Mycologia, 108(2), 2016, pp. 292–302. DOI: 10.3852/15-036

Ancestral state reconstruction infers phytopathogenic origins of sooty blotch and flyspeck fungi on apple

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Abstract: Members of the sooty blotch and flyspeck (SBFS) complex are epiphytic fungi in the Ascomycota that cause economically damaging blemishes of apples worldwide. SBFS fungi are polyphyletic, but approx. 96% of SBFS species are in the Capnodiales. Evolutionary origins of SBFS fungi remain unclear, so we attempted to infer their origins by means of ancestral state reconstruction on a phylogenetic tree built utilizing genes for the nuc 28S rDNA (approx. 830 bp from near the 5′ end) and the second largest subunit of RNA polymerase II (*RPB2*). The analyzed taxa included the well-known genera of SBFS as well as non-SBFS fungi from seven families within the Capnodiales. The non-SBFS taxa were selected based on their distinct ecological niches, including plant-parasitic and saprophytic species. The phylogenetic analyses revealed that most SBFS species in the Capnodiales are closely related to plant-parasitic fungi. Ancestral state reconstruction provided strong evidence that plant-parasitic fungi were the ancestors of the major SBFS lineages. Knowledge gained from this study may help to better understand the ecology and evolution of epiphytic fungi.

Key words: Dothideomycetidae, multilocus phylogeny

Submitted 28 Feb 2015; accepted for publication 7 Nov 2015.
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Introduction

Fungi in the sooty blotch and flyspeck (SBFS) complex are epiphytes that form tightly adhering colonies on the surface of living fruits of apples and many other plant hosts, such as pears, plantains, mangos and persimmons, in humid production regions around the world (Gleason et al. 2011). SBFS fungi colonize the waxy epicuticle with dark superficial mycelia without penetrating the underlying living tissue. In North America heavily infected fruit are not marketable as fresh produce, which can result in economic losses for fresh-market apples of up to 90% (Williamson and Sutton 2000).

Analyses of rDNA sequences coupled with assessment of morphological characteristics has revealed more than 80 putative species in the SBFS complex (Batzer et al. 2005, Díaz Arias et al. 2010, Mayfield et al. 2012). These findings represent a vast expansion of documented diversity in the SBFS complex, because only four species, *Peltaster fructicola*, *Zygophiala jamaicensis*, *Leptodontidium elatius* and *Geastrumia polystigmatis*, had been recognized in the previous 165 y of research (Williamson and Sutton 2000). Survival under prolonged exposure to ultraviolet (UV) radiation, desiccation and limited-nutrient conditions suggests that SBFS taxa share key ecological traits, although they appear to be polyphyletic (Batzer et al. 2005, Diaz Arias et al. 2010, Li et al. 2012, Mayfield et al. 2012). However, no study has addressed the evolutionary history of these fungi or inferred the direction of transitions between SBFS fungi and their parasitic or saprophytic relatives. By studying SBFS fungi we may learn more about the evolution and adaptation of other microbes that dwell on plant surfaces.

In view of rapidly expanding knowledge of the diversity of the complex, as well as improved methods to suppress the economic damage, an investigation of the biodiversity and evolutionary origins of the SBFS complex was undertaken. Nuclear ribosomal RNA genes have proven useful for phylogenetic inferences in fungi (Lutzoni et al. 2004, Schoch et al. 2012), and the 28S large subunit rDNA (28S) is a commonly used marker to study evolution of symbiosis within the Ascomycota (Fell et al. 2000, Lutzoni et al. 2001, Scorzetti et al.

2002). Efforts to reconstruct the evolutionary origins of fungal groups also have used DNA sequences from the second largest RNA polymerase II subunit gene (*RPB2*) (Liu and Hall 2004, James et al. 2006), a single copy protein-coding gene that has slow sequence divergence and can resolve deep phylogenetic relationships with a high level of reliability (Liu et al. 1999, Liu and Hall 2004). Schoch et al. (2006) reported that *RPB2* provided significant support for nodes in the Dothideomycetes and Lecanoromycetes that were not resolved by rDNA data.

There is an increased interest in analyzing morphological evolution and ecological shifts in fungal survival strategies (Lutzoni et al. 2001, Binder and Hibbett 2006, Gueidan et al. 2007, Ekman et al. 2008). Mapping ecological characters on a molecular phylogeny can provide in-depth information on the evolutionary origin of a particular trait. However, simply generating phylogenetic trees and determining the character states of their terminal taxa to assess ecological character transition may not be adequate to confidently infer which state is primitive or derived. Ancestral state reconstruction has been used to analyze the distribution of traits across extant organisms and to infer the most likely ancestral character states (Pagel 1999, Huelsenbeck and Bollback 2001). Phylogenetic reconstruction with maximum likelihood and Bayesian approaches offers advantages of accommodating phylogenetic ambiguity regarding the evolution of a character and includes an estimation of uncertainty in tree topology and branch lengths. In addition, Bayesian approaches facilitate the statistical testing of hypotheses regarding character trait evolution (Huelsenbeck et al. 2000, Ronquist 2004).

For this study we hypothesized that SBFS fungi are polyphyletic and some SBFS lineages arose from plant-parasitic species. We focused on the Capnodiales, because approximately 96% of identified SBFS species fall within this order (Gleason et al. 2011). In this case each SBFS lineage in the Capnodiales would be closely related to plant parasites rather than saprophytes. A rationale for the premise that plant parasitism is an ancestral state for SBFS fungi was derived from observations that SBFS grouped with plant-parasitic fungi within some families in the Capnodiales in rDNA analyses, although these groups appear to be ecologically distinct. Many SBFS fungi appear to be related to tissue-penetrating, necrotrophic plant parasites in the Mycosphaerellaceae, including fungi in the genera *Cercospora*, *Pseudocercospora*, *Ramularia*, *Ramichloridium* and *Septoria* (Arzanlou et al. 2007, Crous et al. 2007a). In contrast, SBFS fungi colonize the waxy cuticle of plant surfaces without causing necrosis and could be considered epiphytes or saprophytes. However, saprophytes obtain nutrition from dead plant material and SBFS fungi are known only to grow on living plant surfaces.

Integrating epiphytic (SBFS), plant-parasitic and saprophytic species into phylogenetic trees will help us better understand the ecological niches of the SBFS fungal complex. The objectives of this study were: (i) to clarify the evolutionary history of SBFS fungi and closely related non-SBFS fungi within the Capnodiales and; (ii) assess the evolutionary origins and survival strategies of SBFS fungi using ancestral state reconstruction analysis.

MATERIALS AND METHODS

Taxon sampling.—To determine phylogenetic placement, our taxon sampling was focused on fungi belonging to the order Capnodiales, subclass Dothideomycetidae, class Dothideomycetes (TABLE I). Although most SBFS fungi reside in the Capnodiales, some representatives of the Dothi deales and Myriangiales were included in this study to show a deeper phylogeny outside Capnodiales. Within the constraints of time and availability of specimens as many taxa were sampled as possible. For SBFS fungi 23 representative species from 15 anamorphic genera of the Mycosphaerellaceae, Dissoconiaceae, Micropeltidaceae, Schizothyriaceae and Teratosphaeriaceae within Capnodiales were selected from the Gleason personal collection (GPC) at Iowa State University. Five additional SBFS species and four representative non-SBFS species in the Pleosporales (Pleosporomycetidae) were also included to further deepen the phylogeny and confirm that there are alternative origins of SBFS fungi outside the Capnodiales (TABLE I).

To select representatives of the closest relatives of SBFS fungi, BLAST nucleotide queries (National Center for Biotechnical Information, Bethesda, Maryland) were performed with 28S sequences of known SBFS fungi as query sequences. The BLASTn queries showed sequences of high similarity between SBFS taxa and plant-parasitic species, and taxa with the closest matches were used for phylogenetic analyses if cultures of those taxa were available. In contrast, the 28S sequences of SBFS fungi did not show close affinity to saprophytic species. However, known representative saprophytic fungi in the Dothideomycetes were included in the taxon sampling.

Cultures of non-SBFS fungi were obtained from the Centraalbureau voor Schimmelcultures (CBS-KNAW Fungal Biodiversity Centre) in Utrecht, the Netherlands, or the working collection of P.W. Crous (CPC), including at least one representative species of each of the Mycosphaerellaceae, Dissoconiaceae, Capnodiaceae, Davidiellaceae and Teratosphaeriaceae in the Capnodiales (TABLE I). No cultures representing the Antennulariellaceae or Metacapnodiaceae were available. Also Piedraiceae was not represented because this family has not been well resolved within the Capnodiales (Crous et al. 2009). In total seven families within the Dothideomycetes were represented. *Saccharomyces cerevisiae* was used as outgroup taxon for the 28S (HQ262270) and *RPB2* datasets (NM_001183570).

TABLE I. Fungal isolates and DNA sequences used in this study

Species[a]	Culture accession No.		GenBank accession Nos.	
	CBS/CPC[b]	GPC[c]	28S[d]	*RPB2*[e]
Capnodium coffeae	CBS 147.52		DQ247800	KT216519[g]
Capnodium salicinum	CBS 131.34		DQ678050	KT216553[g]
Cercospora apii	CBS 118712		GQ852583	KT216554[g]
Cercospora beticola	CBS 116456		DQ678091	KT216555[g]
Cladosporium cladosporioides	CBS 170.54		DQ678057	DQ677952
Colletogloeopsis-like sp. FG2.1	CBS 125300	NY1-3.2F1c	FJ031986	KT223023[g]
Davidiella macrospora	CBS 138.40		DQ008148	KT223022[g]
Delphinella strobiligena	CBS 735.71		DQ470977	DQ677951
Devriesia strelitziae	CBS 122379		EU436763	GU371738
Dissoconium aciculare	CBS 132082	MSTB4b	JQ622089	KT216556[g]
Dissoconium aciculare	CBS 201.89		GU214418	KT216557[g]
Dothidea insculpta	CBS 189.58		DQ247802	DQ247792
Dothidea sambuci	CBS 198.58		AF382387	KT216559[g]
Elsinoe phaseoli	CBS 165.31		DQ678095	KT216560
Geastrumia polystigmatis	NA[f]	NC4-1.8F1a	KF896877[g]	KT223021[g]
Graphiopsis chlorocephala	CBS 100405		EU009456	KT216520[g]
Hortaea acidophila	CBS 113389		GU323202	KT216521[g]
Houjia pomigena	CBS 125224	UIF2b	AY598925	KT216522[g]
Houjia yanglingensis	CBS 125227	TN1-2.2F1d	FJ147166	KT216550[g]
Leptodontium elatius	NA	Le1021	KF896879[g]	KT216551[g]
Lophiostoma crenatum	CBS 629.86		DQ678069	KT216552[g]
Microcyclospora sp. FG1.9	CBS 125308	MA2-3.5F1c	FJ147169	KT216523[g]
Microcyclospora malicola	CPC 16172	GR61fb	KF896879[g]	KT216524[g]
Microcyclospora pomicola	CPC 16173	SP1-49Fa	GU570551	KT216525[g]
Mycosphaerella graminicola	CBS 292.38		DQ678084	KT216526[g]
Teratosphaeria nubilosa	CBS 116005		DQ246228	KT216527[g]
Mycosphaerella punctiformis	CBS 113265		DQ470968	DQ470920
Myriangium duriaei	CBS 260.36		DQ678059	KT216528[g]
Peltaster fructicola	CBS 125304	KY1-12.2E2b	AY598928	KT223020[g]
Phaeothecoidiella illinoisensis	NA	TN-12.4E1d	GU117902	KT216529[g]
Phaeothecoidiella missouriensis	CBS 118959	AHE7c	AY598917	KT216530[g]
Cyphellophora sessilis	NA	SP1-2386Ca	KF896880[g]	KT216531[g]
Pleomassaria siparia	CBS 279.74		DQ678078	KT216532[g]
Pleospora ambigua	CBS 366.52		AY787937	KT216533[g]
Pseudocercospora fori	CBS 113285		DQ204748	KT356874[g]
Pseudocercospora-like sp. LLS1	NA	NC1-3.7A1a	KF896882[g]	KT216535[g]
Pseudcercospora-like sp. LLS2	NA	KY3-2.2D1b	KF896881[g]	KT216534[g]
Pseudoveronaea ellipsoidea	CBS 125648	MI3-3.4F1a	FJ147154	KT921165[g]
Ramichloridium biverticillatum	CBS 190.63		EU041857	KT921166[g]
Ramichloridium cerophilum	CBS 103.59		EU041855	KT921167[g]
Ramichloridium-like sp. FG9	NA	NC1-2.1E2b	FJ031992	KT921168[g]
Ramichloridium-like sp. FG10	CBS 125310	TN1-1.3F1a	FJ031993	KT216536[g]
Ramularia miae	CBS 120121		DQ885902	KJ504672
Ramularia pratensis var. pratensis	CPC 11294		EU019284	KT216537[g]
Ramularia like sp. P5	CBS 119227	UME2a	AY598910	KT216538[g]
Schizothyrium pomi	CBS 125312	VA1-7A1d	FJ147155	KT216539[g]

TABLE I. Continued

Species[a]	Culture accession No.		GenBank accession Nos.	
	CBS/CPC[b]	GPC[c]	28S[d]	*RPB2*[e]
Scleroramularia abundans	CBS 128078	T129A1c	FR716667	KT216540[g]
Scleroramularia pomigena	CBS 128072	MA5-3.5Cs3a	FR716673	KT216541[g]
Scorias spongiosa	CBS 325.33		GU214696	KT216542[g]
Septoria apiicola	CBS 400.54		GQ852674	KT921169[g]
Septoria protearum	CBS 778.97		GU214494	KT216543[g]
Stomiopeltis-like sp. RS4.1	CBS 125314	TN1-6.3E2a	FJ147162	KT216544[g]
Stomiopeltis-like sp. RS5.2	CBS 125317	NC1-1.8C1d	FJ147164	KT216545[g]
Uwebraunia commune	CBS 132091	NC1-3.2C1d	JQ622093	KT216546[g]
Uwebraunia commune	CPC 12920		KF251658	KT216558[g]
Zasmidium anthuriicola	CBS 118742		GQ852732	KT216547[g]
Zasmidium cellare	CBS 892.85		EU041878	KT356875[g]
Zasmidium angulare	CBS 132094	GA2-2.7B1a	JQ622096	KT921170[g]
Zygophiala cryptogama	CBS 125658	OH4-1A1a	FJ147157	KT216548[g]
Zygophiala wisconsinensis	CBS 125659	OH4-9A1c	FJ147158	KT216549[g]

[a] SBFS fungi and non-SBFS fungi used in the taxon sampling.
[b] Accession numbers of strains deposited at the Centraalbureau voor Schimmelcultures (CBS), Crous Personal Collection (CPC) Utrecht, the Netherlands.
[c] SBFS taxa from Mark Gleason's personal collection (GPC) at Iowa State University.
[d] GenBank accession numbers for a partial of the 28S large subunit (28S) of the rDNA sequence.
[e] GenBank accession numbers for a partial of the RNA polymerase II gene (*RPB2*).
[f] Not available.
[g] Newly generated sequences.

DNA extraction, PCR amplification and sequencing.—Genomic DNA was extracted from fresh fungal mycelium with the Wizard® Genomic DNA Purification Kit (Promega Corp., Madison, Wisconsin) following the manufacturer's protocol for plant tissue. DNA concentration was measured by a NanoDrop Spectrophotometer ND-1000 3.3 (NanoDrop Technologies Inc., Wilmington, Delaware). DNA extracted from the fungal isolates was used as template for polymerase chain reaction (PCR) to amplify the targeted genes: (i) a partial fragment (~830 bp near the 5′ end) of 28S; and (ii) a partial gene region (1.2 kb fragment) of *RPB2* (conserved regions 5–7). Our data stem primarily from conserved regions 5–7 of *RPB2*, the partial 1 exon sequence of *RPB2* and one intron (42 bp). However, the distribution of introns across the Capnodiales is still not well known.

The 28S region was amplified with primers LROR and LR5 using PCR mixtures and conditions of Vilgalys and Hester (1990). PCR products were purified with Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Amersham Biosciences, Buckinghamshire, UK) and quantified on a NanoDrop Spectrophotometer (ND-1000 3.3) before sequencing.

Primers used for amplification of the partial *RPB2* gene were fRPB2-5F and fRPB2-7cR (Liu et al. 1999). The PCR amplifications were performed in a total volume of 25 μL containing 10–20 ng template DNA, 1X PCR buffer, 1 μL dimethylsulfoxide (DMSO) (5%), 25 mM $MgCl_2$, 0.4 μM of each primer, 0.2 mM of each dNTP and 0.02 U GoTaq® Flexi DNA polymerase (Promega Corporation). The PCR program for the *RPB2* was: an initial denaturation at 95 C for 5 min, followed by 35 cycles of denaturation at 95 C for 1 min, primer annealing at 55 C for 2 min, primer elongation at 72 C for 90 s and a final extension step at 72 C for 10 min (Liu et al. 1999). For *RBP2* the PCR products with desired fragments were excised and purified from agarose gel using Illustra GFX PCR DNA and Gel Band Purification Kit, and the purified PCR products were cloned using pGEM®-T Easy Vector Systems (Promega Corp., Madison, Wisconsin). The positive recombinants were confirmed by PCR using M13 forward and reverse primers. Plasmids were extracted using Illustra™ PlasmidPrep Mini Spin Kit (GE Healthcare). Plasmid DNA was sequenced with T7-2 forward and SP6 reverse primers with Big Dye Terminator 3.1 Chemistry (Applied Biosystems, Foster City, California) with an ABI Prism 3730xl DNA Analyzer (Applied Biosystems) at the DNA Sequencing and Synthesis Facility of the Iowa State University Office of Biotechnology. New sequences generated in this study were deposited in GenBank (TABLE I).

Sequence alignment and phylogenetic analysis.—For the 28S dataset, retrieved sequences from the NCBI GenBank nucleotide database (http://www.ncbi.nlm.nih.gov) were aligned with generated sequences of the SBFS fungi and related non-SBFS fungi in MAFFT 6.0 (Katoh et al. 2005).

The aligned sequences were manually corrected with BioEdit 7.0.9.0 (Hall 1999). Ambiguously aligned characters were excluded from the analysis. Likewise sequence datasets for *RPB2* were aligned in MAFFT; all introns and regions with ambiguously aligned characters (i.e. regions where characters have more than one equally plausible alignment) were excluded. Alignments were deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S17128).

Bayesian analysis.—Bayesian analysis was performed separately for the 28S and *RPB2* datasets. with MrBayes 3.2.2 (Ronquist and Huelsenbeck 2003). A mixed model was used for nucleotide substitutions, letting us sample across the GTR model space. Rate heterogeneity across sites was modeled with gamma distribution. Two independent analyses with four Markov chains were run for 5 million generations for each dataset, saving a tree every 500 generations. The first 200 trees were removed as burn-in. A maximum clade credibility (MCC) tree of the sampled trees in Bayesian MCMC analysis and posterior probabilities (PP) of the clade were summarized in TreeAnnotator (Drummond and Rambaut 2007).

Bayesian ancestral state reconstruction.—A Bayesian MCMC analysis with BayesTraitsMultiState (Pagel et al. 2004) was used to reconstruct ancestral states for nodes of interest within subclass Dothideomycetidae across the posterior distribution of Bayesian trees. For the ancestral state reconstruction each taxon was assigned an ecological character state: (i) plant parasitic; (ii) SBFS epiphytic; or (iii) saprophytic. We used the ADDNODE command to reconstruct the common ancestor for each node of interest in the phylogeny. We performed a reversible-jump Markov chain Monte Carlo (RJMCMC command) (Pagel and Meade 2006) analysis to find the proportional likelihood of the ecological character states at each node. The hyperprior approach was selected to specify an exponential prior seeded from a uniform on the interval 0–30. The MCMC analysis was conducted to run 10 000 000 iterations, sampling every 1000 iterations and discarding the first 100 000 samples as burn-in. The average of the mean values of the proportional likelihoods for each node from the output files generated from BayesMultiState was calculated with Excel.

To test for significance of support for the ancestral state reconstruction at the Capnodiales nodes (28S, node 7; *RPB2*, node 7), we used the FOSSIL command in BayesMulti State. By fixing the node of interest at plant-parasitic and epiphytic states, likelihoods of the trees can be compared (Pagel and Meade 2006). We performed this method for Capnodiales nodes in both 28S and *RPB2* datasets with a RJMCMC analysis. The RJMCMC analysis was conducted to run 10 000 000 iterations, sampling every 1000 iterations and discarding the first 100 000 samples as burn-in for each node fossilized at each state. We used the log of the harmonic mean of the likelihoods to compute Bayes factor as twice the difference between these two numbers. In interpreting Bayes factor, values > 10 indicate very strong support (Kass and Raftery 1995).

RESULTS

28S phylogeny.—The final alignment for the 28S gene had a total length of 847 bp and included 62 taxa. The Bayesian consensus tree for this dataset is illustrated (FIG. 1).

Seven SBFS species including *Pseudocercospora*-like spp. LLS1 and LLS2, *Ramularia*–like sp. P5, *Zasmidium mali*, *Ramichloridium*-like spp. FG9 and FG10 and *Colletogloeopsis*-like sp. FG2.1 grouped with plant-parasitic species, including *Septoria* spp., *Cercospora* spp., *Pseudocercospora fori*, *Ramularia* spp., *Ramichloridium* spp., *Mycosphaerella* spp. and *Zasmidium* spp. in the family Mycosphaerellaceae, in a strongly supported (PP = 1) clade. *Uwebraunia commune* and *Dissoconium aciculare* are reported as both SBFS species and as plant parasites, and they grouped with the SBFS species *Pseudoveronaea ellipsoidea* with a high support (PP = 1) in a clade representing the Dissoconiaceae. Three SBFS species, *Microcyclospora* sp. FG1.9, *M. malicola* and *M. pomicola*, grouped with the plant-parasitic species *Teratosphaeria nubilosa* in family Teratosphaeriaceae with 0.94 posterior probability. In contrast, the represented Micropeltidaceae comprised only SBFS species in a strongly supported (PP = 1) clade that included *Houjia pomigena*, *H. yanglingensis*, *Phaeothecoidiella missouriensis*, *P. illinoisensis*, *Stomiopeltis*-like spp. RS4.1 and RS5.2. Similarly three SBFS species (*Schizothyrium pomi*, *Zygophiala cryptogama*, and *Z. wisconsinensis*) formed a monophyletic Schizothyriaceae clade with strong support (PP = 1).

Outside the main groups of SBFS fungi three species formed a clade with moderate posterior probability (PP = 0.94) representing the Capnodiaceae. Three plant-parasitic species formed a well-supported clade (PP = 0.99) representing the Davidiellaceae. One unresolved SBFS species, *Peltaster fructicola*, was placed outside the other families within the Capnodiales, but this genus is not placed at the family rank.

Three of the five SBFS species outside the Capnodiales (*Scleroramularia abundans*, *S. pomigena* and *Geastrumia polystigmatis*) were placed in subclass Pleosporomycetidae incertae sedis with a high posterior probability (PP = 1). Two SBFS species (*Leptodontidium elatius* and *Cyphellophora sessilis*) clustered in a well-supported clade within the Chaethotyriales (PP = 1).

RPB2 *phylogeny*.—The aligned sequences of the *RPB2* region had a total length of 1280 nucleotide characters for the 62 taxa. A maximum clade credibility tree and posterior probabilities were summarized based on 50 001 sampled trees (FIG. 2).

Based on the *RPB2* analysis, placement of the major SBFS lineages within the Capnodiales can be asserted with high confidence (PP = 1) (FIG. 2). The recently

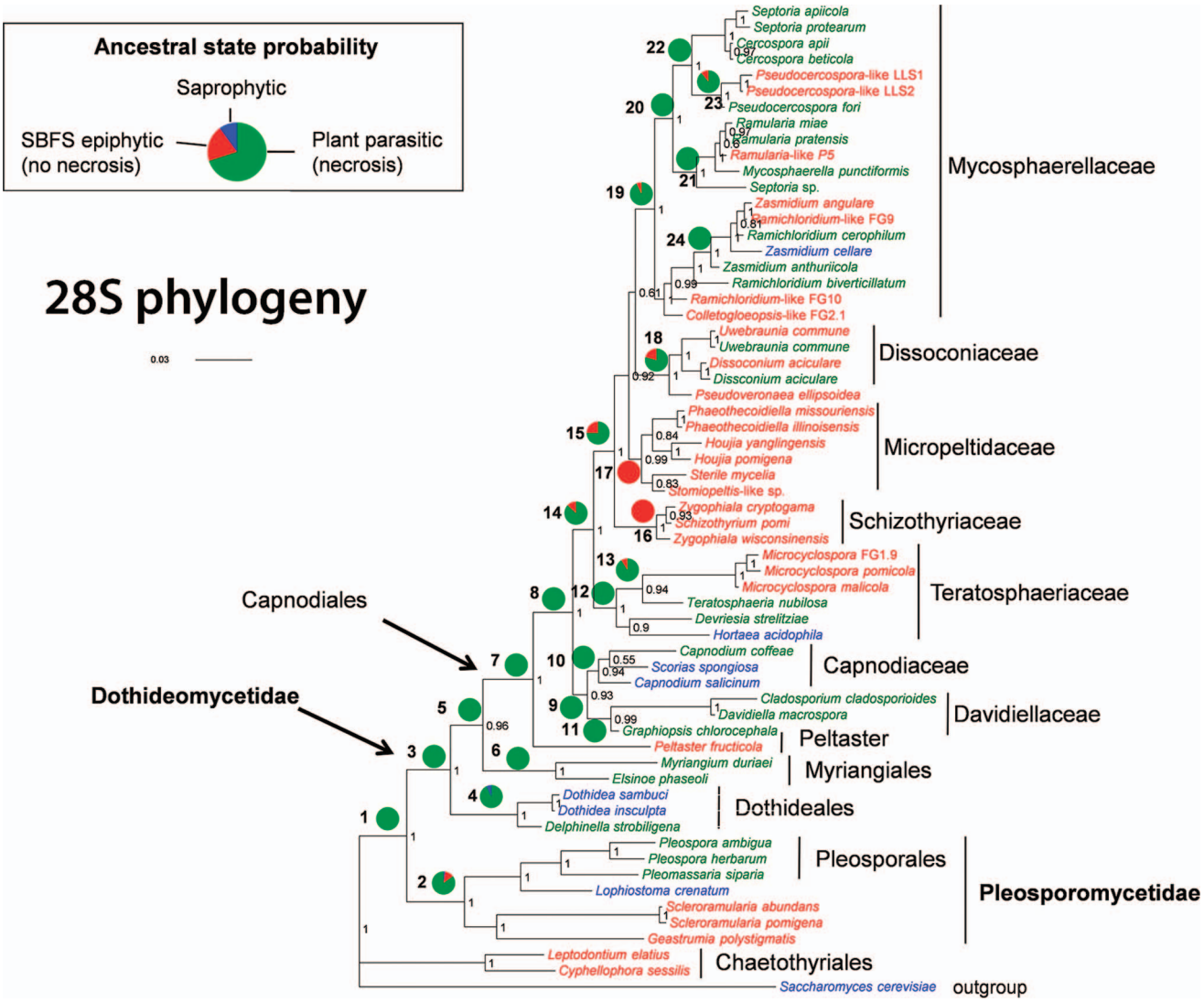

FIG. 1. 28S rDNA phylogeny with a Bayesian MCMC analysis of ancestral state reconstruction of ecological niches. Posterior probabilities for each of the three states are represented in pie charts at each reconstructed node (1–24) (TABLE II).

recognized families and genera were well-supported by the *RPB2* dataset, except Mycosphaerellaceae, which formed a monophyletic clade with high posterior probability for the 28S gene but was polyphyletic with *RPB2*, apparently due to conflicts between the datasets for resolution of three unidentified SBFS species. These three unidentified SPFS species (*Pseudocercospora*-like sp. LLS2, *Ramichloridium*-like sp. FG10, *Colletogloeopsis*-like sp. FG2.1) grouped within the Mycosphaerellaceae based on the 28S sequences but were found sister to the Dissoconiaceae based on the RPB2 tree, which placed other *Ramnochloridium* spp., *Pseudocercospora*-like sp. LLS1 and the plant-parasitic *Pseudocercospora forii* and spp. in the Mycosphaerellaceae (FIG. 2). Similar incongruence among gene trees has been found for genera and species of Mycosphaerellaceae and has been particularly noted in *Pseudocercospora* spp. (Quaedvlieg et al. 2014). Regardless of the incongruence the three unidentified SBFS species grouped with plant parasites in both gene trees.

In the *RPB2* phylogeny most families and genera in Capnodiales included the same mixtures of SBFS fungi and plant-parasitic species found in the 28S tree. In *Dissoconiaceae* two species known to be both plant parasites and SBFS species (*Dissoconium aciculare*, *Uwebraunia commune*) grouped with the SBFS species *Pseudoveronaea ellipsoidea*, consistent with the 28S analyses. SBFS fungi in the genus *Microcyclospora* have a close relationship with the plant-parasitic species *Teratosphaeria nubilosa* in Teratosphaeriaceae with significant support for the family clade (PP = 1). Schizothyriaceae and Micropeltidaceae each exclusively comprised SBFS species as well-supported, monophyletic clades (PP = 1).

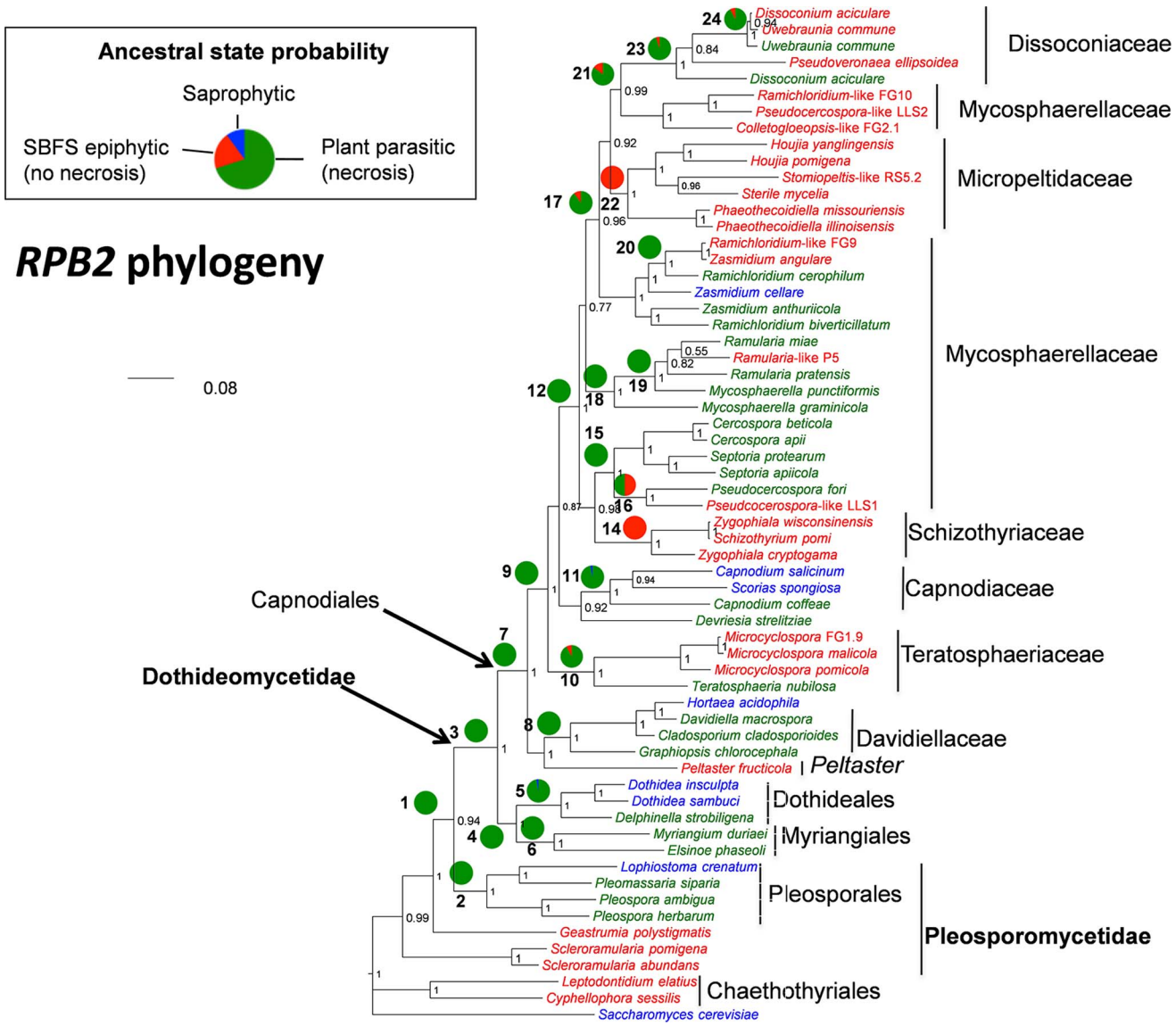

FIG. 2. *RPB2* phylogeny with a Bayesian MCMC analysis of ancestral state reconstruction of ecological niches. Posterior probabilities for each of the three states are represented in pie charts at each reconstructed node (1–24) (TABLE III).

Outside the main SBFS lineages Capnodiaceae and Davidiellaceae had high posterior probability support (PP = 0.92 and PP = 1 respectively). The two orders outside the Capnodiales, Dothideales (PP = 1) and Myriangiales (PP = 1), also formed clades of strong support. Pleosporales, sister to Capnodiales, formed a clade of strong support (PP = 1).

Consistent with the 28S tree, five SBFS species fell outside the Capnodiales based on *RPB2* analyses. *Geastrumia polystigmatis* formed a separate lineage in the Pleosporomycetidae. *Scleroramularia pomigena* and *S. abundans* grouped together (PP = 1) with in the Pleosporomycetidae. *Leptodontium elatius* and *Cyphellophora sessilis* resided in the Chaetothyriales (PP = 1).

Bayesian ancestral state reconstruction.—For the 28S phylogeny 16 nodes were reconstructed as plant parasites with posterior probability = 1 in the BayesTraits analysis (TABLE II, FIG. 1) and eight nodes were reconstructed as such with low to moderate support (PP = 0.76–0.94). Node 3 (Dothideomycetidae) had PP = 1 for plant parasite as the ancestral state. Bayes Factor of the likelihood at node 7 (Capnodiales) indicated strong support for the plant-parasitic state. In contrast, nodes 16 (Schizothyriaceae) and 17 (Micropeltidaceae) each contained only SBFS species, and each was reconstructed with significant support (PP = 1.0) for ancestors with SBFS epiphytic survival strategy. Outside Capnodiales node 4 (Myriangiales) was reconstructed with moderate support (PP = 0.93), whereas

TABLE II. Support values for nodes of the 28S phylogeny for the ancestral state reconstruction of fungal ecological niche

Nodes	Corresponding taxonomic groups	Ancestral state reconstruction of ecological niche (0 = plant parasitic, 1 = epiphytic, 2 = saprophytic) MCMC P(0)	P(1)	P(2)
1	Dothideomycetes	1.00	0.00	0.00
2	Pleosporales	0.85	0.13	0.02
3	Dothideomycetidae	1.00	0.00	0.00
4	Dothideales	0.93	0.00	0.07
5	—	1.00	0.00	0.00
6	Myriangiales	1.00	0.00	0.00
7	Capnodiales	1.00	0.00	0.00
8	—	1.00	0.00	0.00
9	—	1.00	0.00	0.00
10	Capnodiaceae	1.00	0.00	0.00
11	Davidiellaceae	1.00	0.00	0.00
12	Teratosphaeriaceae	1.00	0.00	0.00
13	—	0.91	0.09	0.00
14	—	0.87	0.13	0.00
15	—	0.75	0.25	0.00
16	Schizothyriaceae	0.00	1.00	0.00
17	Micropeltidaceae	0.00	1.00	0.00
18	Dissoconiaceae	0.79	0.21	0.00
19	Mycosphaerellaceae	0.93	0.07	0.00
20	—	1.00	0.00	0.00
21	—	1.00	0.00	0.00
22	—	1.00	0.00	0.00
23	—	0.89	0.11	0.00
24	—	1.00	0.00	0.00

Note: Plant parasitic was coded 0, BFS epiphytic was coded 1, and the state saprophytic was coded 2. For the Bayesian reconstruction analysis (MCMC), the posterior probabilities (PP) of each state are P(0), P(1) and P(2). The nodes refer to those illustrated (FIG. 1).

node 6 (Dothideales) was reconstructed with strong support (PP = 1.0) for a plant-parasitic state.

For the *RPB2* phylogeny 16 nodes were reconstructed with high support (PP = 0.95–1.0) for the plant-parasitic state. Node 3 (Dothideomycetidae) was reconstructed with significant support (PP = 1.0) for the plant-parasitic state. The result of Bayes Factor indicated strong support for the plant-parasitic state at Capnodiales node (28S, node 7, PP = 1.00; *RPB2*, node 7, PP = 1.00). As in the 28S analysis two nodes, 14 (Schizothyriaceae) and 22 (Micropeltidaceae), were reconstructed with high support (PP = 1.0) for an ancestor with a SBFS epiphytic state (TABLE III, FIG. 2). Two nodes outside Capnodiales, 6 (Myriangiales) and 7 (Dothideales), were reconstructed with strong support (PP = 1.0) for the plant-parasitic state.

TABLE III. Support values for nodes of the *RPB2* phylogeny for the ancestral state reconstruction of the fungal ecological niche

Nodes	Corresponding taxonomic groups	Ancestral state reconstruction of ecological niche (0 = plant parasitic, 1 = epiphytic, 2 = saprophytic) MCMC P(0)	P(1)	P(2)
1	—	1.00	0.00	0.00
2	Pleosporales	1.00	0.00	0.00
3	Dothideomycetidae	1.00	0.00	0.00
4	—	1.00	0.00	0.00
5	Dothideales	0.97	0.00	0.03
6	Myriangiales	1.00	0.00	0.00
7	Capnodiales	1.00	0.00	0.00
8	Davidiellaceae	1.00	0.00	0.00
9	—	1.00	0.00	0.00
10	Teratosphaeriaceae	0.92	0.08	0.00
11	Capnodiaceae	0.97	0.00	0.03
12	—	1.00	0.00	0.00
13	—	1.00	0.00	0.00
14	Schizothyriaceae	0.00	1.00	0.00
15	Mycosphaerellaceae	1.00	0.00	0.00
16	—	0.50	0.50	0.00
17	—	0.90	0.10	0.00
18	Mycosphaerellaceae	1.00	0.00	0.00
19	—	1.00	0.00	0.00
20	—	1.00	0.00	0.00
21	—	0.85	0.15	0.00
22	Micropeltidaceae	0.00	1.00	0.00
23	Dissoconiaceae	0.95	0.05	0.00
24	—	0.92	0.08	0.00

Note: The state plant parasitic was coded 0, the state SBFS epiphytic was coded 1 and the state saprophytic was coded 2. For Bayesian reconstruction analysis (MCMC), the posterior probability (PP) of each state is P(0), P(1) and P(2). The nodes refer to those shown illustrated.

DISCUSSION

Phylogenetic analyses based on DNA sequence data from 28S rDNA and *RPB2* indicate that SBFS lineages within the Capnodiales are descendants of plant-parasitic species. Many well-known genera of plant pathogens, as well as most SBFS epiphytic species and

some saprophytic species, belong to the Capnodiales (Crous et al. 2009). Most of the currently recognized families in the Capnodiales, except Mycosphaerellaceae, were supported in both the 28S and *RPB2* trees. In the Mycosphaerellaceae three unidentified species were placed sister to the Dissoconiaceae in the *RPB2* tree, but in both the 28S and *RPB2* trees these unidentified SBFS species were nearest to plant parasites. In both datasets the SBFS fungi were highly polyphyletic and found mostly scattered throughout the Capnodiales. However, these analyses confirm that at least three SBFS species fall within the Pleosporomycetidae and two species within the Chaetothyriales.

The distinction of the SBFS survival strategy from a plant-parasitic state is not clearly seen with most of the SBFS fungi in the Capnodiales. For example within Mycosphaerellaceae SBFS species of *Ramichloridium* shared a close evolutionary history to the plant pathogens *R. cerophilum* and *R. biverticillatum*, which are the causal agents of tropical speckle disease of banana (Arzanlou et al. 2007). SBFS species of *Ramularia* shared a close common ancestor with the plant pathogen *R. miae*, which is the causal agent necrotic of leaf spots disease of *Wachendorfia thyrsiflora* (Gams et al. 1998). In the family Teratosphaericeae, SBFS species in the genus *Microcyclospora* reside with the species *Teratosphaeria nubilosa*, which is a well-known plant pathogen that causes necrotic lesions and blight on *Eucalyptus l*eaves (Hunter et al. 2006).

In the family Dissoconiaceae, sequences of the SBFS species *Uwebraunia commune* and *Dissoconium aciculare* were similar to those of the SBSF fungus *Pseudoveronaea ellipsoidea*. *Uwebraunia commune* causes leaf spots on *Eucalyptus* (Crous et al. 2004) and SBFS on apple and other fruit (Li et al. 2012). However, the niche of *Dissoconium aciculare* is not clear because it originally was described as a hyperparasite on *Erisyphe* (de Hoog et al. 1983) and has been commonly found to cause SBFS in the upper Midwest USA (Batzer et al. 2005). Additionally *D. eucalypti* and *D. australiensis* are leaf pathogens of *Eucalyptus* (Crous et al. 2007b).

The ancestor of two families within the Capnodiales may have been specialized to an epiphytic state. The Micropeltidaceae appear to be a monophyletic group of epiphytes based on our sampling of SBFS species. Although not included in our study, Gregory et al. (2007) identified numerous *Micropeltis* spp. as epifoliar fungi (not causing necrosis) on living leaves of woody tropical plants. Thus formation of colonies on plant surfaces without causing necrosis may be a synapomorphic feature for the Micropeltidaceae, although further phylogenetic analyses are needed to test this hypothesis.

In a second possible case of specialization to an epiphytic state, the family Schizothyriaceae is monophyletic, and all known species form unique, flyspeck-type colonies on fruit and stems of living plants. Flyspecks are clusters of tiny, rounded, black, sclerotium-like bodies without an intercalary mycelial mat (Batzer et al. 2008). Sutton et al. (1998) reported that *Schizothyrium pomi* grew on waxy surfaces of 38 wild plant species near apple orchards in North Carolina. Based on surveys throughout the eastern USA Díaz Arias et al. (2010) also reported that *S. pomi* was a highly cosmopolitan species, in that it was detected in 38 of 39 apple orchards. It is reasonable to speculate that some ancestral lineages of SBFS fungi, especially Schizothyriaceae and Micropeltidaceae, found a successful niche in occupying the waxy surfaces of plants without causing necrosis, which may let them colonize the surfaces of a much broader range of host species.

SBFS fungi apparently have wax-degrading capability, which enables them to dissolve and embed in the epicuticular wax layer of the apple cuticle. Because SBFS fungi do not invade the living tissue below, the infection is only superficial and cosmetic. This behavior of entrenching into the surface medium also can be observed in SBFS cultures grown on agar plates (Batzer et al. 2005). Belding et al. (2000) reported that, based on electron micrographs of flyspeck, *Z. jamaicensis* appeared to metabolize epicuticular wax on apple fruit while avoiding primary contact with the plant's defense response. In contrast, many plant pathogenic species secrete protein effectors that cause disease by altering host cells and the plant immune response (Stergiopoulos and de Wit 2009), and some necrotrophs have acquired a gene-for-gene relationship to interact with the host (Oliver and Solomon 2010).

In addition to cuticular adhesion SBFS fungi would need other characteristics for this epiphytic niche, such as melanization and development of sclerotium-like structures to provide shelter from environmental factors such as periods of low humidity and high UV radiation. Plant parasitism and melanized fungal hyphae, fruiting bodies and spores are common throughout the Ascomycota, but pigmented plant pathogens are particularly prevalent in the Capnodiales. Thus it is not surprising that the SBFS state has arisen repeatedly in the Capnodiales of fungi and that most SBFS reside in this order.

It is evident that species in many lineages of plant parasites in the Capnodiales are capable of causing SBFS on apple fruit, and it appears that in at least two families there may have been a switch from plant parasitism to epiphytism. The genetic basis of the SBFS epiphytic state remains unknown. In the future it will be valuable to use functional genomics approaches to understand how SBFS-epiphytic fungi form tightly adhering colonies and meet the other challenges of

life on plant surfaces. Comparative genomic studies of SBFS fungi and the plant-parasitic species in the *Mycosphaerella* clade can be a potential model system for future study of important biological, ecological and evolutionary questions surrounding adaptation to extreme environments.

ACKNOWLEDGMENTS

We thank the Ministry of Higher Education Malaysia for doctoral scholarship support for first author and Holland Hauenstein for technical support.

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