Insight into plant cell wall degradation and pathogenesis of *Ganoderma boninense* via comparative genome analysis

Ahmad Bazli Ramzi[1*], Muhammad Lutfi Che Me[1], Ummul Syafiqah Ruslan[1], Syarul Nataqain Baharum[1], Nor Azlan Nor Muhammad[1]

[1]Institute of Systems Biology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia

Corresponding Author:
Ahmad Bazli Ramzi*
Institute of Systems Biology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Malaysia
Email address: bazliramzi@ukm.edu.my

ABSTRACT

Background

G. boninense is a hemibiotrophic fungus that infects oil palms (*Elaeis guineensis* Jacq.) causing basal stem rot (BSR) disease and consequent massive economic losses to the oil palm industry. The pathogenicity of this white-rot fungus has been associated with cell wall degrading enzymes (CWDEs) released during saprophytic and necrotrophic stage of infection of the oil palm host. However, there is a lack of information available on the essentiality of CWDEs in wood-decaying process and pathogenesis of this oil palm pathogen especially at molecular and genome levels.

Methods

In this study, comparative genome analysis was carried out using *G. boninense* NJ3 genome to identify and characterize carbohydrate-active enzyme (CAZymes) including CWDE in the fungal genome. Augustus pipeline was employed for gene identification in *G. boninense* NJ3 and the produced protein sequences were analyzed via dbCAN pipeline and PhiBase 4.5 database annotation for CAZymes and plant-host interaction (PHI) gene analysis, respectively. Comparison of CAZymes from *G. boninense* NJ3 was made against *G. lucidum*, a well-studied model *Ganoderma* sp. and five selected pathogenic fungi for CAZymes characterization. Functional annotation of PHI genes was carried out using Web Gene Ontology Annotation Plot (WEGO) and was used for selecting candidate PHI genes related to cell wall degradation of *G. boninense* NJ3.

Results

G. boninense was enriched with CAZymes and CWDEs in a similar fashion to *G. lucidum* that corroborate with the lignocellulolytic abilities of both closely-related fungal strains. The role of polysaccharide and cell wall degrading enzymes in the hemibiotrophic mode of infection of *G.*

40 *boninense* was investigated by analyzing the fungal CAZymes with necrotrophic *Armillaria*
41 *solidipes*, *A. mellea*, biotrophic *Ustilago maydis*, *Melampsora larici-populina* and
42 hemibiotrophic *Moniliophthora perniciosa*. Profiles of the selected pathogenic fungi
43 demonstrated that necrotizing pathogens including *G. boninense* NJ3 exhibited an extensive set
44 of CAZymes as compared to the more CAZymes-limited biotrophic pathogens. Following PHI
45 analysis, several candidate genes including polygalacturonase, endo β-1,3-xylanase, β-glucanase
46 and laccase were identified as potential CWDEs that contribute to the plant host interaction and
47 pathogenesis.
48 **Discussion**
49 This study employed bioinformatics tools for providing a greater understanding of the biological
50 mechanisms underlying the production of CAZymes in *G. boninense* NJ3. Identification and
51 profiling of the fungal polysaccharide- and lignocellulosic-degrading enzymes would further
52 facilitate in elucidating the infection mechanisms through the production of CWDEs by *G.*
53 *boninense*. Identification of CAZymes and CWDE-related PHI genes in *G. boninense* would
54 serve as the basis for functional studies of genes associated with the fungal virulence and
55 pathogenicity using systems biology and genetic engineering approaches.
56
57

58 **INTRODUCTION**
59   Cell wall degrading enzymes (CWDEs) are part of the carbohydrate-active enzymes
60 (CAZymes) produced by plant pathogens for penetrating and degrading the plant cell walls, and
61 these CAZymes have been directly linked to devastating crop diseases (Zhang, Bruton & Biles,
62 2014; Somai-Jemmali et al., 2017; Gawade et al., 2017). Plant pathogenic fungi, namely among
63 the fungal families of Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota, have
64 been reported to contain the highest number of CAZymes (Zhao et al., 2013; Kubicek, Starr &
65 Glass, 2014). Differences in composition and structure of the woody components are commonly
66 mirrored with the types of lignocellulolytic enzymes produced by invading pathogenic fungi
67 (King et al., 2011). In fact, many plant pathogens particularly white rot fungi are well-endowed
68 with high copies of CWDEs as compared to decay-feeding saprotrophs and have been
69 demonstrated to be highly competent producers of lignocellulolytic enzymes for host-specific
70 attack, and subsequent biomass degradation (King et al., 2011; O'Connell et al., 2012).
71   Interestingly, genome and transcriptome analyses indicate different profiles of CAZymes
72 were produced by pathogenic fungi during different stages of pathogenesis transition from
73 biotrophic to necrotrophic lifestyles (O'Connell et al., 2012; M'Barek et al., 2015). Enzymatic
74 production of CWDE by pathogenic fungi has been found to be correlated with the degree of
75 pathogenicity and cell wall disintegration of infected plant hosts (Kang & Buchenauer, 2000;
76 Wanjiru, Zhensheng & Buchenauer, 2002; Lyu et al., 2015; Somai-Jemmali et al., 2017).
77 Considering the importance of CWDE in the uptake of nutrients from plant host, these hydrolytic
78 enzymes are considered as the key pathogenicity determinant among plant pathogens (Brito,

79   Espino & González, 2006; Kubicek, Starr & Glass, 2014; Bravo Ruiz, Di Pietro & Roncero,
80   2016).
81   *Ganoderma boninense* is a causative agent of basal stem rot (BSR) disease that beset the
82   oil palm industries with devastating economic losses due to the reduced lifespan and eventual
83   death of the infected oil palm (Chen et al., 2017). Due to the toxicity and environmental issues of
84   chemical pesticides, the *Ganoderma* disease is currently managed mainly through cultural
85   practices such as the removal of dead trees and infected stumps prior to or during replanting but
86   these strategies remain ineffective in preventing the spread of the *G. boninense* in affected
87   plantations (Hushiarian, Yusof & Dutse, 2013; Sahebi et al., 2015). Recent research works in
88   overcoming the *Ganoderma* disease has been mainly aimed at understanding the oil palm
89   molecular defense response via transcriptional analysis, and profiling of proteins and metabolites
90   of the infected oil palm (Nusaibah et al., 2016; Sahebi et al., 2017; Ho et al., 2018). The spread
91   of *G. boninense* in oil palm plantation has been attributed to two main routes specifically spore
92   dispersal and root contact with *G. boninense* infected palm tissues (trunk, bole, and roots)
93   (Paterson, 2007; Chen et al., 2017). Importantly, root infection via cell wall degradation has been
94   suggested as the main mode of *Ganoderma* infection based on the spread of infection from root
95   to the base of mature palm trees (Rees et al., 2009).
96   Lignocellulolytic enzymes of *G. boninense* have been shown to be predominant in
97   instigating oil palm infection and cell wall-degrading processes (Goh, Ganeson & Supramaniam,
98   2014; Jumali & Ismail, 2017; Surendran et al., 2018). Direct roles of CWDEs in the
99   hemibiotrophic infection of oil palm roots were first demonstrated via macroscopic examination
100   of enzymatically-degraded root outer cell layers and invaded root and stem tissues of *G.*
101   *boninense*-infected tissues (Rees, 2006; Rees et al., 2009). In the initial stage of infection, the
102   fungal mycelia behave as biotrophs that absorb the plant nutrient by penetrating the oil palm root
103   surface and culminating in rapid growth spread in oil palm lower stem. During the necrotrophic
104   stage, the fungus attacks the host cell walls by excreting host of enzymes including CWDEs that
105   led subsequent cell death and multiplication of basidiocarps in decayed palm woods (Rees et al.,
106   2009; Chong, Dayou & Alexander, 2017).
107   Despite the important roles of CWDEs in the oil palm pathogenesis, information about
108   genomic features and mechanisms underlying the pathogenicity of *G. boninense* in oil palm is
109   severely lacking. The necessity for establishing a reliable genetic model for understanding
110   *Ganoderma*-oil palm interactions is highlighted in recent reports of draft genome sequences of
111   different strains of *G. boninense* which could facilitate the identification of CWDEs as
112   pathogenicity factors essential for successful invasion of oil palm cells (Sulaiman et al., 2018;
113   Utomo et al., 2018). A deeper understanding on the genetic composition of CWDEs as part of
114   carbohydrate-acting enzymes of *G. boninense* and the biological mechanisms conferring the
115   fungal ability to produce CWDEs are important in our efforts to elucidate the plant-pathogen
116   interactions at the genome and molecular levels. Therefore, this research work was devised to
117   obtain genomic insight of CWDEs in *G. boninense* through computational and comparative
118   genome analysis. Genome sequence of *G. boninense* NJ3 that was isolated from Malaysian oil

119 palm field was used for the comparative genome analysis (Mercière et al., 2015). In this study,
120 CAZymes specifically auxiliary protein (AA), glycosyltransferase (GT), carbohydrate binding
121 modules (CBMs), carbohydrate esterases (CE), glycoside hydrolases (GH) and polysaccharide
122 lyases (PL) in *G. boninense* NJ3 genome were annotated using CAZy annotation pipeline. Direct
123 comparison of CAZymes was made with close relative and model strain *G. lucidum* as the
124 reference *Ganoderma* strain. Further comparison was carried out with five selected pathogenic
125 Basidiomycetes in the search for genetic patterns underlying *G. boninense* hemibiotrophic
126 infection strategy. Identification of the responsible genes for *G. boninense* CAZymes including
127 CWDEs will broaden genomic understanding on the molecular mechanisms of the fungal wood-
128 decaying abilities and oil palm pathogenesis
129
130 **MATERIALS & METHODS**
131 ***Ganoderma boninense* NJ3 genome**
132 The *G. boninense* NJ3 assembled genome was obtained from the NCBI assembly database
133 (GenBank assembly accession: GCA_001855635.1). The assembly level is at contig level and
134 was sequenced using Illumina HiSeq 454 in 2016. Comparative genome analysis was carried out
135 by comparing CAZymes dataset of *G. boninense* NJ3 with previously-reported genome sequence
136 of *Ganoderma lucidum* (Chen et al., 2012b), *Melampsora larici-populina* (Duplessis et al.,
137 2011), *Ustilago maydis* (Kämper et al., 2006), *Moniliophthora perniciosa* FA553 (Mondego et
138 al., 2008), *Armillaria solidipes* 28-4 (Sipos et al., 2017) and *Armillaria mellea* DSM
139 3731(Collins et al., 2013). Except for *G. lucidum*, archival CAZymes information of the other
140 referenced fungal genomes was retrieved from Joint Genome Institute MycoCosm portal in May
141 2018 (Grigoriev et al., 2014) (https://gp-dge6.jgi.doe.gov/programs/fungi/index.jsf).
142
143 **Prediction of genes**
144 The contigs from the *G. boninense* NJ3 assembly was processed using Augustus gene prediction
145 tool for the identification of genes (http://augustus.gobics.de/). Gene prediction was carried out
146 using "augustus --species=phanerochaete_chrysosporium gboninense_NJ3.fna >
147 gboninense_NJ3_augustus.gff" command where gboninense_NJ3.fna was the assembled contigs
148 file. *Phanerochaete chrysosporium* was chosen as the gene prediction model as it was the closest
149 species to *G. boninense* in Augustus based on the NCBI taxonomy browser
150 (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi). A GFF file containing the
151 genes predicted and their annotations was produced by Augustus and the protein sequences were
152 then extracted via the command "getAnnoFasta.pl gboninense_NJ3_augustus.gff". The predicted
153 genes dataset of the *G. boninense* NJ3 genome has been deposited at European Nuclear Archive
154 (ENA) under the accession number PRJEB34805.
155
156 **dbCAN pipeline analysis**
157 The produced protein sequences from Augustus was searched against the dbCAN: An HMM
158 (Hidden Markov Model) based database for carbohydrate-active enzyme annotation. dbCAN

159 release 6.0 was downloaded in May 2018. The downloaded database was converted into a HMM
160 formatted database using hmmpress (part of HMMER3 software package). hmmscan was run
161 with the following parameters; --domtblout results.out.dm. hmmscan-parser.sh (from dbCAN)
162 was used to process the results table with e-value 1E-3 as filter.
163

164 **Annotation by PhiBase 4.5 database**
165 PhiBase 4.5 was downloaded for local analysis from http://www.phi-base.org. The raw sequence
166 file was converted into a blast database using makeblastdb (part of the ncbi-blast+ software
167 package). A local blastp run was deployed to identify homologs of PhiBase 4.5 in *G. boninense*
168 NJ3 predicted protein sequences. Blastp was run with the parameters -outfmt 6 -max_target_seqs
169 1 -max_hsps 1 -evalue 0.1.
170

171 **Gene ontology and WEGO chart**
172 Gene ontology of PhiBase 4.5 homologs in *G. boninense* NJ3 was obtained using Blast2GO
173 5.2.5 pipeline. Using local blastp function in Blast2GO, the sequences of the homologs were
174 annotated against the NCBI NR (non-redundant) protein database. The NR database was
175 downloaded in April 2019. E-value chosen was 0.1 and number of blast hits was set to 10. Other
176 parameters remained at default values. The gene ontologies were then mapped onto the
177 sequences by mapping the latest Blast2GO database with the blasted sequences. Next,
178 InterProScan 5.33-72 was ran locally to obtain protein domain annotations. The XML file
179 produced was then loaded in Blast2GO. Lastly, the annotation tool in Blast2GO merged and
180 verified the gene ontologies obtained between both gene ontology annotation methods. Blast2GO
181 annotation parameters were left at default values. Functional classification of PHI genes was
182 carried out using Web Gene Ontology Annotation Plot (WEGO) software (Ye et al., 2006). To
183 generate the WEGO chart, we exported the results from Blast2GO annotation in WEGO native
184 format. The WEGO chart was then generated by uploading to http://wego.genomics.org.cn/.
185 Datasets of *G. boninense* NJ3 Augustus gene annotation, GO annotation and protein ID from
186 PhiBase 4.5 analysis are provided in supplementary files.
187

188 **RESULTS & DISCUSSION**
189 **Characterization of carbohydrate-active enzymes (CAZymes) in *G. boninense***
190   In this study, the draft genome of *G. boninense* NJ3 was used for identifying and
191 characterizing the carbohydrate-active enzymes in fungal genome. To identify CAZymes in *G.*
192 *boninense* NJ3, the assembled dataset was further processed using Augustus pipeline to produce
193 predicted genes peptide sequence for CAZymes analysis via the dbCAN pipeline. Comparative
194 analysis for CAZymes characterization in *G. boninense* was carried out using *G. lucidum*
195 genome sequence as the reference *Ganoderma* spp. strain owing to the high-quality genome
196 sequence and well-established genomics studies of this closely-related *Ganoderma* strain. We
197 hypothesized that *G. boninense* is enriched with a high number of CAZymes similar to *G.*
198 *lucidum* that provide wood-degrading capabilities and contribute to the disparate nutritional

199   strategy of hemibiotrophic *G. boninense* and saprophytic *G. lucidum*, respectively.

200   Following analysis, a total of 755 CAZymes was identified in *G. boninense* NJ3 as

201   compared to 489 CAZymes found in *G. lucidum* (Fig. 1). Overall, about 465 copies of cell wall

202   degrading enzymes (CWDEs) comprising of glycoside hydrolase (GH), carbohydrate esterase

203   (CE) and polysaccharide lyase (PL) were found in the *G. boninense* NJ3 genome. From the total

204   CWDEs, 348, 102 and 15 genes were found for GH, CE and PL, respectively (Table S1). The

205   amount of CWDEs found in *G. boninense* is comparatively higher when compared with *G.*

206   *lucidum* (273 GHs, 30 CEs, 10 PLs). A richer and highly similar set of CWDEs was observed in

207   *G. boninense* NJ3 that enables degradation of woody structures such as hemicellulose and pectin

208   for nutrient uptake and growth in similar means to *G. lucidum* which harbored one of the richest

209   sets of polysaccharide-degrading enzymes in the sequenced genome of Basidiomycota fungi

210   (Chen et al., 2012b). Interestingly, *G. boninense* NJ3 possesses a higher number of CEs of about

211   102 copies as compared to *G. lucidum* with 30 copies of CEs that are important for plant cell

212   wall modification. In addition to polysaccharide-deacetylating CE enzymes, *G. boninense* NJ3

213   also harbors a diverse array of GHs that are crucial in the hydrolysis of cellulose and

214   hemicellulose components of the plant biomass. Common GH in white rot fungi such as GH6

215   and GH7, and universal CWDEs of cellulolytic GH1, GH3 and GH5 for degradation of cellulose,

216   hemicellulose and pectin were observed in both *Ganoderma* spp. Importantly, *G. boninense*

217   possesses several GHs that were not found in *G. lucidum* specifically polysaccharides-acting

218   GH109 (α-N-acetylgalactosaminidase), GH145 (L-Rhα-α-1,4-GlcA α-L-rhamnohydrolase),

219   GH135 (α-1,3-galactosaminogalactan hydrolase) and GH131 (broad specific β-glucanase) that

220   degrade both cellulose and hemicellulose. In terms of polysaccharide-active enzymes, both

221   *Ganoderma* spp. contained multiple copies of pectic-acting PL8 and PL14 while PL4

222   (rhamnogalacturonan endolyase), PL12 (heparin-sulfate lyase) and PL15 (alginate lyase) were

223   only found in *G. boninense*.

224   Apart from CWDEs, both fungal genomes harbored other CAZymes including

225   carbohydrate binding module (CBM), auxiliary activity (AA) and glycosyltransferase (GT) that

226   are essential for lignin depolymerization and carbohydrate utilization from the host plant. During

227   wood decaying process, access to the structural woody components was aided by CBMs that

228   formed a two-domain structure together with catalytic domains (CDs) of cellulases by increasing

229   the enzyme concentration on the substrate surfaces. Overall, a total of 290 copies of CAZymes

230   was identified in *G. boninense* NJ3 as compared to 176 copies in *G. lucidum*. Of these, 67

231   CBMs, 145 AAs and 78 GTs were identified from *G. boninense*. Both *Ganoderma* spp. strains

232   have a similar set of CBMs except for CBM19 (chitin-binding function) and CBM32 (pectic-

233   binding) were unique for *G. boninense* while CBM12 (chitin-binding) was only found in *G.*

234   *lucidum*. Although white rot fungi have been associated with lack of CBMs, *G. lucidum* and *G.*

235   *boninense* contained 10 and 12 out of 16 total families of CBMs, respectively. It is notable that

236   *G. boninense* possessed high copies of CBM1, an important fungal CBM that uses cellulose and

237   chitin as substrates for polysaccharide-degrading activities (Mello & Polikarpov, 2014; Várnai et

238   al., 2014). Both fungi shared similar GTs except for GT65 and GT41 that were found only in *G.*

239 *boninense* while GT31 was present only in *G. lucidum*. The ability of *Ganoderma* spp. to utilize
240 nutrient from plant tissues relied heavily on the synergistic actions of cellulolytic and ligninolytic
241 enzymes that include redox AA enzymes (Zhou et al., 2018). Laccase (AA1_A1), ferroxidase
242 (AA1_A2), class II peroxidase (AA2), GMC oxidoreductase (AA3), radical-copper oxidase
243 (AA5), 1,4-benzoquinone reductase (AA6) and iron reductase (AA8) were among AA enzymes
244 identified in both sets of fungal genomes. From this comparative analysis, *G. boninense* was
245 found to be endowed with significantly higher copies of the redox enzymes especially lignin-
246 acting laccase (AA1) and peroxidase (AA2) and oxidoreductase (AA3) enzymes as compared to
247 *G. lucidum*.
248   Lignocellulolytic enzymes production has been well-documented in white rot
249 *Ganoderma* spp. for wood decomposition and subsequent feeding and propagation on the woody
250 substrates (Silva, Melo & Oliveira, 2005; Paterson, 2007; Zhou et al., 2013). Apart from wood-
251 degrading enzyme producing capabilities and plant pathogenicity, *Ganoderma* species are
252 generating much research interest for therapeutic applications through the production of
253 bioactive polysaccharides and terpenoids such as ganoderic acid (Boh, 2013; Wu et al., 2013).
254 Owing to its therapeutic and biotechnological potentials, *G. lucidum* has been developed as
255 model medicinal mushroom through extensive biochemistry, genomics, and genetic engineering
256 research, and this saprophytic mushroom is in fact endowed with an extensive set of CAZymes
257 encoded in its genome (Xu, Xu & Zhong, 2012; Chen et al., 2012b; Liu et al., 2012; Yu et al.,
258 2012). In this study, the genome sequence of *G. boninense* NJ3, a pathogenic fungal isolate from
259 oil palm plantation in Malaysia (Mercière et al., 2015), was employed for identifying genes
260 involved in the production of cell wall degrading and carbohydrate active enzymes. By
261 comparing CAZymes of *G. boninense* NJ3 with model *G. lucidum*, profiles of these close-related
262 *Ganoderma* spp. can be acquired especially in terms of the cell wall degrading abilities of the
263 lesser-studied *G. boninense*. Based on the results obtained, *G. boninense* NJ3 was found to be
264 enriched with an extensive repertoire of CAZymes in similar fashions but with significantly
265 higher numbers of lignocellulosic-degrading enzymes as compared to the non-pathogenic *G.*
266 *lucidum*, hence, underlining the essentiality of CAZymes in cell wall degradation for the fungal
267 growth and nutrient uptake. Differences in CAZymes characteristics especially CWDEs and
268 polysaccharide-active AAs can be linked and predetermined by the nutritional strategy of either
269 *Ganoderma* spp. hence providing genomic insight and characterization of plant cell wall
270 degradation mechanism of these industrially-important fungi.
271

272 **Profiling of CAZymes in selected phytopathogenic fungi**
273   Following characterization of CAZymes in *G. boninense*, the innate ability of this white
274 rot fungus to cause oil palm BSR disease was further investigated by comparative analysis with a
275 few selected disease-causing Basidiomycetes. For this purpose, five phytopathogenic
276 basidiomycetous fungi exhibiting the biotrophic, hemibiotrophic and necrotrophic mode of plant
277 infections were employed for comparison with *G. boninense*. The fungi of interest were *Ustilago*
278 *maydis* (model biotrophic pathogen), *Melampsora larici-populina* (biotrophic poplar pathogen),

279 and *Moniliophthora perniciosa* (hemibiotrophic cocao pathogen). The remaining two fungi were
280 *Armillaria solidipes* and *A. mellea* representing facultative necrotrophic fungi attributed to root
281 rot in many conifers and ornamentals, respectively (Kämper et al., 2006; Duplessis et al., 2009;
282 Meinhardt et al., 2014; Koch et al., 2017). Biotrophs primarily depend on and derive nutrients
283 without killing the hosts while necrotrophs kill the plant and feed nutrients off the dead cells
284 (Mendgen & Hahn, 2002). On the other hand, hemibiotrophs adopt early asymptomatic
285 biotrophic phase and then switched to the host-killing necrotrophic stage with distinct disease
286 symptoms, and decayed tissues (Horbach et al., 2011). Although each fungus may differ in
287 targeting host and infection mechanisms, these plant pathogens have been found to rely on an
288 array of hydrolytic enzymes for complete degradation of plant biomass for colonization and
289 nutrient uptake with or without killing the hosts. In this study, we hypothesized that pathogenic
290 fungi with necrotizing abilities (necrotroph and hemibiotroph) would harbor distinct CAZymes
291 profile as compared to non-necrotizing fungi (biotroph) which may be attributed to specific host
292 preference and interaction.
293   The profile of CWDE in all six pathogenic fungi was illustrated in Figure 2. *G.*
294 *boninense* NJ3 contained glycosyl hydrolase (GH) GH2 and GH10 for specialized hemicellulose
295 degradation in addition to dual cellulose and hemicellulose-degrading activities of GH1, GH3,
296 GH5, GH12, GH51, and GH131. The ability of this oil palm pathogen to hydrolyse the pectin
297 component is further provided by GH28, GH105 and necrotroph-specific GH53 and GH78.
298 Biotrophic *U. maydis* and *M. laricis-populina* lack GH1, GH6, GH78 and GH95 which were
299 prevalent in the necrotizing fungi (*G. boninense* NJ3, *M. perniciosa*, *A. solidipes* and *A. mellea)*
300 (Fig. 2a). Additionally, *U. maydis* lacked GH7 that is common among pathogenic white rot
301 fungi. On the other hand, biotrophic *U. maydis* and *M. laricis-populina* possess GH26 which was
302 not observed in other 4 necrotizing pathogens investigated in this study. The lack of GH1, GH6
303 and GH78 were well-documented in biotrophs which generally harbor less plant cell wall
304 degrading enzymes than necrotrophs and hemibiotrophs (Zhao et al., 2013; Li et al., 2017).
305 Obligate necrotrophs (*A. solidipes* and *A. mellea*) and hemibiotrophs (*G. boninense* NJ3 and *M.*
306 *laricis-populina*) were evidently supplemented with GH3 and GH28 for cellulose, hemicellulose
307 and pectin degradation. From the analysis, *G. boninense* NJ3 exhibited the highest copies of
308 GH18 (Chitinase/endo-β-N-acetylglucosaminidase), GH43 (Hemicellulase), GH79
309 (Glucuronidase), GH10 (Xylanase) and harbored unique GH4 (glycosidase), GH89 (α-N-
310 acetylglucosaminidase) and GH109 (α-N-acetylgalactosaminidase) for polysaccharide
311 depolymerization in comparison to other plant pathogens examined in this study. Taken together,
312 these findings highlighted the essentiality of several GHs specifically GH3 and GH5 for cell wall
313 degradation by the phytopathogens that corroborated with previous reports on the plant host
314 infection interplays by the phytopathogenic fungi (Zhao et al., 2013; Blackman, Cullerne &
315 Hardham, 2014; Chang et al., 2016).
316   In addition to cellulose and hemicellulose, some plants are enriched with pectins
317 comprising of homogalacturonan, xylogalacturonan or rhamnogalacturonan as external barriers
318 against pathogen infections. In *G. boninense* NJ3, the cell wall-degrading GHs could work in

319   tandem with pectic-acting enzymes of polysaccharide lyase 8 (PL8), PL12, PL14 and PL15.
320   Common PL found in pathogens, PL1 and PL3 were not observed in *G. boninense* NJ3 which is
321   interesting considering the abundance of these PLs in necrosis-causing *M. perniciosa, A.*
322   *solidipes* and *A. mellea* (Fig. 2b). Pectin degradation by pectinolytic enzymes particularly PL4 is
323   common among necrotizing fungi examined in this study except *G. boninense* NJ3 and this
324   enzyme had been shown to be highly expressed during crops infection by necrotrophic
325   *Rhizoctonia solani* (Zheng et al., 2013; Chang et al., 2016). Although important for cell wall
326   degradation by fungi, the smaller amount of pectinases in *G. boninense* NJ3 may indicate
327   substrate or host preference specifically monocotyledon-type as compared to dicotyledon-
328   preferred pathogens that have been associated with increased secretion of pectinases (Zhao et al.,
329   2013; Loyd et al., 2018). Hemibiotrophic and necrotrophic fungi are well-equipped with the
330   extended set of CWDEs which enable tailored and extensive production of the cell wall
331   degrading enzymes during infection.
332   In this study, it was found that all 6 pathogenic fungi possess at least one copy of
333   carbohydrate esterase 4 (CE4) as one of the polysaccharide-modifying enzymes in the genomes
334   (Fig. 2c). The genome of *G. boninense* NJ3 was well-represented with CE16 in addition to CE1
335   and CE12 that were also found in necrosis-causing *M. perniciosa, A. solidipes* and *A. mellea*
336   while CE2, CE14 and high copies of CE10 were found only in the pathogenic *Ganoderma* spp.
337   CEs have been associated in the first line of attack during fungal invasion via the removal of
338   acetylated moieties of saccharides that formed parts of plant protection system against hydrolytic
339   enzymes (Ospina-Giraldo, McWalters & Seyer, 2010; Sista Kameshwar & Qin, 2018). The CE10
340   enzyme is involved in the degradation of lignin and cellulosic components of the plant cell wall
341   and was found to be abundant in several pathogenic fungi including *Macrophomina phaseolina*,
342   *Bipolaris cookei* and *Corynespora cassiicola* (Islam et al., 2012; Zaccaron & Bluhm, 2017; Looi
343   et al., 2017). In sum, notable differences in CWDE profiles of hemibiotrophic and necrotrophic
344   fungi can be associated with the less aggressive nature of biotrophic *U. maydis* and *M. laricis-*
345   *populina* that adapted the hydrolytic enzyme production specifically for limiting host cell wall
346   damages hence supporting their host nutrient-dependent growth (Kämper et al., 2006; Duplessis
347   et al., 2009; Olson et al., 2012).
348   Profiling of the remaining CAZymes in the six pathogenic fungi was carried out for
349   comparing and establishing the association between mode of infection and type of genes present.
350   Generally, glycoside transferase (GT) enzymes are mainly responsible for cell wall formation in
351   contrast to the more abundant carbohydrate-hydrolysing GHs in the fungal genomes. As shown
352   in Fig 3a, the six pathogenic fungi harbored highly similar set of GTs while GT71 is unique for
353   biotrophs and GT65 was only found in *G. boninense* NJ3. Metabolism of starch components of
354   the plant biomass is linked to the presence of starch-active carbohydrate binding modules
355   (CBMs) including CBM1, CBM20, CBM48 and CBM50. Only CBM48 and CBM50 were found
356   in all of the studied pathogens while CBM1 was missing in biotrophic *U. maydis* and *M. laricis-*
357   *populina* which in turn, represented the most in *G. boninense* NJ3 genome (Fig. 3b). The
358   occurrence of CBMs was often associated with facilitating the hydrolytic activities of amylolytic

359   GHs such as GH13 and GH15 by increasing cell-substrate attachment and degradation (Chen et
360   al., 2012a). In particular, CBM1-containing proteins have been found mainly in nectrotrophs and
361   hemibiotrophs for promoting cellulose hydrolysis and were shown to elicit plant defense
362   response which is detrimental for fungi with biotrophic lifestyle (Jones & Ospina-Giraldo, 2011;
363   Klosterman et al., 2011; Larroque et al., 2012).
364   For lignin decomposition, all six pathogenic fungi possessed auxiliary activities (AA)
365   enzymes for AA1, AA3, AA5 and AA6 that encode for ligninolytic and redox enzymatic
366   activities while phenolic-active AA4 (vanillyl-alcohol oxidase) was found only in *G. boninense*
367   (Fig. 3c). Another important lignin-modifying enzyme, AA9 also classified as lytic
368   polysaccharide monooxygenases (LPMO), is involved in lignocellulosic degradation by
369   oxidizing cellulose in synergistic reactions with laccase and lignin-modifying peroxidase
370   enzymes. On one hand, biotrophic fungi *M. larici-populina* and *U. maydis* harbored a smaller
371   set of lignolytic AAs with about 36 (*M. larici-populina*) and 23 (*U. maydis*) as compared to the
372   other studied pathogens. These biotrophic strains contained lesser copies of AA1 encoding for
373   laccase and multicopper oxidase enzymes that involved in degradation of lignin barrier. All
374   pathogens possessed AA9 except *U. maydis* while AA2 and AA8 were absent in both biotrophic
375   fungi. High copies of AA9 observed in necrotrophs and hemibiotrophs studied may indicate the
376   importance of these enzymes during the host attack and cell wall deformation. The identification
377   of cellulose-cleaving oxidoreductases LPMOs as part of that AA9 family was previously
378   associated with improved fungal cellulase and wood decaying activities with the presence of
379   reducing agents (Dimarogona et al. 2013; Karnaouri et al. 2014). These auxiliary redox enzymes
380   played an important role in completing hydrolysis of lignin by wood-decomposing saprotrophic
381   fungi and have been associated with increased virulence of parasitic fungi (Hatakka, 1994;
382   Levasseur et al., 2013; Janusz et al., 2017). The abundance of AAs may contribute to the
383   enhanced ability of *G. boninense* NJ3 to invade and penetrate lignin and acetylated saccharides
384   as it switches from biotrophic to necrotrophic parasitism that involves overlapped biological
385   processes as found in forest pathogen and wood decayer *Heterobasidion annosum sensu lato*
386   (Olson et al. 2012). The production of diverse ligninolytic enzymes by *Ganoderma* are therefore
387   important for the fungal proliferation off plant tissues especially in the depolymerizing of the
388   recalcitrant lignin barrier (Hu et al., 2017; Sarah Jumali & Ismail, 2017; Zhou et al., 2018).
389   Expression patterns and production of carbohydrate-acting enzymes have been
390   demonstrated to be correlated with the fungal mode of interactions with host plants.
391   Transcriptome analysis of *G. boninense*-treated oil palm transcripts showed very high expression
392   of a host of distinct up-regulated genes encoding for CAZymes from lignin-degrading AAs
393   (laccase and AA2 manganese peroxidase), carbohydrate-active CBM and CE (CBM13, CE10,
394   CE9) to cell wall-hydrolyzing exo-β-1,3-glucanase, chitinase and polygalacturonase when
395   compared to untreated and *Trichoderma harzianum*-treated control samples (Ho et al., 2016).
396   Similar patterns of highly expressed CAZymes transcripts were observed in necrotrophic *A.*
397   *solidipes* that exhibited high number of homologs of GH18, GH47, CE10, CE4 and
398   polygalacturonase following plant-fungus inoculation (Ross-Davis et al., 2013). Higher

399 expression of cell wall degrading enzymes (GH, PL, GT) were observed in necrotrophic
400 *Leptosphaeria biglobosa* as compared to hemibiotrophic counterpart, *L. maculans* which
401 accumulated higher CBM during early stage of plant infection (Lowe et al., 2014). Similar
402 CAZymes interplays were suggested in the early infection of *G. boninense* that aimed at
403 overcoming the oil palm host defense response mechanisms including hypersensitive response
404 (HR) leading to the switch from biotrophic stage to the more aggressive necrotrophic attacks
405 culminating in host cell death and successful invasion (Bahari et al., 2018). A closer look of the
406 CAZymes in the selected pathogenic fungi would therefore enable genome-wide profiling of
407 carbohydrate-active enzymes that are distinct and correlated with the fungal mode of infection.
408 Importantly, *G. boninense* NJ3 harbored a distinct set of cell wall degrading and polysaccharide
409 depolymerization enzymes that were suited for infecting monocot oil palm host through
410 hemibiotrophic lifestyle.
411
412 **Potential pathogenicity genes among CAZymes of *G. boninense* NJ3**
413   Comparative CAZymes analysis of the selected of phytopathogens indicated the
414 correlation between the fungal nutritional strategy with the profiles of carbohydrate-active
415 enzymes essential for plant host cell wall degradation and nutrient consumption. Considering the
416 lack of information genes related to the pathogenicity of *G. boninense*, further genome-wide
417 analysis of the fungal genome was carried out using the protein sequences in the Pathogen-Host
418 Interaction Database (PHI database) and functionally classified according to molecular function,
419 biological process and cellular component via WEGO analysis. A total of 5,099 annotated PHI
420 genes were obtained from the WEGO analysis of which membrane (1682, 24.8%) and metabolic
421 process (2903, 42.8%) were represented the highest in respective cellular component and
422 biological process categories (Fig. 4). In molecular function category, the PHI genes were
423 predominantly annotated with catalytic activity (3337, 49.2%) including CAZymes-related
424 polygalacturonase (GO:0004650), cellulase (GO:0008810) and endo-1,4-beta-xylanase
425 (GO:0031176).
426   Considering the prevalence of carbohydrate-active enzymes and the high percentage of
427 PHI genes with hydrolase activity, we hypothesized that some of the CAZymes may directly
428 involve in plant pathogenesis via cell wall degradation by the secreted enzymes. As shown in
429 Table 1, several genes of *G. boninense* NJ3 were shown to share PHI homologs with lignin
430 depolymerization and cell wall degrading enzymes specifically pectic-acting polygalacturonase
431 (PG)-coding homolog gene (PHI id:4879), endo-1,4-beta-xylanase GH10 (PHI id:2209), β-
432 glucanase Eng1 (PHI id:6265) and laccase LCC2 (PHI id:552). CWDEs including pectinase,
433 glycosyl hydrolase and laccase mainly serve as primary weaponry for fungal attack causing the
434 plant cell wall becoming less compact and more permeable for consequent digestion by cellulase
435 and hemicellulase enzymes (Chu et al., 2015). Importantly, PG is one of the first enzymes
436 secreted by pathogenic fungi upon contact with plant cell wall and these pectinases have been
437 widely studied for their role in plant pathogenesis especially necrosis and rotting in the infected
438 plants (De Lorenzo & Ferrari, 2002; Kubicek, Starr & Glass, 2014). This finding corroborated

439   with the reported polygalacturonase activities from the transcriptome profile of *G. boninense*-
440   infected oil palm (*Elaeis guineensis*) whereby PG transcript was shown to be elevated as
441   compared to none observed in control unaffected oil palm (Ho et al., 2016). It can be postulated
442   that pectin-acting PG work synergistically with hemicellulases for *Ganoderma* infection in
443   similar fashion to necrotrophic infection and virulence of many phytopathogens including
444   *Fusarium* spp., the main causative agents of vascular wilt and head blight diseases in important
445   crops (Gómez-Gómez et al., 2002; Chen et al., 2012b; Paccanaro et al., 2017). Hemi-cellulosic
446   digestion activities of *G. boninense* were previously demonstrated to assist the fungal growth on
447   oil palm hence supporting the association of these PHI genes as potential pathogenicity factors in
448   oil palm infection (Surendran et al., 2017; Surendran et al., 2018).
449   On the other hand, no cutinase (CE5) homolog was found from the CAZymes and
450   PHIbase analysis of *G. boninense* NJ3 suggesting the lack of cutinase-mediated cell wall
451   modification during wood-decaying process which may be compensated by the high numbers of
452   oxidative AAs and hydrolytic GHs found in the *Ganoderma* spp. examined in this study (Table
453   S2). CE5 was found not prevalent in wood-decaying basidiomycetes including pathogenic *H.*
454   *irregulare* and *Fomitiporia mediterranea* which harbored multiple copies of lignolytic
455   peroxidase enzymes (Floudas et al., 2012; Zhao et al., 2013). Expression of cutinase was also
456   reported to be non-essential during pathogenesis of other necrotizing pathogens such as *F. solani*
457   f. sp. *pisi* and *Botrytis cinerea* (van Kan et al., 1997; Stahl & Schafer, 2007; Zhao et al., 2013).
458   Combined actions of ligninolytic and cellulolytic enzymes including laccase and endoglucanase
459   were previously shown to be directly involved in the wood decaying and infection processes of
460   wheat and cacao by necrotrophic *F. graminearum* and *Moniliophthora roreri,* respectively
461   (Zhang et al., 2012; Meinhardt et al., 2014). Transcriptome analysis of *Ganoderma* infected-oil
462   palm seedling demonstrated the presence of multiple copies of laccase transcripts as compared to
463   none observed in the sample of beneficial fungus, *T. harzianum*, indicating the important role of
464   cell wall degradation in oil palm infection (Ho et al., 2016, 2018). The identification of these cell
465   wall degrading PHI genes further supported the hemibiotrophic mode of infection of *G.*
466   *boninense* conferred by fungal genotypic capabilities to produce a plethora of carbohydrate-
467   acting enzymes. Overall, the comparative genome analysis employed in this study succeeded in
468   characterizing carbohydrate-active enzymes and identifying CWDE genes that are involved in
469   plant cell wall degradation and pathogenesis of *G. boninense*. Further genome analysis of *G.*
470   *boninense* strains can be carried out with the recent report of draft genome of *G. boninense* G3
471   strain isolated from Indonesian region (Utomo et al., 2018). Correlation between the fungal
472   pathogenicity with CWDE production and other factors can be further validated via targeted
473   transcriptome analysis and gene expression profiling of targeted genes (Isaac et al., 2018).
474   Functional studies of the cell wall degrading enzymes in *G. boninense* shall be pursued for
475   greater understanding on the essentiality of the enzymatic capacity in the fungal pathogenesis.
476
477   **CONCLUSIONS**

478 In this study, comparative genome analysis succeeded in the identification of carbohydrate-
479 acting and cell wall degrading enzymes in hemibiotrophic *G. boninense* NJ3. The pathogenic *G.*
480 *boninense* NJ3 genome contained an abundant amount of CAZymes and shared many similar
481 sets of CAZymes to closely-related *G. lucidum* of which the differences between the gene sets
482 can be attributed to the different nutritional strategy of either *Ganoderma* spp. Necrotizing fungal
483 pathogens including *G. boninense* NJ3 exhibited distinct CAZymes profiles as compared to the
484 non-necrotizing counterparts which can be correlated with host preference and parasitic
485 lifestyles. Several CWDE-related genes were identified from PHI analysis including
486 polygalacturonase and laccase which could directly contribute to the fungal pathogenesis
487 especially through degradation of the plant cell wall. These findings provide fundamental
488 knowledge on the fungal genetic ability and capacity to secrete polysaccharide and cell wall
489 degrading enzymes. Greater insight on the fungal phenotype can be obtained through future
490 studies involving functional and gene expression analysis of specific genes in the fungal
491 carbohydrate metabolism.
492

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496

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Figure 1

Comparison of carbohydrate-active enzymes (CAZymes) in *Ganoderma* spp.

Overview of CAZymes profile in (a) *G. boninense* NJ3 and (b) *G. lucidum*. Abbreviations: GH Glycoside hydrolase, CE Carbohydrate esterase, PL polysaccharide lyase, CBM carbohydrate binding module, AA auxiliary activity, GT glycosyltransferase.

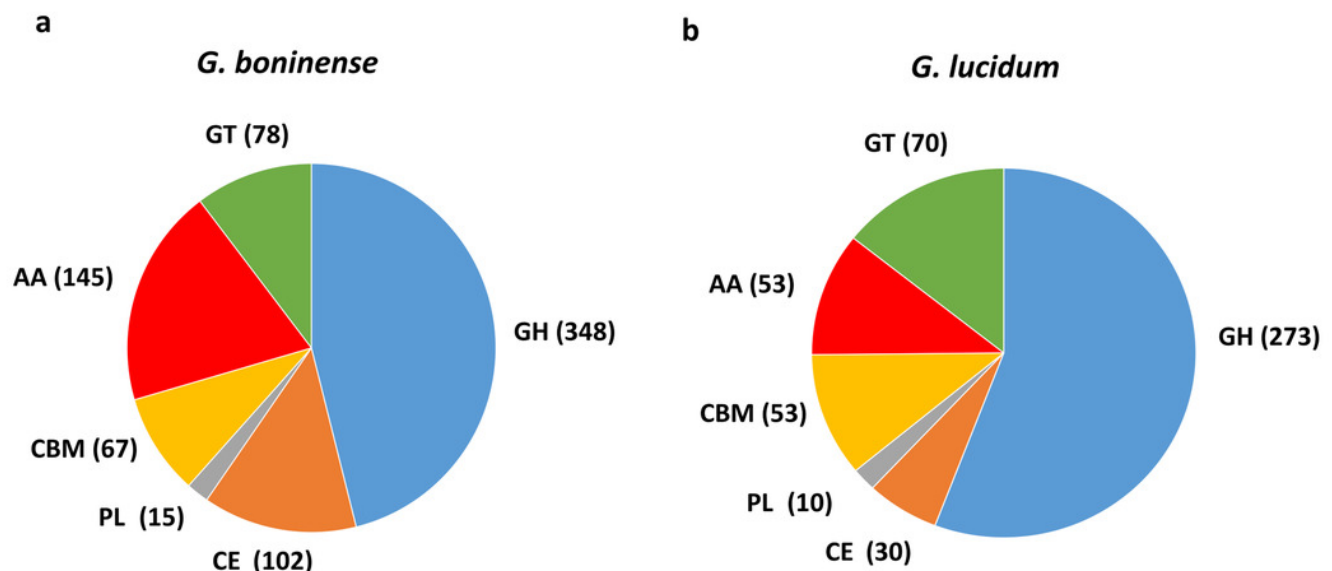



Figure 2

Profiling of CWDEs in selected phytopathogenic Basidiomycota fungi.

Profile of (a) common GH, (b) CE and (c) PL in the pathogenic fungi. Abbreviations: GH Glycoside hydrolase, CE Carbohydrate esterase, PL polysaccharide lyase.

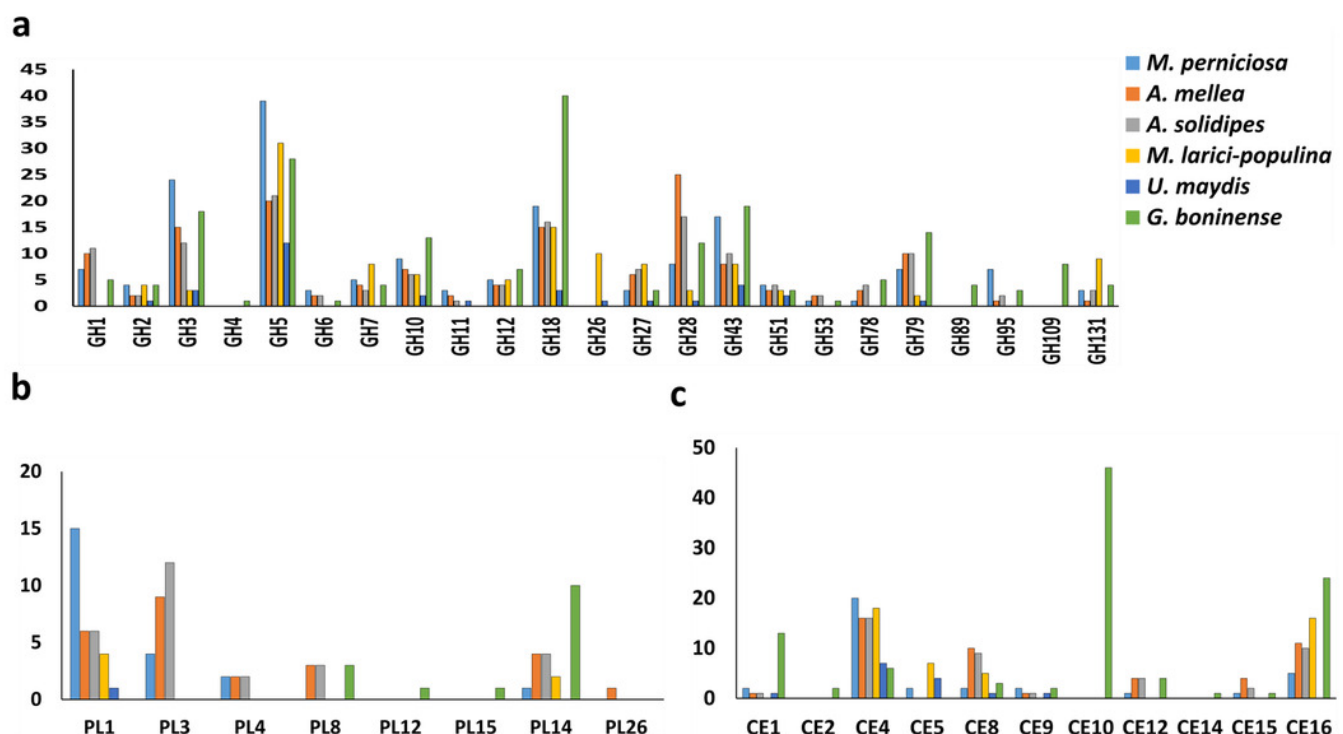



Figure 3

Profiles of carbohydrate-active enzymes (CAZymes) in phytopathogenic Basidiomycota fungi.

Profiles of (a) selected GT, (b) AA and (c) CBM of the compared plant pathogens.

Abbreviations: GT glycosyltransferase, AA auxiliary activity, CBM carbohydrate binding module.

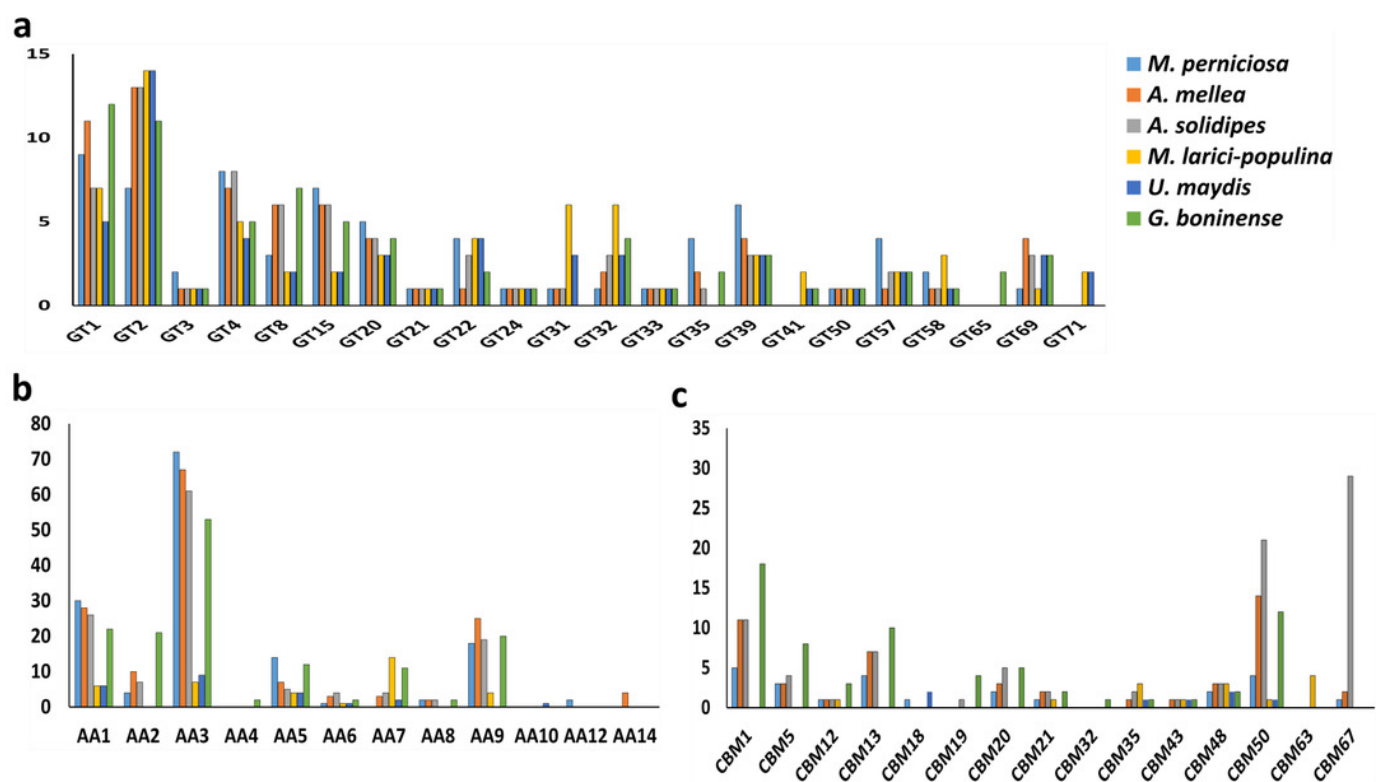
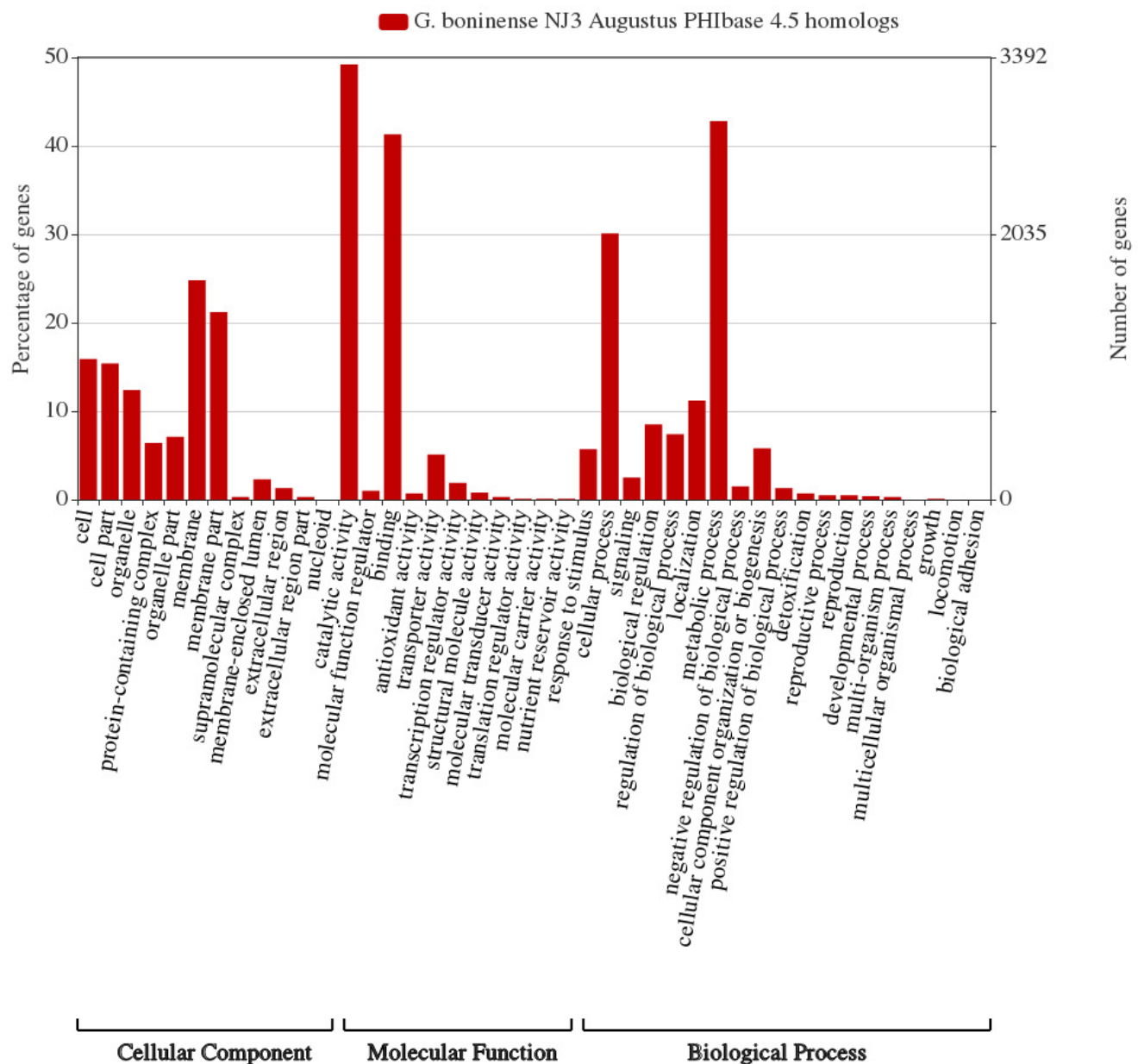



Figure 4

G. boninense NJ3 Augustus predicted amino acid sequences PhiBase 4.5 homologs classified based on gene ontologies

The gene ontology chart was generated using WEGO 2.0 (http://wego.genomics.org.cn/).

List of candidate CWDE-related pathogenicity genes from PHI database analysis.

Information of homolog gene from reference organism is described in the table.

Gene	PHI ID	Protein ID	Organism	Function/Role	Reference
PG1	PHI:4879	A0A0C4DHY2	*Fusarium oxysporum*	Catalysis of the random hydrolysis of (1,4)-alpha-D-galactosiduronic linkages in pectate and other galacturonans.	(Bravo Ruiz, Di Pietro & Roncero, 2016)
Endo-1,4-beta-xylanase (GH10 family)	PHI:2209	G4N1Y8	*Magnaporthe oryzae*	Catalysis of the endohydrolysis of (1,4)-beta-D-xylosidic linkages in xylans	(Nguyen et al., 2011)
Eng1	PHI:6265	C0NFK7	*Histoplasma capsulatum*	Catalysis of the hydrolysis of any O-glycosyl bond	(Garfoot, Andrew L. Shen, Qian, Wüthrich, Marcel, Klein, Bruce S., Rappleye, 2016)
LCC2	PHI:552	Q96WM9	*Botrytis cinerea*	Lignin degradation and detoxification of lignin-derived products	(Schouten et al., 2002)