Real-Time and Conventional PCR Tools for Detection and Discrimination of *Calonectria pseudonaviculata* and *C. henricotiae* Causing Boxwood Blight

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Abstract

Calonectria pseudonaviculata and *C. henricotiae* are the causal agents of boxwood blight, a devastating disease of boxwood that has caused significant economic impact on the nursery and landscape industries in the U.S. and in Europe. The two species are genetically distinct and are found in different geographic areas but are difficult to distinguish based on morphology and pathogenicity. Fast, accurate, and inexpensive methods to detect and differentiate these species is critical in stopping the spread of the disease. We designed primer pairs based on available sequences of four conserved regions—calmodulin, histone H3, internal transcribed spacer, and β -tubulin—and tested their ability to differentiate the two *Calonectria* species. Here we report three primer pairs derived from

sequence differences in the histone H3 region that can be used to specifically detect *C. pseudonaviculata*, *C. henricotiae*, or both species. Specificity of these primers was tested against nine isolates of *C. pseudonaviculata*, three isolates of *C. henricotiae*, 13 other *Calonectria* species, and five isolates from related genera using conventional and real-time PCR. These are the first primers available that can be used with either a multiplexed conventional PCR or SYBR-based real-time PCR to specifically detect and differentiate the two fungal species.

Keywords: disease diagnosis, disease management, fungal pathogen, species-specific primers

Calonectria pseudonaviculata and *C. henricotiae* are the causal agents of boxwood blight, a devastating disease of *Buxus* species (boxwood) that has caused significant economic impact on the nursery and landscape industries in the U.S. and in Europe. The disease was first observed in the U.K. in 1994 (Henricot and Culham 2002) and has since been described throughout Europe, Asia, North America, and New Zealand (Daughtrey 2019; LeBlanc et al. 2018). While both species cause similar symptoms and are morphologically indistinct, they are isolated genetically and geographically—*C. pseudonaviculata* can be found in all areas where boxwood blight has been reported, whereas *C. henricotiae* is found only in Europe (Gehesquière et al. 2016; LeBlanc et al. 2019; Malapi-Wight et al. 2019). Symptoms of boxwood blight include dark lesions on the leaves and stems of infected plants, followed by defoliation and plant death. The fungi are locally transmitted through water splash or contact with infected plants, and are spread geographically through movement of infected nursery stock (bark, leaves, and stems) (Daughtrey 2019; LeBlanc et al. 2018). The two boxwood blight fungi are considered invasive pathogens because they are highly mobile locally (through water splash or direct contact), long lived, fast growing, and utilize an asexual reproductive biology with the potential for rapid multiplication (Daughtrey 2019; LeBlanc et al. 2018). The impact of the fungus can be partially mitigated by cultural and phytosanitary measures (Henricot et al. 2008; Kong and Hong 2017; Miller et al. 2018), or more reliably by fungicides (Henricot et al. 2008; LaMondia

2014). Host plant resistance has not been observed, although variation in the response of boxwood taxa to the fungi has been described (Ganci et al. 2013; Guo et al. 2015; Henricot et al. 2008; LaMondia and Shishkoff 2017; Shanjani et al. 2018).

Early and accurate detection of *C. pseudonaviculata* or *C. henricotiae* on symptomatic and asymptomatic plants is critical in stopping the spread of the disease. Currently, diagnosis of boxwood blight is based on visual symptoms on infected plants, isolation and incubation of the fungus, or lab-based molecular diagnostic assays (Daughtrey 2019; LeBlanc et al. 2018). Visual inspection of plants for disease symptoms is not always reliable because diagnosis of boxwood blight can be confounded by symptoms of other boxwood diseases such as those caused by *Pseudonectria buxi*, *P. foliicola*, *Phytophthora* spp., *Verticillium buxi*, and *Macrophoma candollei* (Daughtrey 2019). Identification based on microscopic examination of conidia is reliable but requires time and expertise to incubate the fungus and make the diagnosis. Lab-based diagnostic assays have been developed that can detect or differentiate *C. pseudonaviculata* and *C. henricotiae* (Dart et al. 2014; Gehesquière et al. 2013, 2016; LeBlanc et al. 2018; Malapi-Wight et al. 2016); however, these assays are generally based on methods that require considerable time and preparation, highly specialized or costly laboratory equipment, or technical expertise (sequence comparison; TaqMan-based PCR; PCR-RFLP). To date, these methods unfortunately have not been adopted for routine detection and identification of boxwood blight on symptomatic and asymptomatic plants. Our objective was to develop a simple and sensitive diagnostic PCR assay that could be routinely used in conventional or SYBR-based real-time PCR systems to detect and differentiate *C. pseudonaviculata* and *C. henricotiae* quickly and accurately in plant tissue.

Materials and Methods

Fungal isolates and leaf infection. Three isolates of *C. henricotiae*, nine isolates of *C. pseudonaviculata*, 14 isolates representing 13 other *Calonectria* species, and species from five related genera (Table 1) were used in this study to evaluate primer specificity. All cultures were grown at 23°C and maintained at 4°C on potato dextrose agar (PDA). Spores were prepared as described by Guo et al. (2015), and experimentally infected boxwood leaves were prepared according to Guo et al. (2016). Briefly, leaves of *Buxus sempervirens*

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'Suffruticosa' were surface sterilized with 70% ethanol, rinsed with sterile water, and each leaf was wounded by gentle pressure with a 200- μ l pipette tip on the abaxial leaf surface, resulting in a 0.1-cm-diameter wound. The wounded spot was then inoculated with 2- μ l of mycelium inoculum prepared as described by Guo et al. (2016). The inoculated leaves were placed inoculation side up on moistened filter paper in 100 $\times$ 10 mm polystyrene Petri dishes at 23°C with a 12-h photoperiod.

DNA extraction. Total genomic DNA was extracted from mycelium, spores, and infected and healthy boxwood leaves following the manufacturer's instructions using a DNeasy Plant Mini Kit in a QIAcube (Qiagen, Valencia, CA), and quantified with a NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific, Wilmington, DE). For DNA extraction from cultured fungus, the isolates were grown on PDA plates for 3 weeks in the dark at 23°C, and then 50 to 100 mg of mycelia or microsclerotia were collected by scraping off the top layer with a sterile spatula. For DNA extraction from boxwood leaves, a single leaf with a lesion covering at least 50% of the leaf area (5 to 7 days post inoculation) or a healthy control leaf was used, and the final DNA concentrations were adjusted to

10 ng/ μ l with water. For spores, DNA from 1 $\times$ 10⁶ spores in 100 μ l water was extracted, and the resulting DNA preparation was suspended in 100 μ l of water. Ten-fold serial dilutions were made with water (for assays with spores or mycelium) or 10 ng/ μ l plant DNA solution extracted from healthy boxwood leaves (for assays with infected leaf tissue).

Primer design. GenBank sequence data of four conserved regions, calmodulin (Cal), histone H3 (His), internal transcribed spacer (ITS), and β -tubulin (Tub) of *C. henricotiae*, *C. pseudonaviculata*, and three closely related species, *C. naviculata*, *C. multiphialidica*, and *C. chinensis* (Lombard et al. 2010), were aligned for primer design using CLC Main Workbench 20.0 (Qiagen, Aarhus, Denmark). Primer pairs were designed with specificity to *C. henricotiae* (Ch), *C. pseudonaviculata* (Cp), and to both species (Chp) using the online Primer BLAST tool (Ye et al. 2012). The primer sequences and related information of all primers designed and tested are shown in Supplementary Table S1. The universal fungal primer pair, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990), was used as a positive fungal PCR control, and the universal plant primer pair

Table 1. Fungal samples and isolates tested in this study, and mean Ct values ($\pm$ standard deviation for three replications) of each primer pair for each sample using real-time PCR with the indicated annealing temperature

Species	Isolate and origin ^a	ITS1/ITS4 (54°C)	His-Cp-F56/R268 (62°C)	His-Ch-F39/R286 (60°C)	His-Chp-F57/R267 (62°C)
<i>Calonectria pseudonaviculata</i>	NCBB1 (NC, U.S.A.)	24.16 $\pm$ 0.76	23.33 $\pm$ 0.07	>40	22.82 $\pm$ 0.03
	CT-1 (CT, U.S.A.)	25.82 $\pm$ 0.17	22.59 $\pm$ 0.26	>40	23.30 $\pm$ 0.03
	ODA#1 (OR, U.S.A.)	22.78 $\pm$ 0.08	24.78 $\pm$ 0.43	>40	24.25 $\pm$ 0.08
	CB042 (Belgium)	22.15 $\pm$ 0.03	22.86 $\pm$ 0.09	>40	22.49 $\pm$ 0.29
	CPS-L2 (CT, U.S.A.)	22.24 $\pm$ 0.79	23.43 $\pm$ 0.06	>40	22.91 $\pm$ 0.03
	CT-15 (CT, U.S.A.)	23.07 $\pm$ 0.17	23.48 $\pm$ 0.04	>40	23.12 $\pm$ 0.15
	ICMP17495 (N.Z.)	21.89 $\pm$ 0.49	24.18 $\pm$ 0.07	>40	23.91 $\pm$ 0.16
	TU001 (Turkey)	23.13 $\pm$ 0.18	23.26 $\pm$ 0.08	>40	22.88 $\pm$ 0.23
	MDSH (MD, U.S.A.)	23.98 $\pm$ 0.08	23.35 $\pm$ 0.11	>40	22.96 $\pm$ 0.24
NCBB1-infected leaf-1		19.47 $\pm$ 0.02	22.02 $\pm$ 0.14	>40	22.65 $\pm$ 0.19
NCBB1-infected leaf-2		21.39 $\pm$ 0.05	23.41 $\pm$ 0.03	>40	23.83 $\pm$ 0.05
<i>Calonectria henricotiae</i>	CB045 (Belgium)	23.10 $\pm$ 0.20	>40	23.55 $\pm$ 0.02	22.42 $\pm$ 0.12
	CB077 (Belgium)	28.30 $\pm$ 0.10	>40	28.05 $\pm$ 0.25	28.27 $\pm$ 0.10
	NL-18 (Netherlands)	22.97 $\pm$ 0.78	>40	27.96 $\pm$ 0.04	27.54 $\pm$ 0.07
CB045-infected leaf-1		22.58 $\pm$ 0.23	>40	28.38 $\pm$ 0.06	25.03 $\pm$ 0.08
CB045-infected leaf-2		22.62 $\pm$ 0.31	>40	30.76 $\pm$ 0.06	30.53 $\pm$ 0.27
<i>C. asiatica</i>	CBS 114073	23.24 $\pm$ 0.06	>40	>40	>40
<i>C. brassicicola</i>	CBS 112756	22.07 $\pm$ 0.09	>40	>40	>40
<i>C. canadiana</i>	CBS 110817	22.90 $\pm$ 0.14	>40	>40	>40
<i>C. chinensis</i>	CBS 114872	24.37 $\pm$ 0.06	>40	>40	>40
<i>C. colombiensis</i>	CBS 112220	28.23 $\pm$ 0.40	>40	>40	>40
<i>C. curvisporum</i>	CBS 116159	28.14 $\pm$ 0.12	>40	>40	>40
<i>C. ilicicola</i>	CBS 115897	23.88 $\pm$ 0.15	>40	>40	>40
<i>C. multiphialidica</i>	CBS 112678	28.17 $\pm$ 0.53	>40	>40	>40
<i>C. naviculatum</i>	CBS 101121	23.23 $\pm$ 0.16	>40	>40	>40
<i>C. naviculatum</i>	CBS 116080	22.71 $\pm$ 0.15	>40	>40	>40
<i>C. pacifica</i>	CBS 114038	23.08 $\pm$ 0.05	>40	>40	>40
<i>C. penicilloides</i>	CBS 174.55	24.06 $\pm$ 0.08	>40	>40	>40
<i>C. sumatrensis</i>	CBS 112829	23.14 $\pm$ 0.06	>40	>40	>40
<i>C. uniseptata</i>	CBS 413.67	27.12 $\pm$ 0.00	>40	>40	>40
<i>Alternaria solani</i>	AS-rj	22.34 $\pm$ 0.29	>40	>40	>40
<i>Botrytis cinerea</i>	BC-rj	17.72 $\pm$ 0.11	>40	>40	>40
<i>Colletotrichum theobromicola</i>	GJS08.51	26.29 $\pm$ 0.09	>40	>40	>40
<i>Phytophthora infestans</i>	PI-rj	27.64 $\pm$ 0.81	>40	>40	>40
<i>Pseudonectria buxi</i>	PNB-2	27.70 $\pm$ 0.20	>40	>40	>40
Control - Uninfected leaf	10 ng DNA	>40	>40	>40	>40
Control - water		>40	>40	>40	>40

^a Isolates of *C. pseudonaviculata* and *C. henricotiae* were provided by J. Crouch, USDA-ARS Mycology and Nematology Genetic Diversity and Biology Lab (Beltsville, MD), with geographic origin as indicated. Isolates preceded by 'CBS' originated from the CBS-KNAW Culture Collection (www.wi.knaw.nl) and were provided by L. Castlebury, USDA-ARS Mycology and Nematology Genetic Diversity and Biology Lab. Isolates followed by "rj" or preceded by GJS were provided by R. Jones or John Stommel, respectively, USDA-ARS Genetic Improvement of Fruits and Vegetables Lab (Beltsville, MD). PNB-2 was isolated by Henry Guo from boxwood plants.

ITS-p3 (5'-YGAAGCTCTCGGCAACGGATA-3') and ITS-u4 (5'-RGTTCCTTTTCTCCGCTTA-3') (Cheng et al. 2016) was used as a positive PCR control in assays using leaf tissue. All primers were manufactured by Integrated DNA Technologies (Coralville, IA, U.S.A.).

Conventional PCR assay. PCR reactions were performed in a 25 µl volume in a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA). The reaction mixture contained 12.5 µl 2xBioline Mango-Mix and 1.25 µl of 50 mM MgCl (Bioline, Taunton, MA), 5 µl each of 5 µM primer, 0.25 µl water, and 1 µl of sample DNA (1 ng). DNA was amplified using the following PCR profile: 10 min of denaturation at 94°C; followed by 35 cycles of 94°C for 10 s; annealing (54°C, 60°C, or 62°C) for 10 s; and 72°C for 30 s; with a final extension step at 72°C for 10 min. Multiplex PCR reactions used the same final reagent concentrations and amplification conditions, with 54°C annealing temperature since this was the highest temperature at which the diagnostic primers and positive control primers all worked. A 10 µl aliquot of amplification product was electrophoresed on 2% agarose plus 1% NuSieve 3:1 agarose (Lonza, Rockland, ME), and visualized with SYBR Safe DNA Gel Stain (Invitrogen). Gels were photographed using the AlphaImager Mini system (ProteinSimple, San Jose, CA). Conventional PCR assays with each primer were run multiple times as we optimized conditions and tested primers. To ensure repeatability using different reagents, we also tested the optimized assay using AccuPower PCR mix (Bioneer, Oakland, CA) and GoTaq Green Master Mix (Promega, Madison, WI) using the same conditions described above.

Real-time PCR assay. PCR reactions were performed in a 10 µl volume in a CFX96TM Real-Time System C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA), and each reaction was repeated three times. PCR reaction mixtures contained 0.5 µl of each 10.0 µM primer, 5.0 µl of SYBR Green Supermix or Precision Melt Supermix (Bio-Rad), 3.0 µl of nuclease-free water, and 1 µl of template DNA (1 ng). Negative controls contained 1 µl of water and/or healthy boxwood leaf DNA (10 ng). The amplification profile consisted of 5 min preheating at 95°C, followed by 40 cycles of 10 s of denaturation at 95°C, 10 s of annealing at 54, 60, or 62°C, and 30 s of extension at 72°C.

The limit of detection (LoD) of the real-time PCR assay was determined for conidia and mycelium for all primer pairs using dilutions of mycelium or spore DNA from *C. pseudonaviculata* isolate NCBB1 and *C. henricotiae* isolate CB045. Tenfold serial dilutions were used for mycelium (10 to 1 × 10⁶ fg DNA) or conidia (1 to 1 × 10⁴ spores). Three replicates of each dilution were analyzed, with experiments replicated three times for mycelium. The threshold cycle (Ct) is the cycle number at which the quantity of amplified PCR product goes above a threshold line. In this study, a Ct of 35 was set as the cutoff for reliable or true target amplification in the real-time PCR assays. Linear regression plots of Ct versus the log of the sample dilution were made to estimate the LoD (Forootan et al. 2017; Gehesquière et al. 2013). Sensitivity of conventional PCR assays was not tested because it depends in part on the methods used to visualize the amplification products (e.g., capillary versus agarose gel electrophoresis; type of stain or dye used to visualize products).

Results

PCR primer specificity. A total of 45 primer pairs were designed and tested based on DNA sequence comparisons of target *C. pseudonaviculata* and *C. henricotiae* from previously published genome regions. The results of these primer assays are presented in Supplementary Table S1 as a resource for other scientists seeking species- or genus-specific primers. Although 19 of these primer pairs amplified a single PCR product unique to Ch, Cp, or both species, we highlight three primer pairs in this assay based on their specificity, repeatability, annealing temperature, amplicon size, and utility in both conventional multiplex PCR and real-time PCR. One primer pair is specific to *C. pseudonaviculata*; one primer pair is specific

Fig. 1. Agarose gel images showing conventional PCR results from four primer pairs targeting fungal ITS (control) or His3 regions. Primer pairs and annealing temperature are specified on each gel. Lanes: m = 100 bp DNA ladder; A = CB045; B = NCBB1; C = negative control (water); D = CBS 5174.55; E = CBS 413.67; F = CBS 110817; G = CBS 112220; H = CBS 112678; I = CBS 112756; J = CBS 112829; K = CBS 114038; L = CBS 114073; M = CBS 114872; N = CBS 115897; O = CBS 116159; P = CBS 101121; Q = CBS 116080; R = AS-rj; S = BC-rj; T = GJS08.51; U = Pl-rj; V = PNB-2; W = boxwood leaf DNA (10 ng); X = NL-18; Y = CB077; Z = CT-1; 1 = ICMP17495; 2 = ODA#1. See Table 1 for full species designations.

Table 2. PCR primer pairs developed in this study. Specificity is based on sequence differences in the Histone H3 region observed in the indicated GenBank accession

Primer pair	Sequence (5' to 3')	Amplicon size (bp)	GenBank accession	Specificity	Optimal annealing temperature (°C)
His-Cp-F56	CTCGCAATTCACCGGCACA	213	KF815170.1	<i>C. pseudonaviculata</i>	62
His-Cp-R268	AGCTGTGCGGTATGCAATCGTTT				
His-Ch-F39	CGGTGAGTACCTGTCCAT	248	KF815185.1	<i>C. henricotiae</i>	60
His-Ch-R286	GGACCTGTTTGTGTTAGG				
His-Chp-F57	TCGCAATTCACCGGCAC	211	KF815170.1	<i>C. pseudonaviculata</i> and <i>C. henricotiae</i>	62
His-Chp-R267	GCTGTGCGGTATGCAATCGTT				

to *C. henricotiae*; and one primer pair amplifies DNA from both species, but not from other related *Calonectria* (Table 2).

For each primer pair, we tested annealing temperatures of 54°C, 60°C, and 62°C. For conventional PCR, we found that a higher annealing temperature (60°C) resulted in the cleanest amplification of target sequence, with no amplification product seen in other species of *Calonectria* (Fig. 1). We observed the same specificity using all isolates of *C. pseudonaviculata* and *C. henricotiae* tested. For real-time PCR assays, all tested annealing temperatures readily differentiated amplification versus lack of amplification for each primer pair and each isolate (Table 1). In addition, in conventional PCR assays, we found that the Taq polymerase systems we tested from different manufacturers (Bioline, Bioneer, and Promega) all reliably produced the same results, although with slightly different intensities of bands in conventional PCR.

Assay sensitivity. In replicated assays to evaluate assay sensitivity by plotting the Ct values against the log of a dilution series, we found high R^2 values (>0.98), meaning that the Ct value is a reliable predictor of the relative number of spores or mycelium DNA detected (Fig. 2). This relationship was true for all three primer pairs and suggests a detection level of ~1 pg of DNA for mycelium and 10 to 100 spores for conidia. While this method gives a reasonable estimate of the sensitivity of the assay, it is by no means absolute. The Ct value can be affected by PCR efficiency, dilution, copy number, and number of replicates; the sensitivity reported here is therefore an estimate only.

Fig. 2. Standard curves showing correlation between cycle threshold (Ct) and log of DNA concentration from 10-fold dilution series. Log scale for mycelium DNA is 1 = 10 fg DNA; 2 = 100 fg DNA; 3 = 1,000 fg DNA; etc. Log scale for spores is 0 = 1 spore; 1 = 10 spores; 2 = 100 spores; etc. The top figure shows Cp-specific primer using isolate NCBB-1. The middle figure shows Ch-specific primer using CBO45. The bottom figure shows Cp/Ch primer using NCBB-1 and CBO45. Each curve is based on three replicates of each dilution. The efficiency (E) is indicated for each curve.

Detection on boxwood leaves. The practicality of this assay depends on how readily it can be used to detect the pathogen directly from boxwood leaves. Therefore, after determining the specificity of the primer pairs on fungal isolates, we tested the assay using DNA from infected and noninfected boxwood leaves, using the ITS-p3 and ITS-u4 as a positive PCR control. The control primers have an optimum annealing temperature of 50 to 52°C, and do not amplify at annealing temperatures higher than 54°C. Therefore, a conventional multiplex assay with a positive PCR control requires the use of 54°C annealing temperature, rather than the higher optimal annealing temperature of the diagnostic fungal-specific primer pairs. The multiplexed PCR resulted in successful amplification of both the fungal DNA (when present) and the positive plant control (ITS-p3 and ITS-u4) for all three primer pairs (Fig. 3). This allows multiplexing each test plant sample with a positive control to ensure that the PCR reaction is working. The use of infected leaves for template DNA also worked well in the real-time PCR assay (Table 1), with strong amplification of target sequence and no amplification in the negative controls. We therefore conclude that the real-time and conventional PCR assays can be applied to fungal isolates or DNA extracted from symptomatic or asymptomatic leaf tissue with no inhibition of PCR by background plant DNA.

Discussion

Our goal in this study was to develop specific diagnostic PCR primers suitable for conventional or real-time PCR that we could use to screen for boxwood blight in field-grown samples. While several other DNA-based assays have been reported previously (Gehesquière et al. 2013, 2016; Malapi-Wight et al. 2016), these assays have not been adopted to routinely screen for the presence of the pathogen in nursery or landscape plant material. Rapid and simple RPA dipstick assays based on LAMP target sequences are currently being developed in other labs (J. Crouch, personal communication). However, until those assays are developed, the assay presented here is the most straightforward, cost-effective, and readily adapted assay for confirming the presence of boxwood blight on plants. The primers published here are the only primers available that work reliably with either conventional PCR or SYBR-based real-time PCR, and can be multiplexed with a positive control in conventional PCR. These primers have the specificity to distinguish between *C. pseudonaviculata* and *C. henricotiae*. Both conventional and SYBR-based real-time PCR offer a time- and cost-saving advantage over TaqMan- or probe-based real-time PCR assays.

Fig. 3. Agarose gel image showing conventional PCR amplification of DNA from boxwood leaves using an annealing temperature of 54°C and the indicated Cp-, Ch-, or Ch/Cp-specific primers multiplexed with the universal plant primers ITS-p3 and ITS-u4. Lanes: m = 100 bp DNA ladder; "0" = Control [healthy uninfected boxwood leaf (*Buxus sempervirens*)]; "Ch" = CB077-infected leaf (*C. henricotiae*); "Cp" = NCBB1-infected leaf (*C. pseudonaviculata*).

These assays were tested using nine isolates of *C. pseudonaviculata* and three isolates of *C. henricotiae*, as well as related *Calonectria* species. The isolates we tested represent genotypes from SSR groups G1, G2, and G3 (LeBlanc et al. 2019). Previous studies have indicated that there is surprisingly little genetic variation across populations of these two species (LeBlanc et al. 2019; Malapi-Wight et al. 2019). Therefore, despite the relatively small number of isolates currently available for testing, this assay will likely be effective across other isolates of *C. pseudonaviculata* or *C. henricotiae* as they are discovered and collected.

Assays using these primers are effective regardless of the source of template DNA, whether from isolated fungal spores or mycelium, or from infected boxwood leaf tissue. By using universal plant ITS primers as a positive PCR control in conjunction with these new primers, including multiplexing in the conventional PCR assay (Fig. 3), false negatives due to failed PCR will not be an issue. We are currently working on a rapid one-step DNA extraction method (e.g., Steiner et al. 1995; Zou et al. 2017), so that this assay can be adapted to screen large numbers of samples for the presence of *C. pseudonaviculata* or *C. henricotiae* quickly and reliably. In addition to the three primer sets highlighted in this report, we also developed, tested, and reported several other primer pairs (Supplementary Table S1) that may be useful to other researchers in developing diagnostic assays to this serious pathogen. These assays will benefit growers, extension agents, regulatory agencies, and managers of plant collections.

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