Single Nucleotide Polymorphism-Based Diagnostic System for Crop-Associated *Sclerotinia* Species[▽]

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A molecular diagnostic system using single nucleotide polymorphisms (SNPs) was developed to identify four *Sclerotinia* species: *S. sclerotiorum* (Lib.) de Bary, *S. minor* Jagger, *S. trifoliorum* Erikss., and the undescribed species *Sclerotinia* species 1. DNAs of samples are hybridized with each of five 15-bp oligonucleotide probes containing an SNP site midsequence unique to each species. For additional verification, hybridizations were performed using diagnostic single nucleotide substitutions at a 17-bp sequence of the calmodulin locus. The accuracy of these procedures was compared to that of a restriction fragment length polymorphism (RFLP) method based on Southern hybridizations of EcoRI-digested genomic DNA probed with the ribosomal DNA-containing plasmid probe pMF2, previously shown to differentiate *S. sclerotiorum*, *S. minor*, and *S. trifoliorum*. The efficiency of the SNP-based assay as a diagnostic test was evaluated in a blind screening of 48 *Sclerotinia* isolates from agricultural and wild hosts. One isolate of *Botrytis cinerea* was used as a negative control. The SNP-based assay accurately identified 96% of *Sclerotinia* isolates and could be performed faster than RFLP profiling using pMF2. This method shows promise for accurate, high-throughput species identification.

Sclerotinia is distinguished morphologically from other genera in the Sclerotiniaceae (Ascomycota, Pezizomycotina, Leotiomycetes) by the production of tuberoid sclerotia that do not incorporate host tissue, by the production of microconidia that function as spermatia but not as a disseminative asexual state, and by the development of a layer of textura globulosa composing the outer tissue of apothecia (8). Two hundred forty-six species of *Sclerotinia* have been reported, most distinguished morphotaxonomically (Index Fungorum [www.indexfungorum.org]). These include the four species of agricultural importance now recognized plus many that are imperfectly known, seldom collected, or apparently endemic to relatively small geographic areas (2, 5, 6, 7, 8, 9, 17).

The main species of phytopathological interest in the genus *Sclerotinia* are *S. sclerotiorum* (Lib.) de Bary, *S. minor* Jagger, *S. trifoliorum* Erikss., and the undescribed species *Sclerotinia* species 1. *Sclerotinia* species 1 is an important cause of disease in vegetables in Alaska (16) and has been found in association with wild *Taraxacum* sp., *Caltha palustris*, and *Aconitum septentrionalis* in Norway (7). It is morphologically indistinguishable from *S. sclerotiorum*, but it was shown to be a distinct species based on distinctive polymorphisms in sequences from internal transcribed spacer 2 (ITS2) of the nuclear ribosomal repeat (7). The other three species have been delimited using morphological, cytological, biochemical, and molecular characters (3, 8, 9, 10, 12, 15). Interestingly, given that the ITS is sufficiently polymorphic in many fungal genera to resolve species, in *Sclerotinia*, only species 1 and *S. trifoliorum* are distinguished by characteristic ITS sequence polymorphisms; *S. scler-*

rotiorum and *S. minor* cannot be distinguished based on ITS sequence (2, 7).

Sclerotinia sclerotiorum is a necrotrophic pathogen with a broad host range (1). *S. minor* has a more restricted host range but causes disease in a variety of important crops such as lettuce, peanut, and sunflower crops (11). *S. trifoliorum* has a much narrower host range, limited to the Fabaceae (3, 8, 9). Sclerotial and ascospore characteristics also serve to differentiate among the three species. *Sclerotinia minor* has small sclerotia that develop throughout the colony in vitro and aggregate to form crusts on the host, while the sclerotia of *S. sclerotiorum* and *S. trifoliorum* are large and form at the colony periphery in vitro, remaining separate on the host (8, 9). The failure of an isolate to produce sclerotia or apothecia in vitro is not unusual, especially after serial cultivation (8). The presence of dimorphic, tetranucleate ascospores characterizes *S. trifoliorum*, while *S. sclerotiorum* and *S. minor* both have uniformly sized ascospores that are binucleate and tetranucleate, respectively (9, 14).

With the apparent exception of *Sclerotinia* species 1, morphological characteristics are sufficient to delimit *Sclerotinia* species given that workers have all manifestations of the life cycle in hand. In cultures freshly isolated from infected plants, investigators usually have mycelia and sclerotia but not apothecia. Restriction fragment length polymorphisms (RFLPs) in ribosomal DNA (rDNA) are diagnostic for *Sclerotinia* species (3, 10), but the assay requires cloned probes (usually accessed from other laboratories) hybridized to Southern blots from vertical gels, an impractical procedure for large samples. We have analyzed sequence data from previous phylogenetic studies (2) and have identified diagnostic variation for the rapid identification of the four *Sclerotinia* species. The single nucleotide polymorphism (SNP) assay that we report here is amenable to a high throughput of samples and requires only PCR amplification with a standard set of primers and oligonucleotide hybridizations to Southern blots in a dot format.

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For additional verification, the following oligonucleotide probes, designed from diagnostic single nucleotide substitutions at a 17 bp sequence of the CAL locus should be used with hybridization temperatures of 45°C.

Locus	Site designation	Probe sequence (5' – 3')	Sclerotinia species
CAL	448/500	GTACGGCT A AGCATG	<i>S. minor</i>
	448/500	GTACGGC G AAGCATA	<i>S. trifoliorum</i>
	448/500	GTACGGC A AAGCATA	<i>S. sclerotiorum</i>
	446/500	GGGTACG A CAAAGCA	<i>Sclerotinia</i> sp.1

FIG. 1. Protocol for the SNP-based identification of *Sclerotinia* species, with diagnostic SNP sites underlined and in boldface type for each hybridization probe.

The SNP assay was performed using two independent sets of species-specific oligonucleotide probes, all with SNP sites shown to differentiate the four *Sclerotinia* species (Fig. 1). A panel of 49 anonymously coded isolates (Table 1) was screened using these species-specific SNP probes, as outlined in Fig. 1. The assay was validated by comparison to Southern hybridizations of EcoRI-digested genomic DNA hybridized with pMF2, a plasmid probe containing the portion of the rDNA repeat with the 18S, 5.8S, and 26S rRNA cistrons of *Neurospora crassa* (4, 10).

MATERIALS AND METHODS

Characterization of SNP sites and probe design. The initial screen of loci that determined the rank of resolution of DNA sequence polymorphisms from which loci were chosen for the present study at the intraspecies level within *Sclerotinia* was based on a total of 465 isolates. These included 341 *S. sclerotiorum* isolates from crops in North America, Northern Europe, and New Zealand; 29 isolates of *S. minor* from North America; 8 isolates of *S. trifoliorum* from North America and Tasmania; 2 isolates of *Sclerotinia* species 1 from wild plants in Norway; an isolate of *Sclerotinia glacialis*; isolates of species that have been assigned to *Sclerotinia* but are likely other genera (*S. tetraspora* and *S. borealis*); isolates of *Dumontinia tuberosa* and *Dumontinia ulmariae*; 67 isolates representing an undescribed species of *Dumontinia* reported in the literature as being *Sclerotinia sclerotiorum*; and isolates of *Sclerotium cepivorum*, *Christulariella moricola*, and *Botrytis cinerea* (2). Sequence data from these isolates at seven loci (2) were analyzed to identify SNP sites that were specific to each of the four main species

of *Sclerotinia*. This reference sample of isolates did not overlap with 49 randomly coded isolates screened in the present study, with the exceptions of the negative and positive controls listed below.

Diagnostic SNP sites were selected for loci that had high relative sequence similarity to ensure robust hybridization results. Primer3 (13) was used to design 15-bp oligonucleotide probes with optimized hybridization temperatures ranging from 45°C to 51°C (Fig. 1).

Isolate sampling. The test sample included 8 isolates of *S. minor*, 14 isolates of *S. sclerotiorum*, and 6 isolates of *S. trifoliorum* from the Kohn laboratory culture collection (Table 1). The screening also included three sets of isolates from localities not sampled in previous studies from the Kohn laboratory: Alaska, Australia, and Finland (Table 1). *Botrytis cinerea* (isolate LMK18) was used as a negative control for all hybridization experiments. *Sclerotinia sclerotiorum* (isolate LMK211), *Sclerotinia trifoliorum* (isolate LMK36), and *Sclerotinia* species 1 (isolate LMK745) were used as positive controls. The screening was blind, as all isolates were randomly coded 1 through 49 to ensure the anonymity of presumed species designations (Table 1).

Fungal cultures and DNA extraction. All isolates were grown on potato dextrose agar (Difco Laboratories, Detroit, MI) for 3 to 5 days and were then transferred into standing-culture potato dextrose broth (Difco Laboratories) for 1 to 2 days. On both solid and liquid media, cultures were grown in the dark at ambient room temperature (20 to 22°C). Cultures were filtered through Miracloth (Calbiochem, EMD Chemicals Inc., Darmstadt, Germany) and lyophilized. DNA was extracted from 10 to 15 mg of lyophilized mycelium using a DNeasy plant minikit (Qiagen, Mississauga, Ontario, Canada). The quantity of DNA was estimated using 1.0% ethidium bromide-stained agarose gels, with known quantities of bacteriophage lambda DNA as standards against which comparisons of band intensity could be made. DNA extraction samples were

TABLE 1. Isolates and hybridization results for all SNP-based oligonucleotide probes^a

Collector's isolate	Anonymous code	Prescreened presumed species identity	Origin	Host	Species-specific SNP									
					IGS50	CAL448 S.trifol	CAL124	CAL448 S.minor	RAS148	CAL446 S.sp1	CAL19A	CAL19B	CAL448 S.sclero	
LMK18	49	<i>Botrytis cinerea</i>	Ontario, Canada	<i>Allium cepa</i>	—	—	—	—	—	—	—	—	—	—
FA2-1	3	<i>Sclerotinia minor</i>	North Carolina	<i>Arachis hypogaea</i>	—	—	—	—	—	—	—	—	—	—
W1	5	<i>Sclerotinia minor</i>	North Carolina	<i>Cyperus esculentus</i>	—	—	—	—	—	—	—	—	—	—
W10	30	<i>Sclerotinia minor</i>	North Carolina	<i>Oenothera lacinata</i>	—	—	—	—	—	—	—	—	—	—
PF1-1	38	<i>Sclerotinia minor</i>	North Carolina	<i>Arachis hypogaea</i>	—	—	—	—	—	—	—	—	—	—
PF18-497	14	<i>Sclerotinia minor</i>	Oklahoma	<i>Arachis hypogaea</i>	—	—	—	—	—	—	—	—	—	—
PF17-482	46	<i>Sclerotinia minor</i>	Oklahoma	<i>Arachis hypogaea</i>	—	—	—	—	—	—	—	—	—	—
PF19-519	48	<i>Sclerotinia minor</i>	Oklahoma	<i>Arachis hypogaea</i>	—	—	—	—	—	—	—	—	—	—
LF-27	20	<i>Sclerotinia minor</i>	United States	<i>Lactuca sativa</i>	—	—	—	—	—	—	—	—	—	—
AR1281	1	<i>Sclerotinia sclerotiorum</i>	Argentina	<i>Arachis hypogaea</i>	—	—	—	—	—	—	—	—	—	—
AR1282	16	<i>Sclerotinia sclerotiorum</i>	Argentina	<i>Arachis hypogaea</i>	—	—	—	—	—	—	—	—	—	—
LMK211	6	<i>Sclerotinia sclerotiorum</i>	Canada	<i>Brassica napus</i>	—	—	—	—	—	—	—	—	—	—
LMK57	25	<i>Sclerotinia sclerotiorum</i>	Norway	<i>Ranunculus ficaria</i>	—	—	—	—	—	—	—	—	—	—
LMK754	15	<i>Sclerotinia sclerotiorum</i>	Norway	<i>Ranunculus ficaria</i>	—	—	—	—	—	—	—	—	—	—
UR19	39	<i>Sclerotinia sclerotiorum</i>	Uruguay	<i>Lactuca sativa</i>	—	—	—	—	—	—	—	—	—	—
UR478	9	<i>Sclerotinia sclerotiorum</i>	Uruguay	<i>Lactuca sativa</i>	—	—	—	—	—	—	—	—	—	—
CA901	32	<i>Sclerotinia sclerotiorum</i>	California	<i>Lactuca sativa</i>	—	—	—	—	—	—	—	—	—	—
CA995	40	<i>Sclerotinia sclerotiorum</i>	California	<i>Lactuca sativa</i>	—	—	—	—	—	—	—	—	—	—
CA1044	41	<i>Sclerotinia sclerotiorum</i>	California	<i>Lactuca sativa</i>	—	—	—	—	—	—	—	—	—	—
1980 ^a	34	<i>Sclerotinia sclerotiorum</i>	Nebraska	<i>Phaseolus vulgaris</i>	—	—	—	—	—	—	—	—	—	—
Ss001	13	<i>Sclerotinia sclerotiorum</i>	New York ^b	<i>Glycine max</i>	—	—	—	—	—	—	—	—	—	—
Ssp005	31	<i>Sclerotinia sclerotiorum</i>	New York	<i>Glycine max</i>	—	—	—	—	—	—	—	—	—	—
H02-V28	33	<i>Sclerotinia species 1</i>	Alaska ^c	Unknown vegetable crop	—	—	—	—	—	—	—	—	—	—
H01-V14	26	<i>Sclerotinia species 1</i>	Alaska	Unknown vegetable crop	—	—	—	—	—	—	—	—	—	—
LMK745	21	<i>Sclerotinia species 1</i>	Norway	<i>Taraxacum</i> sp.	—	—	—	—	—	—	—	—	—	—
02-26	11	<i>Sclerotinia trifoliorum</i>	Finland ^d	<i>Trifolium pratense</i>	+	+	—	—	—	—	—	—	—	—
06-14	29	<i>Sclerotinia trifoliorum</i>	Finland	<i>Trifolium pratense</i>	+	+	—	—	—	—	—	—	—	—
202	2	<i>Sclerotinia trifoliorum</i>	Finland	<i>Trifolium pratense</i>	+	+	—	—	—	—	—	—	—	—
2-L9	45	<i>Sclerotinia trifoliorum</i>	Finland	<i>Trifolium pratense</i>	+	+	—	—	—	—	—	—	—	—
3-A5	24	<i>Sclerotinia trifoliorum</i>	Finland	<i>Trifolium pratense</i>	+	+	—	—	—	—	—	—	—	—
5-L9	12	<i>Sclerotinia trifoliorum</i>	Finland	<i>Trifolium pratense</i>	+	+	—	—	—	—	—	—	—	—
K1	4	<i>Sclerotinia trifoliorum</i>	Finland	<i>Trifolium pratense</i>	+	+	—	—	—	—	—	—	—	—
K2	37	<i>Sclerotinia trifoliorum</i>	Finland	<i>Trifolium pratense</i>	+	+	—	—	—	—	—	—	—	—
L-112	23	<i>Sclerotinia trifoliorum</i>	Finland	<i>Trifolium pratense</i>	+	+	—	—	—	—	—	—	—	—
L-119	44	<i>Sclerotinia trifoliorum</i>	Finland	<i>Trifolium pratense</i>	+	+	—	—	—	—	—	—	—	—
LMK36	19	<i>Sclerotinia trifoliorum</i>	Tasmania	<i>Trifolium repens</i>	+	+	—	—	—	—	—	—	—	—
Ssp001	18	<i>Sclerotinia trifoliorum</i>	New York	<i>Lotus corniculatus</i>	+	+	—	—	—	—	—	—	—	—
Ssp002	10	<i>Sclerotinia trifoliorum</i>	New York	<i>Lotus corniculatus</i>	+	+	—	—	—	—	—	—	—	—
Ssp003	28	<i>Sclerotinia trifoliorum</i>	New York	<i>Lotus corniculatus</i>	+	+	—	—	—	—	—	—	—	—
Ssp004	36	<i>Sclerotinia trifoliorum</i>	New York	<i>Lotus corniculatus</i>	+	+	—	—	—	—	—	—	—	—
LMK47	43	<i>Sclerotinia trifoliorum</i>	Virginia	<i>Medicago sativa</i>	+	+	—	—	—	—	—	—	—	—
MBRS-1	27	Unknown	Australia ^e	<i>Brassica</i> spp.	—	—	—	—	—	—	—	—	—	—
MBRS-2	7	Unknown	Australia	<i>Brassica</i> spp.	—	—	—	—	—	—	—	—	—	—
MBRS-3	42	Unknown	Australia	<i>Brassica</i> spp.	—	—	—	—	—	—	—	—	—	—
MBRS-5	22	Unknown	Australia	<i>Brassica</i> spp.	—	—	—	—	—	—	—	—	—	—
WW-1	35	Unknown	Australia	<i>Brassica</i> spp.	—	—	—	—	—	—	—	—	—	—
WW-2	8	Unknown	Australia	<i>Brassica</i> spp.	—	—	—	—	—	—	—	—	—	—
WW-3	17	Unknown	Australia	<i>Brassica</i> spp.	—	—	—	—	—	—	—	—	—	—
WW-4	47	Unknown	Australia	<i>Brassica</i> spp.	—	—	—	—	—	—	—	—	—	—

^a The annotated genome for *S. sclerotiorum* strain 1980 (ATCC 18683) is publicly available through the Broad Institute, Cambridge, MA (http://www.broad.mit.edu/annotation/genome/sclerotinia_sclerotiorum/Home.html).

^b All isolates from New York were provided by Gary C. Bergstrom, Cornell University, Ithaca, NY. Isolates Ss001 and Ssp005 were submitted as *S. sclerotiorum*, and Ssp001 through Ssp004 were submitted as *S. trifoliorum*.

^c All isolates from Alaska, submitted as *Sclerotinia* species 1, were provided by Lori Winton, USDA-ARS Subarctic Agricultural Research Unit, University of Alaska, Fairbanks.

^d All isolates from Finland, submitted as *S. trifoliorum*, were provided by Tapani Yli-Mattila, University of Turku, Turku, Finland.

^e All isolates from Australia, presumed to be *S. sclerotiorum* but requiring species confirmation, were provided by Martin Barbetti, DAF Plant Protection Branch, South Perth, Australia.

^f The probes that are diagnostic for *S. minor*, *S. sclerotiorum*, *S. trifoliorum*, and *Sclerotinia* species 1 are listed, with a "+" indicating a positive hybridization for the probe and a "—" indicating no hybridization of the probe.

TABLE 2. Primer sets for amplification of loci used in this study

Locus	Definition	Primer	Primer DNA sequence (5'-3')	Annealing temp (°C)
CAL	Calmodulin	CAL-228F CAL-737R	GAGTTCAAGGAGGCCTTCTCCC CATCTTTCTGGCCATCATGG	55
IGS	Intergenic spacer region	PTR-1a PTR-1b	GGCGCATGGATAAACTCTCC GATTATCCATTTGCCCCAC	58
RAS	Ras protein	RAS-264F RAS-565R	GATGAATATGATCCTACGAT AAATCACATTTGTTACCAAC	50

diluted to 10 to 20 ng/μl in elution buffer (10 mM Tris-Cl, 0.5 mM EDTA [pH 9]) for use in PCR.

PCR and sequencing. An approximately 300-bp segment of the intergenic spacer (IGS) and portions of genes encoding calmodulin (CAL) (500 bp) and ras protein (RAS) (350 bp) were amplified using the primer sets listed in Table 2. PCRs were performed by using GoTaq colorless master mix (Promega Corporation, Madison, WI) containing GoTaq DNA polymerase in 1× colorless GoTaq reaction buffer (pH 8.5), 200 μM deoxynucleoside triphosphates, and 1.5 mM MgCl₂; 0.2 μM of each primer; and 10 to 20 ng of DNA template in a total volume of 50 μl per reaction mixture. PCR was performed under the following conditions by using a GeneAmp PCR system 9700 programmable thermal cycler (Applied Biosystems, Foster City, CA): denaturing at 95°C for 2 min followed by 35 cycles of 30 s of denaturing at 95°C, 30 s of annealing at corresponding temperatures for each primer set (Table 2), and 1 min of elongation at 72°C, followed by a final extension step at 72°C for 5 min. PCR fragments were visualized by electrophoresis on a 1.0% ethidium bromide-stained agarose gel, using a 100 bp ladder (BioShop Canada Inc., Burlington, Ontario, Canada) to estimate the sizes of the fragments. All PCR products were purified and sequenced using 3730xl DNA analyzer systems (Applied Biosystems, Foster City, CA) at the McGill University and Genome Quebec Innovation Centre.

SNP-based Southern hybridizations. PCR products were quantified and normalized to ensure the standardization of DNA templates prior to Southern hybridization. Capillary transfer of DNAs from gel to a nylon hybridization membrane (Genescreen Plus; Perkin-Elmer Life Sciences Inc., Waltham, MA) was done according to the manufacturer's recommendations. Labeling reaction mixtures for each SNP probe contained 1 μM oligonucleotide, 10 μCi [γ -³²P]ATP (Perkin-Elmer Life Sciences Inc.), 1× reaction buffer, and 1 unit of T4 kinase (Invitrogen, Carlsbad, CA) in a total volume of 10 μl per reaction mixture. The prehybridization mixture contained 6× saline sodium citrate (SSC) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, and 0.5% sodium dodecyl sulfate (SDS), and prehybridization was performed for 1 h at the appropriate hybridization temperature for each probe (Fig. 1). Hybridization temperatures were initially 5°C below the optimal annealing primer temperature (as indicated by the supplier, Invitrogen). If a strong hybridization signal was not evident, the hybridization temperature of the probe was lowered stepwise by 2°C; conversely, if there was significant background, the hybridization temperature was increased. Labeled probe was added to a hybridization solution containing 6× SSC–0.5% SDS, and membranes were hybridized at the appropriate temperature for 3 h. Membranes were washed in a solution containing 6× SSC–0.5% SDS at hybridization temperatures three times, totaling 5 min. Membranes were exposed for variable times corresponding to signal intensity so that the signal yield was <5% of the saturation capacity of the phosphor screen (Molecular Dynamics, Sunnyvale, CA). The signal intensity of the Southern blots was measured using a Storm phosphorimager (Molecular Dynamics), and band intensities were quantified using scanning densitometry with ImageQuant software (Molecular Dynamics) according to the manufacturer's instructions. Positive hybridization results were recorded for each sample by normalization and comparison to the hybridization signals of bands corresponding to positive and negative controls for each probe.

Southern hybridization with pMF2. In order to compare the efficiencies of our species-specific SNP probes, we screened the 49 isolates for RFLPs in hybridizations with the rDNA-containing plasmid probe pMF2 and compared them with previously published phenotypes that are diagnostic for *S. sclerotiorum*, *S. minor*, and *S. trifoliorum* (3, 10). Genomic DNA was digested with 10 units of EcoRI (Invitrogen) for 3 to 4 h at 37°C and run out on 0.75% agarose gels in

Tris-acetate buffer at 1.5 V/cm for 16 h to allow the optimal separation of DNA fragments. Bacteriophage lambda DNA digested with HindIII was used as a molecular weight standard. The capillary transfer of DNAs from gel to a nylon hybridization membrane (Genescreen Plus) was done according to the manufacturer's recommendations. Plasmid probe pMF2 was labeled with [γ -³²P]dCTP (3,000 Ci/mmol; Perkin-Elmer Life Sciences Inc.) using a nick translation kit (Gibco BRL, Invitrogen) as described previously (10). Membranes were washed three times for 5 min in 2× SSC at 65°C, three times for 20 min in a solution containing 2× SSC–1% SDS at 65°C, and twice for 10 min with 0.1× SSC at room temperature. Membranes were exposed on phosphor screens and analyzed as described above.

Nucleotide sequence accession numbers. Sequences reported here have been deposited in the GenBank database under accession numbers GQ292562 to GQ292708.

RESULTS

Southern hybridizations using species-specific SNP hybridization probes. Hybridization results from both the 15-bp species-specific SNP oligonucleotide probes and the 17-bp segment of the calmodulin gene (Fig. 1) are summarized in Table 1. Results from the procedure in Fig. 1 are shown in Fig. 2. The two oligonucleotide probes that are diagnostic for *S. minor* (CAL124 and CAL448_*S.minor*) hybridized to all *S. minor* isolates, and the results of the Southern blot analysis for CAL124 are shown in Fig. 2. The probes that are diagnostic for *Sclerotinia* species 1 (RAS148 and CAL446_*S.sp1*) hybridized to two isolates of *Sclerotinia* species 1 from vegetable crops in Alaska (Table 1) and to LMK745, an isolate found in association with wild *Taraxacum* species from Norway. The SNP probes for *S. sclerotiorum* (CAL19 [A and B] and CAL448_*S.sclero*) positively hybridized to all *S. sclerotiorum* isolates, including a panel of unknown sclerotiniaceous isolates from Australia (Table 1). The probe, IGS50, hybridized to all isolates of *S. trifoliorum* except isolate 3-A5. The other probe that is diagnostic for *S. trifoliorum*, CAL448_*S.trifol*, failed to hybridize to both isolates 02-26 and 3-A5. Sequencing results confirmed that isolates 02-26 and 3-A5 differed from the other *S. trifoliorum* isolates at this locus. All other positive and negative hybridizations were confirmed by sequence data to ensure no false-negative or -positive scoring.

One of the diagnostic probes for *S. sclerotiorum*, CAL19B, hybridized only to DNA from isolate LMK754 in this assay. This probe was designed based on the sequence of 341 isolates of *S. sclerotiorum* (2), 59 of which had the characteristic sequence of TCATCTTTCTAACTT. We attempted to use a degenerate probe for all *S. sclerotiorum* isolates, but the degeneracy of the probe was too close to the SNP site and

FIG. 2. Examples of SNP-based scoring using Southern hybridizations of *Sclerotinia* DNAs with radiolabeled, species-specific oligonucleotide probes. (A) The CAL124 probe is diagnostic for *Sclerotinia minor*. (B) The RAS148 probe is diagnostic for *Sclerotinia* species 1. (C) The IGS50 probe is diagnostic for *S. trifoliorum*. (D) CAL19A and CAL19B together are diagnostic for *S. sclerotiorum*. DNAs are arranged by anonymous numerical code (Table 1).

resulted in nonspecific hybridization. Both CAL19A and CAL19B SNP-based hybridization probes must be used for the species identification of *S. sclerotiorum* at that site in the calmodulin locus, but they may be applied to the membrane together because they have the same hybridization temperatures (Fig. 2).

Southern hybridization of EcoRI-digested DNAs using rDNA-containing probe pMF2. The results of Southern hybridizations of EcoRI-digested DNAs with a representative panel of isolates of *S. sclerotiorum*, *S. minor*, *S. trifoliorum*, and *Sclerotinia* species 1 are shown in Fig. 3. The sizes of the DNA

fragments that hybridized to rDNA-containing plasmid probe pMF2 were identical to those previously reported for *S. sclerotiorum* and *S. minor* (10). All of the unknown isolates from Australia had DNA fragment sizes of 4.8, 3.9, and 2.2 kb, consistent with the pattern for *S. sclerotiorum*. Previously, intraspecific variation was shown for *S. trifoliorum* isolates (10). Isolates had DNA fragments of different sizes, although the sum of the fragments was consistently 11.3 kb for all isolates (10). In this study, one *S. trifoliorum* isolate, 06-14, had two DNA fragments of 4.1 kb and 7.8 kb, resulting in a total size of 11.9 kb (Fig. 3). Additionally, the two isolates that did not hybridize with the SNP probes that were diagnostic for *S. trifoliorum* (02-26 and 3-A5) produced RFLP patterns that were significantly different from those of the other isolates (data not shown). The size of the rDNA repeat estimated from EcoRI digests is 10.9 kb (7.4- and 3.5-kb DNA fragments) for *Sclerotinia* species 1 (Fig. 3).

DISCUSSION

The objective of this study was to test the accuracy of the SNP-based assay to identify the four common crop-associated *Sclerotinia* species. The assay accurately identified 96% of all *Sclerotinia* isolates, as 46 of the total 48 isolates positively hybridized to their respective species-specific SNP probes (Fig. 1), and these results were consistent both with the additional calmodulin sequence and with the Southern hybridizations using probe pMF2. The two isolates with inconclusive hybridization results were isolates 02-26 and 3-A5, from Finland, originally identified as being *S. trifoliorum* by the collectors (Table

FIG. 3. Southern hybridization of *Sclerotinia* DNAs digested with EcoRI, with radiolabeled rDNA-containing plasmid probe pMF2. From left to right, DNAs were from isolates of *S. sclerotiorum* (scl), *S. trifoliorum* (tri), *S. minor* (min), and *Sclerotinia* species 1 (sp.1). Specific isolate designations are listed below each lane.

1). Neither isolate produced sclerotia under our growing conditions, although a loss of sclerotial production is not uncommon with prolonged serial culture of *Sclerotinia* (8). For example, T4, one of the sequenced strains in the *Botrytis cinerea* genome project, does not produce sclerotia, yet in whole-genome comparisons to the other reference strain, B05.10, it is indisputably *B. cinerea* (<http://urgi.versailles.inra.fr/projects/Botrytis/index.php>). The RFLP phenotypes from DNAs of 02-26 and 3-A5 were considerably different from those of *S. trifoliorum* isolates, yet these isolates had RAS sequences that were identical to those of all other isolates of *S. trifoliorum* (data not shown).

Because mutations continually arise spontaneously in populations, no sample can census all real or potential intraspecific variation in *Sclerotinia sclerotiorum*, *S. minor*, *S. trifoliorum*, or *Sclerotinia* species 1 that may be encountered in nature, nor can it census all emerging or new, undescribed species that may exist in crops. Our SNP-based assay can detect genetic variants within the four crop-infecting species of *Sclerotinia* that may represent mutations within populations or emerging or cryptic species. The sample in the present study revealed two genetic variants within *S. trifoliorum*. The sample of 465 isolates from which the loci for the present study were selected revealed two “cryptic” species, one of which is *Sclerotinia* species 1 and the other of which was reported to be *S. sclerotiorum* but is actually a newly discovered species in another genus, *Dumontinia* (2). Species other than the four species commonly associated with crops might be expected among isolates from wild hosts or understudied geographical areas. A failure to hybridize to one of the diagnostic probes could indicate a genetic variant within one of the four species. Failure to hybridize to the suite of probes that are diagnostic for a species could indicate a genetic variant within the four species or two additional possibilities: (i) the isolate represents a rare but previously described species, or (ii) the isolate might represent a new species. A survey of species epithets associated with the host or location of interest, and the sequencing of herbarium specimens that would establish the connection between the contemporary isolate and the name in the literature, would be required to distinguish the former from the latter.

The SNP-based hybridization method is faster and more robust than the rDNA-RFLP hybridization method and is amenable to the high-throughput screening of samples that might be required for an epidemiological study, in which there could be a high likelihood of a repeated sampling of one or two species. The RFLP method requires a 6-h restriction enzyme digestion of whole genomic DNA, followed by gel electrophoresis for 12 h to ensure the separation of the fragments. A maximum of 20 DNA samples, plus references, can be loaded onto a membrane with acceptable imaging. In contrast, the SNP-based method uses PCR amplification that yields high copy numbers of the desired sequence with a high affinity for the probe. The gel electrophoresis step is short, 15 to 20 min, compared to the RFLP method. With the SNP diagnostic method, hundreds of sample DNAs can be spotted onto one membrane, allowing high-throughput screening of samples. Although the RFLP method requires only one hybridization step, it is 16 to 24 h long, while SNP-based hybridizations take only

3 h per probe. Finally, hybridization results with the SNP diagnostic system are robust even under variable conditions. In the RFLP method, in which the template is restriction-digested whole genomic DNA, there are often differences in signal intensities among hybridizing bands, ranging from very light to highly saturated. Two or more exposure times for a single blot may be required to identify real, repeatable bands worthy of scoring. The dot blot format of our diagnostic-screen Southern blots is chemically quite different from that used for the RFLP screen, as the high copy number of PCR-amplified DNA sequences provides a template for which the probes will have a high affinity, resulting in a bright signal that requires little interpretation regardless of exposure time.

We propose that the SNP-based assay be conducted as outlined in Fig. 1. At least two species-specific SNP probes should be used for positive identification, and use of the entire suite of probes is the best approach for addressing concerns over false-positive or -negative scoring. Comparing all probes and examining them for cross-hybridization with more than one species-specific diagnostic SNP (Fig. 2) would be evidence of a false-positive reaction (not observed in the present study). Concerns over false-negative scoring can be addressed by using both sets of probes; as observed in the present study, a failure to hybridize to both sets of probes is unlikely without actual sequence variation consistent with a true negative result. Although we used a radioactive label, probes can also be ordered with a nonradioactive label, such as biotin, in large quantities and stored indefinitely. The SNP-based assay outlined here is an effective and rapid method for identifying the four described crop-associated *Sclerotinia* species. Importantly, this assay indicates with confidence when an isolate is not one of the described species. The SNP diagnostic system may be particularly useful for epidemiological studies with high-throughput requirements, especially when two or more species are mixed in plants or within fields.

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