Molecular Genetic Characterization of *Olpidium virulentus* Isolates Associated with Big-Vein Diseased Lettuce Plants

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ABSTRACT

Maccarone, L. D., Barbetti, M. J., Sivasithamparam, K., and Jones, R. A. C. 2010. Molecular genetic characterization of *Olpidium virulentus* isolates associated with big-vein diseased lettuce plants. *Plant Dis.* 94:563-569.

Lettuce plants showing symptoms of lettuce big-vein disease were collected from fields in the Perth Metropolitan region of southwest Australia. When root extracts from each plant were tested by polymerase chain reaction (PCR) using primers specific to the rDNA internal transcribed spacer (ITS) region of *Olpidium brassicae* and *O. virulentus*, only *O. virulentus* was detected in each of them. The nucleotide sequences of the complete rDNA ITS regions of isolates from five of the root samples and 10 isolates of *O. virulentus* from Europe and Japan showed 97.9 to 100% identities. However, with the six *O. brassicae* isolates, their identities were only 76.9 to 79.4%. On phylogenetic analysis of the complete rDNA-ITS region sequences of the five Australian isolates and 10 others, the Australian isolates fitted within two clades of *O. virulentus* (I and II), and within clade I into two of its four subclades (Ia and Id). Japanese isolates had greatest sequence diversity fitting into both clades and into all of clade I subclades except Ib, while European isolates were restricted to subclades Ib and Id. When the partial rDNA-ITS region sequences of two additional southwest Australian isolates, four from Europe, and four from the Americas were included in the analyses, the Australian isolates were within *O. virulentus* subclades Ia and Id, the European isolates within subclade Ic, and the American isolates within subclades Ia and Ib. These findings suggest that there may have been at least three separate introductions of *O. virulentus* into the isolated Australian continent since plant cultivation was introduced following its colonization by Europeans. They also have implications regarding numbers of different introductions to other isolated regions. *Lettuce big-vein associated virus* and *Mirafiori lettuce big-vein virus* were both detected when symptomatic lettuce leaf tissue samples corresponding to the root samples from southwest Australia were tested using virus-specific primers in reverse transcription-PCR, so presence of both viruses was associated with *O. virulentus* occurrence.

Lettuce big-vein disease (LBVD) was first found in California (10). It occurs widely in regions of the world with temperate or Mediterranean-type climates and at high altitudes in subtropical regions. LBVD is now known to be associated with a complex of two viruses, *Lettuce big-vein associated virus* (LBVaV; genus *Varicosavirus*) and *Mirafiori lettuce big-vein virus* (MLBVV; genus *Ophiovirus*) (17,18,20). Its reported natural host range is limited to lettuce (*Lactuca sativa*), en-

dive (*Cichorium endive*), spiny sowthistle (*Sonchus asper*), and common sowthistle (*Sonchus oleraceus*) (2,12). The vector for both viruses is a root-infecting chytrid belonging to the genus *Olpidium* (Chytridiomycota). Motile zoospores of this chytrid transmit MLBVV and LBVaV to the roots of healthy plants, and in the absence of susceptible hosts, its resting spores retain the ability to transmit for decades in infested soil (1,17,19,23). In addition to damaging plants' quality through the presence of obvious "big-vein" leaf symptoms (chlorophyll clearing around leaf veins that appear enlarged) from which the disease gets its name, LBVD decreases yield of produce from infected plants substantially (14,15). Symptom expression is most obvious at low temperatures and low light intensities (2-4,17,27,28).

Olpidium spp. are obligate plant pathogens that vector several different soilborne plant viruses. They are found commonly throughout the world infecting the roots of wild and domesticated plants, and until recently only two species were recognized as vectors: *O. brassicae* and *O. bornovanus* (= *O. radicle*) (2,3,9,19). Sahtiyanci (21) separated *O. brassicae* (formerly *Pleotrachelus brassicae*) into two species: *O. brassicae* being crucifer-infecting and *O. virulentus* non-crucifer-infecting. Morphologically, *O. brassicae* and *O. virulentus* varied slightly. Pleomorphism was present in both species with few variations in *O. virulentus* but more variation in *O. brassicae*. Also, the zoosporangium of *O. brassicae* was larger than that of *O. virulentus* (21). However, this study was overlooked by researchers investigating LBVD, who continued to refer to a non-crucifer-infecting strain of *O. brassicae* as the vector of LBVaV (2-5,17,19). Recently, molecular and host range studies confirmed that *O. virulentus* was sufficiently different to be considered a distinct species that differs from *O. brassicae* in infecting lettuce but not *Brassica* species, whereas *O. brassicae* infects *Brassica* species but not lettuce (7,13,23). Nucleotide sequence data for the rDNA internal transcribed spacer (ITS) region of its *Olpidium* sp. vector confirmed that it was *O. virulentus* that transmitted MLBVV and the Tobacco stunt virus (TSV) strain of LBVaV to lettuce roots (23).

In Australia, LBVD is a widespread problem in commercial lettuce producing areas. In southwest Australia, the lettuce production system on the Swan Coastal Plain is intensive and often uses irrigated land that is contaminated with LBVD, sometimes without crop rotation. Also, winter plantings often have >90% of plants with symptoms of the disease (12,14,15). However, no nucleotide sequences of *Olpidium* spp. are available from the Australian continent. This paper reports studies that determined the nucleotide sequences of the rDNA ITS region of southwest Australian *Olpidium* sp. isolates obtained from the roots of lettuce plants with LBVD, and

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Accepted for publication 21 December 2009.

doi:10.1094/PDIS-94-5-0563
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compared their sequence identities and phylogenies with those of the rDNA ITS region of *Olpidium* spp. isolates from elsewhere.

MATERIALS AND METHODS

Collection of field samples. Whole lettuce plants showing LBVD symptoms were sampled from two crops growing in the northern Perth Metropolitan region of southwest Australia. They came from two different commercial lettuce growing farms, four samples from one farm (labeled Nan 6, Nan 7, Nan 8, and T1 23) and three from the other (labeled Col 1, Col 2, and Col 3). On the same day, roots from each sample were washed and ground in liquid nitrogen before DNA was extracted and stored at -20°C.

DNA extraction, polymerase chain reaction (PCR), and agarose gel electrophoresis. Total DNA was extracted from the lettuce root tissue from samples Nan 6 to 8, T1 23, and Col 1 to 3 using a Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) according to manufacturer's instructions. No cultures of *O. brassicae* or *O. virulentus* were available to provide positive control extracts. Primers used to amplify target *Olpidium* spp. came from White et al. (30) (ITS1 and ITS4) or were designed from within the rDNA-ITS region by aligning already known sequences using BioEdit (6) (Ob1, Ob2, Ov1, and Ov2). For amplification of *O. brassicae*,

the primer pairs used were Ob1 Fwd (5' TCA TTA ATA AAT GCT TCA TTG CAT 3') with ITS4 Rev (5' TCC TCC GCT TAT TGA TAT GC 3'), and ITS1 Fwd (5' TCC GTA GGT GAA CCT GCG G 3') (30) with Ob2 Rev (5' TCC AGC AAC AAG TCT TCC CAA 3'). For *O. virulentus* amplification, the primer pairs used were Ov1 Fwd (5' CAA GAC CTG CCC CCA AAA GGG 3') with ITS4, and ITS1 with Ov2 Rev (5' CCA AGT TCG CAA ACG TGG CG 3'). All primer pair combinations were used on tissue extracts from each sample. PCR reactions were done using *Taq* DNA polymerase and 5x polymerization buffer (Fisher Biotech, Perth, Western Australia).

PCR amplification conditions consisted of 1 min denaturation at 94°C followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min followed by a final extension at 72°C for 10 min before being held at 14°C. PCR amplicons were visualized by electrophoresis on 1% agarose gels. Tris-boric acid-EDTA (TBE) electrophoresis buffer was used (22).

Virus RNA extraction and multiplex reverse transcription (RT)-PCR. Symptomatic leaf tissue samples corresponding to six of the seven root samples were used for RNA extraction (isolate T1 23 was not available). Total RNA was extracted from each sample using an UltraClean Plant RNA Isolation Kit (MoBio Laboratories, Inc.) according to manufacturer's instructions. RNA extracts were stored at -80°C.

Three primer pairs developed by Navarro et al. (18) were used for multiplex RT-PCR: VP248 Fwd (5' CGC CAG GAT CTT TGA TCC ATC TG 3') with VP249 Rev (5' TTG CGA CAT GTT CCT CCT CAT CG 3') to amplify LBVaV, VP286 Fwd (5' TAT CAG CTC ACA TAC TCC CTA TCG 3') with VP287 Rev (5' CAA CTA GCT CAG AAT ACA TGC AG 3') to amplify MLBVV, and control primers VP383 Fwd (5' AGC GTG CTA ATC CCT ATG TTC AT 3') with VP389 Rev (5' AAT GAA AAT CTT AAA AGC CGT AG 3') to amplify the mitochondrial NADH dehydrogenase subunit 4 (*nad4*) of lettuce. RT-PCR was done using a OneStep RT-PCR kit (Qiagen, Australia) according to manufacturer's instructions.

PCR amplification conditions consisted of 50°C for 30 min, 95°C for 15 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min followed by a final extension at 72°C for 10 min before being held at 14°C.

Cloning and sequencing of PCR amplicons. PCR amplicons of *Olpidium* were purified by ethanol precipitation and then quantified using a Nanodrop UV-Vis spectrophotometer following the manufacturer's protocol. Next, standard ligation and transformation reactions were done into JM109 competent cells using a pGEM®-T Easy Vector (Promega Corp, Madison, WI) as recommended in the manufacturer's protocol.

Table 1. Details of isolates of three vector *Olpidium* spp. used to determine relationships using the nucleotide sequences of their rDNA internal transcribed spacer regions

Species	Accession no.	Isolate	Geographic origin	Isolated from root of	Reference
<i>O. bornovanus</i>	AB205214	NA	Nagasaki, Japan	Melon	23
<i>O. bornovanus</i>	AB205215	CH	Chiba, Japan	Melon	23
<i>O. brassicae</i>	AB205209	CBG-3	Nagano, Japan	Cabbage	23
<i>O. brassicae</i>	AB205210	NKa-3	Nagano, Japan	Cabbage	23
<i>O. brassicae</i>	AB205211	YR-2	Shimane, Japan	Cabbage	23
<i>O. brassicae</i>	AB205212	ZE-1	Zentsuji, Japan	Cabbage	23
<i>O. brassicae</i>	AB205213	EH-2	Iyo, Japan	Cabbage	23
<i>O. brassicae</i>	AY373015	GBR7	Warwickshire, UK	Cauliflower	7
<i>O. virulentus</i>	AB205203	WT-1	Wakayama, Japan	Lettuce	23
<i>O. virulentus</i>	AB205204	HY-1	Hyogo, Japan	Lettuce	23
<i>O. virulentus</i>	AB205205	KZ-1	Kagawa, Japan	Lettuce	23
<i>O. virulentus</i>	AB205206	TAK-1	Takamatsu, Japan	Tobacco	23
<i>O. virulentus</i>	AB205207	CH-1	Chunan, Japan	Tobacco	23
<i>O. virulentus</i>	AB205208	WOMs-3	Toyama, Japan	Welsh onion	23
<i>O. virulentus</i>	AY373011	GBR1	Warwickshire, UK	Lettuce	7
<i>O. virulentus</i>	AY373012	ITA1	Mirafiori, Italy	Lettuce	7
<i>O. virulentus</i>	AY373013	NLD4	De Lier, the Netherlands	Lettuce	7
<i>O. virulentus</i>	AY373014	GBR10	Humberside, UK	Cucumber	7
<i>O. virulentus</i>	AY997067	633	Duke University Herbarium, USA	Unknown	11
<i>O. virulentus</i>	EU981898	GT-8	Zacapa, Guatemala	Cantaloupe	9
<i>O. virulentus</i>	EU981899	MX-06	Sinaloa, Mexico	Cantaloupe	9
<i>O. virulentus</i>	EU981900	MX-05	Colima, Mexico	Cantaloupe	9
<i>O. virulentus</i>	EU981901	SP-08	Castellon, Spain	Lettuce	9
<i>O. virulentus</i>	EU981902	SP-09	Murcia, Spain	Tomato	9
<i>O. virulentus</i>	EU981903	SP-07	Alicante, Spain	Watermelon	9
<i>O. virulentus</i>	EU981905	SP-06	Seville, Spain	Cantaloupe	9
<i>O. virulentus</i>	GQ304519	Nan6	Perth, Western Australia	Lettuce	This study
<i>O. virulentus</i>	GQ304520	Nan7	Perth, Western Australia	Lettuce	This study
<i>O. virulentus</i>	GQ304521	Nan8	Perth, Western Australia	Lettuce	This study
<i>O. virulentus</i>	GQ304522	T1 23	Perth, Western Australia	Lettuce	This study
<i>O. virulentus</i>	GQ304523	Col1	Perth, Western Australia	Lettuce	This study
<i>O. virulentus</i>	GQ328050	Col2	Perth, Western Australia	Lettuce	This study
<i>O. virulentus</i>	GQ328051	Col3	Perth, Western Australia	Lettuce	This study

Screening for recombinant plasmids was done using M13 primers Fwd (5' CGT CAG GCT TTT CCC AGT CAC GAC 3') and Rev (5' TCA CAC AGG AAA CAG CTA TGA C 3'). Amplification conditions consisted of 1 min denaturation at 94°C followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s before being held at 14°C. Amplicons were then visualized on 1% agarose gels as described above. Recombinant plasmids were inoculated into 5 ml of Luria Bertani (LB) broth with ampicillin (100 mg/liter) and incubated on a shaker at 37°C overnight. Plasmids were then purified from the culture using an Aurum Bio-Rad Plasmid Extraction Kit following the manufacturer's instructions. Presence and size of inserts was confirmed with a restriction digest of the purified plasmid DNA by *Eco*R1 (10 µl of purified plasmid DNA, 2 µl of 10× *Eco*R1 buffer, 1 µl of *Eco*R1, and 7 µl of water), incubating on a 37°C heat block for 1 h, and visualizing on 1% agarose gel.

Dideoxy-termination sequencing was done at the Western Australian State Agricultural Biotechnology Centre (SABC), Murdoch University, Perth, or Royal Perth Hospital, Perth. In both instances, Big Dye Version 3.1 chemistry was used. The complete rDNA-ITS sequences of isolates Nan 6 to 8, T1 23, and Col 1 (each 632 bp) and partial sequences of isolates Col 2 (577 bp) and Col 3 (503 bp) were submitted to GenBank (Table 1).

Analysis of sequence data. Nucleotide sequence data for the 21 complete rDNA-ITS regions (632 bp) of *Olpidium* spp. and the 10 partial rDNA-ITS regions (503 to 577 bp) were assembled and analyzed using Molecular Evolutionary Genetics Analysis (MEGA) program Version 4.1 (24). Sequence data for the rDNA ITS

region of six full-length sequences of *O. brassicae*, 10 full-length sequences of *O. virulentus* from Europe and Japan, and eight partial sequences from the Americas and Europe were retrieved from GenBank (Table 1). Isolates SP-010 from Spain (EU981907) and PT-01 from Portugal (EU981906) were excluded from this analysis, as they were only 176 bp in length (8).

The complete *Olpidium* rDNA-ITS region sequences from five Australian isolates and 16 isolates from other continents were analyzed in direct pairwise comparisons to establish percent identities between them. Nucleotide sequence data for these 21 complete *Olpidium* rDNA-ITS region sequences were then aligned in MEGA 4.1 using Clustal W (26) and bootstrapped

Fig. 1. Unrooted neighbor-joining relationship dendrogram for the complete 632-bp rDNA internal transcribed spacer nucleotide sequences of five *Olpidium* isolates sequenced in this study and 16 others from Europe and Japan available on GenBank. The phylogenetic tree was generated using MEGA 4.1 using the default parameters. Tree branches were bootstrapped with 1,000 replications. Numbers at nodes indicate bootstrap scores >50%. Scale bar represents a genetic distance of 0.02 for horizontal branch lengths. Table 1 shows isolate designations.

Table 2. Estimates of evolutionary percent identity between isolates of *Olpidium virulentus* and *O. brassicae* based on nucleotide sequence alignment of the complete rDNA internal transcribed spacer region

	<i>Olpidium virulentus</i>															<i>Olpidium brassicae</i>					
	KZ-1	Nan7	Hy-1	Nan6	NLD4	GBR10	ITA1	Col1	T123	WOm3	GBR1	CH-1	TAK-1	Nan8	WT-1	YR-2	EH-2	CBG-3	GBR7	ZE-1	NKA3
KZ-1		99.8	99.8	99.4	98.3	98.3	99.3	99.1	98.9	98.9	98.9	99.3	99.1	98.7	98.5	78.2	78.2	77.9	77.7	77.4	77.4
Nan7			100	99.6	98.5	98.5	99.4	99.3	99.1	99.1	99.1	99.4	99.3	98.9	98.7	78.4	78.4	78.2	77.9	77.7	77.6
HY-1				99.6	98.5	98.5	99.4	99.3	99.1	99.1	99.1	99.4	99.3	98.9	98.7	78.4	78.4	78.2	77.9	77.7	77.6
Nan6					98.9	98.5	99.4	99.3	99.1	99.1	99.1	99.4	99.3	98.9	98.7	78.7	78.7	78.5	78.2	77.9	77.9
NLD4						98.1	99.1	98.5	98.3	98.3	98.3	98.7	98.5	98.1	97.9	77.7	77.7	77.4	77.2	76.9	76.9
GBR10							99.1	98.5	98.3	98.3	98.3	98.7	98.5	98.1	97.9	78.4	78.4	78.1	77.9	77.6	77.6
ITA1								99.4	99.3	99.3	99.3	99.6	99.4	99.1	98.9	78.7	78.7	78.4	78.2	77.9	77.9
Col1									99.4	99.4	99.4	99.3	98.9	98.7	98.4	78.4	78.4	78.2	77.9	77.7	77.7
T123										100	100	99.3	99.1	98.7	98.5	78.9	78.9	78.7	78.4	78.2	78.1
WOm3											100	99.3	99.1	98.7	98.5	78.9	78.9	78.7	78.4	78.2	78.1
GBR1												99.3	99.1	98.7	98.5	78.9	78.9	78.7	78.4	78.2	78.1
CH-1													99.8	99.1	98.9	78.9	78.9	78.7	78.4	78.2	78.1
TAK-1														98.9	98.7	78.9	78.9	78.7	78.4	78.2	78.1
Nan8															99.4	79.4	79.4	79.1	78.9	78.6	78.6
WT-1																79.4	79.4	79.2	78.9	78.7	78.6
YR-2																	99.6	99.8	99.6	99.4	99.4
EH-2																		99.8	99.6	99.4	99.4
CBG-3																			99.8	99.6	99.6
GBR7																				99.4	99.4
ZE-1																					99.3
NKA-3																					

with 1,000 resamplings. This process was then repeated to also include the two partial sequences from southwest Australia and the other eight partial sequences. Evolutionary history was inferred using the neighbor-joining method initially for the 21 full-length sequences only and then for all 31 sequences. Evolutionary distances were computed using the Maximum Composite Likelihood method. All positions containing gaps and missing data were

eliminated from the dataset (complete deletion option). Two sequences of *O. bornovanus* were then added to the alignment and a radiation diagram constructed to show the relative genetic distance between the different species of *Olpidium* reported to be virus vectors.

RESULTS

PCR DNA extracts from roots. PCR amplification of the rDNA-ITS nucleotide

sequence from all seven root samples using primers designed specifically for *O. virulentus* always revealed presence of this *Olpidium* species. However, primers designed specifically for *O. brassicae* produced only one very faint amplicon from one sample. Subsequent sequencing of this band failed, suggesting that *O. brassicae* was absent, which is consistent with recent findings that this species does not infect lettuce (7,13,23).

Fig. 2. Unrooted neighbor-joining relationship dendrogram for rDNA internal transcribed spacer nucleotide sequences of the five complete and two partial *Olpidium* isolates sequenced in this study and 16 complete and eight partial ones from Europe, Japan, and the Americas available on GenBank. The phylogenetic tree was generated using MEGA 4.1 using the default parameters. Tree branches were bootstrapped with 1,000 replications. Numbers at nodes indicate bootstrap scores >50%. Scale bar represents a genetic distance of 0.02 for horizontal branch lengths. Table 1 shows isolate designations.

Nucleotide sequence identities. Of the 21 *Olpidium* complete rDNA-ITS region nucleotide sequences analyzed, those of the five southwest Australian isolates showed greatest nucleotide identity with those of *O. virulentus* (four isolates from Europe and six from Japan) (Table 2). Overall, there was 97.9 to 100% identity between the 15 isolates of *O. virulentus*, and 99.3 to 100% identity between the six isolates of *O. brassicae*. However, the identity between *O. virulentus* and *O. brassicae* sequences was only 76.9 to 79.4%.

Phylogenetic analysis. The five *Olpidium* sp. isolates with complete rDNA-ITS nucleotide sequences from southwest Australia all grouped with *O. virulentus* (Fig. 1). They fitted into two *O. virulentus* clades, I and II. Although the rDNA-ITS region of *Olpidium* is highly conserved, it was also possible to distinguish subclades within clade I despite the small overall sequence divergence. Four isolates fitted into clade I within two of its four subclades (Ia-d). Nan 6 and Nan 7 grouped together with Japanese isolates KZ-1 and HY-1 within subclade Ia, while T1 23 and Col 1 grouped with Japanese isolate WOns-3 and UK isolate GBR1 within subclade Id. Subclade Ib contained one isolate each from the Netherlands (NLD4), Great Britain (GBR10), and Italy (ITA1). The third subclade, Ic, contained two isolates from Japan (CH-1 and TAK-1). Australian isolate Nan 8 grouped with Japanese isolate WT-1, and these isolates were more distant genetically from the others forming a distinct clade on their own (clade II).

When the 10 partial sequences are added to the phylogeny (Fig. 2), subclade Ia then also included an Australian isolate (Col 3) and one from Guatemala (GT-08), subclade Ib contained two isolates from Mexico (MX-05 and MX-06) and one from the United States (633), four isolates from Spain (SP-06 to SP-09) were in subclade Ic, and the remaining Australian isolate (Col 2) fitted into subclade Id. None of the partial sequences fell within clade II.

When the relative genetic distances between the three viral vector species of *Olpidium* were compared, *O. virulentus* and *O. brassicae* were much closer to one another than either was to *O. bornovanus* (Fig. 3).

RT-PCR RNA extracts from leaves.

When leaf tissue from samples Nan 6 to 8 and Col 1 were tested by RT-PCR using primers specific for LBVaV or MLBVV, a 496-bp fragment was amplified indicating presence of MLBVV, a 292-bp fragment indicating presence of LBVaV, and a 360-bp control fragment indicating presence of the *nad4* gene of lettuce (Fig. 4). This shows that both viruses were present in each of the samples. Leaf tissue from samples Col 2 and Col 3 were also tested using the same method. Col 2 contained both viruses, while MLBVV was the only virus present in Col 3. As *O. virulentus*, but not

O. brassicae, was associated with the two viruses in the same lettuce plants, this indicates that it may have been responsible for the transmission of LBVaV and MLBVV on the two farms originally sampled.

DISCUSSION

This study revealed that *O. virulentus*, but not *O. brassicae*, was associated with

samples of lettuce plants with LBVD collected from two farms in southwest Australia, and that the same samples were infected either with LBVaV and MLBVV, or MLBVV alone. This association of *O. virulentus* with the two viruses is consistent with previous research which suggested that *O. virulentus*, rather than *O. brassicae*, was the chytrid vector of

Fig. 3. Phylogenetic tree showing relative genetic distance between the three virus vector *Olpidium* species: *O. bornovanus*, *O. brassicae*, and *O. virulentus*. The tree was generated in MEGA 4.1 using default parameters. Scale bar represents a genetic distance of 0.05 for branch lengths.

MLBVV and the TStV strain of LBVaV (23), and that *O. brassicae* does not infect lettuce (7,13,23). However, it contrasts with previous publications (e.g., 4,17,25,27), which named *O. brassicae* as vector of LBVaV and MLBVV.

Sasaya and Koganezawa (23) reported that the extent of nucleotide sequence identity in the complete rDNA-ITS region of *O. virulentus* and *O. brassicae* isolates was 98.5 to 100% and 99.3 to 100%, respectively, while it was 79.7 to 81.8% between the two species. This is consistent with the percent identities found in our study within isolates of *O. virulentus* from Australia, Europe, and Japan (97.9 to 100%), and of *O. brassicae* from Europe and Japan (99.3 to 100%). However, the percent rDNA-ITS region nucleotide sequence divergence between *O. brassicae* and *O. virulentus* isolates that we found was somewhat greater (76.9 to 79.4%). This degree of sequence divergence supports the conclusion made by Sahtiyanci (21) based on morphological evidence, and Sasaya and Koganezawa (23) based on molecular evidence, that they are distinct *Olpidium* species, not simply different strains of the same species. When the nucleotide sequences from the complete rDNA-ITS region of five *O. virulentus* isolates from samples of lettuce roots from Australia and those of the complete rDNA-ITS region of 10 *O. virulentus* isolates from Europe and Japan were analyzed phylogenetically, two clades were found (I and II), and within clade I, four subclades were distinguishable (Ia-Id). Australian isolates grouped within both clades and within clade I, subclades Ia and Id. Japanese isolates fitted within both clades and in all subclades except Ib, while European isolates only fitted in subclades Ib and Id. When the partial rDNA-ITS region se-

quences of two additional southwest Australian isolates, four from Europe, and four from the Americas were included in the analyses, the Australian isolates were within subclades Ia and Id, the European isolates within subclade Ic, and the American isolates within subclades Ia and Ib. One of the Australian farms sampled contained isolate sequences that fitted not only into both of the clades but also into two subclades (Ia and Id), while the second farm contained isolates within the same subclades. This suggests that since European settlement of southwest Australia more than 175 years ago and the subsequent introduction of plant cultivation to the region, three separate introductions of *O. virulentus* have occurred. The data indicate that there have been four separate introductions to Japan. Although Japanese isolates showed the greatest nucleotide sequence diversity for the rDNA-ITS region of *O. virulentus*, this does not mean that this species originated in Japan, as it is not a recognized center of crop domestication. Lettuce was probably domesticated first in Egypt (16). From there, lettuce cultivation spread through the Mediterranean region, first into Italy and then to Greece (29). Assuming that *O. virulentus* evolved with lettuce in its original center of domestication within the Mediterranean Basin, greater nucleotide sequence diversity of *O. virulentus* would be expected in this region than elsewhere. However, unlike Japanese and Australian isolates, European isolates did not fit into both clades, only into three subclades of clade I. Sequencing of more isolates from the Mediterranean region and Europe in general is required to reveal the extent of diversity in the *O. virulentus* population in that part of the world. Further sequencing of the rDNA-ITS region of *O. virulentus*

isolates from the Americas is needed to comment on the number of possible introductions there, but more sequence diversity would be expected than that in Australia given the longer history of plant cultivation since European colonization.

Methyl bromide was formerly used widely as a fungicide to control *Olpidium* spp., but it is no longer available in some countries, including Australia. Environmentally friendly cultural (agronomic) and phytosanitary control measures against *Olpidium* vectors were included within the LBVD integrated disease management strategies for nurseries, nutrient film, infested land, and uninfested land of Jones (12). For example, when infested land is used, decreasing irrigation and/or placing plastic mulch on the soil surface when overhead irrigation is used diminishes the mobility of viruliferous *O. virulentus* zoospores (14).

ACKNOWLEDGMENTS

We thank Stephen Wylie and Craig Webster for their advice on nucleotide sequencing, local farmers for allowing sampling on their farms, and Frances Brigg and Julie Uhlmann for operating sequencing machines and the SABC for providing laboratory facilities. Linda Maccarone received an Australian Postgraduate Award (Industry) jointly funded by the Australian Research Council and the Department of Agriculture and Food Western Australia.

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Fig. 4. Agarose gel resulting from a multiplex reverse transcription-polymerase chain reaction to detect the presence of *Mirafiori lettuce big-vein virus* (MLBVV), *Lettuce big-vein associated virus* (LBVaV), and control gene *nad4* in four samples of lettuce with lettuce big-vein disease from southwest Australia. All samples had mixed infection with LBVaV and MLBVV, and were positive for *nad4*. Lane 1: isolate Nan 6, lane 2: Nan 7, lane 3: Nan 8, lane 4: Col 1. Lane M represents a 100-bp molecular weight marker.

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