Genetic Differentiation of *Verticillium dahliae* Populations Recovered from Symptomatic and Asymptomatic Hosts

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ABSTRACT

Verticillium dahliae is a soilborne fungal pathogen affecting many economically important crops that can also infect weeds and rotational crops with no apparent disease symptoms. The main research goal was to test the hypothesis that *V. dahliae* populations recovered from asymptomatic rotational crops and weed species are evolutionarily and genetically distinct from symptomatic hosts. We collected *V. dahliae* isolates from symptomatic and asymptomatic hosts growing in fields with histories of Verticillium wilt of potato in Israel and Pennsylvania (United States), and used genotyping-by-sequencing to analyze the evolutionary history and genetic differentiation between populations of different hosts. A phylogeny inferred from 26,934 single-nucleotide polymorphisms (SNPs) in 126 *V. dahliae* isolates displayed a highly clonal structure correlated with vegetative compatibility groups, and isolates grouped in lineages 2A, 2B⁸²⁴, 4A, and 4B, with 77% of the isolates in lineage 4B.

The lineages identified in this study were differentiated by host of origin; we found 2A, 2B⁸²⁴, and 4A only in symptomatic hosts but isolates from asymptomatic hosts (weeds, oat, and sorghum) grouped exclusively within lineage 4B, and were genetically indistinguishable from 4B isolates sampled from symptomatic hosts (potato, eggplant, and avocado). Using coalescent analysis of 158 SNPs of lineage 4B, we inferred a genealogy with clades that correlated with geographic origin. In contrast, isolates from asymptomatic and symptomatic hosts shared some of the same haplotypes and were not differentiated. We conclude that asymptomatic weeds and rotational hosts may be potential reservoirs for *V. dahliae* populations of lineage 4B, which are pathogenic to many cultivated hosts.

Keywords: ecology, epidemiology, fungal pathogens, genomics, population biology

Plant-pathogenic microorganisms have a range of interactions with their hosts other than those that result in disease. Microbial species traditionally considered to be plant pathogens have been found interacting with plant hosts as endophytes without causing apparent disease (Bacon and Hinton 1996; Lofgren et al. 2018; Malcolm et al. 2013), living on plant surfaces as epiphytes (Xin et al. 2018), colonizing the plant rhizosphere (Busch et al. 1978; Edel et al. 1997), or as soil inhabitants (Demers et al. 2015). Plant–microbe interactions are often overlooked by plant pathologists in the absence of disease symptoms or outside of an agricultural context, although these asymptomatic interactions have been the subject of significant ecological research (Burdon 1991; Burdon et al. 2006; Gilbert 2002) and are very relevant for disease epidemiology and management. For example, from a phytopathological perspective, plants on which symptoms have not been yet observed for a given disease are considered to be nonhosts of the pathogen. However, some studies indicate that asymptomatic plant

hosts can serve as cryptic or hidden reservoirs of microbial diversity (Evans 1971; Xin et al. 2018), including plant pathogens. This has led us to use interactions with pathogens to classify plants into “symptomatic”—plant species displaying visible disease symptoms as a consequence of microbial infection—and “asymptomatic”—plant species in which endophytic colonization does not result in detectable disease—thus leaving the term “nonhost” to refer to plant species that do not interact with the given microbe (Malcolm et al. 2013). Similarly, some microbial communities found in the environment such as in soil or in water may also be sources of pathogen diversity and inoculum contributing to epidemics in agroecosystems (Monteil et al. 2013, 2016; Tsrer (Lahkim) et al. 2020). However, for most plant–microbe interactions, the role of asymptomatic hosts and the diversity of pathogens associated with them remain relatively unknown. Tsrer (Lahkim) et al. (2020) recently reported alternative hosts of *Spongopora subterranea*, the causal agent of powdery scab on potato, which allow multiplication and survival of the pathogen over time. *S. subterranea* was detected in 16 weed species from eight families and in volunteer plants of wheat, sampled in potato fields with history of powdery scab. Whether asymptomatic plants act as reservoirs that have roles in the evolution of pathogen populations and epidemic dynamics in subsequent susceptible crops (Stergiopoulos and Gordon 2014; Suproniene et al. 2019; Wang et al. 2010) is an important question that still needs to be addressed for many plant pathogens and may be especially relevant for designing effective disease management strategies in agroecosystems.

Verticillium dahliae is a soilborne fungal pathogen of many economically important crops that can also infect weeds and rotational crops without causing apparent disease symptoms (Evans

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1971; Malcolm et al. 2013; Wheeler and Johnson 2016). *V. dahliae* is most prevalent in temperate and Mediterranean climates and has a broad range of susceptible hosts of over 300 plant species (Pegg and Brady 2002). Verticillium wilt is one of the most difficult diseases to manage because of the limited availability of disease-resistant cultivars. Management primarily targets the reduction of inoculum in soil. *V. dahliae* produces long-lived microsclerotia, which are highly melanized structures that can survive in the environment for long periods of time in the absence of a host plant. Reduction in inoculum is achieved primarily by soil disinfestation using fumigants and rotations with crops that do not develop symptoms, whether asymptomatic or true nonhosts (Rowe and Powelson 2002; Rowe et al. 1987; Tsror (Lahkim) et al. 2005). In theory, crop rotations with asymptomatic and nonhost species are important for managing plant diseases to avoid the inoculum build-up resulting from constant cultivation of symptomatic, susceptible crops. Rotations are also a more environmentally sustainable alternative to soil fumigation for managing Verticillium wilts (Davis and Huisman 2004).

Crop rotations often have inconsistent effects for reducing Verticillium wilt of potato, and those effects largely depend on the sequence and length of the rotation and soil type (Busch et al. 1978; Davis et al. 1996, 1999a, b; Joaquim et al. 1988; Larkin et al. 2011). For example, in Israel, potato cultivation includes 3 to 4 years of rotation between potato crops; this relatively short period results in little reduction in Verticillium wilt and requires fumigation, which is done once every few cycles depending on cultivar susceptibility and soil inoculum levels (Cohen et al. 2012). Previous research showed that some rotational crops and weeds can be asymptotically infected by *V. dahliae* in the field (Demirci and Genc 2009, 2011; Evans 1971; Krikun and Bernier 1987, 1990; Malcolm et al. 2013; Wheeler and Johnson 2016; Wheeler et al. 2019). In addition, *V. dahliae* can colonize the surface of roots and produce microsclerotia on rotational crops and weeds even though it may not colonize systemically (Busch et al. 1978; Evans 1971; Evans and Gleeson 1973; Harrison and Isaac 1969; Lacy and Horner 1966; Levy and Isaac 1976; Mol 1995).

Populations of *V. dahliae* comprise a limited number of clonal lineages that correlate with vegetative compatibility groups (VCGs) and can be identified by single-nucleotide polymorphism (SNP) genotyping (Milgroom et al. 2014, 2016; Rafiei et al. 2018). Diversity of *V. dahliae* has been extensively studied in populations associated with symptomatic hosts in different geographical regions (Atallah et al. 2010; Iglesias-Garcia et al. 2013; Jiménez-Díaz et al. 2011, 2012; Joaquim and Rowe 1991; Schnathorst and Mathre 1966; Tsror (Lahkim) et al. 2001). For example, in Israel, lineages 4B, 2A, and 2B (inferred by VCG) were identified among *V. dahliae* isolates causing disease in cotton, potato, tomato, and other vegetable crops, whereas lineage 1A, the defoliating pathotype, was only identified from cotton in the northern region of the country (Korolev et al. 2000, 2001, 2008). Isolates of lineage 2B were recovered both from northern and southern parts of Israel while 4B isolates were found mostly in the south, where potato is primarily grown. In the United States, *V. dahliae* lineages 2A, 2B, 4A, 4B, and 6 (inferred from VCGs) affect potato and other vegetable crops. Isolates from lineages 4A and 4B are highly aggressive toward most potato cultivars and predominant in potato fields (Bhat et al. 2003; Dung et al. 2013; Joaquim and Rowe 1991). In contrast, few studies have investigated the genetic diversity of *V. dahliae* populations found in asymptomatic hosts. For example, Demirci and Genc (2009, 2011) isolated *V. dahliae* lineages 2B and 4B (inferred from VCGs) from asymptomatic weeds in Turkish potato fields, and Wheeler et al. (2019) observed that *V. dahliae* isolates pathogenic to potato were recovered from asymptomatic rotational crops collected from potato fields in Washington State. It is important to elucidate whether asymptomatic hosts in potato fields serve as reservoirs of *V. dahliae* lineages 4A and 4B, which are aggressive on potato. If inoculum is produced in asymptomatic rotational crops and weeds, it could contribute to the initiation of

epidemics in subsequent potato crops. If inoculum is produced in asymptomatic hosts that is effective on a primary crop such as potato, we predict that the same pathogen genotypes would be found in both symptomatic crops and asymptomatic rotational crops or weeds.

The main objective of this study was to test the hypothesis that *V. dahliae* populations recovered from asymptomatic rotational crops and weeds are genetically distinct from those infecting symptomatic susceptible crops such as potato. A second objective was to investigate the genetic structure and evolution of the lineages associated with asymptomatic hosts. These objectives were addressed by using an SNP dataset generated by genotyping-by-sequencing (GBS) to analyze the evolutionary history and genetic differentiation of *V. dahliae* populations collected from fields with histories of Verticillium wilt of potato in Israel and Pennsylvania (United States).

MATERIALS AND METHODS

Isolates of *V. dahliae*. *V. dahliae* was sampled from symptomatic and asymptomatic hosts grown in fields with histories of Verticillium wilt in Israel and Pennsylvania (United States). In Israel, most sampling was done in the southern region of the country which is the main potato-growing area. Sampling was done in fields that had been planted to potato 1 to 3 years prior to sampling. *V. dahliae* was isolated from volunteer potato plants or rotational crops such as sorghum and sunflower, and from weeds (asymptomatic hosts) growing in the same fields. In addition to sampling in southern Israel, we attempted to isolate *V. dahliae* from various crops and weeds in several cultivated fields with diverse cropping histories in central and northern Israel; none had a history of potato production. In Pennsylvania, we sampled *V. dahliae* from potato ‘Snowden’ and ‘Reba’ and oat ‘Armor’ in fields located in Ringtown (Schuylkill County) with a history of Verticillium wilt, as described by Malcolm et al. (2013). Oat was planted as a rotational crop in this field and considered an asymptomatic host. Isolation of *V. dahliae* was performed as described by Tsror (Lahkim) and Levin (2003) and Malcolm et al. (2013). Prior to SNP genotyping, we performed VCG typing on a large subset of the Pennsylvania or Israel isolates to confirm the correlation between SNP-derived lineages and VCGs. VCG typing was done using standard methods for *V. dahliae* (Tsror (Lahkim) and Levin 2003). In addition to sampling from symptomatic and asymptomatic hosts, we included nine additional isolates from various crops as representatives of known VCGs or lineages that we assumed may present larger genetic diversity; therefore, these isolates were only used for the phylogenetic analysis in our study (Supplementary Table S1). These nine isolates originated from cotton (GVd20 = VCG1A, and GVd57 and GVd96 = VCG2B), olive (GVd56 = VCG2A, and GVd97 = VCG2B), tomato (GVd108 = VCG2A, and GVd107 = VCG4B), chrysanthemum (GVd105 = VCG2B), and eggplant (GVd85 = VCG4B).

Genomic DNA and GBS. Single-spore cultures were grown on potato dextrose agar (Difco, Franklin Lakes, NJ, U.S.A.) for 5 to 7 days at room temperature. Sterile water (2 ml) was added to the surface of the plate and the plate was scraped to release mycelia and spores. Mycelia and spores were collected, lyophilized, and ground to fine powder using sterile pestles. Genomic DNA from Pennsylvania isolates was extracted from 25 mg of lyophilized material with the UltraClean Tissue & Cells DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, U.S.A.) following the manufacturer’s instructions. Nucleic acid purity and concentration were assessed on a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, U.S.A.). DNA of isolates from Israel was extracted using Maxwell 16 Tissue DNA Purification Kit (Promega Corporation, Madison, WI, U.S.A.) according to the manufacturer’s protocol. Genomic DNA quality was assessed by enzymatic digestion with *Hind*III (New England BioLabs, Ipswich,

MA, U.S.A.) and electrophoresis on 0.8% agarose gel. Genomic DNA samples were submitted to the Beijing Genomics Institute (BGI, Shenzhen, China) for GBS (Elshire et al. 2011). Briefly, genomic DNA was digested with the restriction enzyme *ApeKI* to generate a reduced-representation library. Oligonucleotide adapters and sample-specific barcodes were ligated to the restriction fragments for PCR and sequencing on Illumina HiSeq 4000. Paired-end reads of 100 bp from the ends of the restriction fragments were generated and separated by taxa (i.e., sample). Barcode- and adapter-free quality sequencing reads were provided by BGI for further bioinformatic analysis.

Variant calling and assignment of *V. dahliae* isolates to clonal lineages based on SNP markers. SNP genotyping and assignment of *V. dahliae* isolates to clonal lineages was performed as described previously (Milgroom et al. 2014, 2016; Rafiei et al. 2018), following the recommendations from Grünwald et al. (2016). Reads were aligned to the *V. dahliae* reference genome VdLs.17 (GenBank accession GCA_000952015.1) (Klosterman et al. 2011), using the Burrows-Wheeler Alignment Tool (BWA *mem* option) (Li 2013) with default parameters for paired-end reads. Samtools v1.4.1 (Li et al. 2009) and Sambamba v0.6.6 (Tarasov et al. 2015) were used to sort aligned read files and mark duplicates using the functions *samtools view-Shu*, *sambamba sort*, and *sambamba markdup*. Joint variant calling was performed using Freebayes v1.1.0-9-g09d4ecf (Garrison and Marth 2012) with *-f* function and default parameters. Sites containing putative variants were filtered with *vcfilter* (*vcflib* from Freebayes) *-f* function for SNP sites with read depth >5 and mapping quality >30 in individual samples. Subsequent analyses were performed on SNP datasets of Pennsylvanian or Israeli isolates alone and together with SNP data from representatives of *V. dahliae* clonal lineages. Unfiltered SNP data from 33 representatives of *V. dahliae* clonal lineages 1A, 2B³³⁴, 4A, 6, 2B^{R1}, 2B⁸²⁴, 2A, and 4B (Milgroom et al. 2014) were included. All heterozygous values (caused infrequently by sequencing or alignment errors) were recorded as missing data, as described previously (Milgroom et al. 2014, 2016; Rafiei et al. 2018), and the merged dataset was filtered using TASSEL V5.2.38 (Bradbury et al. 2007). All sites were filtered for a minimum minor allele frequency of 1% and allele information in at least 75% of the samples. Monomorphic sites and samples that did not have information in at least 50% of the sites were excluded. Filtered SNP positions were used to assign Pennsylvanian and Israeli isolates to clonal lineages previously defined (Milgroom et al. 2014). A neighbor-joining phylogeny was inferred on the filtered SNP dataset using the maximum-composite-likelihood model and 1,000 bootstraps of the data with pairwise deletion option for missing allele data in MEGA7 (Kumar et al. 2016).

Genetic structure of *V. dahliae* lineage 4B populations. Diversity and structure of *V. dahliae* lineage 4B populations from Pennsylvanian and Israeli potato fields were further analyzed. In TASSEL, raw SNP data from lineage 4B isolates from potato fields were filtered for sites with a minimum minor allele frequency of 1% and allele information in 100% of the samples. Monomorphic sites were excluded. To explore the genetic structure of lineage 4B isolates, we used k-means and the Bayesian Information Criterion (BIC) method, a clustering algorithm to identify genetic groups without prior definition after transforming data using a principal component analysis (Jombart et al. 2010). The discriminant analysis of principal components (DAPC) was used to visualize the variation between and within clusters in the R package *ade4* *adegenet* 2.1.2 (Jombart 2008) in R 3.6.2 (R Core Team 2020). Briefly, genetic clusters were identified using *find.clusters* function with options *max.n.clust* = 60 and *n.pca* = 100, and the DAPC graph was built using the genetic clusters previously identified, *dapc* and *scatter.dapc* functions.

Genetic relationships among lineage 4B isolates were explored using a minimum spanning network (MSN) with *bitwise.dist* function set for Hamming distance, which calculates the number of

allelic differences between samples, and *poppr.msn* in the R package *poppr* 2.8.3 (Kamvar et al. 2014). In addition, a coalescent genealogy was inferred to investigate the ancestry of lineage 4B isolates from potato fields. Coalescent analyses were performed on Mobyly SNAP Workbench (Monacell and Carbone 2014) using the program Genetree (Bahlo and Griffiths 2000) and the workflow instructions for Genetree provided at <http://snap.hpc.ncsu.edu/cgi-bin/mobyly/portal.py#welcome>. Only parsimony-informative sites and sites without missing data or gaps were included in the analysis. The data set was analyzed in RDP4 (Martin et al. 2015) to confirm the absence of recombination among the selected sites. In order to reconstruct the mutation history of the alignment, we performed 1 million runs of the data starting from three different seed numbers.

Genetic differentiation of *V. dahliae* populations from symptomatic and asymptomatic hosts. In order to investigate whether *V. dahliae* populations from symptomatic and asymptomatic hosts were genetically differentiated, analyses were focused on lineage 4B because all isolates from asymptomatic hosts sampled from potato fields were placed in this lineage (see Results). Two populations were established based on the symptomatology of the host from which they were isolated: symptomatic (originating from symptomatic hosts) and asymptomatic (originating from asymptomatic hosts). First, analysis of molecular variance (AMOVA) was performed to analyze the genetic differentiation between the symptomatic and asymptomatic populations of *V. dahliae* lineage 4B. Second, AMOVA was conducted to detect the level of differentiation between populations within each of the lineage 4B genetic clusters identified by k-means and BIC.

Analyses were performed on SNPs with at least 1% minor allele frequency and no missing allele information to test the null hypothesis that *V. dahliae* populations were not genetically differentiated. Tests were performed in *poppr* using *poppr.amova* to estimate Φ , analogous to fixation index (F_{ST}) (Excoffier et al. 1992) with threshold = 0.1 and filter = TRUE. Data were randomly permuted 999 times with the function *randtest* from the R package *ade4* 1.7-13 (Chessel et al. 2004). The genetic relationship of isolates from both populations were also explored using the MSN as described above.

Data availability. All files with SNP data and scripts used for the analyses in this study can be accessed at GitHub (https://github.com/laurabau/BautistaJalon_Vdahliae).

RESULTS

Isolates of *V. dahliae*. We obtained a total of 132 *V. dahliae* isolates from symptomatic ($n = 78$) and asymptomatic hosts ($n = 54$) in Israel and Pennsylvania (Table 1; Supplementary Table S1). In Israel, we obtained 27 isolates from symptomatic crops and 41 isolates from asymptomatic crops and weeds. Although central and northern Israel were sampled intensively, most isolates, including all obtained from asymptomatic samples, were collected from fields with recent potato cropping history located in the south ($n = 66$). Despite attempts to isolate from many plants in central and northern Israel, we only obtained two isolates from symptomatic hosts in these regions. *V. dahliae* isolates from Pennsylvania ($n = 64$) were collected from symptomatic potato ‘Snowden’ and ‘Reba’ ($n = 51$) and asymptomatic ‘Armor’ oat plants ($n = 13$). All isolates sampled from Israel were typed to VCG except two (GVd35 and GVd54). All isolates collected from Israel from asymptomatic hosts were in VCG4B ($n = 40$), whereas isolates collected from symptomatic hosts were in VCGs 2A ($n = 3$), 2B ($n = 4$), and 4B ($n = 19$). In total, 30 isolates from the 64 isolates from Pennsylvania were typed to VCG (24 isolates from the symptomatic host potato and 6 from the asymptomatic host oat). Isolates from potato were in VCGs 2A ($n = 2$), 4B ($n = 11$), and 4A ($n = 11$), whereas all isolates from oat were in VCG4B (Supplementary Table S1).

SNP genotyping and phylogenetic structure of *V. dahliae* populations. In total, 136,683 SNP sites were detected with Freebayes and *vcfilter* among *V. dahliae* isolates from Pennsylvania and Israel. After merging and filtering the SNP dataset from

Pennsylvania and Israel with unfiltered SNP data from *V. dahliae* lineage representatives (Milgroom et al. 2014), six isolates (Supplementary Table S1) were excluded because they were missing data at >50% of the SNP loci. We inferred the evolutionary history of *V. dahliae* using 26,934 SNPs distributed throughout the genome of 168 *V. dahliae* isolates (Fig. 1; Supplementary Table S2). This set included 59 isolates from Pennsylvania and 67 from Israel; in addition, it included 33 reference isolates for clonal lineages identified by SNP genotyping (Milgroom et al. 2014) and 9 reference isolates of known VCGs from Israel (Korolev et al. 2000, 2008).

The neighbor-joining phylogeny comprises three main clades with subclades that correlate to VCGs (Fig. 1). It is concordant with the phylogenetic structure observed in previous studies (Milgroom et al. 2014; Raffei et al. 2018), except for the location of 2B^{R1}, which was closely related to 2B⁸²⁴ and 6 in this study instead of 1A/1B and 2B³³⁴ as in Milgroom et al. (2014).

Pennsylvanian isolates from symptomatic potato ($n = 50$) were placed in lineages 2A, 4A, and 4B ($n = 2, 18$, and 30 isolates, respectively), as expected from their VCG phenotypes. Pennsylvanian isolates from oat ($n = 9$), an asymptomatic rotational host, were found only in lineage 4B. Israeli isolates recovered from symptomatic hosts were placed in lineages 2A, 2B⁸²⁴, and 4B ($n = 4, 4$, and 19 isolates, respectively), as expected from their VCG phenotypes. These isolates were obtained from mango, olive, avocado, potato, eggplant, pepper, cantaloupe, watermelon, and sunflower (Table 1). Isolates sampled from asymptomatic hosts ($n = 40$) clustered solely in lineage 4B and were recovered from weeds, peanut, and sorghum (Table 1). Isolates in lineage 4A were obtained only from potato in Pennsylvania (Fig. 1).

Genetic structure of *V. dahliae* lineage 4B populations.

All *V. dahliae* isolates from asymptomatic hosts were placed in lineage 4B together with some isolates from symptomatic

hosts (Table 1). Lineage 4B comprised the largest group of *V. dahliae* individuals in this study, with a total of 98 isolates, 77% of the isolates sampled ($n = 59$, Israel and $n = 39$, Pennsylvania).

We identified 1,343 SNPs with no missing allele information, 1% minor allele frequency, and randomly distributed throughout the genomes of 98 lineage 4B isolates (Supplementary Table S2). Using the k-means clustering algorithm, the BIC values suggested an optimal number of three or four genetic clusters (Fig. 2). Four clusters were selected ($k = 4$), corresponding to three groups containing Pennsylvanian isolates (PA_1, PA_2, and PA_3) and one group of Israeli isolates, independent of the symptomatology of the host of origin.

Variation among the genetic clusters was visualized in DAPC using two principal components and two discriminant functions (DA eigenvalues). Both DAPC and MSN analyses showed three main groups, with PA_3 and Israeli populations plotted close to each other in one large group but distant from PA_1 and PA_2. PA_1 and PA_2 were also distant between themselves (Figs. 2 and 3A) and positioned in different quadrants.

Coalescent analysis was performed for 98 individuals using 158 parsimony-informative SNPs with no missing data to infer the genealogy of lineage 4B isolates (Fig. 4). RDP4 detected no recombination signals in the SNP alignment, which fulfilled necessary assumptions for coalescent analysis. In total, 24 haplotypes were identified. The inferred genealogy showed a topology concordant with four genetic clusters. Isolates in the Israeli and PA_3 clusters formed two distinct groups that coalesced with each other before they coalesced with clusters PA_1 and PA_2. Israeli isolates grouped into 12 haplotypes in a single clade. In all, 38 of the 59 Israeli isolates were characterized as haplotype H6 and were obtained from a variety of symptomatic hosts ($n = 16$) such as potato, tomato, olive, and avocado but also from asymptomatic

TABLE 1. *Verticillium dahliae* isolates sampled in this study from symptomatic and asymptomatic hosts by geographic location and host, and their clonal lineage placement

Location, symptomatology ^a	Host plant family	Number of isolates	Crop	Clonal lineage ^b	Haplotype ^c	
Israel						
Asymptomatic (<i>n</i> = 41)	Amaranthaceae	6	Weed	4B	H6, H10, H11	
	Asteraceae	3	Weed	4B	H6	
	Boraginaceae	11	Weed	4B	H5, H6, H7, H15, H16	
	Brassicaceae	12	Weed	4B	H6, H9, H12, H14	
	Convolvulaceae	1	Weed	4B	H14	
	Fabaceae	1	Peanuts	4B	H6	
	Malvaceae	4	Weed	4B, ND (<i>n</i> = 1)	H6, H13, H14	
	Poaceae	1	Sorghum	4B	H15	
	Solanaceae	2	Weed	4B	H6, H12	
	Symptomatic (<i>n</i> = 27)	Anacardiaceae	1	Mango	4B	H6
Asteraceae		2	Sunflower	4B	H6	
Cucurbitaceae		2	Cantaloupe (<i>n</i> = 1)	4B	H6	
...		...	Watermelon (<i>n</i> = 1)	4B	H6	
Lauraceae		2	Avocado	4B	H6	
Oleaceae		3	Olive	2A (<i>n</i> = 1)	ND	
...		...	4B (<i>n</i> = 2)	H6		
Solanaceae		17	Potato (<i>n</i> = 10)	4B	H6, H8, H9	
...		...	Pepper (<i>n</i> = 5)	2A (<i>n</i> = 1)	ND	
...		...		2B ⁸²⁴ (<i>n</i> = 4)	ND	
...		...	Eggplant (<i>n</i> = 2)	2A	ND	
Pennsylvania, United States						
Asymptomatic (<i>n</i> = 13)		Poaceae	13	Oat	4B (<i>n</i> = 9), ND (<i>n</i> = 4)	H2, H3, H17, H22
Symptomatic (<i>n</i> = 51)		Solanaceae	51	Potato	2A (<i>n</i> = 2)	ND
		...	...	...	4A (<i>n</i> = 18)	ND
		...	...	...	4B (<i>n</i> = 30)	H1, H2, H4, H17, H18, H19, H20, H21, H23, H24
		...	...	...	ND (<i>n</i> = 1)	ND

^a Geographic location and host symptomatology; n = number of isolates.

^b All isolates were assigned to a clonal lineage based on single nucleotide polymorphism (SNP) data generated by genotyping-by-sequencing. ND = clonal lineage was not determined because of missing data for >50% of the SNPs.

^c ND = haplotype not determined because isolates were not included in the coalescence analysis.

hosts ($n = 22$) such as weed species in the families Brassicaceae, Asteraceae, and Boraginaceae. This haplotype can also be considered the ancestral state of the Israeli population of lineage 4B because there are no mutations that differentiate it from the most recent common ancestor of this clade (Fig. 4). Other isolates

obtained from symptomatic hosts were in haplotypes H8 and H9. Isolates from asymptomatic hosts were in haplotypes H6, H9, and nine other haplotypes (H5, H7, H10, H11, H12, H13, H14, H15, and H16). Isolates from Pennsylvania were also found in 12 different haplotypes, with one predominant haplotype (H17 in PA_1 clade)

Fig. 1. Neighbor-joining tree inferred from 26,934 single-nucleotide polymorphisms among 168 *Verticillium dahliae* isolates collected in Pennsylvania (PA) and Israel. Branch bootstrap supports <70% are omitted. Thirty-three *V. dahliae* reference isolates, representative of clonal lineages 1A, 2A, 2B³³⁴, 2B⁸²⁴, 2B^{R1}, 4A, 4B, and 6 (Milgroom et al. 2014), are indicated by solid circles. Israeli lineage or vegetative compatibility group reference isolates are indicated with open circles. Geographic origin, host, and symptomatology of the host of *V. dahliae* isolates are shown on the right side. Only 50 selected isolates of 98 isolates in lineage 4B are included in the figure to reduce the figure size. Missing allele data were excluded from this analysis.

comprising nine isolates from potato (symptomatic host) and five from oat (asymptomatic host). Two haplotypes (H2 and H17 in PA_3 and PA_1, respectively) contained isolates from both symptomatic (potato) and asymptomatic (oat) hosts. Eight haplotypes had isolates only from the symptomatic host, potato (H1, H4, H18, H19, H20, H21, H23, and H24), while two haplotypes included only isolates from the asymptomatic host, oat (H3 and H22).

Genetic differentiation of *V. dahliae* populations from symptomatic and asymptomatic hosts. All *V. dahliae* isolates

recovered from asymptomatic hosts were in lineage 4B; however, isolates recovered from symptomatic hosts were in lineages 2A, 2B⁸²⁴, and 4B (isolates from Israel) and 2A, 4A, and 4B (isolates from Pennsylvania). This disparity in lineage diversity indicates that populations from symptomatic and asymptomatic hosts are differentiated (Table 1). Focusing on lineage 4B, results from AMOVA showed that isolates from symptomatic hosts and asymptomatic hosts were strongly differentiated ($\Phi = 0.1405$, $P = 0.001$) (Table 2) when all isolates were analyzed together without taking population structure into account. However, no genetic differentiation was found between isolates from symptomatic and asymptomatic hosts within three of the genetic clusters Israel, PA_1, and PA_2 ($P > 0.1$); isolates from genetic cluster PA_3 could not be compared because of the small sample size ($n = 3$), and PA_2 only included two isolates from asymptomatic hosts; therefore, we should be cautious when drawing conclusions given the unbalanced number of isolates in this cluster. In addition, the MSN showed that isolates from asymptomatic hosts were genetically highly similar to isolates collected from symptomatic hosts from the same genetic cluster (Fig. 3B). Therefore, the strong differentiation that was initially observed between isolates from symptomatic hosts and asymptomatic hosts when genetic clusters were not taken into account reflects a confounding effect caused by genetic structure.

DISCUSSION

The main goal of this research was to test the hypothesis that *V. dahliae* populations recovered from rotational crops and weeds—asymptomatic hosts—are evolutionarily and genetically distinct from those infecting susceptible crops such as potato—symptomatic hosts. To test this hypothesis, the phylogenetic diversity and the genetic differentiation of *V. dahliae* populations collected from asymptomatic and symptomatic hosts in Israel and Pennsylvania were analyzed with a large dataset of SNPs generated by GBS. Overall, *V. dahliae* isolates from symptomatic hosts were placed in previously known lineages 2A, 2B⁸²⁴, 4A, and 4B. However, isolates recovered from asymptomatic hosts were all characterized as members of lineage 4B, indicated both by SNP genotyping and VCG phenotyping. The high lineage diversity found in populations infecting symptomatic hosts is consistent with previous research in North America and Israel (Bhat et al. 2003; Dung et al. 2013; Joaquim and Rowe 1991; Korolev et al. 2000, 2001, 2008; Tsrör (Lahkim) et al. 2001). A few isolates from Israel recovered from symptomatic eggplant, pepper, and olive were placed in lineages 2B⁸²⁴ and 2A, and a small subset of isolates from Pennsylvania infecting potato were characterized as lineage 2A. Lineages 2A and 2B⁸²⁴ are not as frequently associated with potato as lineages 4A and 4B but, rather, are lineages that generally affect other solanaceous crops (Dung et al. 2013; Tsrör (Lahkim) et al. 2001).

Endophytic or epiphytic fungi that do not cause overt symptoms on asymptomatic hosts may have the potential to affect susceptible crop plants (symptomatic hosts). Such asymptomatic hosts represent hidden ecological niches that could serve as reservoirs of plant pathogens. They are significant for disease management because they have the potential to (i) maintain pathogen diversity and (ii) maintain populations that are pathogenic to other crops and can contribute inoculum to initiate new field-level epidemics, thus bypassing the benefits of crop rotation (Evans 1971; Monteil et al. 2013, 2016; Xin et al. 2018). Our results indicate that asymptomatic weeds and rotational crops did not harbor a diverse *V. dahliae* population, because isolates from these hosts were exclusively in lineage 4B, a lineage with the potential to cause disease to a broad range of susceptible hosts (Bebegali et al. 2010; Chandelier et al. 2003; Iglesias-Garcia et al. 2013; Tsrör (Lahkim) et al. 2001). Lineage 4B was also the predominant lineage among *V. dahliae* isolates infecting a wide range of symptomatic hosts in this study.

There was no significant differentiation of lineage 4B isolates between populations on symptomatic and asymptomatic hosts.

Fig. 2. A, Discriminant analysis of principal components of *Verticillium dahliae* lineage 4B isolates from Pennsylvanian and Israeli potato fields. The analysis was performed on 1,343 single-nucleotide polymorphisms and 98 individuals using the adegenet R package. Genetic clusters were identified using k-means and Bayesian Information Criterion (BIC). Genetic variation was visualized using two principal components and two discriminant functions. B, Values of BIC versus number of genetic clusters in *V. dahliae* lineage 4B isolates from Pennsylvania and Israel. Isolates from asymptomatic hosts grouped in each of the four genetic clusters identified together with isolates from symptomatic hosts.

Isolates infecting host plants as endophytes were often closely related to isolates from susceptible hosts (symptomatic hosts), as illustrated in the MSN and the coalescent analysis (Figs. 2 and 3). Additionally, recent studies in greenhouse conditions showed that *V. dahliae* isolates that are pathogenic to common susceptible hosts could asymptotically infect a variety of rotational crops and weed species typically found in U.S. potato fields (Frederick et al. 2017; Wheeler and Johnson 2016), and that *V. dahliae* isolates collected from asymptomatic hosts caused disease in potato (Wheeler et al. 2019). Therefore, we conclude that the symptomatology of a given host is the result of the interaction between host plant species or genotypes and specific *V. dahliae* lineages, although future experiments should address this.

In this study, asymptomatic hosts included a wide variety of common weeds such as wild mustard species (Brassicaceae); species in the Solanaceae, Boraginaceae, Amaranthaceae, and Malvaceae families; and crops frequently grown in rotation with potato such as oat and sorghum. This is a relevant finding because oat and sorghum are intentionally rotated with potato for the management of Verticillium wilt. The strategy for rotating susceptible crops with ostensibly resistant species or fallow periods is to minimize inoculum build-up in soil by separating in time inoculum-production cycles in susceptible crops. However, an important question raised here is whether or not inoculum is produced on asymptomatic hosts infected with *V. dahliae* and whether this inoculum is effective on susceptible crops. Previous research found *V. dahliae* infecting a variety of host plants (mustards, millet, wheat, corn, sunflower, buckwheat, and barley) in the field without causing symptoms (Demirci and Genc 2009, 2011; Vallad et al. 2006; Wheeler and Johnson 2016; Wheeler et al. 2019). Some studies also showed that, in general, asymptomatic *V. dahliae*-infected rotational crops and weeds produced lower levels of *V. dahliae* inoculum (potentially microsclerotia) compared with infected potato under greenhouse conditions (Frederick et al. 2017; Wheeler and Johnson 2016). Additionally, other studies reported that *V. dahliae* can produce microsclerotia in plant roots even in the absence of systemic infections (Busch et al. 1978; Harrison and Isaac 1969; Lacy and Horner 1966; Mol 1995). If lineage 4B individuals infecting asymptomatic plants produce microsclerotia on roots and aboveground plant tissue, crop rotations may have the unintended effect of carrying over certain lineages between growing seasons and, thus, contributing inoculum to soil, resulting in ineffectual management. We speculate that *V. dahliae* multiplies during wilt epidemics on potato tissue but only a fraction of the lineage diversity is maintained through rotations by endophytic infections or colonization of nonhost roots because only lineage 4B infects asymptomatic hosts. A similar process could be occurring with other *V. dahliae* lineages, as suggested by the experiments previously mentioned (Frederick et al. 2017; Wheeler and Johnson 2016; Wheeler et al. 2019). Wheeler and Johnson (2016) sampled oat crops from two commercial fields with a history of Verticillium wilt of potato or mint in the Columbia Basin of central Washington but failed to isolate *V. dahliae* from oat stems. It is hard to explain the discrepancy in outcomes from Wheeler and Johnson (2016) and this study without speculation; however, one plausible explanation is that it is a consequence of differences in lineage composition of *V. dahliae* populations in the fields sampled.

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Fig. 3. Minimum spanning network of *Verticillium dahliae* lineage 4B isolates from Pennsylvania and Israel. Analyses were performed using 1,343 single-nucleotide polymorphisms and 98 individuals. **A**, Populations are defined by genetic clusters identified by k-means and Bayesian Information Criterion (BIC). **B**, Populations are defined by host symptomatology. Isolates from asymptomatic hosts were genetically highly similar to isolates from symptomatic hosts and grouped in the same genetic cluster.

V. dahliae populations causing Verticillium wilt of mint overwhelmingly comprise members of VCG2B, and populations causing Verticillium wilt of potato in Washington are mainly in VCG4A (Dung et al. 2013). As seen from our results, the lineage associated with asymptomatic infections in Israel and Pennsylvania

is VCG4B. Therefore, the capability of establishing an endophytic interaction with a given asymptomatic host seems to be lineage dependent. These scenarios might explain the mixed outcomes provided by crop rotations in Verticillium wilt management (Busch et al. 1978; Davis et al. 1996, 1999a,b; Joaquim et al. 1988; Larkin

Fig. 4. Inferred coalescent genealogy of *Verticillium dahliae* lineage 4B using 158 single nucleotide polymorphisms (SNPs) among 98 isolates from Pennsylvania and Israel. Black dots represent nucleotide mutations and numbers beside them are their positions in the SNP sequence alignment. The scale on the right shows coalescent time rescaled to 1. Haplotypes defined by Genetree (Haplotype), the number of isolates in each haplotype (n), host symptomatology of isolates in each haplotype (Symptomatology: A = asymptomatic and S = symptomatic), and the lineage-4B genetic clusters (Israel, PA_1, PA_2, and PA_3) are indicated below the tree.

et al. 2011). Consequently, the fact that asymptomatic hosts can be carriers of lineage 4B between seasons should be considered during the management decision-making process.

The lineages identified in this study were differentiated by host of origin. For example, lineages 2A and 2B were found only in symptomatic hosts and lineage 4A was found only in potato. Isolates from Pennsylvania in lineage 4B were divided into three distinct groups whereas 4B isolates from Israel grouped into a single cluster. A small group of Pennsylvanian 4B potato and oat isolates (PA_3) was genetically and evolutionarily more similar to the Israeli 4B group than to the other Pennsylvanian 4B samples, and the coalescent genealogy showed that these two groups coalesced before the rest of the samples from Pennsylvania. The coalescent analysis also indicated that all of the lineage 4B haplotypes in Israel appear to be derived from one introduction or multiple introductions from the same clonal source population. Historically, Israel has imported potato seed tubers from northern European countries. Surveys of Israeli potato seed carried out from 1995 to 1998 found significant levels of infection with major seedborne pathogens, and more than one-third of the domestic certified seed tubers infested or infected by *V. dahliae* (Tsrur et al. 1999). However, in a survey done in 2000 to 2005, 21 of 178 seed lots imported from northern Europe carried *V. dahliae* and all isolates belonged to lineage 4B (L. Tsrur (Lahkim), unpublished results). Because potato seed trade between Israel and northern Europe happens annually, it is probable that lineage 4B was introduced into Israel on potato seed tubers through this commercial route. Studies on *Colletotrichum coccodes*, another common tuberborne potato pathogen, in Israel showed that imported certified seed tubers from Europe were infected with specific VCGs of the pathogen, making it possible to identify the sources of the introductions: VCG6 originated from The Netherlands and VCG7 from Scotland (Shcolnick et al. 2007). However, we cannot rule out the hypothesis that *V. dahliae* lineage 4B was introduced with hosts other than potato because this lineage has a broad host range. One plausible explanation for the relation between 4B populations in Israel and one small cluster of 4B isolates in Pennsylvania could be that they are descendants from the same source population located somewhere else and they were introduced with agricultural commodities. A comparison of lineage 4B isolates from a broader geographical sampling and a larger SNP dataset could help clarify migration routes of this lineage.

We did not find new lineages or lineages associated only with asymptomatic reservoirs as previously hypothesized and described in other host–pathogen systems (Bartoli et al. 2015; Monteil et al. 2013). The cropping history of a field is an important factor that may influence the diversity of *V. dahliae* lineages present in that field (Cohen et al. 2012). It is possible that the cropping history of the fields sampled in this study may have influenced the prevalence of

lineage 4B infecting asymptomatic and symptomatic hosts. For example, lineage 1A, a lineage highly aggressive toward cotton and olive, was prevalent in areas of southern Spain with a long history of cultivation of these crops; however, a higher lineage diversity was found in provinces with a more diverse cropping history that had been more recently planted to olive (Jiménez-Díaz et al. 2011). In many regions of Israel, lineages other than 4B were more common in crops such as cotton and tomato until 15 years ago (L. Tsrur (Lahkim), unpublished results). However, Verticillium wilt incidence in these crops was significantly reduced due to a decrease in cotton-growing area and the introduction of Verticillium wilt-resistant varieties. As a consequence, potato and rotational crops, including orchards cultivated on fields with a history of potato cultivation, became the primary crops that harbor *V. dahliae*. Therefore, we speculate that *V. dahliae* lineage 4B, which is highly prevalent on potato, became the major lineage that can infect both symptomatic and asymptomatic hosts in Israel.

Although there was no significant differentiation of lineage 4B isolates between populations from symptomatic and asymptomatic hosts (as illustrated in the MSN and the coalescent analysis) (Figs. 2 and 3), the pathogenicity of isolates recovered from asymptomatic hosts toward potato or other economically important crops still needs to be determined. However, overall, our study shows that asymptomatic rotational crops and weeds have the potential to be reservoirs of *V. dahliae* lineage 4B, which contains isolates pathogenic to potato and other economically important cultivated species. This may explain the lack of consistency in Verticillium wilt management provided by crop rotations, as discussed above. However, several additional research questions need to be addressed before augmenting current management recommendations. These include (i) how much inoculum is produced on asymptomatic hosts under field conditions; (ii) how crop cultural practices affect production, survival, viability, and genetic diversity of the inoculum; and (iii) how asymptomatic infections affect the dynamics of subsequent epidemics on susceptible hosts. Our study also highlights how little we know about the ecology of *V. dahliae* outside crop production. For instance, it is unknown what roles, if any, this fungus might play outside of agricultural systems; if plant pathogenicity is the main ecological role of the species or just an artifact of agriculture; and what conditions determine whether *V. dahliae* infections result in endophytic or pathogenic interactions. Knowing the range and nature of hosts and lineage diversity in a field is crucial for improving disease management because different *V. dahliae* lineages are associated with meaningful biological attributes such as pathogenicity, aggressiveness, and host range (Iglesias-García et al. 2013; Jiménez-Díaz et al. 2006, 2016; Joaquim and Rowe 1991; Rafiei et al. 2018). To make agroecosystems more resilient and design more efficient and

TABLE 2. Analysis of molecular variance between *Verticillium dahliae* lineage 4B populations from symptomatic (Symp) and asymptomatic (Asymp) hosts collected from potato fields in Pennsylvania (PA) and Israel^a

Sample ^b	Symp	Asymp	Φ^c	P value	Source of variation ^d	df	Sum of squares	Variance components	Variation (%)
Lineage 4B	49	49	0.1405	0.001	Between	1	2,069.63	37.54	14.05
	...	...	...	...	Within	96	22,096.00	230.17	85.95
	...	...	...	...	Total	97	24,165.63	267.71	100.000
Clusters ^e									
Israel	19	40	0.0062	0.165	...	...	...	...	...
PA_1	18	5	0.0449	0.159	...	...	...	...	...
PA_2	11	2	0.1403	0.112	...	...	...	...	...

^a Data were permuted 999 times.

^b In the sample analyzed, isolates were grouped in populations defined by symptomatology of the host they were isolated from: symptomatic (isolates recovered from symptomatic hosts) and asymptomatic (isolates recovered from asymptomatic hosts).

^c Population differentiation statistic (Φ) analogous to fixation index (F_{ST}) (Excoffier et al. 1992).

^d Between populations, within population, and total.

^e Genetic clusters within lineage 4B. Isolates were assigned to genetic clusters identified by k-means and Bayesian Information Criterion (BIC) based on single nucleotide polymorphism data. Level of genetic differentiation between *V. dahliae* isolates from cluster PA_3 was not estimated because of the small sample size ($n = 3$).

sustainable management practices against *Verticillium* wilts, we should take into account the lineage diversity and ecology of *V. dahliae* populations.

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