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# Population analyses of the vascular plant pathogen *Verticillium dahliae* detect recombination and transcontinental gene flow

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ABSTRACT

The fungal pathogen *Verticillium dahliae* has resulted in significant losses in numerous crops in coastal California, but lettuce remained unaffected until the mid-1990s. Since then outbreaks have decimated entire fields, but the causes of this sudden susceptibility of lettuce remain elusive. The population structure of *V. dahliae* isolated from coastal California ($n = 123$) was investigated with 22 microsatellite markers, and compared with strains from tomato in central California ($n = 60$), spinach seed imported from Washington State and Northern Europe ($n = 43$), and ornamentals from Wisconsin ($n = 17$). No significant differentiation was measured among hosts in coastal California or with the spinach and Wisconsin ornamental sampling groups. In contrast, the tomato sampling group was significantly differentiated. Significant gene flow was measured among the various geographic and host sampling groups, with the exception of tomato. Evidence of recombination in *V. dahliae* was identified through gametic disequilibrium and an exceedingly high genotypic diversity. The high incidence of *V. dahliae* in spinach seed and high planting density of the crop are sources of recurrent gene flow into coastal California, and may be associated with the recent outbreaks in lettuce.



## 1. Introduction

Verticillium wilt (caused by *Verticillium dahliae* Kleb.) has plagued the production of numerous small fruits and vegetables in coastal California for decades. Since 1995, the hitherto unaffected lettuce (*Lactuca sativa* L.) production has suffered greatly from the disease (Subbarao et al., 1997). The causes of this sudden broadening of the host range of *V. dahliae* into lettuce are uncertain. Speculations include: (i) a shift or adaptation in the local *V. dahliae* populations toward lettuce; or (ii) a sudden increase in population numbers in the region; and (iii) recurrent introductions of the pathogen into the area.

The genetics of *V. dahliae* populations are poorly understood, and little information is available on the structure of populations in California and elsewhere (Klosterman et al., 2009). Previous research relied on phenotypic or dominant markers (Bhat and Subbarao, 1999; Collado-Romero et al., 2006; Collins et al., 2003; Dobinson et al., 1996; Koike et al., 1996), which restrict re-tracing evolutionary histories. An array of 22 unlinked simple sequence repeat markers (SSR, microsatellite) was recently developed to explore the impact of genetic factors such as recombination, gene flow, genetic drift, etc. (Almany et al., 2009).

*Verticillium dahliae* survives as microsclerotia, which are dispersed through seed, vegetative planting material, soil, water, agricultural equipment, etc. (Klosterman et al., 2009). For example, significant infestation of spinach (*Spinacia oleracea* L.) seed was reported (du Toit et al., 2005). The majority of commercial spinach production in North America is in coastal California, and changes in spinach farming practices coincide chronologically with the first appearance of the disease in lettuce. Therefore, examining the extent of gene flow with spinach seed and its potential impact on the structure of the resident population of coastal California may prove critical. Migration analyses have been successfully employed to assess the evidence for the dispersal and relocation of fungal plant pathogens (Brunner et al., 2007; Ciampi et al., 2008). By comparing allele composition and frequencies among populations, inferences may be made on the movement of contemporary and historical pathogenic populations (Beerli and Felsenstein, 1999; Pearse and Crandall, 2004; Rannala and Mountain, 1997). Re-tracing the source of populations of *V. dahliae* present in coastal California

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may provide needed insight into the mechanisms of pathogen redistribution and dispersal regionally and globally.

The first objective of this research was to test the hypothesis that the sub-population of *V. dahliae* that infects lettuce is differentiated from others based on host or geographic origin. If sampling groups are differentiated, the second objective was to test the hypothesis of lack of gene flow among the host and geographic sampling groups. Because *V. dahliae* has no known sexual stage, the third objective was to test the hypothesis that populations are clonal. Finally, the fourth objective tested the hypothesis of lack of variation in current and historical sizes of the populations of *V. dahliae* evaluated.

## 2. Materials and methods

### 2.1. Isolate collection and DNA extraction

Two hundred and forty-three strains of *V. dahliae* were included in this study (Table 1) to represent the genetic diversity found in coastal California. Strains were isolated from infected plants of lettuce ($n = 65$), strawberry ($n = 18$), bell and chili pepper ($n = 25$), and weeds (five strains from sowthistle and one from coneflower) along with six strains from artichoke and three from marigold in the Asteraceae family ($n = 15$). The latter strains together will be referred to as the non-lettuce Asteraceae group. All strains were collected between 1995 and 2007 from various commercial fields located in the Salinas and Pajaro Valleys of the central coast of California and where Verticillium wilt had occurred. Strains from spinach seed ($n = 43$) produced in the US Pacific Northwest, Denmark and The Netherlands were also included in the study. These seed lots were destined for planting in coastal California and other spinach producing regions. Because a substantial portion of the lettuce seed is produced in the San Joaquin Valley of California, and no Verticillium wilt has been observed on lettuce in this region, the analyses included strains from tomato plants ($n = 60$) from this region. Resistance to race 1 of *V. dahliae*, which is conferred by the *Ve* gene, is prevalent in tomato cultivars currently grown in California, and thus, the *V. dahliae* population from tomato is chiefly composed of race 2 strains (Grogan et al., 1979). Another outgroup population of *V. dahliae* with no anticipated gene flow was assembled from diseased ornamentals in Wisconsin ($n = 17$). These urban and suburban ornamentals included the following genera: *Acer* ($n = 7$), *Rubus* ($n = 1$), *Rosa* ($n = 3$), *Fraxinus* ($n = 4$) and *Echinacea* ($n = 2$).

**Table 1**
Origin of *Verticillium dahliae* strains used in population analysis. Also, the number of genotypes obtained using 22 SSR markers (Almany et al., 2009) and Nei's corrected gene diversity ($H$) are listed.

| Sampling group | # Individuals | # Genotypes | $H_{obs}$[a] |
|---|---|---|---|
| Asteraceae[b] | 15 | 15 | 1 |
| Pepper[c] | 25 | 25 | 1 |
| Strawberry[c] | 18 | 18 | 1 |
| Spinach[d] | 43 | 41 | 0.999 |
| Lettuce[c] | 65 | 65 | 1 |
| Wisconsin[e] | 17 | 17 | 1 |
| Tomato[f] | 60 | 48 | 0.892 |
| Overall | 242 | 229 | 0.994 |

[a] Nei's corrected gene diversity ($H$) values were generated in GenoDive (Meirmans and Van Tienderen, 2004).
[b] Non-lettuce Asteraceae group strains were isolated from diseased artichoke or weeds growing in coastal California.
[c] Pepper, strawberry and lettuce strains were isolated from diseased plants in coastal California.
[d] Spinach strains were isolated from spinach seed produced in the US Pacific Northwest or northern Europe and sold in the coastal California.
[e] Wisconsin strains were isolated from dying-back ornamental trees planted in residential urban and suburban environments.
[f] Tomato strains were isolated from diseased plants grown in the San Joaquin Valley of California where lettuce seed is produced.

Mycelia were harvested from potato dextrose broth cultures and total DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Extracted DNA was 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, Billerca, MA). DNA samples were kept at 4 °C until used.

### 2.2. SSR amplifications

The following 22 simple sequence repeat (SSR) loci (and target repeat motifs) were amplified (Almany et al., 2009): VD1 (CCTG), VD2 (TCTGGC), VD3 (CACGCCCT), VD4 (CTG), VD7 (AG), VD8 (GAT), VD9 (GATGCGT), VD10 (TTGC), VD11 (GTCTGCCT), VD12 (TTTC), VD26 (GCAGAGAG), VD27 (CAATGCCTCG), VD65 (CACATACG), VD69 (TGGCAGA), VD73 (CGTTGC), VD74 (AGGC), VD77 (CCTTCA), VD85 (GA), VD92 (TCCTCT), VD96 (TGC), VD97 (GGCTTTCT), VD98 (GTGCTGG). Polymerase chain reaction (PCR) assays were conducted to amplify individual loci. Reaction mixtures included: 2 ng DNA from *V. dahliae*, and GoTaq colorless PCR mix (Promega, Madison, WI), in 20 μl reactions. Amplifications were performed in Bio-Rad DNA Engine thermocyclers (Bio-Rad Laboratories, Hercules, CA) and used the following cycling parameters: denaturing at 95 °C for 2 min, followed by 35 cycles of denaturing at 95 °C for 10 s, annealing at 58 °C for 10 s and extension at 72 °C for 30 s. A final extension was carried out at 72 °C for 5 min. Subsequently, PCR fragments labeled with the various fluorophores were multiplexed for fragment analysis. A total of 1 μl of each labeled PCR fragment was mixed with fragments labeled with the three remaining fluorophores. Then 1 μl of this multiplex mixture was combined with Hi-Di formamide and 0.3 μl of LIZ-500 size standard and separated on an ABI 3100 sequencer (Applied Biosystems, Foster City, CA). Fragment analysis was completed using the GeneMarker software (SoftGenetics, State College, PA).

This array of SSR markers comprises motifs ranging between 2 and 10 nucleotides. Because of concerns about potential homoplasy due to the length of the SSR motifs, only two markers were designed around bi-nucleotide motifs, and three for tri-nucleotide motifs. Previous sequence analyses confirmed the identity and length of those repeat motifs (Almany et al., 2009). Furthermore, the strain VdLs17 for which a complete genome sequence is available was included in every PCR plate to ensure consistency across runs (http://www.broad.mit.edu/annotation/genome/verticillium_dahliae).

### 2.3. Population analysis

The level of differentiation of *V. dahliae* from various geographic and host populations (termed sampling groups in the remainder of this report) included in this study was evaluated using an analysis of molecular variance (AMOVA) with the software Arlequin ver. 3.11 (Excoffier et al., 2005), which uses an analysis of variance framework based on an analog of Wright's fixation index ($\Phi$) to compare the genetic variability within and between populations and population groups (Excoffier et al., 1992). The sampling groups were divided into four clusters: (1) all strains collected in coastal California, (2) strains from tomato from the San Joaquin Valley of California, (3) strains from Wisconsin, (4) strains from spinach seed produced in the US Pacific Northwest and northern Europe. Pairwise comparisons among populations were conducted and Slatkin's $R_{ST}$ was calculated, which assumes a high rate of stepwise mutation model (Slatkin, 1995). An $R_{ST} = 0$ indicates panmictic populations; an $R_{ST} < 0.05$ denotes populations that are negligibly differentiated; $0.05 \leqslant R_{ST} \leqslant 0.25$ signifies that populations are moderately differentiated; an $R_{ST} > 0.25$ indicates populations that are highly differentiated; and an $R_{ST} = 1$ demonstrates populations that are completely differentiated and do not share migrants. Be-