

Four species of *Zygothiala* (Schizothyriaceae, Capnodiales) are associated with the sooty blotch and flyspeck complex on apple

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Abstract: Sooty blotch and flyspeck (SBFS) is a complex of fungi that cause late-season blemishes of apple and pear fruit that cosmetically damage the cuticle, which result in fruit that are unacceptable to consumers. Previous studies reported that a single, wide-host-range species, *Schizothyrium pomi* (presumed anamorph *Zygothiala jamaicensis*), caused flyspeck on apple. In the present study we compared morphology and DNA phylogeny (ITS, LSU) of 139 fungal strains isolated from flyspeck signs from 39 apple orchards in 14 midwestern and eastern states (USA). Parsimony analysis, supported by cultural characteristics and morphology in vitro, provided support to delimit the flyspeck isolates into four species of *Zygothiala*, two of which are known to be sexual. Three of these species are described as new. Based on DNA phylogeny, species of *Schizothyrium* were shown to cluster with members of the genus *Mycosphaerella* in the Capnodiales, having similar asci and ascospores but morphologically distinct ascospores. These data question the value of ascospore morphology at the ordinal level, although it still appears to be relevant at the family level, delimiting the thyrothecial Schizothyriaceae from other families in the Capnodiales.

Key words: anamorph, plant pathology, SBFS, *Schizothyrium pomi*, *Zygothiala jamaicensis*

INTRODUCTION

Sooty blotch and flyspeck (SBFS) are late-season blemishes on the cuticle of apples and pears in humid regions worldwide, resulting in produce that is unacceptable to fresh market consumers. Fungi in the

SBFS complex grow superficially on the epicuticular wax, do not penetrate the cuticle (Belding 2000) and may use exuded nutrients present on the apple surface (Baker 1977, Nasu and Kunoh 1987b, Wrona 2004, Wrona and Gleason 2005, Le Corronc et al 2006). The term “flyspeck” designates colonies in the SBFS complex that develop clusters of shiny, black, round to ovoid, sclerotium-like bodies and have no visible mycelial mat. *Schizothyrium pomi* (Mont. & Fr.) Von Arx (presumed anamorph *Zygothiala jamaicensis* E.W. Mason) has been described as the cause of flyspeck (Baines 1940, Baker 1977). In contrast the term “sooty blotch” designates fungi in the complex that form a dark mycelial mat with or without sclerotium-like bodies. Several newly described SBFS fungi, referred to as compact speck and discrete speck, closely resemble flyspeck, but they can be distinguished from flyspeck by the absence of ring-like remnants of the sclerotium-like bodies on the apple cuticle when the bodies are removed and by size and density of sclerotium-like bodies (Batzer et al 2005).

What is now recognized as the SBFS complex initially was described from apples collected in Pennsylvania, USA, as *Dothidea pomigena* Schwein. (Schweinitz 1834). Diverse colony morphologies on blemished fruit were thought to be caused by a single species, and flyspeck and sooty blotch were presumed to be developmental stages of the same fungus (Montagne 1834, Sprague 1856, Duggar 1909). Colby (1920) however concluded that sooty blotch and flyspeck were caused respectively by separate fungi, *Gloeodes pomigena* (Schwein.) Colby and *Leptothyrium pomi* A. Selby. The name *L. pomi* was synonymized with *Mycothyriella rubi* Petr. (Baines 1940), but it later was recognized as *Schizothyrium pomi* (Mont. & Fr.) Von Arx (Baker et al 1977). In the past 10 y, the SBFS complex has been further expanded to include as many as 30 species based on a combination of genetic and morphological evidence (Johnson and Sutton 1994; Johnson et al 1996, 1997; Batzer et al 2005).

Schizothyrium pomi was linked to its presumed anamorph, *Z. jamaicensis*, when immature apple fruit inoculated with ascospores produced both the sexual and asexual stages (Durbin et al 1953). Numerous hosts of *Z. jamaicensis* subsequently have been identified, including 120 species in 44 families of seed plants throughout temperate and tropical

regions (Baines 1940, Baker et al 1977, Sutton et al 1988, Nasu and Kunoh 1987a). Although isolates from these diverse hosts were morphologically similar, they were observed to differ in their cultural characteristics (Durbin et al 1953). However cross-inoculation studies gave no evidence for host specialization (Baker et al 1977, Nasu and Kunoh 1987b), and Nasu and Kunoh (1993) conjectured that *Z. jamaicensis* might be able to survive on all plants whose surfaces are covered by a waxy bloom, unless antifungal substances or inadequate nutritional sources prevent fungal growth.

Several *Schizothyrium* species were named for the host from which they were isolated but subsequently were found to be morphologically similar. For example *S. acerinum*, *S. gaultheria* and *S. reticulatum* were shown to be synonymous with the flyspeck fungus *S. pomi* (von Arx 1959). Although 12 *Schizothyrium* species were recognized by von Arx and Müller (1975), only a single anamorph species, *Z. jamaicensis*, has been reported.

Conidiophores of *Zygothiala* arising from superficial hyphae have a distinctive conidiophore morphology, namely a foot cell that gives rise to a twisted, or curved, dark brown, smooth-walled stipe, which tends to be widest in the middle, an angular, subhyaline, finely verruculose terminal cell and at its apex, two (rarely three) laterally divergent, pale brown, finely verruculose, ovate to ampulliform to elongated subcylindrical conidiogenous cells that bear one to several prominently thickened, circular, darkened and somewhat refractive conidial scars. Conidia are produced in pairs, have a slightly granular surface, are medianly or unevenly 1-septate (rarely multiseptate), ellipsoidal to ovate (rarely obclavate), constricted at septa, with prominently thickened, darkened, refractive scars. Although the morphology of diverse *Zygothiala* isolates has been compared, these observations have not been used to distinguish additional species. Nasu et al (1985) distinguished two isolates based on differing growth patterns, colony color, numbers of sclerotium-like body produced, optimal temperature and pH ranges. Lerner (2000) also grouped 30 isolates from six eastern states in the USA based on growth rate and colony morphology.

During a survey in 2000 of nine apple orchards in five states in the midwestern USA, four putative species of *Zygothiala* were delineated based on their morphology on the host and cultural growth characteristics. These isolates and other flyspeck isolates collected during a survey in 2005 covering 30 apple orchards in 10 eastern states were used for taxonomic study. The aim of the present study was to identify and describe species of flyspeck fungi based on DNA phylogeny and phenotype.

MATERIALS AND METHODS

Sources of isolates.—Three isolates of *Schizothyrium pomi* were obtained from the CBS collection (TABLE I). Three isolates identified as *S. pomi* were also kindly provided by Dr Turner B. Sutton of North Carolina State University (NCSU). All other isolates were obtained from orchards surveyed in the eastern and midwestern USA (TABLE I). In autumn 2000 isolates were obtained from SBFS colonies on 40 apples harvested from each of nine orchards in Iowa, Illinois, Missouri and Wisconsin. In autumn 2005 a similar survey was conducted from 30 orchards in 10 eastern states (Georgia, North Carolina, Virginia, Kentucky, Tennessee, New York, Massachusetts, Pennsylvania, Ohio and Michigan). Approximately 12 flyspeck colonies were selected arbitrarily from apples sampled from each orchard. Isolations were made as described by Batzer et al (2005). A total of 139 flyspeck isolates were purified and stored in glycerol at -80°C . Segments of apple peels with flyspeck signs were preserved by pressing the thallus and supporting peel between paper towels until dry. Representative cultures were deposited at the Centraalbureau voor Schimmelfcultures (CBS), Fungal Biodiversity Centre, Utrecht, The Netherlands, and specimens on apple peels were deposited at the Iowa State University Herbarium, Ames, Iowa, and at CBS.

Polymerase chain reaction and sequencing.—The internal transcribed spacer region of the ribosomal DNA (ITS1, 5.8S rDNA gene, ITS2) of 130 isolates from flyspeck-like colonies was sequenced. A portion of the 28S (large subunit, LSU) rDNA gene was sequenced for representative isolates of each clade identified by parsimony analysis of the ITS region.

For isolates obtained in 2000, template DNA for polymerase chain reaction (PCR) was obtained by scraping mycelia with a pipette tip from 4- to 6 wk old cultures grown on PDA (Harrington and Wingfield 1995). For the isolates obtained in 2005, DNA was extracted from mycelia with Prepman Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, California). Primer pairs used for amplification and sequencing of the ITS region were ITS-1F/ITS4 (White et al 1990), and primer pairs used for amplification and sequencing of LSU were respectively LR0R/LR5 and LR0R/LR3 (Vilgalys and Hester 1990). Amplification reactions consisted of 4 mM MgCl_2 , 5% DMSO, $1\times$ Sigma buffer, 200 μM dNTPs, 0.5 μM of the forward and reverse primers, and 3 units of *Taq* polymerase (Sigma Chemical Co., St Louis, Missouri). Cycling conditions (MJ Research Inc. thermocycler, PTC-100 Waltham, Massachusetts) for amplifications were an initial denaturation at 94°C for 95 s followed by 35 cycles of denaturation at 94°C for 35 s, annealing at 49°C for LSU and at 52°C for ITS for 60 s, and extension at 72°C for 2 min. The PCR product was purified with a QIAquick DNA Purification Kit (QIAGEN, Valencia, California) and quantified on a Hoefer DyNA Quant 200 Fluorometer (Amersham Pharmacia Biotech, San Francisco, California). Automated sequencing was performed at the Iowa State University DNA Sequencing and Synthesis Facility.

TABLE I. Accession numbers from Centraalbureau voor Schimmelcultures (CBS), Iowa State University Herbarium and GenBank for partial rDNA sequences of *Zygothiala* spp. occurring on apple fruit

Species	Strain CBS Accession No.	Herbarium Accession No.	GenBank Accession		
			LSU	ITS	
<i>Schizothyrium pomi</i>	CUA1a, CBS 118957	438789, CBS-H19787	AY598895	EF164898	
	ZJ001		AY598894	AY598848	
	ZJ002			AY598849	
	ZJ003			AY598850	
	AHA2a			AY598851	
	GTA1a			AY598852	
	CBS 228.57			EF134947	EF134947
	CBS 406.61			EF134949	EF134949
	CBS 486.50			EF134948	EF134948
<i>Zygothiala cryptogama</i>	FVA2a, CBS 118949	438791, CBS-H19785	AY598896	AY598854	
	MWA8a			EF164899	
	KYI 1.2A1c			EF164902	EF164900
<i>Zygothiala tardicrescens</i>	MWA1a, CBS 118946	438792, CBS-H19788	EF164901	AY598856	
<i>Zygothiala wisconsinensis</i>	MSTA8a, CBS 118950	438790, CBS-H19786	AY598897	AY598853	
	GTA4b			AY598855	

Sequence alignment and phylogenetic analysis.—Sequences were imported into BioEdit (Hall 1999), and the 5'- and the 3'- ends were trimmed to aid alignment. Length of the ITS sequences analyzed was approximately 485 base pairs. Preliminary alignments of the ITS sequences were generated with Clustal X (Thompson et al 1997) with gap opening and gap extension parameters of 50:5, and these alignments were optimized manually. Isolates with redundant ITS and LSU sequences obtained from the same orchard were eliminated from the dataset, reducing the number of isolates in the analyses from 130 to 82 and 45 to 13 respectively. Maximum parsimony (MP) analysis was performed with PAUP v.4.0b10 (Swofford 2002). Heuristic searches were conducted with a 1000 random sequence additions and tree bisection-reconnection (TBR) 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, a strict consensus of the most parsimonious trees was generated and a bootstrap analysis of 1000 replications was performed. We rooted the LSU tree to four species from the Chaetothyriales (*Ceramothyrium carniolicum* [Rehm] Petr., *Exophiala dermatitidis* [Kano] de Hoog, *Rhinocladiella atrovirens* Nannf. and *Ramichloridium anceps* [Sacc. & Ellis] de Hoog). Outgroup for ITS phylogenetic analysis was *Mycosphaerella marksii* Carnegie & Keane. MP analysis, treating gaps as missing data, also was conducted on the LSU alignment because of concerns that gaps could be over-weighted in the analysis where gaps were treated as a fifth character. Alignments and the representative trees (FIGS. 1, 2) were deposited in TreeBASE SN3221.

Morphology of SBFS isolates on apple and in vitro.—Signs of SBFS on preserved apple peels were described, including mycelial growth patterns and fruiting body size and density.

Colony descriptions were made after 1 mo growth on oatmeal agar (OA) at 21–24 C under intermittent ambient light. Fungal structures were mounted in clear lactic acid and examined at 1000× magnification. Thirty measurements were determined for each structure. For conidial measurements, the 95% percentiles are presented and extremes given in brackets.

RESULTS

Phylogenetic analysis.—The ITS alignment contained 83 taxa (including outgroup), and 481 characters were used for the analyses. Of these characters, 33 were parsimony informative, 101 were variable and parsimony uninformative and 347 were constant. The 24 equally parsimonious trees obtained from ITS analysis delimited four putative species of *Zygothiala* (FIG. 1). The largest clade (86% bootstrap support) consisted of 102 isolates and included isolates from all 14 states surveyed and from 30 of the 39 orchards. This clade contained three strains from the CBS culture collection and was identified as *S. pomi*. Three other clades, representing previously undescribed species, also were delimited in the ITS analysis. The first of these was poorly supported but appeared sister of the *S. pomi* clade. Isolates from this clade were obtained from Iowa, Ohio, Michigan and Kentucky, and the species is described as *Zygothiala cryptogama* sp. nov. A well supported clade (89% bootstrap support) contained isolates obtained from Wisconsin, Ohio, Michigan, Virginia and Missouri and is described as *Zygothiala wisconsinensis* sp. nov. Isolates from the last clade (100% bootstrap support and sister of *Z. wisconsinensis*) were obtained from a

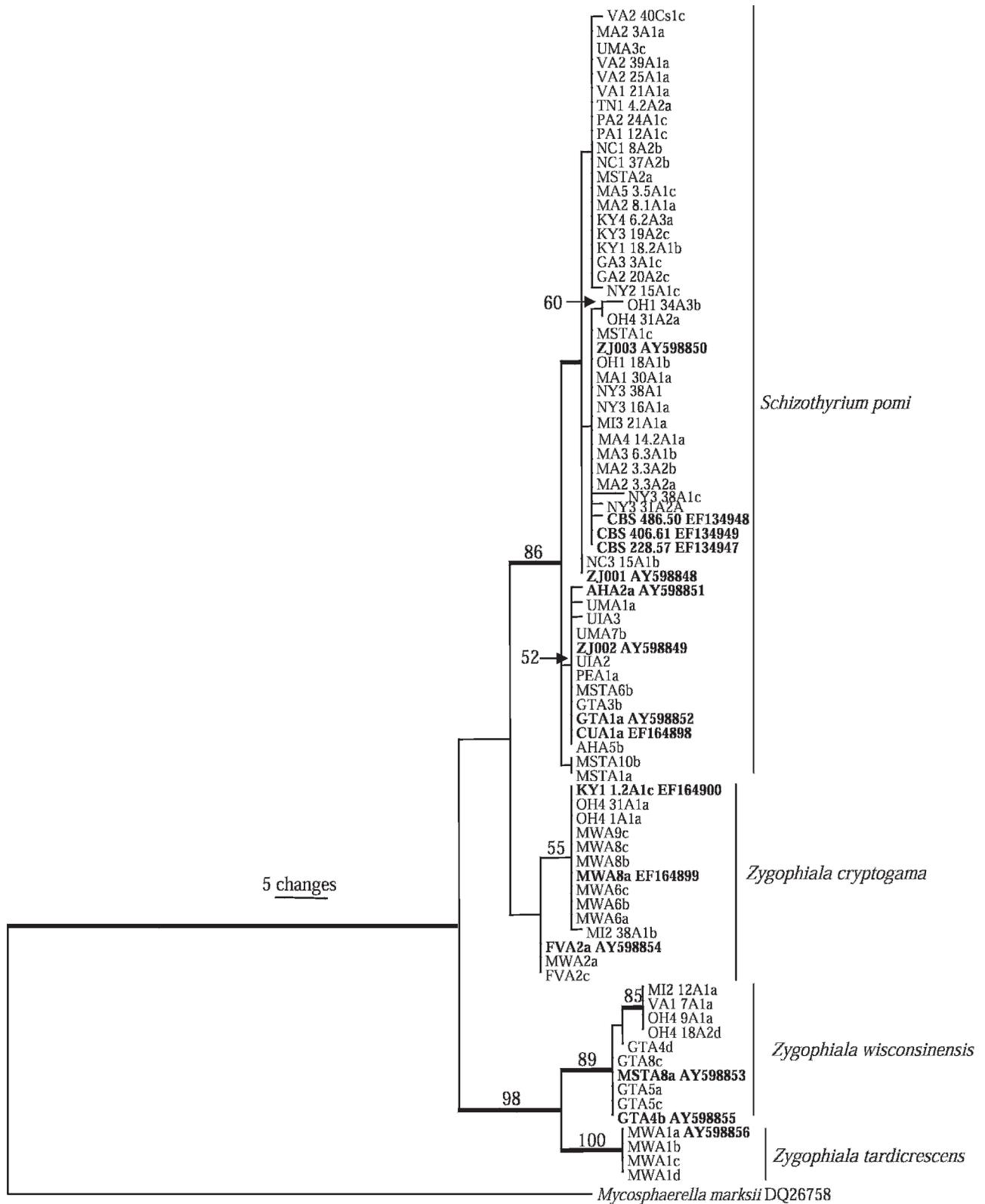


FIG. 1. One of 24 equally most parsimonious trees determined from ITS sequences obtained from isolates taken from flyspeck signs on apple fruit from eastern and midwestern orchards. Bootstrap support values (>50%) based on 1000 replicates are shown at the nodes, and strict consensus branches are thickened. The tree is rooted to *Mycosphaerella marksii* and new sequences deposited in GenBank are printed in boldface. Tree length = 167, consistency index = 0.898, retention index = 0.969, rescaled consistency index = 0.867.

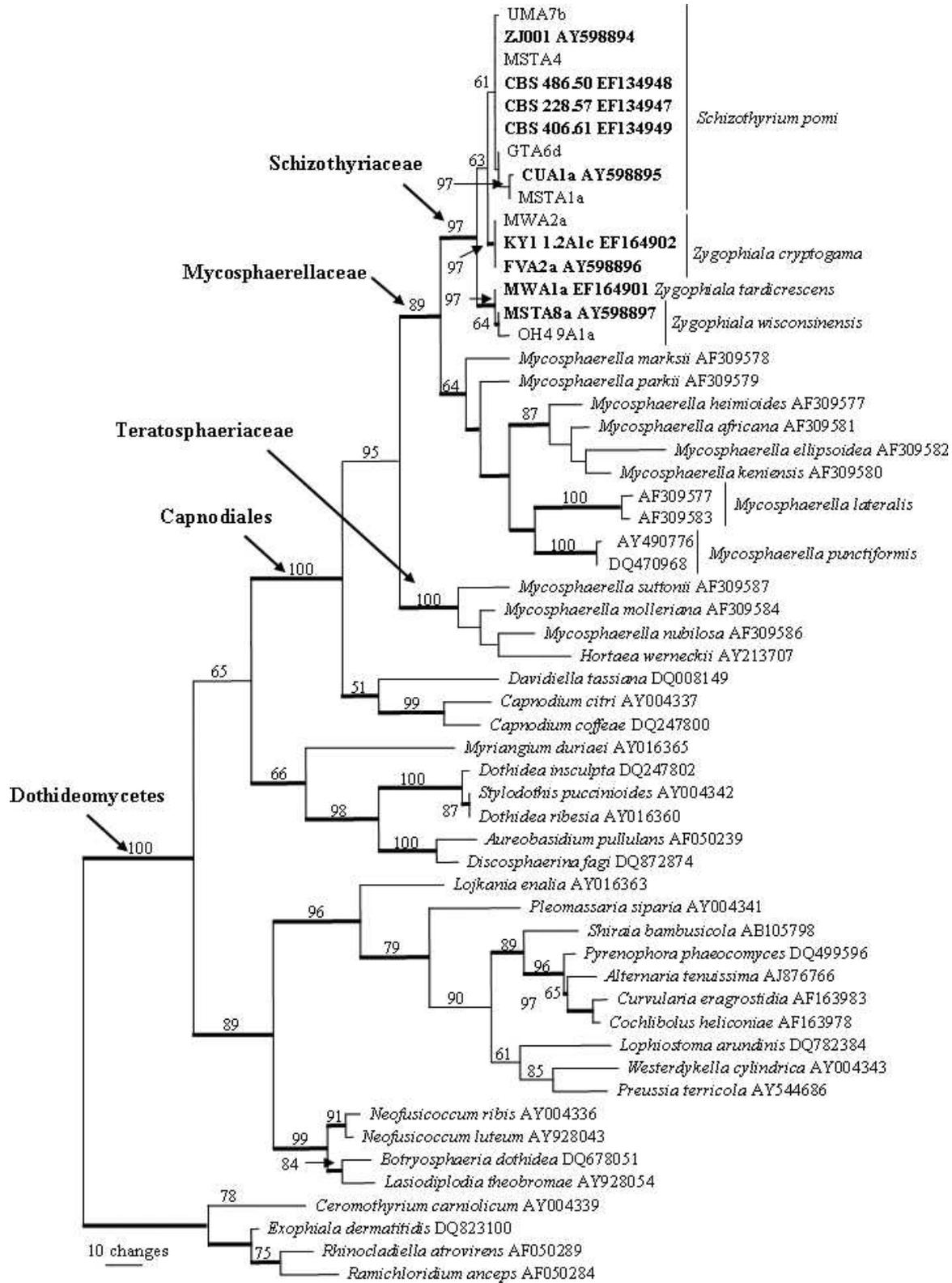


FIG. 2. One of 10 equally most parsimonious trees of partial sequences of the 28S large subunit (LSU) region of rDNA from flyspeck isolates on apple fruit from eastern and midwestern orchards and other ascomycetes. Bootstrap support values (>50%) based on 1000 replicates are shown at the nodes, and strict consensus branches are thickened. The tree is rooted to four species from the Chaetothyriales (*Ceromathyrium carniolicum*, *Exophiala dermatitidis*, *Rhinochadiella atrovirens* and

single Iowa orchard and are described as *Zygothiala tardicrescens* sp. nov.

The LSU alignment contained 56 taxa (including the four outgroup taxa) and 554 characters were used for the analyses. Of these characters 215 were parsimony informative, 42 were variable and parsimony uninformative and 297 were constant. Maximum parsimony analysis of the LSU sequences resulted in 10 equally most parsimonious trees (FIG. 2). Parsimony analysis grouped the *Zygothiala* species within the Capnodiales (Schoch et al 2006) with bootstrap support value of 100%. The Schizothyriaceae formed a well supported (97% bootstrap support) clade within the Mycosphaerellaceae (95% bootstrap support) clade when gaps were treated as a fifth character. When gap treatment was altered to missing data, bootstrap support of the Mycosphaerellaceae was reduced to 63%. However the overall topology of the trees was almost identical when gaps were treated as missing characters.

Taxonomy.—Isolates could be grouped into four species based on their morphology on cultural media, growth characteristics and DNA phylogeny. Sclerotium-like bodies of *Schizothyrium pomi* on apple were round, 250(155–480) μm diam and with a density of 2.4/mm². Sclerotium-like bodies of *Zygothiala cryptogama* were also round but slightly smaller, 230(150–364) μm diam, and more densely arranged, averaging of 3.6 sclerotium-like bodies/mm². *Zygothiala wisconsinensis* sclerotium-like bodies were ovoid, larger, 380(300–450) \times 500(425–600) μm and were more sparsely arranged with a density of 0.8/mm². Sclerotium-like bodies of *Zygothiala tardicrescens* were similar to *S. pomi*, 260(250–270) μm diam and were arranged at a density of 2.8/mm².

Three new species of *Zygothiala* were distinguished and are described below.

Schizothyrium pomi (Mont. & Fr.) Von Arx, Proc. K. Ned. Akad. Wet., Ser. C, Biol. Med. Sci. 62:336. 1959. FIGS. 3, 4.

\equiv *Labrella pomi* Mont. (Fr. in litt.), Ann. Sci. Nat., Sér. 2, Bot. 1:347. 1834.

Anamorph. *Zygothiala* sp. (non *Z. jamaicensis* E.W. Mason).

Ascomata black, shiny, dimidiate, in random clusters, but frequently in circles, superficial on leaves, stems or fruit, appressed to the cuticle, 150–375 μm diam, 30–50 μm high, with irregular margins; upper

layer consisting of interwoven mycelium, forming 2–4 layers of thick-walled, brown, pseudoparenchymatal cells, 4–8 μm thick; ostiole central, but upper layer splitting at maturity via irregular ruptures from the elevated center; ascomata situated on a thin, hyaline, basal stroma. *Hamathecium* hyaline, consisting of branched, septate, pseudoparaphysoid-like filaments, 3–5 μm wide. *Asci* bitunicate, 8-spored, ovoid to subglobose or ellipsoid to clavate, apical chamber present but inconspicuous at maturity, 20–45 \times 8–16 μm ; formed in a single layer in the hamathecial tissue. *Ascospores* hyaline, guttulate, thick-walled, medianly 1-septate, constricted at septum, fusoid-ellipsoidal, widest in the middle of the apical cell, which is acutely rounded, while the lower cell is subobtusely rounded, (10–)12–13(–14) \times (3–)3.5–4(–5) μm . Ascospores germinating after 24 h on MEA, becoming brown and verruculose, with a visible mucoid sheath surrounding the spore on the agar surface, slightly or not constricted at the septum, 4–5 μm wide, not distorting, germinating from both ends, with 2–3 germ tubes; cultures are homothallic.

Conidiophores arising from superficial hyphae, 2–3 μm wide, erect, scattered, 3–4-septate, subcylindrical, rarely straight, mostly flexuous, consisting of a hyaline to subhyaline supporting cell that gives rise to a smooth, dark brown stipe, 25–35 \times 7–8 μm (from basal septum to below phialide), terminating in a finely verruculose, medium brown apical cell, 6–7 \times 6–7 μm , that gives rise to two (rarely three) medium brown, finely verruculose, doliform to ellipsoid or subcylindrical, polyblastic conidiogenous cells, 8–12 \times 6–7 μm ; scars prominent, apical, darkened, thickened, somewhat refractive, with 1(–2) per conidiogenous cell, 2 μm wide. *Conidia* solitary, fusiform to obclavate, hyaline, smooth and thick-walled, transversely 1(–7)-septate, prominently constricted at septa, (20–)22–25(–30) \times 5–7(–8) μm if 1-septate but up to 110 μm long if 7-septate; apex subobtuse, base subtruncate, with a darkened, thickened hilum, 2 μm wide.

Cultural characteristics. Colonies after 2 wk on OA in the dark flat, spreading with sparse aerial mycelium and smooth, regular margins; pale olivaceous gray to olivaceous gray in the center, becoming cream to pale luteous toward the margin; developing erumpent ascomatal initials in older cultures.

Specimen examined. USA. ILLINOIS: Rockford, on apple fruit, Sep 2000, J. Batzer, 438789, CBS-H19787, cultures CUA1 = CBS 118957, GenBank: AY598895.

←

Ramichloridium anceps) and new sequences deposited in GenBank are printed in boldface. Tree length = 1002, consistency index = 0.438, retention index = 0.805, rescaled consistency index = 0.374.

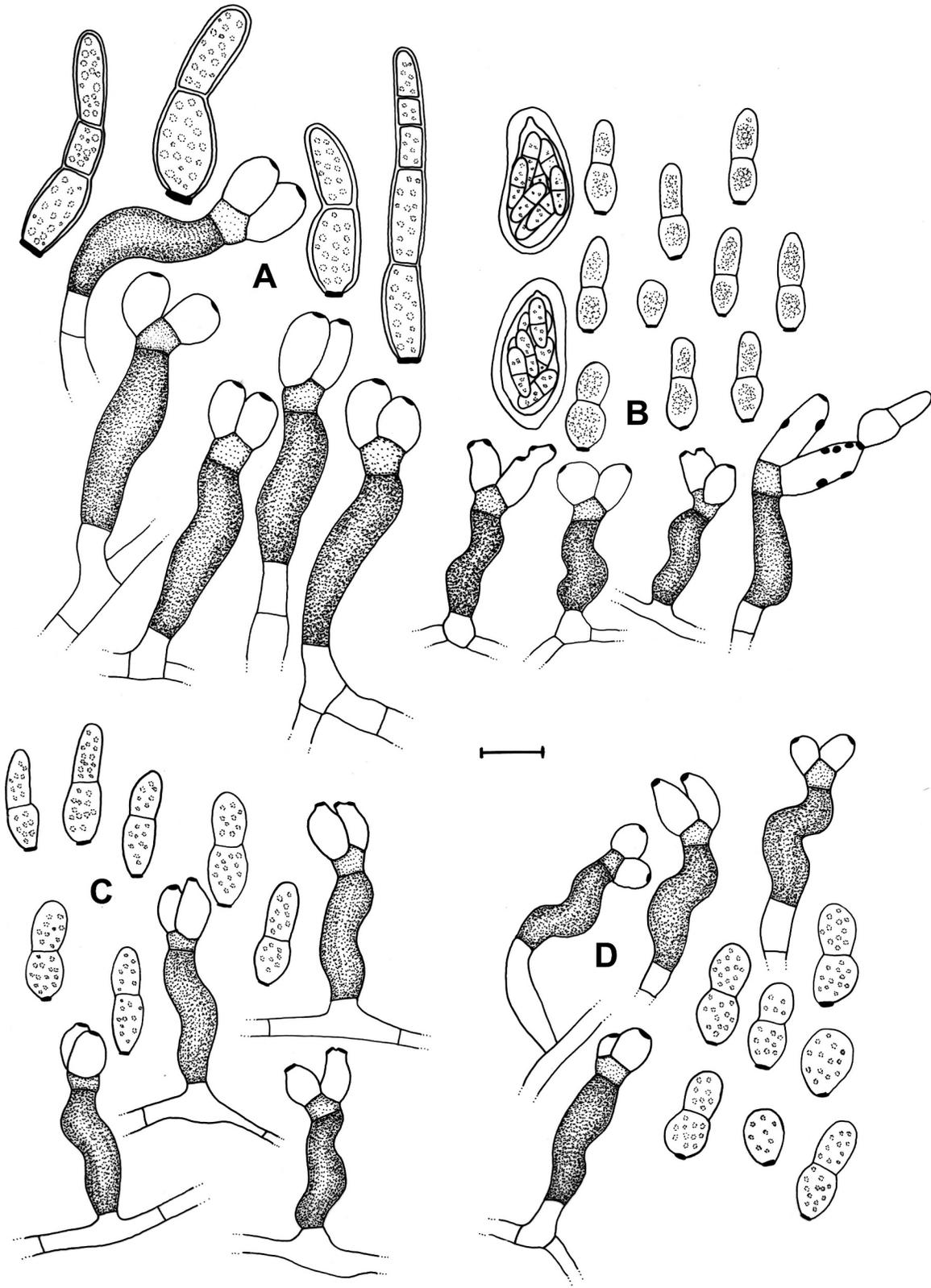


FIG. 3. *Zygophiala* spp. sporulation on oatmeal agar. A. Conidiophores and conidia of the *Zygophiala* anamorph of *Schizothyrium pomi* (CBS 118957). B. Asci, conidiophores and conidia of *Z. cryptogama* (CBS 118949). C. Conidiophores and conidia of *Z. tardicrescens* (CBS 118946). D. Conidiophores and conidia of *Z. wisconsinensis* (CBS 118950). Bar = 10 μ m.

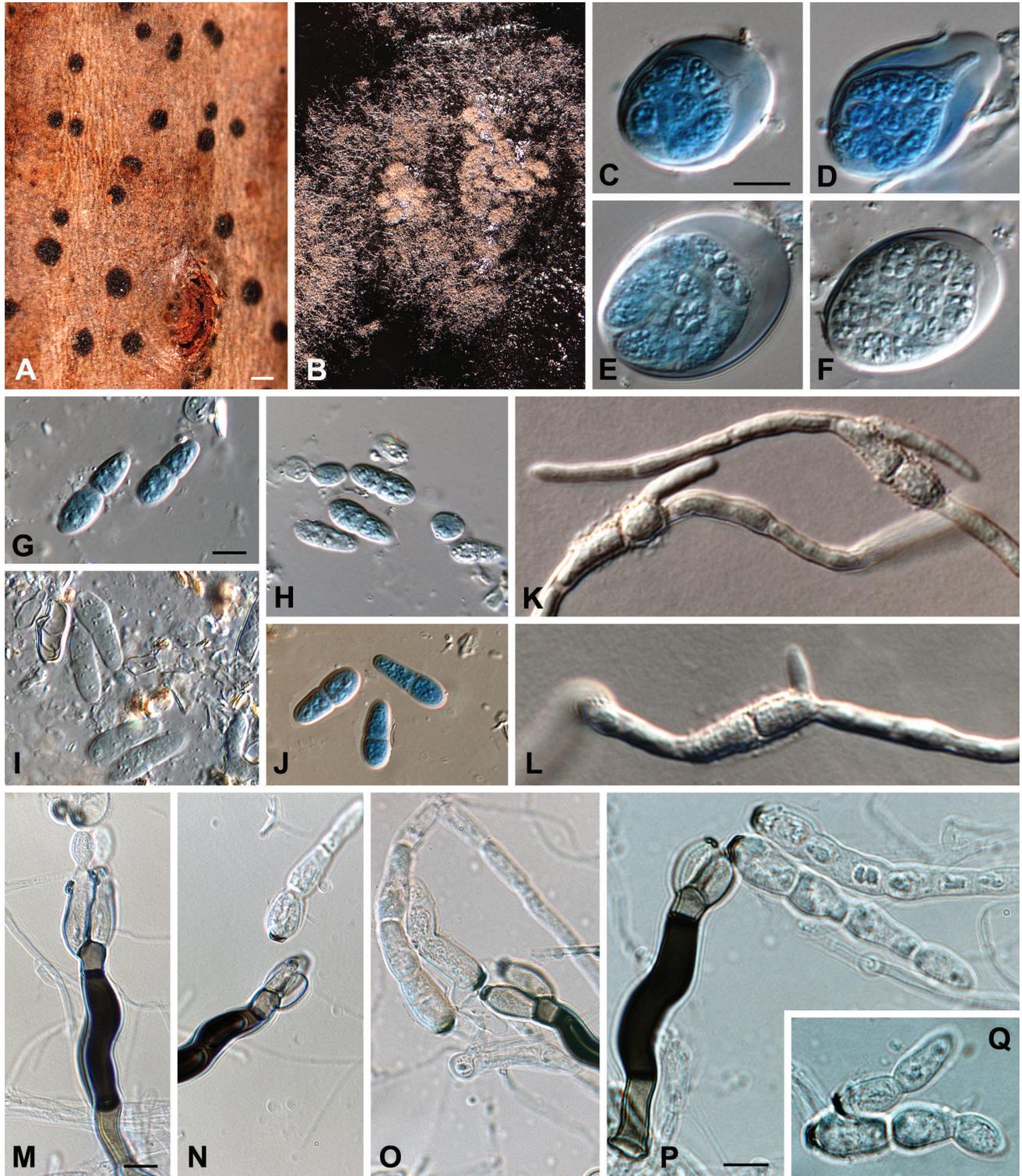


FIG. 4. *Schizothyrium pomi* and its *Zygothiala* anamorph. A. Thyrothecia occurring on a *Rhus* stem. B. Ascomatal initials forming on oatmeal agar. C-F. Asci. G-J. Ascospores. K-L. Germinating ascospores. M-Q. Conidiophores and conidia in vitro. Bars: C = 6, G = 5, M, P = 8 μ m.

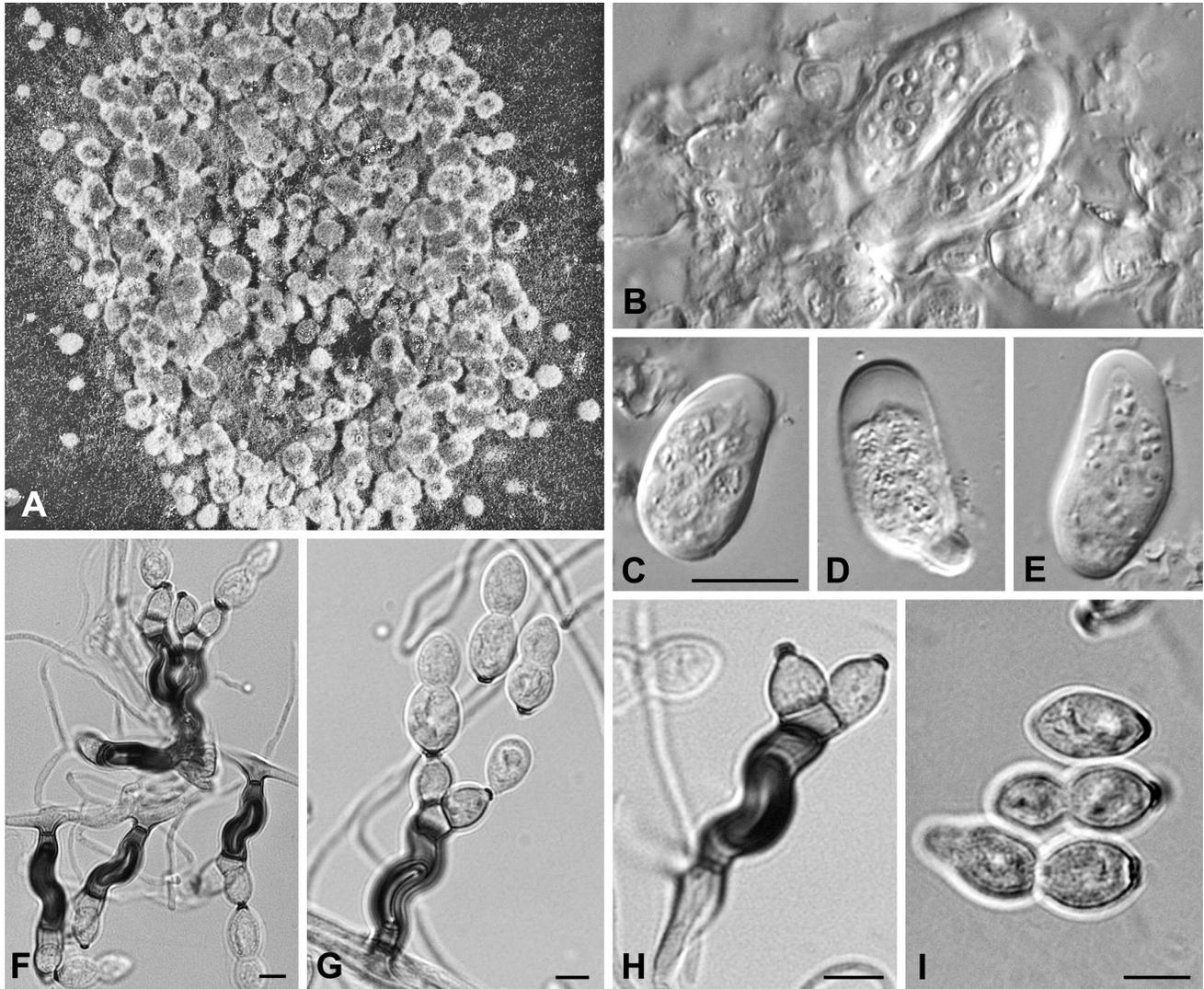


FIG. 5. *Zygophiala cryptogama* on oatmeal agar (CBS 118949). A. Ascomatal initials. B–E. Asci. F–H. Conidiophores. I. Conidia. Bars: C = 13, F = 4, G, H = 5, I = 6 μm .

Notes. The link between *Schizothyrium pomi* and *Zygophiala jamaicensis* was established by Durbin et al (1953), who inoculated apple fruit with ascospores, which resulted in both the teleomorph and anamorph states developing. This relationship has been observed numerous times subsequently and has not yet been questioned. However, when Martyn (1945) described *Z. jamaicensis* from banana leaves collected in Jamaica, conidiophores were observed to be $16\text{--}24 \times 4\text{--}5 \mu\text{m}$ and conidia $15\text{--}18 \times 4\text{--}5 \mu\text{m}$. In the present study we found that neither of these measurements overlapped with those of the *Zygophiala* anamorph of *S. pomi*. Although the relationship between *Schizothyrium* and *Zygophiala* is correct, our data suggest that the anamorph of *S. pomi* is an unnamed species of *Zygophiala* and not *Z. jamaicensis*.

***Zygophiala cryptogama* Batzer & Crous, sp. nov.**

FIGS. 3, 5.

Mycobank MB501243.

Etymology. Named after a hidden sexual cycle observed only in culture.

Zygophialae jamaicensis similis, sed conidiis latoribus, $(12\text{--}14\text{--}18\text{--}20) \times (4\text{--}5\text{--}6\text{--}8) \mu\text{m}$, distinguenda.

Conidiophores arising from superficial hyphae, $1.5\text{--}3 \mu\text{m}$ wide, erect, scattered, 3-septate, subcylindrical, irregularly flexuous, consisting of a hyaline supporting cell that gives rise to a smooth, dark brown stipe, $17\text{--}22 \times 4\text{--}5 \mu\text{m}$ (from basal septum to below phialide), terminating in a finely verruculose, medium brown apical cell, $3\text{--}4 \times 4\text{--}5 \mu\text{m}$, that gives rise to two medium brown, finely verruculose, doliiform to elongated subcylindrical, polyblastic conidiogenous

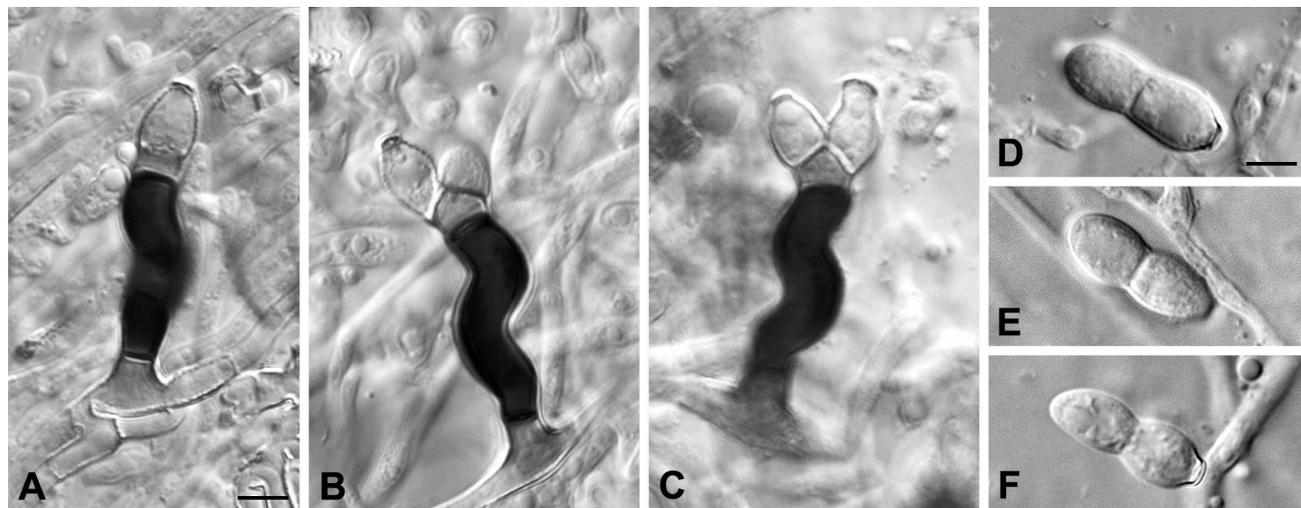


FIG. 6. *Zygophiala tardicrescens* on oatmeal agar (CBS 118946). A–C. Conidiophores. D–F. Conidia. Bars: A = 6, D = 7 μ m.

cells with 1–10 loci, 6–15 \times 5–6 μ m; scars prominent, apical and lateral, darkened, thickened, somewhat refractive, 1–2 μ m wide. *Conidia* solitary, fusiform to obclavate, hyaline, smooth and thick-walled, transversely (0–)1(–2)-septate; aseptate, 6–7(–9) \times 5–6(–7) μ m, 1-septate, (12–)14–18(–20) \times (4–)5–6(–8) μ m, 2-septate, 19–24(–30) \times 5–6(–7) μ m, prominently constricted at septa; apex subobtuse, base subtruncate, with a darkened, thickened hilum, 1–2 μ m wide. Forming fertile, globose ascomata on the surface of OA plates. *Asci* 8-spored, obovoid to ellipsoid, bitunicate, with an apical chamber (note that this is inconspicuous in *S. pomi*), 20–25 \times 12–13 μ m. *Ascospores* multiseriate, hyaline, smooth, fusoid-ellipsoidal, medianly 1-septate, 7–8 \times 3 μ m.

Cultural characteristics. Colonies after 2 wk on OA in the dark flat, spreading, aerial mycelium absent, margins smooth, regular; olivaceous gray throughout; developing submerged to erumpent, globose ascomatal initials.

Specimen examined. USA. IOWA: Iowa Falls, on apple fruit, Sep 2000, J. Batzer, HOLOTYPE 438791, ISOTYPE CBS-H19785, cultures ex-type FVA2a = CBS 118949, GenBank: AY598896, AY598854.

Notes. The globose structures observed embedded and on the surface of OA plates became fertile and were shown to be ascomata. It is interesting to note that all four species form ascomatal initials, although ascospore production was only confirmed in vitro in *Z. cryptogama*.

***Zygophiala tardicrescens* Batzer & Crous, sp. nov.**

FIGS. 3, 6. MycoBank MB501244.

Etymology. Named after its slow growth.

Zygothialae jamaicensi similis, sed coloniis lentius crescentibus et conidiis 20 μ m vel magis longis, 6 μ m vel magis latis distinguenda.

Conidiophores arising from superficial hyphae, 2–3 μ m wide, erect, scattered, 3-septate, subcylindrical, irregularly flexuous, consisting of a hyaline supporting cell that gives rise to a smooth, dark brown stipe, 14–16 \times 5–6 μ m (from basal septum to below phialide), terminating in a finely verruculose, medium brown apical cell, 3–4 \times 4–6 μ m, that gives rise to two medium brown, finely verruculose, doliform to ellipsoidal, polyblastic conidiogenous cells, 7–10 \times 5–6 μ m, with 1–2 prominent scars, apical and lateral, darkened, thickened, somewhat refractive, 2 μ m wide. *Conidia* solitary, fusiform to obclavate, hyaline, smooth and thick-walled, granular, transversely 1-septate (rarely median), (13–)16–20(–23) \times (6–)7–8 μ m, prominently constricted at the septum; apex obtuse, base subtruncate, with a darkened, thickened hilum, 2 μ m wide.

Cultural characteristics. Colonies after 2 wk on OA in the dark flat, spreading, aerial mycelium absent, margins smooth, and somewhat irregular; olivaceous gray in the center, with a thin, white outer margin, and a reddish pigment that diffuses into the agar.

Specimen examined. USA. IOWA: Indianola, on apple fruit, Sep 2000, J. Batzer, HOLOTYPE 438792, ISOTYPE CBS-H19788, cultures ex-type MWA1a = CBS 118946, GenBank: AY598856.

Notes. *Zygophiala tardicrescens* is morphologically distinct from other species of *Zygothiala* by having conidia intermediate in size between those of *S. pomi* and *Z. jamaicensis* (see key below).

***Zygothiala wisconsinensis* Batzer & Crous, sp. nov.**

FIGS. 3, 7. MycoBank MB501245.

Etymology. Named after its type locality, Wisconsin, USA.

Zygothialae jamaicensi similis, sed coloniis celerius

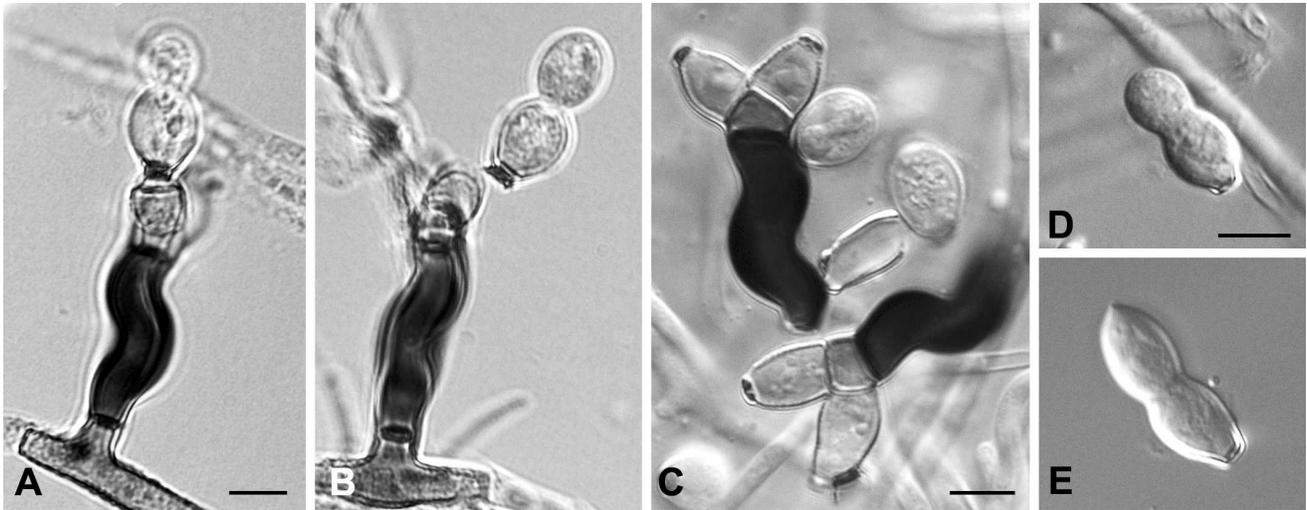


FIG. 7. *Zygophiala wisconsinensis* on oatmeal agar (CBS 118950). A–C. Conidiophores. D–E. Conidia. Bars: A, C = 6, D = 7 μ m.

crescentibus et conidiis 20 μ m vel magis longis, 6 μ m vel magis latis distinguenda.

Conidiophores arising from superficial hyphae, 2–3 μ m wide, erect, scattered, 3–4-septate, subcylindrical, irregularly flexuous, consisting of a hyaline supporting cell that gives rise to a smooth, dark brown stipe, 15–20 \times 4–7 μ m (from basal septum to below phialide), terminating in a finely verruculose, medium brown apical cell, 3–4 \times 4–5 μ m, that gives rise to two medium brown, finely verruculose, dolii-form to ellipsoidal, polyblastic conidiogenous cells, 7–11 \times 5–6 μ m, with 1–2 prominent scars, apical and lateral, darkened, thickened, somewhat refractive, 2 μ m wide. *Conidia* solitary, fusiform to obclavate, hyaline, smooth and thick-walled, granular, aseptate, 6–8 \times 6–8 μ m, or transversely 1-septate (rarely median), (13–)15–18(–23) \times (6–)7–8 μ m, prominently constricted at the septum; apex obtuse, base subtruncate, with a darkened, thickened hilum, 2–3 μ m wide.

Cultural characteristics. Colonies after 2 wk on OA in the dark flat, spreading with moderate aerial mycelium and smooth, regular margins; pale olivaceous gray in the middle, with a large, dirty white to cream outer zone.

Specimen examined. USA. WISCONSIN: New Munster, on apple fruit, Sep 2000, J. Batzer, HOLOTYPE 438790, ISOTYPE CBS-H19786, cultures ex-type MSTA8a = CBS 118950, GenBank: AY598897, AY598853.

Notes. Morphologically *Z. wisconsinensis* is similar to *Z. tardicrescens*. However the two species can be distinguished easily in culture because *Z. wisconsinensis* grows relatively rapidly, reaching 13.5–22.5 mm diam on MEA after 2 wk at 25 C, while *Z. tardicrescens*, reached only 2.5–4.5 mm.

DISCUSSION

The present study has revealed several novel findings. First, flyspeck can be caused by at least four species of *Zygophiala*. Although several papers have commented on cultural variation among isolates of *Zygophiala* (Durbin et al 1953, Baker et al 1977), the genus until now has been accepted as monotypic, having a wide host range and geographic distribution. The fact that several species are involved strongly questions reports on host and geographic distribution of *Z. jamaicensis*. However all strains of *S. pomi* available in the CBS culture collection appear to be a single species, conspecific with the many apple isolates included in this study. It appears therefore that the majority of records reporting *S. pomi* from different hosts could be correct, but that records reporting *Z. jamaicensis* should be considered with care. *Z. jamaicensis* originally was described from banana leaves collected in Jamaica, with conidia cited as being 15–18 \times 4–5 μ m (Martyn 1945). Ellis (1971) reported conidia to be 13–20 \times 5–6 μ m, while Williamson and Sutton (2000) cited them as 13–20 \times 4–6 μ m, whereas the present study found conidia of *S. pomi* to be 1(–)7-septate, prominently constricted at septa, (20–)22–25(–30) \times 5–7(–8) μ m if 1-septate, but up to 110 μ m long if 7-septate. Thus it is likely that there are additional *Zygophiala* species associated with flyspeck signs.

The genus *Mycosphaerella* currently is characterized by pseudothecial ascomata that vary in wall thickness (Crous 1998, Crous et al 2004a), position on or in the host substrate (Crous 1998) and superficial stromatal development, which usually gives rise to an associated cercosporoid anamorph (Crous et al 2004b, 2006).

Although reports have shown that some species of *Mycosphaerella* may form ascospores that are 3-septate (*Sphaerulina* s. str.) (Crous et al 2003), taxa placed in *Mycosphaerella* generally have 1-septate, hyaline to pale brown ascospores, with or without a sheath, and lack any pseudoparaphyses, although some taxa do have remnants of the hamothecium that still could be visible among asci (Crous et al 2004b, 2006). As far as we are aware however ours is the first report of a fungus with a thyrothecial ascoma that is phylogenetically closely related to *Mycosphaerella*. The genus *Schizothyrium*, which is based on *S. pomi*, traditionally has been placed in the family Schizothyriaceae of the Dothideales (von Arx and Müller 1975). The Dictionary of Fungi (Kirk et al 2001) placed *Schizothyrium* (Schizothyriaceae) in the Microthyriales, characterized by strongly flattened, crustose, rounded or elongated ascomata, opening by irregular splits, with bitunicate asci lacking an apical chamber (but see descriptions above), and some interascal tissue composed of remnants of stromatal cells, and transversely 1-septate, hyaline to pale brown ascospores. In Myconet Eriksson (2006) placed *Schizothyrium* (Schizothyriaceae) in the Dothideomycetes, which agrees with phylogenetic data.

Our findings that *Mycosphaerella* was paraphyletic was unexpected. As part of the Fungal Tree of Life project Schoch et al (2006) used a data matrix consisting of 4 loci (nuc SSU rDNA, nuc LSU rDNA, tef1, RPB2), showing that the genus *Mycosphaerella* resides in the Dothideomycetes, subclass Dothideomycetidae, order Capnodiales. *Schizothyrium* appears to be within the Mycosphaerellaceae in our rDNA analyses, but other gene trees need to be examined to confirm this relationship.

Our findings provide the first evidence that one part of the SBFS complex, flyspeck, is caused by at least four species of fungi rather than a single species. Because only a small portion of the geographic range of SBFS fungi was examined in our surveys it is likely that additional flyspeck species remain to be discovered. As the full range of genetic diversity in SBFS causing organisms is revealed the environmental biology and geographic range of each species must be clarified to improve the effectiveness of SBFS management practices.

KEY TO SPECIES OF *ZYGOPHIALA*

- 1. Conidia (0–)1 to multiseptate on OA 2
- 1. Conidia (0–)1-septate on OA 3
- 2. One-septate conidia (20–)22–25(–30) × 5–7(–8) μm. *Schizothyrium pomi*
- 2. One-septate conidia (12–)14–18(–20) × (4–)5–6(–8) μm *Zygothiala cryptogama*

- 3. One-septate conidia shorter than 20 μm, and narrower than 6 μm; conidia 15–18 × 4–5 μm *Zygothiala jamaicensis*
- 3. One-septate conidia 20 μm or longer, and 6 μm or wider; conidia 13–23 × 6–8 μm 4
- 4. Colonies fast-growing, reaching 13.5–22.5 mm diam on MEA after 2 wk at 25 C. *Zygothiala wisconsinensis*
- 4. Colonies slow-growing, reaching 2.5–4.5 mm diam on MEA after 2 wk at 25 C. *Zygothiala tardicrescens*

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