Identification of Species in the *Botryosphaeriaceae* Family Causing Stem Blight on Southern Highbush Blueberry in Florida

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ABSTRACT

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Stem blight of southern highbush blueberry (SHB) results in premature plant mortality and has been identified by Florida blueberry growers as the economically most important disease for the industry. In 2007, plants with stem blight and dieback symptoms were sampled at 4-month intervals from two farms located in Alachua and Polk Co., FL. In all, 30 cane samples (stem blight) and 30 crown segments (dieback) were collected at each sample date and each location. In total, 360 samples were collected; fungal species in the family *Botryosphaeriaceae* were isolated from 85% of the samples. Based on morphology and phylogenetic analysis of the internal transcribed spacer region and elongation factor 1- α (EF1- α) sequences, two dominant species recovered from SHB in Florida were identified: *Lasiodiplodia theobromae* and *Neofusicoccum ribis*. Species isolation was independent of location, symptom type, and time of year. Additional samplings are needed to investigate population change over multiple years and in the rest of the southeastern United States. Breeding for resistance and management of stem blight and dieback in Florida should focus on these two fungal species.

The Florida southern highbush blueberry (SHB; interspecific *Vaccinium corymbosum* and *V. darrowi* hybrid) industry has an early-season high-dollar niche market (34). Blueberry production has doubled since the early 1980s; currently, Florida ranks fifth in the United States for commercial acreage (34,35). Fungal vascular diseases have become an important problem for blueberry growers. Pathogens enter through flower buds, lenticels, stomata, and wounds and colonize the vascular system (5,14,15,19,30).

Florida blueberry growers have identified stem blight and dieback symptoms that result in premature plant mortality as the economically most important disease problems they face. Stem blight typically infects current-season growth and can progress rapidly down the vasculature (15). Symptoms include rapid wilting and reddening of leaves on affected branches. Partial or complete occlusion of the vasculature results in brown discoloration, typically on one side of the infected branch (15,36). A single branch with terminal leaf and stem blight symptoms as described above is referred to as the stem blight in the current study. In severe cases, infection progresses into the crown of the plant and results in systemic branch dieback over a

period of weeks or months, eventually killing the plant. Systemic dieback associated with crown infection is referred to as dieback in this study. Mortality associated with dieback limits production and results in substantial replant costs. Fungicide applications (15), optimizing irrigation practices (8), and aggressive pruning for the control of stem blight increase production costs and cannot adequately manage the disease (32).

Species in the family *Botryosphaeriaceae* typically have a wide host range and geographical distribution (24). These fungi are largely considered drought-stress opportunistic pathogens and live as saprophytes or endophytes most of the time (10,27). Species identification has been difficult because multiple species have been found parasitizing the same host (3,4,30,31). Virulence of and symptoms caused by *Botryosphaeria* spp. have been reported to be different depending on cultivar and location (30).

The anamorphs of *Botryosphaeria* spp. occur frequently on infected tissue and are primarily used for identification (9–11,36). Anamorphic characteristics have phenotypic variation and are similar among species (11,21). Eighteen anamorphic genera have been associated with *Botryosphaeria* spp. and, currently, 10 lineages are recognized within the family *Botryosphaeriaceae* (9). Phylogenetic studies using morphological characterization and genomic data have contributed to the clarification of *Botryosphaeria* spp. taxonomy (9,11,21,39). Molecular data have allowed for the positive and rapid identification of species in the *Botryosphaeriaceae* family parasitizing

grapes (6,29–31), mango (22), and pistachio (12).

Witcher and Clayton (36) described *Botryosphaeria dothidea* as the causal agent of stem blight of blueberry. At the time, *B. ribis* and 23 other named fungal species were considered synonyms of *B. dothidea* (21,36). Pennycook and Samuels (17) accepted the synonyms for *B. dothidea* and described *B. parva*. Based on multigene phylogeny, *B. ribis* (anamorph *Neofusicoccum ribis*) is considered to be a separate species from *B. dothidea* (anamorph *Fusicoccum aesculi*) and *B. parva* (anamorph *N. parvum*) (9,21). However, *N. parvum* and *N. ribis* cannot be distinguished based on internal transcribed spacer (ITS) rDNA sequences (21,25,39) and restriction fragment length polymorphism analysis (23). Conidial morphology between the species is similar but conidia of *N. parvum* are slightly smaller than those of *N. ribis* and turn brown and multi-septate with age (21). Partial sequences of the elongation factor 1- α (EF1- α), a portion of RNA polymerase II subunit, and slight differences in conidial morphology are used to distinguish the two species (16,21,31).

The species of fungi that cause the most important dieback and stem blight symptoms of SHB in Florida are unknown. Fungi such as *Diplodia*, *Macrophoma*, and *Phomopsis* spp. have been reported to cause these types of symptoms (1). The objective of this study was to identify to species the predominant causal agents associated with stem blight and dieback symptoms of SHB in Florida.

MATERIALS AND METHODS

Sample material collection and pathogen isolation. Stem blight samples consisted of partially hardened succulent stems from current-season growth. Dieback samples consisted of a major cane and were taken no further than 12 cm above the crown of a plant. Samples were collected periodically from two farms in Florida: the first was located in Alachua County (north-central Florida) and the second was located 225 km south in Polk County (central Florida). A farm-wide survey of disease was made at each location at four-month intervals (January to February, June to July, and October to November) in 2007; 30 samples of each stem blight and dieback symptom were collected from both farms during a survey period. Other samples outside the survey

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included isolates from *Vaccinium virgatum*, SHB samples from the Florida Extension Plant Disease Clinic, *Ilex* spp. (common near blueberry fields in Florida), and isolates from a foliar ring spot symptom on the SHB cvs. Millennia and Star (Table 1). Samples outside the survey were included for comparison with samples collected from the two farms. Bark of blueberry samples was removed to expose the discolored vasculature and margins were excised and cut into small pieces. Four pieces of each sample were surface disinfested in 10% household bleach (0.615% sodium hypochlorite) for 1 min and rinsed with distilled water. Tissue samples were dried with a paper towel and plated on 85-mm petri dishes containing V8 juice agar (15.0 g of agar, 200 ml of V8 juice, and 2.0 g of CaCO₃ per liter) amended with 0.01 mg of rifampicin and 0.25 g of ampicillin sodium salt per liter. Cultures were incubated at 25°C for 5 days. Fungal isolates were obtained by transferring mycelial fragments from the margins of expanding colonies. Isolates were stored on filter paper disks and maintained in a 5°C refrigerator.

The remaining portions of samples after plating were incubated at 25°C for 2 weeks in sealed plastic bags with a moist paper towel. All samples were checked for the presence of sporulating structures after incubation, and the number of samples per location and sample period were counted. If asexual structures were observed, conidia were identified by morphological characteristics. Likewise, if sexual fruiting bodies were observed, isolations were made from single-spore serial dilutions onto plates of potato dextrose agar (PDA)

(BD, Sparks, MD). Mycelial fragments from the margins of single-spore colonies were excised, transferred, and stored as described above.

DNA extraction, amplification, and phylogenetic analysis. Genomic DNA from 219 isolates randomly selected from storage was extracted using the Qiagen DNeasy Kit (Qiagen, Santa Clarita, CA). After extraction, oligonucleotide primers ITS1 and ITS4 were used to amplify the ITS1 and ITS2 and 5.8s regions of rDNA (33). A partial sequence of EF1- α was amplified using primers EF1-728 and EF1-928 (Integrated DNA Technologies, Inc., Coralville, IA) (7). Polymerase chain reaction (PCR) mixtures included: 10 μ l of RED Extract-N-Amp PCR mix (Sigma-Aldrich, St. Louis), 2 μ l of each primer, 2 μ l of PCR-grade water, and 4 μ l of extracted DNA. The thermocycler program (Brinkman Instruments, Inc., Westbury, NY) used with ITS primers included an initial denaturing step of 3 min at 94°C followed by 35 cycles of denaturing at 94°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 60 s. A final extension at 72°C was held for 10 min. Likewise, the EF1- α program included an initial denaturing step of 3 min at 94°C followed by 40 cycles of denaturing at 94°C for 60 s, and annealing at 60°C for 45 s, and extension at 72°C for 60 s. A final extension at 72°C was held for 10 min. For each PCR product, 5 μ l was separated by gel electrophoresis in 1.2% agarose gels (Fisher Scientific, Fair Lawn, NJ) containing 0.5 μ g per 1 ml of ethidium bromide in a Tris-borate buffer (Sigma-Aldrich). Each unrefined PCR product (5 μ l) was placed on half of a 96-well PCR

plate that was sent to the University of Florida's Interdisciplinary Center for Biotechnology Research for bidirectional sequencing with the PCR primers.

Sequences were edited using the software program Sequencher 4.6 (Gene Codes, Corp., Ann Arbor, MI), locally aligned using Clustal X 2.06-macosx (Conway Institute, UCD., Dublin) (28) and manually aligned using the computer software McClade 4.08 OSX (Sinauer Associates, Inc., Sunderland, MA) (13). Phylogenetic analysis was completed using PAUP 4.0b10 (Sinauer Associates) (26). Alignment gaps were treated as missing data. Twenty-two isolates from the survey (Table 1) were compared using phylogenetic analysis to related sequences published in GenBank (Table 2). Isolate CMW7063 (*Bionectria* sp.) was used as the outgroup for phylogenetic analysis. Separate and combined phylogenetic analyses were run for both data sets. A partition homogeneity test was used to determine whether ITS and EF1- α regions could be combined. Maximum parsimony analysis was performed using the heuristic search option (NNI branch swapping). Bootstrap values were evaluated using 1,000 replicates and 100 random sequence additions to test branch strength. Tree length, consistency index (CI), and retention index (RI) were recorded for all analyses.

Morphological characterization. Thirty *Botryosphaeriaceae* isolates identified based on sequence data were further characterized by conidial morphology and colony growth characteristics. Conidia used for characterization were produced on PDA and in planta following cut-stem inoculations. Isolates taken from storage

Table 1. Representative isolates from Florida sample collections used in phylogenetic analysis

Species ^a	Origin	Collected	Host ^b	Abbreviation	GenBank accession no.	
					ITS ^c	EF1- α ^d
<i>Neofusicoccum ribis</i>	Archer, FL	August 2004	SHB	UF0440	FJ877139	GQ845100
<i>Lasiodiplodia theobromae</i>	Apopka, FL	May 2005	ILL	UF05161	GQ845096	GQ850468
<i>Botryosphaeria dothidea</i>	Wildwood, FL	February 2007	SHB	UF0730	GU595170	GQ869643
<i>B. dothidea</i>	Archer, FL	August 2007	SHB	ARSM	GU595171	GQ869644
<i>N. ribis</i>	Archer, FL	August 2007	SHB	ARS	GQ845088	GQ845108
<i>N. ribis</i>	Waycross, GA	May 2007	SHB	GAC1	GQ336828	GQ845103
<i>N. ribis</i>	Hawthorne, FL	May 2007	REB	Rbe2	GQ845087	GQ845107
<i>N. ribis</i>	Windsor, FL	August 2007	SHB	WRS2	GQ336829	GQ845104
<i>L. theobromae</i>	Polk County, FL	October 2007	SHB	MixFF1	GQ845093	GQ850465
<i>N. ribis</i>	Polk County, FL	October 2007	SHB	MixFF8	GQ336826	GQ845101
<i>N. ribis</i>	Polk County, FL	June 2007	SHB	MixSuC4	GQ845092	GQ845112
<i>N. ribis</i>	Alaucha County, FL	November 2007	SHB	WFC25	GQ845090	GQ845110
<i>N. ribis</i>	Alaucha County, FL	November 2007	SHB	WFF10	GQ845091	GQ845111
<i>N. ribis</i>	Alaucha County, FL	November 2007	SHB	WFF9	GQ845089	GQ845109
<i>L. theobromae</i>	Alaucha County, FL	November 2007	SHB	WFF92	GQ845095	GQ850467
<i>N. ribis</i>	Alaucha County, FL	June 2007	SHB	WsuC17	GQ336827	GQ845102
<i>N. ribis</i>	Alaucha County, FL	June 2007	SHB	WSuF16	GQ336831	GQ845106
<i>N. ribis</i>	Alaucha County, FL	January 2007	SHB	WWC38	GQ336830	GQ845105
<i>L. theobromae</i>	Alaucha County, FL	January 2007	SHB	WWF37	GQ845099	GQ850471
<i>L. theobromae</i>	Alaucha County, FL	February 2007	SHB	WMixC35	GQ845097	GQ850469
<i>L. theobromae</i>	Polk County, FL	February 2007	SHB	WmixF13	GQ845094	GQ850466
<i>L. theobromae</i>	Polk County, FL	February 2007	SHB	WmixF27	GQ845098	GQ850470

^a Fungi identified based on morphology and phylogenetic analyses.

^b SHB = Southern highbush blueberry; ILL = *Illex cassine*; REB = *Vaccinium virgatum*.

^c Internal transcribed spacer region.

^d Elongation factor 1- α .

were plated directly onto PDA and were incubated at 25°C with 12 h of light per day. Conidia production occurred after 5 weeks for most isolates.

Cv. Jewel was used for stem inoculations. An 8-mm mycelium plug was taken from the margin of a 3-day-old colony of selected isolates. Plugs were secured with Parafilm (Pechiney Plastic Packaging, Co., Chicago), and lesions were allowed to develop. After 10 days, pycnidia were visible on plant stems. The length and width of 60 conidia of each isolate pro-

duced on PDA or by stem inoculations was measured with a compound microscope using the imaging software Q-capture pro (QImaging, Surry, Canada). Mean and standard deviation of conidial measurements were analyzed in SAS (SAS institute, Cary, NC) using summary statistics. Conidial color, shape, and presence or absence of septations also were recorded.

Botryosphaeriaceae isolates selected for conidial characterization also were used for temperature growth studies. A 5-mm plug was taken from the margin of a 3-

day-old colony and placed in the center of an 85-mm petri plate. Three replicates of each isolate were incubated separately at 5, 10, 15, 20, 25, 30, 35, and 40°C in the dark. Pigmentation was recorded if present at a particular temperature. Colony diameter was measured after 12, 24, and 36 h of growth. Data were converted to radial growth rate in millimeters using the measurements from 12 and 36 h (mm/day) (20). The experiment was conducted twice. A *t* test determined whether data from both experiments could be combined. Average

Table 2. Isolates not collected in this study but used for comparison in phylogenetic analysis

Isolate ^a	Species	Host	Origin	GenBank accession no.	
				ITS ^b	EF1- α ^c
CBS119047	<i>Botryosphaeria corticis</i>	<i>Vaccinium corymbosum</i>	New Jersey, United States	DQ299245	EU017539
CBS119048	<i>B. corticis</i>	<i>V. corymbosum</i>	New Jersey, United States	DQ299246	EU017540
CBS9075	<i>B. dothidea</i>	<i>Populus nigra</i>	New Zealand	AY236950	AY236899
CBS 116741	<i>B. dothidea</i>	<i>P. nigra</i>	Portugal	AY640254	AY236890
CMW1130	<i>B. parva</i>	<i>Sequoia gigantea</i>	South Africa	AY236945	AY236890
CBS110301	<i>B. parva</i>	<i>Vitis vinifera</i>	Portugal	AY259098	AY573221
CMW7054	<i>B. ribis</i>	<i>Ribis rubrum</i>	New York, United States	AF241177	AY236879
CMW7773	<i>B. ribis</i>	<i>Ribis</i> sp.	New York, United States	AY236936	AY236878
CBS447.62	<i>Lasiodiplodia pseudotheobromae</i>	<i>Citrus aurantium</i>	Suriname	EF622081	EF622060.1
CBS190.73	<i>L. theobromae</i>	<i>Persea americana</i>	Tanzania	EF622068	EF622048
CMW7063	<i>Bionectria</i> sp.	<i>Taxus baccata</i>	Netherlands	AY23656	AY236905

^a Acronyms of culture collections: CMW = Culture Collection Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; and CBS = Centraalbureau Schimmelfcultures, Utrecht, Netherlands.

^b Internal transcribed spacer region.

^c Elongation factor 1- α .

Table 3. Isolation results from sampling effort and preliminary identification of a select group of isolates using the sequences of the internal transcribed spacer region

Survey period, sample type	Colony ^c	Seq. ^d	<i>Neofusicoccum ribis</i> ^a		<i>Lasiodiplodia theobromae</i> ^b		Other	
			No.	Percent	No.	Percent	No.	Percent
Winter (January–February)								
Alachua County								
Stem blight	25	17	14	82	3	18	0	...
Dieback	27	20	13	65	7	35	0	...
Subtotal	52	37	27	73	10	27	0	...
Polk County								
Stem blight	18	18	17	85	2	10	1	5
Dieback	29	21	12	57	9	43	0	...
Subtotal	47	31	29	71	11	27	1	2
Winter total	99	78	56	72	21	27	1	1
Summer (June–July)								
Alachua County								
Stem blight	24	19	18	95	1	5	0	...
Dieback	23	15	9	60	4	27	2	13
Subtotal	47	34	27	79	5	15	2	6
Polk County								
Stem blight	24	16	11	69	3	19	2	12
Dieback	21	14	11	85	2	15	0	...
Subtotal	45	29	22	76	5	17	2	7
Summer total	92	63	49	78	10	16	4	6
Fall (October–November)								
Alachua County								
Stem blight	30	23	20	87	3	13	0	...
Dieback	30	18	14	78	4	22	0	...
Subtotal	60	41	34	83	7	27	0	...
Polk County								
Stem blight	26	20	14	70	4	20	2	10
Dieback	29	17	11	65	6	35	0	...
Subtotal	55	37	25	68	10	27	2	5
Fall total	115	78	59	76	17	22	2	2

^a Sequenced isolates identified as *Neofusicoccum ribis*.

^b Sequenced isolates identified as *Lasiodiplodia theobromae*.

^c Number of samples that *Botryosphaeria*-like fungi were isolated from out of 30 per symptom type.

^d Number of isolates sequenced from total recovered.

radial growth rate versus temperature was plotted in SAS. Multiple regression curves were fit to the data in SAS using the Proc. Reg. function. After the regression equations were derived, the quadratic equation was used to calculate the optimum temperature of the regression curves.

RESULTS

Field survey, fungal isolation, and molecular characterization. Colonies consistent with *Botryosphaeria* spp.-like growth were isolated from 99 of 120 samples in the winter, 92 of 120 samples in the summer, and 115 of 120 samples in the fall (Table 3). Overall, colonies consistent with *Botryosphaeria* spp.-like growth were isolated from 85% of the 360 samples. Other fungal genera were isolated from approximately 15% of blueberry samples, including *Alternaria*, *Pestalotia*, and *Phomopsis* spp.

Identification of isolates from colonies consistent with morphology and growth characteristics of fungi in the family *Botryosphaeriaceae* was based on ITS sequence data and was confirmed by isolate morphology (Tables 3–5). The total number of isolates sequenced from the winter, summer, and fall collection periods were 78, 63, and 78, respectively. Fungi isolated included *Lasiodiplodia theobromae* and *N. ribis*, and other fungi such as *Alternaria* and *Phomopsis* spp. Both species in the *Botryosphaeriaceae* family were recovered from stem blight and dieback samples (Table 3). *B. dothidea* was isolated twice from samples outside the survey area.

Fruiting structures were observed in the fall at each sample location. Pycnidia were observed on 11 and 18 samples from Alachua and Polk Counties, FL. Conidia morphology was used to identify samples with pycnidia. *L. theobromae* was recovered from 10 samples in Alachua County and from 17 samples in Polk County. *N. ribis* was recovered once from each location in the fall. Likewise, perithecia were observed on one sample at each location.

All samples with perithecia were identified as *B. ribis*.

Phylogenetic characterization. ITS and EF1- α sequences of isolates were compared with homologous sequences published in GenBank (Fig. 1). A partition homogeneity test indicated that the ITS and EF1- α datasets could be combined ($P = 0.77$). Of the 867 nucleotides analyzed, 203 were parsimony informative. Maximum parsimony analysis yielded one most parsimonious tree (length = 691, CI = 0.89, RI = 0.95; Fig. 1). Tree topology was consistent with the ITS tree. Species in the *Botryosphaeriaceae* family with hyaline thin-walled conidia formed a monophyletic clade supported by an 84% bootstrap value, and included *B. corticis*, *B. dothidea*, *N. parvum*, and *N. ribis*. Isolates of *L. theobromae* with thick-walled, pigmented conidia grouped in a clade supported by a 99% bootstrap value. Intraspecific variation was present within the clade. *B. dothidea* and *B. corticis* grouped in a clade separate from *N. ribis* and *N. parvum* and were supported by a 100% bootstrap value.

Morphology. All isolates could be separated into two groups based on conidial

characteristics. One group was characterized by the production of brown-pigmented, thick-walled conidia and included *L. theobromae*. The second group was characterized by the production of hyaline, thin-walled conidia and included *F. aesculi* and *N. ribis*. Conidial dimensions recorded for these fungi are shown in Table 4, along with previously reported dimensions.

A cubic polynomial model best fit the data. All isolates grew over a range of temperatures (15 to 35°C) and the optimum temperature of approximately 30°C did not vary between species (Table 5). None of the isolates tested grew at either 5 or 10°C after 36 h. *L. theobromae* was the only species to exhibit slight growth at 40°C, and displayed bright pink pigment at temperatures $\geq 35^\circ\text{C}$. Likewise, *N. ribis* isolates displayed a yellow pigment at temperatures from 20 to 30°C. Among the fungi tested, *L. theobromae* had the highest mycelial growth rate at the optimum temperature after 36 h, followed by *N. ribis* and *B. dothidea* (Table 4). No significant variation was present between isolates of the same species at each temperature.

Table 5. Optimum temperature and growth rate of fungi in the family *Botryosphaeriaceae* from Florida in comparison with those reported from previous studies

Species, isolate	This study		Previous studies		Reference
	Opt. (°C) ^a	Growth rate ^b	Opt. (°C) ^a	Growth rate ^c	
<i>Botryosphaeria dothidea</i>					
UF0730	28	13.6 $\pm$ 0.6	25–30	12.5	17
ARSM	29	11.7 $\pm$ 0.3	28–29	11.1	21
<i>Lasiodiplodia theobromae</i>					
UF05161	30	17.8 $\pm$ 0.8	30–35	10.6	30
WFF92	29	17.5 $\pm$ 0.4	30.8	10.0	31
WmixC35	30	18.3 $\pm$ 1.4			
<i>N. parvum</i>	...	...	28–29	18.2	17
	...	...	27.8	8.8	31
<i>Neofusicoccum ribis</i>					
rbe2	29	11.5 $\pm$ 0.8	25	16	21
WsuF16	27	12.6 $\pm$ 1.3	28	...	36
WRS2	28	13.0 $\pm$ 0.6	...	...	...

^a Optimum temperature is defined as the maximum radial growth after 36 h.

^b Average ($n = 6$) radial growth and standard deviation in millimeters per day at 30°C.

^c Average radial growth in millimeters per day at various temperatures.

Table 4. Dimensions of conidia of fungi in the family *Botryosphaeriaceae* from Florida in comparison with those reported from previous studies

Species, isolate	Isolates from this study (mean and SD [μm]) ^a	Fungi from previous studies (conidia size [μm]) ^b	Reference
<i>Botryosphaeria corticis</i>	...	23.5–32.5 $\times$ 5.5–7	18
<i>B. dothidea</i>			
UF0730	26.0 $\pm$ 1.6 $\times$ 5.5 $\pm$ 0.7 ^c	(15–)20–26(–32) $\times$ (4–)5–6(–9)	17
ARSM	26.2 $\pm$ 2.1 $\times$ 5.8 $\pm$ 0.6 ^c	(20–)23–27(–30) $\times$ 4–6(–6)	21
<i>Lasiodiplodia theobromae</i>			
UF05161	29.9 $\pm$ 1.4 $\times$ 16.4 $\pm$ 0.9 ^c	(–20)23–27(–30) $\times$ (–10)11–13(–14)	30
WFF92	29.4 $\pm$ 3.2 $\times$ 14.3 $\pm$ 1.4 ^c	(18–)20–25 $\times$ (9)11–14	31
WmixC35	25.9 $\pm$ 2.5 $\times$ 14.5 $\pm$ 1.1 ^c		
<i>Neofusicoccum parvum</i>	...	(11–)14–18(–23) $\times$ (5–)6–7(–9)	17
	...	(10–)14.5–17 $\times$ (5)7–9	30
<i>N. ribis</i>			
rbe2	20.7 $\pm$ 2.0 $\times$ 6.6 $\pm$ 0.9 ^d	(16–)19–23(–24) $\times$ 5–6(–7)	21
WsuF16	20.3 $\pm$ 1.9 $\times$ 6.6 $\pm$ 0.7 ^d	16–22.9 $\times$ 4.9–7.8	36
WRS2	20.3 $\pm$ 1.8 $\times$ 6.8 $\pm$ 0.7 ^d	...	...

^a Average length, width, and standard deviation (SD) of 60 conidia from each isolate.

^b Minimum size, most common values, and maximum size for length and width of conidia.

^c Conidia produced directly on potato dextrose agar.

^d Conidia produced by stem inoculations.

DISCUSSION

The Florida SHB industry has two primary pathogens causing stem blight and dieback of blueberry: *L. theobromae* and *N. ribis*. *B. dothidea* was not recovered from any samples in the two-survey farm. Species recovery was not dependent on location, season, or type of symptom (Table 1). Although *L. theobromae* was recovered from sample material less frequently than *N. ribis*, both species were recovered from stem blight and dieback samples. A preliminary study also confirmed that all three species were pathogenic on SHB in Florida (38). Recovery incidence and pathogenicity indicate that both species are economically important for the industry because they have the potential to reduce

yield and field longevity. Therefore, management strategies need to be developed which control both pathogens.

Witcher and Clayton (36) described *B. dothidea* as the causal agent of blueberry stem blight. The morphology of *N. ribis* isolates recovered from SHB in Florida was consistent with Witcher and Clayton's original description of the pathogen. Isolates produced a yellow pigment on PDA, and mycelium growth rate and conidia dimensions for *N. ribis* isolates were similar to the original studies' description (Table 4). In 2004, *N. ribis* was separated from *B. dothidea* (21) and another closely related species, *N. parvum*. In 2009, the *N. parvum*-*N. ribis* complex was further split into several cryptic species using genea-

logical concordance of phylogenetic species recognition (GCPSR) (16). Our results confirm that one of the two causal agents of stem blight of SHB in Florida belongs within the *N. ribis* species complex.

The ITS region alone could not statistically separate *N. ribis* from *N. parvum*, and a partial sequence of the EF1- α region was needed to place our isolates (9,21,31). Within the *N. ribis* clade, our isolates formed a separate group (Fig. 1). Phylogenetic variation could be due to regional adaptation and genotypic recombination. The addition of other phylogenetic informative regions, including a portion of the RNA polymerase II subunit, should be analyzed to confirm the exact grouping of the Floridian isolates within the *N. ribis* clade (16).

The second species identified as causing stem blight of SHB was *L. theobromae*. The association of *L. theobromae* as the casual agent of stem blight of SHB was recently reported (37). Conidial morphology for *L. theobromae* isolates was consistent with the species descriptions (Table 4). Immature conidia were hyaline with a thick cell wall and were single celled with granular contents. Conidia became single septate with age, and dark brown with black longitudinal striations (2,9). Phylogenetic analysis confirmed morphological identification and grouped isolates within the *L. theobromae* clade (Fig. 1). Genetic variation was present in the *L. theobromae* clade, which could be due to regional adaptation.

The sexual state of *L. theobromae* was not observed on any of the sample material. However, the majority of samples found producing conidia were identified as *L. theobromae* and predominantly were observed during the fall of 2007. Observations were inconsistent with previous conidial production studies. Peak sporulation months for *B. dothidea* were June to July for blueberry in North Carolina (8) and August for peaches in Georgia (32). Variation are likely due to differences in climate, geographic location, and fungal species. Additional sporulation studies are needed to verify when conidia of *L. theobromae* and *N. ribis* are produced in Florida.

One *B. dothidea* isolate was obtained from a plant with a foliar ring spot symptom outside the survey. Additional samples with similar symptoms did not yield the fungus. Foliar inoculation studies with *B. dothidea* were not conducted, and additional work is needed to determine whether *B. dothidea* was causing these symptoms or existing as a foliar endophyte.

Surprisingly, *B. dothidea* was identified infrequently and was not recovered from any of the sample material in the survey. Low occurrence could be due to taxonomic reevaluation of the species (21). Recovery frequency of species in the family *Botryosphaeriaceae* has been reported to be

Fig. 1. Most equally parsimonious tree using 1,000 bootstrap and 100 random addition sequence replicates in PAUP. A partial sequence of the internal transcribed spacer (ITS1, 5.8S, and ITS2) and the elongation factor 1- α (EF1- α) from species in the family *Botryosphaeriaceae* were used to generate the consensus tree. Asterisks indicate isolates from previous studies.

different depending on geographic location (30). The survey also includes data from only 1 year; therefore, shifts in populations from year to year are a possibility. Continued periodic sampling of stem blight pathogens in Florida and the southeastern United States will help further define dynamics of the pathogen populations affecting the industry in the region. Sequence data collected to date will be used to develop PCR protocols to help distinguish between these species without the need to obtain sequences of multiple loci per isolate. Such protocols could help diagnosticians that routinely use PCR to differentiate these fungi; however, there are no practical management implications presented here that would support the added expense and effort. Although *N. ribis* and *L. theobromae* are the primary stem blight pathogens of SHB in Florida, additional work is needed before conclusions are drawn concerning other plant species and other blueberry-producing regions.

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