FUNGI IN THE APPLE SOOTY BLOTCH AND FLYSPECK COMPLEX FROM SERBIA AND MONTENEGRO

M.M. Ivanović[1], M.S. Ivanović[1], J.C. Batzer[2], N. Tatalović[2], B. Oertel[3], J. Latinović[4], N. Latinović[4] and M.L. Gleason[2]

[1] *Department of Phytomedicine University of Belgrade, Faculty of Agriculture, Belgrade-Zemun, Serbia*
[2] *Department of Plant Pathology, Iowa State University, Ames, IA 50011, USA*
[3] *INRES/Gartenbauwissenschaften, Universität Bonn, 53121 Bonn, Germany*
[4] *Biotechnical Faculty University of Montenegro, Podgorica, Montenegro*

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.

Corresponding author: M.L. Gleason
Fax: +1.515.294.9420
E-mail: mgleason@iastate.edu

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 21-24ºC 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 -80ºC. 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 24ºC under intermittent ambient light, colony texture, margins and pigment 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 25ºC 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'- CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-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: *Pseudocercospora* sp. based on Arx (1983), *Pseudocercosporella* sp. based on Braun (1994), *Zygophiala* 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 *Pseudocercospora* 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

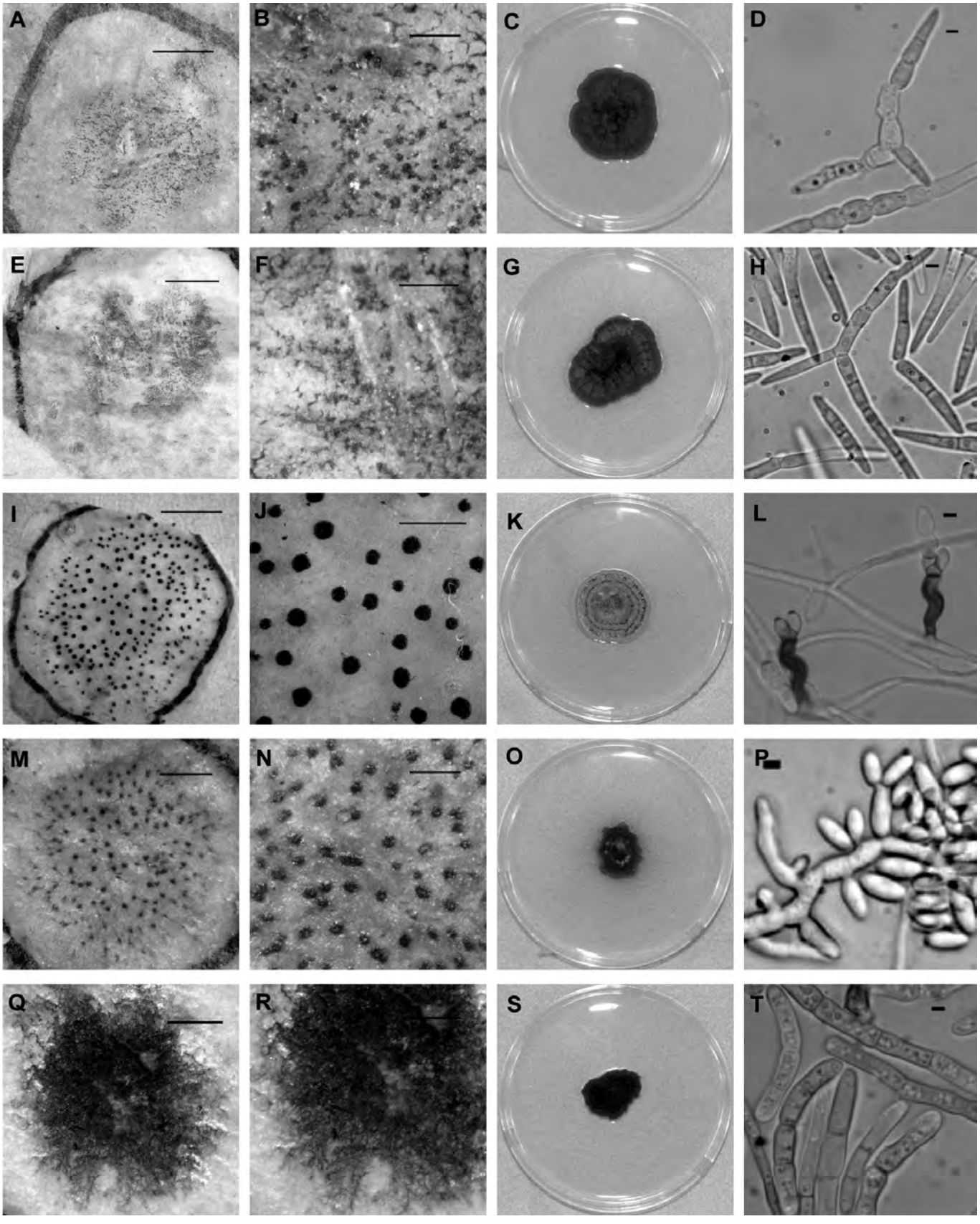

Fig. 1. Morphology of sooty blotch and flyspeck fungi from Serbia and Montenegro. Columns depict mycelial types on apple peel in overview (A, E, I, M, and Q) and close-up view (B, F, J, N, and R), colonies on PDA after 4 weeks (C, G, K, O and S), and conidiphores and conidia (D, H, L, P and T). Rows 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.

Table 1. Putative species, number of isolates, number of orchards detected, and distinguishing morphological characters and growth on MEA at 25ºC of five species of sooty blotch and flyspeck fungi from 14 orchards in Serbia and Montenegro.

Putative species	GenBank accession No.	No. of isolates	Locations detected	Distinguishing morphological characters			
				Mycelial type on apple	Mycelium on PDA	Colony diam on MEA (mm)[a]	
						2 weeks	4 weeks
Pseudocercosporella sp. RH1	FJ808747 FJ808748 FJ808749 FJ808750 FJ808751 FJ808752 FJ808753 FJ808754	66	13	Ridged honeycomb	Deep to dark greyish olive, zonate, mounded	8.1-14.8	11.1-21.2
Pseudocercosporella sp. RH3	FJ808744 FJ808745 FJ808746	6	4	Ridged honeycomb	Deep olive grey and dark olive grey, zonate, slightly mounded	9.0-12.5	12.8-22.0
Schizothyrium pomi	FJ808756 FJ808757	10	3	Flyspeck	Pink, orange, white or green, orange pigment	12.3-19.4	21.0-33.8
Peltaster fructicola	FJ808759	6	3	Punctate	Deep olive grey, felty, mounded irregular	8.1-12.1	12.2-20.4
Pseudocercospora sp.	FJ808755 FJ808758	4	2	Fuliginous	Dark olive grey	6.9-8.4	8.9-9.5

[a]Diameter of colonies, measured after 2 and 4 weeks on MEA at 25ºC.

each taxon (SRBa to SRBk) 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 parsimonyous 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 identi-

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

Location	Geographical coordinates of isolation sites	Putative species				
		Pseudocercosporella sp. RH1	*Pseudocercosporella* sp. RH3	*Schizothyrium pomi*	*Peltaster fructicola*	*Pseudocercospora* sp.
Sabac	44°45' N; 19°41' E	_				_
Valjevo	44°16' N; 19°53' E	_				
Loznica	44°32' N; 19°13' E	_				
Kragujevac	44°10' N; 20°55' E	_		_	_	
Jagodina	43°58' N; 21°15' E	_	_			
Pozarevac	44°36' N; 21°10' E	_				
Smederevo	44°39' N; 20°55' E	_	_			
Zajecar	43°54' N; 22°17' E	_				
Pozega	43°90' N; 20°25' E	_	_		_	
Cacak	43°53' N; 20°20' E					
Leskovac	42°59' N; 21°56' E	_		_		
Vranje	42°33' N; 21°54' E	_	_		_	
Berane*	42°50' N; 19°52' E	_				
Bijelo Polje*	43°20' N; 19°44' E	_		_		_

*Orchards located in Montenegro.

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

The other clade in Fig. 2, containing six isolates from Serbia and Montenegro (61% bootstrap) and one isolate 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 *Pseudocercosporella* sp. RH3 formed distinctive single-celled tapered conidia in creamy gelatinous masses 3 days after transfer to PDA (Tatalović, 2009).

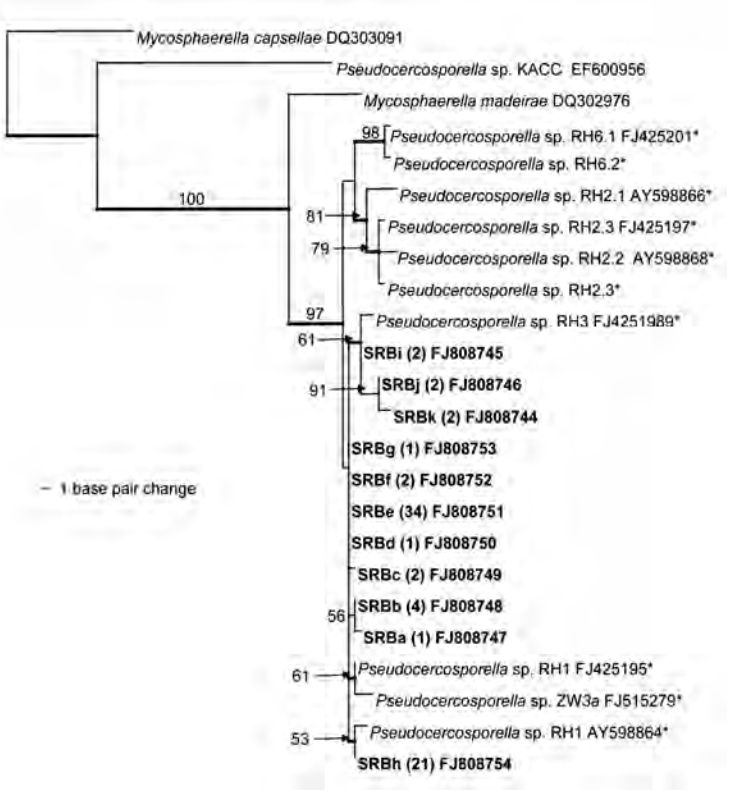

Fig. 2. One of 12 most parsimonious trees determined from ITS sequences obtained from SBFS isolates with *Pseudocercosporella* anamorphs. The tree is rooted to *Mycosphaerella capsella*. Gaps were treated as a fifth base. Parsimony informative characters = 58. Tree length = 182; Consistency index (CI) = 0.8681; Homoplasy index (HI) = 0.1319; Retention index (RI) = 0.7551. Strict consensus branches are shown in bold. Bootstrap values >50% 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 aquatica.*

Both putative species of *Pseudocercosporella* identified 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 olivaceous 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 (including 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 verruculose terminal cell, and two (rarely three) laterally divergent, 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. 1I, 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 outgroup and previously identified species of *P. fructicola* obtained from the U.S. and Germany). Of the 504 characters, 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,

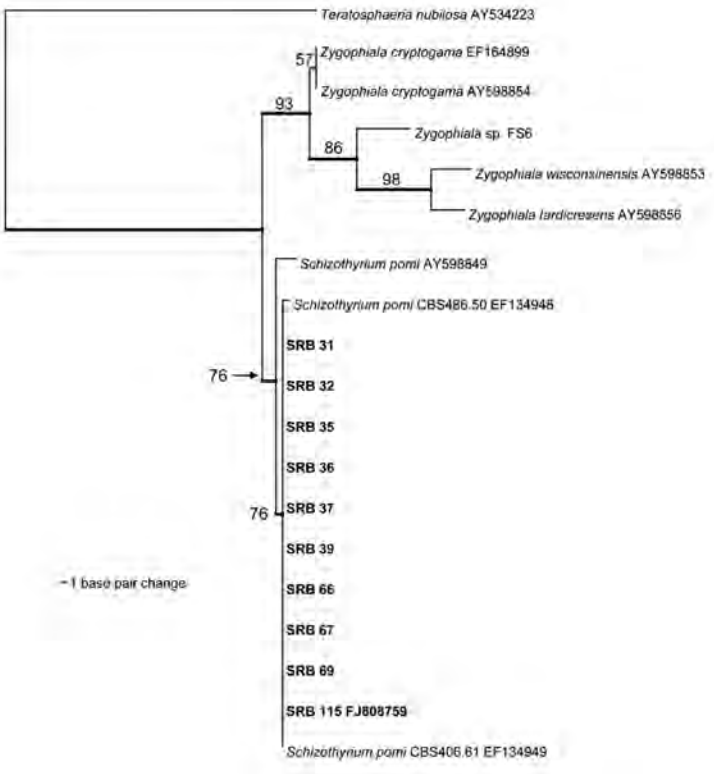

Fig. 3. One of two most parsimonious tree determined from ITS sequences (476 bp) obtained from SBFS isolates with *Zygophiala* anamorphs. The tree is rooted to *Teratosphaeria nubilosa.* Gaps were treated as a fifth base. Parsimony informative characters = 28. Tree length = 136; Consistency index (CI) = 0.9412; Homoplasy index (HI) = 0.0588; Retention index (RI) = 0.8689. Strict consensus branches are shown in bold. Bootstrap values >50% are indicated above branches. Taxa in bold were recovered from apples infested with SBFS in Serbia or Montenegro. Other isolates were recovered from SBFS-infested apples in the U.S. and the Netherlands, and new sequences deposited in GenBank are printed in boldface. Other isolates were recovered from SBFS-infested apples in the U.S. (Díaz, 2007; Batzer *et al.*, 2008).

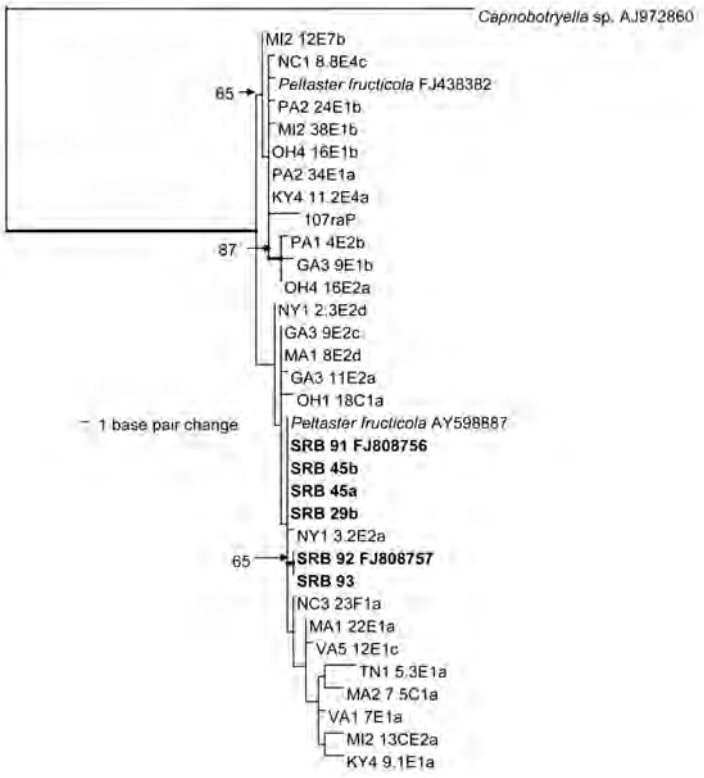

Fig. 4. One of 141 most parsimonious trees determined from ITS sequences obtained from SBFS isolates with *Peltaster* anamorphs. The tree is rooted to *Capnobotryella* sp. Gaps were treated as a fifth base. Parsimony informative characters = 14. Tree length = 187; Consistency index (CI) = 0.8289; Homoplasy index (HI) = 0.1711; Retention index (RI) = 0.5429. Strict consensus branches are shown in bold. Bootstrap values >50% are indicated above branches. Taxa recovered from apples infested with SBFS in Serbia or Montenegro, and new sequences deposited in GenBank are printed in boldface. Other isolates were recovered from SBFS-infested apples in the U.S. or Germany.

velvety, mounded, irregular and produced brown diffusible pigment in the media (Fig. 1O). 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* sp. 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 previously reports for *Pseudocercospora* sp. 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 felty, 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

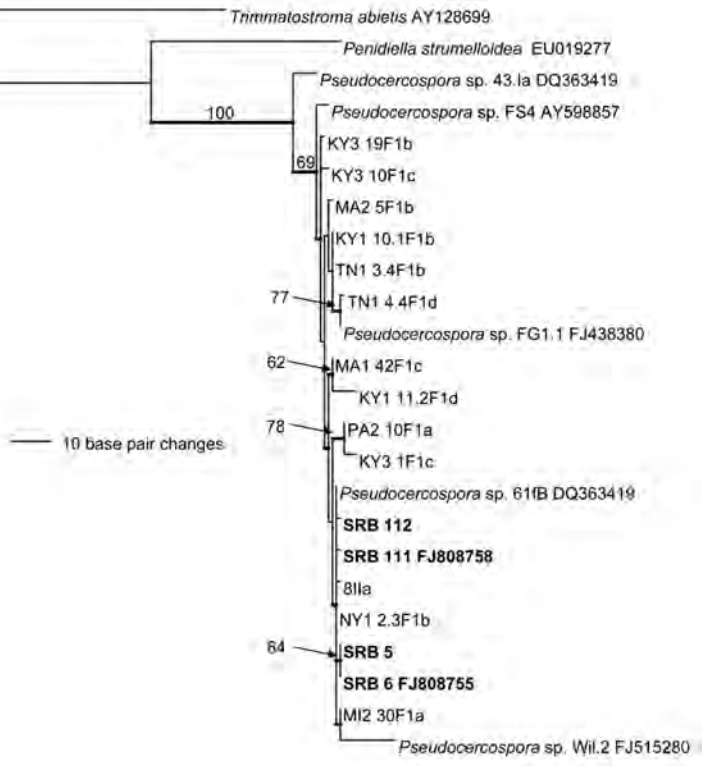

Fig. 5. One of 14 most parsimonious trees determined from ITS sequences obtained from SBFS isolates with *Pseudocercospora* anamorphs (Batzer *et al.,* 2005). The tree is rooted to *Trimmatostroma abietis* . Gaps were treated as a fifth base. Parsimony informative characters = 45. Tree length =256; Consistency index (CI) = 0.9102; Homoplasy index (HI) = 0.0898; Retention index (RI) = 0.7500. Strict consensus branches are shown in bold. Bootstrap values >50% 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. Other *Pseudocercospora* isolates were recovered from SBFS-infested apples in the U.S., Germany or Poland.

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, *Pseudocercopsora* 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 as yet unrecognized patterns of these parameters.

REFERENCES

Arx J.A. von., 1983. Mycosphaerella and its anamorphs. *Proceedings of the Koninklijke Nederlandse Akademie van Wetenschappen, 1983*. C 86: 15-54.

Baines R.C., 1940. Pathogenicity and hosts of the fly-speck fungus of apple. *Phytopathology* **30**: 2.

Baker K.F., Davis L.H., Durbin R.D., Snyder W.C., 1977. Greasy blotch of carnation and flyspeck disease of apple: diseases caused by *Zygophiala jamaicensis*. *Phytopathology* **67**: 580-588.

Barr M.E., 1987. Prodomus to Class Loculoascomycetes. Published by the author. Amherst, MA, USA.

Batzer J.C., Gleason M.L., Weldon B., Dixon P.M., Nutter F.W. Jr., 2002. Evaluation of postharvest removal of sooty blotch and flyspeck on apples using sodium hypochlorite, hydrogen peroxide with peroxyacetic acid, and soap. *Plant Disease* **86**: 1325-1332.

Batzer J.C., Gleason M.L., Harrington T.C., Tiffany L.H., 2005. Expansion of the sooty blotch and flyspeck complex on apples based on analysisof ribosomal DNA gene sequences and morphology. *Mycologia* **97**: 1268-1286.

Batzer J.C., Diaz Arias, M.M., Harrington T.C., Gleason M.L., Groenewald J.Z., Crous P.W., 2008. Four species of *Zygophiala* (Schizothyriaceae, Capnodiales) are associated with the sooty blotch and flyspeck complex on apple. *Mycologia* **100**: 246-258.

Belding R.D., Sutton T.B., Blankenship S.M., Young E., 2000. Relationship between apple fruit epicuticular wax and growth of *Peltaster fructicola* and *Leptodontidium elatius*, two fungi that cause sooty blotch disease. *Plant Disease* **84**: 767-772.

Braun U., 1994. Studies on Ramularia and allied genera (VII). *Nova Hedwigia* **58**: 191-222.

Colby A.S., 1920. Sooty blotch of pomaceous fruits. *Transactions of the Illinois Academy of Sciences* **13**:139-179.

Crous P.W., Braun U., Groenewald J.Z., 2007. Mycosphaerella is polyphyletic. *Studies in Mycology* **58**: 1-32.

Díaz Arias M.M., 2007. Biogeografia y Diversidad Genetica de las Especies del Compleio Fungico Mancha Negra ("Sooty Blotch") y Mancha de la Mosca ("Flyspeck") de la Manzana en la Region Este de los Estados Unidos. Ms.Sc. Thesis. Ciudad Universitaria Rodrigo Facio, San José, Costa Rica.

Groves A.B., 1933. A study of the sooty blotch disease of apples and casual fungus *Gloeodes pomigena*. *Virginia Agricultural Experiment Station Bulletin* **50**: 1-43.

Fisher N.L., Burgess L.W., Tousson T.A., Nelson P.E., 1982. Carnation leaves as a substrate and for preserving cultures of *Fusarium* species. *Phytopathology* **72**: 151-153.

Hall T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-98.

Harrington T.C., Wingfield B.D., 1995. A PCR-based identification method for species of *Armillaria*. *Mycologia* **87**: 280-288.

Hernandez S.M., Batzer J.C., Gleason M.L., Mueller D.S., Dixon P.M., Best V., McManus P.S., 2004. Temperature optima for mycelial growth of newly discovered fungi in sooty blotch and flyspeck complex on apples. *Phytopathology* **94**: S41.

Hickey K.D., 1960. The sooty blotch and flyspeck diseases of apple with emphasis on variation within *Gloeodes pomigena* (SCW.) Colby. Ph.D. Thesis. Pennsylvania State University, University Park, PA, USA.

Johnson E.M., Sutton T.B., 1994. First report of *Geastrumia polystigmatis* on apple and common blackberry in North America. *Plant Disease* **78**: 1219.

Johnson E.M., Sutton T.B., Hodges C.S., 1996. *Peltaster fructicola*: a new species in the complex of fungi causing apple sooty blotch disease. *Mycologia* **88**: 114-120.

Johnson E.M., Sutton T.B., Hodges C.S., 1997. Etiology of apple sooty blotch disease in North Carolina. *Phytopathology* **87**: 88-95.

Knežević T., Jevtić R., Gleason M.L., 2006. First study of sooty blotch and flyspeck complex on apple and pear in Serbia. *Plant Doctor* **2**: 112-117.

Le Corronc F., Batzer J.C., Gleason M.L., 2006. Effect of apple juice on in vitro morphology of four newly discovered fungi in the sooty blotch and flyspeck complex. *Phytopathology* **96**: S65.

Ridgway R., 1912. Color Standards and Color Nomenclature. Press of a Hoen and Company, Baltimore, MD, USA.

Sisson A.J., Batzer J.C., Waxman K.D., Hemnani K., Gleason M.L., 2008. Phenology of sooty blotch and flyspeck fungi on apples in Iowa. *Phytopathology* **98**: S147.

Schweinitz L.D., 1832. *Dothidea pomigena*. Transactions of the American Philosophical Society, pp 232. New Series, Philadelphia, PA, USA.

Swofford D.L., 2002. PAUP* Phylogenetic analysis using parsimony (*and other methods) version 4.0. Sinauer Associates, Sunderland, MA, USA.

Tarnowski T.B., Batzer J.C., Gleason M.L., Helland S., Dixon P., 2003. Sensitivity of newly identified clades in the sooty blotch and flyspeck complex on apple to thiophanatemethyl and ziram. Online. *Plant Health Progress* doi:10.1094/PHP-2003-12XX-01-RS.

Tatalović N., 2009. Delineation of taxa closely grouping with *Mycosphaerella madeirae* in the sooty blotch and flyspeck complex of apples. Ms. Sc. Thesis. Iowa State University, Ames, IO. USA.

Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F., Higgins D.G., 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876-4882.

van de Voort J.L., Batzer J.C., Helland S.J., Gleason M.L., 2003. Agar media affect growth and sporulation of newly discovered sooty blotch fungi. *Phytopathology* **93**: S86.

White T.J., Bruns T., Taylor J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Snisky J.J., White, J.W. (ed.) PCR Protocols: A Guide to Molecular Methods and Amplifications, pp. 315-322. Academic Press, New York, NY, USA.

Williamson S.M., Sutton T.B., 2000. Sooty blotch and flyspeck of apple: Etiology, biology, and control. *Plant Disease* **84**: 714-724.

Williamson S.M., Hodges C.S., Sutton T.B., 2004. Re-examination of *Peltaster fructicola*, a member of the apple sooty blotch complex. *Mycologia* **96**: 885-890.

Wrona B.R., 2004. Influence of fruit surface sugars on the growth of fungi that cause sooty blotch of apple. *Journal of Plant Protection Research* **44**: 283-288.

Wrona B.R., Gleason M.L., 2005. Effect of surface amino acids on the growth of *Peltaster fructicola* – fungus associated with sooty blotch complex. *Journal of Plant Protection Research* **45**: 273-278.

Received March 27, 2009
Accepted September 16, 2009