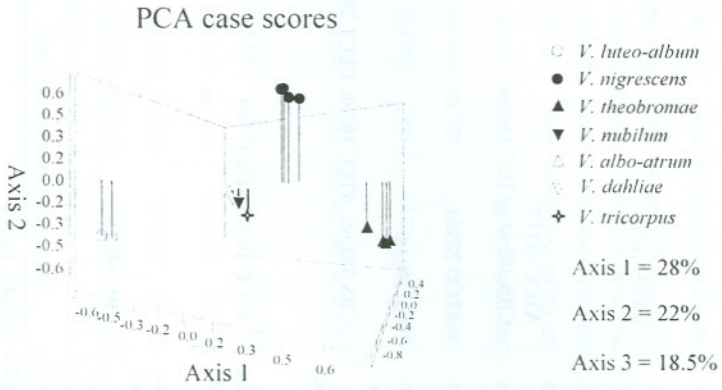

Fig. 7b. Principle component analysis of sections *Verticillium* and *Nigrescentia* based on RFLPs of ITS region, β-tubulin gene and mtDNA.

species *V. theobromae* and *V. nigrescens* are each divided into four subgroups and *V. luteo-album* into two subgroups, while the other four species (*V. nubilum*, *V. tricorpus*, *V. dahliae*, *V. albo-atrum*) are not further subdivided (Fig. 7a). The four major groupings identified are clearly distinguished in the Principal Component Analysis (Fig. 7b).

Conclusion and Discussion

ITS-RFLPs offered a unique pattern for all strains of *V. luteo-album* while they placed the six plant-associated species *V. albo-atrum*, *V. dahliae*, *V. tricorpus*, *V. nubilum*, *V. nigrescens* and *V. theobromae* into three groups with *V. albo-atrum*, *V. dahliae*, *V. tricorpus* and *V. nubilum*, that had identical patterns, in one cluster (Fig. 1, 2a, b). Mitochondrial DNA-RFLPs on the other hand, revealed a large extent of variability and not only separated all morphological species but also divided *V. luteo-album*, *V. nigrescens* and *V. theobromae* into smaller subgroups. Studies by TYPAS *et al.* (1992) on different *Verticillium* species and by KOUVELIS *et al.* (1999) on the *V. lecanii* species complex using mtDNA-RFLPs showed a high degree of variation within the studied taxa. The amount of the variability detected within the two species *V. nigrescens* and *V. theobromae* is nearly equal to those found between the four species *V. albo-atrum*, *V. dahliae*, *V. nubilum* and

Table 1. List of strains examined

Taxon	IMI No.	Host/Substrate	Origin, Date, Collector	Other information
V. luteo-album (Link) Subram.	017438a	*Polygonum sieboldii*	UK, 1947, S.J. Hughes	
V. luteo-album	096206	hay	UK, IMI 1962, M.E. Lacey	
V. luteo-album	182719	dead butterfly	UK, IMI 1974, H.P. O'Neil	
V. luteo-album	054377	*Polyporus squamosus*	UK 1953, C.J. La Touche	
V. albo-atrum Reinke & Berthold	172738	*Lycopersicon esculentum*	UK, 1970, J.W.H. Taylor	NVRS 1670
V. albo-atrum	298097	*Humulus lupulus*	UK, IMI 1985, P. Talboys	
V. albo-atrum	298098	*Humulus lupulus*	UK, IMI 1985, P. Talboys	
V. albo-atrum	298102	*Humulus lupulus*	UK, IMI 1985, P. Talboys	
V. albo-atrum	062131	*Lycopersicon esculentum*	UK, IMI 1956, G.F. Pegg	ATCC 48117
V. albo-atrum	118378	*Solanum tuberosum*	Canada, 1962, W.E. Sackston	CBS 382.66, ATCC 16534
V. dahliae Klebahn	088630	*Lycopersicon esculentum*	Zimbabwe, 1961, A. Rothwell	
V. dahliae	304985	*Gossypium* sp.	IMI 1986, G. Malaguti	ATCC 22924
V. dahliae	090682	*Helianthus annuus*	Canada, 1961, W.E. Sackston	
V. dahliae	088631	*Solanum mammosum*	Zimbabwe, 1961, A. Rothwell	MR17058
V. dahliae	045492	*Mentha* sp.	Zimbabwe, 1951, J. Broadfoot	
V. nigrescens Pethybr.	118380	*Beta vulgaris*	Canada, 1963, W.E. Sackston	CBS 383.66, ATCC 16536, CECT 2696
V. nigrescens	295728	*Avena sativa*	Pakistan, IMI 1985, A.H. Tariq	
V. nigrescens	044575	*Solanum tuberosum*	UK, 1944, R.V. Harris	
V. nigrescens	326064	*Humulus lupulus*	UK, 1988	
V. nigrescens	112791	soil	India, 1962, M.J. Thirumalachar	ATCC 16090, HACC 149
V. nubilum Pethybr.	278734	*Solanum tuberosum*	UK, IMI 1983, E.P. Dashwood	
V. nubilum	130213	mushroom compost	Scotland, 1967	
V. theobromae (Turconi) E. Mason & S. Hughes	172699	*Musa* sp.	Carribean, IMI 1973, J. Burden	
V. theobromae	225818	*Musa* sp.	India, IMI 1978, I. Isaac & E. Bremner	
V. theobromae	031432a	*Musa* sp.	Jamaica, 1948, E.B. Martyn	CBS 397.58
V. theobromae	073524	*Musa cavendishii*	Nigeria, 1958, E. Harris	
V. theobromae	084424	*Musa* sp.	Jamaica, 1960, D.S. Meredith	
V. tricorpus Isaac	071799	*Lycopersicon esculentum*	UK, IMI 1958, D.J. Harrison	
V. tricorpus	271801	*Solanum tuberosum*	Ireland, IMI 1983, L.J. Dowley	NRRL 13753
V. tricorpus	262722	*Humulus lupulus*	UK, IMI 1981, A. Phillips	NRRL 13691
V. tricorpus	335344	*Dianthus caryophyllus*	Holland, IMI 1989, K. Davis & M. Priest	

Table 2. RFLP fragment sizes and patterns of the ITS region, mtDNA and β-tubulin gene. Symbols for different patterns in the second column: ITS patterns = Capital letters, mtDNA = Arabic figures, β-tubulin gene = Roman figures

Taxa and Accession codes	Pattern	Total size (bp)	RFLPs of ITS region (bp)			RFLPs of mtDNA (bp)	Total size (bp)	RFLPs of the β-tubulin gene (bp)			
			Msp I	*Hinf* I	*Hae* III	*Hae* III		*Alu* I	*Cfo* I	*Hinf* I	*Hae* III
F. luteo-album (IMI 017438a, IMI 096206)	A,1,I	600	260,160,140,60	320,120,100, 80	240,220,160	10,6.2,6.1,9.1,8	540	340,100	280,200	360,180	380,180
F. luteo-album (IMI 182719)	A,1',I	600	260,160,140,60	320,120,100, 80	240,220,160	>12,8.2,3.1	540	340,100	280,200	360,180	380,180
F. luteo-album IMI 054377	A,-,I	600	260,160,140,60	320,120,100, 80	240,220,160	-	540	340,100	280,200	360,180	380,180
F. albo-atrum (IMI 172736, IMI 298097, IMI 298098, IMI 298102, IMI 062131, IMI 118378)	B,2,II	580	240,200,140	280,180,120	380,160	10.5,4,2.7,2.4	540	230,120,80	280,170,100	540	180,170,140
F. dahliae (IMI 088630, IMI 304985, IMI 090682, IMI 088631, IMI 045492)	B,3,III	580	240,200,140	280,180,120	380,160	7,4,3.2,2.8,2.4,2.3	540	230,120,80	280,170,100	540	250,180,140
F. oxysporum (IMI 071799, IMI 271801, IMI 262722, IMI 335344)	B,4,IV	580	240,200,140	280,180,120	380,160	7.1,5.5,4.5,3.8	540	230,120,80	270,270	540	240,190,140
F. nubilum (IMI 278734, IMI 130213)	B,5,V	580	240,200,140	280,180,120	380,160	6.5,4.3,2.2	540	230,120,80	460	320,220	360,180
F. nigrescens (IMI 044575, IMI 326064)	C,6,VI	600	240,160,100,80	300,160,140	400,100,80	8,4,3.4,2.8	500	280,120,80	320,100	260,230	320,180
F. nigrescens (IMI 118380)	C,6',VI	600	240,160,100,80	300,160,140	400,100,80	7,4,3.2,2.6,2.4	500	280,120,80	190,140,100	480	320,180
F. nigrescens (IMI 295728)	C,6'',VI	600	240,160,100,80	300,160,140	400,100,80	2.5,2.3,1.9,1.8	500	280,120,80	190,140,100	480	320,180
F. nigrescens (IMI 112791)	C,6''',VI	600	240,160,100,80	300,160,140	400,120,80	6,5.5,4.2,3.5,3,1.6	500	280,120,80	320,100	260,230	320,180
F. theobromae (IMI 073524, IMI 084424)	D,7,VII	600	200,140,120,80,60	300,300	400,100,90	9.2,3.5,3.2,9.2,6.2,4.1,4	580	380,120,80	230,190,130	580	400,180
F. theobromae (IMI 172699)	D,7',VII	600	200,140,120,80,60	300,300	400,100,90	7.1,5.3,6.3,2.5	580	380,120,80	230,190,130	580	220,190,180
F. theobromae (IMI 225818)	D,7'',VII	600	200,140,120,80,60	300,300	400,100,90	7.1,4.7,4.5,3.6,3.2.5	580	380,120,80	230,190,130	580	220,190,180
F. theobromae (IMI 031432a)	D,7''',VII	600	200,140,120,80,60	300,300	400,100,90	8.5,5.3,3.8,3.6,3.4,3, 2.5,2.2,1.8,1.7	580	380,120,80	230,190,130	580	400,180

V. tricorpus. However, these are considered here as distinct species that can be separated from each other on morphological and molecular grounds. When the amplicons of the β-tubulin gene were digested by *Hae* III the two species *V. albo-atrum* and *V. dahliae* produced different banding patterns that here is considered the easiest way to confirm identification in case of morphological uncertainty (Fig. 5, patterns 19, 20; Fig. 6a, b). Distinctive species-specific bands were observed by CARDER & BARBARA (1991) after digestion of the total genomic DNA by *Eco*R I and probing between *V. albo-atrum* and *V. dahliae*. They found that one isolate of *V. tricorpus* was identical to *V. nigrescens* and some others were not distinguishable from *V. nubilum*. CARDER & BARBARA considered the approach they have used as insufficient to identify the four plant-associated species. However, based on a combination of RFLPs obtained from ITS region, β-tubulin gene and mtDNA these were separated in this study. A comparison between different RFLP methods indicate a general concordance of results with resolution of groups in the order mtDNA > β-tubulin gene > ITS.

Acknowledgment

Prof. W. Gams (CBS, the Netherlands) is thanked for critical reading of the manuscript and for his advice on *Verticillium* taxonomy. Dr. A. Culham (Reading University, UK) is thanked for his help and advice on the analysis of molecular data.

References

AOKI, T. and O'DONNELL, K. 1999. Morphological and molecular characterization of *Fusarium pseudograminearum* sp. nov., formerly recognized as the Group 1 population of *F. graminearum*. Mycologia 91: 597-609.

BRUNS, T. D., PALMER. J. D., SHUMARD, D. S., GROSSMAN, L. I. and HUDSPETH, M. E. S. 1988. Mitochondrial DNAs of *Suillus*: three-fold size change in molecules that share a common gene order. Current Genetics 13: 49-56.

BUDDIE, A. G., MARTINEZ-CULEBRAS, P., BRIDGE, P. D., GARCÍA, M. D., QUEROL, A., CANNON, P. F. and MONTE, E. 1998. Molecular characterization of *Colletotrichum* strains derived from strawberry. Mycological Research 103: 385-394.

BUHR, T. L. and DICKMAN, M. B. 1993. Isolation and characterization of a β-tubulin-encoding gene from *Colletotrichum gloeosporioides* f. sp. *aeschynomene*. Gene 124: 121-125.

CARDER, J. H. and BARBARA, D. J. 1991. Molecular variation and restriction fragment length polymorphisms (RFLPs) within and between six species of *Verticillium*. Mycological Research 95: 935-942.

CARDER, J. H., SEGERS, R., BUTT, T. M., BARBARA, D. J., VON MENDE, N. and COOSEMANS, J. 1993. Taxonomy of the nematophagous fungi *Verticillium chlamydosporium* and *V. suchlasporium* based on secreted enzyme activities and RFLP analysis. Journal of Invertebrate Pathology 62: 178-184.

DOMSCH, K. H., GAMS, W. and ANDERSON, T. H. 1980. Compendium of soil fungi I. London: Academic Press.

FRIES, E. 1832. Systema Mycologicum 3. Greifswald: Ernest Mauritii.

GAMS, W. 1971. *Cephalosporium*-artige Schimmelpilze (Hyphomycete). Gustav Fischer Verlag, Stuttgart.

GAMS, W. and VAN ZAAYEN, A. 1982. Contribution to the taxonomy and pathogenicity of fungicolous *Verticillium* species I: Taxonomy. Netherlands Journal of Plant Pathology 88: 57-78.

GAMS, W., O'DONNELL, K., SCHROERS, H. J. and CHRISTENSEN, M. 1998. Generic classification of some more hyphomycetes with solitary conidia borne on phialides. Canadian Journal of Botany 76: 1570-1583.

GAMS, W. and ZARE, R. 2001. A revision of *Verticillium* sect. *Prostrata*. III.

Generic classification. Nova Hedwigia 72:47-55.

GAMS, W. and ZARE, R. 2002. New generic concepts in *Verticillium* sect. *Prostrata*. Mycological Research 106: 130-131.

GARDES, M. and BRUNS, T. D. 1993. ITS primers with enhanced specificity for Basidiomycetes application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113-118.

GLASS, N. L. and DONALDSON, G. C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Applied and Environmental Microbiology 61: 1323-1330.

HAWKSWORTH, D. L. 1970a. *Verticillium tricorpus*. CMI Descriptions of Pathogenic Fungi and Bacteria 260: [2] pp.

HAWKSWORTH, D. L. 1970b. *Verticillium nigrescens*. CMI Descriptions of Pathogenic Fungi and Bacteria 257: [2] pp.

HAWKSWORTH, D. L. and HOLLIDAY, P. 1970. *Verticillium theobromae*. CMI Descriptions of Pathogenic Fungi and Bacteria 259: [2] pp.

HAWKSWORTH, D. L. and TALBOYS, P. S. 1970a. *Verticillium albo-atrum*. CMI Descriptions of Pathogenic Fungi and Bacteria 255: [2] pp.

HAWKSWORTH, D. L. and TALBOYS, P. S. 1970b. *Verticillium dahliae*. CMI Descriptions of Pathogenic Fungi and Bacteria 256: [2] pp.

HUGHES, S. J. 1951. Studies on microfungi. XI: some hyphomycetes which produce phialides. Mycological Papers 45: 1-36.

HUGHES, S. J. 1958. Revisions hyphomycetum aliquot cum appendice de nominibus rejiciendis. Canadian Journal of Botany 36: 323.

ISAAC, I. 1949. A comparative study of pathogenic isolates of *Verticillium*. Transactions of the British Mycological Society 32: 137-157.

ISAAC, I. 1953. A further comparative study of pathogenic isolates of *Verticillium*:

V. nubilum Pethybr. and *V. tricorpus* sp. nov. Transactions of the British Mycological Society 36: 180-185.

ISAAC, I. 1967. Speciation in *Verticillium*. Annual Review of Phytopathology 5: 201-222.

ISAAC, I. and KEYWORTH, G. W. 1948. *Verticillium* wilt of the hop (*Humulus lupulus*) III: a study of the pathogenicity of the isolates from fluctuating and from progressive outbreaks. Annals of Applied Biology 35: 243-249.

JENSEN, A. 1965. *Verticillium*-arter i Danmark. Horticultura 19: 91–97.

JOAQUIM, T. R. and ROWE, R. C. 1990. Reassessment of vegetative compatibility relationship among strains of *Verticillium dahliae* using nitrate-nonutilizing mutants. Phytopathology 80: 1160-1166.

JUN, Y., BRIDGE, P. D. and EVANS, H. C. 1991. An integrated approach to the taxonomy of the genus *Verticillium*. Journal of General Microbiology 137: 1437-1444.

KARAPAPA, V. K., BAINBRIDGE, B. W. and HEALE, J. B. 1997. Morphological and molecular characterization of *Verticillium longisporum* comb. nov., pathogenic to oilseed rape. Mycological Research 101: 1281-1294.

KOIKE, M., WATANABE, M., NAGAO, H. and OSHIMA, S. 1995. Molecular analysis of Japanese isolates of *Verticillium dahliae* and *V. albo-atrum*. Letters in Applied Microbiology 21: 75-78.

KOUVELIS, V. N., ZARE, R., BRIDGE, P. D. and TYPAS, M. A. 1999. Differentiation of mitochondrial subgroups in the *Verticillium lecanii* species complex. Letters in Applied Microbiology 28: 263-268.

LI, K. N., ROUSE, D. I. and GERMAN, T. L. 1994. PCR primers that allow intergeneric differentiation of Ascomycetes and their application to *Verticillium* spp. Applied and Environmental Microbiology 60: 4324-4331.

MESSNER, R., SCHWEIGKOFLER, W., IBL, M. and PRILLINGER, H. 1996. Molecular characterization of the plant pathogen *Verticillium dahliae* Kleb. using RAPD-PCR and sequencing of the 18S rRNA-gene. Journal of Phytopathology 144: 347-354.

MORTON, A., TABRETT, A. M., CARDER, J. H. and BARBARA, D. J. 1995. Sub-repeat sequences in the ribosomal RNA intergenic regions of *Verticillium albo-atrum* and *V. dahliae.* Mycological Research 99: 257-266.

MUGNAI, L., BRIDGE, P. D. and EVANS, H. C. 1989. A chemotaxonomic evaluation of the genus *Beauveria.* Mycological Research 92: 199-209.

MÜLLER, E. and VON ARX, J. A. 1973. Pyrenomycetes: Meliolales, Coronophorales, Sphaeriales. *In*: The Fungi, An Advanced Treatise, Vol. 4A. pp. 87-132. (eds. G. C. Ainsworth, F. K. Sparrow, and A. S. Sussman) Academic Press, New York.

NAZAR, R. N., HU, X., SCHMIDT, J., CULHAM, D. and ROBB, J. 1991. Potential use of PCR amplified ribosomal intergenic sequences in the detection and differentiation of *Verticillium* wilt pathogen. Physiological and Molecular Plant Pathology 39: 1-11.

NEES VON ESENBECK, C. G. 1816. Das System der Pilze und Schwämme. Stahelschen Buchhandlung, Würzburg. XXXVII + 329 pp.

OKOLI, C. A. N., CARDER, J. H. and BARBARA, D. J. 1994. Restriction fragment length polymorphisms (RFLP) and the relationships of some host-adapted isolates of *Verticillium dahliae.* Plant Pathology 43: 33-40.

PEGG, G. F. 1974. *Verticillium* diseases. Review of Plant Pathology 53: 157-182.

PETHYBRIDGE, G. H. 1919. Notes on some saprophytic species associated with diseased potato plants and tubers. Transactions of the British Mycological Society 6: 104-120.

RAEDER, U. and BRODA, P. 1985. Rapid preparation of DNA from filamentous fungi. Letters in Applied Microbiology 1: 17-20.

SCHNATHORST, W. C. 1973. Nomenclature and physiology of *Verticillium* species, with emphasis on the *V. albo-atrum* versus *V. dahliae* controversy. Proceedings of a Work Conference, National Cotton Pathology Research Laboratory, College Station, Texas ARS-S-19: 1–19.

SKADOW, K. 1969. Ein Beitrag zur *Verticillium*-Taxonomie. Archiv für Pflanzenschutz 5: 155–166.

SUNG, G.-H., ZARE, R., SPATAFORA, J. and GAMS, W. 2001. A revision of *Verticillium* sect. *Prostrata*. II. Generic delimitation using ribosomal DNA sequences. Nova Hedwigia 72: 29-46.

TSUNEDA, A, SKOROPAD, W. P. and TEWARI, J. P. 1976. Mode of parasitism of *Alternaria brassicae* by *Nectria inventa*. Phytopathology 66: 1056-1064.

TYPAS, M. A., GRIFFEN, A. M., BAINBRIDGE, B. W. and HEALE, J. B. 1992. Restriction fragment length polymorphisms in mitochondrial DNA and ribosomal RNA gene complexes as an aid to the characterization of species and sub-species populations in the genus *Verticillium*. FEMS Microbiology Letters 95: 157-162.

WAKATABE, D., NAGAO, H., ARAI, H., SHIRAISHI, T., KOIKE, M. and LIJIMA, T. 1997. Vegetative compatibility groups of Japanese isolates of *Verticillium dahliae*. Mycoscience 38: 17-23.

WHITE, T. J., BRUNS, T., LEE, S. and TAYLOR, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes from phylogenetics. In PCR Protocols, pp. 315-322. (eds. M. A. Innes, D. H. Gelfand, J. S. Sninsky and T. J. White). Academic Press, London, UK.

WILLIAMS, M. A. J., BRADY, B. L., BRIDGE, P. D. and PATERSON, R. R. M. 1992. Biochemical and physiological tests as aids to identification of

Verticillium sect. *Nigrescentia*. Mycopathologia 119: 101-114.

YAN, K. and DICKMAN, M. B. 1996. Isolation of β-tubulin gene from *Fusarium moniliforme* that confers cold-sensitive benomyl resistance. Applied and Environmental Microbiology 62: 3053-3056.

ZARE, R. and GAMS, W. 2001a. A revision of *Verticillium* section *Prostrata*. IV. The genus *Lecanicillium* and the new genus *Simplicillium*. Nova Hedwigia 73: 1-50.

ZARE, R. and GAMS, W. 2001b. A revision of *Verticillium* sect. *Prostrata*. VI. The genus *Haptocillium*. Nova Hedwigia 73: 271-292.

ZARE, R., GAMS, W. and EVANS, H. C. 2001. A revision of *Verticillium* sect. *Prostrata*. V. The genus *Pochonia*. Nova Hedwigia 73: 51-86.

ZARE, R., GAMS, W. and CULHAM, A. 2000. A revision of *Verticillium* sect. *Prostrata*. I. Phylogenetic studies using ITS sequences. Nova Hedwigia 71: 465-480.

ZARE, R., KOUVELIS, V. N., TYPAS, M. A. and BRIDGE, P. D. 1999. Presence of a 20 bp insertion/deletion in the ITS1 region of *Verticillium lecanii*. Letters in Applied Microbiology 28: 258-262.

Address of the author: Dr. R. ZARE, Department of Botany, Plant Pests & Diseases Research Institute, P. O. Box 1454, Tehran 19395, Iran.
E-mail: simplicillium@yahoo.com