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Phylogenomics of Plant-Associated *Botryosphaeriaceae* Species

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The *Botryosphaeriaceae* is a fungal family that includes many destructive vascular pathogens of woody plants (e.g., *Botryosphaeria* dieback of grape, Panicle blight of pistachio). Species in the genera *Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neofusicoccum*, and *Neoscytalidium* attack a range of horticultural crops, but they vary in virulence and their abilities to infect their hosts via different infection courts (flowers, green shoots, woody twigs). Isolates of seventeen species, originating from symptomatic apricot, grape, pistachio, and walnut were tested for pathogenicity on grapevine wood after 4 months of incubation in potted plants in the greenhouse. Results revealed significant variation in virulence in terms of the length of the internal wood lesions caused by these seventeen species. Phylogenomic comparisons of the seventeen species of wood-colonizing fungi revealed clade-specific expansion of gene families representing putative virulence factors involved in toxin production and mobilization, wood degradation, and nutrient uptake. Statistical analyses of the evolution of the size of gene families revealed expansions of secondary metabolism and transporter gene families in *Lasiodiplodia* and of secreted cell wall degrading enzymes (CAZymes) in *Botryosphaeria* and *Neofusicoccum* genomes. In contrast, *Diplodia*, *Dothiorella*, and *Neoscytalidium* generally showed a contraction in the number of members of these gene families. Overall, species with expansions of gene families, such as secreted CAZymes, secondary metabolism, and transporters, were the most virulent (i.e., were associated with the largest lesions), based on our pathogenicity tests and published reports. This study represents the first comparative phylogenomic investigation into the evolution of possible virulence factors from diverse, cosmopolitan members of the *Botryosphaeriaceae*.

Keywords: grapevine trunk diseases, *Botryosphaeria* dieback, comparative genomics, gene family evolution, virulence factors, secondary metabolism, cell wall degrading enzymes

INTRODUCTION

The fungal family *Botryosphaeriaceae* (*Botryosphaeriales*, *Dothideomycetes*) was introduced in 1918 by Theissen and Sydow (1918) with *Botryosphaeria* as the type genus. Members of this group have been taxonomically characterized based on the production of large, ovoid to oblong, typically hyaline, aseptate ascospores, which may become brown and septate with age, within bitunicate asci within unilocular or multilocular botryose ascomata known as pseudothecia (Sivanesan, 1984; Phillips et al., 2005). The asexual states of *Botryosphaeriaceae* exhibit a wide range of conidial morphologies that are taxonomically informative (Phillips et al., 2005). Crous et al. (2006) contributed to stabilize the taxonomy of the genera within the *Botryosphaeriaceae* by employing a natural unit classification scheme, which is also referred to as the "genus-for-genus concept" (Seifert et al., 2000). The distinct asexual morphs were linked to unique sexual morphs on a unit-by-unit basis, which was corroborated with phylogenetic analysis of 28S rDNA sequence data revealing 10 generic clades. The *Botryosphaeriaceae* is currently composed of 24 well-defined genera and more than 200 species (Burgess et al., 2019) that are cosmopolitan in distribution and exist primarily as saprobes, endophytes, or pathogens on a wide array of important perennial plant hosts (Slippers and Wingfield, 2007), in both human-altered (agricultural and urban) and natural ecosystems (forests and riparian areas) (Slippers et al., 2009; Lawrence et al., 2017).

The ecology of Botryosphaeriaceous taxa is complex and not fully understood. For example, in spite of being a shoot blight and canker pathogen of pine, *Diplodia sapinea* has been isolated from the bark surface and internal woody tissues of woody twigs from asymptomatic *Pinus* (Petrini and Fisher, 1988), representing what some may consider an "endophytic phase," in which neither the internal plant tissues from which it is isolated, nor other plant tissues/organs showed apparent symptoms, nor were there negative impacts to host growth at the time of isolation. A similar pattern in the ecology of other *Botryosphaeriaceae* species considered pathogenic, but later being isolated during an endophytic phase, has been documented (Slippers and Wingfield, 2007; Luo et al., 2019; Hrycan et al., 2020). In some cases, abiotic stress (water stress, heat stress) has been shown to induce severe symptoms in different host plants infected with seemingly innocuous *Botryosphaeriaceae* (Pusey, 1989; Mullen et al., 1991; Smith et al., 1994). This relationship between abiotic stress and more severe symptoms or more rapid colonization has also been reported for pathogenic species, e.g., *Neofusicoccum parvum* causing *Botryosphaeria* dieback of grape (Luque et al., 2010; Galarneau et al., 2019) and *Botryosphaeria dothidea* causing Pistachio panicle and shoot blight (Ma et al., 2001). Under climate-change scenarios of more frequent temperature extremes and prolonged drought, the interactions between host plants and Botryosphaeriaceae species may transit more readily from endophytic to pathogenic (Desprez-Loustau et al., 2006; Slippers et al., 2007). An increase in *Botryosphaeriaceae* symptom severity in conjunction with other biotic stresses has also been documented in the literature (Old et al., 1990; Lawrence et al., 2018).

Members of the *Botryosphaeriaceae* are probably most well-known as being destructive blight and canker pathogens of planted hosts (Luo et al., 2019). In agricultural settings, for example, they infect a large number of fruit and nut crops, such as almond (Inderbitzin et al., 2010; Gramaje et al., 2012; Nouri et al., 2018; Holland et al., 2020), apple (Phillips et al., 2012), avocado (McDonald et al., 2009), citrus (Linaldeddu et al., 2015), grapevine (Urbez-Torres, 2011), olive (Úrbez-Torres et al., 2013), pistachio (Michailides, 1991; Nouri et al., 2019), and walnut (Chen S. et al., 2014). In forest plantations in Australia and South Africa, for example, they infect *Eucalyptus* spp. and *Pinus* spp. (Slippers et al., 2007). Infection is through either wounds to green and woody tissues or through natural openings in flowers, fruit, leaves, and shoots. The pathogens produce enzymes and/or toxins that kill cells and tissues of the various plant organs they attack. Infections of woody tissues of perennial hosts, either deep in the wood or just below the bark, can lead to stunted shoot growth, with eventual shoot death or "dieback."

Ecological genomic comparisons of phytopathogenic and saprobic fungi suggest that the former possess expanded gene families that generally fall into two main functional categories: (1) lytic capabilities (Massonnet et al., 2018) and (2) putative transporters (Powell et al., 2008). Fungal lignin peroxidases, peroxidases, laccases, and polyphenol oxidases allow fungi to gain access to nutrients and to protect themselves from host defenses while growing in wood (Mayer, 2006; Martínková et al., 2016; Valette et al., 2017). Pathogenic species with the ability to enzymatically decompose a broader diversity of cell wall carbohydrates might be expected to more rapidly colonize, kill, and/or decompose host tissue. Membrane transporters of fungal plant pathogens also play important roles in exporting virulence factors involved in pathogenesis, influx of nutrients, and efflux of host-derived defense antimicrobial compounds (Denny and VanEtten, 1983; Denny et al., 1987). Previous genomic comparisons of phylogenetically diverse wood-infecting pathogens of grape revealed expansions in the repertoire of cell-wall degrading enzymes called carbohydrate-active enzyme (CAZyme) gene families, whose protein products are involved in the synthesis, degradation, and/or modification of glycosidic bonds of plant cell wall constituents, including the main components of wood, cellulose, hemicelluloses, lignin (Morales-Cruz et al., 2015), and significantly so in *Neof*. Further, a recent genomic annotation and *in planta* transcriptomic study of putative virulence factors of *Neof. parvum* during wood colonization revealed 567 protein-coding genes belonging to 52 different CAZyme families with glycoside hydrolases (GHs), which made up approximately 50% of the pathogen's cell-wall degrading repertoire (Massonnet et al., 2018). Likewise, Yan et al. (2018) identified 820 CAZymes with at least 10 families that have experienced expansion in the genome of *Lasiodiplodia theobromae* with GHs representing the largest super family involved in the modification of plant cell wall carbohydrates. Genome comparisons of *B. dothidea*, *L. theobromae*, and *Neof. parvum* revealed that the genome of *L. theobromae*, the most virulent of the three species, is expanded in gene families associated with membrane transport, mainly ATP-binding-cassette (ABC family), and major facilitator super (MFS) families

(Yan et al., 2018). That same study reported 17 membrane transport genes that were significantly up-regulated upon host recognition including amino acid transporters and sugar porters. The largest transporter families reported in the genome of *Neof. parvum* include MFS, Peroxisomal Protein Importer (PPI) family, and the ABC superfamily (Massonnet et al., 2018).

In this study we analyze the genome sequences of seventeen *Botryosphaeriaceae* species representing six genera (*Botryosphaeria*, *Diplodia*, *Dothiorella*, *Lasiodiplodia*, *Neofusicoccum*, and *Neoscytalidium*), which are wood-canker pathogens that attack horticultural crops, namely grape, pistachio, *Prunus* species (almond and stone fruits apricot, peach, and plum), and walnut. Our objective is to examine through phylogenomic comparisons this comprehensive set of species on one host, grape, to better understand the evolutionary trends within this important fungal family, especially as it pertains to the gene space involving pathogenesis of woody tissues and fungal virulence.

MATERIALS AND METHODS

Isolate Collection and Species Confirmation

All fungal isolates utilized in this study were obtained from internal wood cankers of symptomatic hosts following the protocol of Baumgartner et al. (2013) (**Table 1**). Total genomic DNA was extracted following Morales-Cruz et al. (2018). The internal transcribed spacer (ITS) and translation elongation factor (TEF) loci were amplified for each isolate via PCR using primers ITS5/ITS4 (White et al., 1990) and EF1-688F/EF1-1251R (Alves et al., 2008). TEF and ITS sequences of each species (including type specimen sequences downloaded from GenBank) were concatenated and aligned using MUSCLE v3.8.31 (Edgar, 2004) with default parameters. The alignment was cleaned with GBlocks v. 0.91b (Castresana, 2000) with a minimum block's length of 5 bp and half of the gaps allowed. PhyML (Guindon et al., 2010) was used to calculate the maximum likelihood tree using 100 bootstrap replications, HKY85 substitution model and the subtree-pruning-regrafting method for searching for optimal tree topology. The resulting tree was visualized and edited for presentation using FigTree v1.4.1 (Rambaut, 2012).

Sequencing and Genomes Assemblies

DNA extraction was done following the methods used by Morales-Cruz et al. (2015) using the axenic cultures of the isolated fungi and a CTAB protocol. Sequencing libraries were prepared and sequenced as described in Morales-Cruz et al. (2015). After adapter ligation, libraries were size selected to 550–600 bp using a double-sided size selection with Ampure XP magnetic beads (Beckman Coulter, United States) to remove unused adapter and adapter dimer. Sequencing was carried out on an Illumina HiSeq4000 machine at the DNA Technologies Core at UC Davis. Paired-end reads of 150 bp in length were generated. Raw reads were trimmed for quality ($Q > 30$) and adapter removal using Trimmomatic v0.36 (Bolger et al., 2014) with options LEADING:3 TRAILING:3

SLIDINGWINDOW:4:20 MINLEN:100. Assembly of high-quality reads was made using SPAdes v3.9 (Bankevich et al., 2012) with the careful option and automatic read coverage cutoff. Assembly completeness was assessed using the Core Eukaryotic Genes Mapping Approach (CEGMA v.2.5; Parra et al., 2007) and Benchmarking Universal Single-Copy Orthologs (BUSCO v.1.1; Simão et al., 2015) analysis. RepeatMasker v.4.06 (Smit et al., 1996-2015) with default parameters was used to mask repeats. Gene model prediction was performed with Augustus v.3.2.1 (Stanke et al., 2006) with default parameters and using *Neof. parvum* gene model as training set. Sequencing data are available at NCBI (BioProject PRJNA673527). Sequencing data of *Diplodia seriata* (Morales-Cruz et al., 2015) and *Neof. parvum* (Massonnet et al., 2018) can be retrieved from NCBI under BioProject PRJNA261773 and PRJNA321421, respectively. All genome assemblies and gene models are publicly available at Zenodo (doi: 10.5281/zenodo.4417445).

Functional Annotation

The general annotation of the predicted proteins was assigned based on the similarities with peptides in the GenBank with Blast2GO (Conesa et al., 2005), and to conserved domains in Pfam database (Finn et al., 2016). The functional annotation (**Supplementary Table 3**) was assigned based on the databases and parameters presented in **Supplementary Table 4**. CAZymes were annotated with the dbCAN2 (Zhang et al., 2018). The signal peptides were predicted using SignalP 5.0 (Armenteros et al., 2019). The proteins with annotation in both databases were annotated as secreted CAZymes. Secondary metabolites clusters were annotated using antiSMASH 5.0 (Blin et al., 2019). Peroxidases were annotated using a specialized database for fungi called fPoxDB (Choi et al., 2014). CYPED 6.0 was used to annotate the Cytochrome P450 proteins (Fischer et al., 2007). At last, the proteins involved in transportation functions were annotated using the TCDB (Saier et al., 2006, 2016).

Construction of a Clock-Calibrated Phylogenetic Tree

Seventy-three single copy peptides used in Floudas et al. (2012) for fungal phylogeny reconstruction were extracted from the reference strain *Saccharomyces cerevisiae* 2S88C Genome Release 64-2-1 (downloaded from http://www.yeastgenome.org). All these peptides were compared using BLASTP (v.2.6.0+) against the seventeen *Botryosphaeriaceae* species and two wood-decay basidiomycetes that colonize grape: pathogenic, wood-rotting fungus (with characteristics of both white-rot and brown-rot fungi) *Fomitiporia mediterranea* and saprobic, white-rot fungus *Stereum hirsutum*. *Fomitiporia mediterranea* is one of a complex of pathogens that causes the grapevine trunk disease Esca in Europe, whereas the pathogenicity of *St. hirsutum* to grape is not known (Fischer, 2006). Twenty-one proteins had exactly one top hit in all the species. The rest of the seventy-three initial proteins were excluded because they were either not present in all the species or had paralogs. Each set of orthologous proteins was aligned using MUSCLE v3.8.31 (Edgar, 2004). Alignments were concatenated and cleaned using GBlocks v.

TABLE 1 | Genome assembly summary statistics of the *Botryosphaeriaceae* species analyzed.

Species (isolate ID)	Isolated from	Assembly size (Mbp)	N. scaffold	N50 (kbp)	L50 (Scaffold #)	CEGMA***	BUSCO***
Botryosphaeria dothidea (0053)	Grape	46	2,425	506	28	99%	98%
Diplodia mutila (SBen820)	Grape	46	4,003	175	67	98%	98%
Diplodia seriata (DS831)*	Grape	37	811	301	39	98%	98%
Dothiorella iberica (Wolf833)	Apricot	37	636	412	28	97%	98%
Dothiorella sarmentorum (SBen806)	Apricot	42	1,664	242	57	97%	98%
Dothiorella viticola (Wint804)	Grape	37	2,477	482	25	98%	98%
Lasiodiplodia citricola (06I-35)	Walnut	44	216	1,067	13	97%	98%
Lasiodiplodia exigua (UCR-LT5)	Grape	44	340	720	15	98%	98%
Lasiodiplodia missouriana (09-C092)	Grape	44	324	883	17	98%	98%
Lasiodiplodia theobromae (MXBCL28)	Grape	44	193	883	16	99%	98%
Neofusicoccum australe (UCR-NA2)	Grape	42	2,218	385	35	99%	98%
Neofusicoccum hellenicum (02-K91)	Pistachio	43	221	768	19	98%	98%
Neofusicoccum mediterraneum (Wint817)	Grape	42	196	551	24	99%	98%
Neofusicoccum nonquaesitum (05-A04)	Walnut	43	316	667	19	99%	98%
Neofusicoccum parvum (UCD646So)**	Grape	43	2,452	168	74	99%	98%
Neofusicoccum vitifusiforme (05-H02)	Walnut	42	288	506	24	98%	98%
Neoscytalidium dimidiatum (UCR-Neo1)	Grape	42	478	419	31	100%	98%

*Published in Morales-Cruz et al., 2015.
**Published in Massonnet et al., 2018.
***Percentage of complete CEGMA and BUSCO peptides found in the genome assembly.

0.91b (Castresana, 2000; maximum number of contiguous non-conserved positions = 4, minimum length of a block = 10), reducing the initial 21,008 positions to 12,066 informative positions. Clean alignments were imported into BEAUti v1.10.4 to prepare them for BEAST v1.10.4 analysis (Bouckaert et al., 2014). Monophyletic partitions were set for *Ascomycota*, *Basidiomycota*, and *Dothideomycetes* species. Calibrations points were set to 588 and 350 mya on *Ascomycota* and *Dothideomycetes* partition, respectively, according to Beimforde et al. (2014). Six MCMC chains of 1,000,000 steps were launched on BEAST (WAG substitution model, 4 Gamma Categories + Invariant sites, Lognormal relaxed clock, Calibrated Yule Model). The resulting trees were concatenated with LogCombiner v1.10.4 (Bouckaert et al., 2014) and a consensus tree was obtained from TreeAnnotator v1.10.4 (Bouckaert et al., 2014; **Supplementary Figure 1**). FigTree v1.4.1 (Rambaut, 2012) and Inkscape v.1.0.1 (Inkscape Project, 2020) were used to edit the tree for figure presentation.

Computational Analysis of Gene Family Evolution (CAFE)

BLASTP (e-value $< 10^{-6}$) was used to group proteins in families based on sequence similarity followed by Markov clustering with MCL (Van Dongen, 2000; Enright et al., 2002). The 10,158 families with at least one protein in no less than four species were used with the clock calibrated tree as input for the CAFE v.4.2.1 (De Bie et al., 2006) analysis. CAFE was run in the default mode with the option -s to optimize the lambda parameter to 0.00155948837239, and a P-value threshold of 0.01 (option -p). To evaluate significant expansions or contractions of a specific branch, Viterbi P-values were calculated for each significant family.

Phylogenetic Principal Component Analysis

The phyl.pca function of the phytools R package (Revell, 2012) was used to create the phylogenetic PCAs. The inputs for his function were the clock calibrated tree and protein matrices of CAZymes and secondary metabolite clusters.

Pathogenicity Tests

The pathogenicity of 17 species of *Botryosphaeriaceae* was evaluated on potted grapevines (*Vitis vinifera* 'Pinot noir' clone 777) in replicate experiments in the greenhouse (18 treatments × 10 replicate plants per treatment × 2 experiments = 360 total plants). Hardwood cuttings obtained from a commercial nursery were propagated in May/June 2016 following the protocol of Travadon et al. (2013). Inoculations took place after callusing and before planting in pots in the greenhouses. For inoculations, the commonly used agar plug method was employed (e.g., Chacón et al., 2020). This method has been shown to produce lower variability in lesion length than the method with mycelial suspensions for the pathogens *Neof. parvum* and *Di. seriata* (Guan et al., 2016). For inoculations, a power drill was used to wound (5 mm wide × 3 mm deep) the cutting, approximately 2 cm below the apical node. A 5-mm agar plug from a 7-day culture on PDA was aseptically inserted into the wound and sealed with Vaseline and parafilm to prevent inoculum desiccation. Cuttings were coated in melted paraffin wax (Gulf Wax; Royal Oak Enterprises, LLC, Roswell, GA, United States) and potted in a sterile potting mix amended with slow-release fertilizer (Osmocote® Pro 24-4-9, Scotts, Marysville, OH, United States). The plants were watered twice per week for 16 weeks. Ten plants were used for each isolate and ten plants were mock-inoculated with sterile PDA. Plants were

arranged in a completely randomized design in two separate greenhouses at the University of California Experiment Station in Davis from June 2016 to October 2016 [natural sunlight photoperiod, 25 ± 1 C (day), 18 ± 3 C (night)]. The second experiment was initiated 1 week after the first experiment. The length of internal wood discoloration extending out from the inoculation site up and down the stem (lesion length) was measured approximately 16 weeks after inoculation in October 2016. First, plants were inspected for foliar symptoms. Then the newly developed green shoots, roots, and bark of each plant were removed and discarded, and the woody stems were surface sterilized in 1% sodium hypochlorite for 2 min and rinsed with deionized water. The length of each stem was recorded and cut longitudinally to expose wood discoloration, the length of which was measured with a digital caliper. From each plant, ten small wood pieces were cut from the margin of the lesion with flame-sterilized scalpels and plated onto PDA in an attempt to recover the inoculated fungi. Inoculated pathogens, identified based on colony morphology, were re-isolated from all samples.
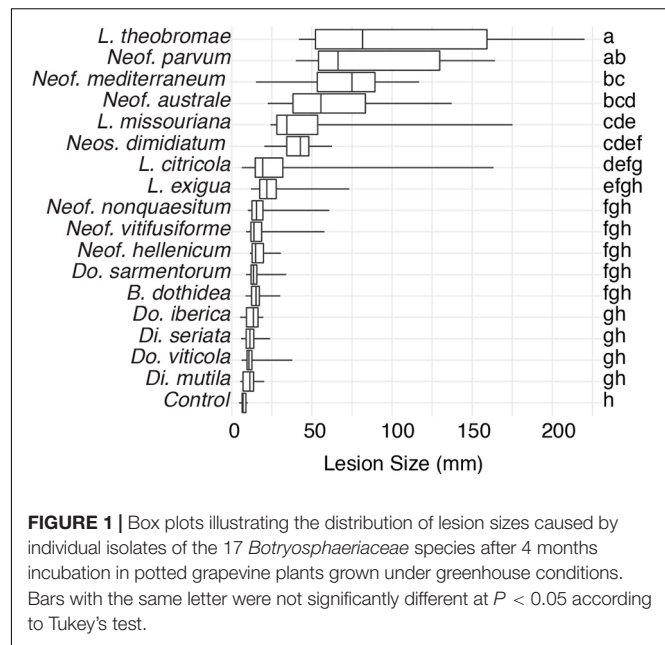
Lesion lengths were used as a measure of pathogenicity. Normality and homogeneity of variances were evaluated using Shapiro–Wilk's and Levene's tests, respectively. ANOVA was used to determine whether there were differences in lesion length among treatments. ANOVA was performed in R using the lesion size as a function of the inoculation treatment and the experiment. Means were compared for significant effects ($P < 0.05$) by Tukey's HSD *post hoc* test.

RESULTS

Genome Assembly, Gene Prediction, and Virulence Factor-Focused Functional Annotation

To expand the genomic information for *Botryosphaeriaceae*, we *de novo* assembled the genomes of fifteen species isolated from multiple hosts. We included the previously published genomes of *Neof. parvum* and *Di. seriata* in the comparative genomics analysis (**Table 1** and **Supplementary Table 1**). For all seventeen species, pathogenicity was evaluated using inoculations of potted grapevines (**Figure 1**). All seventeen species produced dark necrotic lesions in the woody stems extending upward and downward from the point of inoculation at 15 weeks post inoculation. Overall, *Lasiodiplodia* and *Neofusicoccum* spp. were the most aggressive, while *Diplodia* and *Dothiorella* spp. caused the smallest lesions.

All genomes were sequenced using Illumina technology at coverage $171 \pm 10\times$ (**Supplementary Table 1**). On average, sequencing Illumina reads were assembled into $1,066 \pm 308$ scaffolds (N50 length: 577.64 ± 64.53 kbp; L50 scaffold count: 27.9 ± 4 scaffolds). The total genome assembly size varied from 37 Mbp for *Dothiorella viticola* to 46 Mbp for *B. dothidea* with an average of 42.37 ± 0.69 Mbp. The expected and assembled genome size had a discrepancy of less than ten percent on average (4.4 ± 2.2 Mb), which suggests near completeness of the assemblies (**Supplementary Table 1**). This result was confirmed

FIGURE 1 | Box plots illustrating the distribution of lesion sizes caused by individual isolates of the 17 *Botryosphaeriaceae* species after 4 months incubation in potted grapevine plants grown under greenhouse conditions. Bars with the same letter were not significantly different at $P < 0.05$ according to Tukey's test.

by CEGMA (Parra et al., 2007) and BUSCOs (Simão et al., 2015) analyses, which reported an average $98.3 \pm 0.2\%$ and $98.1 \pm 0.1\%$ completeness, respectively (**Table 1**). Interspersed repeats only accounted for $1.87 \pm 0.003\%$ of the genome assemblies. Among the classified elements, long-terminal-repeats (LTR) were the most abundant, ranging from a total of 315 kbp in *B. dothidea* to 26 kbp in *Neoscytalidium dimidiatum* (**Supplementary Table 2**). The predicted protein-coding genes in the seventeen genomes varied from 10,827 in *Dothiorella iberica* to 13,492 in *Dothiorella sarmentorum*. On average $12,193 \pm 193$ CDS were found per species (**Table 2**).

The predicted genes of the seventeen genomes were annotated using general databases for protein domains (Pfam), gene ontology (GO), as well as more specialized databases related to putative virulence factors. The last group included carbohydrate-active enzymes (CAZymes), cytochrome P450s, peroxidases, usually associated with host colonization and wood degradation, and secondary metabolism gene clusters, including toxins production, and cellular transporters (**Supplementary Table 4** and **Table 3**). A total of 229,251 predicted protein-coding genes were annotated (**Table 3**).

Carbohydrate-Active Enzymes Are Especially Abundant in the Genomes of *Neofusicoccum* Species

A wide variety of monosaccharides can be linked to many different types of molecules (proteins, lipids, nucleic acids and, sugar themselves) and converting these glycoconjugates into one of the most structurally diverse substrates (Cantarel et al., 2009). CAZymes are the group of enzymes responsible for the assembly and breakdown of these diverse substrates (Lombard et al., 2014). Not all CAZymes contribute to the pathogenicity of the microorganisms, however, predicting them

TABLE 2 | Gene model predictions statistics of the *Botryosphaeriaceae* species analyzed.

Species	Repeat content (bp)	N. CDS	Mean protein size (AA)	BUSCO*	Mean gene density (genes/10 kbp)	SD
Botryosphaeria dothidea	2,608,021 (5.67%)	12,424	478	98%	2.14	1.9
Diplodia mutila	2,877,891 (6.28%)	11,947	491	98%	1.74	1.9
Di. seriata	1,144,284 (3.05%)	11,085	495	98%	2.93	1.8
Dothiorella iberica	1,350,049 (3.69%)	10,827	491	98%	3.02	1.8
Do. sarmentorum	1,687,353 (4.02%)	13,942	474	98%	2.90	2.5
Do. viticola	950,252 (2.60%)	11,235	473	98%	2.19	1.9
Lasiodiplodia citricola	825,529 (1.89%)	12,376	492	98%	3.19	1.4
L. exigua	771,071 (1.77%)	12,399	492	98%	3.12	1.5
L. missouriana	833,148 (1.89%)	12,448	494	98%	3.12	1.5
L. theobromae	820,166 (1.88%)	12,434	493	98%	3.20	1.4
Neofusicoccum australe	2,055,816 (4.92%)	12,104	488	98%	2.29	1.9
Neof. hellenicum	1,078,449 (2.53%)	12,433	486	98%	3.27	1.4
Neof. mediterraneum	960,302 (2.26%)	12,541	484	98%	3.36	1.4
Neof. nonquaesitum	1,010,201 (2.35%)	12,602	487	98%	3.24	1.5
Neof. parvum	2,170,098 (5.00%)	12,679	485	98%	2.27	1.9
Neof. vitifusiforme	1,405,151 (3.37%)	12,112	485	98%	3.22	1.5
Neoscytalidium dimidiatum	1,099,801 (2.61%)	11,067	478	98%	3	1.5

Percentage of complete BUSCO peptides found in the predicted proteome.

TABLE 3 | Number of protein coding genes annotated per functional category.

Species	Total genes	Annotated genes	Secondary metabolites involved genes	P450s	CAZymes	Secreted CAZymes	Peroxidases	Signal Peptides	Transporters	% Secreted CAZymes of total secreted
Botryosphaeria dothidea	12,424	11,877	809	857	485	280	56	1,341	2,505	20.9%
Diplodia mutila	11,947	11,240	407	675	433	242	55	1,198	2,362	20.2%
Di. seriata	11,085	10,535	300	620	432	251	53	1,114	2,238	22.5%
Dothiorella iberica	10,827	10,237	374	609	399	229	41	1,029	2,185	22.3%
Do. sarmentorum	13,942	13,226	457	652	487	259	50	1,596	3,143	16.2%
Do. viticola	11,235	10,663	283	594	395	218	46	1,053	2,339	20.7%
Fomitiporia mediterranea	11,338	11,338	205	639	315	178	52	780	1,985	22.8%
Lasiodiplodia citricola	12,376	11,831	564	785	465	266	54	1,298	2,492	20.5%
L. exigua	12,399	11,838	578	785	474	271	55	1,287	2,499	21.1%
L. missouriana	12,448	11,902	572	787	470	267	54	1,305	2,507	20.5%
L. theobromae	12,434	11,832	610	784	465	270	54	1,279	2,490	21.1%
Neofusicoccum australe	12,104	11,709	654	800	488	291	61	1,334	2,530	21.8%
Neof. hellenicum	12,433	12,015	662	868	501	281	62	1,369	2,546	20.5%
Neof. mediterraneum	12,541	12,052	658	843	504	288	61	1,372	2,549	21.0%
Neof. nonquaesitum	12,602	12,229	661	918	514	296	60	1,394	2,604	21.2%
Neof. parvum	12,679	12,328	639	908	504	298	59	1,393	2,588	21.4%
Neof. vitifusiforme	12,112	11,715	575	817	501	289	62	1,322	2,492	21.9%
Neoscytalidium dimidiatum	11,067	10,701	489	746	452	247	48	1,139	2,357	21.7%
Saccharomyces cerevisiae	5,917	5,917	48	215	136	44	21	306	1,478	14.4%
Stereum hirsutum	14,066	14,066	426	652	394	230	52	1,070	2,150	21.5%

in conjunction with signal peptides is widely used to obtain information about plant pathogen cell wall degrading enzymes (Floudas et al., 2012; Suzuki et al., 2012; Blanco-Ulate et al., 2014; Jones et al., 2014; Morales-Cruz et al., 2015). An average of 20.9 ± 0.3% of the predicted secreted proteins among all seventeen *Botryosphaeriaceae* genomes shared similarity with

the CAZymes in the dbCAN2 database (Zhang et al., 2018). Glycoside Hydrolases (GH) and Auxiliary Activity CAZymes (AA) were the two groups with the most predicted proteins. GHs were especially abundant in *Neofusicoccum* spp. with an average of 336 ± 4 proteins compared to 303 ± 7 for the rest of the *Botryosphaeriaceae* species in this study (**Supplementary**

Table 5). A total of 15 putative genes of GH3 were present in *Neofusicoccum nonquaesitum* and *Lasiodiplodia missouriana*, as well as 14 in *Neof. parvum*, *Lasiodiplodia citricola*, and *Lasiodiplodia exigua*. GH3 and GH43 families activities include β-glucosidases, β-xylosidases, glucanases, L-arabinofuranosidase, galactanase and others related to the hydrolysis of plant cell wall components into more simple sugars (Faure, 2002; Polizeli et al., 2005; Cairns and Esen, 2010; Knob et al., 2010; Sampedro et al., 2017).

Auxiliary activities (AAs) were also more abundant in *Neofusicoccum* (127 ± 3) than the rest of the *Botryosphaeriaceae* species (100 ± 4). The AA3 family was the most abundant with numerous copies in the genus *Neofusicoccum* (**Figure 2**), ranging from 21 to 26 predicted proteins in *Neof. parvum* (UCD646So*)*. The genome of *B. dothidea* was predicted to possess 23 AA3 proteins. The AA3 and AA9 families include cellobiose dehydrogenases, alcohol oxidases, pyranose oxidase, acting over more complex substrates of the plant cell wall like cellulose and/or lignin (Daniel et al., 1994; Henriksson et al., 2000; Harreither et al., 2011; Hernández-Ortega et al., 2012).

Neofusicoccum, *Botryosphaeria*, and *Lasiodiplodia* Species Encode the Largest Number of Predicted P450s

Cytochrome P450 enzyme evolution is thought to contribute to the adaptation of organisms to new ecological niches. The functions may vary from primary metabolism, detoxification of xenobiotic compounds, to producing a vast variety of secondary metabolites (Črešnar and Petrič, 2011; Moktali et al., 2012; Chen W. et al., 2014). These features sometimes play essential roles in pathogenesis (Črešnar and Petrič, 2011; Moktali et al., 2012). The P450s were classified in superfamilies as described by Fischer et al. (2007). *Neofusicoccum*, *Botryosphaeria*, and *Lasiodiplodia* species encoded a larger number of predicted P450 (859 ± 20, 857, and 785 ± 1, respectively) compared to *Diplodia* and *Dothiorella* species (648 ± 28 and 618 ± 17, respectively). *Neof. nonquaesitum* and *Neof. parvum* showed the highest number of predicted P450s genes with 918 and 908, respectively (**Table 3** and **Supplementary Table 6**). CYP53, CYP51, and CYP504 were the most abundant across all the species. CYP53 was especially numerous in *Neofusicoccum* (137 ± 4 genes), *Lasiodiplodia* (128 ± 1 gene), and *Botryosphaeria* (119 ± 4 genes) species compared to the other genera (93 ± 8 genes). On the other hand, the CYP51 was very consistent in the *Botryosphaeriaceae* family (from 93 to 105 genes). Other superfamilies like CYP706, CYP102, and CYP3 show the same pattern of higher representation in *Neofusicoccum*, *Lasiodiplodia*, and *Botryosphaeria* than in *Diplodia* and *Dothiorella* species.

Peroxidases Are Most Abundant in the Genomes of *Neofusicoccum* Species

Fungal peroxidases are oxidoreductases that catalyze the oxidation of various compounds ranging from ligninolysis to the detoxification of host-derived reactive oxygen species and have been s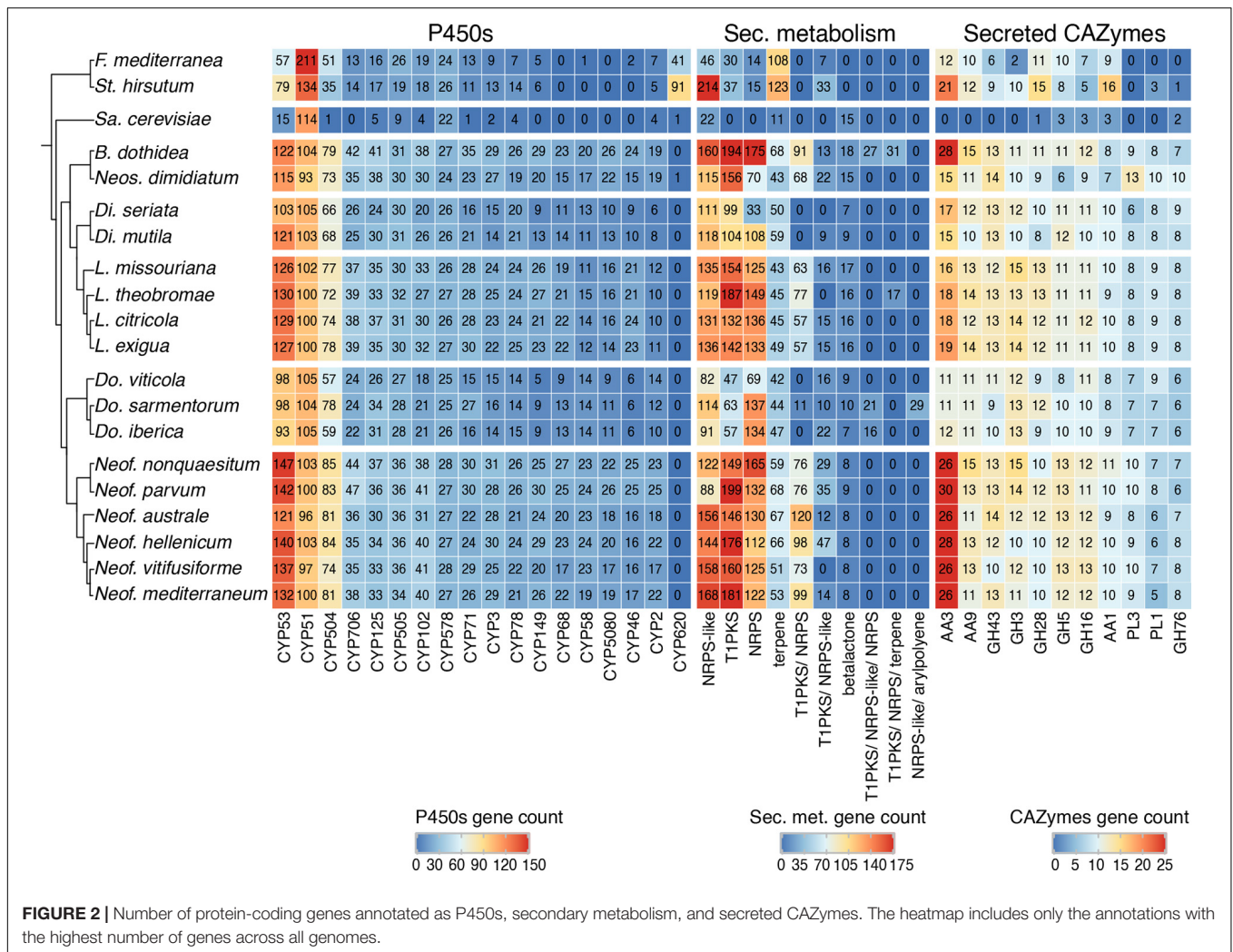hown to contribute to virulence (Molina and Kahmann, 2007; Guo et al., 2010; Choi et al., 2014). The annotation of these peroxidases was based on the manually curated Fungal Peroxidases Database fPoxDB (Choi et al., 2014). *Neofusicoccum* species encoded the largest number of predicted peroxidases (61 ± 0), followed by *Lasiodiplodia*, *Botryosphaeria*, and *Diplodia* with an average of 54 ± 1 annotated genes (**Table 3**). *Dothiorella*, with only 46 ± 3 was the genus with the least number of annotated peroxidases in the *Botryosphaeriaceae* (**Supplementary Table 7**). Hybrid Ascorbate-Cytochrome C peroxidases were more abundant in *Neofusicoccum*, *Botryosphaeria*, and *Lasiodiplodia*, ranging from 8 to 11 genes, while haloperoxidases were more abundant in the genus *Neofusicoccum* (11 ± 0 genes).

The Genomes of *Botryosphaeria*, *Neofusicoccum,* and *Lasiodiplodia* Species Have the Largest Number of Secondary Metabolism Gene Clusters

Secondary metabolites play important roles in fungal development and interactions with other organisms, including plant hosts (Keller, 2019). Phytotoxic metabolites, e.g., melleins, produced by *Neof. parvum* both *in vitro* and in the wood of symptomatic grape are thought to be associated with pathogenesis (Abou-Mansour et al., 2015). In fungi, the genes encoding the functions responsible for the biosynthesis of secondary metabolites are physically grouped in clusters of contiguous genes (Brakhage, 2013; Keller, 2019), which typically comprise a central biosynthetic gene as well as genes involved in post-synthesis modification of the metabolites and cellular transport.

Using antiSMASH 5 (Blin et al., 2019), we detected an average of 43 ± 3 biosynthetic gene clusters (BGCs) in the seventeen *Botryosphaeriaceae*. The Type I Polyketide synthase cluster (T1PKS) and the Non-ribosomal peptide synthetase-like fragment (NRPS-like) together accounted for 47% of all annotated BGCs. BGCs were most abundant in *Botryosphaeria*, *Neofusicoccum*, and *Lasiodiplodia* species with an average of 57 ± 8, 56 ± 1, and 49 ± 1 BGCs, respectively. In these genera, we also found the larger number of genes per BGC (11 ± 1, 12 ± 0, and 12 ± 0, respectively; **Supplementary Table 9**). In *Neofusicoccum* spp., 169 ± 7 genes were associated with T1PKS, 154 ± 12 in *Lasiodiplodia* and 175 ± 19 in *Botryosphaeria* (**Figure 2**). For these secondary metabolites as well as classes, we found fewer genes in the genomes of *Diplodia* and *Dothiorella* species (**Supplementary Table 10**).

Toxins and other secondary metabolites are exported by cellular transporters (Del Sorbo et al., 2000). Homologies with the Transporter Classification Database (TCDB; Saier et al., 2006) were used to annotate hypothetical protein transporters. Overall, the Electrochemical Potential-driven Transporters was the most prominent group across all the species representing 31 ± 1% of the annotated transporters followed by the Primary Active Transporters (19%) and the Incompletely Characterized Transport Systems (19%). More specifically, The Major Facilitator Superfamily (MFS) (TCDB code 2.A.1) represented the highest number in all the species but was especially abundant

FIGURE 2 | Number of protein-coding genes annotated as P450s, secondary metabolism, and secreted CAZymes. The heatmap includes only the annotations with the highest number of genes across all genomes.

in *Neofusicoccum*, *Lasiodiplodia*, and *Botryosphaeria* from 455 to 514 predicted genes (**Supplementary Table 8**). The genome of *Dothiorella sarmentorum* encodes a higher number of genes in the ATP-binding Cassette (ABC; 134 genes) superfamily compared to the other fungi analyzed (59 ± 1 genes). Both MFS and ABC transporters can be involved in toxin secretion and defense responses (Del Sorbo et al., 2000; Perlin et al., 2014).

Estimation of Gene Family Expansion and Contraction and Evaluation of Functional Enrichment

We further evaluated the differences in putative virulence factors to identify gene families that have significantly expanded or contracted in specific lineages by statistical analysis of the evolution of the size of gene families using Computational Analysis of gene Family Evolution (CAFE; De Bie et al., 2006). CAFE estimates the global birth and death rate of gene families and identifies those families that have an accelerated rate of gain or loss (Hahn et al., 2005; De Bie et al., 2006). CAFE uses a clock-calibrated phylogenetic tree and gene family sizes in all

the species' genomes as input. We included *F. mediterranea* and *St. hirsutum*, two well-known wood decay basidiomycetes related to the white-rot symptom in Esca disease of grapevines, and *Sa. cerevisiae*. These additional species were used as calibration points for the estimated dates of monophyletic partition of *Ascomycota* (588 mya) and *Dothideomycetes* (350 mya) as in Floudas et al. (2012) and Beimforde et al. (2014). To construct the phylogenetic tree, we identified twenty-one single-copy protein sequences that were previously used to study phylogenetic relationships across fungi (Floudas et al., 2012). The phylogenetic tree was built using a multiple alignment comprising 12,066 amino acid positions. The topology of the clock-calibrated tree was confirmed independently (**Supplementary Figure 2**) using ITS (Internal Transcribed Spacer) and TEF (Translation Elongation Factor), and was consistent with published ones (Phillips et al., 2008; Chen S. et al., 2014; Thambugala et al., 2014).

The gene families were computed using a Markov Cluster algorithm (MCL) that groups putative orthologs and paralogs (Enright et al., 2002). In total, 237,976 proteins of the 20 fungal genomes were clustered into gene families (e-value $< 1e^{-6}$). These family sizes and the clock calibrated tree produced by

BEAST allowed CAFE to detect 666 families (35,498 genes) across all the species with a significantly higher than expected rate of gene gain/loss ($P \leq 0.01$). The numbers of gene families expanded and contracted for each branch of the phylogeny are shown in **Figure 3**. The parent branches of the *Neofusicoccum*, *Lasiodiplodia*, and *Botryosphaeria* clades show a positive rate of gene gain/losses (+0.45, +0.14, and +0.44, respectively), which suggest an expansion of some set of proteins. On the other hand, the parent branches of *Diplodia*, *Dothiorella*, and Basidiomycetes (*Stereum* and *Fomitiporia*) clades present a negative rate of gain losses (−0.40, −0.49, and −0.70, respectively). *Saccharomyces cerevisiae*, as seen in previous studies (Morales-Cruz et al., 2015), showed the lowest rate with −1.63.

The 35,498 genes in the significantly expanded or contracted families were analyzed with a Fisher's Exact test to identify functional enrichments within those families. We found that these enrichments were not always present in all the species of a clade (**Figure 4**). However, there are some patterns to highlight. The *Lasiodiplodia* clade represents an overall expansion of the transporter proteins. *B. dothidea* also shows a significant expansion of this family. On the other hand, the *Diplodia* and *Neofusicoccum* clades show an overall contraction of transporters. The *Dothiorella* clade and *Neos. dimidiatum* show no specific pattern. The expanded secondary metabolite proteins were specifically abundant in *L. missouriana*, *L. exigua*, *B. dothidea*, *Do. sarmentorum*, and *Neos. dimidiatum*. The P450 family was expanded mostly in *Lasiodiplodia* species and *B. dothidea* but contracted in *Diplodia* species and *Neos. dimidiatum*. *Neofusicoccum* species and *B. dothidea* have an important representation of expanded secreted CAZymes, *Diplodia* and *Dothiorella* represent several expanded proteins, however, the numbers in *Lasiodiplodia* are extremely low.

Transporter related genes in the Major Facilitator Superfamily (MFS-2.A.1) were the most enriched in all the clades analyzed (196 predicted proteins were expanded and 205 contracted). The secondary metabolite related proteins type 1 Polyketide Synthases (T1PKS) were expanded in *Neos. dimidiatum*, *L. missouriana*, *L. exigua*, and *Do. sarmentorum*, whereas Non-Ribosomal Peptide Synthetases (NRPS) were expanded in *B. dothidea* and *Neos. dimidiatum*. For the secreted CAZymes, *Neof. nonquaesitum*, *Neofusicoccum hellenicum*, and *B. dothidea* show an enrichment of the Auxiliary Activity family 3. Also, *Do. iberica* and *Diplodia mutila* show an enrichment of the Glycoside Hydrolase Family 3.

Phylogenetically Informed Principal Component Analysis of the Expanded Gene Families Associated With Virulence Factors

To identify similarities between species in the *Botryosphaeriaceae* family, a phylogenetically informed-principal component analysis (phylo-PCA) was applied to the significantly expanded families of virulence functions. These gene families were grouped into the functional categories based on the specialized databases, and the PCA was carried out using the Phyl.PCA (Revell, 2012). Phyl.PCA considers correlations among species due to

phylogenetic relatedness, while correcting the matrices for non-independence among observations (Revell and Collar, 2009). Two separate analyses were conducted using the clock-calibrated tree presented previously and the tables of the number of genes classified as secreted CAZymes (**Figure 5A**) and Secondary Metabolism (**Figure 5B**).
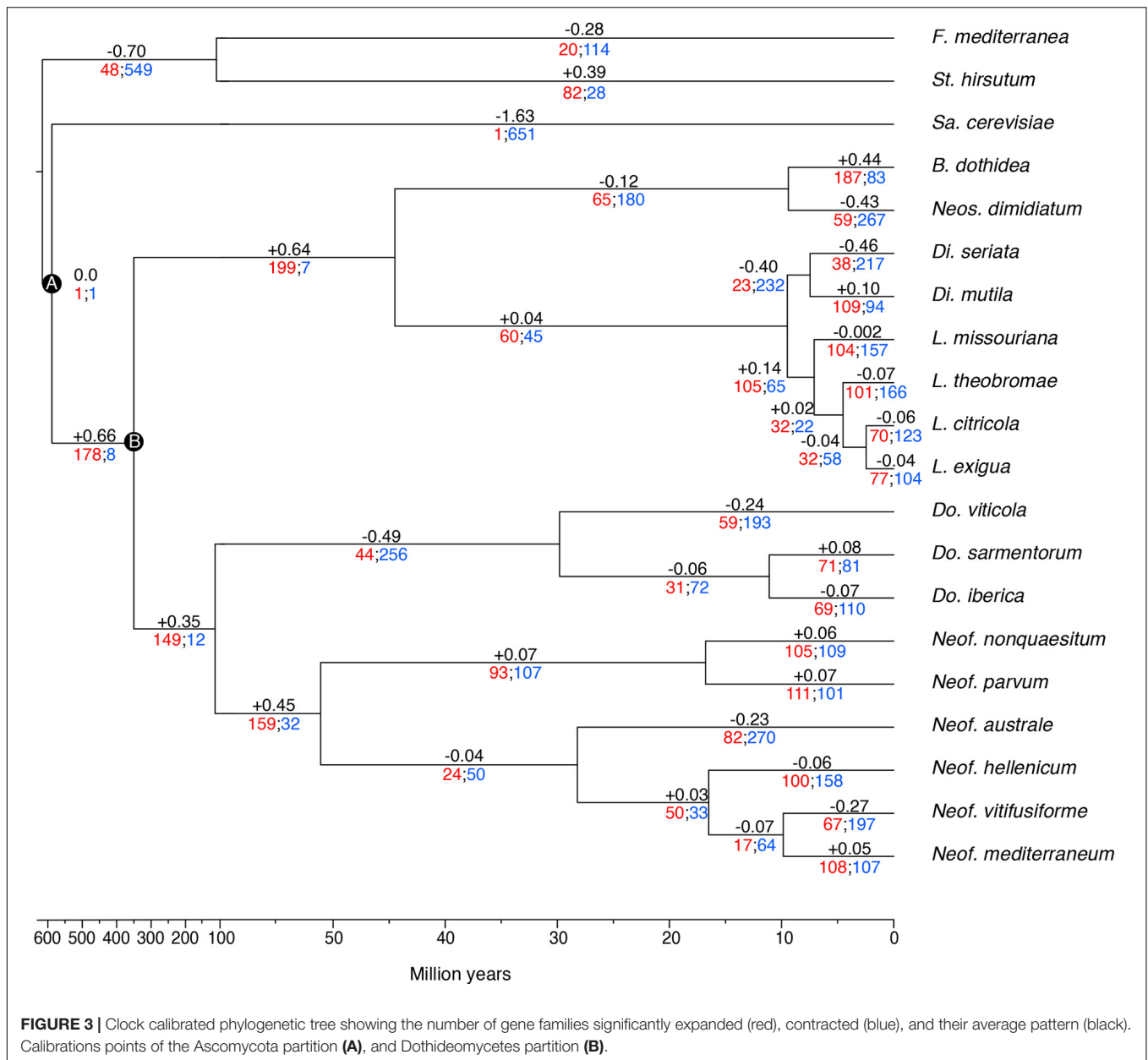
Due to the close phylogenetic relationship of the *Botryosphaeriaceae* family, the set of secreted CAZymes is remarkably similar. However, there is a clear separation of the species that are considered to be more virulent (**Figure 5A**), those belonging to the genera *Neofusicoccum*, *Lasiodiplodia*, and *Botryosphaeria* (Úrbez-Torres et al., 2008; Úrbez-Torres and Gubler, 2009). At the same time, we observe a close cluster of *Neofusicoccum* species which are separated from the other groups mostly by the abundance of AA1, AA3, and GH5. In addition, the genus *Lasiodiplodia* is tightly clustered together with *B. dothidea*. This is driven by the abundance of AA9, GH28, and GH3, with the last family being more abundant in *Lasiodiplodia* species. The close clustering of *Neofusicoccum*, *Botryosphaeria*, and *Lasiodiplodia* is driven mostly by their similar profile of GH16 and AA3. *Neoscytalidium dimidiatum* is well separated from the rest of the species by the higher presence of GH76 and PL3 proteins.

The PCA on secondary metabolite genes shows a similar separation of the most virulent genera from the others (**Figure 5**). *Lasiodiplodia* species are grouped together by similarly high profiles of T1PKS, Beta-lactone and T1PKS/NRPS clusters. *Neofusicoccum* species are grouped due to high numbers of terpene synthases and NRPS-like clusters. *Botryosphaeria dothidea* is separated because of its high abundance of NRPS, T1PKS, Terpenes, Beta-lactone, and NRPS-like clusters.

DISCUSSION

In this study, we describe the genome sequences of seventeen well-known canker-causing fungal species in the *Botryosphaeriaceae*. The genomes assembled coupled with *in-planta* experiments allowed us to start analyzing the pathogenicity levels and the virulence factor profiles within this important fungal family. The level of completeness of the assembled genomes is consistent across all the drafts based on the expected and assembled genome sizes. This behavior is also confirmed by the high representation of conserved genes (Parra et al., 2007; Simão et al., 2015). The completeness of the genomes, as well as the protein-coding genes and the repetitive DNA content, are similar to those of other wood-colonizing fungi of grape, such as *Diaporthe ampelina DA912* (Morales-Cruz et al., 2015), *Di. seriata DS831* (Morales-Cruz et al., 2015), and *L. theobromae LA-SOL3* (Félix et al., 2019). Apart from the estimated completeness of the genomes, it is necessary to understand some of the limitations of the short reads technology, like copy number errors, chimeric contigs, and under-representation of repetitive regions (Alkan et al., 2011; Treangen and Salzberg, 2012).
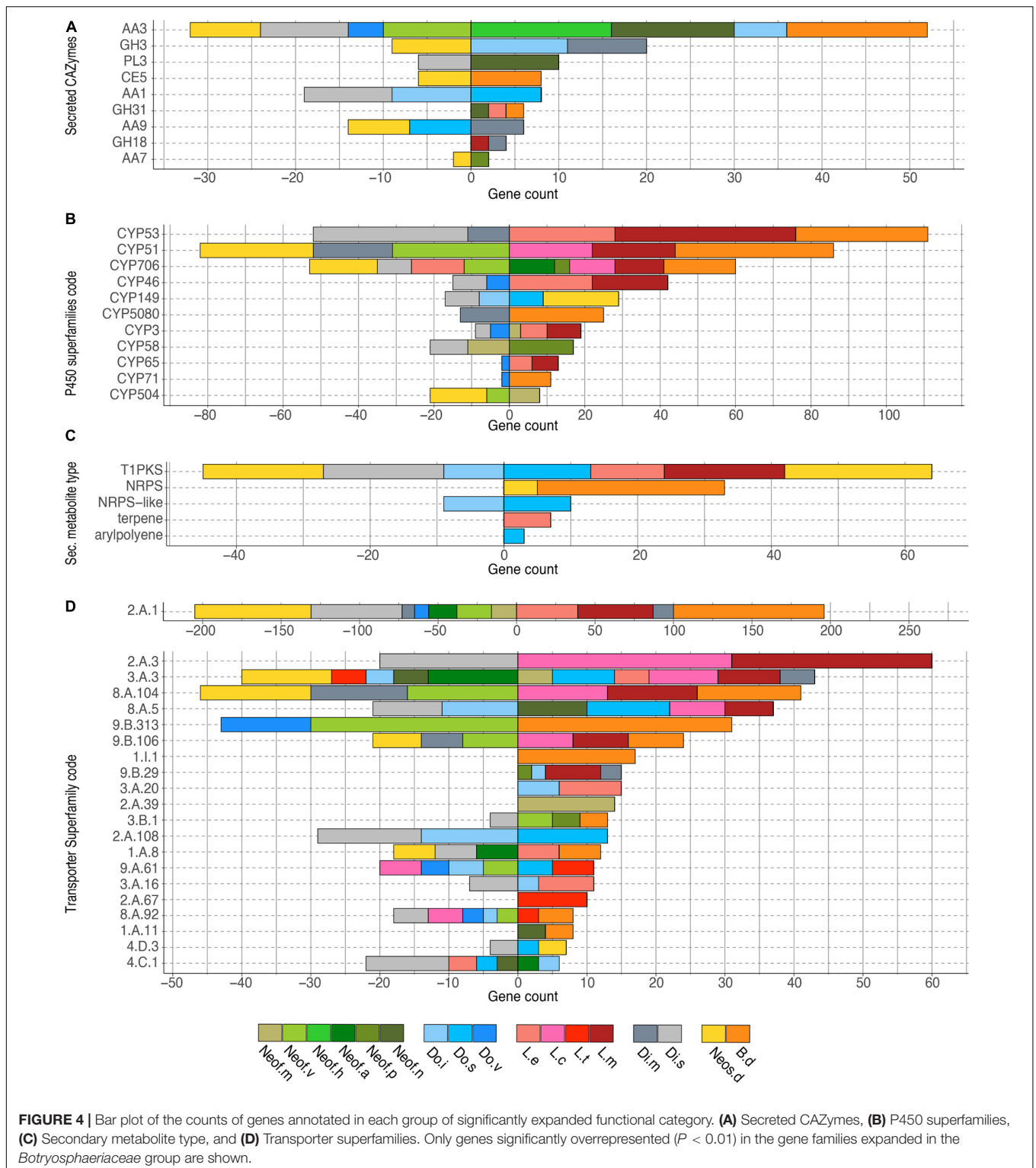
The functional annotation of the seventeen *Botryosphaeriaceae* species presents a broad and variable

FIGURE 3 | Clock calibrated phylogenetic tree showing the number of gene families significantly expanded (red), contracted (blue), and their average pattern (black). Calibrations points of the Ascomycota partition **(A)**, and Dothideomycetes partition **(B)**.

profile of virulence factors that are used in different ways by fungi to colonize and survive in their hosts (Schulze-Lefert and Panstruga, 2011; Peyraud et al., 2019). The results show a great variation in the number of genes identified with a functional category, and these differences were usually associated with the genus of each species like those observed by Baroncelli et al. (2016) in *Colletotrichum* and Morales-Cruz et al. (2015) in other grapevine trunk pathogens. Researchers are inclined to think that the gene content is associated with the lifestyle and the variety of hosts (Zhao et al., 2013; Lo Presti et al., 2015; Baroncelli et al., 2016). The expansion or contraction of a gene family usually occurs on functions that are under positive or negative selection. For instance, the genes related to host colonization and defense are under high pressure, therefore, it is common to encounter
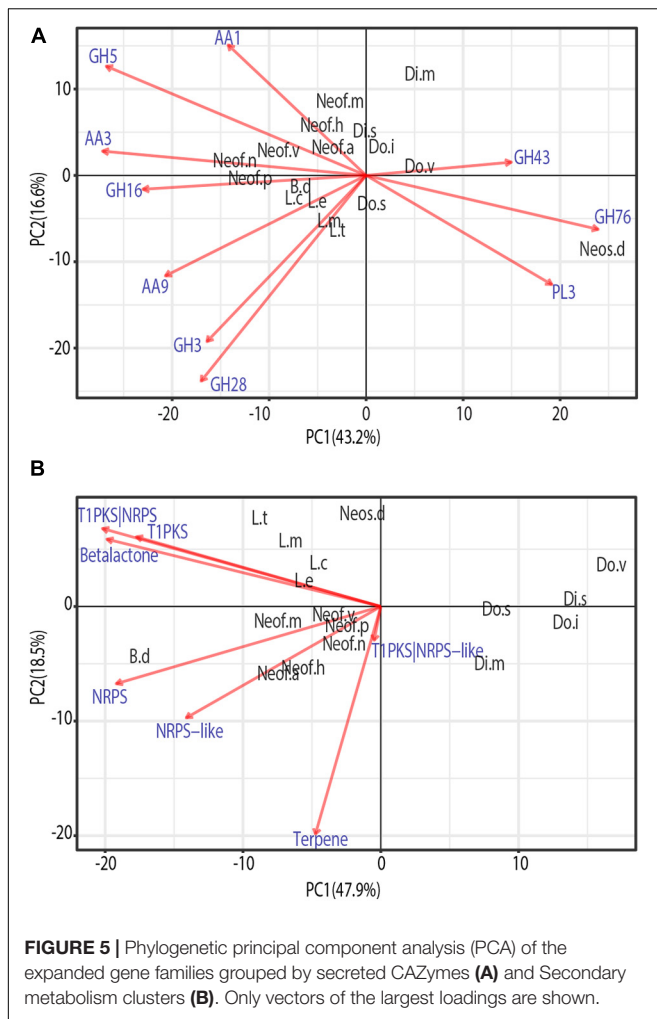
duplications or even losses. On the other hand, genes related to growth are more conserved and usually selected against these changes (Wapinski et al., 2007). Gene duplication events are crucial as they are considered to be one of the main processes that generate functional innovation (Zhang, 2003; Ohno, 2013). This process plays one of the most important roles in fungal adaptation and divergence (Gladieux et al., 2014).

Host colonization during infection is mostly driven by gene expression of some groups of well-known proteins, namely, the secreted CAZymes, cytochrome P450 monooxygenases, peroxidases, and secondary metabolite-producing proteins (Massonnet et al., 2018). The *Botryosphaeriaceae* family has a variable profile of these sets of genes, with the most virulent and aggressive species having, on average, greater

FIGURE 4 | Bar plot of the counts of genes annotated in each group of significantly expanded functional category. **(A)** Secreted CAZymes, **(B)** P450 superfamilies, **(C)** Secondary metabolite type, and **(D)** Transporter superfamilies. Only genes significantly overrepresented (*P* < 0.01) in the gene families expanded in the *Botryosphaeriaceae* group are shown.

numbers of annotated genes in these categories (**Table 3**). In grape and pistachio, species in the genera *Neofusicoccum* and *Lasiodiplodia*, are typically more virulent than species in the genera *Diplodia* and *Dothiorella* (Úrbez-Torres et al., 2008; Úrbez-Torres and Gubler, 2009; Nouri et al., 2019). GH

functions of β-glucosidases, β-xylosidases, glucanases, L-arabinofuranosidase, and galactanase were present in all the pathogens in this study and significantly more in *Neofusicoccum* and *Lasiodiplodia*. In the same way as the GH, AA functions like cellobiose dehydrogenases, alcohol oxidases, pyranose

FIGURE 5 | Phylogenetic principal component analysis (PCA) of the expanded gene families grouped by secreted CAZymes **(A)** and Secondary metabolism clusters **(B)**. Only vectors of the largest loadings are shown.

oxidase were more abundant among *Neofusicoccum* species and *B. dothidea*. GH and AA play a critical role in the degradation of the host cell wall compounds (Kubicek et al., 2014), which is involved with the degree of pathogenicity within these genera, albeit on grape, the host we examined. Marsberg et al. (2017); Massonnet et al. (2018), and Félix et al. (2019), found similar numbers of CAZymes in *Neof. parvum*, *L. theobromae*, and *B. dothidea*, respectively. P450s are instrumental to the development of all organisms. These enzymes are involved in many aspects of primary and secondary metabolisms and are responsible for xenobiotic detoxification and degradation (Črešnar and Petrič, 2011; Moktali et al., 2012). Virulence may in part reflect the ability of some species to better tolerate and, further, to metabolize phenolic compounds produced by the host. Both *Neof. parvum* and *Di. seriata* can eliminate the stilbene piceid and its derivative resveratrol *in vitro* (Stempien et al., 2017), but the former is better able to tolerate resveratrol derivatives ampelopsin A, hopeaphenol, isohopeaphenol, miyabenol C, and ε-viniferin, which are produced at higher levels *in planta* in response to *Neof. parvum* versus *Di. seriata* infection (Lambert et al., 2012). Therefore, it is not unexpected to see a

variable profile amongst genera in the *Botryosphaeriaceae* family and even within a single genus. As presented in **Figure 2**, some superfamilies are abundant in *Neofusicoccum*, *Lasiodiplodia* and *Botryosphaeria* genera, but other superfamilies are especially more numerous in the Basidiomycetes species included in this study. On the other hand, for most of the superfamilies presented, *Sa. cerevisiae* shows a considerable lack of such annotated genes, but CYP53 and CYP578 the counts are comparable with the rest of the species. This variation is sourced by the constant evolution and adaptation of the microorganism and hosts to their specific environment (Yan et al., 2018).

As plants evolve new defense mechanisms and compounds against pathogens, the fungi diversify their methods to degrade these compounds or generate new metabolites to attack their hosts (Deng et al., 2007; Yan et al., 2018). The *Botryosphaeriaceae* species in this study and the two Basidiomycetes present a set of fungal peroxidases that range from 41 to 62. As for the previous putative virulence factors, *Neofusicoccum*, *Lasiodiplodia*, and *Botryosphaeria* genera have the most annotated peroxidases, however, in this case, *Diplodia* also showed a comparable amount. Manganese peroxidase was only found in the two basidiomycetes. This enzyme has a critical role in the degradation of lignocellulose compounds by basidiomycetes (Elisashvili and Kachlishvili, 2009; Liers et al., 2011), therefore it is very common in white-rot fungi such as *F. mediterranea* and *St. hirsutum* (Morgenstern et al., 2010; Lee et al., 2015). Ascomycetes that rot wood are characterized as soft-rot fungi, which do not degrade lignin by producing manganese peroxidase, but instead "alter" lignin (to gain access to cellulose and hemicellulose) by producing lignin peroxidases, peroxidases, polyphenol oxidases, and laccases (Goodell et al., 2008). Haloperoxidases also have roles in lignin degradation and toxic compound resistance (Mayer et al., 2001; Hofrichter et al., 2010; Zámocký and Obinger, 2010). The former enzyme was found in higher numbers in the genus *Neofusicoccum* compared to other genera within the family. The hybrid ascorbate-cytochrome C peroxidase was overrepresented in the genera *Neofusicoccum*, *Lasiodiplodia,* and *Botryosphaeria* and is associated directly with the detoxification of ROS (Zámocký et al., 2014; Wang et al., 2016; Segal and Wilson, 2018).

The wide array of transporters annotated in this study suggests a high adaptation to toxic compounds, either produced by other microorganisms, the host, or potentially chemical synthesized fungicides (Stergiopoulos et al., 2002). The number of proteins in the Major Facilitator Superfamily (MFS) and Superfamily in *Neofusicoccum*, *Lasiodiplodia*, and *Botryosphaeria* were more numerous than the other *Botryosphaeriaceae* species. Protein members of the MFS family may have different functions in the influx/efflux of molecules between cells and the exterior environment, and several cases of fungicide resistances have been associated with the overexpression of certain MFS channels (Stergiopoulos et al., 2002; Gulshan and Moye-Rowley, 2007; Dos Santos et al., 2014; Chen et al., 2017). The former genera have been reported to have lower sensitivities to almost full resistance to different synthetic fungicides (Wang et al., 2010; Tennakoon et al., 2019; Li et al., 2020). Similar behavior was observed in *Do. sarmentorum*, were the ATP-binding Cassette (ABC) is

highly represented. The ABC superfamily plays different roles in fungicide resistance, mycelial growth, and overall pathogenicity (Stergiopoulos et al., 2002; Qi et al., 2018). In addition, the array of secondary metabolite gene clusters is more expanded in the *Botryosphaeriaceae* family than in the Basidiomycetes except for terpene synthase gene clusters. T1PKS, NRPS, and hybrids of T1PKS-NRPS produce toxic polyketides and toxic polypeptides, which kill host cells and leads to disease development (Luini et al., 2010; Andolfi et al., 2011; Morales-Cruz et al., 2015; Pusztahelyi et al., 2015; Blin et al., 2019).

To evaluate the potential differences in virulence within the *Botryosphaeriaceae* family in more detail, we executed a Computational Analysis of gene Family Evolution (De Bie et al., 2006). By identifying species and gene families with higher rates of gain and loss can help us to better understand the differences in pathogenicity as it relates to the numbers of copies of virulence genes (Hahn et al., 2005; Morales-Cruz et al., 2015). Six hundred and sixty-six gene families of the proteins analyzed in this study have a significantly higher than expected rate of gain/loss. The annotation of putative virulence factors in *Neofusicoccum*, *Lasiodiplodia*, and *Botryosphaeria* shows an average expansion of these gene families, even if some of the species shows a contraction, the overall clade rate is positive. Among those expanded or contracted families there is a set of functions that are overrepresented. The secreted CAZymes seem to be expanding in *Neof. hellenicum*, *Neof. nonquaesitum*, *B. dothidea*, *Di. mutila*, *Do. iberica*, and *Do. sarmentorum*, whereas the *Dothiorella* species show contractions in some families. However, almost no significant gain/loss of secreted CAZymes appears to be occurring in the genomes of *Lasiodiplodia* species. The opposite scenario is observed for the P450s, where *Lasiodiplodia* appears to be actively evolving, showing major expansions in three of the four species in this study. Also, *B. dothidea* and three *Neofusicoccum* species (*Neof. parvum*, *Neofusicoccum australe*, and *Neofusicoccum mediterraneum*) show an expansion of these families. On the other side, *Neos. dimidiatum*, *B. dothidea*, *Do. sarmentorum*, *L. exigua*, and *L. missouriana* are actively expanding their secondary metabolite gene clusters. Finally, the wide variety of transporters present in fungi, is the result of the positive selection pressure over them. The need of the fungi to adapt to new environments and hosts had selected for multiple mutations that diversifies the transporters functions (Gladieux et al., 2014). The MFS (2.A.1) displays the largest effect of expansion and contraction among all the species. *Botryosphaeria dothidea*, *L. missouriana*, *L. exigua*, and *Di. mutila* appear to be actively expanding the MFS transporters. However, *Neos. dimidiatum*, *Di. seriata*, *Neofusicoccum vitifusiforme*, *Neof. australe*, and *Neof. mediterraneum* are contracting MFS transporters.

Phylo PCAs results support the idea that within the *Botryosphaeriaceae* family, *Neofusicoccum*, *Lasiodiplodia*, and *Botryosphaeria* genera are the most virulent (Úrbez-Torres et al., 2008; Úrbez-Torres and Gubler, 2009). There was a very clear separation of these species from the *Diplodia*, *Dothiorella*, and *Neoscytalidium*. The secreted CAZymes that cause the clustering of the *Neofusicoccum* species

are usually associated with laccases, cellobiose dehydrogenases, and cellulase activities. These enzymes usually target components of the plant cell wall such as lignin, cellulose, cellobiose (Cameron and Aust, 2001; Zamocky et al., 2006; Fillat et al., 2016; Di Francesco et al., 2020). Among the functions driving the clustering of *Lasiodiplodia* and *Botryosphaeria*, the lytic polysaccharide monooxygenases (LPMOs, AA9) are one of the most important. They have a role in the oxidative degradation of various biopolymers such as cellulose and chitin. LPMOs can increase the activity of cellulases highly, and now, they are used in a mixture for the preparation of biofuels (Frommhagen et al., 2018; Labourel et al., 2020). Therefore, this set of enzymes may facilitate the colonization and infection of their hosts. The separation of *Neos. dimidiatum* from the rest of species is caused by GH78 which includes mannanases, α-glucosidase enzymes and the PL3 family of pectate lyases. As *Neos. dimidiatum* is also known for infecting the fruits and soft tissues of their hosts (Marques et al., 2013; Nouri et al., 2018), this set of enzymes seems to be well developed.

Lasiodiplodia species have a wide array of secondary metabolites. Their profile varies according to the species, isolate, and even the host (Salvatore et al., 2020). These metabolites are often synthesized by clusters of T1PKS, T1PKS/NRPS, and some beta-lactones (Félix et al., 2019; Salvatore et al., 2020), which are some of the major drivers for their clustering in the phylo-PCA (**Figure 5**). *Neofusicoccum*, besides the previous gene clusters, also have lamanypenes and NRPS, which drives their clustering in the PCA. Similar results have been presented by Morales-Cruz et al. (2015) and Massonnet et al. (2018). Very little literature is available about the effect of the secondary metabolites of *B. dothidea* on their plant host; however, this fungus is known for its remarkable ability to produce secondary metabolites *in vitro* (Wang et al., 2018), which recently have been studied for their potential use as commercial antioxidants (Xiao et al., 2014; Druzian et al., 2020; Valente et al., 2020).

Few of the fungi in this study have been characterized in terms of their interactions with wood and individual wood components, their activation of cell-wall degrading enzymes, or their ability to tolerate phenolic compounds. Therefore, it is difficult to connect the pattern of gene family evolution to such aspects of fungal biology, especially in a comparative way among so many species, none of which have all been compared at once on a single host. The pathogenicity test on young, rooted grapevine plants raises some interesting observations. First, *L. theobromae*, *Neof. parvum*, and *Neof. australe* are among the species that induced the most prominent lesions in the plants. These results are consistent with those of Úrbez-Torres and Gubler (2009), who found these species to be highly virulent on grape. This same study found *Di. mutila*, *Di. seriata*, *Do. iberica*, and *Do. viticola* to be weakly virulent, which is congruent with the results presented by this study. Although most *Neofusicoccum* species present high numbers of putative virulence factors, the targets for these may be variable within the genus. In this pathogenicity experiment, the isolates of *Neof. vitifusiforme*, *Neof. nonquaesitum*, and *Neof. hellenicum* were isolated from active cankers in walnut and pistachio trees, and even if some of these

species can develop disease in grapevine, their virulence on *Vitis vinifera* may not be the same. Finally, the reasons why lesions produced by *B. dothidea* were not significantly different from the control are difficult to assess with certainty, but studies had reported *B. dothidea* to be weakly or moderately pathogenic on grapevine (Úrbez-Torres and Gubler, 2009; Pitt et al., 2013). Some researchers remark that in potted plants after 5–6 weeks of inoculation with *B. dothidea*, the plants were not different compared to the control, whereas the other species in that study showed poor bud development and stunted green shoot growth (Úrbez-Torres and Gubler, 2009). Also, in other studies, this species is presented as endophytic and latent pathogen and they suggest that the environmental conditions can have a significant effect on the development of the disease (Piškur and Jurc, 2011; Marsberg et al., 2017).

DATA AVAILABILITY STATEMENT

Sequencing data are available at NCBI (BioProject PRJNA673527). Sequencing data of Diplodia seriata and *Neof. parvum* can be retrieved from NCBI under BioProject PRJNA261773 and PRJNA321421, respectively. All genome assemblies and gene models are publicly available at Zenodo (doi: 10.5281/zenodo.4417445).

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AUTHOR CONTRIBUTIONS

RT, KB, DL, and DC conceived the study. DL and RT carried out the pathogenicity experiments. PR and RH-M contributed to fungal strains. JG, AM-C, AM, DL, and DC carried out the computational analysis. JG, DL, KB, and DC wrote the manuscript. All authors read and approved the final manuscript.

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