Analysis of *Verticillium dahliae* Suggests a Lack of Correlation Between Genotypic Diversity and Virulence Phenotypes

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Abstract

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Verticillium dahliae causes severe wilt and recurring losses in numerous agricultural and ornamental hosts worldwide. Two virulence phenotypes (races) have been identified based on the *Ve* resistance gene and its homologs but their distribution and evolutionary history are unknown. Sequence analyses of the intergenic spacer of the ribosomal DNA and amplified fragment length polymorphism markers suggested an absence of correlation between genotypic diversity and virulence phenotypes. Additionally, both race 1 and 2 phenotypes were isolated in various geographic regions and hosts. Sustained levels of migration of both virulence phenotypes among various geographic regions were evident, and the study also suggested that both virulence

phenotypes infect a variety of hosts, regardless of the availability of resistant cultivars. Given the high genotypic diversity observed in *V. dahliae*, more than the two known virulence phenotypes may be present in nature but not yet identified because of the current lack of sources of resistance other than the *Ve* gene and its homologs. The inclusion of various genotypes exhibiting the same virulence phenotype may greatly improve the long-term effectiveness of resistance to race 2 of *V. dahliae* regardless of the host. This study also confirms the transcontinental gene flow and high genotypic diversity of *V. dahliae* affecting lettuce in coastal California regardless of the molecular markers employed.

Verticillium dahliae Kleb. is a cosmopolitan Ascomycete that causes vascular wilt, and possible death of a range of plant species (45). *Verticillium* wilt has been a major problem on numerous vegetable and small fruit crops in coastal California since the early 20th century but was not observed on lettuce (*Lactuca sativa* L.) until the mid-1990s (56). In lettuce, the disease has only been observed in the Salinas and Pajaro Valleys of coastal California, and has not been reported from other lettuce-producing regions in North America. In lettuce, the disease commonly results in total crop loss, because infected heads or plants, depending on the lettuce type, become unmarketable at or just prior to vegetative maturity. Conversely, *Verticillium* wilt has not been observed in baby leaf spinach (*Spinacia oleracea*) and baby leaf lettuce, both widely grown in coastal California for value-added salad mixes at higher plant densities than fresh market lettuce, because they are harvested well before vegetative maturity. Migration of *V. dahliae* genotypes with imported spinach seed and a lack of differentiation among various hosts grown in coastal California were previously described using simple-sequence repeat (SSR) or microsatellite markers (3). However, it is unclear whether the analysis of additional marker types, such as the dominant amplified fragment length polymorphism (AFLP) markers or intergenic spacer region of the ribosomal DNA (IGS rDNA) sequences, would lead to convergent conclusions. Furthermore, little information is available on the relationship of genotypic diversity with virulence phenotypes, and no knowledge of the evolutionary history of such phenotypes exists.

Two races of *V. dahliae* were previously differentiated on lettuce, sunflower, and tomato based on virulence of strains on differ-

ential cultivars (1,21,62). Further analyses indicated that a single gene is likely involved in conferring resistance to race 1 strain (21,23,29,54,62). The virulence phenotype of *V. dahliae* strains on hosts other than the ones they were isolated from was previously established (2,9,10,40). Sources of resistance to race 2 have not yet been identified in any host. Furthermore, it is not clear whether both races of *V. dahliae* are naturally prevalent in all populations or form distinct groups in separate geographic regions or on separate hosts. Similarly, little is known about the evolutionary history of *V. dahliae* in relation to modern-day breeding and selection efforts.

Extensive information comparing the virulence phenotype (race) of *V. dahliae* strains to the genotypic diversity of this organism is lacking. Thus, the current study was designed to determine the potential of host specialization among genotypes and tested three objectives: (i) investigate the relationship between virulence phenotypes and distinct AFLP and IGS rDNA genotypes, (ii) investigate the relationship between geographic and host origin of *V. dahliae* subpopulations with virulence phenotypes, and (iii) test the convergence of migration and results obtained from the analysis of population structure using AFLP and IGS rDNA with those of previous reports using SSR. All three objectives will have significant implications to the breeding for *Verticillium* wilt resistance in the different hosts.

Materials and Methods

Isolate collection and DNA extraction. Two hundred strains of *V. dahliae* were included in this study (Table 1) to represent the diversity in coastal California. Strains were isolated from infected lettuce (*L. sativa*) ($n = 77$); strawberry (*Fragaria × ananassa*) ($n = 19$); bell and chili pepper (*Capsicum annuum*) ($n = 28$); and weeds (five strains from sowthistle [*Sonchus oleraceus*] and one from coneflower [*Echinacea* spp.]); along with six strains from artichoke (*Cynara scolymus*) and three from marigold (*Tagetes erecta*) in the Asteraceae family ($n = 15$) which, together, will be referred to as the non-lettuce Asteraceae group. All strains were isolated from symptomatic plants collected at random in commercial fields where *Verticillium* wilt had occurred in the Salinas and Pajaro Valleys of coastal California.

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Strains isolated from commercial spinach seed lots ($n = 46$) were also included in the study. These seed lots were produced in the U.S. Pacific Northwest (Washington State) and northern Europe (The Netherlands and Denmark) and were planted in coastal California and other spinach-production regions. Preliminary analyses showed no differentiation between these two distinct geographic spinach seed production regions, and the strains were combined into a single subpopulation.

Because lettuce seed is produced in the San Joaquin Valley of California and no *Verticillium* wilt has been observed in lettuce seed production fields in this region, strains from commercial tomato fields ($n = 20$) were included in the study. Another outgroup population of *V. dahliae* with no anticipated gene flow was obtained from diseased ornamental trees in Wisconsin ($n = 15$). These urban and suburban ornamentals included the genera *Acer* ($n = 7$), *Rubus* ($n = 1$), *Rosa* ($n = 3$), and *Fraxinus* ($n = 4$) (Table 1).

All strains were recovered from plants showing *Verticillium* wilt symptoms that were collected at random from commercial fields (for commercial crops collected in coastal California and the San Joaquin Valley), runoff ditches (for weeds collected in coastal California), and urban and suburban landscapes (for ornamental hosts). *V. dahliae* was isolated on NPX medium and subsequently purified by transferring individual conidia. Conidial suspensions in 20% glycerol were used for long-term strain preservation at -20°C in a freezer. Strains were revived from such suspensions and grown in potato dextrose broth in preparation for DNA extraction.

Mycelia were harvested from cultures and total DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Valencia, CA). DNA extracts were subsequently quantified using a NanoDrop 1000 (NanoDrop Products, Wilmington, DE) spectrophotometer and concentrations were standardized to 2 ng/ μl by dilution in autoclaved purified water (Milli-Q; Millipore, Billerica, MA). DNA samples were kept at 4°C until used.

The identity of utilized strains was confirmed morphologically (presence of microsclerotia and verticillate conidiophores) and by sequencing of the internal transcribed spacer (ITS) region of the rDNA, which were deposited in GenBank (accessions GQ336671 to GQ336792). AFLP haplotypes were generated for all 200 strains, whereas the IGS rDNA was sequenced for 141 strains, and 92 strains were included in the virulence assay. The choice of strains used in each assay was based on sampling group representativeness.

IGS rDNA amplification and sequencing. The IGS rDNA was amplified from each strain using primers SK34 (5'-GGG TCC TGT AAG CAG TAG-3') and SK35 (5'-GAG CCA TTC GCA GTT TCG-3'). Bi-directional direct sequencing of polymerase chain reaction (PCR) products, following an AMPure magnetic bead cleanup (Beckman Coulter Genomics, Danvers MA), was performed at MCLab (South San Francisco, CA) using an ABI 3730XL sequencer and BigDye v.3.1 chemistry (Applied Biosystems, Foster City, CA), edited in BioEdit (28), aligned in Clustal W (59,60), and manually refined in McClade v. 4.06 (39). The amplification and sequencing of two control strains (including the completely sequenced Ls17 strain) were repeated three times.

AFLP data generation. AFLP assays (63) were performed using a fluorescent AFLP kit (Li-Cor, Lincoln, NE) according to the manufacturer's instructions. The template DNA was prepared by digesting with *EcoRI* and *MseI* restriction enzymes, followed by adapter ligation, and by preamplification using *EcoRI* and *MseI* primers without additional selective nucleotides. For selective PCR amplifications, 15 *EcoRI* (labeled with 6-FAM fluorescent dye at the 5' end) and *MseI* primer combinations with one or two selective bases were used (63). Products were separated by capillary electrophoresis in an automatic DNA sequencer (ABI Prism 3100 Genetic Analyzer; Applied Biosystems). Fragment analysis was performed using the software GeneMarker ver. 1.80 (SoftGenetics, State College, PA). Fragments 50 to 500 bp long and showing a minimum intensity of fluorescence of 250 were retained. The binary data were assembled using the AFLPDAT function (16) in the statistical package R (30,34).

To ensure reliability and reproducibility of the AFLP results, three distinct DNA samples were digested, ligated, preamplified, and amplified three times each. Additionally, negative control samples with no DNA templates were included in each PCR plate, and all yielded no results.

Phylogenetic analysis. Sites that violated the infinite-site model were removed from the IGS rDNA sequences, and indels were recoded in SNAP Workbench (49) using SNAP Map (4). Subsequently, all analyses were performed using the edited IGS rDNA sequences.

The Bayesian model as implemented in MrBayes (31) was employed to determine the phylogenetic relationship among the IGS rDNA sequences of the various strains. The most appropriate nucleotide substitution model was determined in Modeltest (46) using the Akaike information criterion (AIC). The general time-reversible (GTR) model fit the nucleotide substitution pattern best. In MrBayes, four runs of 5,000,000 generations each were used and trees were sampled every 100th generation, specifying the substitution model as Nst = 6. The heating temperature was reduced to 0.001 to obtain average standard deviations of the split frequencies smaller than 0.01, which required a minimum of 500,000 generations to achieve. Posterior probabilities were determined from a set of 10,000 trees, 1,000 per run, with sampling after 100,000 generations, allowing the log likelihood values to plateau. Midpoint rooting was used to display the unrooted IGS rDNA tree in FigTree v. 1.2.2 (22).

The evolutionary history of the *V. dahliae* strains was also explored using phylogenetic network analyses applied on IGS rDNA sequences. Network methods effectively show potential recombination or homoplasy events, in addition to identifying ancestral haplotypes (43,55). Haplotypes were generated in SNAP Workbench using SNAP Map. The IGS rDNA sequences were also analyzed using the statistical parsimony network approach (47,58). The parsimony network, which is a character-based method, was applied on IGS haplotypes in the software TCS (13) with the parsimony limit set to nine characters, or 95%. This method separates haplotype groups based on the number of different single substitutions.

The neighbor-net network, which is based on a distance method, was generated in SplitsTree v. 4 (32,33) for AFLP data sets. The Φ_w test, implemented in SplitsTree (11), was used to estimate the probability of recombination among the studied strains.

Population structure analysis. The level of differentiation among strains of *V. dahliae* from various geographic and host populations (termed "sampling groups" in the remainder of the article) included in this study was evaluated using an analysis of molecular variance (AMOVA) with the software Arlequin ver. 3.11 (18), which uses an analysis of variance framework based on an analog of Wright's fixation index (Φ) to compare the genetic variability within and between populations and population groups (19). To analyze the hierarchical structure of IGS rDNA sequence data,

Table 1. Number of *Verticillium dahliae* strains used to generate intergenic spacer of the ribosomal DNA (IGS rDNA) sequences and amplified fragment length polymorphism (AFLP) haplotypes from each geographic or host sampling group, along with the number of strains used to inoculate a panel of lettuce cultivars differing in response to the pathogen

Sampling group	Number of strains ^a		
	IGS rDNA	AFLP	Virulence
Asteraceae ^b	9	15	5
Lettuce	42	77	33
Pepper	14	28	14
Spinach	36	46	15
Strawberry	5	19	5
Wisconsin	15	15	...
Tomato	20	...	20
Total	141	200	92

^a All strains were isolated from symptomatic plants or infected spinach seed.

^b Non-lettuce isolates of Asteraceae.

the sampling groups were nested into four clusters: (i) all strains collected in coastal California from lettuce, pepper, non-lettuce Asteraceae, and strawberry; (ii) strains from tomato collected in the San Joaquin Valley of California; (iii) strains from ornamentals collected in Wisconsin; and (iv) strains from spinach seed produced in the U.S. Pacific Northwest and northern Europe. For AFLP analyses, only three clusters were used because of the lack of data on the tomato sampling group.

Identification of genetic clusters. The affiliation of individual strains of *V. dahliae* from sampling groups to specific genetic clusters and the possibility of admixture were tested using the software Structure v. 2.3.3 (50) on AFLP haplotypes. A lack of significant differentiation between sampling groups may be explained by repeated gene flow. For each number of clusters K ($K = 1$ through 10), defined as genetic clusters, 10 runs were performed with a burn-in period of 50,000 generations and 500,000 Markov Chain Monte Carlo simulations. The admixture model was chosen and the loci were considered independent. The analysis was repeated five times to ensure convergence of results.

The ad-hoc statistic ΔK was computed (17) to estimate the most likely number of genetic clusters K . This method uses the rate of change in the log probability between the successive K values. Accordingly, the results from the software Structure were graphically plotted following permutation and alignment in the program CLUMPP (35), and plotted using the software Distruct (52).

Migration analysis. Sequences of IGS rDNA were used to infer the potential for and rate of migration (gene flow) between sampling groups. Analysis was performed using Migrate ver. 3.0 (<http://popgen.scs.fsu.edu>), which employs the coalescent theory to implement a maximum likelihood estimation of the population size and interpopulation migration (7,8). For haploids $\Theta = 2Ne\mu$, with Ne being the effective population size and μ the mutation rate per locus. Mutation rates of IGS rDNA sequences were assumed to be constant to determine the rate of migration. Short chains were performed with 1,000 samples and 20 sampling increments and 1,000 trees were recorded; whereas long chains were performed with 5,000 samples and a burn-in of 10,000, and 5,000 trees were recorded. Sequences from the strawberry sampling group were not used for the migration analysis because number of entries ($n = 5$) was less than the minimum 8 individuals required for a meaningful estimation of Θ and, subsequently, a reasonable estimate of migration rates (20).

Coalescent analysis. A coalescent analysis was performed in an effort to retrace the evolutionary history of *V. dahliae* and to further clarify the historical relationship among strains in the various sampling groups (5,25,26). The IGS rDNA haplotypes derived above were used for this analysis. First, Tajima's D (57) and Fu and Li's D and F^* (24) neutrality tests were completed in the program DnaSP v. 5.10.01 (38). Growth in the subject populations may affect the assumption of a neutral evolution. Hence, the potential

for population growth was assessed in the program Lamarc v. 2.1.3 (37) using the maximum likelihood method, including 10 initial chains of 5,000 sampled genealogies and 5 final chains of sampled 10,000 genealogies. Four heated chains were also employed, which significantly improved the likelihood of the obtained population growth estimates. Subsequently, population growth and migration rates (calculated above) were incorporated with the ancestral analysis completed in Genetree v. 9.0 (5,26), which is included in SNAP Workbench. The tree exhibiting the highest likelihood and with the highest root probability was chosen to map the chronological mutation events and to identify the time to the most recent common ancestor. Five independent analyses were completed, each including a different random seed number and 1,000,000 coalescent simulations.

Pathogenicity assay. Seedlings of lettuce 'Salinas' (iceberg type, race 1- and race 2-susceptible) and 'La Brillante' (Batavia type, race 1-resistant and race 2-susceptible) (62) were initially grown in 200-well seedling plug trays (Hummert International, St. Louis) containing a pasteurized sand/potting soil mixture (2:1) for 4 weeks in a greenhouse prior to inoculation. Inoculum preparation, inoculation method, and disease rating have been described previously (62). Briefly, strains were grown on potato dextrose agar (PDA) for 4 to 5 days at room temperature to produce conidia that were collected in sterile distilled water (Table 2). Seedlings were inoculated with a 3-ml spore suspension (2.0×10^6 conidia/ml) applied directly to the soil in each plug tray well and repeated after 7 days. Noninoculated controls were treated with autoclaved water. Seedlings were then transplanted 1 or 2 weeks later to 0.5-liter foam-insulated cups filled with a pasteurized sand/soil potting mixture (3:1). Plants were maintained for an additional 8 to 10 weeks in a greenhouse with a 16-h photoperiod and at approximately $24 \pm 5^\circ\text{C}$ during the day and $12 \pm 5^\circ\text{C}$ at night. Experiments were conducted on 12 replications per cultivar and isolate and the entire experiment was repeated. At 8 to 10 weeks after inoculation (plants 12 to 14 weeks old), Verticillium wilt severity was assessed on uprooted and longitudinally cut crown and tap root. Disease severity was rated on a scale of 0 to 5, with 0 = no disease and 5 = 100% of discoloration of vascular and foliar tissues (62).

Strains consistently yielding an average severity exceeding 2.5 were considered virulent to the cultivar. Hence, isolates classified as race 1 consistently caused Verticillium wilt symptoms on Salinas but not on La Brillante. Conversely, isolates classified as race 2 consistently caused Verticillium wilt symptoms on both cultivars. Tap root and crown tissues of all inoculated and non-inoculated plants were sampled randomly and plated on NPX medium (12) to confirm the presence or absence of the pathogen.

Results across replicated experiments were consistent. Therefore, disease severity data from all experiments were pooled and the combined dataset analyzed as a two-way factorial using non-parametric methods. 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 LD_CI macro to generate relative treatment effects and 95% confidence intervals. Replications of experiments and blocks within experiments were considered random effects in the analysis.

Results

Pathogenicity assay. Of the 92 strains tested, 80 caused Verticillium wilt symptoms with average disease severity ratings exceeding 2.5 and were considered virulent on lettuce (Table 2). Forty-four isolates caused disease on Salinas but not on La Brillante, and were assigned to race 1. Thirty-six isolates caused disease on both Salinas and La Brillante and were assigned to race 2. Noninoculated control plants did not exhibit disease symptoms, nor was the typical vascular discoloration associated with infection by *V. dahliae*.

IGS rDNA phylogeny. A PCR product approximately 1,800 bp in size was amplified from all strains, and no evidence of multiple

Table 2. Numbers of strains of *Verticillium dahliae* isolated from five sampling groups that were tested for virulence on 'Salinas' (race 1- and race 2-susceptible) and 'La Brillante' (race 1-resistant and race 2-susceptible) lettuce

Sampling group	Number of strains			
	Total tested	Virulent ^a	Race 1 ^b	Race 2 ^c
Asteraceae ^d	5	3	1	2
Lettuce	33	31	24	7
Pepper	14	7	3	4
Spinach	15	14	12	2
Strawberry	5	5	3	2
Tomato	20	20	1	19
Total	92	80	44	36

^a Virulence was assessed based on the vascular discoloration on the crown and root system of 12- to 14-week-old plants inoculated at 4 weeks of age in the greenhouse.

^b *V. dahliae* strains virulent on Salinas but avirulent on La Brillante.

^c *V. dahliae* strains virulent on Salinas and La Brillante.

^d Non-lettuce isolates of Asteraceae.

products was observed following gel electrophoresis. Sequences of approximately 800 bp were obtained from each end of the PCR product. The reverse sequences obtained using primer SK35 was reverse-complemented and concatenated with the forward se-

quences obtained using primer SK34. The high sequence and size variability among strains was due in large part to numerous indels. All IGS rDNA sequences were deposited in GenBank (GQ336529 to GQ336670). After the removal of the infinite-sites violations and

Fig. 1. Unrooted Bayesian phylogenetic analysis using the intergenic spacer of the ribosomal DNA sequences of 142 strains of *Verticillium dahliae*. Analyses were performed in MrBayes (31) and using the general time reversible (GTR) substitution model identified in Modeltest (46). Values above branches indicate Bayesian posterior probabilities. The tree is displayed with midpoint rooting. Strain abbreviations: non-lettuce Asteraceae = Cs and Te; lettuce = Ls, pepper = Ca and Cf; strawberry = Fca; spinach = So; tomato = Le; and Wisconsin = Acer, Ap, Echin, Frax, Rubus, and Rosa.

recoding the indels, the length of the IGS rDNA sequence alignment was 923 bp.

The Bayesian analysis resulted in three main clades, including two that comprised strains from lettuce (strains Ls) and one that encompassed strains from pepper (strains Ca and Cf) (Fig. 1). Moreover, spinach strains (strains So) were observed in each of these three clades, including the cluster that contained strain Ls17, the representative race 2 strain. Similarly, the statistical parsimony network grouped the majority of the strains into three interior clades (Fig. 2). Only one haplotype, from the non-lettuce Asteraceae sampling group, could not be connected to the network because it fell outside of the 95% confidence limit of the parsimony. No evidence of homoplasy was observed. Haplotypes from spinach were represented in each of the three main clusters, whereas haplotypes from lettuce and pepper were present in two of the three clusters.

AFLP phylogeny. In total, 2,603 AFLP markers were scored from the 200 strains and used in the phylogenetic analysis. Loci were retained only if they were present in all replicates. The neighbor-net network exhibited elevated rates of recombination at the base of the network bifurcations (Fig. 3). No patterns of clustering were identified, and no clones were observed. Significant netting was observed at the center of the phylogenetic network. No correlation with IGS rDNA haplotypes and virulence was observed.

Population structure. Using the IGS rDNA sequences, 90% of the genotypic variability was measured within sampling groups (Table 3), with a $\Phi_{ST} = 0.1$ ($P = 0.005$). The differentiation among clusters (Φ_{CT}) was 0.029, not significantly different from 0 ($P = 0.279$), and amounted to 3% of the total variability (Table 3). The differentiation among sampling groups within clusters (Φ_{SC}) was 0.073 and was not statistically significant ($P = 0.184$).

Using AFLP haplotypes, 91% of the genotypic variability was measured within sampling groups (Table 3). The Φ_{ST} value for the AFLP was 0.087 ($P < 0.0001$); Φ_{CT} was 0.033, not significantly different from 0 ($P = 0.296$), and amounted to 3% of the total variability (Table 3); and Φ_{SC} was 0.055 and was significantly different from 0 ($P < 0.0001$). No significant differences were found when monomorphic loci were either censored or included, possibly because of the small proportion of such monomorphic loci.

The ΔK analysis (*not shown*) suggested that a two-cluster model best explained the structure of the studied sampling groups based on the AFLP data. Both genetic clusters were represented in all six sampling groups, regardless of geographic or host origin (Fig. 4).

Migration and coalescent analyses. Symmetrical migration rates were observed among multiple sampling groups, regardless of geographic distance or host crop. There was no significant gene flow from the spinach sampling group into the tomato and Wisconsin ornamental sampling groups (Table 4). However, symmetrical migration was measured between the spinach sampling group and coastal California sampling groups (non-lettuce Asteraceae, lettuce, and pepper). Gene flow was also identified between coastal California sampling groups and the tomato sampling group. Effective population sizes (Θ) were not significantly different among sampling groups (Table 4).

The hypothesis of neutrality was not substantiated in any of the sampling groups, regardless of the method used (*data not shown*). However, evidence of growth was observed in each of the sampling groups, with the exception of the non-lettuce Asteraceae, which showed a decline (Table 4). This significant change in population size may explain the departure from neutrality.

The coalescent analyses indicated that the majority of the strains exhibited evidence of a relatively recent expansion and a trend toward divergence, regardless of the host of origin (Fig. 5). Eighty

Fig. 2. Statistical parsimony network (58) of the intergenic spacer of the ribosomal DNA sequences from 141 *Verticillium dahliae* strains from non-lettuce Asteraceae (yellow), lettuce (green), pepper (purple), spinach (blue), strawberry (red), tomato (salmon), and ornamentals for Wisconsin (black). Small black circles are putative haplotypes not found in the studied samples. The size of colored circles is proportional to the number of individuals that form each haplotype. Numbers in circles indicate the number of individuals from each sampling group.

haplotypes formed a distinct lineage, which included strains from lettuce, imported spinach seed, tomato, non-lettuce Asteraceae, strawberry, and Wisconsin ornamentals. Similarly, a recently diverged lineage included 18 strains from ornamentals in Wisconsin or marigold (California non-lettuce Asteraceae). Two more ancient diverging groups of particular interest were identified. One included 18 strains from pepper and imported spinach seed, whereas a second, more recent, lineage included 7 strains from lettuce and spinach seed.

Discussion

This study identified both virulence phenotypes of *V. dahliae* (race 1 and race 2) in a variety of hosts from various geographic origins. Furthermore, both virulence phenotypes were identified in all ancestral clades, indicating a possible ancient origin of these phenotypes. These virulence phenotypes were not correlated with genotypic variability regardless of the marker type used. Additionally, the results of the 2,603 scored AFLP loci and the IGS rDNA sequences were concordant with those of SSR markers used on the same *V. dahliae* populations (3). All three marker types employed highlighted the transcontinental gene flow in *V. dahliae*.

Results indicated that *V. dahliae* strains from several hosts are capable of causing severe disease on lettuce. Additionally, strains of race 2 were prevalent in lettuce, pepper, spinach seed, and strawberry subpopulations and accounted for 45% of the 80 strains tested for virulence. The origin of the two virulence phenotypes remains unknown. However, the virulence phenotype was clearly not associated with any particular genotypic cluster. Thus, the occurrence of such virulence phenotypes in the subpopulations is probably not a novel phenomenon associated with modern breeding for resistance, and the evolution of this phenotype may possibly not be affected by the interaction with specific plant hosts (14,42). Nevertheless, the relatively recent deployment of race 1-resistant tomato cultivars has prevented the appearance of race 1 strains in areas where tomato is grown extensively (selective sweep; 15,27).

Previous research (51,62) had suggested a possible correlation between IGS rDNA sequences and the virulence phenotype, which was not apparent in the current study. Portions of the IGS rDNA undergo rapid evolution (6,61), especially in “hot-spot” regions filled with tandem repeats (48). Similar to the findings of this study, Pramteftaki et al. (48) failed to find any association between the IGS rDNA and phenotypic traits. Recombining organisms exhibit such a lack of correlation between various phenotypic and genotypic characters (41).

Similar to the IGS rDNA, the analysis of the AFLP haplotypes highlighted the high genetic diversity of *V. dahliae*. The neighbor-net network suggested that this high variability may be attributed to

recombination (meiotic or mitotic) events that appear as dense webbing at the center of the network. This was also suggested by the Φ_w test ($P = 0.0$). Similarly, gametic equilibrium was observed using microsatellite haplotypes among several of the same *V. dahliae* populations (3). However, it is currently unclear whether such recombination occurred historically and that, currently, the fungus does not recombine; or, conversely, that recombination is contemporary and possibly a ubiquitous phenomenon among *V. dahliae* subpopulations.

Phylogenetic and coalescent analyses of the IGS rDNA concordantly suggested the existence of three main genetic clusters of *V. dahliae* distributed in the United States and northern Europe. Additionally, the information from either type of molecular markers showed no geographic separation based on virulence, thus highlighting the global distribution of both virulence phenotypes. Knowing that distinct genotypes commonly shared identical virulence and aggressiveness traits, it becomes critical for plant breeders to utilize various genotypes of each virulence phenotype in the identification of resistant plant material.

The coalescent genealogy suggested a “recent” diversification in *V. dahliae*, which may be associated with contemporary agricultural practices that foster global movement of such fungi and offers a wide array of overlapping hosts. Coupled with the evidence of gene flow among various geographic regions, these findings highlight the potential significance of the incidental gene flow that accompanies the movement of plant material. Following their introduction, strains of *V. dahliae* have likely established in coastal California, as evidenced by their subsequent isolation from a variety of hosts in infested fields over time. Microsclerotia allow the survival of *V. dahliae* over long periods in the absence of a host but can also perpetuate the same strains across multiple cropping seasons (36,53). Furthermore, it is likely that the observed gene flow contributes to promoting the diversification and growth in most *V. dahliae* populations.

Because of the prevalence of plant material infested with *V. dahliae* that is traded across state and international borders, and the possibility of recombination, it is likely that additional virulence phenotypes exist. Only the availability of plant genotypes varying in genes coding for resistance to *V. dahliae* may assist in clarifying this matter. Furthermore, plant breeders would be greatly served by increasing the number of genotypes associated with each virulence phenotype, especially for race 2, against which resistance is currently not available. Because of the financial risks associated with selective sweeps accompanying the widespread deployment of resistance to one virulence phenotype, it is critical to test such cultivars against the broadest diversity of pathogen genotypes in addition to virulence phenotypes. This has implications for the long-term effectiveness of disease resistance, which will likely

Table 4. Effective population size (Θ) and gene flow (immigration and emigration) between pairs of geographic and host sampling groups of *Verticillium dahliae* from Migrate v. 3.0.3 (7), and estimate of population growth rate (PGR) using Lamarck 2.13 (37), employing intergenic spacer of the ribosomal DNA sequence haplotypes

Sampling group ^b	Θ^c	PGR	Sampling group ^a					
			Asteraceae ^d	Lettuce	Pepper	Spinach	Tomato	Wisconsin
Asteraceae ^d	0.021 (0.007–0.053)	–89.3	...	84.3 (30.2–181.2)	16.9 (1.0–74.3)	206.9 (110.9–346.6)	82.2 (28.0–179.3)	99.6 (38.5–203.5)
Lettuce	0.002 (0.001–0.005)	2,073.3	22.7 (3.8–70.2)	...	11.4 (0.6–50.0)	292.2 (154.3–421.3)	204.2 (122.6–316.4)	22.7 (3.8–70.3)
Pepper	0.008 (0.004–0.012)	9,556.3	0.0 (0.0–62.5)	97.7 (24.3–253.8)	...	195.3 (77.5–396.1)	0.0 (0.0–62.5)	0.0 (0.0–62.5)
Spinach	0.005 (0.003–0.010)	1,077.7	132.9 (76.2–212.6)	146.9 (47.8–231.8)	26.7 (6.6–69.3)	...	55.1 (21.9–111.4)	80.0 (38.4–144.3)
Tomato	0.014 (0.008–0.020)	15,000.0	87.9 (34.9–178.3)	133.3 (64.0–240.4)	58.6 (18.2–136.2)	0.0 (0.0–28.1)	...	29.3 (22.0–90.6)
Wisconsin	0.006 (0.003–0.010)	4,812.5	11.7 (0.7–129.5)	163.9 (92.3–265.6)	0.0 (0.0–22.5)	0.0 (0.0–22.5)	11.8 (8.8–51.8)	...

^a Maximum likelihood estimate of the 95% confidence interval of the migration rate M ($M = m/\mu$) and 95% confidence interval are shown.

^b Source populations are in columns and recipient populations in rows.

^c Maximum likelihood estimate and 95% confidence interval of the effective population size $\Theta = 2Nm$.

^d Non-lettuce isolates of Asteraceae.

become the single most effective management method available to growers, especially when soil fumigants are banned from use in agricultural production owing to their environmental and health concerns.

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Fig. 5. Coalescent genealogy based on the sequence of the intergenic spacer of the ribosomal DNA ($L = 1.96e-62$; $SD = 6.23e-64$) performed in Genetree (5,25). Numbered black circles display the position of mutations. The coalescent was based on 1,000,000 simulations and the analysis also included gene flow and population growth data, and an estimate of Waterston's $\Theta (m) = 3.65$. All 19 identified haplotypes and the frequencies of recovery from sampling groups are shown at the bottom. Asteraceae refers to the non-lettuce Asteraceae sampling group. Strawberry strains were not included in this analysis because of the small number of strains.

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