Widespread Occurrence of a CYP51A Pseudogene in Calonectria pseudonaviculata

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ABSTRACT
Calonectria pseudonaviculata and C. henicotiae are two closely related fungal species responsible for boxwood blight disease of ornamental shrubs (Buxus spp.) in the U.S. and Europe. A previous study has shown isolates of the latter species, which is restricted to Europe, to be less sensitive to tetracozazole, an azole fungicide. In this study, we have analyzed the CYP51 paralogs for polymorphism in 26 genomes, representing geographically disparate populations of C. pseudonaviculata (n = 19) and C. henicotiae (n = 7), from the U.S., Europe, Asia, and New Zealand. The presence of a CYP51A pseudogene and lack of a functional CYP51A paralog in all C. pseudonaviculata genomes examined is a novel discovery for fungi and could have implications for the evolution of resistance to antifungal chemicals.

1. Introduction
Calonectria pseudonaviculata and C. henicotiae are the causal agents of the invasive fungal disease boxwood blight in native and commercial Buxus species [1,2]. This disease manifests itself through foliar and stem lesions, potentially leading to plant death. C. pseudonaviculata causes the disease in Europe, the U.S., New Zealand, and Asia, whereas C. henicotiae has been documented only in Europe [3].

C. pseudonaviculata was originally identified as conspecific with C. henicotiae, though [3] showed that these are two distinct species based on a multigene phylogeny. Phenotypic differences were also reported, including higher thermotolerance in C. henicotiae [3].

Among the antifungal chemistries used to control boxwood blight, and a multitude of other fungal diseases in agriculture and medicine, is the azole chemical class, also known as demethylation inhibitors (DMIs) [4,5]. The azoles bind sterol 14α-demethylase, also known as CYP51, a fungal-specific enzyme which facilitates the synthesis of the fungal membrane sterol ergosterol, a critical component of fungal cell membranes [6,7]. By binding to the active site of this enzyme, the azoles are inhibitors of ergosterol biosynthesis, thereby weakening and disrupting the cell membrane.

Repeated exposure of fungal pathogens to azoles provides a significant selection pressure on populations of fungi [8–10]. Over time, individuals may develop reduced sensitivity and ultimately become differentially resistant to single azoles or to the entire class [10]. Fungi overcome inhibition of ergosterol biosynthesis by azoles via a variety of mechanisms involving CYP51. Many reports have shown that fungi incur nonsynonymous mutations in the CYP51 gene, which alter the conformation of the encoded active-site amino-acid residues. Such changes render the azoles unable to bind to the active site, thereby enabling continued ergosterol biosynthesis. Another commonly reported mechanism involves overexpression of the CYP51 gene in order to compensate for inhibition [10].

Some species of fungi have been shown to harbor CYP51 paralogs, or duplicated copies of the gene, which can accumulate mutations leading to gene loss or a change in protein structure [10,11]. These paralogs are denoted CYP51A, CYP51B, and CYP51C. Differential gene loss of the paralogs has resulted in only a single copy, either CYP51A or CYP51B, being left behind in diverse fungal lineages, with members of these lineages developing resistance and reduced sensitivity to azoles no matter which of the paralogs remain [10]. Recent studies suggest specific roles of the paralogs in the normal
growth of *Fusarium* species, which are known to encode all three genes [11]. Individual gene knockouts of each of the *Fusarium* CYP51 paralogs resulted in increased sensitivity to differentazole fungicides for CYP51A and CYP51B, but not CYP51C. Of the three paralogs, CYP51B was found to be most vital for normal growth.

Select genomes of *C. pseudonaviculata* and *C. henricotiae* species possess three CYP51 paralogs; however, in *C. pseudonaviculata*, CYP51A is a pseudogene due to a premature stop codon [12]. A widespread CYP51A pseudogene has been reported in another fungus, Rhynchosporium commune, where the presence or absence of a functional CYP51A paralog has a population-specific distribution [7,13]. To date, there are no reports of fungal species for which all isolates have only a CYP51A pseudogene and no functional CYP51A paralog. Based on in vitro assays on solid agar media, *C. henricotiae* displays significant reduced sensitivity to the azole tetraconazole when compared to *C. pseudonaviculata*, which could be explained by the presence of a functional CYP51A in the former and only a CYP51A pseudogene in the latter [3].

As a result of these insights, we sought to understand the distribution of the CYP51A pseudogene in *C. pseudonaviculata* isolates. Our specific objectives were to mine the CYP51 paralogs from genome sequences originating from a worldwide collection of *C. pseudonaviculata* isolates and compare these sequences with those of *C. henricotiae* in order to identify CYP51 pseudogenes and/or polymorphisms that could explain the differential sensitivity to tetraconazole reported by [3]. Our findings have implications for the evolution of the CYP51 pseudogenes and paralogs in fungal pathogens, which could inform future studies on the mechanisms underpinning resistance toazole antifungals.

## 2. Methods

Nineteen genomes of *C. pseudonaviculata* and seven genomes of *C. henricotiae* representing geographically disparate populations (Table 1) were generated at the Mycology and Nematology Genetic Diversity Laboratory at the U.S. Department of Agriculture. Genomic data were generated for the fungal isolates as described in [14] using the Illumina MiSeq platform. Sequence reads were further processed to remove adapter traces using cutadapt v1.14 [15]. Draft genome assemblies were generated from the processed paired-end reads using SPAdes v3.10.0 [16]. Gene models were predicted in the previously generated Illumina-PacBio hybrid assembly of isolate CT1 [3,17] using the AUGUSTUS web server with the *Fusarium graminearum* species training set [18].

The CYP51 paralog sequences were obtained by running local blastn searches of the above genome sequences using the CLC Genomics Workbench v11 (QIAGEN, Redwood City, CA, U.S.A.) [19]. Preliminary blastn searches were conducted using

| Isolate Id | Alternate ID | Species* | Continent | Location | Year | Pseudogene |
|------------|--------------|----------|-----------|----------|------|------------|
| CT1        | CpsCT1       | C.p.s.   | United States | Connecticut | 2011 | Y          |
| JAC13-14   | CpsCT10      | C.p.s.   | United States | Connecticut | 2012 | Y          |
| JAC13-118  | CB088        | C.p.s.   | Europe     | Belgium    | 2011 | Y          |
| JAC13-149  | NC BB13      | C.p.s.   | United States | North Carolina | 2012 | Y          |
| JAC13-167  | TU004        | C.p.s.   | Asia       | Turkey     | 2012 | Y          |
| JAC13-172  | 91.9.6 A     | C.p.s.   | Asia       | Iran       | 2013 | Y          |
| JAC13-182  | STE-U 3399   | C.p.s.   | New Zealand | New Zealand | 1998 | Y          |
| JAC18-13   | CDFAS582     | C.p.s.   | United States | California | 2017 | Y          |
| JAC18-14   | CDFAS666     | C.p.s.   | United States | California | 2016 | Y          |
| JAC13-59   | MDSH 7.7     | C.p.s.   | United States | Maryland   | 2013 | Y          |
| JAC13-107  | CB054        | C.p.s.   | Europe     | Belgium    | 2010 | Y          |
| JAC13-110  | CB105        | C.p.s.   | Europe     | Belgium    | 2012 | Y          |
| JAC13-126  | MM2013calTA7 | C.p.s.   | Asia       | Iran       | 2013 | Y          |
| JAC13-127  | 11-416-11    | C.p.s.   | Europe     | Slovenia   | 2011 | Y          |
| JAC14-13   | CB002        | C.p.s.   | Europe     | Belgium    | 2008 | Y          |
| JAC14-15   | RHS 21350    | C.p.s.   | Europe     | United Kingdom | 2007 | Y          |
| JAC14-108  | NY13-370.9a  | C.p.s.   | United States | New York   | 2013 | Y          |
| JAC14-154  | 09-1762      | C.p.s.   | Europe     | France     | 2009 | Y          |
| JAC15-1    | 2015-7-36-0041 | C.p.s. | United States | Pennsylvania | 2015 | Y          |
| JAC13-124  | PD 011/04744201 | C.h. | Europe     | The Netherlands | 2011 | N          |
| JAC13-131  | P-30-3865    | C.h.     | Europe     | Germany    | 2010 | N          |
| JAC13-147  | DFO17        | C.h.     | Europe     | Germany    | 2011 | N          |
| JAC13-185  | JKI 2100     | C.h.     | Europe     | Germany    | 2007 | N          |
| JAC13-216  | RHS 190276   | C.h.     | Europe     | United Kingdom | 2012 | N          |
| JAC14-101  | NLO18        | C.h.     | Europe     | The Netherlands | 2011 | N          |
| JAC14-47   | NLO19        | C.h.     | Europe     | The Netherlands | 2011 | N          |

The presence of the CYP51A pseudogene is indicated for each isolate.
*C.p.s.* designates *C. pseudonaviculata*, while *C.h.* designates *C. henricotiae*. 

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**Table 1. Identification and origin of Calonectria pseudonaviculata and C. henricotiae isolates from which genomes were examined for this study.**
Fusarium graminearum CYP51 paralogs as query sequences [11]. Intron sequences were verified and trimmed from the assembled genome data guided by the gene prediction annotation of the CT1 genome. The sequences were aligned in MEGA v7 and translated into amino-acid sequences [20]. The CYP51 coding sequences were searched for early stop codons or nonsynonymous polymorphisms. The CYP51 paralog sequences for C. hennicotiae were uploaded into GenBank with the following accessions: CYP51A, MN497050; CYP51B, MN497051; CYP51C, MN497052. The CYP51 paralog sequences for C. pseudonaviculata were uploaded into GenBank with the following accessions: CYP51A, MN497053; CYP51B, MN497054; CYP51C, MN497055.

A blastp search against the non-redundant protein sequence database at NCBI was performed in January and February of 2019 using the translated coding sequences for the CYP51 paralogs from isolates of both C. pseudonaviculata and C. hennicotiae [19]. In both species, each paralog was found to have only one unique genotype and thus one unique amino acid sequence per species. Therefore, the blastp search was conducted using the coding sequences of CYP51 paralogs originating from a single genome of each species.

A phylogenetic analysis was carried out with amino acid sequences of the three paralogs, which were aligned and analyzed according to [9]. Briefly, amino acid sequences from this study were aligned to a subset of those from [6]. Sequences were aligned using the MAFFT (Multiple Alignment using Fast Fourier Transform) sequence alignment program [21]. A neighbor joining phylogenetic tree was generated with 1000 bootstrap replicates using MEGA v7 [20].

3. Results

The CYP51A gene had one predicted intron, which began at nucleotide 193 and ended at nucleotide 251. The CYP51B gene had two predicted introns, one beginning at position 247 and ending at position 355; the other beginning at position 556 and ending at position 630. The CYP51C gene had two predicted introns, the first beginning at position 424 and ending at position 478; the second beginning at position 1544 and ending at position 1591.

For the CYP51A amino acid sequence of C. pseudonaviculata, the blastp top hit was eburical 14-alpha-demethylase from Neonectria ditissima (KPM38499.1), with a query coverage of 99% and an E value of 0. The CYP51A sequence had 80% amino acid identity (404 of 506 amino acids) and 89% positive amino acid matches (453 of 506 amino acids) with respect to the N. ditissima protein. For CYP51B of C. pseudonaviculata, the top blastp hit was to a predicted protein from Nectria haematococca (XP_003054236.1), which had a query coverage of 100% and an E value of 0. The CYP51B sequence had 86% amino acid identity (455 of 527 amino acids) and 92% positive amino acid matches (488 of 527 amino acids) with respect to the N. haematococca protein. The top blastp hit for the CYP51C of C. pseudonaviculata was a hypothetical protein of Fusarium euwallaceae (RTE69955.1), which had a query coverage of 100% and an E value of 0. The CYP51C sequence had 83% amino acid identity (427 of 516 amino acids) and 91% positive amino acid matches (473 of 516 amino acids) with respect to the F. euwallaceae protein.

For the CYP51A amino acid sequence of C. hennicotiae, the top blastp hit was to the same eburical 14-alpha-demethylase found in N. ditissima (KPM38499.1), which had a query coverage of 99% and an E value of 0. The CYP51A sequence also had 80% amino acid identity (404 of 506 amino acids) and 89% positive amino acid matches (453 of 506 amino acids) with respect to the N. ditissima protein. For CYP51B of C. hennicotiae, the top blastp hit was to the same predicted protein in N. haematococca (XP_003054236.1), which had a query coverage of 99% and an E value of 0. The CYP51B sequence had 86% amino acid identity (455 of 527 amino acids) and 92% positive amino acid matches (488 of 527 amino acids) with respect to the N. haematococca protein. The top blastp hit for the CYP51C of C. hennicotiae was also a hypothetical protein of F. euwallaceae (RTE69955.1), which had a query coverage of 99% and an E value of 0. The CYP51C sequence had 83% amino acid identity (428 of 516 amino acids) and 91% positive amino acid matches (473 of 516 amino acids) with respect to the F. euwallaceae protein.

Amino acid sequences encoded by the CYP51 gene paralogs were separated into three monophyletic clades along a phylogenetic tree with bootstrap values greater than 70% (Figure 1). The three clades consisted solely of CYP51A, CYP51B, or CYP51C sequences for various fungal lineages. The C. pseudonaviculata and C. hennicotiae paralog sequences were paired together across each of the three clades with high bootstrap support, as expected for sister taxa [3].

All isolates of C. pseudonaviculata displayed a single CYP51A pseudogene, with premature stop codon in the CYP51A nucleotide sequence, thereby confirming the earlier report of this pseudogene [12; Table 2]. The early TGA stop was the result of a nucleotide substitution (C688T). No functional CYP51A paralog was found from blast searches of
the *C. pseudonaviculata* genomes. None of the *C. henricotiae* isolates displayed the premature stop codon in *CYP51A*.

All 19 *C. pseudonaviculata* genomes shared a single genotype for *CYP51B* and *CYP51C*. Similarly, all seven *C. henricotiae* genomes shared a single genotype for these paralogs. With respect to isolates of *C. henricotiae*, *C. pseudonaviculata* exhibited polymorphism in both paralogs. In contrast, *C. pseudonaviculata* and *C. henricotiae* *CYP51B* gene paralogs encoded identical amino acid sequences (Table 2). In *CYP51C*, one nonsynonymous polymorphism was present.

4. Discussion
A *CYP51A* pseudogene was mined from the genomes of all 19 isolates of *C. pseudonaviculata* from four continents, confirming its widespread distribution. The premature stop codon occurred in the *CYP51A*
pseudogene prior to the region encoding the heme-binding site residues of the CYP51A protein [22,23]. We hypothesize that this interrupts function of the CYP51A protein, thus restricting the potential total biosynthesis of ergosterol when compared to *C. henricotiae*, since we did not uncover evidence for a functional CYP51A paralog in *C. pseudonaviculata*. We speculate that this could partially explain the greater in vitro sensitivity of *C. pseudonaviculata* to tetraconazole when compared to *C. henricotiae* and perhaps be linked to differences in thermotolerance between these closely related species [3,7,10].

This is the first report to our knowledge of a ubiquitous CYP51 pseudogene without evidence for a co-occurring functional CYP51A paralog. Brunner et al. [13] examined isolates of *Rhyhnchosporium commune* from barley fields across the globe and found the presence of a CYP51A pseudogene to be absolute; however, the presence of a functional CYP51A paralog was found in many cases to co-occur with the pseudogene, especially in countries where prior introduction ofazole fungicides has occurred. Further, these populations of *R. commune* were shown to consist of both members with only the CYP51A pseudogene and those with a functional CYP51A paralog in addition to the pseudogene [13].

Historical sampling by [7] reported that during the twentieth century *R. commune* populations at an experimental site in the UK displayed the CYP51A pseudogene only at high frequencies and that a functional CYP51A paralog began to increase in frequency during the mid-1980’s, a phenomenon termed “paralog re-emergence”. This occurred during a period which coincided with introduction of newazole fungicides to the United Kingdom mirroring the findings of [13] which noted that the functional CYP51A paralog appeared to be undergoing significant positive selection, likely due to intragenic recombination, in countries with prior azole exposure (2016). In both studies, the presence of the functional CYP51A paralog was definitively associated with a dramatic reduction in sensitivity to azoles.

In our analyses of genomes from nineteen isolates of *C. pseudonaviculata* from across four continents, we did not uncover a functional CYP51A gene paralog, though all isolates of *C. henricotiae* possessed a CYP51A pseudogene. Perhaps with deeper sampling of populations from worldwide collections of *C. pseudonaviculata*, CYP51A paralog reemergence will be documented. LeBlanc et al. [24] characterized a global collection of *C. pseudonaviculata*, a subset of which were applied to this study, with simple sequence repeat markers and concluded that the widespread clonality in this pathogen could be explained by multiple introductions of a single clonal lineage. Given the lack of sexual recombination reported for this fungus, the likelihood of paralog reemergence of CYP51A in *C. pseudonaviculata* is unclear, though results presented here should facilitate future efforts to document this phenomenon [24,25].

Both Brunner et al. [13] and Hawkins et al. [7] concluded that the presence of a functional CYP51A paralog alone was associated with a dramatic decrease in sensitivity to azoles, which was thus not attributed to polymorphism in the CYP51B paralog. The absence

### Table 2. Summary of single nucleotide polymorphisms (SNPs) from the CYP51 paralog coding sequences between *Calonectria pseudonaviculata* and *C. henricotiae.*

| SNP          | Synonymous or nongenomous | SNP          | Synonymous or nonsynonymous | SNP          | Synonymous or nonsynonymous |
|--------------|---------------------------|--------------|-----------------------------|--------------|-----------------------------|
| G109A        | N, V371                   | C384T        | S, 128                      | G45A         | S, 15                       |
| G207C        | S, 69                     | A924G        | S, 308                      | C54A         | S, 18                       |
| G234T        | N, K78N                   | C1218T       | S, 406                      | C204T        | S, 68                       |
| T496C        | N, W166R                  |              |                             | T619C        | S, 207                      |
| T534G        | N, H178Q                  |              |                             | T753C        | S, 251                      |
| A579G        | S, 193                    |              |                             | T7619        | S, 406                      |
| G587A        | N, R196K                  |              |                             | G780A        | S, 340                      |
| G625T        | S, 218                    |              |                             | A1120G       | S, 340                      |
| T688C        | N, *230R                  |              |                             | A1430G       | N, K477R                    |
| C704A        | N, A235E                  |              |                             |              |                             |
| C758T        | N, A235V                  |              |                             |              |                             |
| T847C        | N, Y283H                  |              |                             |              |                             |
| G868A        | N, V299M                  |              |                             |              |                             |
| A1049G       | N, K350R                  |              |                             |              |                             |
| T1271C       | N, V424A                  |              |                             |              |                             |
| T1366C       | N, Y456H                  |              |                             |              |                             |
| G1418A       | N, S473N                  |              |                             |              |                             |
| T1521C       | S, 507                    |              |                             |              |                             |
| A1522C       | N, K508Q                  |              |                             |              |                             |

Amino acid substitutions resulting from nonsynonymous nucleotides are indicated.

aAny nucleotide or amino acid substitution is ordered as *C. pseudonaviculata* first and *C. henricotiae* second.

bS denotes synonymous polymorphism, while N denotes nonsynonymous polymorphism.

Denotes the premature stop encoded by CYP51A from *C. pseudonaviculata*. 

[22,23]
of nonsynonymous substitutions in CYP51B between the two species is further support that the lack of a functional CYP51A in C. pseudonaviculata may at least partially explain the previously reported discrepancy in sensitivity to tetraconazole [3]. A single nonsynonymous polymorphism was identified in CYP51C between the two species, but evidence for a role of this gene in sensitivity to azoles has not been reported for fungi. Future work will examine expression levels of the CYP51 paralogs in response to tetraconazole exposure, in addition to promoter analysis, in order to screen for single nucleotide polymorphisms or repetitive DNA elements, which have been linked to overexpression of CYP51A in plant pathogenic fungi [26]. Additionally, RNA-Seq transcriptomic analyses could be applied to identify detoxification genes from the fungal xenome that share a coordinated role in the reduced sensitivity to an azole fungicide reported for C. hensicioiata [9,27].

In summary, our results contribute to the knowledge base on the evolution of CYP51 gene paralogs in the ascomycete fungi, which may have implications for treating boxwood blight and other fungal diseases. Consequently, the possibility of paralog re-emergence of CYP51A may exist for C. pseudonaviculata, which could reduce the efficacy of azoles in treating future boxwood blight outbreaks. In turn, the reported clonal nature of the pathogen, the ubiquity of the CYP51A pseudogene and apparent lack of a functional CYP51A paralog in C. pseudonaviculata may place constraints on CYP1B mutation under selection of azoles applied in the field. Finally, research on this pathosystem presents a unique future opportunity to examine additional genetic and genomic factors underpinning differential antifungal sensitivity in two recently diverged species.

Disclosure statement
No potential conflict of interest was reported by the authors.

References
[1] Daughtrey ML. Boxwood blight: threat to ornamentals. Annu Rev Phytopathol. 2019;57(1):189–209.
[2] Leblanc N, Salgado-Salazar C, Crouch JA. Boxwood blight: an ongoing threat to ornamental and native boxwood. Appl Microbiol Biotechnol. 2018;102(10):4371–4380.
[3] Gehesquiere B, Crouch J, Marra R, et al. Characterization and taxonomic re-assessment of the box blight pathogen Calonectria pseudonaviculata, introducing Calonectria henriciota sp. Plant Pathol. 2016;65(1):37–52.
[4] LaMondia JA. Fungicide efficacy against Calonectria pseudonaviculata, causal agent of boxwood blight. Plant Dis. 2014;98(1):99–102.
[5] LaMondia JA. Management of Calonectria pseudonaviculata in boxwood with fungicides and less susceptible host species and varieties. Plant Dis. 2015;99(3):363–369.
[6] Becher R, Weihmann F, Deising HB, et al. Development of a novel multiplex DNA microarray for Fusarium graminearum and analysis of azole fungicide responses. BMC Genomics. 2011;12(1):52.
[7] Hawkins NJ, Cools HJ, Sierotzki H, et al. Paralog re-emergence: a novel, historically contingent mechanism in the evolution of antimicrobial resistance. Mol Biol Evol. 2014;31(7):1793–1802.
[8] Chen FP, Fan JR, Zhou T, et al. Baseline sensitivity of Monilinia fructicola from China to the DMI fungicide SYP-Z048 and analysis of DMI-resistant mutants. Plant Dis. 2012;96(3):416–422.
[9] Hulvey J, Popko JT, Jr., Sang H, et al. Overexpression of ShCYP51B and ShatrD in Sclerotinia homoeocarpa isolates exhibiting practical field resistance to a demethylation inhibitor fungicide. Appl Environ Microbiol. 2012;78(18):6674–6682.
[10] Cools HJ, Hawkins NJ, Fraaije BA. Constraints on the evolution of azole resistance in plant pathogenic fungi. Plant Pathol. 2013;62:36–42.
[11] Zheng B, Yan L, Liang W, et al. Paralogous Cyp51s mediate the differential sensitivity of Fusarium oxysporum to sterol demethylation inhibitors. Pest Manag Sci. 2019;75:396–404.
[12] Hulvey JP, Marra RE. Evidence for CYP51-mediated reduced sensitivity to triazole fungicides in Calonectria henriciota. Phytopathology. 2018;108(10):24.
[13] Brunner PC, Stefansson TS, Fountain J, et al. A global analysis of CYP51 diversity and azole sensitivity in Rhynchosporium commune. Phytopathology. 2016;106(4):355–361.
[14] Salgado-Salazar C, Shishkoff N, LeBlanc N, et al. Coccinonectria pachysandriicola, causal agent of a new foliar blight disease of Sarcococca hookeriana. Plant Dis. 2019;103(6):1337–1346.
[15] Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBOcnet. J. 2011;17(1):10–12.
[16] Bankevich A, Nurk S, Antipov D, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 2012;19(5):455–477.
[17] Crouch JA, Malapi-Wight M, Rivera Y, et al. Genome datasets for Calonectria henricicota and C. pseudonaviculata causing boxwood blight disease and related fungal species. Ag Data Commons. 2017. https://doi.org/10.15482/USDA.ADC/1410184
[18] Stanke M, Morgenstern B. AUGUSTUS: a web server for gene prediction in eukaryotes that allows user-defined constraints. Nucleic Acids Res. 2005;33(Web Server):W465–W467.
[19] Altschul SF, Gish W, Miller W, et al. Basic local alignment search tool. J Mol Biol. 1990;215(3):403–410.
[20] Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33(7):1870–1874.
[21] Madeira F, Mi Park Y, Lee J, et al. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res. 2019;47(W1):W636–W641.
[22] Kim D, Lim Y-R, Ohk SO, et al. Functional expression and characterization of CYP51 from dandruff-causing Malassezia globosa. FEMS Yeast Res. 2011; 11(1):80–87.

[23] Hargrove TY, Wawrzak Z, Lamb DC, et al. Structure-function characterization of cytochrome P450 sterol 14α-demethylase (CYP51B) from Aspergillus fumigatus and molecular basis for the development of antifungal drugs. J Biol Chem. 2015;290(39):23916–23934.

[24] LeBlanc N, Gehesquiére B, Salgado-Salazar C, et al. SSRs identify limited genetic diversity across pathogen populations responsible for the global emergence of boxwood blight. Plant Pathol. 2019; 68(5):861–868.

[25] Malapi-Wight M, Veltri D, Gehesquiére B, et al. Global distribution of mating types shows limited opportunities for mating across populations of fungi causing boxwood blight disease. Fungal Genet Biol. 2019;131:103246.

[26] Villani SM, Hulvey J, Hily J-H, et al. Overexpression of the CYP51A1 gene and repeated elements are associated with differential sensitivity to DMI fungicides in Venturia inaequalis. Phytopathology. 2016;106(6):562–571.

[27] Sang H, Hulvey JP, Green R, et al. A xenobiotic detoxification pathway through transcriptional regulation in filamentous fungi. mBio. 2018;9(4):pii: e00457-18.