SUMMARY

Sooty blotch and flyspeck (SBFS) is a late season disease complex of apple and pear caused by epiphytic fungi that blemish the fruit surface, resulting in economic losses in humid regions worldwide. The SBFS disease complex includes more than 60 species of fungi in eastern North America, but species in many other parts of the world have not been identified. From August to October 2007, SBFS-infested apple fruits were collected from 14 orchards in central and southern Serbia and Montenegro. Colony morphology and sequences of the internally transcribed spacer (ITS) regions were assessed for 92 SBFS isolates and compared to previously identified species. From the four SBFS genera that were found in Serbia and Montenegro – Pseudocercosporella, Schizothyrium, Peltaster and Pseudocercospora – five putative species were identified. Most (72 of 92) isolates were Pseudocercosporella spp. All of these species have also been isolated from infested apples and described in North America or Germany.

Keywords: epiphytic fungi, diversity, characterization, morphology.

INTRODUCTION

Fungi in the sooty blotch and flyspeck (SBFS) disease complex colonise the cuticle of apple fruit (Malus domestica Borkh.) in regions with moist climates, during the mid- to late growing season. These fungi grow superficially on the epicuticular wax of the fruit, do not penetrate the cuticle (Belding, 2000) and may utilize exuded nutrients present on the apple surface (Wrona, 2004; Wrona and Gleason, 2005; Le Corronc et al., 2006). The term “sooty blotch” designates fungi that form a dark mycelial mat with or without sclerotium-like bodies, whereas “flyspeck” denotes colonies that develop clusters of shiny, black, round to ovoid, sclerotium-like bodies and have no visible mycelial mat (Batzer et al., 2008). Schizothyrium pomi (Mont. & Fr.) Arx has been described as the cause of flyspeck (Baines, 1940; Baker, 1977; Batzer et al., 2008). Recently, two additional mycelial types of SBFS fungi, referred to as compact speck and discrete speck, have been described; they closely resemble flyspeck but can be distinguished from it by the absence of ring-like remnants of the sclerotium-like bodies on the apple cuticle when the bodies are removed, or by size and density of sclerotium-like bodies (Batzer et al., 2005).

Sooty blotch and flyspeck initially were described as having a single causal agent, Dothidea pomigena Schwein. (Schweinitz, 1832). Colby (1920) determined that sooty blotch and flyspeck were caused respectively by Gloeodes pomigena (Schwein.) Colby and Schizothyrium pomi. In the past 10 years, the SBFS disease complex has been further expanded to include more than 50 species from 11 anamorph genera, based on molecular and morphological evidence (Johnson and Sutton, 1994; Johnson et al., 1996; Johnson et al., 1997; Batzer et al., 2005, 2008; Diaz Arias, 2007). Among SBFS species from North America, mycelial growth varied more than 10-fold on water agar amended with the fungicides thiophanate-methyl or ziram (Tarnowski et al., 2003). Growth rates also differed substantially among putative species in response to nutrient composition of media and temperature (van de Voort et al., 2003; Hernández et al., 2004; LeCorronc et al., 2006). Understanding the biology, ecology and geographic range of each species in the SBFS disease complex should set the stage for developing more effective strategies against this group of pathogens.

In order to reach this goal, it is essential to know which species are present, where each predominates, and how each responds to environmental conditions. So far there have been no reports or publications on the SBFS disease complex from southern Europe. The aim of this study was to identify and describe species in the SBFS disease complex from Serbia and Montenegro based on the internal transcribed spacer region of the rDNA and isolate morphology.

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MATERIALS AND METHODS

Origin of isolates. Apples showing SBFS symptoms were harvested from the end of August to October 2007. Although most of the 14 orchards surveyed were in central Serbia, two were in southern Serbia and two in Montenegro. Apples were rinsed for 30 min in tap water and allowed to dry in a transfer hood. Approximately three colonies were selected arbitrarily from each of three apples sampled from each orchard, transferred aseptically to water agar acidified with a post-autoclave addition of 40 drops of 50% lactic acid per litre (AWA) and incubated at 21-24°C under ambient light. As mycelial growth became visible after 1 to 3 weeks, isolates were transferred to potato dextrose agar (PDA) (Difco, USA). Isolates chosen for additional study were further purified using antibiotic-amended PDA (ABPDA) (post-autoclave addition of 1 ml of 50% lactic acid per liter, 0.15 g streptomycin sulfate, 0.15 g tetracycline hydrochloride, and 18 drops of Tergitol surfactant). A total of 109 isolates were purified and stored in cryotube vials in 15% glycerol at -80°C. Segments of apple peels containing the same colony from which isolates were made were also preserved by pressing the thallus and supporting peel between paper towels until they were dry.

Morphology of SBFS isolates on apple and in vitro. Signs of SBFS preserved on apple peels were described, and representative isolates from each orchard were grown on 1.5% malt extract agar (MEA) and PDA. After 1 month of growth on PDA at 21 to 24°C under intermittent ambient light, colony texture, margins and pigments diffusing into agar were described. Colour of the top and bottom sides of each colony was described using standard colour plates (Ridgway, 1912), and these surfaces were photographed. Diameter growth of colonies was determined on MEA; 5-mm-diameter plugs from 3-week-old colonies on MEA were placed on three plates (three plugs per plate) and incubated upside down at 25°C in darkness. A Watchdog temperature sensor (Spectrum Technologies, USA) was placed in the incubator to monitor temperature. After 14 and 28 days, two perpendicular measurements of each colony diameter were made, and average diameter for each isolate was recorded (Batzer et al., 2005). In order to describe fungal structures, pieces of cellulose membrane (Flexel Inc., Covington, Indiana, USA) were washed, autoclaved and placed on CLA plates (Fischer et al., 1982). Mycelial plugs were transferred to the edge of the cellulose pieces and hyphae were allowed to grow over the cellulose. When fungal structures were evident, the cellulose pieces were transferred from the agar, mounted on glass slides, and examined under a compound microscope.

PCR and sequencing. The ITS region (ITS1, 5.8S rDNA gene, ITS2) of ribosomal DNA was sequenced for each isolate. Isolates with identical ITS sequences were grouped. Template DNA for polymerase chain reaction (PCR) was extracted from mycelia using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, USA). Primer pairs used for amplification and sequencing of the ITS region were ITS-1F (5'- CTTGGTCATT-TAGAGGAAGTAA-3') and ITS4 (5'- TCCTCCGCT-TATTGATATGC-3') (White et al., 1990). The PCR reactions, purifications, and sequencing of products were performed as described in Batzer et al. (2005).

Sequence alignment and parsimony analysis. Sequence alignment and maximum parsimony analysis was conducted as described by Batzer et al. (2005). Because numerous large insertion/deletions in the ITS dataset prevented unambiguous alignment of all sequences, ITS sequences of taxa within the same genus were grouped into separate alignments and analyzed independently to delineate species. Alignable gaps were treated as a “fifth base”. To assess the robustness of clades and internal branches, a strict consensus of the most parsimonious trees was generated and a bootstrap analysis of 1000 replications was performed for each of four ITS data sets.

Putative species designation. Classification of each anamorph taxon was based on the following: Pseudocercosporella sp. based on Arx (1983), Pseudocercosporella sp. based on Braun (1994), Zygothiala sp. based on Batzer et al. (2008), and Peltaster sp. based on Williamson et al. (2004). Isolates were grouped into putative species based on ITS parsimony analysis, conidial characters and colony morphology on apple peels and artificial media. However, anamorph designations for Pseudocercosporella and Pseudocercosporella are provisional and additional taxonomic studies are underway (J.C. Batzer, unpublished data). Putative species within each genus were assigned mycelial types based on their appearance on apple, including ridged honeycomb (RH), flyspeck (FS), punctate (P) and fuliginous (FG) (Fig. 1) (Colby, 1920; Groves, 1933; Batzer et al., 2005). Letter designations of putative species were followed by numbers based on unique ITS sequences and phenotypic differences in culture.

RESULTS

Five putative species were delineated based on ITS sequences and morphological characters. In our survey Pseudocercosporella sp. RH1 was the predominant species, comprising 66 of 92 isolates (Table 1), and occurring in 13 of 14 orchards surveyed (Table 2). Redundant sequences were eliminated from the alignment and
Fig. 1. Morphology of sooty blotch and flyspeck fungi from Serbia and Montenegro. Columns depict mycelial types on apple peel in overview (A, E, I, M, and Q) and close-up view (B, F, J, N, and R), colonies on PDA after 4 weeks (C, G, K, O and S), and conidiphores and conidia (D, H, L, P and T). Rows of images show Pseudocercosporella sp. RH1 (1), Pseudocercosporella sp. RH3 (2), Schizothyrium pomi (3), Peltaster fructicola (4), and Pseudocercospora sp. (5). Bars: A, E, I, M, Q = 1000 µm, B, F, J, N, R = 500 µm, and D, H, L, P and T = 2 µm.
Diversity of the sooty blotch and flyspeck disease complex

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Each taxon (SRB_a to SRB_k) contained 1 to 34 isolates (Fig. 2). Parsimony analysis of the ITS sequences included 24 taxa (including the outgroups) and 471 characters were used for the analysis. Of these characters, 58 were parsimony informative. The 12 most parsimonious trees obtained from parsimony analysis delimited two putative species (Fig. 2). The largest clade was poorly supported by bootstrap analysis and consisted of 66 isolates. This clade contained two strains from the U.S. and one from Poland and was provisionally identified.

Table 2. Prevalence of SBFS species identified from apple fruit in 14 orchards in Serbia and Montenegro.

<table>
<thead>
<tr>
<th>Location</th>
<th>Geographical coordinates of isolation sites</th>
<th>Pseudocercosporella sp. RH1</th>
<th>Pseudocercosporella sp. RH3</th>
<th>Schizothyrium pomi</th>
<th>Peltaster fructicola</th>
<th>Pseudocercospora sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sabac</td>
<td>44°45’ N; 19°41’ E</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Valjevo</td>
<td>44°16’ N; 19°53’ E</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>Loznica</td>
<td>44°32’ N; 19°13’ E</td>
<td>–</td>
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<td>–</td>
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<td>–</td>
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<tr>
<td>Kragujevac</td>
<td>44°10’ N; 20°55’ E</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Jagodina</td>
<td>43°58’ N; 21°15’ E</td>
<td>–</td>
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<tr>
<td>Pozarevac</td>
<td>44°36’ N; 21°10’ E</td>
<td>–</td>
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<tr>
<td>Smocerevo</td>
<td>43°39’ N; 20°55’ E</td>
<td>–</td>
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<tr>
<td>Zajecar</td>
<td>43°54’ N; 22°17’ E</td>
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<tr>
<td>Pozega</td>
<td>43°59’ N; 20°25’ E</td>
<td>–</td>
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<td>–</td>
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<td>–</td>
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<tr>
<td>Cacak</td>
<td>43°53’ N; 20°20’ E</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>Leskovac</td>
<td>42°59’ N; 21°56’ E</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>Vranje</td>
<td>42°33’ N; 21°54’ E</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>Berane_</td>
<td>42°50’ N; 19°52’ E</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>Bijelo Polje*</td>
<td>43°20’ N; 19°44’ E</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</table>

*Orchards located in Montenegro.
fied as Pseudocercosporella sp. RH1. Isolates from this clade were common in all orchards in Serbia except Ca-
cak, as well as in Montenegro. Scolecospores of Pseudo-
cercosporella sp. RH1 were 3 to 4 µm wide, two- to se-
veral-celled, and produced numerous secondary conidia
on the sides and ends that resulted in highly branched
masses; conidiogenous cells often became rounded (Tat-
alović, 2009).

The other clade in Fig. 2, containing six isolates from
Serbia and Montenegro (61% bootstrap) and one iso-
late from the U.S., was identified as Pseudocercosporella
sp. RH3. Although the ITS sequences of taxa in this
clade differed from each other by as many as five base
pairs, consistent differences in morphology among taxa
were not detected. Scolecospores of Pseudocercosporella
sp. RH3 were 2 to 3 µm wide, and conidiogenous cells
did not become rounded. Colonies of Pseudocer-
cosporella sp. RH3 formed distinctive single-celled ta-
pered conidia in creamy gelatinous masses 3 days after
transfer to PDA (Tatalović, 2009).

Both putative species of Pseudocercosporella identi-
fied in our study exhibited the ridged honeycomb
mycelial type on apple (Table 1), with clumps and
ridges of mycelia (Figure 1A, B, E, F). Colony texture of
both species on PDA after 4 weeks was felty, and the
colonies were mounded with radial ridges and lobed
margins. Colour ranged from dark greyish olive to iron
gray (Figure 1C, G). Colony bottoms were oliveaceous
black, and the agar and mycelia were frequently pulled
away from the bottom of the plate. Colony diameter on
MEA after 2 weeks of incubation ranged between 8.1
and 14.8 mm for Pseudocercosporella sp. RH1 and 9.0
and 12.5 mm for Pseudocercosporella sp. RH3. At the
end of the trial (4 weeks) diameter ranged between 11.1
and 21.2 mm for RH1, and 12.8 and 22.0 mm for RH3.

The ITS alignment that included isolates obtained
from flyspeck mycelial types contained 20 taxa (includ-
ing the outgroup); 476 characters were used for the
analysis and 28 characters were parsimony informative.
The two most parsimonious trees grouped all isolates of
the flyspeck mycelial type obtained in this study with
two previously identified strains of Schizothyrium pomi
(Fig. 3). The Zygophiala anamorph of S. pomi produced
conidia on distinctive conidiophores comprising a foot
cell that gives rise to a twisted or curved, dark brown,
smooth-walled stipe, an angular, subhyaline, finely ver-
ruculose terminal cell, and two (rarely three) laterally di-
vergent, pale brown, ovate conidiogenous cells that
bore thickened, circular scars. Solitary conidia were
produced in pairs and measured 22 to 25 x 5 to 7 µm
(Fig. 1L). The flyspeck mycelial type formed clusters of
round to oval, shiny, dark, flattened sclerotium-like
bodies on apple peel without a visible mycelial mat (Fig.
11, J). After 4 weeks on PDA cultures were felty to
smooth, flat on the margins and slightly lumpy in the
mid-region. Colour was zoned with drab feathered
edges, olive grey mid-region and greyish olive centers
(Fig. 1K). Colony diameter on MEA ranged between
12.3 and 19.4 mm after 2 weeks, and 21.0 and 33.8 mm
after 4 weeks. Isolates of S. pomi were recovered from
three orchards (Kragujevac and Leskovac in Serbia, and
Bijelo Polje in Montenegro).

Six isolates of Peltaster fructicola were obtained from
three locations (Kragujevac, Pozega and Vranje). The
ITS alignments contained 34 taxa (including the out-
group and previously identified species of P. fructicola
obtained from the U.S. and Germany). Of the 504 char-
acters, 14 were parsimony informative. The 141 most
parsimonious trees grouped all six isolates in our study
with previously identified strains of P. fructicola (Figure
4). After 4 weeks on PDA cultures were deep olive grey,

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**Fig. 2.** One of 12 most parsimonious trees determined from
ITS sequences obtained from SBFS isolates with Pseudocercosporella anamorphs. The tree is rooted to Mycosphaerella capsella. Gaps were treated as a fifth base. Parsimony informative characters = 58. Tree length = 182; Consistency index (CI) = 0.8681; Homoplasy index (HI) = 0.1319; Retention index (RI) = 0.7551. Strict consensus branches are shown in bold. Bootstrap values >90% are indicated above branches. Taxa in bold were recovered from apples infested with SBFS in Serbia or Montenegro, and new sequences deposited in GenBank are printed in boldface. Lower-case letters following SRB taxa denote groups of isolates having the same ITS sequence, and numbers of isolates are shown in parentheses. Other Pseudocercosporella spp. denoted with * were isolated from SBFS colonies on apple and identified in the U.S. (Batzer et al., 2005; Díaz, 2007), Germany, and Poland. Pseudocercosporella sp. KACC was isolated from Korea on Stellaria aquatic.
velvety, mounded, irregular and produced brown diffusible pigment in the media (Fig. 10). Colony diameter after 2 weeks on MEA ranged between 8.1 and 12.1 mm, and 12.2 and 20.4 mm after 4 weeks. Single-celled hyaline ellipsoid conidia (1.5 to 10.0 x 1.5 to 4.0 µm) were successively produced on indistinct conidiophores through a hyphal pore.

Four isolates (two from each country) were identified as 
Pseudocercospora

based on morphology and ITS sequences. Parsimony analysis of the ITS sequences included 24 taxa (including the outgroup) and 498 characters were used for the analysis. Of these characters, 45 were parsimony informative. The 14 most parsimonious trees grouped these isolates with other isolates of 
Pseudocercospora

found to cause SBFS on apple in the U.S., Germany and Poland (69% bootstrap support) (Fig. 5). All isolates from this clade had fuliginous mycelial type with no fruiting bodies on apple peels (Fig. 1Q, R). The absence of orange and brown pigments when cultured on PDA was consistent with previous reports for 
Pseudocercospora

FG1.1 (Batzer et al., 2005). The long flexuous conidia (20 to 70 x 2 to 3 µm) had 3 to 6 septa, were broader at the base and lacked scars. Subhyaline conidia were borne from pores on swollen hyphal cells (Fig. 1T). Mycelium surface after 4 weeks on PDA was feltly, dark olive grey, and slightly mounded with irregular, feathered margins and olivaceous colony bottom. Diameter of the colonies varied between 6.9 and 8.4 mm after 2 weeks, and 8.9 and 9.5 mm after 4 weeks.

DISCUSSION

This survey provides convincing evidence that the SBFS disease complex in southeastern Europe and...
North America includes many of the same fungi. Knežević et al. (2006) briefly discussed occurrence of SBFS in Serbia, economic importance of the complex, and recommendations for its control, but did not identify the species in the complex. We found five putative species involved in the SBFS disease complex on apples from Serbia and Montenegro. The species obtained in the study have all previously been subjected to Koch’s postulates; suspensions of mycelia were swabbed onto immature apples in the orchard, which were bagged until the apples were mature. Developing signs were compared to original mycelial types, re-isolated and sequenced to verify their identity (Batzer et al., 2005).

The goal of this study was to characterize taxonomic diversity of SBFS isolates from Serbia and Montenegro and to contribute to a worldwide understanding of diversity in this relatively unexplored fungal complex. It is likely that there are undiscovered members of the complex in Serbia and Montenegro because only 14 orchards were sampled in a single year. Northern Serbia is becoming an important region for apple production, but the present study included only orchards from central and southern regions of the country. It is also possible that patterns of species predominance indicated in this paper could be revised as a result of more intensive surveys in the future, since additional sampling over time and region would amplify these preliminary findings.

Preliminary analysis of portions of the 28S region (LSU) of the rDNA indicated that all species isolated in this study were within the order Capnodiales. Species in the genus Pseudocercosporella grouped with the Mycosphaerellaceae, Pseudocercospora spp. grouped with the Teratosphaeriaceae, Schizothyrium pomi grouped with the Schizothyriaceae and Peltaster spp. could not be placed at the family level (Batzer, unpublished data). Additional work is underway to assign Latin binomials and use other regions of DNA to more accurately reflect the lineage of these fungi. Placement of form genera, especially Pseudocercosporella and Pseudocercospora, was provisional based on conidium morphology (Braun, 1994; Arx, 1983). Within the Teratosphaeriaceae and Mycosphaerellaceae, many anamorph genera are polyphyletic (Crous et al., 2007).

The present study creates a foundation for future surveys to more clearly define the diversity, biogeography, and environmental biology of the SBFS complex in the Balkan region. Previous surveys in eastern and North America have disclosed distinct regional patterns in species occurrence, predominance, and ecology (Batzer et al., 2005; Diaz Arias, 2007; Sisson et al., 2008). It is likely that additional surveys in the Balkans will uncover yet unrecognized patterns of these parameters.

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Diversity of the sooty blotch and flyspeck disease complex


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