*Plant Pathology* (2005) **54**, 549–557 Doi: 10.1111/j.1365-3059.2005.01240.x

# Correlation of molecular markers and biological properties in *Verticillium dahliae* and the possible origins of some isolates

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Haploid and amphihaploid *Verticillium dahliae* isolates were studied using PCR-based molecular markers which: (i) discriminate the defoliating and nondefoliating pathotypes (two primer pairs INTD2f/r and INTND2f/r), and (ii) are species-specific (primer pair 19/22). The results were compared with some known biological and other molecular properties of the isolates. Five discrete sequences of the 19/22 amplicon were found. Sequence 4 was associated with both defoliating isolates from Spain and nondefoliating isolates from Spain and USA; these pathotypes were separated by the primer pairs INTD2f/r and INTND2f/r, but the data showed that the primer espdef01 (derived from the 19/22 amplicon) cannot be used for this purpose. Amplicon sizes and sequences with primers 19/22 divided amphihaploid isolates from crucifers (thought to be interspecific hybrids) into those corresponding to the previously reported α and β groups. The β-group isolates had either sequence 4 or 5 (these two differing by a single base). The distinct amplicon sequence 3 given by the α-group isolates demonstrated that the *V. dahliae*-like 'parent' of this group was molecularly unlike any haploid isolate yet studied. The overall results are discussed in relation to phytosanitary considerations and the probability of defoliating or crucifer pathotypes arising *de novo* within Europe, either by selection or by interspecific hybridizations.

*Keywords*: amphihaploids, interspecific hybrids, pathotypes, vegetative compatibility, wilt diseases

## Introduction

Vascular wilts caused by infection by *Verticillium* spp. are important fungal diseases of many herbaceous and woody crops and wild plants worldwide. As a soilborne pathogen primarily infecting roots via a long-lived melanized resting structure, *V. dahliae* seems poorly adapted for medium- to long-distance spread. However, such spread does occur, often with the assistance of humans, in plant debris, soil, infected plants and on infested seeds (Pegg & Brady, 2002). Isolates can differ markedly in pathogenicity and specific pathotypes may be important in particular countries or hosts. For the proper application of phytosanitary measures, it is important to understand whether virulent isolates are most likely to enter at-risk crops by importation or whether they can arise *de novo* in those crops.

Although verticillium wilts once seemed to be only minor problems in cruciferous hosts, they are now major diseases, e.g. in oilseed rape in northern Europe (Heale & Karapapa, 1999) and cauliflower in California (Koike *et al*., 1994).

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*Accepted 22 March 2005*

Most *V. dahliae* isolates have short conidia (and are haploid), but the majority of isolates from cruciferous hosts have longer conidia (Stark, 1963; Karapapa *et al*., 1997; Collins *et al*., 2003) and are thought to be stable, natural interspecific hybrids which retain the majority of the two parental genomes in a fused nucleus. The term 'amphihaploid' has been used to describe these isolates (Barbara & Clewes, 2003; Collins *et al*., 2003). Studies using amplified fragment length polymorphisms (AFLPs) divided these amphihaploid isolates into two groups, designated α and β (Collins *et al*., 2003). Isolates from cotton (*Gossypium hirsutum*) can be classified into either defoliating (D) or nondefoliating (ND) pathotypes according to their ability to defoliate this host or, less damagingly, to cause wilt without defoliation (Bejarano-Alcázar *et al*., 1996; Schnathorst & Mathre, 1966). Isolates from olive (*Olea europaea*) may be similarly classified, and isolates from cotton and olive show cross-virulence (Schnathorst & Mathre, 1966; Schnathorst & Sibbet, 1971; Rodríguez-Jurado *et al*., 1993). Besides Spain and the USA, the D and ND *V. dahliae* pathotypes have also been reported from China (Xia *et al*., 1998) and central Asia (Daayf *et al*., 1995). *Verticillium dahliae* D-pathotype isolates from China, Spain and the USA appear molecularly similar (Pérez-Artés *et al*., 2000).

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For amphihaploid isolates, *de novo* hybrids (at least ones like those already described) will only arise in regions where the 'parental' types occur. Therefore, defining these 'parents' molecularly may allow differentiation of areas at risk from crucifer-pathogenic isolates arising afresh from those at risk only from importation. Similarly, D-pathotype isolates in Spain appear to be clonal and must have been selected or imported from a population including isolates with similar properties. This clonal nature suggests that the importation into Spain of an isolate particularly virulent for both olive and cotton first occurred in one crop and that this isolate then spread to cause problems in other crops. However, it cannot be ruled out that spread between olive and cotton (and possibly other hosts) occurred outside Spain followed by two or more importations. Better definition of D isolates and comparison of them with isolates from other areas may help to clarify which countries are sources of isolates capable of causing severe disease in two very important crops.

Whilst validating the specificity of polymerase chain reaction (PCR) tests based on two primer pairs that have been described elsewhere (Carder *et al*., 1994; Pérez-Artés *et al*., 2000), it became clear that they revealed previously unrecognized variation. This study correlates this variation with biological properties to try to shed light on the origins of both amphihaploid and D-pathotype haploid isolates of *V. dahliae*.

## Materials and methods

### Isolates

The *V. dahliae* isolates used, their origins and other properties are listed in Tables 1 and 2. Karapapa *et al*. (1997) proposed a new species, *V. longisporum*, based on some of the long-spored isolates, but as the full complexity of these isolates is still not known, the name *V. dahliae* will be used here to cover all plant-pathogenic *Verticillium* isolates producing only microsclerotia as their resting structures.

### DNA extraction

At Warwick HRI (W-HRI), DNA was extracted from dried or fresh mycelium either by squash blot (for small amounts) as described by Langridge *et al*. (1991), except that Nytran N (Schleicher & Schuell) was substituted for Hybond-N, or (for larger amounts) by a commercial kit (Qiagen Dneasy) used according to the manufacturer's instructions. At the Instituto de Agricultura Sostenible (IAS), mycelium was harvested and dried as described by Pérez-Artés *et al*. (2000) and DNA extracted using the method of Raeder & Broda (1985).

### PCR, restriction fragment length polymorphism (RFLP) assays and DNA sequencing

All PCR assays used standard procedures. Primer pair 19 (5′-CGGTGACATAATACTGAGAG-3′) and 22 (5′-GACGATGCGGATTGAACGAA-3′), annealing temperature 54°C, are thought to be species-specific for *V. dahliae* (Carder *et al*., 1994). Two pairs of primers, both with an annealing temperature of 64°C, have been used to differentiate isolates of the D and ND pathotypes (Mercado-Blanco *et al*., 2001, 2002). Primer pair INTD2f (5′-ACTGGGTATGGATGGCTTTCAGGACT-3′) and INTD2r (5′-TCTCGACTATTGGAAAATCCAGCGAC-3′) produce an amplicon of around 460 bp with D-pathotype isolates. Primer pair INTND2f (5′-CTCTTCGTACATGGCCATAGATGTGC-3′) and INTND2r (5′-CAATGACAATGTCCTGGGTGTGCCA-3′) produce an amplicon of around 820 bp with ND-pathotype isolates. These two primer pairs may be used in duplex PCR assays. Primer espdef01 (5′-TGAGACTCGGCTGCCACAC-3′), derived from the sequence of the indel in the amplicon produced from some isolates by primer pair 19/22 (Mercado-Blanco *et al*., 2003), was used with primer 19 or simultaneously with both 19 and 22, with an annealing temperature of 62°C. Amplicons were separated by agarose gel electrophoresis, stained with ethidium bromide and visualized with UV light.

Where appropriate, amplicons of primers 19/22 were compared by RFLP analysis with the restriction endonucleases *Hae*III and *Alu*I, used according to the manufacturer's (Invitrogen) instructions. DNA fragments from the digestions were separated and visualized as above. For sequencing of 19/22 amplicons, primers and low-molecular-weight material were removed using a commercial kit (Qiagen). Purified PCR products were then sequenced directly using commercial services (Sequiserve, Vatterstetten, Germany and Newbiotechnic SA, Seville, Spain) employing one or more of the primers used to produce the amplicon. No evidence (i.e. double peaks) of mixed sequences was seen in the data from amphihaploid isolates, suggesting either that gene conversion had occurred or that one putative parent (presumably the non-*V. dahliae* one, as the primer pair 19/22 appeared specific for *V. dahliae* in both published [Carder *et al*., 1994] and extensive unpublished tests on haploid isolates) did not carry this sequence. Most amplicons were sequenced in only one direction. Sequence comparisons and manipulations were made using DNAStar (Lasergene). Any sequence corresponding to PCR primers was removed before comparisons were made. Accession numbers for isolates typical of the five sequences seen (other isolates also deposited, but not listed here) are: sequence 1, isolate 332, AF363250; sequence 2, MD80, AF363251; sequence 3, 90-02, AF363247; sequence 4, 001, AF363245; sequence 5, 9802, AF363243.

## Results

### PCR amplification

The results for PCR reactions using primer pair 19/22 with a collection of haploid *V. dahliae* isolates, mainly tested at IAS, and a collection of amphihaploid *V. dahliae* isolates, mainly tested at W-HRI, are shown in Tables 1 and 2. All isolates in these sets gave amplicons with these primers. Two distinct lengths of amplicon were produced,

Table 1 Source, original host, geographic origin, vegetative compatibility group (VCG), pathotype and results of polymerase chain reaction (PCR) tests with three sets of primers (with sequence of selected amplicons for primers 19/22) for haploid isolates of *Verticillium dahliae*, *V. albo-atrum* and *V. tricorpus* (tests carried out mainly at the Instituto de Agricultura Sostenible)

| Species/ isolate[a] | Original host | VCG[b] | Pt[c] | Country of origin | PCR with: | | | Seq |
|---|---|---|---|---|---|---|---|---|
| | | | | | INT D/ND | 19/22 | espdef | |
| *V. dahliae* | | | | | | | | |
| V135 I[1] | *Olea europea* | 1A | D | Spain | 460 | 550 | 330 | 4 |
| V136 I[1] | *O. europea* | 1A | D | Spain | 460 | 550 | 330 | 4 |
| V138 I[1] | *Gossypium hirsutum* | 1A | D | Spain | 460 | 550 | 330 | 4 |
| V150 I[1] | *O. europea* | 1A | D | Spain | 460 | 550 | 330 | –[d] |
| V153 I[1] | *O. europea* | 1A | D | Spain | 460 | 550 | 330 | 4 |
| V177 I[1] | *G. hirsutum* | 1A | D | Spain | 460 | 550 | 330 | 4 |
| V180 I[1] | *G. hirsutum* | 1A | D | Spain | 460 | 550 | 330 | 4 |
| V184 I[1] | *G. hirsutum* | 1A | D | Spain | 460 | 550 | 330 | – |
| V186 I[1] | *G. hirsutum* | 1A | D | Spain | 460 | 550 | 330 | – |
| V-017[2] | *Cynara cardunculus* var. *scolymus* | 1A | D | Spain | 460 | 550 | 330 | 4 |
| 1990–1[3] | *Acer* spp. | 1B | ND | USA | NA[e] | 550 | 330 | 4 |
| 9-6[3] | *Cladrastis lutea* | 1B | ND | USA | NA | 550 | 330 | 4 |
| V-001[2] | *Prunus amygdala* | 2B | ND | Spain | NA | 550 | 330 | 4 |
| V-016[2] | *C. cardunculus* | 2B | ND | Spain | NA | 550 | 330 | 4 |
| V-021[2] | *C. cardunculus* | 2B | ND | Spain | NA | 550 | 330 | 4 |
| ep4[4] | *Solanum melongena* | 2A | ND | Israel | 820 | 530 | NA | – |
| ep53[4] | *S. melongena* | 2A | – | Israel | 820 | 530 | NA | – |
| pt44·3G[4] | *S. tuberosum* | 2A | ND | Israel | 820 | 530 | NA | – |
| pt71[4] | *S. tuberosum* | 2A | – | Israel | 820 | 530 | NA | – |
| tom1[4] | *Lycopersicon esculentum* | 2A | ND | Israel | 820 | 530 | NA | – |
| tom18[4] | *L. esculentum* | 2A | – | Israel | 820 | 530 | NA | – |
| V018 I[2] | *C. cardunculus* | 2A | ND | Spain | 820 | 530 | NA | 2 |
| V143 I[1] | *O. europea* | 2A | ND | Spain | 820 | 530 | NA | 2 |
| V144 I[1] | *O. europea* | 2A | ND | Spain | 820 | 530 | NA | 2 |
| V147 I[1] | *O. europea* | 2A | ND | Spain | 820 | 530 | NA | – |
| V148 I[1] | *O. europea* | 2A | ND | Spain | 820 | 530 | NA | – |
| V152 I[1] | *O. europea* | 2A | ND | Spain | 820 | 530 | NA | 2 |
| V176 I[1] | *G. hirsutum* | 2A | ND | Spain | 820 | 530 | NA | 2 |
| V200 I[1] | *G. hirsutum* | 2A | ND | Spain | 820 | 530 | NA | – |
| V213 I[1] | *G. hirsutum* | 2A | ND | Spain | 820 | 530 | NA | 2 |
| V217 I[1] | *G. hirsutum* | 2A | ND | Spain | 820 | 530 | NA | 2 |
| cot56[5] | *G. hirsutum* | 2B | ND | Israel | 820 | 530 | NA | – |
| cot59[5] | *G. hirsutum* | 2B | ND | Israel | 820 | 530 | NA | – |
| cot117[5] | *G. hirsutum* | 2B | PD | Israel | 820 | 530 | NA | 1 |
| ep1[4] | *S. melongena* | 2B | ND | Israel | 820 | 530 | NA | – |
| ep22[4] | *S. melongena* | 2B | – | Israel | 820 | 530 | NA | – |
| tom20[4] | *L. esculentum* | 2B | – | Israel | 820 | 530 | NA | – |
| tom28[4] | *L. esculentum* | 2B | ND | Israel | 820 | 530 | NA | – |
| V-008[2] | *C. cardunculus* | 2B | ND | Spain | 820 | 530 | NA | 1 |
| V-013[2] | *C. cardunculus* | 2B | ND | Spain | 820 | 530 | NA | – |
| V-022[2] | *P. amygdala* | 2B | ND | Spain | 820 | 530 | NA | 1 |
| MD71[6] | *Matricaria chamomilla* | 2B | – | Germany | 820 | 530 | – | 1 |
| MD124[6] | *M. chamomilla* | 2B | – | Germany | 820 | 530 | – | (1)[f] |
| cot22[5] | *G. hirsutum* | 4B | PD | Israel | 820 | 530 | NA | – |
| cot23[5] | *G. hirsutum* | 4B | PD | Israel | 820 | 530 | NA | – |
| cot24[5] | *G. hirsutum* | 4B | ND | Israel | 820 | 530 | NA | – |
| cot40[5] | *G. hirsutum* | 4B | PD | Israel | 820 | 530 | NA | 2 |
| cot92[5] | *G. hirsutum* | 4B | ND | Israel | 820 | 530 | NA | 2 |
| cot120[5] | *G. hirsutum* | 4B | PD | Israel | 820 | 530 | NA | – |
| cot129[5] | *G. hirsutum* | 4B | ND | Israel | 820 | 530 | NA | 2 |
| ep17[4] | *S. melongena* | 4B | – | Israel | 820 | 530 | NA | – |
| ep47[4] | *S. melongena* | 4B | – | Israel | 820 | 530 | NA | – |
| pt15[4] | *S. tuberosum* | 4B | ND | Israel | 820 | 530 | NA | – |
| pt63[4] | *S. tuberosum* | 4B | – | Israel | 820 | 530 | NA | – |
| tom5[4] | *L. esculentum* | 4B | ND | Israel | 820 | 530 | NA | – |
| tom26[4] | *L. esculentum* | 4B | – | Israel | 820 | 530 | NA | – |
| V188 I[5] | *G. hirsutum* | 4B | ND | Spain | 820 | 530 | NA | – |

Table 1 *Continued*

| Species/ isolate[a] | Original host | VCG[b] | Pt[c] | Country of origin | PCR with: INT D/ND | 19/22 | espdef | Seq |
|---|---|---|---|---|---|---|---|---|
| V192 I[5] | *G. hirsutum* | 4B | ND | Spain | 820 | 530 | NA | – |
| V227 I[5] | *G. hirsutum* | 4B | ND | Spain | 820 | 530 | NA | – |
| Vd128[7] | *Brassica oleracea* var. *botrytis* | 4[g] | – | Germany | 820 | 530 | NA | 2 |
| VdCa147a[8] | *Capsicum annuum* | 6 | – | USA | 820 | 530 | NA | 1 |
| Fv343[9] | *P. amygdala* | – | – | Italy | 820 | 530 | NA | 2 |
| 0190[9] | *O. europea* | – | – | Italy | 820 | 530 | NA | 2 |
| P14[10] | *L. esculentum* | – | – | Brazil | 820 | 530 | – | 2 |
| 332[11] | *Fragaria ananassa* | – | – | UK | 820 | 530 | – | 1 |
| MD80[6] | *B. napus* ssp. *oleifera* | (si)[g] | – | Germany | – | 530 | – | 2 |
| *V. albo-atrum* | | | | | | | | |
| V48 I[2] | *Humulus lupulus* | – | – | UK | NA | NA | NA | |
| 1974[11] | *H. lupulus* | – | – | UK | NA | NA | NA | |
| STR3[12] | *Medicago sativa* | – | – | Canada | 820 | NA | NA | |
| *V. tricorpus* | | | | | | | | |
| V53 I | Soil | – | – | USA | NA | NA | NA | |

[a]Supplier or reference: 1, Pérez-Artés *et al.* (2000) ; 2, IAS collections ; 3, Dr T. Katan ; 4, Korolev *et al.* (2000); 5, Korolev *et al.* (2001); 6, Dr H. Prillinger; 7, Professor M. Cirulli; 8, Dr K. Subbarao; 9, Dr F. Nigro; 10, A. Soares; 11, HRI collection; 12, Dr K. Broersma.
[b]VCG determined using nit mutants as described by Korolev & Katan (1997).
[c]Pathotype, previously determined by García-Andrés *et al.* (2001), Korolev *et al.* (2000) or Korolev *et al.* (2001): D, defoliating; ND, nondefoliating; PD, partially defoliating.
[d]–, not known or not tested in this work.
[e]NA, no amplification.
[f]Sequence type determined by RFLP analysis only.
[g]VCGs 4A and B not differentiated in test at HRI.
[h]si, self-incompatible in VCG tests.

estimated by agarose gel electrophoresis at 530 and 550 bp. Sequencing of amplicons from selected isolates produced five distinct sequences. Sequences 1 and 2 were found only in haploid isolates, and sequences 3 and 5 only in amphihaploid isolates. Sequence 4 was found in both types of isolates (Tables 1 and 2). In BLAST searches of nucleotide databases, none of these five sequences produced significant matches. RFLP analysis of the 19/22 amplicon suggested that the majority of the amphihaploid isolates gave amplicons similar to sequence 3, although minor differences may not have been detected. This was certainly the case with the amplicons with sequences 4 and 5, which were not differentiated by RFLP analysis (Table 2), but actually differed by a single base. Sequences 1–3 differed from each other by 5–10 bp (Fig. 1). Sequences 4 and 5 differed from sequences 1–3 by eight to 12 individual base changes and also by the presence of a 15-bp indel (Fig. 1). The sequence of primer espdef01 was based on this indel (Mercado-Blanco *et al.*, 2003); as expected when tested on haploid *V. dahliae* isolates with either primer 19 or primers 19 and 22 simultaneously, this primer gave 330-bp amplicons only with isolates that gave the larger (~550 bp) amplicon with primers 19/22 alone (Table 1).

The primer pairs INTD2f/r and INTND2f/r gave three types of results with haploid isolates viz. amplicons estimated by electrophoresis at either 460 (INTD2f/r) or 820 (INTND2f/r) bp, or no amplification (Table 1). All 50 isolates tested which had given the smaller amplicon with primers 19/22 gave the larger amplicon with INTD2/INTND2. The 15 isolates giving the 550-bp amplicon with primers 19/22 gave either a 460-bp amplicon (10 isolates) or no amplification (five isolates) with INTD2/INTND2 (Table 1).

## Correlation with other properties

Data, according to isolate supplier or as in references given, for original host, vegetative compatibility group (VCG), pathotype and country of origin are shown for the haploid *V. dahliae* isolates in Table 1 and summarized in Table 3, and for country of origin, host and AFLP type for the amphihaploid *V. dahliae* isolates in Table 2. Amongst the amphihaploid isolates there was a clear correlation of amplicon size (estimated by gel electrophoresis at 530 or 550 bp) produced with primers 19/22 with AFLP group (determined by Collins *et al.*, 2003). All α-group isolates gave amplicons with sequence 3 (determined by complete sequencing or by RFLP analysis). There was no differentiation of these isolates by geographic source or host (all came from *Brassica* spp., except 86207 from *Raphanus raphanistrum*). With the same primers, the four AFLP β-group isolates gave the larger amplicon. The two isolates of this group with sequence 5 came from Germany and the other two from the USA. However, with such a small number of isolates, the importance of this correlation, or even of the single base difference distinguishing the sequences, is arguable. Three of these isolates came from

Table 2 Source, original host, geographic origin and results of PCR tests (with RFLP analysis and sequence of selected amplicons from primer pair 19/22) for amphihaploid isolates of *Verticillium dahliae* (tests mainly carried out at HRI)

| | | | | PCR | | Amplicon | |
|---|---|---|---|---|---|---|---|
| Isolate[a] | Original host | Country of origin | AFLP group[b] | D/ND | 19/22 | RFLP | Sequence |
| 9802[2] | *Armoracia rusticana* | Germany | β | 820 | 550 | 4/5[c] | 5 |
| MD73[3] | *Brassica napus* ssp. *oleifera* | Germany | β | NA[d] | 550 | 4/5 | 5 |
| 001[1] | *A. rusticana* | USA | β | NA | 550 | 4/5 | 4 |
| 004[1] | *A. rusticana* | USA | β | NA | 550 | 4/5 | –[e] |
| 162[4] | *B. napus* ssp. *oleifera* | Sweden | α | – | 530 | 3 | – |
| 334[4] | *B. napus* ssp. *oleifera* | Germany | α | – | 530 | 3 | – |
| 617[4] | *B. napus* ssp. *oleifera* | France | α | – | 530 | 3 | – |
| 668[4] | *B. napus* ssp. *oleifera* | France | α | – | 530 | 3 | – |
| 855[4] | *B. napus* ssp. *oleifera* | Germany | α | – | 530 | 3 | 3 |
| 892[4] | *B. napus* ssp. *oleifera* | France | α | – | 530 | 3 | – |
| 84020[5] | *B. campestris* spp. *rapifera* | Japan | α | – | 530 | 3 | – |
| 84120[5] | *B. campestris* spp. *pekinenesis* | Japan | α | – | 530 | 3 | 3 |
| 84122[5] | *B. campestris* spp. *pekinenesis* | Japan | α | – | 530 | 3 | – |
| 86207[5] | *Raphanus raphinistrum* | Japan | α | – | 530 | 3 | – |
| 90-02[6] | *B. oleracea* var. *botrytis* | USA | α | – | 530 | 3 | 3 |
| 90-03[6] | *B. oleracea* var. *botrytis* | USA | α | NA | 530 | 3 | – |
| 90-10[6] | *B. oleracea* var. *botrytis* | USA | α | NA | 530 | 3 | 3 |
| MD57[3] | *B. napus* ssp. *oleifera* | Germany | α | – | 530 | 3 | – |
| MD123[3] | *B. napus* ssp. *oleifera* | Germany | α | – | 530 | 3 | – |
| Vd1[7] | *B. napus* ssp. *oleifera* | Sweden | α | – | 530 | 3 | 3 |
| Vd4[7] | *B. napus* ssp. *oleifera* | Sweden | α | – | 530 | 3 | – |
| Vd11[7] | *B. napus* ssp. *oleifera* | Sweden | α | – | 530 | 3 | – |
| Vd191[8] | *B. campestris* spp. *utilis* | Italy | α | – | 530 | 3 | – |
| Vd292[8] | *B. oleracea* var. *botrytis* | Italy | α | – | 530 | 3 | – |
| VdII[9] | *B. napus* ssp. *oleifera* | Germany | α | NA | 530 | 3 | – |
| VdIII[9] | *B. napus* ssp. *oleifera* | Germany | α | – | 530 | 3 | – |
| VdIV[9] | *B. napus* ssp. *oleifera* | Germany | α | – | 530 | 3 | – |

[a]Suppliers of isolates: 1, Professor D. Eastburn; 2, Belgium Co-ordinated Collections of Microorganisms; 3, Dr H. Prillinger; 4, Dr H. Brun; 5, Dr S. Horiuchi; 6, Dr S. Koike; 7, Dr I. Happstadius; 8, Professor M. Cirulli; 9, Dr B. Holtschulte.
[b]AFLP group as reported in Collins *et al.* (2003).
[c]RFLP analysis used did not distinguish sequences 4 and 5.
[d]NA, no amplification.
[e]–, not tested.

*Armoracia rusticana* (horseradish) and the fourth from *B. napus* ssp. *oleifera* (oilseed rape).

For the haploid isolates, the larger amplicons produced by primer pair 19/22 were all sequence 4 (although amplicons from three isolates were not sequenced). As mentioned, this larger amplicon was associated with either the 460-bp product or with no amplification with INTD2f/r and INTND2f/r. Isolates from Spain of the D pathotype and VCG1A all gave the combination of 550/460-bp products. The five isolates giving 550 bp/no amplification came from the USA or Spain and were of the ND pathotype. The American isolates were VCG 1B (the only two of this VCG subgroup found) and the Spanish VCG 2B; VCG 2B was also found amongst isolates giving the smaller amplicon with primers 19/22. The combination of the smaller (530 bp) 19/22 product with the larger INTND2f/r (820 bp) was found with all the VCGs other than 1A/1B (i.e. VCG2A, 2B, 4B and 6) and, where known, were all ND or partially defoliating (PD, highly virulent isolates that induce partial defoliation affecting mainly lower to mid-leaves [Korolev *et al.*, 2001] ) on cotton. There was no clear correlation with country of origin, except that the all the VCG 1A/defoliating isolates came from Spain (Table 1). The smaller 19/22 amplicon was sequenced for 21 isolates and these all gave sequences 1 or 2. All four VCG 2B isolates with this smaller amplicon and the VCG 6 isolate gave sequence 1, and all 11 VCG2A/4B(4) isolates gave sequence 2.

## Discussion

Molecular variation in short-spored, haploid isolates of *V. dahliae* has been reported previously and some association of this variation with other properties recognized, mainly within isolates from particular countries, e.g. pathotype in Japan (Carder & Barbara, 1994). These associations may reflect the vagaries of past movements of isolates; for example, in the UK, the close association of VCG and RFLP group in the majority of isolates probably reflects two main importations (Carder & Barbara, 1994).

The D pathotype of *V. dahliae* in Spain has previously been shown to be correlated with VCG1 (Daayf *et al.*,

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Seq5 CATAATACTGAGAGAAGCATGCGACAGGAGCGATGAGCCCCTCAGTCGCGCCGACTTCGT 60
Seq4 ...........................................G................
Seq3 ...........................................G................
Seq2 ...........................................G................
Seq1 ...........................................G................

Seq5 CCCGAGCTCTGAAGTAGTGGCAAGCGTGCATGTCGGCGAGTTTGGCATGTTTAGCATCCC 120
Seq4 ............................................................
Seq3 ..............C.............G...............................
Seq2 ..............C.............G...............................
Seq1 ............................................................

Seq5 GCTCGTCATGGGTTTCGGCAACTCCAGCAAAGTTCCCCTGCACGGGTCCTGCAGCTTTCT 180
Seq4 ............................................................
Seq3 .....................................-...........C..........
Seq2 .....................................-...........C..........
Seq1 ..........T......................................C..........

Seq5 GGTTCAGATGGGCGCGGGCCTGAAGAATATGCGGCAGTCTATCGACCATGTCCTCGAGGG 240
Seq4 ............................................................
Seq3 ..............................................T.............
Seq2 ..............................................T....T........
Seq1 ..............................................T....T........

Seq5 AGGCTTAAGTTAACTACGGCACTAAAGGGTCAGCCAGGTATGAGGTCCATATCCAACACG 300
Seq4 ............................................................
Seq3 .....C......................................................
Seq2 .....C...................................A..................
Seq1 .....C...................................A..................

Seq5 AGCTGGAGCGTGTGGCAGCCGAGTCTCAGCTTCTTCTATGACCTCGTCTGCATCCAGTAG 360
Seq4 ............................................................
Seq3 ........---------------.....................................
Seq2 ........---------------.....................................
Seq1 ........---------------.....C...............................

Seq5 TATCTTATATACATGACAGCGATGAGACTGTCGAGCACCTCAGCCATCGCAGGTCAGTGC 420
Seq4 ............................................................
Seq3 ............................................................
Seq2 ........................T................G..................
Seq1 ........................T................G..................

Seq5 TATGGGAATTAAATTGGATTATATCGTCAACAAAAATATCAGCATTAAGAAGACTAACAT 480
Seq4 ............................................................
Seq3 ..............G..........................................G.
Seq2 ..............G.............................................
Seq1 ..............G.............................................

Seq5 TTTTAATAATGGAACAGTAGTCCCAC 506
Seq4 ..........................
Seq3 ..........................
Seq2 ..........................
Seq1 ..........................
```

Figure 1 Sequence alignment of the five distinct sequences found for the amplicon produced by primer pair 19/22. See Tables 1 and 2 for *Verticillium* isolates showing each sequence. Identical bases are indicated by dots (.) and the absence of bases by dashes (-).

1995). However, assessing either VCG or pathotype for a new isolate is labour-intensive and a PCR test using primer pairs INTD2f/r and INTND2f/r is much more rapid and can be used directly on plants to distinguish the D and ND pathotypes (Mercado-Blanco *et al*., 2001, 2002). As expected, these primer pairs gave amplicons of two sizes. The smaller amplicon was correlated with the defoliating pathotype, but some isolates did not give a product with these primers. The second marker (primer pair 19/22) gave, as expected, amplicons with all the microsclerotial isolates (i.e. *V. dahliae*) tested here. These primers consistently give no amplification with other *Verticillium* spp., including the closely related *V. albo-atrum*, either here or in other tests (unpublished). Unexpectedly, these primers gave amplicons of two sizes and three distinct sequences with haploid isolates. For these isolates, the larger amplicons all had the same sequence (4), distinct from the other two sequences by seven or 10 single base changes and the presence of a 15-bp indel. The group giving the larger amplicon was divided by the primer pairs INTD2f/r and INTND2f/r, which gave amplicons with all the D isolates, but not with five ND isolates from the USA or Spain. It has been reported (Mercado-Blanco *et al*., 2003) that using 19/espdef01 improves PCR tests intended to distinguish the D and ND isolates. An important practical outcome of the studies here is that this primer pair cannot be used reliably for this purpose. Although relatively few amplicons were sequenced for those isolates giving the smaller amplicon, for the well-established VCG groups there was a correlation of 2A and 4B with sequence 2, and of 2B with sequence 1. The newly described VCG 6 isolate (Bhat *et al*., 2003) also had sequence 1 and was not molecularly distinct from VCG 2B isolates.

Table 3 Summary of main results in Table 1 for *Verticillium dahliae* haploid isolates grouped by 19/22 amplicon sequence, amplicons produced, vegetative compatibility group (VCG) and country

| | Amplicon size after PCR | | | | | | |
|---|---|---|---|---|---|---|---|
| Seq of 19/22 | 19/22 | INTD/ND | espdef | Pt[a] | Source VCG | No. of country | Isolates[b] |
| 4 | 550 | 460 | 330 | D | 1A | Spain | 10 |
| 4 | 550 | NA[c] | 330 | ND | 1B | USA | 2 |
| | | | | ND | 2B | Spain | 3 |
| 2 | 530 | 820 | NA | ND | 2A | Spain | 4 |
| | | | | ND/PD | 4B | Israel | 13 |
| | | | | –[d] | (4[e]) | Germany | 1 |
| | | | | – | – | Italy | 2 |
| 1 | 530 | 820 | NA | ND/PD | 2B | Israel | 10 |
| | | | | ND | 2B | Spain | 3 |
| | | | | – | 2B | Germany | 2 |
| | | | | – | 6 | USA | 1 |
| – | 530 | 820 | NA | ND | 2A | Israel | 6 |
| | | | | ND | 4B | Spain | 3 |

[a]Pathotype: D, defoliating; ND, nondefoliating; PD, partially defoliating.
[b]Not all isolates tested for all properties (see Tables 1 and 2 for details).
[c]NA, no amplification.
[d]–, not known.
[e]Subgroup not known.

Long-spored isolates from crucifers have been suggested to be interspecific hybrids between haploid *V. dahliae* isolate(s) and isolate(s) of *V. albo-atrum* from lucerne (Karapapa *et al*., 1997). Understanding the identity of the 'parents' of these crucifer pathogens is important; if the 'parents' are common and widespread then new crucifer-pathogenic isolates might arise wherever these crops are grown. On the other hand, if they have limited distribution, then movement of existing isolates forms the main threat of wilt of crucifers occurring in new regions.

Earlier AFLP studies have divided the long-spored isolates into two groups (Collins *et al*., 2003). It was further shown that in some ways the non-*V. dahliae* 'parents' of the α-AFLP-group isolates and two of the β-group isolates (001 and 004) were very distinct molecularly from either of the two recognized pathotypes/molecular groups of *V. albo-atrum* (Collins *et al*., 2003). Sequence 3 found for the 19/22 amplicon from α-group *V. dahliae* amphihaploids is quite distinct from that from haploid *V. dahliae* isolates, implying that the *V. dahliae* 'parent' (presumed to be haploid) is molecularly unlike any of the haploid isolates so far studied. However, for 001 and 004, the *V. dahliae* 'parent' is, at least according to this marker, indistinguishable from those haploid isolates with sequence 4. Neither of the β-group isolates 001 or 004 gave a product with INTD2f/r or INTND2f/r, making them similar to five either Spanish or American ND isolates and suggesting that the *V. dahliae* 'parent' of these isolates may resemble these five. The remaining two β-group isolates so far identified did not retain the *V. albo-atrum*-like rRNA markers used previously, and at present nothing definite can be said about their non-*V. dahliae* 'parent'. However, these isolates showed small differences from haploid isolates in a *V. dahliae*-derived rRNA marker (Collins *et al*., 2003) and in the 19/22 amplicon studied here. The significance of small differences is arguable until more information is available on the range of variation in these markers. These two isolates gave different results with the INTD2f/r and INTND2f/r primers pairs. For isolate 9802, the combination of the 550-bp 19/22 amplicon with the 820-bp product (INTND2f/r) was unique among the isolates studied here. The *V. albo-atrum* lucerne isolate STR3 also gave an 820-bp product. If the amplicon is derived from the *V. dahliae* 'parent' then this is molecularly distinct from all *V. dahliae* isolates studied. However, it may also suggest that the non-*V. dahliae* 'parent' of this isolate resembles the lucerne pathotype in this respect. Like 001 and 004, MD73 gave no product with the INTD2f/r and INTND2f/r primers, possibly suggesting their 'parent' was similar to, but slightly distinct from, haploid isolates with 19/22 sequence-4 amplicons. It may also be that MD73 had the same origin as 9802, but has subsequently lost the 820-bp marker amplified by the INTND2f/r primers. As MD73 was the only amphihaploid isolate which consistently seemed to have retained a minor rRNA repeat sequence (Collins *et al*., 2003), perhaps the differentiation from 9802 was not entirely surprising.

On the basis of being somewhat distinct from the other haploid isolates tested in AFLP analysis, it was suggested that the two short-spored isolates Vd128 and MD80 from crucifers, and possibly also the Brazilian isolate P14 from tomato, were derived from amphihaploid isolates by rehaploidization (Collins *et al*., 2003). The 19/22 amplicons from these isolates gave sequence 2. Whilst not completely ruling out their being derived from other hybrid isolates, this strongly suggests that these three isolates are not derived from amphihaploids in either AFLP group studied here. Why the only two short-spored isolates

from crucifers studied in this way are so divergent in AFLP analysis and distinct in the rRNA related 'V-region' (Collins *et al*., 2003) remains unexplained.

Some groups of virulent *V. dahliae* isolates have been shown to have multiple origins, e.g. Canadian isolates able to overcome the *Ve* resistance gene in tomato (Dobinson *et al*., 1998). The important conclusion from the work reported here with regard to phytosanitary policy in Europe is that the main threat of *V. dahliae* D or crucifer-pathogenic isolates emerging in new areas is the result of importation (rather than selection from existing populations) or new hybridization events. The damaging D-pathotype isolates from Spain are clearly distinct by one or two molecular markers and VCG from the other European isolates studied here. They also appear homogenous, suggesting that this pathotype may have been introduced from an unknown source country outside Europe, possibly on a single occasion. It is known that isolates molecularly and pathologically similar to these do occur elsewhere (Pérez-Artés *et al*., 2000). Similarly, both the 'parents' of the widespread α-AFLP-group of hybrid crucifer-pathogenic isolates are unlike any studied here for the *V. dahliae* 'parent' or by Collins *et al*. (2003) for the non-*V. dahliae* 'parent'. These hybrid isolates presumably arose in some unknown region where both these unusual 'parent' types occur. The same can be said, although with less certainty because the sequence differences are small and less well documented, for the two European β-group isolates; the *V. dahliae* 'parent' appears distinct by one or two markers from European haploid isolates. These amphihaploid isolates are currently under further study for molecular clues as to the identity of the second 'parent'. The apparent difference here in one marker between these two isolates, and the fact that they were already known possibly to differ in the retention of an infrequent second rRNA repeat (Collins *et al*., 2003), emphasizes the apparently complex origins of long-spored isolates from crucifers and the need for caution in categorizing them. Only four β-AFLP-group isolates are known and it is necessary to identify and study more of them before the full complexity of this group can be known. Although one haploid isolate molecularly resembling the *V. dahliae*-like 'parent' of the two American β-group isolates has been found in the USA, the results of Collins *et al*. (2003) suggest the other 'parent' has not yet been described. It is possible that these hybrid isolates arose in the USA and a targeted search for isolates similar to the putative non-*V. dahliae* 'parent' could provide support for this suggestion.

## Acknowledgements

AC was supported by a studentship funded by Harper Adams University College. JMB is a contract holder under the Ramón y Cajal Programme of the Ministerio de Educación y Ciencia. EC was supported by a Biotechnology and Biological Sciences Research Council studentship. Research support at both IAS and HRI was provided by the European Commission (Framework Programme 5; project reference number QLRT-1999-1523).

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