Complex molecular relationship between Vegetative Compatibility Groups in *Verticillium dahliae:* VCGs do not always align with clonal lineages

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ABSTRACT

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Verticillium wilts caused by the soilborne fungus *Verticillium dahliae* are amongst the most challenging diseases to control. Populations of this pathogen have been traditionally studied by means of vegetative compatibility groups (VCGs) under the assumption that VCGs comprise genetically related isolates that correlate with clonal lineages. We aimed to resolve the phylogenetic relationships among VCGs and their subgroups based on sequences of the intergenic spacer region of the rDNA (IGS) and six anonymous polymorphic sequences (VdSNP) containing single nucleotide polymorphisms (SNPs). A collection of 68 *V. dahliae* isolates representing the main VCGs and subgroups (VCGs 1A, 1B, 2A, 2B, 3, 4A, 4B, and 6) from different geographic origins and hosts was analyzed using the seven DNA regions. Maximum parsimony (MP) phylogenies inferred from IGS and VdSNP sequences showed five and six distinct clades, respectively. Phylogenetic analyses of individual and combined datasets indicated that certain VCG subgroups (e.g., VCGs 1A and 1B) are closely related and share a common ancestor; however, other subgroups (e.g., VCG 4B) are more closely related to members of a different VCG (e.g., VCG 2A) than to subgroups of the same VCG (VCG 4B). Furthermore, MP analyses indicate that VCG 2B is polyphyletic with isolates placed in at

least three distinct phylogenetic lineages based on IGS sequences, and two lineages based on VdSNP sequences. Results from our study suggest the existence of main VCG lineages that contain VCGs 1A and 1B; VCGs 2A and 4B; and VCG 4A, for which both phylogenies agree; and the existence of other VCG or VCG subgroups that seem to be genetically heterogeneous or show discrepancies in their phylogenetic placement: VCG 2B, VCG 3 and VCG 6. These results raise important caveats regarding the interpretation of VCG analyses: genetic homogeneity and close evolutionary relationship between members of a VCG should not be assumed.

Vegetative compatibility in fungi refers to the ability of hyphae to anastomose and form a stable heterokaryon. This allows organizing isolates that are vegetatively compatible into vegetative compatibility groups (VCGs). Based on knowledge derived from model fungal systems, vegetative compatibility is controlled by *het*erokaryon incompatibility (*het*) or *ve*getative *in*compatibility (*vic*) loci (21,31). For hyphal anastomosis to occur between two fungal isolates, alleles at those *het/vic* loci must be identical, or compartmentalization and hyphal cell death will occur at the point of fusion (20,21,31). The apparent need of allele homozygosis at *het/vic* loci has led to the assumption that isolates within a VCG must be genetically related, and consequently VCGs are generally conceived as clonal lineages that comprise individuals related by common descent with variation within clonal lineages arising mainly through mutations (1,19).

The VCG structure in fungal populations has been used to estimate genetic diversity in many fungi (38,47), including *Verticillium dahliae*, a soilborne anamorphic fungus in the

Phylum Ascomycota, that causes vascular wilt diseases in hundreds of important crops worldwide (10,23,29,42,48). Puhalla first identified VCGs in *V. dahliae* using UV-induced microsclerotial color mutants (44). Based on this assay, four VCGs were initially identified (44) although this number increased to 16 VCGs when a diverse collection of 86 isolates was analyzed (45). Later, Joaquim and Rowe (26) reclassified 15 of the 16 VCGs identified by Puhalla (44) into four main VCGs: VCG 1, VCG 2, VCG 3, and VCG 4, using an assay that relies on the use of nitrate-nonutilizing (*nit*) auxotrophic mutants that arise naturally in medium containing chlorate (26). The use of nitrogen sources and the wild type growth are reestablished following positive complementation between different *nit* mutants of paired isolates, and those isolates are assigned to the same VCG. Since 1990, VCG groups based on complementation assays with *nit* mutants have been slightly updated. Several VCGs have been further subdivided into subgroups based on vigor and frequency of complementation (i.e., VCG1A and VCG1B, VCG2A and VCG2B, VCG4A and VCG4B) (6,24,26,51). More recently, isolates causing disease on pepper in California were characterized as a new VCG 6 (9).

The advancement of molecular tools and their increased availability in phytopathology has led to a steady replacement of VCG testing by molecular assays and protocols to resolve fungal population diversity. However, VCGs are still important markers for studies that focus on *V. dahliae* (24,41). Different VCGs can be linked to host adaptation shown by populations of *V. dahliae* and therefore these groups bear significant biological information (8,22,24,28,48). In some cases, virulence to a given symptomatic host plant (34) can be correlated to different VCGs. For example, isolates of VCG 4A,

which has only been found in North America mainly associated to potato crops (41,48), are more virulent on potato than VCG 4B and 2B isolates, which occur in North America and elsewhere (27,51). In Israel, VCG 4B isolates are more virulent to potato, and VCG2A isolates are more virulent to tomato, than VCG 2B isolates (55). In Spain, *V. dahliae* isolates from artichoke in VCG2B were more virulent to this host plant compared with isolates in VCG1A and VCG2A (24). Another example regarding the phytopathogenic significance of VCGs concerns *V. dahliae* infecting cotton and olive in Spain. Isolates in VCG 1A correlate to the highly virulent defoliating pathotype in olive and cotton crops, whereas isolates of VCGs 2A, 2B, or 4B cause the lesser virulent non-defoliating syndrome in these two symptomatic hosts (22,23). Those differences in virulence on cotton and olive between defoliating and non-defoliating isolates have been shown consistently (4,5,23,32,37).

Previous work utilizing a suite of molecular tools suggests that VCGs in *V. dahliae* may not represent distinct clonal lineages and may be more diverse than previously expected (12,13,24). For instance, studies using amplified polymorphic fragment length polymorphic (AFLP) markers revealed that VCG subgroups are not necessarily closely related (12,13). This finding was supported by analyses of mitochondrial sequences (35). Similarly, studies on Verticillium wilt in artichoke crops at eastern-central Spain indicated that *V. dahliae* isolated from affected plants and typed as VCG 2B were genetically diverse since some Isolates complemented with international reference VCG 2B tester isolates but others did not complement with these latter testers but did with local VCG 2B isolates (24). PCR-based markers previously associated with cotton- and

olive-defoliating or non-defoliating *V. dahliae* pathotypes, as well as AFLP analysis also revealed this genetic heterogeneity (14,24,37).

The objective of this study was to analyze phylogenetic relationships between VCGs in *V. dahliae* to better understand the population structure in this important pathogen. This has been attempted before using AFLPs (12,13), conserved, house-keeping genes (12) and mitochondrial DNA regions (35). However, results from those studies may be undermined by AFLP markers being potentially subjected to significant homoplasy (17), and by the little resolution within VCGs provided by other DNA regions tested (12,35). Also, not all known VCGs were included in previous studies (12,35). A region that has shown promising in *V. dahliae* is the nuclear ribosomal DNA gene cluster intergenic spacer (IGS). Qin et al. (46) showed significant variability in IGS sequences in *V. dahliae* populations from numerous symptomatic hosts, but VCG information was not included in the analyses. Other sequence-based regions of potential use for studying VCG phylogenetic relationships are those containing single nucleotide polymorphisms (SNPs) (15,54). In the present study we analyzed evolutionary relationships between VCGs using IGS sequences and six previously characterized anonymous polymorphic regions containing SNPs (7). We hypothesized that clonality within VCGs of *V. dahliae* will be supported by topological concordance between the different regions analyzed; that is, if VCGs comprise clonal lineages, the topologies of phylogenetic trees inferred from the different datasets (IGS and the six polymorphic regions) should be congruent in the absence of recombination.

MATERIALS AND METHODS

Fungal isolates, culture conditions, and DNA extraction. A collection of 68 isolates of *V. dahliae* and one of *V. albo-atrum* originally obtained from diverse hosts, geographic origins, and VCG groups were used in this study (Table 1). The collection included isolates from China, Greece, Israel, Italy, Japan, Spain, Swaziland, Syria, UK, and the USA. The host of origin of the isolates was also diverse, including artichoke, chickpea, cotton, eggplant, elm, green ash, muskmelon, olive, pepper, pistachio, potato, pumpkin, strawberry, tomato, watermelon, and yellowwood (Table 1). All *V. dahliae* isolates had been previously typed to VCG in earlier studies or by the supplier (Table 1). The collection studied includes all known VGCs and subgroups described, except for VCG 5. VCG 5 was only described once for a single isolate from Catalpa in Illinois (USA) (26,45,52), which is no longer available. Isolates are long-term stored in sterile soil at 4^{o}C in the dark, in the culture collection of M. M. Jiménez-Gasco, Department of Plant Pathology and Environmental Microbiology, The Pennsylvania State University, PA, USA. Active cultures were obtained by plating colonized soil onto potato dextrose agar (PDA, Difco Laboratories, Detroit, MI), followed by incubating at 26^{o}C in the dark. Efforts were made to assure purity of isolates' cultures. Mycelium was generated from 2- to 4-week-old PDA cultures by gently scraping it off the media. This mycelium was used directly for DNA extraction using the Mo Bio Ultraclean Microbial DNA Isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA) following the manufacturer's protocol, with the addition

of 10 minutes at 65°C after step 4 to increase DNA yield through improved cell lysis. DNA solutions were stored at -20°C until used.

Amplification and sequencing of IGS and VdSNP regions. All 69 *Verticillium* isolates were subjected to phylogenetic analyses using IGS sequences and six polymorphic regions containing SNPs (VdSNP sequences) (7). Conditions used for DNA amplification of the complete IGS region were as described by Qin et al. (46) using primers VdIGSF1 and VdIGSR1 (46). For amplification of the IGS region, the reaction mix (25 μl) consisted of 20 ng of DNA, 0.5 μM each primer, 0.2 mM dNTP, 2.5 μl 10x reaction buffer, and 0.65 U DNA polymerase (Denville Scientific, Metuchen, NJ). All PCR reactions were performed in a Mastercycle©ep Thermocycler (Eppendorf North America, Hauppauge, NY), with an initial denaturation step at 95°C for 5 min followed by 35 cycles of 1 min at 95°C, 50 sec at 65°C, and 1 min at 72°C, and a final step of 10 min at 72°C. All polymorphic regions containing SNPs (VdSNP1, 2, 3, 4, 5, and 7) were amplified using primers and protocols described in Berbegal et al. (7). Amplification products were visualized in agarose gels prior to sequencing. PCR products were cleaned using ExoSap (Affimetrix USB, Cleveland, OH), and sequenced at the Genomics Core Facility, The Pennsylvania State University, University Park, PA, USA. Sequencing of the complete IGS region was achieved by using primers VdIGSF1 and VdIGSR1 (46), as well as primers VdIGSInt1F (5'-3', CTTTCGGCTGCAGCGGCGTGCC), VdIGSInt1R (5'-3', GGCACGCCGCTGCAGCCGAAAG), and VdIGSInt2R (5'-3', AATTCCCGGGTAGCTTTCCACC), which were designed in this study based on sequences internal to the IGS region. Both

strands were sequenced for all VdSNP regions using the same primers used for PCR amplifications and consensus sequences for IGS and VdSNP regions were generated using Sequencher v.4.7 (Gene Codes, Ann Arbor, MI).

DNA sequence analysis. For each region, sequences were aligned using Sequencher v.4.7 followed by careful and extensive manual alignment, especially for the IGS region. Manual alignment was advisable because the IGS region contains numerous large indels as described by Pramateftaki et al. (43). Congruence between all datasets was estimated with the incongruence length difference (ILD) test implemented as the partition homogeneity test in PAUP v.4.0.b10 (53), with 1,000 replicate partitions subjected to heuristic parsimony searches.- Phylogenetic analyses were conducted on each dataset individually, all VdSNP sequences combined, and on the combined dataset. Maximum parsimony (MP) analyses were conducted using the parsimony ratchet (39) in PAUPRat (50) as implemented in PAUP v.4.0.b10 with the following settings: set seed = 0, nreps = 200, pct = 15, set wtmode = uniform, with simple sequence addition and heuristic searches. Maximum likelihood (ML) analyses were done in PAUP using the HKY85 model and the heuristic search option with 1,000 random addition sequences and the subtree-punning-regrafting (SPR) algorithm. An isolate of *V. albo-atrum* was used as outgroup. MP and ML bootstrapping were conducted using 1,000 replicates. The Shimodaira-Hasewaga test (49) was used to evaluate the alternative phylogenetic hypothesis of monophyly for VCG 2B under ML. This test was implemented in PAUP with 1,000 replicates.

RESULTS

Amplification and sequencing of IGS and VdSNP regions. Amplification of the complete IGS region of the nuclear rDNA resulted in single amplicons ranging 1,700 to 2,200 bp in size, depending on the isolate. After editing sequences and alignments the IGS region ranged from 1,743 to 2,328 bp for all *Verticillium* isolates tested, including gaps and indels. The length of the VdSNP sequences did not vary among isolates and coincided with the values described in Berbegal et al. (7). After editing of sequences and completion of alignments the length for each polymorphic sequence dataset including gaps was as follows: VdSNP1, 244 bp; VdSNP2, 411 bp; VdSNP3, 262 bp; VdSNP4, 298 bp; VdSNP5, 417 bp; and VdSNP, 209 bp. All sequences generated in this study were submitted to NCBI GenBank and have been deposited under accession numbers KF295831-KF296313.

Phylogenetic analyses. Partition homogeneity tests between all pair wise combinations of datasets were conducted to assess concordance between the different regions (Supplemental Table 1). ILD tests showed that certain datasets were congruent, but incongruence was statistically significantly for the following dataset combinations: VdSNP1/VdSNP2, VdSNP1/VdSNP5, VdSNP1/VdSNP7, IGS/VdSNP1, IGS/VdSNP5, IGS/VdSNP7, VdSNP5/VdSNP7 ($P < 0.05$). Phylogenetic analyses were done on: a) the IGS dataset alone; b) each of the VdSNP polymorphic regions individually, c) all the VdSNP regions combined, and d) all regions (IGS and VdSNP) combined. For all datasets both

MP and ML analyses were performed; however, since trees generated with both types of analyses were nearly identical (data not shown), only MP phylogenetic trees are shown (Figs. 1 and 2). MP analysis of the IGS region yielded five distinct clades (clades 1-5, Figure 1A). Clade 1 included exclusively all isolates of VCG 4A (74% bootstrap support). Clade 2, grouped seven of the 13 VCG 2B isolates included in the analysis (isolates were named 2B-I in Fig. 1A) (61% bootstrap support). Clade 3 comprised all isolates typed as VCG 1 (including VCG 1A and VCG 1B), as well as three VCG 2B isolates from artichoke in Spain, named 2B-II in Fig. 1A (85% bootstrap support). Clade 4 was the most complex one including all isolates typed as VCG 2A and VCG 4B, the three isolates of VCG 3, and one isolate of VCG 2B (isolate CS1) (79% bootstrap support). Finally, clade 5 comprised isolates of VCG 6 originating from pepper in California, and three VCG 2B isolates from artichoke in Spain, named 2B-III in Fig. 1A (69% bootstrap support). There were two isolates included in the analyses that failed to self-anastomose and were typed as heterokayon self-incompatible (HSI). Those two isolates were placed in different clades in the IGS MP tree, one (V547) was placed within clade 3, closely related to VCGs 1A and 1B, and the second one (PU) was placed within clade 5, together with VCG 6 and VCG 2B-III isolates (Fig. 1A).

Results of analyses of individual VdSNP polymorphic sequences are shown in Supplemental Figure 1. Since for individual regions clade resolution was poor due to the limited number of polymorphic sites (2-10 parsimony-informative characters), all VdSNP regions were combined into one dataset, even though some regions were incongruent. A MP tree of all six VdSNP polymorphic sequences combined is shown in Figure 1B and

yielded six main clades, namely I through VI. Clade I included all isolates of VCG 4A (63% bootstrap support) and it was closely related to clade II that comprised the two isolates of VCG 6 analyzed (75% bootstrap support). Clade III was closely related to clades I and II and included eight of the 13 isolates of VCG 2B and the HSI isolate PU (bootstrap support of 85%). Clade IV comprised all isolates of VCGs 2A and 4B (70% bootstrap). Clade V contained all isolates of VCGs 1A, 1B, and 3 (68% bootstrap). A last clade, VI, included three isolates of VCG 2B and the HSI isolate V547 (72% bootstrap). Two isolates from VCG 2B, isolates 115 and V652 remained unresolved (Fig. 1B).

With the exception of VCG 2B, isolates within a VCG subgroup were closely related to each other. For most VCGs (i.e., VCGs 4A, 2A, 4B, 1A, and 1B), the IGS and VdSNP phylogenies were congruent. For these phylogenies, isolates within a VCG subgroup were indeed closely related to each other sharing a common ancestor. For example, all isolates of VCG 4A were placed in the same clade for both phylogenies (clades 1 and I for IGS and VdSNP, respectively) (Fig. 1). Regarding relationships between subgroups of a VCG, results showed that VCGs 1A and 1B are closely related and isolates from both subgroups are indistinguishable based on either phylogeny. However, this was not the case for other VCG subgroups. VCGs 4A and 4B were not phylogenetically related, and isolates of VCG 4B were indistinguishable phylogenetically from isolates of VCG 2A. For other groups (i.e., VCGs 3, 6 and 2B) the IGS and VdSNP phylogenies were not concordant and these isolates were placed in different clades. VCG 3 isolates were nested within clade 4 based on IGS, and indistinguishable from isolates of VCGs 2A and 4B (Fig. 1A). However, isolates of VCG 3 were grouped together with isolates of VCG 1A

and 1B in clade V based on VdSNP sequences (Fig. 1B). A similar situation occurred for VCG 6, which grouped with VCG 2B-III isolates in IGS clade 5 (Fig. 1A), but they were placed in clade II, closely related to VCG 4A (clade I) based on VdSNP sequences (Fig. 1B). IGS and VdSNP phylogenies showed the most complex relationships for isolates of VCG 2B. These were divided into three groups, named 2B-I, 2B-II, and 2B-III in the IGS phylogeny (clades 2, 3 and 5), and into two groups in the VdSNP one (in clades III and VI) (Figs. 1 and 3). Isolates of VCG 2B-I, placed in clade 2 based on IGS, were included in VdSNP clade III together with isolates of VCG 2B-III (IGS clade 5). All isolates of 2B-II were indistinguishable from VCG 1A and 1B isolates and placed in IGS clade 3. However, 2B-II isolates were grouped in VdSNP clade VI, distinct from VCGs 1A and 1B (Fig. 1). Also, isolate 2B-I V652 was closely related to VdSNP clade VI. A comparison of both IGS and VdSNP phylogenies is shown in Figure 3, indicating the lack of congruence in the placement of VCGs 2B, 3 and 6 in both phylogenies.

Finally, a MP tree was inferred from the combined dataset including IGS and the six VdSNP polymorphic sequences (Fig. 2), although IGS was not congruent with the combined VdSNP dataset ($P < 0.001$). Overall, the MP tree inferred from the combined data (Fig. 2) was very similar to the IGS tree (Fig. 1A), which was not unexpected since the IGS region contributed 94 parsimony-informative characters to the analysis, whereas the combined VdSNP dataset only contributed 30 parsimony-informative characters of a total of 123. Three main well-supported clades were resolved in the combined phylogenetic tree: one comprised all VCG 4A isolates (97% bootstrap support), a second included all isolates of VCGs 1A, 1B, and some isolates of VCG 2B

(termed earlier VCG 2B-II isolates) (93% bootstrap support), and a third clade included isolates of VCGs 2A, 2B-III, 3, 4B and 6 (79% bootstrap support). The rest of VCG 2B isolates (called earlier VCG 2B-I) remained unresolved in a poorly-supported clade closely related to VCG 4A. One important observation is that bootstrap values were higher in the combined analysis (Fig. 2) compared to the IGS tree (Fig. 1A) or the combined VdSNP tree (Fig. 1B), but only for those clades with a concordant phylogenetic placement in both individual phylogenies (i.e., VCG 4A or VCGs 1A and 1B). However, bootstrap values dropped for the analysis of combined datasets in the rest of the clades (Figs. 1 and 2). As indicated by the IGS and VdSNP trees, isolates of VCG 2B were present in different well-supported clades of the combined dataset indicating polyphyly of this group. The alternative hypothesis of monophyly for VCG 2B was tested using the Shimodaira-Hasewaga test by comparing a tree in which isolates of VCG 2B were constrained to be monophyletic, to the unconstrained tree. The analysis showed that the constrained tree was significantly less likely (ln L = -8821.399) than the unconstrained tree (ln L = -9339.372) (P = 0.000), strongly supporting polyphyly for VCG 2B.

DISCUSSION

For the last two decades, VCG analysis has been used to estimate genetic variation in *V. dahliae* populations (6,9,16,18,24,26,27,28,30,51), under the assumption that isolates within a given VCG comprise genetically related isolates that descend from a common

ancestor; that is, VCGs align with clonal lineages. This has been shown to be true for many fungi (3,25,36,47). However, results from this and other studies indicate that for some VCGs in *V. dahliae* this assumption is not necessarily true, and a VCG may comprise a genetically heterogeneous group of isolates that are phylogenetically distant, as shown herein for VCG 2B based on IGS and VdSNP analyses.

Another clear indication from the present study is that, with the exception of VCGs 1A and 1B that are closely related, all other subgroups for a given VCG are not phylogenetically related, do not share a common ancestor, and are indeed closer to subgroups of other VCGs than to each other. For example, VCG 4A isolates are distinct from VCG 4B isolates, and VCG 4B isolates are phylogenetically indistinguishable from VCG 2A isolates, regardless of the genomic region analyzed (Figs. 1 and 2). This is consistent with analyses based on mitochondrial sequences (34), but not with results from AFLP analyses that clearly distinguished between VCG 2A and VCG 4B (12). Isolates of VCG 2A and 4B have been shown to establish weak complementation and some of these isolates have been said to be “bridge isolates” (6,28). Whether this is an artifact of the *nit*-mutant VCG assay itself (6), or some “transitory” heterokaryosis (27) is unknown, and more research targeting *het*/*vic* genes and mechanisms of incompatibility in *V. dahliae* is needed.

We had hypothesized that clonality within VCGs of *V. dahliae* would be supported by topological concordance between the different regions analyzed. Although for several VCGs the phylogenies for IGS and VdSNP were congruent, this was not the case for all groups since there was significant disagreement in their phylogenetic placement. For

example, VCGs 4A, 2A, 4B, 1A, and 1B were grouped similarly based on IGS and VdSNP sequences (Fig. 2). However, VCG 3 isolates were nested within the clade containing isolates of VCGs 2A and 4B based on IGS (Fig. 1A, clade 4), whereas VCG 3 was closely related to VCG 1A and 1B according to the VdSNP phylogeny (Fig. 1B, clade V). Similarly, VCG 6 isolates grouped with some VCG 2B isolates in IGS clade 5, but they were closely related to VCG 4A according to VdSNP sequences (Fig. 1B). The most striking differences in both phylogenies involved VCG 2B isolates. While isolates of VCG 2B were placed in three different clades based on IGS, there were only two VdSNP clades containing VCG 2B isolates. Isolates 2B-I were placed in IGS clade 2, and within clade III in the VdSNP tree. This VdSNP clade also contained 2B-III isolates, placed in a separate distant clade (clade 5) in the IGS phylogeny. All 2B-II isolates were in clade VI (Fig. 2). Isolates named 2-II in this study contained isolate V574, characterized as VCG 2Ba by Jiménez-Díaz et al. (24). VCG 2Ba isolates infected artichoke in eastern-central Spain and were unable to complement with international VCG 2B testers but produced positive complementation with local VCG 2B testers. Those VCG 2Ba isolates were thus genetically different from other VCG 2B isolates [called VCG 2Br in Jiménez-Díaz et al. (24)] that did complement with international reference VCG 2B testers (24,30). There was also diversity in PCR-based amplicons of 334 and 824 bp, markers previously associated with cotton and olive defoliating and non-defoliating *V. dahliae* pathotypes, respectively (14,24,37). Some VCG 2B isolates from artichoke amplified the defoliating-associated marker of 334bp, whereas others amplified the non-defoliating marker of 824 bp (7,24). All 2B-II isolates, including the HSI isolate V547, grouped in IGS clade 3 and VdSNP clade VI, amplified the

334-bp marker, which is consistent with these isolates being closely related to cotton and olive defoliating VCG 1A isolates. All other VCG 2B isolates analyzed amplified the 824-bp marker (7,12,24). An earlier study that included some of the VCG 2B isolates that amplified the 824-bp marker showed identical haplotypes for five microsatellites (7), which was different from the haplotypes displayed by VCG 2B isolates that amplified the 334-bp marker. The discrepancies between phylogenies generated by IGS and VdSNP sequences could also be explained by the fast-evolving nature of the IGS region. Similar incongruencies between IGS and other regions have been reported for the *Fusarium oxysporum* complex (19,40).

Overall, comparisons of results from the present study with those from AFLP analysis (12) indicate that some of the groups generated with AFLP phylogenies coincide with the VCG relationships determined by the IGS phylogeny, but some others are closer to the clades displayed by the VdSNP phlylogeny. Since AFLP markers are widespread throughout the whole genome, it would be logical to expect that the AFLP tree would look like a combination of the phylogenies inferred from IGS and VdSNP polymorphic regions in the present study. Although Collado-Romero et al. (12) included a sequence analysis of various other regions (housekeeping genes actin, β-tubulin, calmodulin, histone 3, as well as the ITS2 region of the rDNA), these regions resolved very little diversity and most of the phylogenetic signal in the analysis combining AFLPs and other regions was contributed by the AFLP markers. Thus, in the AFLP study (12) there were two main evolutionary groups identified: lineage I that corresponds to IGS clade 3 and a second lineage II that includes the rest of IGS clades identified in the present research.

In the AFLP dendrogram, VCG 6 isolates grouped with the only isolate of VCG 4A included in that study, which coincides with the close placement of these two VCGs using VdSNP sequences (Fig. 1B).

Our results indicate that VCG 2B is clearly polyphyletic, which was also noted by Collado-Romero et al. (12) using AFLP markers, although only two VCG 2B groups were identified by that study. Dung et al. (18) also identified two genetically distinct groups within VCG 2B isolates using microsatellite analysis: one group contained isolates from tomato and pistachio, and the other comprised isolates from potato, watermelon and mint. There are several possible explanations for polyphyly in VCGs. One is that convergent mutations in *het*/*vic* loci may have resulted in the same functional alleles that allow anastomosis and the establishment of stable heterokaryons between isolates of otherwise distinct genetic background. A second potential scenario is the exchange of genetic material between different VCGs due to parasexuality, especially during transitory heterokaryosis between different VCGs as it has been shown to be possible experimentally in *Verticillium* (56) and other fungi (33). In the latter case, the resulting genotypes would share genomic regions with both parental isolates. Finally, these isolates of conflicting phylogenetic placement may represent remnants of ancient, or current but rare, recombination events. *V. dahliae* populations have been shown to harbor *MAT1-1* and *MAT1-2* idiomorphs similar to the genetic mating system of other Ascomycota (2,57). Also, a departure from clonality shown by microsatellite analysis of *V. dahliae* populations was attributed to potential sexual recombination (2); although a major predominance of *MAT1-2* was found in populations of *V. dahliae,* with only one

isolate carrying the *MAT1-1* idiomorph in 286 isolates tested (18). In any case and regardless of the evolutionary mechanism that resulted in polyphyly of VCGs, results from this and other studies advice for an important caveat regarding the interpretation of VCG analyses: genetic homogeneity or close evolutionary relationships between members of a VCG should not be assumed.

In our study, we included some of Puhalla's original isolates (44,45) that were used to perform early VCG testing (6,27,45,51,52). One of those isolates, isolate PCW, was first typed as VCG 16 by Puhalla and Hummel (45), it was later reclassified as VCG 3 by Joaquim and Rowe (26), and finally was identified as VCG 4 by Bell (6) and Strausbaugh et al. (52). Interestingly, in our study this PCW isolate as well as others typed as VCG 3 *sensu* Joaquim and Rowe (26) were placed with VCGs 2A and 4B within IGS clade 4, and together with VCG 1A and 1B in clade V, based on VdSNP sequences. VCG 3 was reported as being an interspecific hybrid between *V. dahliae* VCG1B and an unidentified non-*Verticillium* parent (11). This was due to the presence of two different sequences for actin, calmodulin and histone 3 genes in the VCG tester isolate 70.21; one sequence that matched other *V. dahliae* sequences and another sequence that matched an unidentified non-*Verticillium* member of the Ascomycota (11). Isolate 70.21 was also included in our analyses together with two other VCG 3 isolates. However, in contrast with Collado-Romero et al. (11), in our study all sequences associated with the three VCG 3 isolates were clean and there was no indication of multiple sequences contained in one amplicon. Based on our analysis there is no indication of a hybrid origin for VCG 3.

Our study suggests the existence of main VCG clonal lineages that contain the isolates of VCGs 1A and 1B; VCGs 2A and 4B; and VCG 4A; and the existence of other VCG or VCG subgroups that have been found scarcely and seem to be genetically different: VCG 2B (2B-I, 2B-II and 2B-III), VCG 3 and VCG 6. This is consistent with a population structure consisting of some recombination events (maybe extant or rare) with a clonal expansion of certain successful individuals, possibly associated with agriculture. It is possible that these latter groups have emerged through the evolutionary mechanisms described above, but the one question arising is: where are these variants originating from? We believe that there are aspects of *V. dahliae* biology that have not yet been explored appropriately, and it is possible that there is a more complex diversity in *V. dahliae* that has not been targeted. For example, the diversity of soil *V. dahliae* populations has not been properly studied, and the fungus has been found in endophytic associations with asymptomatic hosts (34). Bell (6) suggested that the early VCG work done by Puhalla (44) using melanin-deficient mutants probably overestimated VCG diversity since the following studies based on *nit* mutant assays lumped the 16 early VCGs into four VCGs (26,27,51,52). However, the increasing use of molecular tools is resolving more finely the diversity in *V. dahliae* populations and identifying complex associations between VCGs. Although we are closer to understanding these relationships, and the impact they have in *V. dahliae* biology, there is a clear need for more powerful molecular markers used in an ecologically broad context to resolve the origin and evolution of diversity in *V. dahliae* and its impact on the biology of this important fungus.

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Table 1. Isolates used in this study with reference number(s), geographic origin, host source, vegetative compatibility group (VCG) and referenced study were the VCG testing was reported.

Isolate[a]	Geographic origin	Host/substrate of origin	VCG[b]	Reference[e]
Verticillium dahliae				
DX2	USA (AZ)	Cotton (*Gossypium hirsutum*)	1	
V-EMS	USA (OH)	Elm (*Ulmus* sp.)	1	
V403 (V-017)	Spain	Artichoke (*Cynara cardunculus* var. *scolymus*)	1A	14
V138	Spain	Cotton (*G. hirsutum*)	1A	30
V610	Spain	Cotton (*G. hirsutum*)	1A	13
T9*	USA (CA)	Cotton (*G. hirsutum*)	1A	26
V44*	USA (TX)	Cotton (*G. hirsutum*)	1A	26
V661	Greece	Cotton (*G. hirsutum*)	1B	13
V666	Greece	Cotton (*G. hirsutum*)	1B	13
V517 (9-6)	USA (IL)	Yellowwood (*Cladrastis kentukea*)	1B	14
V607 (R04)	USA (MN)	Green Ash (*Fraxinus pennsylvanica*)	1B	
V488 (pt71)	Israel	Potato (*Solanum tuberosum*)	2A	14
V720 (V39)	Italy	Olive (*Olea europaea*)	2A	13
492	Japan	Tomato (*Solanum lycopersicum*)	2A	
V542	Spain	Artichoke (*C. cardunculus* var. *scolymus*)	2A	24
V185	Spain	Cotton (*G. hirsutum*)	2A	30
V20	Spain	Muskmelon (*Cucumis melo*)	2A	8
A11	Spain	Potato (*S. tuberosum*)	2A	7

A2	Spain	Potato (*S. tuberosum*)	2A	7
A38	Spain	Potato (*S. tuberosum*)	2A	7
V161	Spain	Potato (*S. tuberosum*)	2A	12
V10	Spain	Pumpkin *Cucurbita pepo*	2A	8
V25	Spain	Tomato (*S. lycopersicum*)	2A	8
V2	Spain	Watermelon (*Citrullus lanatus*)	2A	8
CS1*	Swaziland	Cotton (*G. hirsutum*)	2B	26
SS4	USA (CA)	Cotton (*G. hirsutum*)	2A	26
PH*	USA (CA)	Pistachio (*Pistacia vera*)	2A	26
442	USA (OH)	Tomato (*S. lycopersicum*)	2A	
443	USA (OH)	Tomato (*S. lycopersicum*)	2A	
V357 (JY)	China	Cotton (*G. hirsutum*)	2B	13
V652	Greece	Cotton (*G. hirsutum*)	2B	13
V285 (cot 72)	Israel	Cotton (*G. hirsutum*)	2B	30
V258 (CECE)	Italy	Chickpea (*Cicer arietinum*)	2B	12
V534	Spain	Artichoke (*C. cardunculus* var. *scolymus*)	2B	24
V549	Spain	Artichoke (*C. cardunculus* var. *scolymus*)	2B	24
V574	Spain	Artichoke (*C. cardunculus* var. *scolymus*)	2B[c]	24
V593	Spain	Artichoke (*C. cardunculus* var. *scolymus*)	2B	24
V613	Spain	Artichoke (*C. cardunculus* var. *scolymus*)	2B	24
115*	Syria	Cotton (*G. hirsutum*)	2B	26
V552 (332)	UK	Strawberry (*Fragaria × ananassa*)	2B	12
P19	USA (OH)	Potato (*S. tuberosum*)	2B	26
S92	USA (OH)	Potato Soil	2B	26

70.21	USA (AZ)	Bell Pepper (*Capsicum annuum*)	3	26
PCW*	USA (CA)	Pepper (*Capsicum annuum*)	3	26
VMD9	USA (OR)	Peppermint (*Mentha × piperita*)	3	
V25R	USA (ID)	Potato (*S. tuberosum*)	4A	
V27	USA (ID)	Potato (*S. tuberosum*)	4A	
318	USA (ND)	Potato (*S. tuberosum*)	4A	27
319	USA (ND)	Potato (*S. tuberosum*)	4A	27
320	USA (ND)	Potato (*S. tuberosum*)	4A	27
321d	USA (ND)	Potato (*S. tuberosum*)	4A	
VA102	USA (ND)	Potato (*S. tuberosum*)	4A	
VA49	USA (ND)	Potato (*S. tuberosum*)	4A	
V304 (cot 120)	Israel	Cotton (*G. hirsutum*)	4B	30
V683	Spain	Artichoke (*C. cardunculus* var. *scolymus*)	4B	24
V684	Spain	Artichoke (*C. cardunculus* var. *scolymus*)	4B	24
V158	Spain	Eggplant (*Solanum melongena*)	4B	8
V789	Spain	Olive (*O. europaea*)	4B	13
A54	Spain	Potato (*S. tuberosum*)	4B	7
V61	Spain	Potato (*S. tuberosum*)	4B	7
V551 (330)	UK	Strawberry (*F. ananassa*)	4B	12
V553 (1875)	UK	Strawberry (*F.ananassa*)	4B	12
S39	USA (OH)	Potato soil	4B	26
WS4	USA (WI)	Potato (*S. tuberosum*)	4B	
V560 (VdCa.83a)	USA (CA)	Pepper (*Capsicum annuum*)	6	9
V561 (VdCa147a)	USA (CA)	Pepper (*Capsicum annuum*)	6	9

V547	Spain	Artichoke (*C. cardunculus* var. *scolymus*)	HSI	24
PU*	UK	Potato (*S. tuberosum*)	HSI	26
V. albo-atrum				
462	USA (MN)	Potato (*S. tuberosum*)	n/a[d]	

[a] Isolate reference. In parenthesis, codes given by supplier or by previous studies. An asterisk indicates that this isolate was used in the original VCG classification by Puhalla and Hummel (45). Isolates from Greece were provided by E. Paplomatas, Agricultural University of Athens, Athens, Greece; isolates from Israel and the USA (CA) were provided by T. Katan, The Volcani Center, Bet Dagan, Israel; isolates from UK, California, and Brazil provided by D. Barbara, Warwick HRI, England; isolates from the USA and Japan were provided by R. Rowe, OARDC, The Ohio State University, Wooster, Ohio; isolates from Italy provided by F. Nigro, Università degli Studi di Bari, Bari, Italy, or A. Zazzerini, Università degli Studi di Perugia, Perugia, Italy.

[b] Vegetative compatibility group was determined in the study referenced in the next column or by the supplier of the isolate. HSI, heterokaryon self-incompatible.

[c] VCG 2Ba, artichoke isolate able to complement with other artichoke isolates assigned to VCG2B but not with international VCG 2B testers.

[d] n/a, not applicable.

[e] References of studies where isolates were assigned to VCG. For isolates with no reference, VCG typing was performed by the supplier.

FIGURE LEGENDS

Figure 1. Phylogenetic relationships between *Verticillium dahliae* vegetative compatibility groups (VCG)**. A,** One of 200 most parsimonious phylogenetic trees inferred from sequences of the intergenic spacer region of the rDNA (IGS) (93 parsimony-informative characters; 327 steps; consistency index, CI = 0.930; retention index, RI = 0.966; rescaled consistency index, RC = 0.898). **B,** One of 200 most parsimonious phylogenetic trees inferred from the combined VdSNP polymorphic sequences (30 parsimony-informative characters; 163 steps; CI = 0.791; RI = 0.901; RC = 0.713). Numbers above branches are bootstrap values (>50%) based on 1,000 replicates. Each taxon label indicates isolate reference, VCG, geographical origin, and host source. Trees are rooted with *V. albo-atrum* 462. VCG 2B isolates are further subdivided into VCG 2B-I, VCG 2B-II, and VCG 2B-III according to their phylogenetic placement in the IGS tree (A).

Figure 2. Phylogenetic relationships between *Verticillium dahliae* vegetative compatibility groups (VCG). One of 200 most parsimonious phylogenetic trees inferred from the combined dataset of the intergenic spacer region of the rDNA (IGS) and six polymorphic sequences (VdSNP) (123 parsimony-informative characters, 504 steps, consistency index, CI = 0.859; retention index, RI = 0.931; rescaled consistency index, RC = 0.8). Numbers above branches are bootstrap values (>50%) based on 1,000 replicates. Each taxon label indicates isolate reference, VCG, geographical origin, and host source. Trees are rooted with *V. albo-atrum* 462. Arrows indicate VCG 2B isolates.

Figure 3. Comparative analysis between maximum parsimony phylogenetic trees derived from intergenic spacer region of the rDNA (IGS) sequences (**A**), and six anonymous polymorphic sequences VdSNP combined (**B**). Lines indicate discrepancies between the phylogenetic placement of vegetative compatibility groups (VCG) in both inferred phylogenies. Dotted lines correspond to discrepancies regarding isolates of VCG 2B; the black line corresponds to discrepancies regarding isolates of VCG 3; and the double line indicates discrepancies regarding isolates of VCG 6. VCG 2B isolates are further subdivided into VCG 2B-I, VCG 2B-II, and VCG 2B-III according to their phylogenetic placement in the IGS tree (Figure 1A).

Supplemental Table 1. Analysis of congruence between all datasets estimated with the incongruence length difference (ILD) test implemented as the partition homogeneity test in PAUP v.4.0.b10 with 1,000 replicate partitions subjected to heuristic parsimony searches.

	IGS	VdSNP1	VdSNP2	VdSNP3	VdSNP4	VdSNP5	VdSNP7
IGS	—	0.01*	0.08	0.055	0.607	0.001*	0.001*
VdSNP1	—	—	0.08	0.02*	0.14	0.01*	0.01*
VdSNP2	—	—	—	0.32	0.871	0.552	0.239
VdSNP3	—	—	—	—	1.00	0.769	0.136
VdSNP4	—	—	—	—	—	0.258	0.230
VdSNP5	—	—	—	—	—	—	0.001*
VdSNP7	—	—	—	—	—	—	—

* indicates statistically significant incongruence between datasets at $P < 0.05$.

Supplemental Figure 1. Maximum parsimony phylogenies inferred from individual anonymous polymorphic sequences VdSNP. **A**, One of 200 most parsimonious phylogenetic trees inferred from VdSNP1 sequences (7 parsimony-informative characters, 23 steps; consistency index, CI = 0.522; retention index, RI = 0.911; rescaled consistency index, RC = 0.475). **B**, One of 200 most parsimonious phylogenetic trees inferred from VdSNP2 sequences (5 parsimony-informative characters, 32 steps; CI = 0.938; RI = 0.959; RC = 0.899). **C**, One of 200 most parsimonious phylogenetic trees inferred from VdSNP3 sequences (2 parsimony-informative characters, 2 steps; CI = 1; RI = 1; RC = 1). **D**, One of 200 most parsimonious phylogenetic trees inferred from VdSNP4 sequences (2 parsimony-informative characters, 34 steps; CI = 1; RI = 1; RC = 1). **E**, One of 200 most parsimonious phylogenetic trees inferred from VdSNP5 sequences (10 parsimony-informative characters, 18 steps; CI = 0.415; RI = 0.899; RC = 0.410). **F**, One of 200 most parsimonious phylogenetic trees inferred from VdSNP7 sequences (3 parsimony-informative character, 6 steps; CI = 1; RI = 1; RC = 1). Numbers above branches are bootstrap values (>50%) based on 1,000 replicates. Each taxon label indicates isolate reference, VCG, geographical origin, and host source. Trees are rooted with *V. albo-atrum* 462.

Figure 1

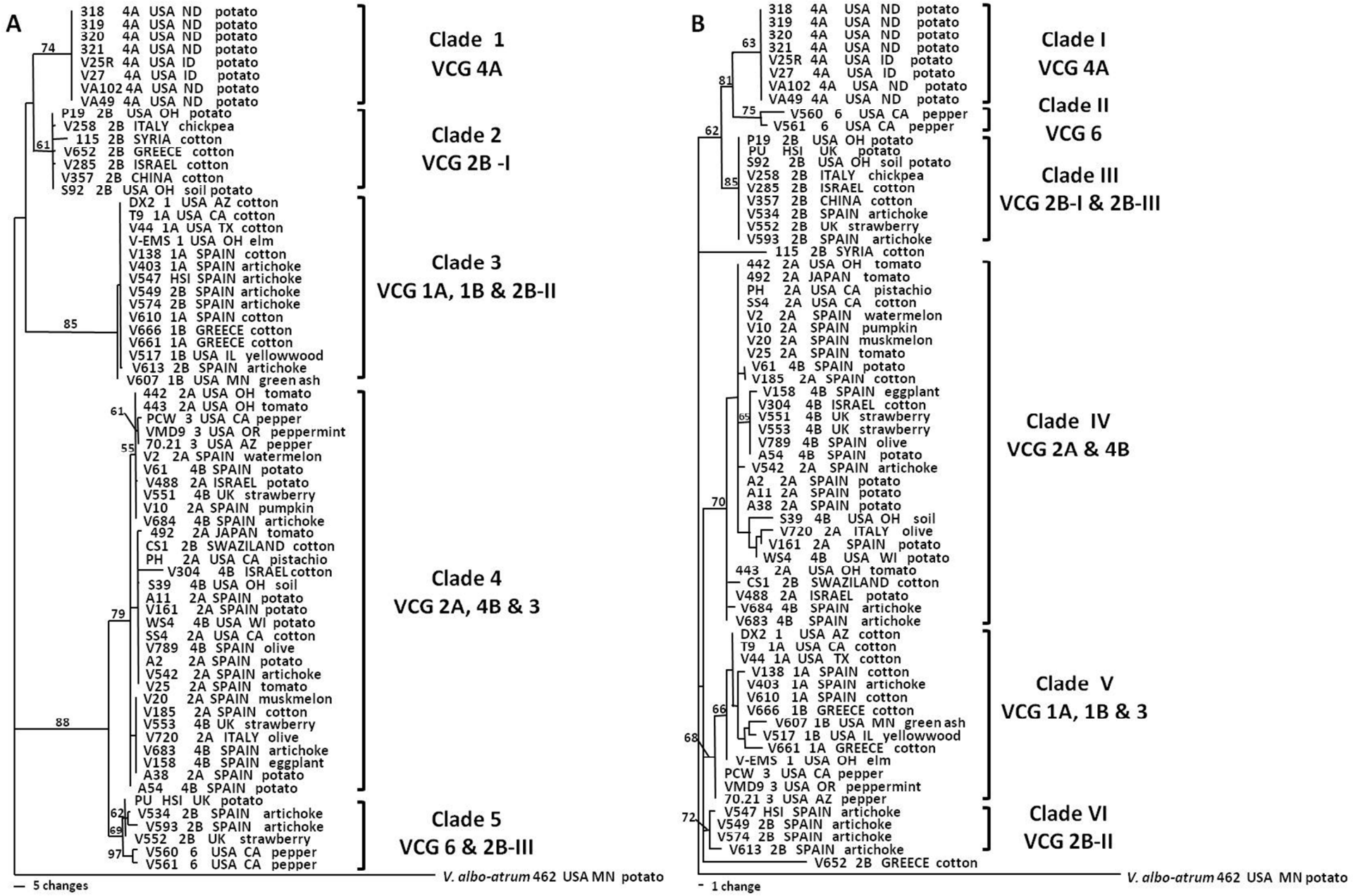


Figure 2

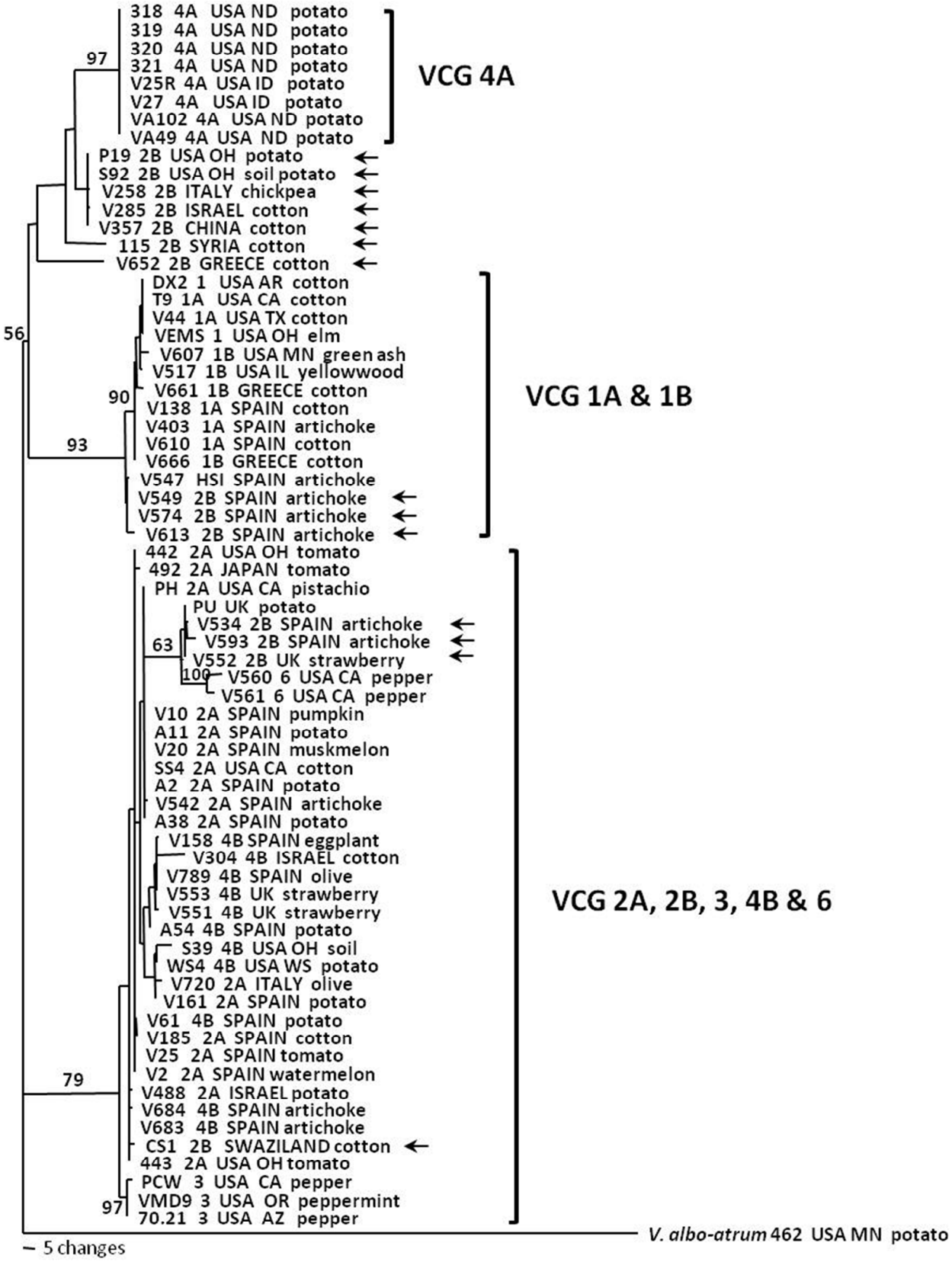
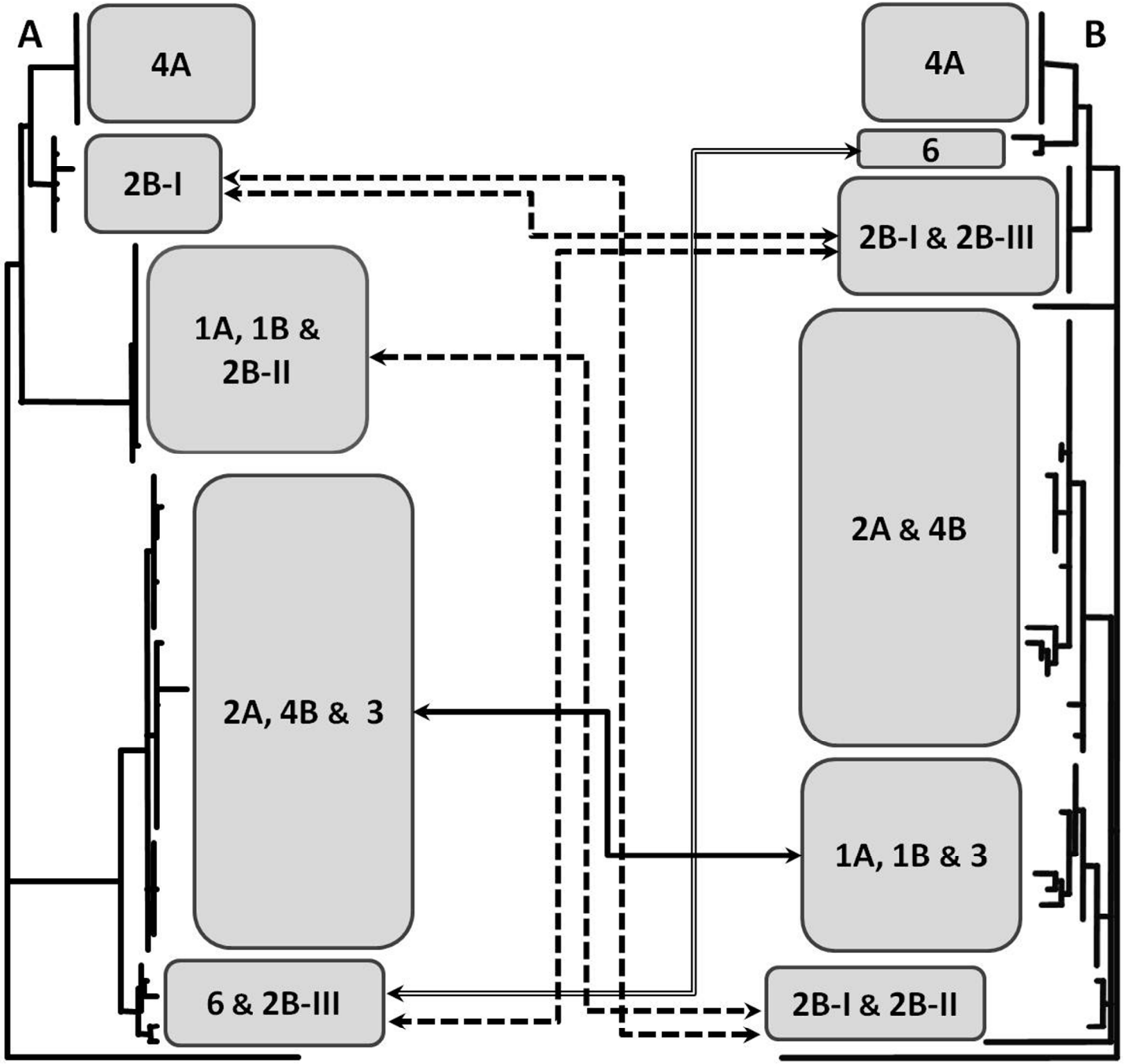


Figure 3



Supplemental Figure 1