Phylogenetic Analysis
of *Verticillium dahliae* Vegetative Compatibility Groups

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ABSTRACT

Collado-Romero, M., Mercado-Blanco, J., Olivares-García, C., and Jiménez-Díaz, R. M. 2008. Phylogenetic analysis of *Verticillium dahliae* vegetative compatibility groups. Phytopathology 98:1019-1028.

The evolutionary relationships among *Verticillium dahliae* vegetative compatibility (VCG) subgroups VCG1A, VCG1B, VCG2A, VCG2B, VCG4A, VCG4B, and VCG6 were investigated by parsimony analysis of amplified fragment length polymorphism (AFLP) fingerprints and sequences of six DNA regions (*actin*, β-*tubulin*, *calmodulin*, and *histone* 3 genes, the ITS 1 and 2 regions of the rDNA, and a *V. dahliae*-specific sequence), using 101 isolates of diverse host and geographic origin. Polymorphisms in gene sequences among isolates of different VCGs were very low and individual gene genealogies provided very little resolution at the VCG level. The combined analysis of all DNA regions differentiated all VCG subgroups except for isolates in VCG1A and VCG1B. VCG clonal lineages in *V. dahliae* and evolutionary relationships among them were resolved independently by analyses of AFLP fingerprints, multiple gene genealogies, and the combined data set of AFLP fingerprinting and multiple gene genealogies. Two main lineages (I and II) were identified with lineage II comprising two closely related subgroups of VCGs. Lineage I included VCG1A, VCG1B, and VCG2B[334]; and lineage II included, VCG2A and VCG4B (subclade 1); and VCG2B[824], VCG4A, and VCG6 (subclade 2). VCG subgroups were monophyletic except for VCG2B that appeared polyphyletic. Limiting the parsimony analysis either to AFLP fingerprints or DNA sequences would have obscured intra-VCG differentiation. Therefore, the dual approach represented by the independent and combined analyses of AFLP fingerprints and DNA sequences was a highly valuable method for the identification of phylogenetic relationships at the intraspecific level in *V. dahliae*.

Additional keywords: defoliating pathotype, genetic diversity, Verticillium wilt.

Knowledge of the genetic variation within and among pathogen populations, as well as the genetic relationships among these populations, is essential for development of disease management strategies (39). For populations of *Verticillium dahliae* Kleb., the causal agent of Verticillium wilts of hundreds of herbaceous and woody crops (44), studies during the last decade have demonstrated that genetic variation in the pathogen is higher than previously thought. Moreover, limited understanding of the nature of such variation and the genetic relationships among *V. dahliae* populations has hampered our understanding of Verticillium wilt development and the efficacy of their management (4,33,47). In addition, severe Verticillium wilts have newly appeared on plants previously considered to be nonhosts of *V. dahliae* (i.e., cauliflower and lettuce) and studies demonstrated that isolates causing severe disease in one host are potentially derived from those infecting other hosts (34,46,53).

Genetic variation in *V. dahliae* has been studied mainly by vegetative compatibility analysis and, to a lesser extent, analyses of fungal DNA. Vegetative compatibility refers to the *het* (heterokaryon incompatibility) loci-controlled ability of strains of a fungal species to undergo hyphal anastomosis and form stable heterokaryons. Fungal isolates that are vegetatively compatible are placed in the same vegetative compatibility group (VCG) (33,38). Because *V. dahliae* is a haploid, asexually reproducing fungus, hyphal anastomosis is a prerequisite to genetic exchange and eventual parasexual genetic recombination among different isolates of the pathogen (26,44). Consequently, *V. dahliae* isolates in different VCGs are often thought of as genetically isolated populations which may vary in a number of ecological, physiological, and virulence traits (33,47).

Four main *V. dahliae* VCGs (VCG1, VCG2, VCG3, and VCG4) have been identified using complementing nitrate-nonutilizing (*nit*) mutants of isolates from diverse hosts and geographic origin worldwide (5,13,30,33,52). Recently, a new VCG, VCG6, was identified among *V. dahliae* isolates infecting pepper in California (6). Based on the frequency and vigor of complementation, each of VCG1, VCG2, and VCG4 were further divided into two subgroups, designated A and B (5,31,51). Moreover, isolates of VCG2B infecting artichoke in eastern-central Spain were differentiated further into two additional subgroups, designated VCG2Br if the isolates complemented with international reference VCG2B testers and VCG2Ba if the complementation occurred with VCG2Br isolates but not with the reference testers (28).

Asexual reproduction in *V. dahliae*, which results in the whole genome being transmitted as a unit from one generation to the next, together with the low number of VCGs found so far in *V. dahliae* populations generally have been considered as an indication of clonal structure in this fungus. However, as for other asexually reproducing fungi such as *Fusarium oxysporum*, clonality of isolates within a VCG should be recognized with caution since the occurrence of an identical set of *het* loci may be coincidental, possibly arising by convergence rather than common descent (1,2,38).

Phenetic analysis of amplified fragment length polymorphism (AFLP) fingerprinting using a large collection of *V. dahliae* isolates from different hosts demonstrated that isolates within a VCG subgroup (A and B) are genetically similar and AFLP

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clustering of isolates correlates with VCG subgroups (14). Based on molecular characterization, VCG subgroups were as distinct as were different VCGs. However, high molecular variability occurred within VCGs as indicated by phenetic analysis of AFLP fingerprinting and amplification of specific polymerase chain reaction (PCR) markers (14). For example, most VCG2B isolates produce an 824-bp marker previously associated with the cotton-nondefoliating (ND) *V. dahliae* pathotype (designated VCG2B[824]) whereas other ND VCG2B isolates (designated VCG2B[334]) and isolates of VCG1B amplify a 334-bp marker previously associated with the cotton-defoliating (D) pathotype belonging to VCG1A (14,15,41). Furthermore, ND VCG2B[334] isolates were molecularly more similar to isolates in VCG1A than to those in VCG2B[824] (14,28). Molecular variability among *V. dahliae* isolates within a VCG might derive from mutational processes, thus we would expect variants to result in a clonal lineage. Conversely, this variability might result from genetic recombination and those isolates would not be placed in the same clonal lineage (6). Also, the existence of bridging isolates able to form stable heterokaryons between isolates in different VCGs would eventually facilitate some gene flow between them (4). Therefore, the evolutionary potential within *V. dahliae* may be intimately related to VCGs (24,39). This is relevant to disease control in terms of potential for emergence of new virulence or pathogenicity traits in natural populations of *V. dahliae*. However, whether or not genetic recombination occurs between *V. dahliae* isolates in different VCGs has not yet been addressed by phylogenetic analysis.

Phylogenetic analyses in fungal populations have been carried out using genealogies of highly conserved genes such as actin (*Act*), β-tubulin (β-*tub*), calmodulin (*Cal*), histone-3 (*H3*), and especially the internal transcribed sequences (ITS) of the nuclear ribosomal RNA gene cluster (rDNA) (10,12,23,29). In particular, β-*tub* and the ITS have been used for phylogenetic analyses of *Verticillium* spp. (8,19,43,46). On the other hand, analysis of AFLP fingerprints is an alternative approach to investigate molecular phylogenetics of closely related taxa (20,25,45). Furthermore, reconstruction of evolutionary history in clonal fungal populations using gene genealogies can both identify patterns of descent in other types of molecular markers, such as whole genome AFLP analysis, and be combined with them to infer more finely resolved phylogenies (3,9,22).

The main objective of this study was to determine the evolutionary relationships among the currently identified VCG subgroups of *V. dahliae* (i.e., VCG1A, VCG1B, VCG2A, VCG2B, VCG4A, VCG4B, and VCG6). A second objective was to test the hypothesis of monophyletic origin of each of those VCG subgroups. To that aim, we used a collection of isolates of diverse host source and geographic origin to carry out phylogenetic analyses of data from AFLP fingerprints or sequences of the six conserved DNA regions: *Act*, β-*tub*, *Cal*, *H3*, and ITS1 and 2 of the rDNA and a *V. dahliae*-specific DNA sequence (11); we also included analysis of the combined data.

MATERIALS AND METHODS

***Verticillium* spp. isolates, culture conditions, and DNA extraction.** A collection of 101 isolates of *V. dahliae* and one of *V. albo-atrum* from diverse host and geographic origins were used in this study (Table 1). Isolates were stored on plum lactose yeast extract agar (PLYA) (54) covered with liquid paraffin at 4°C in the dark (7). Isolates are deposited in the culture collection of the Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible, CSIC, Córdoba, Spain. Active cultures of isolates were obtained on water agar amended with chlorotetracycline (0.3 g liter⁻¹) and subsequent subculturing on potato dextrose agar (PDA) (250 g of unpeeled potato, 20 g of agar, and 20 g of glucose per liter of distilled water) or, alternatively, PLYA (54). DNA was extracted from mycelia obtained from 6- to 7-day-old

cultures of *Verticillium* isolates in Czpeck-Dox broth (Difco Laboratories, Detroit, MI) or PLYA, according to Collado-Romero et al. (14). DNA purity and concentration were determined spectrophotometrically using a Biophotometer (Eppendorf AG, Hamburg, Germany) and by agarose gel electrophoresis according to standard procedures. DNA solutions were stored at –20°C until used.

Vegetative compatibility grouping of isolates. Of the 101 *V. dahliae* isolates, 69 were characterized to a VCG in previous studies (14,15,28,36,37) and 28 were newly assigned to a VCG according to Korolev and Katan (35) and Collado-Romero et al. (14). Briefly, nitrate-nonutilizing (*nit*) mutants were generated on water agar chlorate and further tested on Czapek-Dox agar (CDA). *nit* mutants were phenotyped on CDA amended with sodium nitrite (0.5 g liter⁻¹) or hypoxanthine (0.2 g liter⁻¹) (17). Then, *nit*1 and NitM mutants from each isolate were used to test for heterokaryon self-compatibility as well as for complementation with *nit* mutants of the international Ohio Agricultural Research and Development Center (OARDC, The Ohio State University, Wooster, OH) reference strains of *V. dahliae* VCGs (VCG1, VCG3, and VCG4A) and Israeli *nit* testers (VCG1A, VCG2A, VCG2B, and VCG4B) (36) in all possible combinations. *nit* mutants derived from reference strain T9 of D pathotype, kindly provided by T. Katan (The Volcani Center, Bet Dagan, Israel), were used to identify VCG1A. Since T9 belongs to VCG1A of Bell (1994), we assumed that isolates strongly compatible with it should be assigned to VCG1A. No complementation tests were done in this work to type isolates to VCG1B. VCG1B isolates provided and used in the study were tested for virulence to cotton and proved of the ND pathotype (R. M. Jiménez-Díaz, *unpublished data*). Israeli *nit* testers that were previously demonstrated to correlate with the international OARDC reference strains of *V. dahliae* VCGs were used for identification of VCG2A, VCG2B, and VCG4B (33,36,47). Cultures were incubated at 25°C and a 12-h photoperiod of fluorescent and near-UV light at 36 μEm⁻² for up to 4 weeks. Unknown isolates were assigned to a VCG when a dense, aerial growth or black microsclerotia formed where mycelia from the unknowns and the VCG tester strain had met.

AFLP assays, scoring of AFLP fragments and binary matrix generation. Eighty-nine *V. dahliae* isolates representative of *V. dahliae* VCG1A, VCG1B, VCG2A, VCG2B, VCG4A, VCG4B, and VCG6, and the single isolate of *V. albo-atrum* were selected for phylogenetic analysis using AFLP markers (Table 1). AFLP profiles of 40 *Verticillium* isolates (39 *V. dahliae* and 1 *V. albo-atrum*) were available from a previous study (14). Profiles of the remaining 50 *V. dahliae* isolates were obtained in this study.

AFLP assays were performed using a fluorescent procedure as described in Collado-Romero et al. (14). Briefly, DNA was digested with *Mse*I and *Eco*RI endonucleases and the restricted DNA fragments were ligated to *Mse*I and *Eco*RI adaptors using T4 DNA ligase. A 5% dilution of the ligation reaction in TE$_{0.1}$ buffer was used for preselective amplification using 0.5 μl of preselective primers *Mse*I-C and *Eco*RI-0 and 15 μl of the AFLP Core Mix included in the kit. Selective PCR amplifications were done using four *Eco*RI-XX-Dye/*Mse*I-C combinations in independent reactions using fluorescently labeled dyes NED, JOE, and FAM: *Eco*RI-AT-NED, *Eco*RI-AG-JOE, *Eco*RI-GC-FAM, and EcoRI-GA-FAM. Artifacts in the AFLP profiles were avoided by replicating the assays twice for arbitrarily selected *V. dahliae* isolates V562I and 1F20I. Products of selective amplifications were separated by capillary electrophoresis in an automatic DNA sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems). The size of products was determined using GeneScan-500 [ROX] (Applied Biosystems, Foster City, CA) as internal size standard (35 to 500 bp). One AFLP profile for each of the four selective primer combinations used, ranging from 50 to 490 bp,

were established for each of isolates in the study using the GeneScan v. 2.0 analysis software (Applied Biosystems).

AFLP profiles were assessed individually for each isolate. Only unambiguous peaks were scored for presence or absence (1 = presence or 0 = absence) using Genotyper software version 2.5 (Applied Biosystems). All DNA fragments within the range of 50 to 490 bp were selected first using the 'unmark overlapping peaks' option and AFLP profiles were rescaled using the 'normalized scale option'. Unambiguous, consistent peaks, which scaled higher than 100 fluorescent units, were then selected and those inconsistent were deleted manually. A binary character matrix was developed by combining all data and used for subsequent phylogenetic analysis. Thereafter, data of AFLP and DNA sequence where used jointly for phylogenetic analysis (see below).

Phylogenetic analysis of AFLP patterns. Phylogenetic analysis of the AFLP patterns were conducted by maximum parsimony (MP) using phylogenetic analysis using parsimony (PAUP*) version 4.0beta10 (Sinauer Associates, Inc., Publishers, Sunderland, MA). Unweighted MP analysis was conducted with the heuristic search option and 10 random addition sequences with the MulTrees option on and TBR branch swapping. A strict consensus tree was inferred from most all parsimonious trees. Bootstrap support was calculated with 1,000 bootstrap replicates, using the same settings as for the general heuristic search analysis. The tree derived from MP analysis was rooted to *V. albo-atrum*.

Specific DNA sequences amplification and sequencing. Fifty *V. dahliae* isolates representing diversity in AFLP profiles among and within VCGs were selected for phylogenetic analysis using the polymorphic *V. dahliae*-specific sequence of 523/539 bp (11,15,40) (Table 1). Then, 17 of the 50 isolates representative of all molecular groups and VCGs and the single *V. albo-atrum* isolate were arbitrarily selected for phylogenetic analysis using sequences of genes actin (*Act*), calmodulin (*Cal*), β-tubulin (β-*tub*), and histone 3 (*H3*), as well as the ITS 1 and 2 regions of the rDNA and the *V. dahliae*-specific polymorphic DNA sequence. All sequences were considered independent loci. Primers used for DNA amplification and sequencing were: *Act*, ACT-512F (ATG-TGCAAGGCCGGTTTCGC) and ACT-783R (TACGAGTCCTT-CTGGCCCAT) (10); β-*tub*, Bt1a (TTCCCCCGTCTCCACTT-CTTCATG) and Bt1b (GACGAGATCGTTCATGTTGAACTC) (23); *Cal*, CAL-228F (GAGTTCAAGGAGGCCTTCTCCC) and CAL-737R (CATCTTTCTGGCCATCATGG) (10); *H3*, H3-1a (ACTAAGCAGACCGCCCGCAGG) and H3-1b (GCGGGCGA-GCTGGATGTCCTT) (23); and ITS1 and 2 regions, Tavo1 (CTCATAACCCTTTGTGAACC) and Tavo2 (CCGAGGTCAA-CCGTTGCCG) (56). The translation elongation factor 1α (*EF1α*) gene was also tested but amplification was unsuccessful (EF1: TGGGTAAGGAA/GGACAAGAC and EF2: GGAA/GGTAC-CAGTC/GATCATGTT) (42). Primers DB19 (CGGTGACATA-ATACTGAGAG) and DB22 (GACGATGCGGATTGAACGAA) (11) were used for the amplification and sequencing of the *V. dahliae*-specific sequence.

For all primer pairs, the reaction mixture (25 μl) consisted of: 20 ng of fungal DNA, 400 nM of each primer, 200 nM of each dNTP, 2 mM MgCl₂, 2.5 μl 10× reaction buffer and 0.75 U DNA polymerase (Biotools, B&M Labs, Madrid, Spain). The PCR cycling protocols were: denaturation at 94°C for 5 min followed by 35 cycles of 1 min at 94°C, 1min at 55°C (for primer pairs ACT-512F/ACT783R, Bt1a/Bt1b, CAL228F/CAL737R, and DB19/DB22) or 58°C (for primers pairs H3-1a/H3-1b and Tavo1/Tavo2), 1 min at 72°C, and a final step of 6 min at 72°C. Amplification with primer pair EF1/EF2 was tested at an annealing temperature range of 52 to 64°C. The amplification products (5 μl) were separated by electrophoresis on 1% agarose gels in 1× TAE buffer, stained with ethidium bromide and visualized under UV light. The 0.1-kb DNA ladder XIV size marker was used for

electrophoresis (Roche Diagnostics, Mannheim, Germany). If only one band was identified in agarose gels, the amplicon was excised from the gel and purified using the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). Amplification products were sequenced at Servicio de Secuenciación de ADN, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid, Spain. Forward primers were used in all cases and both forward and reverse sequencing was done occasionally. Sequences were edited with EditSeq (Lasergene, Madison, WI). A search for sequence similarities was performed with the BLAST version 2.2.10 program of the National Center for Biotechnology Information (NCBI) network service.

DNA sequence analysis. All assayed loci except *EF1α* produced amplicons. Sequences of each of the loci were aligned using the CLUSTALW implemented in the MegAlign software (Lasergene, Madison, WI). For comparative purposes, two phylogenetic analyses were performed independently with loci *Act*, β-*tub*, *Cal*, *H3*, and ITS, as well as the *V. dahliae*-specific DNA sequence. First, data were analyzed by neighbor joining (NJ) (49) using TREECON version 1.3b software and genetic distances (32) with indels being considered as changes in the sequence (55). Then, MP analysis was done with PAUP* version 4.0beta10 software using the same parameters as for AFLP analysis but increasing to 1,000 the number of random repeats in the heuristic search and bootstrap replicates. All trees, except those derived from the *V. dahliae*-specific sequence, were rooted using the *V. albo-atrum* sequences. Trees derived from analyses using the *V. dahliae*-specific sequence were rooted with the sequence of *V. dahliae* isolate V138I.

Trees for each of the loci were evaluated simultaneously for congruence using the partition homogeneity test (PHT) (21,27) implemented in PAUP* version 4.0beta10. *Act*, β-*tub*, *Cal*, *H3*, and ITS sequences were combined to perform MP analysis using PAUP* version 4.0beta10 and the same parameters considered in the analysis of each locus. For the combined analysis, indels within each loci were considered numeric characters (i.e., 0, 1, and 2): 0, deletion; 1, presence of nucleotide or nucleotides (insertion); and 2, presence of polymorphism in the insertion. Similarly, the combined sequences of loci *Act*, β-*tub*, *Cal*, *H3*, and ITS, and the *V. dahliae*-specific sequence were used to perform MP analysis.

Phylogenetic analysis of the combined data set of AFLP fingerprints and DNA sequences. Congruence of phylogenetic signals between AFLP data and DNA sequences was studied performing the PHT implemented in PAUP* version 4.0beta10. The combined set of AFLP and DNA sequence data from *V. dahliae* isolates was used for phylogenetic MP analysis using PAUP* version 4.0beta10 with bootstrap analysis of 500 replicates. Parameters used in that analysis were the same as those used in the analysis of each locus. Indels within loci were coded as numerical characters (0, 1, and 2).

RESULTS

Vegetative compatibility grouping of isolates. Of the 28 *V. dahliae* isolates newly characterized to VCG, 10 were assigned to VCG1A, 9 to VCG2A, 3 to VCG2B[824], and 6 to VCG4B (Table 1). Six of the 10 VCG1A isolates originated from cotton in Greece and four were from olive in Spain. VCG2A isolates were of diverse host and geographic origin, including one from each of cotton soil, melon, olive, potato, pumpkin, and watermelon in Spain; one from potato in Israel, one from broccoli in Germany, and one from Japan. The host of the isolate from Japan was unknown. The three isolates of VCG2B originated from chickpea in Italy, or strawberry in England. Finally, of six isolates assigned to VCG4B, one originated from tomato in Brazil, one from olive in Greece, one each from potato and eggplant from Israel, and two from strawberry in England.

Polymorphisms in the *V. dahliae*-specific sequence. An isolate of *V. albo-atrum* did not yield any PCR product using primer pair DB19/DB22. Sequencing of the 523/539-bp amplicons from the 50 selected *V. dahliae* isolates produced three of the five polymorphic sequences described by Collins et al. (15), i.e., *seq1*, *seq2*, and *seq4*, as well as a new polymorphic sequence, namely *seq7* (Table 1). The complete sequences were deposited in the GenBank nucleotide database with accession numbers: *seq1* (527 bp), DQ2666246; *seq2* (526 bp), DQ2666247; DQ2666248; *seq4* (542 bp), DQ2666249 and DQ2666244; and *seq7* (541 bp), DQ2666245. The *seq* sequences and VCG of isolates were correlated as follows: *seq1* was found only in isolates of VCG2B[824] and VCG6, *seq2* was amplified from isolates of VCG2A and VCG4B; and *seq4* occurred in isolates of VCG1A, VCG1B, and VCG2B[334]. The new sequence *seq7* was only amplified from the two VCG4A isolates used in the study (Table 1).

Phylogenetic analysis of the four *seq* sequences yielded one most parsimonious tree only (not shown). In that tree, *seq1* and *seq2* were the most closely related, *seq7* was intermediate and *seq4* was most distant. This suggests that VCG2B[824] and VCG6 are closely related to VCG2A and VCG4B, whereas VCG1A, VCG1B, and VCG2B[334] appear to be related to each other but represent a different, more distant clade, and VCG4A occupying an intermediate position between these two larger groups.

Phylogenetic analysis of the AFLP patterns. AFLP analysis of the complete set including 89 *V. dahliae* isolates and one *V. albo-atrum* in this study yielded 578 unambiguous and easily discernible AFLP fragments, of which 375 were informative for the MP analysis. Only 20,000 of a larger number of most parsimonious trees were retained to generate a consensus tree (Fig. 1). Clades resolved by MP analysis correlated with VCGs and were supported by bootstrap values higher than 70%, except for the clade comprising isolates of VCG2B[824], VCG6, and VCG4A (bootstrap 57%). VCG1A and VCG1B were not clearly resolved but the VCG1A/1B group was closely related to the VCG2B[334] clade. Isolates of VCG2A were separated into two clades, each of them with high bootstrap support. There was no correlation of the two VCG2A clades with host or geographic origin of isolates. VCG4B isolates comprised a main clade closely related to the VCG2A clades, except for the VCG4B isolate, V684I, from artichoke, which was actually placed in one of VCG2A clades. Isolates of VCG2B[824] were placed in a clade closely related to that comprising isolates of VCG4A and VCG6. It was noteworthy that isolates of VCG2 subgroups A and B were less related to each other than they were to other VCG subgroups (i.e., VCG4B or VCG4A and VCG6), and same occurred with isolates of VCG4 subgroups.

Characterization of amplified gene sequences. PCR assays using DNA from *Verticillium* spp. isolates produced a single amplification product for each of genes *Act*, β-*tub*, *Cal*, and *H3*, and the ITS regions of rDNA. Gene identity of the amplified sequences was confirmed by comparison with the GenBank databases. None of the *Verticillium* spp. isolates yielded any PCR product using primer pair EF1/EF2. Amplifications from *V. albo-atrum* isolate V048I from hops yielded DNA fragments of 200 bp (GenBank Accession no. (AN): DQ266104), 446 bp (AN: DQ266147), 518 bp (AN: DQ266165), 439 bp (AN: DQ266200), and 452 bp (AN: DQ266223) for genes *Act*, β-*tub*, *Cal*, *H3*, and ITS regions of rDNA, respectively. The amplification product of gene β-*tub* was polymorphic relative to that of *V. albo-atrum* isolates from lucerne and the amplicon of the ITS regions of rDNA was identical to that identified in nonlucerne *V. albo-atrum* isolates (46).

V. dahliae isolates amplified an identical sequence of about 200 pb for gene *Act* (AN: DQ266108-DQ266124) as well as an identical β-*tub* sequence of 448 bp (AN: DQ266129-DQ266138, DQ266140-DQ266145), except for isolates of VCG6. The latter amplified a sequence of 449 bp (AN: DQ266139) for gene β-*tub*. Amplification of gene *Cal* from isolates of *V. dahliae* yielded a product of 523 bp (ANs: DQ266154-DQ266163, DQ266166-DQ266168, and DQ266174-DQ266177). All *V. dahliae* isolates but those of VCG2B[334] amplified a fragment of 439 bp (ANs: DQ266180-DQ266182, DQ266184, DQ266185, and DQ266187-
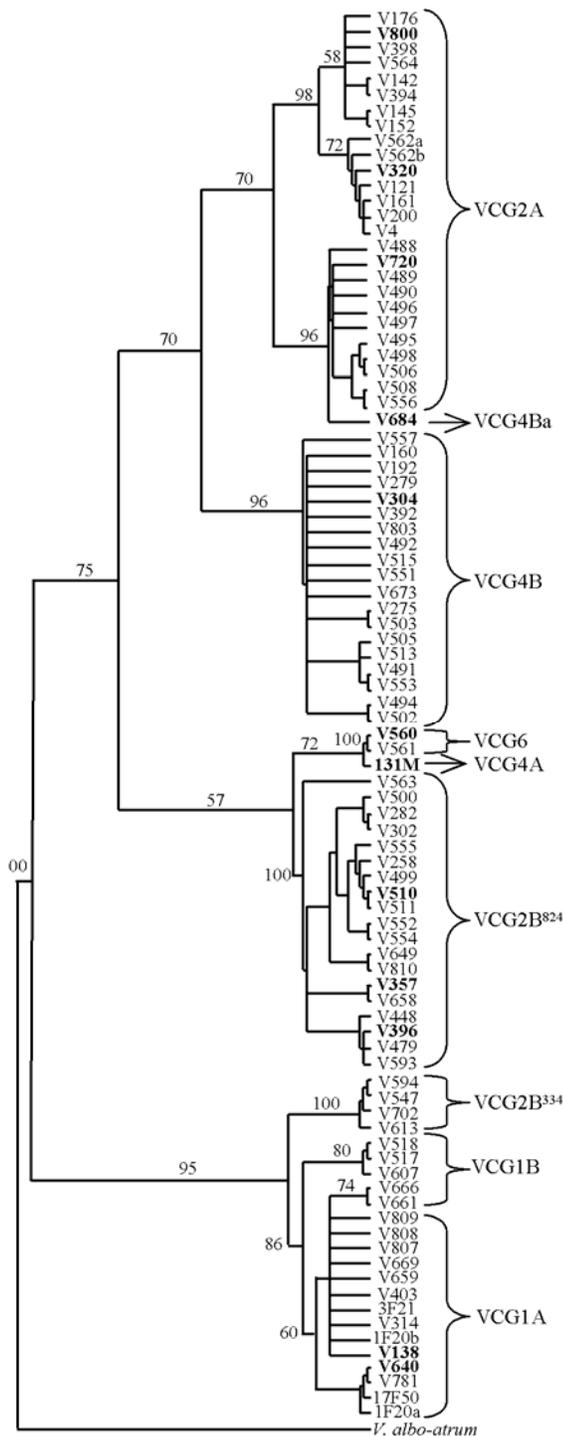
Fig. 1. Consensus tree inferred from maximum parsimony analysis using PAUP version 4.0beta10 of 578 amplified fragment length polymorphism markers from 89 *Verticillium dahliae* isolates representative of different vegetative compatibility groups (VCG), *V. albo-atrum* was used to root the trees. Bootstraps values (1,000 replicates) are indicated in nodes. Parameters defining the tree are: tree length (TL) = 1,263 steps, consistency index (CI) = 0.4537, retention index (RI) = 0.8129, and rescaled consistency index (RC) = 0.3688. VCG4Ba, artichoke isolate of VCG4B; VCG2B[334], artichoke isolates of VCG2Br and VCG2Ba that amplified a 334-bp polymerase chain reaction (PCR) marker (28;40); VCG2B[824], isolates of VCG2B that amplified a 824-bp PCR marker (14,41). In bold, isolates that were used for phylogenetic analysis using DNA sequences.

DQ266197) for gene *H3*. VCG2B[334] isolates amplified a sequence of 438 bp (ANs: DQ266183 and DQ266186) for this gene.

Amplification of the ITS regions of rDNA produced a DNA fragment of 452 bp (ANs: DQ266204-DQ266216 and DQ266220-Q266222) from all *V. dahliae* isolates except those of VCG2A. These latter isolates yielded a 452-bp polymorphic sequence (AN: DQ266217-DQ266219) that was identical to that found in "short-spored" *V. dahliae* isolates from crucifers (16). Conversely, the ITS sequence from the remaining *V. dahliae* isolates showed 100% similarity with ITS sequences of other isolates of *V. dahliae* and some of *V. dahliae* var. *longisporum* (16).

Phylogenetic analysis of DNA sequences and the combined data set of AFLP fingerprints and DNA sequences. Phylogenetic trees for each DNA region derived from MP analysis (not shown) indicated that *V. dahliae* isolates were identical for most of the sequences, regardless of the VCG of isolates. Thus, individual gene genealogies provided very little resolution at the VCG level. The partition homogeneity test (PHT) for congruence among phylogenetic trees for sequences of *Act*, β-*tub*, *Cal*, *H3*, and ITS, and the *V. dahliae*-specific sequences indicated that trees were not significantly incongruent (*P* = 1). Therefore, data of all sequences were combined and subjected to MP analysis. First, the analysis was done using the *Act*, β-*tub*, *Cal*, *H3*, and the ITS sequences from *V. dahliae* and *V. albo-atrum* isolates. Results grouped isolates of VCG1A, VCG1B, VCG2B[824], VCG4A, and VCG 4B; but placed isolates of VCG2B[334], VCG2A, and VCG6 each in separate clades (Fig. 2A). However, two main clades (lineages I and II) were identified when a similar analysis was done adding the *V. dahliae*-specific sequence to the above genes; lineage II being formed by two closely related subclades (Fig. 2B and C). Furthermore, subgroups differentiated within each clade correlated with VCGs. Lineage I included isolates of VCG1A, VCG1B, and VCG2B[334]. Lineage II comprised isolates of VCG 2B[824] and VCG6 in one subclade and isolates of VCG4B and VCG2A in another. Location of VCG4A isolate 131M in those phylogenies varied among the four most parsimonious trees inferred by MP analysis, of which only two are shown in Figure 2B and C. In one case, VCG4A isolate 131M was placed close to subgroups comprising VCG2A and VCG4B isolates (Fig. 2B) but in another it was placed between lineage I (i.e., VCG1A, VCG1B, and VCG2B[334]) and lineage II (i.e., VCG 2B[824], VCG6, VCG4B, and VCG2A (Fig. 2C).

MP analysis of AFLP fingerprints from 18 *Verticillium* spp. isolates used for DNA sequencing (Table 1) led to inferring an MP phylogenetic tree (Fig. 3A). The tree topology was similar to that obtained from MP analysis of AFLP fingerprints of 90 *Verticillium* spp. isolates (Fig. 1). PHT for congruence of trees derived from analysis of DNA sequences and AFLP fingerprints showed no incongruence between them (*P* = 0.3) and allowed a subsequent phylogenetic analysis using the combined data sets of DNA sequences and AFLP fingerprints (Fig. 3B and C). The isolate of *V. albo-atrum* was excluded from that analysis because it did not amplify the *V. dahliae*-specific sequence. Results from analysis of the combined data of AFLP fingerprints and DNA sequences were similar to those obtained with AFLP data only and also concordant with results obtained with genealogies of all DNA sequences. Thus, the same two evolutionary lineages of *V. dahliae* VCGs could be inferred from analysis of the combined data sets, whereby: lineage I includes isolates of VCG1A, VCG1B, and VCG2B[334]; and lineage II comprises isolates of VCG2B[824], VCG4A, and VCG6 in one subclade, and isolates of VCG4B and VCG2A in another (Fig. 3B and C).

DISCUSSION

In a previous phenetic analysis of AFLP fingerprints from a large collection of *V. dahliae* isolates (14), we concluded that *V. dahliae* is a highly structured, clonal pathogen in which correla-

tion between neutral genetic markers and vegetative compatibility can be used to infer relationships among VCGs. Such inference could not be done at that time because of the type of analysis performed with AFLP data. In the present work, phylogenetic analysis of AFLP fingerprints using 39 profiles from the previous study and 50 new profiles, as well as multiple gene genealogies not included in the earlier study, for the first time: (i) demonstrate evolutionary relationships among *V. dahliae* VCGs; (ii) identify two main evolutionary lineages that comprise different VCGs (i.e., lineage I: VCG1A, VCG1B, and VCG2B[334]; and lineage II: VCG2A VCG2B[824], VCG4A, VCG4B, and VCG6); and (iii) show that VCGs have a monophyletic origin with the exception of VCG2B, that appears to be polyphyletic. Therefore, our results support Leslie's claim that assumptions of fungal isolates in a VCG sharing a common ancestor descent even if they are geographically isolated from one another should be taken with caution (38).

The VCG clonal lineages in *V. dahliae* and evolutionary relationships among them were resolved independently by analyses either of AFLP fingerprints, multiple gene genealogies, or the combined data set of AFLP fingerprints and multiple gene genealogies. AFLP markers are based on the simultaneous analysis of many loci representing the whole genome and bear high potential for phylogenetic studies (20,25,45). However, these markers have not been widely used for phylogenetic inference presumably because they are considered highly homoplasic (18). In our study, the large number of AFLP markers analyzed and the moderate to high bootstrap values supporting the analysis indicate that AFLP fingerprints contained a sufficient phylogenetic signal to be used for the purpose of phylogenetic inferences at the intraspecific level in *V. dahliae*. In other studies, a qualitatively similar phylogenetic signal existed within AFLP fingerprint topologies generated by analyses of AFLP fingerprints and DNA sequences (45). Comparative use of different molecular markers in phylogenetic studies can provide resolution on different temporal scales. Thus, use of nucleotide variation of conserved sequences examines ancient patterns of population divergence (i.e., inter specific level) and AFLP fingerprints resolves population genetic structure on a more recent time scale (i.e., intra specific level). In our study, polymorphism level in five nuclear
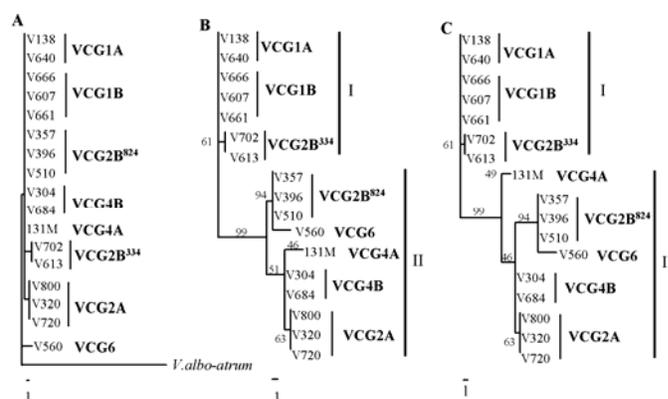
Fig. 2. A, The most parsimonious tree inferred from maximum parsimony analysis using PAUP* of the combined set of sequences of genes actin, β-tubulin, calmodulin, and histone 3, and the ITS2 regions of rDNA from 17 *Verticillium dahliae* isolates representative of different vegetative compatibility groups (VCG). Tree length (TL) = 80, consistency index (CI) = 1, retention index (RI) = 1, rescaled consistency index (RC) = 1. *V. albo-atrum* was used to root the tree. **B and C,** Two of the four most parsimonious trees inferred by maximum parsimony analysis using PAUP of the combined set sequences of the five loci in **A** and a *V. dahliae*-specific sequence (11) (TL = 25, CI = 0.88, RI = 0.963, and RC = 0.847). The *V. dahliae*-specific sequence from isolate V138 was used to root the trees. The trees were inferred using base substitutions and indels. The scale bar below each tree represents a single character change. Bootstrap values (1,000 replicates) are indicated in nodes. I, lineage I; II, and lineage II.

DNA regions (i.e., *actin*, β-*tubulin*, *calmodulin*, and *histone* 3 genes, and the ITS 1 and 2 regions of the rDNA) among isolates of different VCGs was very low and provided little resolution at the VCG level individually; even combined, these makers provided little phylogenetic resolution (Fig. 2A). However, use of the *V dahliae*-specific *seq* sequences amplified by primer pair DB19/DB22 was much more productive than the other nuclear DNA markers and contributed to improved resolution (Fig. 2B and C). Of the four *seq* sequences identified in this work, *seq1*, *seq2*, and *seq3* correlated with the VCG of isolates in agreement with an earlier report (15), indicating that this association is reproducible regardless of the variation in host source and geographic origin. The association of the newly reported sequence *seq7* with VCG4A would need confirmation using a larger set of diverse VCG4A isolates.

Bell (5) proposed that isolates of VCG1 and VCG4 may have evolved from VCG2 isolates. This proposition was based on the fact that VCG2 is distributed widely and VCG2 isolates occasionally establish weak complementation with those of VCG1 or VCG4, yet complementation between VCG1 and VCG4 isolates never occurs. Results of our study would support that hypothesis because each of the two evolutionary lineages identified comprises isolates of VCG2 (i.e., VGC2B^{334} and VCG2B^{824}). However, the evolutionary history of current *V. dahliae* VCGs had not been yet addressed. In filamentous fungi, such as *V. dahliae*, vegetative compatibility is governed by up to 10 *het* loci which most frequently determine allelic systems with two to four alleles per locus (24,38,50). Incompatibility in allelic systems is triggered by co-expression of two different alleles at the same locus, whereas incompatibility in nonallelic systems results from the interaction of two genes belonging to different loci. Assuming asexual reproduction and clonality in *V. dahliae*, and that either genetic system governs vegetative compatibility of this fungus, two simple mechanisms are possible to evolve new VCGs. First,

TABLE 1. *Verticillium* isolates used in this study with vegetative compatibility grouping (VCG), previous reference, host source, geographic origin, and type of *V. dahliae*-specific sequence amplified

VCG[r]	Isolate[s]	Ref.[t]	Host	Geographic origin[u]	V. dahliae-specific sequence type[v]
			Source		
V. dahliae					
VCG1A	**V138I**[wx]	37	*Gossypium hirsutum*	Spain	4[y]
	V181I[z]				4
	V807I (301 v-1), V808I (360 v-1)		*G. hirsutum*	Greece	Ns
	V659I (304 v-1), V641I (313 v-1), V663I[w] (332 v-1), V809I (362 v-1)		*G. hirsutum*	Greece	4
	V640I[wx] (307 v-1)	14	*G. hirsutum*	Greece	4
	V669I[w] (406 v-1)	14	*G. hirsutum*	Turkey	4
	V781I, 3F21I, 17F50I		*Olea europaea*	Spain	Ns
	1F20I		*O. europaea*	Spain	4
	V314I[w]	14	*O. europaea*	Spain	4
	V403I[w] (V-017)	15	*Cynara cardunculus* var. *scolymus*	Spain	4[y]
VCG1B	**V661I**[wx] (328 v-1), **V666I**[wx] (347 v-1)	14	*G. hirsutum*	Greece	4
	V518I[w] (9-6)	15	*Acer palmatum*	USA	4[y]
	V517I[w] (1990-1)	15	*Cladrastris lutea*	USA	4[y]
	V607I[wx] (R04)	14	*Fraxinus pennsylvanica*	USA	4
VCG2A	V142I[w]	37	*G. hirsutum*	Spain	Ns
	V564I (soil)		*G. hirsutum*	Spain	Ns
	V176I[w], V213I[z]	37	*G. hirsutum*	Spain	2[y]
	V200I[w]	37	*G. hirsutum*	Spain	2
	V320I[wx]	14	*G. hirsutum*	California	2
	V152I[w], **V800I**[wx]	14	*O. europaea*	Spain	2
	V144I[z]		*O. europaea*	Spain	2
	V145I[w]	14	*O. europaea*	Spain	Ns
	V720I[wx] (V39)	14	*O. europaea*	Italy	2
	V161I		*Solanum tuberosum*	Spain	2
	V488I (pt71)	15	*S. tuberosum*	Israel	Ns
	V490I (pt44.3G)	36	*S. tuberosum*	Israel	2
	V489I (pt30.2G)		*S. tuberosum*	Israel	Ns
	V506I (tom1)	36	*Lycopersicon esculentum*	Israel	2
	V508I (tom18)	15	*L. esculentum*	Israel	Ns
	V495I (ep4), V496I (ep8), V497I (ep30)	36	*Solanum melongena*	Israel	Ns

(Continued on next page)

[r] Vegetative compatibility was determined using *nit* mutants according to Korolev and Katan (35). VCG assessment in previous studies (6,14,15,28,36,37) or carried out by other authors as unpublished results (ur) (R. Rowe; N. Korolev, J. Katan, R. M. Jiménez-Díaz, and T. Katan). HSI, heterokaryon self incompatible; 2Ba, artichoke isolates able to complement with other artichoke isolates assigned to VCG2B but not with the international VCG testers; Nd, not determined.

[s] Isolate reference code according to IAS Collections. In brackets, code assigned to these isolates in previous studies or by the supplier.

[t] Reference of studies where these isolates were assigned to VCG. Isolates where no reference is given were assigned to VCG in this present study.

[u] Isolates from Greece provided by E. Paplomatas, Agricultural University of Athens, Athens, Greece; artichoke and almond isolates provided by J. Armengol, Universidad Politécnica de Valencia, Valencia, Spain; Isolates from Israel and VCG1B isolates from USA provided by T. Katan, The Volcani Center, Bet Dagan, Israel; Isolates from UK, California, and Brazil provided by D. Barbara, Warwick HRI, England; VCG4A isolates 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.

[v] *V. dahliae*-specific sequence amplified in polymerase chain reaction assays using primer pair DB19/DB22 (11). 1, seq1 (527 pb); 2, seq2 (526 pb); 3, seq3 (526 pb); 4, seq4 (542 pb); 5, seq5 (15); 7, seq7 (541 pb). Ns, not sequenced; –, there was no amplification.

[w] Isolates in which amplified fragment length polymorphism (AFLP) patterns were obtained by Collado-Romero et al. (14).

[x] Bold isolates used for sequencing and phylogenetic analysis of: actin, tubulin, calmodulin, and histone 3 genes; internal transcribed sequences of nuclear ribosomal RNA genes; and *V. dahliae*-specific DNA sequence (11).

[y] Sequenced by Collins et al. (15).

[z] Isolates that were not used for AFLP analysis in this work.

mutations might occur in a *het* locus that determine incompatibility with the parent isolate but allow formation of stable heterokaryons with its progeny. Alternatively, mutations might take place in genes that do not exist to limit heterokaryosis and thus cannot be considered *het* loci (50), but are representative of genes for which polymorphisms can bring about allelic variants whose co-expression determines deleterious effects. These two mechanisms would explain the appearance of monophyletic VCGs; i.e., once a new VCG becomes established others could be generated either directly from it or indirectly from those derived afterward once they become more or less differentiated.

Once several *V. dahliae* VCGs develop, new combinations of *het* loci could potentially develop as a result of parasexual recombination between isolates of different VCGs (26). The existence of 'bridging isolates' in *V. dahliae*, reportedly compatible with isolates from different VCGs (4,33), would offer oppor-

tunities for the development of heterokaryons and transient diploidy. However, our results cannot conclude whether parasexuality and generation of new *het* loci combinations have happened since in our study gene genealogies provided little resolution between VCGs.

VCG2B is a rather heterogeneous group of *V. dahliae* isolates in morphological and physiological traits as well as in host range and virulence (28,33,36,37). Such heterogeneity could be associated with VCG2B polyphyly that suggests multiple independent origins of the set of *het* loci determining vegetative compatibility among isolates of that VCG. If changes in those loci were a common phenomenon, it might be expected that new *V. dahliae* VCGs would appear rather frequently. In reality, the VCG of a *V. dahliae* isolate is rather stable since, in general, an isolate does not change the ability to establish vegetative complementation with isolates of other VCGs (33,47). Acquisition of a common set

TABLE 1. (*Continued from previous page*)

| VCG[r]	Isolate[s]	Ref.[t]	Source — Host	Source — Geographic origin[u]	*V. dahliae*-specific sequence type[v]
VCG2A	V498I (ep53)	15	*S. melongena*	Israel	Ns
	V404I[z] (V-018)	28	*C. cardunculus* var. *scolymus*	Spain	2[z]
	V398I		*Cucurbita pepo*	Spain	Ns
	V394I		*Cucumis melo*	Spain	Ns
	V121I		*Citrullus lanatus*	Spain	Ns
	V556I (128)		*Brassica oleracea* var. *italica*	Germany	2
	V562I (JB84015a)		Unknown	Japan	Ns
VCG2B	**V357I**[wx] (JY)	14	*G. hirsutum*	China	1
	V649I[w] (361 v-1)	14	*G. hirsutum*	Greece	Ns
	V282I[w] (cot67)	36	*G. hirsutum*	Israel	1
	V302I (cot117)				1[y]
	V510I[w] (tom20)	36	*L. esculentum*	Israel	1
	V511I (tom28)	15	*L. esculentum*	Israel	Ns
	V499I (ep1)	15	*S. melongena*	Israel	Ns
	V500I (ep22)				1
	V594I[w], **V613I**[wx], V700I[z]	28	*C. cardunculus* var. *scolymus*	Spain	4
	V396I[wx] (V-008)	15	*C. cardunculus* var. *scolymus*	Spain	1[y]
	V479I[w], V593I[w]	28	*C. cardunculus* var. *scolymus*	Spain	1
	V552I (332)		*Fragaria ananassa*	England	1[y]
	V554I (MD71)	15	*Matricaria chamomilla*	Germany	1[y]
	V555I (MD124)				1
	V390I[z] (V-001)	15	*Prunus amigdalus*	Spain	4[y]
	V408I[z] (V-022)	15	*P. amigdalus*	Spain	1[y]
	V258I (CECE)		*Cicer arietinum*	Italy	Ns
	V563I (JC84160a)		Unknown	Japan	1
2Ba	**V702I**[wx]	28	*C. cardunculus* var. *scolymus*	Spain	4
VCG4A	**131-M**[x], 171-4A[z]	31, ur	*S. tuberosum*	USA	7
VCG4B	V192I[w]	37	*G. hirsutum*	Spain	Ns
	V160I[w] (cot24), V275I (cot23)	36	*G. hirsutum*	Israel	Ns
	V279I[w] (cot40), V294I[z] (cot92), **V304I**[wx] (cot120)	37	*G. hirsutum*	Israel	2
	V673I (431 v-1)		*O. europaea*	Greece	Ns
	V392I[w], V803I[w]	14	*O. europaea*	Spain	2
	V491I (pt15)	36	*S. tuberosum*	Israel	Ns
	V492I (pt63)	15	*S. tuberosum*	Israel	2
	V494I (pt103.1G)		*S. tuberosum*	Israel	2
	V515I (tom5)	36	*L. esculentum*	Israel	2
	V513I (tom26)	37	*L. esculentum*	Israel	Ns
	V557I (P14)		*L. esculentum*	Brazil	2[y]
	V502I (ep17)	37	*S. melongena*	Israel	2
	V505I (ep47)				Ns
	V503I (ep24)		*S. melongena*	Israel	Ns
	V684I[wx]	28	*C. cardunculus* var. *scolymus*	Spain	2
	V551I (330), V553I (1875)		*F. ananassa*	England	Ns
VCG6	**V560I**[wx] (VdCa83a)	6	*Capsicum annuum*	California	1
	V561I[w] (VdCa147a)				1[y]
HSI	V4I[w]	37	*G. hirsutum*	Spain	2
	V537I[z], V547I[w]	28	*C. cardunculus* var. *scolymus*	Spain	4
	V658I[w] (398 v-1)	14	*O. europaea*	Cyprus	Ns
Nd	V810I (340 v-1)		*G. hirsutum*	Greece	Ns
Nd	V448I (Vd-307)		*L. esculentum*	Italy	1
V. albo-atrum					
Nd	**V048I**[wx] (V-48 I)		*Humulus lupulus*	England	–

of *het* loci that determines vegetative compatibility between two isolates could occur by means of independent mutation events. Alternatively, an isolate might have a regressive change to recover ability of complementing with isolates of an ancestral VCG with which it was previously compatible. In that scenario, our results would suggest that VCG2B[334] isolates probably derived from VCG1A isolates (or from a recent common ancestor) because, as a group, VCG2B[334] is molecularly more similar to VCG1A and VCG1B than to VCG2B[824]. Also, VCG2B[334] isolates have probably emerged in eastern-central Spain because they have not been identified yet in any other place. Polyphyly in some *V. dahliae* VCG subgroups may be important for the control of Verticillium wilt diseases. That some isolates in polyphyletic VCG subgroups have evolved more than once in different places implies that their ecological, physiological, and/or virulence traits might vary across locations as compared with those of original populations.

VCG2B[334] is also rather genetically heterogeneous because it comprises a group of isolates (namely VCG2Br[334]) that are compatible with the international reference VCG2B testers, and another group of isolates (namely VCG2Ba) that did not complement with the reference testers but complemented with VCG2Br[334] isolates. However, VCG2Ba and VCG2Br[334] subgroups could not be molecularly differentiated by Collado-Romero et al. (14); therefore, the loss (or gain) of ability of their isolates to establish vegetative complementation with isolates of the corresponding VCG2B subgroup must be a recent event. The evolutionary history of VCG2B[824] isolates differed from that of VCG2B[334]. VCG2B[824] might have originated from the common VCG ancestor and evolved in lineage II together with the recently described VCG6. Isolates of VCG6 probably originated from VCG2B[824] as suggested by molecular similarities and analysis of gene genealogies (Fig. 2B and C). This might have happened in the Salinas Valley (California) where VCG6 recently was identified as a new VCG and both VCG2B and VCG4B have been reported to occur extensively (6). Alternatively, VCG6 might have

originated together with VCG4A as suggested by the combined analysis of AFLP markers and DNA sequences (Fig. 3).

VCG1A, VCG1B, and VCG2B[334] comprised in lineage I were the least related to other VCGs and thus must have diverged prior to differentiation of lineage II. Differentiation between VCG1A and VCG1B was possible only by analysis of AFLP fingerprints which would suggest a recent event of differentiation between them. Furthermore, VCG1B isolates originating from the United States were molecularly differentiated from those originating from Greece; these two groups of isolates were separated in the trees derived from MP analysis of combined AFLP and DNA sequence data (Fig. 3B and C) as well as individual AFLP data (Fig. 1). This would suggest that VCG1B might have originated from VCG1A isolates in North America and Greece independently and thus could have a polyphyletic origin. However, further studies of a larger number of VCG1B isolates representatives from the two locations are needed to confirm that hypothesis.

Lineage II comprised two closely related groups of VCGs, whereby *V. dahliae* isolates of VCG2B[824] and VCG6 were clearly separated from those of VCG2A and VCG4B. *V. dahliae* isolates of VCG4B and VCG2A appear to be closely related but they were not differentiated into two clear subgroups in the analysis of combined data (Fig. 3B and C). These two VCGs were placed in two different subgroups of lineage II by MP analysis of gene genealogies (Fig. 2B and C), but such a differentiation was less clear from AFLP analysis of a larger number of isolates which suggested that VCG2A isolates formed three molecular groups (Fig. 1). These molecular differences could be due to recent evolutionary events and probably are associated with the geographic localization and/or host selection of isolates or homoplasy of AFLP data. This also concerns location of VCG4B isolates from artichoke, that were closer to VCG2A than to other VCG4B isolates when phylogenetic analysis of a large number of isolates was carried out with AFLP data only (Fig. 1), whereas use of gene genealogies supports monophyly of VCG4B
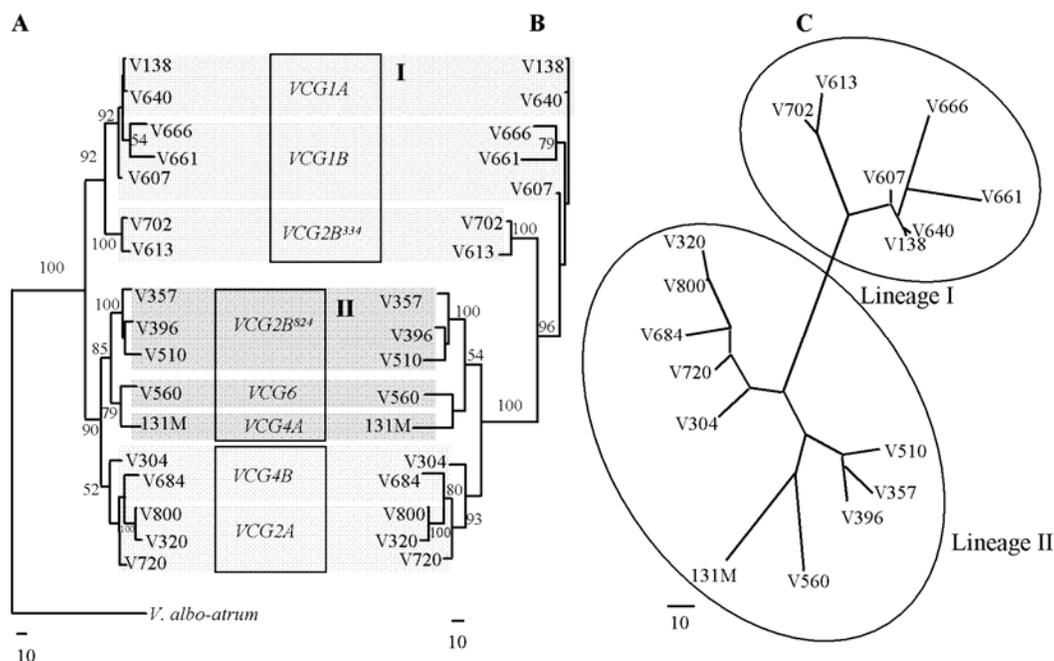
Fig. 3. Comparative analysis between phylogenetic trees inferred by maximum parsimony analysis of **A,** either 578 amplified fragment length polymorphism (AFLP) markers; **B and C,** or the combined data set of AFLP markers and DNA sequences of six loci (genes actin, β-tubulin, calmodulin, and histone 3, ITS2 region of rDNA, and a *Verticillium dahliae*-specific sequence (11). Bootstrap values (200 replicates) are indicated in nodes. I, lineage I; II, and lineage II. The scale bar below each tree represents 10 character changes. **A,** One of eight most parsimonious trees rooted with *V. albo-atrum*; tree length (TL) = 1,038, consistency index (CI) = 0.607, retention index (RI) = 0.656, rescaled consistency index (RC) = 0.398. **B,** One of 10 most parsimonious trees rooted with *V. dahliae* isolate V138 (TL = 378, CI = 0.611, RI = 0.761, and RC = 0.847). Tree in **B** is placed as a mirror image and lined up with tree in **A** to show congruence of the phylogenies estimated from AFLP and combined analysis of AFLP and DNA sequence data. **C,** The same tree as in **B**, but unrooted.

(including artichoke isolate) (Fig. 2B and C). The use of AFLP marker alone could mask phylogenetic relationships among *V. dahliae* VCGs because of homoplasy and comparisons of phylogenies obtained by the two approaches may reveal important relationships.

The phylogenetic relationships of the single VCG4A isolate 131M in the study with other VCGs is uncertain. Thus far, VCG4A has been found only in North America in association with potato crops (48). The VCG4A isolate was placed between lineages I and II or assigned to same subgroup of VCG2A and VCG4B in lineage II by MP analysis of gene genealogies, and assigned to a subgroup with VCG2B[824] and VCG6 in lineage II by MP analysis of AFLP fingerprints (Figs. 2B and C and 3A). Such inconsistency would be in line with isolates of VCG4A sharing some molecular features that differentiate among other VCGs. While isolates of other *V. dahliae* VCGs yielded either the 334- or 824-bp markers in PCR assays using specific primers (14,28), use of these same primers for assays of VCG4A isolates yielded those two amplicons (Collado-Romero, Mercado-Blanco, and Jiménez-Díaz, *unpublished data*). Nevertheless, data from our work do not rule out the possibility of VCG4A isolates were derived from hybridization events between isolates of VCG1A and those in subgroups of lineage II (VCG2B[824] and VCG6, or VCG2A and VCG4B).

In summary, our study demonstrates that a phylogenetic relationship exists between groups of *V. dahliae* VCGs, with most of them being monophyletic and VCG2B being polyphyletic. Moreover, if our study had been limited to results derived from analysis of AFLP fingerprints some interesting conclusions would not have been revealed (e.g., the origin of VCG2B[334] isolates). Similarly, limiting our study to analysis of DNA sequences would have obscured intra-VCG differentiation (e.g., the fingerprint data resolve genotypes within recently evolving clonal lineages as VCG1 and VCG2A that were not resolved by analysis of sequence data of six loci). Therefore, analysis of AFLP fingerprints or DNA sequences or a combination of the two was a highly valuable method for the identification of phylogenetic relationships at the intraspecific level in *V. dahliae*.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Apple, D., and Gordon, T. R. 1995. Intraspecific variation within populations of *Fusarium oxysporum* based on RFLP analysis of the intergenic spacer region of the rDNA. Exp. Mycol. 19:120-128.
2. Apple, D., and Gordon, T. R. 1996. Relationships among pathogenic and nonpathogenic isolates of *Fusarium oxysporum* based on the partial sequence of the intergenic spacer region of the ribosomal DNA. Mol. Plant-Microbe Interact. 9:125-138.
3. Baayen, R. P., O'Donnell, K., Bonants, P. J. M., Cilgenik, E., Kroon, L. P. N. M., Roebroeck, E. J. A., and Waalwijk, C. 2000. Gene genealogies and AFLP analyses in the *Fusarium oxysporum* complex identify monophyletic and nonmonophyletic formae speciales causing wilt and root rot diseases. Phytopathology 90:891-900.
4. Barbara, D. J., and Clewes, E. 2003. Plant pathogenic *Verticillium* species: How many of them are there. Mol. Plant Pathol. 4:297-305.
5. Bell, A. A. 1994. Mechanisms of disease resistance in *Gossypium* species and variation in *Verticillium dahliae*. Pages 225-235 in: Proc. World Cotton Res. Conf.1. G. A. Constable and N. W Forrester, eds. CSIRO, Melbourne.
6. Bhat, R. G., Smith, R. F., Koike, S. T., Wu, B. M., and Subbarao, K. V. 2003. Characterization of *Verticillium dahliae* isolates and wilt epidemics of pepper. Plant Dis. 87:789-797.
7. Bejarano-Alcázar, J., Blanco-López, M. A., Melero-Vara, J. M., and Jiménez-Díaz, R. M. 1996. Etiology, importance, and distribution of Verticillium wilt of cotton in southern Spain. Plant Dis. 80:1233-1238.
8. Bidochka, M. L., Leger, R. J. S., Stuart, A., and Gowanlock, K. 1999. Nuclear rDNA phylogeny in the fungal genus *Verticillium* and its relationship to insect and plant virulence, extracellular proteases and carbohydratases. Microbiology 145:955-963.
9. Carbone, I., Anderson, J. B., and Kohn, L. M. 1999. Patterns of descent in clonal lineages and their multilocus fingerprints are resolved with combined gene genealogies. Evolution 53:11-21.
10. Carbone, I., and Kohn, L. M. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91:553-556.
11. Carder, J. H., Morton, A., Tabrett, A. M., and Barbara, D. J. 1994. Detection and differentiation by PCR of subspecific groups within two *Verticillium* species causing vascular wilts in herbaceous hosts. Pages 91-97 in: Modern Assays for Plant Pathogenic Fungi. A. Schots, F. M. Dewey, and R. Oliver, eds. CAB International, Oxford.
12. Coleman, A. W. 2003. ITS2 is a double-edged tool for eukaryote evolutionary comparisons. Trends Genet. 19:370-375.
13. Chen, W. 1994. Vegetative compatibility groups of *Verticillium dahliae* from ornamental woody plants. Phytopathology 84:214-219.
14. Collado-Romero, M., Mercado-Blanco, J., Olivares-García, C., Valverde-Corredor, A., and Jiménez-Díaz, R. M. 2006. Molecular variability within and among *Verticillium dahliae* vegetative compatibility groups determined by fluorescent amplified fragment length polymorphism and polymerase chain reaction markers. Phytopathology 96:485-495.
15. Collins, A., Mercado-Blanco, J., Jiménez-Díaz, R. M., Olivares, C., Clewes, E., and Barbara, D. J. 2005. Correlation of molecular markers and biological properties in *Verticillium dahliae* and the possible origins of some isolates. Plant Pathol. 54:549-557.
16. Collins, A., Okoli, C. A. N., Morton, A., Parry, D., Edwards, S. G., and Barbara, D. J. 2003. Isolates of *Verticillium dahliae* pathogenic to crucifers are of at least three distinct molecular types. Phytopathology 93:364-376.
17. Correll, J. C., Klittich, C. J. R., and Leslie, J. F. 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. Phytopathology 77:1640-1646.
18. Deprés, L., Gielly, L., Redoutet, B., and Tabarlet, P. 2003. Using AFLP to resolve phylogenetic relationships in a morphologically diversified plant species complex when nuclear and chloroplast sequences fail to reveal variability. Mol. Phylogenet. Evol. 27:185-196.
19. Fahleson, J., Hu, Q., and Dixelius, C. 2004. Phylogenetic analysis of *Verticillium* species based on nuclear and mitochondrial sequences. Arch. Microbiol. 181:435-442.
20. Fahleson, J., Lagercrantz, U., Hu, Q., Stevenson, L. A., and Dixelius, C. 2003. Estimation of genetic variation among *Verticillium* isolates using AFLP analysis. Eur. J. Plant Pathol. 109:361-371.
21. Farris, J. S., Källersjö, A. G., Kluge, A. G., and Bult, C. 1995. Testing significance of incongruence. Cladistic 10:315-319.
22. Garzón, C. D., Geiser, D. M., and Moorman, G. W. 2005. Amplified fragment length polymorphism analysis and internal transcribed spacer and *cox*II sequences reveal a species boundary within *Pythium irregulare*. Phytopathology 95:1489-1498.
23. Glass, N. L., and Donaldson, G. C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microbiol. 61:1323-1330.
24. Glass, N. L., and Kaneko, I. 2003. Fatal attraction: Nonself recognition and heterokaryon incompatibility in filamentous fungi. Eukaryot. Cell 2:1-8.
25. Groenewald, S., Van den Berg, N., Marasas, W. F. O., and Vijloen, A. 2006. The application of high-throughput AFLP's in assessing genetic diversity in *Fusarium oxysporum* f. sp. *cubense*. Mycol. Res. 110:297-305.
26. Hastie, A. C. 1981. The genetics of conidial fungi. Pages 511-543 in: Biology of Conidial Fungi Vol. 2. G. T Cole and W. B Kendrick, eds. Academic Press, New York.
27. Huelsenbeck, J. P., Bull, J. J., and Cunningham, C. W. 1996. Combining data in phylogenetic analysis. Trends Ecol. Evol. 11:152-158.
28. Jiménez-Díaz, R. M., Mercado-Blanco, J., Olivares-García, C., Collado-Romero, M., Bejarano-Alcázar, J., Rodríguez-Jurado, D., Giménez-Jaime, A., García-Jiménez, J., and Armengol, J. 2006. Genetic and virulence diversity in *Verticillium dahliae* populations infecting artichoke in eastern-central Spain. Phytopathology 96:288-298.

29. Jiménez-Gasco, M. M., Milgroom, M. G., and Jiménez-Díaz, R. M. 2002. Gene genealogies support *Fusarium oxysporum* f. sp. *ciceris* as a monophyletic group. Plant Pathol. 51:72-77.
30. Joaquim, T. R., and Rowe, R. C. 1990. Reassessment of vegetative compatibility relationships among strains of *Verticillium dahliae* using nitrate-nonutilizing mutants. Phytopathology 80:1160-1166.
31. Joaquim, T. R., and Rowe, R. C. 1991. Vegetative compatibility and virulence strains of *Verticillium dahliae* from soil and potato plant. Phytopathology 81:552-558.
32. Jukes, T. H., and Cantor, C. R. 1969. Evolution of protein molecules. Pages 21-132 in: Mammalian Protein Metabolism. H. H. Munro, ed. Academic Press, New York.
33. Katan, T. 2000. Vegetative compatibility in populations of *Verticillium*–An overview. Pages 69-86 in: Advances in Verticillium: Research and Disease Management. E. C. Tjamos, R. C. Rowe, J. B. Heale, and R. D. Fravel, eds. American Phytopathological Society Press, St. Paul, MN.
34. Koike, S. T., Subbarao, K. V., Davis, R. M., Gordon, T. R., and Hubbard, J. C. 1994. Verticillium wilt of cauliflower in California. Plant Dis. 78:1116-1121.
35. Korolev, N., and Katan, T. 1997. Improved medium for selecting nitrate nonutilizing (*nit*) mutants of *Verticillium dahliae*. Phytopathology 87:1067-1070.
36. Korolev, N., Katan, J., and Katan, T. 2000. Vegetative compatibility groups of *Verticillium dahliae* in Israel: Their distribution and association with pathogenicity. Phytopathology 90:529-566.
37. Korolev, N., Pérez-Artés, E., Bejarano-Alcázar, J., Rodríguez-Jurado, D., Katan, J., Katan, T., and Jiménez-Díaz, R. M. 2001. Comparative study of genetic diversity and pathogenicity among populations of *Verticillium dahliae* from cotton in Spain and Israel. Eur. J. Plant Pathol. 107:443-456.
38. Leslie, J. F. 1993. Fungal vegetative compatibility. Annu. Rev. Phytopathol. 31:127-150.
39. McDonald, D. A., and Linde, C. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. Annu. Rev. Phytopathol. 40:349-379.
40. Mercado-Blanco, J., Rodríguez-Jurado, D., Parrilla-Araujo, S., and Jiménez-Díaz, R. M. 2003. Simultaneous detection of the defoliating and nondefoliating *Verticillium dahliae* pathotypes in infected olive plants by duplex, nested polymerase chain reaction. Plant Dis. 87:1487-1494.
41. Mercado-Blanco, J., Rodríguez-Jurado, D., Pérez-Artés, E., and Jiménez-Díaz, R. M. 2001. Detection of the nondefoliating pathotype of *Verticillium dahliae* in infected olive plants by nested PCR. Plant Pathol. 50:609-619.
42. O'Donnell, K., Kistler, H. C., Cigelnik, E., and Ploetz, R. C. 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Proc. Natl. Acad. Sci. USA 95:2044-2049.
43. Pantou, M. P., Strunnikova, O. K., Shakhnazarova, V. Y., Vishnevskaya, N. A., Papalouka, V. G., and Typas, M. A. 2005. Molecular and immuno-chemical phylogeny of *Verticillium* species. Mycol. Res. 109:889-902.
44. Pegg, G. F., and Brady, B. L. 2002. Verticillium Wilts. CAB International, Oxford.
45. Pelser, P. B., Gravendeel, B., and van der Meijden, R. 2003. Phylogeny reconstruction in the gap between too little and too much divergence: the closest relatives of *Senecio jacobaea* (Asteraceae) according to DNA sequences and AFLPs. Mol. Phylogenet. Evol. 29:613-628.
46. Qin, Q.-M., Vallad, G. E., Wu, B. M., and Subbarao, K. V. 2006. Phylogenetic analyses of phytopathogenic isolates of *Verticillium* spp. Phytopathology 96:582-592.
47. Rowe, R. C. 1995. Recent progress in understanding relationships between *Verticillium* species and subspecific groups. Phytoparasitica 23:31-38.
48. Rowe, R. C., and Powelson, M. L. 2002. Potato early dying: Management challenges in a changing production environment. Plant Dis. 86:1184-1193.
49. Saitou, N., and Nei, M. 1987. The neighbour-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.
50. Saupe, S. J., Clavé, C., and Bégueret, J. 2000. Vegetative incompatibility in filamentous fungi: *Podospora* and *Neurospora* provide some clues. Curr. Opin. Microbiol. 3:608-612.
51. Strausbaugh, C. A. 1993. Assessment of vegetative compatibility and virulence of *Verticillium dahliae* isolates from Idaho potatoes and tester strains. Phytopathology 83:1253-125.
52. Strausbaugh, C. A., Schroth, M. N., Weinhold, A. R., and Hancock, J. G. 1992. Assessment of vegetative compatibility of *Verticillium dahliae* tester strains and isolates from California potatoes. Phytopathology 82:61-68.
53. Subbarao, K. V., Hubbard, J. C., Greathead, A. S., and Spencer, G. A. 1997. Verticillium wilt. Pages 26-27 in: Compendium of Lettuce Diseases. R. M. Davis, K. V. Subbarao, R. N. Raid, and E. A. Kurzt, eds. American Phytopathological Society Press, St. Paul, MN.
54. Talboys, P. W. 1960. A culture medium aiding the identification of *Verticillium albo-atrum* and *V. dahliae*. Plant Pathol. 9:58-59.
55. Van de Peer, Y. 2003. Analysis of nucleotide sequences using TREECON. Pages 101-136 in: The Phylogenetic HandBook: A Practical Approach to DNA and Protein Phylogeny. M. Salemi and A. M Vandamme, eds. Cambridge University Press, Cambridge.
56. Volossiouk, T., Robb, E. J., and Nazar, R. N. 1995. Direct DNA extraction for PCR-mediated assays of soil organisms. Appl. Environ. Microbiol. 61:3972-3976.