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Phylogenetic Analyses of Phytopathogenic Isolates of *Verticillium* spp.

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ABSTRACT

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To better understand the genetic relationships between *Verticillium dahliae* isolates from lettuce and other phytopathogenic *Verticillium* spp. isolates from various hosts and geographic locations, the complete intergenic spacer (IGS) region of the nuclear ribosomal RNA gene (rDNA) and the β-tubulin gene were amplified and sequenced. The sequences of the complete IGS region and the β-tubulin gene were used alone and in combination to infer genetic relationships among different isolates of *Verticillium* with the maximum-likelihood distance method. Phylogenetic analyses set sequences into four distinct groups comprising isolates of *V. albo-atrum*, *V. tricorpus*, and *V. dahliae* from cruciferous and noncruciferous hosts. Within the four Verticillium groups, isolates of *V. dahliae* from cruciferous hosts displayed the closest affinity to *V. dahliae* from noncruciferous hosts. Isolates of *V. dahliae* from noncruciferous hosts could be further divided into four subgroups based on sequence similarities within the IGS region. Cross-pathogenicity tests demonstrated that most *Verticillium* isolates were as virulent on other hosts as on their hosts of origin. A phenogram based on the cross pathogenicity of individual isolates resembled those derived from the IGS and β-tubulin sequence comparisons. On the basis of the data presented, the potential origin of some isolates of *V. dahliae* pathogenic on lettuce is proposed.

Additional keywords: cross pathogenicity, host range.

The genus *Verticillium* is extremely heterogeneous and has a broad host range including trees, herbaceous plants, plantation crops, and mushrooms extending from subtropical and tropical regions to cool and warm regions (36). Although the genus has been recently reorganized on the basis of molecular and morphological characters (15,52,58–60), the species infecting plants or living as saprophytes, viz. *Verticillium albo-atrum*, *V. dahliae*, *V. tricorpus*, *V. nigrescens*, *V. nubilum*, and *V. theobromae* are still assigned to *Verticillium* (17–19,36,38). Of the six species, *V. dahliae* and *V. albo-atrum* are the predominant plant pathogens worldwide; whereas the others are considered economically less important (5,18,36).

Phytopathogenic species of *Verticillium* invade the vascular tissues in roots and stems and cause vascular wilt in a large number of economically and agriculturally important plants. So far, nearly 80 plant genera, including more than 410 plant species, have been reported as susceptible to Verticillium wilt worldwide (36). In recent years, some of the plants once considered nonhosts of *Verticillium* spp. have succumbed to Verticillium wilt. More than 60 additional plant species were reported to be infected by *Verticillium* spp. in the world during the past decade (36). In California, sudden outbreaks of Verticillium wilt affected cauliflower in 1990 (25) and lettuce in 1995 (51), both were previously considered nonhosts of *V. dahliae*, even though the pathogen was widely distributed in agricultural soils in California (7,8,25,51).

Lettuce is the principal vegetable crop grown year-round in coastal California. The value of lettuce topped $1.73 billion in California during 2003 (1). To date, isolates of *V. dahliae* recovered from diseased lettuce have shown great morphological, physiological, and pathogenic differences (unpublished data). However, little is known about the genetic relationships among lettuce *V. dahliae* isolates or their relationship to isolates from other hosts. For effective disease management, this information is essential to better understand the distinctiveness and the potential origin of isolates of *V. dahliae* infecting lettuce. In addition, comparison of genetic relationships and cross pathogenicity to isolates from other crops could influence disease management practices, such as crop rotation, to reduce the accumulation of *V. dahliae* microsclerotia in soil (57).

The nuclear ribosomal RNA genes (rDNA) and spacer regions have been extensively used in fungal genetic studies to examine the relationships between closely related genera, species, or isolates of a single species (28). In most phylogenetic studies of *Verticillium* and other related genera, the internal transcribed spacer (ITS) regions of the rDNA have been frequently used (10,13). However, a recent study revealed that intraspecific heterogeneity is greater within the intergenic spacer (IGS) region between rDNA genes (39), and subsequently, the IGS region has been used to discriminate different isolates of *Verticillium* spp. (10). Besides rDNA, other genes are often used in phylogenetic studies as well. The β-tubulin gene has also been quite useful in delineating relationships between fungi (28), including phylogenetic studies of phytopathogenic fungi such as *Fusarium* (32,44) and the fungus-like oomycete *Phytophthora* (26). In addition, phylogenetic analysis based on multiple genes or a combination of DNA sequences has been reported for many fungal species (26,30–33,46), including *Verticillium* (13).

In previous genetic studies of *Verticillium* spp., the relationships among isolates based on the molecular markers sometimes were associated with other properties of isolates, such as geographical origin (34), originating host (34,35), pathotype (9,24, 37,62), and so on. However, other molecular studies are in disagreement with these findings (4,8,27,41).

Isolates of *V. dahliae* usually produce short conidia (2.6 to approximately 5.5 μm long) (10; this study), but a long-spored isolate (approximately twice as long as short conidia) of *V. dahliae* from horseradish was first reported and named *V. dahliae* var. *longisporum* (47,48). Since then, many similar isolates from crucifer hosts have been reported (20,22,40,49,50). Because the ma-

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jority of these long-spored isolates from cruciferous hosts possess nearly double the nuclear DNA content of short-spored isolates (average 1.8-fold greater than that of short-spored isolates) (10,20,22,49), they have also been referred to as diploid (20), near-diploid (22), allodiploid, or amphidiploid (10). On the basis of the differences in morphology, physiology, pathogenicity, and random amplified polymorphic DNA (RAPD) band profiles, Karapapa et al. concluded that long-spored isolates from cruciferous hosts formed a distinct new species and named it *V. longisporum* (22). However, the work of Collins et al. has discouraged the use of the new nomenclature since the full complexity and the derivation of those long-spored isolates are still unknown (10). Some reports indicated that short-spored isolates could be grouped with long-spored isolates at the molecular level (49) and that long-spored isolates were not truly host-specific (8,21,50,56) as previously described (22). Because of the inconsistencies in the results of the above studies with those of Karapapa's (22), the appropriateness of the name *V. longisporum* has been questioned even if it is regarded as a distinct new species (13,56).

Since the genetic relationships among *Verticillium* spp. infecting lettuce and other hosts are unclear and uncertainties and discrepancy still exist in the genetic relationships among isolates of *V. dahliae*, the principal objective of the current study was to determine the genetic relationships among *Verticillium* isolates from lettuce and other crops and determine the potential origin of some isolates of *V. dahliae* infecting lettuce. The second objective was to further understand the genetic relationship between long-spored isolates from cruciferous hosts and other phytopathogenic *Verticillium* spp., and the third objective was to determine the association of molecular groupings among isolates with their pathogenicity and/or host.

MATERIALS AND METHODS

Phytopathogenic isolates of *Verticillium* species. Eighty-eight phytopathogenic isolates of *Verticillium* spp. from different hosts and geographical locations were used in this study (Table 1). Prior to their use, all isolates were purified from single-spored isolates and maintained on potato dextrose agar (PDA) at 4°C or at –20°C as a spore suspension in PDYP broth (24 g of PD broth medium, 2.5 g of yeast extraction, and 5 g of peptone per liter; DIFCO Laboratories, Sparks, MD) with 12% glycerol.

DNA and RNA extraction. DNA and total RNA were extracted from frozen mycelia harvested from liquid cultures. Cultures were established by placing 1.0 ml of spore suspension (approximately 1.0×10^9 conidia) in 100 ml of PDYP broth and incubating in an orbital shaker (25°C and 200 rpm) for 3 to 4 days. Mycelia were harvested by filtration with a vacuum pump and washed with two volumes of distilled water under filtration. The harvested mycelia were then frozen in liquid nitrogen and kept at –20°C (for DNA extraction) or at –80°C (for RNA extraction). One hundred milligrams of frozen mycelia was ground to powder, and DNA was extracted by the method described by Al-Samarrai and Schmid (2) Total RNA was extracted by using the RNeasy plant mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Amplification of IGS region and sequencing. To amplify the complete IGS region of rDNA, two sets of primers, VdIGSF1 and VdIGSR1 and VdIGSF2 and VdIGSR2 (Table 2), were designed on the basis of the rDNA complex sequence of *V. dahliae* (GenBank Accession No. AF104926). Polymerase chain reaction (PCR) amplifications were performed in a PT-200 thermocycler (MJ Research, Waltham, MA) in 25-µl volumes as previously described (8). Amplifications were performed using the following conditions: an initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 65°C for 50 s, and extension at 72°C for 2 min; and a final extension at 72°C for 10 min and then stored at 4°C until used.

PCR products were checked on a 0.8% agarose gel in 0.5× TAE buffer (43), and the fragments were recovered from agarose gel and purified with the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. The purified fragments were then ligated to the pGEM-T easy vector and transformed into JM109 (*E. coli*) competent cells (Promega, Madison, WI) according to the manufacturer's instructions. Four to six positive (white or light blue colonies with expected sizes of insertion [verified via PCR amplification with primers mentioned above]) colonies were picked, plasmid extracted, and digested with *Eco*RI to confirm insert presence. A single cloned insert was selected for sequencing (MCLAB, South San Francisco, CA).

Amplification of the β-tubulin gene and sequencing. Total RNA was used as template to conduct cDNA synthesis. Reverse transcription (RT) and RT-PCR were performed with cMaster RT plus PCR system and cMaster RT kit (Eppendorf, Germany) according to the manufacturer's instructions. The two primers used in RT-PCR were forward primer VdBTF1 and anchored reverse primer oligo(dT)19VN (Table 2). VdBTF1 was designed on the basis of two sequences of expressed sequence tags (ESTs) encoding the β-tubulin protein of *V. dahliae* in the Phytopathogenic Fungi EST Database (clone ID: VD0108G10 and VD0206E08, repectively; http://cogeme.ex.ac.uk) (29). RT-PCR amplifications were performed using an initial denaturation at 94°C for 2 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 68°C for 2 min; and final extension at 68°C for 10 min and then stored at 4°C until used. The amplified RT-PCR product (5 µl) was checked on a 1.0% agarose gel in 0.5× TAE buffer. The expected fragments were recovered, purified, ligated to the pGEM-T easy vector, and transformed into JM109 competent cells as described above. Bacterial colony PCR (http://www.cbs.umn.edu/~kclark/protocols/bactPCR.html) amplification was used to identify the positive colonies with expected cDNA insertions with the primers VdBTF1 and VdBTR1 (Table 2). VdBTR1 was designed on the basis of four conserved sequences of other fungal β-tubulin genes (GenBank Accession Nos. AAA62875, CAE85615, EAA76646, and AF115396). A clone with the cDNA insertion of the expected size was selected for sequencing from both directions.

On the basis of cDNA sequence of the β-tubulin gene of *V. tricorpus* (isolate Ls.442), a reverse primer, VdBTR, and a set of inner primers, VdBTF2 and VdBTR2, were designed to amplify more than 90% of the β-tubulin-coding region using genomic DNA of different *Verticillium* isolates as template (Table 2). A second reversed primer, VdBTFR, was also designed to amplify the full length of the β-tubulin gene of isolate Ls.442 with VdBTF1 (Table 2). PCR amplifications were performed in 25-µl volumes as described above for the amplification of the IGS region except 100 to approximately 150 ng of total genomic DNA was used as template and annealing was at 65°C for 1 min.

PCR products were checked on a 0.8% agarose gel in 0.5× TAE buffer, recovered, and purified as described above. The purified PCR products were either sequenced directly from both strands or cloned as described above prior to sequencing.

Phylogenetic analysis based on DNA sequences. Sequences of the complete IGS region (1,622 to 1,919 bp), the β-tubulin gene (1,674 to 1,790 bp), and the combined sequences of the complete IGS region and partial β-tubulin gene sequences were aligned using DNAman 5.2.2 (Lynnon BioSoft 1994-2001). Since the sequences of the β-tubulin gene at the 3′ end of different isolates showed less variation, a 560-bp region containing four introns at the 5′ end was used to combine with the complete IGS sequence (total length 2,180 to 2,479 bp) to infer the phylogenetic relationships among different isolates of *Verticillium* spp. On the basis of the previous alignments, phylogenetic trees were constructed using the maximum-likelihood distance method implemented in DNAman 5.2.2. The method searches for an estimate of the phylogenetic distance between two sequences with

mutation rates estimated from the actual sequences (16,53). The default parameters (Alpha/Beta ratio = 4, AlphaY/R ratio = 1, and Beta12 ratio default = 1) were used to construct phylogenetic trees. The distance matrices were calculated using the neighbor-joining method (42) and 1,000 bootstrap replicates. The distance tree of isolates of *V. dahliae* was constructed from the distance matrix using the UPGMA method (45), and the distance matrix was built up with observed divergence.

Cross-pathogenicity tests and cluster analysis. Tests were performed on alfalfa, artichoke, bell pepper, cabbage, cauliflower, chili pepper, cotton (cvs. Tamcot, Sphint, and Acala SJ-2), eggplant, mint, lettuce, potato, strawberry, tomato, and watermelon. Seeds or other propagating materials were placed into 200 well-seedling trays with an autoclaved sand/potting soil mix (3:1, vol/vol). Seedlings were maintained on benches in a greenhouse (15 to approximately 25°C, 12-h photoperiod) until inocu-

TABLE 1. Original hosts, geographic locations, and year of isolation along with the lengths of the complete intergenic spacer (IGS) sequences and accession numbers of the sequences of the complete IGS region and the β-tubulin gene of *Verticillium* isolates used in this study

Isolate[a]	Originating host	Location	Year isolated	IGS length (bp)	AN[b] for IGS region	AN for β-tubulin gene
Verticillium dahliae isolates from noncruciferous hosts						
Cs.80	*Cynara scolynus* L. (artichoke)	CA, USA	NK[c]	1721	DQ165201	
Ca.35[d], 59, 61[d], 63, 66	*Capsicum annuum* L. (bell pepper)	CA, USA	1996	1741 ~ 1842	DQ165195 ~ 165197	DQ166864 ~ 166865
Cf.31, 36[d], 40, 45, 56[d]	*C. annuum* L. (chili pepper)	CA, USA	NK	1779 ~ 1842	DQ165198 ~ 165200	DQ166866 ~ 166867
Gh.75[d], 77, 101	*Gopsicum hirsutum* L. (cotton)	CA, USA	NK	1753 ~ 1754	DQ165201 ~ 165203	DQ166868 ~ 166869
Sm.113	*Solanum melongena* L. (eggplant)	MD, USA	1997	1816	DQ165205	DQ166870
Sm.129[e]	*Solanum melongena* L. (eggplant)	CT, USA	1997	1865	DQ165204	
Ls.1, 7, 14, 16, 17, 316, 321, 326, 331, 336, 429, 431, 438, 439, 446, 448, 471, 472, 473, 474, 476	*Lactuca sativa* L. (lettuce)	CA, USA	1995 ~ 2001	1622 ~ 1919	DQ165206 ~ 165226	DQ166871 ~ 166882
Mp.89[f], 95[d,f], 97[d,f], 513[g], 518[g]	*Mentha* × *piperita* L. (mint)	OR, USA	NK, 2002	1622 ~ 1817	DQ165227 ~ 165230	DQ166883 ~ 166884
St.81[h], 91[i], 92[d,i], 93[d,i], 94[d,i], 03193[i]	*Solanum tuberosum* L. (potato)	ID and OR, USA	NK, 2003	1703 ~ 1820	DQ165233 ~ 165235	DQ166885 ~ 166887
Fca.21, 22[d], 23, 27[d], 29	*Fragaria* × *ananassa* Duchesne (strawberry)	CA, USA	NK	1820		DQ166888 ~ 166890
Le.78, 88, 109, 110[d], 112[d]	*Lycopersicon esculentum* Mill. (tomato)	CA, USA	NK	1703 ~ 1865	DQ165236 ~ 165238	DQ166891 ~ 166892
Cv.79, 85, 111	*Citrullus vulgaris* Schrader (watermelon)	CA, USA	1993 ~ 1994	1721 ~ 1820	DQ165239 ~ 165241	DQ166893 ~ 166894
V. dahliae isolates from cruciferous hosts (long-spored isolates)						
Ar.136[j], 138[j], 139[d,j]	*Armoracia rusticana* (horseradish)	IL, USA	1997	1691	DQ165183 ~ 165184	DQ166856 ~ 166857
Boc.74	*Brassica oleracea* var. *capitata* L. (cabbage)	CA, USA	NK	1730	DQ165189	DQ166858
Bob.69[d], 70, 71, 73[d], 127[d]	*B. oleracea* var. *botrytis* L. (cauliflower)	CA, USA	NK and 1997	1730 ~ 1847	DQ165187 ~ 165188	DQ166859 ~ 166860
Bp.1[k], 2[k]		CA, USA	2003	1683 ~ 1730	DQ165190 ~ 165191	DQ166861 ~ 166862
Bno.188[l]	oilseed rape	Germany	1989	1691	DQ165185	DQ166863
V. dahliae isolate from cruciferous host (short-spored isolate)						
Bno.197[m]	oilseed rape	Poland	1989	1717	DQ165186	
V. albo-atrum isolates from alfalfa						
Ms.102[d,n], 103[n], 106[d,n], 107[n], 108[n]	*Medicago sativus* L. (alfalfa)	PA, USA	1986	1846 ~ 1847	DQ165192 ~ 165194	DQ166895 ~ 166897
V. tricorpus isolates from artichoke and lettuce						
Cs.225[d], 234, 236, 456[d]	*Cynara scolynus* L. (artichoke)	CA, USA	1999	1814	DQ165242 ~ 165243	DQ166898 ~ 166900
Ls.183[d], 432, 441, 442, 443	*Lactuca sativa* L. (lettuce)	CA, USA	1997 ~ 2001	1742 ~ 1815	DQ165244 ~ 165247	DQ166901 ~ 166904

[a] Mean of conidial lengths and widths of isolates of *V. dahliae* from noncruciferous hosts, cruciferous hosts, *V. albo-atrum*, and *V. tricorpus* was 5.2 × 2.4 (length × width) μm, 8.3 × 2.9 μm (not including short-spored isolate Bno.197 from oilseed rape from Poland, it's conidial size was 5.1 × 2.4 μm), 5.1 × 2.2 μm, and 6.2 × 2.6 μm, respectively. For each individual isolate, 40 to 50 conidia were measured after incubation on potato dextrose agar (25°C) for 2 weeks.
[b] AN = accession number in the GenBank.
[c] NK = not known.
[d] The sequences of the IGS region or the β-tubulin gene or both were not detected.
[e] Provided by W. H. Elmer, Department of Plant Pathology and Ecology, The Connecticut Experiment Station, CT, USA.
[f] Provided by K. Johnson, Department of Botany and Plant Pathology, Oregon State University, OR, USA.
[g] Provided by M. L. Putnam, Department of Botany and Plant Pathology, Oregon State University, OR, USA.
[h] Provided by W. C. Schnathorst, University of California, Davis, CA, USA.
[i] Provided by M. L. Powelson, Department of Botany and Plant Pathology, Oregon State University, OR, USA.
[j] Provided by D. M. Eastburn, Department of Plant Pathology, University of Illinois, IL, USA.
[k] Provided by H. Scheck, Agricultural Commissioner's Office, Weights and Measures, County of Santa Barbara, CA, USA.
[l] Provided by K. Zeise, Institute of Phytomedicine, University of Rostock, Germany.
[m] Provided by J. B. Heale, Microbial Physiology, Division of Life Sciences, King's College London, Kensington Campus, UK.
[n] Provided by B. W. Pennypacker, Department of Agronomy, The Pennsylvania State University, PA, USA.

lation. Inoculum from conidial suspensions was prepared as previously described (8) and adjusted to a concentration of 1×10^7 conidia/ml. The cleaned roots of 4-week-old seedlings were dipped into a conidial suspension of each isolate. For each isolate, 5 to 10 seedlings were dipped in each inoculum suspension (50 ml) for 30 min and then transplanted in 16-oz. foam-insulated cups filled with pasteurized sand/potting soil mix. Seedlings dipped in sterile distilled water were used as controls. Plants were arranged in a randomized block design on greenhouse benches.

After 6 to 10 weeks of incubation in the greenhouse, all plants were gently uprooted, washed free of sand and potting soil, and cut longitudinally along the main root and crown of each plant to visually assess the extent of vascular discoloration, in addition to foliar symptoms, as an indicator of disease severity. A 0 to 5 scale was adopted to assess vascular discoloration and foliar symptoms in which 0 = no vascular discoloration, 1 = 1 to 25% vascular area discolored, 2 = 26 to 50% vascular area discolored, 3 = 51 to 75%, 4 = 76 to 100% vascular area discolored, and 5 = 100% vascular area discolored with foliar symptoms (angular chlorosis, defoliation, stunting, and wilting). Isolates causing a mean rating of ≥2 on a certain host were considered pathogenic (8). The experiment was repeated twice. The pathogenicity of isolates on different plant hosts formed a data matrix, and cluster analysis with the matrix was performed using the CLUSTER procedure of SAS (Version 9.1; SAS Institute Inc., Cary, NC) to analyze the cross-pathogenicity relationships among the isolates.

RESULTS

Sequencing of the complete IGS region and phylogenetic analysis. The complete IGS region ranging from 1.62 to 1.92 kb long was sequenced from 65 *Verticillium* isolates (Table 1). The *Eco*RI-digestion pattern of the IGS region varied among isolates, however, the digestion patterns did not differ for clones of the same isolate (data not shown). The pairwise sequence identity for the IGS region ranged from 76.1 to 99.9% between isolates within the same species and 60.2 to 83.8% between isolates of different species.

On the basis of the alignment of the IGS region and maximum-likelihood distances, the resulting phylogenetic tree clustered the *Verticillium* isolates into four main groups; *Vaa*, *Vd*Cr, *Vd*NC, and *Vtr* (Fig. 1). Group *Vaa* included the three isolates of *V. albo-atrum* from alfalfa. Group *Vd*Cr contained only isolates of *V. dahliae* from cruciferous hosts. Group *Vd*NC contained the remaining isolates of *V. dahliae* from several noncruciferous hosts, and group *Vtr* included the six isolates of *V. tricorpus* from lettuce and artichoke. *Verticillium* isolates in group *Vd*Cr shared a closer relationship to isolates in group *Vd*NC than to isolates in group *Vaa* (Fig. 1). Isolates of group *Vtr* were distinct from those of group *Vaa, Vd*NC, and *Vd*Cr.

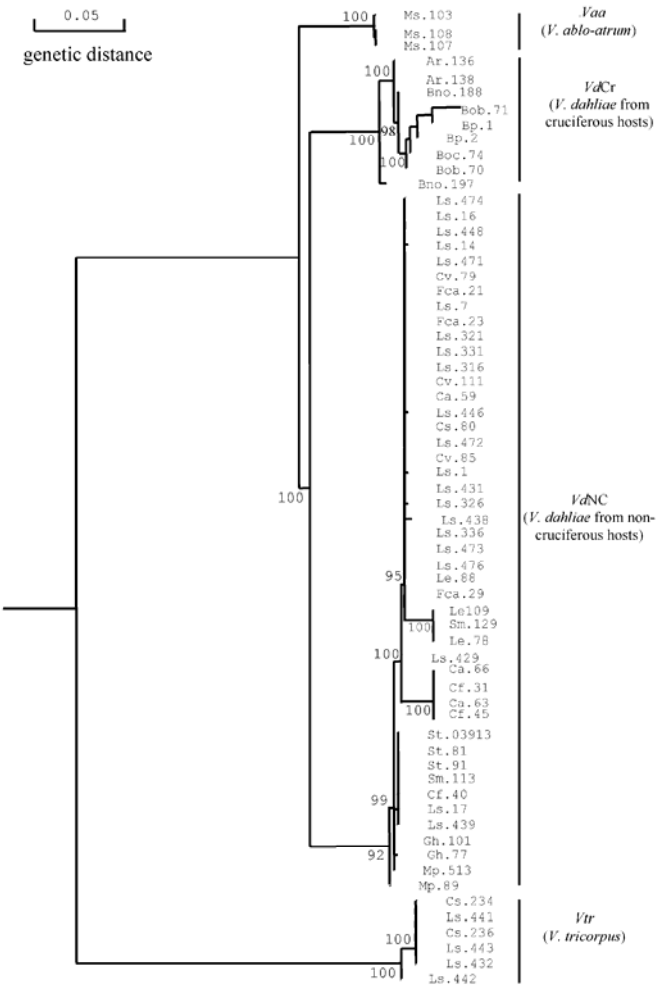

Fig. 1. Phylogenetic tree of phytopathogenic *Verticillium* isolates based on nucleotide sequences of the complete intergenic spacer region. The tree constructed with the maximum-likelihood distance method and bootstrap values based on 1,000 replicates are indicated. *Vaa* = *Verticillium albo-atrum*, *Vd*Cr and *Vd*NC = *V. dahliae* from cruciferous and noncruciferous hosts, respectively, and *Vtr* = *V. tricorpus*.

TABLE 2. Primers used for polymerase chain reaction or reverse transcription-polymerase chain reaction amplifications

Primer	Sequence	Used with primer	Amplified DNA fragment	Expected product size (kb)	Reference
VdIGSF1	5′ GGGTCCTGTAAGCAGTAG 3′	VdIGSR1	Intergenic spacer (IGS) region	1.8~2.3	AF104926[a]
VdIGSR1	5′ GAGCCATTCGCAGTTTCG 3′	VdIGSF1	IGS region		
VdIGSF2	5′ AGCCTTGTTGTTACGATCTGC 3′	VdIGSR2	IGS region	1.7~2.2	AF104926[a]
VdIGSR2	5′ ACTACTGGCAGCATCAACCAG 3′	VdIGSF2	IGS region		
VdBTF1	5′ TCAGCTTCCTCAGCCTTCC 3′	VdBTR1	IGS region		VD0108G10[b], VD0206E08[b]
VdBTR1	5′ AGGGAACTCCTCGCGGATC 3′	VdBTF1	β-tubulin gene	0.8	AAA62875[a], CAE85615[a], EAA76646[a], AF115396[a]
VdBTF2	5′ CAAGATCCGCGAGGAGTTC 3′	VdBTR2	β-tubulin gene	0.9	cDNA of the β-tubulin gene in this study
VdBTR2	5′ CATACCCTCACCAGTGTAC 3′	VdBTF2	β-tubulin gene		
VdBTR	5′ TCCTGGTACTGCTGGTACTCC 3′	VdBTF1	β-tubulin gene	1.7~1.8	
VdBTRF	5′ AATCGTGATGCAGTGTCGTAC 3′	VdBTF1	β-tubulin gene	1.8~2.2	
$d(T)_{19}VN$	5′ TTTTTTTTTTTTTTTTTTTVN 3′	VdBTF1	cDNA of the β-tubulin gene	>1.5	

[a] Accession number in GenBank.
[b] Clone ID in the Phytopathogenic Fungi EST Database; website: http://cogeme.ex.ac.uk (29).

Great variation was found among the isolates within group *Vd*Cr. Horseradish isolates Ar.136 and Ar.138 from Illinois, oilseed rape isolates Bno.188 from Germany, and Bno.197 from Poland were distinct from other cruciferous isolates from California (Boc.74 from Chinese cabbage, Bob.70 and Bob.71 from cauliflower, and Bp.1 and Bp.2 from Napa cabbage). In addition, a short-spored isolate, Bno.197 (5.1 × 2.4 μm), was grouped together with the long-spored isolates (8.3 × 2.9 μm) (Table 1; Fig. 1).

Within group *Vtr*, isolate Ls.442 showed the greatest sequence divergence from the other isolates (Fig. 1). Within group *Vd*NC, isolates could be divided further into four subgroups on the basis of sequence similarities within the IGS region (Fig. 2). The majority of the lettuce isolates were grouped together with isolates from strawberry and watermelon to form subgroup *Vd*NC I. Isolates from lettuce in subgroup *Vd*NC I exhibited 88.6 to 99.9% sequence identity with isolates from strawberry and watermelon. Within this subgroup, lettuce isolates Ls.7, Ls.321, and Ls.476, strawberry isolates Fca.21 and Fca.23, and watermelon isolates Cv.79 and Cv.111 displayed high pairwise sequence identity within the IGS region.

Two isolates from lettuce Ls.17 and Ls.439, along with isolate Cf.40 from chili pepper and some isolates from mint, potato, and eggplant clustered into subgroup *Vd*NC III. Within *Vd*NC III, the highest pairwise sequence identity of 99.7% existed between lettuce isolate Ls.17 and chili pepper isolate Cf.40. Sequence identity among isolates from pepper and cotton ranged from 92.9 to 99.9% to form subgroup *Vd*NC II. Two isolates from tomato and one isolate from eggplant formed subgroup *Vd*NC IV with sequence identity ranging from 99.7 to 99.8% (Fig. 2).

Amplification of the β-tubulin gene and phylogenetic analysis. The full-length β-tubulin gene from isolate Ls.442 contained five introns, each marked by intron/exon junction sequences (3) conserved in other filamentous fungi (Fig. 3). The cDNA region encoding the β-tubulin protein from isolate Ls.442 shared as much as 90.0% identity with that of *Neurospora crassa* and *Pestalotiopsis microspora* (GenBank Accession Nos. XM323372 and AF115396, respectively), and the deduced amino acid sequence shared 94.9 and 95.3% identity with the β-tubulin of *Colletotrichum gloeosporioides* f. sp. *aeschynomene* and *N. crassa* (GenBank Accession Nos. AAA62875 and CAE85615, respectively) (data not shown).

Variation in the β-tubulin gene sequences among *Verticillium* isolates within the same species was minimal, for example, the β-tubulin gene of 25 of 26 isolates of *V. dahliae* exhibited 100% identity. However, several deletions, insertions, transitions, and transversions were found among isolates in different species, such as a 19-bp deletion at nucleotide position 49 to 67 shared among isolates of *V. tricorpus* and a 17-bp insertion at nucleotide position 129 to 145 shared among isolates of *V. albo-atrum* (Fig. 3). *V. dahliae* isolates from cruciferous and noncruciferous hosts exhibited the highest identity in the β-tubulin gene sequence, as much as 98.2% versus the 95.1 and 85.3% identity between sequences from *V. dahliae* and *V. albo-atrum* and *V. tricorpus*, respectively. The deduced β-tubulin protein sequence from *V. dahliae* and *V. albo-atrum* were identical and shared 98.2% sequence identity with that of *V. tricorpus*.

A phylogenetic tree constructed on the basis of the β-tubulin gene sequences of 43 *Verticillium* isolates indicated that the isolates could be split into four main groups similar to the phylogenetic tree based on the IGS sequences (Fig. 1). Group *Vaa* contained isolates of *V. albo-atrum*, group *Vd*Cr and *Vd*NC contained isolates of *V. dahliae* from respective cruciferous and noncruciferous hosts, and group *Vtr* contained isolates of *V. tricorpus* (Fig. 4). The relationships among the four *Verticillium* groups based on sequencing of the β-tubulin gene were quite similar to those based on sequences of the IGS region: isolates of *V. dahliae* from cruciferous hosts (group *Vd*Cr) showed a closer affinity to isolates of *V. dahliae* from noncruciferous hosts (group *Vd*NC) than to isolates of *V. albo-atrum* (group *Vaa*); and isolates of *V. tricorpus* formed a distinct group (*Vtr*) separate from the aforementioned three groups (Fig. 4). The high sequence identity of the β-tubulin gene among isolates of *V. dahliae* and *V. albo-atrum* (99.9 to 100%) prevented further subdivision of isolates within these groups. Within the *Vtr* group, isolates could be further divided on the basis of sequence variation (Fig. 4).

Phylogenetic relationships based on combined sequences. Sequences for the IGS region and the β-tubulin gene were obtained from 45 isolates. A dendrogram based on the combined sequences of the IGS region and the β-tubulin gene displayed the differences not only among *Verticillium* species but also isolates within the same species (Fig. 5). Similar to the previous phylogenetic trees based on the sequences from the IGS region and the β-tubulin gene, the *Verticillium* isolates were again divided into the same four main groups, *Vaa*, *Vtr*, *Vd*Cr, and *Vd*NC, spanning a total taxonomic distance of 0.12. (minimum average distance that can separate the four groups). Once again, isolates from cruciferous hosts of *V. dahliae* formed group *Vd*Cr, distinct from the isolates of *V. albo-atrum* (group *Vaa*) and the other isolates of *V. dahliae* from noncruciferous hosts (group *Vd*NC). Group *Vd*Cr still displayed a closer phylogenetic relationship with other isolates of *V. dahliae* in Group *Vd*NC than to the isolates of *V. albo-atrum* in Group *Vaa*, and Group *Vtr* was still distinct from Groups *Vaa*, *Vd*Cr, and *Vd*NC (Fig. 5).

Cross-pathogenicity test and cluster analysis. Fifteen host plants were evaluated for their reactions to different phytopathogenic isolates of *V. albo-atrum* and *V. dahliae* (Table 3). All isolates, including isolates from cruciferous hosts, caused symptoms of root and crown discoloration and foliar wilt on hosts other than the hosts they were originally collected from (Table 3). Isolates of *V. dahliae* from lettuce, strawberry, and watermelon exhibited a similar host range and caused disease on 8 to 10 hosts of the 15

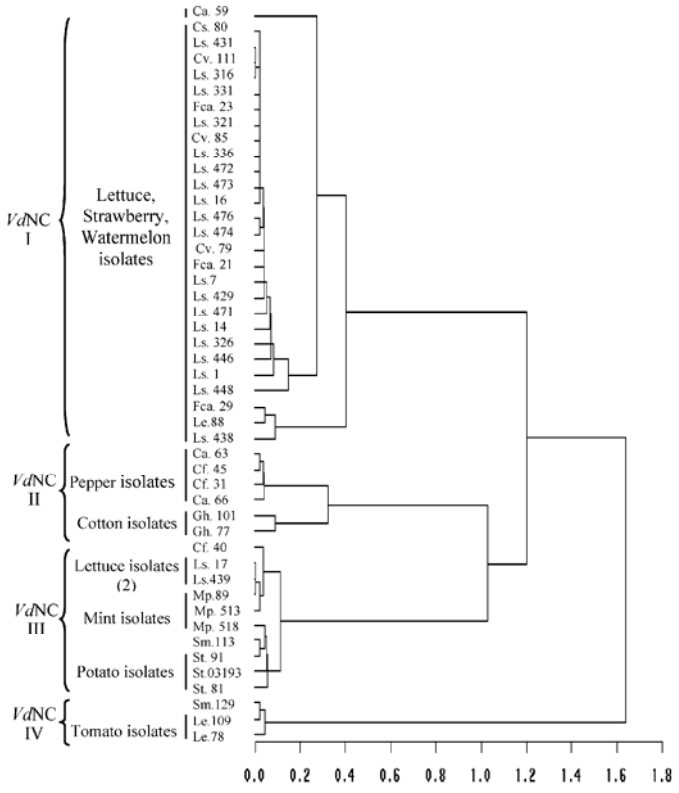

Fig. 2. Dendrogram of isolates of *Verticillium dahliae* from noncruciferous hosts based on the sequence variations of the intergenic spacer region among isolates. Dendrogram generated with distance matrix using the unweighted pair group method of analysis (average linkage joining). *Vd*NC = *V. dahliae* from noncruciferous hosts. Scale is average distance between clusters.

hosts tested. For example, the lettuce isolate Ls.17 was quite virulent toward chili pepper, cotton, eggplant, lettuce, and watermelon, the strawberry isolates Fca.23 and Fca.27 were highly virulent on artichoke, cotton, eggplant, mint, lettuce, potato, and watermelon, while the watermelon isolates Cv.79, Cv.85, and Cv.111 were quite virulent on artichoke, cotton, eggplant, lettuce, potato, strawberry, and tomato (Table 3). Isolates of *V. dahliae* from mint were generally less virulent on hosts other than mint. Most of the isolates of *V. albo-atrum* from alfalfa exhibited high virulence on 5 of the 14 hosts tested (Table 3). The isolates caused root vascular discoloration in more than 80% of plants, some of the plants showed severe wilting and stunting within 3 weeks postinoculation as in cotton and watermelon. The height and biomass (fresh weight and root weight) of plants were significantly reduced (data not shown).

Cluster analysis of the cross pathogenicity of individual isolates indicated that isolates originating from the same host often exhibited a similar host range among the 14 alternative hosts tested.

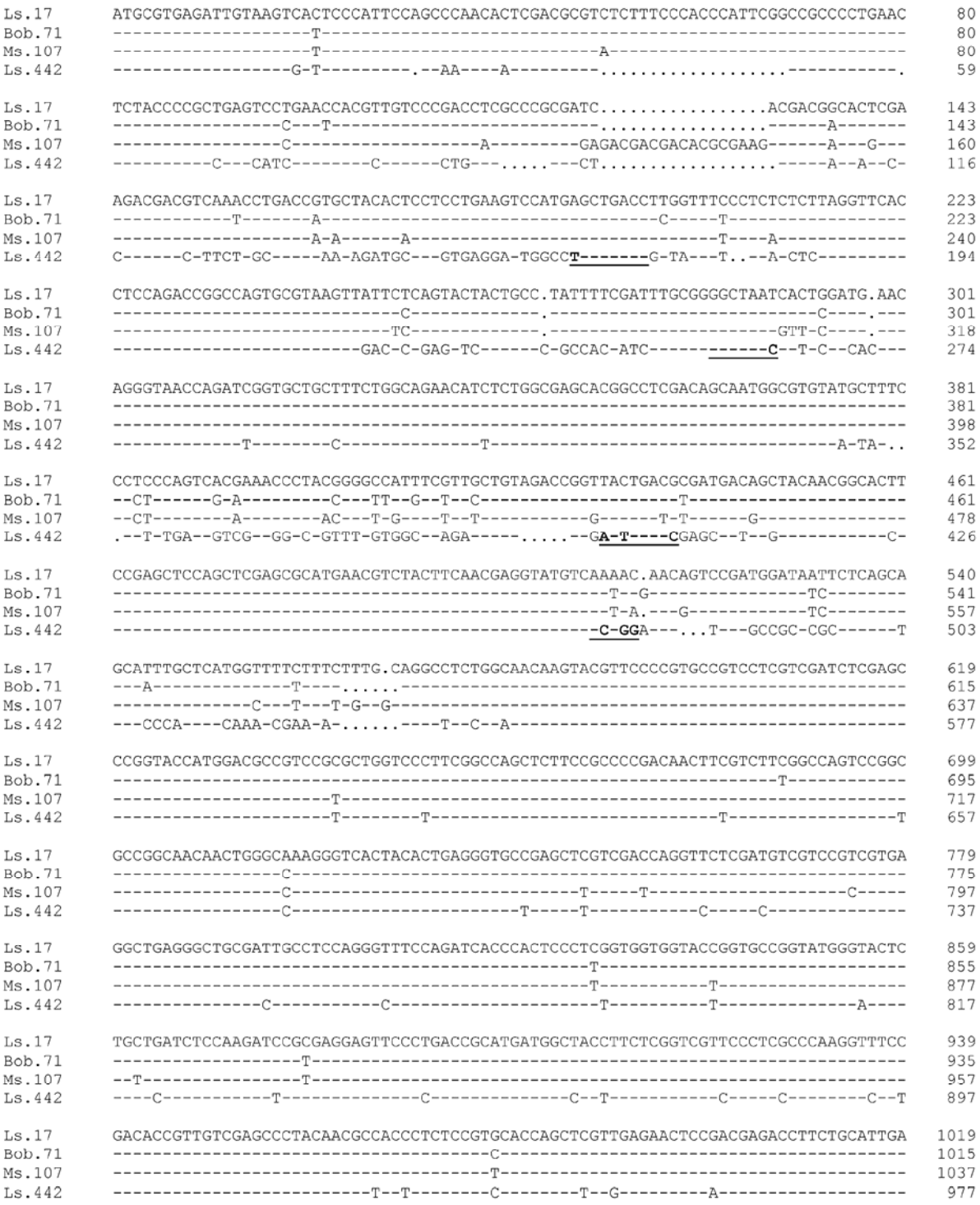

Fig. 3. A comparison of the partial β-tubulin gene sequences from Ls.17 (isolate of *Verticillium dahliae* from lettuce), Bob.71 (long-spored isolate of *V. dahliae* from cauliflower), Ms.107 (isolate of *V. albo-atrum* from alfalfa), and Ls.442 (isolate of *V. tricorpus* from lettuce). Identical bases are indicated by dashes (–) and the absence of bases by dots (…). Some internal conserved intron/exon junction sequences within introns are underlined.

With an average linkage distance of 0.82, the tested isolates clustered into five main groups (Fig. 6). Group *Vaa* contained all the isolates of *V. albo-atrum* from alfalfa; group *Vd*NC_V included isolates of *V. dahliae* from a variety of noncruciferous hosts including lettuce, strawberry, potato, tomato, and watermelon; group *Vd*NC_P consisted of isolates of *V. dahliae* from bell pepper and chili pepper; group *Vd*NC_M included isolates of *V. dahliae* from mint; and group *Vd*Cr consisted of isolates originating from two cruciferous hosts, cabbage, and cauliflower (Fig. 6). The range of pathogenicity within each group of isolates differed, with isolates in Group *Vd*NC_V exhibiting the broadest host range and isolates in Group *Vd*NC_M exhibiting the narrowest host range (Table 3). Within group *Vd*NC_V, isolates could be further subgrouped by their originating hosts with an average distance at 0.48 (Fig. 6).

DISCUSSION

V. dahliae is a pathogen that lacks host specificity, although some isolates are regarded as host-specific (22) or classified as distinct pathotypes (24,37,54). Usually, isolates of *V. dahliae* are considered to be host-adapted rather than host-specific, since isolates have the potential to infect a wide range of hosts but often seem to be most virulent toward certain hosts (11). Great morphological, physiological, pathogenic, and molecular differences were found among isolates from lettuce (this study; K. V. Subbarao, Q.-M. Qin, and G. E. Vallad, *unpublished data*). Isolates from lettuce, strawberry, and watermelon grouped together phylogenetically based on the sequence similarities of the IGS region and the combined sequences of the IGS region and the β-tubulin gene, and also grouped together on the basis of cross pathogenicity. Of the isolates tested, only isolates from lettuce, strawberry, and watermelon exhibited pathogenicity toward each other's host of origin. Previous molecular profiling with RAPD analysis also concluded that an isolate from lettuce (Ls.7) and strawberry (Fca.21) displayed the closest phylogenetic relationship relative to the other host-adapted isolates tested (8). In coastal California lettuce production, fields infested with *V. dahliae* were usually preceded by strawberry, and strawberry isolates were virulent to lettuce. Molecular and pathogenicity data suggest that some lettuce isolates were perhaps derived from strawberry isolates that adapted to lettuce under selection pressure. The results reported in this study may provide clues in regards to the ability of *V. dahliae* to expand its host range; in this case, causing disease on the hitherto nonhost lettuce in 1995. It appears that isolate Cf.40 from chili pepper is the likely progenitor of lettuce isolate Ls.17 because the two isolates exhibited high levels of virulence toward lettuce and chili pepper, similar patterns of cross pathogenicity, and the highest pairwise identity (99.7%) within the IGS region in subgroup *Vd*NC III. The possible genetic relationships among isolates from lettuce and strawberry and chili pepper mentioned above may also explain the increased incidence of Verticillium wilt on pepper in coastal California (6) and the sudden appearance of wilt on lettuce.

Interestingly, recent pathogenicity tests using an expanded collection of lettuce varieties found that pathogenic isolates from subgroup *Vd*NC I and *Vd*NC III also exhibited differential virulence patterns toward the tested varieties, with only isolates in subgroup VdNC III able to cause disease on all varieties tested (55; G. E. Vallad, R. J. Hayes, and K. V. Subbarao, *unpublished data*). While the existence of host-adapted isolates of *V. dahliae*

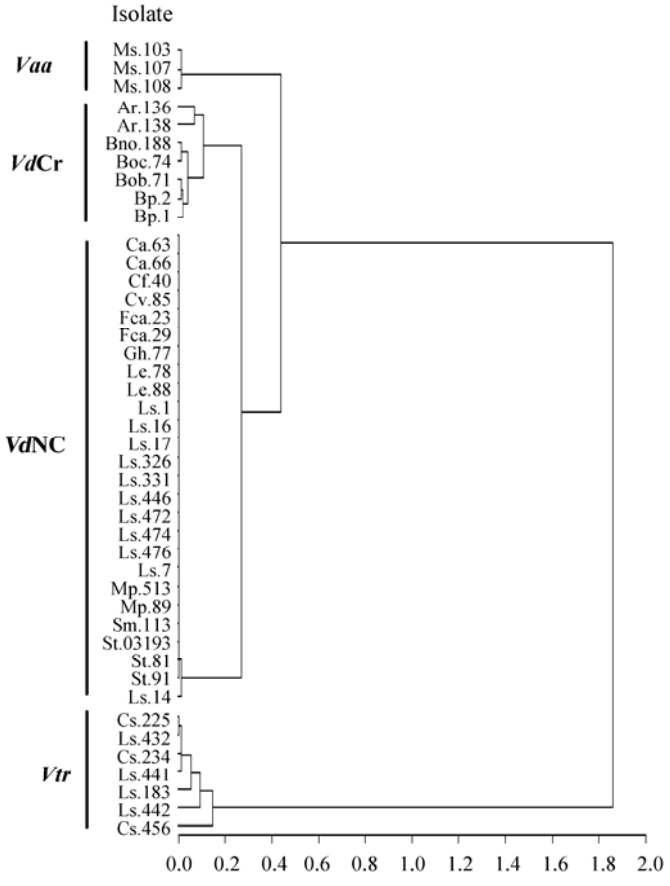

Fig. 4. Phylogenetic tree of *Verticillium* isolates based on the sequences of the β-tubulin gene. Dendrogram generated with distance matrix using the unweighted pair group method of analysis (average linkage joining). *Vaa* = *Verticillium albo-atrum*, *Vd*Cr and *Vd*NC = *V. dahliae* from cruciferous hosts and noncruciferous hosts, respectively, and *Vtr* = V. *tricorpus*. Scale represents the average distance between clusters.

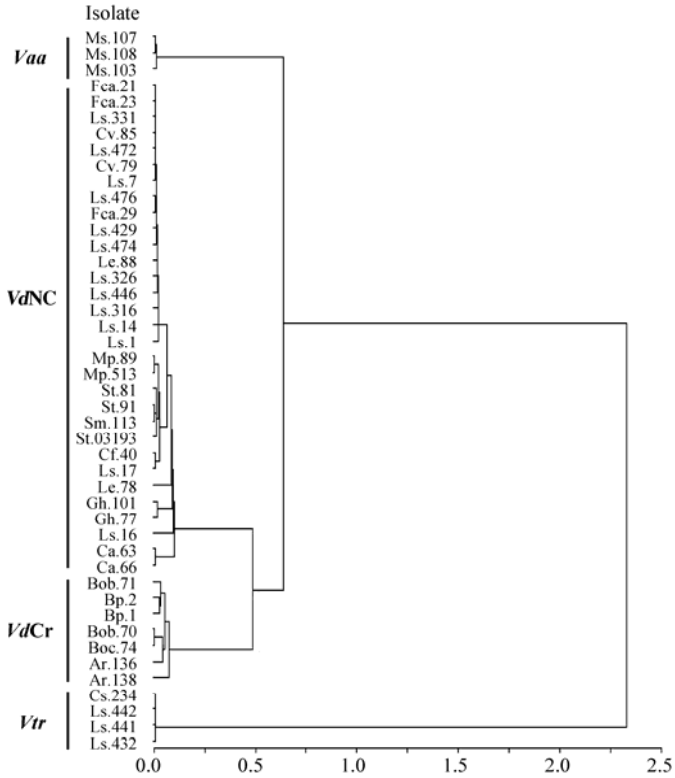

Fig. 5. Dendrogram of *Verticillium* isolates based on the combined sequences of the intergenic spacer region and partial β-tubulin gene. Dendrogram generated with distance matrix using the unweighted pair group method of analysis (average linkage joining). *Vaa* = *Verticillium albo-atrum*, *Vd*Cr and *Vd*NC = *V. dahliae* from cruciferous hosts and noncruciferous hosts, respectively, and *Vtr* = *V. tricorpus*. Scale represents the average distance between clusters.

are reported for some plants, such as horseradish (12) and mint (11), it seems likely that cross pathogenicity and the ability of certain isolates from an established host to adapt to a new host under selection pressure is advantageous to the fungus and possibly explains the sudden appearance of Verticillium wilt in once regarded nonhosts like lettuce.

Both nucleotide and deduced amino acid sequences showed a high degree of similarity among the β-tubulin sequences of *Verticillium* spp. and of other filamentous fungi. To our knowledge, this is the first such study to employ the β-tubulin gene for the phylogenetic analysis of phytopathogenic *Verticillium* spp. Compared with the IGS region, the β-tubulin gene showed less sequence variation among isolates within the same species. Although the variation among the β-tubulin gene sequences was insufficient to differentiate most isolates within the same species, especially among isolates of *V. dahliae* from noncruciferous hosts, it was sufficient enough to easily distinguish isolates from the different *Verticillium* species examined.

The genetic relationships among the phytopathogenic isolates of *Verticillium* spp. inferred from the phylogenetic analysis of sequences of the IGS region and the β-tubulin gene indicated that isolates of *V. dahliae* group *Vd*Cr from cruciferous hosts formed a

TABLE 3. Pathogenicity rating of different isolates of *Verticillium* spp. from various hosts

		Tested hosts[a]															
Isolates	Origin	Alf	Art	BP	Bro	Cab	Cau	CP	Cot[b]	Cot[c]	EP	Mint	Let	Pot	Str	Tom	Wat
Verticillium albo-atrum																	
Ms.102	Alfalfa	5.0[d]	5.0	0.4	0.3	3.8	2.0	3.8	5.0	5.0	3.4	0	0.9	3.8	5.0	0.9	5.0
Ms.103		5.0	3.4	0.8	0	0.6	0.6	3.4	5.0	5.0	1.4	0.4	0.4	4.2	3.2	2.8	5.0
Ms.106		5.0	4.2	0.4	0	0.6	1.0	3.4	5.0	5.0	2.6	0.6	1.4	4.4	3.2	2.6	5.0
Ms.107		5.0	3.0	0.6	0	0.4	0.6	4.0	5.0	5.0	1.6	0.4	0.6	4.6	3.6	2.6	5.0
Ms.108		5.0	3.8	0.4	0	0.2	0.8	3.4	4.6	3.6	2.6	0.2	1.0	3.8	2.8	3.4	5.0
V. dahliae isolates from noncruciferous hosts																	
Cs.80	Artichoke	2.3	5.0	0.3	0.6	3.8	0.6	2.5	5.0	5.0	3.8	3.8	1.8	5.0	3.3	3.8	5.0
Ca.35	Bell pepper	0.8	2.2	5.0	0.6	0.6	0.6	5.0	4.4	1.8	5.0	0.2	1.6	4.6	1.4	2.6	5.0
Ca.59		1.1	3.8	5.0	0.4	0.5	2.3	5.0	1.4	0.6	3.8	0.3	0.4	3.8	3.8	2.3	5.0
Ca.61		1.0	4.2	5.0	0.8	0.2	0.2	5.0	3.4	2.0	5.0	0	3.2	4.2	1.6	3.8	5.0
Ca.63		1.2	3.8	5.0	0.8	0.2	0.6	5.0	5.0	4.4	5.0	0.2	1.0	4.0	2.4	3.4	5.0
Ca.66		1.0	4.8	5.0	0.8	0.4	1.0	5.0	5.0	4.4	5.0	0.2	1.4	5.0	3.2	3.6	5.0
Cf.36	Chili pepper	0.8	4.0	5.0	0.8	0.4	0.8	5.0	5.0	4.0	5.0	1.6	2.8	4.4	1.6	4.0	5.0
Cf.40		0.8	2.8	0.8	0.4	0.2	1.0	4.0	5.0	4.4	5.0	1.0	4.2	4.4	2.0	1.6	5.0
Cf.45		1.2	4.8	5.0	0.6	0.4	0.2	5.0	5.0	4.6	5.0	1.8	1.4	5.0	0.8	4.0	5.0
Cf.56		1.0	4.8	5.0	0.6	0	0.6	5.0	5.0	3.8	5.0	1.0	1.6	5.0	2.8	4.0	5.0
Gh.75	Cotton	0.2	1.4	0.4	0	0	0.2	3.6	4.2	5.0	5.0	0.8	4.0	3.0	1.4	2.0	5.0
Sm.113	Eggplant	1.0	4.5	2.0	0.5	0.3	0.6	3.8	2.2	4.0	5.0	0.3	2.3	5.0	4.5	0.5	5.0
Mp.89	Mint	1.5	3.0	0.8	0.8	1.3	2.0	2.5	5.0	5.0	2.5	5.0	1.5	0.6	3.8	0.1	0.6
Mp.95		0.8	1.2	0.6	0	0.2	0.2	3.6	4.2	2.4	0.8	5.0	0.8	0.8	2.8	0.8	1.0
Mp.97		0.8	2.0	1.4	0	0.6	0.4	3.6	3.6	2.8	0.8	5.0	0.2	0.8	2.4	0	2.0
Ls.1	Lettuce	0	3.0	0	0.4	0.2	0	1.6	5.0	3.4	5.0	0.6	4.0	4.8	0.4	2.0	5.0
Ls.7		1.3	5.0	0.1	0	1.5	0.6	3.8	5.0	5.0	5.0	3.8	5.0	4.5	2.3	1.8	5.0
Ls.14		0	1.6	0.6	0.4	0.4	0.2	2.0	3.6	3.8	4.8	0	4.4	4.2	0.2	3.0	4.2
Ls.16		0.4	4.0	0.4	0.4	0.4	0	1.8	4.0	3.4	5.0	0	4.2	4.4	0	3.8	5.0
Ls.17		0.6	3.6	0.8	0.4	0.4	0.8	4.0	5.0	4.0	5.0	1.8	5.0	4.0	2.8	2.0	5.0
St.81	Potato	2.4	5.0	1.8	0.2	0.4	1.0	4.0	4.8	4.2	5.0	4.2	0.6	5.0	3.6	3.4	5.0
St.91		3.4	5.0	1.0	0.6	2.0	3.8	2.5	3.8	5.0	5.0	3.8	2.5	5.0	5.0	0.6	5.0
St.92		2.2	5.0	2.8	0.6	1.2	1.2	4.0	4.8	3.6	5.0	2.2	1.2	5.0	4.0	3.2	5.0
St.93		2.2	4.6	1.8	0.6	0.2	0.4	4.0	4.2	2.6	5.0	2.4	2.4	5.0	3.8	3.0	5.0
St.94		2.4	4.8	2.2	0.6	0.6	1.2	4.0	4.8	3.0	5.0	1.4	1.2	4.8	2.6	3.0	5.0
Fca.21	Strawberry	0.8	5.0	0	0.3	2.3	0.6	2.5	5.0	5.0	5.0	3.8	1.5	5.0	5.0	3.8	5.0
Fca.22		1.2	4.8	0.4	0.4	0.2	0.2	2.4	5.0	4.6	5.0	3.2	2.8	4.6	2.8	4.0	5.0
Fca.23		1.4	5.0	0.2	0.6	0.8	0	2.2	5.0	5.0	5.0	4.0	4.2	4.4	3.6	3.2	5.0
Fca.27		1.0	5.0	0.2	0.4	0.4	0.6	2.6	5.0	5.0	5.0	4.4	4.2	4.2	3.4	1.2	5.0
Fca.29		1.2	4.6	1.0	0.6	0.6	0	1.6	5.0	4.8	5.0	2.0	3.0	4.6	3.6	1.0	5.0
Le.88	Tomato	3.4	5.0	0.8	0.6	3.8	3.8	3.8	5.0	5.0	5.0	1.3	2.0	5.0	3.8	3.8	5.0
Le.109		2.0	4.6	0.6	0.2	0.6	0.8	2.8	5.0	5.0	5.0	0.6	1.6	4.8	3.8	3.8	5.0
Le.110		1.8	4.8	0.4	0.2	0.2	1.0	3.0	5.0	5.0	5.0	1.4	4.0	5.0	4.2	3.6	5.0
Le.112		2.0	5.0	0.6	0.4	0.6	0.6	3.0	5.0	5.0	5.0	1.2	2.0	5.0	4.0	3.2	5.0
Cv.79	Watermelon	2.6	4.4	1.2	0.2	0.6	0.2	2.8	4.6	4.2	5.0	2.4	4.4	3.8	3.6	4.0	5.0
Cv.85		2.5	5.0	0.8	0.5	3.8	3.8	3.8	5.0	5.0	5.0	3.8	3.4	5.0	3.3	3.8	5.0
Cv.111		2.6	5.0	1.0	0.2	0.2	1.2	2.6	4.4	4.8	5.0	2.2	3.0	4.4	4.2	3.6	5.0
V. dahliae isolates from cruciferous hosts																	
Bob.69	Cauliflower	1.4	0.8	0.6	2.0	4.6	5.0	0.4	4.4	2.2	1.0	0.8	0.8	2.8	3.0	0.6	4.4
Bob.70		2.5	2.0	0.3	3.8	5.0	5.0	2.5	2.2	0.8	0.8	0.4	0.4	3.8	3.8	0.1	3.8
Bob.71		1.0	2.4	0	3.0	4.4	4.8	1.2	2.6	1.0	2.2	0.4	0.2	2.0	2.2	1.0	5.0
Bob.73		2.0	1.5	0	2.3	5.0	5.0	1.3	2.4	0.5	0.6	0	0.3	2.3	3.3	0	3.8
Bob.127		2.6	1.6	0	2.8	4.2	4.8	1.2	3.4	1.6	1.4	0.2	0	2.2	3.6	0	5.0
Boc.74	Cabbage	2.3	3.8	0	2.3	3.8	5.0	1.0	0.6	0.6	0.6	0.3	0	2.0	3.8	0	3.8
Control		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Host Alf, Art, BP, Bro, Cab, Cau, CP, Cot, EP, Mint, Let, Pot, Str, Tom, and Wat represents alfalfa, artichoke, bell pepper, broccoli, cabbage, cauliflower, chili pepper, cotton, eggplant, mint, lettuce, potato, strawberry, tomato, and watermelon, respectively.

[b] Cotton cv. Tamcot Sphint.

[c] Cotton cv. Acala SJ-2.

[d] Mean vascular discoloration of 10 to 20 tested plants. A 0 to 5 scale was adopted to assess disease severity in which 0 = no vascular discoloration, 1 = 1 to 25% vascular area discolored, 2 = 26 to 50% vascular area discolored, 3 = 51 to 75% vascular area discolored, 4 = 75 to 100% vascular area discolored, and 5 = 100% vascular area discolored with foliar wilting. Isolates causing a mean disease severity of ≥2 on a certain host were considered pathogenic.

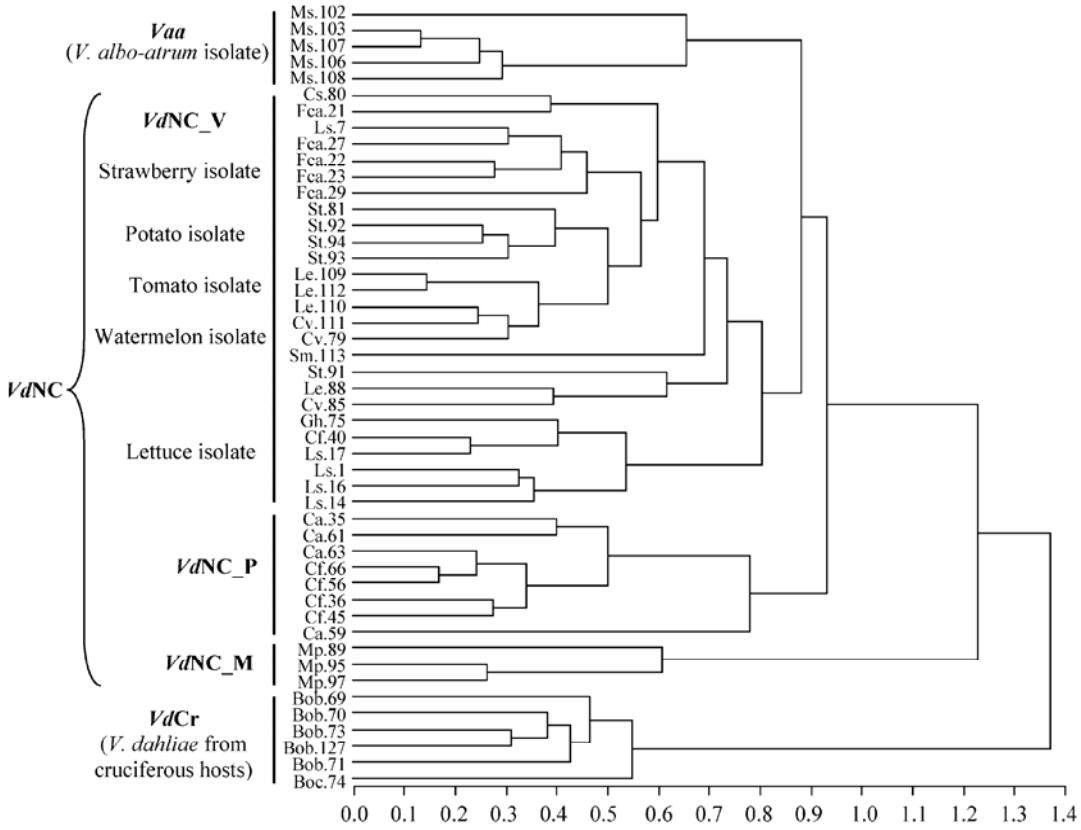

Fig. 6. Phenogram of *Verticillium* isolates constructed from the matrix of pathogenicity of isolates on 15 hosts based on the unweighted pair group method (average linkage joining) method. *Vaa* = *Verticillium albo-atrum*, *Vd*Cr = *V. dahliae* from cruciferous hosts; *Vd*NC_V = *V. dahliae* from noncruciferous hosts (various hosts), *Vd*NC_P = *V. dahliae* from noncruciferous hosts (bell and chili pepper), *Vd*NC_M = *V. dahliae* from noncruciferous hosts (mint), and *Vtr* = *V. tricorpus*. Scale represents the average distance between clusters.

distinct group and shared a closer relationship with the isolates of group *Vd*NC than group *Vaa*. These data are consistent with those of previous reports (10,13,14,22,23,34,49,61). On the basis of the combined partial sequences of the mitochondrial cytochrome gene (*cob*), the mitochondrial small subunit rRNA gene (*rns*), and the nuclear ITS2 region (total 838 bp), Fahleson et al. (13), indicated that isolates of *V. dahliae* from cruciferous hosts and *V. albo-atrum* displayed the closest relationship. However, the cruciferous isolates in our study had a closer relationship to *V. dahliae* than to *V. albo-atrum* based on IGS and β-tubulin gene sequences, which is consistent with studies using a genome-wide approach (10,14,62). This discrepancy may result from the limited variations in the selected and combined sequences to show the real relationships among different isolates. In addition, the isolates of group *Vd*Cr were also distinct from other isolates of group *Vd*NC and group *Vaa* in the phenogram based on their cross pathogenicity among various hosts. Karapapa et al. (22) reported that isolates of *V. dahliae* from cruciferous hosts were host-specific. However, cross-pathogenicity tests in the current study demonstrated that the long-spored isolates of Group *Vd*Cr collected from cabbage and cauliflower in California, were not host-specific and caused severe disease and even death to hosts outside the Brassicaceae family, such as watermelon, cotton, and strawberry. The extended cross pathogenicity observed among the cruciferous isolates of *V. dahliae* is consistent with previous reports (8,21). Interestingly, one of the cruciferous isolates of *V. dahliae* used in this study (Bno.197) was a short-spored isolate originally collected from oilseed rape in Poland. This short-spored isolate also phylogenetically clustered with Group *Vd*Cr along with the other long-spored isolates of *V. dahliae* based on sequence similarity at the IGS region, a result similar to those obtained by Steventon et al. (49). Karapapa et al. (22) concluded that the long-spored isolates of *V. dahliae* (including several long-spored isolates in this study) were a new species, *V. longisporum* comb. nov. Since the full complexity of the long-spored isolates and their derivation is still unclear, the establishment of a new species and the appropriateness of the name *V. longisporum* remain questionable (10,13,21,56). More work is necessary to clarify the genetic relationships of these long-spored isolates with other *Verticillium* spp. before their taxonomic fate is decided.

Comparing the dendrogram derived from sequence data to the phenogram derived from pathogenicity data revealed similarities in the clustering of isolates: in both cases, isolates clustered on the basis of species, and in the case of *V. dahliae*, whether they were derived from cruciferous or noncruciferous hosts. Within Group VdNC, isolates could be further subgrouped by cross pathogenicity or by their host of origin. These results further indicated the existence of genetically distinct subpopulations of *V. dahliae* adapted to specific hosts, but each was cross pathogenic to various crops. Some of the past inconsistencies in the associations between *Verticillium* isolates and molecular subgroupings (4,7,27,41) may have resulted from pathogenicity tests that utilized a limited number of hosts, underestimating the full pathogenic potential of the tested isolates.

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