Clonal Spread of *Sclerotium cepivorum* in Onion Production with Evidence of Past Recombination Events

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Accepted for publication 24 January 2000.

ABSTRACT

Couch, B. C., and Kohn, L. M. 2000. Clonal spread of *Sclerotium cepivorum* in onion production with evidence of past recombination events. *Phytopathology* 90:514-521.

Three field populations of *Sclerotium cepivorum* from Ontario and Quebec in Canada, one field sample from the North Island of New Zealand, as well as isolates from other onion- and garlic-growing areas were classified in mycelial compatibility groups (MCGs) and further characterized by DNA fingerprints and DNA sequence polymorphisms at six genomic regions. MCGs in the population samples were genetically distinct clones.

Members of a clone belonged to a unique MCG with an associated multi-locus genotype and DNA fingerprint. The structure in each field population was predominantly clonal based on the repeated sampling of genotypes over a wide geographic area. Only three clones were identified within each of the population samples. The hypothesis that these clones were introduced to the three agricultural production areas is supported by their recovery from additional locations. Some evidence for past recombination was detected when genotypes from the three population samples and from other locations were pooled.

Sclerotium cepivorum Berk., cause of white rot of *Allium* spp., would be expected to disseminate clonally with relatively low genetic diversity in field populations. *S. cepivorum* has no known teleomorph, reproducing through copious production of small sclerotia that serve as both propagules and inoculum (8,9). Sclerotia can remain viable in the soil for up to 20 years in the absence of a host (11) and germinate, specifically in response to sulfides present in *Allium* spp. (10), to produce an infective mycelium. High inoculum loads in some soil types can result in yield reductions over many seasons, precluding further cultivation of onion, leeks, or garlic (13). On a local scale, sclerotia are disseminated by high winds, flooding, and irrigation, as well as by movement of agricultural equipment and materials (34). Macrogeographic dissemination is thought to occur with the movement of infected plant material (34).

Although *S. cepivorum* is only known to reproduce asexually, a sexual cycle likely existed at one time and may still occur somewhere. *S. cepivorum* produces microconidia (9) that are indistinguishable from those known to function as spermatia in the family Sclerotiniaceae (16). *S. cepivorum* is related to the ascomycete *Sclerotinia sclerotiorum* (7) and is thought to be an asexual member of the family Sclerotiniaceae (30). This has been confirmed in a recent phylogenetic study (5) of separate gene genealogies of seven genomic regions, also termed loci, that indicated that *S. cepivorum* is an asexual member of the family Sclerotiniaceae but is not in the *Sclerotinia* clade. Based on this evidence, *S. cepivorum* does not appear to be a member of the genus *Sclerotinia*.

The origin of asexual fungal species has been hypothesized to require the loss of a sexual cycle in a sexual ancestor (2), as evidenced in the genera *Penicillium* and *Aspergillus* (17,33). This predicts that asexual lineages are recent rather than ancient. In *S. cepivorum*, the continued production of microconidia is either a vestige of past spermatization in sexual reproduction or an indication of contemporary spermatization that would precede recombination. While ongoing, contemporary recombination should result in

relatively high genetic diversity, historical recombination might be difficult to distinguish against a pattern of predominant clonality.

Phylogenetic approaches to detecting recombination complement tests for linkage disequilibrium or association (20). Three main approaches are used to infer phylogenies from DNA sequence data: distance, parsimony, and maximum likelihood (15). Parsimony methods reconstruct phylogenetic relationships based on the concept of minimum evolution, under which the best phylogeny (tree) is the one in which characters change state a minimum number of times. The total number of character state changes necessary to construct the tree is termed tree length. Of course, a character, such as a particular nucleotide site, may change state, such as a particular nucleotide, more than once in evolutionary history (i.e., through 'multiple hits'). A character state may, therefore, be the outcome of parallel or convergent evolutionary events in descendants of different ancestors. Both situations result in homoplasy, and such characters are termed homoplasious. Homoplasy causes uncertainty by both increasing the number of possible trees that can be inferred from the data set and by increasing the tree lengths. The consistency index (CI) is an index of conflict in a phylogeny. $CI = R/L$, in which R is the number of steps in a minimum length of a tree and L is the actual length (25). Where the phylogeny is well supported by the data with little conflict, only one most parsimonious tree (MPT) is inferred from a data set, with a CI close to 1.0.

Phylogenetic analysis using parsimony, based on multiple genomic regions (loci), can be used to detect recombination in the history of a sample. A phylogeny inferred from a sample of sequences from one locus is often termed a gene genealogy. Under the condition of no recombination, all genomic regions are linked and share the same evolutionary history. Under this condition, gene genealogies from two or more physically unlinked loci should be congruent. If the gene genealogies are congruent, a tree inferred from the combined data sets of two or more loci should not be significantly longer (i.e., have branches defined by more character state changes) than the sum of the lengths of trees inferred for each locus alone. From the combined data sets from these loci, it should then be possible to infer a well-supported phylogeny, i.e., one or a few MPTs with a CI no lower than the CIs of trees inferred from the single data sets of each locus.

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Publication no. P-2000-0316-01R

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In contrast, recombination shuffles loci with different evolutionary histories. Under the condition of recombination, gene genealogies from several loci will likely be incongruent. If the data sets from these loci are combined, more than one MPT will be inferred (termed equally parsimonious trees if they have the same tree length), and the CI will be lower than the CIs of each of the trees inferred from the data sets of single loci. If a strict consensus tree is inferred from this combined data set, an option that produces one tree as the consensus of the many conflicting trees, the consensus tree will look more like a comb than a branching tree. This would contrast to the more highly branched trees inferred from each locus alone. Such a loss of resolution of branches would not be expected in a consensus tree inferred from combined data under the condition of clonality.

A simple method for detecting incongruence is to identify pairs of loci in which all four possible combinations of alleles exist. This is known as Hudson's four gamete test, which is not a statistical test but rather an objective method for interpreting data under the assumption that multiple hits occur infrequently (22). In addition, the partition homogeneity test (PHT) (23) and a maximum likelihood test (3) can be used to determine if significant incongruence exists among phylogenies (genealogies) inferred from different loci. The PHT compares the length of the observed phylogeny to a distribution of tree lengths generated under the null hypothesis of random association among loci (23), as would be expected if recombination were occurring. In the maximum likelihood test, likelihoods of observed data are calculated under two hypotheses. Under the hypothesis of recombination, the best tree topology is inferred separately for each of the loci. Tree topologies are allowed to vary among loci. Under the hypothesis of no recombination, one best tree topology is inferred from the combined data of all loci. The hypothesis with the higher overall likelihood is accepted if the difference in likelihoods is significantly large (3).

In the absence of recent recombination, if *S. cepivorum* has been introduced to onion-growing regions, local clonal genotypes should predate the introduction of the fungus. The occurrence of the same clones in more than one location would provide strong evidence for movement between locations. If recombination is contemporary and ongoing, populations from different areas would share alleles but not genotypes. Past recombination events may have given rise to new genotypes that have been amplified by asexual reproduction and disseminated to new locations. Contemporary recombination events would generate new genotypes within localized populations that may not have had time to become widely disseminated, due to the limited dispersal ability of sclerotia.

The goals of this study were to determine whether *S. cepivorum* disseminates clonally without recombination or with some element of recombination and whether clonal genotypes in three field population samples predate or postdate the introduction of *S. cepivorum* to these areas. Polymorphic DNA sequences from each of six genomic regions (loci) were screened in field samples from three onion production areas. Isolates were also DNA-fingerprinted with a dispersed repetitive element from *Sclerotinia sclerotiorum* (32) and grouped according to mycelial compatibility (31). To determine if genotypes identified in the three field localities occur elsewhere, additional isolates were characterized from Canada, the South Island of New Zealand, Europe, Mexico, and South America.

MATERIALS AND METHODS

Field samples and additional isolates. Table 1 lists isolates from the three field samples used in this study. A total of 194 isolates of *S. cepivorum* was obtained from 71 infected onions in muck soil at the Holland Marsh, Ontario, on 18 September and 2 October 1996. The eight fields sampled formed a rough transect, approximately 7.5 km long, along the length of the marsh from north to south. A total of 146 isolates of *S. cepivorum* was obtained in a random sample from 56 infected onions from six fields in muck soil

TABLE 1. Mycelial compatibility groups (MCG) and genotypes from field population samples of *Sclerotium cepivorum* from three onion-growing regions

Location ^a /field no.	Onion cultivar	MCG	Genotype ^b	Frequency of MCG/genotype combination ^c
Holland Marsh, Ontario, Canada				
1	Hamlet	2	S	6
		3	T	2
2	Hamlet	3	T	2
	Headliner	2	S	4
	Prince	2	S	4
	Red Man	2	S	3
	Ruby	2	S	4
3	Corona	1	R	2
		2	S	2
	Gazette	2	S	2
	Headliner	2	S	1
4	Fortress	2	S	1
	Hamlet	1	R	8
5	Corona	1	R	6
	Hamlet	1	R	2
6	Blitz	1	Unknown	1
	Corona	1	Unknown	4
	Hamlet	1	R	1
	Corona	1	R	2
7	Hamlet	1	R	5
	Eskimo and Advancer	1	R	9
Sherrington, Quebec, Canada				
1	Fortress	3	T	5
	Mars	3	T	6
		6	V	1
	T383	3	T	3
	Express Pak and Norstar	3	T	9
2		6	V	1
	Hamlet and XPH	3	T	5
3	Fortress and Prince	3	T	5
	Prince	3	T	7
4		6	V	1
	Prince	1	R	2
		3	T	3
		6	V	1
5		6	V	5
	Fortress	6	V	5
Pukekohe, North Island, New Zealand				
1	Unknown ^d	3	T	5
		3	T	5
		1	R	5
		1	R	5
		1	R	5
		1	R	3
2		3	T	1
		7	W	1
		1	R	2
		7	W	3
		1	R	1
3	Unknown	3	T	2
		7	W	2
		7	W	2

^a Holland Marsh isolates collected by B. Couch, L. Kohn, and M. Hovius, September and October 1996; Sherrington isolates collected by B. Couch and A. Gagnon, August 1997; Pukekohe isolates collected by A. Stewart, December 1997 and March 1998.

^b Genotype determined from multilocus haplotype and DNA fingerprint.

^c Frequency of MCG/genotype combination is the number of onions of each cultivar sampled from the field; only one MCG/genotype was recovered from each onion.

^d The cvs. Pukekohe Longkeeper and Pukekohe Longkeeper (Early Variety) are the principal cultivars grown in the area. The specific cultivars from which these isolates were sampled is unknown.

TABLE 2. Mycelial compatibility groups (MCG) and genotypes of additional isolates of *Sclerotium cepivorum* supplementing the field samples

Location ^a	Isolate	MCG	Genotype ^b
Brazil	LMK-73	3	T
British Columbia	Kel-1	2	S
	Clo-2	1	R
Chile	1-1	8	T
	1-2	8	T
England	2-FD30	12	Y
Ontario ^c	DE 1	1	R
	DE 2	2	S
Italy	Sc 1	2	S
	Sc 6	2	S
	Sc 11	8	T
	Sc 12	8	T
	Sc 15	8	T
	Sc 16	11	W
	Sc 21	6	V
	Sc 24	9	X
	Sc 26	6	V
	Sc 28	6	V
	Sc 30	6	V
	Sc 31	2	S
	Sc 33	2	S
	Sc 35	2	S
	Sc 36	6	V
	Sc 37	9	X
	Sc 41	9	X
	Sc 50	9	X
	Sc 52	8	T
	Sc 72	9	X
	Sc 79	10	T
	Sc 81	6	V
	Sc 82	9	X
	Sc 94	2	S
	Sc 92	8	T
	Sc 97	8	T
	Sc 102	10	T
Mexico	1-B	8	T
	1-Vo14	8	T
	2-FD1	8	T
	2-FD11	8	T
	2-FD15	8	T
	2-FD20	8	T
	2-FD22	8	T
	2-FD26	8	T
	2-FD28	8	T
	2-FD32	8	T
	2-FD33	8	T
	2-FD35	8	T
	2-FD42	8	T
Netherlands	LMK-1	4	R
New Jersey	LMK-71	5	U
New Zealand, South Island	Sc 186	1	R
	Sc 187	1	R
	Sc 188	3	T
	Sc 189	1	R
	Sc 191	7	W
	Sc 192	7	W
	Sc 193	7	W
	Sc 194	7	W
	Sc 195	1	R
	Sc 197	7	W
	Sc 198	7	W
	Sc 201	1	R
	Sc 203	7	W

^a Isolates from Mexico, Chile, and England from R. Galdames, CINVESTAV, Irapuato, Mexico; isolates from British Columbia, Canada, collected by J. Rahe, University of British Columbia, September 1997; Italian isolates from A. Carlucci, Università di Bari; isolate from Netherlands from W. Gams, CBS, Baarn; Brazilian isolate from P. Adams, USDA, Beltsville, MD; South Island, New Zealand, isolates collected by A. Stewart, Lincoln University, December 1997 and March 1998.

^b Genotype determined by multilocus haplotype and DNA fingerprint.

^c Voucher isolates for MCG-1 and MCG-2 (14) collected by D. Earnshaw.

outside of Sherrington, Quebec, on 18 August 1997. For both the Holland Marsh and Sherrington samples, the sampling target was three isolates per onion, five onions per cultivar, of all cultivars planted in each field. A total of 40 isolates from five onions in each of eight fields was collected in a random sample from clay loam soil at Pukekohe, North Island, New Zealand, in December 1997 by A. Stewart.

Sclerotia from infected onions were surface-sterilized in 70% ethanol for 5 min, followed by 2% NaOCl for 10 min, and then rinsed in sterile distilled water and plated onto potato dextrose agar (PDA) (Difco Laboratories, Detroit). If sclerotia were not present, mycelium or infected tissue was plated directly onto PDA. Colonies were subcultured and maintained on PDA at room temperature. Additional isolates from other locations are listed in Table 2. All cultures were stored at -70°C for future use.

Mycelial compatibility groupings. Mycelial compatibility tests were performed following the procedure of Kohn et al. (31). Pairings were evaluated 7 and 14 days after inoculation. Pairings were scored as incompatible if a strip of thin, sparse mycelium formed between the colonies, visible on the colony surface, or if a dark reaction line formed in the interaction zone, visible when viewed through the bottom of the plate. Pairings were scored as compatible if no reaction line was observed and the isolates grew together. All isolates were evaluated. Isolates that grew slowly or did not interact within 14 days were paired 2 cm apart instead of 3.5 cm apart as in the standard protocol. Incompatible reactions intensified between days 7 and 14.

DNA extraction. Isolates were grown on liquid complete yeast medium (0.46 g of KH_2PO_4 , 1.00 g of $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.50 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20.00 g of D-glucose, 2.00 g of yeast extract, and 2.00 g of Bacto peptone [Difco Laboratories] per liter) in standing culture in 9-cm petri dishes for 3 to 5 days. Mycelium was harvested by filtration through Miracloth (Calbiochem, La Jolla, CA), dried in a Savant Speed Vac (Savant Instruments, Inc., Farmingdale, NY), and stored at -20°C . DNA was extracted from 15 mg of dried mycelium following the procedure of Zolan and Pukkila (41).

Polymerase chain reaction (PCR), DNA sequencing, and primer design. DNA amplification by means of PCR was performed in either a Perkin-Elmer 9600 or 9700 thermocycler (Perkin-Elmer, Foster City, CA), following the protocol provided with PCR core reagents (Roche Molecular Systems Inc., Branchburg, NJ). Reaction volumes were either 12 or 20 μl with 6 or 10 μl of template DNA at a 1:200 dilution of the DNA extraction.

PCR products to be used as templates for DNA sequencing were purified using the GeneClean II kit (Bio 101, Inc., Vista, CA), following the manufacturer's instructions. Sequencing was carried out using the protocol from a dsDNA cycle sequencing kit (Gibco BRL, Gaithersburg, MD). The thermal program was 95°C for 8 min; 20 cycles of 95°C for 30 s, 55 or 53°C for 30 s, and 70°C for 1 min; 10 cycles of 95°C for 30 s and 70°C for 1 min; and a 4°C hold. Products were separated on Sequagel XR polyacrylamide gels (National Diagnostics, Atlanta, GA), following the manufacturer's in-

TABLE 3. Polymerase chain reaction primers used for sequencing and genotyping of *Sclerotium cepivorum* isolates

Locus	Primer sequences
EF-1 α	5'-TCACTTGATCTACAAGTGGGG-3' 5'-TTGATTGTAGTCGGTGCTGC-3'
Locus 2	5'-ACGTCGTCTAGCCAAAGAACC-3' 5'-GACGTGGAACCTATTGGCATGC-3'
Locus 3	5'-CCACCAGCAGTACCAATACG-3' 5'-GAATGATGGCGAAGTAAACG-3'
Locus 4	5'-GAGTTTGATTTCTGGGGTGG-3' 5'-AGCAATTTTGCTATGCTTGG-3'
Locus 5	5'-GCCTTTCGTGAACAATGTCG-3' 5'-ATGCTCATTGAGCTCGGACC-3'
Locus 6	5'-CCCTTCTCCTGAGAAGTGACC-3' 5'-TTTGTATATCCCGGCGTCTG-3'

structions. Gels were blotted onto filter paper and dried. Autoradiography was performed using Kodak BioMax film (Eastman Kodak Co., Rochester, NY) at room temperature for 24 to 72 h.

DNA sequences were compiled and edited with the aid of Geneworks (IntelliGenetics, Inc., Mountain View, CA). PCR primers (Table 3) were designed for randomly amplified regions and a portion of the EF-1 α gene.

Single-strand conformational polymorphisms (SSCPs). SSCP were used to screen for DNA sequence variation (35). PCR was performed using primers end-labeled with ^{32}P using T4 kinase (Gibco BRL), following the manufacturer's instructions. After thermocycling, 0.3 volume of stop dye (95% [vol/vol] formamide, 10 mM EDTA [pH 8.0], 0.1% [wt/vol] bromophenol blue, and 0.1% [wt/vol] xylene cyanol) was added. PCR products were then denatured at 95°C for 8 min and chilled to 4°C. One to two microliters of reaction mix was loaded onto a 30 cm $\times$ 39 cm $\times$ 0.4-mm vertical polyacrylamide gel (6% polyacrylamide, 0.12% bisacrylamide, and 0.5 $\times$ Tris-borate-EDTA). Gels were prepared either with or without the addition of glycerol to a final concentration of 7 or 10% glycerol. Products were separated at 150 V for 26 to 30 h. Gels were blotted onto filter paper and dried. Autoradiography was performed with Kodak BioMax film at room temperature for 12 to 48 h.

Identification of intraspecific DNA sequence variation. Genomic regions potentially harboring DNA sequence variation were identified by a modification of the sequencing with arbitrary primer pairs (SWAPP) technique (4) and, for one region, by direct sequencing of part of the EF-1 α gene using conserved primers (EF-1 α primer sequences provided by S. Rehner, University of Puerto Rico). To identify phylogenetically informative DNA polymorphisms, a set of six reference strains likely to be divergent was screened (DE-1, DE-2, LMK-1, LMK-71, LMK-73, and HM106011 from the Holland Marsh sample).

Modified SWAPP (4) was performed with one isolate, DE-1, in order to identify primer pairs that amplified a product 400 to 500 bp in length that was unique to the primer pair. PCR cycling was 95°C for 8 min; 3 cycles of 95°C for 3 min, 36°C for 3 min, and 72°C for 3 min; and 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, and 72°C for 5 min; followed by a 4°C hold. These primer pairs were then used in amplifications on the set of reference isolates described above. Amplicons from each isolate that were unique to the primer pair were excised from the gel with a pipette tip, eluted in 50 μl of glass distilled water, and used as a template for PCR, followed by SSCP to identify DNA sequence variation. Alternately, amplifications were performed on the set of reference isolates using ^{32}P -labeled primers, followed directly by SSCP.

Amplicons were sequenced that were unique to a primer pair and had SSCP among isolates. A template for sequencing was produced by eluting DNA of each allele from the dried SSCP gel and amplifying it using PCR. DNA was recovered from the dried SSCP gels by excising the band of interest, placing the gel fragment in 40 μl of filter-sterilized Tris-EDTA buffer, heating in a boiling water bath for 5 min, and incubating at 37°C for 3 h; 10 μl of elutant was used as a template for PCR, and the resulting product was sequenced.

Genotyping by multilocus haplotype and DNA fingerprint. The Holland Marsh and Sherrington samples were subsampled to detect intra- and inter-mycelial compatibility group (MCG) genotypic variation. One subsample of 30 isolates from the Holland Marsh consisted of 10 isolates of each of three MCGs originating from eight fields. A second subsample included 15 isolates of the most frequently sampled MCG at each location (MCG-1 from the Holland Marsh and MCG-3 from Sherrington). The third subsample of 25 isolates from Sherrington included 10 isolates each from MCG-3 and MCG-6, and five isolates from MCG-1. Forty-five isolates from the Holland Marsh, forty isolates from Sherrington, and all isolates from New Zealand, Italy, Mexico, Chile, and British Columbia were genotyped, for a total of 185 isolates. Each new allele identified by the SSCP screen was characterized by sequencing.

DNA fingerprinting was performed with the plasmid probe pLK44.20 containing a dispersed repetitive element from *Sclerotinia sclerotiorum* (27,29,32). Genomic DNA was digested with *Bam*HI. Gels were run at 2 V/cm for 36 h. Autoradiography was performed with Kodak X-Omat AR film (Eastman Kodak Co.) with an intensifying screen at -70°C for 1 to 7 days.

Phylogenetic analysis. Intraspecific genealogies were produced from variable sites from EF-1 α , six anonymous genomic regions, and DNA fingerprint data. Polymorphic sites in sequenced genomic regions (loci) included both substitutions and insertion/deletion motifs (indels). In parsimony analysis, indels within loci and hybridizing bands in DNA fingerprints were coded as present or absent. An unrooted tree was inferred using the branch and bound search option in PAUP 4.0b1 (D. L. Swofford, Smithsonian Institution, Washington, DC).

Hudson's four gamete test (22) assumes an infinite sites model (characters change state only once, no multiple hits) under which convergent mutation should be infrequent. This is not a statistical test but rather a criterion for detecting recombinant genotypes.

Trees for each locus were evaluated simultaneously for congruence using the PHT (23) in PAUP 4.0b1. A maximum likelihood approach (3) was also used to evaluate congruence among phylogenies (genealogies) inferred for each of several loci. First, the likelihoods for each of the six gene genealogies were calculated using the tree scores option in PAUP 4.0b1 and then summed. For the combined data set, the genealogy with the highest likelihood was chosen. Likelihoods were evaluated under two models: model 1, trees inferred from each of several loci have the same topology;

Fig. 1. Representative isolates of *Sclerotinia cepivorum* belonging to mycelial compatibility groups 1 and 2 showing the two DNA fingerprint polymorphisms identified in this study. *Sclerotinia sclerotiorum* (LMK-211) is included as a size standard; molecular weights are as presented by Kohli et al. (27). Fingerprints were obtained by Southern hybridization of *Bam*HI-digested genomic DNA with the plasmid pLK44.20 containing a dispersed repetitive element from *Sclerotinia sclerotiorum* (32).

and model 2, trees inferred from each of several loci have different topologies. The standard deviation of the difference in likelihood between the sum of likelihoods for each genealogy and the likelihood of the combined genealogy under each model was calculated as described by Kishino and Hasegawa (24). If all loci have the same topology, the sum of the likelihoods for genealogies produced from each locus should equal the likelihood of the genealogy produced from the combined data set. If loci have different topologies, the likelihood value calculated as the sum of the $-\ln$ likelihoods for each genealogy will be smaller than the $-\ln$ likelihood for the combined genealogy (3).

Genotypic diversity. Genotypic diversity, G , was calculated as $G = 1/\sum p_i^2$, in which p_i is the frequency of the i th genotype (37).

RESULTS

Mycelial compatibility groupings. Multiple isolates from each onion all belonged to the same MCG, even in fields containing more than one MCG. Table 1 shows the frequency of each MCG from each field sampled. MCG-1 was the most frequent in samples from the Holland Marsh and Pukekohe. MCG-3 was the most frequent in the sample from Sherrington.

Chi-square tests, MCG-by-field for each of the three samples, indicated that the spatial distribution of MCGs on onions sampled from the Holland Marsh, Sherrington, and Pukekohe was significantly nonrandom ($P < 0.001$).

Identification of intraspecific DNA sequence variation. To identify primer pairs suitable for SWAPP, 126 randomly chosen PCR primer pairs were used to amplify DNA from isolate DE-1. By using primers both singly and in combination, 28 primer pairs were identified that produced amplification products unique to the primer pair and were the optimal length, 400 to 500 bp. These 28 primer pairs were then used to amplify DNA from six reference strains of *S. cepivorum*. Amplification products were screened for DNA sequence variation using SSCPs; 12 amplicons containing DNA sequence variation were identified. These variable amplicons were eluted from the SSCP gel, amplified by PCR, and sequenced. Of the 12 amplicons, only five were successfully sequenced due to the presence of more than one sequencing template in the reaction mix. One variable site was also identified by direct sequencing a portion of the EF-1 α gene from the six reference isolates. DNA sequences were accessioned to GenBank, accession numbers AF163670 to AF163681.

DNA fingerprinting. Figure 1 shows a Southern hybridization of pLK44.20 to *Bam*HI-digested DNA from *S. cepivorum* and *Sclerotinia sclerotiorum* (LMK 211). pLK44.20 hybridized strongly

to three restriction fragments from *S. cepivorum*. Only two fingerprint phenotypes were identified in samples of *S. cepivorum*.

Genotyping. Table 4 presents the genotypes associated with each MCG. Seven multilocus genotypes were identified from 185 isolates of *S. cepivorum*. All members of a MCG had the same multilocus genotype. In three cases (genotypes R, T, and W), a single multilocus genotype was shared by more than one MCG.

Twelve variable sites were identified from the genomic regions that were analyzed. One variable site, at position 4 within locus 3, was not detectable by SSCP and was not scored. In total, eight phylogenetically informative characters were identified from four of the six regions analyzed. EF-1 α , locus 3, and locus 5 each provided one phylogenetically informative character. Locus 4 provided five phylogenetically informative characters. Although they did not provide phylogenetically informative characters, loci 2 and 6 delimited the single isolate representing MCG-5.

A single MPT with a CI of 1.0 was inferred for each of these regions as well as for the fingerprint data (Fig. 2). Phylogenies estimated from each locus were incongruent with each other based on results from the PHT performed on all six variable regions simultaneously ($P = 0.201$). Maximum likelihood values for the data set are shown in Table 5. The hypothesis of recombination, under which the best tree topology is inferred separately for each of the loci, is most likely. From the combined data from all six loci, five equally MPTs were inferred (Fig. 2), each two steps longer than the theoretical minimum length tree and each with a CI of 0.846.

Alleles at all loci are shown in Table 4. All four combinations of the two EF-1 α alleles and two locus 4 alleles were observed, an indication of recombination based on Hudson's four gamete test (22). These potential recombinant genotypes were identified when all genotypes were compared in the entire pooled sample, but not when each sampling site was considered separately.

Genotypic diversity for the *S. cepivorum* samples from the Holland Marsh, Sherrington, and Pukekohe were 2.3, 1.5, and 2.5, respectively.

DISCUSSION

Three conclusions can be drawn from this study: (i) *S. cepivorum* has disseminated clonally in Canada and New Zealand, (ii) there

TABLE 5. Maximum likelihood values for the data set calculated under two models, all loci have the same topology and all loci have different topologies

Model	Maximum $-\ln$ likelihood	Difference	Standard deviation
1. Same topology	-1565.9	53.6	5.3
2. Different topologies	-1512.3		

TABLE 4. *Sclerotium cepivorum* mycelial compatibility groups (MCG) and genotypes identified in this study

MCG	Genotype ^a	EF-1 α ^{b,c}	Locus 2	Locus 3	Locus 4					Locus 5	Locus 6		DNA fingerprint
					c	d	d	d	d		e	f	
1	R	<u>1</u>	G	T	<u>A</u>	<u>G</u>	<u>G</u>	<u>G</u>	<u>C</u>	C	1	T	01
4	R	1	G	T	A	G	G	G	C	C	1	T	01
2	S	<u>0</u>	G	T	<u>C</u>	<u>A</u>	<u>A</u>	<u>C</u>	<u>A</u>	T	1	T	10
3	T	<u>1</u>	G	T	<u>C</u>	<u>A</u>	<u>A</u>	<u>C</u>	<u>A</u>	C	1	T	10
8	T	1	G	T	C	A	A	C	A	C	1	T	10
10	T	1	G	T	C	A	A	C	A	C	1	T	10
5	U	1	A	T	A	G	G	G	C	T	0	C	01
6	V	1	G	C	A	G	G	G	C	C	1	T	10
7	W	<u>0</u>	G	T	<u>A</u>	<u>G</u>	<u>G</u>	<u>G</u>	<u>C</u>	T	1	T	01
11	W	0	G	T	A	G	G	G	C	T	1	T	01
9	X	1	G	T	A	G	G	G	C	C	1	T	10
12	Y	1	G	T	A	G	G	G	C	T	1	T	01

^a Genotype determined by multilocus haplotype and DNA fingerprint.
^b EF-1 α contains a three base pair insertion/deletion. The allele containing the deletion is indicated by a 0.
^c Underlining indicates each of two alleles at locus EF-1 α and locus 4 observed in the sample; presence of all four combinations of two alleles at two loci indicates recombination in Hudson's four gamete test (22).
^d Different polymorphic sites within locus 4.
^e Locus 6 contains a 63 base pair insertion/deletion. The allele containing the deletion is indicated by a 0.
^f A different polymorphic site within locus 6.

is evidence consistent with genetic exchange and recombination that predated immigration to the field populations sampled, and (iii) *S. cepivorum* was introduced to each of the three field populations.

Clonal dissemination was supported by three criteria (40): the association of several presumably physically unlinked characteristics (MCG, multilocus haplotype, and DNA fingerprint); the absence of recombinant genotypes on a local scale; and the occurrence of the same multilocus genotype over a wide geographic area. The association between MCG, multilocus haplotypes from six genomic regions, and one of two DNA fingerprint phenotypes was used to identify clones. Within the Holland Marsh, Sherrington, and Pukekohe samples, there was no evidence for genetic exchange and recombination based on Hudson's four gamete test (22), a result consistent with the predominantly clonal population structure at each location. When genotypes from all locations were pooled, however, potential recombinant types were identified. No recombinant types were identified within locus 4, which included more than one phylogenetically informative site; if recombination were frequent, some intragenic recombination might be expected. The same MCGs were repeatedly identified in samples from the Holland Marsh, Sherrington, and Pukekohe. Recovery of MCG-1 and MCG-2 in 1996 from the Holland Marsh, the same MCGs that were previously identified by Earnshaw and Boland (14), plus the association of haplotypes with MCGs in the current study, is an indication that MCGs are clones that are temporally stable and that the two studies can be compared.

Phylogenetic methods provided additional evidence of recombination in the history of *S. cepivorum*. The null hypothesis of recombination could not be rejected due to the presence of recombinant types within the sample. The PHT indicated conflict among genealogies inferred from the six variable loci. Such conflict can result from recombination, convergent, or parallel mutation. Re-

combination is the most likely explanation for these results, since multiple substitutions are unlikely to have occurred in sequences with low levels of divergence (21). This conflict among genealogies is also clear when the strict consensus tree, a tree containing only branches present in all five MPTs from the combined analysis of six loci, is considered. The poor resolution of the consensus tree is indicative of incongruence, as a result of recombination, among genealogies inferred from different loci (1). The likelihood test also indicated significant conflict among genealogies inferred from the six variable loci, since the model in which loci are allowed to have different topologies is the most likely.

Hudson's four gamete test on the pooled data set and evidence of homoplasy in the combined analysis of all six genealogies are consistent with some recombination in *S. cepivorum*, but the four gamete test indicates predominant clonality within the field samples. Evidence of past recombination is not inconsistent with a predominantly clonal mode of contemporary reproduction. Assuming that the ancestor of *S. cepivorum* was sexual, as indicated by the continued production of spermatia, evidence of past recombination events would be expected in contemporary population samples. Even though *S. cepivorum* produces spermatia, sexual reproduction with genetic exchange and recombination appears to be infrequent. It should be noted that recombination has been detected, using these and other tests, in a number of fungi with no observed sexual state (1,39). In *S. cepivorum*, with its copiously produced spermatia, such evidence cannot be ignored.

In the current study, MCGs and multilocus genotypes could be compared, offering some insight into the origins of clones in *S. cepivorum*. The sample from Italy (from an original field sample of 100 isolates) represented six MCGs (Table 2); it is possible that genetic diversity is higher in Europe and that Europe has been a center of origin for the spread of *S. cepivorum* to onion production

Fig. 2. A to E, The five most parsimonious trees and F, the strict consensus tree for *Sclerotium cepivorum* mycelial compatibility groups inferred from all six variable loci. Each of the five most parsimonious trees has a consistency index (CI) of 0.846.

areas elsewhere. In three cases, more than one MCG had the same multilocus genotype and DNA fingerprint, suggesting that, in this species, MCGs have diverged more rapidly than the DNA sequences of the genomic regions screened. In contrast, in the Canadian canola population of *Sclerotinia sclerotiorum*, DNA fingerprints diverged more rapidly than did MCGs (6).

Clonal genotypes at the Holland Marsh, Sherrington, and Pukekohe predate the introduction of *S. cepivorum* to these geographic localities. Evidence for this is the widespread occurrence of the same genotypes. If, for example, MCG-1 originated independently at the Holland Marsh, Sherrington, Pukekohe, the South Island of New Zealand, and British Columbia, it would have had to evolve at least five times. It has been suggested that *S. cepivorum* has moved with infested plant material (34), resulting in introductions to new geographic areas. This is consistent with anecdotal information provided by growers in the Holland Marsh. *S. cepivorum* is believed to have been introduced to the Holland Marsh in 1954 or 1955. Onions from Egypt were imported after Hurricane Hazel ruined the local onion crop (M. R. McDonald, *personal communication*). These onions were graded locally and any culls were discarded in a farmer's field to fill in low spots that remained after the water receded. Even if introduction occurred before the 1950s, given that drainage of the Holland Marsh began in 1925 and the first crops were planted in 1927, it is safe to assume that this pathogen is a recent introduction. Since its introduction, movement within the Holland Marsh has likely occurred as a result of movement of sclerotia-infested soil by high winds and on farm machinery, footwear, and pallet boxes used to transport onions from fields to packing plants (M. R. McDonald, *personal communication*). Similarly, *S. cepivorum* was first reported from Sherrington in 1969 (12) and from Pukekohe in 1922 (36).

The spatial distribution of genotypes at the three locations was nonrandom, consistent with an introduction to a field of a small number of genotypes, as soilborne or onion-borne sclerotia, followed by movement in the course of agricultural practices. Random spatial distribution of genotypes within sampling sites has been reported in *Sclerotinia sclerotiorum* (28), as would be expected for a pathogen with windborne ascospore inoculum (as well as soilborne sclerotia). Nonrandom distribution has been reported in soilborne pathogens from native soils, such as *Fusarium oxysporum* (19) and *Sclerotinia sclerotiorum* associated with the wild plant *Ranunculus ficaria* (27).

Very little genotypic variation was identified within samples of *S. cepivorum* from the Holland Marsh, Sherrington, and Pukekohe. Genotypic diversity within these samples approached the theoretical minimum of 1. This is consistent with the findings of Earnshaw and Boland (14), who identified two MCGs in a sample of 146 isolates from the Holland Marsh. In this study, three MCGs were identified in samples from each location. Only one new allele was identified among the six genomic regions in addition to those already identified in the suite of six reference strains.

The low genetic diversity found in some phytopathogenic fungi such as *Fusarium oxysporum* (26,38) and *Phytophthora infestans* (18) may be due to factors such as epidemic amplification of a few pathogenic genotypes within agricultural settings, bottlenecks associated with dispersal of plant pathogens, intrinsically low genetic diversity within some asexual fungi, or limited movement of genotypes between locations. The loss of genotypic diversity as a result of a bottleneck would be expected given the evidence that *S. cepivorum* was introduced to each of these locations.

The results of this study underscore the importance of cultural practices in controlling the spread of *S. cepivorum*. The careless introduction of *S. cepivorum* to the Holland Marsh on imported, infected onions is a perfect example of the danger associated with moving plant material among agricultural production areas. Identification of centers of genetic diversity, including determinants of pathogenicity and fungicide resistance, could be used to formulate appropriate and timely quarantine measures. The limited potential

for dispersal of soilborne or onion-borne sclerotia of *S. cepivorum* suggests that maintaining good quarantine measures could prevent the introduction of new genotypes.

ACKNOWLEDGMENTS

This project was supported by the program Research Grants to L. M. Kohn from the Natural Sciences and Engineering Research Council of Canada. We thank A. Gagnon (PRISME, Sherrington, Quebec), M. Hovius and M. R. McDonald (University of Guelph–Muck Crops Research Station, Kettleby, Ontario), and A. Stewart (Lincoln University, Canterbury, New Zealand) for providing important background information and assistance in acquiring isolates; and I. Carbone for advice on optimizing techniques.

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