New Populations of *Sclerotinia sclerotiorum* from Lettuce in California and Peas and Lentils in Washington

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Accepted 22 November 2006.

ABSTRACT

Malvárez, M., Carbone, I., Grünwald, N. J., Subbarao, K. V., Schafer, M., and Kohn, L. M. 2007. New populations of *Sclerotinia sclerotiorum* from lettuce in California and peas and lentils in Washington. Phytopathology 97:470-483.

Four populations of *Sclerotinia sclerotiorum* in North America were inferred previously, based on analyses of both rapidly evolving markers (DNA fingerprint and mycelial compatiblity), and multilocus DNA sequence spanning the range between fast and slow evolution. Each population was defined as an interbreeding unit of conspecific individuals sharing a common recent ancestor and arising in a unique evolutionary event. The present study applies this standard to extend characterization of *S. sclerotiorum* populations to the Western United States. Isolates of *S. sclerotiorum* (N = 294) were determined to represent three genetically differentiated populations: California (CA, lettuce), Washington (WA, pea/lentil), and Ontario (ON, lettuce). CA was the most diverse population yet sampled in North America. Clonality was detected in ON and WA. No DNA fingerprints were common among the populations. The index of association (I_A), based on fingerprint, was closer to zero (0) for CA than it was for the other populations. High diversity and lack of association of markers in California are consistent either with genetic exchange and recombination, or with large population size and high standing genetic variation. Intra- and interlocus conflict among three DNA sequence loci was consistent with recombination. The coalescent IGS genealogy confirmed subdivision and showed CA to be older than WA or ON. The Nearest Neighbor statistic on combined data confirmed subdivision among all present and previously defined populations. All isolates had both *MAT1-1* and *MAT1-2*, consistent with uniform homothallism.

Additional key words: migration, MDIV, mating type, multilocus haplotype, MCG, population genetics, phylogeny, vegetative compatibility.

The plurivorous, globally distributed, necrotrophic pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary has been an exemplar of epidemic clonality in central and eastern North America, where cold winters break the cycle of cropping, the history of agriculture is relatively recent, and multiple cropping is standard agronomic practice. Host specificity is unknown among isolates of this species and although there is population subdivision, there is no association of populations with crop species. There is at least one geographically associated population in the southeastern United States on crops, and another in Norway that is associated with a wild buttercup species (10,11). The clonality observed in crops is not surprising given the extensive asexual reproduction by long-lived, soilborne sclerotia and the self-fertilized sexual reproduction in this haploid ascomycete (27,16). Although self-fertilization in fungi is not obligate, and outbreeding is possible at least in the laboratory (5,44), the expected outcome of this life history would be predominant clonality, at least on the scale of contemporary field populations (200 years to the present). This assumption has had substantial support from population surveys of crops such as canola, soybean, cabbage, and kiwifruit in North America, Europe, New Zealand, and Australia (9,10,11,13,14,15, 21,31,33,35,38,45). As evidence of clonality, these studies have shown association of unlinked markers, vegetative (mycelial) compatibility, and DNA fingerprint (35) and repeated recovery of mycelial compatibility groups or unique DNA fingerprints in sampling in some studies over large geographical distances and over as many as 15 years of sampling. In all studies to date, DNA fingerprints and mycelial compatibility groups (MCGs) have been shown to diversify on a local scale; where population boundaries have been established by genetic or phylogenetic methods, no fingerprints of MCGs have been recovered in more than one population (11,14,45). The questions that naturally follow these studies are whether additional populations (i) await discovery, (ii) are associated with specific hosts or geographic distributions, and (iii) conform to the expectation of clonality rather than panmixia.

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doi:10.1094/PHYTO-97-4-0470
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Although a pattern of ancient recombination was detected (9,10), the predominance of clonality in contemporary field populations has been evidenced by the association of unlinked markers such as DNA fingerprints, microsatellite alleles, amplified fragment length polymorphisms (AFLPs) and MCGs, as well as the presence of a small number of clonal genotypes at high frequency, over repeated sampling, and over wide geographical areas. Clone frequencies have shown a skewed distribution, with a few clones represented at high frequency, and many clones or genotypes recovered only once or a few times (e.g., 14,15,21,30,32,35,38,45). Each clone is a mitotic lineage that can accumulate mutations. Intraclonal variation in aggressiveness has been reported in frequently sampled, widely distributed clones (38), and intraclonal variation in susceptibility to the fungicide, benomyl, and in high temperature tolerance have been observed (L. Kohn, *unpublished data*). The question remains whether new clones originate from mutation alone or also from outcrossing and recombination at some unknown, and likely low, frequency.

A pattern and process of population divergence was inferred from multilocus sequence data of ancestral recombination suc-

ceeded by divergence of mainly clonal populations through population fragmentation, following, for example, glacial retreat, followed by dispersal and isolation by distance, such as through the expansion of agriculture in North America (10). Two analytic approaches, hierarchical nesting with cladistic inference and coalescent inference, yielded the same population structure. Five populations were identified from samples on diverse crops and hosts. The most recently diverged populations, 3-1 and 3-2, dispersed in both the temperate and subtropical areas, diverging via dispersal/isolation by distance. Population 3-5 was limited to the temperate areas, and 3-4 was a metapopulation restricted to the wild buttercup, *Ranunculus ficaria*, in Norway (10,11). The oldest, population 3-3, appears to be endemic in the subtropical region of the southeastern United States (10,45). The Southeast, like much of the western United States, has characteristically mild winters with potential carryover of pathogens on weeds, high crop diversity, and multiple crops in a field per year. There is also evidence for more recombination in this predominantly clonal population than in other North American populations (9,14,31).

Deviations from predominant clonality have been observed as new groups of researchers sample new locations. In Washington State's Columbia Basin, where crop diversity is high and the seasonal variation is between wet and dry conditions, rather than between hot and cold, Atallah et al. (2) found that diversity of *S. sclerotiorum* in potato fields was high, and was intermediate on a gradient from strict clonality to panmixia, with observed indices of association for two fields falling within the range for simulated recombination and two fields falling outside this range. Hard evidence for outbreeding and recombination were sibling ascospores from each of three apothecia segregating ≥3 MCGs, while ascospores of the other 9 field apothecia analyzed each represented one MCG. Two MCGs were recovered at relatively high frequency, but microsatellite alleles were not associated with MCGs. Differences in aggressiveness on potato plants or in vitro response to fungicides and temperature gradients were not significant among genotypes. The high diversity of host crops and milder winter conditions were suggested to be more favorable for outcrossing than were conditions for other populations in the Northern Hemisphere (2). The same markers were used to genotype samples from Australian canola (52). Genotype diversity was high, and linkage disequilibrium measures indicated both clonal and recombinant components in the samples. Again, there were no significant differences in virulence among genotypes. Whether these samples were part of divergent populations on a global scale was not established in either of these two studies. Bear in mind that a population is not necessarily a sampling location, but is defined more formally as a group of conspecific individuals that are at least relatively genetically isolated and that share a common evolutionary origin.

Did the apparently recombining Washington samples represent a new population of *S. sclerotiorum* with an origin distinct from the other five mainly clonal populations? Was recombination more frequent than has been observed in central and eastern North America? Are populations distinct along the West Coast of the United States, with its lack of a hard winter and generally mild winter temperatures, its intensive and diversified agriculture, and less stringent crop rotation? In many ways, lettuce production in California represents an ideal system to address these questions.

Lettuce (*Lactuca sativa* L.) is grown throughout the year in the United States. The area encompassing California and Arizona has many climatic zones that provide nearly optimal lettuce-growing conditions, with up to two successive crops per year in some locations. Lettuce is present in at least some areas of the two states throughout the year. For example, the cool coastal climate during the summer and moderate temperatures during the winter make California's Salinas Valley the most important growing area throughout the year. From September through March, lettuce is also grown in the San Joaquin and Imperial Valleys in California, coinciding with production in the Yuma area of Arizona, but both areas are too hot for lettuce production in the summer (49,55).

One of the major production constraints in all growing areas, and particularly in California, is lettuce drop, caused by *S. sclerotiorum* and *S. minor*. Losses of up to 15% due to *Sclerotinia* species are common and losses of up to 60% have been reported (53). Infection of lettuce by *S. sclerotiorum* is primarily by airborne ascospores in California. Infection of the stem and lower leaves from myceliogenic germination of sclerotia seldom occurs in California, but is the predominant type of infection in other regions such as Ontario (22,41). MCG diversity of *S. sclerotiorum* on California lettuce has been reported to be high (58).

In the present study, the objective was to determine whether *S. sclerotiorum* on lettuce in California represents a divergent population in North America. Implicit in this objective is the hypothesis that there is a unique western United States population undetected in studies to date. Samples from pea and lentil in Washington were also included in the study for comparison. Both California and Washington have predominantly short crops (60 to 120 days) cycling throughout the year. We hypothesized that these conditions would present more evidence of recombination than had been previously observed in extensive crops such as canola or soybean in central and eastern North America, where the growing season ends with harsh winters and where populations of the pathogens have been shown to be predominantly clonal. To address the question of contemporary recombination within populations, we examined association of independent markers and a measure of association of hybridizing fragments in DNA fingerprints. We also used fingerprint data to test for population subdivision. To statistically infer population structure within an evolutionary framework, we employed a series of DNA-sequence-based analyses to determine subdivision and migration. This culminated in the inference of coalescent genealogies that provide estimates of time to the most recent ancestor for each DNA haplotype, from which divergence times of populations could be inferred. Using this coalescent evidence, each group of haplotypes with a path to a recent common ancestor distinct from other groups of haplotypes can be interpreted to be a population. Given that short crops and mild winters are also characteristic in the southeastern United States, a comprehensive analysis of population subdivision and migration was performed to include all North American populations previously inferred by Carbone and Kohn (10). In addition to data from samples in the previous study (10), a new sample was made from lettuce in Ontario for comparison with samples from lettuce in California.

MATERIALS AND METHODS

Sampling and isolates used in this study. Isolates and sampling locations are indicated in Table 1. In California, fields were

TABLE 1. Geographic location, host, and isolate designation of *Sclerotinia sclerotiorum* samples for population-level analysis

Location	Number of fields sampled	Host	Isolate Designations[a]	Total number of isolates sampled
California				
Salinas	5	Lettuce	CA 900 -1025	126
Bakersfield	3	Lettuce	CA 1026-1041	32
Ontario				
Holland Marsh	3	Lettuce	ON 1535-1618	83
Washington				
Quincy	1	Pea	WA 1660 – 1698	48
Palouse	1	Lentil	WA 1700 – 1704	5

[a] California = CA; Ontario = ON; and Washington = WA.

approximately 64 ha and were sampled in a V-shaped transect, with sclerotia from 24 to 30 plants sampled from each field. Disease incidence was ≤1.0%. Fields in Ontario were small, (market-garden size [<4 ha]), and in a muck region supporting diversified vegetable production. Disease incidence was 15 to 25%. Sclerotia from each of 30 diseased plants at least 5 m apart were collected along three to four transects. One sclerotium from each sample (group of sclerotia from one plant) was surface- sterilized and placed on PDA (potato dextrose agar), then incubated in the dark at 20 ± 2°C (35).

Fingerprinting and mycelial compatibility grouping, and calculation of index of association (I_A) and fixation index (F_{ST}). Samples were characterized by MCG, and by DNA fingerprinting. In mycelial compatibility grouping (35,52), isolates were paired on modified Patterson's medium (MPM) amended with 75 µl/liter of McCormick's red food coloring (McCormick Corp., Dallas, TX). After 7 days pairings were scored as incompatible or compatible. Reactions were scored as incompatible if a red line was observed in the encounter zone between the two developing colonies. Reactions were scored as compatible if the two developing colonies merged to form one mycelium with no interaction zone. All isolates were paired in groups of 10, including self-self pairings, until all isolates were assigned to an MCG or determined to be incompatible with representatives of all MCGs, including MCGs with only one isolate. In *S. sclerotiorum*, self-self incompatibility has not been observed; self-self pairings were used as a positive (compatible) control.

For DNA fingerprinting, genomic DNA was extracted using the small-scale protocol (34,35) and digested with the endonuclease *Bam*H1. Southern hybridizations were probed with pLK 44.20, which contains a 4.5kb repeated dispersed element of nuclear DNA from *S. sclerotiorum* (35). From previous studies, pLK 44.20 is expected to hybridize to 4 to 20 fragments (35). A Canadian canola isolate, LMK211, used as a standard in all previous studies using fingerprinting in the Kohn laboratory (35), was included in three lanes in all blots. For each fingerprint, 55 fragments (bins) were scored as either present (1) or absent (0). Genetic distance trees were calculated using fingerprint data based on the Neighbor Joining (NJ) method implemented in PAUP*4.0b10 for Unix (Swofford, D. L. 2003. Sinauer Assoc. Inc., Sunderland, MA). The same procedure was applied to compare the fingerprints of the samples with those on a database compiled from previous studies in the Kohn laboratory (35). The database contains the fingerprint data of over 3,000 samples of S. *sclerotiorum* from different hosts, locations, and years.

The degree of association between the two independent markers, MCG and fingerprint, was used as a rough indicator of the extent of clonality/panmixia in the population. In clonal populations, isolates form vegetative compatibility groups of two or more isolates that are transitive, i.e., isolate *a* is compatible with *b*, *b* with *c*, and *a* with *c*. Isolates that form vegetative compatibility groups also share the same fingerprint or differ in relatively few, i.e., ≤5, hybridizing fragments (21,31,35).

To measure genetic diversity in the fingerprints, the program MultiLocus (1) was used. Each bin was scored as a locus with two alleles (1 for present or 0 for absent). The genotypic diversity and linkage disequilibrium analysis option of MultiLocus was applied to each sample separately. This option includes several measures of genotypic diversity and linkage disequilibrium. The index of association (I_A) is used as a measure of multilocus linkage disequilibrium (1). I_A compares the observed variance in the number of mismatches between isolates with the expected variance based on allelic frequencies for a randomly mating (and recombining) system. In a randomly mating system, the observed variance in mismatches should not significantly exceed that of a calculated variance. Therefore, if I_A differs significantly from 0, then the system shows linkage disequilibrium. The expected/observed ratio minus one should be 0 in a randomly mating system. To estimate the expected variance under random mating, 1,000 randomizations were implemented. Analysis of molecular variance (AMOVA) and pairwise fixation indices (F_{ST}) estimations of the fingerprint data were conducted using Arlequin 3.01 (Schneider S., Roessli, D., and Excoffier, L. 2000. Dept. of Anthropol., Genetics and Biometry Lab., University of Geneva). The significance testing was done by comparing the observed statistic to a null distribution produced by permuting haplotypes to populations and calculating the statistic 1,000 times.

Mating type detection. The sequence of the *S. sclerotiorum* mating type genes has been determined from the genome sequence, available online (The Broad Inst. of MIT, Cambridge, MA). The sequence strain 1980 is homothallic and has both *MAT1-1* alpha and *MAT1-2* HMG genes together at the same single *MAT* locus. Probes for detecting the mating type were generated by amplifying fragments within the *MAT1-1* alpha and the *MAT1-2* HMG box. Polymerase chain reaction (PCR) for mating type locus pair 100µl) contained 0.5µl of each primer, 200 µM dNTP, 2× PCR Buffer II with 2mM $MgCl_2$, 0.5U of AmpliTaq DNA polymerase (Applied Biosystems, Branchburg, NJ), and 50 µl of a 100-fold dilution of genomic DNA extracted using the small-scale protocol (35). Primers *MAT1-1*-F (5′-ATACAGCCACTTACCTACCATACAGC) and *MAT1-1*-R (5′-TCTGAGTGGAAGCAATGTGTTGTG) flank a 673-bp region in the *MAT1-1* alpha gene and primers MAT2-F (5′-AGCCTATCCAGGTATCACCAATAACG) and *MAT1-2*-R, (5′-TGCCGAGGAGAGAAGAAGTCATAGAG) flank a 530-bp region in the HMG-box region of *MAT1-2* HMG. Amplifications were carried out in a 9700 Thermocycler (Applied Biosystems) programmed for an initial denaturation at 95°C for 8 min, followed by 35 cycles of 95, 60, and 72°C for 20, 30, and 60 sec respectively, ending with a 10 min extension at 72°C. Following amplification, PCR products were purified using QIAquick PCR Purification Kit (QIAGEN Inc. Mississauga, ON) according to the manufacturer's instructions. The purified product was then quantified on a 1% agarose gel. The blots used for fingerprinting were stripped and probed for mating type determination. Southern blots were probed with 2 µl of each probe (~150 ng/µl). After probing with DNA for *MAT1-1*, blots were stripped and probed with *MAT1-2*. *Bam*HI does not have a restriction site located between the hybridization sites of the mating types, therefore both probes hybridized to the same 4.5-kb fragment in the blot. As a further test, whole genomic DNA from a panel of isolates including California samples was restriction-digested with *Pvu*II, which has one site between the two *MAT* gene loci. DNA was run on 0.8% agarose gel for 24 hrs, and then hybridized with a mixture of both probes.

Multilocus haplotyping. Three loci were used in the present study, with the goal of building on the population/species study of Carbone and Kohn (10). In the previous study, the intergenic spacer of the nuclear ribosomal repeat (IGS) was an especially useful locus at the population level. This large locus (4 kb) presented 55 polymorphic sites for the entire sample of five populations, with prominent recombination blocks, i.e., sites of interblock but not intrablock recombination, in two populations. The subtropical population (3-3) and the wild population (3-4) each presented two similar recombination blocks, approximately at the midpoint of the locus. In the previous study, the 4-kb IGS was subdivided into 10 smaller overlapping regions of 300- to 400-bp each. In the present study, four of the most variable of these subregions (approximately 1,600 bp) were selected for characterization, with between 3 and 10 polymorphic sites in each region (Fig. 1). IGS 1a and 2a are on one side of the break between recombination blocks, and IGS 9a and 10a are on the other side of the block (Table 2). This approach provided a more efficient screening of the relatively large sample. The other loci used in the analysis were a 300-bp portion of the gene encoding translation elongation factor 1 alpha, EF-1α, and a 400-bp portion of an anonymous region from *S. sclerotiorum*, pLK 44.11 (10).

Isolates characterized by fingerprinting were screened by single-strand conformation polymorphisms (SSCPs) for the four selected subregions of the IGS, the EF-1α, and pLK44.11 (Table 2). The forward primer for each locus was endlabeled with $\gamma\ ^{33}$P ATP (60 fmol, 3000 µCi/mmol, and 0.1U of polynucleotide kinase (Invitrogen, Burlington, ON) at 37°C for 30 min. PCR reactions (12µl) contained 0.5µl of each primer, 200 µM dNTP, 2× PCR Buffer II with 2mM $MgCl_2$, 0.5U of AmpliTaq DNA polymerase (Applied Biosystems), and 6 µl of a 100-fold dilution of genomic DNA extracted using the small-scale protocol (35). Amplifications were carried out in a 9700 Thermocycler (Applied Biosystems) programmed for an initial denaturation at 95°C for 8 min, followed by 35 cycles of 95, 55, and 72°C for 15, 20, and 60 sec, respectively, ending with a 5-min extension at 72°C. After cycling was completed, 4 µl of stop solution (95% formamide, 10mM EDTA pH 8.0, 0.1% bromophenol blue, and 0.1% xylene cyanol) was added to reaction mixtures. The reactions were heat-denatured (95°C for 8 min), and 1.5 µl/lane was applied to 6% nondenaturing polyacrylamide gels with low cross-linking (2% bis-acrylamide in 0.5× TBE (Tris-borate-EDTA buffer). Standard sequencing rigs and plates (33 x 40 x 0.04 cm) were used. Electrophoresis was carried out at 150 V for 19 hours. Gels were transferred to filter paper, dried for 2 hours at 80°C, and then exposed to X ray film (Kodak BioMax, VWR, Mississauga, ON) for 16 hours at room temperature (23 ± 2°C). Autoradiograms were analyzed by grouping the samples according to the alleles present for each region. For each allele, three or four representative isolates were sequenced to characterize the allele and to confirm uniformity in scoring.

PCR products of each isolate, obtained with the same conditions as described in the previous section for the SSCP analysis in 20 µl reactions and without primer labeling, were purified using QIAquick PCR Purification Kit (QIAGEN Inc.) following the manufacturer's instructions. The purified product was then quantified on a 1% agarose gel and 3 to 10 ng of PCR product was used in the sequencing reaction. Sequencing was done using ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to manufacturer protocols.

Chromatograms were analyzed with Sequencher Version 4.5 (Gene Codes Corp., Ann Arbor, MI). Multiple alignments were inspected and adjusted manually using Sequence Alignment Editor version 2.0 (Rambaut, A. 2002. Dept. of Zool., University of Oxford). Alignments were exported to SNAP (Suite of Nucleotide Analysis Programs) Workbench for analysis (12,46). SNAP Workbench compiles and gives a suitable platform for the implementation of several programs. The layout of the modules in the program ensures that individual program assumptions and limitations are explored before their implementation (46). Unless specified, all analyses were performed from Workbench. Imported sequences were aligned using CLUSTAL W version 1.7 (54). SNAP Combine (3) was used for concatenating multiple aligned sequences from different loci, (e.g., IGS 1a, 2a, 9a, and 10a) into one combined alignment, and SNAP Map (3) was then used to collapse the sequences into haplotypes recoding insertions and deletion (indels) or excluding indels and infinite site violations.

Determination of population subdivision, migration, and coalescent analysis from multilocus data set. Most coalescent-based approaches for the estimation of genealogies and reconstruction of the ancestral history of samples assume neutrality and no recombination. Therefore, these assumptions were tested by different methods. To initially screen the data for conflict, phylogenetic analyses were performed with unweighted parsimony using PAUP*4.0b10 for Unix (Swofford, D. L. 2003. Sinauer Assoc. Inc., Sunderland, MA). Heuristic searches for datasets with no conflict should result in one most parsimonious tree, while the outcome of many equally parsimonious trees is indicative of conflict. To test for the overall support of conflict among variable sites in the sequence and to identify blocks of recombination, compatibility matrices (26) were generated using SNAP Clade (Markwordt, J. D., Doshi, R., and Carbone, I. 2003. Dept. Plant Pathol., North Carolina State Univ., Raleigh). The clusters of two or more incompatible sites in a matrix define recombination blocks; these blocks were visualized using SNAP Matrix (Markwordt, J. D., Doshi, R., and Carbone, I. 2003. Dept. Plant Pathol., North Carolina State Univ., Raleigh) The presence or absence of recombination blocks determined the subsequent analysis. If conflict was revealed in the compatibility matrix, and the incompatible sites were clustered in blocks, the dataset was partitioned and the analysis done for the blocks separately.

For each nonrecombining block or data set, the hypothesis of no genetic differentiation was tested among the different locations. SNAP Map was used to generate the combined file of haplotypes and geographic locations by collapsing sequences with phenotype (file-listing the geographic locations of each individual) excluding indels and infinite site violations (3). Seqmatrix (25) was used to convert the sequence file to a distance matrix, and Permtest (25) and the Nearest Neighbor statistic, S_{nn}, (24) were used to test for geographic subdivision in the sequences for the three localities: California (CA), Washington (WA), and Ontario (ON). The S_{nn} statistic is a measure of how often the nearest neighbors (i.e., the most similar sequences) are from the same geographic locality. An S_{nn} value ~1 indicates that populations are highly differentiated and values ~0.5 indicate that populations at two localities are part of the same population. The S_{nn} statistic is estimated for the overall sample and also in pairwise comparisons if there are more than two localities. Significance testing was done by comparing the observed statistic to a null distribution produced by randomly permuting (100,000 times) haplotypes to populations and calculating the test statistic. The P value was determined by the proportion of the null distribution with values greater than the observed statistic. The selection of subsequent steps in the analysis depended on whether the hypothesis of no geographic differentiation was rejected or not ($P < 0.05$). When

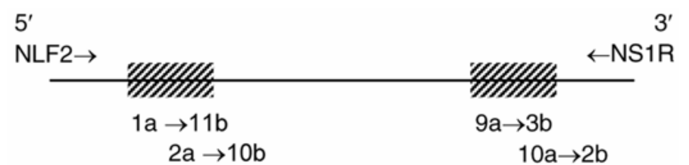


Fig. 1. The IGS locus, showing primers NLF2 and NS1R flanking the 4-kb region. Shaded areas represent the regions amplified in the present study. To amplify each of these regions, two sets of primers comprising two overlapping regions were used, e.g., IGS 1a-11b and IGS 2a-10b for the 5′ region.

TABLE 2. Loci and primers used in this study[a]

Locus	Primer	Primer Sequence (5′ – 3′)[b]
IGS	IGS-1a	CTCTCACCAAGGCATTGAGC
	IGS-11b	TACACTCACCTCACCGGTAG
	IGS-2a	GTGCTCTTAAGGTGCGAACC
	IGS-10b	GATTATTATTCTCCTAAATACCC
	IGS-3b	GGCTTACAGTGTGAATGAGG
	IGS-10a	CTACTATTCCTAGAGCTTC
	IGS-2b	AGAGCTGCAACGTGCTATGC
EF-1α	EF1-728F	CATCGAGAAGTTCGAGAAGG
	EF1-986R	TACTTGAAGGAACCCTTACC
44.11	44.11-908F	GCCTCGATAAAACTGTCTCC
	44.11-R1	ATATCGATCAGCAGATGATG
CAL	CAL-228F	GAGTTCAAGGAGGCCTTCTCCC
	CAL-737R	CATCTTTCTGGCCATCATGG
RAS	RAS-264-F	GATGAATATGATCCTACGAT
	RAS-565R	AAATCACATTTGTTACCAAC

[a] Internal transcribed spacer 1 primers are reported elsewhere (57).
[b] Reference (8).

there was population subdivision, Migration with Division, (MDIV) (42) was used to estimate time of divergence and integrated migration rates between two populations and the results were plotted using **gnuplot** (Williams, T., and Kelley, C., 2004; available online). MDIV implements likelihood and Bayesian methods using Markov Chain Monte Carlo (MCMC) coalescent simulations to estimate the population mean mutation rate (θ), divergence time (T), migration rate (M), and time since the most recent common ancestor (TMRCA) between two subdivided populations. The method uses either an infinite sites or a finite sites model without recombination. If migration rates estimated using MDIV were significantly different than 0, the Isolation with Migration (IM) program was used to estimate whether migration rates were symmetrical (equal in both directions) or asymmetrical between populations (23). This program applies the isolation with migration model to genetic data and estimates the marginal posterior probability densities for the parameters of the model: population size, divergence time, migration, and mutation (θ).

Coalescent analyses to reconstruct the ancestral history of the sample for the nonrecombining blocks or data sets were performed using Genetree (4,19). Genetree computes the joint likelihood surfaces with respect to θ and the migration matrix of backward rates. It also computes the distribution of the time to TMRCA and ages of mutation on the topology of the tree. The geographical information is integrated as the distribution of where the MRCA of the population was, where the subpopulation MRCAs were, and in which subpopulation mutations occurred (4). In each case, a minimum of 10 simulations of 1 million runs each, with different random seed numbers, were run until convergence of the results in the same rooted tree with the highest likelihood. For the rooted tree with the highest likelihood, the ages of mutations were estimated and a picture of the tree was generated with coalescent time units using Treepic (4,19).

Confirmation of California population as *S. sclerotiorum*. To confirm that the samples were *S. sclerotiorum,* four loci were sequenced for a panel of 12 isolates and analyzed in conjunction with representative isolates of the five populations defined in Carbone and Kohn (10), as well as representative isolates of *S. minor*, *S. trifoliorum,* and an unnamed cryptic species, Species 1, for a total of 20 isolates (Table 3). The loci used for this analysis were the internal transcribed spacer 1 (ITS1) of the nuclear rDNA repeat (57), and portions of genes encoding the translation elongation factor 1 alpha (EF-1α), calmodulin (CAL), and ras protein (RAS) (Table 2). For each locus, the 20 sequence alignments were collapsed into haplotypes using SNAP Map (3) and maximum parsimony analyses were performed for each locus, using the branch-and bound search option in PAUP*4.0b10 for Unix (Swofford, D. L. 2003. Sinauer Assoc. Inc., Sunderland, MA).

Determination of the nearest neighbor statistic from combined data from present and previous study. To compare the populations from the present study with those identified previously in the Americas, IGS 9a-10a sequence (approximately 800 bp of the 4 kb of the entire IGS analyzed by Carbone and Kohn (10) was used from samples from the following locations: North Carolina (NC), New York (NY), Louisiana (LA), Alberta (AB,) and Ontario (ON). This was the only portion of the IGS common to the present and previous studies. The other loci common to both studies do not provide additional resolution and cannot be combined at the population level due to interlocus conflict (data not shown). The list of isolates from Carbone and Kohn (10), used in the combined analysis for North America, is shown in Table 4. The sequences of isolates from all the locations were combined and the determination of S_{nn} was as described above.

RESULTS

Fingerprinting and mycelial compatibility grouping, and calculation of index of association (I_A) and fixation index (F_{ST}). Only fingerprints from samples from Ontario matched any

TABLE 4. Samples of *Sclerotinia sclerotiorum* from the Carbone and Kohn study of populations in North America used in this study (10)

Geographic Region	Code[a]	Host	*N*	Population designation[b]
North Carolina	NC	Cabbage *Brassica oleracea*	51	3-2; 3-1; 3-3
New York	NY	Cabbage *B. oleracea*	31	3-5; 3-2
Louisiana	LA	Cabbage *B. oleracea*	22	3-3; 3-1
Alberta	AB	Canola *B. napus* or *B. rapa*	39	3-5; 3-1; 3-2
Ontario	ON	Canola *B. napus*	1	3-5

[a] Designation of each geographic location.
[b] Population designation from previous study (10). Each isolate was assigned to one population but samples employed in the present study may include isolates from one, two, or three populations.

TABLE 3. Panel of isolates used in species determination in this study

Isolate	Species	Host	Geographic region	Source
M-1	*Sclerotinia minor*	*Lactuca sativa*	Canada	I. Carbone
LMK-36	*S. trifoliorum*	*Trifolium repens*	Tasmania	ATCC34327
1358.P	*Sclerotinia species 1*	*Caltha pallustris*	Norway	T. Shumacher, Univ. of Oslo
UR3	*S. sclerotiorum*	*Lactuca sativa*	Uruguay	G. Malvárez
UR8	*S. sclerotiorum*	*Lactuca sativa*	Uruguay	G. Malvárez
UR129	*S. sclerotiorum*	*Lactuca sativa*	Uruguay	G. Malvárez
UR481	*S. sclerotiorum*	*Lactuca sativa*	Uruguay	G. Malvárez
AR312	*S. sclerotiorum*	*Lactuca sativa*	Uruguay	G. Malvárez
AR319	*S. sclerotiorum*	*Lactuca sativa*	Argentina	G. Malvárez
CA904	*S. sclerotiorum*	*Lactuca sativa*	California (CA), United States	B.M. Wu, Univ. of CA, Davis
CA1020	*S. sclerotiorum*	*Lactuca sativa*	California, United States	B.M. Wu
AR1100	*S. sclerotiorum*	*Arachis hypogea*	Argentina	G. Malvárez
AR1150	*S. sclerotiorum*	*Arachis hypogea*	Argentina	G. Malvárez
AR1160	*S. sclerotiorum*	*Arachis hypogea*	Argentina	G. Malvárez
AR1170	*S. sclerotiorum*	*Arachis hypogea*	Argentina	G. Malvárez
LMK211	*S. sclerotiorum*	*Brassica napus*	Canada	Population 3-2[a]
LOU3-5	*S. sclerotiorum*	*Brassica oleracea*	Louisiana, United States	Population 3-3[a]
Ss12	*S. sclerotiorum*	*Brassica* oleracea	N. Carolina, United States	Population 3-3[a]
IC258	*S. sclerotiorum*	*Ranunculus ficaria*	Norway	Population 3-4[a]
825	*S. sclerotiorum*	*Brassica napus*	S. Carolina, United States	Population 3-3[a]

[a] Isolates representative of known populations (10).

fingerprints from previous studies (Fig. 2; Table 5); there were no matches with the Kohn laboratory database to fingerprints from Washington or California (14,30,32,35). No fingerprints were shared between Washington and California, California and Ontario, or Washington and Ontario samples. Ontario and Washington samples were predominantly clonal as evidenced by the repeated sampling of fingerprints and the association of each unique fingerprint with an MCG. In contrast, 90% of the isolates from California presented a unique fingerprint.

All California isolates were tested for mycelial compatibility in subsets of 10 isolates. The usual procedure in a population with some clonality is to take representatives of MCGs observed in the subsets and pair them until all isolates in the sample have been assigned to an MCG or have been determined to be an MCG of one isolate, i.e., incompatible with all other MCGs. In the present sample, when most isolates were determined to be incompatible with all others in the subset, with a negligible number of groups emerging among the subsets, we did not proceed with thousands of additional pairings to test all isolates in all combinations. In the 11 cases in which 2 isolates were scored as compatible and the isolates were fingerprinted, fingerprints differed by as few as 3 bands, within the range of fingerprint variation in a clone (21) to as many as 30 bands in one case. All self-self pairings were compatible.

The observed I_A (Table 5) for the fingerprint loci for the California population ($I_A = 0.34$, $P = 0.0018$) was closer to the expected $I_A = 0$ of a panmitic, randomly mating population than those of Ontario and Washington samples. The results of the AMOVA showed that California, Washington and Ontario populations were highly significantly differentiated ($P < 0.0001$), with 81% variation within populations and 19% among populations. Pairwise estimations of F_{ST} (Table 5) were also highly significant for each population ($P < 0.0001$).

Determination of mating types. All of the isolates were homothallic, each with both *MAT1-1* and *MAT1-2* sequences. When genomic DNA was restriction-digested with *Pvu*II and probed with a mixture of both *MAT1-1* and *MAT1-2*, two hybridizing bands were observed among all isolates screened. With *Bam*HI digestion, followed by probing of *MAT1-1*, then stripping and probing with *MAT1-2*, one band hybridized to both probes. DNAs of all isolates used in this study hybridized to these probes.

Determination of population subdivision, migration, and coalescent analysis from multilocus data set. The IGS DNA sequence was analyzed first, with results compared with the other two loci. The compatibility matrix for IGS1a, 2a, 9a, and10a revealed conflict (Fig. 3A) consistent with recombination, which would lead to the violation of the assumption of no recombination for the subsequent analysis. This outcome was expected since the subregions were selected to span recombination blocks previously detected in the IGS (10). Much of the conflict was resolved by separating the regions in two blocks, IGS 1a-2a (which still presents internal conflict, [Fig. 3B]) and IGS 9a-10a (no internal conflict, [Fig. 3C]). For analyses with the assumption of no recombination, only IGS9a-10a was used. From a total of 754 bp, IGS 9a-10a presented 43 variable sites that grouped the 275 samples from the three locations (CA, ON, and WA) in 15 haplotypes (Fig. 4A). The distributions of the haplotypes for each geographic location are shown in the last three columns of Figure 4A.

The Nearest Neighbor statistic, S_{nn}, (24) indicated population subdivision, with an overall S_{nn} of 0.75 ($P = 0.004$; P values <0.05 indicate significant geographic differentiation). S_{nn} values for the pairwise comparisons of the locations indicated subdivision

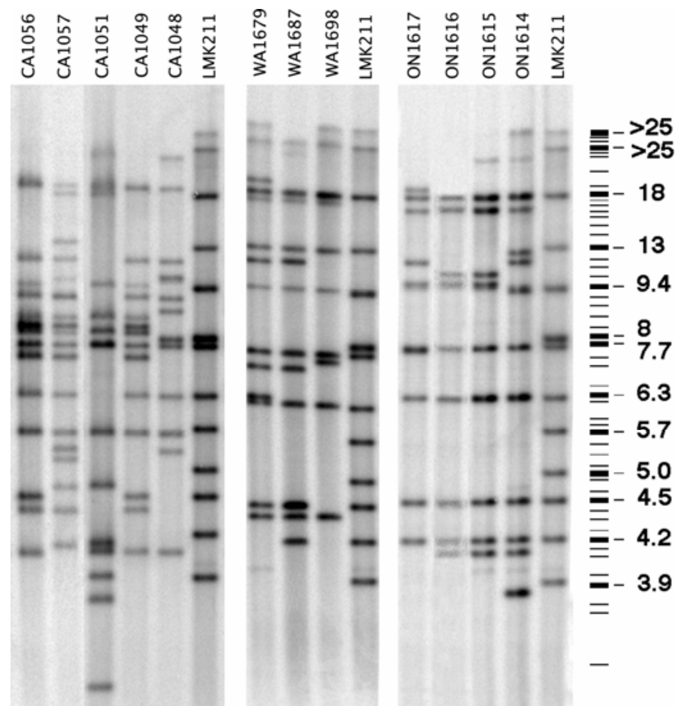


Fig. 2. Representative DNA fingerprints of *Sclerotinia sclerotiorum* from California (CA), Ontario (ON), and Washington (WA), obtained by Southern hybridization of *Bam*HI-digested genomic DNAs, and probed with the plasmid pLK 44.20 that contains a dispersed repetitive element from *S. sclerotiorum.* Isolate LMK211, from canola (35), is used as the reference isolate. Isolates ON1615 and 1616 are mycelially compatible but have fingerprints differing by two bands; they belong to the same clone.

TABLE 5. Comparison of DNA fingerprint results from Ontario, California, and Washington samples with index of association (I_A) and fixation index (F_{ST}), and comparison of fingerprints to database

Location[a]	N	Fingerprints	Unique Fingerprints[b]	Mean number of fragments (range)[c]	Database match[d]	I_A[e]	F_{ST}[f]
Ontario	78	31	20	10 (7-14)	16	1.43 ($P < 0.001$)	(CA) 0.190 ($P < 0.0001$)
California	113	100	91	12 (4-19)	0	0.34 ($P < 0.001$)	(WA) 0.181 ($P < 0.0001$)
Washington	48	21	11	12 (8-15)	0	2.20 ($P < 0.001$)	(ON) 0.176 ($P < 0.0001$)

[a] Ontario = ON; California = CA; and Washington = WA.
[b] Number of fingerprints represented by only one isolate.
[c] Mean number of hybridizing bands. Range is in parenthesis. Each band is scored as a bin; 55 bins are scored.
[d] Number of fingerprints that matched fingerprints in the database from previous studies.
[e] Index of association determined using MultiLocus (1). The observed I_A for the fingerprint loci for the California population was closer to the expected $I_A = 0$ of a panmictic, randomly mating population than those of the Ontario and Washington samples.
[f] Fixation index estimated using Arlequin 3.01 (Schneider, S., Roessli, D., and Excoffier, L. 2000. Dept. of Anthropol., Genetics and Biometry Lab., University of Geneva). The values under the F_{ST} column are the estimates from the pairwise comparisons for the population indicated in the first column of the table and the population indicated in. Significance was tested using 1,000 nonparametric permutations; all values were highly significant ($P < 0.0001$). Populations were significantly differentiated, with 81% variation within populations and 19% among populations.

among all three samples (Table 6). The geographic differentiation of the samples was further confirmed by estimation of population parameters using MDIV. Ten independent runs (each run with different starting random number seeds) of 2 million steps in the chain for estimating the population parameters gave the same overall distribution for migration, time of divergence, and mutation rate (θ). Figure 5 shows the posterior distribution for the migration among the three samples; in all cases migration is extremely low, less than 0.5. Based on these results we generated a backward migration matrix with migration rates close to 0 (0.01) for CA-ON, CA-WA, and ON-WA. Because the populations are strongly subdivided with no significant migration between them we did not need to estimate asymmetrical population migration rates using IM. The coalescent simulations, performed for each of 44 possible rooted trees, taking into account the population subdivision and our backward migration matrix resulted in tree 21 having the highest probability, (likelihood = 1.121×10^{-56}; SD = $8{,}870 \times 10^{-55}$ [Fig. 6A]).

The 44.11 locus alignment presented six variable sites and was collapsed in six distinct haplotypes. As in the case of the IGS locus, the S_{nn} statistic showed population subdivision overall (Snn = 0.80, P = 0.004) and for all pairwise comparisons (Table 6). Coalescent analysis for seven possible rooted gene genealogies was performed for population subdivision, and tree 3 was selected as having the highest probability (likelihood = 1.698×10^{-15}, SD = 1.500×10^{-13}, Fig. 6B).

The alignment for the EF-1α locus presented 34 variable sites that were grouped in three distinct haplotypes when collapsed, excluding indels and infinite site violations (selected as an option on the SNAP map). Population subdivision tests showed overall geographic differentiation between the three regions, S_{nn} = 0.45, P = 0.033. In the pairwise comparisons (Table 5), CA and ON presented values indicative of subdivision, as did CA and WA. In contrast, the comparison between ON and WA showed that these samples were part of the same population for this locus, so these two samples were pooled for further analysis. The S_{nn} values for the CA versus ON/WA was S_{nn} = 0.54, P = 0.026). Based on the subdivision analysis, the coalescent simulations were done for two subdivided populations (CA and ON/WA) and 35 possible rooted tree configurations assuming unequal size. Tree 18 (Fig. 6C) had the highest probability (likelihood = 1.007×10^{-21}, SD 2.848×10^{-20}).

For the EF-1α as well as the 44.11 locus, the coalescent tree showed a similar pattern to the one obtained for the IGS 9a-10. Despite the lower resolution of these two loci in identifying population subdivision, a unique, California isolate (haplotype 14) represents one of the two oldest lineages in the coalescent-based gene genealogy. It was not possible to combine the three loci to create a multilocus coalescent genealogy due to compatibility conflicts present when the loci were concatenated. This interlocus conflict is most likely evidence of recombination, in addition to the intralocus conflict in the IGS.

Given the genetic isolation of the California population from other populations previously identified in North America, the posibility that this sample represented a cryptic species rather than a divergent, conspecific population required further analysis. Reasoning that phylogenetic or coalescent distance among population lineages should be shorter than that for species lineages, we utilized the same relative measure of genetic distance employed by Carbone and Kohn (10). A multilocus analysis of a sample of isolates, including representatives of all North American populations, indicated that branch lengths for representatives of the CA sample were in the range for divergent populations of *S. sclerotiorum,* i.e., not one or more cryptic species of *Sclerotinia* (Table 7; Fig. 7).

Determination of the Nearest Neighbor statistic from combined data from present and previous study. For the comparison of the populations across North America, the isolates from California, Ontario, and Washington were combined with six samples, plus one Ontario isolate from canola from the earlier study of Carbone and Kohn (10). For the analysis of the combined data set, only the IGS 9a-10a was used. The haplotype map of the combined data set, excluding indels and infinite site violations, showed 46 variable sites. The sequences were grouped in 19 haplotypes. The compatibility matrix for the complete data set (data not shown) had incompatibilities that were resolved by excluding sites 19, 288, 606, 646, and 756 of the alignment. Once the sites were excluded, the alignment was collapsed into 14 haplotypes based on 41 variable sites (Fig. 8).

The Nearest Neighbor analysis for the combined samples determined six significantly distinct populations, CA, WA, NY, AB, ON, and NC-LA (Table 8). Samples from North Carolina and Louisiana, consistent with previous results (10), were part of the same population (S_{nn} = 0.57, P = 0.8). One canola isolate sampled in Ontario previously was part of the same population as the lettuce isolates sampled in Ontario in the present study (S_{nn} = 0.50, P = 1.0).

All previously unaccessioned sequences are accessioned in GenBank as EF152586- EF152649.

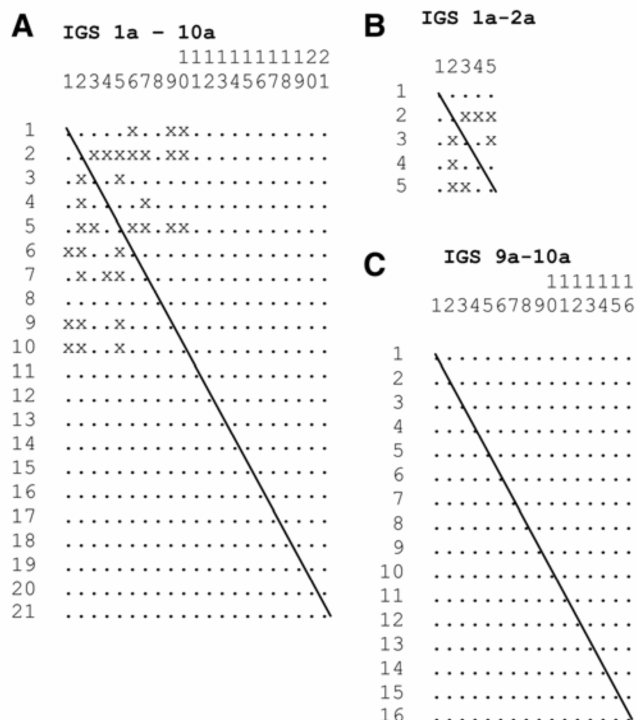


Fig. 3. Site compatibility matrices for the locus IGS, generated using SNAP Clade (Markwordt, J. D., Doshi, R., and Carbone, I. 2003. Dept. Plant Pathol., North Carolina State Univ., Raleigh). The diagonal lines emphasize the symmetry in the matrices. Numbers designate variable sites in the sequence. Incompatible sites are indicated by the letter x and compatible sites are indicated by dots. Incompatibility indicates that for each pair of incompatible sites, two equally parsimonious trees are inferred; it would not be possible to infer one most parsimonious tree. **A,** Compatibility matrix for the combined IGS 1a, 2a, 9a, and 10 sequence. Incompatible sites are concentrated in the first half of the sequence, identifying two blocks. **B and C,** Compatibility matrices for the IGS 1a-2a and IGS 9a-10a sequences, respectively.

DISCUSSION

Based on this study there are two populations of *S. sclerotiorum* on crops in the western U.S. in addition to the four populations previously determined in North America (10). The California population from lettuce is highly diverse, highly divergent from the other populations, and old compared with Washington pea/lentil and Ontario lettuce samples representing two other populations. The high diversity in the California population is con-

A) IGS 9a-10a

```
Position ᵃ             111111122222444444444445555555556666666
                    779014678812248013344456661223334588888999
                    011852642302506863434963474593787824589012

Site Number ᵇ                111111111122222222223333333333444
                    123456789012345678901234567890123456789012

Consensus ᶜ         GCTGTGCCCGCTCGCTTTTTCTTTGCCGGGAGCCTCCTTAGC
Site Type ᵈ         tvtvtvvvvvvvvvvtttvtttttvtvvvtvvvtvtvvtvvt   ʰ CA    WA    ON
Character Typeᵉ     --i--iiiiiii--i------------ii---i---------

H1ᶠ       (162)ᵍ    ............................T...G.........   114     0    48
H2        (  1)     AG...T....................................     0     1     0
H3        ( 17)     ..C.........................T...G.........     0     0    17
H4        ( 17)     ..C..............CA.........T...G.........     0     0    17
H5        (  1)     ..C.........................T...G.GTGGCTCT     0     0     1
H6        (  1)     ...C.T....................................     0     1     0
H7        (  1)     ....CT..................C..C..............     0     1     0
H8        ( 29)     .....T....................................     0    29     0
H9        (  1)     .....T.....................C..............     0     1     0
H10       (  2)     .....T.........................C..........     0     2     0
H11       (  3)     ......AGGCGGGCA...........................     0     3     0
H12       (  1)     ......AGGCGG..A...........................     0     1     0
H13       ( 38)     ...............C............T...G.........    38     0     0
H14       (  1)     ................C..CTCCC.TG..AT.GT........     1     0     0
```

B) EF-1α

```
Position                   111111111111111111111111111
                    1222349000001222223344555555566667
                    6125381456798013565702123456813786

Site Number                  1111111111222222222233333
                    1234567890123456789012345678901234

Consensus           CTCCGAAATGATGCCGTGGCAGTCGGCTCACAAC
Site Type           tvvttvtvtttvvvtvvtttvtvtvvtttvvvtt     CA     WA     ON
Character Type      ----------------------------------
H1        (229)     ..................................    111     37     81
H2        ( 45)     T.................................     41      2      2
H3        (  2)     .GGTATGCCAGATGTCGAATTAATTTTCTCACGT      1      0      1
```

C) 44.11

```
Position            112222
                    881129
                    394820

Site Number         123456

Consensus           GCGGTT
Site Type           vtvvtt     CA     WA     ON
Character Type      --ii--
H1        (142)     ......    113      0     29
H2        ( 79)     C.....      0     24     55
H3        ( 15)     .T....      0     15      0
H4        ( 29)     ..TT..     29      0      0
H5        (  8)     ..TTC.      8      0      0
H6        (  3)     .....C      3      0      0
```

Fig. 4. Haplotype maps for the three loci generated using SNAP Map (3). **A,** IGS 9a-10a; **B,** EF-1α; and **C,** 44.11. Superscript "a" = position of variable sites in the sequence; "b" = designation of variable sites; "c" = consensus haplotype sequence ; "d" = t, transitions, or v, transversions ; "e" = i, parsimony informative sites, or (-), parsimony uninformative sites; "f" = haplotype designations; "g" = frequency of the haplotype in the sample; and "h" = frequency of the haplotype in each geographic location: California (CA), Washington (WA), and Ontario (ON).

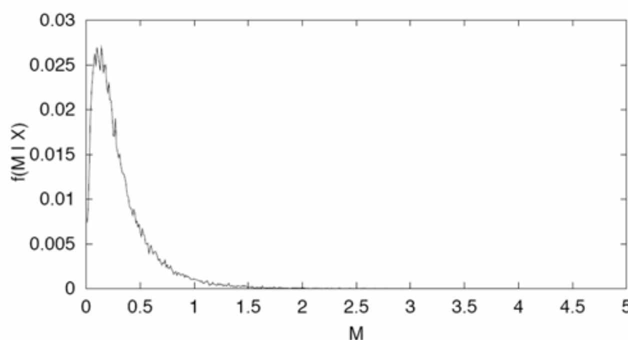
TABLE 6. Nearest Neighbor statistic, S_{nn}, (24), of pairwise tests of geographic differentiation among the samples from California, Ontario, and Washington for the loci IGS 9a-10a, EF-1α, and 44.11

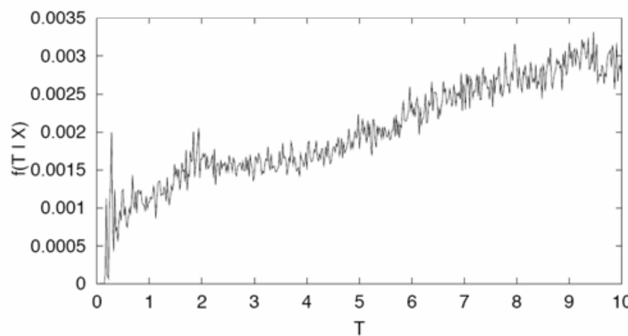
	IGS		EF-1α		44.11	
	California	Ontario	California	Ontario	California	Ontario
Ontario	0.711 (0.012)[a]	-	0.578 (0.030)	-	0.804 (0.025)	-
Washington	1 (0.005)	1 (0.008)	0.687 (0.042)	0.559 (0.911)	1 (0.005)	0.724 (0.008)

[a] Significance testing was done by comparing the observed statistic to a null distribution produced by randomly permuting haplotypes to populations and calculating the test statistic 100,000 times. The *P* value, in parentheses, was determined by the proportion of the null distribution with values greater than the observed statistic.

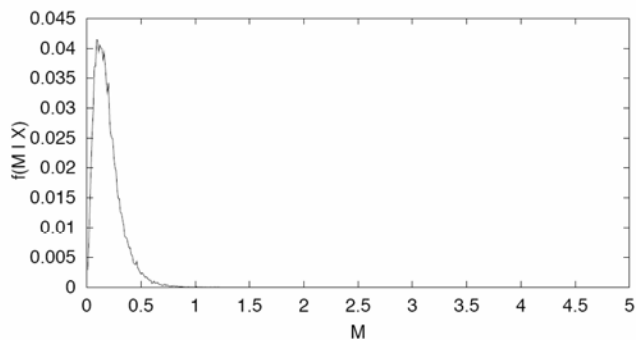
MIGRATION **DIVERGENCE TIME**

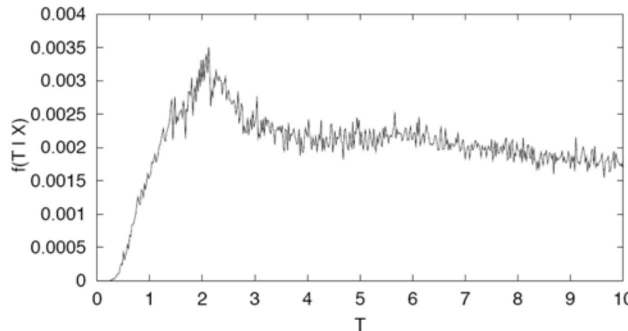
A) CA vs. ON



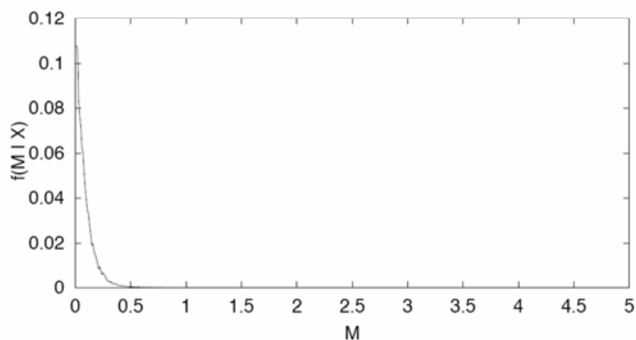


B) CA vs. WA





C) ON vs. WA



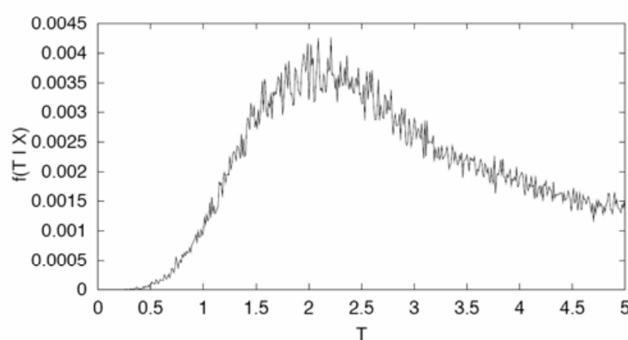


Fig. 5. Migration and time of divergence posterior probability distribution between samples, generated using MDIV (42) for the IGS 9a-10a. **A,** California versus Ontario; **B,** California versus Washington, and **C,** Ontario versus Washington. The data were simulated assuming an infinite sites model: two million steps in the chain for the estimation of the posterior distribution and an initial 500,000 steps to ensure that enough genealogies were simulated before approximating the posterior distribution. Ten independent replicates, using different starting random number seeds, were simulated under the same model and parameters with similar results. Likelihood function for migration f(M|X) and time of divergence f(T|X) are presented in the Y axes while the X axes represent time measured in 2*N* generations. Data points from MDIV were plotted using **gnuplot** (Williams, T., and Kelley, C., 2004; available online).

sistent with the report based on MCGs of Wu and Subbarao (58). The California sample does not represent a new species, despite genetic isolation relative to other populations.

As demonstrated in the multilocus phylogeny, the California population is recently derived from a common ancestor in *S. sclerotiorum*, along with representatives of populations 3-3 (southeastern United States), 3-2 (northcentral United States and adjacent Canada), and 3-4 (Norwegian wild buttercup), as well as isolates from Uruguay and Argentina, while the representatives of other named and cryptic *Sclerotinia* species are derived from a more distant, more ancient, common ancestor. Three of the four loci combined in the species-level phylogenetic analysis (ITS1, RAS, and CAL) were more highly conserved, i.e., more slowly-evolving with fewer polymorphisms than the loci used in the population-level analysis, IGS, EF1-α, and 44.11. The four combined loci therefore did not resolve all populations as lineages (branches), and the three branches resolved were short, compared with species lineages. The IGS was an especially rich source of phylogenetically informative characters for population analysis, but is too variable to align among species of *Sclerotinia* (10). Only the IGS provided enough characters to differentiate a Washington population from the Ontario sample. Both the IGS and 44.11 differentiated a Washington population from the Ontario sample by the statistic for subdivision, S_{nn}. Polymorphisms in the IGS provided higher resolution than the other two loci in the coalescent genealogies, clearly indicating differentiation of Washington and Ontario.

All of the measures of population subdivision point to clear subdivision of the newly discriminated California and Washington populations with reference to previously inferred populations in North America. Both populations of *S. sclerotiorum* are inferred to have different origins from other temperate populations previously delimited. A longer coalescence to the most recent common ancestor was inferred for California samples than for samples from Washington or Ontario. The California population was characterized by lack of immigration from the other populations; high diversity, with almost every isolate a different genotype; recombination within the IGS locus; and interlocus recombination ($I_A = 0$).

TABLE 7. Summary information on sequence data for four loci used to confirm the identification of *Sclerotinia sclerotiorum*

Locus	Haplotypes[a]	Sites (IS)[b]	TL (*N*)[c]	CI[d]
ITS1	2	1 (0)	-	-
EF-1α	7	85 (20)	89 (2)	0.9551
CAL	4	54 (6)	28 (1)	0.9818
RAS	5	26 (12)	28 (1)	0.9286
Combined[e]	7	166 (44)	173 (1)	0.9595

[a] Total number of haplotypes.
[b] Total number of sites per haplotype (number of parsimony informative sites).
[c] Tree length (Number of trees).
[d] Consistency index.
[e] Combined loci: EF-1α , ITS1, CAL, and RAS.

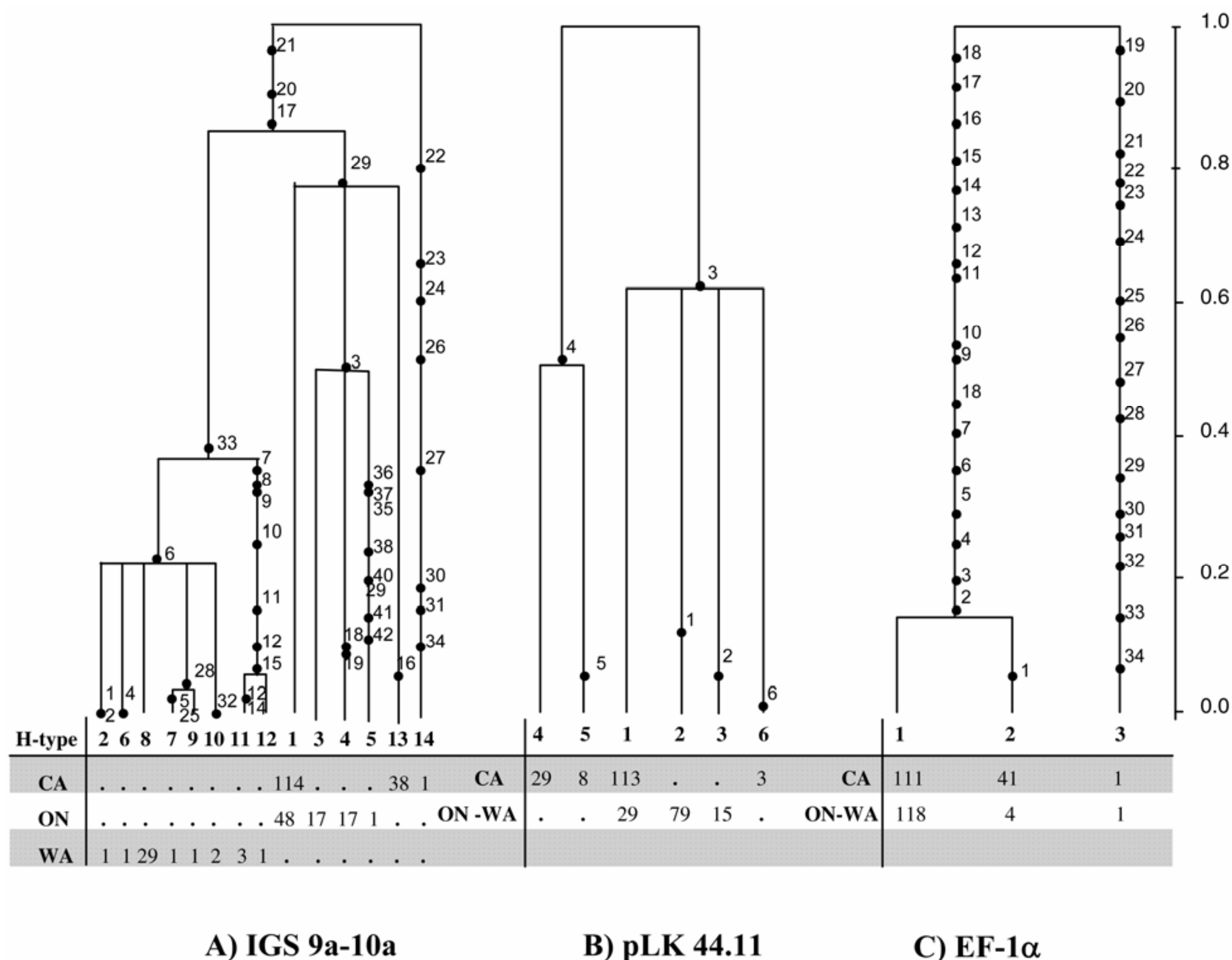


Fig. 6. Coalescent-based gene genealogies inferred using Genetree (4,19) for the loci IGS 9a-10a, 44.11, and EF-1α. For each locus the rooted genealogy was inferred based on 10 different starting random number seeds with 10 million simulations of the coalescent for each run, assuming population subdivision and unequal sample size. The time scale is in coalescent units of effective population size. The direction of divergence is from the top of the genealogy (oldest) to the bottom (present), while the coalescent is from the bottom (present) to the top (past). Solid circles indicate the distribution of the mutations in the genealogy. **A,** IGS 9a-10a; **B,** 44.11; and **C,** EF-1α. H-type = haplotype.

The population represented by the Washington sample from pea/lentil has some history of gene flow with the population represented by the Ontario lettuce sample, mainly population 3-2 in previous studies (10). It is not known whether the Columbia River Basin sample from potato (2) represents the same population as our pea/lentil sample.

Genetic exchange and recombination could explain the high diversity in California, but the evidence is not straightforward and alternatives cannot be rejected. With genetic exchange and recombination, the expectation for a heterothallic species would be a 1:1 ratio of mating types with either *MAT1-1* or *MAT1-2* per individual. If any outcrossing occurs in a homothallic species, however, progeny will still have both mating type idiomorphs. Expectations from outcrossing in *S. sclerotiorum*, a homothallic species, include a lack of association of markers, such as nontransitive MCGs, fingerprints associated with more than one MCG or no association at all, and single apothecia with sister ascospores evidencing segregation of markers, such as >3 MCGs per apothecium or different DNA fingerprints among sister ascospores.

On the side of recombination we have the following evidence from the California population. The I_A based on the fingerprints was closer to expectations for panmixia than was the I_A for the Ontario or Washington populations. Almost every isolate had a unique fingerprint and was compatible only with itself, not forming MCGs, and the few MCGs included isolates with fingerprints differing by as many as 30 bands. We can conclude that there is negligible association between MCGs and fingerprints. One caveat is that with more hierarchical sampling, clonality on single plants or close clusters of plants would likely have been detected, although detection of clones was not the objective. Another caveat is that sister spores of apothecia were not screened in this study. Since ascospores are the primary inoculum in lettuce drop in California, then, if there is disease, there should be apothecia.

The high diversity and lack of association of markers is also consistent with a large population, supporting high standing genetic variation with little selection or drift, an alternative to genetic exchange and recombination. Note that the two explanations are not mutually exclusive. Given the lack of host specificity in *S. sclerotiorum* and the lack of resistance in its many hosts, as well as the intense cultivation of lettuce in California, this is not a far-fetched explanation. Atallah et al. reported segregation of ≥3 MCGs in 3 of 12 apothecia of *S. sclerotiorum* from Washington (2). Despite repeated efforts, we have not observed such evidence. What we have observed is more complex. Two fingerprints associated with two MCGS were observed from single-ascospore progeny of 2 of 42 apothecia from a peach orchard in California (29,31) and 4 of 50 apothecia collected in cabbage and celery fields in South America where *S. sclerotiorum* populations have similar characteristics to those of California (G. Malvárez, *unpublished data*). We can only conclude that these ascospores represented the two parental phenotypes and not recombinants. We hypothesize that the apothecia were mosaic for the two parental phenotypes, or that a diploid was not formed, or that there was a failure in meiosis. Formation of homokaryotic dikaryons from a heterothallic mating was reported when mating type knockouts of *Podospora anserina* were mated with wild type strains. Without functional mating types only uniparental dikaryons were formed, hence uniparental diploids and nonrecombinant progeny (61). This report raises the possibility of a failure in function in mating type idiomorphs of *S. sclerotiorum.* Dimorphism in ascospore size among a low frequency of asci in some, but not all apothecia examined, has been recently reported in *S. sclerotiorum* and *S. minor* (16), Unlike *S. trifoliorum*, which has uniform dimorphism with homothallic large ascospores and heterothallic small ascospores, in *S. sclerotiorum* and *S. minor*, both large and small ascospores were found to be homothallic. As more dimorphic asci are examined further in future studies, it is possible that patterns more similar to that in *S. trifoliorum* may yet be observed. It is not known whether *S. sclerotiorum* is polyploid (a potential obstacle to normal meiosis), although we have not seen evidence of polyploidy, such as multiple PCR products. Presumably the *S. sclerotiorum* genome project will enable us to clarify this point. We anticipate that in highly diverse populations such as that associated with California lettuce production areas, considerably more than 50 apothecia must be screened either to detect evidence of genetic exchange and recombination in meiosis or to provide convincing evidence of its absence.

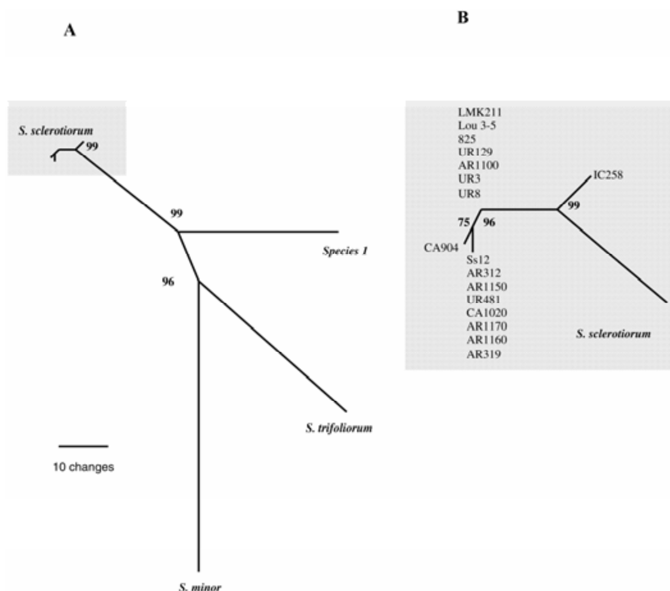


Fig. 7. A, Unrooted parsimony tree of the combined EF1-α, ITS1, CAL, and RAS loci for *Sclerotinia* species. Consistency index (CI): 0.959. **B,** Enlarged view of the *S. sclerotiorum* cluster. The California, Washington, and South American samples are on short branches indicative of different populations, relative to the long branches for other species of *Sclerotinia.* Bootstrap values from 1 million replications are indicated for each interior branch.

All isolates used in this study were demonstrated by Southern hybridization to have both *MAT1-1* and *MAT1-2* genes, which are at one locus in *S. sclerotiorum* (L. Kohn, J. van Kan, and P. Dyer, *unpublished data*). This is confirmatory evidence of homothallism, although it is not yet known whether the mating system is functional and whether outbreeding is possible. The evidence for occasional outbreeding in a homothallic species is stronger in *S. minor*. Among apothecia collected from peanut debris and weeds, 4 of 50 apothecia segregated ≥3 MCGs among sister ascospores, with a different DNA fingerprint from each sister ascospore sampled. The probes for *S. sclerotiorum MAT* genes hybridized to these Southern blots, and as in *S. sclerotiorum*, every isolate had both *MAT* genes (L. Kohn, *unpublished data*).

Factors supporting high standing genetic diversity could include a mild climate lacking a hard winter, lack of crop rotation to nonhosts, highly diverse crops with many potential hosts, poor weed control, and a diversified life cycle with both myceliogenic and carpogenic germination (probably during the same crop cycle). Although experimental work, such as reciprocal transplant studies, could identify effects of specific factors on contemporary crops and pathogen populations, for retrospective study it will be difficult to isolate the effect of each of these factors in the history of the population. It will be helpful to learn more about standing variation in quantitative and qualitative resistance against *S. sclerotiorum* on a population level. The *S. sclerotiorum* genome project, along with the steady progress made by breeders and molecular biologists, has the potential to provide the tools. The *Bremia lactucae*–lettuce pathosystem with its strong gene-for-gene element (unlike *S. sclerotiorum*), is a model in California and Europe of how frequencies of new pathogen races and geographic distribution of virulence factors are strongly driven by dis-

A Haplotype map for IGS 9a-10a

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Position^a               11111112222444444444444455555556666666 6
                        77014678812240013344556661233334588888899
                        01963753413615974545074585604898913478901

Site Number^b                    11111111112222222222333333333344
                        12345678901234567890123456789012345678901

Consensus^c             GCGTGCCCGCTCGATTTTTCTTTGCCGGGAGCCTCCTTAGC
Site Type^d             tvvtvvvvvvvvvvtttvtttttvtvvvtvvvtvtvvtvvt
Character Type^e        ----iiiiiii---------------ii---i---------
H1^f        (  2)^g     ....T.........................C..........
H2          (  1)       AG..T....................................
H3          (  1)       ..C.T....................................
H4          (  1)       ...CT..................C..C..............
H5          (261)       ...........................T...G.........
H6          (  3)       .....AGGCGGGC............................
H7          (  1)       .....AGGCGG..............................
H8          ( 20)       .............T.............T...G.........
H9          ( 57)       ..............C............T...G.........
H10         (  1)       ...............C..CTCCC.TG..AT.GT........
H11         ( 40)       ................CA.........T...G.........
H12         (  1)       ...........................T...G.GTGGCTCT
H13         (  1)       ....T.....................C..............
H14         ( 29)       ....T....................................
```

B Frequency of the haplotype in each geographic region

	NC	NY	LA	AB	CA	WA	ON
H1	0	0	0	0	0	2	0
H2	0	0	0	0	0	1	0
H3	0	0	0	0	0	1	0
H4	0	0	0	0	0	1	0
H5	39	12	14	16	114	0	66
H6	0	0	0	0	0	3	0
H7	0	0	0	0	0	1	0
H8	0	0	0	20	0	0	0
H9	11	0	8	0	38	0	0
H10	0	0	0	0	1	0	0
H11	1	19	0	3	0	0	17
H12	0	0	0	0	0	0	1
H13	0	0	0	0	0	1	0
H14	0	0	0	0	0	29	0

Fig. 8. A, Haplotype map for the IGS 9a-10a locus for the combined data set for North America: North Carolina (NC), New York (NY), Alberta (AB), Louisiana (LA), California (CA), Ontario (ON), and Washington (WA) (3). **B,** Frequency of the haplotype in each geographic region. Superscript "a" = position of variable sites in the sequence; "b" = designation of variable sites; "c" = consensus haplotype sequence ; "d" = t, transitions, or v, transversions ; "e" = i, parsimony informative sites, or (-), parsimony uninformative sites; "f" = haplotype designations; and "g" = frequency of the haplotype in the sample.

TABLE 8. Nearest Neighbor Statistic, S_{nn}, (24), of pairwise tests of geographic differentiation among the samples of the combined data set for North America for locus IGS 9a-10a

	ON[a]	AB	NC	LA	NY	CA	WA
ON	-	1 (0.008)[b]	1 (0.007)	1 (0.022)	1 (0.013)	0.71 (0.012)	1 (0.008)
AB		-	1 (0.009)	1 (0.016)	1 (0.014)	1 (0.002)	1 (0.025)
NC			-	0.57 (0.80)	1 (0.012)	1 (0.002)	1 (0.011)
LA				-	1 (0.013)	1 (0.014)	1 (0.016)
NY					-	1 (0.011)	1 (0.005)
CA						-	1 (0.005)

[a] Ontario = ON; AB = Alberta; NC = North Carolina; LA = Louisiana; NY = New York; CA = California; and WA = Washington.

[b] Significance testing was done by comparing the observed statistic to a null distribution produced by randomly permuting haplotypes to populations and calculating the test statistic 100,000 times. The P value, in parentheses, was determined by the proportion of the null distribution with values greater than the observed statistic.

tribution of resistance factors in cultivar deployment, as well as long-term host–pathogen interactions and geographical isolation (39).

There are notable precedents for fungal population divergence and speciation in California among fungi associated with humans or plants. Kerrigan et al. (28) and Xu et al. (59) found four distinct, natural populations of *Agaricus bisporus*, including one in coastal California and another in desert California. The indigenous coastal population showed evidence of competitive pressure from cultivated genotypes likely introduced in the last century from Europe, both under indigenous Monterey cypress where most *A. bisporus* individuals were sampled, and in other more extensive habitats. There was some evidence of hybrization (28). The human pathogen *Coccidioides immitis* has two well-characterized California populations that together represent the core species concept as distinct from non-California genotypes in Arizona, Texas, Mexico, and South America, now recognized as *C. posadisii* (36,37). The two populations are distinguished by a deep divergence in the California clade that corresponds to the geographical division between the Central Valley and the rest of California associated with a mountain range separating the two areas. Differences in microsatellite allele diversity between these two populations were consistent with genetic drift; a similar pattern of drift was observed among the non-California populations, from North America to South America (17). Native California populations of *Pinus radiata* have been under attack from a distinct population of *Fusarium circinatum* with low genetic diversity relative to the southeastern United States, consistent with a relatively recent introduction followed by clonal spread in California after introduction from a sexually recombining population in the Southeast. Molecular marker diversity was consistent with clonal lineages of Vegetative Compatibility Groups (VCGs), and the origin of new VCGs by somatic mutation, not recombination, in California. Interestingly, endophytes/latent pathogens of *P. radiata* such as *Sphaeropsis sapinea* and *Diplodia scrobiculata* also form highly divergent California populations, but have worldwide distributions (6,7). Of course, not all fungi form divergent California populations. While species complexes of *Fusarium solani* and *F. oxysporum* show plant–host specificity and geographical association, no such specificity is observed in human infections (43,60). Not all fungi form subdivided populations. *Aspergillus fumigatus* is an example of a ubiquitous, soil-associated saprophyte and opportunistic human pathogen with both low genetic diversity and no apparent population subdivision (47,50).

Why would genetically distinct fungal populations arise in California? Clearly, long-term association with native plant species such as Monterey pine or cypress could lead to endemism in fungal endophytes or weak pathogens (6,7). Epidemic spread via mainly asexual or selfing clonal lineages could also result in population isolation and divergence through somatic mutation, as suggested for *Fusarium circinatum* (18). The distance of mycelial growth or spore dispersal could isolate populations as they can isolate genets (48). Adaptation to environmental conditions, including local adaptation of both the fungus and the soil microbiota or host plant, could reduce the chance of successful migration, as demonstrated in a reciprocal transplant experiment of arbuscular mycorhizal fungi on both sides of the Great Basin, in San Diego, California, and in Reno, Nevada. In this study, the fungi survived and increased for three seasons when transplanted in exotic soil with the native host, but declined in the exotic soil with the exotic host of the same species (56).

The most attractive hypothesis for the subdivision of a California population of *S. sclerotiorum* would not be adaptation to lettuce, as *S. sclerotiorum* shows negligible host specificity, although there are probably differences in resistance among cultivars, as has been shown for *S. minor* (20). Rather, we seek to test other hypotheses, such as movement with human migration, as has occurred with many plant pathogens, such as *Phytophthora infestans*, and the human pathogen, *Coccidioides immitis*. Once established, the population isolation could have been maintained by intensive and geographically isolated crop production. In addition, there is a potential role for adaptation to a suite of environmental and agronomic conditions. Finally, for this interesting new population from California, we have yet to clarify the contribution of the mating system, affording the advantages of both effective, durable soilborne sclerotia maintaining successful genotypes, and airborne ascospores, potentially contributing diversity from genetic exchange and recombination.

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