Morphology, Molecular Identity, and Pathogenicity of *Verticillium dahliae* and *V. longisporum* Associated with Internally Discolored Horseradish Roots

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Abstract

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During 2008 to 2009, 255 isolates of *Verticillium* were obtained from internally discolored horseradish roots collected from California, Illinois, and Ontario. Twenty representative isolates were selected according to morphological features and geographic origin for further characterization. Based on the conidial size, the isolates were divided into two groups: *Verticillium dahliae* ($4.4 \pm 1.23 \mu\text{m}$) and *V. longisporum* ($7.8 \pm 1.76 \mu\text{m}$). Genetic diversity of the isolates was determined by sequence analysis of the internal transcribed spacer (ITS) and two mitochondrial genes (cytochrome oxidase subunit III [*cox3*] and NADH dehydrogenase subunit I [*nad1*]). Based on ITS analysis, *Verticillium* isolates were

divided into two clades: *V. dahliae* and *V. longisporum*. However five isolates of *V. longisporum* (identified based on conidial size) were clustered with a *V. dahliae* clade, whereas the other five isolates formed a distinct *V. longisporum* clade. Combined analysis of the mitochondrial genes *cox3* and *nad1* showed that the two genetic clades of *V. longisporum* in ITS region analysis corresponded to the previously reported *V. longisporum* lineage A1/D3 and A1/D2. Pathogenicity tests revealed that all tested *Verticillium* isolates caused internal discoloration of horseradish roots, and there were no significant differences in either incidence or severity of root discoloration among the genetic groups.

Horseradish (*Armoracia rusticana* Gaertn., Mey. & Scherb.) is a member of the Brassicaceae family and is grown for its white, fleshy, and pungent roots, which are used primarily as a condiment (Babadoost et al. 2004; Courter and Rhodes 1969). The major areas of commercial cultivation of horseradish in North America are California, Illinois, and Wisconsin in the United States and Ontario in Canada (Babadoost et al. 2004; Yu and Babadoost 2013). Approximately 50% of the total horseradish crop in the United States is grown in the Mississippi River Valley, near East St. Louis, IL (Babadoost et al. 2004; Rhodes 1977; Yu and Babadoost 2013). Eau Claire, WI and Tule Lake, CA are two other major horseradish production areas in the United States (Babadoost et al. 2004; Yu and Babadoost 2013).

Internal discoloration of roots occurs worldwide and affects production of horseradish in Illinois as well as other areas (Babadoost et al. 2004; Eastburn and Chang 1994; Potschke 1923; Yu and Babadoost 2013). The disease begins with dark-brown to black discoloration of the vascular system and gradually spreads inward (core) and outward (cortex) (Babadoost et al. 2004; Eastburn and Chang 1994; Yu and Babadoost 2013). Internally discolored horseradish roots are not acceptable for processing and, thus, are discarded (Babadoost et al. 2004; Eastburn and Chang 1994).

Internal root discoloration of horseradish was first observed in Germany in 1860s and was reported by Sorauer (1899). In the United States, internal discoloration of horseradish was first described in 1931 in Michigan, where 20% of the yield was lost due to root infection (Boning 1938). Since the early 1980s, horseradish producers in Illinois have experienced substantial reductions in marketable yield of horseradish due to discoloration and rotting of roots (Atibalentja and Eastburn 1998; Babadoost et al. 2004; Eastburn and Chang 1994). Yield losses of up to 100% due to the internal root discoloration have frequently been reported in Illinois (Babadoost et al. 2004; Yu and Babadoost 2013).

A species of *Verticillium* Nees was reported to be associated with the internally discolored roots of horseradish (Potschke 1923), which was identified as *Verticillium dahliae* Kleb. in both Europe and the United States (Korff and Boning 1934; Mueller et al. 1982). Mueller et al. (1982) reported *V. dahliae* as the primary agent associated with internally discolored horseradish roots in Wisconsin. However, Percich and Johnson (1990) isolated *V. dahliae*, *Fusarium roseum* 'Acuminatum', and *Pseudomonas fluorescens* from internally discolored horseradish roots in Wisconsin and reported the disease as a root rot complex of horseradish. Further, Babadoost et al. (2004) reported that internal discoloration of horseradish root is a disease complex caused by at least three fungal species: *V. dahliae*, *V. longisporum*, and *F. solani*. Finally, Yu and Babadoost (2013) reported that *F. commune* and *F. oxysporum* also cause internal root discoloration of horseradish.

V. dahliae is a well-known soilborne pathogen that causes wilt in a broad range of plant species, including brassicas (Isaac 1957; Koike et al. 1994; Svensson and Lerenius 1987). In 1961, a variant of *V. dahliae* from horseradish was described by Stark that produced conidia approximately twice as long as those of typical *V. dahliae* strains. This variant was named *V. dahliae* var. *longisporum*. Based on distinct morphological, molecular, and virulence characteristics, Karapapa et al. (1997) suggested that this fungus should be classified as a new species, *V. longisporum*. In addition, several researchers have demonstrated molecular differences between *V. longisporum* and *V. dahliae* isolates that support recognition of the newly erected species (Fahleson et al. 2004; Pantou et al. 2005; Zeise and von Tiedemann 2001). However, considerable intraspecific diversity has been identified within isolates of *V. longisporum*, giving rise to a controversy regarding the proper taxonomic status of *V. longisporum* versus *V. dahliae* var. *longisporum* (Barbara and Clewes 2003; Collado-Romero et al. 2010; Collins et al. 2005; Karapapa et al. 1997; Pantou et al. 2005; Zeise and von Tiedemann 2001). Thus, some researchers have questioned the recognition of the new species because of less homogeneity among isolates of the group (Clewes et al. 2008; Collins et al. 2003, 2005; Klosterman et al. 2009).

Recent molecular analyses have suggested that *V. longisporum* is the result of hybridization between *V. dahliae* and *V. albo-atrum* (Clewes et al. 2008; Collins et al. 2003). Based on the phylogenetic analyses of seven nuclear loci and a dataset of 203 isolates of *V. longisporum*, *V. dahliae*, and related species, Inderbitzin et al. (2011a,b)

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reported that *V. longisporum* is an allodiploid hybrid that originated at three separate times from four different lineages representing three different parental species: species A1, species D1, and *V. dahliae*.

Although genetic diversity within and among *Verticillium* spp. from several host species has been elucidated using molecular genetic techniques (Carder and Barbara 1994; Clewes et al. 2008; Inderbitzin et al. 2011a,b; Klosterman et al. 2009; Pantou et al. 2005; Steventon et al. 2002), comprehensive studies on the genetic diversity within and among *Verticillium* spp. from horseradish have not been conducted. The objective of this study was to characterize *Verticillium* spp. associated with horseradish roots showing internal discoloration in California, Illinois, and Ontario using molecular phylogenetic analysis along with morphological grouping and their pathogenicity.

Materials and Methods

Root samples. In total, 496 internally discolored horseradish roots, including 75, 306, and 115 roots from commercial fields in California, Illinois, and Ontario, respectively, were collected. Each root was washed and sectioned at one-third length from the top and one-third from bottom of the root. Roots with visible discoloration at both cross sections were selected for isolating pathogens. Selected roots were tested immediately for the presence of pathogens or stored at 4°C until they were tested within 2 weeks.

Isolation of *Verticillium*. Fungal isolation was carried out as previously described by Babadoost et al. (2004). A root segment was cut

from the middle of each collected symptomatic root. The outer layers (approximately 1 mm) of the segments were peeled to remove fungal and bacterial contaminants on the root surface. The peeled root segments were surface sterilized by immersing roots in a 6% NaClO (full-strength commercial bleach) solution for 1 min, followed by dipping in 95% ethanol for 3 min and rinsing in sterile-distilled water (SDW) three times, each time for 1 min. The segment was then blotted with sterilized blotter paper and cut into 3- to 5-mm pieces. Five pieces from each root were placed onto potato dextrose agar (PDA; Difco, Sparks, MD) with 4 ml of 25% lactic acid in 1,000 ml of culture medium (A-PDA) in petri plates. The plates were then incubated at 22 to 24°C in the dark for 15 days. The plates were examined 5, 10, and 15 days after incubation, and developing fungal colonies were transferred aseptically onto PDA in petri plates. The cultures were incubated under a cycle of 12 h of near-UV light and 12 h of darkness per day for 8 to 10 days and growing colonies were identified following their sporulation. In total, 255 *Verticillium* colonies (each representing one root) were subcultured onto PDA in petri plates until pure cultures were obtained. Based on the morphological features and geographic origin (Table 1), 20 isolates were selected for further studies. Single conidial cultures were prepared from these 20 isolates (Babadoost et al. 2004) and maintained as stock cultures on PDA slants in the dark at 4°C. The reference isolates examined are listed in Table 2 with their National Center for Biotechnology Information GenBank accession numbers.

Morphological characteristics for identification of the isolates. For morphological observations, in total, 20 isolates of *Verticillium* (Table 2) were grown on PDA in petri plates at 25°C in the dark for 20 days. The isolates were identified based on morphological characteristics of conidia and microsclerotia, as described by the previous investigators (Hawksworth and Talboys 1970; Karapapa et al. 1997; Steventon et al. 2002). For conidia, spore suspensions in SDW were prepared from cultures after 7 days and the lengths of 30 conidia from each isolate were measured. Morphological characteristics of microsclerotia of all isolates were examined using light microscopy after growing them on PDA in petri plates for 14 days.

DNA extraction. A small plaque of each isolate was transferred into 150 ml of potato dextrose broth (PDB; Difco) in a 250-ml flask and the flask was placed onto a shaking incubator (150 rpm) at 25°C for 7 to 10 days. Fungal mycelia were recovered from the PDB culture by centrifugation and filtration through sterilized filter paper (Whatman, Maidston, UK). Total DNA was extracted from fungal

Table 1. Frequency of isolation of *Verticillium dahliae* and *V. longisporum* from roots of horseradish cultivars grown in California, Illinois, and Ontario in 2008 and 2009

Location	Cultivar	N ^z	Isolation frequency ^y	
			<i>V. dahliae</i>	<i>V. longisporum</i>
California	Czechoslovakia	45	2	19
	Tule Lake	30	0	15
Illinois	Big Top Western	72	39	8
	1573	234	71	18
Ontario	1590	115	77	6

^y Identification of the species was based on the morphological characteristics of conidia, conidiophores, and microsclerotia of the isolate.

^z Number of roots assayed.

Table 2. Geographic origin (Origin), year of isolation (Year), conidial length (Length; $n = 30$), and GenBank accession numbers for internal transcribed spacer (ITS), cytochrome oxidase subunit III (*cox3*), and NADH dehydrogenase subunit I (*nad1*) sequences of *Verticillium* isolates used in this study

Species	Isolate code	Origin	Year	Length (μm) ^z	GenBank accession numbers		
					ITS	<i>cox3</i>	<i>nad1</i>
<i>Verticillium dahliae</i>	CAV1003	Ontario	2008	4.8 (1.34)	KC834724	KC852087	KC852100
<i>V. dahliae</i>	CAV1004	Ontario	2008	5.0 (1.47)	KC834725	KC852088	KC852101
<i>V. dahliae</i>	CBV1006	Ontario	2008	3.6 (0.86)	KC834726	KC852089	KC852102
<i>V. dahliae</i>	CBV1014	Ontario	2008	4.7 (1.28)	KC834727	KC852090	KC852103
<i>V. dahliae</i>	CBV1046	Ontario	2008	3.5 (1.16)	KC834728	KC852091	HM988737
<i>V. dahliae</i>	SCV 1005	Illinois	2008	5.0 (1.28)	KC834729	KC852092	KC852104
<i>V. dahliae</i>	SCV1010	Illinois	2008	3.6 (0.73)	KC834730	HM804952	HM988738
<i>V. dahliae</i>	SDV1002	Illinois	2008	4.8 (1.34)	KC834731	HM804953	HM988736
<i>V. dahliae</i>	SDV1006	Illinois	2008	5.2 (1.54)	KC834732	KC852093	KC852105
<i>V. dahliae</i>	SDV1025	Illinois	2008	4.3 (1.33)	KC834733	KC852094	KC852106
<i>V. longisporum</i>	CBV2034	Ontario	2008	8.5 (2.18)	KC834714	HM988730	HM988739
<i>V. longisporum</i>	SCV2008	Illinois	2008	8.0 (2.12)	KC834715	KC852082	KC852095
<i>V. longisporum</i>	SDV2041	Illinois	2008	7.4 (1.48)	KC834716	KC852083	KC852096
<i>V. longisporum</i>	SDV2064	Illinois	2008	8.5 (2.09)	KC834717	HM988732	HM988740
<i>V. longisporum</i>	SDV2066	Illinois	2008	8.3 (2.19)	KC834718	KC852084	KC852097
<i>V. longisporum</i>	TAV3001	California	2009	7.0 (1.39)	KC834719	HM988734	HM988743
<i>V. longisporum</i>	TBV3014	California	2009	6.7 (1.51)	KC834720	HM988733	HM988742
<i>V. longisporum</i>	VAV3003	California	2009	7.1 (1.79)	KC834721	HM988735	HM988744
<i>V. longisporum</i>	VAV3021	California	2009	6.8 (1.54)	KC834722	KC852085	KC852098
<i>V. longisporum</i>	VBV3004	California	2009	6.7 (1.26)	KC834723	KC852086	KC852099

^z Values in parentheses are the standard deviations of measurements of conidial length.

mycelia using a Fast DNA Spin kit (MP Bio, Solon, OH), according to the manufacturer's recommended protocol.

Polymerase chain reaction test. A polymerase chain reaction (PCR) test was performed to amplify the internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2) using the primer pair ITS5/ITS4 (GGAAGTAAAGTCGTAACAAGG/TCCTCCGCTTATTGATATGC) (White et al. 1990). Also, two mitochondrial genes, cytochrome oxidase subunit III (*cox3*) and NADH dehydrogenase subunit I (*nad1*), were amplified using primer pairs *cox3A/cox3B* (TGATTAGAGATST AATATCAGAAG/CCGTGGAAACCTGTSCCAAATA) and *nad1A/nad1B* (ATGGCSAGTATGCAAAGAAGA/GCATGTTC TGTCAATAAASCCACT AAC), respectively, following methods developed by Kouvelis et al. (2004) and Pantou et al. (2003). A PCR mixture with a total volume of 50 µl was prepared, which contained 10× PCR buffer, 2.0 mM MgCl₂, 2.5 mM dNTP, 10 µM each primer, 30 ng of *Taq* polymerase (Promega Corp., Madison, WI), and 2 µl of DNA extract. PCR conditions for ITS were initial denaturation at 95°C for 2 min; 35 cycles of denaturation at 94°C for 45 s, annealing at 54°C for 1 min, and extension at 72°C for 1.5 min; followed by a final extension at 72°C for 5 min. The PCR cycles for the two mitochondrial genes were initial denaturation at 94°C for 3 min; 30 cycles of denaturing at 94°C for 1 min, annealing at 48°C for *cox3* primers and 50°C for *nad1* primers for 1 min, and extension at 74°C for 2 min; and final extension at 74°C for 5 min.

PCR was conducted using a PCT-200 thermal cycler (MJ Research Inc., Waltham, MA). All PCR amplifications were verified by electrophoresis with 10 µl of each PCR product in a 1% agarose gel with 1× Tris-acetate-EDTA buffer stained with ethidium bromide. Gels were visualized and documented under UV light.

DNA sequencing and phylogenetic analyses. PCR products were purified using the Wizard SV gel and PCR Clean-Up system (Promega Corp.), according to the manufacturer's protocol. Purified PCR products were sequenced using an automated sequencer at the Core DNA Sequencing Facility of the University of Illinois at Urbana-Champaign. Sequencing was performed using the same forward and reverse primer sets that were used for PCR amplification.

The results of the sequence were first edited using Clustal X (Thompson et al. 1997) and BioEdit version 7.0.9 (Hall 1999), after which the alignment was refined using PHYDIT, version 3.2 (Jeon et al. 2005). All sequences used for phylogenetic analyses were deposited into GenBank (Table 2).

Phylogenetic analyses were performed on PAUP 4.0 10b (Swofford 1998). Ambiguously aligned regions and sequence gaps were treated as missing data and, thus, excluded from analyses. Maximum-parsimony (MP) analysis was estimated using heuristic searches consisting of random addition order and tree bisection-reconnection branch swapping. Maximum-likelihood (ML) analyses were performed with RAXML 7.2.8 HPC BlackBox (Stamatakis. 2006; Stamatakis et al. 2008) using the GTRGAMMA model of nucleotide substitution. Mega v 5.05 (Tamura et al. 2011) was used for editing of the obtained trees. Trees were rooted with *Gibellulopsis nigrescens* used as the outgroup. Parsimony and ML bootstrapping were performed with 1,000 replicates to assess the relative stability of the branches. The information of the reference strains was obtained from the National Center for Biotechnology Information (NCBI) GenBank database (<http://www.ncbi.nlm.nih.gov/>) and listed in Table 3.

Pathogenicity tests. Pathogenicity tests of the isolates were conducted as previously described (Babadoost et al. 2004; Kouvelis et al. 2004). Two horseradish cultivars, '1573' and 'Big Top Western' (BTW), which are susceptible to internal root discoloration (Babadoost et al. 2004; Yu and Babadoost 2013), and two isolates of each species or group of *Verticillium* were used for the pathogenicity tests.

Asymptomatic horseradish roots were collected from commercial fields and the roots were treated at 47°C for 20 min prior to inoculation to eliminate the possible set-borne inoculum (inoculum carried in symptomatic and asymptomatic propagative roots; Eranthodi et al. 2010). Treated roots were then planted in pots (50 by 35 by 10 cm) containing a pasteurized mix of soil-peat-perlite (1:1:1) (Babadoost et al. 2004). The pots were placed in a greenhouse at 20 to 26°C. After 30 days, plants were removed from the pots, soil was gently cleaned from the main root and lateral roots, and the roots were inoculated with one isolate of *V. dahliae* and two isolates of *V. longisporum* separately and also combined together. The experiment was repeated once.

To produce *Verticillium* inoculum, 2-week-old cultures of *Verticillium* spp. on PDA in petri plates were macerated with 15 ml of SDW. The conidial suspension (1 ml) was spread onto the surface of PDA in each petri plate. The inoculated plates were then incubated at 22 to 24°C under a cycle of 12 h of fluorescent light and 12 h of darkness per day for 3 weeks. The cultures were then blended with 200 ml of distilled water (DW) at high speed for 30 s. The resulting suspension was passed through a series of 500-, 180-, and 38-µm sieves. Material (microsclerotia) caught on the 38-µm sieve was rinsed with DW

Table 3. Reference strains of *Verticillium* spp. and *Gibellulopsis nigrescens* (with their GenBank accession numbers) used for phylogenetic analyses

Species	Isolate code	Host, substrate	Origin ^z	GenBank accession numbers ^y		
				ITS	<i>cox3</i>	<i>nad1</i>
<i>Verticillium dahliae</i>	76	Cotton	Greece	–	AY555919	AY555975
<i>V. dahliae</i>	M5	Egg plant	Moldova	AY555948	AY555921	AY555977
<i>V. dahliae</i>	PD332	Lettuce	California	HQ206718	–	–
<i>V. dahliae</i>	PD727	Horseradish	Illinois	HQ206917	–	–
<i>V. dahliae</i>	PD728	Horseradish	Illinois	HQ206918	–	–
<i>V. dahliae</i>	UZ132	Cotton	Uzbekistan	AY555949	AY555922	AY555978
<i>V. longisporum</i>	G19	Oilseed rape	Germany	AY555950	AY555923	AY555979
<i>V. longisporum</i>	K2	Oilseed rape	Russia	AY555951	AY555924	AY555980
<i>V. longisporum</i>	K12	Oilseed ape	Russia	AY555952	AY555925	AY555981
<i>V. longisporum</i>	PD687	Horseradish	German	HQ206893	–	–
<i>V. longisporum</i>	PD730	Horseradish	Illinois	HQ206920	–	–
<i>V. albo-atrum</i>	1776	Hop	United Kingdom	AY555953	AY555926	AY555982
<i>V. albo-atrum</i>	V90	Cotton	Middle East	AY555954	AY555927	AY555983
<i>V. albo-atrum</i>	PD670	Potato	Wisconsin	JN187990	–	–
<i>V. albo-atrum</i>	PD748	Soil	Canada	JN188017	–	–
<i>V. klebahnii</i>	PD347	Artichoke	California	JN187965	–	–
<i>V. nubilum</i>	IMI278734	Potato	United Kingdom	AY935948	–	–
<i>V. tricorpus</i>	PD593	Potato	Japan	JN187974	–	–
<i>V. tricorpus</i>	PD685	Larkspur	Japan	JN187992	–	–
<i>G. nigrescens</i>	193	Unknown	Unknown	AY555959	AY555932	AY555987
<i>G. nigrescens</i>	PD595	Anemone	Japan	JN187976	–	–

^y ITS = internal transcribed spacer, *cox3* = cytochrome oxidase subunit III, *nad1* = NADH dehydrogenase subunit I, and – = not available.

^z Geographic origin in countries, regions, and the states of California and Illinois in the United States.

and the collected microsclerotia were resuspended in 0.5% sterilized agar solution to prepare sticky inoculums (Babadoost et al. 2004). An inoculum suspension of 500 microsclerotia/ml was prepared using a spore-counting chamber.

Roots of plants grown in the greenhouse were dipped in the 0.5% agar suspension of microsclerotia for 15 s, after which the inoculated plants were planted in plastic pots containing the pasteurized soil-peat-perlite (1:1:1) mix. Roots of control plants were dipped in sterilized 0.5% agar solution. Pots were placed in the greenhouse at 20 to 26°C under a combination of 1,000-W high-pressure sodium and mercury vapor lamps with a photoperiod of 12-h/day. Each inoculation treatment included four replications, each with six plants, in a randomized complete block design.

Plants were removed at 4-week intervals until 4 months after inoculation. Roots were washed, cut, and evaluated for both incidence and severity of internal discoloration in the cross-section of the middle of the root. Severity of root discoloration (percent area of root at cross section discolored) was assessed using a 0-to-4 scale, where 0 = 0, 1 = 1 to 10 (average 5), 2 = 11 to 25 (17.5), 3 = 26 to 50 (37.5), and 4 = 51 to 100% (75%). Root sections were surface sterilized by soaking in 6% NaClO solution for 1 min, dipping in 95% ethanol for 2 min, and rinsing in SDW three times, each time for 1 min. Sections (5 mm thick) of roots were cut and placed on A-PDA in petri plates for reisolation of the pathogens. Plates were incubated at 22 to 24°C under a cycle of 12 h of fluorescent light and 12 h of darkness per day. Growing fungal colonies were transferred onto PDA, after which the species were identified base on morphology of their conidia and conidiophores. The pathogenicity test was repeated once. Statistical analyses of the data were performed using the analysis of variance procedure of SAS (version 9.4; SAS Institute, Cary, NC).

Results

Isolated fungi. Fungi were isolated from 421 of 496 roots, which included 65 of 75, 250 of 306, and 106 of 115 of roots collected from California, Illinois, and Ontario, respectively (Table 1). In total, 586 fungal isolates comprising 106, 288, and 192 isolates from California, Illinois, and Ontario, respectively, were collected. Up to three different fungi were isolated from some of the roots. The majority of the isolates were *Verticillium* (44% of the total isolates) and *Fusarium* (25% of the total isolates) species based on the morphological characteristics and ITS sequence analysis. *Verticillium* spp. were isolated from 34, 47, and 43% of roots collected from California, Illinois, and Ontario, respectively. In total, 255 isolates were identified as *Verticillium*.

Morphological identification of *Verticillium* isolates. Based on conidial length and microsclerotial shape, *Verticillium* isolates were divided into two groups. One group produced a white colony with

abundant fluffy mycelium on PDA. The colony was creamy white initially but gradually became dark and densely compacted and appressed to agar. Abundant conidia were produced by the colonies, which were hyaline, cylindrical to oval, measuring $<6.0\ \mu\text{m}$ in length ($4.4 \pm 1.23\ \mu\text{m}$) (Fig. 1A). Conidiophores were hyaline, verticillately branched, and had 2 to 4 phialides per node (Fig. 1B). Microsclerotia were dark brown to black, spherical and compact, immersed in agar, measuring 20 to 100 μm in diameter (Fig. 1C). Aggregates of microsclerotia up to 200 μm in diameter developed. These characteristics fit the description of *V. dahlia* (Collins et al. 2003; Hawksworth and Talboys 1970; Karapapa et al. 1997). Ten isolates, including CAV1003, CAV1003, CAV1004, CBV1006, CBV1014, CBV1046, SCV1005, SCV1010, SDV1002, SDV1006, and SDV1025, belonged to this group.

Isolates that belonged to another group produced whitish to gray colonies, with sparse aerial mycelium on PDA. Conidia produced by the colonies were hyaline, cylindrical to ellipsoid, measuring $>6.5\ \mu\text{m}$ in length ($7.8 \pm 1.76\ \mu\text{m}$) (Fig. 1D). Conidiophores were hyaline and had 2 to 4 (mostly 3) phialides per node (Fig. 1E). Microsclerotia were variable in shape (elongate to irregularly spherical) and variable in size (30 to 250 μm in diameter), with aggregates of microsclerotia measuring $\geq 300\ \mu\text{m}$ in diameter (Fig. 1F). These characteristics fit the description of *V. longisporum* (Collins et al. 2003; Karapapa et al. 1997). Ten isolates, including CBV2034, SCV2008, SDV2041, SDV2064, SDV2066, TAV3001, TBV3014, VAV3003, VAV3021, and VBV3004, belonged to this group.

Molecular characteristics of *Verticillium* isolates. The sequences from PCR products of ITS regions were analyzed with retrieved sequences from GenBank. Sequences were deposited in GenBank under accession numbers KC834714 to KC834733 (Table 2). Amplification of the ITS regions of the *Verticillium* isolates generated 480-bp fragments, and alignment of the sequences resulted in a dataset of 468 characters. Based on 25 parsimony-informative characters, the three most parsimonious trees were located with a tree length of 82 steps (consistency index (CI) = 0.9268, retention index (RI) = 0.9492, homoplasy index (HI) = 0.0732, released consistency index (RC) = 0.8801). ML analysis produced a tree with a log likelihood (ln) of -1034.035568 (Fig. 2).

Based on phylogenetic analysis of the ITS region, the *Verticillium* isolates from horseradish were ambiguously divided into two clades, *V. dahliae* and *V. longisporum*. All 10 isolates of *V. dahliae* (CAV1003, CAV1004, CBV1006, CBV1014, CBV1046, SCV1005, SCV1010, SDV1002, SDV1006, and SDV1025), identified based on their morphological characteristics, fell into one clade group. Five isolates (TAV3001, TBV3014, VAV3003, VAV3021, and VBV3004), which were identified as *V. longisporum*, were also clustered into the *V. dahliae* clade along with reference strains of

Fig. 1. Morphological characteristics of A to C, *Verticillium dahliae* and D to F, *V. longisporum* grown on potato dextrose agar. A and D, conidia; B and E, conidiophores; and C and F, microsclerotia. Scale bars: A and D = 20 μm , B and E = 20 μm , and C and F = 300 μm .

V. dahliae (strains D322, M5, PD727, PD728, and UZ132) and reference strains of *V. longisporum* (strains G19 and PD687) with strong bootstrap percentage (BP) support (96% ML BP, 97% MP BP). The other five isolates (CBV2034, SCV2008, SDV2041, SDV2064, and SDV2066), identified as *V. longisporum*, and reference strains of *V. longisporum* (K2 and K12) were clustered together into a separate clade with bootstrap support of 100% ML and 62% MP (Fig. 2). Overall, the results showed that the 10 isolates identified as *V. longisporum* based on morphological characteristics were divided into two groups. One group (group A: five isolates from California) formed a monophyletic clade with all isolates identified as *V. dahliae* based on morphological characteristics. ITS sequences of these isolates and reference strain *V. longisporum* PD687 were identical to those of most *V. dahliae* isolates. The other group (group B), including four isolates from Illinois and one isolate from Ontario, formed a distinct clade (Fig. 2).

The sequences of the PCR products of *cox3* were analyzed with sequences retrieved from GenBank. Sequences of the products were deposited in GenBank under accession numbers KC852082 to KC852094 and HM804952 to HM988735 (Table 2). Amplification of the *cox3* gene generated 380-bp fragments, and alignment of the sequences resulted in a dataset of 364 characters. Based on 32 parsimony-informative characters, the three most parsimonious trees were generated with a tree length of 84 steps (CI = 0.9167, RI = 0.9014, HI = 0.0833, RC = 0.8263). The sequences of the PCR products of *nad1* were also analyzed with sequences retrieved from GenBank. Sequences were deposited in GenBank under accession numbers KC852095 to KC852106 and HM988735 to HM988744 (Table 2). Amplification of the *nad1* gene generated 500-bp fragments, and alignment of the sequences resulted in a dataset of 484 characters. Based on 39 parsimony-informative characters, the two most parsimonious trees generated were with a tree length of 157 steps

Fig. 2. Maximum-likelihood (ML) tree based on internal transcribed spacer sequence data of the examined 20 isolates. ML and maximum-parsimony bootstrap values $\geq 50\%$ are shown at nodes. Negative log likelihood of the ML tree = -1,034.035568. Outgroup: *Gibellulopsis nigrescens*. *Verticillium longisporum* isolates in group A were from California, and *V. longisporum* isolates in group B and *V. dahliae* isolates were from Illinois and Ontario. *V. longisporum* isolate SDV-2066^(z) is the same isolate as reference strain *V. longisporum* PD730, which also was reported by Inderbitzin et al. (2011b).

(CI = 0.8726, RI = 0.8148, HI = 0.1274, RC = 0.7110). The combined sequence data for *cox3* and *nad1* resulted in a dataset of 848 characters, with 71 parsimony-informative characters. For MP analysis, the four most parsimonious trees generated were with a tree length of 243 steps (CI = 0.8807, RI = 0.8380, HI = 0.1193, RC = 0.7380). ML analysis produced a tree with log likelihood (ln) of -2,281.445825 (Fig. 3).

Based on phylogenetic analysis of the combined sequence of *cox3* and *nad1*, the *Verticillium* isolates from horseradish were divided into three clades. All 10 isolates (CAV1003, CAV1004, CBV1006, CBV1014, CBV1046, SCV1005, SCV1010, SDV1002, SDV1006, and SDV1025) identified as *V. dahliae* based on morphological characteristics, and the reference strains of *V. dahliae* (76, M5, and UZ132) grouped together and formed the *V. dahliae* clade with moderate bootstrap support (67% ML BP and 52% MP BP). Ten isolates identified as *V. longisporum* based on morphological characteristics were divided into two clades in this tree. One isolate (CBV2034) from Ontario and four isolates (SCV2008, SDV2041, SDV2064, and SDV2066) from Illinois were grouped together (group B) and formed a distinct clade (94% ML BP, <50% MP BP). This clade formed a sister group with the *V. dahliae* clade supported by moderately strong ML BP values, which means that this group was closely related to *V. dahliae*. Five isolates (TAV3001, TBV3014, VAV3003, VAV3021, and VBV3004) from California, identified

as *V. longisporum*, and reference strains of *V. longisporum* (G19, K2, and K12) were grouped together and formed a clade with strong bootstrap support (100% ML BP and 75% MP BP). Interestingly, reference strains of *V. longisporum* K2 and K12, which grouped with *V. longisporum* group B in the ITS tree, were clustered together with group A in this tree. The five isolates in this clade (group A) showed a closer relationship (75% ML BP, and 67% MP BP) to *V. albo-atrum* than to *V. dahliae*, although they were clearly distinct from both species (Fig. 3).

Pathogenicity test. All of the *Verticillium* isolates tested caused internal discoloration of roots. Symptoms of internal root discoloration developed over a period of 4 months in inoculated plants (Table 4). After 1 month, 33.3 and 16.7% of horseradish 1573 and BTW plants inoculated with *V. longisporum* group B isolates developed internal root discoloration. Both the incidence and severity of root discoloration increased gradually and, after 4 months, 83 to 100% of inoculated roots exhibited symptoms of internal discoloration. There were no significant differences in either the incidence or severity of internal discoloration of roots inoculated with *V. dahliae* and the two genetic groups (group A and B) of *V. longisporum* after 4 months from inoculation (Table 4). Also, there was no significant difference in either the incidence or severity of root discoloration between 1573 and BTW horseradish. The same *Verticillium* spp. used to inoculate roots was reisolated from the discolored roots.

Fig. 3. Maximum-likelihood (ML) tree based on combined cytochrome oxidase subunit III and NADH dehydrogenase subunit I sequence data of the examined 20 isolates. ML and maximum-parsimony bootstrap values $\geq 50\%$ are shown at nodes. Negative log likelihood of the ML tree = -2,281.445825. Outgroup: *Gibellulopsis nigrescens*. *Verticillium longisporum* isolates in group A were from California, and *V. longisporum* isolates in group B and *V. dahliae* isolates were from Illinois and Ontario. *V. longisporum* isolate SDV-2066^z is the same isolate as reference strain *V. longisporum* PD730, which also was reported by Inderbitzin et al. (2011b).

Control plants did not develop any symptoms and no pathogen was isolated from them.

Discussion

Conidial size is an important characteristic used to separate *V. dahliae* and *V. longisporum* (Hawksworth and Talboys 1970; Inderbitzin et al. 2011a; Karapapa et al. 1997; Stark 1961; Steventon et al. 2002). Other characteristics proposed by Karapapa et al. (1997) were micro-sclerotial morphology and number of phialides per node. They reported that *V. dahliae* produces relatively spherical microsclerotia and has 4 to 5 phialides on each conidiophore node, whereas *V. longisporum* produces elongate microsclerotia and usually has 3 phialides on each node. However, Inderbitzin et al. (2011a) reported that both *V. dahliae* and *V. longisporum* produce similar numbers of phialides in each node: 2 to 4 for *V. dahliae* and 2 to 5 for *V. longisporum*. In our study, we measured two different sizes of conidia but found similar numbers (2 to 4) of phialides per node for both *V. dahliae* and *V. longisporum*.

In the present study, conidial length of *V. dahliae* averaged 4.4 μm ($<6.0 \mu\text{m}$), while conidial length of *V. longisporum* averaged 7.8 μm ($>6.5 \mu\text{m}$), similar to the studies of Karapapa et al. (1997) and Steventon et al. (2002). Conidial lengths in our study were slightly shorter than that reported by Inderbitzin et al. (2011a). Most of microsclerotia of *V. dahliae* were rounded and measured 20 to 100 μm , while microsclerotia of *V. longisporum* were elongate to irregularly spherical and measured 30 to 250 μm . Thus, morphological characteristics of microsclerotia had some similarities, as reported by Inderbitzin et al. (2011a).

For phylogenetic analysis of *Verticillium* isolates, we used the ITS region (ITS1-5.8S-ITS2 region) and two mitochondrial genes, *cox3* and *nad1*. The ITS region has been extensively used for phylogenetic analysis of *Verticillium* spp. (Fahleson et al. 2004; Inderbitzin et al. 2011b; Pantou et al. 2005) and other fungi (Suga et al. 2000). Mitochondrial genes *cox3* and *nad1* have been used in phylogenetic studies of *Verticillium* isolates (Pantou et al. 2005) to differentiate species of *Verticillium* from each other, including *V. dahliae* and *V. longisporum*. In this study, using ITS1-5.8S-ITS2 did not differentiate *V. dahliae* from *V. longisporum*. When assessing the ITS tree, isolates of *Verticillium* from horseradish were divided into two clades: *V. dahliae* clade and *V. longisporum* clade. However, five isolates of *V. longisporum* from California (identified based on the morphological characteristics) and two reference strains of *V. longisporum* (G19 and PD687) clustered with *V. dahliae*. Similar results were also obtained by Pantou et al. (2005) and Inderbitzin et al. (2011a). According to Inderbitzin et al. (2011b), ITS alleles of *V. longisporum* isolates can be divided into two groups in correlation with the *V. longisporum* lineages D1, D2, and D3. Isolates of *V. longisporum*

lineages D1 and D2 were shown to have identical ITS alleles and formed a sister group with *V. dahliae*, while ITS alleles of *V. longisporum* lineage D3 were identical to those of the majority of *V. dahliae* isolates. In this study, *V. longisporum* group A, which clustered to the *V. dahliae* clade, corresponded to *V. longisporum* lineage D3 of Inderbitzin et al. (2011b).

Several investigators have suggested that *V. longisporum* is an interspecific hybrid whose parents include *V. dahliae*, *V. albo-atrum*, or close relatives of these two species (Clews et al. 2008; Collado-Romero et al. 2010; Collins et al. 2003; Karapapa et al. 1997), and their genetic diversities have been characterized (Barbara and Clews 2003; Collins et al. 2003, 2005; Karapapa et al. 1997; Pantou et al. 2005; Zeise and von Tiedemann 2001). Previously, Inderbitzin et al. (2011b) conducted phylogenetic analyses of seven nuclear loci and a dataset of 203 isolates of *V. dahliae*, *V. longisporum*, and related species. They suggested that *V. longisporum* is an allopolyploid hybrid that originated three different times through independent hybridization events, involving four different parental lineages and three different species. Also, according to Inderbitzin et al. (2011b), all of the hybrids share a common parent, species A1, that hybridized separately with species D1, *V. dahliae* lineage D2, and *V. dahliae* lineage D3, giving rise to the three different lineages, including *V. longisporum* lineage A1/D1, *V. longisporum* lineage A1/D2, and *V. longisporum* lineage A1/D3, respectively. Another study at the same time (Inderbitzin et al. 2011a) suggested that *V. longisporum* lineage A1/D1 corresponds to Zeise and von Tiedemann's group 1sp (Zeise and von Tiedemann 2001) and Clews et al. (2008) group α , whereas *V. longisporum* lineage A1/D2 corresponds to group $\beta\gamma$ of Clews et al. (2008). Also, the *V. longisporum* lineage A1/D3 corresponds to group 1sp of Zeise and von Tiedemann (2001) and group β of Clews et al. (2008).

In our study, we obtained two genetic groups (group A and group B) of *V. longisporum* based on phylogenetic analysis of the ITS1-5.8S-ITS2 region as well as the combined sequence of *cox3* and *nad1*. These two groups of *V. longisporum* were similar to the two different lineages of *V. longisporum* reported by Inderbitzin et al. (2011b). The *V. longisporum* genetic groups A and B corresponded to *V. longisporum* lineages A1/D3 and A1/D2, respectively, of Inderbitzin et al. (2011b). Furthermore, *V. longisporum* group A of our isolates and *V. longisporum* lineage A1/D3 of Inderbitzin et al. (2011b) clustered together with the *V. dahliae* clade in the ITS tree. *V. longisporum* reference strains K2 and K12 clustered with group B in the ITS tree, even though they clustered into group A in the combined *cox3* and *nad1* tree. Phylogenetic analysis of the combined sequence of the ITS region, *cox3*, and *nad1* showed that strains K2 and K12 formed a distinct clade (data not shown). According to the results, we concluded that *V. longisporum* reference strains K2 and K12

Table 4. Occurrence of internal root discoloration caused by artificial inoculation of *Verticillium dahliae* and *V. longisporum* on horseradish '1573' and 'Big Top Western'

Treatment ^y	Occurrence ^z	Disease development in months ^x									
		1573					Big Top Western				
		1	2	3	4	LSD	1	2	3	4	LSD
<i>V. dahliae</i>	Incidence	0 a	50.0 b	83.3 bc	100.0 c	41.4	0 a	50.0 b	83.3 b	83.3 b	47.9
	Severity	0 a	1.8 ab	21.7 bc	37.6 c	16.3	0 a	10.2 a	18.3 a	37.9 b	18.6
<i>V. longisporum</i> (group A)	Incidence	0 a	33.3 a	83.3 b	100 b	39.6	0 a	33.3 a	83.3 b	100.0 b	39.6
	Severity	0 a	6.0 a	21.7 b	31.3 b	12.1	0 a	3.8 ab	18.3 b	37.6 c	15.2
<i>V. longisporum</i> (group B)	Incidence	33.3 a	66.7 ab	83.3 ab	100.0 b	50.4	16.7 a	50.0 ab	66.7 ab	100.0 b	51.6
	Severity	1.7 a	5.5 a	18.3 b	34.7 c	9.7	0.8 a	2.5 a	25.3 b	28.0 b	13.7
Control	Incidence	0	0	0	0	NS	0	0	0	0	NS
	Severity	0	0	0	0	NS	0	0	0	0	NS

^x Values within each row and for each cultivar with a letter in common are not significantly different from each other according to Fisher's protected least significant difference (LSD; $P = 0.05$). NS = not significant.

^y *V. dahliae* isolates from Ontario (CBV 1014 isolate), *V. longisporum* isolates from California (group A, TAV3001 isolate), and *V. longisporum* isolates from Illinois (group B, SCV2008 isolate).

^z Disease occurrence. Incidence = percentage of plants with discolored root. Data represent the average of two experiments, each with 24 plants. Severity = percent root area at the cross-section discolored. Severity of discoloration was based on a 0-to-4 scale, where 0 = 0, 1 = 1 to 10 (average 5), 2 = 11 to 25 (17.5), 3 = 26 to 50 (37.5), and 4 = 51 to 100% (75%).

from oilseed rape belong to another separate lineage. In this study, we could not isolate the third group corresponding to *V. longisporum* lineage A1/D1 of Inderbitzin et al. (2011b) from horseradish.

Inderbitzin et al. (2011b) reported that *V. longisporum* lineage A1/D2 was confined to horseradish in Illinois, whereas lineage A1/D3 was restricted to Europe and Japan, and lineage A1/D1 occurred in Europe, Japan, and North America. In this study, we found that *V. longisporum* lineage A1/D2 (group B in this study) occurred not only in Illinois but also in Ontario, whereas lineage A1/D3 (group A in this study) occurred only in California. The predominant species of *Verticillium* varied according to the horseradish growing area. Specifically, *V. dahliae* was the most prevalent in root samples from Illinois and Ontario, whereas *V. longisporum* group A (lineage A1/D3 of Inderbitzin et al. [2011b]) was the most prevalent in root samples from California.

Both *V. dahliae* and *V. longisporum* have been isolated from oilseed rape but *V. longisporum* was determined to be the cause of *Verticillium* wilt in oilseed rape (Eynck et al. 2007; Heale and Karapapa 1999). Subbarao et al. (1995) reported that *V. longisporum* caused stunting, chlorosis, and discoloration of vessels in leaves in oilseed rape. Novakazi et al. (2015) conducted comparative pathogenicity tests using *V. dahliae* and three *V. longisporum* lineages on 11 different hosts, including artichoke, cabbage, cauliflower, cotton, eggplant, horseradish, lettuce, linseed, oilseed rape, tomato, and watermelon. This study demonstrated that *V. longisporum* was more virulent than *V. dahliae* on brassica hosts, including horseradish. They also showed that virulence of each *V. longisporum* lineage varied with host. For example *V. longisporum* A1/D2 was the most virulent group on horseradish and cabbage, whereas *V. longisporum* A1/D1 showed higher virulence than *V. dahliae* on oilseed rape and cauliflower (Novakazi et al. 2015). *V. longisporum* A1/D3 was not highly virulent, and *V. dahliae* was less virulent than three *V. longisporum* lineages tested on all of the hosts they evaluated. In our study, pathogenicity tests revealed that both *V. dahliae* and two identified genetic groups of *V. longisporum* were pathogenic to horseradish and caused internal discoloration of roots. However, we did not establish species-specific symptom characteristics of our isolates. The roots inoculated with *V. longisporum* group A (corresponding to *V. longisporum* lineage A1/D2) showed disease symptoms earlier (1 month after inoculation) than other *Verticillium* groups, and showed higher disease incidence at 2 months after inoculation. However, there were no significant differences in either the incidence or severity of root discoloration among isolates of *V. dahliae* and two genetic groups of *V. longisporum* 4 months after inoculation. This finding explains that *V. longisporum* lineage A1/D2 was more virulent than other groups in the pathogenicity tests conducted for 9 weeks by Novakazi et al. (2015).

International root discoloration of horseradish is a disease complex. This study provided additional information about the pathogens causing the internal root discoloration of horseradish. However, for developing strategies for effective management of the disease, further studies of the etiology of the disease are needed.

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