Diversity and Biogeography of Sooty Blotch and Flyspeck Fungi on Apple in the Eastern and Midwestern United States

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ABSTRACT

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Sooty blotch and flyspeck (SBFS) fungi on apple fruit were sampled from nine orchards in four midwestern U.S. states during 2000 and 30 orchards in 10 eastern U.S. states during 2005 in order to estimate taxonomic diversity and discern patterns of geographic distribution. Forty apple fruit per orchard were arbitrarily sampled and colonies of each mycelial phenotype were counted on each apple. Representative colonies were isolated, cultures were purified, and DNA was extracted. For representative isolates, the internal transcribed spacer (ITS) and large subunit (LSU) regions of ribosomal DNA were amplified and sequenced. In total, 60 SBFS putative species were identified based on ITS sequences and morphological characteristics; 30 of these were discovered in the 2005 survey. Modified Koch's postulates were fulfilled for all 60 species in an Iowa orchard; colonies resulting from inoculation of apple fruit were matched to the original isolates on the basis of mycelial type and ITS sequence. Parsimony analysis for LSU sequences from both surveys revealed that 58 putative SBFS species were members of the Dothideomycetes, 52 were members of the Capnodiales, and 36 were members of the Mycosphaerellaceae. The number of SBFS species per orchard varied from 2 to 15. Number of SBFS species and values of the Margalef and Shannon indexes were significantly ($P < 0.05$) lower in 21 orchards that had received conventional fungicide sprays during the fruit maturation period than in 14 unsprayed orchards. Several SBFS species, including *Schizothyrium pomi*, *Peltaster fructicola*, and *Pseudocercosporella* sp. RH1, were nearly ubiquitous, whereas other species, such as *Stomiopeltis* sp. RS5.2, *Phialophora sessilis*, and *Geastrumia polystigmatis*, were found only within restricted geographic regions. The results document that the SBFS complex is far more taxonomically diverse than previously recognized and provide strong evidence that SBFS species differ in geographic distribution. To achieve more efficient management of SBFS, it may be necessary to understand the environmental biology of key SBFS species in each geographic region.

Additional keywords: polymerase chain reaction.

Sooty blotch and flyspeck (SBFS) is a disease caused by a complex of saprophytic fungi that colonize the epicuticular wax layer of apple (*Malus* × *domestica* Borkh.) and several other fruit crops in humid production regions worldwide (5,15,46,48). In the eastern half of the continental United States, SBFS is a major problem for commercial apple growers because the dark blemishes of SBFS colonies result in downgrading fruit from fresh-market to processing use, with economic losses as high as 90% (9,31, 38,46).

To suppress SBFS and fruit rots, most apple growers in this region apply fungicide sprays every 1 to 2 weeks from 7 to 10 days after petal fall until shortly before harvest. This strategy entails as many as 10 sprays per season and is costly, time consuming, and potentially hazardous to both environmental quality and human health (7,8). Warning systems for SBFS (7,18) have potential to reduce fungicide use substantially but their performance has been erratic (1,11), possibly because of insufficient knowledge of the identity and environmental biology of SBFS fungi.

Identification based solely on morphological criteria is impracticable because few SBFS species sporulate readily (5,12,20, 26,36). The disease was initially attributed to a single fungus, *Dothidea pomigena*; later, however, sooty blotch (the term denoting colonies that produce dark mycelial mats on the apple cuticle) and flyspeck (groupings of black dots lacking a visible mycelial matrix) were determined to have distinct causal agents, *Gloeodes pomigena* and *Schizothyrium pomi*, respectively (2,9, 44). In the 1990s, Sutton and co-workers presented morphological evidence that at least three species caused sooty blotch in North Carolina: *Peltaster fructicola, Leptodontidium elatius*, and *Geastrumia polystigmatis* (23,25,26,38,46).

Molecular tools are useful for studying the phylogenetic relationships of filamentous fungi and relating them to host specificity, geographical distribution, and phenotype (14,17,28,29). The internal transcribed spacer region (ITS) of the ribosomal DNA (rDNA) is often used to delineate putative species in combi-

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nation with morphology, whereas the large subunit (LSU) of rDNA is used to place taxa at the family and ordinal level (3,5, 13,32,40,43,45). By combining morphological description with parsimony analysis of rDNA, Batzer et al. (5) determined that SBFS colonies sampled from nine orchards in four midwestern U.S. states were caused by at least 30 putative species of fungi in 11 anamorph genera. Several of the newly discovered SBFS species differed significantly from others in phenology (34) and sensitivity to widely used fungicides (41). These findings suggested that identifying SBFS species accurately could lead to more effective disease management.

The first indication that assemblages of SBFS fungi differed among geographic regions emerged from a survey of mycelial types in North Carolina apple orchards (38). In a survey of apple fruit from 10 midwestern and eastern U.S. states in 1992 and 9 states in 1993, Johnson et al. (26) documented that *Geastrumia polystigmatis* occurred in Michigan and New York and that *L. elatius* occurred in New York and Illinois as well as in North Carolina. However, many of the SBFS isolates in these surveys could not be identified solely on the basis of morphological evidence. Based on these pioneering studies, we hypothesized that broader surveys utilizing both morphological and genetic evidence would reveal previously undiscovered SBFS species and regional differences in SBFS species assemblages. Therefore, the objectives of the present study were to (i) characterize diversity and taxonomic relationships of the SBFS fungal complex in the eastern and midwestern United States and (ii) discern biogeographic patterns of species distribution. A portion of the data was published previously (5).

MATERIALS AND METHODS

Sampling sites. Within 2 weeks before harvest during September to October, apple fruit exhibiting SBFS signs were arbitrarily sampled from 9 sites in four midwestern U.S. states (Iowa, Wisconsin, Missouri, and Illinois) in 2000 (5) and 30 sites in 10 states in the eastern U.S. (Michigan, Ohio, New York, Massachusetts, Pennsylvania, Virginia, North Carolina, Georgia, Tennessee, and Kentucky) in 2005 (Fig. 1). Sites included managed and abandoned commercial orchards as well as home orchards. Forty apple fruit were arbitrarily sampled from ≤40 trees per orchard. The most commonly sampled cultivar was Golden Delicious (Table 1). Apple fruit were transported to Iowa State University (ISU) in padded cardboard boxes.

Colony characterization. At ISU, apple fruit were inspected under a dissecting microscope. On each apple, the number of SBFS colonies of each of the following mycelial types was counted: flyspeck, discrete speck, ramose, ridged honeycomb, punctate, fuliginous, and compact speck (5). Additional characteristics, such as morphology of colony margins and size, shape, and density of sclerotium-like bodies, were also noted. In all, 5 to 15 representative colonies of each mycelial type were selected and labeled from a subsample of 10 to 15 apple fruit from each orchard, for a total of ≈500 and 2,000 colonies in 2000 and 2005, respectively. To minimize contamination of cultures by multiple species, colonies selected for isolation did not visibly overlap with other SBFS colonies on the apple surface. Isolation, purification, and long-term storage of 399 and 1,461 fungal strains from the 2000 and 2005 surveys, respectively, were performed as described by Batzer et al. (5) and Díaz Arias (10). Segments of apple peel containing remnants of the colonies that had been sampled for isolation were preserved by excising the peel and pressing it between paper towels until dry; dried peels were stored at room temperature in multiwell tissue culture plates (Costar; Corning Inc., Corning, NY). The SBFS colony on each peel was digitally photographed under a dissecting microscope.

Putative species designation. Putative species were delineated using ITS sequences and morphological characteristics on apple

Fig. 1. Location of the 39 orchards from which apple fruit infested with signs of sooty blotch and flyspeck were sampled in 2000 and 2005.

and in culture (5,10). One to four representative isolates of each mycelial type on apple from each sampled orchard were sequenced, for a total of 1,069 sequences (358 and 711 for the 2000 and 2005 surveys, respectively). Length of ITS sequences was 458 to 534 bp (5,10). Subsets of taxa with similar sequences were analyzed separately to prevent ambiguous alignments due to large insertions or deletions in the ITS data set. Sixteen alignments of ITS sequences were used to delineate putative species (10). Maximum parsimony analyses were performed for each alignment using PAUP* version 4.0b10 for 32-bit Microsoft Windows (5,10,40).

Morphology of isolates grouped into putative species based on ITS parsimony analysis was characterized on apple fruit and on artificial media. Genus designations were determined by anamorph morphology (5,10).

For each putative species, a modified Koch's postulates procedure was performed during four growing seasons in an orchard at the ISU Horticulture Research Farm near Gilbert, IA (5,10). Briefly, after immature apple fruit were surface sterilized with ethanol, a suspension of mycelial fragments and conidia of representative isolates was swabbed onto the fruit, and the fruit were immediately enclosed in Fuji bags (Kobayshi Bag Manufacturing Co. Ltd., Iasa, Nagano, Japan). At harvest, bags were removed, mycelial type of fungal colonies was compared with that of the inoculated isolate, and identity of the reisolated fungus was confirmed by comparing the ITS sequence with that of the original isolate.

TABLE 1. Orchard location, cultivar, summary of isolate recovery from sooty blotch and flyspeck (SBFS) colonies sampled from apple fruit, number of putative species identified, and species diversity in 39 orchards in the eastern and midwestern United States

Orchard[r]											Species diversity[s]	
Code	Long.	Lat.	Cultivar	Spray[t]	Colonies[u]	Types[v]	Attempted[w]	Successful[x]	Cultures[y]	SBFS[z]	MI	SI
GA2	–84.50925	34.71445	Golden Delicious	1	51	6	72	17	17	5	0.53	1.95
GA3	–84.41414	34.64939	Golden Delicious	1	30	7	90	25	24	11	1.41	2.56
IA1	–93.31043	42.51855	Golden Delicious	1	27	6	72	20	15	7	0.94	2.67
IA2	–93.48052	41.34888	Golden Delicious	2	91	7	90	26	25	13	1.43	3.19
IA3	–92.87919	41.39307	Golden Delicious	1	95	7	90	15	7	6	0.60	2.27
IL1	–89.15663	42.32575	Golden Delicious	1	48	5	61	12	9	5	0.55	1.11
IL2	–89.87836	37.95379	Golden Delicious	2	33	7	90	30	20	10	1.21	2.68
IL3	–88.65504	37.45984	Golden Delicious	2	28	6	72	21	19	12	1.52	2.77
KY1	–84.69616	37.99534	Golden Delicious	2	Tmtc	5	65	62	52	15	1.79	2.89
KY2	–85.05524	38.45239	Golden Delicious	1	30	4	50	9	6	6	0.71	1.43
KY3	–88.69779	37.08633	Golden Delicious	2	147	5	60	45	41	11	1.11	2.11
KY4	–83.27374	36.78287	Golden Delicious	1	*	7	108	58	34	10	1.27	2.70
MA1	–71.51437	42.41031	Grimes Golden	1	11	6	72	26	22	7	1.00	1.88
MA2	–71.61803	42.38998	McIntosh	1	7	6	72	37	24	8	1.25	1.38
MA3	–71.56911	42.50095	McIntosh	1	6	3	45	22	6	3	0.37	1.37
MA4	–71.61056	42.44418	McIntosh	1	7	2	24	12	3	2	0.18	0.12
MA5	–72.62319	42.56459	Golden Delicious	1	115	4	60	58	21	4	0.49	1.67
MI2	–86.10859	42.24392	Golden Delicious	2	Tmtc	6	80	37	29	8	0.85	2.66
MI3	–86.19000	42.55214	Golden Delicious	2	4	5	60	20	20	8	1.57	2.39
MO1	–93.65358	39.74801	Golden Delicious	Nd	Tmtc	5	60	19	16	7	0.78	2.66
MO2	–92.76435	39.02161	Golden Delicious		38	6	72	58	28	10	1.19	2.93
NC1	–78.49260	35.66980	Golden Delicious	2	114	7	90	58	46	13	1.43	2.90
NC2	–82.39420	35.29890	Golden Delicious	1	Tmtc	4	60	27	14	4	0.40	0.54
NC3	–82.39660	35.31320	Golden Delicious	1	12	3	45	22	13	3	0.35	0.82
NC4	–82.55888	35.42721	Golden Delicious	1	17	2	20	14	6	2	0.16	0.73
NY1	–77.02251	42.87385	Jonagold	2	70	6	72	53	34	10	1.13	2.60
NY2	–74.09064	41.67057	GoldRush	3	24	7	84	56	27	8	1.02	1.62
NY3	–77.06855	43.25849	Greening	1	16	7	90	33	29	9	1.22	1.25
OH1	–81.91842	40.77872	Golden Delicious	2	87	6	72	32	27	8	0.87	2.01
OH3	–82.53267	40.15306	GoldRush	2	88	6	72	30	16	8	0.86	1.97
OH4	–82.66374	40.05870	Prime Gold	1	57	4	60	27	23	6	0.65	1.64
PA1	–77.23092	39.97889	Ginger Gold	1	12	6	75	55	23	9	1.29	1.07
PA2	–77.24760	39.98757	NW Greening	3	38	4	50	23	22	7	0.85	1.56
TN1	–86.74932	36.06322	Golden Delicious	2	530	7	90	41	33	12	1.13	2.25
VA1	–78.89500	37.73052	Golden Delicious	1	47	5	60	32	27	5	0.53	0.15
VA2	–78.32941	38.49756	Golden Delicious	1	13	5	60	26	18	10	1.68	2.79
VA3	–78.15307	39.18531	Golden Delicious	1	37	4	50	36	14	4	0.47	0.58
VA5	–78.28537	39.11640	Granny Smith	2	76	5	60	48	21	6	0.62	2.06
WI1	–88.25142	42.57697	Golden Delicious	Nd	34	4	50	28	20	8	0.94	2.06

[r] Nine orchards in Iowa, Illinois, Missouri, and Wisconsin were sampled in 2000; 30 orchards in Georgia, Kentucky, Massachusetts, Michigan, New York, North Carolina, Ohio, Pennsylvania, Tennessee, and Virginia were sampled in 2005. Codes: states abbreviated as follows: Georgia = GA; Iowa = IA; Illinois = IL; Kentucky = KY; Massachusetts = MA; Michigan = MI; Missouri = MO; North Carolina = NC; New York = NY; Ohio = OH; Pennsylvania = PA; Tennessee = TN; Wisconsin = WI. Long. = longitude and Lat. = latitude (degrees).

[s] MI = Margalef's Index, derived using a combination of the number of species derived and the total number of individuals summed over all species (30). Higher numbers indicate greater diversity. SI = Shannon Index, which incorporates species richness with the estimated proportion of individuals of a given species to the total number of individuals in the orchard (relative abundance) (33). Higher numbers indicate greater diversity.

[t] Spray programs: 1 = fungicides sprayed on protectant schedule during fruit maturation period; 2 = no fungicides sprayed during the same period; 3 = organic orchard; Nd = information not determined.

[u] Mean colonies per apple; Tmtc = too many SBFS colonies to count. Therefore, number of colonies of each mycelial type was estimated visually as the percentage of fruit area covered by that mycelial type.

[v] Total number of SBFS mycelial types noted on 40 apple fruit per orchard.

[w] Number of attempted isolations.

[x] Number of successful isolations.

[y] Cultures sequenced: internal transcribed spacer (ITS) region of ribosomal DNA.

[z] Number of putative species per subsample of 10 to 15 apple fruit per orchard, based on ITS genotype and isolate morphology, then verified using modified Koch's postulates.

Analysis of LSU region. Putative species were taxonomically characterized by analyzing a portion of the LSU 28S region of rDNA. DNA was extracted from representative isolates of each putative species within each orchard (5,10). The primer pair used for amplification and sequencing was LROR/LR5 (43). Procedures described by Batzer et al. (5) were employed to amplify, purify, and quantify 518 polymerase chain reaction products (177 from the 2000 survey and 341 from the 2005 survey). Automated sequencing was performed at the ISU DNA Sequencing and Synthesis Facility. The length of partial LSU sequences was ≈850 bp, including gaps. Preliminary alignments were generated using CLUSTAL-X (42) with gap opening and gap extension parameters of 50:5, and these alignments were manually optimized using Bioedit (16). Taxa with identical LSU sequences were eliminated from the data block, reducing the number of SBFS taxa in the analysis to 60 sequences. Sequences of isolates from previously identified SBFS species (26) were included in the alignment.

Preliminary trees were generated that included all 60 SBFS taxa recovered from our surveys. To enhance readability of trees, the taxa were divided into two data sets of 24 and 36 putative species, respectively, and sequences from GenBank of related taxa were added to generate two trees. For the first data set, *Agaricus bisporus* (DQ071710) (a Basidiomycete) was chosen to be the outgroup, because preliminary analysis placed the SBFS taxa in two classes of Ascomycota (Sordariomycetes and Dothideomycetes). This alignment also contained *Capnodium coffea* (DQ247800) and several sequences of *Mycosphaerella* spp. that served to place the 36 putative SBFS species used in the second alignment to family, order, and subclass. The outgroup for the second data set was *Capnodium coffea*; this matrix contained the putative species that grouped with *Mycosphaerella* spp. in preliminary trees. For both trees, maximum parsimony analysis was performed using PAUP (40). Heuristic searches were conducted with random sequence addition and tree bisection-reconnection branch swapping algorithms, collapsing zero-length branches, and saving all minimal length trees. Maxtrees was set at 10,000. Alignable gaps were treated as a fifth base. All characters were given equal weight. To assess the robustness of clades and internal branches for data sets, a strict consensus of the most parsimonious trees was generated and a bootstrap analysis of 1,000 replications was performed.

Species diversity. After putative species were delineated based on ITS genotype and culture morphology, the number of species found at each of the 39 sites was determined. Two additional diversity indexes were calculated for each site: the Shannon index (33), a measure of proportional species abundance (30); and Margalef's richness index, which accounts for situations in which one or a few species are dominant and the rest are rare (30).

To estimate relative abundance of each putative species in an orchard, the number of colonies identified as belonging to a particular putative species was multiplied by the proportion of colonies of each corresponding mycelial type. For apple fruit that had too many colonies to count individually, the proportion of each mycelial type was estimated visually. A spreadsheet was constructed to determine frequency with which a given mycelial type was associated with more than one putative species in each sampled orchard. Based on mycelial type identification and sequence analysis, 78% of mycelial types in an orchard were associated with a single putative species. In the 22% of instances where a mycelial type was associated with >1 putative species in a sampled orchard, relative abundance of each species was determined by multiplying the percentage of isolates in the sample with a given mycelial type by the proportion of each putative species exhibiting that mycelial type.

RESULTS

Isolation and species designation. In total, 60 putative species of the SBFS complex were delineated using ITS sequences and morphological characteristics on apple and in culture (5,10); 30 of these species were found in the 2000 survey and 30 additional species were isolated in the 2005 survey. Previously reported SBFS complex members that were also found included *Schizothyrium pomi, Peltaster fructicola*, *Geastrumia polystigmatis*, and *Stomiopeltis versicolor* (23,25,26,38,46). However, no isolates of *L. elatius* were recovered in the survey. Furthermore, no isolates resembled the description of *Gloeodes pomigena* made by Colby in 1920 (9). The survey results and the process for identifying putative species in the SBFS complex for each orchard are summarized in Table 1. The estimated mean number of SBFS colonies per apple was 180. The number of mycelial types per apple was 2 to 7. Of 2,516 attempts to isolate pure cultures from signs on apple, ≈50% were successful. Success of isolation was 17 to 96% per orchard, depending on culturability of individual species and the condition of the fruit when isolations were attempted. Of the 68 genotypes determined from parsimony analysis of the ITS sequences obtained from pure cultures, 88% (60 putative species) were verified as members of the SBFS complex using the modified Koch's postulates procedures.

Phylogenetic placement of putative species. The first LSU data set contained 51 taxa (including the outgroup taxon *A. bisporus*) and 824 characters. Of these characters, 286 were parsimony informative, 111 were variable and parsimony uninformative, and 427 were constant. Maximum parsimony analysis of the LSU sequences resulted in 546 equally informative trees, one of which is shown in Figure 2. Parsimony analysis grouped two putative species, *Phialophora sessilis* and Yeast sp. UI-10, within the Sordariomycetes (29). The remaining 58 taxa were classified as Dothideomycetes (28) (Figs. 2 and 3). Although four putative species, Sterile mycelia sp. FG6, *Geastrumia polystigmatis*, *Ramularia* sp. CS2, and *Sybren* sp. CS1, fell within the Dothideomycetes, they could not be placed to order (Fig. 2). There was strong support (82% bootstrap support) for placing the remaining 55 SBFS taxa in the Dothideomycetidae subclass (Fig. 2). Three isolates of Sterile mycelia sp. UI-6 from Pennsylvania and New York grouped with *Myriangium duriaei* in the Myrangiales.

Parsimony analysis grouped 53 putative species within the Capnodiales (32) with bootstrap value of 100% (Figs. 2 and 3). Within the Capnodiales, the five *Peltaster* spp. formed a strongly supported clade (100% bootstrap support) but this genus did not group with other taxa at the family level (Fig. 2). The remaining 48 putative species were grouped with 98% bootstrap support. These taxa segregated into three distinct clades: Teratosphaeriaceae, Schizothyriaceae, and Mycosphaerellaceae (Figs. 2 and 3). Five putative species, including Sterile mycelia sp. MB1 and *Pseudocercospora* spp. FS4, FG1.1, FG1.9, and FG1.2, resided in the Teratosphaeriaceae with 89% bootstrap support. Parsimony analysis also grouped two sister clades (76% bootstrap support) that included two families, the Schizothyriaceae (96% bootstrap support) and the Mycosphaerellaceae (54% bootstrap support) (Fig. 2). Six putative species in the Schizothyriaceae had *Zygophiala* anamorphs; *Pseudocercospora* sp. FS5 differed from *Zygophiala* in conidia morphology but produced the flyspeck mycelial type on apple fruit.

Over half (36 of 60) of the putative species grouped in the Mycosphaerellaceae and were included in the second alignment (Fig. 3). This LSU alignment contained 50 taxa (including the outgroup taxon *Capnodium coffea*), and 842 characters were used for the analyses. Of these, 149 characters were parsimony informative, 105 characters were variable and parsimony uninformative, and 588 were constant. Maximum parsimony analysis of the LSU sequences resulted in 383 equally parsimonious informative trees, one of which is shown in Figure 3.

Seven putative species formed a strongly supported clade (98% bootstrap) with *Dissoconium aciculare* and *D. commune*. Four of these putative species produced fuliginous signs on apple and three species produced the discrete speck mycelial type. Fourteen

putative species from the surveys formed a strongly supported clade (84%) with two previously identified species of *Stomiopeltis*. With the exception of *Geastrumia polystigmatis* and Sterile mycelia sp. RS6, the *Stomiopeltis* clade contained all of the ramose mycelial types identified in this survey. Seven putative species in the *Stomiopeltis* clade produced anamorphs in culture, including *Phaeothecoidiella* spp. P3 and P4; *Houjia* spp. FG7.1, FG7.2, FG7.3; *Sporidesmajora* FG7.4; and *Passalora* sp. FG3, and these species exhibited either punctate or fuliginous mycelial types on apple. The remaining 15 putative species were grouped with six *Mycosphaerella* spp. (59% bootstrap support) whose LSU sequences were obtained from GenBank. Parsimony analysis grouped *Ramularia* sp. P5 with *M. punctiformis* with 94% bootstrap support. *Pseudocercospora* spp. LLS1 and LLS2 were

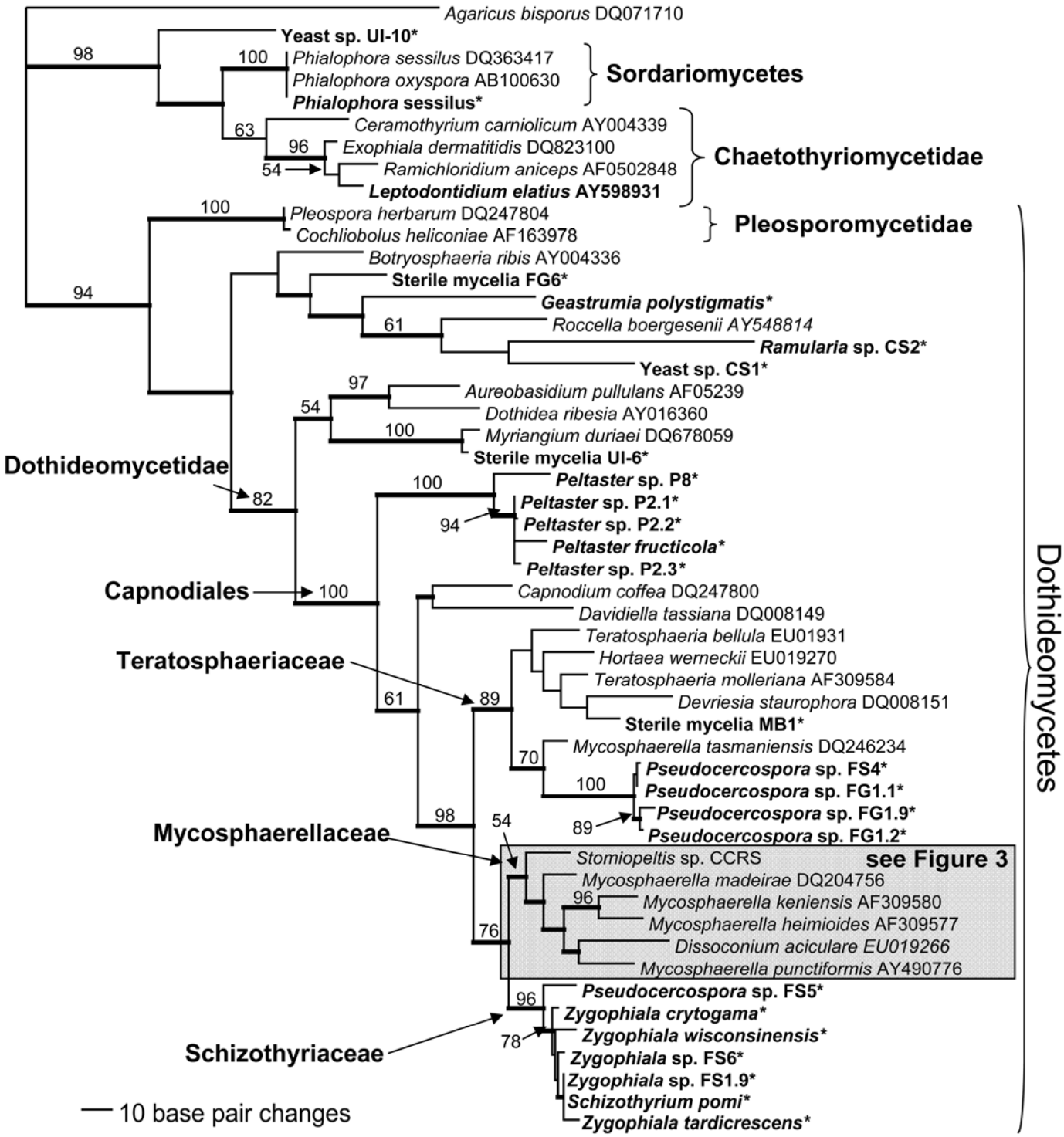

Fig. 2. One of 546 most parsimonious trees determined from partial large subunit sequences (874 bp) obtained from sooty blotch and flyspeck (SBFS) isolates on apple from eastern and midwestern U.S. orchards. One isolate for each putative species of SBFS is included, except for some of the SBFS species in Figure 3. Putative species denoted in bold have been documented to cause SBFS on apple; those also denoted with asterisks were isolated during the 2000 and 2005 surveys. Gaps were treated as a fifth base and 47 characters were excluded from the data set. Parsimony informative characters = 273. Bootstrap values >50 derived from 1,000 replications are shown and branches in bold are derived from strict consensus of most parsimonious trees. Tree length =1,614; consistency index (CI) = 0.4399; homoplasy index (HI) = 0.5601; retention index (RI) = 0.7135. The tree is rooted to an *Agaricus bisporus* sequence (DQ071710) obtained from GenBank. The shaded box denotes the branch that includes most of the SBFS complex groups and is presented in greater detail in Figure 3.

grouped with a strain of *Pseudocercospora* isolated from eucalyptus leaves in Thailand (94% bootstrap support); and *Colletogloeum* sp. FG2 was grouped with *M. marksii* (79% bootstrap support). Three species having *Ramichloridium* anamorphs formed an unsupported cluster with *M. madeirae* and *Ramichloridium* sp. FG9 grouped with *Ramichloridium cerophilum* with 100% bootstrap support. Moreover, strict consensus grouped eight putative species in the *Pseudocercosporella* clade.

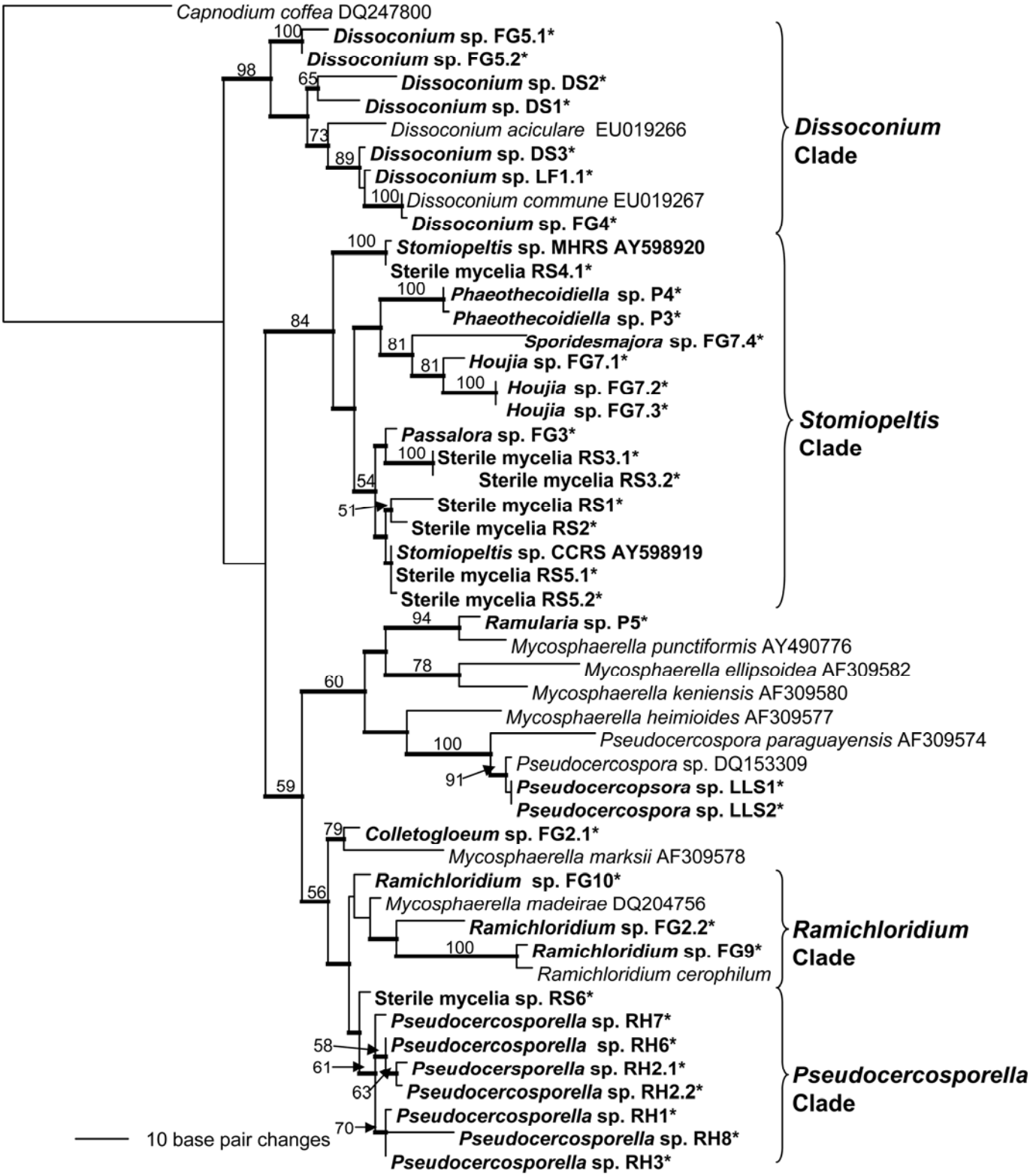

Fig. 3. One of 383 most parsimonious trees determined from partial large subunit sequences (842 bp) obtained from sooty blotch and flyspeck (SBFS) isolates on apple from eastern and midwestern U.S. orchards that formed a well-supported clade in Figure 2. One isolate for each putative species of SBFS that groups with the Mycosphaerellaceae is included. Putative species denoted in bold have been documented to cause SBFS on apple; those also denoted with asterisks were isolated during the 2000 and 2005 surveys. Gaps were treated as a fifth base. Parsimony informative characters = 149. Bootstrap values >50 derived from 1,000 replications are shown and branches in bold are derived from strict consensus of 383 most parsimonious trees. Tree length =581; consistency index (CI) = 0.5938; homoplasy index (HI) = 0.4062; retention index (RI) = 0.7837. The tree is rooted to a *Capnodium coffea* sequence (DQ247800).

Sterilemycelia sp. RS6 was basal to this clade and, in addition to the lack of conidia in culture, produced a ramose mycelial type on apple (Table 2). The remaining seven *Pseudocercosporella* spp. were grouped with 61% bootstrap support and exhibited the ridged honeycomb mycelial type on apple (Fig. 3).

Species prevalence and diversity. The most prevalent SBFS species in the 2000 and 2005 surveys were *Schizothyrium pomi,*

TABLE 2. Putative species of sooty blotch and flyspeck (SBFS) fungi, mycelial type on apple, prevalence, number of isolates, representative strain, and accession numbers from surveys of 39 apple orchards in the eastern and midwestern United States in 2000 and 2005

Putative species[t]	Mycelial type[u]	Prevalence[v]	Isolates[w]	Representative strain	Accession numbers		
					ITS[x]	LSU[y]	CBS[z]
Colletogloeum sp. FG2.1	Fuliginous	11	32	NY1 3.2F1c	FJ425193	FJ031986	CBS 125300
Dissoconium aciculare (DS1)	Discrete speck	10	16	MSTB4b	AY598874	AY598912	CBS 118967
Dissoconium sp. DS2	Discrete speck	1	2	MWB6	na	AY598914	CBS 118948
Dissoconium sp. DS3	Discrete speck	2	4	GA2 38A1a	FJ425203	FJ147152	CBS 125301
Dissoconium sp. FG5.1	Fuliginous	4	7	UIF3	AY598877	AY598916	CBS 118961
Dissoconium sp. FG5.2	Fuliginous	1	2	MI3 34F1a	FJ425205	FJ147154	CBS 125648
Dissoconium sp. LF1.1	Fuliginous	3	5	OH3 37E1d	FJ425204	FJ147153	CBS 125302
Dissoconium commune (FG4)	Fuliginous	5	9	MSTF2	AY598876	AY598915	CBS 118962
Geastrumia polystigmatis	Ramose	24	131	NC4 1.8F1a	FJ438389	FJ147177	CBS 125303
Houjia pomigena (FG7.1)	Fuliginous	1	1	UIF2b	AY598885	AY598925	CBS 125224
Houjia sp. FG7.2	Fuliginous	1	1	KY3 13F1d	FJ438377	FJ147165	CBS 125228
Houjia yanglingensis (FG7.3)	Fuliginous	1	1	TN1 2.2F1d	FJ438378	FJ147166	CBS 125227
Passalora sp. FG3	Fuliginous	2	6	GTF3a	na	AY598926	CBS 118964
Peltaster sp. P2.1	Punctate	3	4	GTE9a	AY598888	AY598929	CBS 119464
Peltaster sp. P2.2	Punctate	5	9	UIE11b	AY598930	AY588930	CBS 118953
Peltaster sp. P2.3	Punctate	1	1	KY2 16E1b	FJ438383	FJ147170	CBS 125649
Peltaster sp. P8	Punctate	2	3	KY3 8E1a	FJ438384	FJ147171	CBS 125304
Peltaster fructicola	Punctate	29	121	KY1 12.2E2b	FJ438382	AY598928	CBS 125304
Phaeothecoidiella missouriensis (P3)	Punctate	1	2	AHE7c	AY598878	AY598917	CBS 118959
P. illinoisensis (P4)	Punctate	2	3	UIE3	AY598879	AY598918	CBS 118947
Phialophora sessilis	Punctate	5	6	NY2 4C1a	FJ438386	FJ147173	CBS 125306
Pseudocercospora sp. FG1.1	Fuliginous	9	25	MA2 5F1b	FJ438380	FJ147168	CBS 125307
Pseudocercospora sp. FG1.2	Fuliginous	1	1	MSTF5a	na	AY598899	CBS 118954
Pseudocercospora sp. FG1.9	Fuliginous	2	2	MA2 3.5F1c	FJ438381	FJ147169	CBS 125308
Pseudocercospora sp. FS4	Flyspeck	1	2	MWA4b	AY598857	AY598900	CBS 118945
Pseudocercospora sp. FS5	Flyspeck	1	3	MA3 1.3B1a	FJ438371	FJ355914	CBS 125309
Pseudocercospora sp. LLS1	Fuliginous	2	3	NC1 22F2d	FJ425192	FJ025897	na
Pseudocercospora sp. LLS2	Fuliginous	1	1	KY3 22D1b	EU605812	FJ025898	CBS 125650
Pseudocercosporella sp. RH1	Ridged honeycomb	23	60	KY3 20D1a	FJ425195	FJ031988	CBS 124417
Pseudocercosporella sp. RH3	Ridged honeycomb	12	17	OH1 34D2a	FJ425196	FJ031989	CBS 125651
Pseudocercosporella sp. RH2.1	Ridged honeycomb	4	5	UMD1a	AY598866	AY598902	CBS119462
Pseudocercosporella sp. RH2.2	Ridged honeycomb	10	16	PA1 31D1a	FJ425197	FJ031990	CBS 125652
Pseudocercosporella sp. RH6	Ridged honeycomb	1	2	MI3 20F1a	FJ425201	FJ031994	CBS 125653
Pseudocercosporella sp. RH7	Ridged honeycomb	1	1	GA3 3D1b	FJ425202	FJ031995	CBS 125654
Pseudocercosporella sp. RH8	Ridged honeycomb	1	1	NY1 3.2D1b	na	FJ147151	CBS 125655
Ramichloridium sp. FG2.2	Fuliginous	2	5	GA3 25G1c	FJ425194	FJ031987	CBS 125656
Ramichloridium sp. FG9	Fuliginous	4	8	NC1 3F1a	FJ425199	FJ031992	na
Ramichloridium sp. FG10	Fuliginous	3	4	TN1 1.3F1a	FJ425200	FJ031993	CBS125310
Ramularia sp. P5	Punctate	3	3	UME2	AY598873	AY598910	CBS 119227
Ramularia sp. CS2	Compact speck	6	11	OH3 9H1c	FJ438390	FJ147176	CBS125311
Schizothyrium pomi	Flyspeck	38	108	VA1 7A1d	FJ425206	FJ147155	CBS125312
Sporidesmajora pennsylvaniensis (FG7.4)	Fuliginous	1	1	PA1 9F1a	FJ438379	FJ147167	CBS125229
Sterile mycelia sp. MB1	Ramose	2	2	VA1 29D1c	na	FJ355913	CBS125313
Sterile mycelia sp. RS1	Ramose	4	4	PEC6a	AY598882	AY598921	CBS118955
Sterile mycelia sp. RS2	Ramose	3	7	AHC3a	AY598883	AY598922	CBS119228
Sterile mycelia sp. RS3.1	Ramose	2	2	MI3 24F1a	FJ438372	FJ147160	CBS 125657
Sterile mycelia sp. RS3.2	Ramose	1	1	KY4 11.2F2b	FJ438373	FJ147161	na
Sterile mycelia sp. RS4.1	Ramose	4	13	TN1 6.3E2a	FJ438374	FJ147162	CBS125314
Sterile mycelia sp. RS6	Ramose	3	12	OH1 6C1b	FJ425198	FJ031991	CBS125315
Sterile mycelia sp. FG6	Fuliginous	1	1	MWF4b	na	AY598924	CBS 125418
Sterile mycelia sp. UI-6	Punctate	2	3	PA1 3E1d	FJ438385	FJ147172	CBS125316
Sterile mycelia sp. RS5.2 (*Stomiopeltis)*	Ramose	5	28	NC1 18C1d	FJ438376	FJ147164	CBS125317
Sterile mycelia sp. RS5.1 (*Stomiopeltis)*	Ramose	2	2	GA3 23C2b	FJ438375	FJ147163	na
Sybren sp. CS1	Compact speck	14	47	KY3 15E1b	FJ438388	FJ147175	CBS 125660
Yeast sp. UI-10	Punctate	1	2	MI2 34E2b	FJ438387	FJ147174	CBS 125661
Zygophiala sp. FS1.9	Flyspeck	4	4	NY3 16A1a	FJ425207	FJ147156	CBS125318
Zygophiala cryptogama	Flyspeck	5	14	OH4 1A1a	FJ425208	FJ147157	CBS 125658
Zygophiala sp. FS6	Flyspeck	3	6	KY4 17.2A1a	na	FJ147159	CBS125319
Zygophiala tardicrescens	Flyspeck	1	4	MWA1a	AY598856	EF164901	CBS 118946
Zygophiala wisconsinensis	Flyspeck	5	11	OH4 9A1c	FJ425209	FJ147158	CBS 125659

[t] SBFS species recovered from 2000 and 2005 surveys of 39 orchards in 14 U.S. states. Identity of each species was verified with modified Koch's postulates (5).
[u] Mycelial type on apple: appearance of colonies on apple peel (5).
[v] Number of orchards in which detected out of 39 orchards surveyed.
[w] Number of pure isolates with internal transcribed spacer (ITS) sequences; na = not available.
[x] GenBank accession numbers for the ITS region of the ribosomal DNA (rDNA) sequence.
[y] GenBank accession numbers for a portion of the large subunit (LSU) of the rDNA sequence.
[z] Accession numbers of strains deposited at the Centraalbureau voor Schimmelcultures (CBS), The Netherlands.

Peltaster fructicola, and *Pseudocercosporella* sp. RH1, each of which occurred in more than half of the orchards sampled (Table 2). *Geastrumia polystigmatis* was one of the most prevalent species in the 2005 survey but was not found in the 2000 survey. Only 9 of the 60 species (15%) occurred in >10 orchards, whereas 41 species (70%) each occurred in <4 orchards, 30 species (50%) occurred in 2 orchards, and 19 species (31.7%) were found in only a single orchard.

The number of species per orchard varied from 2 to 15, with a mean of 7.7 (Table 1). Number of SBFS species and values of diversity indexes were significantly ($P < 0.0001$) lower in orchards that had received synthetic chemical fungicide sprays on a protectant schedule during the fruit maturation period than in orchards that had not received fungicide sprays during this period. No diversity comparisons were made with orchards that had received sprays of organically certified fungicides during the fruit maturation period due to the small sample size (two orchards), and fungicide-spray records could not be obtained for 2 of the 39 orchards sampled.

For the entire data set, orchards located west of longitude 84.6°W (west of the Appalachian Mountains) had significantly ($P = 0.0171$) more SBFS species per orchard and higher values of Shannon and Margalef indexes ($P = 0.0015$ and 0.0512, respectively) than orchards east of that meridian. Unsprayed western orchards had an average of 11.0 species whereas unsprayed eastern orchards averaged 8.6 species. Orchards south of latitude 39.5°N did not differ significantly from orchards to the north of this parallel in number of species or Shannon and Margalef index values ($P = 0.2931$, 0.6010, and 0.7924, respectively). Mean number of species per orchard was 7.2 in the north compared with 8.3 in the south, and unsprayed northern orchards had an average of 9.6 species compared with 11.0 species in southern orchards.

These regions were then further subdivided by the same latitude and longitude lines into four quadrants designated as South Central (SC), North Central (NC), Southeast (SE), and Northeast (NE) (Table 3). Results of *F* tests comparing all orchards in the four geographic regions differed significantly for number of species as well as values of Shannon and Margalef indexes ($P = 0.0351$, 0.0086, and 0.0007, respectively). The SC region had the most species per orchard, whereas the SC and NC regions had the highest Shannon Index values (Table 3). The average Margalef Index value was significantly higher in the SC than the SE region. When comparing only orchards that had been sprayed with fungicides, however, the number of SBFS species per orchard did not differ among the four regions.

Biogeography. Several SBFS species, including *Schizothyrium pomi*, *Peltaster fructicola*, and *Pseudocercosporella* sp. RH1, were nearly ubiquitous throughout the area of the two surveys (Fig. 4). Species in the same genus sometimes had sharply different geographic ranges. For example, *Zygophiala cryptogama* did not occur outside the Midwest and Upper South (Tennessee and Kentucky), *Peltaster* sp. P2.2 had a similar distribution, and *Pseudocercosporella* sp. RH3 was not found in the Upper Midwest (M. M. Díaz Arias, *unpublished data*). Examples of other species that were found only within restricted geographic regions included *Stomiopeltis* sp. RS5.2 (southern states only), *Phialophora sessilis*, *Geastrumia polystigmatis* (Southeast, Mid-Atlantic, and Northeast), *Ramularia* sp. P5 (Ohio River Valley to New England), *Sybren* sp. CS1 (all regions except New England), and *Colletogloeum* sp. FG2.1 (Midwest and Upper South) (Fig. 5). It was not possible to generalize about geographic distribution of SBFS species that were found in only one or two orchards.

DISCUSSION

The results document that the SBFS complex is far more taxonomically diverse than previously recognized. Using molecular genetic analysis in combination with morphological characterization, we documented 60 putative species in the eastern half of the United States alone, compared with 4 that had been identified previously using morphological criteria (46). The 2005 survey doubled the number of SBFS species from the 30 previously documented in the 2000 survey (5). More intensive surveys of U.S. orchards are likely to reveal additional SBFS species (12, 34), and surveys in other countries indicate that the worldwide SBFS complex is substantially more diverse than the assemblage documented in the United States (4,36,37).

Several consistent patterns are evident in SBFS taxonomic diversity. Most U.S. species of SBFS are in the order Capnodiales, with some widely occurring exceptions, including *Geastrumia polystigmatis* and *Sybren* sp. CS1. Many of the putative species recovered in the 2005 survey were in the same genera as those from the 2000 survey. Furthermore, preliminary surveys in China and Europe have found some of the same or closely related species, based on ITS sequences, as those reported from the United States (4,22,35). This suggests that the majority of the SBFS worldwide complex can be expected to be Capnodiales.

Our findings offer strong support for revising the long-standing perception of SBFS as two distinct diseases, "sooty blotch" and "flyspeck," each caused by one or a few species of fungi (39,46). It is now evident that SBFS is caused by a highly diverse assemblage of fungi, with as many as 20 species sharing a single mycelial type on apple fruit. Although mycelial type appears to be a consistent character for each species based on current evidence, the seven recognized mycelial types constitute a continuum between so-called sooty blotch and flyspeck morphologies rather than two distinct groups (5). Furthermore, although the ecology and epidemiology of most SBFS species has not been studied,

TABLE 3. Biodiversity of sooty blotch and flyspeck fungi in apple orchards in four subregions of eastern North America[v]

	Average no. of species per orchard[x]			Average Shannon Index[y]			Average Margalef Index[z]		
Region[w]	All	Spray	No spray	All	Spray	No spray	All	Spray	No spray
SC	10.86 a	6.00	11.67 a	2.48 a	1.43	2.60 a	1.22 a	0.71	1.31 a
NC	7.75 b	6.00	9.67 ab	2.38 a	2.02	2.75 a	0.96 ab	0.69	1.28 a
SE	6.64 b	6.00	9.50 ab	1.62 b	1.42	2.48 ab	0.80 b	0.76	1.02 b
NE	6.85 b	6.00	8.67 b	1.55 b	1.30	1.95 b	0.86 ab	0.80	0.95 b
LSD	2.74	…	2.59	0.71	…	0.63	0.38	…	0.25

[v] Orchards were divided on the basis of spray programs: All = all 39 orchards regardless of spray program; Spray = fungicides sprayed during fruit maturation period; No spray = no fungicides sprayed during this period. Numbers in a column followed by the same letter are not significantly different (least significant difference [LSD], $P < 0.05$). An *F* test showed no significant differences among regions for orchards sprayed with fungicides.

[w] Orchards were grouped into four regions, divided by longitude 84.6°W and latitude 39.5°N: South Central (SC) (orchards IL2, IL3, KY1,KY2, KY3, MO2, and TN1), Southeast (SE) (orchards GA2, GA3, KY4, NC1, NC2, NC3, NC4, VA1, VA2, VA3, and VA5), North Central (NC) (orchards IA1, IA2, IA3, IL1, MI2, MI3, MO1, and WI1), and Northeast (NE) (orchards MA1, MA2, MA3, MA4, MA5, NY1, NY2, NY3, OH1, OH3, OH4, PA1, and PA2).

[x] Number of putative species based on internal transcribed spacer genotype and isolate morphology, then verified using modified Koch's postulates.

[y] Shannon Index incorporates species richness with the estimated proportion of individuals of a given species to the total number of individuals in the orchard (relative abundance) (33).

[z] Margalef Index is derived using a combination of the number of species derived and the total number of individuals summed over all species (30).

some are distinct from each other in temperature tolerance, nutritional response, fungicide sensitivity, phenology, and tendency to be dislodged from the apple surface by postharvest dip treatments followed by brushing (6,19,24,27,34,41,47). To achieve more efficient management of SBFS on apple fruit, it may be necessary to account for interspecies differences in environmental biology and discard the outmoded two-disease paradigm.

This report is the first to describe patterns of SBFS species diversity in and among orchards. In previous studies, knowledge of species diversity was constrained by inability to conclusively identify some of the component species. Our findings regarding the impact of fungicide use on species diversity are robust because the differences were statistically significant for all three diversity indexes. Fungicide sensitivity can vary at least 20-fold among SBFS species (41); therefore, SBFS species that are relatively sensitive to commonly used fungicides may be rare or absent in regularly sprayed orchards. Indirect evidence supporting this idea is the fact that unsprayed orchards were the source of 13 of the 19 SBFS species (68%) that were found in only a single orchard; in other words, rare species occurred primarily in unsprayed orchards.

Although higher SBFS diversity was found in western than eastern orchards, the reasons for this difference are unclear. More intensive regional surveys, incorporating larger numbers of sprayed and unsprayed orchards, will be needed to clarify possible regional patterns of SBFS biodiversity.

Our study is also the first to clearly describe biogeographic patterns of individual SBFS species occurrence on a regional scale. Hints of these patterns were evident in results of previous surveys (26,38) but combining morphological and genetic data allowed us to identify a larger segment of the SBFS complex. In our surveys, certain species were cosmopolitan throughout much of the eastern half of the United States, including *Schizothyrium pomi* and *Peltaster fructicola.* Johnson et al. (26) also found that *Peltaster fructicola* occurred widely in the southeastern, midwestern, and northeastern United States. In contrast, other species in our surveys were regionally restricted in geographic range (e.g., *Geastrumia polystigmatis* and *Colletogloeum* sp. FG2.1). The factors determining region-specific geographic patterns are uncertain. One possible contributing factor could be interspecific differences in temperature tolerance. The fact that SBFS historically has been uncommon in the northern margin of the apple-growing region of northeastern North America—central and

Fig. 4. Distribution of **A, B, and C,** cosmopolitan; and **D, E, and F,** regionally restricted putative species within the same genera of the sooty blotch and flyspeck (SBFS) complex, based on results of surveys of 39 orchards in 2000 and 2005. **A,** *Schizothyrium pomi*; **B,** *Peltaster fructicola*; **C,** *Pseudocercosporella* sp. RH1; **D,** *Zygophiala cryptogama*; **E,** *Peltaster* sp. P2.2; and **F,** *Pseudocercosporella* sp. RH3.

Fig. 5. Distribution of six regionally-limited putative species in the sooty blotch and flyspeck (SBFS) complex, based on results of surveys of 39 orchards in 2000 and 2005. **A,** *Sybren* sp. CS1; **B,** *Geastrumia polystigmatis*; **C,** *Stomiopeltis* sp. RS5.2; **D,** *Ramularia* sp. CS2; **E,** *Colletogloeum* sp. FG2.1; and **F,** *Phialophora sessilis.*

northern portions of Minnesota, Wisconsin, Michigan, New York, and northern New England—despite adequately warm and moist summer conditions for SBFS development suggests either inability of SBFS species to survive extremely cold winter temperatures or a growing season that is too short to produce visible SBFS colonies. Regional differences in the assemblage of reservoir host species surrounding orchards could also potentially impact SBFS species distribution. The present study provides a foundation for future research by establishing that some SBFS species display marked patterns of geographic distribution.

By combining morphological characterization with genetic analysis tools, it should now be possible to pinpoint which SBFS species pose the greatest economic threat in each region, and then to focus epidemiological studies on these key species. Using specific primers and RFLPs, for example, phenological studies of the SBFS assemblage in six Iowa orchards showed that the same two species were the first to appear on apple fruit in all the surveyed orchards (34). Because disease-warning systems for SBFS (7,11) are built around timing fungicide sprays to avoid outbreaks of these early-appearing species, identifying them is likely to be an important step toward achieving more cost-effective disease management.

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