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Molecular Characterization of the Plant Pathogen *Verticillium dahliae* Kleb. Using RAPD-PCR and Sequencing of the 18SrRNA-Gene

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With 4 figures

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Abstract

Thirty-four isolates of *Verticillium dahliae* Kleb. from nine different genera of dicotyledonous host plants and a broad range of geographic regions were analysed genotypically. Random amplified polymorphic DNA (RAPD) markers were used for the estimation of the genetic variability within the species. Using four primers for the analysis, 79 distinct fragments were obtained. The derived phenogram clustered the isolates in two main groups: one consisted almost entirely of *V. dahliae* isolates from oilseed rape (*Brassica napus napus*), the other group comprised isolates from a wide range of host plants. No correlation between geographic location of the isolates and the RAPD-pattern was observed.

Sequencing of the gene for the 18SrRNA and calculation of the phylogenetic tree integrated the deuteromycetous fungus *V. dahliae* into the sexual system of the filamentous ascomycetes.

Zusammenfassung

Molekulare Charakterisierung des Pflanzenpathogens *Verticillium dahliae* Kleb. mittels RAPD-PCR und Sequenzierung des 18SrRNA-Gens.

Vierunddreißig Isolate von *Verticillium dahliae* Kleb. aus neun verschiedenen dikotylen Wirtspflanzen und unterschiedlichen geographischen Regionen wurden genotypisch analysiert. Zur Bestimmung der genetischen Variabilität innerhalb der Art wurden "Random-Amplified-Polymorphic-DNA" (RAPD)-Marker verwendet. Durch den Einsatz von vier Primern wurden 79 distinkte Fragmente erhalten, das daraus berechnete Phenogramm gruppiert die Isolate in zwei Cluster: der erste enthält *V. dahliae* Isolate aus Raps (*Brassica napus napus*), der zweite umfaßt Isolate aus einem breiten Wirtsspektrum. Die geographische Herkunft der Isolate zeigt keine Korrelation zum erhaltenen RAPD-Muster.

Sequenzierung des Gens für die 18SrRNA und anschliessende Stammbaumberechnung integrierte den Deuteromyceten *V. dahliae* in das sexuelle System der filamentösen Ascomyceten.

Introduction

The hyphomycete *Verticillium dahliae* Kleb. is a root-associated fungus of worldwide distribution and has been cultured from blackleg-affected oilseed rape (*Brassica napus napus*), paprika (*Capsicum annuum*), flax (*Linum usitatissimum*), grape (*Vitis vinifera*) and a wide range of other plants of economic value in which it causes tracheomycosis (Verticillium-wilt). Host-specific isolates of *V. dahliae* are indistinguishable on the basis of morphological characters. Distinctive characters of *Verticillium* species within the section *Nigrescentia* are the formation of dark resting mycelium by *V. albo-atrum*, of dark microsclerotia by *V. dahliae* and of resting mycelium, microsclerotia and chlamydospores by *V. tricorpus*. Unfortunately, members of this genus may loose some of the distinctive characters after prolonged maintenance in culture or when kept on an inappropriate medium.

There have been many attempts to separate *Verticillium albo-atrum* and *V. dahliae* and other *Verticillium* species at the species level recently (Carder and Barbara, 1991; Nazar et al., 1991; Williams et al., 1992; Carder et al., 1993; Li et al., 1994). On the basis of the nucleotide sequences of an amplified mitochondrial small rRNA gene region Li et al. (1994) developed specific primers that amplified a 140-bp region of *V. dahliae* DNA. The *V. dahliae*-specific PCR primer may aid in more rapid and specific detection of the pathogen directly in plant and/or soil samples. Further differentiation of specific pathotypes from *V. albo-atrum* and *V. dahliae* was reported from Okoli et al. (1993, 1994) using restriction fragment length polymorphisms (RFLPs). Two distinct groups were shown in *V. albo-atrum*: isolates from lucerne formed one group and those from all other hosts the second group. In *V. dahliae* four distinct groups were

found: diploid isolates comprised a group (D) only distantly related to either haploid isolates of *V. dahliae* or to *V. albo-atrum*. Most *V. dahliae*-isolates were haploid and fell into three further groups (A, B and C) (Horiuchi et al., 1990). Additional studies from Carder and Barbara (1994) using isolates from Japan and North America showed an even broader range of variation in the RFLP-patterns.

Recent work by Morton et al. (1995a,b) using subrepeat sequences in the RNA intergenic regions and sequences of the internal transcribed spacers (ITS) showed a remarkable extent of differences between haploid and diploid isolates of *V. dahliae*, may be even enough to put them into two separate species.

Attempts to use fungicide-resistance-markers (e.g. Johnson et al., 1994) and virulence markers (e.g. Schreiber and Domir, 1994) that describe genetic variation in plant pathogen populations are obviously important because they provide direct information concerning the effects of selection and the potential effectiveness of resistance genes. However, virulence and resistance markers may represent only a small portion of the total genetic variation present in the population. As shown by Samborski and Dyck (1976) for the *Puccinia recondita* f. sp. *tritici–Triticum aestivum*-system, virulence/avirulence was most often inherited as a single gene that corresponded to a specific resistance gene. Conclusions drawn exclusively on race phenotypes of plant pathogenic fungi which were derived by this gene-for-gene concept (Flor, 1971) may not give sufficient information on the level of other genetic differences either between races or between independent collections of a race.

In recent years 'scanning' the DNA of isolates at loci arbitrarily selected across the genome using random amplified polymorphic DNA (RAPD) analysis, has become a widespread approach in plant pathology (e.g. Schäfer and Wöstemeyer, 1992).

Cluster analysis of resulting bands are able to distinguish between geographical races (e.g. Assigbetse et al., 1994; Kolmer et al., 1995; Maclean et al., 1995), pathotypes restricted to a sole host plant (e.g. Hayden et al., 1994) and somatic hybrids following parasexual recombination in *Penicillium roqueforti* (Durand et al., 1993).

The clear genotypic differentiation of parasitic fungi into pathotypes of variable aggressiveness is a prerequisite for biological plant protection using resistance induction in the host plant. For example, elm trees which were pre-treated with a less aggressive strain of *Ophiostoma novo-ulmi* survived a subsequent inoculation with a highly aggressive type (Scheffer, 1990).

The introduction of deuteromycetes into the system of ascomycetes and basidiomycetes nowadays seems possible using molecular techniques. Using the sequence of the gene for the 18S subunit of ribosomal RNA, Berbee and Taylor (1992a,b) showed, that the anamorphic human pathogen *Sporothrix schenkii* lies phylogenetically within the sexual genus *Ophiostoma*. Rehner and Samuels (1994), working with the nuclear large subunit ribosomal DNA (28SrDNA), showed members of the genus *Gliocladium* to be polyphyletic and generically distinct, although showing great morphological similarities. The importance of these findings for the future design of molecular plant protection is obvious.

The present study investigated molecular heterogeneity and phylogenetic position of the plant pathogen *Verticillium dahliae* using RAPD-PCR and sequencing of the 18SrDNA.

Materials and Methods

Fungal strains

The strains examined are listed in Table 1. All strains are maintained at the culture collection of the Institute of Applied Microbiology, IAM, University of Agriculture, Vienna; Austria.

DNA extraction

Fungal strains were cultured in 100 ml Erlenmeyer-flasks containing 20 ml Mandels-Andreotti-Medium (per liter: 10 g glucose; 2 g peptone; 2.8 g ammonium sulfate; 4 g KH_2PO_4; 10 g Na_2HPO_4, pH 5.0; 10 ml of a simplified Czapek conc.: 7 g $MgSO_4$; 0.05 g $CuSO_4 \times 5H_2O$; 0.1 g $FeSO_4 \times 7H_2O$; 0.1 g $ZnSO_4 \times 7H_2O$) for five days using a rotary shaker (30°C; 150 rpm).

The mycelium was collected by filtration and ground to a fine powder in liquid N_2. Fifty mg of the ground were transferred to a 1.5 ml Eppendorf tube and mixed with 0.4 ml lysis buffer (0.1 M Tris; 1.4 M NaCl; 50 mM EDTA; pH 8.0). The suspension was frozen at −20°C and thawed three times. After addition of 0.4 ml phenol freezing and thawing was repeated. The suspension was heated at 55°C for 10 min, 0.4 ml of chloroform were added and heated again. The resulting mixture was centrifugated at 15 500 × *g* for 30 min, and 0.3 ml of the clear supernatant was mixed with 0.45 ml of propan-2-ol. After incubation at room temperature for 5 min the mixture was centrifuged at 15 500 × *g* for 10 min and the resulting pellet washed twice with 0.8 ml of 70% ethanol. The pellet was dried under vacuum and dissolved in 50 µl TE-buffer. Samples chosen for sequencing were digested with 2.5 µl RNAse (10 mg/ml) for 3 h at 37°C. DNA concentrations were evaluated by agarose gel electrophoresis.

RAPD-analysis

The RAPD-PCR was performed as described in Messner et al. (1994), using the primers M13 (5'dGAGGGTGGCGGTTCT); GACA (5'dGACAGACAGACAGACA); (both Lieckfeldt et al., 1993); V1 (5'dACGGTCTTGG; Schäfer and Wöstemeyer, 1992) and V5 (5'dTGCCGAGCTG; Caetano-Anolles et al., 1992). The temperature profile for primers M13 and GACA was as follows: denaturation at 98°C for 15 s; annealing at 50°C for 60 s and extension at 72°C for 100 s for a total of 40 cycles. For decamer primers V1 and V5 annealing was performed at 32°C for 90 s. Synthesis of primers was performed by Codon Genetic Systems (Vienna; Austria) using a model 392 DNA synthesizer (Applied Biosystems, Foster City, CA, USA).

Computer analysis of RAPD patterns was performed as in Halmschlager et al. (1994). Basically, the infor-

Table 1
List of fungal strains studied

Species	IAM no.	Other no.	Host	Geographic region[a]
V. dahliae	MD15		*Capsicum annum*	Burgenland/Austria
	MD44		*Linum usitatissimum*	Rostock/Germany
	MD45		*Linum usitatissimum*	Rostock/Germany
	MD46		*Linum usitatissimum*	Rostock/Germany
	MD47		*Linum usitatissimum*	Rostock/Germany
	MD48		*Linum usitatissimum*	Rostock/Germany
	MD49		*Capiscum annuum*	Burgenland/Austria
	MD53	VD372	*Brassica napus napus*	
	MD54	VD383	*Humulus lupulus*	The Netherlands
	MD55	VD185	*Gossypium hirsutum*	
	MD56	VD375	*Brassica napus napus*	Sweden
	MD57	VD374	*Brassica napus napus*	
	MD58	VD183	*Piper nigrum*	
	MD59	VD186	*Solanum melongea*	
	MD68	ELV1	*Trifolium repens*	Malchow/Germany
	MD69	ELV13	*Gossypium hirsutum*	Cordoba/Spain
	MD70	ELV16	*Solanum tuberosum*	Rostock/Germany
	MD71	ELV17	*Matricaria chamomilla*	Rostock/Germany
	MD73	ELV19	*Brassica napus napus*	Rosenhagen/Germany
	MD74	ELV22	*Brassica napus napus*	Kappeln/Germany
	MD75	ELV23	*Brassica napus napus*	Schönberg/Germany
	MD76	ELV25	*Brassica napus napus*	Rostock/Germany
	MD77	ELV26	*Matricaria chamomilla*	Rostock/Germany
	MD80	ELV29	*Brassica napus napus*	Friedrichsfeld/Germany
	MD81	ELV34	*Brassica napus napus*	Schönberg/Germany
	MD82	PV41	*Brassica napus napus*	Rostock/Germany
	MD123	ELV10	*Brassica napus napus*	Rostock/Germany
	MD124	ELV11	*Matricaria chamomilla*	Rostock/Germany
	MD125	ELV24	*Brassica napus napus*	Bondelsdorf/Germany
	MD126	ELV44	*Brassica napus napus*	Fehmarn/Germany
	MD127	ELV12/3	*Gossypium hirsutum*	Cordoba/Spain
	MD128	ELV12/7	*Gossypium hirsutum*	Cordoba/Spain
	MD167		*Capiscum annum*	Stockerau/Austria
	MD179		*Capiscum annum*	Stockerau/Austria

[a]No information means that the origin is not known.

mation obtained from agarose gel electrophoresis was digitalized by hand to a two-discrete-character-matrix (0 and 1 for absence and presence of RAPD-markers). Phenograms were calculated by using the Jukes-Cantor option in DNADIST program and application of the FITCH program (Fitch and Margoliash, 1967) to the computed distance matrix (PHYLIP package; Felsenstein, 1989). For running DNADIST, the two discrete characters 0 and 1 had to be converted to Guanine and Thymine in the RAPD data matrix.

Sequencing of the 18SrRNA-Gene

RNAse treated DNA of the haploid isolate MD15 was amplified using the primers NS0 and ITS2 (White et al., 1990). Thirty-one cycles of the program 98°C/15 s; 58°C/60 s and 72°C/120 s were performed.

For removing the amplification primers, PCR products were diluted by 3 volumes of TE-buffer and precipitated overnight by 4 volumes of PEG-buffer (13% w/v polyethylene glycol 6000; 1.6 M NaCl). After centrifugation, the pellets were washed in 70% ethanol, dried and dissolved in 20 μl water. DNA concentrations were estimated by agarose gel electrophoresis.

The sequencing reactions were performed by Codon Genetic Systems (Vienna, Austria) using a 373A automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA). Both strands of the 18SrRNA gene were sequenced, using a total of 14 primers, including the primers NS0 and ITS2 engaged in DNA amplification. Up to 400 bases were recorded per sequencing reaction. Sequence primers were optimized by the OLIGO Primer Analysis Software (National Biosciences, MA, USA). The sequence of the 18SrRNA-gene from *Verticillium dahliae* is accessible from GenBank under the accession number U33637.

Sequence analysis

The DNA sequence obtained and selected reference sequences derived from GenBank were aligned using the ClustalW program (Thompson et al., 1994). Because of slightly different gene lengths, 'overhanging' nucleotides at the 3' end had to be cut off for some species. Phylogenetic computation of alignments was done using the programs DNADIST (Parameter 'Kimura 2'; Kimura, 1980), FITCH, SEQBOOT and CONSENSE in the PHYLIP package. The sequence of *Saccharomyces cerevisiae* was used as the outgroup. Bootstrap confidence values were calculated from 1000 repeats.

Results

Cluster analysis of RAPDs

For RAPD-analysis crude preparations of total nucleic acids were obtained without removing RNA from the

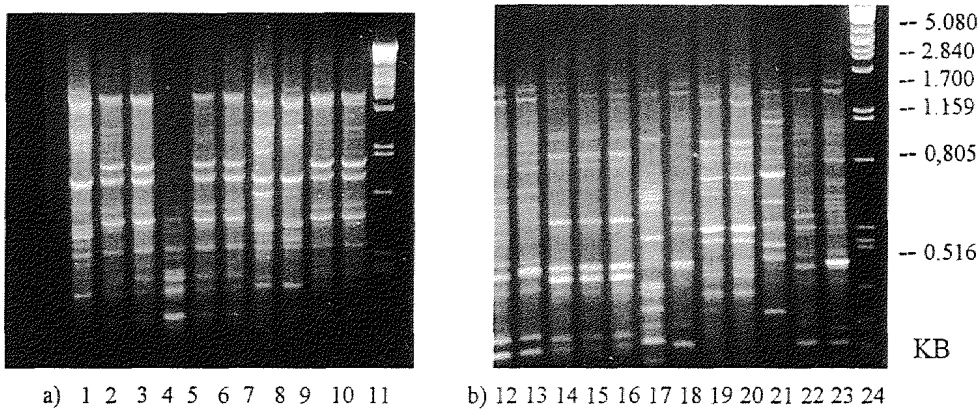

Fig. 1 RAPD patterns of *Verticillium* strains. Lane 1, MD73; lane 2, MD74; lane 3, MD75; lane 4, MD76; lane 5, MD81; lane 6, MD82; lane 7, MD80; lane 8, MD123; lane 9, MD125; lane 10, MD126; lane 11, lambda DNA digested with *Pst*1; lane 12, MD15; lane 13, MD49; lane 14, MD53; lane 15, MD57; lane 16, MD56; lane 17, MD65; lane 18, MD69; lane 19, MD127; lane 20, MD128; lane 21, MD63; lane 22, MD55; lane 23, MD71; lane 24, lambda DNA digested with *Pst*1. (a) Strains isolated from *Brassica napus*, primed with oligonucleotide M13. Note the distinctive pattern of the strains MD73, MD80 and MD123. (b) Strains from *Brassica napus* (MD53, 57, 56) compared with strains from other host plants. Primed with oligonucleotide V5. MD65 (lane 17) is *V. tenerum*, MD63 (lane 20) is *V. lecanii*, both isolated from *G. hirsutum*. These two strains were not included in the construction of the dendrogram

DNA. PCR-amplifications of DNA from all listed isolates were done using the four primers mentioned in the Materials and Methods section. After separation on a 1.3% (w/v) agarose-gel, fragments were obtained for each isolate and primer, giving a total of 79 different bands. Examples of the patterns seen are given in Fig. 1. For further resolution, a matrix of the 34 strains by absence (0) or presence (1) of RAPD-markers at a given position was created by hand (Fig. 2) and analysed with the computer programs DNADIST and FITCH for estimation of the branch lengths [set to Jukes and Cantor (1969) distance methods within DNADIST, and global rearrangements within FITCH]. The resulting phenogram is shown in Fig. 3.

Clusters of *Verticillium* isolates showed no strong correlation to geographic origin. For example some of the isolates from Rostock, Germany, clustered together with isolates either from Burgenland, Austria (MD 44, 46, 47, 48–MD 15, 49), others with strains from Cordoba, Spain (MD 70–MD 127, 128), or from Malchow, Germany (MD 77–MD 68).

On the other hand, a distinct, host-specific cluster was obtained for pathogens of the oilseed rape *Brassica napus napus*, independently of local origin. Out of 13 examined strains, 10 fell into a clearly distinguished group, representing isolates from different parts in Germany and Sweden. The remaining three *Brassica*-isolates (MD 73, 80 and 123) showed no clear group-specific clustering.

Phylogenetic analysis

For determining the position of *V. dahliae* among the phylogenetic system of the ascomycetes, sequencing of the whole 18SrRNA-gene was chosen because this represents the most conserved ribosomal gene and therefore it is the most convenient for assessing 'large scale' evolutionary distances.

For alignment purposes, 18SrRNA-gene sequences from 18 filamentous ascomycetes and deuteromycetes and 2 yeasts (*Saccharomyces cerevisiae* and *Kluyveromyces lactis*) were obtained from GenBank (listed in Table 2).

Comparison of the sequences using PHYLIP-computer-package leads to a phylogenetic tree shown in Fig. 4. *V. dahliae* clusters in a clade with the order *Hypocreales* (*Hypomyces chrysospermus* and *Hypocrea lutea*), *Microascales* (*Microascus cirrosus* and *Pseudallescheria boydii*) and *Phyllachorales* (*Colletotrichum gloeosporiodes*, Teleomorph: *Glomerella cingulata*). Although the internal resolution of the group would need further analysis, this clade is well supported by statistical analysis: bootstrap confidence calculated with 1000 cycles was 99.6% for this grouping, giving a good separation from the closest clade of the *Sordariales*, containing *Podospora*, *Sordaria*, *Neurospora* and *Chaetomium*.

The *Ophiostomatales* (*O. stenoceras* and *O. ulmi*) cluster together with the *Diaporthales* (*Endothia gyrosa* and *Cryphonectria cubensis*), building up a clade, which is well separated from the former mentioned sister group by 97.6% bootstrap confidence.

The grouping of the *Dothideales* (*Septoria nodorum*, *Alternaria alternata*, *Aureobasidium pullulans*) and the *Eurotiales* (*Eupenicillium javanicum* and *Penicillium notatum*) as sister taxa in the phenogram does not necessarily reflect a close phylogenetic affinity between these two clades since the short branch leading to this group is not sufficiently supported. However, the clear distinction between this group which includes taxa without known teleomorphs (*A. pullulans*) and the clade which includes *Verticillium* clearly demonstrates the need for integration of the deuteromycetes into the ascomycetous system.

The monophyly of the studied filamentous species was

Strain	Primer M13	Primer GACA	Primer VI	Primer V5
MD73	0101001000111101000	1100000000011001000	1010001010000100	0111011110000011000000
MD74	1000011100100100000	0001001000010001011000	0010000011000010	1111011100000010001000
MD75	1000011100100100000	0001001000010001011000	0010000011000010	1111011100000010001000
MD76	0000000100100100100	0001001000010001011000	0000000010000110	1111011100000010001000
MD81	1000011100100100000	0001001000010001011000	0010000011000010	1111011100000010001000
MD82	1000011100100100000	0001001000010001011000	0010000011000000	0000011100000010001000
MD80	0101100010101101000	1100000000011001000	1010001010000100	0111011110000011000000
MD123	0100100010011101000	1100000000000010001000	1010001010010100	0111011110000011010000
MD125	1000011100100100000	0001001000010001011000	0010000011000000	1111011100000010001000
MD126	1000011100100100000	0000001000010001011000	0010000011000000	0111011100000010000000
MD53	1000011000100000000	0001001000010001011000	0010000011000000	1111011000000010001000
MD57	1000011000100000000	0001001000010001011000	0010000011000000	1111011000000010001000
MD56	1000011000100000000	0001001000010001011000	0010000011000000	1111011000000010001000
MD69	0000001000111101000	1100001000011001000	0010100010000100	0010001110010010000000
MD127	0001100101001001000	0110101010001100010000	0100100010010000	0111110010010000000010
MD128	0001100101001001000	0110101010001100010000	0100100010010000	0111110010010000000010
MD55	0101001000111101000	1100000000011000000 1000	1010001111000100	0011011110000011000000
MD71	0101001000110101000	1100000000000010000 1000	1010001010000100	1111011110000011000000
MD77	0101001000110000000	1100000000000010100 01000	1010001010000100	0110011110010000000000
MD124	1000011000100000000	0001001000010001011000	0010000011000000	1110011100000000000000
MD68	0101001000110000000	1100000000000010000 1000	1010001010000101	0110011110010000000000
MD70	0001100001001001000	0011000010001110000 1000	0110001010010100	0110111001001000000010
MD54	0101001000000000000	1100000000000011000 1000	1010001010010100	1010001110010010000000
MD58	0101001000000000001	1100000000000101100 01000	1010001010010100	1010001110010010000000
MD59	0101001000000000001	1100000000011011000 1000	1010001010000100	0010011110010000000000
MD44	0101001000111100000	1100000000000011000 1000	1010001010000100	0111011110000011000000
MD45	0101101100110000000	1100000000000101100 01000	1010001010000100	0111011110001110000000
MD46	0101001000111100000	1100000000000011000 1000	1010001010000100	0111011110000011000000
MD47	0101001000111100000	1100000000000011000 1000	1010001010000100	0111011110000010000000
MD48	0101001000111100000	1100000000000011000 1000	1010001010000100	0111011110000010000000
MD49	0101001000111100000	1100000000000011000 1000	1010001010000100	0111011110000010000000
MD15	0101001000111100000	1100000000000011000 1000	1010001010000100	0111011110000010000000
MD167	0101001001111100000	1100000000000011000 1000	1010001010000100	0111011110000010000000
MD179	0101001001111100000	1100000000000011000 1000	1010001010000100	0111011110000010000000

Fig. 2 Binary presence (1)—absence (0) data matrix for RAPD fragments. The matrix shown is a subset of a work which also included non-*V. dahliae***-strains and therefore includes positions with unitorm information**

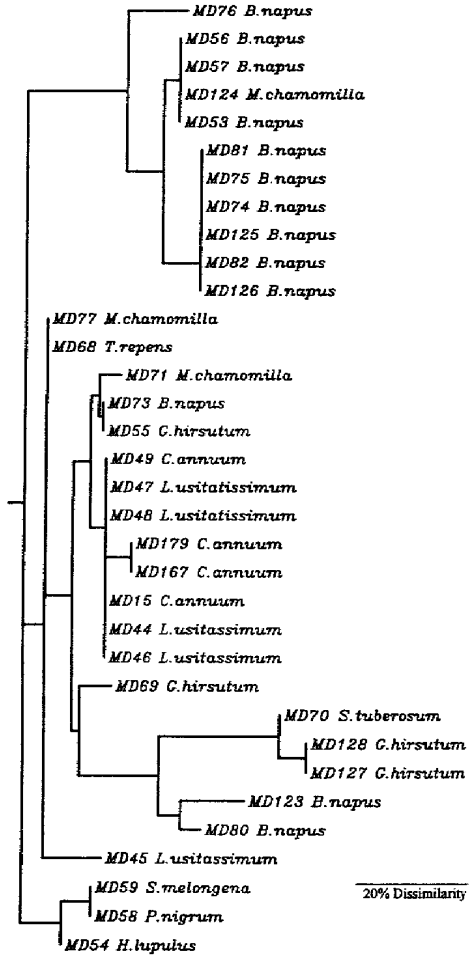

Fig. 3 Dendrogram of 34 isolates of *V. dahliae* Kleb. from different host plants based on data from RAPD analysis. The dendrogram was calculated using DNADIST (Jukes-Cantor option) and FITCH program of the PHYLIP package. Species names of host plants are indicated

corroborated by 100% bootstrap confidence in respect to the unicellular yeasts *S. cerevisiae* and *K. lactis*.

Discussion

Thirty-four isolates of *Verticillium dahliae* from nine genera of dicotyledonous host plants were studied using RAPD-PCR. Approximately 90% of the *V. dahliae* isolates fell into two groups (see Fig. 1). One group consists almost entirely of *V. dahliae* isolates from *Brassica napus* (10 strains) but also includes one isolate from *Matricaria chamomilla*. The isolates were collected in Germany and Sweden. The second group comprises isolates from different host plants such as *Capiscum*, *Linum*, *Solanum melongena*, *S. tuberosum*, *Trifolium*, *Humulus*, *Piper*, and *Gossypium* collected from different european regions. The second group also contains three isolates from *Brassica napus*. Rohde (1995) recently tested these *Brassica napus* isolates for virulence. Isolates MD 73 and MD 80 showed a significantly lower aggressiveness than did the other isolates from *B. napus* when inoculated into seedlings of *B. napus* grown on quartz-sand. Similar data were reported from Horiuchi et al. (1990). Their isolates of group D were only pathogenic to brassicaceous host plants. Jackson and Heale (1985) demonstrated natural stable diploids of *V. dahliae* from *Brassica* spp. Measurements of the conidium length by Rohde (1995) indicate that conidiospores of all the *Brassica* isolates except MD 80 may belong to the var. *longisporum* which was considered to be a diploid race of *V. dahliae* by Jackson and Heale (1985). In *V. albo-atrum*, host specificity appears to be the basis of a differentiation in two groups. Okoli et al. (1993) included in group L all the isolates from lucerne (alfa-alfa), whereas group NL (= non-lucerne) consists of the isolates of several other host plants.

According to Domsch et al. (1980) and Gams and van Zaayen (1982) the anamorphic genus *Verticillium* is heterogenous. The type species *Verticillium tenerum* (Nees) Link possesses a teleomorph in *Nectria inventa* Pethy. (*Hypocreales*). *Cordyceps* and *Torrubiella* are two clavicipitalien genera with a *Verticillium* anamorph. In a first phylogenetic approach we have been able to grow *V. dahliae* from *Brassica* and *Capsicum* in a unicellular yeast-like growth stage using a Czapek-Dox medium. Purified cell walls of this unicellular growth stage showed the Glucose Mannose Galactose pattern (Glc: 72%, Man: 10%, Gal: 18%) and a ubiquinone Q-10 H2. A similar cell wall composition and ubiquinone spectrum was found in *Pringsheimia chamaecyparidis* (Glc: 76, Man: 11, Gal: 13), a dothidealean fungus (unpublished data). To clarify if the carbohydrate pattern of yeast stages from filamentous Ascomycetes are phylogenetically useful, we have sequenced the whole 18SrDNA of *V. dahliae* and created a phylogenetic tree (Fig. 4). *V dahliae* clusters in a clade with the order *Hypocreales*.

This close relation to the *Hypocreales* was further corroborated with the finding, that a 1044 bp-fragment of the 18SrRNA-gene from *Hypocrea schweinitzii* showed the highest score in a blast-search of the *V. dahliae*-gene through the GenBank. Because of non-completeness of the sequence, *H. schweinitzii* was not included in the construction of the phylogenetic tree.

No affinities of *V. dahliae* to the *Dothideales* were found. Although yeast cell wall sugars and the ubiquinone system are very useful characters in the taxonomy of yeasts, their significance is probably rather low in the taxonomy of filamentous Ascomycetes.

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Table 2
Sources of 18SrRNA-gene sequence data

Species	GenBank accession no.	Reference
Ophiostoma stenoceras	M85054	Berbee and Taylor (1992c)
Ophiostoma ulmi	M83261	Berbee and Taylor (1992c)
Endothia gyrosa	L42443	Chen et al., unpublished
Cryphonectria cubensis	L42439	Chen et al., unpublished
Hypocrea lutea	D14407	Yoshimura and Sugiyama, unpublished
Hypomyces chrysospermus	M89993	Berbee and Taylor (1992b)
Verticillium dahliae	U33637	This paper
Pseudallescheria boydii	M89782	Berbee and Taylor (1992b)
Microascus cirrosus	M89994	Berbee and Taylor (1992b)
Colletotrichum gloeosporiodes	M55640	Illingworth et al. (1991)
Podospora anserina	X54864	Sogin, unpublished
Sordaria fimicola	X69851	Wilmotte et al.(1993)
Neurospora crassa	X04971	Sogin et al. (1986)
Chaetomium elatum	M83257	Berbee and Taylor (1992a)
Septoria nodorum	U04236	Morales et al., unpublished
Alternaria alternata	U05194	Jasalavich et al., unpublished
Aureobasidium pullulans	M55639	Illingworth et al. (1991)
Eupencillium javanicum	U21298	Berbee et al., unpublished
Pencillium notatum	M55628	Sogin et al., unpublished
Saccharomyces cerevisiae	J01353	Mankin et al. (1986)
Kluyveromyces lactis	X51830	Maleszka and Clark-Walker (1990)

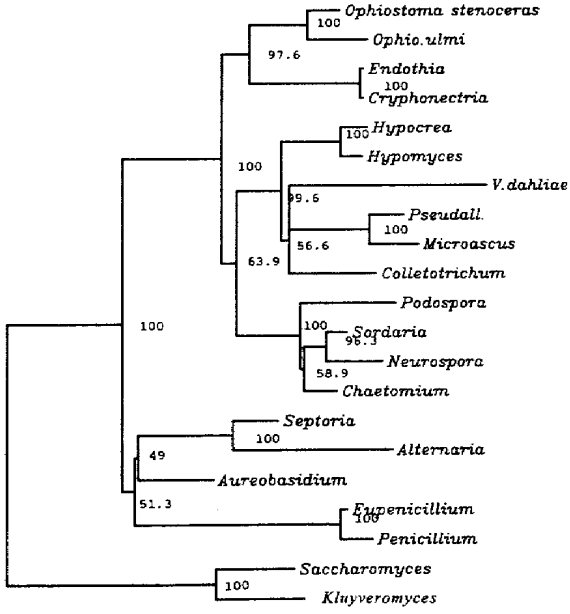

Fig. 4 An estimation of the phylogenetic tree of 21 fungi and yeasts (see Table 2) derived from complete 18SrRDNA sequences. The algorithms used for tree construction are referred to in Materials and Methods. Bootstrapping values were calculated from 1000 cycles

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