Plant Pathology (2011) **60,** 866–877 Doi: 10.1111/j.1365-3059.2011.02432.x

Development and application of new molecular markers for analysis of genetic diversity in *Verticillium dahliae* populations

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The aim of this study was to develop new polymorphic markers for analysis of genetic diversity in the fungal soilborne plant pathogen *Verticillium dahliae*. Twelve polymorphic markers (five microsatellites and seven polymorphic sequences) were developed from a genomic library enriched for microsatellites. Screening of polymorphic loci was done using a collection of 25 *V. dahliae* isolates of diverse geographic origins, host sources and vegetative compatibility groups (VCGs). Three methods were used to score alleles: polyacrylamide gel electrophoresis (PAGE), sequencing of PCR-amplified loci, and capillary electrophoresis. The new markers were used to assess genetic differentiation between isolates associated with different host plants. Two collections of isolates were analysed, obtained from artichoke (30 isolates) and potato (20 isolates) from crops grown in rotation located in the same area in eastern-central Spain. The resolution of genetic differentiation between these two collections using the new markers was compared to that provided by other often-used markers (SCARs and VCGs). Sequence analysis of the alleles proved to be the most unambiguous technique for scoring microsatellite data. The relatively high genetic differentiation observed between isolates from different crops (genetic differentiation coefficient, G_{ST} = 0·24) and their high genotypic diversity suggest a divergence between *V. dahliae* from artichoke and potato. It is hypothesized that evolution of *V. dahliae* from the local resident population in association with the two host crops has occurred. The new markers are useful for resolving population structure within *V. dahliae* and may contribute to a better understanding of the population biology of this fungus.

Keywords: fungal ecology, host adaptation, molecular markers, SSRs, verticillium wilt

Introduction

Verticillium dahliae is a strictly asexually reproducing soilborne pathogen that causes wilt diseases in hundreds of important crops worldwide (Pegg & Brady, 2002). Asexual reproduction results in offspring that are genetically identical to each other and to the parental isolate, but which accumulate diversity through mutations, leading to populations comprised of clonal lineages (Milgroom, 1996). Genetic diversity in *V. dahliae* has traditionally been studied by means of vegetative compatibility grouping (Strausbaugh *et al.*, 1992; Daayf *et al.*, 1995; Korolev *et al.*, 2000; Jiménez-Díaz *et al.*, 2006). Five main vegetative compatibility groups (VCGs) have been identified in *V. dahliae* isolates from different hosts and geographic origins (VCG1, VCG2, VCG3, VCG4 and VCG6). Several VCGs were later subdivided into subgroups (i.e. VCG1A and VCG1B, VCG2A and VCG2B, and VCG4A and VCG4B) based on vigour and frequency of complementation to tester isolates (Joaquim & Rowe, 1990; Strausbaugh *et al.*, 1992; Rowe, 1995; Bhat *et al.*, 2003). Recently, VCG2B isolates infecting artichoke in eastern-central Spain were found to be genetically heterogeneous and were further subdivided into two subgroups, namely VCG2Ba and VCG2Br, based on differential complementation with tester isolates (Jiménez-Díaz *et al.*, 2006). VCG2Br isolates were defined by complementation of nitrate-nonutilizing (*nit*) mutants of the isolates with the complementary mutants of an international collection of tester isolates from the Ohio Agricultural Research and Development Center, Ohio State

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Published online 23 February 2011

University (OARDC-OSU), and from Israel (Rowe, 1995; Korolev *et al.*, 2000). Conversely, VCG2Ba isolates were characterized by positive complementation of *nit1* mutants of the tested isolates with *nit* mutants of VCG2B isolates from artichoke but failure to establish complementation with VCG2B *nit* mutants of tester isolates in the OARDC-OSU and Israeli collections (Jiménez-Díaz *et al.*, 2006). Furthermore, these VCG2B subgroups showed diversity in polymerase chain reaction (PCR)-based amplicon markers of 334 and 824 bp previously associated with cotton and olive defoliating (D) or nondefoliating (ND) *V. dahliae* pathotypes, respectively (Mercado-Blanco *et al.*, 2003; Collins *et al.*, 2005; Jiménez-Díaz *et al.*, 2006). As a result of new molecular analyses of *V. dahliae* populations, it is now known that VCGs, previously thought to comprise groups of isolates with limited genetic variability, may represent more diversity than anticipated (Collado-Romero *et al.*, 2006, 2008; Jiménez-Díaz *et al.*, 2006).

Previous studies of genetic diversity in *V. dahliae* using amplified fragment length polymorphism (AFLP) markers indicated a positive relationship between genetic groups and VCG subgroups, regardless of geographic origin or host source (Collado-Romero *et al.*, 2006, 2008), but intraclonal lineage resolution was poor. However, genetic diversity studies examining the relationship between VCGs and host specificity have often yielded contradictory results. For example, restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) analyses grouped *V. dahliae* isolates from different hosts and geographic origins according to VCGs (Okoli *et al.*, 1994; Dobinson *et al.*, 2000; Zeise & von Tiedemann, 2002). However, similar correlations were not found in other studies. For example, RAPD analyses revealed minor genetic variation among isolates from different hosts or VCGs (Bhat & Subbarao, 1999; Bhat *et al.*, 2003).

Resolving variation within clonal lineages in *V. dahliae* populations requires the use of highly polymorphic, easily scorable molecular markers. Microsatellite [simple sequence repeat (SSR)] markers and sequences containing single nucleotide polymorphisms (SNP) provide powerful tools for population studies (Taylor *et al.*, 1999). Microsatellites are single-locus, codominant markers that are highly variable, easy to score and reproducible; thus, they offer suitable resolution for the analysis of individual isolates within a population and phylogenetic relationships between closely related taxa (Cooke & Lees, 2004). Microsatellite markers were previously developed for *Verticillium* sp. from an interspecific, long-spored, amphihaploid isolate (*V. longisporum*) (Barbara *et al.*, 2005). However, haploid *V. dahliae* isolates yielded unsuccessful amplifications (Jiménez-Gasco, unpublished results), suggesting that the microsatellite markers were developed from the portion of the *V. longisporum* genome that corresponds to an unknown *Verticillium* sp. and not from *V. dahliae*. A problem often associated with the use of microsatellite markers is lack of accuracy in determination of allele size, mainly because of small size differences between alleles (Gobbin *et al.*, 2003).

Artichoke and potato crops can be severely affected by verticillium wilt in vegetable-producing areas of eastern-central Spain where both crops are repeatedly grown in rotation (Berbegal *et al.*, 2007; Ortega & Pérez, 2007). Moreover, *V. dahliae* isolates from artichoke and potato plants in this region have been demonstrated to be cross-pathogenic under controlled conditions (controlled inoculations and optimal environment for disease development); thus, these isolates have the potential to contribute to epidemics of verticillium wilt on both crops (Ortega & Pérez, 2007). However, the extent of the contribution of inoculum generated by the pathogen in one crop to verticillium wilt epidemics in another crop remains unknown. Therefore, analysis of *V. dahliae* associated with artichoke and potato from this region may provide an important tool for addressing epidemiological questions regarding possible sources of inoculum, the role of different hosts in epidemic development and the evolution of virulence in *V. dahliae*.

The main goal of this study was to develop highly polymorphic and easily scorable molecular markers for use in resolving genetic diversity and population structure in *V. dahliae*. The specific objectives of this research were: (i) to develop reproducible polymorphic markers for genetic analysis of *V. dahliae* populations and to compare available techniques for scoring allelic variation; and (ii) to analyse the potential of these markers for assessing genetic diversity by studying two collections of *V. dahliae* obtained from artichoke and potato crops located in eastern-central Spain. In order to establish the potential of these new markers for resolving population differentiation, they were compared with markers that are currently available for *V. dahliae* population analysis: VCGs and sequence-characterized amplified regions (SCAR markers) (Jiménez-Díaz *et al.*, 2006; Collado-Romero *et al.*, 2009).

Materials and methods

Fungal isolates, culture conditions and DNA extraction

A set of 75 monoconidial *V. dahliae* isolates from diverse host sources and geographic origins was used in this study. This set included a collection of 25 isolates from artichoke, aubergine, chickpea, cotton, muskmelon, olive, pepper, potato, pumpkin, strawberry, tomato and watermelon of diverse geographic origin (China, Greece, Israel, Italy, Spain, UK and USA) and VCGs (i.e. VCG1A, VCG1B, VCG2A, $VCG2Br_{834}$, $VCG2Br_{334}$, $VCG2Ba_{334}$, VCG4B and VCG6), that were used for marker development and screening for polymorphic markers (Table 1). The newly developed markers were subsequently used to analyse the genetic diversity of collections of *V. dahliae* isolates obtained from artichoke (30 isolates) and potato (20 isolates) (Table 1).

Table 1 *Verticillium dahliae* culture collections used in this study, including vegetative compatibility group (VCG), host source, geographic origin and multilocus genotype generated by combining alleles of four microsatellite markers and seven polymorphic sequences generated in this study

Isolate code	Host	Geographic origin	VCG[ab]	Multilocus genotype[c]										
Multi-host isolate collection used for marker development														
V2	Watermelon	Spain	2A	2	1	1	2	3	4	1	1	2	2	3
V10	Pumpkin	Spain	2A	2	1	1	2	3	4	1	1	2	2	3
V20	Muskmelon	Spain	2A	2	1	1	2	3	4	1	1	2	2	3
V25	Tomato	Spain	2A	2	1	1	2	3	4	1	1	2	2	3
V135	Olive	Spain	1A	2	1	1	1	4	1	2	1	2	1	1
V138	Cotton	Spain	1A	2	1	1	1	4	1	2	1	2	1	1
V158	Aubergine	Spain	4B	1	1	1	2	3	4	3	1	2	2	3
V185	Cotton	Spain	2A	2	1	1	2	3	3	1	1	2	2	3
V258	Chickpea	Italy	$2Br_{824}$	2	3	2	2	1	7	1	2	1	2	3
V285	Cotton	Israel	$2Br_{824}$	2	3	2	2	1	7	1	2	1	2	3
V304	Cotton	Israel	4B	1	1	1	2	3	4	3	1	2	2	3
V357	Cotton	China	$2Br_{824}$	2	3	2	2	1	7	1	2	1	2	3
V473	Artichoke	Spain	$2Br_{334}$	2	4	1	1	2	5	1	3	2	1	1
V488	Potato	Israel	2A	1	2	1	2	3	4	1	1	2	2	2
V536	Artichoke	Spain	$2Br_{824}$	2	3	2	2	1	8	1	2	1	2	3
V547	Artichoke	Spain	HSI[d]	2	4	1	1	2	9	1	3	2	1	1
V551	Strawberry	UK	4B	1	1	1	2	3	4	3	1	2	2	3
V552	Strawberry	UK	$2Br_{824}$	2	3	2	2	1	7	1	2	1	2	3
V553	Strawberry	UK	4B	1	1	1	2	3	4	3	1	2	2	3
V561	Pepper	USA	6	2	1	1	2	5	7	1	2	3	2	3
V610	Cotton	Spain	1A	2	1	1	1	4	2	2	1	2	1	1
V666	Cotton	Greece	1B	2	1	1	1	4	2	2	1	2	1	1
V675	Artichoke	Spain	$2Ba_{334}$	2	4	1	1	2	6	1	3	2	1	1
V701	Artichoke	Spain	$2Ba_{334}$	2	4	1	1	2	5	1	3	2	1	1
V789	Olive	Spain	4B	1	1	1	2	3	4	3	1	2	2	3
Artichoke isolate collection														
403	Artichoke	Spain	1A	2	1	1	1	4	2	2	1	2	1	1
404	Artichoke	Spain	2A	2	1	1	2	3	2	1	1	2	2	3
534	Artichoke	Spain	$2Br_{824}$	2	3	2	2	1	7	1	2	1	2	3
535	Artichoke	Spain	$2Br_{824}$	2	3	2	2	1	7	1	2	1	2	3
537	Artichoke	Spain	HSI	2	4	1	1	2	9	1	3	2	1	1
540	Artichoke	Spain	$2Br_{334}$	2	4	1	1	2	5	1	3	2	1	1
542	Artichoke	Spain	2A	2	1	1	2	3	2	1	1	2	2	3
549	Artichoke	Spain	$2Br_{334}$	2	4	1	1	2	5	1	3	2	1	1
574	Artichoke	Spain	$2Ba_{334}$	2	4	1	1	2	5	1	3	2	1	1
575	Artichoke	Spain	2A	2	1	1	2	3	4	1	1	2	2	3
576	Artichoke	Spain	2A	2	1	1	2	3	4	1	1	2	2	3
591	Artichoke	Spain	2A	2	1	1	2	3	4	1	1	2	2	3
593	Artichoke	Spain	$2Br_{824}$	2	3	2	2	1	7	1	2	1	2	3
613	Artichoke	Spain	$2Br_{334}$	2	4	1	1	2	6	1	3	2	1	1
676	Artichoke	Spain	$2Ba_{334}$	2	4	1	1	2	6	1	3	2	1	1
677	Artichoke	Spain	$2Ba_{334}$	2	4	1	1	2	5	1	3	2	1	1
680	Artichoke	Spain	$2Ba_{334}$	2	4	1	1	2	5	1	3	2	1	1
683	Artichoke	Spain	4B	2	2	1	2	3	4	1	1	2	2	2
684	Artichoke	Spain	4B	2	2	1	2	3	3	1	1	2	2	2
687	Artichoke	Spain	4B	2	2	1	2	3	3	1	1	2	2	2
691	Artichoke	Spain	2A	2	1	1	2	3	4	1	1	2	2	3
692	Artichoke	Spain	2A	2	1	1	2	3	4	1	1	2	2	3
693	Artichoke	Spain	$2Ba_{334}$	2	4	1	1	2	5	1	3	2	1	1
698	Artichoke	Spain	$2Br_{334}$	2	4	1	1	2	5	1	3	2	1	1
699	Artichoke	Spain	$2Ba_{334}$	2	4	1	1	2	5	1	3	2	1	1
704	Artichoke	Spain	$2Ba_{334}$	2	4	1	1	2	5	1	3	2	1	1
705	Artichoke	Spain	$2Ba_{334}$	2	4	1	1	2	5	1	3	2	1	1
917	Artichoke	Spain	2A	2	1	1	2	3	4	1	1	2	2	3
921	Artichoke	Spain	$2Ba_{334}$	2	4	1	1	2	5	1	3	2	1	1
925	Artichoke	Spain	$2Ba_{334}$	2	4	1	1	2	5	1	3	2	1	1

Table 1 *Continued*

Isolate code	Host	Geographic origin	VCG[ab]	Multilocus genotype[c]										
Potato isolate collection														
A2	Potato	Spain	2A	2	1	1	2	3	4	1	1	2	2	3
A7	Potato	Spain	4B	1	1	1	2	3	4	3	1	2	2	3
A8	Potato	Spain	4B	1	1	1	2	3	4	3	1	2	2	3
A11	Potato	Spain	2A	2	1	1	2	3	4	1	1	2	2	3
A12	Potato	Spain	2A	1	2	1	2	3	4	1	1	2	2	2
A21	Potato	Spain	4B	1	1	1	2	3	4	3	1	2	2	3
A24	Potato	Spain	2A	2	1	1	2	3	4	1	1	2	2	3
A28	Potato	Spain	2A	2	1	1	2	3	4	1	1	2	2	3
A33	Potato	Spain	4B	1	1	1	2	3	4	3	1	2	2	3
A38	Potato	Spain	2A	2	1	1	2	3	4	1	1	2	2	3
A45	Potato	Spain	4B	1	1	1	2	3	4	3	1	2	2	3
A49	Potato	Spain	4B	1	1	1	2	3	4	3	1	2	2	3
A52	Potato	Spain	$2Ba_{334}$	2	4	1	1	2	5	1	3	2	1	1
A54	Potato	Spain	4B	1	1	1	2	3	4	3	1	2	2	3
V3	Potato	Spain	$2Ba_{334}$	2	4	1	1	2	5	1	3	2	1	1
V7	Potato	Spain	4B	1	1	1	2	3	4	3	1	2	2	3
V9	Potato	Spain	2A	2	1	1	2	3	4	1	1	2	2	3
V13	Potato	Spain	2A	2	1	1	2	3	4	1	1	2	2	3
V61	Potato	Spain	4B	2	1	1	2	3	3	1	1	2	2	3
V80	Potato	Spain	2A	2	1	1	2	3	4	1	1	2	2	3

[a]VCG2Br is defined by complementation of nitrate-nonutilizing (*nit*) mutants of the tested isolates with complementary mutants from the international OARDC-OSU reference cultures and Israeli tester isolates (Rowe, 1995; Korolev *et al.*, 2000); VCG2Ba is defined by complementation of *nit*1 mutants of the tested isolates with selected *NitM* mutants of isolates from artichoke previously assigned to VCG2Br. Isolates assigned to VCG2Ba failed to complement with *nit* mutants from the international OARDC reference and Israeli tester isolates.
[b]$2Br_{824}$, $2Br_{334}$ and $2Ba_{334}$ are amplicons produced by sequence-characterized amplified region (SCAR) markers using primer sets DB19/DB22/espdef01 (334 bp) and INTND2f/INTND2r (824 bp). Amplicons of 334 and 824 bp were previously associated with cotton-defoliating and -nondefoliating pathotypes of *V. dahliae*, respectively (Mercado-Blanco *et al.*, 2001, 2002, 2003).
[c]Multilocus genotypes were generated by combining alleles of four microsatellite markers (VdSSR1, VdSSR2, VdSSR4 and VdSSR5) and the seven polymorphic sequences (VdSNP1, VdSNP2, VdSNP3, VdSNP4, VdSNP5, VdSNP6 and VdSNP7) developed in this study. Allele codes are given in Table 3.
[d]HSI = heterokaryon self-incompatible isolate.

Isolates in these two collections were collected from commercial artichoke (Jiménez-Díaz *et al.*, 2006) and potato fields located in eastern-central Spain, where intensive vegetable production is concentrated along the Mediterranean coast. Cropping practices in this area are identical and potato and artichoke crops are commonly grown in rotation. VCGs of the artichoke isolates were determined in an earlier study by Jiménez-Díaz *et al.* (2006). VCG typing of the potato isolates was performed by Ortega, Guirao, Olivares and Jiménez-Díaz (unpublished). One isolate of each of two additional species closely related to *V. dahliae* were included to test potential transferability of the markers developed in this study: *V. albo-atrum* and *Gibellulopsis nigrescens* (formerly known as *V. nigrescens*). The *G. nigrescens* isolate was obtained from an artichoke-cultivated soil in Castellón, Spain, and the *V. albo-atrum* isolate (V-123B) was obtained from *Ailanthus altissima* in Pennsylvania and provided by Dr Donald Davis (The Pennsylvania State University).

Isolates were stored on plum lactose yeast extract agar (PLYA) (Talboys, 1960), covered with liquid paraffin at 4°C in the dark, and deposited in the culture collection of the Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible, CSIC, Córdoba, Spain. Cultures of the isolates were maintained on water agar amended with 0·3 g chlorotetracycline L^{-1} (Sigma Chemical Co.) and subsequently subcultured on potato dextrose agar (PDA) (Sigma Chemical Co.). DNA was extracted from mycelia obtained from 6- to 10-day-old cultures grown in potato dextrose broth (PDB) (Difco Laboratories), using the DNeasy Plant Mini kit (Qiagen) following the manufacturer's instructions. DNA quality and concentration were determined by agarose gel electrophoresis according to standard procedures. DNA solutions were stored at −20°C until further use.

Development of genomic DNA libraries

A genomic DNA library highly enriched for microsatellites was constructed from *V. dahliae* VCG2B isolate V285 collected from cotton in Israel [labelled cot-72 in Korolev *et al.* (2001)], using the Dynabead biotin-enrichment strategy described by Glenn & Schable (2005). Isolate V285 was grown in PDB and DNA was extracted from mycelia using the DNeasy Plant Mini kit (Qiagen). Total DNA (2 μg) of the isolate was digested separately with the restriction enzymes *Rsa*I and *Alu*I (New England Biolabs). Restriction fragments were ligated to double-stranded SuperSNX24 (5′-GTTTAAGGCCTAGCTAG

CAGAATC-3′) and SuperSNX24 + 4P (5′-pGATTCT GCTAGCTAGGCCTTAAACAAAA-3′) linkers (Glenn & Schable, 2005). Ligated fragments were subsequently amplified by PCR assays using primers based on the SuperSNX24 linkers before enrichment of the microsatellite motifs. The enrichment process for microsatellite-containing DNA was performed using streptavidin M-280 Dynabeads (Invitrogen) and 3′-biotinylated $(AC)_{12}$, $(AG)_{12}$, $(GT)_{12}$, $(CT)_{12}$, $(AAC)_6$, $(AAT)_{12}$, $(ACT)_{12}$, $(GGT)_{12}$, $(AAG)_8$ and $(CTT)_8$ probes. Microsatellite-enriched single-stranded DNA fragments were PCR-amplified using primers based on the SuperSNX24 linkers to recover double-stranded DNA. PCR products were ligated into the cloning vector pCR2·1-TOPO (Invitrogen), which was then transformed into *E. coli* (chemically competent TOP10 cells; Invitrogen). Transformed bacteria were stored at −80°C in 15% glycerol.

Library screening, primer design and PCR amplification

Screening for microsatellite repeats in the enriched library was done by direct-colony PCR amplifications and sequencing of amplicons with standard M13 primers. A total of 576 insert-containing clones were analysed by PCR amplification of the insert DNA, purification of amplicons (ExoSap, USB) and sequencing using an ABI Hitachi 3730XL DNA analyser at the Genomics Core Facility, The Pennsylvania State University (GCF-PSU). The presence of microsatellite core motifs in cloned sequences was determined both visually and by using Staden Package software with the module and tandem repeat occurrence locator (TROLL) (Castello *et al.*, 2002). Primers flanking repeat motifs were designed using Primer3 (Rozen & Skaletsky, 2000) and PrimerSelect 3.11 (DNAStar package, Lasergene). All primers were initially tested with a selection of 25 isolates representing the different *V. dahliae* VCGs (VCG1A, VCG1B, VCG2A, $VCG2Br_{834}$, $VCG2Br_{334}$, $VCG2Ba_{334}$, VCG4B and VCG6, Table 1). Each PCR reaction mixture (25 μL) contained 1 × standard buffer (1·5 mM final concentration of $MgCl_2$) (Gene Choice), primers at 0·25 μM each (Table 2) (GCF-PSU), dNTPs at 250 μM each, 1·25 U *Taq* DNA polymerase (Gene Choice) and 10–50 ng template DNA. Reactions were performed in a Mastercycle® ep (Eppendorf AG), with an initial denaturation step at 94°C for

Table 2 Polymorphic molecular markers developed for *Verticillium dahliae* isolates evaluated in this study

Marker	Locus	GenBank accession no.	Primer sequence (5′–3′)	T_a (°C)[a]	Size (bp)[b]
Microsatellite					
	VdSSR1	EU264160	F: TTGATCTAGGAAAGCGCATGAC R: CCAGGCAACAATGCAGACA	55	200
	VdSSR2	EU264161	F: CCCCGACCTCTCTTCTTTCTAT R: AGCAGAATCACTCATAGGGTGC	47	261
	VdSSR3	EU264162	F: GCAGAATCCTTTCACATAATTGGAC R: CCCTGTTTTGATCTCTCCTCT	55	123
	VdSSR4	EU264163	F: TCAGAACCGAAGTAACCAA R: GAATCACGCTCCTTCACGGTA	47	270
	VdSSR5	EU264164	F: GCAGGGGGTGGCCTCAGCACT R: CGCAGGCATGCCTGGGTGTG	59	285
Polymorphic sequence					
	VdSNP1	EU264165	F: TGACACTGAAAAGGATACACCG R: TGGAACACCCATAAGACAACTG	55	318
	VdSNP2	EU264166	F: GATCATGAAGCGAGTGCGAAGAAC R: TTGGGGCGTGTGGAAAGGAT	59	768
	VdSNP3	EU264167	F: TGCGCAGCGAACAGAGAC R: CTAAGCCACCCAGCAACACA	59	367
	VdSNP4	EU264168	F: GAAGGCGCTCCACCCAGTCGTC R: GCTAATCGCCCCGTCGCAACA	50	463
	VdSNP5	EU264169	F: ACGGGGGATGGGTGTGAT R: TTCTGGCAAGTTTGACATGACTG	59	437
	VdSNP6	EU264170	F: GCGGCGGCATGCATCACCTC R: CCATGGCCTCGGGCGTTCG	59	267
	VdSNP7	EU264171	F: TCAGCTGCGCAACTGCTCAA R: TCACCGCTTTCCGCTACTACCTT	55	231

[a]T_a = annealing temperature used in PCR amplifications.
[b]Length of original PCR-amplified cloned allele.

4 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at T_a (Table 2) for 1 min and elongation at 72°C for 1 min, plus a final elongation step of 72°C for 6 min. For polyacrylamide gel electrophoresis (PAGE) allele identification, microsatellite alleles were separated on 6% polyacrylamide gels in 0·5 × Tris-borate-EDTA (TBE) buffer. Products were stained with ethidium bromide and visualized under UV light, and molecular weights were estimated using a 25-bp ladder (Invitrogen).

Allele determination for microsatellites and polymorphic sequences

Allelic variation was assessed for 25 *V. dahliae* isolates from diverse geographic origins and host sources, representative of VCG1A, VCG1B, VCG2A, VCG2B (2Br and 2Ba), VCG4B and VCG6 (Table 1). In addition to PAGE, allele size and variation in the number of repeats for microsatellites were assessed by sequencing the amplified loci and by capillary electrophoresis of amplicons. For SNP location and type, amplification products were purified and sequenced as described above (GCF-PSU). Allele sequences of each locus were aligned and compared using Sequencher version 4.7 (Gene Codes). DNA from the 25 representative *V. dahliae* isolates (Table 1) was PCR-amplified with fluorochrome FAM (6-carboxy-fluorescein)-labelled SSR primers (Integrated DNA Technologies). Sizes of the PCR products were established using an automatic ABI prism 3730XL capillary sequencing system (CGF-PSU) and analysed using GeneMapper® software version 4.0 (Applied Biosystems). Alleles for isolates from the artichoke and potato isolate collections were determined by sequencing of the PCR-amplified loci as described above.

Allele determination for previously developed SCAR markers

Verticillium dahliae isolates were characterized by duplex- or multiplex-PCR assays using several primer pairs as previously described (Mercado-Blanco *et al.*, 2003; Collado-Romero *et al.*, 2006). SCAR primer pair DB19/DB22 (Carder *et al.*, 1994) yields *V. dahliae*-specific polymorphic DNA bands of 539 or 523 bp (Mercado-Blanco *et al.*, 2003). SCAR primer pairs INTD2f/INTD2r and INTND2f/INTND2r produce PCR markers of 462 and 824 bp, respectively, that are associated with cotton and olive defoliating (D) and nondefoliating (ND) *V. dahliae* pathotypes, respectively (Mercado-Blanco *et al.*, 2001, 2002, 2003). In contrast, SCAR primer pair DB19/espdef01 amplifies a 334-bp marker present in D isolates as well as some ND isolates of VCG1B and VCG2B (Collins *et al.*, 2005; Collado-Romero *et al.*, 2006). All PCR assays were performed in a Peltier Thermal Cycler-200 (MJ Research). Amplification products were separated on 1% agarose gels stained with ethidium bromide and visualized under UV light.

Marker data analysis

In order to establish the potential of the new markers (microsatellites and polymorphic sequences) to resolve genetic diversity in *V. dahliae*, genetic diversity parameters were calculated for two collections of *V. dahliae* isolates obtained from artichoke and potato plants. In addition, the 25 isolates of *V. dahliae* from different host sources and geographic origins previously used for marker development were considered together as a third group (referred to as the 'multi-host' isolate collection) for comparison with *V. dahliae* isolates from artichoke and potato. Some data were missing for microsatellite marker VdSSR3 so this locus was excluded from the analysis. A comparative analysis was performed in which genetic parameters for the three collections of isolates (artichoke, potato and multi-host) were generated with other markers that have been previously used for *V. dahliae* population analysis: VCGs and three SCAR markers (Jiménez-Díaz *et al.*, 2006; Collado-Romero *et al.*, 2009). In this way, three marker sets were defined: new microsatellite markers and polymorphic sequences (set I), VCGs and three SCAR markers (set II), and the same three SCAR markers alone (set III).

Gene diversity and genetic differentiation

For each of the three sets of markers, the degree of polymorphism estimated by gene diversity (Nei, 1987) was calculated for all 75 isolates of *V. dahliae* using POPGEN version 1.32 (University of Alberta, Edmonton, Canada). Subsequently, gene diversity in each of the individual isolate collections (i.e. the artichoke, potato and multi-host collections) was calculated using the same software. A genetic differentiation coefficient (G_{ST}) for the isolate collections from artichoke and potato was calculated as: $G_{ST} = (H_T - H_S)/H_T$, where H_S = the average of the estimated gene diversities of each of the two populations, and H_T = the estimated gene diversity of the total population (Nei, 1973). Differentiation among the artichoke and potato isolate collections was also tested using Weir's θ (Weir, 1996). The null hypothesis of no population differentiation ($\theta = 0$) was tested by comparing observed θ values to values obtained from 1000 artificially randomized datasets where multilocus genotypes were permuted among collections using MULTILOCUS 1.3 (Agapow & Burt, 2001).

Genotypic diversity

Multilocus genotypes were assigned to each isolate in the three *V. dahliae* isolate collections described above (artichoke, potato and multi-host), by combining alleles across the new polymorphic markers (set I), as well as for the other two marker sets used for comparison (set II, VCG and three SCAR markers; and set III, SCAR markers alone). The total number of unique genotypes and the frequency of the most common genotype in each isolate collection were calculated using MULTILOCUS 1.3. Genotypic diversity (D), was estimated according to the formula

Table 3 Characteristics of 12 polymorphic molecular markers developed for *Verticillium dahliae* isolates in this study

Locus	Repeat motif[a]	Microsatellite core repeats	Allele size (bp) or genotype[b]	*H*[c]
VdSSR1	$(GTCG)_3$	1, 3	1- 192 2- 200	0·36
VdSSR2	$(CAG)_7$	7, 8, 9, 12	1- 261 2- 264 3- 267 4- 276	0·57
VdSSR3	$(GA)_{26}$	11, 13, 20, 24, 25, 26, 33, 34, 47	1- 95 2- 99 3- 115 4- 121 5- 123 6- 125 7- 139 8- 141 9- 167	0·83
VdSSR4	$(GT)_5(GA)_8$	5-8, 5-7	1- 268 2- 270	0·32
VdSSR5	$(CATTGGA)_4$	3, 4	1- 278 2- 285	0·43
VdSNP1	—	—	1- CTCCTGA 2- GTCTTCA 3- CTCCCGA 4- GTTTTCT 5- GCCCTGA	0·71
VdSNP2	—	—	1- TGGCG 2- TGGCT 3- CGGCG 4- CGGCT 5- CGCCT 6- CGCCG 7- CAGCT 8- CAGCG 9- CGCGT	0·81
VdSNP3	—	—	1- CT 2- CC 3- TC	0·52
VdSNP4	—	—	1- GG—AA 2- GA—AA 3- GGGAA	0·56
VdSNP5	—	—	1- GGTGTA 2- GGTGAA 3- AA— —TG	0·38
VdSNP6	—	—	1- TGTG 2- TGAG	0·44
VdSNP7	—	—	1- CCT 2- —CT 3- CTA	0·49

[a]Repeat motif of core microsatellite sequence of original PCR-amplified allele.
[b]Allele size based on microsatellite amplicon. For polymorphic sequences, allele genotypes were determined based on nucleotides at the following positions of the corresponding GenBank accessions (Table 2): VdSNP1 (positions 78, 97, 98, 181, 189, 222, 292); VdSNP2 (positions 328, 329, 334, 348, 386); VdSNP3 (positions 80, 313); VdSNP4 (positions 288, 289); VdSNP5 (126, 175, 197, 199, 223, 294); VdSNP6 (position 204); VdSNP7 (positions 82, 109, 113).
[c]*H* = Gene diversity for 25 *V. dahliae* isolates used for marker development (Table 1; Nei, 1973).

$D = \mathrm{n/n} - 1(1 - \sum P_{\mathrm{i}}^2)$ (Pielou, 1969) using MULTILOCUS. The null hypothesis of no genotypic diversity was tested by comparing observed D values with those calculated from 1000 artificially randomized datasets where individual multilocus genotypes were permuted within each collection.

Results

Marker development

A total of 576 cloned fragments from the enriched genomic library were sequenced and screened for the presence

of microsatellites. Of the clones, 121 (21%) contained microsatellites. Primers were designed based on regions flanking the repeat cores for those microsatellites that were distant enough from the end of the sequences. Initially, 26 primer pairs were tested, of which 22 produced clear single amplicons. Five loci from the 22 amplified microsatellite regions were polymorphic, that is, they displayed allelic diversity in the microsatellite core repeats (Table 2) among the 25 *V. dahliae* isolates of diverse VCGs, host sources and geographic origins used for marker development; and 17 loci were monomorphic (did not display allelic diversity in the microsatellite core repeats) based on sequence data of the amplified loci. Seven of 17 monomorphic microsatellite markers were identified as polymorphic sequences as they contained one to seven nucleotide polymorphisms located in regions flanking the core repeats (Tables 2 and 3). Eleven of the loci tested showed PCR amplification signals for *G. nigrescens* and *V. albo-atrum* (data not shown) and alleles were within the size range observed for alleles in *V. dahliae*. One locus (VdSNP2) displayed amplification only from the *V. dahliae* isolates but not from the *G. nigrescens* or *V. albo-atrum* isolates (data not shown).

Genetic analyses

Microsatellite markers and polymorphic sequences

Analysis of the five microsatellite markers and seven polymorphic (SNP-based) sequences showed a total of 47 alleles (Table 3) for the 25 *V. dahliae* isolates initially studied (Table 1). The loci displayed from two to nine alleles, and more than two alleles were identified for eight of the loci. The size of alleles based on capillary electrophoresis concurred with that estimated from PAGE, and differences of two or three nucleotides between alleles were resolved clearly. Sequence analysis of PCR products was necessary for allele determination of the polymorphic sequences. For microsatellites, sequence analysis showed a clear agreement between allele sizes determined using capillary electrophoresis and the number of microsatellite core repeats (Table 3). Gene diversity (H) ranged from 0·32 to 0·83 among loci (Table 3), with an average of 0·46 (Table 4). The highest gene diversity was displayed by loci VdSSR3 and VdSNP2 (Table 3).

SCAR and VCG markers

SCAR primer sets DB19/DB22/espdef01, INTD2f/INTD2r, and INTND2f/INTND2r produced amplicons of 334, 462 and 824 bp, respectively (data not shown). Gene diversity ranged from 0·10 to 0·45 among loci, with an average of 0·33 (data not shown). Amplification of the 334- and 462-bp markers was observed in 5% of the *V. dahliae* isolates studied (four out of 75), while 30% (22 out of 75) produced only the 334-bp marker and 65% (49 out of 75) produced only the 824-bp marker (data not shown). Marker set II (SCAR and VCG markers) showed gene diversity values ranging from 0·10 to 0·78 among loci, with an average of 0·47 (data not shown). Four of the 75 *V. dahliae* isolates studied (5%) belonged to VCG1A, only one isolate belonged to VCG1B and 23 isolates (31%) belonged to VCG2A. Thirteen of the 75 isolates (17%) belonged to VCG2Ba, 14 isolates (19%) to VCG2Br and 17 isolates (23%) to VCG4B. Two isolates (3%) were heterokaryon self-incompatible (HSI), and only one was VCG6 (Table 1).

Population structure

Diversity estimated by microsatellite markers and polymorphic sequences

The number of alleles observed per locus for the *V. dahliae* isolates from artichoke and potato ranged from two to eight (data not shown). Gene diversity in the isolate group from artichoke was greater than that from potato (Table 4). The genetic differentiation coefficient (G_{ST}) between collections from artichoke and potato in eastern-central Spain was 0·24, i.e. 24% of the genetic variation among the isolates of *V. dahliae* was attributable to variation between the two groups and 76% was within the two host-associated groups. Differentiation among the artichoke and potato isolates (θ = 0·31 at

Table 4 Gene and genotype diversity parameters derived from new microsatellite markers and polymorphic sequences developed in this study for *Verticillium dahliae*, and parameters calculated using previously published molecular markers

	Gene and genotype diversity by isolate collection																		
	Multi-host (N = 25)					Artichoke (N = 30)					Potato (N = 20)					Total (N = 75)			
Marker set	H[a]	Ng[b]	Nu[c]	Fm[d]	D[e]	H	Ng	Nu	Fm	D	H	Ng	Nu	Fm	D	H	Ng	Fm	D
I: new[f]	0·50	12	3	5	0·92*	0·42	10	3	12	0·80*	0·23	5	1	8	0·70*	0·46	16	18	0·86*
II: VCG- and PCR-based	0·46	8	2	6	0·86*	0·41	6	0	14	0·71*	0·23	3	0	9	0·62*	0·43	9	23	0·78*
III: PCR-based	0·36	3	0	17	0·50*	0·35	3	0	15	0·55*	0·12	2	0	18	0·19*	0·33	3	49	0·49*

[a]Gene diversity (Nei, 1973).
[b]Number of multilocus genotypes observed.
[c]Number of unique multilocus genotypes observed.
[d]Frequency of most common genotype.
[e]Genotypic diversity (Pielou, 1969). The null hypothesis of no genotypic diversity was tested by comparing D values obtained from 1000 randomized datasets to those estimated from the observed dataset. *P < 0·001.
[f]Marker set I: VdSSR1, VdSSR2, VdSSR4, VdSSR5, VdSNP1, VdSNP2, VdSNP3, VdSNP4, VdSNP5, VdSNP6 and VdSNP7.

$P < 0{\cdot}001$) was significant as indicated by the randomization test.

The number of multilocus genotypes in the three *V. dahliae* collections studied ranged from five to 12 (Table 4). In the multi-host, artichoke and potato collections, three, three and one of 16 multilocus genotypes, respectively, were unique. The most common genotype of *V. dahliae* was detected 12 times within the artichoke isolates (Table 4). Genotypic diversity in the multi-host and artichoke groups of *V. dahliae* were similar and greater than that observed in the potato group (Table 1). Genotypic diversities were significantly greater ($P < 0{\cdot}001$) than 0 in all three isolate collections studied.

Diversity estimated by SCAR markers (set III) and by SCAR markers combined with VCG markers (set II)

Gene diversity values obtained with the microsatellites and polymorphic sequences (set I) and those obtained with SCAR markers combined with VCGs (set II) were similar for the *V. dahliae* isolates studied (Table 4).

In the multi-host isolate group that included the original 25 isolates of *V. dahliae* used for marker development, the estimated number of genotypes was eight when VCGs were combined with SCAR markers (set II) vs. three with SCAR markers alone (set III) (Table 4). The estimated number of genotypes was six for marker set II versus three for marker set III in the artichoke group of isolates, and three for marker set II versus two for marker set III in the potato group of isolates (Table 4). Only two of eight unique genotypes were detected by marker set II in the multi-host collection, with no unique genotypes detected in either the artichoke or potato isolates (Table 4). Genotypic diversity values (D) obtained from the SSR and polymorphic sequence markers (set I) and the SCAR markers combined with VCGs (set II) were similar for the three groups of isolates studied (Table 4). Considering D values obtained from marker sets II and III, genotypic diversity in the multi-host and artichoke *V. dahliae* isolates were similar (Table 4). For all the marker sets used, genotypic diversity was significantly greater than 0 ($P < 0{\cdot}001$) in all three groups of isolates of *V. dahliae* studied.

Discussion

The main goal of this study was to develop new molecular markers for analysis of genetic diversity in populations of *V. dahliae*. Twelve new polymorphic markers were identified, consisting of five microsatellites and seven polymorphic sequences, which showed high levels of polymorphism among 75 *V. dahliae* isolates included in this study, as indicated by relatively high gene diversity. The study also indicated the usefulness of these markers for differentiating two groups of the pathogen associated with different host plants, potato and artichoke, from fields located in the same region of intensive vegetable production in eastern-central Spain, where both crops are commonly grown in rotation.

The level of variation detected by the new markers, as indicated by Nei's gene diversity index (Table 4), was comparable to that generated by VCG typing in combination with use of SCAR markers, a practice often used for analysis of diversity in *V. dahliae* (Jiménez-Díaz *et al.*, 2006; Collado-Romero *et al.*, 2009). Furthermore, the number of genotypes identified was greater using the new markers for 75 *V. dahliae* isolates than when combining data from VCG and SCAR markers or from SCAR markers alone (Table 4). These results support the potential of the new molecular markers for resolving population structure within *V. dahliae*.

Variation in the allelic size of microsatellites is generally assumed to reflect differences in the number of core repeats. However, different types of interruptions within the tandem-repeat array, as well as nucleotide insertions and deletions in regions flanking the repeat motif, can also account for variation in allele size (Ortí *et al.*, 1997). For the microsatellite markers identified in this study, sequence analysis showed that allele variation of each marker was caused by variation in the number of repeats. Capillary electrophoresis of microsatellite alleles is widely used and may be the most cost-efficient way to analyse large pathogen populations. However, such detection methods for microsatellites can obscure the origins of allele size variation. In this study, determination of the size of microsatellites alleles by PAGE yielded results similar to those obtained with capillary electrophoresis. Visualizing allele size using PAGE was a technically easy, rapid and an inexpensive method to initially identify polymorphic markers. Nevertheless, data obtained from sequence analyses of the microsatellites were more informative, especially for the samples for which it was difficult to differentiate allele sizes using gels or capillary electrophoresis. Despite the higher cost of sequencing versus PAGE or capillary electrophoresis, screening techniques for allele size determination indicated that sequence analysis was the most unambiguous of the methods evaluated in this study for analysis of microsatellite markers.

The novel markers developed in this study also generated amplicons from two other fungal species closely related to *V. dahliae*, viz. *V. albo-atrum* and *G. nigrescens*, which indicates the potential of transferability of markers to other species, although this was not examined further in the present study. It has been reported that amplification of microsatellite loci from other fungi different from the original species they were developed from is generally possible only among species within a genus, and even then success at cross-amplification can be low (Dutech *et al.*, 2007). The potential to use the markers developed in this study on these other two fungi is consistent with *V. albo-atrum* and *G. nigrescens* being closely related to *V. dahliae* (Barbara & Clewes, 2003).

For asexually reproducing fungi such as *V. dahliae*, identification of population subdivision within a particular geographic area can be associated with the epidemiology of the disease, such as sources of inoculum and host or tissue specificity (Ureña-Padilla *et al.*, 2002; Milgroom

& Peever, 2003). The relatively high G_{ST} value (0·24) between the *V. dahliae* isolates from artichoke and potato evaluated in this study indicated high differentiation among the two groups within the same cropping area of eastern-central Spain (Balloux & Lugon-Moulin, 2002). This was consistent with the statistically significant θ value for these two groups that indicated differentiation between them. Furthermore, the high genotype diversity and the greater number of unique genotypes observed in the artichoke isolates than the potato isolates suggest a degree of divergence between *V. dahliae* in association with these two hosts. Divergence as a result of mutations and random changes caused by genetic drift may be favoured by agricultural practices in the area of study, where artichoke and potato have been cultivated in the same small fields for centuries, possibly harbouring limited effective population sizes that would favour random changes in allele frequencies (Slatkin, 1987). The association of different genotypes with artichoke and potato could also be caused, in part, by the introduction of divergent genotypes with planting material. However, artichoke planting material comes from northern Spain, where the disease has not been encountered. Further research would be required to fully rule out that possibility.

The hypothesis and evidence for divergence between *V. dahliae* isolates from different host crops is supported also by the high number and frequencies of multilocus genotypes specific to each of the artichoke and potato groups of isolates studied. In contrast with the genetic differentiation observed between the *V. dahliae* isolates evaluated in this study, bioassays for cross-pathogenicity under controlled conditions failed to demonstrate host specificity among *V. dahliae* isolates from artichoke and potato, and genotypes of the same pathogen caused wilt on both hosts (Ortega & Pérez, 2007; Berbegal *et al.*, 2010). Ortega & Pérez (2007) tested 11 of the 20 isolates from potato used in this study for pathogenicity on artichoke; and Berbegal *et al.* (2010) tested nine of the 30 isolates from artichoke for pathogenicity on artichoke and potato. The consistent association under field conditions of certain portions of the *V. dahliae* population with specific host plants, while maintaining the ability of causing disease in a wide host range under controlled conditions (artificial inoculations and optimal environmental conditions for disease development), has been reported. For example, in North America, VCG4A is nearly exclusively associated with potato crops, even when other VCGs are present in the fields (Rowe & Powelson, 2002; Omer *et al.*, 2008). Furthermore, even if VCG4A isolates are also pathogenic to other hosts under controlled conditions (Rowe & Powelson, 2002; Dung *et al.*, 2010), isolates of this VCG are rarely associated with crops other than potato (Rowe & Powelson, 2002). It is hypothesized that *V. dahliae* associated with potato and artichoke crops probably originated from a common diverse resident *V. dahliae* population established or native to the area of study. After years of continuous artichoke–potato crop rotations, *V. dahliae* isolates may have adapted to the host species from which they were isolated, resulting in divergence of the two groups that occupy the same fields. Divergence may have occurred as a result of genetic drift and/or local adaptation to increase relative fitness in local microenvironments, the different host plants artichoke and potato (Kawecki & Ebert, 2004).

Acknowledgements

MB was a recipient of a FPU fellowship from the Ministry of Education and Science of Spain (AP2002-3818); we thank Jeniffer Yanez and Rachel Leonard for very valuable technical support and D. Davis for providing the *V. albo-atrum* isolate. This research was partially supported by The Pennsylvania State University Agricultural Experiment Station and the Fundación Ramón Areces grant 'Verticilosis del olivo' from Spain. Editorial improvement of the manuscript by anonymous reviewers and the editor is gratefully acknowledged.

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