مروری بر گونه‌های ورتیکیلیوم مرتبط با گیاهان

A revision of plant-associated Verticillium species

رسول زارع

بخش تحقیقات رستنی‌ها، موسسه تحقیقات آفات و بیماری‌های گیاهی

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روش‌های مولکولی جهت ارزیابی معیارهای تاکسونومیک ورتیکیلیوم‌های مرتبط با گیاهان مورد استفاده قرار گرفتند. سپس ویک استریفت‌زدن ورتیکیلیوم به‌همراه مقدارهای ITS-RFLPs وسیله مقایسه‌ای بود. کلاس‌تیری شدن را به‌چنار کلاس‌تیر خاص و ژنتیکی یافتند. کلاس‌تیر اول در بر گیرنده کلاس‌تیر دوم شامل V. luteo-album (Link : Fries) Subramanian دریافت، V. dahliae Klebahn، V. albo-atrum Reinke & Berthold، V. theobromae (Turconi) E. Mason & S. Hughes و V. tricorpus Isaac نیز شامل بود. گواهی E. Pethybridge درون کلاس‌تیر چهار شامل V. nigrescens Pethybridge به دست آمد. از روش بتا توبولین (β-tubulin gene) تشخیص دادند. هر هفت گونه مورد آزمایش را از DNA RFLP فکر می‌کنند. با قدرت فنکیک از اکثر V. nigrescens و V. theobromae استریفت‌زدن گونه‌های دو کلاس‌تیر نمود. بر اساس نقشه انتزایی بر شکل انتخاب دو کلاس‌تیر به‌صورت Hae III و روش II و مقایسه اکثریت گونه‌های به‌دست آمده روشن کننده برای انتخاب دو گونه مهم اقتصادی و بحث بر انگیز می‌باشد. متن کامل مقاله در قسمت انجیلیسی ارزشی شده است.

واژه‌های کلیدی: ورتیکیلیوم، گونه‌های مولکولی، تاکسونومی
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A REVISION OF PLANT-ASSOCIATED

VERTICILLIUM SPECIES

R. Zare

Plant Pests & Diseases Research Institute

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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*, *Nigrescentia*, taxonomy, mitochondrial DNA, ITS-RFLPs

Introduction

NEES (1816) introduced the genus *Verticillium* for a saprotopholic 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 (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 Phyllochaorean genus *Glomerella* (MESSNER et al., 1996) while members of section *Prostrata* are anamorphs of Clavicipitalean genera, *Cordyceps* and *Torrubiella*.

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; SCHRATHORST, 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, *Verticillium 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 focusing 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 H₂O 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 and 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 BamH I, Sal I, Pst I, EcoR I, EcoR 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 H₂O. 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 (NH₄)₂ SO₄, 100 µM of each dATP, dCTP, dGTP, dTTP, 30 pmol of each primer, 2 mM MgCl₂, 1 unit Tth DNA polymerase, and the total volume was adjusted to 50 µl by adding HPLC grade H₂O. 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, 1970; 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 .......... 6

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 region (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. lateo-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

![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*).](image)

![Fig. 2a. Cluster analysis of sections *Verticillium* and *Nigrescentia* based on ITS-RFLPs using Jaccard’s Coefficient with UPGMA method.](image)
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. dahlieae*. 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. dahlieae* and *V. albo-atrum*. Pattern 5 (Fig. 3) which represents strain IMI 118380 has four (out of five) bands in common with *V. dahlieae* 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. dahlieae* and strains IMI 118380 and IMI 295728 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. dahlieae* 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* had an identical profile, with some fragments 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.

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.

PCA case scores

Fig. 4b. Principle component analysis of sections *Verticillium* and *Nigrescentia* based on mtDNA-RFLPs.
(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. niirens*, 540-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).

![Fig. 5. Schematic representation of β-tubulin gene-RFLP patterns of sections Verticillium and Nigrescentia.](image)

(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* 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.

PCA case scores

![PCA diagram]

Axis 1 = 28.1%
Axis 2 = 20.2%
Axis 3 = 19%

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

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).

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.
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 *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 17, 18; Fig. 6a, b). Distinctive species-specific bands were observed by Carder and BARBARA (1991) after digestion of the total genomic DNA by *EcoR* 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 and 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.
<table>
<thead>
<tr>
<th>Taxon</th>
<th>IMI No.</th>
<th>Host/Substrate</th>
<th>Origin, Date, Collector</th>
<th>Other information</th>
</tr>
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<tr>
<td><em>V. luteo-album</em> (Link) Subram.</td>
<td>017438a</td>
<td><em>Polygonum sieboldii</em></td>
<td>UK, 1947, S.J. Hughes</td>
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<td>182719</td>
<td>dead butterfly</td>
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<td><em>V. luteo-album</em></td>
<td>054377</td>
<td><em>Polyergus squamosus</em></td>
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<td><em>V. albo-atrum</em></td>
<td>298097</td>
<td><em>Humulus lupulus</em></td>
<td>UK, IMI 1985, P. Talboys</td>
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<td><em>Humulus lupulus</em></td>
<td>UK, IMI 1985, P. Talboys</td>
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<td><em>Solanum tuberosum</em></td>
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<td>CBS 382.66, ATCC 16534</td>
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<td><em>V. dahliae</em> Klebahn</td>
<td>088630</td>
<td><em>Lycopersicon esculentum</em></td>
<td>Zimbabwe, 1961, A. Rothwell</td>
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<td><em>V. dahliae</em></td>
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<td><em>Gossypium sp.</em></td>
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<td>ATCC 22924</td>
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<td><em>Helianthus annuus</em></td>
<td>Canada, 1961, W.E. Sackton</td>
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<td>088631</td>
<td><em>Solanum mammosum</em></td>
<td>Zimbabwe, 1961, A. Rothwell</td>
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<td><em>Mentha sp.</em></td>
<td>Zimbabwe, 1951, J. Broadfoot</td>
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<td><em>Avena sativa</em></td>
<td>Pakistan, IMI 1985, A.H. Tariq</td>
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<td><em>Solanum tuberosum</em></td>
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<td>India, 1962, M.J. Thirumalachar</td>
<td>ATCC 16090, HACC 149</td>
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<td><em>V. rubrum</em> Pethybr.</td>
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<td><em>Solanum tuberosum</em></td>
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<td><em>V. theobromae</em> (Turconi) E. Mason &amp; S. Hughes</td>
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<td><em>V. tricuspus</em></td>
<td>335344</td>
<td><em>Dianthus caryophyllus</em></td>
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</table>
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.

<table>
<thead>
<tr>
<th>Taxa and Accession codes</th>
<th>Pattern</th>
<th>Total size</th>
<th>RFLPs of ITS region</th>
<th>RFLPs of mtDNA</th>
<th>Total RFLPs of the β-tubulin gene</th>
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<td>Hinf1</td>
<td>HaeIII</td>
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<td>A,1,1</td>
<td>600</td>
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<td>320,120,100,80</td>
<td>240,220,160</td>
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<td>280,180,120</td>
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<td>600</td>
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<td>400,100,90</td>
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</tbody>
</table>
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References


OKOLI, C.A.N., CARDER, J.H. and BARBARA, D.J. 1994. Restriction fragment length polymorphisms (RFLP) and the relationships of some host-adapted


Addresses of the authors: