Molecular Variation Among Isolates of *Verticillium dahliae* and Polymerase Chain Reaction-Based Differentiation of Races

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ABSTRACT

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Verticillium dahliae is a soilborne fungal pathogen that causes vascular wilt in a variety of economically important crops worldwide. There are two races of *V. dahliae* that infect tomato and lettuce. Although race-1-specific resistance has been identified in both tomato and lettuce, no resistant sources are available for race 2. Molecular analyses were employed to characterize the genetic variability and race structure of 101 isolates of *V. dahliae* from a variety of hosts, mainly from central and coastal California, and 10 isolates exotic to this area. Analyses of the 16 simple sequence repeat (SSR) markers illustrated that tomato subpopu-

lations from central California were distinct relative to the marigold subpopulations. In contrast, cotton and olive isolates showed admixture with tomato isolates. Analyses of both the ribosomal DNA intergenic spacer regions and SSR markers revealed high genetic variability among isolates but were unable to delineate races of *V. dahliae*. However, a polymerase chain reaction (PCR) assay was applied to amplify a race-1-specific amplicon from the isolates in many hosts from different geographic areas, and was coupled with virulence assays for validation of the data. Results of the PCR assay showed 100% concordance with the virulence assay to differentiate race 1 from race 2 of 48 isolates from tomato. The results indicate that the PCR assay can be applied to differentiate the two races to support our related aim of breeding host resistance, and further reveal insights into the distribution of races in tomato and lettuce cropping systems in California.

Verticillium dahliae Kleb. is a soilborne fungal pathogen that causes vascular wilt in a wide range of hosts, including trees, herbaceous ornamentals, and economically important vegetable and field crops (23). The fungus survives for prolonged periods in soil as microsclerotia, which serve as the principal propagule of pathogen spread between fields and production regions. Pathogen spread also occurs via infested soil and plant material. The fungus is also seedborne in some hosts and weeds, and the latter may produce wind-dispersed seed (37,39).

Sources of resistance to *Verticillium* wilt have been identified in a small number of crops, with strains of *V. dahliae* capable of overcoming resistance in the cases of tomato and lettuce (12, 16,20,23,39). In these two crops, strains that are controlled by host resistance are called race 1, whereas those capable of causing disease on race-1-resistant host cultivars are identified as race 2. In lettuce, a single dominant genetic locus confers resistance to race 1 in the cv. La Brillante (16). In tomato, the dominant *Ve* locus confers resistance to *Verticillium* wilt (31). The *Ve* locus consists of two genes, *Ve1* and *Ve2*, which encode cell surface receptor proteins belonging to the extracellular leucine-rich repeat receptor-like protein (RLP) class of disease resistance proteins (19). Subsequent analyses showed that only *Ve1* provides resistance against race 1 strains of *V. dahliae* and *V. albo-atrum* (12). All processing tomato cultivars currently grown in California harbor the *Ve* allele that confers resistance to race 1 of *V. dahliae*.

Following the introduction of race-1-resistant tomato cultivars, race 2 strains gained prevalence in production fields in California

(14). Nevertheless, nearly 15% of all plants collected by Grogan et al. (14) were asymptotically infected with race 1 strains. Since the initial assessment of the field populations of *V. dahliae* from tomato by Grogan et al. (12) in the mid-70s, potential changes in the pathogen populations have not been evaluated, despite the deployment of cultivars from different genetic backgrounds. *Verticillium* wilt incidence has seen an incremental increase in production fields during this period and the question of whether this is due to potential changes in the pathogen population has required further scrutiny.

Genetic variation among populations of *V. dahliae* has been studied via vegetative compatibility groups (VCGs) (18,30), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), multigene phylogenies (5,8,27), and simple sequence repeats (SSRs or microsatellites) (3). The intergenic spacer region of the ribosomal DNA (IGS rDNA) has been useful in demonstrating a high intraspecific genetic variability of *V. dahliae* (24,27,28). Furthermore, phylogenetic analyses of IGS rDNA sequences coupled with virulence data suggested a relationship between phylogenetic grouping and virulence for some strains of *V. dahliae* (27).

The ability to rapidly identify and differentiate the two races of *V. dahliae* pathogenic on tomato and lettuce is critically important in breeding programs that aim to identify host resistance and to deploy resistant cultivars. Thus far, isolates of *V. dahliae* have been differentiated into two races by virulence assays, which may take up to 90 to 110 days (5,38). Because these assays can be laborious, resource intensive, and time consuming, the development of a more rapid method of differentiating races may obviate the need for cumbersome virulence assays. Recently, Usami et al. (36) identified a race-1-specific DNA sequence (B68-TV) and validated a limited number of *V. dahliae* isolates from tomato in

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Japan (36). As indicated by Usami et al. (36), it was not known whether the assay is equally effective on a population of *V. dahliae* from multiple hosts from different geographic locations.

The objectives of this study were to (i) measure the genetic variability and reassess the frequency of recovery of race 1 and 2 virulence phenotypes of *V. dahliae* from tomato and other hosts; (ii) test the hypothesis that there is a lack of clustering based on host, given that isolates are often cross-pathogenic on several hosts; (iii) test the hypothesis of a correlation between the virulence phenotype and the genotype of individual isolates because *V. dahliae* is described as an asexual (clonal) fungus; and, finally, (iv) validate race-1-specific primers for the differentiation of *V. dahliae* races on a large collection of isolates from different hosts.

MATERIALS AND METHODS

Isolate collection and DNA extraction. In all, 101 isolates of *V. dahliae* from different hosts and geographical locations were collected from symptomatic plants. Seventy isolates were collected from tomato in central California (Table 1; Fig. 1) during 2008 and 2009 cropping seasons. A race 1 tomato isolate, Le1087, was received from Campbell Soup Company, Davis, CA. Seven tomato isolates (race 1 [$n = 5$] and race 2 [$n = 2$]) from Canada and three isolates from Japan (race 1 [$n = 2$] and race 2 [$n = 1$]) were also included in this study. Isolates were grown on potato dextrose broth in an orbital shaker at 25°C at 200 rpm for 4 to 5 days. Mycelia were harvested by filtration, freeze dried, ground with liquid nitrogen, and stored at 4°C. Total DNA of the individual isolates was extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA).

Internal transcribed spacer and IGS rDNA amplification and sequencing. The complete sequence of the internal transcribed spacer (ITS) rDNA was used to confirm the identity of the *V. dahliae* strains used in this study (GenBank accessions FJ900156 to FJ900238 and GU461607 to GU461634). Primers VdIGSF1 and VdIGSR1 (27) were used for IGS rDNA amplification with an initial denaturation at 94°C for 5 min, followed by 35 cycles of 1 min at 51°C, extension at 72°C for 2 min, and a final extension step of 72°C for 10 min. All polymerase chain reaction (PCR) amplifications were performed in a Bio-Rad DNA Engine thermocycler (Bio-Rad Laboratories, Hercules, CA). PCR was carried out in 30- μ l reactions containing 10 ng of genomic DNA, 2 $\times$ GoTaq master mix (Promega Corp., Madison, WI), and 5 pmol each primer. Amplified IGS rDNA products were treated using EXOSAP-IT (Affymetrix/USB, Cleveland, OH) and used for direct sequencing. The sequencing reactions were performed using both VdIGSF1 and VdIGSR1 primers as well as a nested primer, VdIGSKF2 (5'-CAAGGTGGAAAGCTACCCG-3'), to obtain a complete sequence at the University of California, Davis

Genomics Facility, Davis. IGS rDNA sequences were deposited in GenBank (accessions FJ931476 to FJ931531, FJ937758 to FJ937778, and GU461642 to 461657).

SSR amplification. Amplification reactions for 16 SSR primers (1) were performed in 20- μ l mixtures, each of which contained 10 ng of genomic DNA, 2 $\times$ GoTaq master mix (Promega Corp.), 0.5 M each forward and reverse primer, and 4.5 mM MgCl₂. The reaction program included 5 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C; and 5 min at 72°C for the final extension. The SSR loci included in this study were VD1, VD 2, VD 3, VD 7, VD 9, VD 11, VD 12, VD 26, VD 27, VD 65, VD 69, VD 73, VD 85, VD 96, VD 97, and VD 98 (1). All of the SSR primers were labeled with four different fluorophores (HEX, FAM, ROX, and TAMRA) and overlapping loci were amplified with primers labeled with different dyes. The size standard 500 LIZ was employed and the PCR samples were separated on ABI 3100 genetic analyzer (Applied Biosystem Inc., Foster City, CA). Fragment analysis was performed using GeneMarker V. 1.85 (SoftGenetics, State College, PA).

Virulence assays. Virulence assays to differentiate race 1 and race 2 isolates of *V. dahliae* were performed on race-1- and race-2-susceptible (*Ve*-) tomato cv. Earlypak 7 and race-1-resistant (*Ve*+) cv. Beefsteak, and on race-1- and race-2-susceptible lettuce cv. Salinas and race-1-resistant lettuce cv. La Brillante (38). Seedlings were maintained in plug flats in a greenhouse at 22 to 26°C with a 16-h photoperiod. Inoculations were performed by drenching 30-day-old seedlings with 3 ml of a conidial suspension adjusted to 5.0×10^6 conidia/ml or sterile water for the control seedlings. After 1 week, seedlings were transplanted into 16-oz. foam insulated cups filled with pasteurized soil/potting soil mix (3:1). The plants were arranged in a randomized block design on greenhouse benches, with three replications and five plants per replication. Disease severity was evaluated 6 weeks after inoculation. Vascular discoloration was rated visually using a scale of 0 to 4 (5) after slicing the crown and main roots of each plant longitudinally for examination of discoloration. Isolates causing a mean rating of ≥ 1.5 were considered virulent. Experimental units consisted of groups of five plants for each isolate-cultivar combination. Each experimental unit was replicated three times in total. Recorded values were averaged across plants within each experimental unit for further data analysis. Results across replicated experiments were consistent. Therefore, disease severity data from all experiments were pooled and the combined data set analyzed as a two-way factorial using nonparametric methods (32). The overall effect of *Verticillium* isolates on the severity of disease on each lettuce cultivar was analyzed by the analysis of variance type statistics of ranked data using the PROC Mixed procedure in SAS v. 9.2 (SAS Institute, Cary, NC), and the

TABLE 1. Host origin, locations, and number strains of *Verticillium dahliae* used for internal transcribed spacer (ITS), intergenic spacer (IGS), and simple sequence repeat (SSR) analyses

Location	Host	Region	No. of isolates	No. of strains used		
				ITS	IGS	SSR
San Joaquin Co., CA	Tomato	Central California	27	26	21	27
Yolo Co., CA	Tomato	Central California	38	37	26	38
Glenn Co., CA	Cotton and olive	Central California	21	21	19	21
Solano Co., CA	Tomato	Central California	2	1	1	2
Stanislaus Co., CA	Tomato	Central California	1	1	1	1
San Benito Co., CA	Tomato	Central California	1	1	1	1
Fresno Co., CA	Tomato	Central California	1	1	1	1
Tulare Co., CA	Cotton and marigold	Central California	7	5	4	7
Merced Co., CA	Cotton	Central California	1	1	0	1
Santa Cruz Co., CA	Lettuce	Coastal California	2	3	2	2
Canada ^a	Tomato	...	7	7	2	7
Japan ^b	Tomato	...	3	3	1	3

^a Provided by K. Dobinson.

^b Provided by T. Usami.

LD_CI macro to generate relative treatment effects and 95% confidence intervals (7). Replications of experiments and blocks within experiments were considered random effects in the analysis.

Molecular differentiation of races. The applicability of primers VdTr1 and VdTr2 (36) was tested on race 1 and race 2 strains of *V. dahliae* determined by inoculation on differential cultivars of tomato and lettuce (6,27) (*unpublished data*). This study included isolates from tomato ($n = 48$), lettuce ($n = 42$), cotton ($n = 16$), olive ($n = 4$), marigold ($n = 5$), pepper ($n = 19$), spinach ($n = 10$), watermelon ($n = 4$), and artichoke ($n = 3$).

PCR amplifications were performed in 20- μ l reaction volumes, containing 10 ng of genomic DNA, 2 $\times$ GoTaq master mix, (Promega Corp.), and 5 pmol each primer VdTr1 and VdTr2 (36). Amplification conditions included an initial 94°C denaturation for 5 min; 35 cycles of 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. The identities of the amplified PCR products were confirmed by sequencing (GenBank accessions HM582414 to HM582420).

Data analysis. Sequences of the IGS rDNA regions were aligned using ClustalW (35) and refined manually. Subsequently, the sites that violated the infinite-site model were removed from the IGS rDNA sequences and indels were recoded in SNAP Workbench (25) using SNAP Map (4). Subsequently, all analyses were performed using the edited IGS rDNA sequences.

Phylogenetic analyses were conducted using PAUP v. 4b10 (34) using the maximum parsimony method. A heuristic search was

performed with sequences added randomly, and the tree bisection and reconnection (TBR) branch swapping was enforced with the steepest descent. Nucleotide characters were equally weighted and gaps were treated as a fifth state. Statistical support for the calculated topology was completed in a 1,000-replication majority-rule bootstrap analysis. A midpoint rooting was enforced on the tree but no outgroup was assigned.

The neutrality of the variability in the SSR data was assessed using the Ewens-Watterson test in Arlequin version 3.11 (11) and by implementing 1,000 simulations. The distribution of the genetic variability (population structure) was tested by an analysis of the molecular variance (AMOVA) within and among subpopulations (host or geographic region of origin) in Arlequin version 3.11. Subsequently, the level of genetic differentiation among the various hosts or region of origin were compared in all pairwise combinations, using Slatkin's R_{ST} (33) as implemented in Arlequin version 3.11. The potential for structuring in the studied subpopulation was also investigated using the principal coordinates analysis (PCA) in GenAlEx version 6.2 (22). The structure of the population and the level of potential admixture among subpopulations were further tested in the software Structure version 2.2 (26). A lack of significant differentiation between populations may be explained by repeated gene flow. For each number of clusters ($K = 1-10$), 10 runs were performed with a burn-in period of 10,000 generations and 100,000 Markov Chain Monte Carlo (MCMC) simulations. The ΔK method (10) was used to identify

Fig. 1. Map showing the locations of the collections of isolates of *Verticillium dahliae* from different counties in California.

the most likely *K* value. The membership of individual haplotypes acquired from Structure was plotted using the software Distruct (29).

RESULTS

Phylogenetic analyses of ITS and IGS rDNA regions. The ITS rDNA sequences obtained for the strains used in this study confirmed their identity as *V. dahliae*. Amplification of the IGS rDNA yielded a single PCR product 1.7 to 1.9 kb long. Sequence homology was 86.7 to 100%. Interestingly, a 17-bp subrepeat motif was also present in variable numbers in the sequences (up to 11 repeats), and explains some of the overall length variation among sequences.

The phylogenetic analysis of IGS rDNA sequences from the isolates of *Verticillium* spp. identified three main groups (Fig. 2). The vast majority of the tomato isolates, including both race 1 and race 2 types, clustered in the main larger group (group I) with isolates from cotton, olive, and lettuce. This group also included three tomato strains from Canada and Japan (Dvd-S100, Dvd-T3, and PD570) and from lettuce in coastal California (Ls16). Group II included strains from marigold, olive, and cotton but none of the isolates from tomato clustered with group II. Group III included Ls17 from lettuce in coastal California and Le1103 from tomato in central California. No evidence of correlation between IGS rDNA sequences and virulence was observed.

SSR analysis. The number of alleles detected per locus ranged from two to seven. The Ewens-Watterson test indicated that the evolution of the SSR loci was neutral in all three subpopulations. Several clones were recovered from the tomato population but not in non-tomato populations. The AMOVA analysis showed that variability among subpopulations was 21% and the variability within subpopulations was 79% (Table 2). Both PCA and pairwise comparison analysis using Slatkin's *R*_{ST} showed that tomato and non-tomato subpopulations from California were minimally differentiated (*R*_{ST} = 0.079 and *P* = 0.011) (Table 3). In contrast, the non-California tomato subpopulation was highly differentiated from both central California subpopulations (Table 3). Two genetic clusters (*K* = 2) were determined to be the most likely genetic affiliation of individual genotypes using the analysis in Structure (Fig. 3A and B). The latter analysis confirmed the results of the PCA and AMOVA and indicated that significant admixture occurs between various hosts of *V. dahliae* in central California, whereas such admixture is less frequent with the non-California subpopulation (Fig. 4).

Virulence phenotyping of *V. dahliae* on tomato and lettuce differential cultivars. Across three independent experiments, the average disease severity was 0.53 to 4.0 for Earlypak 7 and Salinas and 0.33 to 4.0 for Beafsteak and La Brillante based on a 0 to 4 scale. Although there were minor differences in the overall level of disease between experiments, these differences did not influence the rankings of cultivar-isolate interactions. None of the noninoculated control plants developed the disease.

Significant differences in the mean disease severity rankings were detected between lettuce and tomato cultivar differentials (*P* < 0.0001) and between isolates of *V. dahliae* (*P* < 0.0001), and significant two-way interactions of differentials and isolates (*P* < 0.0001) (Table 4). Across all the isolates tested, the race-1-susceptible Earlypak 7 and Salinas significantly differed from Beafsteak and La Brillante (*P* < 0.0001) in mean disease severity rankings, whereas the differences between the two susceptible differentials was not as apparent (*P* = 0.0921). Of the 15 isolates of *V. dahliae* tested from lettuce, 1 isolate was avirulent on all differentials, 6 were virulent on all differentials, and the remaining 8 were virulent only on race-1-susceptible differentials (Table 5). Similarly, of the 20 isolates of *V. dahliae* tested from tomato, 2 isolates were virulent only on Salinas, 1 isolate was only virulent on Earlypak 7, 1 isolate was virulent only on tomato differentials,

Fig. 2. Single most parsimonious phylogram of 79 *Verticillium dahliae* strains based on sequences of the intergenic spacer region of the ribosomal DNA conducted in PAUP v. 4b10 (34). Values above branches indicate bootstrap statistical support based on 1,000 replicates, and the tree is rooted at the midpoint. Number of parsimony-informative characters was 26, tree length was 28, consistency index was 1.0, homoplasy index was 0.0, and retention index was 1.0. Strain abbreviations: Dvd and PD: tomato strains from Canada and Japan; Gh: cotton from central California; Le: tomato from central California; Ls: lettuce from coastal California; Oe: olive from central California; Te: marigold from central California.

and the virulence response of other isolates was similar between the two crops (Table 5). Thirteen isolates were characterized as race 2 based on their virulence to both lettuce and tomato differentials and three isolates were characterized as race 1 (Table 5). Of the three cotton isolates evaluated, there was one race 1 isolate and one race 2 isolate, while the other isolate was avirulent to either tomato or lettuce differentials (Table 5). Race 2 isolates were highly virulent on all lettuce and tomato cultivars and caused root discoloration in >90% of the plants inoculated. Some plants showed severe wilting and significant reduction in root and shoot growth 4 weeks postinoculation. Overall, race 1 isolates

TABLE 2. Analysis of the molecular variance (AMOVA), using Arlequin version 3.11 (11), of three subpopulations of *Verticillium dahliae* (central California tomato, central California non-tomato, and non-California tomato) based on 16 simple sequence repeat markers (1)

Source of variation	df	Percentage	Φ	<i>P</i> value
Among subpopulations	2	21	0.379	<0.0001
Within subpopulations	105	79	...	...
Total	107	...	...	...

TABLE 3. Pairwise comparisons of three *Verticillium dahliae* subpopulations, using Slatkin's R_{ST} (33) (below diagonal), and respective *P* values (above diagonal), performed in Arlequin ver. 3.11 (11) on 16 simple sequence repeat markers (1)

Subpopulation	Central California	Tomato Central California	Tomato outgroup
Central California	...	0.011	0.000
Tomato Central California	0.079	...	0.001
Tomato outgroup	0.583	0.307	...

from lettuce, tomato, and cotton caused a more severe stunting in the race-1-susceptible tomato cultivar (Earlypak 7) relative to the race 2 isolates. Also, height of the plants was significantly reduced in the inoculated plants (data not shown). Water-inoculated control plants did not show symptoms in any of the tomato and lettuce plants.

Molecular differentiation of races of *V. dahliae*. PCR with primer pair VdTr1 and VdTr2 yielded a specific DNA amplicon of 680 bp from nine different hosts. There was no amplification from any of the strains that exhibited the race 2 virulence phenotype in greenhouse assays, including tomato, cotton, olive, marigold, pepper, spinach, and watermelon (Table 6; Fig. 5). Four isolates (Dvd-T3, Dvd-T5, Dvd-S90, and Dvd-S100) received from

Fig. 4. Principal coordinates analysis of 102 strains of *Verticillium dahliae* from three subpopulations (central California tomato, central California non-tomato, and non-California tomato) using 16 simple sequence repeat loci. Analysis was performed in GenAlEx v. 6.2 (22) and only two principal components were detected.

Fig. 3. Affiliation of individual genotypes of *Verticillium dahliae* as assessed using 16 polymorphic simple sequence repeat loci. **A**, Individuals are separated into discrete vertical bars that are organized by sampling groups. Differences in color within a vertical bar indicate a multi-population affiliation of an individual genotype (admixture). The height of each color with an individual is the measure of proportional affiliation. **B**, $K = 2$ was determined to be the accurate number of genetic populations.

Canada were identified as race 1 by this PCR assay and belonged to VCG2A. In addition, PCR products were not obtained from the race 2 strains Dvd-2 (VCG4B) and Dvd-S85 (VCG2A), which were collected from soil samples collected in Ontario, Canada (13). The sequences of the amplified PCR products matched the sequence of TomR1 (GenBank accession AB095266), which includes the B68-TV sequence (36). There was a strong concor-

dance between the results of the virulence assays and the results of the race-1-specific PCR test (Table 6).

DISCUSSION

The principal objectives of this work were to measure the genetic variability of *V. dahliae* from tomato and other hosts from central California, to reassess the frequency of recovery of race 1 and 2 virulence phenotypes of *V. dahliae* from processing tomato in central California, and to assess the validity of a molecular marker system to rapidly differentiate between these two virulence phenotypes. The use of the IGS rDNA and SSR markers revealed that genotypic variability in the tomato, cotton, lettuce, olive, and marigold populations of *V. dahliae* was not correlated with virulence phenotype. However, the PCR markers derived from B68-TV (36) revealed a close correlation ($\approx 96\%$) between the genotypes obtained and the virulence phenotype (race 1 or race 2). Additionally, given the importance of rapidly differentiating the two races of *V. dahliae* that are pathogenic on tomato, we assessed the wider applicability of PCR markers (36) for differ-

TABLE 4. Statistical analysis of variance (ANOVA) based on the ranked means of Verticillium wilt severity on lettuce and tomato differential cultivars caused by isolates of *Verticillium dahliae* combined across three independent experiments

Effect	ANOVA-type statistic (ATS) ^a			
	df _{Num}	df _{Den}	ATS	P value
Cultivar (C)	2.85	122	16.84	<0.001
Isolate (I)	28.40	122	10.19	<0.001
C $\times$ I	53.60	122	3.07	<0.001

^a Abbreviations: df_{Num} and df_{Den} = numerator and denominator degrees of freedom, respectively.

TABLE 5. Severity rating of different isolates of *Verticillium dahliae* on tomato cultivars Earlypak 7 (susceptible) and Beefsteak (resistant) and lettuce cultivars Salinas (susceptible) and La Brillante (resistant)^a

Host, cultivar	Tomato differential cultivars				Lettuce differential cultivars			
	Ve- (Earlypak 7)		Ve+ (Beefsteak)		Salinas		La Brillante	
	Median	RME	Median	RME	Median	RME	Median	RME
Lettuce								
Ls16	2.8	0.48	0.0	0.11	3.0	0.45	1.4	0.23
Ls17	3.2	0.51	3.0	0.46	3.7	0.71	3.7	0.68
Ls474	3.7	0.73	0.8	0.19	4.0	0.68	1.0	0.15
Ls483	1.3	0.26	0.0	0.12	0.0	0.33	1.0	0.18
Ls791	3.3	0.64	0.7	0.12	3.0	0.54	0.0	0.13
Ls797	3.3	0.68	4.0	0.79	4.0	0.68	3.0	0.61
Ls807	4.0	0.82	0.0	0.09	3.0	0.61	0.0	0.11
Ls850	4.0	0.88	0.0	0.09	2.0	0.48	0.0	0.13
Ls851	4.0	0.88	0.0	0.06	4.0	0.48	0.0	0.13
Ls898	4.0	0.82	1.3	0.42	3.0	0.48	2.0	0.48
Ls899	3.5	0.72	2.0	0.24	3.0	0.47	1.0	0.15
Ls902	4.0	0.77	4.0	0.80	4.0	0.74	4.0	0.88
Ls905	4.0	0.79	4.0	0.79	4.0	0.68	4.0	0.68
Ls907	3.0	0.57	4.0	0.72	2.0	0.48	4.0	0.74
Ls910	4.0	0.88	3.4	0.71	4.0	0.74	4.0	0.68
Tomato								
Le78	3.6	0.66	3.4	0.62	2.8	0.44	3.0	0.48
Le88	2.8	0.42	3.2	0.54	2.2	0.36	2.2	0.36
Le112	1.6	0.25	0.6	0.25	2.8	0.37	2.4	0.36
Le1070	3.8	0.62	4.0	0.88	3.0	0.49	3.5	0.62
Le1073	3.7	0.67	3.3	0.57	3.6	0.65	2.8	0.44
Le1074	0.6	0.13	0.8	0.19	3.0	0.50	2.3	0.39
Le1075	2.8	0.36	0.8	0.19	3.3	0.63	2.6	0.34
Le1087	4.0	0.88	0.0	0.06	3.5	0.65	0.5	0.16
Le1092	3.2	0.45	4.0	0.69	3.0	0.50	3.4	0.59
Le1096	3.2	0.59	3.6	0.64	3.0	0.44	3.3	0.60
Le1103	3.2	0.64	4.0	0.79	3.5	0.61	3.6	0.60
Le1108	4.0	0.60	3.5	0.70	3.8	0.60	2.8	0.61
Le1110	3.5	0.61	3.4	0.69	4.0	0.83	3.6	0.57
Le1111	4.0	0.74	4.0	0.77	3.5	0.64	3.4	0.66
Le1121	3.0	0.56	3.5	0.70	2.8	0.44	3.5	0.67
Le1122	4.0	0.83	3.2	0.67	3.5	0.67	2.6	0.37
Le1123	4.0	0.88	3.0	0.56	3.8	0.64	3.8	0.66
Le1348	4.0	0.88	0.0	0.09	2.0	0.48	0.0	0.20
Le1349	4.0	0.88	4.0	0.88	4.0	0.74	3.0	0.55
Le1350	3.3	0.62	0.0	0.11	3.0	0.61	1.0	0.24
Cotton								
Gh1002	3.3	0.65	0.0	0.10	2.4	0.30	0.8	0.25
Gh1003	1.0	0.28	0.0	0.06	2.3	0.35	1.2	0.25
Gh1013	2.5	0.41	2.4	0.27	3.6	0.74	3.0	0.45
Control	0.0	0.06	0.0	0.06	0.0	0.06	0.0	0.06

^a Median = median disease severity from three independent experiments. Disease severity scored on a 0–4 scale in which 0 = no vascular discoloration, 1 = 1–25% vascular area discolored, 2 = 26–50% vascular area discolored, 3 = 51–75% vascular area, and 4 = 75–100% vascular area discolored. Isolates causing a mean disease severity of ≥ 1.5 were considered as virulent. Estimated relative marginal effects (RMEs) for tomato cultivars Earlypak 7 (race 1 susceptible) and Beefsteak and lettuce cultivars Salinas (race 1 susceptible) and La Brillante were calculated from three independent experiments.

entiating races of *V. dahliae* from tomato and other hosts (which include a *Ve* resistance gene homolog). The PCR-based assay was able to discriminate race 1 from race 2 isolates with 96% reliability, regardless of host or geographic origin.

The IGS rDNA sequences revealed significant genetic variability among the populations studied. The subpopulation of *V.*

dahliae isolated from tomato in central California was not unique to this host, and many strains isolated from other hosts in the region, or from Canada, were found in the same clades of the IGS rDNA phylogram. The *R*_{ST}, PCA, and Structure analyses converged to indicate that the central California subpopulations were minimally differentiated, whereas the tomato outgroup-subpopulation was highly differentiated and exhibited little evidence of gene flow with central California. Two genetic clusters were identified using the Structure analysis from the SSR marker data, one of which was not observed in the tomato outgroup subpopulation. Furthermore, the SSR markers indicated a lack of significant differentiation between the central California tomato and non-tomato subpopulations, with clear evidence of admixture. The geographic range of cotton seed and olive rootstock production overlaps with that of tomato grown for processing in central California. Therefore, it is possible that the isolates were carried in the germplasm produced in the latter region. Furthermore, the rotation between tomato and cotton is prevalent in central California. Nevertheless, the small number of strains obtained from outside of California prevented more meaningful conclusions regarding the global status of *V. dahliae* populations in Canada and Japan compared with those in California.

A correlation between virulence phenotypes and genotypes was observed using both VdTr PCR-based markers (36) and virulence assays to differentiate races of *V. dahliae* isolates. However, the virulence phenotype of the isolates was not correlated with either the IGS rDNA or SSR genotypes. Both virulence phenotypes were recovered from olive and cotton but not from tomato, from which only race 2 was recovered. This may be because of the

TABLE 6. Validation of race-1-specific polymerase chain reaction (PCR) primers on different hosts strains of *Verticillium dahliae*

Hosts	Race ^a	No. of isolates		Concordance (%)
		Virulence assay	PCR	
Lettuce	1	31	29	93.5
	2	11	0	100.0
Tomato	1	8	8	100.0
	2	40	0	100.0
Cotton and olive	1	4	4	100.0
	2	16	0	100.0
Marigold	1	0	0	0.0
	2	5	0	100.0
Pepper	1	11	9	81.8
	2	8	1	87.5
Spinach	1	0	0	0.0
	2	10	0	100.0
Watermelon	1	2	1	50.0
	2	2	0	100.0
Artichoke	1	3	3	100.0
	2	0	0	0.0

^a Race determined by virulence assays on lettuce and tomato differential cultivars (see Materials and Methods).

Fig. 5. Polymerase chain reaction amplification of race-1-specific fragment from tomato, lettuce, cotton, olive, pepper, marigold, spinach, watermelon, and artichoke using VdTr1 and VdTr2 primers (37); no amplifications were observed on race 2 isolates. M = 1-kb DNA molecular weight marker (Promega Corp.).

biased sampling of the diseased tomato plants from cultivars grown in central California that inevitably possess the resistance allele of the *Ve* gene, which would decrease the likelihood of recovering race 1 strains (14). The agreement between in planta virulence testing and PCR-based testing to discriminate between races of *V. dahliae* reached 96%. It is possible that the variability in the greenhouse assay may be the reason for the 4% incongruence, rather than the PCR assay itself. This also may highlight the significance of PCR- or DNA-based assays that remove the inconsistencies that may occur in plant inoculations involving *V. dahliae*.

The finding of a few race 1 isolates (as determined on tomato and lettuce) associated with the hosts examined in this study is of relevance for implementing control of Verticillium wilt of tomato and other crops. The resistance to race 1 of *V. dahliae* conferred by the *Ve* gene in tomato was estimated to have little impact on the overall population, especially given that crops or weeds that lack the *Ve* allele for resistance will succumb to both race 1 and race 2 strains (14). In the current study, all strains recovered from symptomatic tomato plants were characterized as race 2 by both the PCR and virulence assays on differential cultivars. It is plausible that three decades of selection in favor of race 2 may have significantly decreased the prevalence of race 1. This is notwithstanding the fact that crops grown in rotation with tomato, including cotton, are likely to provide a means for race 1 to rebound. Cotton cultivars tolerant to Verticillium wilt may select for strains of *V. dahliae* with increased aggressiveness (2). Taken together, these results are in agreement with the notion that the deployment of resistant cultivars can affect the dynamic of a pathogen population; in this case, selection against race 1 of *V. dahliae*. Current breeding efforts in lettuce, for instance, seek to transfer race 1 resistance into commercially acceptable cultivars, all the while screening diverse *Lactuca* accessions for race 2 resistance (17). Although the VdTr PCR-based marker may allow targeting production of race-1-resistant lettuce cultivars into fields that appear to be exclusively race 1 infested, the current data from tomato supports previous assertions that race 1 resistance in lettuce will not be durable (38). Additionally, the high genotypic diversity and lack of correlation between the phenotype and genotype of strains using IGS rDNA- and SSR-based analyses suggest that *V. dahliae* populations may be undergoing recombination. Evidence for recombination was identified in other reports (9), and recombination occurs frequently enough to generate significant and periodic changes in the populations of the fungus (21). Even though structures associated with clonality, such as microsclerotia, play a major role in the dispersal and survival of *V. dahliae* strains, only the prevalent population is spread to new areas. Taken together, aggressive screening for race 2 resistance or race-nonspecific resistance is warranted.

Based on the limited VCG information available on the isolates examined, there was no correlation between VCG and virulence phenotype (race). The VCG2A *V. dahliae* strains Dvd-T5 and Dvd-S85 (15) were characterized as race 1 and race 2, respectively, both in this study and by Harrington and Dobinson (15). If the VCGs represent clonal lineages of an asexually reproducing population, then these lineages are to be further subdivided by an additional virulence phenotype (race 1 or race 2).

There was a high correlation between the genetic marker amplified by the PCR primers VdTr1 and VdTr2 (36) and virulence phenotype in planta. This indicates that this amplified marker is either directly associated with virulence or closely linked with virulence determinants (race specificity) in *V. dahliae*. The molecular determinants of race in the pathogen are not known (20); however, further research is currently underway to elucidate this relationship. Most importantly, however, these data indicate that the VdTr-based marker system can be useful in differentiating races of *V. dahliae* not only among the tomato isolates previously characterized (36) but also for tomato and lettuce isolates obtained from populations of *V. dahliae* from California and

elsewhere. The ability to rapidly differentiate the two races of *V. dahliae* pathogenic on tomato and lettuce is critical for our related aim of breeding host resistance.

The current study is the first investigation of the diversity of *V. dahliae* on tomato in California since Grogan et al. more than three decades earlier (14). Strains exhibiting the race 2 virulence phenotype apparently have supplanted race 1 strains in infected tomato plants in central California, as exhibited by our failure to isolate a single race 1 strain. The selection against race 1 strains is especially more probable given that processing tomato cultivars grown in this region invariably carry the *Ve* gene for resistance to race 1. However, race 1 could still be isolated from other California-grown hosts that do not incorporate a *Ve* homolog resistance gene. Additionally, the genetic variability and evidence of gene flow among hosts in central California suggests that, apart from differences in virulence, no specialization is found among strains of *V. dahliae* isolated from various hosts.

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