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Additional Information

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

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1 **ABSTRACT**

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

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

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

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

1 Phylum Ascomycota, that causes vascular wilt diseases in hundreds of important crops  
2 worldwide (10,23,29,42,48). Puhalla first identified VCGs in *V. dahliae* using UV-induced  
3 microsclerotial color mutants (44). Based on this assay, four VCGs were initially  
4 identified (44) although this number increased to 16 VCGs when a diverse collection of  
5 86 isolates was analyzed (45). Later, Joaquim and Rowe (26) reclassified 15 of the 16  
6 VCGs identified by Puhalla (44) into four main VCGs: VCG 1, VCG 2, VCG 3, and VCG 4,  
7 using an assay that relies on the use of nitrate-nonutilizing (*nit*) auxotrophic mutants  
8 that arise naturally in medium containing chlorate (26). The use of nitrogen sources and  
9 the wild type growth are reestablished following positive complementation between  
10 different *nit* mutants of paired isolates, and those isolates are assigned to the same  
11 VCG. Since 1990, VCG groups based on complementation assays with *nit* mutants have  
12 been slightly updated. Several VCGs have been further subdivided into subgroups based  
13 on vigor and frequency of complementation (i.e., VCG1A and VCG1B, VCG2A and  
14 VCG2B, VCG4A and VCG4B) (6,24,26,51). More recently, isolates causing disease on  
15 pepper in California were characterized as a new VCG 6 (9).

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

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

13 Previous work utilizing a suite of molecular tools suggests that VCGs in *V. dahliae* may  
14 not represent distinct clonal lineages and may be more diverse than previously expected  
15 (12,13,24). For instance, studies using amplified polymorphic fragment length  
16 polymorphic (AFLP) markers revealed that VCG subgroups are not necessarily closely  
17 related (12,13). This finding was supported by analyses of mitochondrial sequences  
18 (35). Similarly, studies on Verticillium wilt in artichoke crops at eastern-central Spain  
19 indicated that *V. dahliae* isolated from affected plants and typed as VCG 2B were  
20 genetically diverse since some Isolates complemented with international reference VCG  
21 2B tester isolates but others did not complement with these latter testers but did with  
22 local VCG 2B isolates (24). PCR-based markers previously associated with cotton- and

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

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

22

## MATERIALS AND METHODS

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**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 VCGs 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°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°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



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

3

4 **Amplification and sequencing of IGS and VdSNP regions.** All 69 *Verticillium* isolates  
5 were subjected to phylogenetic analyses using IGS sequences and six polymorphic  
6 regions containing SNPs (VdSNP sequences) (7). Conditions used for DNA amplification  
7 of the complete IGS region were as described by Qin et al. (46) using primers VdIGSF1  
8 and VdIGSR1 (46). For amplification of the IGS region, the reaction mix (25 µl) consisted  
9 of 20 ng of DNA, 0.5 µM each primer, 0.2 mM dNTP, 2.5 µl 10x reaction buffer, and 0.65  
10 U DNA polymerase (Denville Scientific, Metuchen, NJ). All PCR reactions were  
11 performed in a Mastercycle® Thermocycler (Eppendorf North America, Hauppauge,  
12 NY), with an initial denaturation step at 95°C for 5 min followed by 35 cycles of 1 min at  
13 95°C, 50 sec at 65°C, and 1 min at 72°C, and a final step of 10 min at 72°C. All  
14 polymorphic regions containing SNPs (VdSNP1, 2, 3, 4, 5, and 7) were amplified using  
15 primers and protocols described in Berbegal et al. (7). Amplification products were  
16 visualized in agarose gels prior to sequencing. PCR products were cleaned using ExoSap  
17 (Affimetrix USB, Cleveland, OH), and sequenced at the Genomics Core Facility, The  
18 Pennsylvania State University, University Park, PA, USA. Sequencing of the complete IGS  
19 region was achieved by using primers VdIGSF1 and VdIGSR1 (46), as well as primers  
20 VdIGSInt1F (5'-3', CTTTCGGCTGCAGCGGCGTGCC), VdIGSInt1R (5'-3',  
21 GGCACGCCGCTGCAGCCGAAAG), and VdIGSInt2R (5'-3', AATCCCGGGTAGCTTCCACC ),  
22 which were designed in this study based on sequences internal to the IGS region. Both

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

4

5 **DNA sequence analysis.** For each region, sequences were aligned using Sequencher  
6 v.4.7 followed by careful and extensive manual alignment, especially for the IGS region.

7 Manual alignment was advisable because the IGS region contains numerous large indels  
8 as described by Pramateftaki *et al.* (43). Congruence between all datasets was

9 estimated with the incongruence length difference (ILD) test implemented as the  
10 partition homogeneity test in PAUP v.4.0.b10 (53), with 1,000 replicate partitions

11 subjected to heuristic parsimony searches.- Phylogenetic analyses were conducted on  
12 each dataset individually, all VdSNP sequences combined, and on the combined dataset.

13 Maximum parsimony (MP) analyses were conducted using the parsimony ratchet (39) in  
14 PAUPRat (50) as implemented in PAUP v.4.0.b10 with the following settings: set seed =

15 0, nreps = 200, pct = 15, set wtmode = uniform, with simple sequence addition and  
16 heuristic searches. Maximum likelihood (ML) analyses were done in PAUP using the

17 HKY85 model and the heuristic search option with 1,000 random addition sequences  
18 and the subtree-punning-regrafting (SPR) algorithm. An isolate of *V. albo-atrum* was

19 used as outgroup. MP and ML bootstrapping were conducted using 1,000 replicates. The  
20 Shimodaira-Hasegawa test (49) was used to evaluate the alternative phylogenetic

21 hypothesis of monophyly for VCG 2B under ML. This test was implemented in PAUP with  
22 1,000 replicates.

## 1 RESULTS

2

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

14

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

1 MP and ML analyses were performed; however, since trees generated with both types  
2 of analyses were nearly identical (data not shown), only MP phylogenetic trees are  
3 shown (Figs. 1 and 2). MP analysis of the IGS region yielded five distinct clades (clades 1-  
4 5, Figure 1A). Clade 1 included exclusively all isolates of VCG 4A (74% bootstrap  
5 support). Clade 2, grouped seven of the 13 VCG 2B isolates included in the analysis  
6 (isolates were named 2B-I in Fig. 1A) (61% bootstrap support). Clade 3 comprised all  
7 isolates typed as VCG 1 (including VCG 1A and VCG 1B), as well as three VCG 2B isolates  
8 from artichoke in Spain, named 2B-II in Fig. 1A (85% bootstrap support). Clade 4 was the  
9 most complex one including all isolates typed as VCG 2A and VCG 4B, the three isolates  
10 of VCG 3, and one isolate of VCG 2B (isolate CS1) (79% bootstrap support). Finally, clade  
11 5 comprised isolates of VCG 6 originating from pepper in California, and three VCG 2B  
12 isolates from artichoke in Spain, named 2B-III in Fig. 1A (69% bootstrap support). There  
13 were two isolates included in the analyses that failed to self-anastomose and were  
14 typed as heterokayon self-incompatible (HSI). Those two isolates were placed in  
15 different clades in the IGS MP tree, one (V547) was placed within clade 3, closely related  
16 to VCGs 1A and 1B, and the second one (PU) was placed within clade 5, together with  
17 VCG 6 and VCG 2B-III isolates (Fig. 1A).

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

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

9 With the exception of VCG 2B, isolates within a VCG subgroup were closely related to  
10 each other. For most VCGs (i.e., VCGs 4A, 2A, 4B, 1A, and 1B), the IGS and VdSNP  
11 phylogenies were congruent. For these phylogenies, isolates within a VCG subgroup  
12 were indeed closely related to each other sharing a common ancestor. For example, all  
13 isolates of VCG 4A were placed in the same clade for both phylogenies (clades 1 and I  
14 for IGS and VdSNP, respectively) (Fig. 1). Regarding relationships between subgroups of  
15 a VCG, results showed that VCGs 1A and 1B are closely related and isolates from both  
16 subgroups are indistinguishable based on either phylogeny. However, this was not the  
17 case for other VCG subgroups. VCGs 4A and 4B were not phylogenetically related, and  
18 isolates of VCG 4B were indistinguishable phylogenetically from isolates of VCG 2A. For  
19 other groups (i.e., VCGs 3, 6 and 2B) the IGS and VdSNP phylogenies were not  
20 concordant and these isolates were placed in different clades. VCG 3 isolates were  
21 nested within clade 4 based on IGS, and indistinguishable from isolates of VCGs 2A and  
22 4B (Fig. 1A). However, isolates of VCG 3 were grouped together with isolates of VCG 1A

1 and 1B in clade V based on VdSNP sequences (Fig. 1B). A similar situation occurred for  
2 VCG 6, which grouped with VCG 2B-III isolates in IGS clade 5 (Fig. 1A), but they were  
3 placed in clade II, closely related to VCG 4A (clade I) based on VdSNP sequences (Fig.  
4 1B). IGS and VdSNP phylogenies showed the most complex relationships for isolates of  
5 VCG 2B. These were divided into three groups, named 2B-I, 2B-II, and 2B-III in the IGS  
6 phylogeny (clades 2, 3 and 5), and into two groups in the VdSNP one (in clades III and VI)  
7 (Figs. 1 and 3). Isolates of VCG 2B-I, placed in clade 2 based on IGS, were included in  
8 VdSNP clade III together with isolates of VCG 2B-III (IGS clade 5). All isolates of 2B-II  
9 were indistinguishable from VCG 1A and 1B isolates and placed in IGS clade 3. However,  
10 2B-II isolates were grouped in VdSNP clade VI, distinct from VCGs 1A and 1B (Fig. 1).  
11 Also, isolate 2B-I V652 was closely related to VdSNP clade VI. A comparison of both IGS  
12 and VdSNP phylogenies is shown in Figure 3, indicating the lack of congruence in the  
13 placement of VCGs 2B, 3 and 6 in both phylogenies.

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

1 (termed earlier VCG 2B-II isolates) (93% bootstrap support), and a third clade included  
2 isolates of VCGs 2A, 2B-III, 3, 4B and 6 (79% bootstrap support). The rest of VCG 2B  
3 isolates (called earlier VCG 2B-I) remained unresolved in a poorly-supported clade  
4 closely related to VCG 4A. One important observation is that bootstrap values were  
5 higher in the combined analysis (Fig. 2) compared to the IGS tree (Fig. 1A) or the  
6 combined VdSNP tree (Fig. 1B), but only for those clades with a concordant phylogenetic  
7 placement in both individual phylogenies (i.e., VCG 4A or VCGs 1A and 1B). However,  
8 bootstrap values dropped for the analysis of combined datasets in the rest of the clades  
9 (Figs. 1 and 2). As indicated by the IGS and VdSNP trees, isolates of VCG 2B were present  
10 in different well-supported clades of the combined dataset indicating polyphyly of this  
11 group. The alternative hypothesis of monophyly for VCG 2B was tested using the  
12 Shimodaira-Hasegawa test by comparing a tree in which isolates of VCG 2B were  
13 constrained to be monophyletic, to the unconstrained tree. The analysis showed that  
14 the constrained tree was significantly less likely ( $\ln L = -8821.399$ ) than the  
15 unconstrained tree ( $\ln L = -9339.372$ ) ( $P = 0.000$ ), strongly supporting polyphyly for VCG  
16 2B.

## 18 DISCUSSION

19  
20 For the last two decades, VCG analysis has been used to estimate genetic variation in *V.*  
21 *dahliae* populations (6,9,16,18,24,26,27,28,30,51), under the assumption that isolates  
22 within a given VCG comprise genetically related isolates that descend from a common

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

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

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



1 example, VCGs 4A, 2A, 4B, 1A, and 1B were grouped similarly based on IGS and VdSNP  
2 sequences (Fig. 2). However, VCG 3 isolates were nested within the clade containing  
3 isolates of VCGs 2A and 4B based on IGS (Fig. 1A, clade 4), whereas VCG 3 was closely  
4 related to VCG 1A and 1B according to the VdSNP phylogeny (Fig. 1B, clade V). Similarly,  
5 VCG 6 isolates grouped with some VCG 2B isolates in IGS clade 5, but they were closely  
6 related to VCG 4A according to VdSNP sequences (Fig. 1B). The most striking differences  
7 in both phylogenies involved VCG 2B isolates. While isolates of VCG 2B were placed in  
8 three different clades based on IGS, there were only two VdSNP clades containing VCG  
9 2B isolates. Isolates 2B-I were placed in IGS clade 2, and within clade III in the VdSNP  
10 tree. This VdSNP clade also contained 2B-III isolates, placed in a separate distant clade  
11 (clade 5) in the IGS phylogeny. All 2B-II isolates were in clade VI (Fig. 2). Isolates named  
12 2-II in this study contained isolate V574, characterized as VCG 2Ba by Jiménez-Díaz *et al.*  
13 (24). VCG 2Ba isolates infected artichoke in eastern-central Spain and were unable to  
14 complement with international VCG 2B testers but produced positive complementation  
15 with local VCG 2B testers. Those VCG 2Ba isolates were thus genetically different from  
16 other VCG 2B isolates [called VCG 2Br in Jiménez-Díaz *et al.* (24)] that did complement  
17 with international reference VCG 2B testers (24,30). There was also diversity in PCR-  
18 based amplicons of 334 and 824 bp, markers previously associated with cotton and olive  
19 defoliating and non-defoliating *V. dahliae* pathotypes, respectively (14,24,37). Some  
20 VCG 2B isolates from artichoke amplified the defoliating-associated marker of 334bp,  
21 whereas others amplified the non-defoliating marker of 824 bp (7,24). All 2B-II isolates,  
22 including the HSI isolate V547, grouped in IGS clade 3 and VdSNP clade VI, amplified the

1 334-bp marker, which is consistent with these isolates being closely related to cotton  
2 and olive defoliating VCG 1A isolates. All other VCG 2B isolates analyzed amplified the  
3 824-bp marker (7,12,24). An earlier study that included some of the VCG 2B isolates that  
4 amplified the 824-bp marker showed identical haplotypes for five microsatellites (7),  
5 which was different from the haplotypes displayed by VCG 2B isolates that amplified the  
6 334-bp marker. The discrepancies between phylogenies generated by IGS and VdSNP  
7 sequences could also be explained by the fast-evolving nature of the IGS region. Similar  
8 incongruencies between IGS and other regions have been reported for the *Fusarium*  
9 *oxysporum* complex (19,40).

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

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

4 Our results indicate that VCG 2B is clearly polyphyletic, which was also noted by Collado-  
5 Romero *et al.* (12) using AFLP markers, although only two VCG 2B groups were identified  
6 by that study. Dung *et al.* (18) also identified two genetically distinct groups within VCG  
7 2B isolates using microsatellite analysis: one group contained isolates from tomato and  
8 pistachio, and the other comprised isolates from potato, watermelon and mint. There  
9 are several possible explanations for polyphyly in VCGs. One is that convergent  
10 mutations in *het/vic* loci may have resulted in the same functional alleles that allow  
11 anastomosis and the establishment of stable heterokaryons between isolates of  
12 otherwise distinct genetic background. A second potential scenario is the exchange of  
13 genetic material between different VCGs due to parasexuality, especially during  
14 transitory heterokaryosis between different VCGs as it has been shown to be possible  
15 experimentally in *Verticillium* (56) and other fungi (33). In the latter case, the resulting  
16 genotypes would share genomic regions with both parental isolates. Finally, these  
17 isolates of conflicting phylogenetic placement may represent remnants of ancient, or  
18 current but rare, recombination events. *V. dahliae* populations have been shown to  
19 harbor *MAT1-1* and *MAT1-2* idiomorphs similar to the genetic mating system of other  
20 Ascomycota (2,57). Also, a departure from clonality shown by microsatellite analysis of  
21 *V. dahliae* populations was attributed to potential sexual recombination (2); although a  
22 major predominance of *MAT1-2* was found in populations of *V. dahliae*, with only one

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

6 In our study, we included some of Puhalla's original isolates (44,45) that were used to  
7 perform early VCG testing (6,27,45,51,52). One of those isolates, isolate PCW, was first  
8 typed as VCG 16 by Puhalla and Hummel (45), it was later reclassified as VCG 3 by  
9 Joaquim and Rowe (26), and finally was identified as VCG 4 by Bell (6) and Strausbaugh  
10 et al. (52). Interestingly, in our study this PCW isolate as well as others typed as VCG 3  
11 *sensu* Joaquim and Rowe (26) were placed with VCGs 2A and 4B within IGS clade 4, and  
12 together with VCG 1A and 1B in clade V, based on VdSNP sequences. VCG 3 was  
13 reported as being an interspecific hybrid between *V. dahliae* VCG1B and an unidentified  
14 non-*Verticillium* parent (11). This was due to the presence of two different sequences  
15 for actin, calmodulin and histone 3 genes in the VCG tester isolate 70.21; one sequence  
16 that matched other *V. dahliae* sequences and another sequence that matched an  
17 unidentified non-*Verticillium* member of the Ascomycota (11). Isolate 70.21 was also  
18 included in our analyses together with two other VCG 3 isolates. However, in contrast  
19 with Collado-Romero et al. (11), in our study all sequences associated with the three  
20 VCG 3 isolates were clean and there was no indication of multiple sequences contained  
21 in one amplicon. Based on our analysis there is no indication of a hybrid origin for VCG  
22 3.

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

22

1

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

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

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



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

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

1

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

8 <sup>b</sup> Vegetative compatibility group was determined in the study referenced in the next column or by the supplier of the isolate. HSI,  
9 heterokaryon self-incompatible.

10 <sup>c</sup> VCG 2Ba, artichoke isolate able to complement with other artichoke isolates assigned to VCG2B but not with international VCG 2B  
11 testers.

12 <sup>d</sup> n/a, not applicable.

13 <sup>e</sup> References of studies where isolates were assigned to VCG. For isolates with no reference, VCG typing was performed by the  
14 supplier.

## 1 FIGURE LEGENDS

2 **Figure 1.** Phylogenetic relationships between *Verticillium dahliae* vegetative  
3 compatibility groups (VCG). **A,** One of 200 most parsimonious phylogenetic trees  
4 inferred from sequences of the intergenic spacer region of the rDNA (IGS) (93  
5 parsimony-informative characters; 327 steps; consistency index, CI = 0.930; retention  
6 index, RI = 0.966; rescaled consistency index, RC = 0.898). **B,** One of 200 most  
7 parsimonious phylogenetic trees inferred from the combined VdSNP polymorphic  
8 sequences (30 parsimony-informative characters; 163 steps; CI = 0.791; RI = 0.901; RC =  
9 0.713). Numbers above branches are bootstrap values (>50%) based on 1,000 replicates.  
10 Each taxon label indicates isolate reference, VCG, geographical origin, and host source.  
11 Trees are rooted with *V. albo-atrum* 462. VCG 2B isolates are further subdivided into  
12 VCG 2B-I, VCG 2B-II, and VCG 2B-III according to their phylogenetic placement in the IGS  
13 tree (A).

14

15 **Figure 2.** Phylogenetic relationships between *Verticillium dahliae* vegetative  
16 compatibility groups (VCG). One of 200 most parsimonious phylogenetic trees inferred  
17 from the combined dataset of the intergenic spacer region of the rDNA (IGS) and six  
18 polymorphic sequences (VdSNP) (123 parsimony-informative characters, 504 steps,  
19 consistency index, CI = 0.859; retention index, RI = 0.931; rescaled consistency index, RC  
20 = 0.8). Numbers above branches are bootstrap values (>50%) based on 1,000 replicates.  
21 Each taxon label indicates isolate reference, VCG, geographical origin, and host source.  
22 Trees are rooted with *V. albo-atrum* 462. Arrows indicate VCG 2B isolates.

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

10

11 **Supplemental Table 1.** Analysis of congruence between all datasets estimated with the  
 12 incongruence length difference (ILD) test implemented as the partition homogeneity  
 13 test in PAUP v.4.0.b10 with 1,000 replicate partitions subjected to heuristic parsimony  
 14 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	—	—	—	—	—	—	—

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

16

17

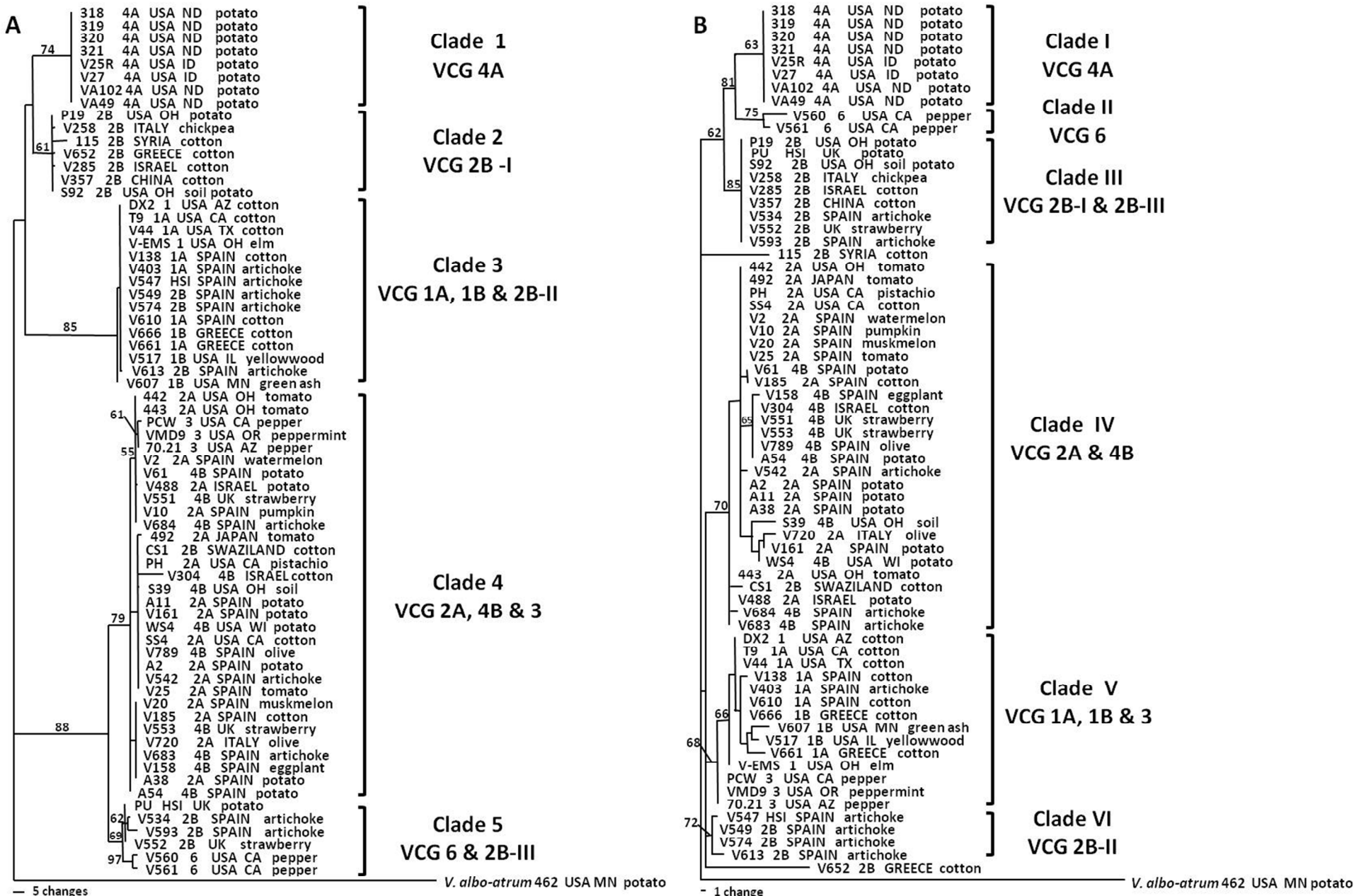
1 **Supplemental Figure 1.** Maximum parsimony phylogenies inferred from individual  
2 anonymous polymorphic sequences VdSNP. **A**, One of 200 most parsimonious  
3 phylogenetic trees inferred from VdSNP1 sequences (7 parsimony-informative  
4 characters, 23 steps; consistency index, CI = 0.522; retention index, RI = 0.911; rescaled  
5 consistency index, RC = 0.475). **B**, One of 200 most parsimonious phylogenetic trees  
6 inferred from VdSNP2 sequences (5 parsimony-informative characters, 32 steps; CI =  
7 0.938; RI = 0.959; RC = 0.899). **C**, One of 200 most parsimonious phylogenetic trees  
8 inferred from VdSNP3 sequences (2 parsimony-informative characters, 2 steps; CI = 1; RI  
9 = 1; RC = 1). **D**, One of 200 most parsimonious phylogenetic trees inferred from VdSNP4  
10 sequences (2 parsimony-informative characters, 34 steps; CI = 1; RI = 1; RC = 1). **E**, One  
11 of 200 most parsimonious phylogenetic trees inferred from VdSNP5 sequences (10  
12 parsimony-informative characters, 18 steps; CI = 0.415; RI = 0.899; RC = 0.410). **F**, One of  
13 200 most parsimonious phylogenetic trees inferred from VdSNP7 sequences (3  
14 parsimony-informative character, 6 steps; CI = 1; RI = 1; RC = 1). Numbers above  
15 branches are bootstrap values (>50%) based on 1,000 replicates. Each taxon label  
16 indicates isolate reference, VCG, geographical origin, and host source. Trees are rooted  
17 with *V. albo-atrum* 462.

18

19

20

Figure 1



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Figure 2

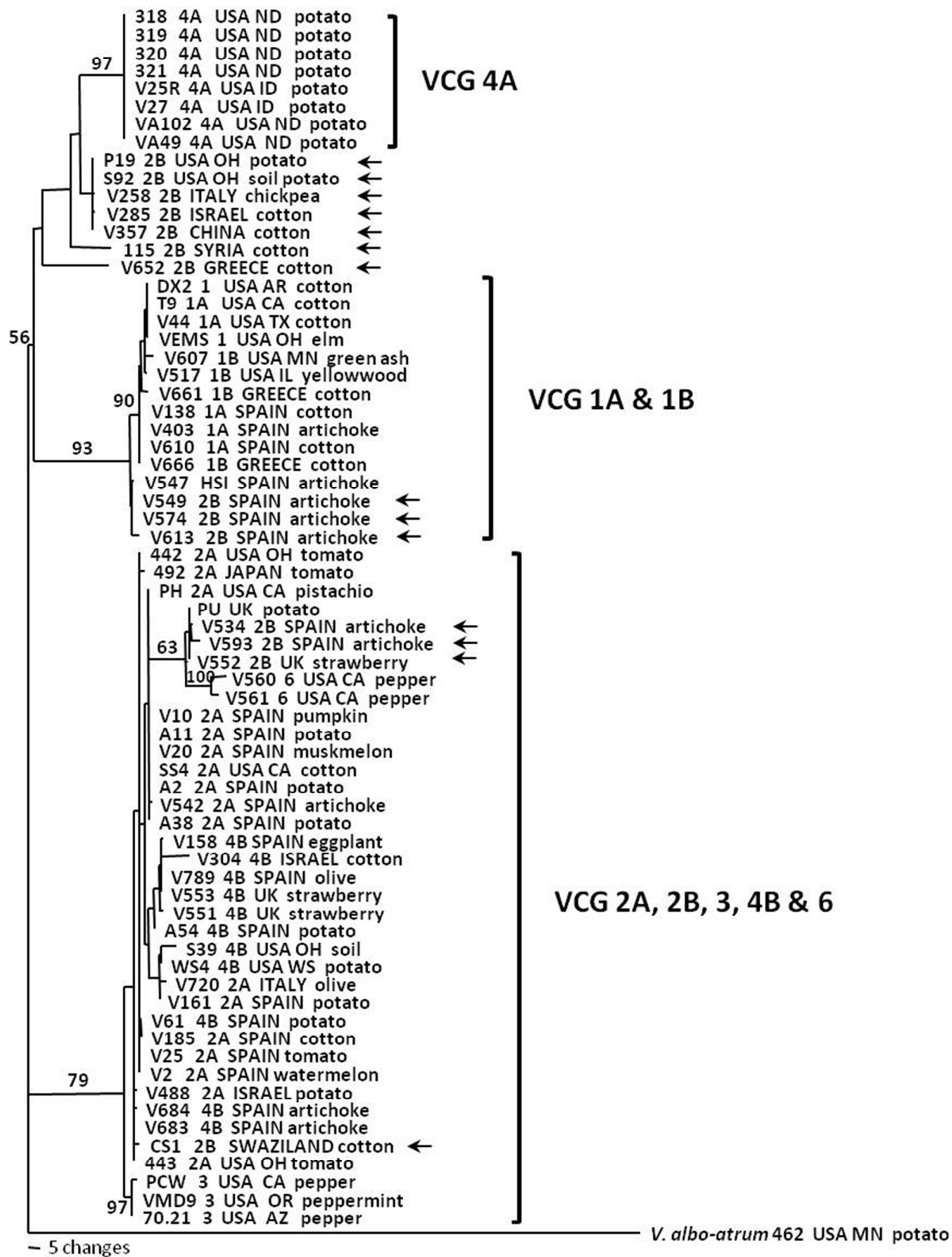
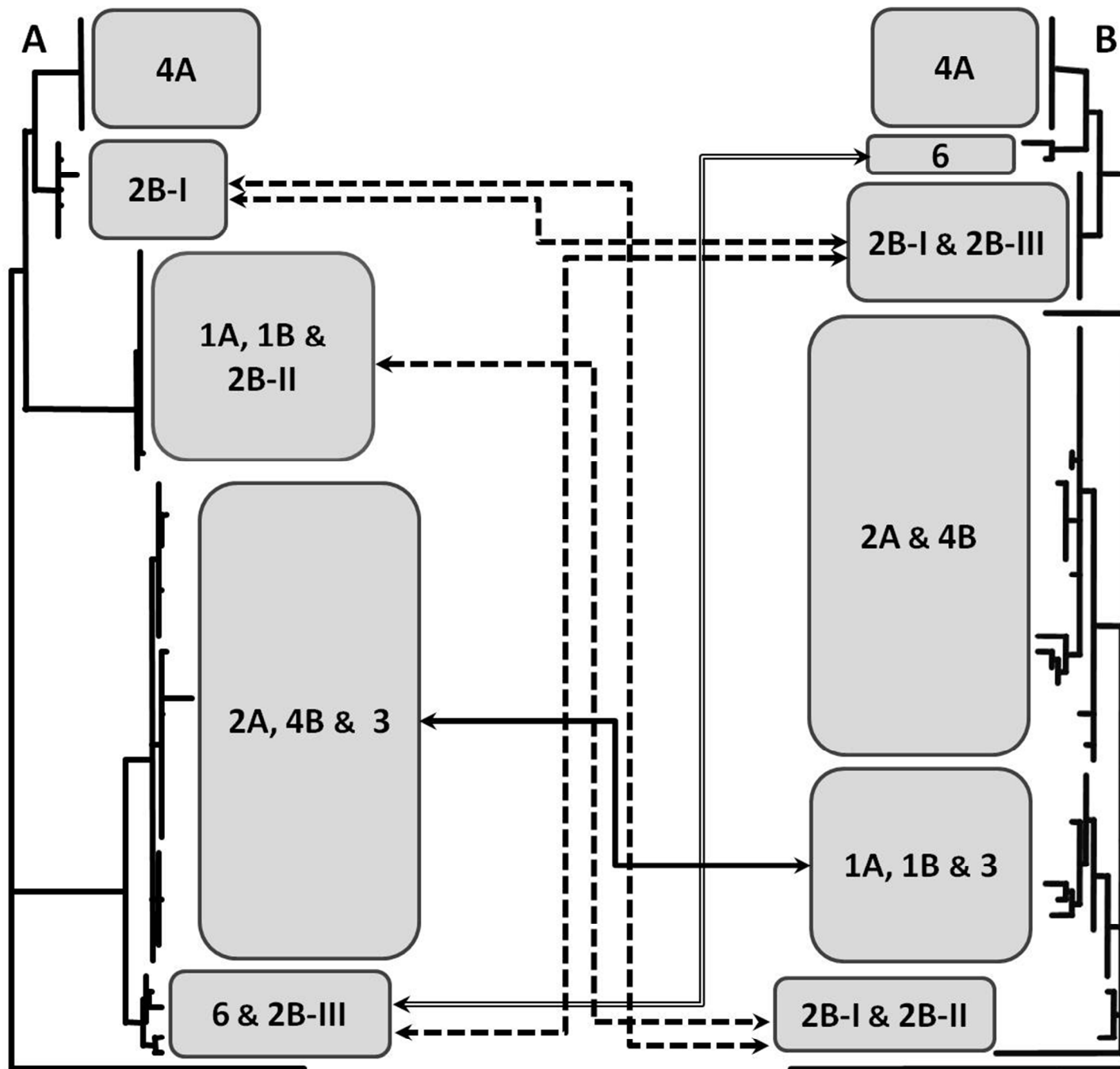


Figure 3





## Supplemental Figure 1

