An RFLP-Based Technique for Identifying Fungi in the Sooty Blotch and Flyspeck Complex on Apple

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ABSTRACT

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A restriction fragment length polymorphism (RFLP)-based technique was developed to identify members of the sooty blotch and flyspeck (SBFS) disease complex on apple because these fungi are difficult to identify using agar-plate isolation and morphological description. The method includes polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) using a fungal-specific forward primer (ITS1-F) and an SBFS-specific reverse primer (Myc1-R), followed by digestion of the PCR product by the *Hae*III restriction enzyme. When applied to previously identified isolates of 24 SBFS-causing species in nine genera, the PCR-RFLP assay produced 14 unique banding patterns. Different genera never shared the same RFLP pattern. To evaluate performance in vivo, the technique was applied to DNA extracted directly from SBFS colonies on apple fruit from three Iowa orchards. The primers amplified the rDNA of only SBFS fungi, with the exception of a *Cladosporium* sp.; however, its RFLP banding pattern was distinct from those of SBFS fungi. The majority (60%) of SBFS colonies in the in vivo trial were identified to genus by RFLP analysis. The PCR-RFLP assay greatly streamlined the identification process by minimizing the need for culturing, indicating its value as a tool for field studies of the SBFS complex.

Additional keywords: Capnodiales, epiphytic fungi, Mycosphaerellaceae

Sooty blotch and flyspeck (SBFS) is a disease complex of apple (*Malus × domestica* Borkh.) caused by epiphytic fungi that blemish the fruit surface. Signs of SBFS fungi appear worldwide in regions with moist climates, during the mid- to late growing season. Because the dark blemishes characteristic of the disease are unappealing to customers, fresh market apple fruit are downgraded to processing use, decreasing crop value by as much as 90% (4,31). In addition, colonization by SBFS fungi increases desiccation of apple fruit during post-harvest storage (22).

In the 1800s, SBFS was attributed to a single fungus, *Dothidea pomigena* Schwein. (24). By the 1920s, sooty blotch and flyspeck were determined to have distinct causal agents, *Gloeodes pomigena* (Schwein.) Colby and *Schizothyrium pomi* (Mont. & Fr.) Arx, respectively (5,31). Several sooty blotch mycelial types were described in 1933 by Groves (9). In the 1990s, it was suggested that the variation in mycelial types was not due to environ-

mental influence on a single pathogen (the previously prevailing hypothesis) but rather to multiple species of fungi causing SBFS signs (15,16). Based on morphological data, three species then were reported to cause sooty blotch: *Peltaster fruticola* Eric M. Johnson, T. B. Sutton & Hodges, *Leptodontium elatius* (de Hoog) de Hoog, and *Geastrumia polystigmati* Bat. & M. L. Farr (31).

Further investigation revealed a surprisingly large number of fungi involved in the SBFS disease complex. By coupling morphological description with phylogenetic analysis of ribosomal DNA (rDNA), Batzer et al. (3) determined that SBFS in the Midwest is caused by at least 30 species of fungi in 11 anamorph genera. All the Midwestern SBFS species were Dothideomycetes, with the vast majority belonging to the family Mycosphaerellaceae in the order Dothideales, which subsequently has been reclassified to the order Capnodiales (23). Each species was associated with a single mycelial type on apple. This study also redefined SBFS mycelial types to include discrete speck, compact speck, punctate, ramose, ridged honeycomb, and fuliginous (3). The number of species in the disease complex continues to increase as SBFS fungi from Europe, China, and the eastern United States are surveyed (2,6). In 2006, the first SBFS species belonging to the phylum Basidio-

mycota, *Wallemia sebi* (Fr.) Arx, was identified in China (26).

Taxonomic barriers have held back progress in understanding the environmental biology of SBFS fungi, because many identifications made prior to the 1990s now appear to be incorrect (3,31). A major impediment is that these fungi are challenging to culture and identify by traditional techniques. Most SBFS fungi grow slowly in culture and are highly sensitive to surface disinfectants; therefore, they often are overgrown by contaminants. Morphology of SBFS fungi in culture often differs radically from that on apple, and most sporulate sporadically or not at all in culture. Mycelial types on apple can be difficult to differentiate, further complicating identification (1,3,9). These limitations, coupled with the immense diversity of the complex, mean that alternative techniques are needed to facilitate rapid and reliable identification.

Current polymerase chain reaction (PCR)-based identification methods for SBFS fungi assess variation in the large subunit (LSU) and internal transcribed spacer (ITS) regions of rDNA (3,27). However, species identification by sequence homology demands substantial time and expense. Species-specific primers could aid the identification process but would be cumbersome to apply to all the species in the complex.

An alternative approach involves the use of restriction fragment length polymorphism (RFLP) of PCR products, as has been done with various plant pathogens and mycorrhizal fungi (8,11,17,20,21). In a preliminary study, Sun et al. (27) developed a PCR-RFLP identification technique for SBFS members that amplified the ITS region using a reverse primer specific to the most commonly identified SBFS fungi paired with a fungal-specific forward primer, followed by digestion with *Hae*III or *Alu*I restriction enzymes. However, this assay was performed on only 14 species in seven genera and used only pure cultures. Identification by RFLP analysis of SBFS colonies sampled directly from apple could greatly facilitate field studies by circumventing the need for agar-plate isolation. Several other plant-pathogenic fungi have been identified by PCR-RFLP analysis from in vivo samples (8,11,17,20).

The objective of this study was to determine the ability of a PCR-RFLP assay

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to distinguish members of the SBFS complex in culture and in vivo.

MATERIALS AND METHODS

SBFS isolates. SBFS fungi were isolated from apple in 2000 from nine orchards in Illinois, Iowa, Missouri, or Wisconsin and identified by morphological

characters and rDNA sequence analysis (3; Table 1). These isolates, which had been stored in glycerol at -80°C , were grown on potato dextrose agar (PDA; Difco Laboratories, Detroit) at room temperature prior to DNA extraction.

PCR and restriction enzyme digestion. For PCR assays, DNA was extracted

from 1- to 6-week-old mycelium on PDA using the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA). A minute amount of aerial mycelium was transferred from the PDA plate to a microcentrifuge tube containing 50 μl of the PrepMan Ultra extraction fluid using a pipette tip. The complete ITS region

Table 1. Origin, morphology on apple, and size of *Hae*III restriction fragments of the internal transcribed spacer (ITS) region of ribosomal DNA of sooty blotch and flyspeck (3)

Putative species	Acc. no. ^w	Mycelial type ^x	Isolate	Origin	Fragment sizes (bp) ^y	RFLP type ^z
<i>Schizothyrium pomi</i> (FS1)	AY598851	Flyspeck	GTA2a	Illinois	262, 258, 91, 81	A
			MSTA1a	Wisconsin	259, 205, 91, 82	B
			MSTA2a	Wisconsin	262, 258, 91, 81	A
			UMA7b	Missouri	262, 258, 91, 81	A
<i>Zygophiala</i> sp. FS2	AY598854	Flyspeck	FVA2a	Iowa	261, 258, 90, 82	A
			MWA6a	Iowa	261, 258, 90, 82	A
			MWA8b	Iowa	261, 258, 90, 82	A
<i>Zygophiala</i> sp. FS3.1	AY598853	Flyspeck	GTA5c	Illinois	259, 205, 91, 82	B
			GTA8c	Illinois	259, 205, 91, 82	B
			MSTA8a	Wisconsin	259, 205, 91, 82	B
<i>Zygophiala</i> sp. FS3.2	AY598856	Flyspeck	MWA1a	Iowa	261, 259, 91, 82	A
			MWA1d	Iowa	261, 259, 91, 82	A
<i>Dissoconium</i> sp. DS1.1	AY598874	Discrete speck	CUB2c	Illinois	552, 91, 86, 80	C
			MSTB5b	Wisconsin	552, 91, 86, 80	C
			MWB7b	Iowa	552, 91, 86, 80	C
<i>Dissoconium</i> sp. DS1.2	AY598875	Discrete speck	UMB2a	Missouri	552, 91, 86, 80	C
			UMB4b	Missouri	552, 91, 86, 80	C
<i>Dissoconium</i> sp. FG4	AY598876	Fuliginous	MSTF2	Wisconsin	247, 292, 99	D
			MSTF3b	Wisconsin	247, 292, 99	D
<i>Dissoconium</i> sp. FG5	AY598877	Fuliginous	UIF3a	Illinois	555, 88	C
<i>Pseudocercospora</i> sp. FG1.1	AY598858	Fuliginous	MWF7a	Iowa	285, 251, 91, 90	E
			MSTF5b	Wisconsin	285, 251, 91, 90	E
<i>Pseudocercospora</i> sp. FS4	AY598857	Flyspeck	MWA4b	Iowa	285, 251, 91, 81	E
<i>Pseudocercospora</i> sp. RH1	AY598862	Ridged honeycomb	AHD1a	Missouri	357, 193, 90, 80	F
			GTD2a	Illinois	357, 193, 90, 80	F
			MSTD1a	Wisconsin	357, 193, 90, 80	F
			UMD7a	Missouri	357, 193, 90, 80	F
<i>Pseudocercospora</i> sp. RH2.1	AY598866	Ridged honeycomb	AHE9a	Missouri	359, 195, 91, 81	F
			GTE5b	Illinois	359, 195, 91, 81	F
			UMD1a	Missouri	359, 195, 91, 81	F
<i>Pseudocercospora</i> sp. RH2.2	AY598868	Ridged honeycomb	GTC1a	Illinois	357, 194, 91, 81	F
			GTC4a	Illinois	357, 194, 91, 81	F
			UMD8b	Missouri	357, 194, 91, 81	F
<i>Colletogloeum</i> sp. FG2.1	AY598870	Fuliginous	AHF3a	Missouri	331, 193, 168, 90	G
			AHF4a	Missouri	331, 193, 168, 90	G
			UMF4a	Missouri	331, 193, 168, 90	G
<i>Colletogloeum</i> sp. FG2.2	AY598871	Fuliginous	CUF3c	Illinois	329, 193, 168, 91	G
			MWF1a	Iowa	329, 193, 168, 91	G
			UMF2a	Missouri	329, 193, 168, 91	G
<i>Colletogloeum</i> sp. FG2.3	AY598872	Fuliginous	CUF2d	Illinois	329, 193, 168, 90	G
			UIF1	Illinois	329, 193, 168, 90	G
<i>Peltaster fructicola</i> (P1)	AY598887	Punctate	GTE1a	Illinois	713, 72	H
			MSTE10b	Wisconsin	713, 72	H
			UME4a	Missouri	713, 72	H
<i>Peltaster</i> sp. P2.1	AY598888	Punctate	GTE5a	Illinois	367, 261, 163	I
			GTE6d	Illinois	367, 258, 228, 179, 146	J
			AHE5d	Missouri	367, 258, 228, 179, 146	J
<i>Peltaster</i> sp. P2.2	AY598889	Punctate	CUE2b	Illinois	367, 258, 228, 179, 146	J
<i>Xenostigmina</i> sp. P3	AY598878	Punctate	AHE3a	Missouri	282, 173, 146	K
			AHE7a	Missouri	282, 173, 146	K
<i>Xenostigmina</i> sp. P4	AY598879	Punctate	UIE3a	Illinois	282, 173, 145	K
<i>Sterile mycelia</i> sp. RS1	AY598882	Ramosae	PEC6a	Iowa	281, 174, 146, 91	L
			UMC4	Missouri	281, 174, 146, 91	L
<i>Sterile mycelia</i> sp. RS2	AY598882	Ramosae	AHC1a	Missouri	280, 145, 92, 90	M
			AHC4b	Missouri	280, 145, 92, 90	M
			UMC2	Missouri	280, 145, 92, 90	M
			UMC7a	Missouri	280, 145, 92, 90	M
<i>Ramularia</i> sp. P5	AY598873	Punctate	UME2a	Missouri	417, 249, 83	N

^w GenBank accession numbers are from Batzer et al. (3), representing the ITS sequence of the species for one specific isolate but not necessarily the isolates used in the testing of the assay.

^x Mycelial type on apple.

^y Fragment sizes were determined from sequence analysis of ITS1-F/Myc1-R amplicons.

^z Patterns of restriction fragment length polymorphism (RFLP) fragments are classified by letters A to N.

(ITS1-5.8S-ITS2) along with a portion of the LSU of rDNA was amplified using the fungal-specific ITS1-F primer (30) and the Myc1-R primer (27). The reverse-primer Myc1-R was designed to represent the most commonly observed species of SBFS fungi in the Midwestern United States based on 14 species in seven genera (3). This primer (5'-ACTCGTCCGAAGGAGCTACG-3') was designed from the first 20 bp of 28S rDNA gene by selecting sequences homologous within the SBFS fungi. The universal fungal primer ITS1-F was selected as the forward primer. Selectivity of the primer was tested with 19 non-SBFS species; 4 species were fungi frequently isolated from apple epicuticle and 15 were cosmopolitan species obtained from culture collections.

The 50- μ l PCR reaction mixture contained 4 mM MgCl₂, 10 $\times$ Promega buffer, 200 μ M dNTPs, 0.25 pM each primer, 2 units of Taq polymerase (Promega Corp.,

Madison, WI), and 2 μ l of DNA extract. To improve amplification for some isolates, the DNA extract was diluted 10-fold and 5% dimethyl sulfoxide (DMSO) was included in the PCR reaction. Cycling conditions for PCR (Model PCT-100; MJ Research Inc., Waltham, MA) included hot start at 85°C; initial denaturation at 95°C for 95 s; 35 cycles of denaturation at 95°C for 60 s, annealing at 58°C for 60 s, and extension at 72°C for 60 s; followed by final annealing at 58°C for 60 s and final extension at 72°C for 5 min. Amplification was verified by running 10 μ l of the PCR product in a 2% agarose gel (BioRad, Hercules, CA) with 1 $\times$ Tris-borate EDTA (TBE), staining with ethidium bromide, and visualizing with ultraviolet light.

Amplified DNA was digested with the restriction enzyme *Hae*III (Invitrogen, Carlsbad, CA). Three units of *Hae*III were added directly to 20 μ l of PCR product, which then was incubated at 37°C for 2 h.

Digested PCR products were observed in a 2% agarose gel, run for 1.75 h at 150 V in 1 $\times$ TBE buffer, stained with ethidium bromide, and visualized with ultraviolet light. Band patterns were determined against a 1-kb-plus ladder using the band-matching feature of the Quantity One software (BioRad). The PCR-RFLP analysis was performed at least twice for all isolates. PCR products were sequenced at the Iowa State University DNA Sequencing and Synthesis Facility. Sequences were edited using Sequence Navigator (Applied Biosystems) and analyzed using BioEdit (10).

SBFS colonies in vivo. SBFS samples were collected from apple fruit in fall 2003 from three orchards near Fort Dodge, Gilbert, and Jefferson, IA. After classification by mycelial type (discrete speck, flyspeck, fuliginous, punctate, ramose, or ridged honeycomb; 3), samples were preserved by pressing individual SBFS colonies and supporting apple epidermis between sheets of paper until dry, and then photographed under a dissecting microscope. Each of the 318 preserved samples consisted of a single SBFS colony. The dried samples were stored for 1 to 12 months at room temperature in plastic petri plates before DNA extraction. Fungal DNA was extracted after transferring mycelium scraped from the apple cuticle with a scalpel to PrepMan Ultra Sample Preparation Reagent. Conditions for DNA extraction, PCR, and enzymatic digestion were the same as those described above. Resulting RFLP patterns were compared with patterns observed from cultures of previously identified SBFS species. The PCR products from apple samples that yielded previously unidentified RFLP patterns were sequenced as previously described and subjected to a BLAST search (National Center for Biotechnology Information, Bethesda, MD). To check accuracy and reliability of resulting RFLP patterns, the ITS products of a subsample of colonies on apple were sequenced, edited, and subjected to a BLAST search.

The DNA of each SBFS sample that produced weak or no PCR product with the ITS1-F/Myc1-R primer set was subjected to a second attempt to amplify the ITS region, replacing the Myc1-R primer with a nonspecific reverse primer, ITS4 (30). Other than replacement of the reverse primer, the PCR protocol was performed as described above. The DNA of SBFS samples that did not amplify with either ITS primer set was subjected to another round of PCR in an attempt to amplify the LSU region. The PCR protocol was performed as described above, except that the primer set was LROR/LR5 (29), 5% DMSO was included, and the annealing temperature was 49°C. Amplified samples then were sequenced with primers LROR and LR3 and analyzed as described above.

Variation within colonies. To test repeatability of the technique, we evaluated

Fig. 1. Fourteen distinct restriction fragment length polymorphism patterns (A–N) after *Hae*III digestion of the internal transcribed spacer ribosomal DNA products amplified from pure cultures of sooty blotch and flyspeck fungi. Unmarked lanes are 100-bp-ladders.

whether subsamples of mycelium from single colonies gave consistent results. Seven SBFS colonies on apple were collected and preserved from SBFS-infested fruit in fall 2004 from three orchards in Iowa and stored at room temperature for 18 months. The colonies represented six different mycelial types, and each was divided into four quadrants. Three of the four quadrants from each colony were sampled separately and subjected to the RFLP assay as described above.

RESULTS

The ITS1-F/Myc1-R primer set amplified the ITS region of each of the 59 SBFS isolates. The amplification products ranged from 784 to 834 bp, except that two of the three *Peltaster* sp. P2.1 isolates and the *Peltaster* sp. P2.2 isolate produced amplicons of approximately 1,400 bp due to a large insert in the ITS1 region.

Digestion of the amplified products of the pure cultures with *Hae*III yielded 14 distinct RFLP patterns. We designated each of the 14 RFLP types by its own letter (Table 1; Fig. 1). All of the RFLP patterns were unique to genus, three were unique to species, and one was unique to an individual isolate. More than one RFLP pattern was observed for four of the nine genera tested. Two pairs of band patterns, RFLP types D and E and K and L (Table 1), were difficult to distinguish visually unless fragments of 100 bp or less were clearly seen (Fig. 1). Intraspecific variability was observed for only two putative species, *Zygophiala jamaicensis* and *Peltaster* sp. P2.1. Each RFLP pattern corresponded to a single SBFS mycelial type on apple; however, a mycelial type sometimes corresponded to more than one RFLP pattern.

Validation of RFLP identification in vivo. Of the 318 unidentified SBFS colonies sampled from apple peels, 213 (67%) yielded amplification product using the ITS1-F/Myc1-R primer pair, and 191 (60% of all colonies, 85% of those that amplified) produced RFLP patterns matching those of previously identified SBFS fungi (27). Of the 14 RFLP patterns identified from pure culture, 8 (including RFLP types A, C, F, G, K, L, M, and N) were observed among the samples on apple. For each of the observed SBFS RFLP patterns from apple samples, the ITS region of two to nine colonies was sequenced and subjected to a BLAST search. For seven of the eight patterns, the sequences aligned with those of the SBFS species as predicted by RFLP patterns. The only unexpected outcome occurred when the sequences from three SBFS colonies exhibiting the RFLP band pattern of *Mycelia sterilia* sp. RS2 aligned more closely to *M. sterilia* sp. RS1. Direct comparison of the sequences showed that the majority of the sequences matched *M. sterilia* sp. RS1 and that the variation in RFLP pattern was due to the presence of an extra *Hae*III restriction site, as found in

M. sterilia sp. RS2, that was not previously observed.

Nineteen SBFS colonies on apple (6% of all colonies, 9% of the samples that amplified) produced RFLP patterns that did not match those of SBFS fungi. A single amplicon of approximately 790 bp was observed before digestion of all 19 SBFS samples. Of the 19 samples, 6 produced RFLP patterns with a single fragment of approximately 790 bp, 8 produced RFLP patterns with two fragments of approximately 790 and 620 bp, and 5 produced RFLP patterns with three fragments of approximately 790, 710, and 620 bp. The ITS regions of four samples with the single 790-bp RFLP pattern were identical and matched (517/517 bp) *Cladosporium tenuissimum* (GenBank accession no. AY904056). The ITS regions of two samples with the RFLP pattern including three band fragments were identical to each other, matched closely with the same *C. tenuissimum* (479/480 bp), but shared a single point mutation differentiating these sequences from those with a single-band RFLP pattern.

Of all the apple samples, 71 (37%) produced RFLPs that included fragment patterns of both a known SBFS fungus and of *Cladosporium* spp., indicating that these samples included an SBFS species and a *Cladosporium* sp. The 11 SBFS RFLP patterns observed in the in vivo survey were clearly distinguishable from those of the *Cladosporium* fragments (Fig. 2).

Three SBFS colonies from apple produced RFLP band patterns that had not been observed previously. Their respective sequences matched most closely with *Pseudocercospora* sp. 4ra (466/468 bp; GenBank accession no. DQ363411), *Pseudocercospora* sp. 43.1a (441/484 bp; GenBank accession no. DQ363419), and an unnamed fungal endophyte species (421/431 bp, GenBank accession no. AM262372), all of which have been observed to cause SBFS signs in surveys outside the Midwestern United States (2,6).

Of the SBFS colonies on apple, 105 (33%) either did not amplify an ITS product with the ITS1-F/Myc1-R primer pair or the amplified product did not produce discernible RFLP patterns. Of the 105 samples subjected to PCR using the general ITS or LSU primers, 8 ITS and 24 LSU regions were amplified. The amplified products were sequenced and included multiple incidence of *Penicillium* spp. (nine LSU and three ITS samples), *Athelia* spp. (five LSU samples), *Tilletiopsis* spp. (two ITS and two LSU samples), and single LSU samples of a *Exophiala* sp., a *Stropharia* sp., an *Acremonium* sp., a *Bulleromyces* sp., and an *Aureobasidium* sp. The sequences of five samples matched those of known SBFS species, *Colletogloeum* sp. FG2.3 (one ITS and one LSU sample), *Mycelia sterilia* sp. RS1 (one LSU sample), a *Dissiconium* sp. (one ITS sample), and a *Xenostigmata* sp. (one LSU sample); and one sequence matched a *Cladosporium* sp. (one ITS sample).

Fig. 2. Lanes 1 to 5 are examples of restriction fragment length polymorphism patterns after *Hae*III digestion of the internal transcribed spacer ribosomal DNA products amplified from colonies on apple including both sooty blotch and flyspeck fungi and *Cladosporium* spp.; unmarked lanes are 1-kb-plus ladders.

The results of the validation of RFLP identification *in vivo* are summarized in Table 2.

Variation within colonies. In each of the seven SBFS colonies on apple that were assayed, all three of the quadrants sampled from the same colony produced identical RFLP patterns (K. B. Duttweiler, *unpublished data*).

DISCUSSION

The tested PCR-RFLP assay is substantially faster, simpler, and more reliable than culturing for identification of colonies of SBFS fungi. The assay rapidly and accurately identified the genera of SBFS fungi found on apple in the Midwestern United States. The RFLP band patterns corresponded to the known SBFS morphology and were useful for distinguishing among similar-looking mycelial types on apple (3). As indicated by sequence analysis of a subsample of SBFS colonies from the *in vivo* study, the technique was consistently accurate. By circumventing agar-plate isolation, the *in vivo* assay reduced the time needed for identification from weeks or months to hours. The RFLP-based identification method is also simpler than traditional methods, which require extensive training in recognizing subtle morphological differences in cultures. In addition, the identification rate of SBFS fungi from colonies on apple was increased from <35% using agar-plate isolation (3) to at least 60% using the RFLP assay.

Traditional identification methods, including determination of growth rate, morphology, and sporulation *in vivo* and *in vitro*, have contributed to misidentification of SBFS fungi for 180 years (1,5). For the vast majority of SBFS fungi, it is impossible to identify to genus (let alone species) based on morphology *in vivo*, and it can be done *in vitro* only when sporulation occurs (a rare event in many species) or if the rDNA of samples is sequenced (3). Knowing the genera of SBFS fungi populating an orchard may prove to be important for management of the disease, because different genera vary in fungicide sensitivity (28) as well as other aspects of environmental biology (14,25).

The RFLP assay described here takes a valuable first step toward genus and species identification, and could be refined by use of additional restriction enzymes to reveal more species-specific banding patterns. For example, some colonies of *M. sterilia* spp. RS1 and RS2 had the same banding patterns after *Hae*III digestion but they were differentiated clearly after digestion with the restriction enzyme *Alu*I (27; K. B. Duttweiler, *unpublished data*). The RFLP technique also could be an essential first step to guide efficient selection of specific primers to confirm species identity.

For identification of SBFS members directly from the apple epidermis, using the ITS1-F/Myc1-R primer pair proved to be a valuable tool that avoids the frustrations and failures that often accompany agar-plate isolation of these fungi. The technique was robust to the vast majority of non-SBFS fungi, as observed after using nonspecific fungal primers for the amplification and sequencing of the ITS or LSU regions of the *in vivo* samples that did not amplify with Myc1-R. Of these PCR products sequenced, 85% matched most closely to sequences of members of non-SBFS fungi, indicating that the Myc1-R primer did not amplify the non-SBFS fungal epiphytes present in this study. Although the remaining 15% were SBFS fungi, the Myc1-R primer did not appear to selectively exclude any SBFS species, because the sequences matched SBFS fungi from a diverse array of genera and mycelial types.

Amplification of a *Cladosporium* sp., which does not cause SBFS, was not surprising because it is closely related to the seven genera of SBFS fungi used to design the Myc1-R primer (within the same order, Capnodiales) and is a common epiphyte (19). Analysis of ITS regions revealed that the sequences of *Cladosporium* spp. producing a single fragment after digestion with *Hae*III differed from those that produced multiple fragments after digestion with *Hae*III, and may explain the variability of the observed *Cladosporium* RFLP patterns. It is likely that future surveys will encounter non-SBFS fungi in the order Capnodiales, in addition to *Cladosporium* spp. If so, it would be necessary to deter-

mine their RFLP patterns in order to exclude them from SBFS surveys.

This RFLP analysis consistently distinguished SBFS fungi from the non-SBFS *Cladosporium* sp., even when both were present in the same sample. Therefore, the assay appears to be robust to nontarget organisms, whereas pure SBFS cultures are required for identification using agar-plate isolation (3,15).

Three new SBFS RFLP patterns were identified from the survey of *in vivo* colonies. The ITS sequences of the three samples displaying new band patterns matched closely with fungi previously identified as members of the SBFS complex; however, these fungi had not yet been assayed using the RFLP technique. This finding indicates that the RFLP assay is likely to be useful for identification of SBFS fungi other than those included in this study.

As the RFLP library expands to SBFS members in regions other than the Midwestern United States, the increasing number of band patterns may complicate visual differentiation among similar patterns. In some cases, it may be difficult to distinguish between similar SBFS RFLP patterns unless they are run adjacent to each other in the same gel, which can require additional time and expense (7). It also is possible that additional RFLP patterns of SBFS fungi may resemble those of *Cladosporium* sp. or other non-SBFS epiphytes. Therefore, it may be helpful to investigate use of additional restriction enzymes in order to increase differentiation among RFLP patterns.

The PCR-RFLP assay has the potential to minimize the misidentification of SBFS species that previously stymied research on this pathosystem. The assay also could be used for rapid identification of samples taken directly from field studies and to expedite identification of pure cultures. Therefore, the technique can become a valuable tool for improving the ecological understanding of the relatively unexplored SBFS complex. For example, SBFS fungi on plum were identified by coupling sequence information, obtained directly from colonies on plum peels using the ITS1-F/Myc1-R primer pair, with morphological description of pure cultures from the same peels (18). The RFLP assay also was used to identify SBFS fungi taken directly from stems of woody plants to determine potential reservoir hosts (12). Third, efficacy of artificial inoculation of apple with recently discovered SBFS isolates was evaluated using this assay, and it proved to be especially useful when pure cultures could not be recovered from colonies on apple fruit (13). Additionally, in a field study investigating phenology of appearance of SBFS species on apple in three Iowa orchards, the RFLP assay identified 85% of 96 samples in 6 days, and up to 92% after either the addition of 5% DMSO in the PCR reaction or 10-fold dilution of extracted

Table 2. Summary of the polymerase chain reaction and restriction fragment length polymorphism (RFLP) analysis results of sooty blotch and flyspeck (SBFS) colonies taken directly from apple peels

Analysis results ^y	No. of colonies ^z	Percentage of colonies
Amplified using ITS1-F/Myc1-R	213	67
RFLPs matched previously identified SBFS patterns	191	60
RFLPs matched non-SBFS <i>Cladosporium</i> patterns	19	6
RFLPs did not match previously identified SBFS patterns	3	1
Not amplified using ITS1-F/Myc1-R	105	33
No amplification with ITS1-F/ITS4 or LROR/LR5 primer sets	71	22
Sequences matched non-SBFS apple epiphytes	29	9
Sequences matched previously identified SBFS patterns	5	2

^y ITS = internal transcribed spacer.

^z Total number assessed was 318.

DNA (25). The increased rate of identification noted in the latter study may be due in part to improving the technique of transferring in vivo SBFS samples to DNA extract fluid, which reduced the presence of plant tissue contaminants during DNA extraction.

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