Clonal Expansion and Migration of a Highly Virulent, Defoliating Lineage of *Verticillium dahliae*

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Accepted for publication 31 March 2016.

ABSTRACT

Milgroom, M. G., Jiménez-Gasco, M. M., Olivares-García, C., and Jiménez-Díaz, R. M. 2016. Clonal expansion and migration of a highly virulent, defoliating lineage of *Verticillium dahliae*. Phytopathology 106:1038-1046.

We used a population genomics approach to test the hypothesis of clonal expansion of a highly fit genotype in populations of *Verticillium dahliae*. This fungal pathogen has a broad host range and can be dispersed in contaminated seed or other plant material. It has a highly clonal population structure, with several lineages having nearly worldwide distributions in agricultural crops. Isolates in lineage 1A are highly virulent and cause defoliation in cotton, okra, and olive (denoted 1A/D), whereas those in other lineages cause wilting but not defoliation (ND). We tested whether the highly virulent lineage 1A/D could have spread from the southwestern United States to the Mediterranean basin, as predicted from historical records. We found 187 single-nucleotide polymorphisms (SNPs), determined by genotyping by sequencing, among 91 isolates of lineage 1A/D and 5 isolates in the closely related lineage 1B/ND. Neighbor-joining and maximum-likelihood analyses on the 187 SNPs showed a clear divergence between 1A/D and 1B/ND haplotypes. Data for only 77 SNPs were obtained for all 96 isolates (no missing data); lineages 1A/D and 1B/ND differed by 27 of these 77 SNPs, confirming a clear divergence between the two lineages. No evidence of recombination was detected within or between these two lineages. Phylogenetic and genealogical analyses resulted in five distinct subclades of 1A/D isolates that correlated closely with geographic origins in the Mediterranean basin, consistent with the hypothesis that the D pathotype was introduced at least five times in independent founder events into this region from a relatively diverse source population. The inferred ancestral haplotype was found in two isolates sampled before 1983 from the southwestern United States, which is consistent with historical records that 1A/D originated in North America. The five subclades coalesce with the ancestral haplotype at the same time, consistent with a hypothesis of rapid population expansion in the source population during the emergence of 1A/D as a severe pathogen of cotton in the United States.

Many plant-pathogenic fungi and oomycetes are well known to have clonal population structures (Anderson and Kohn 1998; Milgroom 2015). Several clonal genotypes are distributed across broad geographic areas (Grünwald et al. 2012; Jiménez-Gasco et al. 2002), including some that may have arisen from sexually reproducing populations (Ali et al. 2010; Goodwin et al. 1994; Milgroom et al. 2008). The term "epidemic population structure" was proposed for the clonal expansion of highly fit, novel pathogen genotypes that arise in recombining populations and are then amplified because of selection by asexual reproduction to the point where they dominate the population (Smith et al. 1993). This phenomenon is well known among bacterial pathogens of humans (Achtman et al. 1999; Caugant et al. 1987) but less well known among clonal plant pathogens. Population structures of some plant-pathogenic fungi reflect regular sexual reproduction followed by multiple generations of asexual reproduction (Cowger et al. 2008; Sommerhalder et al. 2010) but they are seldom dominated by only a few genotypes because of frequent recombination. By contrast, other fungi have highly clonal populations in which clonal lineages arose by historical, if not ongoing, recombination (Couch and Kohn 2000; Kohli and Kohn 1998; Milgroom et al. 2014).

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*The *e*-Xtra logo stands for "electronic extra" and indicates that three supplementary tables are published online.

http://dx.doi.org/10.1094/PHYTO-11-15-0300-R

Verticillium dahliae is an asexual, soil- and seedborne fungus that causes vascular wilt in approximately 400 plant species (EFSA PLH 2014; Inderbitzin et al. 2011; Klosterman et al. 2009). *V. dahliae* has no known sexual stage and reproduces mitotically by conidia and persistent soilborne dormant structures (microsclerotia). It is known to disperse clonally by the movement of soil and irrigation water, and with seed and vegetative propagation (Atallah et al. 2011; du Toit et al. 2005; Duressa et al. 2012; Göre et al. 2011, 2014; Jiménez-Díaz et al. 2012; Short et al. 2015) and has a distinctly clonal population structure (Collado-Romero et al. 2006, 2008; Dung et al. 2013; Gurung et al. 2014; Jiménez-Gasco et al. 2014; Milgroom et al. 2014). Clonality in *V. dahliae* was originally described by vegetative compatibility groups (VCGs), which comprise isolates that can form stable heterokaryons (Katan 2000; Rowe 1995). Five main VCGs (VCG1 through VCG4, plus VCG6) have been identified in *V. dahliae*, of which VCG1, VCG2, and VCG4 were further divided into subgroups A and B based on the frequency, speed, and vigor of complementation (Bell 1994; Bhat et al. 2003; Joaquim and Rowe 1991; Strausbaugh 1993). Molecular genetic markers correlate almost perfectly with VCGs, with the major exception of VCG2B, which was subdivided into genetically distinct lineages $2B^{334}$ and $2B^{824}$ (Collado-Romero et al. 2006, 2008; Dung et al. 2013; Gurung et al. 2014; Jiménez-Gasco et al. 2014, Milgroom et al. 2014), and a recently discovered recombinant lineage, $2B^{R1}$ (Milgroom et al. 2014). Individuals in VCGs 1A, 2A, 2B, and 4B are widely distributed geographically across several continents (EFSA PLH 2014).

In contrast to its current clonal population structure, recent evidence suggests that *V. dahliae* has reproduced sexually in the past and may retain this potential currently. For example, the *V. dahliae* genome contains genes necessary for meiosis (Milgroom et al.

2014; Short et al. 2014), which is considered molecular evidence for the potential of sexual reproduction (Schurko et al. 2009). Moreover, population genomic analyses of *V. dahliae* revealed that clonal lineages historically arose by recombination (Milgroom et al. 2014). In particular, lineages $2B^{334}$, $2B^{R1}$, and 6 are clearly recombinants. All other lineages were also involved with recombination, although it was not possible to infer parental and recombinant lineages because of repeated recombination events that obscured the exact recombination history (Martin et al. 2011). The widespread distribution of some clonal lineages that arose from recombination strongly suggests that *V. dahliae* has an epidemic population structure.

A highly virulent lineage of *V. dahliae* may exemplify the dynamics associated with clonal expansion and epidemic population structure. Individuals in VCG1A cause rapid defoliation of cotton, okra, and olive, in contrast to those in other VCGs, which generally cause wilting but not defoliation (Bell 1994; Daayf et al. 1995; Dervis et al. 2007, 2010; Jiménez-Díaz et al. 2011; Korolev et al. 2001, 2008; Schnathorst and Mathre 1966). These highly virulent individuals are referred to as having a defoliating (D) pathotype, and are denoted here as 1A/D to indicate both VCG and pathotype. Individuals of VCG1B, which is closely related to VCG1A (Collado-Romero et al. 2008; Jiménez-Gasco et al. 2014), cause leaf necrosis and wilting but not defoliation when tested on cotton (Bell 1994) (R. M. Jiménez-Díaz, unpublished data). Isolates that cause wilting without defoliation are referred to as having a nondefoliating (ND) pathotype regardless of the VCGs to which they belong. The haplotypes of all 1A/D individuals determined by genotyping by sequencing (GBS) were closely related to nondefoliating individuals in VCG1B (denoted here as 1B/ND), forming lineage 1A/1B (Milgroom et al. 2014). Whereas 1B/ND isolates have been reported only from woody plants in North America (Bell 1992; Chen 1994; Collado-Romero et al. 2006), lineage 1A/D has a wide geographic distribution and is known to infect at least 11 crop plants (artichoke, celery, cotton, eggplant, flax, okra, olive, safflower, tomato, sunflower, and watermelon) with a range of virulence but causing defoliation only on cotton, okra, and olive (Jiménez-Díaz et al. 2006; Korolev et al. 2008; Schnathorst and Mathre 1966).

The historical occurrence of the D pathotype has been extensively documented and reviewed (Bell 1992; Jiménez-Díaz et al. 2012). The D pathotype was first described as a genetically distinct strain in California in the early 1960s (Schnathorst and Mathre 1966). Not long afterward, the D pathotype was reported defoliating olive in California (Schnathorst and Sibbett 1971b) and was found to be the predominant pathotype in the High Plains of Texas, New Mexico, and southeastern Arizona (Bell 1992; Mathre et al. 1966). Subsequently, the D pathotype was reported on cotton in Mexico, Peru, China, Spain, Iran, Tajikistan, Greece, Turkey, and Israel (Bell 1992; Jiménez-Díaz et al. 2012). Based on these historical records, Bell (1992) hypothesized that the D pathotype was indigenous to the southwestern United States and northern Mexico and caused severe wilt when cotton was planted under irrigation in virgin desert soils in these areas. Moreover, he hypothesized that it was subsequently introduced to other countries with cotton seed from the United States. The occurrence of nearly identical 1A/D genotypes found in widespread geographic locations (Collado-Romero et al. 2006; Jiménez-Gasco et al. 2014; Milgroom et al. 2014) is consistent with the hypothesis that 1A/D constitutes a highly fit clonal lineage that dispersed from North America and, therefore, that *V. dahliae* has an epidemic population structure.

The objective of this study was to describe the clonal expansion and diversification of the highly virulent lineage 1A/D of *V. dahliae* in the Mediterranean basin. Specifically, our objectives were to (i) determine the origin of lineage 1A/D with respect to the closely related ND pathotype in VCG1B; (ii) determine whether the D pathotype arose independently in the Mediterranean basin or was introduced from North America and, if so, how many introductions occurred; and (iii) given the rapid spread of the D pathotype in Spain from cotton to olive since 1984 (Blanco López et al. 1989), we wanted to test whether genetic variation within lineage 1A/D in Spain was correlated with host, geographic locations, or time of isolation since this lineage was first reported.

MATERIALS AND METHODS

***V. dahliae* isolates.** In a previous study using GBS, we genotyped 73 isolates of *V. dahliae* in lineage 1A/D and 5 isolates in lineage 1B/ND (Milgroom et al. 2014); we used published data for 69 of these isolates in the present analysis. Isolates in this study were not randomly sampled; they were arbitrarily selected to maximize the geographic and temporal sampling diversity in extensive collections of *V. dahliae* in the Department of Crop Protection, Institute for Sustainable Agriculture, CSIC, Córdoba, Spain, and the Department of Plant Pathology and Environmental Microbiology, the Pennsylvania State University (Supplementary Table S1). Using exactly the same techniques as previously (Milgroom et al. 2014), we genotyped an additional 27 isolates from southern Spain, for a total of 96 (Table 1). The additional Spanish isolates were selected from the same *V. dahliae* collection in Córdoba, Spain, to investigate the spatial and temporal patterns of genetic variation of the D pathotype in Andalusia since it was first reported in 1984 (see below). Among the additional isolates genotyped, cotton isolates were selected from the current geographic range of the D pathotype in Andalusia (Jiménez-Díaz et al. 2011), representative of its early detection in 1984, 1985, and 1991. We also genotyped olive isolates sampled in 1994, 2005, and 2006. All isolates had previously been characterized as belonging to VCG1A, genotyped with molecular markers diagnostic for the D pathotype, and shown experimentally to cause defoliating symptoms on cotton.

SNP genotyping. GBS was performed at the Cornell University Institute for Genomic Diversity (IGD) following the methods described by Elshire et al. (2011). Briefly, GBS is done by digesting genomic DNA with a restriction enzyme (*Ape*KI) and sequencing the ends of restriction fragments on an Illumina platform to obtain many short reads. Sequence reads were aligned to a reference genome sequence for *V. dahliae* isolate VdLs.17 (Klosterman et al. 2011) and scored for single-nucleotide polymorphisms (SNPs) when differences were found among the test isolates (differences with the reference genome are irrelevant in this study). Methods for culturing, DNA isolation, and GBS were reported in detail previously (Milgroom et al. 2014).

SNPs produced by GBS were initially filtered by IGD for 80% coverage in all isolates per nucleotide site and a minimum minor allele frequency of 1%. Monomorphic sites were not used in any analyses; only variable sites were used. SNPs were further edited by recoding sites scored as heterozygous (caused rarely by sequencing or alignment errors) as missing data. To evaluate the reproducibility

TABLE 1. Samples of *Verticillium dahliae* from lineages 1A/D and 1B/ND[a]

Lineage	*n*	Location	Host
1A/D	27	Spain	Olive
	27	Spain	Cotton
	1	China	Cotton
	8	Greece	Cotton
	12	Israel	Cotton
	13	Turkey	Olive
	3	United States	Cotton
1B/ND	5	United States	Woody hosts[b]

[a] All isolates were previously determined to be in VCG1A or VCG1B, and either in the defoliating (D) or nondefoliating (ND) pathotype (Dervis et al. 2007; Jiménez-Díaz et al. 2006, 2011; Korolev et al. 2001, 2008; Pérez-Artés et al. 2000; Xu et al. 2012). More precise information on locations and dates of sampling for all isolates is found in Supplementary Table S1.

[b] Woody hosts included yellowwood (*Cladrastis lutea*), Japanese maple (*Acer palmatum*), velvet leaf (*Lavatera arborea*), green ash (*Fraxinus pennsylvanica*), and sugar maple (*A. saccharum*).

of GBS in *V. dahliae*, we genotyped several of the same isolates multiple times, in different GBS runs, to determine how much polymorphism might be due to sequencing or other errors. In all cases, we found identical SNP genotypes except for the loci with missing data, a common feature in GBS (results not shown). Data were filtered using Tassel 4.0 (http://www.maizegenetics.net/) to remove isolates with <90% of the scored nucleotide sites. Different filtering criteria did not fundamentally change any of the results (results not shown). The filtering criteria we used are somewhat arbitrary; however, there are currently no agreed-upon "best practices" for this type of SNP genotyping (Andrews et al. 2016).

Data analysis. We analyzed SNP data using Recombination Detection Program v4.46 (Martin et al. 2010). Recombination was previously detected because some isolates or lineages had genotypes with a mixture of SNPs commonly found in genotypes of two different lineages (Milgroom et al. 2014). The same analysis was used in this study to search for evidence of recombination within and between lineages 1A/D and 1B/ND. One 1A/D isolate (VEMS481) previously found to be a recombinant between lineages 1A/1B and $2B^{334}$ (Milgroom et al. 2014) was not used in the present study. Recombinant genotypes were not used because recombination violates the assumptions in coalescent analyses that haplotypes evolve only by mutation (Grünwald and Goss 2011; Rosenberg and Nordborg 2002).

To get an overview of the genetic relationships among all isolates in this study, we constructed a neighbor-joining tree with 1,000 bootstrap replications from the filtered dataset using Molecular Evolutionary Genetic Analysis v6.06 (MEGA6) (Tamura et al. 2013). We used a distance-based analysis like neighbor-joining because of the missing data inherent in GBS. The distance matrix for this analysis was constructed with pairwise deletion of sites when data were missing between any pair of isolates. In addition, we constructed a maximum-likelihood tree in MEGA6 using the partial deletion option for missing data.

A reduced dataset with no missing data was used for inferring a coalescent genealogy in the program Genetree (Bahlo and Griffiths 2000). This inference was conducted using the Mobyle SNAP Workbench (Monacell and Carbone 2014), accessed at http://snap.hpc.ncsu.edu/cgi-bin/mobyle/portal.py. For this analysis, SNPs with missing data in any individual were eliminated completely. We conducted 2 million runs for each analysis, starting from three different random number seeds to confirm convergence on the same genealogy.

Molecular epidemiology in 1A/D populations from cotton and olive in southern Spain. To test for a correlation between genetic distance and geographic distance of 1A/D isolates sampled from Spain, we conducted a Mantel test using the program GenAlEx 6.5 (Peakall and Smouse 2006, 2012). Genetic distance was calculated between pairs of haplotypes in MEGA6 as described for the neighbor-joining analysis. Pairwise geographic distances were estimated in GenAlEx 6.5 from latitude and longitude of the approximate locations where samples were collected. We also conducted a Mantel test in GenAlEx 6.5 for a correlation between genetic distance and the difference in time (years) between sampling dates of all pairs of isolates.

RESULTS

Divergence between lineages 1A/D and 1B/ND. The final dataset comprised 187 SNPs among 96 isolates (Supplementary Table S2). Among the 96 isolates, 117 of the 187 SNPs were parsimony-informative; the remaining 70 SNPs occurred only in one isolate each (singletons). No recombination was detected among the 96 1A/D isolates; therefore, all divergence among haplotypes is due to mutation. The neighbor-joining analysis on 187 SNPs shows a clear divergence between 1A/D and 1B/ND haplotypes (Fig. 1). A maximum-likelihood tree showed almost exactly the same topology as the neighbor-joining tree (results not shown).

For the coalescent analysis to infer the genealogy, we used a reduced dataset of 77 SNPs, omitting nucleotide sites with missing data. These 77 variable sites defined 24 haplotypes among 96 isolates (Supplementary Table S3). As in the neighbor-joining tree, the inferred coalescent genealogy shows a marked divergence between lineages 1A/D and 1B/ND (Fig. 2). More than half (27 of 50) of the parsimony-informative SNPs were mutations that resulted in the divergence of lineages 1A/D and 1B/ND (Fig. 2).

Variation within lineage 1A/D. The neighbor-joining tree (Fig. 1) and coalescent genealogy (Fig. 2) show that the geographic origins of 1A/D isolates are associated with five distinct subclades (Table 2). The genealogy in Figure 2 is not well resolved; all five subclades coalesce with the most recent common ancestor (MRCA) of lineage 1A/D at one point in time. With a few exceptions, each of four subclades comprises isolates mainly from one location (e.g., Greece, Israel, western Turkey, or central Turkey). The fifth subclade (subclade 1) includes all isolates from Spain, seven from Israel, and one from China. These correlations of geographic origins with subclades have a few exceptions. For example, isolates from Israel are found in subclades 1 and 5. Subclade 5 includes five isolates from Israel with a haplotype (H4) (Fig. 2) identical to one from Turkey; it also includes a haplotype (H13) in U.S. isolate T9, which originated from cotton in California in 1964 as a single-spore isolate from isolate T-1, which was the first D pathotype isolate reported (Puhalla and Mayfield 1974; Schnathorst and Mathre 1966). Seven other isolates from Israel (three in haplotype H1 and four in haplotype H19) are in subclade 1 with those from Spain; haplotype H1 also includes a single isolate from China. Finally, subclade 2 includes isolate V1904 from central Turkey, with the same haplotype (H9) as an isolate from western Turkey.

The three isolates from the United States (isolates T9, V44, and DX2), sampled before 1983, are near the base of the 1A/D clade in the neighbor-joining tree (Fig. 1). The haplotype for U.S. isolate V44 is the inferred root of lineage 1A/D in the bootstrap consensus tree (results not shown). In a coalescent genealogy of lineage 1A/D, haplotype H16 is the ancestral haplotype (Fig. 2). H16 is considered the ancestral haplotype because there are no mutations on the branches for this haplotype that are not shared by all other 1A/D haplotypes (Fig. 2); that is, H16 is the same haplotype as that of the MRCA for this lineage. This ancestral haplotype was found in two U.S. isolates (DX2 and V44) sampled from cotton in Arizona and Texas, respectively, before 1983 (exact isolation dates are not known).

Molecular epidemiology in 1A/D populations from cotton and olive in southern Spain. Only 47 of the 187 SNPs were variable among 54 isolates from Spain (all in subclade 1) (Figs. 1 and 2; Table 2), only 8 of which were parsimony informative (the remaining 39 were singletons). Most of the isolates from Spain had identical haplotypes in the reduced data set (Table 2), regardless of being isolated from cotton or olive, indicating no genetic differentiation by host. We found a weak positive correlation between genetic distance and the logarithm of geographic distance ($r = 0.136$, $P = 0.018$) among isolates from Spain, providing some marginal evidence of isolation by distance (Fig. 3). However, no correlation was found between genetic distance and year of isolation ($r = 0.01$, $P = 0.378$).

DISCUSSION

Our analyses revealed marked genetic differentiation between *V. dahliae* individuals with 1A/D and 1B/ND phenotypes that was not reported previously in a larger-scale population genomic analysis (Milgroom et al. 2014). This differentiation into discrete lineages is consistent with phenotypic differences based on VCGs and pathotypes, and with amplified fragment length polymorphism markers (Collado-Romero et al. 2006). The only evidence of recombination within lineages 1A/D and 1B/ND (as opposed to well-documented recombination between other lineages) was caused by the presence of a single isolate (VEMS481), which was reported previously (Milgroom et al. 2014). This isolate was not used in the present study to avoid complications of recombination in inferring genealogies (Rosenberg and Nordborg 2002). Therefore, our analysis

confirms that these two phenotypes represent distinct clonal lineages that diverged by mutation and not recombination.

In conjunction with historical records (Bell 1992; Jiménez-Díaz et al. 2012), results of this study support the hypothesis that the D pathotype arose in North America and was dispersed clonally to widespread geographic locations. Our results show that lineage 1A/D arose by mutation from a common ancestor of lineage 1B/ND. Isolates from lineage 1B/ND have been sampled only from North America (Bell 1992; Chen 1994; Collado-Romero et al. 2006). One 1B/ND isolate in this study (V518) was isolated in 1972, more than 10 years before the D pathotype was reported in the Mediterranean basin (Blanco López et al. 1989), further implicating North America as the source. The strongest genetic evidence for inferring North America as a source for introduction elsewhere is the coalescent genealogy shown in Figure 2. Haplotype H16 is inferred as the ancestral haplotype for lineage 1A/D because no mutations are found along the branches of this haplotype in the genealogy (Fig. 2), making it identical to the MRCA of this lineage. All other subclades of lineage 1A/D differ from the MRCA by three or more mutations. The ancestral haplotype (H16) was sampled from Texas (isolate V44) and Arizona (isolate DX2) in the southwestern United States before 1979 and 1983, respectively (precise dates are not known). These isolates predate the earliest reports of 1A/D in the Mediterranean basin, which were from Spain in 1984 (Blanco López et al. 1989). Altogether, these results are consistent with the hypothesis that lineage 1A/D originated in the United States and was later introduced to the Mediterranean basin. In addition, this conclusion is in agreement with Bell's early hypothesis that the D pathotype is indigenous to the southwestern United States and northern Mexico and was dispersed to other locations with contaminated cotton seed (Bell 1992).

Although our sample includes only a small number of isolates from North America, coalescent analyses do not require large samples (Grünwald and Goss 2011). Although small samples may seem contrary to conventional wisdom in statistics, in which larger samples provide more robust estimates and smaller variances, sample size has a negligible effect on coalescent analyses (Grünwald and Goss 2011; Rosenberg and Nordborg 2002). The bulk of the coalescent events that define the basic structure of the genealogy can be captured by samples on the order of approximately 15 individuals, whereas larger samples mainly add resolution to the tips of the inferred genealogy (Grünwald and Goss 2011). Moreover, large samples are not necessary for ensuring that the ancestral type is found. The probability that a sample from an unstructured population contains the MRCA is given by $(n-1)/(n+1)$, where n is the sample size (Rosenberg and Nordborg 2002). For example, for a sample size of 18, this probability is 0.90. Although our sample of lineage 1A/D, taken from a culture collection, was not necessarily from a single unstructured population, we found 18 haplotypes among 91 isolates, with a high probability of finding the MRCA. These properties of coalescent analyses make it a powerful approach for evolutionary inferences, especially in situations like ours in which it is difficult to obtain random samples of additional isolates (Grünwald and Goss 2011; Rosenberg and Nordborg 2002).

The lack of resolution in the inferred genealogy, in which all 1A/D subclades coalesce at the same time (Fig. 2), may be indicative of rapid population expansion. In the absence of recombination, genealogies with long branches and many singleton SNPs typically

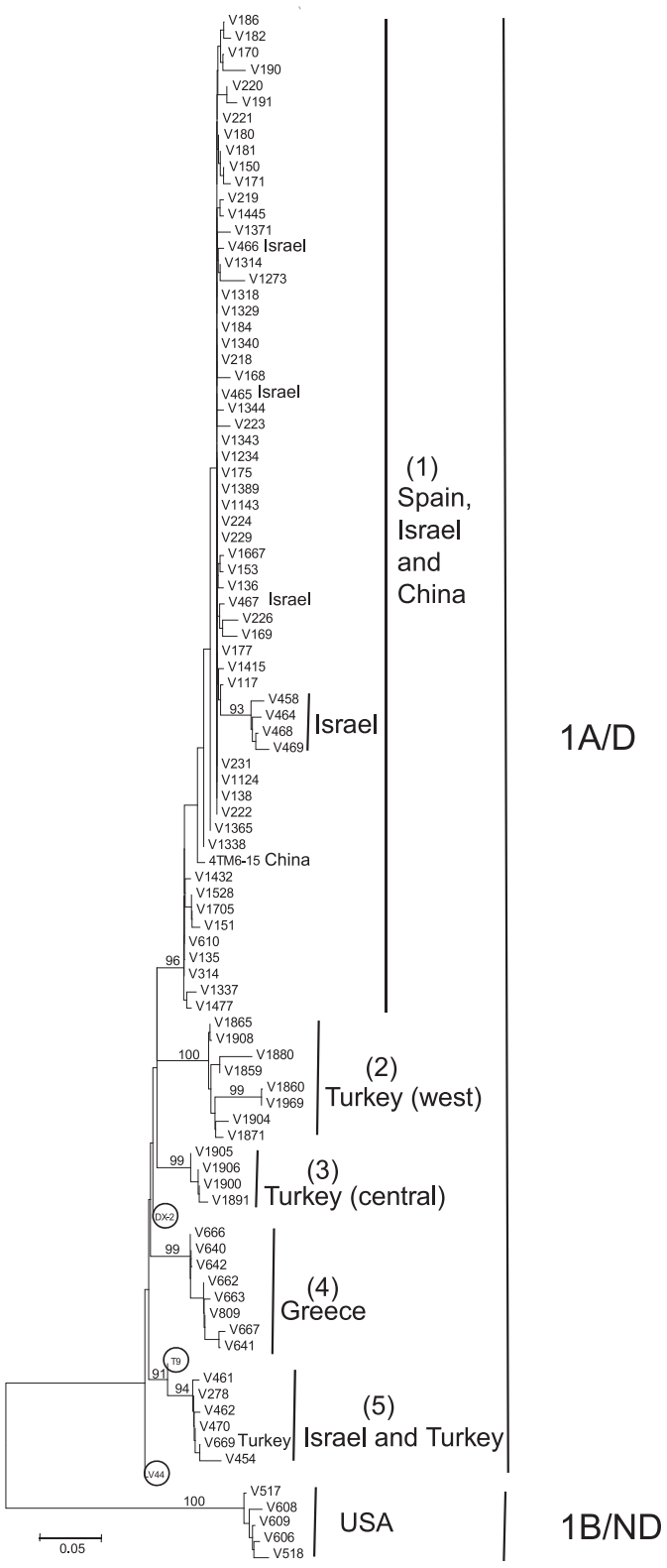

Fig. 1. Neighbor-joining tree showing the divergence and geographic distribution of lineages 1A/D and 1B/ND in *Verticillium dahliae*. The two main clades are indicated by vertical lines labeled as lineages 1A/D and 1B/ND. Five subclades within lineage 1A/D (numbered 1 to 5) and their countries of origin are also indicated by vertical lines. Isolate names followed by country names indicate their origins; isolates without country names are from the country listed first for each subclade. The three isolate names circled near the base of the tree (T9, DX2, and V44) are in lineage 1A/D and were sampled in the southwestern United States before 1983. This neighbor-joining tree was constructed in MEGA6 (Tamura et al. 2013) from 187 SNPs in 96 isolates, each with a unique haplotype because of missing data. Missing data were pairwise deleted between haplotypes and not used in constructing the distance matrix for constructing this neighbor-joining tree. Bootstrap values >90% from 1,000 replications are shown above the branches. The scale bar at the bottom left indicates genetic distance using default parameters in MEGA6 with pairwise deletion of sites with missing data.

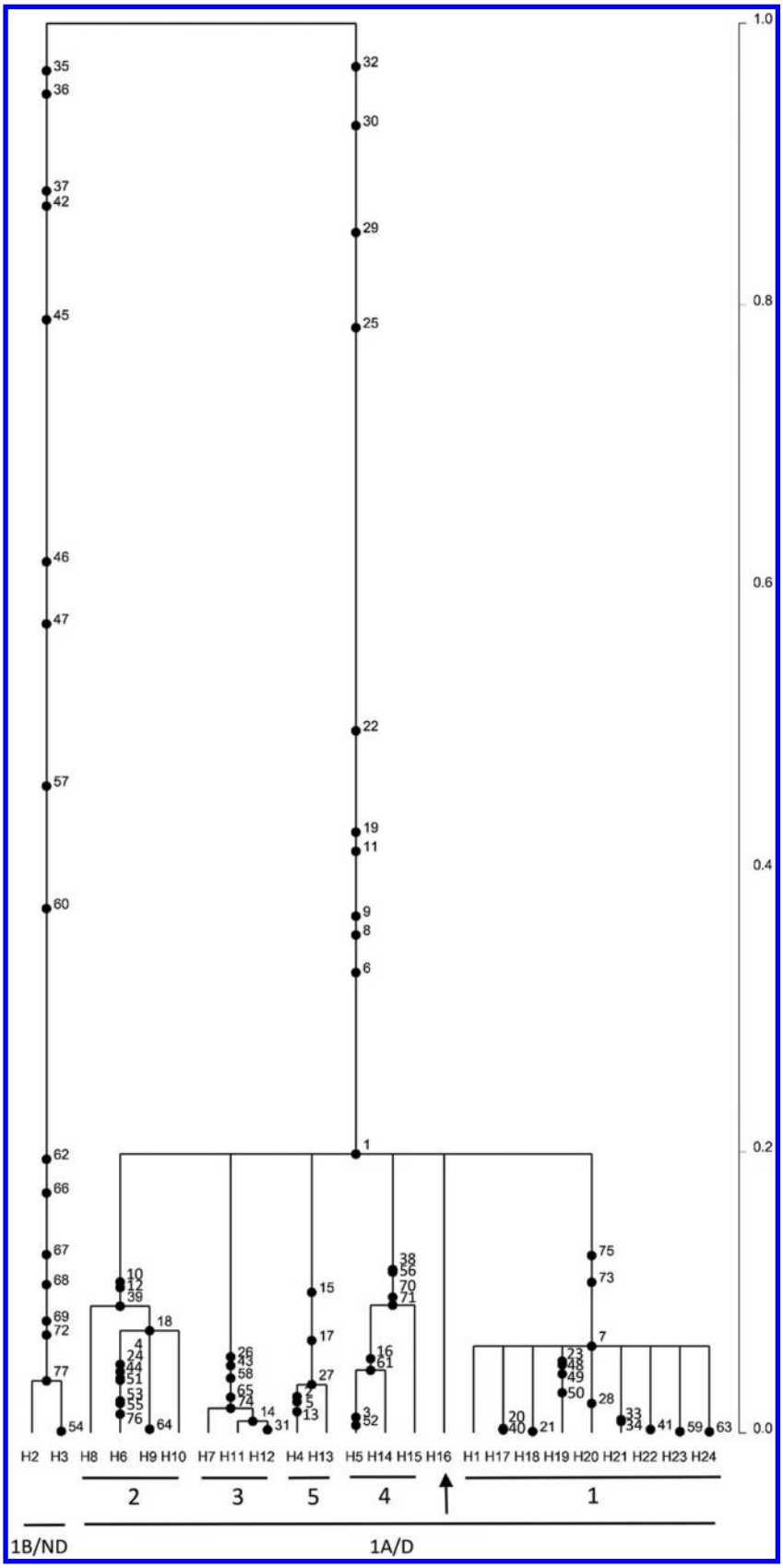

Fig. 2. Inferred coalescent genealogy of lineages 1A/D and 1B/ND of *Verticillium dahliae*. Haplotypes H2 and H3 comprise isolates in lineage 1B/ND; all others are in lineage 1A/D, with subclades numbered corresponding to those in Figure 1 and Table 2. The inferred ancestral haplotype for lineage 1A/D is H16 (indicated by an arrow). This genealogy was inferred from 77 SNPs in 96 isolates (no missing data) using Genetree (Bahlo and Griffiths 2000), facilitated by Mobyle SNAP Workbench (Monacell and Carbone 2014), accessed at http://snap.hpc.ncsu.edu/cgi-bin/mobyle/portal.py. Mutations defining each haplotype are shown as black dots on the branches; the number beside each dot indicates the mutation that occurred, as defined in Supplementary Table S3. The scale on the right indicates coalescent time rescaled to 1. This genealogy is one of three independent replicates (each starting from a different random number seed) that converged on the same genealogy after 2 million runs.

result from rapid population expansion, as might be expected for a pathogen with an epidemic population structure. Verticillium wilt was reported throughout the southwestern United States when cotton was first cultivated under irrigation in virgin desert soils (Bell 1992). Therefore, *V. dahliae* was indigenous in these soils, and populations of those genotypes capable of infecting cotton expanded rapidly when large areas of desert were planted to highly susceptible cotton monocultures. Bell (1992) hypothesized that the D pathotype is indigenous throughout this area and caused Verticillium wilt on cotton as early as the 1920s, even though it was not described as a genetically unique strain until the 1960s, when it became a major factor for severe defoliation in previously resistant cotton varieties and, later, in olive (Schnathorst and Mathre 1966; Schnathorst and Sibbett 1971a). The lack of availability of additional isolates from the United States in this study is an obvious weakness of our sampling. However, we speculate that the five subclades of lineage 1A/D found in the Mediterranean basin represent a subset of the diversity of 1A/D genotypes of *V. dahliae* present in indigenous populations in the southwestern United States at the time they were introduced. For example, subclade 5 comprises haplotype H4 from isolates sampled in Israel (and one isolate from Turkey) and haplotype H13 found in isolate T9, which is an isolate derived from the original T-1 strain in California in 1964 (Puhalla and Mayfield 1974). We speculate that an individual with haplotype H13 (or one with a closely related haplotype) founded a population in Israel. Under this scenario, North America is likely to have a diverse population of lineage 1A/D indigenous to the southwestern United States and was the source for at least four additional independent introductions documented in the Mediterranean basin.

The lack of sampling of North American haplotypes (except haplotype H4, which is closely related to haplotype H13) in the

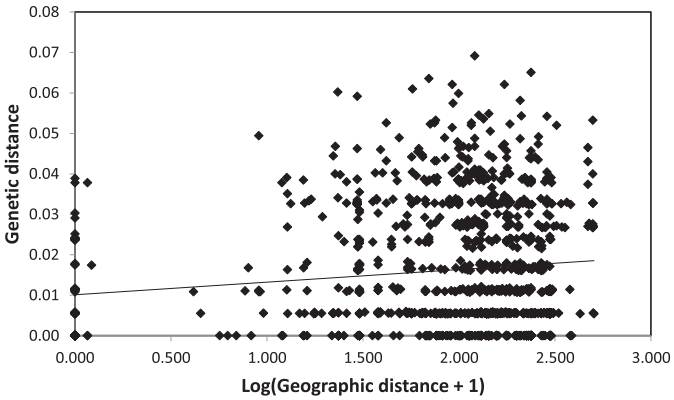

Fig. 3. Isolation by distance within lineage 1A/D of *Verticillium dahliae* sampled from cotton and olive in southern Spain, as shown by the significant correlation between genetic distance and the logarithm of geographic distance between pairs of 54 isolates ($r = 0.136$, $P = 0.018$). In total, 1,431 (= 54·53/2) pairwise comparisons were used in this analysis. Isolates were sampled equally from cotton and olive from 1984 to 2006. Genetic distance was calculated with pairwise deletion of SNPs with missing data as in the neighbor-joining analysis (Fig. 1).

TABLE 2. *Verticillium dahliae* isolates in lineages 1A/D and 1B/ND assigned to haplotypes (Hap) defined by 77 SNPs[a]

Lineage	Subclade	Haplotype	*n*	Isolates	Country	Host
1B/ND	N/A	H2	4	V517, V518, V606, V609	United States	Woody hosts
		H3	1	V608	United States	Green ash
1A/D	2	H8	3	V1859, V1865, V1908	Western Turkey	Olive
		H6	2	V1860, V1969	Western Turkey	Olive
		H9	2	V1880, V1904	Central and western Turkey	Olive
		H10	1	V1871	Western Turkey	Olive
	3	H7	1	V1905	Central Turkey	Olive
		H11	2	V1900, V1906	Central Turkey	Olive
		H12	1	V1891	Central Turkey	Olive
	5	H4	5	V278, V454, V461, V462, V470	Israel	Cotton
			1	V669	Turkey	Olive
		H13	1	T9	United States	Cotton
	4	H5	2	V641, V667	Greece	Cotton
		H14	3	V662, V663, V809	Greece	Cotton
		H15	3	V642, V640, V666	Greece	Cotton
	N/A	H16	2	DX2, V44	United States	Cotton
	1	H1	25	V135, V136, V150, V151, V153, V314, V1124, V1143, V1234, V1273, V1314, V1318, V1337, V1338, V1340, V1343, V1344, V1365, V1389, V1415, V1432, V1477, V1528, V1667, V1705	Spain	Olive
			22	V117, V175, V177, V180, V181, V220, V221, V222, V226, V231, V138, V170, V182, V184, V186, V191, V218, V219, V224, V229, V610, V1329	Spain	Cotton
			3	V465, V466, V467	Israel	Cotton
			1	4TM6-15	China	Cotton
		H17	1	V1371	Spain	Olive
		H18	1	V1445	Spain	Olive
		H19	4	V458, V464, V468, V469	Israel	Cotton
		H20	1	V169	Spain	Cotton
		H21	1	V223	Spain	Cotton
		H22	1	V190	Spain	Cotton
		H23	1	V168	Spain	Cotton
		H24	1	V171	Spain	Cotton

[a] Haplotypes are listed in the order corresponding to the positions of subclades (from left to right) in the inferred coalescent genealogy in Figure 2. Subclades of lineage 1A/D (numbered 1 to 5) are the same as those showing geographic groupings in the neighbor-joining tree in Figure 1. N/A = not applicable.

Mediterranean basin suggests the alternative hypothesis that some subclades evolved after introduction. We consider this hypothesis unlikely for two reasons. First, we genotyped an extensive collection of 1A/D isolates (n = 54) sampled from southern Spain between 1984 and 2006. This large sample allowed us to get a picture of how fast this lineage evolved over the 22 years since introduction. The ancestral haplotype for subclade 1 in Spain is H1 (Fig. 2), which is found in 47 of the 54 isolates. All other haplotypes in Spain are represented by single isolates and, therefore, defined by singleton SNPs, typical in populations that experience rapid expansion, such as happened after the introduction and subsequent spread of *V. dahliae* in southern Spain (Jiménez-Díaz et al. 2011). Given the divergence among the different subclades, in which each subclade is at least three mutations different from the MRCA, it would be highly unlikely that the different subclades evolved within the 30 or so years that have elapsed since the D pathotype was first reported in the Mediterranean basin. Second, if all sampled haplotypes were derived by a series of mutations from the ancestral type after its introduction, then all intermediate haplotypes for each subclade were lost and only the descendants of the mutants survived to be sampled. Given that ancestral types (including to specific subclades) have a high probability of being included in relatively small samples, this explanation seems unlikely. Thus, we are left with the hypothesis that at least five independent introductions occurred into the Mediterranean basin, most likely from a diverse population indigenous to North America.

Based on our interpretations, separate introductions occurred into Spain, Greece, Israel, central Turkey, and western Turkey (corresponding to the five subclades), with some evidence of secondary migrations between Turkey and Israel and between central and western Turkey. Subclade 1, with the largest number of isolates, includes isolates from Spain, Israel, and China; haplotype H1 is the ancestral type (or founder) for this subclade. This pattern could emerge from a single introduction into Spain or Israel, with secondary migration occurring between them. Alternatively, by chance, the same haplotype could have been introduced independently into the two countries. The disparity in the number of isolates genotyped from the two countries reflects our bias for addressing epidemiological questions in Spain (see below) but does not give any indication of the direction of migration. However, 1A/D was first detected in Spain in 1984 (Blanco López et al. 1989) and spread rapidly throughout Andalusia (Jiménez-Díaz et al. 2011), whereas 1A/D was reported from only one location in Israel in 1997 (Korolev et al. 2008). The D pathotype was the predominant type sampled in a severe outbreak of Verticillium wilt in the Hula Valley in 1997; however, it had not been found previously in an extensive survey across the country between 1992 and 1997 (Korolev et al. 2000, 2001). The occurrence of a single isolate with haplotype H1 in China may have resulted from a secondary introduction from the Mediterranean basin, although it is impossible to make any firm conclusion from these results.

Given the nature of the sampling and bias associated with GBS, we did not attempt to estimate a date for the coalescence of lineages 1A/D and 1B/ND or the subclades within lineage 1A/D because it is impossible to estimate a mutation rate accurately from these data. GBS data are biased by sampling relatively conserved regions of the genome, at least with respect to the conservation of restriction sites (Arnold et al. 2013). Moreover, SNP data in the output of GBS include only variable sites, which would markedly inflate mutation rate estimates. Finally, any differences between 1A/D and 1B/ND might also be under selection because of their differences in virulence and host ranges, further biasing estimates of divergence. Previous attempts to date the divergence of *V. dahliae* lineages from microsatellite data (Short et al. 2015) are equally fraught because of homoplasy and recombination and, potentially, selection. These latter authors used hypervariable markers, with an average of almost 20 alleles per locus, making it unlikely that all alleles identical in state are identical by descent. More importantly, *V. dahliae* lineages arose by recombination, not divergence due to mutation (Milgroom et al. 2014), making estimates of divergence times based on mutation and drift inappropriate. Finally, selection for host preferences or virulence, although not controlled by microsatellite markers themselves, can affect the divergence of entire genomes in clonal organisms, further biasing estimates assumed to be caused only by mutation.

Genotyping at many thousands of SNP loci is becoming the method of choice in population genetics (Andrews et al. 2016), especially as a way of finding genetic polymorphisms among closely related genotypes within a clonal lineage (Grünwald et al. 2016). Although microsatellite markers have been used for genotyping *V. dahliae* (Short et al. 2015), we did not use them because relatively few loci can be genotyped compared with SNPs, making it unlikely to find enough polymorphisms to answer population genetic questions. However, we had to genotype a large numbers of SNPs by GBS to detect enough diversity to investigate phylogeography within a lineage. We found 187 variable sites, only 117 of which were parsimony informative; half of these separated lineages 1A/D from 1B/ND. Because of missing data inherent in GBS, we were left with only 77 SNPs for genealogical analyses, with relatively little variation within lineages 1A/D and 1B/ND. Nonetheless, enough variation was detected to infer the origin of 1A/D and independent introductions into the Mediterranean basin.

In general, geographic origins of *V. dahliae* isolates were confounded with hosts of origin in our samples (Table 1), except in Spain, where we sampled equally from the two main agricultural hosts of lineage 1A/D, olive and cotton. No genetic differentiation could be detected between these two hosts; in fact, most isolates in Spain have nearly identical haplotypes regardless of host, location, or year of sampling. Nonetheless, even with relatively little nucleotide diversity, we found a weak but significant, positive correlation between genetic distance and the logarithm of geographic distance within southern Spain, indicating some evidence of isolation by distance developing over approximately 30 years since lineage 1A/D was first reported. No correlation was found, however, between genetic distance and temporal differences in sampling dates. In southern Spain, the D pathotype was first found in 1984 in cotton crops within a limited area in southwestern Seville province in Andalusia; by 2012, it had spread more than 320 km and become the predominant pathotype infecting olive throughout 1.5 million ha of olive-growing area in southern Spain (Jiménez-Díaz et al. 2011, 2012).

In conclusion, our results suggest that the highly virulent lineage 1A/D of *V. dahliae* arose once in North America and was introduced at least five times into the Mediterranean basin. The most likely means of migration was in cotton seed contaminated with *V. dahliae*, as hypothesized previously (Bell 1992). The extent of seed transmission of *V. dahliae*, including for the D pathotype, was recently demonstrated (Göre et al. 2011, 2014). Once introduced within a region, the D pathotype can become well established and spread to large areas by several means of dispersal, including infected planting stocks, infested plant parts, and infested water (Jiménez-Díaz et al. 2012). Our results reinforce the need for certifying planting material free from infection by *V. dahliae*, and especially free of genotypes in lineage 1A/D, to avoid introduction of highly virulent pathogen strains into areas where they are not yet found.

ACKNOWLEDGMENTS

This project was funded by grants P10-AGR 6082 from 'Consejería de Economía, Innovación y Ciencia', Regional Government of Andalusia and AGL2011-24935 (Vertigen project) from Ministerio de Economía y Competitividad, Spain, and co-financed with FEDER funds from the European Union. We thank J. Armengol, S. Dervis, T. Katan, E. Paplomatas, and R. C. Rowe and for sharing *V. dahliae* isolates; J. L. Trapero-Casas for technical support; P. Castillo for help with figures; N. Grünwald for advice on analyses; and anonymous reviewers who made helpful suggestions.

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