Isolates of *Verticillium dahliae* Pathogenic to Crucifers Are of at Least Three Distinct Molecular Types

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ABSTRACT

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Diverse isolates of the soilborne wilt fungi *Verticillium dahliae* and *V. albo-atrum* were studied to understand the nature and origins of those infecting cruciferous hosts. All isolates from cruciferous crops produced microsclerotia, and the majority produced long conidia with a high nuclear DNA content; these isolates were divided into two groups by amplified fragment length polymorphism (AFLP) analysis. One group could be subdivided by other criteria such as rRNA sequences and mitochondrial DNA restriction fragment length polymorphism (RFLP) analysis. Two crucifer isolates were short spored and had a low nuclear DNA

content. The results are consistent with the crucifer isolates being interspecific hybrids. The long-spored isolates are best regarded as amphihaploids (or allodiploids) with the AFLP groups probably each representing separate interspecific hybridization events. The short-spored crucifer isolates appear to be derived from interspecific hybrids and are here called 'secondary haploids'. Molecular evidence suggests that one parent in the crosses was similar to *V. dahliae*. The other parent of the amphihaploids seems to have been more similar to *V. albo-atrum* than to *V. dahliae*, but was distinct from all isolates of either species so far studied. The implications for the taxonomy of crucifer isolates are discussed and the use of the name *V. longisporum*, proposed elsewhere for just some of these isolates, is discouraged.

Additional keywords: interspecific hybrids, vascular wilts.

The fungal genus *Verticillium* contains two well-studied species of widespread and common soilborne plant pathogens, *V. dahliae* and *V. albo-atrum* (13). The primary characteristic used to differentiate these two species is resting structure; *V. dahliae* produces microsclerotia, and *V. albo-atrum* produces dark resting mycelium. Two weak pathogens or saprophytes are closely related to these major pathogenic species; *V. nubilum* produces only chlamydospores while *V. tricorpus* produces all three resting structures (17). Some *Verticillium* isolates producing dark resting mycelium and particularly associated with potato have been recently shown to be very distinct, mainly on the basis of molecular data and are referred to as *V. albo-atrum* Group II (23,24,29). Although not formally described as such, these isolates are best regarded as forming a separate species. Other saprophytes (e.g., *V. nigrescens*) or less widespread pathogens (e.g., *V. theobromae*) are relatively distantly related by molecular measures (37) and are not considered here.

The majority of isolates of the two main plant pathogenic species of *Verticillium* are not host adapted but are able to infect a wide range of hosts. Pathotypes do occur with perhaps the clearest example being *V. albo-atrum* isolates from lucerne (alfalfa), which are molecularly and pathologically distinct from isolates from all other hosts (5,12). Infection of cruciferous hosts once seemed unusual with only isolated occurrences reported from brussels sprouts in 1957 (16) and horseradish in 1961 (32,33). Recently, infections of crucifers by microsclerotial isolates have been found much more frequently, e.g., in oilseed rape in Europe (14), cauliflower in California (20), horseradish in Illinois (8), and various crucifers in Japan (15). Isolates of *V. dahliae* and *V. albo-atrum*

from most hosts are short spored and have a low nuclear DNA content, but the majority of isolates from cruciferous hosts are long spored and have a high nuclear DNA content (14,32,33).

All crucifer isolates produce microsclerotia and have usually been classified as *V. dahliae*, often with the subspecific addition of 'var. *longisporum*', the name C. Stark gave to the first isolate from horseradish (32,33). On the basis of molecular and other studies, Karapapa et al. (18) suggested that long-spored isolates should be classified as a separate species, *V. longisporum*. This new taxon was based mainly on European oilseed rape isolates and was not entirely satisfactory in that it excluded some long-spored isolates, including the original *V. dahliae* var. *longisporum*, which were described with the undefined term 'recombinant'. The new taxon also left unclear the status of short-spored crucifer isolates (18). It was further suggested by Karapapa et al. (18) that *V. longisporum* was an interspecific hybrid between a lucerne isolate of *V. albo-atrum* and *V. dahliae*, and that genetically the new species was 'near-diploid'.

The work reported here had a number of objectives linked to the overall aim of understanding the origins of *V. dahliae* isolates from crucifers, their genetic status, and the most appropriate nomenclature. The first objective was to establish whether isolates from crucifers form a homogenous group by simultaneously examining, using molecular techniques, a broad range of isolates. Generally, previous studies have focused on isolates from specific cruciferous hosts and geographic regions (e.g., oilseed rape from Europe [18]), which may have effectively led to multiple sampling and comparison of single clones (or closely related groups of isolates). The second objective was to compare spore size and DNA content of isolates from crucifers with other isolates as guides to the genetic status and stability of the crucifer-infecting isolates. Since it has been suggested that long-spored isolates are interspecific hybrids (18), the third objective was to ask whether the molecular properties of long- and short-spored isolates support

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such an origin and allow identification of the original 'parental' species/pathotypes. For artificial hybrids, the rate of haploidization was lower for the offspring of interspecific crosses than for those from intraspecific crosses (11). This greater stability and the apparent longevity of natural long-spored isolates might be due to problems in reductive nuclear division arising ultimately from differing chromosome numbers in the parental isolates. The fourth objective was to study chromosome numbers in the two main candidate species for being the parents of naturally long-spored isolates through interspecific hybridization. Some terms with no clear meaning have been used to discuss long-spored isolates (e.g., near-diploid and recombinant isolates [18]), and a new species proposed which includes only some of the long-spored isolates (18). The final objective of this paper is to suggest, in the light of the results presented, more appropriate terminology and approaches to taxonomy.

MATERIALS AND METHODS

Fungal isolates and culture. The identity and sources of isolates representing the four species (*V. albo-atrum*, *V. dahliae*, *V. tricornis*, and *V. albo-atrum* GpII) used in this study are given in Table 1. Imported isolates were maintained under the conditions of UK DEFRA license PHL 166/4025. Isolates were maintained on prune lactose yeast agar (PLYA) (34) plates in the dark at 4°C with subculturing every 8 months or stored frozen in liquid nitrogen at -80°C. For freezing, conidial suspensions were prepared by washing the surface of a plate with 1 ml of sterile distilled water (SDW). Suspensions were mixed 1:1 with 200 ml liter⁻¹ glycerol in water and 150 µl stored in cryovials containing approximately 15 ceramic craft beads of 3 mm diameter (Creative Beadcraft Ltd., Amersham, UK). Mono-conidial isolates were prepared from cultures grown on plates in the dark at 25°C.

Conidial dimensions and nuclear DNA content. Fresh conidial suspensions in water were prepared from 14-day-old cultures grown on PLYA plates, and the width and length of 30 conidia were measured for each isolate using a micrometer eyepiece (×16) and a 20× phase contrast objective.

The nuclear DNA content of conidia was measured in 29 isolates from cruciferous hosts, 16 of *V. dahliae* from noncruciferous hosts, and six of *V. albo-atrum* by flow cytometry (courtesy C. Dixelius, Uppsala University, Sweden) as described by De Lucas et al. (7), except that the concentration of propidium iodide was increased to 50 µg ml⁻¹. Examination by fluorescence microscopy of propidium iodide stained conidia showed no staining of cell components other than the nucleus. Quantitation of DNA was by comparison with *Aspergillus nidulans* (Glasgow wild type from the Fungal Genetics Stock Center) with a genome size reported to be 31 Mbp, equivalent to 33 fg per haploid genome (4). Approximate genome sizes were calculated from the estimates of nuclear DNA contents based on the assumption that short-spored isolates are haploid; the same calculation was used for long-spored isolates on the basis that they are interspecific hybrids maintaining both parental haploid genomes (described below) that together effectively form a single haploid genome.

DNA extraction. Small-scale extractions were by a squash blot method (22) from mycelium taken from 2-week-old plate cultures. Initially Hybond-N (Amersham Pharmacia Biotech, Buckinghamshire, UK) membrane was used but after manufacturing changes rendered this unsuitable, Nytran N (Schleicher & Schuell, Dassel, Germany) was substituted. DNA was eluted into 60 µl of reverse osmosis (RO) water and stored at -20°C. For larger scale extractions, the DNeasy plant mini kit (Qiagen GmbH) was used. One million conidia were used to inoculate 125 ml of sterile modified Czapek-Dox liquid medium and incubated for 1 week at 23 to 25°C in an orbital shaker set at 100 rpm. Mycelium harvested by filtration with muslin was rinsed in water, frozen at -20°C, and lyophilized. Thirty milligrams of dry mycelium was

ground with sterilized sand, and DNA extracted according to the manufacturer's instructions.

DNA was quantified by comparison of fluorescence following staining with ethidium bromide with known standards in agarose electrophoresis and stored at -20°C.

Polymerase chain reaction (PCR) amplification and restriction endonuclease analysis. PCR amplifications were routinely performed in a total volume of 25 µl containing 5 µl of squash blot DNA solution or 10 ng of genomic DNA with 10 pmol of each primer (Invitrogen, Paisley, UK) as listed elsewhere, 0.2 mM of each dNTP (Amersham Pharmacia Biotech), 1.5 mM MgCl₂, 1× PCR buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4), and 0.65 U of *Taq* polymerase (Invitrogen). Thermocycling used annealing temperatures appropriate to the primers.

For routine restriction endonuclease analysis, 2 to 5 µl of unpurified PCR products were digested according to the enzyme manufacturer's instructions for 2.5 h in a total volume of 10 µl, and DNA fragments from digestions were separated by electrophoresis on appropriate agarose gels in 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). Staining was with ethidium bromide.

DNA sequencing. DNA sequencing of PCR products and plasmid DNA was carried out by a commercial service (Sequise, Germany) with appropriate primers. Sequences were edited and aligned using the computer package DNASTAR (version 4.03, Madison, WI). Amplicons and clones were generally sequenced initially in one direction; for at least one sequence of each type the result was confirmed by sequencing in the reverse direction. If possible, PCR products were sequenced directly, but if necessary, were cloned into pMOSBlue in accordance with the manufacturer's instructions (Amersham Pharmacia Biotech). Plasmid DNA was extracted from overnight cultures (in 3 ml of Luria-Bertani medium/broth (Invitrogen)) using a Qiagen plasmid mini-kit used according to the manufacturer's instructions.

Amplified fragment length polymorphisms (AFLP) analysis. AFLP analysis with unlabeled primers used the AFLP Analysis System II kit (Invitrogen) in accordance with the manufacturer's instructions and starting with 250 ng of DNA. Selective amplification was performed with *MseI* primers containing two selective nucleotides (*MseI*-CA i.e., GATGAGTCCTGAGTAA-CA; *MseI*-CT; *MseI*-CC; *MseI*-CG) and *EcoRI* primers containing one selective nucleotide (*EcoRI*-A, i.e., GAC TCGGTACCAATTC-A; *EcoRI*-C; *EcoRI*-G; *EcoRI*-T). Three sets of pairings of these primers were used: (i) set 1 was all possible combinations except *MseI*-CC/*EcoRI*-C; (ii) set 2 was *MseI*-CA with *EcoRI*-C or *EcoRI*-G and *MseI*-CT with *EcoRI*-C or *EcoRI*-T; (iii) set 3 was all pairings except *MseI*-CC was omitted as were *MseI*-CA with *EcoRI*-T and *MseI*-CG with *EcoRI*-A. Three microliters of the selective amplification reaction was loaded on a Spreadex 800 gel (Elchrom Scientific, Cham, Switzerland) and separated by electrophoresis at 50 to 55°C for 2 h at 120 V. Gels were stained in SYBR Gold (Molecular Probes, Eugene, OR) diluted 1:10,000 in 0.33× TAE (1× TAE is 30 mM Tris, 0.5 mM EDTA, 14 mM glacial acetic acid) for 45 min and destained in RO water for 2 h. Bands were visualized using UV and scored manually. Data analysis was performed with Genstat (version 5, Lawes Agricultural Trust, Rothamsted, UK) using an average linkage cluster analysis with Jaccard's coefficient.

Analysis of the internal transcribed spacer (ITS) regions of the rRNA genes. ITS sequences were amplified using primers ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCT-CCGCTTATTGATATGC) (36) with an annealing step of 54°C. The possible presence of ITS sequences occurring as a minor component (i.e., occurring in a low proportion of the rRNA repeats in an isolate) was tested by amplification with species-specific primers (26) (for *V. dahliae*-like sequences 363, CCGGTC-CATCAGTCTCTCTG and for *V. albo-atrum*-like sequences 361, CCGGTACATCAGTCTCTTTA) used in conjunction with ITS4

with an annealing temperature of 56°C. Under our conditions, these primers are not highly specific, and the identity of amplicons was confirmed by enzyme digestion using *AluI*, *HaeII*, and *TspEI*. From the known sequence of the ITS regions of the

four main species studied (24) these were expected to give species-specific patterns following gel electrophoresis. The small number of nucleotide differences between *V. albo-atrum* and *V. dahliae* in the ITS regions (described below) precluded develop-

TABLE 1. Original host, geographic origin, year of isolation, and supplier of *Verticillium* isolates used in this study, along with conidial lengths and widths (with ratio between them), nuclear DNA content, sequences of the major internal transcribed spacer (ITS) and intergenic spacer (IGS) subrepeats, and presence of group I intron in the small subunit rRNA gene

Species/ Isolate ^a	Country of Origin	Original host	Year isolated	Spore length (µm)	Spore width (µm)	Length/ width	DNA content (fg)	AFLP group ^b	ITS sequence ^c	IGS repeats amplicon sequence		Intron present ^f
										(bp) ^d	Type ^e	
<i>V. dahliae</i> cruciferous isolates												
001 ¹	USA	<i>Armoracia rusticana</i> (horseradish)	NK ^g	7.3 (0.8) ^h	2.2 (0.3)	3.3	66	β	1(Vaa)	650	39(C) ⁱ	Yes
004 ¹	USA	<i>A. rusticana</i>	NK	7.0 (0.8)	2.1 (0.4)	3.3	57	β	1(Vaa) ^j	650	39(C) ⁱ	Yes
162 ²	Sweden	<i>Brassica napus</i> ssp. <i>oleifera</i> (oilseed rape)	NK	8.2 (1.0)	3.0 (0.3)	2.7	70	α	1(Vaa) ^j	650	39(C)	Yes
334 ³	Germany	<i>B. napus</i> ssp. <i>oleifera</i>	1985	7.1 (1.1)	2.2 (0.2)	3.2	51	α	1(Vaa) ^j	650	39(C)	Yes
617 ³	France	<i>B. napus</i> ssp. <i>oleifera</i>	1988	6.8 (0.9)	2.3 (0.3)	3.0	55	α	1(Vaa) ^j	740	39(C)	Yes
668 ³	France	<i>B. napus</i> ssp. <i>oleifera</i>	1989	6.8 (0.9)	2.3 (0.3)	3.0	50	α	1(Vaa) ^j	690	39(C)	Yes
855 ³	Germany	<i>B. napus</i> ssp. <i>oleifera</i>	1988	6.8 (0.9)	2.3 (0.3)	3.0	67	α	1(Vaa) ^j	650	39(C) ⁱ	Yes
892 ³	France	<i>B. napus</i> ssp. <i>oleifera</i>	1991	7.0 (0.7)	2.3 (0.3)	3.0	60	α	1(Vaa) ^j	650/690 ^k	39(C)	Yes
9802 ⁴	Germany	<i>A. rusticana</i>	NK	7.5 (1.0)	2.6 (0.3)	2.9	64	β	5(Vd)	740	81(Vd)	No
84020 ⁵	Japan	<i>B. campestris</i> spp. <i>rapifera</i> (turnip)	NK	7.4 (0.9)	2.7 (0.3)	2.7	60	α	1(Vaa) ^j	850 ^k	39(C)	Yes
84120 ⁵	Japan	<i>B. campestris</i> ssp. <i>pekinensis</i> (Chinese cabbage)	1980	7.7 (0.9)	3.1 (0.3)	2.5	58	α	1(Vaa) ^j	850/900 ^k	39(C)	Yes
84122 ⁵	Japan	<i>B. campestris</i> ssp. <i>pekinensis</i>	NK	8.0 (1.2)	2.9 (0.2)	2.8	80	α	1(Vaa)	870 ^k	39(C)	Yes
86207 ⁵	Japan	<i>Raphanus</i> <i>raphanistrum</i> (wild radish)	1980	7.5 (0.7)	2.5 (0.2)	3.0	70	α	1(Vaa)	650	39(C)	Yes
90-02 ⁶	USA	<i>B. oleracea</i> var. <i>botrytis</i> (cauliflower)	NK	7.3 (0.8)	2.9 (0.4)	2.5	70	α	1(Vaa)	690	39(C) ⁱ	Yes
90-03 ⁶	USA	<i>B. oleracea</i> var. <i>botrytis</i> (cauliflower)	NK	7.5 (0.8)	2.9 (0.3)	2.6	60	α	1(Vaa)	690	39(C)	Yes
90-10 ⁶	USA	<i>B. oleracea</i> var. <i>botrytis</i> (cauliflower)	NK	8.0 (1.0)	3.1 (0.4)	2.6	61	α	1(Vaa)	690	39(C) ⁱ	Yes
K3 ⁷	Russia	<i>B. napus</i> ssp. <i>oleifera</i>	NK	8.0 (1.1)	2.4 (0.3)	3.3	—	α	1(Vaa)	—	—	Yes
K12 ⁷	Russia	<i>B. napus</i> ssp. <i>oleifera</i>	NK	6.9 (1.1)	2.3 (0.3)	3.0	—	α	1(Vaa)	—	—	Yes
MD57 ⁸	Germany	<i>B. napus</i> ssp. <i>oleifera</i>	NK	6.6 (0.8)	2.9 (0.3)	2.3	67	α	2(Vaa)	650	39(C) ⁱ	Yes
MD73 ⁸	Germany	<i>B. napus</i> ssp. <i>oleifera</i>	1989	7.6 (1.2)	2.6 (0.2)	2.9	70	β	3(Vd)	740	81(Vd)	No (Minor) ^l
MD123 ⁸	Germany	<i>B. napus</i> ssp. <i>oleifera</i>	1987	7.8 (1.0)	2.8 (0.4)	2.8	59	α	1(Vaa)	650	39(C) ⁱ	Yes
Vd1 ⁹	Sweden	<i>B. napus</i> ssp. <i>oleifera</i>	NK	7.8 (0.8)	3.0 (0.3)	2.6	60	α	1(Vaa) ^j	650	39(C) ⁱ	Yes
Vd4 ⁹	Sweden	<i>B. napus</i> ssp. <i>oleifera</i>	NK	8.3 (1.5)	3.0 (0.3)	2.8	70	α	1(Vaa) ^j	650	39(C) ⁱ	Yes
Vd11 ⁹	Sweden	<i>B. napus</i> ssp. <i>oleifera</i>	NK	8.0 (0.8)	2.9 (0.4)	2.8	59	α	1(Vaa) ^j	650	39(C) ⁱ	Yes
Vd191 ¹⁰	Italy	<i>B. campestris</i> spp. <i>utilis</i> (broccoli raab)	1986	7.5 (0.6)	2.5 (0.2)	3.0	60	(α)	1(Vaa)	650	39(C) ⁱ	Yes
Vd292 ¹⁰	Italy	<i>B. oleracea</i> var. <i>botrytis</i> (broccoli)	1995	7.3 (1.0)	2.7 (0.3)	2.7	60	α	1(Vaa)	650	39(C) ⁱ	Yes

(Continued on next page)

^a Suppliers: 1 = D. Eastburn; 2 = J. B. Heale; 3 = H. Brun; 4 = Belgium Coordinated Collections of Micro-organisms; 5 = S. Horiuchi; 6 = S. T. Koike; 7 = L. G. Portenko/M. G. Guranowska; 8 = H. Prillinger; 9 = I. Happstad; 10 = M. Cirulli; 11 = B. Holtshulte; 12 = A. Soares; 13 = K. Subbarao; 14 = HRI culture collection; 15 = D. C. Harris; 16 = K. Broersma; 17 = D. C. Erwin; 18 = M. Cavalier; 19 = A. Termorshuizen; and 20 = J. E. Robb.

^b AFLP = amplified fragment length polymorphism. Parenthesis = similar to but divergent from group indicated, α and β = groups among cruciferous isolates (see text for details), nc = noncruciferous group, l = lucerne group, nl = nonlucerne group, t = *V. tricorpus*, and gpII = *V. albo-atrum* gpII.

^c Numerals represent internal transcribed spacer (ITS) sequences, in parentheses Vd or Vaa indicate the previously reported species they most resemble (24) from which they differ by a maximum of two bases. Sequence 1 is identical to the previously reported sequence for long-spored isolates (24) and is a single base different from that for *V. albo-atrum* lucerne isolates. Sequence 2 is the same as that reported for *V. albo-atrum* nonlucerne isolates (24), and the two control isolates sequenced here. Sequence 3 is identical to that previously reported for the majority of noncruciferous *V. dahliae* isolates (24) (and the three controls here) while Vd4 and Vd5 are both single bases different from this. The *V. tricorpus* and *V. albo-atrum* GpII isolates gave sequences quite distinct from those above but similar or identical to those already reported (24).

^d Estimated molecular size by electrophoresis of amplicon produced by primer pair 723/727.

^e Subrepeat sequence length (bp) and isolate type from which this repeat length first reported; C = crucifer isolate 162, Vd = noncruciferous isolate 2341, Vaa = isolate 1974 (25).

^f Presence of intron in small subunit rRNA indicated by 2.5-Kbp amplicon (19); absence indicated by 1.65-Kbp amplicon.

^g NK = not known.

^h Standard error of difference of measurements in parentheses.

ⁱ Type determined by enzyme digestion and comparison with sequenced isolates.

^j Determined by single-strand conformation polymorphism analysis (SSCP) rather than sequencing.

^k Multiple size products; for three Japanese isolates the PCR gave weak multiple bands and sizes given are for clearest bands; for all four isolates the PCR product was cloned before sequencing.

^l Intron present in minor repeat as shown by specific primers.

^m Intron present but smaller size.

ment of more specific primers and altering the conditions of amplification did not give improved specificity (presumably due to the high concentration of the majority sequence type) (data not shown).

Single-strand conformation polymorphism analysis (SSCP) (10) was used to identify the sequence type of major (i.e., occurring in a high proportion of the rRNA repeats) ITS sequences, which were not sequenced. Preliminary experiments using either *Verticil-*

TABLE 1. (continued from preceding page)

Species/ Isolate ^a	Country of Origin	Original host	Year isolated	Spore length (µm)	Spore width (µm)	Length/ width	DNA content (fg)	AFLP group ^b	ITS sequence ^c	IGS repeats amplicon sequence		Intron present ^f
										(bp) ^d	Type ^e	
VdII ¹¹	Germany	<i>B. napus</i> ssp. <i>oleifera</i>	NK	7.9 (0.9)	3.1 (0.3)	2.5	70	α	1(Vaa)	650	39(C) ⁱ	Yes
VdIII ¹¹	Germany	<i>B. napus</i> ssp. <i>oleifera</i>	NK	7.6 (1.1)	2.7 (0.3)	2.8	70	α	1(Vaa) ^j	650	39(C) ⁱ	Yes
VdIV ¹¹	Germany	<i>B. napus</i> ssp. <i>oleifera</i>	NK	7.5 (0.9)	3.0 (0.2)	2.5	57	α	1(Vaa) ^j	650	39(C) ⁱ	Yes
Mean				7.5	2.7	2.8	63					
MD80 ⁸	Germany	<i>B. napus</i> ssp. <i>oleifera</i>	1990	5.5 (0.6)	2.3 (0.3)	2.4	40	(nc)	4(Vd)	870	81(Vd)	No
Vd128 ¹⁰	Germany	<i>B. oleracea</i> var. <i>botrytis</i> (broccoli)	1978	5.3 (0.8)	2.8 (0.3)	1.9	30	(nc)	4(Vd)	900	81(Vd)	No
Mean				5.4	2.5	2.1	35					
<i>V. dahliae</i> noncruciferous isolates												
38 ¹²	Brazil	<i>Fragaria × ananassa</i> (strawberry)	NK	4.6 (0.8)	2.0 (0.2)	2.3	46	—	—	—	—	—
147 ¹³	USA	<i>Capsicum annuum</i> (bell pepper)	NK	—	—	—	—	nc	—	—	—	(Yes) ^m
318 ¹⁴	UK	<i>F. × ananassa</i>	1985	4.8 (0.5)	2.3 (0.4)	2.1	45	—	—	—	—	—
327 ¹⁴	UK	<i>F. × ananassa</i>	NK	—	—	—	—	—	—	—	—	—
330 ¹⁴	UK	<i>F. × ananassa</i>	1989	4.9 (1.3)	2.1 (0.3)	2.3	24	—	—	—	—	—
332 ¹⁴	UK	<i>F. × ananassa</i>	1989	4.4 (0.9)	2.4 (0.3)	1.8	35	—	—	—	—	—
1964 ¹⁴	UK	<i>F. × ananassa</i>	1972	—	—	—	—	—	—	—	—	—
1877 ¹⁴	UK	<i>F. × ananassa</i>	1966	4.6 (0.8)	2.4 (0.3)	1.9	38	—	—	—	—	—
2341 ¹⁴	NK	<i>Humulus lupulus</i> (hop)	NK	4.6 (0.8)	2.4 (0.3)	1.9	28	—	—	—	—	—
3440 ¹²	Brazil	<i>Solanum melongena</i> (aubergine)	NK	4.7 (0.8)	2.0 (0.2)	2.4	36	—	3(Vd)	—	—	—
12078 ¹⁵	UK	Soil	1998	4.1 (0.5)	2.4 (0.3)	1.7	28	—	—	—	—	—
12086 ¹⁵	UK	<i>F. × ananassa</i>	1998	4.4 (0.8)	2.6 (0.6)	1.7	37	—	—	—	—	—
12087 ¹⁵	UK	<i>F. × ananassa</i>	1998	4.2 (0.5)	2.3 (0.4)	1.8	34	nc	—	—	—	No
DC59 ¹⁴	UK	<i>H. lupulus</i>	1983	4.5 (0.8)	2.3 (0.3)	2.0	40	—	—	—	—	—
IMI ¹⁴	Zimbabwe	<i>Mentha piperita</i> (mint)	1951	4.6 (1.0)	2.4 (0.4)	1.9	46	—	—	—	—	—
MD71 ⁸	Germany	<i>Matricaria chamomilla</i> (camomile)	1989	4.8 (0.9)	2.5 (0.3)	1.9	31	nc	3(Vd)	870	81(Vd)	No
MD124 ⁸	Germany	<i>M. chamomilla</i>	1987	5.9 (0.6)	2.8 (0.4)	2.1	40	nc	3(Vd)	870	81(Vd)	—
Omega ¹⁴	UK	<i>H. lupulus</i>	NK	—	—	—	—	—	—	—	—	—
P14 ¹²	Brazil	<i>Lycopersicon</i> <i>esculentum</i> (tomato)	NK	5.4 (1.2)	2.7 (0.3)	2.0	44	nc	—	—	—	—
SS2 ¹⁴	USA	<i>Mentha piperita</i>	1991	4.4 (0.6)	2.4 (0.3)	1.8	33	—	—	—	—	—
Mean				4.7	2.4	2.0	37					
<i>V. albo-atrum</i> lucerne isolates												
220 ¹⁴	USA	<i>Medicago sativa</i> (lucerne)	NK	—	—	—	—	—	—	—	—	—
234 ¹⁴	France	<i>Medicago sativa</i>	1981	5.0 (0.7)	2.2 (0.3)	2.3	25	—	—	—	—	—
235 ¹⁴	France	<i>Medicago sativa</i>	1981	5.1 (0.6)	2.6 (0.3)	2.0	—	—	—	—	—	—
1906 ¹⁴	UK	<i>Medicago sativa</i>	1968	5.0 (0.5)	2.3 (0.2)	2.2	34	—	—	—	—	—
SWB1 ¹⁴	UK	<i>Medicago sativa</i>	1987	4.7 (0.6)	2.1 (0.2)	2.2	—	1	6(Vaa)	—	—	—
STR1 ¹⁶	Canada	<i>Medicago sativa</i>	2000	—	—	—	—	1	6(Vaa)	850	18(Vaa)	No
STR3 ¹⁶	Canada	<i>Medicago sativa</i>	2000	—	—	—	—	—	6(Vaa)	900	18(Vaa)	No
STR4 ¹⁶	Canada	<i>Medicago sativa</i>	2000	—	—	—	—	—	6(Vaa)	—	—	—
KRS1 ¹⁶	Canada	<i>Medicago sativa</i>	2000	—	—	—	—	—	6(Vaa)	—	—	—
Vaa5 ¹⁷	USA	<i>Medicago sativa</i>	NK	—	—	—	—	—	6(Vaa)	—	—	—
Mean (L)				5.0	2.3	2.2	30					
<i>V. albo-atrum</i> nonlucerne isolates												
72 ¹⁴	NK	<i>F. × ananassa</i>	NK	5.1 (0.6)	2.5 (0.3)	2.0	—	—	—	—	—	—
231 ¹⁴	UK	<i>S. tuberosum</i> (potato)	NK	5.7 (1.0)	2.7(0.6)	2.1	—	—	—	—	—	—
1602 ¹⁴	UK	<i>L. esculentum</i>	NK	—	—	—	—	—	—	—	—	—
1831 ¹⁴	UK	<i>Chenopodium</i> sp.	1956	5.1 (0.6)	2.5 (0.3)	2.0	—	—	—	—	—	—
1844 ¹⁴	UK	<i>H. lupulus</i>	1961	5.0 (0.6)	2.5 (0.2)	2.0	40	—	—	—	—	—
1953 ¹⁴	UK	<i>H. lupulus</i>	1972	4.9 (0.5)	2.3 (0.2)	2.1	33	nl	—	800	18(Vaa)	No
1974 ¹⁴	UK	<i>H. lupulus</i>	1980	5.1 (0.6)	2.3 (0.2)	2.2	43	nl	—	800	18(Vaa)	No
2101 ¹⁸	Belgium	<i>H. lupulus</i>	NK	4.6 (0.4)	2.3 (0.2)	2.0	—	nl	2(Vaa)	800	—	—
11041 ¹⁵	UK	<i>H. lupulus</i>	NK	—	—	—	—	nl	—	—	—	—
VA1 ¹⁹	Netherlands	<i>L. esculentum</i>	NK	4.9 (0.7)	2.5 (0.3)	2.0	31	—	2(Vaa)	—	—	—
Mean (NL)				5.0	2.4	2.0	37					
Mean All <i>V. albo-atrum</i>				5.0	2.4	2.1	34					
<i>V. tricolor</i>												
1988 ¹⁴	England	<i>L. esculentum</i>	1979	—	—	—	—	t	8	—	—	—
<i>V. albo-atrum</i> Group II												
151 ²⁰	Canada	Soil	1989	—	—	—	—	gpII	—	—	—	—
166 ²⁰	Netherlands	<i>S. tuberosum</i>	1989	—	—	—	—	gpII	7	—	—	—

lium-specific primers based on conserved sequences in the internal transcribed spacers and adjoining sequences of the rRNA gene repeats or primers ITS5 and ITS4 did not give good separation of fragments covering the region of interest (data not shown). However, following *Eco*RI digestion of the ITS5/ITS4 PCR products, it was possible to distinguish single base differences in the amplicons using SSCP. Before electrophoresis, restriction endonuclease digest products were purified using Prep-A-Gene (Bio-Rad, Hertfordshire, UK) in accordance with manufacturer's instructions. For electrophoresis, 3 µl of the purified products were

mixed with 7 µl of formamide containing 10 mM NaOH, heated to 30°C for 5 min, and placed immediately on ice to avoid renaturation. Eight microliters of the mixture was loaded on a Gene Mutation Analysis gel (Elchrom Scientific, Switzerland) and separated by electrophoresis at 6 V cm⁻¹ for 15 h in 1× TAE. Preliminary experiments showed 9°C to give best resolution of all four strands from the two fragments produced from the amplicon. Gels were stained with SYBR Gold as above.

Analysis of rRNA intergenic spacer (IGS) sequences. IGS sequences were amplified using primers 727 (TCCTACCATCTA-TGGAGG) and 723 (CACGGAACCAACTCTGCC). The majority of isolates tested gave single amplicons, which were sequenced directly. Three isolates (84020, 84120, and 84122) gave weak amplification and multiple products, and material from the strongest fluorescing band was cloned before sequencing. A fourth isolate (892) gave strong amplification of two similarly sized products, and material from these was also cloned prior to sequencing. When required, *Alu*I was used to digest 723/727 amplicons, and the resulting fragments were analyzed by electrophoresis on 30 g liter⁻¹ Metaphor agarose (FMC) gels. This enzyme has single recognition sites in each IGS subrepeat (25) and clearly distinguishes subrepeat lengths by giving a single strong band of the appropriate size.

Testing for the possible presence of a small proportion of *V. dahliae*-like IGS sequences in isolates with predominately *V. albo-atrum*-like IGS sequences was carried out using PCR amplification with primers 719 (GGGTAGCTGGTCTCTGGAA) and 720 (TGCGCTCTTTCTAATGCACTA) or 833 (CGGCGCTC-GATGTCGG) and 834 (AGGTGCCCCACGCACGCC). All IGS primers were used with an annealing temperature of 58°C.

Group I introns. Small-subunit (SSU) rRNA gene specific primers 18SVDF (GCGAACTGCGAATGGCT) and 18SVDR (G-TAATGATCCCTCCGCTG) and group I intron specific primers INT1 (CCAGGGGTAAACGGACTG) and INT2 (GGGCCAT-GCATTCGATTGC) (19) were used with annealing temperatures of 60°C to amplify and locate group I introns. Amplicons were separated on 10 g liter⁻¹ agarose gels in TBE.

Mitochondrial DNA (mtDNA) analysis. One to two micrograms of genomic DNA from each isolate were digested separately overnight with two enzymes with four base recognition sites rich in G+C (*Hae*III, GGCC, and *Hpa*II, CCGG) in accordance

Fig. 1. Diagrammatic representation as an unrooted tree of the nucleotide differences between the 320-bp nonrepetitive sequences located between the main subrepeat regions and the two downstream imperfect repeats of the rRNA intergenic spacer (IGS). Figures by each line give the number of base differences between sequences. Tree built by manual placement of nucleotide changes; 87 seven single-base changes and presumed double changes in eight positions were placed unambiguously; presumed double changes in three positions were ambiguous and not used but would not materially affect the outcome. * = identical to the published sequences for two noncruciferous *V. dahliae* isolates (TR12; AF144005 and Vd76; 104926). ** = published sequence (AF144006) for *V. albo-atrum* nonlucerne isolate 1776 has a single insertion relative to these sequences.

TABLE 2. Significance testing (*t* test) of differences in conidial length, width, and nuclear DNA content in groups of *Verticillium dahliae* and *V. albo-atrum*

	SED	Test statistic	df	Significance ^a
Mean conidial lengths				
Long-spored <i>V. dahliae</i> versus <i>V. dahliae</i> NC ^b	0.143	19.702	43	<i>P</i> < 0.001***
Long-spored versus short-spored <i>V. dahliae</i>	0.136	20.221	47	<i>P</i> < 0.001***
Long-spored <i>V. dahliae</i> versus <i>V. albo-atrum</i>	0.144	17.191	39	<i>P</i> < 0.001***
<i>V. dahliae</i> NC versus <i>V. albo-atrum</i>	0.145	2.300	26	<i>P</i> < 0.05*
All short-spored <i>V. dahliae</i> versus <i>V. albo-atrum</i>	0.149	1.785	30	<i>P</i> < 0.1
Mean conidial widths				
Long-spored <i>V. dahliae</i> versus <i>V. dahliae</i> NC	0.090	3.226	43	<i>P</i> < 0.01**
Long-spored versus short-spored <i>V. dahliae</i>	0.084	2.850	47	<i>P</i> < 0.01**
Long-spored <i>V. dahliae</i> versus <i>V. albo-atrum</i>	0.098	2.698	39	<i>P</i> < 0.05*
<i>V. dahliae</i> NC versus <i>V. albo-atrum</i>	0.077	0.322	26	<i>P</i> < 0.4
All short-spored <i>V. dahliae</i> versus <i>V. albo-atrum</i>	0.080	0.313	30	<i>P</i> < 0.4
Mean ratio length/width				
Long-spored <i>V. dahliae</i> versus <i>V. dahliae</i> NC	0.064	12.410	43	<i>P</i> < 0.001***
Long-spored versus short-spored <i>V. dahliae</i>	0.063	12.794	47	<i>P</i> < 0.001***
Long-spored <i>V. dahliae</i> versus <i>V. albo-atrum</i>	0.064	10.972	39	<i>P</i> < 0.001***
<i>V. dahliae</i> NC versus <i>V. albo-atrum</i>	0.065	1.526	26	<i>P</i> < 0.2
All short-spored <i>V. dahliae</i> versus <i>V. albo-atrum</i>	0.058	1.726	30	<i>P</i> < 0.1
Mean nuclear DNA content				
Long-spored <i>V. dahliae</i> versus <i>V. dahliae</i> NC	2.162	12.226	41	<i>P</i> < 0.001***
Long-spored versus short-spored <i>V. dahliae</i>	2.065	12.887	43	<i>P</i> < 0.001***
Long-spored <i>V. dahliae</i> versus <i>V. albo-atrum</i>	3.082	9.302	31	<i>P</i> < 0.001***
<i>V. dahliae</i> NC versus <i>V. albo-atrum</i>	3.203	0.696	20	<i>P</i> < 0.5
All short-spored <i>V. dahliae</i> versus <i>V. albo-atrum</i>	3.097	0.664	22	<i>P</i> < 0.6

^a * = significant at *P* < 0.05, ** = significant at *P* < 0.01, and *** = significant at *P* < 0.001.

^b *V. dahliae* NC = noncruciferous isolates.

with the manufacturer's instructions. DNA fragments were separated at 1.5 V cm⁻¹ for 18 h on 7 g liter⁻¹ agarose gels. Gels were stained with SYBR gold diluted 1:10,000 in TBE for 2 h and destained in water for 4 h to overnight. Fluorescent bands of greater than 1.95 kbp were scored. Similar approaches have been used to study many fungi, including the entomopathogen *V. lecanii* (21) on the basis that the bands revealed are mtDNA in origin but this may not be true for all bands, especially some smaller ones.

Electrophoretic karyotyping. Ten flasks, each containing 125 ml of Czapek-Dox medium, were inoculated with 2×10^8 conidia and incubated at 25°C for 36 to 40 h with shaking (100 rpm). Mycelium was harvested by centrifugation ($4,000 \times g$ for 5 min) and washed twice in 0.6 M magnesium sulfate. Protoplasts were prepared by digestion (3 to 4 h at 30°C) with 3.5 mg ml⁻¹ lysing enzymes from *Trichoderma harzianum* (Novozym 234, Sigma-Aldrich, but no longer available) in magnesium sulfate (20 ml g⁻¹ wet weight mycelium). After filtering through muslin, digests were overlaid with 0.6 M sorbitol, 100 mM Tris-HCl, pH 7.0, and centrifuged ($4,000 \times g$ for 30 min). Protoplasts were collected from the interface, mixed with an equal volume of 1 M sorbitol, 10 mM Tris-HCl, pH 7.5, and recovered by centrifugation ($2,400 \times g$ for 15 min). After washing three times in 0.6 M sorbitol, 10 mM Tris-HCl, pH 7.5, the protoplasts were resuspended at 1×10^8 ml⁻¹ in 0.6 M sorbitol, 0.1 M EDTA pH 8.0, and embedded in agarose blocks by mixing 3:1 with 10 g liter⁻¹ low melting point agarose (Bio-Rad) in 0.6 M sorbitol, 10 mM Tris-HCl, pH 7.5, 125 mM EDTA, which was cast into polystyrene molds. DNA was released in situ by sequential incubating blocks in: (i) 0.5 M EDTA, pH 8.0, 10 mM Tris-HCl pH 7.5, 0.1 M mercaptoethanol (24 h at 37°C); (ii) 0.5 M EDTA, pH 8.0, 10 mM Tris-HCl, pH 7.5, 10 g liter⁻¹ laurylsarcosine, 1 mg ml⁻¹ proteinase K (twice for 24 h at 50°C); and (iii) 50 mM EDTA, pH 8.0, (twice for 15 min and twice for 24 h, all at room temperature).

For pulse-field gel electrophoresis (PFGE) (30), blocks from above were soaked in 0.5× TBE (twice for 30 min), and slices containing 0.5 to 2 µg of DNA (estimated visually after staining sample slices with ethidium bromide) were cut. Slices were loaded in a 6.5 g liter⁻¹ agarose gel (Bio-Rad) in 0.5× TBE in a CHEF DR-II apparatus (Bio-Rad). *Verticillium* chromosomes were separated by electrophoresis at 50 V for 190 h with a pulse time of 2,500 s ramped to 2,100 s with buffer at 11°C. After electrophoresis, gels were stained in ethidium bromide and visualized under UV. *Schizosaccharomyces pombe* chromosomal DNA (FMC) was used as size standards and these gave a straight line when log₁₀MW was plotted against reciprocal of distance migrated.

***V. albo-atrum* nonlucerne pathotype specific primers.** DNA from all crucifer isolates was tested with primers 2 (ATGGACCGAACAGCTAGGTA) and 3 (TCTCAGATATATGCTGCTGC), which are specific for the nonlucerne pathotype of *V. albo-atrum* (6). The annealing temperature used was 54°C.

Accession numbers (Genbank) for DNA sequences. For ITS/5.8S sequences: *V. dahliae* isolates 001 - AF363986, 3440 - AF364001, 9802 - AF364007, 84122 - AF363987, 86207 - AF363988, 90-02 - AF363989, 90-03 - AF363990, 90-10 - AF363991, K3 - AF363997, K4-1 - AF364002, K12 - AF363996, L1 - AF364003, MD71 - AF363999, MD73 - AF364000, MD80 - AF364004, MD123 - AF363992, Md124 - AF363998, Vd128 - AF364005, Vd191 - AF363995, Vd292 - AF363994, and VdII - AF363993; *V. albo-atrum* isolates 2101 - AF364015, KRS1 - AF364008, STR1 - AF364009, STR3 - AF3640010, STR4 - AF364011, SWB1 - AF364012, Vaa5 - AF364013, and VA1 - AF364014; *V. albo-atrum* GpII isolate 166 - AF364013; and *V. tricornutus* isolate 1988 - AF364017. For rRNA IGS sequences accession numbers are listed in Figure 1 except for the less frequent variant in *V. dahliae* MD73 which is AF363270: published rRNA IGS sequences used; *V. dahliae* TR12 - AF144005 and Vd76 - AF104926; and *V. albo-atrum* 1776 - AF144006 (28). For group I intron: *V. dahliae* MD123 - AY056820 and

MD73 - AF153421; published sequence of crucifer isolate 86207 was AF153421, and of *V. dahliae* bell pepper isolate 147 was AY056822 (19).

RESULTS

Conidial size and nuclear DNA contents. Conidial dimensions and nuclear DNA contents are shown in Table 1. Based on spore length, the majority of cruciferous isolates (mean length 7.5 µm) formed a separate population from noncruciferous isolates of *V. dahliae* (4.7 µm) and *V. albo-atrum* (5.0 µm). These differences were significant (Table 2). Four isolates of *V. dahliae* from cruciferous hosts were clearly shorter spored (mean length 5.0 µm) than the majority of crucifer isolates. Conidial width similarly differentiated the isolates (Tables 1 and 2). Length and width were correlated within long-spored (>6.5 µm) isolates ($r = 0.65$, $P < 0.001$), within *V. albo-atrum* ($r = 0.70$, $P < 0.01$), and within all short-spored (<6 µm) isolates of *V. dahliae* ($r = 0.45$, $P < 0.05$) but not within just the noncruciferous isolates ($P < 0.1$, >0.05). The long- and short-spored *V. dahliae* isolates were different in average length/width ratios (2.8 versus 2.0; Table 1), and formed distinct populations as shown by parallel regression lines giving a significantly better fit ($P < 0.001$) than a single line. On the basis of conidial length/width ratio, *V. albo-atrum* similarly formed a discrete population from long-spored isolates ($P < 0.001$).

Measurement of nuclear DNA gave distinct populations of isolates of *V. dahliae* and *V. albo-atrum* with a low nuclear DNA content and of most crucifer isolates with a high nuclear DNA content (Tables 1 and 2). By this measure, *V. albo-atrum* was not significantly different from the noncruciferous *V. dahliae* isolates. The two short-spored cruciferous *V. dahliae* isolates tested had DNA contents comparable with those of the noncruciferous *V. dahliae* isolates. There was a significant correlation of conidial length with nuclear DNA content within the long-spored isolates ($r = 0.41$, $P < 0.05$) but not within short-spored isolates of *V. dahliae* or within *V. albo-atrum*. Overall, the isolates studied form two clear and distinct populations viz. short-spored/low nuclear DNA isolates (which can be subdivided into forms producing microsclerotia and those forming dark resting mycelium) and long-spored/high nuclear DNA (Fig. 2).

Fig. 2. Scatter diagram of conidial length against nuclear DNA content for *V. dahliae* and *V. albo-atrum* isolates from various hosts.

The mean nuclear DNA contents in Table 1 were calculated to be equivalent to genome sizes of 57 Mbp for long-spored crucifer isolates, 32 Mbp for short-spored crucifer isolates, 34 Mbp for noncruciferous isolates, and 31 Mbp for *V. albo-atrum* (27 Mbp for lucerne isolates and 34 Mbp for nonlucerne isolates but few lucerne isolates were available for testing).

AFLP analysis. Initially, seven selected *V. dahliae* crucifer isolates and 12 other isolates were tested using 15 primer combinations (set 1). As expected, cluster analysis clearly differentiated the *V. tricornis* and *V. albo-atrum* GpII isolates from all others (Fig. 3). The four *V. albo-atrum* isolates were clearly differentiated into two pairs, those from lucerne and those from nonlucerne hosts, with both pairs clearly separated from the *V. dahliae* isolates. The long-spored cruciferous isolates split into two clear groups with little variation within them relative to the separation between them; these will be referred to as α (isolates 90-02/VdII/86207) and β (9802/MD73). The short-spored cruciferous and noncruciferous *V. dahliae* isolates formed a further, more diverse, group within the species with the two short-spored crucifer isolates being somewhat distant from each other and the noncruciferous isolates. Using the four primer pairs revealing most polymorphisms in the first analysis (set 2), a further 22 cruci-

ferous isolates were compared with the five long-spored isolates initially tested. The majority of the 22 isolates clearly fell into the α group; 15 isolates were identical with isolates VdII and 86207 on the basis of 38 bands scored (162, 334, 668, 855, 892, 84020, 84120, 84122, MD57, MD123, VdIII, VdI, Vd4, Vd11, and Vd292), one isolate (617) had a single extra band, and three lacked a single band (90-03, 90-10, and VdIV). Although more similar to the β group, the remaining three isolates (001, 004, and Vd191) were not identical with 9802 or MD73 using these four primer pairs. These three isolates, a further two crucifer (K3 and K12) and one noncrucifer (P14) *V. dahliae* isolates, and two *V. albo-atrum* isolates (2101 and 11401) with unusual properties were compared to control isolates using the 10 primer pairs (set 3) showing the most polymorphisms from the initial 15 primers tested. The two *V. albo-atrum* isolates were identical to each other and similar to the nonlucerne isolate 1953. The two new crucifer isolates were identical to VdII in the α group. Isolates 001 and 004 were similar to MD73 and clearly in the β group. Vd191 was somewhat distinct from VdII but closer to the α group than the β group. The noncruciferous isolate P14 was somewhat distinct from the other noncruciferous isolates but more similar to them than to Vd128.

When individual AFLP bands, shown by those cruciferous isolates tested with the full 15 primers, were compared with those from noncruciferous isolates of *V. dahliae*, *V. albo-atrum*, *V. tricornis*, and *V. albo-atrum* GpII it was clear that few were common with the last two species (data not shown). Therefore, all bands recorded for seven *V. dahliae* crucifer isolates (and two chamomile isolates as controls) were classified as corresponding to *V. dahliae* or to *V. albo-atrum* or as isolate/group specific (Table 3). For all isolates, many bands (39% or more of the total) corresponded to those shown by *V. dahliae* noncruciferous isolates. For isolates in the α and β groups, a slightly lower proportion corresponded to *V. albo-atrum* bands. However, only a few bands could be attributed to the lucerne or nonlucerne pathotypes specifically. For the short-spored crucifer isolates, the number of bands corresponding to *V. albo-atrum* was low and equal to, or only slightly more than the number seen in the chamomile *V. dahliae* isolates. In all seven crucifer isolates some bands appeared to be crucifer-isolate-specific. For the five long-spored isolates, the total of crucifer and *V. albo-atrum*-specific bands was greater than the number of *V. dahliae*-specific bands but somewhat less for the two short-spored isolates. Many of the crucifer-specific bands were shared by all the long-spored isolates but not the short-spored isolates. Three bands were shared between the short-spored crucifer isolates and the group β long-spored isolates (Table 3).

Analysis of the rRNA gene ITS regions. The crucifer isolates were screened initially by restriction fragment length polymorphism (RFLP) analysis of amplicons containing the 5.8S gene and ITS 1/2 regions; 27 isolates gave patterns indistinguishable from *V. albo-atrum* controls, and four gave patterns indistinguishable from *V. dahliae* noncruciferous controls. The amplicons from 17

Fig. 3. Cladogram of similarity of isolates based on amplified fragment length polymorphism (AFLP) bands from 15 primer pairs. *VaaGpII* = *V. albo-atrum* group II; *Vt* = *V. tricornis*; *VdNC* and *VdCr* = *V. dahliae* from noncruciferous and cruciferous hosts; *VaaL* and *VaaNL* = *V. albo-atrum* lucerne and nonlucerne pathotypes. Cluster analysis by an unweighted pair group method of analysis (average linkage joining) using Jaccard's coefficient of similarity. Scale is proportion similarity.

TABLE 3. Number of bands seen in amplified fragment length polymorphism (AFLP) studies with crucifer isolates, which can be classified as also present in *V. dahliae* noncruciferous (NC) isolates or *V. albo-atrum* (lucerne [L] or nonlucerne [NL] pathotype or in both pathotypes) or as crucifer specific with results for two noncruciferous *V. dahliae* isolates included for comparison

Species(pathotype)	Number of specific bands ^b	Long-spored group α^a		Long-spored group β^a		Short-spored ^a		Chamomile ^a	
		VdII/86207	90-02	MD73/9802		MD80	Vd128	MD71	MD124
<i>V. dahliae</i> (NC)	53	27 (39 ^c)	26 (39)	30 (41)		39 (78)	36 (72)	46 (94)	45 (94)
<i>V. albo-atrum</i> (L)	37	5 (7)	5 (7)	5 (7)		1 (2)	0 (0)	0 (0)	0 (0)
<i>V. albo-atrum</i> (NL)	38	5 (7)	4 (6)	3 (4)		2 (4)	1 (2)	1 (2)	1 (2)
<i>V. albo-atrum</i> (L/NL)	97	14 (20)	13 (19)	11 (15)		3 (6)	2 (4)	2 (4)	2 (4)
Crucifer	...	18 (26)	19 (28)	24 (33)		5 (10)	11 (22)	0 (0)	0 (0)
Total for isolate		69	67	73		50	50	49	48

^a Number in parentheses is percent of total bands for each isolate.

^b Number of bands given by isolates of each haploid species.

crucifer isolates and 13 other isolates were sequenced. The overall ITS1/5.8S/ITS2 sequences were 450 to 452 bases. The 17 crucifer isolates contained five different sequences, two of which were identical or similar to the previously reported sequences for long-spored isolates and *V. albo-atrum* (24) (Fig. 4) and are referred to as *V. albo-atrum*-like (Table 1). The remaining three sequences were *V. dahliae*-like. The *V. tricornutus* and *V. albo-atrum* GpII isolates gave sequences distinct from those above but similar or identical to those already reported (24) (Fig. 4). The amplicons from the remaining crucifer isolates were tested by SSCP (which can distinguish the five sequence types found in crucifer isolates above; data not shown). Overall, 27 crucifer isolates had *V. albo-atrum*-like ITS sequences (26 sequence 1; one sequence 2). Four crucifer isolates had *V. dahliae*-like ITS sequences with the two short-spored crucifer isolates being identical and distinct from the two long-spored isolates (Table 1; Fig. 4).

All the long-spored crucifer isolates with *V. albo-atrum*-like ITS sequences were in the α AFLP group except for two horseradish isolates (001 and 004) which were in the β group (Table 1). Both long-spored isolates with *V. dahliae*-like ITS sequences were in the β AFLP group. The two short-spored crucifer isolates (MD80 and Vd128) had *V. dahliae*-like ITS sequences.

Assaying for multiple forms of the rRNA gene ITS regions. The possibility of a low proportion of rRNA genes repeats having distinct sequences (minor genes) was tested in the cruciferous isolates and controls by PCR using species-specific primers. Of 27 tested, no isolates with *V. albo-atrum*-like ITS sequences in the majority of rRNA repeats (i.e., as the major gene type) contained minor *V. dahliae*-like ITS repeats. The results for four cruciferous isolates with *V. dahliae*-like major ITS sequences and the chamomile isolates MD71 and MD124 were less clear-cut. All gave positive amplification with the *V. albo-atrum*-specific primers but with variable frequencies. Isolate 9802 gave product indicative of *V. albo-atrum*-like sequences being present on one of five times tested, Vd128 on two of four, and MD71 and 124 on four of five occasions; it is not clear whether this variability is because of contamination or weak amplification due to competition in the PCR from the major ITS sequence present. Only MD73 gave amplification on all five occasions it was tested, including when grown and tested in a separate facility. Thus, while it is possible that all isolates with *V. dahliae*-like major ITS sequences may maintain a minor *V. albo-atrum*-like rRNA component, this is not certain, with MD73 as the most likely candidate to do so.

Repeat-associated rRNA gene IGS sequences. Subrepeat sequences within the IGS region of the rRNA tandem arrays of *Verticillium* spp. have been reported previously (25,28). During the current work, two 18-bp imperfect repeats were found in all isolates 320 bp downstream of the conserved *Eco*RI restriction endonuclease site occurring at the end of the subrepeats. The non-repetitive region between the main subrepeats and the two reported here was designated the V-region. The sequences of both sets of subrepeats and the V-region were compared for a range of isolates.

Crucifer isolates with *V. dahliae*-like ITS sequences gave IGS related amplicons, which were generally larger (740 to 900 bp) than those from isolates with *V. albo-atrum*-like ITS sequences (650 to 900 bp but with the majority either 650 or 690 bp) (Table 1). For four crucifer isolates with *V. dahliae*-like ITS sequences (two long-spored, 9802 and MD73 and two short-spored, Vd128 and MD80), the sequences of the main subrepeats could be arranged into 81-bp units similar to those previously reported for haploid *V. dahliae* isolates (25). Variations in amplicon lengths were due mainly to differences in numbers of subrepeats. The long-spored isolates gave amplicons with the same lengths, but the short-spored isolates differed from these and each other. For 10 isolates with *V. albo-atrum*-like ITS sequences, the IGS subrepeat structures were similar to each other in mainly comprised varying numbers of repeated 39-bp elements. When tested by

RFLP analysis, the amplicons from 15 isolates with *V. albo-atrum*-like ITS sequences were indistinguishable from the 10 sequenced (Table 1). Subrepeat sequences from *V. albo-atrum* isolates were more variable but distinct from either of the above. No crucifer isolates gave *V. albo-atrum*-like subrepeat structures (Table 1). Slight variations were seen in the amplicon length for the *V. albo-atrum* isolates. Minor sequence variants, mainly single nucleotide polymorphisms, were found in the main subrepeats, but these were not correlated with any property and are not detailed here. Similarly, minor variants were found in the downstream subrepeats; MD80 and Vd128 were identical to each other but different from 9802 and MD73.

Ninety-eight polymorphic positions were seen 320-bp V-region sequences; 87 occurred as single base changes, but double base changes were assumed at the remaining 11 positions. The majority of the polymorphisms were used to draw an unrooted tree showing the interrelationships of the sequences (Fig. 1). The sequences of the noncruciferous *V. dahliae* isolates, MD71 and MD124, were identical to the published sequences for other noncruciferous *V. dahliae* isolates (25). Of the cruciferous isolates with *V. dahliae*-like ITS sequences, the two short-spored isolates were identical, as were the two long-spored isolates and these were four and one bases different, respectively, from the noncruciferous isolates (Fig. 1). For 10 crucifer isolates with *V. albo-atrum*-like major ITS sequences, the V-region sequences were similar to each other and different from those of any isolate with *V. dahliae*-like ITS sequences or any of the *V. albo-atrum* isolates, for which the lucerne and nonlucerne isolates were also distinct. Four isolates among this 10 had given weak or double bands in the initial PCR, and the amplicon had been cloned before sequencing. One clone (of two sequenced) from isolate 84120, and the single clone of 84122 sequenced had a one and two base differences, respectively, from the sequences obtained from the other eight isolates (Fig. 1),

Fig. 4. Diagrammatic representation as an unrooted tree of the nucleotide differences between the combined rRNA internal transcribed spacer (ITS) 1/5.8S/ITS 2 sequences. Isolates giving each sequence are listed in Table 1 except for *V. nubilum*, which was taken from a database (GenBank Accession No. AJ292463). Figures against lines give the number of base differences between sequences, and figures in parenthesis after the sequence identification number are the number of isolates from crucifers giving these sequences. Origins of equivalent sequence given in Morton et al. (24) are shown in square parenthesis. Tree built by manual placement of nucleotide changes; all changes were used and were positioned unambiguously except for an assumed double change on the branches to *V. nubilum* and Seq8.

but as these small differences were unconfirmed they are not shown here. The two horseradish isolates, 001 and 004, were identical to each other and differed by two bases from the 10 isolates above (Fig. 1).

Assaying for multiple forms of the rRNA gene IGS regions. Two pairs of primers, designed from the *V. albo-atrum*-like IGS sequences from crucifer isolates, were used to test for the possible presence of less common, minor IGS sequences in four crucifer isolates with *V. dahliae*-like major ITS sequences (9802, MD73, MD80, and Vd128) and two noncruciferous *V. dahliae* controls (MD71 and MD124). Only isolate MD73 gave amplicons of the expected sizes. This isolate had consistently shown the presence of a minor ITS type. The minor IGS amplicons were sequenced and found to be identical in the main subrepeat region to the major IGS sequence from isolates 162, 334, 86207, and VdII. The V-region of the MD73 minor component amplicon was identical to that of 162 and similar isolates except for a single base difference, but as only a single PCR product covering the region was sequenced the significance of this is not clear.

Presence of a group I intron. Primers 18SVDF and 18SVDR (19) were used to amplify DNA from 31 crucifer and three noncruciferous *V. dahliae* isolates and four *V. albo-atrum* isolates (two from lucerne). Four crucifer isolates, two of the noncrucifer isolates, and the *V. albo-atrum* isolates gave products of approximately 1.65 kbp (Table 1). The remaining crucifer isolates gave amplicons of approximately 2.5 kbp indicating the presence of a group I intron in the small subunit rRNA genes. Primers INT1 and INT2 based on the reported sequence of the intron (19) were used to confirm this; these primers gave an 800-bp product with all those isolates, which had given the larger amplicon with the 18SVDF/R primers but not with those which had given the smaller amplicon with the exception of MD73. This isolate gave an 800-bp product despite having given only a 1.65-kbp amplicon

with primers 18SVDF and 18SVDR. That this intron was present in the rRNA genes was confirmed by using two combinations of primers, 18SVDF/INT2 and 18SVDR/INT1, that gave amplicons of the expected sizes. The amplicons from MD73 and MD123 were sequenced and found to be identical (MD123) to or only two bases different (MD73) from the sequence of the intron reported for the Japanese crucifer isolate 86207 and were apparently inserted at the same point in the SSU rRNA gene (equivalent to 1165 nt of *Saccharomyces cerevisiae* [19]).

The remaining noncruciferous *V. dahliae* isolate tested, 147, gave a product of approximately 2.1 kbp with primers 18SVDF/R. Neither of the primer pairs 18SVDF/INT1 or INT1/INT2 gave a product from this isolate but 18SVDF/INT2 did. The amplicon from this last pair was sequenced. Comparison with the other sequences indicated the presence of a 475-bp intron inserted at the same point in the rRNA genes. There were two large deletions and 16 base substitutions relative to the larger intron (data not shown), but these changes did not affect the major conserved regions of the intron. A substitution and two insertions were responsible for the primer INT1 being unable to amplify from DNA from isolate 147.

MtDNA analysis. MtDNA RFLP patterns were established for 13 cruciferous *V. dahliae* isolates, five noncruciferous isolates, and four isolates of *V. albo-atrum* by restriction endonuclease digestion of total genomic DNA followed by agarose gel electrophoresis. The results are summarized as a cladogram in Figure 5. *V. albo-atrum* lucerne isolates were clearly differentiated from the nonlucerne isolates and both of these isolates were differentiated from the *V. dahliae* noncruciferous isolates and the short-spored cruciferous isolates. The majority of cruciferous isolates, including all those tested in the α AFLP group and two in the β group, clustered together and were more similar to *V. albo-atrum* than they were to *V. dahliae* but were clearly distinct from both. The short-spored crucifer isolates, Vd128 and MD80, and the β AFLP group isolates, 001 and 004, were similar to the noncruciferous *V. dahliae* isolates. As with rRNA ITS sequences the patterns, ignoring minor variations, are classified from the cladogram into broad types (i.e., *V. dahliae* noncruciferous, *V. albo-atrum* NL and L pathotypes and crucifer-isolate type).

Electrophoretic karyotyping. For three isolates of *V. albo-atrum*, seven bands were seen in PFGE (Table 4) ranging from 2.5 to 2.8 Mbp for the smallest band to 5.8 to 6.6 Mbp for the largest. Total implied genome size was 28 to 30 Mbp. For *V. dahliae*, six bands were seen in preparations from four isolates ranging from 3.3 to 3.5 up to 6.3 to 6.8 Mbp, implying genome sizes of approximately 28 to 29 Mbp (Table 4). Using the same methods, two long-spored isolates (161, an isolate from sugar beet and 84120) did not give clearly resolved bands and no estimate could be made of the genome size (data not shown).

***V. albo-atrum* nonlucerne pathotype specific primers.** PCR using primers specific for the nonlucerne pathotype of *V. albo-atrum* (6) did not give an amplicon with any of the crucifer isolates listed in Table 1 (K3 and K12 were not tested) but did give a product with *V. albo-atrum* 1974.

DISCUSSION

All the crucifer isolates known, either those studied here or reported elsewhere, produce microsclerotia and are traditionally classified as *V. dahliae*. The majority of these are long spored and it has been suggested (18) that some of these should form the basis of a new, 'near-diploid' species, *V. longisporum*. In the current work, a diverse range of crucifer and other isolates were studied with a number of techniques. Based on our results, we will argue that: (i) the erection of the new species is premature and does not truly reflect the complexity of the situation; and (ii) that the undefined terms 'near-diploid' and 'recombinant' are inappropriate and their use should be discontinued.

Fig. 5. Cladogram showing the similarities between mtDNA patterns from restriction fragment length polymorphism (RFLP) analysis using two restriction endonucleases (*Hae*III and *Hpa*II). Cladogram generated by unweighted pair group method analysis (nearest neighbor joining) using Jaccard's coefficient. Bar represents 40% dissimilarity.

Resting structure, conidial lengths, and nuclear DNA contents clearly divide *V. dahliae* and *V. albo-atrum* isolates into three main types viz. short-spored/low nuclear DNA and long-spored/high DNA types producing microsclerotia, and short-spored/low nuclear DNA isolates producing dark resting mycelium. Within two of these three populations, both conidial shape and nuclear DNA contents are associated with conidial length. However, test statistics showed no meaningful overall association of DNA content with spore length, although this has been suggested (18), and long- and short-spored isolates appear to form discrete populations. No isolates had intermediate properties, suggesting that high and low nuclear DNA content forms are stable in nature or that transition of isolates between forms is rapid, possibly occurring in a single nuclear division. As expected from earlier reports (14,18, 32,33), the majority of isolates from cruciferous hosts were long spored and had a high nuclear DNA content.

Molecular analysis lead to further subdivision of the three broad categories defined above. As an aid to understanding, the results for some isolates are summarized in Table 5 along with an interpretation of the possible origins and genetic status of the isolates from crucifers.

AFLP analysis divided the long-spored isolates in two groups (α and β) with only limited variation within each group. One group (β) could be subdivided on the basis of rRNA gene type (as shown by isolates having *V. dahliae*-like or *V. albo-atrum*-like ITS sequences in the majority of repeats). Further subdivision of the β group could be made on the basis of ITS sequence variants (although as these comprised single base changes, their significance may be arguable) and the retention of a second, infrequently occurring rRNA gene ITS sequence (as has been reported for

Fusarium spp. [31]). This AFLP group is also subdivided by mtDNA type. Only relatively few isolates fell in this AFLP group, and more isolates need to be identified and studied to better define the variability that occurs within it. The second AFLP group (α) appeared more homogenous. We propose that the simplest interpretation of this data is that long-spored crucifer-pathogenic interspecific hybrids have been formed on at least two separate occasions, each represented by one of the AFLP groups. Subdivision of the β group then arose due to the differential loss/retention of one or other parental type of highly duplicated genes (e.g., the tandemly repeated rRNA gene arrays) and mitochondria. Whether this process of choosing parental types is selectively neutral or is driven by some unknown advantage being conferred is not clear. The possible role of host selection within the crucifers is also not clear, but it is notable that β AFLP group isolates with both *V. dahliae*-like and with *V. albo-atrum*-like rRNA genes were found in horseradish. It is equally possible that association with this host (and possibly adaptation to it) occurred before parental types were selected or as two events after gene/mitochondrial choice. The degree of host adaptation to different cruciferous hosts by isolates in the AFLP groups and subgroups needs clarification as no host testing has been done which specifically covers all these categories.

Two short-spored crucifer isolates studied here, MD80 and VD128, had low nuclear DNA contents and are presumably haploid. By AFLP analysis, they are both distinct from non-cruciferous *V. dahliae* isolates and from each other. We propose that these isolates are derived (by rehaploidisation) from interspecific hybrid isolates. Their relatively close affinity by AFLPs to the noncruciferous *V. dahliae* isolates suggests that they have

TABLE 4. Number of bands seen in pulse-field gel electrophoresis (PFGE) separations of DNA from isolates of *Verticillium dahliae* and *V. albo-atrum* with estimated molecular sizes (Mbp) of the bands and the total genome size implied

Species Isolate (host)	Number of bands		Sizes ^a							Total
<i>V. dahliae</i>										
318 (strawberry)	6		6.3	5.2	4.8	4.3	3.8	3.5	...	27.9
327 (strawberry)	6		6.3	6.0	5.1	3.7	3.3	3.1	...	27.5
1877 (strawberry)	6		6.6	5.0	4.4	3.9	3.5	3.2	...	26.6
Omega (hop)	6		6.8	5.7	5.2	4.1	3.9	3.4	...	29.1
Mean										27.8
<i>V. albo-atrum</i>										
220 (lucerne)	7		6.0	5.1	4.8	4.1	3.7	3.2	2.5	29.4
1602 (tomato)	7		5.8	5.4	4.5	3.9	3.2	2.8	2.5	28.1
1974 (hop)	7		6.6	5.7	4.6	3.8	3.3	3.1	2.8	29.9
Mean										29.1

^a Band sizes were calculated from relative mobility when compared to *Schizosaccharomyces pombe* chromosomal DNA markers.

TABLE 5. Summary of data for main species/pathotypes and molecular characteristics of *V. dahliae* and *V. albo-atrum* and the proposed hybridization events giving rise to them and genetic status

Species/pathotype ^a	Typical isolates	Spore length	DNA content	Major		Minor		Mt DNA	AFLP type	Proposed	
				ITS ^b	IGS ^c	ITS ^b	IGS ^c			Hybridization	Genetic status
<i>V. albo-atrum</i> /NL	1974	Short	Low	Vaa ^d	VaaNL	nt ^e	nt	VaaNL	VaaNL		Haploid
<i>V. albo-atrum</i> /L	STR1	Short	Low	Vaa	VaaL	nt	nt	VaaL	VaaL		Haploid
<i>V. dahliae</i> /NC	MD71/MD124	Short	Low	Vd	Vd	nt	nt	Vd	VdNC		Haploid
<i>V. dahliae</i> /Cr	MD80/VD128	Short	Low	Vd	Vd	–	–	Vd	(VdNC) ^f	(?) ^g	Secondary haploid
<i>V. dahliae</i> /Cr	90-02/VDIII	Long	High	Vaa	Cr	–	–	Cr	α	1	Amphihaploid
<i>V. dahliae</i> /Cr	001/004	Long	High	Vaa	Cr	–	–	Vd	β	2	Amphihaploid
<i>V. dahliae</i> /Cr	MD73	Long	High	Vd	Vd	Vaa	Cr	Cr	β	2	Amphihaploid
<i>V. dahliae</i> /Cr	9802	Long	High	Vd	Vd	–	–	Cr	β	2	Amphihaploid

^a Pathotypes are NL = nonlucerne; L = Lucerne; NC = noncruciferous; Cr = cruciferous.

^b ITS = internal transcribed spacer.

^c IGS = intergenic spacer.

^d Minor variants of characteristics are grouped into broad types as resembling *V. dahliae*, *V. albo-atrum* (with pathotype where distinguishable) or crucifer type.

^e nt = not tested; – = not detected.

^f Amplified fragment length polymorphism (AFLP) results place these isolates as close to but distinct from *V. dahliae* noncruciferous isolates and from each other.

^g ? = these may represent one or more further hybridization events or may be derived from the amphihaploid types listed.

inherited the majority of their genome from the *V. dahliae* parent of the hybrid, but their distinctness suggests that they have retained part of the second parental genome. Whether these isolates are molecularly distinct from each other because they have retained different parts of the same parental genomes (i.e., they represent one hybridization) or because they come from separate origins is not clear. We therefore, propose that at least three distinct types of crucifer isolates exist. In our studies, the long-spored isolates appeared stable, but apparent breakdown of a long-spored isolate to a short-spored isolate in culture has been reported (18). We have no evidence whether the short-spored crucifer isolates from the field are derived from a stable hybrid, which had existed for some time (as presumably have the long-spored isolates studied) or from more transient forms.

Both the long- and short-spored crucifer isolates appear molecularly distinct from *V. albo-atrum* and noncruciferous *V. dahliae* isolates, and the basis of their host adaptation is presumably associated with that distinctness. It is an important question as to whether the novel host specificity arises as a direct result of their hybrid nature (i.e., because novel combinations of genes have been brought together) or simply comes from one of the parents. Karapapa et al. (18) suggested on the basis of limited RAPD analysis that the *V. albo-atrum* parent was of the lucerne pathotype. The inability here to produce an amplicon with primers specific for the nonlucerne pathotype of *V. albo-atrum* (6) from crucifer isolates provides some direct evidence that an isolate of this pathotype was not a parent (although this genome section may have been lost). Generally, the data presented here on rRNA gene sequences and associated introns and from the AFLP studies suggests that the *V. albo-atrum* parent was quite distinct from any isolate of any species studied here or elsewhere. In summary, for crucifer isolates with *V. albo-atrum*-like rRNA genes: (i) the ITS sequences are distinct, although only by a small number of base changes (data presented here; 24); (ii) the IGS subrepeats have distinct structures (data presented here; 25); (iii) the IGS associated V-region sequence is clearly different from that of both pathotypes; (iv) RFLP analysis of mtDNA show a distinct type for these isolates; (v) all have an intron in the SSU rRNA gene not found in *V. dahliae* (data presented here; 19). Karapapa and Typas (19) suggested that because they did not find this intron in noncruciferous *V. dahliae* isolates or *V. albo-atrum*, it had been horizontally transferred into cruciferous isolates either from another fungus or from a host plant and one of these might well represent the ultimate origin of the intron. However, its close association in crucifer isolates with the *V. albo-atrum*-like ITS sequence both when this occurs as the most frequent type, as in most isolates, and when as the minor repeat type seen in isolate MD73, makes it more likely that in the shorter term it was derived from the *V. albo-atrum* parent as part of the complete repeat unit. Only the intron from MD73 was sequenced here, and the importance of the two base differences seen when this was compared with the published sequence of isolate 86207 (19) is not known. To date, no similarly placed intron has been reported for *V. albo-atrum* isolates. The possibility of finding introns in as yet unstudied isolates of *Verticillium* is underlined by the presence of a similar (albeit with two large deletions and several other base changes) intron in *V. dahliae* 147, an example of a new vegetative compatibility group from pepper (2); and (vi) in the AFLP studies, for both α and β group, isolates with either rRNA gene type, approximately 40% of the bands seen for the group corresponded to those seen with a noncruciferous *V. dahliae* and 26 to 35% corresponded to bands seen with *V. albo-atrum*. The majority of these *V. albo-atrum* bands did not correspond to bands seen with representatives of either main pathotype but rather to bands common to both types. Crucifer isolate AFLP group specific bands comprised 26 to 33% of the total. These novel bands might possibly have arisen by recombination within the hybrid isolates, but it seems more likely that the majority probably represent

bands specific to the parental isolates. The proportion of these attributable to each of the parents is not known, but even if it is assumed that equal proportions come from both, it implies that the *V. albo-atrum* parent isolate was not of either known *V. albo-atrum* pathotype.

At this stage, we can only predict on the basis of ITS sequence similarity that the unknown parent is *V. albo-atrum*-like. However, only when isolates with the required combination of rRNA intron/V-region sequence/ITS sequence/mtDNA etc. have been identified will the nature of the resting structure and other properties be known, making it possible to classify these parental isolates as known or new species. The presence of an almost identical intron in the less frequent rRNA gene repeats of MD73 suggests that the same (or similar) as yet unrecognized type of *V. albo-atrum* was involved in generating this isolate. The presence of minor type rRNA genes in this isolate presents direct evidence for it having a hybrid origin.

Data on the nature of the *V. dahliae* involved as the parent is less clear. For crucifer isolates with *V. dahliae*-like ITS sequences, no intron was present, and the IGS V-region sequence was only differentiated from that of noncruciferous isolates by one to four nucleotide changes. It may be argued as above that some of the crucifer-specific AFLP bands are derived from the *V. dahliae* parent, which would suggest major differences between noncruciferous isolates studied and the parent but equally all the crucifer-specific bands may be derived from the other parent. However, other evidence is emerging (manuscript in preparation) that the *V. dahliae* parent is also distinct from most isolates examined.

All the AFLP α group isolates appear similar and may be clonal, arising from one line selected from one hybridization event. The β group isolates are more variable and may actually represent several separate hybridization events. The overall similarity of the AFLP patterns for β group isolates suggest that even if several events were involved, the parents were generally similar in them all. An important crop protection question relating to crucifer isolates is whether they might arise de novo in countries where they do not currently occur or whether the danger is from importation. If it can be confirmed that the involvement of a *V. albo-atrum*-like isolate, unlike any yet described, is a necessary prerequisite to crucifer pathogenicity, then the geographic distribution of this hypothetical isolate and a suitable *V. dahliae* parent determines the possible sites of hybridization. Few *V. albo-atrum* isolates have been tested in ways that would clearly identify them as an appropriate parental type. Only relatively recently was evidence presented that some isolates particularly, but not exclusively associated with potato, were sufficiently distinct to be regarded as new species (24,29). Testing a range of *V. albo-atrum* isolates with suitable PCR primers specific for the V-region sequence of crucifer isolates would provide a rapid preliminary screen for isolates similar to the hybrids' parents.

The isolates placed in *V. longisporum* (18) were described as 'near-diploid', but this term was undefined and seems unsatisfactory. Long-spored isolates appear to represent interspecific hybrids where the entire (or almost entire) haploid genomes of the two parents have combined in a single nucleus. Different chromosome numbers were found in *V. albo-atrum* and *V. dahliae*. If we assume that the unknown *V. albo-atrum*-like parent similarly has seven chromosomes, then the disparity between the parents carried through to the hybrid nucleus would probably prevent chromosome pairing and the hybrids acting genetically as diploids, thus playing a role in hybrid stability by preventing simple reductive nuclear division. In diploid species of plant, the terms amphidiploid or allotetraploid are used for this specific form of allopolyploidy e.g., in *Brassica* (35) and tobacco (9) where amphidiploids form novel hybrid species. Amphihaploid, a term that has been used to describe interspecific *Brassica* hybrids of various origins with single copies of each chromosome (1), or allodiploid therefore appear to be more appropriate and informative terms to use to

describe the *Verticillium* hybrids studied here than does 'near-diploid'.

The genome sizes estimated for the haploid species by two separate methods are in good general agreement at approximately 30 Mbp. Both the flow cytometry results here and the estimations of Karapapa et al. (18) suggest that the long-spored isolates have slightly less than twice the nuclear DNA content of short-spored noncruciferous *V. dahliae* isolates (at 1.7- and 1.8-fold, respectively). The approximate size estimates derived by electrophoretic karyotyping suggest this would not be the case for a theoretical *V. dahliae/V. albo-atrum* hybrid but too few isolates were studied to draw a firm conclusion. Similarly, by flow cytometry, the disparity in the average sizes of the *V. albo-atrum* and *V. dahliae* noncruciferous isolate genomes was insufficient to give this ratio. Moreover, we have no estimate for the genome size of the theoretical *V. albo-atrum*-like parent by either method. It remains, therefore, an open question whether the shortfall between the approximate 57-Mbp genome size seen, and the greater than 60 Mbp expected is due to some loss of genetic material in the hybrid or because one or other of the actual parents has a smaller genome than seen here.

The short-spored crucifer isolates, MD80 and Vd128, appear molecularly distinct from haploid noncrucifer isolates and are probably derived through the rehaploidisation or breakdown of one or more amphihaploid parents. The term 'secondary haploids' is proposed as a pragmatic way of distinguishing these and similar isolates. However, while molecular data and crucifer pathogenicity served to clearly identify the two isolates studied here it may be that some, or even most, secondary haploids are not crucifer pathogens, and it is not clear what level of molecular differentiation from ordinary haploid isolates would be necessary for their unambiguous identification. For example, *V. dahliae* P14 from Brazil was almost as distinct from other noncruciferous isolates as are the two short-spored crucifer isolates, but as it came originally from a noncruciferous plant there is no host evidence to suggest it is a secondary-haploid. The proposed genetic status of the selected isolates is shown in Table 5.

It was recently stated in relation to allopolyploid *Phytophthora* isolates that an "issue, which has yet to be tested in mycology, is how to taxonomically define a hybrid or hybrid complex in a way that is of practical use in quarantine legislation and diagnosis" (3). This caution applies equally to the complexity of morphological and molecular types of crucifer-infecting *Verticillium* isolates. At present, isolates in one AFLP group appear much more frequent than do other crucifer pathogenic forms but whether this merely reflects some chance event (such as dispersal of effectively one isolate with contaminated seed), sampling bias, or some fundamental property related to pathology is not known. It seems premature to grant species status to only one of the AFLP groups, largely on the basis that they are important pathogens in European oilseed rape. Currently, we favor a single species covering all plant pathogenic interspecific *Verticillium* isolates, possibly including short-spored ones with a name reflecting their novel pathogenicity. However, we suggest that until we have a much fuller understanding of the relative importance of the different forms, the use of the name *V. longisporum* for just some isolates is inappropriate. Similarly the undefined term recombinant to describe some isolates (18) is not useful.

Although known examples of interspecific fungal hybrids are apparently infrequent (3) it has been shown that novel pathogenic properties may arise in this way (e.g., *Melampsora* × *columbiana* [27]). *Phytophthora* isolates infecting alders have been shown to be a collection of allopolyploids (3), but in other hybrids there is no change in genetic status. As examples of amphihaploid fungi with novel pathogenicity arising from asexual parental species, these long-spored crucifer-infecting *Verticillium* isolates appear to be highly unusual and possibly unique. It is somewhat ironic that in Europe and elsewhere they are major pathogens of amphidiploid plant species.

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