Identification and Characterization of Fungal Pathogens Causing Fruit Rot of Deciduous Holly

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Abstract

Cut branches of deciduous holly (*Ilex* spp. L.) harboring colorful berries are traditionally used as ornaments in holiday decorations. Since 2012, a fruit rot of unspecified cause has resulted in significant yield reduction and economic losses across Midwestern and Eastern U.S. nurseries. In this study, symptomatic fruit samples collected from nine different locations over five years were analyzed, and several fungal species were isolated. A combination of morphological characterization, multilocus phylogenetic analyses, and pathogenicity assays revealed that *Alternaria alternata* and *Diaporthe ilicicola* sp. nov. were the primary pathogens associated with symptomatic fruit. Other fungi including *A. arborescens*, *Colletotrichum fioriniae*, *C. nymphaeae*, *Epicoccum nigrum*, and species

in the *D. eres* species complex appeared to be minor pathogens in this disease complex. In detached fruit pathogenicity assays testing the role of wounding and inoculum concentration on disease development, disease incidence and severity increased when fruit was wounded and inoculated with a higher inoculum concentration. These findings indicate that management strategies that can protect fruit from injury or reduce inoculum may lower disease levels in the field. This research established the basis for further studies on this emerging disease and the design of research-based management strategies. To our knowledge, it also represents the first report of species of *Alternaria*, *Colletotrichum*, *Diaporthe*, and *Epicoccum* causing fruit rot of deciduous holly.

Members of the genus *Ilex* are deciduous or evergreen ornamental shrubs or trees, ranging in height from 1 to over 30 m (Galle 1997). The deciduous species are popularly used in the cut flower industry for winter holiday decorations because of the beautiful display of colorful fruit they present, varying from red to orange or yellow depending on the hybrid and cultivar, that can persist on the plants throughout the winter (Fig. 1A). The overall sale value of *Ilex* spp. in the cut flower industry is estimated at \$571,279 nationally (NASS 2014). Sales of the cut woody stems allows revenue in late fall and early winter when there is little activity and little other income for this segment of the nursery industry, also contributing to extended employment for seasonal employees.

The three most common deciduous holly species grown in North America are *Ilex decidua* (possumhaw holly), *I. serrata* (Japanese winterberry), and *I. verticillata* (common winterberry), along with their many cultivars and hybrids. Plants are functionally polygamodioecious (mostly dioecious, but with either a few flowers of the opposite sex or a few bisexual flowers on the same plant [Fernald 1950]), requiring both male and female plants with similar flowering periods in the same field for pollination. The fruit of *Ilex* is composed of chartaceous exocarp, juicy mesocarp, and separated coriaceous endocarp. From a botanical perspective, it is neither a berry nor a drupe, and there is a lack of specific terminology for its designation. Although the term “bacco-drupe” was proposed by Hu in 1950 (Galle

1997), there has been a lack of agreement on the use of this term. Therefore, the general term “fruit” will be used throughout this manuscript to refer to the fruit of *Ilex*.

Since 2012, a fruit rot of unspecified cause has been challenging holly growers across the Midwestern and Eastern United States. Affected plants defoliate earlier in the season (September rather than October) and carry undersized fruit that fails to turn color, loses its normal gloss, shrivels, and eventually becomes necrotic (Fig. 1B and C). Some popular cultivars, including Bonfire, Sparkleberry, and Winter Red, are particularly affected. As a consequence, nursery growers are reporting decreased crop yield and, in some cases, complete crop loss. While an official value that quantifies these losses is not available in the literature, in the winter of 2014, several Ohio growers who participated in this project lost their entire crop to this fruit rot (Peduto Hand, personal observation).

In the winter of 2013, at the time of branch harvest, a few symptomatic fruit samples from three Ohio nurseries were sent to the C. Wayne Ellett Plant and Pest Diagnostic Clinic at The Ohio State University for diagnosis. The involvement of bacteria and oomycetes was ruled out through the diagnostic process but several different fungi were isolated from the symptomatic tissues, belonging to the genera *Alternaria* Nees, *Colletotrichum* Corda, *Diaporthe* Nitschke., and undetermined genera in the Botryosphaeriaceae Theiss. & P. Syd. Several species within these fungi are well known and economically important fruit rot pathogens that have been extensively studied on many fruit crops, including apple (Biggs 2004; Gao et al. 2013; Munir et al. 2016), blueberry (Milholland and Daykin 1983; Zhu and Xiao 2015), cranberry (Olatinwo et al. 2003), and strawberry (Curry et al. 2002; Howard et al. 1992). To date, there is no official record of the occurrence of the above fungal genera as pathogens causing fruit rot on deciduous holly.

Understanding this disease problem, including elucidating the components of the disease triangle (pathogen-host-environment), is a critical step toward providing nursery growers with research-based recommendations for effective management. Thus, the initial objectives of our investigation were to (i) confirm identification of the fungal pathogen(s) associated with the disease from samples collected over multiple years and across multiple locations by conducting morphological and molecular studies, and (ii) determine factors that might be contributing to fruit infection. Future research will build upon these findings and will focus on elucidating the sources of pathogen inoculum in the field, the timing of host susceptibility

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Funding: This publication was financed in part or totally through a grant from the Ohio Department of Agriculture Specialty Crop Promotion Program, the State of Ohio, and the United States Department of Agriculture under the provisions of the Specialty Crop Block Grant. This project was supported by the Specialty Crop Block Grant Program at the U.S. Department of Agriculture through grants AGR-SCG-14-08 and AGR-SCG-16-09. This study was also funded by the USDA-NIFA Hatch project #1004939, the T. J. Kavanagh Foundation, and The Ohio State University Department of Plant Pathology.

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Accepted for publication 11 May 2018.

to infection, as well as the role of several environmental factors in disease development. This information will be critical to identify potential management measures, including appropriate timing for horticultural practices and fungicide treatments.

Materials and Methods

Sample collection and fungal isolation. Between 2013 and 2017, 623 symptomatic fruit of deciduous holly comprising cultivars Bonfire, Oosterwijk, Sparkleberry, and Winter Red were received or collected from commercial nurseries, landscapes, and arboreta across the Midwestern and Eastern U.S., including Ohio ($n = 593$), Massachusetts ($n = 10$), North Carolina ($n = 15$), and Pennsylvania ($n = 5$). Samples received from out of state were processed immediately upon receipt, while those collected from field sites in Ohio were transported to the laboratory within a cooler and then kept at 4°C for a maximum of 24 h before being processed. Samples were surface disinfected with 2% Clorox household bleach (The Clorox Company) for 2 min, then rinsed in sterile water three times. Following disinfection, samples were air dried on sterile paper towels in a laminar flow hood. Two pieces of symptomatic tissue comprising the exocarp and mesocarp were excised from the edge of each lesion with a sterile scalpel and transferred to potato dextrose agar (PDA; Difco Laboratories, Sparks, MD) amended with 0.01% tetracycline hydrochloride and 0.015% streptomycin sulfate (Fisher Scientific, Fair Lawn, NJ). All plates were incubated at 25°C under constant light for up to 10 days. Plates were checked daily and any observed fungal growth was subcultured onto PDA. Pure cultures of each isolate were obtained by transferring hyphal tips from the margin of the fungal colonies onto new PDA plates. All isolates were then grown on sterile filter paper and stored at -20°C until further use.

Morphological characterization. Isolates were first identified to genus based on comparison of morphological characteristics of the spores and spore-producing structures with descriptions available in the literature (Barnett and Hunter 1998). Two to 14 isolates of each of the most represented fungal genera (i.e., >4% isolation frequency) were then selected to include all locations ($n = 9$) and years ($n = 5$) of sampling and subject to further characterization (Table 1). Pure cultures of all isolates except *Alternaria* spp. were transferred to fresh PDA and incubated at 23°C with a 12-h photoperiod for up to 4 weeks to induce sporulation. Isolates tentatively identified as *Alternaria* spp. were grown on V8 juice agar as described by Simmons (2007).

The length and width of 30 randomly selected conidia from each isolate included in the study (Table 1) were measured using a Leica DM750 compound microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) and the Leica Application Suite EZ software (version 3.0.0). Mean and standard error of the conidial measurements were calculated for each isolate. Conidial color, shape, and presence or absence of septation were also recorded. Additionally, fungal colony diameter on each of three plates per fungal isolate was measured 3, 5, and 7 days post-inoculation (DPI) to calculate mycelial growth rates (mm day^{-1}). All data were analyzed independently using PROC GLM in SAS (version 9.4; SAS Institute Inc., Cary NC). Tukey's honest significant difference ($\alpha = 0.05$) was used for mean comparisons among isolates.

DNA extraction, amplification, and sequencing. Total genomic DNA was extracted from pure cultures of isolates grown on PDA at 25°C for 7 days using the DNeasy Plant Mini Kit (Qiagen Inc., Hilden, Germany) according to manufacturer's instructions. All PCR amplifications were carried out in a final volume of 20 μl using the Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA). All amplified loci, primers, and PCR conditions are summarized in Table 2. Amplified PCR products were resolved by electrophoresis at 100 V/cm in 1.5% agarose gels in 1 $\times$ Tris-borate-EDTA buffer stained with 0.005% GelRed (Bio-tium Inc., Fremont, CA) and visualized under a UV transilluminator. The size of the fragments was estimated using a 1-kb DNA ladder (New England BioLabs Inc., Ipswich, MA). PCR products were purified using ExoSAP-IT (Affymetrix, Inc., Santa Clara, CA) according to manufacturer's instructions and both strands were sequenced at the Genomics Shared Resource at the Ohio State University Comprehensive Cancer Center using an ABI Prism 3730 DNA analyzer (Applied Biosystems, Foster City, CA). Sequences were manually edited using the software Chromas (v 2.6.4, Technelysium Pty Ltd, Australia) and assembled using MUSCLE in MEGA7 (Kumar et al. 2016). Consensus sequences of each locus were exported and compared with those available in GenBank using the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, Bethesda, MD).

Phylogenetic analyses. The same isolates selected for morphological characterization were subject to phylogenetic analyses (Table 1). Sequences of reference strains recently published in the literature were retrieved from GenBank and included in the analysis for

Fig. 1. Fruit rot symptoms observed on deciduous holly cultivar Sparkleberry in an Ohio nursery. **A**, healthy plants; **B**, overview of symptomatic berries on an entire plant; **C**, close-up of fruit exhibiting rot symptoms on twigs.

comparison (Table 3; Andrew et al. 2009; Damm et al. 2012; Dissanayake et al. 2017; Du et al. 2016; Gomes et al. 2013; Huang et al. 2013; Huang et al. 2015; Machingambi et al. 2015; Mostert et al. 2001; Peever et al. 2004; Rotondo et al. 2012; Tan et al. 2013; Thompson et al. 2015; Udayanga et al. 2012, 2014a, b, 2015; Valenzuela-Lopez et al. 2018; Woudenberg et al. 2015; Yang et al. 2017). Concatenated sequences of different loci for each fungal genus (endoPG, OPA1, and OPA10 for *Alternaria*; TUB and GAPDH for *Colletotrichum*; ITS, HIS, and TUB for *Diaporthe*) were aligned using MUSCLE in MEGA7 (Kumar et al. 2016). The nucleotide substitution model for each dataset was determined using Find-Model (<https://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html>) based on the Akaike Information Criterion (AIC). Each concatenated dataset was subjected to maximum likelihood (ML) analysis in MEGA7 and to Bayesian inference (BI) analysis using MrBayes as implemented in siMba (Mishra and Thines 2014; Ronquist and Huelsenbeck 2003). The heuristic method in ML analysis used subtree-pruning-regrafting with SPR level 5, and branch strength was evaluated using 1,000 bootstrap replications. All gaps and missing data

were deleted. For BI, a Metropolis Coupled Markov Chain Monte Carlo (MCMCMC) analysis was carried out with two independent runs with four chains each for one million generations. The analysis was stopped when the average standard deviation of split frequencies fell below 0.01. Trees were sampled every 500 generations and the first 25% of samples were discarded as “burn-in.” Trees were visualized in FigTree v. 1.4.3 (Rambaut 2016).

Representative sequences for each locus and pathogen species included in this study were deposited into GenBank (Table 1). All isolate cultures are maintained in the fungal collection of the Department of Plant Pathology at The Ohio State University. The novel taxon described in this study was also deposited in the Westerdijk Fungal Biodiversity Institute Collection (CBS-KNAW, Utrecht, The Netherlands) and registered in MycoBank (www.mycobank.org).

Pathogenicity tests. Three sets of pathogenicity experiments, each conducted twice, were carried out to further characterize the isolates included in Table 1. In the first experiment (A), healthy fruit were collected from potted plants of cultivar Sparkleberry and surface-disinfested as previously described. Ten individual fruit were

Table 1. Fungal isolates recovered from symptomatic holly fruit used in this study for morphological characterization, phylogenetic analyses, and pathogenicity tests

Species	Origin ^x	Year	Isolate ^y	Host, cultivar	GenBank accession no. ^z		
					EPG	OPA1	OPA10
<i>Alternaria alternata</i>	MA	2015	FPH2015390	<i>Ilex verticillata</i> , Oosterwijk	MH003571	MH003585	MH003599
<i>A. alternata</i>	MA	2015	FPH2015396	<i>I. verticillata</i> , Oosterwijk	MH003573	MH003587	MH003601
<i>A. alternata</i>	OH	2015	FPH2015338	<i>I. verticillata</i> × <i>I. serrata</i> , Sparkleberry	MH003570	MH003584	MH003598
<i>A. alternata</i>	OH	2015	FPH2015468	<i>I. verticillata</i> × <i>I. serrata</i> , Bonfire	MH003574	MH003588	MH003602
<i>A. alternata</i>	OH	2015	FPH2015470	<i>I. verticillata</i> × <i>I. serrata</i> , Sparkleberry	MH003575	MH003589	MH003603
<i>A. alternata</i>	OH	2015	FPH2015507*	<i>I. verticillata</i> × <i>I. serrata</i> , Sparkleberry	MH003576	MH003590	MH003604
<i>A. alternata</i>	OH	2015	FPH2015567	<i>I. verticillata</i> × <i>I. serrata</i> , Sparkleberry	MH003577	MH003591	MH003605
<i>A. alternata</i>	OH	2015	FPH2015589	<i>I. verticillata</i> × <i>I. serrata</i> , Bonfire	MH003578	MH003592	MH003606
<i>A. alternata</i>	OH	2015	FPH2015591	<i>I. verticillata</i> × <i>I. serrata</i> , Bonfire	MH003579	MH003593	MH003607
<i>A. alternata</i>	OH	2015	FPH2015592	<i>I. verticillata</i> × <i>I. serrata</i> , Bonfire	MH003580	MH003594	MH003608
<i>A. alternata</i>	OH	2016	FPH2015597	<i>I. verticillata</i> × <i>I. serrata</i> , Sparkleberry	MH003581	MH003595	MH003609
<i>A. alternata</i>	OH	2016	FPH2016709	<i>I. verticillata</i> × <i>I. serrata</i> , Bonfire	MH003582	MH003596	MH003610
<i>A. alternata</i>	OH	2016	FPH2016711	<i>I. verticillata</i> × <i>I. serrata</i> , Bonfire	MH003583	MH003597	MH003611
<i>A. arborescens</i>	MA	2015	FPH2015395	<i>I. verticillata</i> , Oosterwijk	MH003572	MH003586	MH003600
					GAPDH	TUB	
<i>Colletotrichum fioriniae</i>	MA	2015	FPH2015392	<i>I. verticillata</i> , Oosterwijk	MH003615	MH003623	MH003615
<i>C. fioriniae</i>	OH	2013	FPH201317	<i>I. verticillata</i> × <i>I. serrata</i> , Sparkleberry	MH003613	MH003621	MH003613
<i>C. fioriniae</i>	OH	2013	FPH201318	<i>I. verticillata</i> × <i>I. serrata</i> , Sparkleberry	MH003614	MH003622	MH003614
<i>C. fioriniae</i>	OH	2015	FPH2015466*	<i>I. verticillata</i> × <i>I. serrata</i> , Bonfire	MH003616	MH003624	MH003616
<i>C. fioriniae</i>	OH	2015	FPH2015564	<i>I. verticillata</i> × <i>I. serrata</i> , Bonfire	MH003617	MH003625	MH003617
<i>C. fioriniae</i>	OH	2016	FPH2016720	<i>I. verticillata</i> × <i>I. serrata</i> , Sparkleberry	MH003619	MH003627	MH003619
<i>C. fioriniae</i>	OH	2013	FPH201314	<i>I. verticillata</i> × <i>I. serrata</i> , Sparkleberry	MH003612	MH003620	MH003612
<i>C. nymphaeae</i>	OH	2015	FPH2015590	<i>I. verticillata</i> × <i>I. serrata</i> , Bonfire	MH003618	MH003626	MH003618
					HIS	ITS	TUB
<i>Diaporthe eres</i>	MA	2015	FPH2015394	<i>I. verticillata</i> , Oosterwijk	MH003629	MH003632	MH003635
<i>D. eres</i>	OH	2013	FPH201322	<i>I. verticillata</i> × <i>I. serrata</i> , Bonfire	MH003628	MH003631	MH003634
<i>D. eres</i>	PA	2017	FPH2017229	<i>I. verticillata</i> × <i>I. serrata</i> , Bonfire	MH003630	MH003633	MH003636
<i>D. ilicicola</i> sp. nov.	OH	2015	FPH2015472	<i>I. verticillata</i> × <i>I. serrata</i> , Sparkleberry	MH171082	MH171062	MH171072
<i>D. ilicicola</i> sp. nov.	OH	2015	FPH2015473	<i>I. verticillata</i> × <i>I. serrata</i> , Sparkleberry	MH171083	MH171063	MH171073
<i>D. ilicicola</i> sp. nov.	OH	2015	FPH2015502*	<i>I. verticillata</i> × <i>I. serrata</i> , Bonfire	MH171084	MH171064	MH171074
<i>D. ilicicola</i> sp. nov.	OH	2015	FPH2015509	<i>I. verticillata</i> × <i>I. serrata</i> , Sparkleberry	MH171085	MH171065	MH171075
<i>D. ilicicola</i> sp. nov.	OH	2015	FPH2015558	<i>I. verticillata</i> × <i>I. serrata</i> , Sparkleberry	MH171086	MH171066	MH171076
<i>D. ilicicola</i> sp. nov.	OH	2015	FPH2015598	<i>I. verticillata</i> × <i>I. serrata</i> , Sparkleberry	MH171087	MH171067	MH171077
<i>D. ilicicola</i> sp. nov.	NC	2016	FPH2016671	<i>Ilex</i> sp.	MH171088	MH171068	MH171078
<i>D. ilicicola</i> sp. nov.	OH	2016	FPH2016702	<i>I. verticillata</i> × <i>I. serrata</i> , Bonfire	MH171089	MH171069	MH171079
<i>D. ilicicola</i> sp. nov.	OH	2016	FPH2016706	<i>I. verticillata</i> × <i>I. serrata</i> , Bonfire	MH171090	MH171070	MH171080
<i>D. ilicicola</i> sp. nov.	OH	2016	FPH2016725	<i>I. verticillata</i> × <i>I. serrata</i> , Bonfire	MH171091	MH171071	MH171081
					ITS		
<i>Epicoccum nigrum</i>	OH	2015	FPH2015417*	<i>I. verticillata</i> × <i>I. serrata</i> , Bonfire	MH003637		
<i>E. nigrum</i>	OH	2015	FPH2015505	<i>I. verticillata</i> × <i>I. serrata</i> , Bonfire	MH003638		

^x MA = Massachusetts; NC = North Carolina; OH = Ohio; PA = Pennsylvania.

^y Isolates with an asterisk (*) were selected for use in pathogenicity experiments B and C.

^z EPG = endopolygalacturonase; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HIS = histon H3; OPA1 and OPA10 = anonymous noncoding region; TUB = β -tubulin; ITS = internal transcribed spacer.

used for each isolate. Each fruit was wounded using a sterile needle and point inoculated using a micropipette with 8 µl of a sterile water-Tween 20 solution (0.05% v/v) containing 10⁵ conidia/ml of each isolate. Ten wounded fruit inoculated with the same amount of sterile water-Tween 20 solution served as controls. All fruit were placed on a plastic tray in a completely randomized design and the entire tray was enclosed in a moist chamber made of a clear plastic bag containing a wet sterile paper towel. Moist chambers were incubated at 25°C for up to 4 weeks and fruit rot incidence was recorded for each isolate. Visual examination and identification of spores on symptomatic tissues as well as fungal reisolation and morphological identification of the isolates were performed to fulfill Koch's postulates.

One isolate per fungal species was selected from those used in experiment A. In two additional experiments (B and C), fruit were wounded, inoculated, and incubated as previously described. In both experiments, wounded and unwounded fruit inoculated with sterile water served as controls. Experiment B was conducted to test the effects of spore concentration and wounding on fruit infection. Experiments were carried out using a series of spore suspensions of each fungal species ranging from 10³ to 10⁶ conidia/ml. Inoculum was prepared by suspending spores from 7- to 21-day-old PDA cultures in sterile distilled water and by adjusting the concentration using a hemacytometer. Ten individual fruit per treatment were arranged in a complete randomized block design with six blocks. Fruit incidence and severity were recorded weekly for up to 6 weeks post inoculation. Finally, experiment C was conducted to test the effects of combined pathogen inoculum, spore concentration, and wounding on fruit infection. Spore suspensions at a concentration of 10², 10³, and 10⁵ conidia/ml were initially obtained for each fungal species. For each spore concentration, two pathogen species were combined (1:1 v/v) to obtain the fungal treatment. All combinations of fungal species and spore concentrations were tested using six single fruit per treatment arranged in a complete randomized block design with six blocks. Disease evaluation was the same as previously described.

In all experiments, treatment effects were analyzed using PROC MIXED in SAS (version 9.4; SAS Institute Inc., Cary NC). Combined datasets from the two runs of each experiment were analyzed when results were in congruence. All data were arcsine transformed before statistical analyses were performed. Treatment means and 95% confidence intervals were calculated in a transformed scale and back-transformed to present results. Least squares means

(LS-means; $\alpha = 0.05$) were used for mean comparisons among treatments.

Results

Sample collection and fungal isolation. Three hundred and four symptomatic fruit samples out of the 623 processed resulted in the isolation of a total of 121 fungal isolates. Species of *Diaporthe* and *Alternaria* were the most frequently isolated fungi (39.47 and 32.89%, respectively), followed by the genera *Colletotrichum*, *Epicoccum* Link, *Cladosporium* Link, *Botryosphaeriaceae*, *Fusarium* Link, and *Phoma* Sacc. (<10%; Table 4). In a few cases, two fungi were isolated from the same lesion (Table 4). No fungal growth was observed from the remaining 319 samples.

Isolate characterization. For clarity of reporting, combined morphological and molecular characterization of the isolates is reported below.

Alternaria spp. Fourteen isolates tentatively identified as *Alternaria* sp. were assigned to four morphological groups based on colony and conidial morphology as well as sporulation patterns (Barnett and Hunter 1998). Ten isolates in the first group developed distinctive concentric rings of growth and sporulation. Isolates produced a primary chain of 3 to 11 conidia and abundant lateral secondary chains of 2 to 5 conidia. Conidia were brown, ellipsoid, with 3 to 6 transverse septa and sometimes one longitudinal septum. Characteristics were consistent with the description of *Alternaria alternata* (Fr.) Keissl. (Simmons 2007). Two isolates (FPH2015470 and FPH2015591) in the second group formed long chains of 4 to 9 conidia and occasionally secondary chains of 2 to 4 conidia. Conidia were wide ellipsoid or ovoid, constricted, with 4 to 7 transverse septa and rarely 1 longitudinal septum. Isolates in this group were tentatively identified as *A. tenuissima* (Nees) Wiltshire (Simmons 2007). One isolate (FPH2015338) in the third group was characterized by short primary chains of 4 to 7 conidia and occasionally secondary chains of 1 to 3 conidia. Conidia were wide ellipsoid or ovoid, constricted, and with 3 to 6 transverse septa and 1 to 2 longitudinal septa. These characteristics were consistent with the description of *A. toxicogenica* E. G. Simmons (Simmons 2007). One isolate (FPH2015395) in the fourth group produced long conidiophores and primary chains of 3 to 7 conidia and abundant secondary and tertiary chains of 1 to 3 conidia. Conidia were ellipsoid with 3 to 5 transverse septa and 0 to 2 longitudinal septa. This isolate was tentatively identified as *A. arborescens* E. G. Simmons (Simmons 2007). The

Table 2. Amplified loci, primers, PCR conditions, and references for each fungal genus characterized in this study

Fungal genus	Locus ^y	Primers (fw/rev)	PCR conditions	References	
				Primers	PCR ^z
<i>Alternaria</i>	endoPG	PG3/PG2b	95°C 1 min; (95°C 30 s, 57°C 30 s, 72°C 30 s) × 35 cycles; 72°C 7 min	Andrew et al. 2009	Andrew et al. 2009
	OPA1	OPA1-3L/OPA1-3Rb	94°C 1 min; (94°C 20 s, 56°C 30 s, 72°C 40 s) × 35 cycles; 72°C 7 min	Peever et al. 2004	Peever et al. 2004
	OPA10	OPA10-2L/OPA10-2R	95°C 1 min; (95°C 30 s, 57°C 30 s, 72°C 30 s) × 35 cycles; 72°C 7 min	Andrew et al. 2009	Andrew et al. 2009
<i>Colletotrichum</i>	ITS	CaInt2/ITS4	95°C 5 min; (95°C 30 s, 60°C 30 s, 72°C 40 s) × 30 cycles; 72°C 5 min	McKay et al. 2009; White et al. 1990	McKay et al. 2009
	TUB	T1/Bt-2b	95°C 4 min; (95°C 30 s, 55°C 30 s, 72°C 45 s) × 35 cycles; 72°C 7 min	Glass and Donaldson 1995; O'Donnell and Cigelnik 1997	Damm et al. 2012
	GAPDH	GDF/GDR	95°C 1 min; (95°C 30 s, 57°C 30 s, 72°C 30 s) × 35 cycles; 72°C 7 min	Templeton et al. 1992	Damm et al. 2012
<i>Diaporthe</i>	ITS	ITS5/ITS4	94°C 2 min; (94°C 30 s, 58°C 60 s, 72°C 60 s) × 40 cycles; 72°C 3 min	White et al. 1990	Gomes et al. 2013
	HIS	CYLH3F/H3-1b	95°C 1 min; (95°C 30 s, 62°C 60 s, 72°C 60 s) × 40 cycles; 72°C 3 min	Crous et al. 2004; Glass and Donaldson 1995	Gomes et al. 2013
	TUB	T1/CYLTUB1R	95°C 1 min; (95°C 30 s, 60°C 60 s, 72°C 60 s) × 40 cycles; 72°C 3 min	Crous et al. 2004; O'Donnell and Cigelnik 1997	Gomes et al. 2013
<i>Epicoccum</i>	ITS	ITS1/ITS4	94°C 5 min; (94°C 45 s, 57°C 45 s, 72°C 90 s) × 35 cycles; 72°C 10 min	White et al. 1990	White et al. 1990

^y endoPG = endopolygalacturonase, GAPDH = glyceraldehyde-3-phosphate dehydrogenase, HIS = histon H3, OPA1 and OPA10 = anonymous noncoding region, TUB = β -tubulin, ITS = internal transcribed spacer.

^z In this study, DreamTaq DNA Polymerase (5U/µl) (Thermo Scientific, Waltham, MA) was used in all the PCR reactions.

Table 3. Reference sequences for *Alternaria*, *Colletotrichum*, *Diaporthe*, and *Epicoccum* retrieved from GenBank that were included in the phylogenetic analyses

Species	Strain ^y	Origin	Host	GenBank accession no. ^z		
				endoPG	OPA1-3	OPA10-2
<i>Alternaria alternata</i>	CBS 102596	USA	<i>Citrus jambhiri</i>	KP124030	AY295047	KP124637
<i>A. alternata</i>	CBS 102598	USA	<i>Citrus jambhiri</i>	AY295026	AY295048	KP124638
<i>A. alternata</i>	CBS 102600	USA	<i>Citrus reticulata</i>	KP124033	AY295042	KP124640
<i>A. alternata</i>	CBS 102602	Turkey	<i>Minneola tangelo</i>	AY295023	AY295052	KP124641
<i>A. alternata</i>	CBS 102604	Israel	<i>Minneola tangelo</i>	KP124035	AY631443	KP124643
<i>A. alternata</i>	CBS 104.26	Unknown	Unknown	KP123995	-	KP124603
<i>A. alternata</i>	CBS 106.24	USA	<i>Malus sylvestris</i>	AY295020	JQ800572	JQ800620
<i>A. alternata</i>	CBS 115152	China	<i>Psychotria serpens</i>	KP124049	-	KP124658
<i>A. alternata</i>	CBS 117143	Italy	<i>Capsicum annuum</i>	KP124055	-	KP124665
<i>A. alternata</i>	CBS 911.97	India	<i>Artemisia brevifolia</i>	KP124027	-	KP124634
<i>A. alternata</i>	CBS 916.96	India	<i>Arachis hypogaea</i>	JQ811978	AY295043	KP124632
<i>A. alternata</i>	CBS 918.96	UK	<i>Dianthus chinensis</i>	KP124026	JQ859820	KP124633
<i>A. alternata</i>	CBS 966.95	India	<i>Solanum lycopersicum</i>	KP124024	-	KP124630
<i>A. alternata</i>	EGS 44-159	Turkey	<i>Citrus reticulata</i> × <i>C. paradisi</i>	AY295022	AY295051	EF504051
<i>A. arborescens</i>	CBS 102605	USA	<i>Solanum lycopersicum</i>	AY295028	AY295035	KP124712
<i>A. betae-kenyensis</i>	CBS 118810	Kenya	<i>Beta vulgaris</i>	KP124123	-	KP124733
<i>A. eichhorniae</i>	CBS 489.92	India	<i>Eichhornia crassipes</i>	KP124130	-	KP124740
				GAPDH	TUB	
<i>Colletotrichum acutatum</i>	CBS 112996	Australia	<i>Canica papaya</i>	JQ948677	JQ005860	
<i>C. cosmi</i>	CBS 853.73	Netherlands	<i>Cosmos</i> sp.	JQ948604	JQ949925	
<i>C. fioriniae</i>	CBS 128517	USA	<i>Fiorinia externa</i>	JQ948622	JQ949943	
<i>C. fioriniae</i>	CBS 200.35	USA	<i>Rubus</i> sp.	JQ948623	JQ949944	
<i>C. fioriniae</i>	CBS 293.67	Australia	<i>Persea americana</i>	JQ948640	JQ949961	
<i>C. guajavae</i>	IMI 350839	India	<i>Psidium guajava</i>	JQ948600	JQ949921	
<i>C. nymphaeae</i>	CBS 515.78	Netherlands	<i>Nymphaea alba</i>	JQ948527	JQ949848	
<i>C. scovillei</i>	CBS 126529	Indonesia	<i>Capsicum</i> sp.	JQ948597	JQ949918	
<i>C. walleri</i>	CBS 125472	Colombia	<i>Coffea</i> sp.	JQ948605	JQ949926	
<i>C. johnstonii</i>	CBS 128532	New Zealand	<i>Solanum lycopersicum</i>	JQ948775	JQ950095	
				HIS	ITS	TUB
<i>Diaporthe acaciigena</i>	CBS 129521	Australia	<i>Acacia retinodes</i>	KC343489	KC343005	KC343973
<i>D. acerina</i>	CBS 137.27	-	<i>Acer saccharum</i>	KC343490	KC343006	KC343974
<i>D. alleghaniensis</i>	CBS 495.72	Canada	<i>Betula alleghaniensis</i>	KC343491	KC343007	KC343975
<i>D. alnea</i>	CBS 146.46	Netherlands	<i>Alnus</i> sp.	KC343492	KC343008	KC343976
<i>D. ampelina</i>	CBS 114016	France	<i>Vitis vinifera</i>	-	AF230751	JX275452
<i>D. amygdali</i>	CBS 126679	Portugal	<i>Prunus dulcis</i>	KC343506	KC343022	KC343990
<i>D. apiculata</i>	CGMCC 3.17533	China	<i>Camellia sinensis</i>	-	KP267896	KP293476
<i>D. australafricana</i>	CBS 111886	Australia	<i>Vitis vinifera</i>	KC343522	KC343038	KC344006
<i>D. beckhausii</i>	CBS 138.27	-	<i>Viburnum</i> sp.	KC343525	KC343041	KC344009
<i>D. betulae</i>	CFCC 50469	China	<i>Betula platyphylla</i>	KT732999	KT732950	KT733020
<i>D. betulicola</i>	CFCC 51128	China	<i>Betula albosinensis</i>	KX024661	KX024653	KX024657
<i>D. bincta</i>	CBS 121004	USA	<i>Juglans</i> sp.	KC343618	KC343134	KC344102
<i>D. biguttusis</i>	CGMCC 3.17081	China	<i>Lithocarpus glabra</i>	-	KF576282	KF576306
<i>D. brasiliensis</i>	CBS 133183	Brazil	<i>Aspidosperma tomentosum</i>	KC343526	KC343042	KC344010
<i>D. carpinii</i>	CBS 114437	Sweden	<i>Carpinus betulus</i>	KC343528	KC343044	KC344012
<i>D. caulivora</i>	CBS 127268	Croatia	<i>Glycine max</i>	KC343529	KC343045	KC344013
<i>D. celsastrina</i>	CBS 139.27	USA	<i>Celastrus scandens</i>	KC343531	KC343047	KC344015
<i>D. cf. heveae</i> 1	CBS 852.97	Brazil	<i>Hevea brasiliensis</i>	KC343600	KC343116	KC344084
<i>D. cf. heveae</i> 2	CBS 681.84	India	<i>Hevea brasiliensis</i>	KC343601	KC343117	KC344085
<i>D. cf. nobilis</i>	CBS 587.79	Japan	<i>Pinus pentaphylla</i>	KC343637	KC343153	KC344121
<i>D. charlesworthii</i>	BRIP 54884m	Australia	<i>Rapistrum rugosum</i>	-	KJ197288	KJ197268
<i>D. citri</i>	CBS 135422	USA	<i>Citrus</i> sp.	KJ420881	NR145304	KC843187
<i>D. citrichinensis</i>	ZJUD34	China	<i>Citrus unshiu</i>	KJ490516	JQ954648	KJ490396
<i>D. convolvuli</i>	CBS 124654	Turkey	<i>Convolvulus arvensis</i>	KC343538	KC343054	KC344022
<i>D. crataegi</i>	CBS 114435	Sweden	<i>Crataegus oxyacantha</i>	KC343539	KC343055	KC344023
<i>D. cynaroidis</i>	CBS 122676	South Africa	<i>Protea cynaroides</i>	KC343542	KC343058	KC344026
<i>D. decedens</i>	CBS 109772	Austria	<i>Corylus avellana</i>	KC343543	KC343059	KC344027
<i>D. detrusa</i>	CBS 109770	Austria	<i>Berberis vulgaris</i>	KC343545	KC343061	KC344029
<i>D. ellipicola</i>	CGMCC 3.17084	China	<i>Lithocarpus glabra</i>	-	KF576270	KF576294

(Continued on next page)

^y Ex-holotype, ex-epitype, ex-type, authentic, or representative cultures are in bold. AR = Collection of Systematic Mycology and Microbiology Laboratory, USDA-ARS, Beltsville; ATCC = American Type Culture Collection; BRIP = Plant Pathology Herbarium, Dutton Park, Queensland, Australia; CBS: Centraalbureau voor Schimmelmicrocultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; CFCC = China Forestry Culture Collection Center, Beijing, China; CGMCC = China General Microbial Culture Collection Center, China; CMW = Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; EGS = Collection of E.G. Simmons; IMI: Culture collection of CABI Europe UK Centre, Egham, UK; LC: Collection of L. Cai; PD: Plant Protection Service, Wageningen, the Netherlands; ZJUD = Zhejiang University, China.

^z endoPG = endopolygalacturonase; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HIS = histon H3; OPA1-3 and OPA10-2 = anonymous noncoding region; TUB = β -tubulin; ITS = internal transcribed spacer.

Table 3. (Continued from previous page)

Species	Strain ^y	Origin	Host	GenBank accession no. ^z		
				endoPG	OPA1-3	OPA10-2
<i>D. eres</i>	CBS 101742	Netherlands	<i>Fraxinus</i> sp.	KC343557	KC343073	KC344041
<i>D. eres</i>	CBS 109767	Austria	<i>Acer campestre</i>	KC343559	KC343075	KC344043
<i>D. eres</i>	CBS 122.82	Netherlands	<i>Skimmia japonica</i>	KC343561	KC343077	KC344045
<i>D. eres</i>	CBS 129168	Latvia	<i>Rhododendron</i> sp.	KC343562	KC343078	KC344046
<i>D. eres</i>	CBS 138594	Germany	<i>Ulmus laevis</i>	KJ420850	KJ210529	KJ420799
<i>D. eres</i>	CBS 186.37	UK	<i>Picea abies</i>	KC343563	KC343079	KC344047
<i>D. eres</i>	CBS 250.38	UK	<i>Fraxinus excelsior</i>	KC343564	KC343080	KC344048
<i>D. eres</i>	CBS 267.32	-	-	KC343565	KC343081	KC344049
<i>D. eres</i>	CBS 267.55	Netherlands	<i>Laburnum × watereri</i>	KC343566	KC343082	KC344050
<i>D. eres</i>	CBS 283.85	Netherlands	<i>Allium giganteum</i>	KC343567	KC343083	KC344051
<i>D. eres</i>	CBS 287.74	Netherlands	<i>Sorbus aucuparia</i>	KC343568	KC343084	KC344052
<i>D. eres</i>	CBS 297.77	Netherlands	<i>Osmanthus aquifolium</i>	KC343569	KC343085	KC344053
<i>D. eres</i>	CBS 365.97	Netherlands	<i>Opuntia</i> sp.	KC343570	KC343086	KC344054
<i>D. eres</i>	CBS 370.67	Netherlands	<i>Ilex aquifolium</i>	KC343571	KC343087	KC344055
<i>D. eres</i>	CBS 375.61	-	<i>Malus sylvestris</i>	KC343572	KC343088	KC344056
<i>D. eres</i>	CBS 422.50	Netherlands	<i>Phaseolus vulgaris</i>	KC343573	KC343089	KC344057
<i>D. eres</i>	CBS 439.82	UK	<i>Cotoneaster</i> sp.	KC343574	KC343090	KC344058
<i>D. eres</i>	CBS 445.62	Netherlands	<i>Alliaria officinalis</i>	KC343575	KC343091	KC344059
<i>D. eres</i>	CBS 485.96	Netherlands	<i>Rumex hydrolapathum</i>	KC343576	KC343092	KC344060
<i>D. eres</i>	CBS 528.83	Netherlands	<i>Wisteria sinensis</i>	KC343577	KC343093	KC344061
<i>D. eres</i>	CBS 688.97	Netherlands	<i>Abutilon</i> sp.	KC343578	KC343094	KC344062
<i>D. eres</i>	CBS 694.94	Netherlands	<i>Ilex aquifolium</i>	KC343579	KC343095	KC344063
<i>D. eres</i>	CBS 791.68	Netherlands	<i>Magnolia × soulangeana</i>	KC343580	KC343096	KC344064
<i>D. eres</i>	CBS 841.84	Netherlands	<i>Hordeum</i> sp.	KC343581	KC343097	KC344065
<i>D. fibrosa</i>	CBS 109751	Austria	<i>Rhamnus cathartica</i>	KC343583	KC343099	KC344067
<i>D. foeniculina</i>	CBS 187.27	Italy	<i>Camellia sinensis</i>	KC343591	KC343107	KC344075
<i>D. fraxini-angustifoliae</i>	BRIP 54781	Australia	<i>Fraxinus angustifolia</i>	-	JX862528	KF170920
<i>D. gardeniae</i>	CBS 288.56	Italy	<i>Gardenia florida</i>	KC343597	KC343113	KC344081
<i>D. goulteri</i>	BRIP 55657a	Australia	<i>Helianthus annuus</i>	-	KJ197290	KJ197270
<i>D. helianthi</i>	CBS 592.81	Serbia	<i>Helianthus annuus</i>	KC343599	KC343115	KC344083
<i>D. heliciis</i>	AR 5211	France	<i>Hedera helix</i>	KJ420875	KJ210538	KJ420828
<i>D. hickoriae</i>	CBS 145.26	USA	<i>Carya glabra</i>	KC343602	KC343118	KC344086
<i>D. hongkongensis</i>	CBS 115448	China	<i>Dichroa febrifuga</i>	KC343603	KC343119	KC344087
<i>D. impulsu</i>	CBS 114434	Sweden	<i>Sorbus aucuparia</i>	KC343605	KC343121	KC344089
<i>D. juglandicola</i>	CFCC 51134	China	<i>Juglans mandshurica</i>	KX024622	KU985101	KX024634
<i>D. longicicola</i>	CGMCC 3.17089	China	<i>Lithocarpus glabra</i>	-	KF576267	KF576291
<i>D. longicolla</i>	ATCC 60325	USA	<i>Glycine max</i>	KJ659188	NR144924	KJ610883
<i>D. longispora</i>	CBS 194.36	Canada	<i>Ribes</i> sp.	KC343619	KC343135	KC344103
<i>D. lusitanicae</i>	CBS 123212	Portugal	<i>Foeniculum vulgare</i>	KC343620	KC343136	KC344104
<i>D. macintoshii</i>	BRIP 55064a	Australia	<i>Rapistrum rugosum</i>	-	KJ197289	KJ197269
<i>D. mahothocarpus</i>	CGMCC 3.15181	China	<i>Lithocarpus glabra</i>	-	KC153096	KF576312
<i>D. manihotia</i>	CBS 505.76	Rwanda	<i>Manihot utilisima</i>	KC343622	KC343138	KC344106
<i>D. masirevicii</i>	BRIP 54256	Australia	<i>Glycine max</i>	-	KJ197277	KJ197257
<i>D. mayteni</i>	CBS 133185	Brazil	<i>Maytenus ilicifolia</i>	KC343623	KC343139	KC344107
<i>D. melonis</i>	CBS 507.78	USA	<i>Cucumis melo</i>	KC343626	KC343142	KC344110
<i>D. miriciae</i>	BRIP 54736j	Australia	<i>Helianthus annuus</i>	-	KJ197282	KJ197262
<i>D. musigena</i>	CBS 129519	Australia	<i>Musa</i> sp.	KC343627	KC343143	KC344111
<i>D. neilliae</i>	CBS 144.27	USA	<i>Spiraea</i> sp.	KC343628	KC343144	KC344112
<i>D. neoarctii</i>	CBS 109490	USA	<i>Ambrosia trifida</i>	KC343629	KC343145	KC344113
<i>D. nomurai</i>	CBS 157.29	Japan	<i>Moru</i> sp.	KC343638	KC343154	KC344122
<i>D. nothofagi</i>	BRIP 54801	Australia	<i>Nothofagus cunninghamii</i>	-	JX862530	KF170922
<i>D. novem</i>	CBS 127270	Croatia	<i>Glycine max</i>	KC343640	KC343156	KC344124
<i>D. ocoteae</i>	CBS 141330	France	<i>Ocotea obtusata</i>	-	KX228293	KX228388
<i>D. oncostroma</i>	CBS 809.85	Germany	<i>Ilex aquifolium</i>	KC343647	KC343163	KC344131
<i>D. oxe</i>	CBS 133186	Brazil	<i>Maytenus ilicifolia</i>	KC343648	KC343164	KC344132
<i>D. padi</i>	CBS 114200	Sweden	<i>Prunus padus</i>	KC343653	KC343169	KC344137
<i>D. paranensis</i>	CBS 133184	Brazil	<i>Maytenus ilicifolia</i>	KC343655	KC343171	KC344139
<i>D. penetratrum</i>	CGMCC 3.17532	China	<i>Camellia sinensis</i>	KP714493	KP267879	KP293459
<i>D. perijuncta</i>	CBS 109745	Austria	<i>Ulmus glabra</i>	KC343656	KC343172	KC344140
<i>D. phaseolorum</i>	AR 4203	USA	<i>Phaseolus vulgaris</i>	KJ659220	KJ590738	KJ610893
<i>D. pseudomangiferae</i>	CBS 101339	Dominican	<i>Mangifera indica</i>	KC343665	KC343181	KC344149
<i>D. pulla</i>	CBS 338.89	Yugoslavia	<i>Hedera helix</i>	KC343636	KC343152	KC344120
<i>D. pustulata</i>	CBS 109742	Austria	<i>Acer pseudoplatanus</i>	KC343669	KC343185	KC344153
<i>D. pustulata</i>	CBS 109760	Austria	<i>Acer pseudoplatanus</i>	KC343670	KC343186	KC344154
<i>D. pustulata</i>	CBS 109784	Austria	<i>Prunus padus</i>	KC343671	KC343187	KC344155

(Continued on next page)

Table 3. (Continued from previous page)

Species	Strain ^y	Origin	Host	GenBank accession no. ^z		
				endoPG	OPA1-3	OPA10-2
<i>D. raonikayaporum</i>	CBS 133182	Brazil	<i>Spondias mombin</i>	KC343672	KC343188	KC344156
<i>D. rudis</i>	CBS 113201	Portugal	<i>Vitis vinifera</i>	KC343718	KC343234	KC344202
<i>D. sackstonii</i>	BRIP 54669b	Australia	<i>Helianthus annuus</i>	-	KJ197287	KJ197267
<i>D. salicicola</i>	BRIP 54825	Australia	<i>Salix purpurea</i>	-	JX862531	KF170923
<i>D. schini</i>	CBS 133181	Brazil	<i>Schinus terebinthifolius</i>	KC343675	KC343191	KC344159
<i>D. scobina</i>	CBS 251.38	UK	<i>Fraxinus excelsior</i>	KC343679	KC343195	KC344163
<i>D. subclavata</i>	ZJUD95	China	<i>Citrus</i> sp.	KJ490572	KJ490630	KJ490451
<i>D. terebinthifolii</i>	CBS 133180	Brazil	<i>Schinus terebinthifolius</i>	KC343700	KC343216	KC344184
<i>D. toxica</i>	CBS 534.93	Australia	<i>Lupinus angustifolius</i>	KC343704	KC343220	KC344188
<i>D. vaccinii</i>	CBS 160.32	USA	<i>Oxycoccus macrocarpos</i>	KC343712	KC343228	KC344196
<i>D. vawdreyi</i>	BRIP 57887a	Australia	<i>Psidium guajava</i>	-	KR936126	KR936128
<i>D. virgiliae</i>	CMW 40755	South Africa	<i>Virgilia oroboides</i>	-	KP247573	KP247583
<i>D. wollworthii</i>	CBS 148.27	-	<i>Ulmus americana</i>	KC343729	KC343245	KC344213
<i>Diaporthella corylina</i>	CBS 121124	China	<i>Corylus</i> sp.	KC343488	KC343004	KC343972
ITS						
<i>Epicoccum brasiliense</i>	CBS 120105	Brazil	<i>Amaranthus</i> Sp.	GU237760		
<i>E. camelliae</i>	CGMCC 3.18343	China	<i>Camellia sinensis</i>	KY742091		
<i>E. keratinophilum</i>	CBS 142455	USA	Human superficial tissue	LT592930		
<i>E. laticollum</i>	CGMCC 3.18346	China	<i>Sorghum bicolor</i>	KY742101		
<i>E. nigrum</i>	CBS 125.82	Netherlands	Human toe nail	FJ426995		
<i>E. nigrum</i>	CBS 173.73	USA	<i>Dactylis glomerata</i>	FJ426996		
<i>E. nigrum</i>	LC 8158	USA	<i>Poa annua</i>	KY742111		
<i>E. nigrum</i>	LC 8159	USA	<i>Poa annua</i>	KY742112		
<i>E. ovisporum</i>	CBS 180.80	South Africa	<i>Zea mays</i>	FJ427068		
<i>E. pinprinum</i>	CBS 246.60	India	Soil	KY742113		
<i>E. pinprinum</i>	PD 77/1028	India	Soil	KY742114

Fig. 2. Maximum likelihood (ML) phylogenetic tree based on the Kimura 2-parameter model plus a gamma distribution model (Kimura 1980) obtained from combined endoPG, OPA1, and OPA10 datasets of *Alternaria* spp. The ML bootstrap support (BS) ≥ 70 and Bayesian posterior probability (PP) ≥ 0.95 values are indicated at the nodes (BS/PP). The tree was rooted with *A. eichhorniae* (CBS 489.92).

concatenated datasets of three loci (endoPG, OPA1, and OPA10) were analyzed using ML and BI and similar tree topologies were obtained. Thirty-one sequences were included in the phylogenetic analysis consisting of 17 reference strains (Table 3; Andrew et al. 2009; Peever et al. 2004; Rotondo et al. 2012; Woudenberg et al. 2015) and 14 isolates from this study. Isolate FPH2015395 grouped with *A. arborescens* reference strain CBS 102605 with a high bootstrap support value (BS = 96) and a high Bayesian posterior probability (PP = 0.97). All other isolates clustered with several *A. alternata* reference sequences (BS = 75, PP = 0.96; Fig. 2).

Diaporthe spp. In total, 13 isolates were identified as *Diaporthe* spp. Of these, three isolates (FPH201322, FPH2015394, and FPH2017229) were characterized by white, fluffy, aerial mycelium, gray to brown on the reverse side. Pycnidia in culture were dark brown to black, globose or subglobose, erumpent, solitary or aggregated, arranged in concentric rings. Creamy to yellow conidial masses were globose or exuded in cirrhi. Alpha conidia were hyaline, aseptate, smooth, guttulate, and ellipsoid. Beta conidia were hyaline, aseptate, slightly curved, hamate or filiform, and less common than alpha conidia. Even though these three isolates had similar morphological characteristics, the width of both alpha and beta conidia of isolate FPH2017229 was significantly smaller (Table 5; significance reference not shown). All three isolates were tentatively assigned to the *Diaporthe eres* Nitschke species complex (Baumgartner et al. 2013; Dissanayake et al. 2015; Udayanga et al. 2014a). The 10 remaining isolates presented morphological characteristics that differed from the three isolates previously described as well as from other *Diaporthe* species described in the literature, including those previously reported from *Ilex* spp. (Dissanayake et al. 2017; Du et al. 2016; Gomes et al. 2013; Huang et al. 2013, 2015; Machingambi et al. 2015; Mostert et al. 2001; Tan et al. 2013; Thompson et al. 2015; Udayanga et al. 2015; Wehmeyer 1933; Yang et al. 2017). Detailed morphological features of these isolates are reported in the new species description below (Fig. 3).

Two sets of multilocus phylogenetic analyses were conducted for *Diaporthe* spp. isolates. In the first analysis, the concatenated HIS, ITS, and TUB datasets consisted of 102 isolates from 82 taxa (Dissanayake et al. 2017; Du et al. 2016; Gomes et al. 2013; Huang et al. 2013, 2015; Machingambi et al. 2015; Mostert et al. 2001; Tan et al. 2013; Thompson et al. 2015; Udayanga et al. 2012, 2014a, b, 2015; Yang et al. 2017). In both ML and BI analyses, the 13 isolates from this study grouped into three different clades (Fig. 4). Three isolates (FPH201322, FPH2015394, and FPH2017229), in accordance with their morphology, clustered within the *D. eres* species complex, and grouped with reference strains CBS 138594, CBS 287.74, CBS 370.67, and CBS 587.79. The remaining 10 isolates clustered with strong support (BS = 85 and PP = 1) as a unique and unnamed clade sharing a strongly supported phylogenetic

relationship (BS = 99 and PP = 1) near the reference strain of *D. cf. heveae* 1 Petch (CBS 852.97; Fig. 4). Morphological and molecular data revealed that these 10 isolates represented a unique and undescribed species of *Diaporthe* with no apparent species name. Thus, we hereby propose a new species name to properly identify this unique taxon:

D. ilicicola S. Lin, Taylor & Peduto Hand, sp. nov.

Mycobank No. MB824842

Etymology – named according to the host it was first isolated from, namely *Ilex verticillata* × *I. serrata*.

Conidiomata pycnidial, dark brown to black, erumpent, solitary or aggregated, ostiolate, creamy to light salmon conidial mass globose or exuding in cirrhi, arranged in circle at the edge of colony. Conidiophores hyaline, subcylindrical, reduced to conidiogenous cells. Conidiogenous cells hyaline, subcylindrical, tapering toward the apex, 7.64 to 14.14 × 1.9 to 3.82 µm (av. ± SD = 11.2 ± 0.4 × 2.7 ± 0.14 µm). Alpha conidia hyaline, aseptate, smooth, ovate to

Table 4. Isolation frequency of the different fungi recovered from the 304 symptomatic holly fruit that yielded fungal growth between 2013 and 2017 across all sampling locations

Fungal species	Isolation frequency (%)
<i>Diaporthe</i> spp.	39.47
<i>Alternaria</i> spp.	32.89
<i>Colletotrichum</i> spp.	7.24
<i>Epicoccum</i> spp.	4.61
<i>Cladosporium</i> spp.	3.68
<i>Alternaria</i> sp. + <i>Diaporthe</i> sp.	2.34
<i>Botryosphaeriaceae</i>	2.01
<i>Alternaria</i> sp. + <i>Colletotrichum</i> sp.	1.67
<i>Fusarium</i> spp.	1.00
<i>Phoma</i> spp.	1.00
<i>Epicoccum</i> sp. + <i>Diaporthe</i> sp.	1.00
<i>Alternaria</i> sp. + <i>Fusarium</i> sp.	0.33

Fig. 3. Morphology of *Diaporthe ilicicola* sp. nov. strain FPH2015502. **A**, colony on PDA after 28 days at 23°C; top and bottom of the plate; **B**, conidiogenous cells and alpha conidia; **C**, alpha conidia; **D**, sporulation from conidiomata on PDA.

ellipsoid, guttulate, on PDA 7.13 to 9.43 × 2.91 to 4.16 μm (av. ± SD = 8.41 ± 0.1 × 3.63 ± 0.05 μm). Beta conidia not observed. Sexual morph not observed.

Culture characteristics – Colonies on PDA at 23°C with 12-h photoperiod slow growing, 2.89 ± 0.08 mm/day, flat and compact, irregular edge, originally white to olive, turning to grayish brown while aging, reverse dark brown.

Host – *I. verticillata* × *I. serrata*

Distribution – United States

Specimens examined. United States, Ohio, Madison, on rotten fruit of *I. verticillata* × *I. serrata*, cultivar Bonfire, December 2015, Shan Lin, holotype FPH2015502 (CBS 144318).

In the second analysis, the concatenated HIS, ITS, and TUB datasets consisted of 39 isolates from 16 taxa that are within or closely related to the *D. eres* species complex (Gao et al. 2016; Gomes et al. 2013; Udayanga et al. 2014a) and three *Diaporthe* isolates from this study (FPH201322, FPH2015392, and FPH2017229). According to the new analysis, isolates FPH201322 and FPH2015392 grouped with *D. eres* reference strains (BS = 99, PP = 1.0 and BS = 97, PP = 0.95, respectively), while isolate FPH2017229 grouped with a reference strain of *D. cf. nobilis* (CBS 587.79; BS = 90, PP = 0.99; Fig. 5).

Colletotrichum spp. Eight isolates tentatively identified as *Colletotrichum* spp. were divided into two morphological groups. All but

one isolate belonged to the first group and were characterized by cottony mycelium that was light gray to gray on the upper side of the plate, and brownish pink with abundant scattered melanized spots on the reverse side. Orange conidial masses formed in the center of the colony after 21 days. Conidia were hyaline, aseptate, fusiform to cylindrical, and slightly pointed at one or both ends. These features were consistent with the description of *Colletotrichum fioriniae* (Marcelino & Gouli) Pennycook (Shivas and Tan 2009). The isolate in the second morphological group (FPH2015590) presented white to gray colored mycelium on the upper side of the plate and salmon on the reverse side. Conidia were hyaline, aseptate, fusiform to cylindrical, with one or both ends slightly acute. This isolate was identified as *C. nymphaeae* (Pass.) Aa (Velho et al. 2015). DNA from all *Colletotrichum* isolates was successfully amplified using *C. acutatum* species complex-specific primers targeting the ITS region, and sequence analysis confirmed that they belonged to the *C. acutatum* species complex (McKay et al. 2009). In the multigene phylogenetic analyses of combined GAPDH and TUB datasets using ML and BI, isolates grouped into two clades. Isolate FPH2015590 clustered with *C. nymphaeae* strain CBS 515.78, while the rest of the isolates clustered with *C. fioriniae* strains CBS 128517, CBS 200.35, and CBS 293.67 (Damm et al. 2012; Fig. 6).

Epicoccum spp. Two isolates tentatively identified as *Epicoccum* sp. were characterized by yellow to orange colonies producing an

Table 5. Conidial measurements and mycelial growth rate of select isolates of *Alternaria*, *Colletotrichum*, *Diaporthe*, and *Epicoccum* retrieved from symptomatic winterberry fruit used in this study (*Diaporthe ilicicola* sp. nov. data not included)

Morphospecies	Isolate	Conidium length (μm)	Conidium width (μm)	Mycelial growth rate (mm/day)
<i>Alternaria alternata</i>	FPH2015390	32.58 ± 0.63 (27.86–42.8) ^v	11.09 ± 0.26 (8.79–13.9)	5.39 ± 0.06 (5.31–5.54) ^w
	FPH2015396	31.85 ± 0.67 (31.49–40)	9.39 ± 0.24 (6.93–12.28)	5.4 ± 0.04 (5.3–5.49)
	FPH2015468	30.85 ± 0.92 (23.84–39.71)	11.11 ± 0.3 (8.29–13.75)	5.52 ± 0.03 (5.48–5.59)
	FPH2015507	32.9 ± 0.65 (25.79–40.7)	10.05 ± 0.2 (8.05–12.17)	5.32 ± 0.004 (5.32–5.33)
	FPH2015567	31.66 ± 0.74 (25.2–40.46)	11.87 ± 0.24 (8.01–13.56)	5.17 ± 0.05 (5.06–5.25)
	FPH2015589	31.31 ± 0.73 (25.36–41.1)	11.08 ± 0.32 (8.25–13.67)	5.28 ± 0.02 (5.25–5.31)
	FPH2015592	31.26 ± 0.55 (27.26–38.06)	10.07 ± 0.27 (7.95–13.54)	5.63 ± 0.02 (5.6–5.67)
	FPH2015597	32.92 ± 0.83 (25.46–42.75)	10.37 ± 0.2 (8.76–13.72)	5.16 ± 0.05 (5.06–5.27)
	FPH2016709	32.98 ± 0.77 (26.88–42.28)	11.8 ± 0.29 (9.15–15.19)	5.23 ± 0.02 (5.19–5.28)
	FPH2016711	31.95 ± 0.84 (25.85–43.78)	10.85 ± 0.23 (8.19–13.5)	5.58 ± 0.02 (5.53–5.62)
	Mean	32.09 ± 0.23 C ^x	10.77 ± 0.09 B	5.37 ± 0.03 A
<i>A. arborescens</i>	FPH2015395	29.05 ± 0.67 (22.47–35.37)	11.84 ± 0.21 (9.21–15.11)	5.26 ± 0.05 (5.2–5.37)
	Mean	29.05 ± 0.67 D	11.84 ± 0.21 A	5.26 ± 0.05 A
<i>A. tenuissima</i>	FPH2015470	43.19 ± 1.1 (35.73–59.01)	10.76 ± 0.28 (8.46–13.52)	5.58 ± 0.02 (5.54–5.62)
	FPH2015591	39.04 ± 0.88 (32.57–51.34)	10.3 ± 0.18 (8.66–12.3)	5.31 ± 0.02 (5.29–5.35)
	Mean	41.11 ± 0.52 A	10.53 ± 0.2 B	5.45 ± 0.06 A
<i>A. toxicogenica</i>	FPH2015338	37.29 ± 0.66 (30.38–44.79)	10.7 ± 0.23 (8.81–13.88)	5.31 ± 0.01 (5.29–5.32)
	Mean	37.29 ± 0.66 B	10.7 ± 0.23 B	5.31 ± 0.01 A
<i>Colletotrichum fioriniae</i>	FPH201314	10.57 ± 0.21 (8.9–13.24)	3.71 ± 0.11 (2.94–5.72)	3.02 ± 0.03 (2.97–3.08)
	FPH201317	9.02 ± 0.19 (7.32–10.61)	4.01 ± 0.11 (3.15–5.47)	2.83 ± 0.02 (2.77–2.85)
	FPH201319	9.86 ± 0.13 (8.25–11.93)	4.39 ± 0.07 (3.51–5.12)	2.51 ± 0.08 (2.39–2.69)
	FPH2015392	6.13 ± 0.17 (4.37–8.41)	3.74 ± 0.08 (3.06–5.17)	2.69 ± 0.11 (2.52–2.96)
	FPH2015466	11.17 ± 0.14 (9.77–12.74)	4.81 ± 0.09 (4.03–5.77)	2.99 ± 0.01 (2.97–3.01)
	FPH2015564	11.17 ± 0.13 (9.88–12.5)	4.81 ± 0.09 (4.03–5.75)	3.02 ± 0.02 (2.97–3.06)
	FPH2016720	10.45 ± 0.24 (8.01–12.38)	4 ± 0.11 (2.94–5.52)	3.25 ± 0.1 (3.02–3.43)
	Mean	9.77 ± 0.13 A	4.21 ± 0.05 B	2.9 ± 0.06 A
<i>C. nymphaeae</i>	FPH2015590	10.13 ± 0.17 (8.31–13.6)	4.54 ± 0.1 (3.65–6.31)	2.87 ± 0.11 (2.59–3.02)
	Mean	10.13 ± 0.17 A	4.54 ± 0.1 A	2.87 ± 0.11 A
<i>Diaporthe eres</i>	FPH201322	7.58 ± 0.09 (6.74–8.61) ^y	2.99 ± 0.04 (2.59–3.53)	4.7 ± 0.19 (4.25–4.96)
		24.73 ± 0.52 (18.91–29.65) ^z	1.68 ± 0.05 (1.27–2.13)	
	FPH2015394	6.38 ± 0.12 (5.04–8.01)	2.89 ± 0.06 (2.21–3.82)	5.59 ± 0.12 (5.31–5.8)
		24.39 ± 0.38 (21.66–29.79)	1.8 ± 0.03 (1.48–2.09)	
	FPH2017229	6.87 ± 0.08 (6–7.58)	2.59 ± 0.05 (2.15–3.32)	4.89 ± 0.09 (4.76–5.05)
<i>Epicoccum nigrum</i>		24.81 ± 0.44 (18.76–29.66)	1.63 ± 0.03 (1.27–2.05)	
	FPH2015417	20.27 ± 0.27 (17.37–23.26)	18 ± 0.3 (14.12–22.18)	3.43 ± 0.02 (3.38–3.45)
	FPH2015505	19.85 ± 0.31 (17.05–23.78)	17.23 ± 0.32 (13.17–21.71)	3.57 ± 0.05 (3.48–3.69)

^v Values reported are mean ± standard error (minimum-maximum size) of 30 randomly selected conidia.

^w Values reported are mean ± standard error (minimum-maximum mycelia growth rate).

^x Values with the same letter in the same column within each fungal genus are not significantly different (α = 0.05).

^y Alpha conidia.

^z Beta conidia.

orange to brown diffusible pigment in PDA. Dark pigmented conidia were produced in sporodochia, and were multicellular and globose to pyriform. These features were consistent with the description of *Epicoccum nigrum* Link (Mahadevakumar et al. 2014; Wu et al. 2017). Both ML and BI analyses using the ITS gene were in accordance with the results of morphological characterization as both isolates from this study clustered with *E. nigrum* reference strains (Chen et al. 2017; Valenzuela-Lopez et al. 2018; Fig. 7).

Pathogenicity tests. In experiment A, all isolates of *A. alternata*, *C. fioriniae*, *D. ilicicola* sp. nov., and *E. nigrum* were able to infect detached wounded mature holly fruit. Brown to black lesions developed from the inoculation point on all fruit inoculated with *Alternaria* and *Colletotrichum* isolates 2 DPI (= 100% incidence; data not shown). After 3 to 5 days, lesions enlarged to cover the whole fruit and sporulation occurred on the surface of the fruit (Fig. 8A and B). *D. ilicicola* sp. nov. caused a light salmon discoloration of the

Fig. 4. Maximum likelihood (ML) analysis tree based on the General Time Reversible plus a gamma distribution model obtained from concatenated sequences of combined HIS, ITS, and TUB datasets of *Diaporthe* spp. The ML bootstrap support (BS) ≥ 70 and Bayesian posterior probability (PP) ≥ 0.95 values are indicated at the nodes (BS/PP). The *Diaporthe ilicicola* sp. nov. isolates from this study are highlighted. The tree was rooted to *Diaporthe corylina* (CBS 121124).

fruit 1 week post-inoculation. Pycnidia exuding light yellow conidial masses (Fig. 8C) formed on the entire fruit 14 DPI (= 100% incidence; data not shown). Isolates of *D. eres* induced similar symptoms but after a longer period of time (3 to 4 weeks). Fruit rot incidence for *D. eres* isolates FPH201322, FPH2015394, and FPH2017229 was 45, 70, and 65%, respectively (data not shown). *Epicoccum* isolates were able to induce similar symptoms to those caused by *Alternaria* and *Colletotrichum*; however, lesions remained limited to the point of inoculation (Fig. 8D) and disease incidence was relatively lower overall (60%; data not shown).

In experiment B, all four pathogens, regardless of the concentration of inoculum tested, were able to infect both wounded and unwounded detached fruit (data not shown). Disease incidence and severity on wounded fruit, however, were significantly higher than on unwounded fruit. On wounded fruit, disease incidence was above 88% for all pathogens 16 DPI (data not shown). Except for *Epicoccum*, disease severity increased along with inoculum concentration. *Epicoccum* inoculations resulted in low fruit rot severity, ranging from 5 to 11% at all concentrations (data not shown). On unwounded fruit, symptoms started to develop 24 DPI. No correlation pattern was observed between disease incidence and inoculum concentration. Disease incidence and severity for all treatments 42 DPI were 33 to 66% and 5 to 41%, respectively. Among all the pathogen-inoculum combinations tested, *D. ilicicola* sp. nov. at 10^6 conidia/ml caused the highest fruit rot incidence and severity.

Fig. 5. Maximum likelihood (ML) phylogenetic tree based on the Hasegawa-Kishino-Yano plus a gamma distribution model obtained from combined HIS, ITS, and TUB datasets of *Diaporthe* spp. The ML bootstrap support (BS) ≥ 70 and Bayesian posterior probability (PP) ≥ 0.95 values are indicated at the nodes (BS/PP). The tree was rooted with *Diaporthe nomurai* (CBS 157.29).

In experiment C, all pathogen inoculum combinations at all concentrations were able to infect both wounded and unwounded fruit (Figs. 9 and 10). However, disease incidence and severity of combined pathogens on wounded and unwounded fruit were not significantly different from those infected by individual pathogens (Figs. 9 and 10). With the exception of *E. nigrum*, disease incidence and severity on wounded fruit for all the pathogen-concentration treatments were significantly higher than on unwounded fruit. Regardless of the presence/absence of wounding and the type of pathogen inoculum, fruit infected with 10^5 conidia/ml resulted in significantly higher fruit rot incidence and severity compared with that inoculated with 10^3 and 10^2 conidia/ml. No rot symptoms developed on fruit in the control treatments in any of the three sets of experiments.

Discussion

Fruit rot is an emerging problem on deciduous holly that in recent years has been responsible for limiting crop production in ornamental nurseries across the Midwestern and Eastern U.S. Rot symptoms are characterized by black lesions on the surface of the fruit that eventually cause the whole fruit to soften and deteriorate. This study represents the first attempt to elucidate the etiology of this disease through characterization of the fungi associated with symptomatic tissues.

Seven different fungal species including *A. alternata*, *A. arborescens*, *C. fiorinae*, *C. nymphaeae*, *D. ilicicola* sp. nov., *D. eres*, and *E. nigrum* that were commonly associated with symptomatic deciduous holly samples were studied using morphological, phylogenetic, and pathogenicity characterizations. Among these, *A. alternata* and *D. ilicicola* sp. nov. were predominant. Overall, *Colletotrichum* and *Epicoccum* species were only occasionally isolated from fruit lesions, indicating that their role in the disease complex may be limited. Findings were supported by extensive sampling over multiple years and locations, including commercial nurseries, landscapes, and arboreta, mainly in Ohio but also in a few other eastern U.S. states.

The different fungal species recovered from the symptomatic samples did not seem to be associated with a specific sampling location. Instead, multiple species were found within the same planting at all locations except one arboretum in North Carolina, where only *D. ilicicola* sp. nov. was isolated. In particular, *A. alternata* and *D. ilicicola* sp. nov. were found within the same planting from more than half of the sampling locations across Ohio, Pennsylvania, and Massachusetts (data not shown). These findings contribute to supporting

Fig. 6. Maximum likelihood (ML) phylogenetic tree based on the Hasegawa-Kishino-Yano plus a gamma distribution model obtained from combined GADPH and TUB datasets of *Colletotrichum* spp. The tree with the highest log likelihood (-1,486.05) is shown. The ML bootstrap support (BS) ≥ 70 and Bayesian posterior probability (PP) ≥ 0.95 values are indicated at the nodes (BS/PP). The tree was rooted with *Colletotrichum johnstonii* (CBS 128532).

our conclusion that *A. alternata* and *D. ilicicola* sp. nov. are the primary pathogens associated with fruit rot of deciduous holly.

Symptoms caused by the individual pathogens were not distinct. Additionally, fungi were not always recovered from rotten fruit, which indicates that some of the symptoms may have been due to other causes, possibly including bacterial infection, environmental stresses, physiological disorders, or mechanical injury. It has been observed that discoloration or blackening of deciduous holly fruit may occur due to cold weather or frost (Galle 1997). In our study, we consistently observed that early defoliation was associated with fruit rot in the field. Loss of leaves could expose fruit directly to the sunlight and possibly result in sunscald (Barber and Sharpe 1971). In this study, however, sunscald on fruit was not observed. Olatinwo et al. (2003) also found no fungi associated with some rotten fruit in the cranberry fruit rot disease complex, and speculated that it could be due to physiological breakdown or mechanical injury. While the focus of this study was to characterize the fungal pathogens associated with fruit rot of deciduous holly, the roles of other factors causing fruit rot should be further investigated.

Morphological characteristics were used to initially assign the isolated fungi to different genera. Within each genus, detailed morphological comparisons allowed us to separate isolates into several groups. *Alternaria* isolates in this study were identified as small-spored and grouped into four morphological groups: *A. alternata*, *A. arborescens*, *A. tenuissima*, and *A. toxicogenica* mainly based on conidial morphology and sporulation patterns (Simmons 2007). However, it was impossible to rely solely on morphological characteristics for species identification because of the extensive number of species, overlapping morphological characteristics, and variable growth conditions. Several studies emphasize the importance of using combined morphological and molecular characterization for accurate identification of *Alternaria* species (Andrew et al. 2009; Armitage et al. 2015; Hartevelde et al. 2013; Lawrence et al. 2013). In this study, we selected the endopolygalacturonase gene (endoPG) and two anonymous genomic regions OPA1-3 and OPA10-2 that were successfully used in previous studies to distinguish small-spored *Alternaria* species (Andrew et al. 2009; Peever et al. 2004; Woudenberg et al. 2015) and we used concatenated sequences of these three loci to carry out phylogenetic analyses. Isolates morphologically identified as *A. arborescens* and *A. toxicogenica* clustered with reference strains of these same species included in the analysis. However, isolates morphologically identified as *A. alternata* and *A. tenuissima* distributed throughout the phylogenetic tree and were not grouped into any specific clade. These results were in agreement with other studies that used multilocus phylogenetic analyses in which *A. alternata* and *A. tenuissima* could not be separated (Andrew et al. 2009; da Cruz Cabral et al. 2017; Luo et al. 2017; Woudenberg et al. 2015). Therefore, Andrew et al. (2009) proposed the separation of *A. alternata* and *A. arborescens*, but combined other morpho-species including *A. alternata*, *A. tenuissima*, and *A. toxicogenica* into one single species, namely *A. alternata*. A recent taxonomic study by Woudenberg et al. (2015) using multilocus phylogeny supported the aforementioned study and suggested merging 35 morphospecies of *Alternaria* into *A. alternata*. Accordingly, we concluded that species of *Alternaria* associated with fruit rot of deciduous holly included *A. alternata* and *A. arborescens*.

The use of a combination of morphological and multilocus phylogenetic analyses is strongly suggested in the literature to delimit species boundaries of *Diaporthe* (Gomes et al. 2013; Santos et al. 2017; Udayanga et al. 2012, 2014a). In our study, morphological characterization and phylogenetic studies of concatenated sequences of three loci (ITS+HIS+TUB) were used to identify *Diaporthe* spp. associated with fruit rot of deciduous holly. Several species of *Diaporthe* have been previously reported from dead twigs or leaves of *Ilex* spp., including *D. eres*, *D. hongkongensis* R.R. Gomes, C. Glienke & Crous, *D. ilicis* (Ellis & Everh.) Wehm., *D. oncostoma* (Duby) Fuckel, *D. oxyspora* (Peck) Sacc., *D. pardalota* (Mont.) Nitschke ex Fuckel, and *D. rudis* (Fr.) Nitschke (Cash 1952; Gao et al. 2016, 2017; Gomes et al. 2013; Guba and Stevenson 1963;

Wehmeyer 1933). However, only a few sequences of reference strains of these species from *Ilex* are available in the literature (i.e., *D. eres* [CBS 370.67 and CBS 694.94]; *D. oncostoma* [CBS 809.85]; Gomes et al. 2013). For *D. rudis* and *D. hongkongensis*, sequences of isolates from *Ilex* were not available; therefore the ex-type and ex-epitype of these two species (CBS 113201 and CBS 115448) were included in the analyses (Gomes et al. 2013). *D. ilicis*, *D. oxyspora*, and *D. pardalota* were originally reported from *Ilex* in 1933. Sequences of these three species, regardless of the host on which they were found, were also not available and were not included in the phylogenetic studies. As a result of multilocus ML and BI analyses, 10 of 13 isolates included in the present study did not cluster with any of the above species, but grouped together in an unnamed monophyletic clade closely related to a reference strain of *D. cf. heveae* 1 (CBS 852.97) isolated from *Hevea brasiliensis* in Brazil. Sequences from a total of six *D. cf. heveae* isolates, comprising unpublished sources, were initially included in the phylogenetic analysis and subsequently removed to include published sources only. It is to be noted that even in the initial analysis all the isolates from our study grouped in a different clade. The two *D. cf. heveae* reference isolates that were left (CBS 852.97 and CBS 681.84), however, were reported to be sterile (Gomes et al. 2013), so morphological comparisons between isolates was not feasible. Additionally, morphological characterizations of our 10 isolates differed from the original descriptions of *D. ilicis*, *D. oxyspora*, and *D. pardalota* available in the literature (Wehmeyer 1933). At the time this manuscript is being prepared, no new record of morphological or molecular characterization of these three species has been found. Additionally, these species had not been found to infect fruit of *Ilex*, either. Based on detailed morphological characterization and molecular analysis of our isolates, we hereby propose a new species description, namely *D. ilicicola* sp. nov. The genus *Diaporthe* consists of species that range from host specific to those with a wide host range. In this study, only *Ilex* fruit was inoculated to test pathogenicity of our *D. ilicicola* sp. nov. isolates. Cross-inoculation of *D. ilicicola* sp. nov. on other fruit crops may provide valuable information for further species characterization.

The remaining three *Diaporthe* isolates included in our study were identified as belonging to the *D. eres* species complex based on both morphological and molecular analyses. To better understand

Fig. 7. Phylogenetic tree from maximum likelihood (ML) analysis based on the Hasegawa-Kishino-Yano plus a gamma distribution model obtained from ITS datasets of *Epicoccum* spp. The ML bootstrap support (BS) ≥ 70 and Bayesian posterior probability (PP) ≥ 0.95 values are indicated at the nodes (BS/PP). The tree was rooted with *Epicoccum keratinophilum* (CBS 142455).

the relationship of these three isolates within the *D. eres* species complex, a second phylogenetic analysis was conducted using additional reference strains that were within or closely related to the *D. eres* species complex. According to the new analysis, two of the three isolates (FPH201322 and FPH2015394) clustered with *D. eres* reference strains, including one recovered from *Ilex* (CBS 370.67), while one isolate (FPH2017229) grouped with *D. cf. nobilis* (CBS 587.79). It is to be noted that alpha and beta conidia of FPH2017229 were significantly narrower compared with FPH201322 and FPH2015394, possibly indicating species differences. Our findings are in agreement with previous phylogenetic

studies where separate clades for *D. eres* and *D. nobilis* were obtained (Baumgartner et al. 2013; Gomes et al. 2013; Lawrence et al. 2015), but are in disagreement with a study that specifically addressed the *D. eres* species complex (Udayanga et al. 2014a), in which *D. nobilis* was not recognized as a separate species. It is to be noted that in the latter case, ITS was not included in the multi-locus phylogenetic analysis as this gene was considered as having too much variability within the genus and was discordant to other trees generated in the study. While the analysis of additional loci may be needed to further differentiate our three isolates within the *D. eres* species complex, due to the relatively low frequency

Fig. 8. Symptoms and signs on detached holly fruit used in the pathogenicity experiments 10 days postinoculation. **A**, *Alternaria alternata*; **B**, *Colletotrichum fioriniae*; **C**, *Diaporthe ilicicola* sp. nov.; **D**, *Epicoccum nigrum*.

of isolation of these fungi in our study, we did not pursue additional identification. Both *D. eres* and *D. nobilis* are reported as minor pathogens causing blight, cankers, leaf spots, and fruit rot on a broad range of host families of woody plants worldwide (Baumgartner et al. 2013; Choi et al. 2017; Farr and Rossman 2018; Kaliterna et al. 2012; Lawrence et al. 2015; Li et al. 2017; Lorenzini and Zapparoli 2018; Thomidis and Michailides 2009; Zhang et al. 2016). Indeed, in our pathogenicity experiments, *D. eres* isolates caused relatively lower fruit rot incidence compared with *D. ilicicola* sp. nov. and it took a longer period of time for the inoculated fruit to develop symptoms. All *D. ilicicola* sp.

nov. isolates tested resulted in 100% disease incidence with symptoms visible in less than a week, compared with 45 to 70% incidence seen for *D. eres* that took 21 to 28 days to become evident. Based on these observations, fungi in the *D. eres* species complex should be considered minor pathogens causing fruit rot of deciduous holly.

Selected isolates belonging to the two predominantly isolated pathogen species (*A. alternata* and *D. ilicicola* sp. nov.), along with possibly minor pathogens such as *C. fioriniae* and *E. nigrum*, were included in three sets of pathogenicity experiments that not only tested their ability to cause disease but also aimed to understand the role of wounding and inoculum concentration on disease

Fig. 9. Fruit rot incidence recorded on wounded and unwounded fruit inoculated in pathogenicity experiment C with individual and combined pathogens at all inoculum concentrations. Bars indicate 95% confidence intervals.

Fig. 10. Fruit rot severity recorded on wounded and unwounded fruit inoculated in pathogenicity experiment C with individual and combined pathogens at all inoculum concentrations. Bars indicate 95% confidence intervals.

development. All isolates were able to infect detached mature holly fruit and induce rot symptoms, and all were reisolated from the symptomatic tissues, which fulfilled Koch's postulates. Disease incidence and severity levels, however, were overall much higher in fruit that had been wounded compared with unwounded fruit, indicating that fruit injury, either biotic or abiotic in nature, could result in higher disease levels in the field. Higher inoculum concentration always resulted in more severe disease, suggesting that practices that can decrease inoculum concentration (e.g., removal of infected plant material, pruning, or chemical applications) could lower disease levels in the field. Since occasionally multiple pathogens were isolated from the same lesion on the original field samples, individual inoculum and combination of pathogens were also tested. Similar disease incidence and severity resulted from single versus combined pathogen inoculations. It is to be acknowledged that all inoculation experiments in this study were conducted in the laboratory where fruit was maintained in a controlled environment at optimal temperature and humidity levels, which may have favored pathogen activity. However, a separate set of inoculations whose results are partially reported elsewhere (Lin et al. 2017) were made in the winter of 2016 on wounded and unwounded fruit of deciduous holly cultivar Sparkleberry maintained outdoors in a container trial. In that experiment, fruit was inoculated with some of the same isolates used in the detached fruit assays reported in this study, and naturally exposed to fluctuating environmental conditions. While disease incidence was overall lower in the outdoor trial, all four pathogens (*A. alternata*, *C. fioriniae*, *D. ilicicola* sp. nov., and *E. nigrum*) successfully infected wounded mature fruit, caused the same symptoms observed on the detached fruit assays, and were reisolated from the lesions, which fulfilled Koch's postulates. However, no infection was observed on unwounded fruit *in planta*. Further studies investigating the possible pathways of fruit infection in natural settings are underway and will complement these preliminary observations.

This study identified and characterized various fungal pathogens associated with fruit rot of deciduous holly, which represents a first step in the understanding of this disease complex. Further research is being conducted to investigate the sources of pathogen inoculum in the field, to understand when pathogens infect the plant during the growing season, and to identify the environmental factors that favor disease development. Precise information about the disease cycle and disease epidemiology is needed to ultimately provide nursery growers with effective management recommendations.

Acknowledgments

The research described in this paper represents a portion of the dissertation submitted by S. Lin to the Office of Graduate Studies of The Ohio State University to partially fulfill requirements for the Ph.D. degree in Plant Pathology. The authors thank all the winterberry growers in Ohio, Pennsylvania, and Massachusetts who provided many of the fruit samples used in this study. The authors also thank Dr. F. P. Trouillas for assistance in the description of the new *Diaporthe* species, as well as Dr. J. R. Urbez-Torres and Dr. G. Marchi for critical review of this manuscript prior to submission.

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