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Estimation of genetic variation among *Verticillium* isolates using AFLP analysis

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Abstract

Amplified fragment length polymorphism (AFLP) analysis was used to study genetic variation among 76 isolates of *Verticillium*. A dendrogram based on the AFLP data revealed three main groups. One group consisted of 35 European isolates derived from *Brassica napus* together with five Californian isolates taken from *B. oleracea*. This group displayed a high degree of genetic similarity and included three isolates earlier classified as *Verticillium longisporum*, indicating that all isolates in this group probably should be regarded as members of *V. longisporum*. *V. dahliae* isolates constituted the second group while the third group contained four *V. albo-atrum* isolates. In addition to these three groups, a cluster of six *V. nigrescens* isolates was observed. However, the genetic distances between the isolates of *V. nigrescens* were much higher than those between members in the other groups and the bootstrap value for the *V. nigrescens* cluster was subsequently low. Four isolates classified as *V. tricorpus* were highly diverse and did not cluster together. Analysis of molecular variance revealed that the isolates of *V. longisporum* were separated into four subgroups, based on geographic origin. The study furthermore shows that AFLP is a suitable method for studying population structure in *Verticillium*.

Introduction

Fungi in the genus *Verticillium* cause vascular wilting on a large number of host plants, including agriculturally important crops such as lucerne, hop, tomato, potato, cotton, eggplant, peppers, strawberry, cucurbits, mints, woody ornamentals, fruit tree species (Heale, 1988) and crucifer crops (Svensson and Lerenius, 1987; Subbarao et al., 1995; Xiao and Subbarao, 1998). *Verticillium* fungi are soil-borne pathogens and are spread from field to field mainly by infested soil (Xiao and Subbarao, 1998). Microsclerotia are stimulated by root exudates to germinate and to produce conidia (Schnathorst, 1981). The conidia infect the roots and invade the plant via the vascular system (Deacon, 1997). A sexual stage has not yet been discovered among *Verticillium* species, although it has been suggested that a parasexual cycle may be operating (Hastie and Heale, 1984; Clarkson and Heale, 1985a,b; Heale, 1988; Karapapa et al., 1997). Since the extent to which the parasexual cycle occurs in nature is not known (Heale, 1988), recombination in *Verticillium* might be limited.

Verticillium wilt on oilseed crops has increased in the northern European countries (Svensson and Lerenius, 1987; von Krüger, 1989; Zielinski and Sadowski, 1995). An important basis for limiting the losses caused by Verticillium wilt is to dissect and characterise the pathogen population. Knowledge of the geographical distribution of different fungal genotypes is vital from a plant breeding perspective and also for crop management.

During the last decade, molecular markers, such as restriction fragment length polymorphism (RFLP) (Carder and Barbara, 1991; Okoli et al., 1993; 1994), random amplified polymorphic DNA (RAPD), repetitive DNA sequences (Messner et al., 1996; Griffen et al., 1997; Karapapa et al., 1997;

Dobinson et al., 1998) and differences in organisation of sub-repeats in the rRNA gene clusters (Morton et al., 1995a,b) have been used to differentiate isolates of *Verticillium*. Since molecular markers are considered to be mainly selectively neutral, they are also well suited for population studies. Subsequently, RFLPs (Harrington and Dobinson, 2000; Steventon et al., 2002a) and RAPDs (Barasubiye et al., 1995; Koike et al., 1996; Cherrab et al., 2000) have been used for investigating population structures in *Verticillium.*

In the context of molecular markers, amplified fragment length polymorphisms (AFLPs) have been very useful for studying genetic variation in fungi (Majer et al., 1996). Due to its capacity to generate a large number of dispersed genetic markers, the AFLP technique has been used in population studies of different fungal species, such as *Cercospora zeae-maydis* (Wang et al., 1998; Dunkle and Levy, 2000), *Colletotrichum lindemuthianum* (O'Neill et al., 1997; Gonzalez et al., 1998), *Eutypa* spp. (DeScenzo et al., 1999) and *Leptosphaeria maculans* (Pongam et al., 1999; Purwantara et al., 2000).

The objective of the present study was to optimise the AFLP procedure for *Verticillium* with the purpose of studying genetic variation and population structure among *Verticillium* isolates from different geographical areas and hosts. Specifically, we wanted to focus on the *Verticillium* isolates attacking *Brassica napus* (oilseed rape) in Sweden and Germany. The indicated specialisation of *V. longisporum* on *Brassica* crops (Karapapa et al., 1997; Steventon et al., 2002a,b; Zeise and Tiedemann, 2002) made it interesting to investigate whether this specialisation was present also among a larger collection of *Verticillium* isolates derived from *B. napus*.

Materials and methods

Fungal cultures

In total, 76 *Verticillium* isolates were studied (Table 1). Thirty-six isolates were collected from *B. napus*, while five isolates were derived from *B. oleracea*. Three isolates in this group, 90–10, VD11 and VD18 had earlier been classified as *V. longisporum* (Karapapa and Typas, 2001; Steventon et al., 2002a,b). The remaining 35 isolates were collected from various other host species and included 20 isolates of *V. dahliae* isolates, five isolates of *V. albo-atrum*, six of *V. nigrescens* and four of *V. tricorpus*. The Swedish and German isolates from *B. napus* were collected in the southern and central parts of Sweden, and various parts of Germany (Figure 1). All isolates were cultured from single spores and grown routinely on potato dextrose agar (Difco Laboratories, USA) or malt extract agar (Difco Laboratories) plates in darkness at 18–22 °C. For long-term storage of isolates cultures were grown on media slants, and maintained at 4 °C, or stored in glycerol at −20 or −70°C.

DNA isolation

Liquid fungal cultures were initiated by inoculating 100 ml of potato dextrose broth medium (Difco Laboratories) with a small amount of fungal tissue. After a culture period of 3–4 weeks on a shaker (90 rpm), the tissue was lyophilised. DNA was extracted from 30–60 mg of lyophilised material (Möller et al., 1992) as modified by Steventon et al. (2002a) or by using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA).

AFLP analysis

All primers used are listed in Table 2. The procedure for restriction enzyme digestion, ligation, preselective amplification, selective amplification and sample preparation were as described by Samils et al. (2001) with the following modifications. To the restriction-ligation mix, approximately 50 ng of DNA was added. In the preselective amplification step, 0.6 U of polymerase (DynaZymeII, Finnzymes, Finland) per reaction, was added. In the selective amplification step, the volume of the reaction mix was decreased to 50%. The resulting fragments were separated via electrophoresis (constant voltage at 3000 V for 2.5 h) using an automated DNA sequencer (ABI Prism 377, XL upgrade, Perkin Elmer/Applied Biosystems, USA). After electrophoresis, a digitised gel picture was created by the computer using the GeneScan Analysis software (Perkin Elmer/Applied Biosystems). The DNA fragments in each sample lane were tracked manually and accurately sized by an internal size standard (ROX 500, Perkin Elmer/Applied Biosystems) using the local southern size calling method. The peak amplitude thresholds were set to 50 for the internal standard and 10 for the analysed signal. Remaining threshold values were kept as default.

The reproducibility of the AFLP analysis was tested in two ways. First, the isolates CBS11 and P14 were run twice, together with the remaining samples. DNA from one extraction of isolate CBS11 was used for producing two replicate samples while isolate P14 was

Table 1. Designation, geographical origin, and host species of *Verticillium* isolates used in the study

Isolate	Place of origin	Plant host or substrate
V. albo-atrum		
A1	the Netherlands	*Lycopersicon esculentum*
CBS7 (394.91)	Belgium	*Humulus lupulus*
1974	England, Kent	*Humulus lupulus*
234	France	*Medicago sativa*
1906	England, Bristol	*Medicago sativa*
V. dahliae		
CBS1 (384.49)	the Netherlands	*Solanum tuberosum*
CBS2 (388.49)	the Netherlands	*Anthirrhinum majus*
CBS3 (389.49)	Unknown	*Humulus lupulus*
DC59	England	*Humulus lupulus*
2341	Unknown	*Humulus lupulus*
IMI	Zimbabwe	*Mentha x piperita*
SS-2	USA, Indiana	*Mentha x piperita*
NOVA 1	France	*Beta vulgaris*
NOVA 2	Sweden	*Beta vulgaris*
NOVA 4	France	*Beta vulgaris*
P14	Brazil	*Lycopersicon esculentum*
P14.2 (replicate of P14)	Brazil	*Lycopersicon esculentum*
38	Brazil	*Fragaria x ananassa*
330	England, Norfolk	*Fragaria x ananassa*
332	England, Kent	*Fragaria x ananassa*
1877	Scotland, Edinburgh	*Fragaria x ananassa*
1964	England	*Fragaria x ananassa*
12087	England, Kent	*Fragaria x ananassa*
12086	Scotland	*Fragaria x ananassa*
3440	Brazil	*Solanum melongena*
INRA544A	France	*Brassica napus*
VD17	Germany, Malchow	*Trifolium pratense*
V. longisporum		
Bob70	USA, California	*Brassica oleracea*
Bob73	USA, California	*Brassica oleracea*
Bob127	USA, California	*Brassica oleracea*
Boc74	USA, California	*Brassica oleracea*
90-10	USA, California	*Brassica oleracea*
G1-1	Germany, Großenstein	*Brassica napus*
G4	Germany, Mettman	*Brassica napus*
G5-1	Germany, Albstadt	*Brassica napus*
G9	Germany, Northeim	*Brassica napus*
G10	Germany, Fehmarn	*Brassica napus*
G17	Germany, Grebin	*Brassica napus*
G20-1	Germany, Herrentierbach	*Brassica napus*
G22	Germany, Bremervörde	*Brassica napus*
G25-1	Germany, Simmern	*Brassica napus*
S2	Sweden, Södra Sandby	*Brassica napus*
S3	Sweden, Malmö	*Brassica napus*
S6	Sweden, Bjuv	*Brassica napus*
S9	Sweden, Vallkärra	*Brassica napus*
S14	Sweden, Hammenhög	*Brassica napus*
S22	Sweden, Fågelstad	*Brassica napus*
S28	Sweden, Ljungsbro	*Brassica napus*
S29	Sweden, Svanshals	*Brassica napus*
S30	Sweden, Vadstena	*Brassica napus*
S31	Sweden, Vadstena	*Brassica napus*
S33	Sweden, Svanshals	*Brassica napus*

Table 1. (*Continued*)

Isolate	Place of origin	Plant host or substrate
S40	Sweden, Ljungsbro	*Brassica napus*
S42	Sweden, Vikingstad	*Brassica napus*
S44	Sweden, Linköping	*Brassica napus*
S46	Sweden, Fågelstad	*Brassica napus*
S47	Sweden, Södra Sandby	*Brassica napus*
S48	Sweden, Södra Sandby	*Brassica napus*
S49	Sweden, Södra Sandby	*Brassica napus*
S51	Sweden, Malmö	*Brassica napus*
VD11*	Sweden, Skara	*Brassica napus*
VD18*	Germany, Rostock,	*Brassica napus*
36-1*	Sweden, Vellinge	*Brassica napus*
40-2*	Sweden, Furulund	*Brassica napus*
42-1*	Sweden, Hörby	*Brassica napus*
43-3*	Sweden, Eslöv	*Brassica napus*
44-4*	Sweden, Kristianstad	*Brassica napus*
V. nigrescens		
CBS15 (747.83)	the Netherlands	*Apium graveolens*
CBS16 (387.35)	Italy	*Amaranthus tricolor*
CBS17 (345.39)	Sweden	Wood pulp
CBS18 (455.51)	UK	*Solanum tuberosum*
CBS19 (470.64)	France	*Medicago*
CBS20 (100826)	Israel	*Solanum tuberosum*
V. tricorpus		
CBS11 (447.54)	England	*Lycopersicon esculentum*
CBS11.2 (replicate of CBS11)	England	*Lycopersicon esculentum*
CBS12 (127.79B)	New Zealand	*Nicotiana tabacum*
CBS13 (384.84)	the Netherlands	*Solanum tuberosum*
CBS14 (101218)	Israel	Soil from potato field

Isolates marked with an asterisk were obtained from Ingrid Hapstadius, Svalöf-Weibull AB, Svalöv, Sweden. The remaining Swedish isolates were kindly provided by Gunilla Berg, Alf Djurberg and Anna-Karin Kuusk, Plant Protection Service, Swedish Board of Agriculture. The CBS isolates were obtained from Centraalbureau voor Schimmelcultures, the Netherlands. Numbers within brackets denote the CBS accession number. The INRA isolate was a gift from H. Brun (INRA, Le Rheu, France). The three isolates taken from sugar beet (NOVA1, NOVA2 and NOVA4) were a gift from M. Nihlgaard, Novartis, Landskrona, Sweden. The isolates Bob70, Bob73, Boc74 and Bob127 were kindly provided by Dr K.V. Subbarao, Department of Plant Pathology, University of California, Davis, California, USA. The remaining isolates were kindly provided by Dr D. Barbara, Department of Plant Pathology and Microbiology, Horticulture Research International, Wellesbourne Warwickshire, United Kingdom.

recultured and DNA was extracted two times. Second, AFLP runs of a subset of the isolates (32 isolates) were repeated twice, in total run three times (DNA was only extracted once from each of these isolates). From these 32 isolates, 5 isolates (three replicated samples from each, in total 15 samples) were randomly chosen and analysed. The isolates selected were CBS3, CBS14, G20-1, VD11 and 332.

Scoring of AFLP fragments and data analysis

After the analysis in GeneScan, the sample files were imported into Genotyper™ version 2.0 (Perkin Elmer/Applied Biosystems) where the selected potential markers (fragments or categories), were subjected to analysis of overlapping categories to eliminate artefacts. This type of analysis can be explained as follows. The category tolerance is normally set to ±0.50 base pairs (bp) which means that two fragments that differ by 1 bp can be distinguished. However, when a large number of isolates of diverse origin are analysed, an overall high density of peaks can be obtained, as in the present study, and several overlapping categories are formed due to slight shifts of peak positions in the different samples. As a consequence, the Genotyper program cannot place some fragments

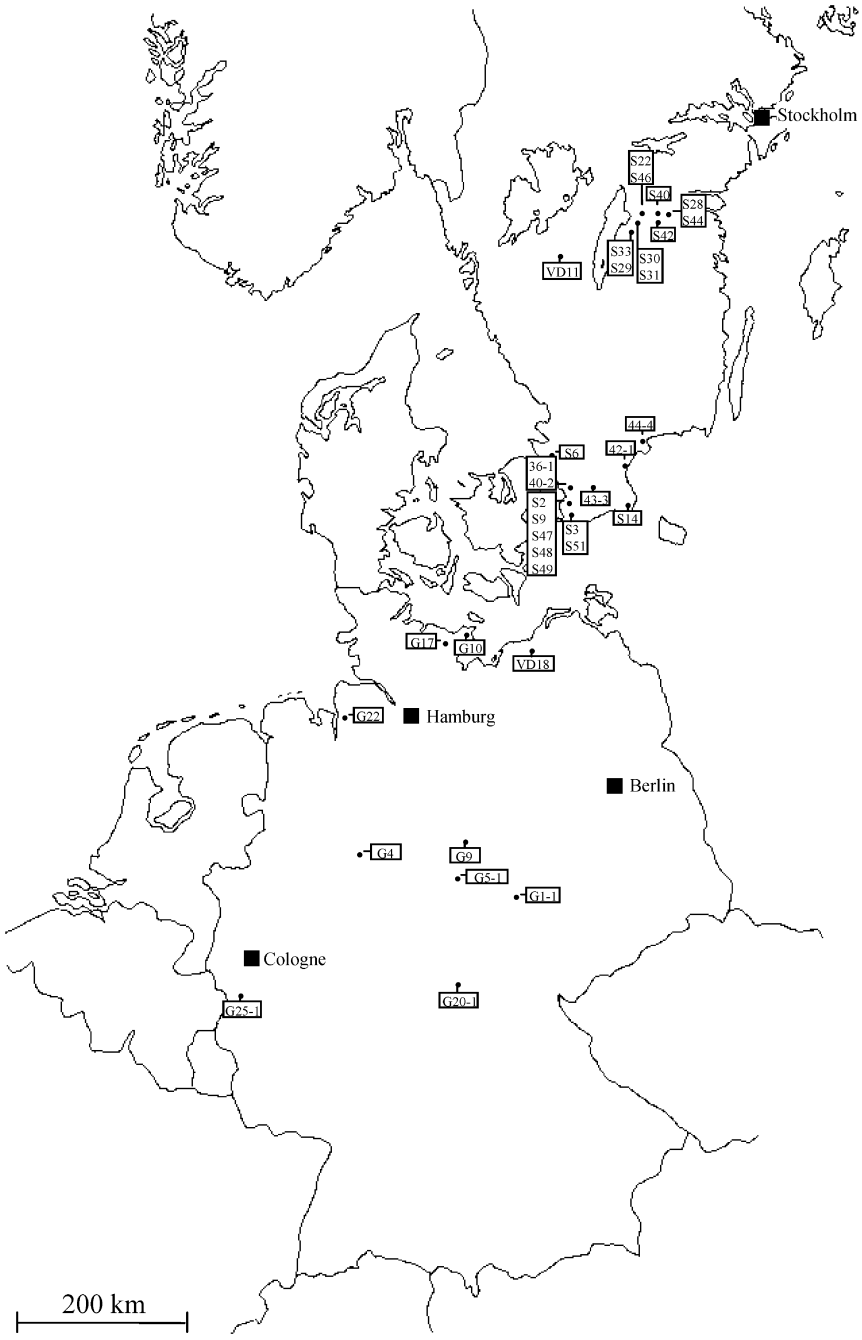

Figure 1. Map showing the collection places for the Swedish and German isolates of *Verticillium* species from *B. napus* plants.

in a single category and instead assigns these fragments to two categories, thus creating artefacts. By identifying these overlapping categories and changing the category tolerance, the artefacts can be eliminated. This cannot be done by looking at the whole dataset but replicated samples can be used to check the accuracy of the procedure. After the analysis of overlapping categories, parameters were set to ensure that the

Table 2. Description of the primers used for AFLP analysis of *Verticillium* isolates

Primer	Function	Sequence
E-ad1	*Eco*RI-adapter	5′ CTCGTAGACTGCGTACC 3′
E-ad2	*Eco*RI-adapter	5′ AATTGGTACGCAGTC 3′
M-ad1	*Mse*I-adapter	5′ GACGATGAGTCCTGAG 3′
M-ad2	*Mse*I-adapter	5′ TACTCAGGACTCAT 3′
E-00	Preamplification	5′ AGACTGCGTACCAATTC 3′
M-C00	Pre-selective amplification	5′ GATGAGTCCTGAGTAAC 3′
E-GA	Selective amplification	5′ AGACTGCGTACCAATTCGA 3′ NED*
E-GC	Selective amplification	5′ AGACTGCGTACCAATTCGC 3′ HEX*

*Fluorescent label at the 5′-end.

fragments in the selected categories reached a scaled peak height of at least 200 or they were not included. Fragments between 100 and 450 bp were scored and a binary data matrix (Samils et al., 2001), was produced.

The resulting data set was analysed with distance-based methods. A phenogram was constructed using the TreeCon program (Van de Peer and De Wachter, 1994) based on the similarity coefficient of Nei and Li (1979) and the neighbour-joining method (Saitou and Nei, 1987). Bootstrap analysis was based on 100 replicates. The pairwise distances in the matrix were calculated using the formula $D_{xy} = 1 - S_{xy}$ where S_{xy} is the similarity coefficient of Nei and Li (1979). Similarity $S_{xy} = 2n_{xy}/(n_x + n_y)$, where n_{xy} is the number of fragments in common between isolates x and y, and n_x and n_y are the total numbers of fragments in isolates x and y, respectively.

An AMOVA analysis (Excoffier et al., 1992) was conducted using the Arlequin software (Schneider et al., 2000) to test the existance of different populations within species. For testing the influence of geographic origin, 40 isolates from *Brassica* host species (INRA544A was excluded) were divided into three groups, the Swedish group (25 isolates), the German group (10 isolates) and the Californian group (5 isolates). In a second AMOVA test, the Swedish group was divided into two subgroups. Eleven isolates were included in the Sweden-Central group (S22, S28, S29, S30, S31, S33, S40, S42, S44, S46 and VD11) while 14 isolates were included in the Sweden-Southern group (36-1, 40-2, 42-1, 43-3, 44-4, S2, S3, S6, S9, S14, S47, S48, S49 and S51). The significance level for all AMOVA tests was set to 0.05.

PCR amplification using species-specific primers

DNA from the isolates 234 (classified as *V. albo-atrum*), CBS12 and CBS13 (classified as *V. tricorpus*) were utilised for PCR amplification with species-specific primers. As *V. albo-atrum*-specific primers we used the ones described by Morton et al. (1995b), while the *V. dahliae* and *V. tricorpus*-specific primers used were the same as described by Carder et al. (1994) and Moukhamedov et al. (1994), respectively.

Results

AFLP analysis, scoring of fragments and data analysis

In an initial test of the applicability of the AFLP technique on *Verticillium*, 69 primer combinations were evaluated and 6 were found to be suitable, based on the number of fragments generated (data not shown). Two of these six primer combinations were used on all isolates in this study (Table 2). The total number of markers was 349 for the two primer combinations. Thus, each sample was assessed for the presence or absence of a fragment at 349 putative loci. All markers were polymorphic.

Within each of the two replicated samples, CBS11 and P14, a very similar fragment pattern was observed, but due to overlapping categories, several differences were noted. Analysis of overlapping categories by changing the category tolerance (see Materials and methods), together with a high threshold level, eliminated the majority of the artefacts. In the interval 100–450 bp, the total number of differences for the replicated isolates (CBS11 and P14), before analysis, was 52. After adjustment, the number of differences was lowered to 13, of which 12 were due to peaks of too low height and were thus present in both samples of a replicate but only scored for one sample. A lower threshold level was therefore tested for one of the primer combinations. This did not

change the number of artefactual differences. The similarity coefficients for the CBS11 replicates was 99.6% while for the P14 replicates the similarity was 99.7%.

The banding pattern for the five samples that were run 3 times (CBS3, CBS14, G20-1, VD11 and 332) was compared for each isolate. In total, 154 peaks were studied and 2 differences were noted.

Dendrogram construction and AMOVA analysis

A dendrogram for the 76 isolates was constructed based on 349 putative AFLP loci (Figure 2). Three main groups were distinguished. One group (A) consisted of isolates from *B. napus* and *B. oleracea* (except INRA544A). A second group (B) contained all the *V. dahliae* isolates. A third group (C) was made up of four *V. albo-atrum* isolates. A cluster consisting of isolates of *V. nigrescens* (D) was also observed but the genetic diversity within this group was high and the cluster was not supported by a high bootstrap value. The four *V. tricorpus* isolates were genetically diverse, with one isolate (CBS12) clustering with group B isolates (*V. dahliae*), while two isolates, CBS11 and CBS14, clustered together. The fourth *V. tricorpus* isolate (CBS13) clustered with isolate 234 (*V. albo-atrum*).

The AMOVA analysis revealed significant differences between the Swedish, German and Californian isolates in group A (Table 3). This was also the case for the Central and Southern groups of the Swedish isolates (Table 3).

PCR amplification using species-specific primers

The isolates 234 (classified as *V. albo-atrum*), CBS12 and CBS13 (classified as *V. tricorpus*) were utilised for PCR amplification with species-specific primers. DNA from isolates 234 and CBS13 produced a clearly visible band of about 800 bp when using the *V. albo-atrum*-specific primers but only faint bands were produced with the two other species-specific primer pairs. According to Carder et al. (1994), the *V. dahliae*-specific primers should produce a DNA fragment of 580 bp. DNA from isolate CBS12 was readily amplified with the *V. dahliae*-specific primers, producing a fragment of the predicted size, but only faint bands were seen when using the other two primer pairs (data not shown).

Discussion

The present study focused on a large collection of Swedish and German *Verticillium* isolates with the aim of describing the population structure and genetic variability within this group of agriculturally important plant pathogens. In the dendrogram, the 25 Swedish and ten German isolates from *B. napus* clustered together with the five Californian isolates from *B. oleracea* and formed group A. AMOVA analysis revealed that geographic structuring was present between the isolates from the three countries. The Swedish isolates were divided into two subgroups that represent the two main areas where oilseed rape is cultivated in Sweden. The distance between the Southern location (Skåne) and the Central location (Östergötland) is approximately 200 km and separated by large areas of forest. The distance between these locations is probably large enough to result in a geographic structure, providing there is limited exchange of infected material. The water barrier between Sweden and Germany may in a similar way account for the difference between the Swedish and the German isolates.

The isolates 90-10, VD11 and VD18 in group A have, in previous studies, been classified as *V. longisporum* (Karapapa and Typas, 2001; Steventon et al., 2002a). Accordingly, we suggest that all 40 isolates clustering in the group A should be regarded as *V. longisporum.* All *V. dahliae* isolates including INRA544A, which was isolated from *B. napus* were found in group B. This isolate has, together with other French isolates, been considered as *V. dahliae* (Steventon et al., 2002a). The four *V. tricorpus* isolates were genetically diverse and did not cluster together. Isolate CBS12, which clustered together with the *V. dahliae* isolates, was morphologically similar to *V. dahliae* since it produced microsclerotia. This was not observed for the other *V. tricorpus* isolates in the study. Since CBS12 produced a strong band of the expected size with the *V. dahliae*-specific primers, we speculate that CBS12 was misclassified. Another isolate that did not cluster together with its respective species was isolate 234 (*V. albo-atrum*). This isolate has in earlier AFLP experiments been shown to be only distantly related to the other *V. albo-atrum* isolates (Fahleson et al., 2000) which was also found in this extended study. However, PCR analysis confirmed the *V. albo-atrum* affiliation. CBS13, denoted as *V. tricorpus*, on the other hand, both clustered together and gave the same result as isolate 234

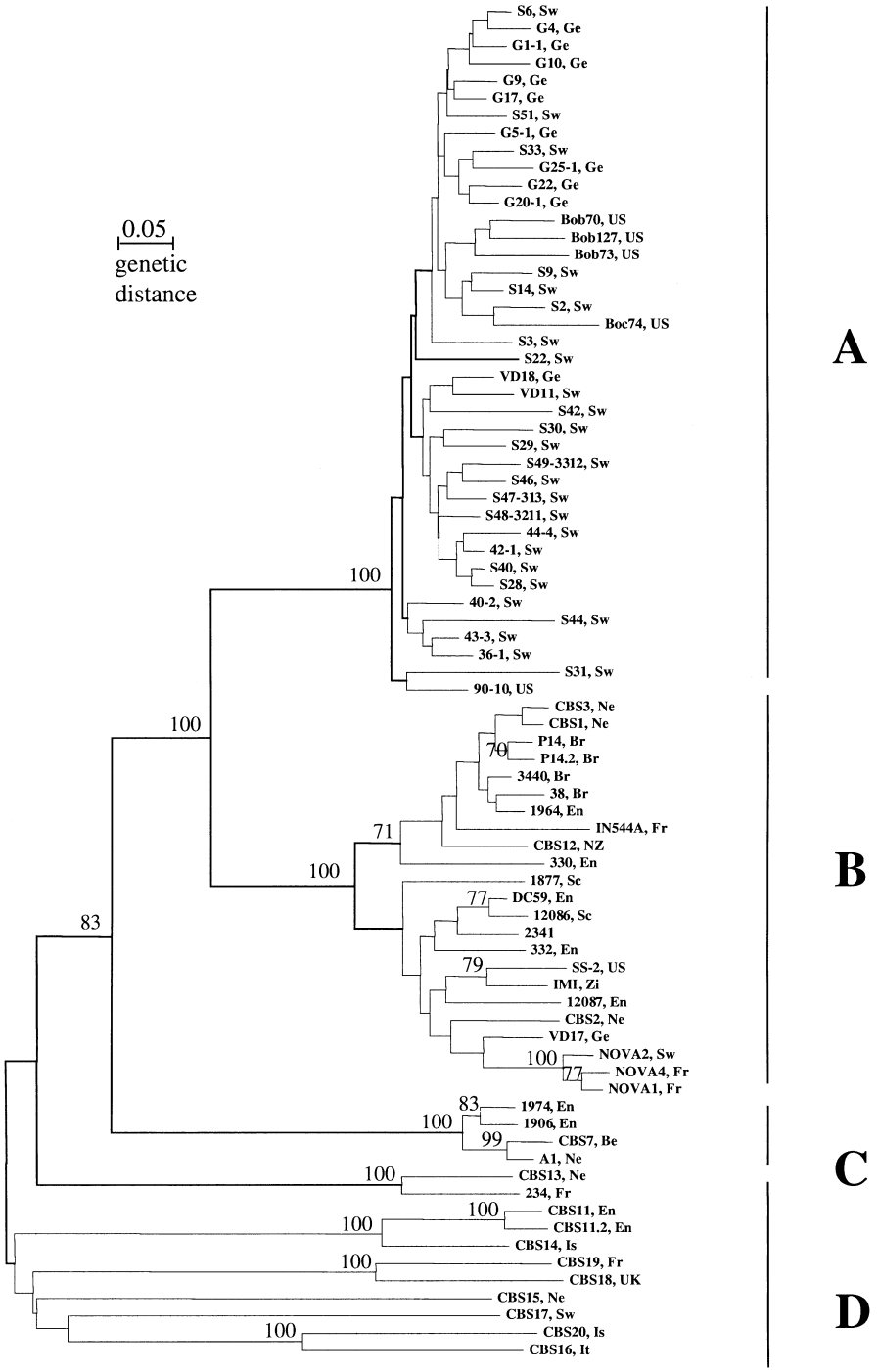

Figure 2. Dendrogram based on 76 *Verticillium* isolates (replicates of CBS11 and P14, CBS11.2 and P14.2, are included) and 349 AFLP loci. The numbers near the nodes denote bootstrap values (100 replicates). Bootstrap values equal to or above 70 are shown. The tree was constructed using the neighbour-joining method (Saitou and Nei, 1987). Clusters are denoted with letters. A = *V. longisporum*, B = *V. dahliae*, C = *V. albo-atrum*, D = *V. nigrescens*. Origins of isolates are denoted with letters after each isolate. Be = Belgium, Br = Brazil, En = England, Fr = France, Ge = Germany, Is = Israel, It = Italy, Ne = the Netherlands, NZ = New Zealand, Sc = Scotland, Sw = Sweden, US = United States, UK = United Kingdom, Zi = Zimbabwe.

Table 3. Analysis of molecular variance (AMOVA) of 349 AFLP loci among 40 isolates from *Verticillium longisporum*

Source of variation	d.f.[a]	Variance components	Percentage of total variance	Φ-statistics (Φ_{ST})	Probability[b] (P)
Among locations in group A (Sweden, Germany, California)	2	1.55	14.4	0.144	<0.001
Within locations in group A	37	9.27	85.6		
Among Swedish locations in group A	1	0.49	5.1	0.051	0.03
Within Swedish locations in group A	23	9.35	94.9		

[a]d.f. = degrees of freedom.
[b]Probability of a larger value obtained by chance, determined by 1000 permutations of the data set.

when testing different species-specific primers. These observations suggest that CBS13 has also been misclassified and that both 234 and CBS13 belong to the *V. albo-atrum* species but are distantly related to the other *V. albo-atrum* isolates included in this study.

AFLP data are normally generated using either radioactively (O'Neill et al., 1997; Gonzalez et al., 1998; Wang et al., 1998; Pongam et al., 1999; Dunkle and Levy, 2000; Purwantara et al., 2000) or fluorescently labelled DNA primers (DeScenzo et al., 1999; Schwarz et al., 2000, Samils et al., 2001). In the study of Schwarz et al. (2000) the use of fluorescent molecules and subsequent computerised analysis was shown to produce a higher resolution than analysing an autoradiogram obtained using radioactively labelled oligonucleotides. Also, since the computer directly processes the data obtained by fluorescent AFLP, any mistakes made in optical scoring and manual input in table sheets can be avoided. Despite this, within each of the two replicates included in the present study, several differences were found. A slight shift of the peak positions was sometimes observed between the replicated samples, which had to be adjusted. This adjustment together with a suitable threshold level gave a more accurate analysis since the number of artefacts between each of the replicates was drastically reduced and subsequently clustered the replicates close to each other. However, artefacts were not eliminated completely, suggesting that a more detailed analysis of individual peaks is required to be able to identify clones. A computerised analysis can rapidly identify putative clones, but the detailed analysis must be done manually as with procedures using radioactively labelled oligonucleotides.

In conclusion, the AFLP procedure adopted in this study was highly reproducible since only 2 differences out of 154 peaks (1.3%) were observed when comparing the five samples that were replicated three times. By using AFLP, we have shown that there is a limited genetic variation among Swedish and German isolates of *V. longisporum* attacking *B. napus*. Still, for these isolates, a geographic structure exists between countries from which the isolates originate as well as between two separate regions in Sweden. These findings might have implications for future plant breeding activities.

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References

Barasubiye T, Parent J-G, Hamelin RC, Laberge S, Richard C and Dostaler D (1995) Discrimination between alfalfa and potato isolates of *Verticillium albo-atrum* using RAPD markers. Mycological Research 12: 1507–1512

Carder JH and Barbara DJ (1991) Molecular variation and restriction fragment length polymorphisms (RFLPs) within and between six species of *Verticillium*. Mycological Research 95: 935–942

Carder JH, Morton A, Tabrett AM and Barbara DJ (1994) Detection and differentiation by PCR of subspecific groups within two *Verticillium* species causing vascular wilts in herbaceous hosts. In: Modern Assays for Plant Pathogenic Fungi: Identification, Detection and Quantification (pp 91–97) CAB International, Oxford, UK

Cherrab M, Serrhini MN and Charest PM (2000) Characterization of Moroccan isolates of *Verticillium dahliae* Kleb using RAPD markers. Journal of Phytopathology 148: 243–249

Clarkson JM and Heale JB (1985a) Heterokaryon compatibility and genetic recombination within a host plant between hop wilt isolates of *Verticillium albo-atrum*. Plant Pathology 34: 129–138

Clarkson JM and Heale JB (1985b) A preliminary investigation of the genetics of pathogenicity in hop wilt isolates of *Verticillium albo-atrum*. Transactions of the British Mycological Society 85: 345–350

Deacon JW (1997) Fungi as plant pathogens. In: Modern Mycology (pp 239–247) Blackwell Science, Oxford, UK

DeScenzo RA, Engel SR, Gomez G, Jackson EJ, Munkvold GP, Weller J and Irelan NA (1999) Genetic analysis of *Eutypa* strains from California supports the presence of two pathogenic species. Phytopathology 89: 884–893

Dobinson KF, Patterson NIIA, White GJ and Grant S (1998) DNA fingerprinting and vegetative compatibility analysis indicate multiple origins for *Verticillium dahliae* race 2 tomato isolates from Ontario, Canada. Mycological Research 102: 1089–1095

Dunkle LD and Levy M (2000) Genetic relatedness of African and United States populations of *Cercospora zeae-maydis*. Phytopathology 90: 486–490

Excoffier L, Smouse PE and Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. Genetics 131: 479–491

Fahleson J, Butterworth LA and Dixelius C (2000) AFLP and RFLP analyses as tools to characterize different *Verticillium* isolates. 12th Crucifer Genetics Workshop, September 5–9, 2000, HRI, Wellesbourne, UK

Gonzalez M, Rodriguez P, Zavala ME, Jacobo JL, Hernandez F, Acosta J, Matinez O and Simpson J (1998) Characterization of Mexican isolates of *Colletotrichum lindemuthianum* by using differential cultivars and molecular markers. Phytopathology 88: 292–299

Griffen AM, Bainbridge BW and Heale JB (1997) Ribosomal, mitochondrial and amplified DNA polymorphisms in *Verticillium albo-atrum* pathogenic to hops, lucerne and other plants. Mycological Research 101: 1085–1091

Harrington MA and Dobinson KF (2000) Influences of cropping practices on *Verticillium dahliae* populations in commercial processing tomato fields in Ontario. Phytopathology 90: 1011–1017

Hastie AC and Heale JB (1984) Genetics of *Verticillium*. Phytopathology Mediterrenean 23: 130–162

Heale JB (1988) *Verticillium* spp.: The cause of vascular wilts in many species. Advances in plant pathology. In: Sidhu GS (ed) Genetics of Plant Pathogenic Fungi, Vol 6 (pp 291–312) Academic Press, London, UK

Karapapa VK and Typas MA (2001) Molecular characterization of the host-adapted pathogen *Verticillium longisporum* on the basis of a group-I intron found in the nuclear SSU-rRNA gene. Current Microbiology 42: 217–224

Karapapa VK, Bainbridge BW and Heale JB (1997) Morphological and molecular characterization of *Verticillium longisporum* comb. nov., pathogenic to oilseed rape. Mycological Research 101: 1281–1294

Koike M, Fujita M, Nagao N and Ohshima S (1996) Random amplified polymorphic DNA analysis of Japanese isolates of *Verticillium dahliae* and *V. albo-atrum*. Plant Disease 80: 1224–1227

Krüger von W (1989) Untersuchungen zur Verbereitung von *Verticillium dahliae* Kleb. und anderen Krankheits und Schaderegerm bei Raps in der Bundesrepublik Deutschland. Nachrichtenblatt des Deutschen Pflanzenschutzdienstes 41: 49–56

Majer D, Mithen R, Lewis BG, Vos P and Oliver RP (1996) The use of AFLP fingerprinting for the detection of genetic variation in fungi. Mycological Research 9: 1107–1111

Messner R, Schweigkofler W, Schweigkofler M, Berg G 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

Moukmahedov R, Hu X, Nazar RN and Robb J (1994) Use of polymerase chain reaction-amplified ribosomal intergenic sequences for the diagnosis of *Verticillium tricorpus*. Phytopathology 84: 256–259

Möller EM, Bahnweg G, Sandermann H and Geiger HH (1992) A simple and efficient protocol for the isolation of high molecular weight DNA from filamentous fungi, fruit bodies and infected plant tissues. Nucleic Acids Research 20: 6115–6116

Morton A, Carder JH and Barbara DJ (1995a) Sequences of the internal transcribed spacers of the ribosomal RNA genes and relationships between isolates of *Verticillium alboatrum* and *V. dahliae*. Plant Pathology 44: 183–190

Morton A, Tabrett AM, Carder JH and Barbara DJ (1995b) Subrepeat sequences in the ribosomal RNA intergenic regions of *Verticillium alboatrum* and *V. dahliae*. Mycological Research 99: 257–266

Nei M and Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Sciences of the USA 76: 5269–5273

O'Neill NR, van Berkum P, Lin J-J, Kuo J, Ude GN, Kenworthy W and Saunders JA (1997) Application of amplified restriction fragment length polymorphism for genetic characterization of *Colletotrichum* pathogens of alfalfa. Phytopathology 87: 745–750

Okoli CAN, Carder JH and Barbara DJ (1993) Molecular variation and sub-specific groupings within *Verticillium dahliae*. Mycological Research 97: 233–239

Okoli CAN, Carder JF and Barbara DJ (1994) Restriction fragment length polymorphisms (RFLPs) and the relationships of some host-adapted isolates of *Verticillium dahliae*. Plant Pathology 43: 33–40

Pongam P, Osborn TC and Williams PH (1999) Assessment of genetic variation among *Leptosphaeria maculans* isolates using pathogenicity data and AFLP analysis. Plant Disease 83: 149–154

Purwantara A, Barrins JM, Cozijnsen AJ, Ades PK and Howlett BJ (2000) Genetic diversity of isolates of *Leptosphaeria maculans* species complex from Australia, Europe and North America using amplified fragment length polymorphism analysis. Mycological Research 104: 772–781

Saitou N and Nei M (1987) The neighbour-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406–425

Samils B, Lagercrantz U, Lascoux M and Gullberg U (2001) Genetic structure of *Melampsora epitea* populations in Swedish *Salix viminalis* plantations. European Journal of Plant Pathology 107: 399–409

Schnathorst WC (1981) Life cycle and epidemiology of *Verticillium*. In: Mace ME, Bell AA and Bechman CH (eds) Fungal Wilt Diseases of Plants (pp 81–111) Academic Press, New York, USA

Schneider S, Roessli D and Excoffier L (2000) Arlequin ver. 2.000: A software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland

Schwarz G, Herz M, Huang XQ, Michalek W, Jahoor A, Wenzel G and Mohler V (2000) Application of fluorescence-based semi-automated AFLP analysis in barley and wheat. Theoretical and Applied Genetics 100: 545–551

Steventon LA, Fahleson J, Hu Q and Dixelius C (2002a) Identification of the causal agent of *Verticillium* wilt of winter oilseed rape in Sweden as *V. longisporum*. Mycological Research 106: 570–578

Steventon LA, Happstadius I, Okori P and Dixelius C (2002b) Development of a rapid technique for the evaluation of the response of *Brassica napus* to Verticillium wilt. Plant Disease 86: 854–858

Subbarao KV, Chassaot A, Gordon TR, Hubbard JC, Bonello P, Mullin R, Okamoto D, Davis RM and Koike ST (1995) Genetic relationships and cross pathogenicities of *Verticillium dahliae* isolates from cauliflower and other crops. Phytopathology 85: 1105–1112

Svensson C and Lerenius C (1987) An investigation on the effect of *Verticillium* wilt (*Verticillium dahliae* Kleb.) on oilseed rape. Bulletin-SROP 10: 30–34

Van de Peer Y and De Wachter R (1994) TREECON for Windows: A software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Computer Applications in the Biosciences 10: 569–570

Wang J, Levy M and Dunkle LD (1998) Sibling species of *Cercospora* associated with grey leaf spot in maize. Phytopathology 88: 1269–1275

Xiao CL and Subbarao KV (1998) Relationships between *Verticillium dahliae* inoculum density and wilt incidence, severity, and growth of cauliflower. Phytopathology 88: 1108–1115

Zielinski D and Sadowski C (1995) A preliminary study on *Verticillium dahliae* Kleb. in winter oilseed rape in Poland. 9th International Rapeseed Congress, 4–7 July Cambridge F28, pp 649–651

Zeise K and von Tiedemann A (2002) Host specialization among vegetative compatibility groups of *Verticillium dahliae* in relation to *Verticillium longisporum*. Journal of Phytopathology 150: 112–119