



# Comparative Genomics Reveals Evolutionary Traits, Mating Strategies, and Pathogenicity-Related Genes Variation of *Botryosphaeriaceae*

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*Botryosphaeriaceae*, as a major family of the largest class of kingdom fungi *Dothideomycetes*, encompasses phytopathogens, saprobes, and endophytes. Many members of this family are opportunistic phytopathogens with a wide host range and worldwide geographical distribution, and can infect many economically important plants, including food crops and raw material plants for biofuel production. To date, however, little is known about the family evolutionary characterization, mating strategies, and pathogenicity-related genes variation from a comparative genome perspective. Here, we conducted a large-scale whole-genome comparison of 271 *Dothideomycetes*, including 19 species in *Botryosphaeriaceae*. The comparative genome analysis provided a clear classification of *Botryosphaeriaceae* in *Dothideomycetes* and indicated that the evolution of lifestyle within *Dothideomycetes* underwent four major transitions from non-phytopathogenic to phytopathogenic. Mating strategies analysis demonstrated that at least 3 transitions were found within *Botryosphaeriaceae* from heterothallism to homothallism. Additionally, pathogenicity-related genes contents in different genera varied greatly, indicative of genus-lineage expansion within *Botryosphaeriaceae*. These findings shed new light on evolutionary traits, mating strategies and pathogenicity-related genes variation of *Botryosphaeriaceae*.

**Keywords:** *Botryosphaeriaceae*, phytopathogen, evolution traits, mating strategies, pathogenicity-related genes

## INTRODUCTION

*Dothideomycetes* represents the largest and most important class of ascomycete fungi, including 23 orders, 110 families, 1,261 genera, and 19,000 species (Wijayawardene et al., 2017). The members of *Dothideomycetes* comprise both phytopathogenic (Ohm et al., 2012) and non-phytopathogenic fungi with diverse lifestyles (Ruibal et al., 2009) as well as many mycorrhizal fungi (Peter et al., 2016). Among these 110 families, *Botryosphaeriaceae* is an important and distinctive family.

This is because it includes saprobes, endophytes, and phytopathogens, and it is one of the most widely geographically distributed groups of opportunistic plant pathogens. The host range of this family is very wide, and many economically important plants worldwide can be infected by them (Slippers and Wingfield, 2007). These pathogenic fungi can infect plants through wounds or natural openings, such as lenticels and stomata. Once they enter host tissues, they may survive as endophytes to stay at a biotrophic stage for a long time and turn into the destructive necrotrophic stage when the host is stressed (Yan et al., 2013; Morales-Cruz et al., 2015). The members of *Botryosphaeriaceae* can infect many woody plants and cause serious disease symptoms, such as dieback, branch canker, leaf spots, and fruit and seed rot (Marsberg et al., 2017). But the interaction of some of *Botryosphaeriaceae* fungi like *Botryosphaeria dothidea* (*B. dothidea*) with host plants includes a latent or endophytic phase, which makes the fungal infection easily be neglected (Bostock et al., 2014; Marsberg et al., 2017; Wang et al., 2018). Therefore, it is of substantial biological significance to explore the evolutionary characteristics of *Botryosphaeriaceae* at the level of *Dothideomycetes*.

The exceptional feature of *Botryosphaeriaceae* fungi is that it is difficult to observe their sexual structure under both natural and experimental conditions, but this does not mean that sexual reproduction does not occur for these fungi (Phillips et al., 2013). With more in-depth research being conducted, the mating strategies (homothallism or heterothallism) of increasing members of the *Botryosphaeriaceae* family have been reported (Bihon et al., 2012a,b, 2014; Billiard et al., 2012; Lopes et al., 2017, 2018), revealing some unresolved questions regarding their sexual reproduction. For example, how conservative are the nucleic acid and protein sequences of the mating type determination genes; how conservative are the genes and their arrangement at the mating type determination loci; how has the mating type evolved, and what is the origin type. In addition, the host range of different *Botryosphaeriaceae* fungi varies greatly. *B. dothidea* is a common pathogen with a wide range of hosts. Generally, the infection becomes symptomatic when the host is subjected to drought, physical damage, waterlogging, or freezing stress (Bostock et al., 2014; Marsberg et al., 2017; Wang et al., 2018). The symptoms primarily include canker on young seedlings, branches, and stems; the necrosis of branches; and fruit decay, which may lead to the death of the host in extreme cases (Kim et al., 2005; Tang et al., 2012). *Botryosphaeria kuwatsukai* (*B. kuwatsukai*) can also cause symptoms, such as fruit softening and decay, and severe canker of branches and stems (Wang et al., 2021). However, *B. kuwatsukai* has a relatively narrow host range and primarily infects apple and pear trees (Xu et al., 2015). Therefore, a systematic study of the mating strategies and differences of pathogenicity-related genes in *Botryosphaeriaceae* fungi will lead to a better understanding of their molecular evolutionary history and pathogenic characteristics.

Currently, genomics technology has been widely used to study many pathogenic fungi of plants, and greatly promoted the understanding of their evolution and pathogenic mechanisms (Islam et al., 2012; Blanco-Ulate et al., 2013;

van der Nest et al., 2014; Nagel et al., 2018; Félix et al., 2019; Landi et al., 2020; Meile et al., 2020). For example, gene family expansion associated with virulence factors in wood-colonizing pathogenic fungi in the *Botryosphaeriaceae* was revealed via phylogenomic comparisons (Garcia et al., 2021); the comparative genome analyses of latent plant pathogens in the *Botryosphaeriaceae* were conducted to define their genomes (Nagel et al., 2021). In addition, a large-scale comparative genomic analysis can better reveal the physiological characteristics and evolutionary history of fungi (Guttman et al., 2014; Haridas et al., 2020; Miyauchi et al., 2020). Therefore, in this study, 167 *Dothideomycetes* fungi, including 19 *Botryosphaeriaceae* species, were fully sequenced, and a comparative genomics approach was used to comprehensively analyze the molecular evolution characteristics, mating strategies and pathogenicity within *Botryosphaeriaceae* fungi. The sequence differences of related genes benefit our understanding of the evolutionary history of *Botryosphaeriaceae* fungi and provide useful information for the prevention and control of the diseases caused by these fungi.

## MATERIALS AND METHODS

### Fungal Strains

A total of 167 *Dothideomycetes* fungal strains, including 160 *Botryosphaeriaceae* fungi (19 species) (**Supplementary Table 1**), from the Fungal Strain Library of Shandong Agricultural University, Tai'an, China, were sequenced in this study. The sequencing data are found at NCBI (PRJNA777748).

### Sequencing and Genome Assembly

The CTAB (hexadecyltrimethylammonium bromide) method (Murray and Thompson, 1980) was used to extract high-quality DNA. A 500 bp DNA fragment library was constructed, and PE150 sequencing was performed using Illumina HiSeq 4,000 (San Diego, CA, United States). The raw data obtained from sequencing were inputted into Trimmomatic v0.39 (Bolger et al., 2014) for quality control, and reads with an average quality of less than 30 were filtered. Jellyfish v2.3.0 (Marçais and Kingsford, 2011) was used to calculate k-mer distribution and GenomeScope v2.0 (Ranallo-Benavidez et al., 2020) was then used to assess genome size of each fungus. The assembly was carried out using SPAdes v3.13.1 (Bankevich et al., 2012).

### Gene Prediction and Genome Annotation

RepeatModeler v2.0.1<sup>1</sup> was used to construct custome repeat libraries for each assembly, and RepeatMasker v4.1.1<sup>2</sup> was used to determine repeat contents with the custom repeat libraries. For *ab initio* gene prediction, GeneMark-ES v4.48\_3.60 (Lomsadze et al., 2014) and AUGUSTUS v3.2.1 (Stanke and Morgenstern, 2005) were used with default parameters. First, GeneMark-ES was used to predict gene models, then the models were used to train AUGUSTUS. Exonerate v2.2.0

<sup>1</sup><http://www.repeatmasker.org/RepeatModeler/>

<sup>2</sup><http://www.repeatmasker.org/>

(Slater and Birney, 2005) was used for homology comparison prediction. Finally, MAKER v3.01.03 (Campbell et al., 2014) was used to predict protein-coding genes by combining the gene models from GeneMark-ES, AUGUSTUS, and Exonerate. BUSCO v4.1.4 was used to evaluate the completeness of genomes and genome annotations based on *pezizomycotina\_odb9* (3156 core ortholog genes) (Waterhouse et al., 2018). Functional annotations on the putative genes were performed using the following softwares: BLAST v2.8.1+ (Camacho et al., 2009) for the NCBI non-redundant protein (NR, 2020-06), SwissProt and FunSecKB2 databases (E-value threshold of 1E-05); HMMER v3.2.1 (Eddy, 2008) for the Pfam 32.0 and TransportDB 2.0 databases; KofamKOALA v1.2.0 (Aramaki et al., 2020) for KEGG annotation; and dbCAN2 (Zhang et al., 2018) for annotating carbohydrate active enzymes (CAZy).

## Evolutionary Analysis of *Botryosphaeriaceae* Fungi

To construct an accurate evolutionary tree, eight outgroups (**Supplementary Table 1**) were selected and OrthoFinder v2.3.11 (Emms and Kelly, 2015) was used for gene family clustering. According to the clustering results, single copy orthogroups were extracted and aligned using MAFFT v7.427 (Katoh et al., 2002). These processed single copy orthogroups were then concatenated using a self-written Perl script and filtered using Gblock v0.91b (Castresana, 2000). PartitionsFinder v2.1.1 (Lanfear et al., 2017) and RAxML v.8.2.2 (Stamatakis, 2014) were used to determine the optimal amino acid substitution model and build the Maximum Likelihood (ML) tree with 1,000 bootstrap replicates, respectively.

The known ecologies states (multistate and binary) and FUNGuild were used to determine the ecologies of each species (Nguyen et al., 2016). Mesquite v3.61 software was then used to infer ancestral ecological character states (Haridas et al., 2020).

## Functional Enrichment Analysis of *Botryosphaeriaceae*

To find the differences in gene function annotation of *Botryosphaeriaceae* fungi with different lifestyles, *scipy.stats* package in Python was used to perform the two-tailed Fisher exact test for functional annotation with *p*-value threshold of 0.01. To reduce false positives, functional annotations with the number of genes less than 100 were filtered.

## Mating Strategies of *Botryosphaeriaceae*

### Mating Type Genes and Surrounding Genes

To determine the presence of mating type genes in the *Botryosphaeriaceae* fungal genome, the mating type genes of *Diplodia sapinea* (*D. sapinea*) and some neighboring genes (KF551229 and KF551228) (Bihon et al., 2012a) were used as templates to search homologous genes from the putative *Botryosphaeriaceae* fungal genes that has been generated in this study, using the partial alignment mode in BLASTx (Camacho et al., 2009). The mating type genes obtained were then inputted into the NCBI's conserved domain database (Marchler-Bauer et al., 2015) to determine their functional domains.

## Comparison of the Arrangement of Mating Type Loci

To compare the arrangement of mating type genes and their surrounding genes in the *Botryosphaeriaceae* fungal genome, a BLASTn alignment was conducted and then EasyFig version 2.2.2 (Sullivan et al., 2011) was used for collinearity analysis, with the *E* value threshold set to  $1e^{-4}$ .

## Phylogenetic Comparison and Ancestral State Reconstruction

To study the mating strategies of *Botryosphaeriaceae* fungi, ML evolutionary trees of 24 *Botryosphaeriaceae* fungi were constructed with single-copy genes (using the same method described in section: Comparison of the Arrangement of Mating Type Loci). Briefly, OrthoFinder v2.3.11 (Emms and Kelly, 2015) was used to cluster gene families and extract single copy orthogroups. Each orthogroup was aligned and concatenated with MAFFT v7.427 (Katoh et al., 2002). Gblock v0.91b (Castresana, 2000) was used for filtering, and finally, RAxML v.8.2.2 (Stamatakis, 2014) was used to build an ML tree with the LG + I + G + F model. Mesquite v3.61<sup>3</sup> and the Mk1 likelihood model were used to reconstruct the evolutionary process of homothallism (hom) and heterothallism (het).

## Changes in Genes Related to the Pathogenicity of *Botryosphaeriaceae* Fungi

The production of phytotoxic compounds in pathogenic fungi, such as secondary metabolite, secreted proteins, and carbohydrate-active enzymes, is one of the important infective weapons (Amselem et al., 2011). The genomes of *Botryosphaeriaceae* were searched for genes encoding the phytotoxic compounds and then the number difference of these genes was statistically analyzed as previously described (Wang et al., 2018).

Specifically, 23 *Botryosphaeriaceae* fungi (7 genera) and 10 other representative fungi were included to analyze changes in genes related to the pathogenicity in *Botryosphaeriaceae*. The 10 representative fungi contained 1 biotrophic fungus (*Puccinia graminis*), 2 necrotrophic fungi (*Valsa mali* and *Pyrenophora tritici-repentis*), 2 saprophytic fungi (*Neurospora crassa* and *Rhizopus oryzae*), 3 hemibiotrophic fungi (*Pyricularia oryzae*, *Colletotrichum higginsianum*, and *Zymoseptoria tritici*), 1 symbiotic fungus (*Laccaria bicolor*), and 1 endophytic fungus (*Peltaster fructicola*).

## RESULTS

### Genome Sequencing, Assembly, and Annotation

In this study, 167 genomes of *Dothideomycetes*, including 160 *Botryosphaeriaceae* (19 species), were sequenced, assembled, and annotated (**Supplementary Table 1**). All genomes were sequenced at average coverage  $166 \pm 48$  x. The average assembled genome lengths of 167 *Dothideomycetes* ranged between 28.85 and 61.78 Mb, which were consistent with the sizes estimated by

<sup>3</sup><http://www.mesquiteproject.org/>

k-mer counting approach. The contig N50 values of assembled genomes varied from 41.81 to 779.02 kb with a mean of 241.77 kb. The repeat contents of assembled genomes varied from 0.95 to 10.98% with a mean of 5.18%. All assembled genomes have a high completeness with an average of  $94.8 \pm 4.3\%$ , and the similar result was also found in genome annotations (average of  $98.0 \pm 2.9\%$ %).

## Classification Based on Whole-Genome Data

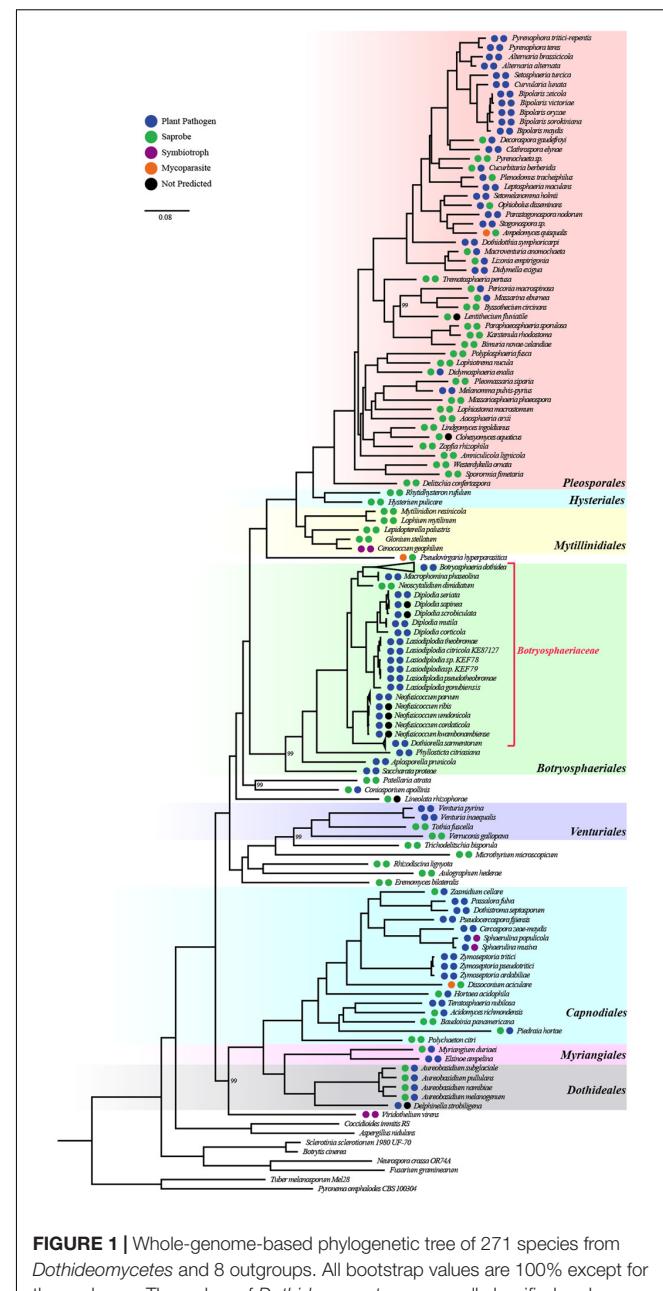
To better understand the evolutionary characteristics of *Botryosphaeriaceae*, we used 167 newly sequenced in this study and 112 genomes available in public database to construct a whole-genome phylogenetic tree using 480 single-copy gene families. The phylogenetic tree was clearly divided into 2 clades, corresponding to 2 subclasses, namely *Pleosporomycetidae* and *Dothideomycetidae* (Supplementary Figure 1). A comprehensive phylogenetic analysis revealed that the members of *Botryosphaeriaceae* that belonged to *Botryosphaerales* were added to *Pleosporomycetidae* (Figure 1 and Supplementary Table 2).

## Saprophytic Fungi Have a Larger Genome Size Than Phytopathogenic Fungi

The genome sizes of *Dothideomycetes* ranged from 17 (*Piedraia hortae*) to 177 Mbp (*Cenococcum geophilum*), and 7,896–34,881 protein-coding genes were detected (Supplementary Figures 2, 3 and Supplementary Table 1). Compared with non-pathogenic fungi, pathogenic fungi usually have a smaller genome (Supplementary Figure 2). The genome size of *Botryosphaeriaceae* is in the range of 28.85 (*Aureobasidium pullulans*) – 61.78 Mbp (*Dothiorella sarmientorum*) (47.19 Mbp on average), and 11,505–16,851 proteion-coding genes were predicted (13,765 genes in average).

## Saprophytic Fungi Are the Possible Evolutionary Ancestors of *Botryosphaeriaceae* Fungi

To infer the ecological characteristics of ancestors of *Botryosphaeriaceae* fungi, we analyzed their lifestyle evolution process at the class level. The results showed that the ancestral lifestyle was likely to be the saprophytic type (Figure 2), which is also supported by the maximum likelihood analysis (Supplementary Figure 4 and Supplementary Table 3). During the evolution of *Dothideomycetes* fungi, at least 6 transitions from non-phytopathogenic (NPP) to phytopathogenic (PP) were detected, including 4 major transitions, which are presented at the MRCA node of *Mycosphaerellaceae* (*Dothideomycetidae*) (Node 225: NPP = 0.1868, PP = 0.8132), *Venturia* (*Pleosporomycetidae*) (Node 189: NPP = 0.0039, PP = 0.9961) and *Botryosphaerales* (Node 20: NPP = 0.0984, PP = 0.9016), along with the branching point of *Setomelanomma-Bipolaris* (Node 98: NPP = 0.0074, PP = 0.9926). In addition, the *Botryosphaeriaceae* fungi have undergone at least 3 transitions from saprophytic to pathogenic fungi, including Node 176 (NPP = 0.9859, PP = 0.0141), Node

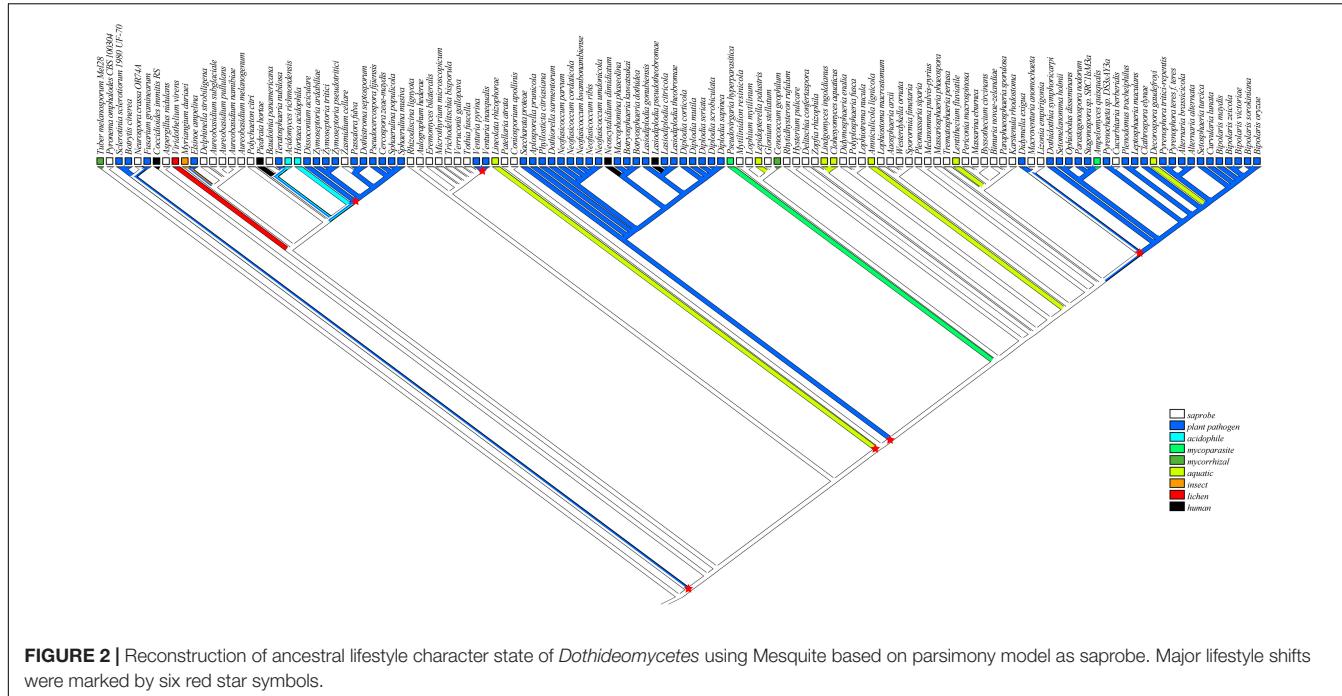


**FIGURE 1 |** Whole-genome-based phylogenetic tree of 271 species from *Dothideomycetes* and 8 outgroups. All bootstrap values are 100% except for those shown. The orders of *Dothideomycetes* were well classified and were displayed by different colors. The two circles left of species names stand for lifestyle classification according to organism data and FunGuild, respectively.

180 (NPP = 0.9992, PP = 0.0008) and Node 246 (NPP = 0.9101, PP = 0.0899) (Supplementary Table 3).

## Differences in the Gene Families of Plant Pathogenic and Saprophytic Fungi

To investigate the differences between plant pathogenic and saprophytic fungi from the perspective of gene family contractions and expansions, we used Fisher's exact test to perform these analyses on gene families. Differences in 78 Pfam and 58 GO annotations were found between saprophytic and



**FIGURE 2 |** Reconstruction of ancestral lifestyle character state of *Dothideomycetes* using Mesquite based on parsimony model as saprobe. Major lifestyle shifts were marked by six red star symbols.

plant pathogenic fungi. In the saprophytic fungi, 42 Pfam and 35 GO terms showed more than 20% of expansion, and 31 Pfam and 14 GO terms displayed more than 20% of contraction. In plant pathogenic fungi, 32 Pfam and 17 GO terms showed greater than 20% of expansion, and 35 Pfam and 21 terms displayed more than 20% of contraction. Compared with saprophytic fungi, the plant pathogenic fungi contained more gene families that showed contraction. Compared with saprophytic fungi, 94 Pfam and 67 GO terms of *Botryosphaeriaceae* fungi showed greater than 20% of expansion, while 52 Pfam and 44 GO terms displayed contractions (> 20%) (Supplementary Table 4).

## Mating Strategies of *Botryosphaeriaceae* Fungi

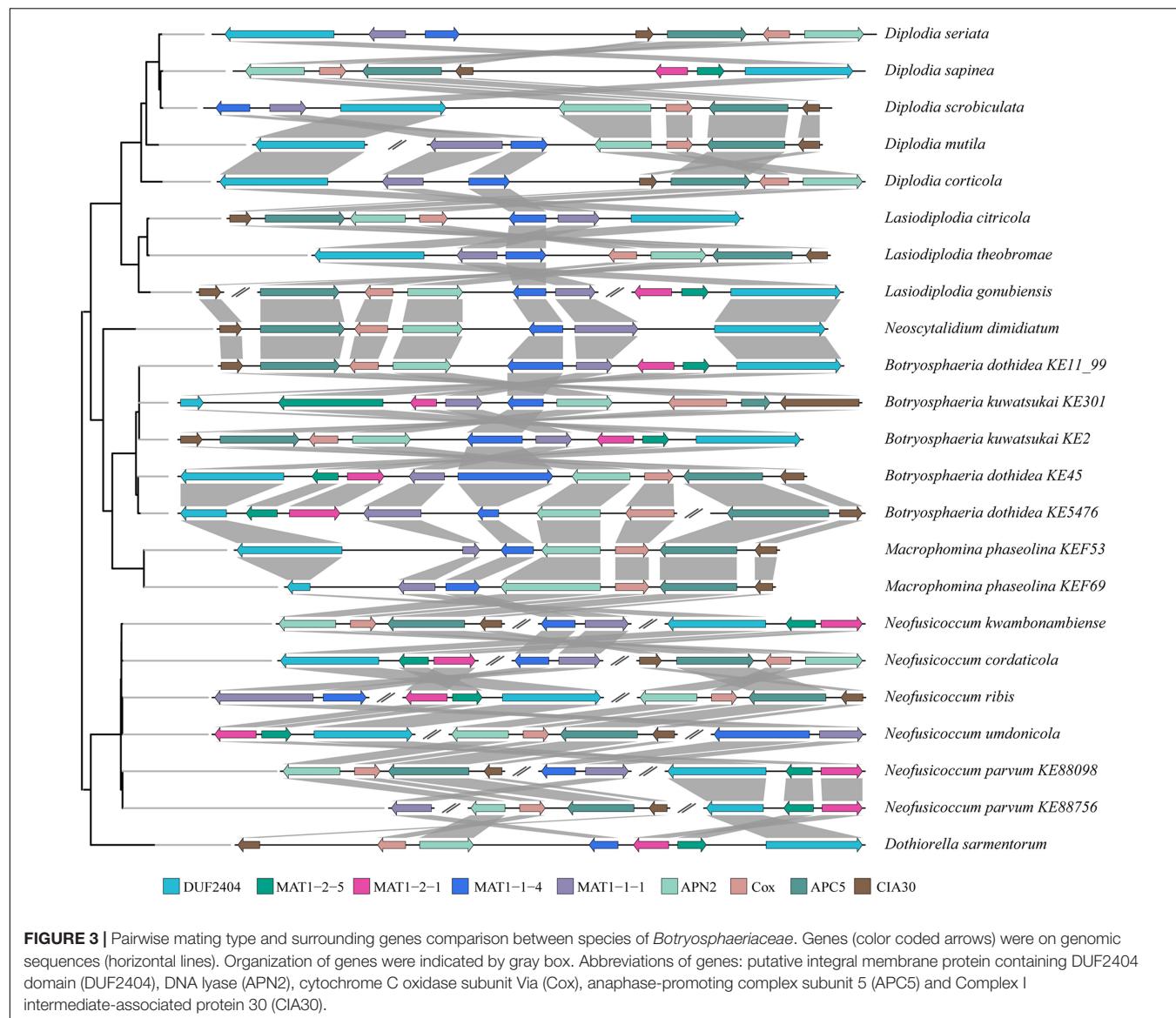
### Mating Type Genes

To determine the mating type genes, *MAT1-1* and *MAT1-2* of 24 fungi (7 genera and 19 species) were analyzed in the *Botryosphaeriaceae* family. The results showed that the *Botryosphaeria*, *Neofusicoccum*, and *Dothiorella* genomes harbored both *MAT1-1* and *MAT1-2*, indicating that they were homothallic. *Diplodia*, *Macrophomina*, and *Neoscytalidium* genomes harbored either *MAT1-1* or *MAT1-2*, indicating that they were heterothallic fungi. *Lasiodiplodia* fungi had various mating strategies, including homothallism (*L. gonubensis*) and heterothallism (*Lasiodiplodia citricola*, *L. pseudotheobromae*, and *L. theobromae*) (Supplementary Table 5). In addition, the protein domains of *MAT1-1-1* and *MAT1-2-1* were highly conservative. All the *MAT1-1-1* proteins contain MATalpha domains, and all the *MAT1-2-1* proteins contain MAT\_HMG-box domains (Supplementary Table 6). Compared with the neighboring genes, the nucleic acid sequences of the mating type genes were poorly conserved (Supplementary Table 7), while the length of

the coding sequence and the position and size of the intron were largely conservative.

### Arrangement of Mating Loci

Arrangement analysis of mating loci showed that there were three types of arrangements at the mating type determining loci of *Botryosphaeriaceae* (Figure 3). In most *Botryosphaeriaceae* genomes (e.g., *Diplodia sapinea*), the *MAT1* gene was primarily located between a collinear region that contains 4 protein-coding genes and 1 putative integral membrane (PIM) protein (Figure 3). The PIM contains pleckstrin homology domains and DUF2404. The four genes in the collinear region encode, in order, a DNA lyase (APN2), cytochrome c oxidase subunit VIa (CoxVIa), anaphase-promoting complex subunit 5 (APC5), and complex I intermediate-associated protein 30 (CIA30). According to the positional relation between the *MAT1* and APN2 genes (the *MAT1* gene was located at the proximal end of APN2), the second type of arrangement was observed in *Botryosphaeria*, *L. gonubensis* (*Lasiodiplodia*), *M. phaseolina* (*Macrophomina*), and *N. dimidiatum* (*Neoscytalidium*). For example, the *MAT1* gene of *M. phaseolina* and *B. dothidea* was located upstream of the APN2 gene, while in *L. gonubensis* and *N. dimidiatum*, the *MAT1* gene was located downstream of the APN2 gene, and the four genes in the collinear region were arranged in reverse order. However, the opposite arrangement was observed in the two strains of *B. kuwatsukai*—the *MAT1* gene of the PG2 strain was located downstream of the APN2 gene, while LW030101 was located upstream. A similar type of reversed arrangement as the one found for *B. kuwatsukai* was also identified in two *B. dothidea* strains. The third type of arrangement was primarily observed for *Neofusicoccum* species in which the two subtypes of *MAT1*, *MAT1-1*, and *MAT1-2*,



were not located in conjunction; they were either located both distantly from the collinear region or at different chromosomes (or scaffolds) (Figure 3).

#### Reconstruction of the Ancestral State of the Mating Strategy

To understand the evolutionary characteristics of the mating type of *Botryosphaeriaceae*, we selected 24 representative *Botryosphaeriaceae* strains to reconstruct the evolutionary process of homothallism and heterothallism mating strategies in *Botryosphaeriaceae* (Supplementary Figure 5). Our analysis showed that het mating was likely to be the ancestral type (Node 2, het = 0.6198, hom = 0.3802) (Supplementary Figure 5 and Supplementary Table 8). During the course of evolution, *Botryosphaeriaceae* fungi have undergone at least 2 major transitions to their hom strategy. The first was located at the branching point of *Lasiodiplodia theobromae* and *L. citricola*

(Node 14: het = 0.8291, hom = 0.1709), and the other transition was primarily observed at the branching point of *Neofusicoccum* and *Dothiorella* (Node 26: het = 0.4344, hom = 0.5656) (Supplementary Figure 5 and Supplementary Table 8).

#### Changes in Genes Related to the Pathogenicity of *Botryosphaeriaceae* Secondary Metabolism

To find the key enzymes involved in the synthesis of secondary metabolites in *Botryosphaeriaceae* fungi, we used Pfam annotations to find 3 types of genes that encode these key enzymes, such as polyketide synthase (PKS), non-ribosomal peptide synthetase (NRPS), and dimethylallyl tryptophane synthase (DMATS). The PKS genes of *Botryosphaeriaceae* fungi differed significantly from each other ( $p = 2.0 \times 10^{-16}$ ) (Figure 4A). Among all the species, the *Macrophomina* fungi contained the largest number of PKS genes (31 on average),

while the *Dothiorella* fungi contained the least number (8 on average) (**Supplementary Table 9**). The genes related to secondary metabolites in fungi also included those that encode cytochrome P450 enzymes, regulatory factors, and transporters. Cytochromes P450 can catalyze the transformation of hydrophobic intermediates in the primary and secondary metabolic pathways and plays an important role in fungi. Compared with other fungi, *Botryosphaeriaceae* fungi contained the largest number of genes that encode cytochromes P450 (133–267). Among all the species, *Neofusicoccum* fungi contained the highest number of cytochromes P450 (267), followed by *Botryosphaeria* ( $n = 251$ ) (**Figure 4B**). The ATP-binding cassette (ABC) or major facilitator superfamily (MFS) gene families played important roles in the transport of secondary metabolites. The numbers of ABC or MFS in *Botryosphaeriaceae* fungi were higher than those of other species (ABC: 268–353; MFS: 16–62). Among all the species, *Botryosphaeria* fungi contained the largest number of MFS ( $n = 62$ ), while *Neoscytalidium dimidiatum* fungi contain the least amount ( $n = 16$ ) (**Figure 4B**).

### Secreted Proteins

Pathogens can secrete a battery of proteins, which are deployed to the host-pathogen interface during infection, and these secreted proteins played important roles in fungal pathogenicity (O’Connell et al., 2012; Fernandes et al., 2014; Félix et al., 2016). In this study, we predicted the secreted proteins of *Botryosphaeriaceae* (**Figure 5**). The number of secreted proteins in *Botryosphaeria* (the *B. dothidea* fungi contain 1,034, and the *B. kuwatsukai* fungi contain 939) was not significantly different from *Macrophomina* ( $n = 1,118$ ), and the *B. kuwatsukai* fungi seemed to secrete less proteins. The number of proteins secreted by the *Neofusicoccum* fungi varied greatly (922–1,028). Among all the species, the *N. parvum* fungi contained the largest number ( $n = 1,028$ ), and *N. cordaticola* fungi contained the least ( $n = 922$ ). Compared with other fungal genera, the *Diplodia* genus contained fewer secreted proteins (671–866) (**Figure 5** and **Supplementary Table 10**).

The transfer of secreted effector proteins to host plant cells was the key to pathogenesis of many plant pathogenic microorganisms. Secreted proteins less than 200 amino acids in length and rich in cysteine were considered as candidate secreted effectors (Wang et al., 2018). The *Botryosphaeriaceae* family contained two genera *Macrophomina* and *Botryosphaeria* that may release a large number of secreted proteins, and the number of small, secreted proteins of these two genera was also higher than that of other genera. For example, *M. phaseolina* (261), *B. dothidea* (253), and *B. kuwatsukai* (230) displayed significant expansions of gene families. Small secretory proteins rich in cysteine were the most abundant in *Botryosphaeria* fungi and the least in *Diplodia* fungi (79–113) (**Figure 5** and **Supplementary Table 10**). In *Botryosphaeria*, both *B. dothidea* and *B. kuwatsukai* contained 154 small secretory proteins rich in cysteine, and the Pfam annotations of them included 19 and 21 known functional domains, respectively (**Supplementary Table 11**). By comparing the Pfam annotations of *B. dothidea* and *B. kuwatsukai* secretory proteins, we found that ribonuclease, the cerato-platinin family, and cysteine-rich secretory protein

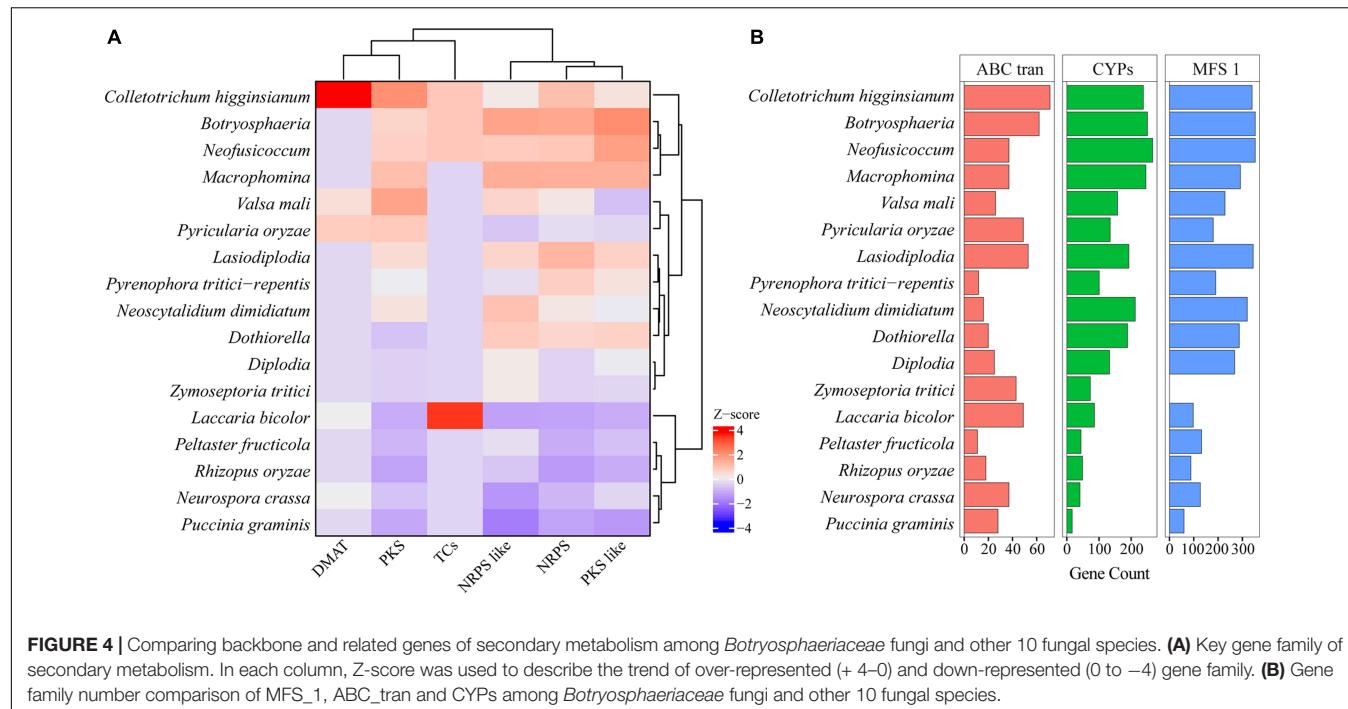
(CAP) family were only present in *B. kuwatsukai*, while the cell wall integrity and stress response component (WSC) domain, a putative carbohydrate binding domain was only present in *B. dothidea* (**Supplementary Table 11**).

Pathogenic and saprophytic fungi can secrete peptidases into their surroundings to degrade a variety of host-related proteases (Wang et al., 2018). This degradation mechanism has potential benefits in eliminating the activity of antifungal host proteins and providing nutrients. Compared with other fungi, *Botryosphaeriaceae* fungi contained a higher number of secretory peptidases. Among all the species, the *M. phaseolina* (*Macrophomina*) fungi contained the largest number of secretory peptidases ( $n = 298$ ), followed by *B. dothidea* ( $n = 264$ ) and *B. kuwatsukai* ( $n = 235$ ), but both were similar to the semi-biotrophic phytopathogenic pathogens *C. higginsianum* ( $n = 233$ ) and *P. oryzae* ( $n = 240$ ). *Diplodia* fungi contain the lowest number of secreted proteins ( $n = 192$ ;  $p = 0.8 \times 10^{-3}$ ) (**Figure 5** and **Supplementary Table 10**).

### Carbohydrate Active Enzymes

The ability to degrade complex carbohydrates in plants is an important aspect of the lifestyle of phytopathogenic fungi (Wang et al., 2018). Compared with other species, the fungi in *Botryosphaeriaceae* family contained a higher number of carbohydrate active enzyme-related gene families, such as genes that encode glycoside hydrolases (GHs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), auxiliary activities (AAs), and carbohydrate binding modules (CBMs). Some representative species include *N. parvum* (753), *B. dothidea* (750), *N. kwambonambiense* (724), *L. theobromae* (719), *N. ribis* (716), *N. umdonicola* (716), and *N. cordaticola* (709). Within the *Botryosphaeriaceae* family, *N. parvum* has the largest number of carbohydrate active enzyme-related gene families (753), followed by *B. dothidea* (750), and *B. kuwatsukai* (675). Most *Diplodia* fungi contain fewer carbohydrate activity enzyme-related gene families, such as *D. scrobiculata* (522), *D. corticola* (548), *D. mutila* (577), and *D. sapinea* (556). However, for some species, such as *D. seriata* (662), there was a small increase in the number (**Figure 6A** and **Supplementary Table 12**).

The plant cell wall is a complex network structure composed of different polysaccharides, including cellulose, hemicellulose, pectin, and lignin (Terrett and Dupree, 2019). Proteins encoded by fungal carbohydrate active genes and related auxiliary genes can degrade plant cell walls into simple monomers that are absorbed as the carbon source to provide energy for fungi (Islam et al., 2012; Wang et al., 2018). Compared with other fungi, those of *Botryosphaeriaceae* contain expanded gene families involved in the degradation of lignin, cellulose, hemicellulose, and pectin. For example, the gene family involved in the degradation of lignin (107 genes in average) was significantly larger than that in other species (54 genes in average;  $p = 2.0 \times 10^{-4}$ , *t*-test). The number of gene families involved in the degradation of hemicellulose and pectin also expanded significantly, with 67 vs. 34 ( $p = 2.0 \times 10^{-3}$ ) and 70 vs. 32 ( $p = 2.0 \times 10^{-3}$ ), respectively. Among the *Botryosphaeriaceae* fungi, compared with other genera, it was found that *Neofusicoccum* fungi contain the largest number of gene families involved in the degradation of



plant cell walls (287–329), followed by *Botryosphaeria* (219–314) and *Macrophomina* (291–306), while *Diplodia* fungi contain the least number of gene families (215–259) (**Figures 6B,C** and **Supplementary Table 13**).

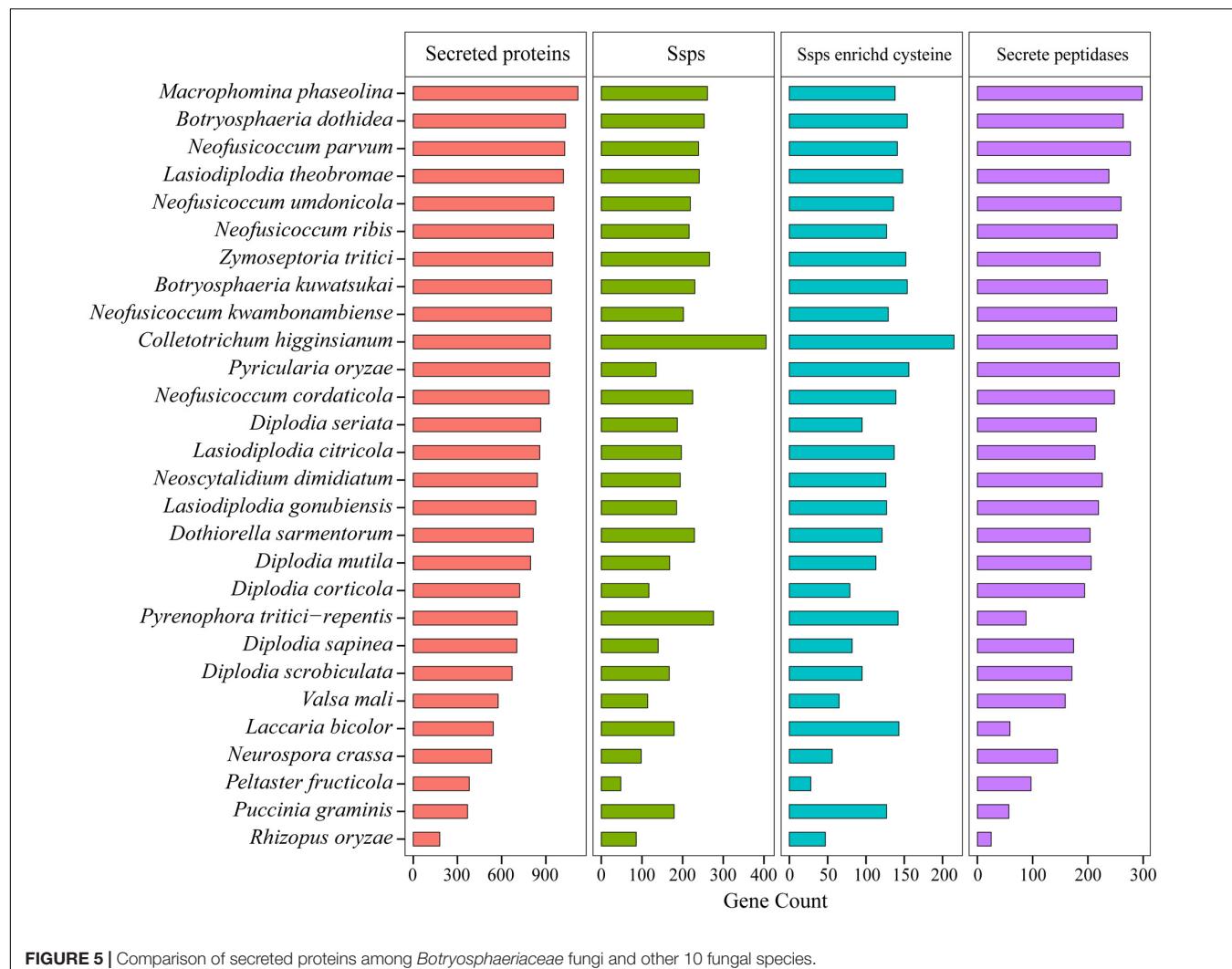
## DISCUSSION

In this study, we conducted a large-scale whole-genome sequencing of 167 *Dothideomycetes* fungi, including 19 species, and comprehensively analyzed evolutionary traits, mating strategies and changes in pathogenic genes in *Dothideomycetes* fungi. Our results can advance our understanding of the evolutionary history of *Botryosphaeriaceae* fungi.

Our results also confirmed that the 167 *Dothideomycetes* fungi can be divided into two subclasses, *Pleosporomycetidae* and *Dothideomycetidae*, and *Botryosphaeriaceae* belongs to *Pleosporomycetidae*, indicating that it is accurate to classify fungi using phylogenies based on phylogenomics (Haridas et al., 2020). Our results inferred the ancestral lifestyle of *Botryosphaeriaceae* fungus as saprobe, and these fungi have undergone at least three transitions from saprophytic to phytopathogenic states. This result is consistent with Schoch et al. (2009). They inferred that the *Dothideomycetes* fungi have experienced multiple transitions from saprophytic pathogens to lichens to phytopathogens, along with multiple transitions from terrestrial to aquatic lifestyles (Schoch et al., 2009). In addition, a larger genome size and a higher number of protein-coding genes are usually associated with saprophytic fungi compared with phytopathogenic fungi. This result is consistent with the findings of Schuelke et al. (2017). They found that in the *Geosmithia* genus, compared with

non-pathogenic fungi, the pathogenic fungi in this family have smaller genomes (Schuelke et al., 2017).

Previous studies have shown that phytopathogenic fungi have a smaller genome and contain fewer protein-coding genes compared with saprophytic fungi, which is related to the expansion and contraction of gene families (Ohm et al., 2012; Haridas et al., 2018). In this study, many gene families of phytopathogenic fungi showed contractions, primarily including genes which contain the Pfam domain tetratricopeptide repeat (TPR). TPR can interact with a variety of proteins, such as the anaphase-promoting complex, NADPH oxidases, and HSP90-binding proteins (Das et al., 1998; Kaneko et al., 2006; Kondo et al., 2010). The TPR protein is also part of the plant hormone signaling pathways (Schapire et al., 2006). The family enrichment results found that multiple members of the TRP family proteins (TPR 1, 3, 8, 10, 11, 12, 16, and 19) contracted in phytopathogenic fungi. This contraction may be due to a reduction of signal related TPR proteins in pathogenic fungi. This is because during the process of pathogenic fungal infection, fungal signal related TPR proteins will be affected by plant hormone signals (Haridas et al., 2020). Some other Pfam domains also contracted significantly in phytopathogenic fungi, including PF17111 (the fungal N-terminal domain of STAND proteins), PF05729 (the NACHT domain), and PF14479 (involved in prion inhibition and propagation). These domains are in heterokaryons and play an important role in incompatibility (Paoletti and Saupe, 2009; Greenwald et al., 2010; Daskalov et al., 2012). The contraction of Pfam domains associated with incompatibility in the heterokaryons suggests that other strategies to reduce the level of incompatibility in heterokaryons also exist to improve the adaptability of pathogenic fungi (Ishikawa et al., 2012). Simultaneously, compared with saprophytic fungi, many GO

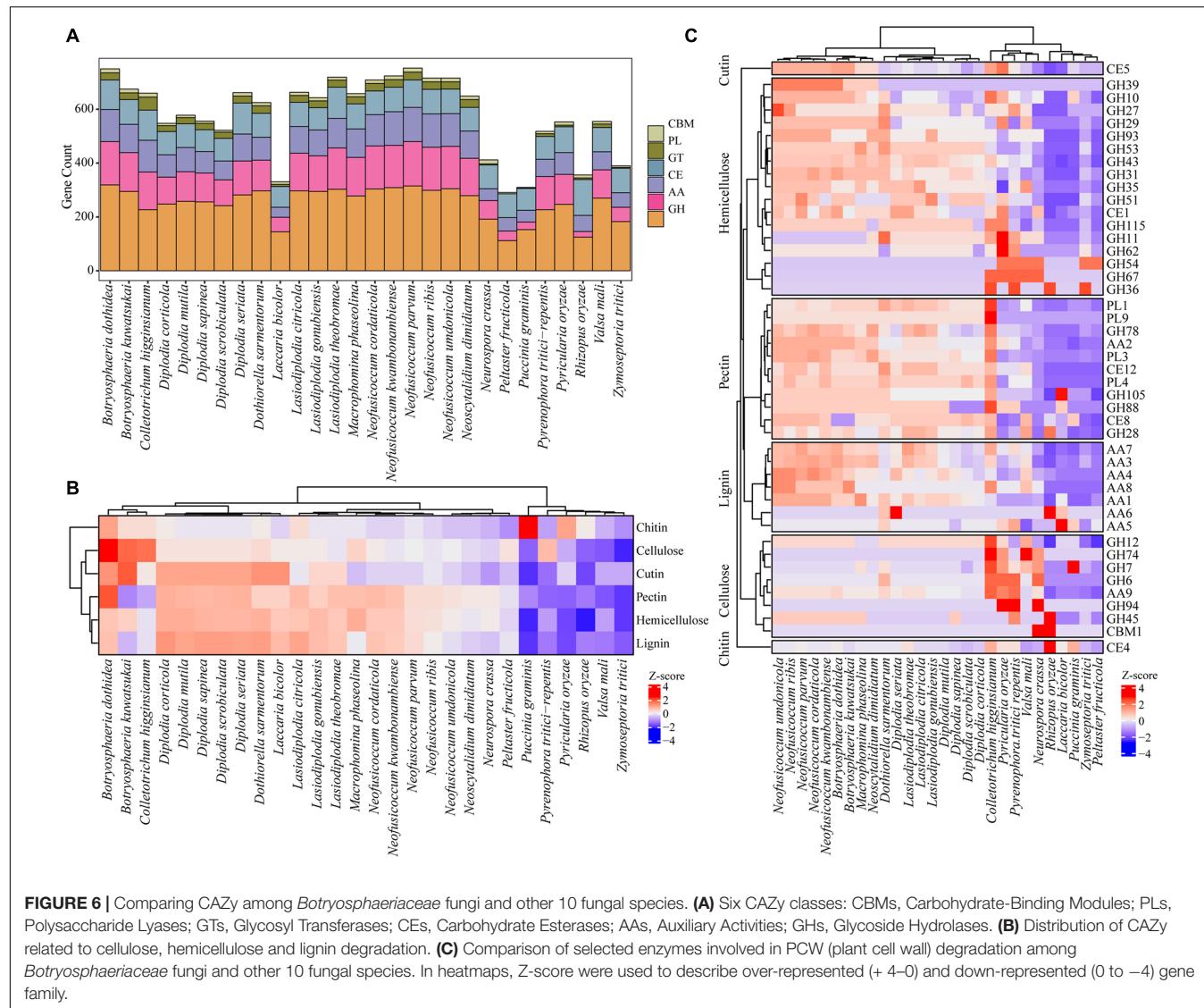


**FIGURE 5 |** Comparison of secreted proteins among *Botryosphaeriaceae* fungi and other 10 fungal species.

terms are contracted in phytopathogenic fungi, including protein kinases (GO: 0004672), transcription factors (GO: 0003700), zinc ion binding (GO: 0008270), and regulation of nitrogen utilization (GO: 0006808). Given the complexity of amino acid biosynthetic pathways and energy requirements, fungi rely on the absorption of plant amino acids to conserve their own energy. This could be one of the reasons for the contraction of GO terms in plant pathogens (Staats et al., 2013; Mur et al., 2017).

*Botryosphaeriaceae* fungi display both types of mating strategies, homothallism and heterothallism, which are determined by the single locus *MAT1* (Mur et al., 2017). In this study, the mating type genes of 24 *Botryosphaeriaceae* fungal isolates (19 species) were analyzed, and we found that these genes were not highly conserved in terms of their nucleotide and amino acid sequences. However, for two subtype genes, two domains, MATalpha\_HMGbox (*MAT1-1-1*) and MATA\_HMG-box (*MAT1-2-1*), are more conservative. This is consistent with the fact that the mating type genes of fungi have almost no differences within a species, but they are highly divergent

between species (Turgeon, 1998). In many ascomycete fungi, it is a conservative trend that the MATalpha and MATA\_HMG-box regions contain introns (Arie et al., 2000; Paoletti et al., 2005; Duong et al., 2013; de miccolis et al., 2016). In *D. sapinea* and *D. seriata*, the introns in the MATA\_HMG-box region of the *MAT1-2-1* gene were lost, but the amino acid sequences that flank the lost intron site remain intact (Nagel et al., 2018). This phenomenon is consistent with the intron loss model derived from Poly-A primed mRNA (Sharpton et al., 2008). In this study, with the exception of *Neofusicoccum*, the arrangement of *MAT1-1* and *MAT1-2* genes is highly conserved in six other genera. They all are located between a collinear region that contains four protein-coding genes and one PIM protein, which is the same arrangement as found in most *Phyllostictaceae* fungi (Bihon et al., 2014; Wang et al., 2016). The arrangement of mating type genes of *Neofusicoccum* fungi is exceptional. The locations of the two subtypes of mating type genes are not in conjunction; they are either both located distantly from the collinear region or at different chromosomes (or scaffolds) (Lopes et al., 2017). Similarly, this discontinuous arrangement



**FIGURE 6 |** Comparing CAZy among *Botryosphaeriaceae* fungi and other 10 fungal species. **(A)** Six CAZy classes: CBMs, Carbohydrate-Binding Modules; PLs, Polysaccharide Lyases; GTs, Glycosyl Transferases; CEs, Carbohydrate Esterases; AAs, Auxiliary Activities; GHs, Glycoside Hydrolases. **(B)** Distribution of CAZy related to cellulose, hemicellulose and lignin degradation. **(C)** Comparison of selected enzymes involved in PCW (plant cell wall) degradation among *Botryosphaeriaceae* fungi and other 10 fungal species. In heatmaps, Z-score were used to describe over-represented (+ 4 to 0) and down-represented (0 to -4) gene family.

of mating type genes also exists in *Aspergillus nidulans* (Galagan et al., 2005), *Curvularia cymbopogonis* (Galagan et al., 2005), and *Neosartorya fischeri* (Rydholm et al., 2007).

The mating type gene *MAT1* has two subtypes—*MAT1-1* and *MAT1-2*. When two subtypes are both harbored in the genome, the mating strategy is hom, and if only one is present, it is het (Idnurm, 2011). The repetitive sequence-mediated deletion of one or more mating type genes can cause non-directional changes in mating types, such as the transition from self-fertility to self-sterility (Wilken et al., 2014; Xu et al., 2016; Yun et al., 2017). Such repetitive sequences were not observed in this study. Therefore, *Botryosphaeriaceae* fungi are unlikely to undergo a non-directional change regarding mating types (Nagel et al., 2018). In addition, to further understand the evolutionary characteristics of the fungal mating type in *Botryosphaeriaceae*, we reconstructed the ancestral state of the *Botryosphaeriaceae* fungal mating type and found that the het mating strategy is the ancestral type. Moreover, the fungi in this family experienced a

number of transitions to the homothallism strategy, a shift that is common in ascomycete fungi (Inderbitzin et al., 2005; Yokoyama et al., 2006; Nygren et al., 2011; Gioti et al., 2012).

In this study, many gene families of the *Botryosphaeriaceae* fungi have shown significant expansions and contractions, and this change is conducive to the adaptation of fungi to the living environment (Alkan et al., 2013). These contracted gene families include genes that encode secondary metabolite synthases, secreted proteins, and carbohydrate active enzymes. In the family of genes that encode secondary metabolite synthases, *NPRS* and *PKS* gene clusters are responsible for the synthesis of toxic peptides and the production of naphthalenone pentaketides, respectively, in *Botryosphaeriaceae* fungi (*D. seriata*, *L. theobromae* and *N. parvum*) and other pathogenic fungi (*A. fumigatus*, *Diaporthe ampelina*, *Phaeomoniella chlamydospora*, and *Togninia minima*) (Andolfi et al., 2011; Paolinelli-Alfonso et al., 2016). Although the gene clusters of secondary metabolites are not regulated during infection, a

large number of products of these gene clusters, which are significantly expanded in the genome, may be involved in the induction of disease symptoms and host adaptation (Yan et al., 2018). Cytochrome P450 is a superfamily of monooxygenases. In addition to participating in the post-synthesis modification of a variety of metabolites, it can also promote the adaptation of fungi to specific ecological niches by altering potentially harmful chemicals in the environment (Siewers et al., 2005). Here, we found the expansion of this gene family, which can explain the wide host range of *Botryosphaeriaceae* fungi. This is because these genes are also associated with some physiological characters; thus, their expansions are likely to promote pathogenic evolution (Nierman et al., 2005). In this study, expansions are obvious for the genes that encode ABC transporters in *Botryosphaeriaceae* fungi, indicating that these fungi have evolved stronger virulence and capacity against plant defense compounds (Han et al., 2001; Luini et al., 2010). Sugar synergistic transporters belong to the MFS family, and they play important roles in fungal spore formation, intercellular communication, and pathogenicity (Gaur et al., 2008). In the *Botryosphaeriaceae* fungi, we found that the sugar synergistic transporter gene family showed different degrees of expansion, which probably makes it more adaptive to different host environments (e.g., different pH values), and more resistant when interacting with various plant pathogens (Zhang et al., 2012; Yin et al., 2015).

Previous studies have showed that secreted proteins play important roles in the infection process of pathogenic fungi (Cobos et al., 2010; O'Connell et al., 2012; Fernandes et al., 2014; Félix et al., 2016, 2019). In this study, the secretory protein gene families of *Botryosphaeriaceae* fungi have expanded to different degrees, but there are large differences between different genera, which may be related to the different infection ranges of *Botryosphaeriaceae* fungi (Wang et al., 2018). In addition, the gene families of carbohydrate active enzymes in *Botryosphaeriaceae* fungi also showed different degrees of expansion. Among these families, the glycoside hydrolase family GH33 is composed of sialidase, which can hydrolyze the glycosidic bonds of terminal sialic acid residues in oligosaccharides. Sialidase can function as a pathogenic factor, facilitating the adaption to the host by evading host recognition or inhibiting host defense responses (Alviano et al., 2004). The cell wall of most dicotyledonous plants is composed of approximately 35% pectin. Pectin-degrading enzymes contribute to the degradation of the cell wall. This local degradation of the cell wall is necessary for fungi to enter the plant cytoplasm and replicate in the host tissue (ten Have et al., 1998). Similar to the highly pathogenic *Colletotrichum higginsianum*, *Neofusicoccum*, and *Botryosphaeria* both possess a larger number of genes encoding pectin-degrading enzymes, which may explain the difference in pathogenicity between *Botryosphaeriaceae* fungi (Wang et al., 2018). Many genes encoding cellulase (AA9 and GH12) and hemicellulase (GH31 and GH43) have been significantly expanded in *Neofusicoccum* and *Botryosphaeria* fungi. These expansions may explain the rapid infection and colonization of *Botryosphaeriaceae* fungi in woody plants (Yan et al., 2018).

## CONCLUSION

In conclusion, we constructed a phylogenetic tree using whole-genome data and clarified the taxonomic position of *Botryosphaeriaceae* in *Dothideomycetes*. Heterothallism is the ancestral mating type of *Botryosphaeriaceae* fungi, and these fungi have undergone at least 3 transitions from heterothallism to homothallism. The host range of *Botryosphaeriaceae* infection is closely related to the changes in the number of pathogenic genes. Our results provide important insights into the evolutionary history, mating strategies and pathogenicity-related genes variation in *Botryosphaeriaceae*.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://zenodo.org/record/5184447#.YRUFTci0yAc>; the sequencing data are found at NCBI (PRJNA777748).

## AUTHOR CONTRIBUTIONS

CY and HL developed the concept of this study and were main contributors to writing the manuscript. CY, YD, QL, and JZ performed all experiments, data analysis, and prepared figures. CY, HL, SC, XX, AL, and XZ contributed to the manuscript edit and review. All authors read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.800981/full#supplementary-material>

**Supplementary Figure 1** | Whole-genome-based phylogenetic tree of 271 species from Dothideomycetes and 8 outgroups. The two subclass *Pleosporomycetidae* and *Dothideomycetidae* were well resolved.

**Supplementary Figure 2** | Comparison of genome size between pathogen and saprobe fungi.

**Supplementary Figure 3** | Comparison of gene count between pathogen and saprobe fungi.

**Supplementary Figure 4** | Reconstruction of ancestral binary character (non-plant pathogen and plant pathogen) of *Dothideomycetes* using Mesquite based on Mk1 model.

**Supplementary Figure 5** | Reconstruction of ancestral mating type (heterothallism and homothallism) of *Dothideomycetes* using Mesquite based on Mk1 model as heterothallism.

**Supplementary Table 1** | List of 271 *Dothideomycetes* fungi and 8 outgroups.

**Supplementary Table 2** | Classification of 19 *Botryosphaeriaceae* fungi.

**Supplementary Table 3** | Reconstruction of ancestral binary character (non-plant pathogen and plant pathogen) of *Dothideomycetes* using Mesquite based on Mk1 model.

**Supplementary Table 4** | Pfam and GO enrichment of *Dothideomycetes* fungi.

**Supplementary Table 5** | Mating type of *Botryosphaeriaceae* fungi.

**Supplementary Table 6** | Conserved protein domains of *Botryosphaeriaceae* fungi mating type genes.

**Supplementary Table 7** | Conservation of mating type genes of *Botryosphaeriaceae* fungi.

**Supplementary Table 8** | Reconstruction of ancestral mating type of *Botryosphaeriaceae* fungi.

**Supplementary Table 9** | Gene number of backbone and related genes of secondary metabolism of *Botryosphaeriaceae* fungi and 8 outgroups.

**Supplementary Table 10** | Gene number of secreted proteins of *Botryosphaeriaceae* fungi and 8 outgroups.

**Supplementary Table 11** | Pfam annotations of secreted proteins of *B. dothidea* and *B. kuwatsukai*.

**Supplementary Table 12** | Gene number of CAZy of *Botryosphaeriaceae* fungi and 8 outgroups.

**Supplementary Table 13** | Genes number of CZAy related to cellulose, hemicellulose and lignin degradation of *Botryosphaeriaceae* fungi and 8 outgroups.

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