Pathogenic Adaptations Revealed by Comparative Genome Analyses of Two \textit{Colletotrichum} spp., the Causal Agent of Anthracnose in Rubber Tree

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\textit{Colletotrichum} \textit{siamense} and \textit{Colletotrichum australisinense} cause \textit{Colletotrichum} leaf disease that differ in their symptoms in rubber tree (\textit{Hevea brasiliensis}), and pathogenicity of these two fungal species is also not identical on different cultivars of rubber tree. This divergence is often attributed to pathogen virulence factors, namely carbohydrate-active enzymes (CAZymes), secondary metabolites (SM), and small-secreted protein (SSP) effectors. The draft genome assembly and functional annotation of potential pathogenicity genes of both species obtained here provide an important and timely genomic resource for better understanding the biology and lifestyle of \textit{Colletotrichum} spp. This should pave the way for designing more efficient disease control strategies in plantations of rubber tree. In this study, the genes associated with these categories were manually annotated in the genomes of \textit{C. australisinense} GX1655 and \textit{C. siamense} HBCG01. Comparative genomic analyses were performed to address the evolutionary relationships among these gene families in the two species. First, the size of genome assembly, number of predicted genes, and some of the functional categories differed significantly between the two congeners. Second, from the comparative genomic analyses, we identified some specific genes, certain higher abundance of gene families associated with CAZymes, CYP450, and SM in the genome of \textit{C. siamense}, and Nep1-like proteins (NLP) in the genome of \textit{C. australisinense}.

**Keywords:** cell wall degrading enzymes, \textit{Colletotrichum}, genome, \textit{Hevea brasiliensis}, necrosis- and ethylene-inducing peptides, secondary metabolites associated genes, symptoms

**INTRODUCTION**

\textit{Hevea brasiliensis} (Willd. ex A. Juss.) Müll. Arg., commonly known as the Para rubber tree, is native to the tropical rain forests of Central and South America, and the primary source of natural rubber. \textit{Colletotrichum} leaf disease (CLD) caused by \textit{Colletotrichum} species has become a serious biotic factor limiting rubber tree cultivation in plantations. The disease has been recorded and confirmed in most rubber growing countries such as China, Malaysia, Sri Lanka (Waste, 1967; Liu et al., 1987; Jayasinghe et al., 1997). The fungus causing this disease had been originally
identified as *Colletotrichum heveae* Petch, but then presumed to be *Colletotrichum gloeosporioides* (Penzig & Sacc.). Yet *C. gloeosporioides* as well as *Colletotrichum acutatum* are often associated with anthracnose disease occurrences in rubber trees (Brown and Soepena, 1994; Jayasinghe et al., 1997). Furthermore, *C. dematium*, *C. crassipes*, *C. karstii*, and *C. boninense* specimens have also been isolated from infected rubber leaves, but were deemed of less economic importance than the aforementioned two *Colletotrichum* species because they happen sporadically (Wastie and Janardhanan, 1970; Cai et al., 2016; Jiang et al., 2016).

Taxonomically, much of the recent research and treatments have primarily focused on studying the different *Colletotrichum* species complexes. According to the latest work and updates, the genus consists of fourteen species complexes (Jayawardena et al., 2016; Marin-Felix et al., 2017; Damm et al., 2018). Within just the last decade, many cryptic and new species have been reported from rubber leaves with CLD symptoms; for example, five *Colletotrichum* species belonging to the *C. acutatum* species complex were reported from Sri Lanka (Damm et al., 2012; Hunupolagama et al., 2017). In China, however, *Colletotrichum siamense*, *Colletotrichum fructicola*, and *Colletotrichum ledongense* of the *C. gloeosporioides* species complex were newly associated with CLD of rubber tree (Liu et al., 2018). Two new species (*Colletotrichum australisinense* and *C. bannaense*) of the *C. acutatum* species complex were also discovered, although *C. siamense* (the *C. gloeosporioides* species complex) and *C. australisinense* (the *C. acutatum* species complex) are currently recognized as the predominant species in that country. However, the infectious symptoms on rubber tree arising from *C. siamense* and *C. australisinense* are significantly different (Liu et al., 2018). The thorough characterization of species causing anthracnose and knowledge of their respective molecular mechanisms of infection is critical for devising strategies to effectively control the spread of the disease in rubber tree plantations.

The *Colletotrichum* species have evolved a diverse range of lifestyles to colonize and obtain nutrients from their host species of monocot and/or dicot plants, ranging functionally from biotrophs, nectrotrophs, hemibiotrophs to endophytes (De Silva et al., 2017). In recent years, progress has advanced toward a better understanding of their infection process at the molecular level and its correlation with the life history of each fungus (Villa-Rivera et al., 2017). The entire genomic sequence of some *Colletotrichum* species belonging to different species complexes, are now publicly available, providing an impetus to research of *Colletotrichum*–host tree interactions. Genus-wide comparative genome analyses of *Colletotrichum* species focusing on important gene classes, such as secretory proteases and carbohydrate-active enzymes (CAZymes), candidate effectors, and secondary metabolite (SM) biosynthesis genes, have revealed the core genes conserved in the genus *Colletotrichum*. Additionally, information on the amplification and contraction of certain gene families that could be attributed to their specific life histories and host range have been provided (O’Connell et al., 2012; Gan et al., 2013; Baroncelli et al., 2016; Gan et al., 2016; Buiate et al., 2017; Rao and Nandineni, 2017; Liang et al., 2018).

In this study, we report on the genome sequences of *C. siamense* in the *C. gloeosporioides* species complex (CGsc) and of *C. australisinense* in the *C. acutatum* species complex (CAsc), which, respectively, cause different symptoms in diseased rubber trees. Comparative genomic analyses were carried out between the two species. We laid special emphasis on analyzing the gene families encoding CAZymes, SM biosynthesis, and necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs), given their already known roles in plant–pathogen interactions, and characterized the content of these genes and their variation. Comparative genomics revealed reductions of gene families encoding CAZymes and SM biosynthesis within *C. australisinense* belonging to the CAsc. We also found evidence for an expansion of NLPS. Based on these patterns, we hypothesize that for *Colletotrichum* species with a broad host range, particularly those within CAsc, Lineage Specific Effector protein Candidates (LSECs) have diminished roles in interactions with plants, perhaps instead relying mainly on CAZymes, proteases, and NLPS for successful host colonization.

**MATERIALS AND METHODS**

**Fungal Cultures and Infection Conditions**

*Colletotrichum australisinense* GX1655 and *C. siamense* HBCG01 isolates were obtained from distinctive leaf lesions associated with the different disease symptoms (the brown conical or raised spots vs. the anthracnose spots) they, respectively, cause in rubber trees. Their species identity was confirmed by a polyphasic approach (Liu et al., 2018). The GX1655 isolate was cultured on potato dextrose agar (PDA) and preserved as conidial suspension amended with 15% glycerol at –80°C. It was afterward deposited in the China General Microbiological Culture Collection center (CGMCC) under the accession number CGMCC3.18886.

**Genome Sequencing and Assembly**

Genomic DNA of freshly collected mycelia was extracted with the QIAGEN® Genomic DNA kit. The BluePippin system was used for the library construction. Genome sequencing was performed on a GridION X5 platform at the Nextomics Genomic Sequencing Center (Wuhan, China). Raw reads were trimmed with GraphMap and Minimap software tools1 to remove low quality reads and any reads having adaptor contamination (mean qscore >7). Clean reads were then de novo assembled using Canu v1.3 (Koren et al., 2017), for which the correction of each assembly was then assessed using Bwa 0.7.12-r1039 (Walker et al., 2014) and “Benchmarking Universal Single-Copy Orthologs” (BUSCO v3.0) (Simão et al., 2015; Seppey et al., 2019). The generated genomes were deposited at NCBI as BioProjects under accession PRJNA597657 and PRJNA597926.

**Gene Prediction**

The transposable elements present in assembled genome were identified using a combination of de novo and homology-based approaches. Specifically, RepeatMasker v4.0.5

1https://github.com/lbcb-sci/graphmap2
search against the current assembly of genomes, by using the six identified and classed based on a search for the conserved and ethylene-inducing peptide 1-like proteins (NLPs) genes were domains) of PKS proteins and PKS-NRPS hybrids were separately domains (KS) (also referred to as N- and C-terminal docking and polyketide synthases (PKS)-NRPS hybrids, the keto-synthase domain) of non-ribosomal peptide synthetases (NRPS) proteins.

5.0.0rc1 (Medema et al., 2011). The adenylation domain (A) of non-ribosomal peptide synthetases (NRPS) proteins and polyketide synthases (PKS)-NRPS hybrids, the keto-synthase domains (KS) (also referred to as N- and C-terminal docking domains) of PKS proteins and PKS-NRPS hybrids were separately used for the phylogenetic analyses (Buiate et al., 2017). Necrosis and ethylene-inducing peptide 1-like proteins (NLPs) genes were identified and classed based on a search for the conserved motif GHRHDWE in the predicted proteins and a TBLASTN search against the current assembly of genomes, by using the six previously published NLP sequences belonging to three different types (i.e., type-1, type-2, and type-3) (Kleemann et al., 2012; Oome and Van den Ackerveken, 2014). Only full-length protein sequences were used in that analysis. Those proteins transported out of the cell and into the extracellular space were identified using a battery of tools: namely, Signal-P (v4.1) (Petersen et al., 2011), TargetP5 TMHMM (v2.0) (Krogh et al., 2001), GPISOM (Fankhauser and MaÈser, 2005), and WoLF PSORT (v0.2) (Horton et al., 2007), which were run sequentially.

**Construction of Phylogenetic Trees**

For phylogenetic analysis, the genomes and/or proteomes of 10 *Colletotrichum* species (Gan et al., 2013; Baroncelli et al., 2014a,b, 2016; Hacquard et al., 2016; Zampounis et al., 2016) were downloaded from NCBI. Predicted proteins encoded by a total of 12 *Colletotrichum* species genomes were filtered, and clustered into orthologous groups by Orthofinder version 2.3.8 (Emms and Kelly, 2019). Single copy orthogroups were then extracted for phylogenomic tree construction. Independent ortholog groups were aligned with MAFFT version 7β and then concatenated (Baroncelli et al., 2016). A substitution model and its parameter values were selected using ProtTest version 3.4 (Abascal et al., 2005; Baroncelli et al., 2016). A maximum-likelihood (ML) phylogenetic tree with 1000 bootstraps was constructed with RAxML version 8.1.1 (Stamatakis, 2006; Liang et al., 2018). *Verticillium alfae* (Klosterman et al., 2011) was used as outgroup. In the phylogenetic analysis of SM clusters and NLP between *C. australisine* and *C. siamense*, amino acid sequences were aligned using MUSCLE of MEGA 6, for which the best-fitting amino-acid substitution model and parameter settings were chosen based on the “model test” of MEGA 6. To construct each phylogenetic tree, the maximum likelihood algorithm implemented in MEGA 6 was used (Kumar et al., 2016), with n = 1000 bootstrap replications.

**RESULTS**

**Infection Conditions**

In rubber tree plantations, *C. australisine* mainly infects tender and immature leaves (phenophase: copper brown and light green leaves), as revealed initially by many brown conical or raised spots. As the disease progresses, the infected leaves become wrinkled and twisted, eventually falling off the petiole (Figures 1A–C). On a mature leaf, the symptoms appear only as conical or raised spots, latterly perforated (Figure 1D). By contrast, the anthracnose spots caused by *C. siamense* infection appear as concentric rings that occur generally along the leaf margins, and occasionally in the middle of a mature leaf (Saha et al., 2002). These lesions are large, and may coalesce to form discernable larger-sized spots. The central portion of this spot is light brown and papyraceous. Another noteworthy leaf spot symptom associated with *C. siamense* infection is the almost circular papyraceous lesions having
a dark brown center surrounded by a yellow halo (Saha et al., 2002; Figures 1E,F).

**Genome Sequencing and Assembly and Gene Prediction**

The genome assembly size of *C. australisinense* GX1655 is distinctly different from *C. siamense*. BUSCO v3.0 was used to provide an estimate of assembly completeness. According to this analysis, the assembly of the *C. australisinense* genome covered 95.5% of the total gene space while that of *C. siamense* covered 99.3% of it (Table 1).

For the *C. australisinense* GX1655 genome, a total of 15,189 protein-coding genes were predicted, of which 26.9% (4095), 46.8% (8870), 0.84% (127), and 98.2% (14,915) of the putative proteins could be, respectively, annotated based on COG, GO, Swiss-Prot, KEGGs, and NR, respectively. In the BUSCO analysis, 95.5% of the fungal core genes had “complete” hits and 95.2% had “complete and single-copy” hits, and ca. 5.65% of the assembly consisted of repeat sequences. From the *C. siamense* HBCG01 genome a total of 15,493 protein-coding genes were predicted—slightly more than obtained for the *C. australisinense* GX1655—of which 29.1% (4,519), 50.4% (7,812), 62.4% (9,672), 2.76% (427), and 99.0% (15,438) could be annotated based on COG, GO, Swiss-Prot, KEGGs and NR, respectively. According to BUSCO, 98.3 and 99.6% of this species fungal core genes had hits of “complete” and “complete and single-copy,” respectively, with repeat sequences amounting to ca. 1.85% of the assembly. For both species, the thoroughness of their genes’ annotation was demonstrated by the high percent values (Table 1). In the categorization of all genes based on GO, *C. siamense* HBCG01 in general has more genes in cellular component, molecular function, biological process. *C. australisinense* GX1655 has only higher abundance of genes involved in signal transducer activity and molecular transducer activity (molecular function), membrane-enclosed lumen (cellular component), biological regulation

| TABLE 1 | Assembly and gene prediction information of Colletotrichum spp. genomes. |
|----------|------------------|
|           | *C. siamense*    | *C. australisinense* |
| Assembly size (Mb) | 58.4            | 55.3            |
| Max contig (Mb) | 7.62            | 10.04           |
| N50 (Mb)   | 4.58            | 5.69            |
| BUSCO complete | 99.3%          | 95.5%          |
| BUSCO partial | 99.0%          | 95.5%          |
| Repeat elements | 1.85%          | 5.65%          |
| Number of predicted genes | 15493      | 15189       |
| Average gene length (bp) | 1612.97    | 1586.30     |
| Average exons number per gene | 2.90      | 3.17       |
| Average introns number per gene | 1.90       | 2.17       |
and cellular component organization or biogenesis (biological process) (Supplementary Figure 1).

**Phylogenetic Analyses**

In orthogroups analysis, we found 13,018 orthogroups in the *C. australisinense*, and 12,930 orthogroups in the *C. siamense*. A total of 4,782 single-copy orthologs were found in all species used in this study. 42 orthogroups had no homologs in any other species used in this study, and hence represented the species-specific orthologs that are unique to *C. australisinense*. However, 5 orthogroups are species-specific to *C. siamense*.

To understand the evolutionary relationships among species within the genus *Colletotrichum*, a phylogenomic tree was constructed based on the concatenated alignment of 4,782 single-copy orthologs obtained from the genomes of 12 *Colletotrichum* species. The inferred phylogeny showed that *C. australisinense* was most closely related to *C. nymphaeae*. *C. siamense* clustered with *C. fructicola*. The CAsc and CGsc appear to be evolutionary very distant (Figure 2).

**Secreted CAZymes**

The plant cell wall primarily consists of three polysaccharide types (cellulose, hemicelluloses and pectins). Carbohydrate-metabolizing enzymes are vital for the degradation and utilization of these polysaccharides during host colonization by pathogenic fungi. In all, 451 genes of *C. australisinense* GX1655 associated with 88 CAZyme families were predicted to have secreted proteins; however, *C. siamense* HBCG01 harbored a larger repertoire of CAZymes in which 507 genes associated with 92 CAZyme families were predicted to have secreted proteins. The key classes of CAZymes, such as auxiliary activities (AA), carbohydrate esterases (CE), glycoside hydrolases (GH), and polysaccharide lyases (PL), were compared between species (Figure 3A). Evidently, *C. siamense* HBCG01 had higher copy numbers of these families, especially of CE and GH. Among the most amplified gene families were those involved in pectin degradation, such as PL1, GH28, and GH78, as well as those participating in the degradation of hemicelluloses and cellulose, such as CE1, CE3, CE6, GH16, GH35, GH71, and GH92 (Figure 3B). Hence, *C. siamense* evolution underwent a strong amplification of plant cell wall-degrading enzymes (PCWDEs).

**Cytochrome P450s**

Cytochromes P450 (CYPs) are proteins of the superfamily containing heme as its cofactor, otherwise known as hemoproteins (Gonzalez and Gelboin, 1992). A total of 674 and 817 genes were predicted, respectively in *C. australisinense* and *C. siamense*, for which both numbers were associated with 57 families of CYPs (Figure 4A), which pointed to a significant higher abundance in P450 gene families of *C. siamense*. The family that expanded most was CYP53. Research on fungal P450s has revealed that the P450 family CYP53 can serve as a novel

**FIGURE 2** | Phylogenetic tree based on single-copy orthologous genes of different *Colletotrichum* species, with *Verticillium alfalfae* as the outgroup to root the tree. All branches have 100% bootstrap support values. Two *Colletotrichum* species in this study are in bold.
Alternative anti-fungal drug target (Jawallapersand et al., 2014), and the CYP53 gene was earlier shown to be essential for fungal species survival (Fraser et al., 2002). The CYPI49, CYP68, and CYP65 families corresponding to cytochrome P450s related to SM biosynthesis (Jimbo et al., 2000; Liang et al., 2018) also have obvious higher abundance (Figure 4B).

**SM Synthesis Capacity**

The fungal genus *Colletotrichum* produces an enormous array of SM that could figure prominently in its pathogenesis of plants. The biosynthesis of SM requires precursor genes, most of which are located inside fungal gene clusters. It is imperative to identify the genes involved in the codification of these enzymes, because these genes are often involved in different mechanisms of pathogenicity and/or virulence (Boettger and Hertweck, 2013; Moraga et al., 2018). Here, by using antiSMASH, 85 SM gene clusters in the *C. siamense* genome were predicted: 31 type 1 polyketide synthases (t1PKS) and 18 NRPS genes, 7 t1PKS-NRPS hybrid genes, 19 terpene (TS) and 7 indole genes, and one each of t1PKS-type 3 polyketide synthases (t3PKS), NRPS-TS, and t1PKS-indole hybrid genes. Yet there were fewer SM gene clusters in the *C. australisinense* genome, whose 55 clusters comprised 15 t1PKS and 16 NRPS genes, 7 t1PKS-NRPS hybrid genes, 10 TS and 5 indole genes, and one each of NRPS-betalactone and NRPS-TS hybrid genes. The t1PKSs commonly responsible for the biosynthesis of macrolides were the most abundant clusters detected in the *C. siamense* genome (Figure 5). Some of these genes showed homology to known clusters in other fungi: eight gene clusters of *C. siamense* showed 100% similarity with alternapyrone (*Alternaria solani*), dimethylcoprogen (*Alternaria solani*), chrysogine (*Fusarium graminearum*), clavaric acid...
FIGURE 5 | Number of secondary metabolite backbone genes predicted by antiSMASH in the two species of *Colletotrichum*. PKS, polyketide synthase; NRPS, non-ribosomal peptide synthetase; PKS-NRPS, PKS-NRPS hybrid containing at least one PKS and one NRPS domain; TS, terpene synthase. Cs, *C. siamense*; Ca, *C. australisinense*.

(Hypholoma sublateritium), fusarin (*Fusarium verticillioides*), phomopsins (*Phomopsis leptostromiformis*), gibberellin, and 1,3,6,8-tetrahydroxynaphthalene (*Nodulisporium* sp. ATCC74245) biosynthetic gene clusters. Of these clusters, in *C. australisinense* genome only alternapyrone and 1,3,6,8-tetrahydroxynaphthalene biosynthetic gene clusters were found, which showed 100% similarity. Furthermore, two species-specific gene clusters of *C. australisinense* showed 100% similarity with the pyranonigrin E (*Aspergillus niger* ATCC 1015) and deoxysambucinol/sambucinol/oridin E (*Fusarium sambucinum*) biosynthetic gene clusters.

We also investigated the relationships among the putative SM-associated proteins using the adenylation domain (A domain) of NRPS proteins and the keto-synthase (KS) of PKS in the two *Colletotrichum* spp. This phylogenetic analysis revealed a rich diversity, with relatively few NRPS-associated protein ortholog families conserved across both species (*Figures 6A,B*). Nonetheless, 11 PKS gene clusters—but none that included the PKS-NRPS hybrid—were apparently shared between *C. siamense* and *C. australisinense*. Interestingly, *C. siamense* HBCG01 had a larger repertoire of keto-synthase (KS) in these PKS clusters (*Figures 7A,B*).

FIGURE 6 | (A) Phylogenetic tree of the adenylation domain (A domain) amino acid sequences of putative NRPS and PKS-NRPS hybrids. Sequences were aligned using MEGA 6.0, and the phylogenies inferred by maximum-likelihood (as calculated by the LG + G Model) in MEGA 6.0. The numbers on branch nodes indicate support values above 50%. Red branches show the A domain of *Colletotrichum siamense* only; blue branches show the A domain of *C. australisinense* only; green branches the A domain shared by both species. (B) Venn diagram summarizing the numbers of conserved and non-conserved sequences between the two fungal species.

Necrosis and Ethylene-Inducing Peptide 1 (Nep1)-Like Proteins (NLPs)

Nep1-like proteins (NLP) are perhaps best known for their cytotoxic activity in dicot plants (Oome and Van den Ackerveken, 2014). These proteins function as effectors that boost pathogen virulence during the host colonization process by disintegrating the plasma membrane of plant cells. NLP are taxonomically widespread among microbes having very different lifestyles (Oome and Van den Ackerveken, 2014). Here, NLPs were identified through a search for the conserved motif GHRHDWE in the predicted proteins. Eleven proteins were predicted in *C. australisinense*, and likewise eight in *C. siamense*, which belonged to the NEP1-like protein (NLP) family (Oome and Van den Ackerveken, 2014). Two NLPs of *C. siamense*,
In our study, the whole-genome sequencing, assembly, and functional annotation of *C. australisinense* and *C. siamense* characterized by distinct lifestyles on rubber tree were carried out. Pathogenicity-related genes were classified and compared, and marked duplication/reduction events were also identified. *Colletotrichum* is a large genus of *Ascomycete* fungi, containing many species with different life-histories capable of causing anthracnose or blight to a wide range of host plants (Jayawardena et al., 2016). The majority of *Colletotrichum* spp. are known for their hemibiotrophic lifestyle, which includes two successive stages of infection, biotrophy followed by necrotrophy. The particular life history of species in this genus depends on multiple factors, including the host identity, type of host tissue infected, and local environmental conditions (Perfect et al., 1999; De Silva et al., 2017). Although *C. australisinense* and *C. siamense* form the dominant populations causing *Colletotrichum* leaf disease in rubber trees, they have a different life history. *C. australisinense* has only been found on the rubber tree. By contrast, the *C. siamense* has a wide host range, and can colonize many host plant species, such as those in the genera *Carica*, *Coffea*, *Mangifera*, *Annona*, *Musa*, and *Camellia* (Udayanga et al., 2013; Liu et al., 2015). Genus-wide comparative genome analyses indicate that *Colletotrichum* species have tailored profiles for some enzymes according to their specific lifestyle, including CAZymes, SM synthetases, and peptidases (Gan et al., 2016; Buiate et al., 2017; De Silva et al., 2017; Liang et al., 2018). In the pathogenic process, these genes are expressed dynamically to fulfill stage-specific pathogenic functions (O’Connell et al., 2012; Gan et al., 2013; Rao and Nandineni, 2017). Effectors and SM enzymes are induced before penetration and during biotrophy, whereas most degradative enzyme and transporters are upregulated later, at the necrotrophic stage of growth (O’Connell et al., 2012). Hemibiotrophy in *C. orbiculare* is characterized by distinct stage-specific gene expression profiles of expanded classes of potential pathogenicity genes (Gan et al., 2013). The comparative genomic research has ever yielded key insights into the *Colletotrichum* fungi’s evolution (Liang et al., 2018). The gene family expansions and contractions
are thought to be critical for shaping the host specificity of members of this genus (Gan et al., 2016; Baroncelli et al., 2016).

When compared with *C. australisinense*, our results suggest that the major polysaccharide degrading enzyme (CAZyme) families underwent a species-specific increase of pectin degrading CAZymes (GH28, GH78, PL1), cutinases (CE5), and hemicelluloses and cellulose degrading CAZymes (CE1, CE3, CE6, GH16, GH35, GH71, GH92) in *C. siamense*, the species that causes anthracnose symptoms in a wide variety of hosts. Thus, *C. siamense* has a stronger ability to degrade plant cell wall components, such as xyloglucan, xylan, pectin, and cellulose. This offensive trait may help explain why, of the two *Colletotrichum* spp. studied, it is *C. siamense* which mainly infects the mature leaves of rubber tree, causing large anthracnose lesions. In contrast, the *C. australisinense* infection of leaves only causes little raised spots that slowly expand.

P450s are diverse, heme-thiolate proteins, which enable primary and SM synthesis, fungal pathogenicity, and detoxification of plant-derived antimicrobial compounds. In this respect, there seems to be little difference between *C. siamense* and *C. australisinense*, both of which contain nearly the same P450 categories. However, the gene number of particular P450 families has strongly expanded in *C. siamense*, especially that of CYP65, while CYP68 was found closely associated with the SM synthesis, and CYP102, CYP149, and CYP3 with toxic substance metabolism. These results correspond well with the number of SM gene clusters predicted in two species, which may explain how these two pathogens can detoxify phytoalexins (Soby et al., 1996).

The SMs produced by fungal phytopathogens are typically associated with their respective pathogenicity and host range (Rao and Nandineni, 2017). When applied to host leaves, these phytotoxic metabolites will induce symptoms similar to those of anthracnose caused by *Colletotrichum* species, and they have been shown to play a significant role in pathogenesis and infection mechanisms (García-Pajón and Collado, 2003). The comparative analysis of the SM gene clusters, as predicted through antiSMASH, revealed considerably more of such clusters in *C. siamense*, especially PKS (31 vs. 15) and TS (19 vs. 10) are twofold higher than in *C. australisinense*. Although most of the KS genes in the predicted *C. australisinense* PKS clusters were shared with *C. siamense*, the latter did harbor many specific KS genes that could contribute to its genetic potential to generate diverse SMs. It has been appreciated for some time now that PKSs are important in facilitating host penetration by *Colletotrichum* species (Takano et al., 1995, 1997).

Nep1-like proteins (NLPs) induce necrosis and ethylene production when they infiltrate the extracellular space in leaves of dicot plant species (but not monocots) (Oome and Van den Ackerveken, 2014). The NLPs are taxonomically widespread among microbes having very different lifestyles. Based on their phylogenetic analysis of NLP sequences, Oome and Van den Ackerveken (2014) defined four groups belonging to three phylogenetic types. A great number of newly identified NLP
sequences, from a broad range of diverse organisms, have been studied to date, providing much valuable insight into the evolution and functions of these remarkable proteins (de Oliveira et al., 2012; Kleemann et al., 2012; Zhou et al., 2012; Oome and Van den Ackerveken, 2014; Chen et al., 2018). Importantly, each NLP type differs in its ability to induce necrosis. It has been suggested that an NLP acts in a manner extracellular to the plant cell to confer its cytotoxic activity, which may be achieved via different mechanisms (Kleemann et al., 2012; Oome and Van den Ackerveken, 2014). In this study, we found that the number and categories of NLPS in C. australisinense exceeded those of C. siamense: In the previous work, it was confirmed that ChNLP1 and ChNLP2 of C. higginsianum were upregulated during the transition from biotrophy to necrotrophy, the former having been shown to induce necrosis in plants. Most of the NLPS in C. siamense belong to subgroup II, including ChNLP3 and ChNLP5, which are unable to induce necrosis in infected host plants. Since the genes encoding non-cytotoxic NLP are expressed during the biotrophic and early stages of infection by these (hemi)biotrophic pathogens, this suggests they play a role in the penetration or establishment of infection (Kanneganti et al., 2006; Kleemann et al., 2012). Previous studies have revealed lineage-specific expansions of NLP families within the C. acutatum species complex. It may well be that a lineage specific evolution is unique to members of the C. acutatum species complex (Baroncelli et al., 2016; Liang et al., 2018).

The remarkable repertoire of NLPS found in the C. acutatum species complex might be linked to their potent ability to infect multiple host species. Some of these proteins may function as necrosis-inducing proteins, whereas other NLPS may contribute to overcoming early plant defense responses (Baroncelli et al., 2016). We suggest that C. australisinense—which belongs to the C. acutatum species complex—has a stronger ability to induce necrosis than does C. siamense of the C. gloeosporioides species complex. Species within the acutatum complex, infection strategies are usually based on intracellular hemibiotrophy in addition to subcuticular, intramural necrotrophy (De Silva et al., 2017). Nonetheless, infection by C. gloeosporioides was reportedly hemibiotrophic, in that both intracellular hemibiotrophic and intramural necrotrophic infections can occur (Kim et al., 2004; O’Connell et al., 2012; Moraes et al., 2013; De Silva et al., 2017). For the intracellular hemibiotrophic strategy, transitioning from one state to another can vary depending on the host plant identity and ontogenic stage and the particular infecting Colletotrichum species, of which some can engage in rather unique infection behaviors. Species in the acutatum complex commonly begin their life cycle with a short biotrophic phase before switching to a necrotrophic stage (De Silva et al., 2017). These developmental aspects may help to explain why C. australisinense mainly infects tender and immature leaves of rubber tree, leading to rapidly wrinkled lesions.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the GenBank, accession nos. PRJNA597657 and PRJNA597926.

AUTHOR CONTRIBUTIONS

XL conducted the experiments and analyzed the results. BL, YY, JC, TS, and XZ collected Colletotrichum isolates and performed the experiments. GH revised and approved the final version of the manuscript. All authors reviewed the 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.2020.01484/full#supplementary-material

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