Genetic Diversity of *Verticillium dahliae* Populations From Olive and Potato in Lebanon

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Abstract

Verticillium dahliae is widely distributed in potato and olive fields in Lebanon, causing serious economic losses. However, little is known about the inoculum source, population structure, and genetic diversity of the pathogen or the mechanisms of dissemination within Lebanon. To understand the population structure, a total of 203 isolates sampled from olive ($n = 78$) and potato ($n = 125$) were characterized for species, mating type, and race, and the genetic relationships were delineated using 13 microsatellite markers. All isolates except one from potato were *V. dahliae*, with 55.1 and 12.1% race 1, and 43.6 and 83.1% race 2 in olive and potato, respectively. The genetic structure of the studied

population was best described by two large and two small clusters. Membership in the two large clusters was determined by the presence or absence of the effector gene *Ave1*. Furthermore, genetic structure was moderately associated with the host of origin but was weakly associated with the geographic origin. All but four isolates represented by three multilocus haploid genotypes were *MAT1-2*. This study identified a clear lack of gene flow between virulence genotypes of *V. dahliae* despite the proximity of these cropping systems and the wide distribution of genetic diversity among hosts and geographic regions in Lebanon.

Potato and olive represent two of the most important sources of sustenance and income in Lebanon. Potato ranked first with a total production of 443,000 tons from a 17,352-ha area in 2014 (FAO-STAT 2014). Olive has been cultivated in Lebanon for thousands of years (Chalak et al. 2015), and in 2014 was produced on 58,496 ha (FAO-STAT 2014). Overall potato production is concentrated in Bekaa (70%) and Akkar (27%) districts, whereas olive production is spread among North Lebanon (23%), Nabatiyeh (21%), Akkar (18%), and South Lebanon (15%). In the last decade, a gradual increase in area under olive cultivation has expanded into Bekaa (Anonymous 2012).

Verticillium dahliae Kleb. causes vascular wilts of nearly 200 plant species (Inderbitzin and Subbarao 2014), including olive and potato. *V. dahliae* is a soil-borne fungus that produces microsclerotia that survive in soil for at least 14 years (Wilhelm 1955). As a result, once microsclerotia infest the soil and increase to thresholds that affect different crops, control of *Verticillium* wilt is difficult. *Verticillium* wilt causes billions of dollars of damage annually to a variety of crops throughout the world (Pegg and Brady 2002). Recent surveys indicated high incidence of *Verticillium* wilt in both olive (25.7%) and potato (61.2%) fields in Lebanon, and were widely distributed among fields (46.2 and 77.5%, respectively) (Baroudy et al. 2016; Habib et al. 2017). However, little information is available on whether the populations from one crop affect the other and the structure of these populations. Knowledge of the genetic structure of *V. dahliae* in Lebanon may help explain how the pathogen became widely distributed

on these two crops that are in most cases separated geographically, and would lay the basis for practices to prevent further spread of the pathogen.

Microsatellite markers have been used to characterize the population structure of *V. dahliae* from a single field (Puri et al. 2017) or from a global collection (Short et al. 2015). Microsatellite genotyping was also used to identify clonal lineages in the haploid *V. dahliae* that can help in the determination of several phytopathological traits such as race and pathotype (Rafiei et al. 2018).

First, *V. dahliae* is known to exhibit qualitative differences in virulence governed by the gene-for-gene interaction between the effector gene *Ave1* in the pathogen and the R gene *Ve1* in tomato (de Jonge et al. 2012) or its homolog in Solanaceous or non-Solanaceous plants, including potato (Fick and Zimmer 1974; Hayes et al. 2011; Simko et al. 2003; Song et al. 2016; Zhang et al. 2013). Race 1 isolates of *V. dahliae* possess *Ave1*, the product of which is recognized by hosts with *Ve1* or a homolog, rendering these isolates avirulent. In contrast, race 2 isolates lack *Ave1* and are therefore able to evade recognition and cause disease on hosts with *Ve1* or a homolog. Thus, race is an important context in which to understand the population biology of *V. dahliae*.

Second, the mating type (*MAT*) locus of *V. dahliae* is organized in a manner typical of heterothallic fungi in which individuals possess only one of the *MAT* genes *MAT1-1-1* or *MAT1-2-1*, found in the dissimilar idiomorphs *MAT1-1* and *MAT1-2*, respectively. A heterothallic individual generally must encounter an individual of the opposite mating type to sexually reproduce. The meiotic recombination that occurs during sexual reproduction may combine favorable alleles and result in genotypes of the pathogen that are more fit and/or aggressive (Usami et al. 2009). Although no sexual stage has been found in any *Verticillium* species, the proximity of opposite mating types or their migration into a single field represents a risk of sexual recombination. In addition, there is a lack of information on the distribution of races and mating types among *V. dahliae* isolates within Lebanon. Even though Short et al. (2015) analyzed a global population of over 1,100 isolates from a variety of hosts, isolates from Lebanon were not represented in this study.

The objectives of the present study were to: i) characterize the population genetic structure of *V. dahliae* from olive and potato in Lebanon using microsatellites, mating type, and race designation; and

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ii) determine if this structure is associated with host plant and geographic location of origin.

Materials and Methods

Isolates collection and DNA extraction. A total of 203 isolates collected from potato plants ($n = 125$) and olive trees ($n = 78$) during nationwide disease surveys in Lebanon were analyzed in this study (Baroudy et al. 2016; Habib et al. 2017). Briefly, both symptomatic and asymptomatic tissue samples from olive trees, and only symptomatic tissue from potato plants were collected from the Northern, Southern, and Bekaa areas in Lebanon. The Northern area consisted of three Lebanese governorates: North Lebanon, Mount Lebanon, and Akkar; the Southern area consisted of South Lebanon and Nabatiyeh governorates; and the Bekaa area included Bekaa and Baalback-Hermel governorates (Table 1). The *Verticillium* spp. isolates were obtained from the surface-disinfested stem tissue plated on potato dextrose agar (PDA) amended with 500 ppm streptomycin sulfate (Sigma-Aldrich, Milan, Italy). Following isolation of *Verticillium* from the plated tissue, single conidia cultures were obtained and cultured on PDA for future processing. The single spore cultures were grown in 9-cm PDA plates, parafilm, and shipped from Italy to Dr. Subbarao's lab at University of California, Davis. Further isolate handling, DNA extraction, and PCR amplification were done following the APHIS permit requirements. All molecular work, unless mentioned otherwise, was done at the University of California, Davis.

For DNA extraction, two or three agar plugs (3 mm diameter) from fresh cultures were transferred to 50 ml of potato dextrose broth and incubated for 10 days at room temperature. Mycelium was collected using vacuum filtration, washed with sterile distilled water, dried using paper towels, and placed in 2-ml tubes for storage at -80°C for later use. Mycelium was then lyophilized and ground to fine powder using FastPrep instrument (MP Biomedicals, Irvine, CA) for 1 min at maximum speed. The FastDNA Kit (MP Biomedicals LLC, Solon, OH, USA) was used to extract genomic DNA from all isolates following the manufacturer's instructions, and the quality and quantity of DNA were assessed using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). An aliquot of the DNA of each isolate was adjusted to 30 ng/ μl for all the polymerase chain reactions (PCR).

Verticillium species identification. To identify isolates to species within *Verticillium* sensu stricto (Inderbitzin et al. 2011), all isolates were tested with previously developed *V. dahliae* – *V. longisporum* multiplex PCR assays (Inderbitzin et al. 2013). Briefly, each PCR reaction consisted of 100 ng DNA, 0.5 μM of each primer (D1f/AlfD1r, Alf1A1r, DfD1r), and 12.5 μl of 2 $\times$ GoTaq Green Master Mix (Promega, Madison, WI) in a total volume of 25 μl . PCR was performed in a ProFlex 2 $\times$ 96-well PCR System (Thermo Fisher Scientific, Inc.) programmed for 2 min at 94°C for initial denaturation followed by 35 cycles of 10 s at 94°C , 20 s at 64°C , and 1 min at 72°C , and a final 7-min extension at 72°C . Six microliters of SYBR Gold 1 $\times$ (Invitrogen, Carlsbad, CA) was added for each PCR amplicon, loaded to 1.5% (wt/vol) agarose gels in 0.5% TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA) and referenced to 100-bp DNA ladder (Invitrogen Life Technologies, Carlsbad, CA). Gels were run 45 min at 100 V and visualized with a transilluminator (UVP, Upland, CA). The band expected for *V. dahliae* is 490 bp. Any isolates that did not amplify with the previous multiplex PCR were tested with *V. nubilum* primers (Nuf/NoNuR) using the same PCR mix and conditions described previously for the multiplex (including 0.5 μM of each of two primers) except the annealing temperature used was 62°C .

Mating type determination. A multiplex PCR assay was used to screen for the presence of the mating type genes *MAT1-1-I* (primers Alf3/MAT11r) and *MAT1-2-I* (primers HMG21f/MAT21r) (Inderbitzin et al. 2011). A 25- μl mixture was prepared per DNA sample containing 60 ng genomic DNA, 0.5 μM of each primer, and 12.5 μl of 2 $\times$ GoTaq Green Master Mix. Each PCR had the following thermal profile: 3 min at 94°C for initial denaturation, 35 cycles of 1 min at 94°C for denaturation, 30 s at 57°C for annealing, 1 min at 72°C for extension, and a final 10 min extension at 72°C . PCR

amplicons were visualized as above. The bands expected for *MAT1-1-I* and *MAT1-2-I* are ~ 592 bp and 330 bp, respectively.

Race determination. The primers VdAve1F and VdAve1R were used to detect the presence of *Ave1*, the effector gene possessed by *V. dahliae* race 1 isolates (de Jonge et al. 2012), whereas the VdR2F/VdR2R primer pair was used to identify race 2 isolates (Short et al. 2014). PCR reactions for race 1 and race 2 were done separately as explained by Gurung et al. (2014). Briefly, each 25 μl PCR reaction contained 20 ng genomic DNA, 0.4 μM of each primer, and 12.5 μl of 2 $\times$ GoTaq Green Master Mix. For race 1, each PCR had the following thermal profile: 2 min at 94°C for initial denaturation, followed by 35 cycles of 30 s at 94°C , 45 s at 62°C , and 1 min at 72°C followed by a final extension of 10 min at 72°C . For race 2, each PCR had the following thermal profile: 2 min at 94°C for initial denaturation, followed by 35 cycles of 1 min at 94°C , 1 min at 64°C , and 1 min at 72°C followed by a final extension of 10 min at 72°C .

Microsatellite genotyping. Isolates were genotyped using 13 previously described polymorphic microsatellite loci (VD1, VD2, VD3, VD8, VD9, VD10, VD11, VD12, VD27, VD69, VD73, VD92, and VD97) (Molecular Ecology Resources Primer Development Consortium 2009) (Supplementary Table S1). Forward primers were labeled at the 5' end with one of the following fluorophores: FAM, PET, NED, and VIC. Briefly, PCR was performed in 25 μl total volumes containing 8.5 μl of sterile distilled water, 2 μl of genomic DNA at 30 ng/ μl , 0.5 μl of 10 μM of each reverse and forward primer, and 12.5 μl of GoTaq Colorless Master Mix (Promega Corp., Madison, WI). PCR was carried out in ProFlex 2 $\times$ 96-well PCR System (Thermo Fisher Scientific Inc.) that was programmed for 5 min at 94°C for initial denaturation and 35 cycles consisting of 30 s at 95°C , 20 s at 55°C , and 30 s at 72°C , followed by a final 10 min extension at 72°C . For fragment analysis, the PCR amplicons labeled with different fluorophores (FAM, PET, NED, and VIC) were comigrated by adding 2 μl of each labeled PCR product into a 96-well PCR plate. Fragment analysis was performed on an ABI 3100 sequencer (Applied Biosystems, Foster City, CA) at the UC Davis DNA Sequencing Facility following addition of Hi-Di formamide and 0.3 μl of LIZ-500 size standard to 1 μl of the pooled PCR product. The GeneMarker software (SoftGenetics, State College, PA) was used to determine alleles from the peaks for each fragment. The strain VdLs17 (http://www.broad.mit.edu/annotation/genome/verticillium_dahliae), for which a complete genome sequence is available, was included in every PCR plate to ensure consistency across runs.

Data analysis. Exploratory analysis of microsatellite allele data using distance and multivariate methods was performed in R v3.3.2 (R Core Team 2016). First, allele calls and metadata were loaded into R as a *genind* object and subjected to quality control steps in the *poppr* v2.3.0 package (Kamvar et al. 2014, 2015) that removed the VD97 locus and any isolates having more than two missing loci. Other R packages used for minor functions in the analysis pipeline include: *cowplot* v0.9.3 (Wilke 2018) and *fields* v8.10 (Nychka et al. 2015) for grouping figures into panels; *dplyr* v0.5.0 (Wickham and Francois 2016), *plyr* v1.8.4 (Wickham 2011), *stringr* v1.3.1 (Wickham 2018), and *tidyr* v0.6.0 (Wickham 2016) for data manipulation; *phangorn* v2.4.0 (Schliep 2011) for rooting the distance tree; and *RColorBrewer* v1.1-2 (Brewer 2013; Neuwirth 2014).

Poppr was used to produce two clone-corrected datasets, consisting of a single allele at each locus for the haploid *V. dahliae*, for downstream population genetic analyses. The first is 'MLG' (multilocus haploid genotype), which was clone-corrected strictly for the 12 microsatellite loci in which missing data serve as an extra allele. However, numerous multilocus genotypes were shared between metadata factors, ensuring that metadata would be lost if isolates were clone-corrected using microsatellite loci only. To associate population genetic data with these factors while still ameliorating the confounding influence of clonality, a second 'MLG+meta' dataset was made that was clone-corrected for the 12 microsatellite loci plus the metadata factors host, geographic region of origin (Bekaa, North, South), and race. For example, an isolate obtained from Bekaa and an isolate obtained from the North region that possess identical alleles at all 12 microsatellite loci would collapse to one multilocus genotype

Table 1. Isolates of *Verticillium dahliae* used in this study and their corresponding origin, race, mating type (*MAT*), idiomorph, and multilocus genotype

Isolate	Host	Geographical area ^a	Field	Race ^b	Mating type	Multilocus genotype ^c
O1	Olive	Bekaa	O-L1	2	<i>MAT1-2-1</i>	H43
O2	Olive	Bekaa	O-L1	2	<i>MAT1-2-1</i>	H34
O3	Olive	Bekaa	O-L1	2	<i>MAT1-2-1</i>	H35
O4	Olive	Bekaa	O-L1	2	<i>MAT1-2-1</i>	H43
O5	Olive	Bekaa	O-L2	2	<i>MAT1-2-1</i>	H31
O6	Olive	Bekaa	O-L2	2	<i>MAT1-2-1</i>	H30
O7	Olive	Bekaa	O-L2	2	<i>MAT1-2-1</i>	H33
O8	Olive	Bekaa	O-L2	2	<i>MAT1-2-1</i>	H33
O9	Olive	Bekaa	O-L2	2	<i>MAT1-2-1</i>	H43
O10	Olive	Bekaa	O-L2	2	<i>MAT1-2-1</i>	H43
O11	Olive	North	O-L4	1	<i>MAT1-2-1</i>	N.C.
O12	Olive	North	O-L5	1	<i>MAT1-2-1</i>	H25
O13	Olive	North	O-L5	1	<i>MAT1-2-1</i>	H25
O14	Olive	North	O-L6	2	<i>MAT1-2-1</i>	H43
O15	Olive	North	O-L9	2	<i>MAT1-2-1</i>	N.C.
O17	Olive	Bekaa	O-L10	2	<i>MAT1-2-1</i>	H6
O19	Olive	Bekaa	O-L10	N.A.	<i>MAT1-2-1</i>	H12
O20	Olive	Bekaa	O-L13	1	<i>MAT1-2-1</i>	H13
O21	Olive	Bekaa	O-L13	1	<i>MAT1-2-1</i>	N.C.
O22	Olive	Bekaa	O-L15	2	<i>MAT1-2-1</i>	H47
O23	Olive	Bekaa	O-L15	1	<i>MAT1-2-1</i>	H16
O24	Olive	Bekaa	O-L15	2	<i>MAT1-2-1</i>	H50
O26	Olive	Bekaa	O-L15	2	<i>MAT1-2-1</i>	H43
O27	Olive	Bekaa	O-L16	1	<i>MAT1-2-1</i>	H17
O28	Olive	Bekaa	O-L16	1	<i>MAT1-2-1</i>	H13
O29	Olive	Bekaa	O-L17	1	<i>MAT1-2-1</i>	H13
O30	Olive	Bekaa	O-L18	1	<i>MAT1-2-1</i>	H13
O31	Olive	Bekaa	O-L19	2	<i>MAT1-2-1</i>	H43
O33	Olive	Bekaa	O-L19	2	<i>MAT1-2-1</i>	H43
O34	Olive	Bekaa	O-L20	1	<i>MAT1-2-1</i>	H25
O35	Olive	Bekaa	O-L20	1	<i>MAT1-2-1</i>	H13
O36	Olive	Bekaa	O-L18	1	<i>MAT1-2-1</i>	H13
O37	Olive	Bekaa	O-L22	2	<i>MAT1-2-1</i>	N.C.
O38	Olive	Bekaa	O-L22	2	<i>MAT1-2-1</i>	N.C.
O39	Olive	Bekaa	O-L26	2	<i>MAT1-2-1</i>	H7
O42	Olive	Bekaa	O-L30	2	<i>MAT1-2-1</i>	N.C.
O43	Olive	Bekaa	O-L30	1	<i>MAT1-2-1</i>	H5
O44	Olive	South	O-L32	1	<i>MAT1-2-1</i>	H13
O45	Olive	South	O-L32	1	<i>MAT1-2-1</i>	H54
O46	Olive	South	O-L34	1	<i>MAT1-2-1</i>	H13
O47	Olive	South	O-L35	1	<i>MAT1-2-1</i>	H13
O48	Olive	South	O-L35	1	<i>MAT1-2-1</i>	H9
O49	Olive	South	O-L36	2	<i>MAT1-2-1</i>	H43
O50	Olive	South	O-L37	1	<i>MAT1-2-1</i>	H19
O51	Olive	South	O-L37	1	<i>MAT1-2-1</i>	H11
O52	Olive	South	O-L44	1	<i>MAT1-2-1</i>	H13
O54	Olive	South	O-L47	2	<i>MAT1-1-1</i>	H27
O55	Olive	North	O-L52	1	<i>MAT1-2-1</i>	H13
O56	Olive	North	O-L52	2	<i>MAT1-2-1</i>	H43
O57	Olive	North	O-L52	1	<i>MAT1-2-1</i>	H13
O58	Olive	North	O-L54	1	<i>MAT1-2-1</i>	H13
O59	Olive	North	O-L54	1	<i>MAT1-2-1</i>	H13
O60	Olive	North	O-L55	2	<i>MAT1-2-1</i>	H26
O62	Olive	North	O-L56	1	<i>MAT1-2-1</i>	H10
O63	Olive	North	O-L58	2	<i>MAT1-2-1</i>	H43
O64	Olive	North	O-L63	2	<i>MAT1-2-1</i>	H43
O65	Olive	North	O-L51	1	<i>MAT1-2-1</i>	H13
O66	Olive	North	O-L51	1	<i>MAT1-2-1</i>	H13
O67	Olive	North	O-L57	1	<i>MAT1-2-1</i>	N.C.

(Continued)

^a **North**, isolate was obtained within the Lebanese governorates North Lebanon, Mount Lebanon, or Akkar. **South**, from South Lebanon or Nabatiyeh. **Bekaa**, from Bekaa or Baalback-Hermel.

^b **N.A.**, did not amplify with either race 1 or race 2 primers pairs.

^c **N.C.**, isolates removed from SSR analysis due to missing data at more than two loci.

Table 1. (Continued)

Isolate	Host	Geographical area ^a	Field	Race ^b	Mating type	Multilocus genotype ^c
O68	Olive	North	O-L59	2	<i>MAT1-2-1</i>	H43
O70	Olive	South	O-L73	2	<i>MAT1-2-1</i>	H23
O71	Olive	South	O-L75	1	<i>MAT1-2-1</i>	H13
O72	Olive	South	O-L75	1	<i>MAT1-2-1</i>	H13
O73	Olive	South	O-L77	1	<i>MAT1-2-1</i>	H13
O74	Olive	South	O-L78	1	<i>MAT1-2-1</i>	H17
O75	Olive	South	O-L78	1	<i>MAT1-2-1</i>	H20
O76	Olive	South	O-L79	1	<i>MAT1-2-1</i>	H18
O77	Olive	South	O-L79	2	<i>MAT1-2-1</i>	H6
O78	Olive	South	O-L79	1	<i>MAT1-2-1</i>	H15
O79	Olive	South	O-L79	1	<i>MAT1-2-1</i>	H13
O80	Olive	South	O-L81	1	<i>MAT1-2-1</i>	H13
O81	Olive	North	O-L85	1	<i>MAT1-2-1</i>	H13
O82	Olive	North	O-L105	2	<i>MAT1-1-1</i>	H28
O83	Olive	Bekaa	O-L120	2	<i>MAT1-2-1</i>	H46
O85	Olive	South	O-L129	1	<i>MAT1-2-1</i>	H18
O86	Olive	South	O-L130	2	<i>MAT1-2-1</i>	H43
O87	Olive	South	O-L130	1	<i>MAT1-2-1</i>	H13
O88	Olive	North	O-L132	1	<i>MAT1-2-1</i>	H19
P89	Potato	North	NP-L52	2	<i>MAT1-2-1</i>	H43
P101	Potato	North	NP-L1	2	<i>MAT1-2-1</i>	H42
P102	Potato	North	NP-L2	2	<i>MAT1-2-1</i>	H43
P104	Potato	North	NP-L4	2	<i>MAT1-2-1</i>	N.C.
P105	Potato	North	NP-L4	2	<i>MAT1-2-1</i>	H43
P108	Potato	North	NP-L14	2	<i>MAT1-2-1</i>	N.C.
P109	Potato	North	NP-L14	2	<i>MAT1-2-1</i>	H43
P110	Potato	North	NP-L15	2	<i>MAT1-2-1</i>	H43
P111	Potato	North	NP-L15	2	<i>MAT1-2-1</i>	H36
P113	Potato	North	NP-L17	2	<i>MAT1-2-1</i>	H1
P116	Potato	North	NP-L18	2	<i>MAT1-2-1</i>	H49
P119	Potato	North	NP-L20	2	<i>MAT1-2-1</i>	H43
P121	Potato	North	NP-L20	2	<i>MAT1-2-1</i>	H44
P127	Potato	North	NP-L21	2	<i>MAT1-2-1</i>	H43
P129	Potato	North	NP-L21	2	<i>MAT1-2-1</i>	H41
P136	Potato	North	NP-L22	2	<i>MAT1-2-1</i>	H43
P137	Potato	North	NP-L22	2	<i>MAT1-2-1</i>	H52
P138	Potato	North	NP-L24	2	<i>MAT1-2-1</i>	H43
P140	Potato	North	NP-L24	2	<i>MAT1-2-1</i>	H43
P142	Potato	North	NP-L26	2	<i>MAT1-2-1</i>	H39
P143	Potato	North	NP-L26	2	<i>MAT1-2-1</i>	H43
P150	Potato	North	NP-L27	2	<i>MAT1-2-1</i>	N.C.
P152	Potato	North	NP-L29	2	<i>MAT1-2-1</i>	H43
P153	Potato	North	NP-L29	2	<i>MAT1-2-1</i>	H43
P154	Potato	North	NP-L36	1	<i>MAT1-2-1</i>	H22
P155	Potato	North	NP-L36	1	<i>MAT1-2-1</i>	H18
P156	Potato	North	NP-L37	2	<i>MAT1-2-1</i>	H43
P157	Potato	North	NP-L37	2	<i>MAT1-2-1</i>	H43
P158	Potato	North	NP-L38	2	<i>MAT1-2-1</i>	H43
P162	Potato	North	NP-L40	2	<i>MAT1-2-1</i>	H43
P166	Potato	North	NP-L40	2	<i>MAT1-2-1</i>	H43
P168	Potato	North	NP-L41	2	<i>MAT1-1-1</i>	H27
P169	Potato	North	NP-L41	2	<i>MAT1-2-1</i>	H3
P175	Potato	North	NP-L42	2	<i>MAT1-2-1</i>	H2
P176	Potato	North	NP-L46	2	<i>MAT1-2-1</i>	H43
P181	Potato	South	SP-L1	2	<i>MAT1-2-1</i>	H43
P183	Potato	South	SP-L1	N.A.	<i>MAT1-2-1</i>	H4
P186	Potato	South	SP-L3	2	<i>MAT1-2-1</i>	H32
P191	Potato	South	SP-L4	2	<i>MAT1-2-1</i>	H43
P197	Potato	Bekaa	BP-L3	2	<i>MAT1-2-1</i>	H43
P198	Potato	Bekaa	BP-L3	2	<i>MAT1-2-1</i>	H43
P199	Potato	Bekaa	BP-L5	2	<i>MAT1-2-1</i>	H45
P203	Potato	Bekaa	BP-L6	2	<i>MAT1-2-1</i>	H38
P205	Potato	Bekaa	BP-L7	2	<i>MAT1-2-1</i>	H43
P206	Potato	Bekaa	BP-L7	1	<i>MAT1-2-1</i>	H13
P207	Potato	Bekaa	BP-L8	2	<i>MAT1-2-1</i>	H29
P208	Potato	Bekaa	BP-L8	1	<i>MAT1-2-1</i>	H20

(Continued on next page)

Table 1. (Continued from previous page)

Isolate	Host	Geographical area ^a	Field	Race ^b	Mating type	Multilocus genotype ^c
P209	Potato	Bekaa	BP-L9	1	<i>MATI-2-1</i>	H24
P210	Potato	Bekaa	BP-L9	2	<i>MATI-2-1</i>	H55
P214	Potato	Bekaa	BP-L10	2	<i>MATI-2-1</i>	H55
P215	Potato	Bekaa	BP-L11	2	<i>MATI-2-1</i>	H40
P216	Potato	Bekaa	BP-L11	2	<i>MATI-2-1</i>	H43
P217	Potato	Bekaa	BP-L12	2	<i>MATI-2-1</i>	H43
P219	Potato	Bekaa	BP-L12	2	<i>MATI-2-1</i>	H40
P220	Potato	Bekaa	BP-L13	1	<i>MATI-2-1</i>	H13
P222	Potato	Bekaa	BP-L13	1	<i>MATI-2-1</i>	H21
P223	Potato	Bekaa	BP-L14	2	<i>MATI-2-1</i>	H43
P225	Potato	Bekaa	BP-L14	2	<i>MATI-2-1</i>	H43
P227	Potato	Bekaa	BP-L15	1	<i>MATI-2-1</i>	H14
P228	Potato	Bekaa	BP-L16	2	<i>MATI-2-1</i>	H43
P230	Potato	Bekaa	BP-L16	2	<i>MATI-2-1</i>	H43
P231	Potato	Bekaa	BP-L17	1	<i>MATI-2-1</i>	H13
P234	Potato	Bekaa	BP-L19	2	<i>MATI-2-1</i>	H43
P235	Potato	Bekaa	BP-L20	2	<i>MATI-2-1</i>	H55
P236	Potato	Bekaa	BP-L20	2	<i>MATI-2-1</i>	H43
P244	Potato	Bekaa	BP-L23	2	<i>MATI-2-1</i>	H43
P245	Potato	Bekaa	BP-L23	1	<i>MATI-2-1</i>	H20
P248	Potato	Bekaa	BP-L24	2	<i>MATI-2-1</i>	N.C.
P249	Potato	Bekaa	BP-L24	1	<i>MATI-2-1</i>	H25
P254	Potato	Bekaa	BP-L26	2	<i>MATI-2-1</i>	H43
P256	Potato	Bekaa	BP-L26	1	<i>MATI-2-1</i>	H13
P259	Potato	Bekaa	BP-L27	2	<i>MATI-2-1</i>	H43
P260	Potato	Bekaa	BP-L28	2	<i>MATI-2-1</i>	H43
P263	Potato	Bekaa	BP-L29	2	<i>MATI-2-1</i>	H43
P265	Potato	Bekaa	BP-L29	2	<i>MATI-2-1</i>	H44
P267	Potato	Bekaa	BP-L30	2	<i>MATI-2-1</i>	H55
P268	Potato	Bekaa	BP-L31	2	<i>MATI-2-1</i>	H43
P281	Potato	Bekaa	BP-L34	2	<i>MATI-2-1</i>	H43
P282	Potato	Bekaa	BP-L34	1	<i>MATI-1-1</i>	H8
P284	Potato	Bekaa	BP-L35	2	<i>MATI-2-1</i>	H53
P285	Potato	Bekaa	BP-L36	2	<i>MATI-2-1</i>	H43
P287	Potato	Bekaa	BP-L36	2	<i>MATI-2-1</i>	H43
P288	Potato	Bekaa	BP-L36	2	<i>MATI-2-1</i>	H43
P292	Potato	Bekaa	BP-L37	N.A.	<i>MATI-2-1</i>	N.C.
P293	Potato	Bekaa	BP-L38	2	<i>MATI-2-1</i>	H43
P296	Potato	Bekaa	BP-L39	2	<i>MATI-2-1</i>	H43
P300	Potato	Bekaa	BP-L40	2	<i>MATI-2-1</i>	H36
P301	Potato	Bekaa	BP-L50	2	<i>MATI-2-1</i>	H55
P302	Potato	Bekaa	BP-L50	N.A.	<i>MATI-2-1</i>	H48
P307	Potato	Bekaa	BP-L53	2	<i>MATI-2-1</i>	H43
P311	Potato	Bekaa	BP-L55	1	<i>MATI-2-1</i>	H53
P315	Potato	Bekaa	BP-L58	2	<i>MATI-2-1</i>	H43
P316	Potato	Bekaa	BP-L58	2	<i>MATI-2-1</i>	H55
P317	Potato	Bekaa	BP-L58	2	<i>MATI-2-1</i>	H55
P318	Potato	Bekaa	BP-L59	2	<i>MATI-2-1</i>	H46
P319	Potato	Bekaa	BP-L59	N.A.	<i>MATI-2-1</i>	H43
P321	Potato	Bekaa	BP-L61	1	<i>MATI-2-1</i>	H13
P324	Potato	Bekaa	BP-L61	2	<i>MATI-2-1</i>	H43
P326	Potato	Bekaa	BP-L62	2	<i>MATI-2-1</i>	H37
P327	Potato	Bekaa	BP-L63	2	<i>MATI-2-1</i>	H51
P328	Potato	Bekaa	BP-L64	2	<i>MATI-2-1</i>	N.C.
P329	Potato	Bekaa	BP-L65	2	<i>MATI-2-1</i>	H43
P334	Potato	Bekaa	BP-L1	2	<i>MATI-2-1</i>	H43
P338	Potato	Bekaa	BP-L2	2	<i>MATI-2-1</i>	H52
P341	Potato	Bekaa	BP-L2	2	<i>MATI-2-1</i>	H43
P347	Potato	Bekaa	BP-L4	2	<i>MATI-2-1</i>	H40
P348	Potato	Bekaa	BP-L4	2	<i>MATI-2-1</i>	H44
P356	Potato	Bekaa	BP-L41	2	<i>MATI-2-1</i>	H43
P357	Potato	Bekaa	BP-L42	2	<i>MATI-2-1</i>	H43
P360	Potato	Bekaa	BP-L42	2	<i>MATI-2-1</i>	H43
P362	Potato	Bekaa	BP-L43	2	<i>MATI-2-1</i>	H43
P364	Potato	Bekaa	BP-L43	N.A.	<i>MATI-2-1</i>	H43
P366	Potato	Bekaa	BP-L44	2	<i>MATI-2-1</i>	H43

(Continued)

Table 1. (Continued)

Isolate	Host	Geographical area ^a	Field	Race ^b	Mating type	Multilocus genotype ^c
P367	Potato	Bekaa	BP-L44	2	<i>MATI-2-1</i>	H55
P368	Potato	Bekaa	BP-L45	N.A.	<i>MATI-2-1</i>	H56
P369	Potato	Bekaa	BP-L45	2	<i>MATI-2-1</i>	H43
P373	Potato	Bekaa	BP-L46	2	<i>MATI-2-1</i>	H43
P374	Potato	Bekaa	BP-L47	2	<i>MATI-2-1</i>	H43
P375	Potato	Bekaa	BP-L47	2	<i>MATI-2-1</i>	N.C.
P376	Potato	Bekaa	BP-L48	2	<i>MATI-2-1</i>	H43
P377	Potato	Bekaa	BP-L48	2	<i>MATI-2-1</i>	N.C.
P378	Potato	Bekaa	BP-L49	2	<i>MATI-2-1</i>	H43
P381	Potato	Bekaa	BP-L49	2	<i>MATI-2-1</i>	N.C.
P383	Potato	Bekaa	BP-L51	2	<i>MATI-2-1</i>	H39

in the MLG dataset and two multilocus genotypes in the MLG+meta dataset. Mating type was not included in this clone-correction because all but a few isolates were of one mating type. For the MLG dataset, a minimum spanning network of Bruvo's genetic distance (Bruvo et al. 2004) was calculated using the *bruv.msn* function in *poppr*. The network was visualized using the *layout_with_kk* function in the package *igraph* v1.2.1 (Csardi and Nepusz 2006), with the Kamada-Kawai vertex attraction constant (*kkconst*) set at 58 to reduce crossing edges, and the *ggnetwork* function in the package *ggnetwork* v0.5.1 (Briatte 2016). The network was annotated with the distribution of levels of metadata factors (host, geographic region of origin, and race) among isolates within each multilocus genotype using the *geom_scatterpie* function of the *scatterpie* v0.1.2 package (Yu 2018). To allow support of edges in this network to be evaluated, a neighbor-joining tree with 1,000 bootstrap replicates was constructed from the MLG dataset using the *bruv.boot* function in *poppr*, with bootstrap values <50 discarded. Several branches with negative lengths were changed to zero. The tree was visualized using the package *ggtree* v1.10.5 (Yu et al. 2017).

Next, discriminant analysis of principal components (DAPC) (Jombart et al. 2010) was performed on the 'MLG+meta' dataset using the *ade4* package v2.0.1 (Jombart 2008; Jombart and Ahmed 2011). DAPC consists of three steps: 1) principal components analysis (PCA); 2) assigning individuals to groups; and 3) discriminant analysis to summarize structure among groups. Here, two DAPC analyses were formed in parallel that used either unsupervised *k*-means clustering or the metadata factors for the group assignment step. For *k*-means clustering, the function *find.clusters* was run 10 replicate times with the parameters *max.n.clust* = 20, *n.iter* = 100,000, *n.start* = 50 to infer the optimal number of clusters to describe the data as measured by Bayesian information criterion. Next, a single run of *find.clusters* was performed with the chosen optimal number of clusters *n.clust* = 4 (Supplementary Fig. S1) to assign individuals to groups for the DAPC step. For the second parallel analysis, the metadata factors used for a priori group assignment were host, geographic region, race, and host-region, host-race, region-race combined factors. To compare these two analyses and visually determine how well the population structure can be summarized by metadata factors, the results from the *k*-means analysis was color coded according to each of the six metadata factors or combinations. Thus, the *k*-means clustering analysis consisted of one cluster analysis, whereas the metadata group assignment consisted of six separate analyses. For the DAPC step, the number of principal component axes needed to reach 90% of variation was retained, and up to three discriminant axes were retained in each analysis. To compare this analysis with the Bruvo's distance described above, the *k*-means cluster assignment of each MLG was visualized on the minimum spanning network.

Based on this exploratory analysis, further analysis was performed on the host and race metadata factor groups as well as the *k*-means clusters from the DAPC analysis. To assess allelic diversity, two measures of richness and two measures of heterogeneity were calculated for all three datasets (full, MLG+meta, MLG). Allelic richness measures were: A_R (allelic richness), which was calculated with

rarefaction to the smallest sample size across all populations and loci within each metadata factor using the *allel.rich* command in the R package *PopGenReport* v3.0.0 (Adamack and Gruber 2014); and N_p (number of private alleles), which was determined with the *private_alleles* command in *poppr*. Allelic heterogeneity measures were calculated using the ‘HDP’ panel in GenAIEx v6.503 (Peakall and Smouse 2006, 2012) and consisted of: I (information index), which is analogous to H' (Shannon-Wiener Index) in ecology; and N_e (number of effective alleles). In addition, the allelic heterogeneity measure I (information index) was calculated for each locus of the MLG+meta dataset using the ‘HDL’ panel in GenAIEx.

To assess multilocus genotype diversity, one measure of clonal richness, two measures of richness, two measures of heterogeneity, and one measure of evenness were calculated for the full 186 isolate

dataset manually (clonal richness) or in the *poppr* package using the *poppr* command (all other measures). The measures were selected largely based on recommendations by Arnaud-Haond et al. (2007). The measure of clonal richness used was R , which is modified by Dorken and Eckert (2001) from P_d (index of clonal diversity) proposed by Ellstrand and Roose (1987). Haplotype richness measures were: MLG, which is simply the raw number of multilocus genotypes; and eMLG (number of expected multilocus genotypes), which was calculated with rarefaction to the smallest sample size that is ≥ 10 . Genotype heterogeneity measures were: $e^{H'}$, which is H' (Shannon-Wiener Index) converted to the effective number of genotypes, or the number of equally abundant genotypes needed to result in the value of H' calculated from the given population; and $1/\lambda$ (inverse Simpson’s index), which has also been called G

Fig. 1. Minimum spanning network of *Verticillium dahliae* from Lebanon as inferred by Bruvo’s genetic distance. Dataset consisted of 56 multilocus haploid genotypes (MLGs), which was achieved by subjecting raw data to individual (isolates with more than two loci missing removed) and locus (VD97 removed) quality control steps and then strictly clone-correcting remaining isolates based on alleles at 12 microsatellite loci. Edge length (labeled for two edges in bottom-left Region panel) is proportional to Bruvo’s genetic distance. Pies represent MLGs, are centered on nodes of the network, and are annotated with the distribution of isolates among levels of the given metadata factor (race, host, geographic region of origin) or the cluster each MLG was assigned to by k -means. Area of pies are scaled to the number of isolates that belong to each MLG ($G1 = 1$ isolate, $G43 = 76$ isolates).

(Stoddart and Taylor's index). The genotype evenness measure was J' (Pielou's evenness), which is also known as the Shannon evenness index or E.5 (modified Hill's evenness). The conversion of an index to an effective number of genotypes, which are also called numbers equivalents or Hill numbers, ameliorates numerous problems with standard diversity indices (Chao et al. 2014; Ellison 2010). For example, indices converted to effective numbers are in the same units as richness measures, thus allowing intuitive comparisons in the form of a q profile (Sherwin et al. 2017). The inverse Simpson index ($1/\lambda$) due to its mathematical definition is the effective number conversion of the Gini-Simpson index ($1 - \lambda$) (Arnaud-Haond et al. 2007; Chao et al. 2014).

Also from the *poppr* command, linkage equilibrium was assessed with the null hypothesis of sexual reproduction using 999 permutations of r_D (standardized index of association) for the MLG dataset only.

Genetic fixation among clusters, races, hosts, and geographic origins in both the full and clone-corrected MLG+meta datasets was assessed by estimation of the distance-based fixation index Φ'_{PT} , which is standardized for mean within-population diversity (Meirmans 2006), using the analyses of molecular variance (AMOVA) framework (Excoffier et al. 1992; Michalakis and Excoffier 1996). Φ'_{PT} was calculated via the 'PhiPT' panel in GenAlEx using the 'Haplotype-SSR' distance measure on raw data with the 'interpolate missing'

Fig. 2. Association of population structure of *Verticillium dahliae* with the metadata factors race and combined host of origin and race by discriminant analysis of principal components (DAPC). Dataset was the 79 isolate multilocus haploid genotype (MLG)+meta dataset, which was achieved by subjecting raw data to individual (isolates with more than two loci missing) and locus (VD97 removed) quality control steps and then clone-correcting remaining isolates using 12 microsatellite loci plus the metadata factors host, region, and race. **Left column of panels:** clones identified by race (0 = missing). **Right column:** combined host and race factor (O_0 = olive, race missing; O_1 = olive race 1; O_2 = olive race 2; P_0 = potato, race missing; P_1 = potato race 1; P_2 = potato race 2). **Top row of panels:** Discriminant analysis (DA) was initialized using an unsupervised k -means clustering algorithm at $k = 4$. **Bottom row:** DA was initialized using the respective metadata factor. Ellipses represent variation inertia for each cluster (1-4) or metadata group (e.g., Race 1 or Olive Bekaa). Inset bar charts are scree plots that show eigenvalues of DA axes; shaded bars identify which axes are plotted.

option selected. Genetic differentiation was assessed by estimation of sH (Shannon information index) (Smouse et al. 2015) in the ‘Tot SHT’ panel GenAlEx using the log base 2 option. Shannon information complements traditional population genetic indices, and compared with AMOVA indices offers advantages that include greater sensitivity to less frequent alleles, additivity, and allows explicitly hierarchical calculations (Chao et al. 2015; Sherwin et al. 2006, 2017). Both Φ^*_{PT} and sH were estimated using 999 permutations.

Results

Verticillium species, mating type, and race identification. All isolates included in the study were identified as *V. dahliae* according to the new taxonomic system of classification (Inderbitzin et al. 2011), except for one isolate from potato, which was identified as *V. nubilum* Pethybr. and was therefore excluded for further genotypic study. The majority of *V. dahliae* isolates from olive (97.4%) and potato (98.4%) amplified a 400-bp fragment in the MAT-specific PCR assay and were thus designated as MAT1-2-1 mating type. Two isolates from olive (O54 and O82) and two isolates from potato (P168 and P282) amplified a 600-bp band and were considered of MAT1-1-1 type (Table 1).

Out of the 202 *V. dahliae* isolates analyzed, 137 (67.8%) belonged to race 2 and 58 (28.7%) to race 1. PCR products were not obtained from seven isolates (six from potato and one from olive) with either race 1- or race 2-specific primers. The frequencies of the two races differed according to the host and geographical origin (Table 1). On olive, 55.1% of isolates were race 1 and 43.6% were race 2, whereas among potato isolates, race 2 (83.1%) was more frequent than race 1 (12.1%). In North and Bekaa, the frequency of race 2 was 73.2 and 74.6%, and that of race 1 was 26.8 and 20.3%, respectively. Conversely, 67.9% of the isolates were characterized as race 1 and 28.6% as race 2 in the South.

Microsatellite analysis and data handling. Before quality control, the mean incidence of missing data was 5.8%. Locus VD97 was discarded due to a high rate of missing data (17.8%) and because missing data were unevenly concentrated in particular host and geography groups. Other loci with an incidence of missing data above the mean were: VD01 (10.9%), VD03 (8.9%), and VD27 (6.9%). Next, 16 isolates having more than two missing loci were discarded from the dataset, leaving a total of 186 isolates with an overall mean of 2.8% missing data (Supplementary Tables S2 and S3).

The ‘MLG’ dataset consisted of 56 MLG, and the ‘MLG+meta’ dataset consisted of 79 unique combinations of MLGs and the metadata host, geographic region of origin, and race factors. Over

half of all isolates in the study belonged to one of two MLGs, G13 (15.1% of all isolates) and G43 (40.9%). Haplotypes represented by one isolate covered 21.0% of the population studied, and MLGs with only two to three isolates representing them belonged to 16.7% of this population (Table 1).

Population genetic structure. Both DAPC and the minimum spanning network of Bruvo’s genetic distance, carried out on the MLG+meta and MLG datasets, respectively, revealed that the genetic structure of *V. dahliae* in Lebanon is best described by four clusters (Figs. 1 and 2). These clusters are comprised of 17, 31, 3, and 5 MLGs (Fig. 1). However, bootstrap values supporting these clusters were generally low (<50) (Supplementary Fig. S2). The minimum spanning network revealed that one genotype in cluster 4, G8, did not ‘cluster’ with the others, indicating its assignment to that cluster via *k*-means may be the result of the equivalent of long branch attraction. The two abundant genotypes G13 and G43 were assigned to clusters 1 and 2, respectively. The genotype richness of these clusters as measured by eMLG and the genotype heterogeneity as measured by $e^{H'}$ were similar (Table 2). However, $1/\lambda$, which is more sensitive to abundant genotypes than $e^{H'}$, was slightly lower for cluster 2 compared with cluster 1, which likely reflects the slightly greater dominance of G43 in cluster 2 compared with G13 in cluster 1. In the ‘MLG’ dataset, there was weak evidence for rejection of linkage equilibrium within clusters 1, 2, and 3 ($P = 0.933, 0.132, \text{ and } 0.644$, respectively), whereas there was

Table 3. Allelic diversity and linkage equilibrium of the multilocus haploid genotype (MLG) dataset

Factor	Group	MLG	Allelic diversity			Linkage equilibrium ^c	
			Richness ^a	Heterogeneity ^b		Value	$P < H_0$
				I	N_e		
Clusters	1	17	13.4	0.21 (0.09) ^d	1.17 (0.09)	-0.071	0.933
	2	31	13.9	0.32 (0.12)	1.26 (0.11)	0.032	0.132
	3	3	13.7	0.11 (0.08)	1.15 (0.10)	0.500	0.644
	4	5	18.7	0.73 (0.14)	2.15 (0.28)	0.191	0.016
	Total	56	-	0.34 (0.06)	1.43 (0.10)	0.297	0.001

^a A_R : Total allelic richness with rarefaction.

^b I: Shannon’s information index, N_e : mean number of effective alleles.

^c r_D : index of association.

^d Value in parentheses represents the standard error of the corresponding mean value.

Table 2. Haplotype diversity of *Verticillium dahliae* from Lebanon using the full dataset

Factor	Group	Isolates	Cl. Richness ^a	Richness ^b		Heterogeneity ^c		Evenness ^d
			R	MLG	eMLG	$e^{H'}$	$1/\lambda$	J'
Host	Olive	71	0.40	29	29.0 (0.00) ^e	13.20	6.50	0.450
	Potato	115	0.29	34	24.0 (2.18)	8.94	3.31	0.292
	Total	186	0.30	56	27.6 (2.87)	-	-	-
Race	1	55	0.33	19	5.5 (1.34)	7.69	3.61	0.389
	2	125	0.27	34	4.9 (1.42)	7.54	2.79	0.274
	Missing	6	0.80	5	5.0 (0.00)	4.76	4.50	0.930
	Total	186	0.30	56	6.1 (1.43)	-	-	-
Cluster	1	53	0.31	17	5.3 (1.31)	6.89	3.36	0.401
	2	124	0.24	31	4.6 (1.40)	6.69	2.60	0.282
	3	4	0.67	3	3.0 (0.00)	2.83	2.67	0.912
	4	5	1.00	5	5.0 (0.00)	5.00	5.00	1.000
	Total	186	0.30	56	6.1 (1.43)	14.73	5.12	0.300

^a Clonal Richness, R: modified index of clonal diversity.

^b MLG: Total number of multilocus haploid genotype (MLG), eMLG: Expected number of MLG at the lowest common sample size within each factor.

^c $e^{H'}$: Shannon-Weiner Diversity index (H') converted to a number equivalent (Hill’s number) (e^x), or the effective number of genotypes, $1/\lambda$: inverse Simpson index (or G, Stoddart and Taylor index).

^d J': Pielou’s evenness (or evenness derived from Shannon-Weiner index, E5: Modified Hill’s evenness).

^e Value in parentheses represents the standard error of the corresponding mean value.

evidence against linkage equilibrium in cluster 4 ($P = 0.016$) and in the entire dataset ($P = 0.001$) (Table 3).

Population genetic structure was most strongly associated with race. Except for G53 that belonged to cluster 2 and was shared by one isolate each of race 1 and race 2, all MLG and the major clusters 1 and 2 were fixed for a single race (Table 4 and Fig. 1). A priori initialization of DAPC with the race factor revealed that a large portion of the variation was explained by this factor (Fig. 2). An estimated 41.6% of molecular variance ($P = 0.001$) (Table 5) and 37.8% of the Shannon information ($P = 0.001$) was associated with race in the MLG+meta dataset (Table 6). These values were numerically higher in the full dataset (by 27.7 and 18.3 percentage points, respectively), which likely reflects the dominance of the two highly represented G13 and G43 that are fixed for a different race. Haplotype heterogeneity (e^H and $1/\lambda$) was slightly higher among race 1 isolates compared with race 2 (Table 2), which may be reflective of the lower dominance of G13 among race 1 isolates (51%) compared with G43 among race 2 (59%). Indices of allelic diversity (A_R , I , and N_e) were slightly higher among race 2 isolates compared with race 1 (Table 7).

Exploratory analysis using both DAPC (Figs. 2 and 3) and Bruvo's genetic distance clustering (Fig. 1) revealed a weak to moderate association of host with genetic structure. This structure was confined to a single discriminant axis for the second parallel DAPC analysis initialized using the host factor. Numbers of MLGs and isolates were skewed toward olive in cluster 1 and potato in cluster 2 (Table 4). In particular, 82.1% of the isolates belonging to G13 are from olive, and

81.5% of the isolates belonging to G43 are from potato (Table 1). Although genotype richness was slightly higher and genotype heterogeneity was significantly higher for olive than potato (Table 2), in the MLG+meta dataset allelic richness was higher for potato than olive and heterogeneity for potato was similar to olive (Table 7). For the MLG+meta dataset, a total of 5.5% ($P = 0.015$) of the observed molecular variance (Table 5) and 11.7% ($P = 0.001$) of the Shannon information (Table 6) was associated with host of origin. In the full dataset, these values were 18.0 and 6.2 percentage points higher, respectively. This suggests that an appreciable portion of the among-host variation is due to the highly represented G13 and G43 MLGs, which as described above, predominantly represent different hosts. In addition, per-locus calculations of the allelic heterogeneity measure I showed that all loci were polymorphic within isolates from potato, whereas isolates from olive were fixed for a single allele at three loci (Table 8).

The abundant G13 and G43 were identified in isolates collected from all three geographic regions (Table 4), and exploratory analyses detected a low degree of structure associated with geography (Figs. 1 and 3). For differentiation in the MLG+meta dataset, 8.4% ($P = 0.017$) of the observed molecular variance (Table 5) and 11.9% ($P = 0.021$) of the Shannon information (Table 6) was associated with geographic origin. These values were somewhat higher in the full dataset. In addition to numerical differences between the datasets, the order of factors as determined by among-factor variation is different between the full and MLG+meta datasets. In the MLG+meta dataset, host exhibits the lowest among-factor variation values, whereas geographic origin has the lowest value in the full dataset. This

Table 4. Number of multilocus genotypes (and number of isolates in parentheses) among various factors and population genetic clusters

Factor	Host		Race			Mating type		Region			Total
	Olive	Potato	1	2	Missing	1	2	Bekaa	North	South	
Olive	-	-	13 (40)	15 (30)	1 (1)	2 (2)	27 (69)	17 (29)	7 (18)	13 (24)	29 (71)
Potato	-	-	10 (15)	22 (95)	4 (5)	2 (2)	32 (113)	23 (79)	14 (32)	3 (4)	34 (115)
Race 1	-	-	-	-	-	1 (1)	18 (54)	23 (80)	14 (37)	5 (8)	34 (125)
Race 2	-	-	-	-	-	2 (3)	32 (122)	11 (23)	6 (13)	9 (19)	19 (55)
Missing	-	-	-	-	-	0 (0)	5 (6)	4 (5)	0 (0)	1 (1)	5 (6)
Cluster 1	13 (40)	8 (13)	16 (52)	0 (0)	1 (1)	0 (0)	17 (53)	9 (21)	6 (13)	9 (19)	17 (53)
Cluster 2	13 (27)	21 (97)	1 (1)	29 (119)	3 (4)	2 (3)	29 (121)	23 (83)	11 (34)	4 (7)	31 (124)
Cluster 3	3 (4)	0 (0)	1 (1)	2 (3)	0 (0)	0 (0)	3 (4)	3 (3)	0 (0)	1 (1)	3 (4)
Cluster 4	0 (0)	5 (5)	1 (1)	3 (3)	1 (1)	1 (1)	4 (4)	1 (1)	3 (3)	1 (1)	5 (5)
Total ^a	29 (71)	34 (115)	19 (55)	34 (125)	5 (6)	3 (4)	53 (182)	36 (108)	20 (50)	15 (28)	56 (186)

^a Within the host, race, and mating type factors, marginal totals may not sum to the actual total number of genotypes because some are shared among multiple levels of a factor.

Table 5. Estimation of Φ^*_{PT} in the analysis of molecular variance framework of *Verticillium dahliae* isolates in the full and multilocus haploid genotype plus metadata (MLG+meta) datasets grouped by clusters, race, host, and geographic area

Factor	Source	Full				MLG+meta ^a			
		df ^b	% Molecular variance	Φ^*_{PT}	$P > \Phi^*_{PT}$ ^c	df	% Molecular variance	Φ^*_{PT}	$P > \Phi^*_{PT}$
Cluster	Among	3	92.0			3	85.0		
	Within	182	8.0			75	15.0		
	Total	185	100.0	0.920	0.001	78	100.0	0.850	0.001
Race	Among	2	69.3			2	41.6		
	Within	183	30.7			76	58.4		
	Total	185	100.0	0.693	0.001	78	100.0	0.416	0.001
Host	Among	1	23.5			1	5.5		
	Within	184	76.5			77	94.5		
	Total	185	100.0	0.235	0.001	78	100.0	0.055	0.015
Geography	Among	2	16.4			2	8.4		
	Within	183	83.6			76	91.6		
	Total	185	100.0	0.164	0.001	78	100.0	0.084	0.022

^a The MLG+meta dataset consisted of clone-correction for the 12 microsatellite loci plus the metadata factors host, geographic region of origin (Bekaa, North, South), and race.

^b df = degrees of freedom.

^c The probability of obtaining a random Φ^*_{PT} value greater than the observed data was evaluated by standard permutation over the full dataset.

difference likely reflects the low contribution of clonality to among-geographic region fixation or differentiation because the highly represented MLGs G13 and G43 were obtained at similar frequencies from all three geographic regions. However, while isolates from the North geographical area belonged to a range of MLGs, approximately two-thirds of MLG representing the Bekaa and South geographical areas were assigned to cluster 2 and cluster 1, respectively (Table 4). Evaluation of pairwise population differentiation using Φ'_{PT} revealed that the South sampling group was significantly differentiated from the Bekaa group ($\Phi'_{PT} = 0.186$), but the North group was less differentiated from the South and Bekaa groups (Table 9).

Discussion

The diversity of *Verticillium* populations from two strategic cropping systems involving potato and olive in Lebanon were investigated. Except for one isolate, *V. dahliae* was the only species identified on both crops. Races 1 and 2 were detected in both cropping systems but with a higher frequency of race 2 in potato. The *MAT1-2* idiomorph was found to be predominant in *V. dahliae* population from Lebanon, in agreement with previous studies from other geographic regions (Dung et al. 2013a; Gurung et al. 2014; Inderbitzin et al. 2011).

Table 6. Shannon Diversity Partition statistics summary table of *Verticillium dahliae* isolates in the full and multilocus haploid genotype plus metadata (MLG+meta) datasets grouped by clusters, race, host, and geographic area

Factor	Source	Full				MLG+meta ^a			
		df ^b	sH ^c	% sH	P > sH ^d	df	sH	% sH	P > sH
Cluster	Among	3	0.768	79.4	0.001	3	0.910	70.7	0.001
	Within	181	0.199	20.6	1.000	74	0.377	29.3	1.000
	Total	184	0.966	100.0		77	1.287	100.0	
Race	Among	2	0.543	56.1	0.001	2	0.486	37.8	0.001
	Within	182	0.424	43.9	1.000	75	0.801	62.2	1.000
	Total	184	0.966	100.0		77	1.287	100.0	
Host	Among	1	0.173	17.9	0.001	1	0.151	11.7	0.001
	Within	183	0.793	82.1	1.000	76	1.136	88.3	1.000
	Total	184	0.966	100.0		77	1.287	100.0	
Geography	Among	2	0.127	13.1	0.001	2	0.153	11.9	0.021
	Within	182	0.840	86.9	1.000	75	1.134	88.1	0.980
	Total	184	0.966	100.0		77	1.287	100.0	

^a The MLG+meta dataset consisted of clone-correction for the 12 microsatellite loci plus the metadata factors host, geographic region of origin (Bekaa, North, South), and race.

^b df = degrees of freedom.

^c sH = Shannon information.

^d The probability of obtaining a random sH value greater than the observed data was evaluated by standard permutation over the full dataset.

This study revealed that genetic structure in *V. dahliae* in Lebanon is strongly associated with the presence or absence of its virulence gene *Ave1*. Assignment to the two large clusters 1 and 2 exhibited an almost perfect 1:1 association with races 1 and 2, respectively, except for a single genotype belonging to cluster 2. Several explanations for this association could be tested in future work. First, these results could suggest that there are two main *V. dahliae* populations that are genetically distinct, relatively homogeneous, and fixed for race 1 or 2 that serve as sources for the ongoing, nondiscrete introduction of inoculum into Lebanon. Alternatively, the population structure observed in Lebanon could be the result of founder effects from a limited number of introductions from various types of source population(s), which have then undergone amplification and some diversification into two main clonal lineages. This latter proposed hypothesis is supported by the clustering pattern in the minimum spanning network in which several MLGs represented by only one to a few isolates each are closely related to the abundant G43 and G13.

Because these two clusters and races were distributed among both crops and all three regions of Lebanon, there are likely active mechanisms that have prevented gene flow between the two race groups. These mechanisms could include a lack of sexual reproduction, which has never been reported for *V. dahliae*, and vegetative incompatibility. The existence of vegetative compatibility groups (VCGs) within populations of pathogens can contribute to genetic drift and differentiation and are thought to limit gene flow among fungal populations (Leslie 1993). *V. dahliae* isolates in different VCGs have long been considered as genetically isolated populations varying in ecological, physiological, and virulence traits (Katan 2000). Outside its recognition by host R genes, *Ave1* is a virulence factor for *V. dahliae* because it provides higher quantitative disease-causing ability to isolates infecting a host lacking *Ve1* or a homolog compared with isolates without *Ave1* (de Jonge et al. 2012). The concentration of race 1 into one clonal lineage is consistent with the proposed origin of the race 1 trait. There is strong evidence that *V. dahliae* acquired *Ave1* from plants by horizontal gene transfer from a single event (de Jonge et al. 2012), and with few exceptions race 1 isolates have been shown to be restricted to a single clonal lineage delimited by VCG 2A (Jiménez-Díaz et al. 2017; Rafiei et al. 2018). A similar pattern has been observed for a second type of pathogenic variation in *V. dahliae*, the ability of isolates to cause a severe defoliation symptom of cotton, okra, and olive (Jiménez-Díaz et al. 2012; Schnathorst and Mathre 1966), which is found only within the VCG 1A clonal lineage (Jiménez-Díaz et al. 2017; Milgroom et al. 2016). Thus, the presence of *Ave1* or the association of an isolate with the VCG 1A or 2A clonal lineage can serve as a marker for elevated virulence of *V. dahliae* in selected hosts or host genotypes (Jiménez-Díaz et al. 2017; Rafiei et al. 2018).

Table 7. Allelic diversity of *Verticillium dahliae* isolates from Lebanon using full and multilocus haploid genotype plus metadata (MLG+meta) datasets

Factor	Group	Isolates	Full				MLG+meta			
			Richness ^a		Heterogeneity ^b		Richness ^a		Heterogeneity ^b	
			A _R	N _p	I	N _e	MLG+meta ^c	A _R	I	N _e
Host	Olive	71	38.4	13	0.67 (0.12) ^d	1.87 (0.15)	37	37.9	0.77 (0.14)	2.09 (0.20)
	Potato	115	43.3	27	0.47 (0.06)	1.32 (0.05)	42	48.3	0.81 (0.10)	1.84 (0.16)
	Total	186	-	-	0.57 (0.07)	1.59 (0.10)	79	-	0.79 (0.08)	1.96 (0.13)
Race	1	55	14.7	23	0.25 (0.05)	1.13 (0.03)	28	15.9	0.41 (0.08)	1.28 (0.07)
	2	125	14.9	9	0.30 (0.06)	1.14 (0.03)	46	17.7	0.63 (0.11)	1.50 (0.13)
	Missing	6	23.9	2	0.64 (0.05)	1.70 (0.08)	5	23.1	0.71 (0.06)	1.87 (0.10)
	Total	186	-	-	0.40 (0.04)	1.32 (0.05)	79	-	0.58 (0.05)	1.55 (0.07)
Cluster	1	53	12.6	7	0.10 (0.04)	1.05 (0.02)	26	13.0	0.17 (0.07)	1.11 (0.05)
	2	124	12.6	11	0.13 (0.05)	1.06 (0.02)	44	13.6	0.28 (0.10)	1.20 (0.08)
	3	4	13.2	9	0.10 (0.07)	1.12 (0.08)	4	13.2	0.10 (0.07)	1.12 (0.08)
	4	5	18.7	20	0.73 (0.14)	2.15 (0.28)	5	18.7	0.73 (0.14)	2.15 (0.28)
	Total	186	-	-	0.27 (0.06)	1.34 (0.10)	79	-	0.32 (0.06)	1.40 (0.10)

^a A_R: Total allelic richness with rarefaction; N_p: number of private alleles. N_p was omitted from the MLG+meta dataset because the values are identical to the nonclone corrected dataset.

^b I: Shannon's information index; N_e: mean number of effective alleles.

^c The MLG+meta dataset consisted of clone-correction for the 12 microsatellite loci plus the metadata factors host, geographic region of origin (Bekaa, North, South), and race.

^d Value in parentheses represents the standard error of the corresponding mean value.

Fig. 3. Association of population structure of *Verticillium dahliae* with the metadata factors host of origin and region of origin by discriminant analysis of principal components (DAPC). Dataset consisted of 186 isolates after individual (isolates with more than two loci missing) and locus (VD97 removed) quality control steps that was strictly clone-corrected to multilocus genotype and the combined host-region-race factor. **Left column of panels:** clones identified by host of origin. **Right column:** geographic region of origin. **Top row of panels:** Discriminant analysis (DA) was initialized using an unsupervised *k*-means clustering algorithm at *k* = 4. **Bottom row:** DA was initialized using the respective metadata factor. Ellipses represent variation inertia for each cluster (1-4) or metadata group (B = Bekaa, N = northern Lebanon, S = southern Lebanon). Inset bar charts are scree plots that show eigenvalues of DA axes; shaded bars identify which axes are plotted. The bottom-left panel contains a single dimension density plot because only one DA axis is obtainable from the data.

Table 8. Per-locus estimates of I (information index) within *Verticillium dahliae* isolates from olive or potato using the multilocus haploid genotype plus metadata (MLG+meta) datasets

Factor	Group	Locus											
		VD01	VD02	VD03	VD08	VD09	VD10	VD11	VD12	VD27	VD69	VD73	VD92
Host	Olive	1.121	1.061	0.000	1.284	0.684	0.922	0.000	0.965	1.172	1.022	1.008	0.000
	Potato	0.978	0.970	0.496	0.914	0.562	0.792	0.320	1.086	0.800	0.770	1.585	0.421
	Mean	1.049	1.016	0.248	1.099	0.623	0.857	0.160	1.025	0.986	0.896	1.297	0.211
Race	1	0.531	0.574	0.168	0.980	0.469	0.630	0.000	0.315	0.315	0.420	0.499	0.000
	2	1.103	0.650	0.320	0.519	0.182	0.528	0.249	0.685	0.950	0.641	1.434	0.283
	Missing	0.950	0.950	0.500	0.950	0.562	0.562	0.500	0.950	0.673	0.673	0.637	0.562
	Mean	0.861	0.725	0.329	0.816	0.404	0.573	0.250	0.650	0.646	0.578	0.856	0.282

On a broader scale than these two factors, numerous reports have documented host adaptation within *V. dahliae*, in which isolates cause quantitatively more disease in their host of origin compared with other hosts (Bhat and Subbarao 1999). Several of these studies found that the host adaptation was correlated with isolate VCG (Bergal et al. 2010; Douhan and Johnson 2001; Dung et al. 2013a; Jiménez-Díaz et al. 2006; Korolev et al. 2000). *V. dahliae* isolates infecting olive have so far been found to belong to VCG1A, VCG2A, VCG2B, and VCG4B (Dervis et al. 2010; Navas-Cortés et al. 2009). In contrast, the majority of *V. dahliae* isolates associated with potato are in VCGs 4A and 4B (Joaquim and Rowe 1991; Strausbaugh 1993). Characterizing the virulence of our isolates, including for the defoliating/nondefoliating pathotype, was beyond the scope of this study. However, because isolate VCG strongly correlates with molecular markers (Milgroom et al. 2016), host adaptation may contribute to population structure as inferred by molecular markers.

The combined effect of race and host adaptation may have combined to create the moderate association of genetic structure with host that we observed in this study. The two races were distributed almost equally within isolates from olive but were skewed approximately 1:2 toward race 2 among isolates from potato. This skewed distribution of races among hosts is roughly similar to the distribution of genetic clusters among hosts, and may be explained by the gene-for-gene interaction between pathogen and hosts. Homologs to *Ve1* are present in potato (Song et al. 2016), and the planting of cultivars that possess a *Ve1* homolog would exert a selection pressure on *V. dahliae* from potato to shift toward race 2 isolates which lack *Ave1* and thus evade detection by *Ve1* homologs. Similarly, repeated cultivation of a crop may select for *V. dahliae* strains adapted to that host. These selection pressures could explain the moderate association of genetic structure with host and may confound further inference of population genetic forces such as gene flow.

An alternative proposed hypothesis for future testing is that the genetic structure-host association is due to differences between the crops in propagation practices and geographic production patterns. Material for planting potato in Lebanon can originate from certified seed tubers from the European Union or locally produced tubers that in turn originated from Europe. These activities could be providing mechanisms for the introduction and dissemination of new genotypes of *V. dahliae*. Certified seed tubers imported to Lebanon (Saad et al. 2004), certified seed tubers for use in the United States (Dung et al. 2013b), and uncertified tuber seeds obtained from fields in Akkar, Lebanon (Baroudy et al. 2016) have been shown to be infected by *V. dahliae*. In contrast, olive seedlings had been locally produced until the recent introduction of foreign varieties. In addition to propagation practices, olive and potato have historically been grown in separate geographic regions of Lebanon. However, longtime potato-growing fields in the Bekaa area have recently been planted with olive. Indeed, of 12 MLG (excluding G43) representing olive isolates and belonging to cluster 2 (which is skewed toward potato), nine represent isolates obtained exclusively from the Bekaa area.

Genetic structure was also weakly associated with the isolate's geographic region of origin and is almost entirely due to differentiation of isolates from the South, where 24 of the 28 isolates were obtained from olive, when compared with the Bekaa area. Olive trees

have been planted for hundreds of years in Lebanon (Chalak et al. 2015), and they are cultivated on a greater geographical scale and in more microclimates than potato. In addition, the higher diversity of *V. dahliae* subpopulation isolated from olive compared with potato could partially be explained by the longer cultivation history.

This research found that the population of *V. dahliae* in Lebanon is dominated by two clonal lineages that are fixed for either race 1 or race 2. Because genetic diversity is generally well distributed geographically throughout Lebanon and among two major hosts, measures that focus on reducing the introduction of new genotypes via foreign inoculum may limit the ability of the endemic *V. dahliae* population to increase in virulence, damage other hosts, or adapt to management measures.

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Table 9. Pairwise fixation of *Verticillium dahliae* isolates among geographic regions estimated by Φ'_{PT} in an analysis of molecular variance framework from the multilocus haploid genotype plus metadata (MLG+meta) datasets

Area	Bekaa	$\Phi'_{PT} \setminus P\text{-value}^a$	
		North	South
Bekaa	-	0.018	0.002
North	0.080	-	0.205
South	0.186	0.034	-

^a Φ'_{PT} (below diagonal): Distance-based fixation index standardized for mean within-population diversity. *P* value (above diagonal): evaluation of Φ'_{PT} against the null hypothesis of no difference between populations using 999 permutations.

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