Population Genetics of *Verticillium dahliae* in Iran Based on Microsatellite and Single Nucleotide Polymorphism Markers

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ABSTRACT

Verticillium dahliae is a plant pathogenic fungus that reproduces asexually and its population structure is highly clonal. In the present study, 78 *V. dahliae* isolates from Iran were genotyped for mating type, single nucleotide polymorphisms (SNPs), and microsatellites to assign them to clonal lineages and to determine population genetic structure in Iran. The mating type of all isolates was *MAT1-2*. Based on neighbor-joining analysis and minimum spanning networks constructed from SNPs and microsatellite genotypes, respectively, all but four isolates were assigned to lineage 2B⁸²⁴; four isolates were assigned to lineage 4B. The inferred coalescent genealogy of isolates in

lineage 2B⁸²⁴ showed a clear divergence into two clades that corresponded to geographic origin and host. Haplotypes of cotton and pistachio isolates sampled from central Iran were in one clade, and those of isolates from *Prunus* spp. sampled from northwestern Iran were in the other. The strong divergence in haplotypes between the two clades suggests that there were at least two separate introductions of lineage 2B⁸²⁴ to different parts of Iran. Given the history of cotton and pistachio cultivation and *Verticillium* wilt in Iran, these results are consistent with the hypothesis that cotton was historically a likely source inoculum causing *Verticillium* wilt in pistachio.

The life cycles of plant pathogenic fungi are diverse, and can have very different consequences for genetic structure. Many fungal pathogens reproduce asexually, resulting in clonal population structures. In clonal reproduction, the entire genome is transmitted to the next generation as a single linkage group resulting in strong associations between alleles at different loci because they share a common evolutionary history (Balloux et al. 2003; Taylor et al. 1999). Lack of recombination among loci results in linkage disequilibrium (LD) and correlations of phenotypes to genotypes defined by molecular markers (Milgroom 2015; Tibayrenc et al. 1991).

Because of strong LD in asexual organisms, identifying clonal lineages makes it possible to predict biological traits (phenotypes) in asexual populations of fungal or oomycete pathogens. For example, isolates within a lineage of *Phytophthora infestans* share the same phenotypic traits such as fungicide sensitivity, aggressiveness and host specificity (Danieles et al. 2013; Goodwin et al. 1995). For many fungal pathogens, clonal lineages have been identified based on genetic markers that are highly correlated with phenotypic traits such as vegetative compatibility groups (VCGs) or races. For example, VCGs and the production of aflatoxin in *Aspergillus flavus* are correlated with clonal lineages and multilocus genotypes (MLGs) (Horn et al. 1996). In *Puccinia triticina*, molecular markers identified clonal lineages that are associated with races (Goyeau et al. 2007). However, VCGs and genetic markers do not always describe the overall genetic diversity that may exist within fungal populations. The advent of

population genomic analyses, which enable the inference of genome-wide distribution of polymorphisms of many individuals within a species, has provided new insights into biology, genetics, mating systems, and evolution of plant pathogens (Grünwald et al. 2016; Stukenbrock and Bataillon 2012). Recent advances in sequencing technologies have made it possible to genotype thousands of single nucleotide polymorphisms (SNPs), and therefore to address more refined population genetics questions (Grünwald et al. 2016).

Verticillium dahliae is a highly clonal fungal plant pathogen that is distributed worldwide. *V. dahliae* causes *Verticillium* wilt of many dicotyledonous host plants and can be a major constraint on crop production by reducing yield and quality (du Toit et al. 2005; Pegg and Brady 2002). Lack of host specificity and long-term survival of microsclerotia in soil make this pathogen difficult to manage (Pegg and Brady 2002; Qin et al. 2006). Historically, VCGs have been used to characterize population structure. *V. dahliae* has a clonal structure with strong correlations of VCGs to molecular markers. Therefore, VCGs represent distinct clonal lineages. Nine clonal lineages have been described, which were named after the original VCGs, e.g., isolates in VCG2A are in lineage 2A (Milgroom et al. 2014). The exception is VCG2B, which comprises at least three distinct lineages: 2B⁸²⁴, 2B³³⁴, and 2B^{R1} (Collado-Romero et al. 2008; Collado-Romero et al. 2006; Milgroom et al. 2014). Clonal lineages in *V. dahliae* defined by VCGs and molecular markers are also associated with pathogenic phenotypes. For example, race 1 is found almost exclusively in lineage 2A (Jiménez-Díaz et al. 2017) and the defoliating (D) pathotype is found only in lineage 1A (Korolev et al. 2008; Milgroom et al. 2014).

V. dahliae is only known to reproduce asexually, however the presence of both mating-type idiomorphs, orthologs of eight meiosis-specific genes in the genome, and evidence that clonal lineages arose by recombination suggest that the potential exists for sexual reproduction wherever both mating types are present (Milgroom et al. 2014; Short et al. 2014). However, *MAT1-1* is extremely rare, whereas *MAT1-2* is found in almost all *V. dahliae* isolates from diverse hosts and geographic locations (Dung et al. 2013; Milgroom et al. 2014; Short

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et al. 2014; Usami et al. 2009a; Usami et al. 2009b). Such a skewed mating-type ratio reduces the chances for sexual reproduction in *V. dahliae*.

In Iran, Verticillium wilt was first reported in 1964, in cotton (*Gossypium hirsutum*) in the northwest (East-Azerbaijan Province) (Cauquil and Rahmani 1964) (Fig. 1). Within 5 years, it was reported in many other regions, including the north (Golestan and Mazandaran Provinces), the east (Khorasan Province), and central Iran (Esfahan, Markazi, Fars, and Kerman Provinces) (Banihashemi 1983; Scharif and Ershad 1966). Nowadays the disease can be found in many fields and orchards throughout Iran (Ershad 2009). Cotton cultivation has a long history in many regions (more than 2000 years), including the central Provinces of Kerman, Yazd, and Fars, which are also the main pistachio (*Pistacia vera*) production regions. Although cultivation of pistachio goes back hundreds of years, Verticillium wilt was not reported as a major problem until 1999 (Aminae and Ershad 1999). It is now considered to be one of the main limiting factors for cotton and pistachio production in Iran (Arabsalmani and Banihashemi 2000; Hadizadeh and Banihashemi 2007).

Genetic diversity of *V. dahliae* populations from various host plants throughout Iran has been studied by VCGs. In one study, 45 isolates collected from various hosts were assigned to VCG2B (88%) or VCG2A (12%). These results indicated that genetic diversity of *V. dahliae* was relatively low (Rouzbeh and Banihashemi 2006). In another study 69 *V. dahliae* isolates from pistachio and 34 from cotton were reported to belong to VCG2B, VCG4A, or VCG1, and greenhouse pathogenicity tests showed that isolates in VCG1 were of the D pathotype (Hadizadeh and Banihashemi 2007). Based on the similarity of distributions of VCGs and virulence of isolates from the two hosts, and the fact that Verticillium wilt was reported in cotton much earlier than in pistachio, it was hypothesized that cotton was the likely source of *V. dahliae* for pistachio trees (Hadizadeh and Banihashemi 2007). This situation may be analogous to the predominance of D pathotype isolates in VCG1A reported in Spain, where olive trees were cultivated in areas previously planted to cotton; both of these hosts are highly susceptible to the D pathotype (Jiménez-Díaz et al. 2011). Populations of *V. dahliae* on olive and

cotton were genetically indistinguishable, consistent with epidemiological evidence that cotton was the source for populations on olive (Milgroom et al. 2016). However, populations of *V. dahliae* in Iran have not been studied using molecular markers and knowledge regarding mating type, genetic diversity, and differentiation within and among populations of *V. dahliae* from different hosts is lacking. Such information is likely to provide additional insights into the potential origin of *V. dahliae* populations infecting pistachio.

In a previous study, microsatellites and SNPs were used to identify clonal lineages in *V. dahliae* (Rafiei et al. 2018). Microsatellite genotyping from the same isolates that were previously assigned to clonal lineages using genotyping by sequencing (GBS) (Milgroom et al. 2014) made it possible to identify SNP-defined lineages using microsatellite markers. One of the aims of the present study was to test the assignment of *V. dahliae* isolates to lineages with an independent sample of isolates from Iran.

The overall aim of the present work was to study the genetic diversity of *V. dahliae* sampled from various hosts and geographic regions in Iran. The specific objectives were to (i) determine the distribution of mating types and therefore the potential for sexual reproduction, (ii) infer clonal lineages of *V. dahliae* isolates based on microsatellite genotyping, (iii) infer clonal lineages of *V. dahliae* isolates based on GBS (SNPs), (iv) test if cotton and pistachio wilt are caused by the D pathotype, and (v) test whether the population of *V. dahliae* on pistachio could have been derived from the population on cotton.

MATERIALS AND METHODS

Sampling of *V. dahliae* isolates and DNA extraction.

Seventy-eight *V. dahliae* isolates were obtained from infected plants (herbaceous plants and fruit trees) from various geographical areas in Iran (Fig. 1). Infected roots and stems were washed with tap water, cut into 3- to 5-cm segments, surface-sterilized for 5 s in 0.5% NaOCl, blotted dry on paper towels and plated on petri dishes containing a Verticillium-selective medium (Kabir et al. 2004). All cultures were incubated in the dark at room temperature for 2 weeks. Single spores of each isolate were transferred to petri dishes

Fig. 1. Map of Iran showing the locations (solid triangles) where *Verticillium dahliae* was isolated from different host plants.

containing potato dextrose agar (PDA). Additional isolates were obtained from culture collections of the Soil-borne Disease Laboratory, Department of Plant Protection, Shiraz University, Shiraz, Iran, and the Department of Plant Protection, Urmia University, Urmia, Iran. These latter isolates were sampled from infected plants between 2000 and 2014 from various fields in central and northwestern Iran, respectively. Some of these isolates were previously determined as being in VCG2B or VCG4A (Hadizadeh and Banihashemi 2007). Information about hosts and locations of origin for all isolates is summarized in Supplementary Table S1.

Single-spore isolates were cultured on PDA and incubated for 7 to 10 days at room temperature. Mycelia plugs were taken from the colony margin, transferred to potato dextrose broth and incubated for 10 days, at which time mycelia were harvested and lyophilized. Fifty milligrams of lyophilized mycelia was ground to a fine powder using an autoclaved glass rod in 1.5-ml tubes. Genomic DNA was extracted from powdered mycelia for microsatellite genotyping and mating-type analyses using a modified CTAB method (Zhang et al. 2010). To prepare high molecular weight DNA samples for GBS, genomic DNAs were extracted from lyophilized mycelia using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). The quantity and quality of DNAs were measured using NanoDrop (ND-1000 Spectrophotometer; Thermo Fisher Scientific, Wilmington, DE) and by visualization using 1% agarose gel electrophoresis. DNA was diluted in water to a final concentration of 10 ng μl^{-1} and stored at -20°C .

PCR amplification of the mating-type idiomorphs. Mating-type idiomorphs were genotyped using multiplex PCR with two primer pairs: VdMAT1-1a/VdMAT1-1b, which amplifies a 400-bp fragment from *MAT1-1*; and VdMAT1-2a/VdMAT1-2b, which amplifies a 600-bp fragment from *MAT1-2* (Usami et al. 2009a; Usami et al. 2009b). The PCR reaction contained 10 ng of fungal genomic template DNA, 100 pmol of each primer, 25 mM MgCl_2 , 10 mM dNTP, and 1.5 U of GoTaq DNA polymerase (Promega, Madison, WI) in a 20- μl reaction. PCR amplifications were performed with conditions as described previously (Usami et al. 2009a, b). The PCR products were electrophoresed in 1.5% (wt/vol) agarose gel containing 1 $\times$ TAE and visualized using ethidium bromide.

Microsatellite genotyping. Ten microsatellite loci (VD1, VD2, VD3, VD8, VD9, VD10, VD27, VD85, VD96, and VD92) (Atallah et al. 2010), were genotyped in this study. Genotyping was performed using fluorescently labeled M13 primers (Schuelke 2000) and capillary electrophoresis on an ABI 3730xl DNA Analyzer (Applied Biosystems) at the Cornell University Life Sciences Core Laboratories, as described previously (Rafiei et al. 2018). Results from the fragment analysis were viewed and determined using Peak Scanner software (Applied Biosystems). To assess the reliability of microsatellite data, fragments of each size class (27 microsatellite amplicons representative of all 10 microsatellite loci) were sequenced in both directions at the Cornell University Life Sciences Core Laboratories as described previously (Rafiei et al. 2018).

GBS. GBS was performed on a subset of 48 isolates representing each microsatellite-inferred lineage. GBS was performed as previously described (Milgroom et al. 2014) except that sequencing was conducted by BGI Americas (Cambridge, MA) using an Illumina MiSeq instrument with 100-bp paired-end reads. Sequences from Iranian isolates were analyzed along with sequences of 37 reference isolates representative of clonal lineages identified from previous GBS analyses (Milgroom et al. 2014; Milgroom et al. 2016). Quality-filtered and adaptor-trimmed reads were aligned to the reference genome VdLs.17 (GenBank assembly accession GCA_000952015.1) using the Burrows-Wheeler aligner (bwa mem) with default parameters for paired end reads (Li and Durbin 2009). SAMtools v1.4.1 (Li et al. 2009) and Sambamba v0.6.6 (Tarasov et al. 2015) were used to sort aligned-reads files and mark duplicates. Joint variant calling was performed using FreeBayes v1.1.0-9-g09d4ecf with default parameters. Raw variant sites were later filtered with vcfFilter (FreeBayes) for SNP sites with read depth greater than 5 and mapping quality greater than 30.

Assignment of Iranian *V. dahliae* isolates to clonal lineages based on SNP markers. To analyze GBS data for *V. dahliae* from two different sequencing platforms, previous GBS sequencing reads (Milgroom et al. 2014) were reanalyzed using the 2015 assembly of the reference genome of isolate VdLs.17 (GenBank assembly accession GCA_000952015.1). SNP sites were filtered for a minimum of 10% coverage among isolates in the GBSv2 pipeline of Tassel v5.2.37 (Bradbury et al. 2007). SNPs from Iranian *V. dahliae* isolates were merged with the previous GBS data from reference isolates in Tassel. All sites scored as heterozygous were recoded as missing data and the merged dataset was filtered using Tassel for sites with a minimum of 75% coverage in all isolates and a minimum allele frequency of 1%. Isolates with missing data at 50% or more of the sites were also excluded from further analysis.

To assign Iranian isolates to clonal lineages previously defined by GBS (Milgroom et al. 2014), a phylogenetic tree was constructed from SNP data using the neighbor-joining method with 1,000 bootstrap replications using MEGA, version 7 (Kumar et al. 2016), with pairwise deletion of missing data.

Analysis of genetic diversity and population structure. Microsatellite analyses were carried out with methods used previously (Rafiei et al. 2018). The “Poppr” package in R version 3.0.1 (Kamvar et al. 2014) was used for calculating the number of multilocus genotypes (MLGs) and the number of unique alleles per locus among microsatellite data. Poppr was also used to draw minimum spanning networks based on Bruvo’s genetic distance (Kamvar et al. 2014, 2015).

For all isolates in lineage 2B⁸²⁴, a coalescent genealogy was inferred using GENETREE (Bahlo and Griffith 2000) as implemented by the MobyLe SNAP Workbench (Monacell and Carbon 2014), available at <http://snap.hpc.ncsu.edu/cgi-bin/mobyLe/portal.py>. For coalescent analyses, we used unfiltered SNP data from isolates in lineage 2B⁸²⁴. SNPs with missing data in any isolate or that occurred in only one isolate (singletons) were not used. Therefore, only parsimony-informative sites were used for analysis. Moreover, eight sites were removed manually using Tassel to resolve phylogenetic incompatibilities (homoplasy). One million runs were conducted in GENETREE three times, each with a different random number seed, to ensure results converged on the same genealogy.

Virulence tests on cotton to determine pathotypes. Fifteen Iranian *V. dahliae* isolates sampled from cotton, pistachio, and other hosts were inoculated on cotton cultivar Acala SJ-2 and a local variety derived from Acala SJ-2 (cultivar Bakhtegan) using a stem injection method (Jiménez-Díaz et al. 2017) to identify pathotype. Acala SJ-2 and Bakhtegan seeds were provided by the Cotton Research Institute, Gorgan, Iran. Seeds were washed in tap water, disinfested (1% NaOCl for 2 min) and sown in 15-cm pots filled with a nonsterilized potting mixture (soil/sand, 2:1 vol/vol) and maintained in a greenhouse at 20 to 28°C for 6 weeks. Mycelia from 7- to 10-day-old colonies of *V. dahliae* grown on PDA were flooded with 30 to 50 ml of sterile distilled water (SDW) to dislodge conidia, and the suspension was filtered through cheesecloth. Inoculum concentrations were determined using a hemocytometer and diluted to 1×10^7 conidia per ml with SDW. Plants were inoculated using 5 μl of the inoculum suspension with a Hamilton syringe on opposite sides at the base of first and second stem internodes (Jiménez-Díaz et al. 2017). *V. dahliae* isolate V138, which is in lineage 1A (Jiménez-Gasco et al. 2014; Milgroom et al. 2016) (provided by the Department of Plant Pathology and Environmental Microbiology, The Pennsylvania State University) was used as a positive control for the defoliating (D) pathotype. Each isolate was inoculated on five seedlings. Control plants were mock inoculated similarly by injection of SDW. Plants were examined for foliar symptoms and defoliation for 4 weeks after inoculation.

RESULTS

Mating type. Mating type-specific primers amplified a 600-bp *MAT1-2* fragment from all 78 isolates. No *MAT1-1*-specific product was amplified from any isolate.

Assignment of Iranian *V. dahliae* isolates to clonal lineages based on microsatellite genotyping. Eighty-one alleles were observed at 10 microsatellite loci from 78 isolates resulting in 51 MLGs. Nucleotide sequences of representative fragments of each size class confirmed allele sizes determined by capillary electrophoresis (data not shown).

To determine the correspondence of each isolate to a clonal lineage, microsatellite data for 78 Iranian isolates were combined with data for 104 reference isolates representative of all nine clonal lineages in *V. dahliae* (Milgroom et al. 2014; Rafiei et al. 2018). After combining the two datasets, 81 MLGs were found among 182 isolates. Minimum spanning networks based on 10 microsatellite markers in common between the two datasets were used to visualize the correspondence of microsatellite genotypes of Iranian isolates to the previously determined clonal lineages. Genotypes belonging to different clonal lineages grouped in distinct clusters (Fig. 2). Isolates from central Iran were placed in a distinct cluster, in which MLGs 65 and 75 were the most frequent among cotton and pistachio isolates, respectively. Although MLGs from these isolates did not match those of any reference isolates, some MLGs in this cluster were close to those in lineage 2B⁸²⁴ (Fig. 2). Isolates from northwestern Iran, all from *Prunus* spp., were grouped in a cluster with other reference isolates in lineage 2B⁸²⁴.

Microsatellite haplotypes of four isolates sampled from *Prunus* spp., two from Chaharmahal and Bakhtiari, Shahrekord region (AL42 and AL 32) and two from Fars (KHA1 and KHA2) in central Iran, clustered with MLGs in lineage 4B (Fig. 2). These four isolates had the same microsatellite MLG (MLG 52) as the most common reference isolates for lineage 4B.

Assignment of Iranian *V. dahliae* isolates to clonal lineages based on GBS. A total of 79,592 SNPs were detected among 48 Iranian isolates. After filtering the GBS data, 26,934 SNPs were retained for phylogenetic analysis from a combined set of 86

isolates, 48 from Iran and 38 reference isolates representative of known clonal lineages. The neighbor-joining tree (Fig. 3) constructed from these SNPs shows distinct clusters of haplotypes corresponding to known lineages, and is concordant with the tree constructed from SNPs by Milgroom et al. (2014). Iranian isolates clustered unambiguously with lineages 2B⁸²⁴ and 4B. Haplotypes of Iranian isolates from cotton, pistachio and other hosts were almost identical to those of lineage 2B⁸²⁴ reference isolates. Haplotypes of four Iranian isolates from *Prunus* spp. (AL42, AL37, KHA1, and KHA2) clustered with isolates in lineage 4B. Isolate assignment to clonal lineages based on SNP genotyping was consistent with the results from microsatellite analyses, but with much less variation in SNP haplotypes among isolates in the same lineage compared with microsatellites.

Population genetic structure. After removing nucleotide sites with missing data, and sites responsible for phylogenetic incompatibility, we found 22 haplotypes based on 242 SNPs among 44 Iranian isolates in lineage 2B⁸²⁴ (Table 1). Three replications of GENETREE simulations, each with one million simulations, converged on the same genealogy with minor variations in branch lengths (data not shown).

The inferred coalescent genealogy (Fig. 4) indicated divergence between isolates based on geographic origin. Haplotypes of most isolates from West-Azerbaijan Province in the northwest (from *Prunus* spp.) were placed in a distinct clade. Haplotypes from the rest of the country, including Golestan, Fars, and Kerman Provinces in the northeast, south and south-central parts of Iran, respectively (from cotton, pistachio, olive, and okra), formed another clade. All isolates from West-Azerbaijan were sampled from *Prunus* spp., confounding host and geographic origin. Only two isolates were sampled from *Prunus* spp. outside of West-Azerbaijan. These isolates (BSHA1 and BSHA152) were from almond trees in Shahrekord (Chaharmahal and Bakhtiari Province, in the southwest), and were in haplotype H15, which was placed in the same clade as haplotypes of

Fig. 2. Minimum spanning network showing the relationships among Iranian isolates and known clonal lineages (Milgroom et al. 2014) of *Verticillium dahliae*, based on 10 microsatellite loci in common between the datasets. Multilocus genotypes (MLGs) of Iranian isolates are indicated by the locations and hosts from which they were isolated (cotton, pistachio, okra, and *Prunus* spp.); those of reference isolates are indicated by "Lineage." The most frequent MLG in each lineage is shown. The sizes of circles are proportional to the number of individuals in each MLG. Thick and dark lines show MLGs that are more closely related to each other than thin and lighter lines (see scale at bottom).

the clade comprised of south-central isolates before they coalesced with the main West-Azerbaijan clade. All haplotypes from south and south-central Iran sampled from cotton and pistachio, and one haplotype from cotton in Golestan Province, were placed in the same clade. There were no clear differences in haplotypes between cotton and pistachio isolates.

Overall, it was not possible to infer the ancestral haplotype for all isolates in lineage 2B⁸²⁴ because there were multiple mutations on branches leading to all haplotypes (Fig. 4). Haplotype H19, which was found in one isolate (UP1) sampled from Urmia (plum tree), however, could be inferred as the ancestral haplotype for the West-Azerbaijan clade since no mutations occurred on the branches of H19 in this clade. However, there are 19 or more mutations between H19 and the other haplotypes in the same clade.

Pathotypes of *V. dahliae* isolates. All Iranian isolates in lineages 2B⁸²⁴ (13 isolates) and 4B (two isolates) induced mild to moderate leaf chlorosis and necrosis on cotton cultivars Acala SJ-2 and Bakhtegan 3 weeks after inoculation. In contrast, the positive control for the D pathotype (isolate V138 in lineage 1A) induced severe disease symptoms, epinasty followed by severe chlorosis and necrosis, 12 days after inoculation, and caused complete defoliation and death 3 weeks after inoculation. These results indicate that none of the Iranian isolates sampled in this study were of the D pathotype (Supplementary Fig. S1).

Lineage 2B⁸²⁴

Fig. 3. Neighbor-joining tree based on data from 26,934 single nucleotide polymorphisms (SNPs) showing the assignment of Iranian *Verticillium dahliae* isolates to clonal lineages. Except for four isolates in lineage 4B (indicated by solid circles), all Iranian isolates are in lineage 2B⁸²⁴. Solid squares indicate 2B⁸²⁴ reference isolates that cluster with Iranian isolates. The numbers on the branches represent bootstrap values ≥ 50 . SNP data for the reference isolates in each known clonal lineage were from Milgroom et al. (2014).

Microsatellites and SNPs are the most popular markers for describing genetic variation among fungal populations (Milgroom 2015; Tsykun et al. 2017). We previously compared the utility of SNPs and microsatellites in *V. dahliae* populations, and showed that both types of markers were useful for identifying clonal lineages (Rafiei et al. 2018). In the present study, we used both markers to identify lineages of isolates sampled from various hosts and locations in Iran (with unknown VCGs). Almost all Iranian isolates clustered with reference isolates in lineage 2B⁸²⁴ in the neighbor-joining analysis (SNPs) and minimum spanning networks (microsatellites); four other isolates were assigned to lineage 4B. However, 48 microsatellite MLGs were detected in lineage 2B⁸²⁴ among 74 isolates (Fig. 2), and none were identical to any reference isolates from lineage 2B⁸²⁴, even though they were assigned unambiguously to lineage 2B⁸²⁴ based on SNPs (Fig. 3). In the previous study, using microsatellite data from a geographically diverse collection of *V. dahliae* isolates, we also found that genotypic variation and phylogenetic incompatibility (homoplasmy) were higher in lineage 2B⁸²⁴ than in other lineages. However, one of the major limitations of microsatellite markers is their rapid mutation rates, which can increase the potential for homoplasmy (Guichoux et al. 2011). In general, mutations are more likely to occur at microsatellite loci than SNPs (Guichoux et al. 2011; Morin et al. 2004). The results from this study indicate that microsatellites cannot be effectively used for identifying lineage 2B⁸²⁴, unless a sufficiently large number of reference isolates is used representing the range of variability found within this lineage. Therefore, our results corroborate the finding in our previous study (Rafiei et al. 2018) that SNPs are more reliable than microsatellites for discriminating some clonal lineages in *V. dahliae*, especially lineage 2B⁸²⁴. In addition, the high rate of mutation and variation of microsatellites make them poor markers for making evolutionary inferences (Guichoux et al. 2011), although, in theory, these same properties make these markers more useful for tracking specific genotypes over shorter time scales. However, microsatellite genotyping of 113 isolates of *V. dahliae* in lineage 1A from Spain (Rafiei et al. 2018) did not reveal any greater diversity than SNP genotyping of 54 of these same isolates (Milgroom et al. 2016). The type of question being asked and the amount of diversity in each lineage are critical for choosing between microsatellite or SNP genotyping of *V. dahliae* populations.

Previous studies of *V. dahliae* isolated from different crops and woody plants in Iran indicated that VCG2B was the predominant VCG (Hadizadeh and Banihashemi 2007; Rouzbeh and Banihashemi 2006). Consistent with this result, lineage assignments based on SNPs and microsatellite markers in the present study found that lineage 2B⁸²⁴ was predominant: 74 out of 78 isolates were assigned to lineage 2B⁸²⁴. Moreover, three isolates assigned to VCG2B previously (VP39, VP64, and VP22) were genotyped in this study and assigned to lineage 2B⁸²⁴, reinforcing the correlation between VCGs and lineages described previously (Milgroom et al. 2014). In contrast, SNP and microsatellite genotypes of three isolates (VP29, VP27, and CS57), which were previously determined to be in VCG4A (Hadizadeh and Banihashemi 2007), clustered unambiguously with lineage 2B⁸²⁴. VCG4A is known exclusively in isolates from potato or soil from potato fields in North America (Joaquim and Row 1991; Omer et al. 2008; Rowe and Powelson 2002). Although VCGs have the potential to predict clonal lineages in *V. dahliae*, the VCG assay often yields ambiguous results (Dobinson et al. 1998; Dung et al. 2013; Joaquim and Rowe 1990; Strausbaugh 1993; Strausbaugh et al. 2016), further supporting the need for robust molecular markers.

Similarly, two isolates from pistachio and 10 isolates from cotton were previously assigned to VCG1 and were reported to have D pathotypes based on virulence tests in the greenhouse (Hadizadeh and Banihashemi 2007). Although none of these isolates were

available for genotyping, we sampled cotton isolates from the same cotton-growing areas (Estahban region, Fars Province) as the previous study, but we did not identify any isolates in lineage 1A. We also found no defoliating isolates in our virulence tests, although our samples from cotton fields were not large. Therefore, more sampling is needed to provide additional information on the presence of isolates in lineage 1A and D pathotype in Iran.

All isolates in this study had only the *MAT1-2* idiomorph, which is consistent with the predominance of this mating type worldwide (Dung et al. 2013; Milgroom et al. 2014; Short et al. 2014; Usami et al. 2009b). In contrast, *MAT1-1* has only been reported in a small number of isolates even though large numbers of *V. dahliae* isolates have been genotyped for mating type (Dung et al. 2013; Milgroom et al. 2014; Short et al. 2014; Usami et al. 2009b). The presence of a single mating type supports the notion that sexual reproduction is unlikely to occur in Iran.

Most *V. dahliae* isolates from Iran were assigned unambiguously to lineage 2B⁸²⁴ based on SNPs (Fig. 3). Nonetheless, SNP variation within this lineage showed a pattern of geographic differentiation (Fig. 4), although geographic origin was confounded with host in our samples. Considering the lack of host specialization in *V. dahliae* (Pegg and Brady 2002; Qin et al. 2006), differentiation of these populations is more likely to be a result of founder effects and geographic isolation than host specialization. The strong divergence between populations in the north and central regions of

TABLE 1. Single nucleotide polymorphism haplotypes of *Verticillium dahliae* isolates from Iran assigned to lineage 2B⁸²⁴ (Fig. 4)

Haplotype	Isolates	Host of origin	Location of origin
H1	U16A	<i>Prunus armeniaca</i>	Salmas, West Azerbaijan
H1	UP6	<i>Prunus armeniaca</i>	Khoy, West Azerbaijan
H1	14Bj	<i>Prunus domestica</i>	Salmas, West Azerbaijan
H2	CS57	<i>Gossypium hirsutum</i>	Estahban, Fars
H2	EBK8	<i>Gossypium hirsutum</i>	Estahban, Fars
H2	EG42	<i>Gossypium hirsutum</i>	Estahban, Fars
H3	EBM21	<i>Abelmoschus esculentus</i>	Estahban, Fars
H3	EZ2	<i>Olea europaea</i>	Estahban, Fars
H4	EBK63	<i>Gossypium hirsutum</i>	Estahban, Fars
H5	EBK17	<i>Gossypium hirsutum</i>	Estahban, Fars
H5	EBKH171	<i>Gossypium hirsutum</i>	Estahban, Fars
H6	EBM13	<i>Abelmoschus esculentus</i>	Estahban, Fars
H7	EG1	<i>Gossypium hirsutum</i>	Estahban, Fars
H8	EBKH22	<i>Gossypium hirsutum</i>	Estahban, Fars
H8	OLF	<i>Olea europaea</i>	Estahban, Fars
H9	CKG1	<i>Gossypium hirsutum</i>	Kordkuy, Golestan
H9	CKG2	<i>Gossypium hirsutum</i>	Kordkuy, Golestan
H10	15Bj	<i>Prunus domestica</i>	Salmas, West Azerbaijan
H10	U15H	<i>Prunus persica</i>	Salmas, West Azerbaijan
H11	VP52	<i>Pistacia vera</i>	Kerman, Kerman
H11	VP22	<i>Pistacia vera</i>	Rafsanjan, Kerman
H11	VP29	<i>Pistacia vera</i>	Rafsanjan, Kerman
H11	VP27	<i>Pistacia vera</i>	Rafsanjan, Kerman
H11	VP39	<i>Pistacia vera</i>	Rafsanjan, Kerman
H12	S.H	<i>Prunus persica</i> var. <i>nucipersica</i>	Urmia, West Azerbaijan
H13	Togh	<i>Xanthium strumarium</i>	Urmia, West Azerbaijan
H13	U19.2	<i>Prunus persica</i> var. <i>nucipersica</i>	Salmas, West Azerbaijan
H14	3fi	<i>Prunus domestica</i>	Khoy, West Azerbaijan
H14	TB2.VR	<i>Prunus armeniaca</i>	Khoy, West Azerbaijan
H14	UEggpl	<i>Solanum melongena</i>	Urmia, West Azerbaijan
H15	BSHA.1	<i>Prunus dulcis</i>	Shahrekord, Chaharmahal, and Bakhtiari
H15	BSHA152	<i>Prunus dulcis</i>	Shahrekord, Chaharmahal, and Bakhtiari
H16	PRF	<i>Pistacia vera</i>	Rafsanjan, Kerman
H16	PBa2	<i>Pistacia vera</i>	Rafsanjan, Kerman
H16	Pka	<i>Pistacia vera</i>	Rafsanjan, Kerman
H17	UP8	<i>Prunus domestica</i>	Khoy, West Azerbaijan
H17	UP3	<i>Prunus domestica</i>	Khoy, West Azerbaijan
H18	U-pep	<i>Capsicum annuum</i>	Urmia, West Azerbaijan
H18	U7H	<i>Prunus persica</i> var. <i>nucipersica</i>	Salmas, West Azerbaijan
H19	UP1	<i>Prunus domestica</i>	Khoy, West Azerbaijan
H20	UFH	<i>Prunus persica</i>	Salmas, West Azerbaijan
H20	U15.2H	<i>Prunus persica</i>	Salmas, West Azerbaijan
H21	UN	<i>Prunus persica</i> var. <i>nucipersica</i>	Urmia, West Azerbaijan
H22	TB1	<i>Prunus armeniaca</i>	Khoy, West Azerbaijan

Iran suggests that these regions were founded by separate introductions of individuals in lineage 2B⁸²⁴ that were genetically divergent before being introduced. Verticillium wilt was first observed on cotton in northwest provinces in 1964 (Cauquil and Rahmani 1964), but there was little information on its impact on crop production. A more widespread outbreak of the disease was observed between 1966 and 1969 (Banihashemi 1983; Scharif and Ershad 1966), when it was reported in many regions including north and central Iran, making it possible that lineage 2B⁸²⁴ was introduced separately in different locations.

Fig. 4. Inferred coalescent genealogy of 44 isolates in lineage 2B⁸²⁴ from GENETREE (Bahlo and Griffiths 2000) based on 242 single nucleotide polymorphisms showing divergence between haplotypes correlating with geographic regions in Iran. Black dots on the branches indicate unique mutations. The number of isolates represented in each haplotype is shown below the tree.

In addition, as shown in Figure 4, 10 haplotypes in the south-central clade (including H15 from southwestern Iran) coalesced more recently than those from the northwest. Most of these haplotypes were sampled from Fars, Kerman, and Chaharmahal and Bakhtiari (from cotton, okra, olive, and pistachio) that are near each other in south-central Iran, whereas all haplotypes in the other clade were sampled from West-Azerbaijan, far from the other locations in the northwest (*Prunus* spp.) (Fig. 1). Southern and central parts of Iran are suitable for cultivation of many crops and woody plants that are susceptible to *V. dahliae*. For example, in many regions of Fars and Kerman Provinces, both cotton and pistachio are grown. Verticillium wilt is also a major problem in these areas; therefore, soils where cotton and okra were previously grown may have been the source of *V. dahliae* that infected pistachio orchards planted later in these same fields. Soils, vegetative material and irrigation water carrying microsclerotia of *V. dahliae* may also have spread the fungus in southern and central areas, just as *V. dahliae* spread throughout olive in southern Spain after being infected from cotton (Jiménez-Díaz et al. 2011).

Limited gene flow between founder populations is likely to have maintained the divergence between these populations. The positions in the coalescent genealogy (Fig. 4) of haplotypes H14 and H17, from isolates sampled in Northwestern Iran, have SNP haplotypes more similar to those found in central Iran. These haplotypes may represent a third founder event in Iran, although we cannot test this hypothesis with results from this study. Alternatively, a limited amount of gene flow may have occurred between the two populations. Khorasan, Golestan, and Fars Provinces are the main cotton-growing areas in Iran (about 80% of total arable land is planted to cotton) (Ministry of Agriculture 2015). Cotton fields are also cultivated with the same local varieties in these provinces, suggesting that *V. dahliae*-infested seeds could have been a source for its dispersal to these areas (e.g., haplotype H9 in Fig. 4) (Göre et al. 2011). Although, cotton is grown in East-Azerbaijan (Moghan region), it is not cultivated in West-Azerbaijan. However, this province is one of the major fruit producing areas in the country. Considering the differences of haplotypes in the West-Azerbaijan (*Prunus* spp.) clade from those in the south-central clade (containing mostly cotton isolates), gene flow of *V. dahliae* appears to be restricted, probably because of limited trade in *Prunus* rootstocks or cotton seeds (Göre et al. 2011) between the two locations.

Cotton and pistachio isolates were closely related in our analyses (Figs. 2 and 3). The previous study on *V. dahliae* isolates from cotton and pistachio in Iran indicated that isolates from both hosts were in VCG2B and had similar virulence on susceptible hosts (Hadizadeh and Banihashemi 2007). In the coalescent genealogy, haplotypes from cotton and pistachio isolates were placed in the same clade, but it was difficult to determine which evolved first since there were so many mutations, and no clear ancestral haplotype. Cultivation of cotton and pistachio has a long history in Iran. The most important pistachio-growing areas in the country are in Kerman Province (especially in Rafsanjan region). Historically, cotton was grown in some regions of this province long before the cultivation of pistachio. Verticillium wilt was reported in cotton fields in Kerman province in 1966 (Scharif and Ershad 1966), however it was not considered as a serious problem in pistachio trees in Kerman until 1999 (Aminae and Ershad 1999). Therefore, based on the history of cotton and pistachio cultivations and historical reports of Verticillium wilt, cotton is likely the source of *V. dahliae* in pistachio trees in Iran as hypothesized previously (Hadizadeh and Banihashemi 2007).

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