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Conserved white rot enzymatic mechanism for wood decay in the *Basidiomycota* genus *Pycnoporus*.

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48 The authors declare that they have no competing interests

49 **Abstract**

50 White-rot fungi are pivotal decomposers of dead organic matter in forest ecosystems which
51 typically use a large array of hydrolytic and oxidative enzymes to deconstruct lignocellulose.
52 However, the extent of lignin and cellulose degradation varies between fungal species and wood
53 type. Here we combined genomics, transcriptomics and secretomics to assess the diversity of the
54 enzymatic systems related to wood decay within the Basidiomycota genus *Pycnoporus*. We
55 observed strong conservation in the genome structures and the repertoires of protein coding genes
56 across the four *Pycnoporus* species described to date, despite the species having evolved
57 independently in distinct geoclimatic areas. The *Pycnoporus cinnabarinus*, *Pycnoporus*
58 *coccineus*, and *Pycnoporus sanguineus* strains analysed here had varied abilities to grow on
59 (ligno)cellulosic substrates on agar plate assays. At the onset of the response to (ligno)-cellulosic
60 substrates, the three species secreted however a common set of plant cell wall degrading
61 enzymes. The co-occurrence in the secretomes of H_2O_2 producing enzymes (GMC-
62 oxidoreductases and glyoxal oxidases) with H_2O_2 consuming enzymes (class II peroxidases and
63 AA9 LPMOs) was a common feature of three *Pycnoporus* species, although the genes displayed
64 independent transcriptional regulation. In the three species, cellobiose dehydrogenase-coding
65 genes were co-regulated with at least one AA9 LPMO gene copy, indicative of enzymatic
66 synergy in vivo. This study highlights a conserved core intra-genus enzymatic mechanism behind
67 the wood decaying process.

68

69 **Author Summary**

70 Due to their unique ability to degrade all constituents of wood and litter, including lignin, white-
71 rot fungi are the ultimate decomposers of dead organic matter in forest ecosystems. As such, they
72 play a role in the dynamics of carbon cycling. These saprobes produce and secrete a plethora of
73 enzymes able to degrade the main polymers in plant biomass; cellulose, hemicellulose, pectin and
74 lignin by diverse hydrolytic and oxidative mechanisms. Those fungi are thereby a source of
75 enzymes of interest for sustainable manufacturing aimed at a bioeconomy based on renewable
76 carbon sources. White-rot fungi are notably known for the diversity of enzymatic systems they
77 use for lignocellulose breakdown. However, intra-genus diversity has not been extensively
78 explored. Here we investigated the functional diversity within the genus *Pycnoporus.* We
79 developed a methodology for cross-species comparison of gene expression and identified a
80 conserved set of genes responsive to lignocellulosic substrates that outlines key enzymatic
81 mechanics for wood decomposition activity in these fungi.

82

83 **Introduction**

84 Northern Hemisphere forests and tropical forests play a major role in the carbon cycling
85 through a combination of atmospheric carbon (C) capture by plant photosynthesis, C release by
86 autotrophic (plant) and heterotrophic (predominantly fungal) respiration and C storage in wood
87 and litter Dead Organic Matter (DOM) (Bonan, 2008; Mitchard, 2018; Leonhardt *et al.*, 2019).
88 The dynamics of deadwood decay are related to successions of fungal communities and to
89 lignolytic and cellulolytic activities of wood decayers (Rajala *et al.*, 2012; Ottosson *et al.*, 2014;
90 Valentín *et al.*, 2014; Mäkipää *et al.*, 2017). White-rot (WR) fungi are wood decayers with the
91 capacity to mineralize lignin with ultimate formation of CO_2 and H_2O (Blanchette, 1991). A
92 substantial role for WR fungi in DOM decay in temperate regions was demonstrated by the
93 abundance of WR species and concomitant class II heme peroxidase activities in decomposing

94 logs (Leonhardt *et al.*, 2019). The pivotal role WR fungi play in forest ecosystems has stimulated
95 research efforts to understand the enzymatic mechanisms involved in wood degradation. WR
96 fungi deploy a wide arsenal of hydrolytic and oxidative enzymes to degrade wood and their
97 genomes typically contain genes coding for glycoside hydrolases, carbohydrate esterases and
98 polysaccharide lyases that collectively cleave cellulose, hemicellulose and pectin backbones and
99 lateral chains, and oxidative enzymes that target the highly recalcitrant lignin, crystalline
100 cellulose or cellulose-bound xylan (Floudas *et al*., 2012; Riley *et al*., 2014; Couturier *et al*.,
101 2018). Beyond these shared features, several studies have highlighted significant polymorphism
102 between WR fungi regarding their ability to selectively degrade lignin over cellulose (Hastrup *et*
103 *al*., 2012; Fernandez-Fueyo *et al.*, 2012; Kuuskeri *et al.*, 2015) and in the gene portfolios
104 involved in lignocellulose breakdown (Levasseur *et al*., 2013; Ohm *et al*., 2014; Miyauchi *et al*.,
105 2018). Scarce studies at the intra-genus level have shown that functional diversity between
106 species may arise from diversity in gene content. The preferential occurrence of *Phanerochaete*
107 *carnosa* on softwood was proposed to be related to enrichment in manganese peroxidase and
108 P450 monoogygenase genes as compared to *Phanerochaete chrysosporium* which is mostly
109 found on hardwood (Suzuki *et al.*, 2012). Recently, Dai *et al*. observed remarkable genome size
110 variation across the genus *Auricularia*, which was related to differences in repetitive element
111 contents, gene numbers and gene lengths (Dai *et al*., 2019).
112 Among WR fungi, the genus *Pycnoporus* (Basidiomycota, Agaricomycotina) has been studied for
113 the efficiency of lignin degradation, the capacity to secrete laccases and biotechnological
114 applications related to aromatic compound functionalization, biopolymer synthesis and biomass
115 pre-treatment in the pulp and paper industry (Lomascolo *et al.*, 2002). Four *Pycnoporus* species
116 have been differentiated (Nobles & Frew, 1962; Ryvarden & Johansen, 1980) which form a
117 monophyletic group within the Trametes clade (Justo & Hibbett, 2011). The four species are

118 found in different geo-climatic areas with limited geographical overlap; *Pycnoporus*
119 *cinnabarinus* is widely distributed in the Northern hemisphere, *Pycnoporus coccineus* is found in
120 countries bordering the Indian and Pacific Oceans, *Pycnoporus sanguineus* is found in the tropics
121 and subtropics of both hemispheres, and *Pycnoporus puniceus* is found in paleotropical areas
122 (Nobles & Frew, 1962; Ryvarden & Johansen, 1980). The four species are found on stumps and
123 either standing or fallen trunks of deciduous trees.
124
125 Our aim was to investigate the *Pycnoporus* intra-genus genomic and functional diversity focusing
126 on lignocellulose breakdown. We examined whether independent evolution in distinct geographic
127 areas had led to genomic diversity, and if there was a signature of conserved enzymatic
128 mechanics in terms of transcriptomic and secretomic responses to lignocellulosic substrates. We
129 sequenced the genomes of *P. coccineus*, *P. sanguineus* and *P. puniceus* monokaryotic strains. We
130 overviewed the genomic features among the three species, in comparison to the previously
131 sequenced genome of *P. cinnabarinus* (Levasseur *et al.*, 2014) and to other evolutionarily related
132 wood decay fungi. Then, we captured the transcriptomic and secretomic responses of three
133 *Pycnoporus* species to a panel of cellulosic and lignocellulosic substrates representative of
134 Gramineae and hardwoods. The focus was the early adaptive responses to the substrates in order
135 to minimize inter-species differences influenced by varied growth abilities on the substrates. Our
136 omics integrative approach enabled to identify a common set of lignocellulose degrading
137 enzymes mobilized by the fungi at the initial stage of lignocellulose degradation, leading to
138 discoveries of genus-wide conserved expression patterns indicative of conserved enzymatic
139 synergies.
140
141

142 **Results**

143

144 **Four *Pycnoporus* species share similar genomic features and CAZomes**

145 To assess the genomic diversity in the genus *Pycnoporus*, the genome of *P. coccineus* BRFM 310
146 (herein named Pycco), *P. sanguineus* BRFM 1264 (Pycsa) and *P. puniceus* BRFM 1868
147 (Pycpun) were sequenced and compared to that of *P. cinnabarinus* BRFM 137 (herein named
148 Pycci (Levasseur *et al.*, 2014). The genome size of *P. coccineus*, *P. sanguineus* and *P. puniceus*,
149 ranging from 30 to 36 Megabases (Mb) were in line with that of *P. cinnabarinus* (33.67 Mb) and
150 WR relatives (Table 1; Supp. Information Fig. S1, Table S1). We observed low amounts of repeat
151 sequences (1.8% to 12.3%) and the absence of major rearrangements in the genomes (Fig. S2,
152 S3). The genes coding for mating type, Class II peroxidases, CAZymes, peptidases, GSTs,
153 hydrophobins, proteins from the secretory pathway, the glycosylation pathway, the carbon
154 catabolism pathway and Small Secreted Proteins (SSPs) were inspected by expert annotation
155 (Table S2-S15, Fig S4-S13). We observed a high proportion of conserved protein coding genes
156 across the genomes (82.3% of the *P. cinnabarinus* protein coding genes) and a low proportion of
157 species-specific genes (4 to 5%; Fig. S14). Inspection of mating type genes showed high
158 sequence identity between the alleles of the four *Pycnoporus* species (Table S4). In particular, *P.*
159 *coccineus* BRFM 310 and *P. sanguineus* BRFM 1264 alleles were much more similar to each
160 other than to that of the two other *Pycnoporus* species, in support of the notion that these two
161 species are closely related (Nobles & Frew, 1962; Lesage-Meessen *et al.*, 2011).

162

163 The CAZyme gene repertoires (CAZome) in the three newly sequenced genomes were similar to
164 that of *P. cinnabarinus* (mean 436 CAZymes classified into 108 CAZy families). As a common
165 feature of white-rotters (Hori *et al.*, 2014; Riley *et al.*, 2014), *Pycnoporus* genomes contained a

166 large number of genes coding for PCW-active CAZymes as compared to brown-rotters, which
167 use Fenton chemistry in combination with a limited number of CAZymes for PCW breakdown,
168 or fungi of uncertain rot type, which have intermediate numbers of genes coding for PCW-active
169 CAZyme (Fig. 1, Fig S15-S17). The gene counts for FCW-degrading enzymes was similar to that
170 of other wood decay fungi (Fig. S18). The genomes were rich in Lytic Polysaccharide
171 Monooxygenases (LPMOs) active on crystalline cellulose and β-(1,4)-linked hemicellulose
172 polysaccharides (CAZy family AA9; 13 to 17 gene copies), and in Class II peroxidases active on
173 lignin (in total 9 to 11 gene copies for manganese peroxidases (MnP), versatile peroxidases (VP)
174 and lignin peroxidases (LiP; Fig. S19; Table S5). We observed a high number of predicted
175 secreted oxidoreductases that could act as Auxiliary Activity (AA) enzymes for the oxidative
176 degradation of PCWs. Among them, GMC-oxidoreductases from CAZy subfamily AA3_2 (20 to
177 22 gene copies) have pivotal roles in PCW degradation. Secreted AA3_2s are involved in the
178 generation of H_2O_2, a co-factor for class II peroxidases and LPMOs. AA3_2s also contribute to
179 the oxidation of saccharides and to the redox cycling of aromatic alcohols and quinones. In
180 addition, we identified three glyoxal oxidase gene copies in each of the genomes (AA5_1), which
181 are copper radical oxidases involved in extracellular H_2O_2 production (Fig. S9-S13, Table S6).
182
183 In short, the four *Pycnoporus* genomes were very similar in size, organization and gene content.
184 The repertoires of genes coding for PCW-active enzymes were highly conserved and rich in
185 oxidoreductase enzymes involved in oxidative cleavage of polysaccharide and lignin polymers.
186
187 ***Pycnoporus* species show diverse responses to lignocellulosic substrates**
188 To assess the functional diversity within the genus *Pycnoporus*, we compared the ability of the
189 most closely related species; *P. cinnabarinus*, *P. coccineus* and *P. sanguineus* to grow on a

190 variety of plant-derived carbon sources on agar plate assays. In these conditions, we observed
191 differences in fungal growth on complex lignocellulosic substrates (Fig. 2a, Fig. S20). We next
192 analyzed the early response of the three species to cellulose, wheat straw and woody substrates in
193 agitated liquid culture media and compared their transcriptomes and exo-proteomes (secretomes)
194 collected from the media. Maltose was used as a control, as it was shown not to induce carbon
195 catabolic repression in ascomycete fungi (Brown *et al.*, 2014). Avicel was used as a cellulose-
196 enriched substrate, and wheat straw, pine and aspen were used as representatives of Gramineae,
197 softwood and hardwood respectively. At day 3, most cultures had initiated growth and consumed
198 all initial maltose found in the medium. This time-point was therefore selected to analyze the
199 early response of each species to the substrates (Fig. 3; Fig. S21-S22).
200
201 The global transcriptome responses varied among the strains. Especially, *P. coccineus* showed
202 the highest proportion of regulated genes with up to 7.6% of the genes up-regulated on pine (fold
203 change in transcript abundance ≥4; Fig. 2b). Similarity between the transcriptomes of the three
204 species was assessed by analyzing the differential expression of one-to-one orthologous genes
205 (co-orthologs) after 3-day growth on cellulose, wheat straw or aspen as compared to maltose. To
206 identify co-orthologs, in silico deduced proteomes of the three species were clustered into 13836
207 orthogroups using OrthoFinder, of which 6524 represented co-orthologs. Similar transcript
208 regulations were frequent between co-orthologs of two species. Surprisingly however, we
209 observed poor conservation of transcript regulation of co-orthologs across the three species,
210 including for genes with high transcription induction or repression on particular carbon sources
211 (Fig. 4a). We examined the transcript read counts of CAZyme coding genes. Transcriptome
212 profiles were more similar within the species cultured under the different conditions than between
213 the species cultured on the same substrates (Fig. S23). Also, we observed in each species that

214 approximately half of the regulated CAZymes (41-51%) were up-regulated in response to
215 cellulose, wheat straw and aspen, highlighting the presence of core regulations to
216 (ligno)cellulosic substrates with diverse compositions (Fig. S24).
217
218 **Conserved gene regulations in response to lignocellulosic substrates**
219 In search for conserved enzymatic mechanisms involved in the initiation of lignocellulose
220 breakdown, we analyzed the gene sets sharing similar transcription profiles at the onset of the
221 response to cellulose, wheat straw or aspen. We focused on genes coding for proteins typically
222 found in fungal secretomes, i.e. CAZymes, peptidases, small secreted proteins and hydrophobins
223 (Alfaro *et al.*, 2014). A total of 2,227 manually curated genes from the genomes of *P.*
224 *cinnabarinus*, *P. coccineus* and *P. sanguineus* were analyzed. In order to combine all RNA-seq
225 data in a single cross-species analysis, transcript read counts were normalized using the DESeq2
226 package (Love *et al.*, 2014), the normalized read counts were log2 transformed and subjected to
227 removal of batch effects and to quantile normalization. We checked the impact of each
228 normalization step on the distribution of the data (Fig. S25-S28). Inspecting the one-to-one co-
229 orthologs from this set of genes (405 orthology groups), we observed low conservation of the
230 normalized transcript read counts across the species, except for growth on maltose and cellulose,
231 and for *P. coccineus* and *P. sanguineus* grown on aspen, indicating that complex lignocellulosic
232 substrates induced more diverse responses across the species (Fig. 4b).
233
234 We grouped genes with similar transcript profiles into nodes (clusters of co-regulated genes)
235 using the Self-organizing maps (SOM) unsupervised learning method. SOM is a data-driven
236 clustering method constructing a topographic organization of nodes in which neighbouring nodes
237 share similar transcriptome patterns, and thereby alleviates the requirement for arbitrary

238 clustering thresholds. In addition, SOM allows the co-localization on the SOM map of genes with
239 similar transcript levels on the four analyzed substrates and provides indication for gene co-
240 regulation within each strain. We produced 72 nodes containing on average 31 genes per node
241 (Fig. 5a,b). Secretomic data (Tables S16-S18) were combined to the SOM map to integrate gene
242 transcription and protein secretion information.
243
244 We first examined the transcriptional response to cellulose. We identified 19 nodes containing
245 250 genes highly transcribed (mean normalized log2 read count ≥ 12) or up-regulated (mean fold
246 change ≥ 4 compared to maltose; Table S19). Inspecting the gene content for these nodes (e.g.
247 Fig. 5c), we looked for shared gene differential expression across the three species. In the three
248 species we found up-regulation for CAZyme coding genes involved in β-(1,4)-glucan linkage
249 breakdown including endoglucanases from family 5 subfamily 5 (GH5_5), GH45 and GH131,
250 cellobiohydrolases (GH6, GH7), cellobiose dehydrogenases (CDH) and AA9 LPMOs (Fig. 6a).
251 Also, there was a wide panel of enzymes active on hemicelluloses from families GH10, GH74,
252 GH5_7, GH12, GH115, CE1, CE15, CE16, enzymes active on pectin CE8, GH28 and enzymes
253 with promiscuous activities on glycosidic bounds GH1 and GH3. Among the 20 CAZyme
254 families identified, 14 were represented by co-orthologous genes from the three species, showing
255 conservation of their regulation in response to cellulose across the genus. Globally, 50% of the
256 enzymes encoded by the up-regulated genes were detected in the culture medium (Fig. 6a).
257
258 To identify the genes specifically regulated for the complex lignocellulosic substrates, we
259 selected genes up-regulated on wheat straw or aspen, not on cellulose, as compared to maltose.
260 There were 13 nodes including 225 genes that met at least one of the following criteria; (i)

261 average normalized log2 read counts >12 on wheat straw or aspen and <12 on maltose and
262 cellulose, or (ii) mean fold change ≥4 on wheat straw or aspen, and ≤4 on cellulose as compared
263 to maltose. From these nodes we identified the gene families with homologs among the three
264 species. These included genes coding for enzymes that target hemicelluloses (CE16 and CE4
265 acetylesterases, GH2 β-mannosidases or β-glucuronidases, GH16 xyloglucan hydrolase, GH27 α-
266 galactosidases, GH30 β-glucosidase/β-xylosidase, GH51 α-L-arabinofuranosidases and GH31 α-
267 xylosidases/α-glucosidases), enzymes that target pectin (GH28 polygalacturonases, PL14_4 β-
268 1,4-glucuronan lyases), AA1_1 laccases and AA3_2 GMC-oxidoreductases (Fig. 6b, Table S20).
269 Genes coding for glycoside hydrolases active on the fungal cell wall (GH18 chitinases, GH76 α-
270 mannanases) and peptidases were also specifically up-regulated in the three strains.
271
272 In response to lignocellulosic substrates, we observed up-regulation of the various genes for class
273 II heme peroxidases (AA2), AA1_1 laccases, AA3 GMC-oxidoreductases, and AA5_1 glyoxal
274 oxidases (Fig. 7a). The genomes of the three strains encoded the three types of class II
275 peroxidases involved in lignin breakdown; MnP, LiP and VP (Table S5). Orthologous MnP and
276 LiP genes (defined by protein sequence phylogeny; Fig. S6) did not show conserved regulation
277 across the strains (Fig. 7b). The highest induction factors were found in *P. coccineus* and an MnP
278 (protein ID #1468611) and a LiP (#1431101) coding gene reached 800- and 1500-fold induction
279 on aspen respectively. For the three strains, we observed no up-regulation of the VP coding
280 genes. Putative intracellular AA3s (non secreted AA3_2s, AA3_3 alcohol oxidases, AA3_4
281 pyranose oxidases) were also up-regulated among the three strains, suggesting a conserved role
282 for GMC-oxidoreductases in the cycling of redox mediators in the intracellular compartment.
283

284 **Co-regulated genes indicative of enzymatic synergies**

285 We investigated potential enzymatic synergies conserved in the three *Pycnoporus* strains through
286 co-regulated gene transcription with co-secreted corresponding proteins. In each species, one
287 single cellobiose dehydrogenase (CDH) gene was present in the genome, which shared a similar
288 transcription pattern with AA9 LPMO coding genes. CDHs are secreted by many fungal
289 saprotrophs in response to cellulose. *In vitro*, CDHs behave as electron donors for AA9 LPMOs
290 and boost the LPMO activity (reviewed in Berrin *et al.*, 2017). CDHs are composed of a flavin
291 adenine dinucleotide (FAD)-binding dehydrogenase domain (AA3_1) connected via a flexible
292 linker to a haem b-binding cytochrome domain (Cytb; AA8). The three strains had AA9 LPMO
293 genes co-regulated with the *cdh* genes (nodes 39, 57 and 58; Fig. 5c, Fig. 8). As expected, these
294 AA9 LPMO and CDH genes were up-regulated on cellulose and the corresponding proteins were
295 identified in the secretomes. These results suggest CDH as a biologically relevant electron donor
296 for AA9 LPMOs.
297
298 Genes coding for predicted class II peroxidases (AA2s) were specifically up-regulated on wheat
299 straw or aspen, but not on cellulose (Fig. 7b). Node 40, 41 and 32 contained three *mnp* genes
300 from *P. coccineus* co-regulated with the three predicted glyoxal oxidase genes identified in this
301 genome, suggesting that glyoxal oxidases might provide these MnPs with the H_2O_2 required for
302 their activity (Fig. 8). In contrast, *P. cinnabarinus* and *P. sanguineus* did not show any co-
303 regulations between class II peroxidases and glyoxal oxidases at this time point.
304

305 **Differentially regulated genes indicative of detoxification**

306 Lignocellulose degradation leads to the release of toxic degradation products and extractives
307 (Mäkelä *et al.*, 2014; Fernández-González *et al.*, 2018). We investigated if the regulation of genes

308 involved in detoxification was conserved across the three *Pycnoporus* species. We examined
309 genes coding for putative glutathione transferases (GST) in the cross-species SOM analysis.
310
311 One single GST from class GTT2 of *P. cinnabarinus* and *P. sanguineus* was selected from the
312 nodes filtered for up-regulation on wheat straw or aspen (Table S20). One isoform of this class
313 from *Phanerochaete chrysosporium* has been functionally characterized and has been shown to
314 reduce high-molecular weight peroxides (Morel *et al.*, 2013; Thuillier *et al.*, 2014). In contrast, a
315 total of four GST genes of *P. coccineus* were regulated on the tested substrates; two GST class A
316 (FuA) genes were up-regulated on all the tested substrates (Table S19) whereas one S-
317 glutathionyl hydroquinone reductase (GHR) and one GST Omega were specifically up-regulated
318 in response to wheat straw or aspen (Table S20). For the case of *P. chrysosporium*, these classes
319 of GSTs have potential roles in the detoxification of wood-derived molecules acting as ligandins
320 for wood extractives or as catalysts for deglutathionylation of various substrates, including
321 hydroquinone conjugates and terpenes (Mathieu *et al.*, 2012; Meux *et al.*, 2013; Morel *et al.*,
322 2013). These findings suggest that different GST-related detoxification systems may exist in *P.*
323 *coccineus*.
324
325 Cytochrome P450 (CytP450) monooxygenases are commonly involved in the first step of
326 eliminating toxic molecules including molecules released from lignocellulose. CytP450 can
327 initiate the modification of these molecules via hydroxylation, epoxidation or monooxygenation.
328 The genomes of *P. cinnabarinus*, *P. coccineus* and *P. sanguineus* contain 107, 132 and 113
329 predicted CytP450 respectively. Inspection of the transcript profiles for these genes showed that
330 *P. coccineus* had the highest number of up-regulated CytP450 genes in response to the tested
331 substrates (17 genes) compared to *P. cinnabarinus* and *P. sanguineus* (14 and 6 up-regulated

332 genes respectively). Some CytP450 genes were commonly up-regulated in at least two species,
333 which were from families CYP63, CYP5035, CYP5139, CYP5144 and CYP5150 According to
334 the Fungal cytochrome P450 database (Moktali *et al.*, 2012; Table S13). Of these, CYP63,
335 CYP5139 and CYP5144 were shown to be active on multiple xenobiotic compounds such as
336 polycyclic aromatic hydrocarbons (PAHs), alkylphenols and alkanes (Ichinose, 2013; Syed *et al.*,
337 2014; Qhanya *et al.*, 2015). CYP5035s oxidize plant chemicals (i.e. resin and flavonoids), and the
338 number of gene copies for CYP5035 and CYP5150 expanded in basidiomycetes that grow on
339 wood or litter (Syed *et al.*, 2014). Our results suggest that these Cyp450 families could be
340 differently utilised by the strains for detoxification of molecules released from the lignocellulosic
341 substrates.
342
343 **Discussion**
344
345 **Phenotype diversity outreaches diversity of the predicted proteome across the genus**
346 ***Pycnoporus***
347 The four *Pycnoporus* species studied here are found in different geo-climatic areas (Nobles &
348 Frew, 1962; Ryvarden & Johansen, 1980) covering the Northern hemisphere (*P. cinnabarinus),*
349 countries bordering the Indian and Pacific Oceans (*P. coccineus),* or the tropics and subtropics of
350 both hemispheres (*P. sanguineus*) and paleotropical areas (*P. puniceus*). Accordingly, we
351 collected the parental strains of the monokaryons used in this study from Europe, China, South
352 America and Indonesia.
353
354 We found that the genomes sequenced from geographically distant strains did not show any
355 major rearrangements in structure and protein coding gene composition. In particular, we

356 observed strong conservation of CAZomes over the four species. Our findings suggest that
357 geographical isolation of the species did not lead to significant genome diversity. Limited
358 genome plasticity within the genus *Pycnoporus* was congruent with low amounts of transposable
359 elements (1.8% to 12.3%). The observed similarities in CAZyme gene portfolios could be related
360 to the fact that the four *Pycnoporus* species can accommodate similar wood types, which include
361 trunks or dead logs from broad-leaved trees and occasionally from coniferous trees.
362 Alternatively, the conservation in genome structure and gene repertoires could be due to recent
363 speciation as suggested by the high sequence identity between mating type genes of the four
364 species, particularly between *P. coccineus* and *P. sanguineus*.
365
366 Despite conserved CAZomes, the *P. cinnabarinus*, *P. coccineus,* and *P. sanguineus* strains
367 analyzed here showed varied abilities to grow on (ligno)cellulosic substrates on agar plate assays.
368 The comparison of the early responses of the fungi to the substrates showed that gene
369 transcription profiles were more correlated with the species than with the substrates. A similar
370 trend was observed in the WR fungus *Pleurotus ostreatus* among monokaryotic strains issued
371 from a same parental dikaryotic strain. In *P. ostreatus*, these differences in gene regulation were
372 partly attributed to the presence of Transposable Elements (TEs) near the differentially regulated
373 genes (Castanera *et al.*, 2016). A closer analysis of TE localization or methylation status of each
374 monokaryon haplotype would be helpful to understand the transcription regulation we observed.
375

376 **Key enzymatic players for wood decay activity**

377 We identified a set of CAZyme genes with shared transcriptional regulations in response to
378 cellulose among three *Pycnoporus* species. We found enzymes from a sub-set of these genes
379 were commonly present in the secretomes of the three species, strengthening a role for these

380 enzymes as key players in lignocellulose breakdown by *Pycnoporus* fungi. These included genes
381 coding for enzymes active on; β-1,4-glucan linkages (GH5_5 and GH131 endo-β-1,4-glucanases,
382 GH6 and GH7 cellobiohydrolases and AA9 LPMOs); the hemicellulose backbone (GH10
383 endoxylanases, GH12 and GH74 xyloglucan hydrolases, GH5_7 β-mannosidases) or branched
384 groups (CE1 feruloyl esterases); and pectin (GH28 polygalacturonases). In the presence of
385 lignocellulosic substrates, additional genes were commonly induced and the corresponding
386 enzymes secreted such as GH16 xyloglucan hydrolase, GH27 β-galactosidases, GH51 α-L-
387 arabinofuranosidases, AA1_1 laccases, and peptidases for breaking down hemicelluloses.
388
389 The detection of wood decay activity in environmental samples largely relies on a few enzymatic
390 assays, including 1,4-β-glucosidase**,** cellobiohydrolase, 1,4-β-xylosidase, laccase and manganese
391 peroxidase (e.g. Valentín *et al.*, 2014; Angst *et al*., 2018). The additional conserved set of genes
392 we found in this study could further extend the existing list of functional markers for wood
393 decomposition activity by *Pycnoporus* sp.
394
395 **AA9 LPMOs and their enzymatic partners**
396 AA9 LPMOs are known as key players in oxidative cellulose depolymerization by wood decay
397 fungi (Berrin *et al.*, 2017). AA9 LPMOs oxidatively cleave glycosidic chains at the cellulose
398 surface thereby creating new substrate binding sites for hydrolytic cellulases (Tandrup *et al.*,
399 2018). Although a large number of gene copies for AA9 is a common feature of the WR fungal
400 genomes (Nagy *et al.*, 2017), exact roles for the members of the AA9 gene family during wood
401 decay remains to be elucidated. We identified 17, 16 and 11 gene copies coding for AA9 LPMOs
402 in *P. cinnabarinus*, *P. coccineus* and *P. sanguineus*, from which 11 (64%), 13 (81%) and 10

403 (90%) respectively were up-regulated (≥ 4 fold induction) during the early response to cellulose-
404 containing substrates. In each species, several AA9 LPMO gene copies shared similar
405 transcription profiles, and several AA9 LPMOs were concomitantly secreted, which might act
406 simultaneously on the substrate.
407
408 The identification of enzymatic partners for LPMOs is a very active field of research (Garajova *et*
409 *al.*, 2016; Kracher *et al.*, 2016; Bissaro *et al.*, 2018; Sützl *et al.*, 2018). Looking at conserved co-
410 regulations across the three species, we identified CDHs as invariably co-regulated with at least
411 one AA9 LPMO gene copy. CDHs have been shown to reduce O_2 and generate H_2O_2 which can
412 fuel, via the Fenton reaction, the production of hydroxyl radicals that disrupt lignocellulose
413 polymers (Hyde & Wood, 1997). More recently, CDHs have been shown to activate AA9
414 LPMOs *in vitro* through electron transfer (Tan *et al.*, 2015) and it has been proposed that CDHs
415 could also activate AA9 LPMOs through the generation of H_2O_2, a co-substrate for the enzyme
416 (Bissaro *et al.*, 2017). Our findings support the hypothesis of synergy between AA9 LPMOs and
417 CDHs *in vivo* as a conserved mechanism for cellulose degradation in the genus *Pycnoporus* spp.
418
419 **Co-secretion of extracellular H_2O_2-generating and -consuming enzymes**
420 Extracellular H_2O_2 is a central factor in oxidative lignocellulose breakdown. It was proposed that
421 WR fungi could avoid oxidative damages due to H_2O_2 accumulation in the vicinity of the hyphae
422 by the co-secretion of H_2O_2-generating and H_2O_2-consuming enzymatic partners (Bissaro *et al.*,
423 2018). Some secreted GMC-oxidoreductases such as glucose dehydrogenases and aryl-alcohol
424 quinone oxidoreductases (AA3_2) are able to prime AA9 LPMO activity *in vitro* (Garajova *et al.*,
425 2016; Sützl *et al.*, 2018). Similarly, glyoxal oxidases can generate H_2O_2 and prime MnP and LiP
426 activity in vitro (Kersten & Cullen, 2014). A biological relevance for these synergetic activities

427 was previously suggested from the co-occurrence in fungal secretomes of class II peroxidases
428 with GMC-oxidoreductases (Fernandez-Fueyo *et al.*, 2012; Salvachúa *et al.*, 2013; Hori *et al.*,
429 2014; Fernández-Fueyo *et al.*, 2016; Kuuskeri *et al.*, 2016; Miyauchi *et al.*, 2018; Moody *et al.*,
430 2018) or glyoxal oxidases (Kersten, 1990; Martinez *et al.*, 2009; Salvachúa *et al.*, 2013; Hori *et*
431 *al.*, 2014; Kuuskeri *et al.*, 2016; Daou & Faulds, 2017; Miyauchi *et al.*, 2018). We confirmed the
432 co-occurrence in the secretomes of GMC-oxidoreductases and glyoxal oxidases with class II
433 peroxidases and AA9 LPMOs as a common feature of three *Pycnoporus* species. However, we
434 detected no evidence for co-regulation of the corresponding genes at the transcript level except
435 for a set of three MnPs and two glyoxal oxidase encoding genes in *P. coccineus*. We hypothesize
436 that independent tight regulations of gene expression for H_2O_2-generating and H_2O_2-consuming
437 enzymes at the transcription level, enable these fungi to rapidly adapt extracellular reactive
438 oxygen species concentrations and avoid oxidative damage to the hyphae.
439
440 In summary, we combined fungal omics to characterize conserved responses and inter-species
441 diversities in response to lignocellulosic substrates across the genus *Pycnoporus*. It would be
442 possible to explore a wide range of wood decayers using our integrative omics approach. The
443 next step forward would be to clarify the ecological roles of wood decayers in forest microbiota,
444 leading to the improvement of forest management and innovative carbon recycling technologies,
445 which contribute to the prevention of the global climate change and crisis.
446
447 **Materials and Methods**
448
449 **Genome sequencing and assembly**

450 The monokaryotic strains *P. coccineus* BRFM 310, *P. sanguineus* BRFM 1264 and *P. puniceus*
451 BRFM 1868 were generated after fruiting of the parental strains BRFM 66 (IMB WOO6-2),
452 BRFM 902 and BRFM 1856 respectively, as described previously (Lomascolo *et al.*, 2002;
453 Supplementary Information). All strains were maintained at the International Centre of Microbial
454 Resources (CIRM; https://www6.inra.fr/cirm/). The *P. coccineus* BRFM 310 genome was
455 sequenced using the Illumina platform (99.4X) and assembled with AllPathsLG version R46652
456 (Gnerre *et al.*, 2011) (GenBank accession number: NCSW00000000). The *P. sanguineus* BRFM
457 1264 genome was sequenced using 454 (16.8X) and Solexa (87X) technologies and assembled
458 with CABOG (Miller *et al.*, 2008) (GenBank accession number: VOCM00000000). The *P.*
459 *puniceus* BRFM 1868 genome was sequenced using PacBio technology (97X) and assembled
460 with FALCON, improved with finisherSC (Ka-Kit *et al.*, 2015), polished with Quiver (GenBank
461 accession number: VICQ00000000). The three genomes were annotated using the JGI annotation
462 pipeline (Grigoriev *et al.*, 2014), which takes multiple inputs (scaffolds, ESTs, and known genes)
463 and runs several analytical tools for gene prediction and annotation, and deposits the results in the
464 JGI Genome Portal MycoCosm (http://genome.jgi.doe.gov/fungi). The previously sequenced and
465 annotated genome of *P. cinnabarinus* BRFM 137 (Levasseur *et al.*, 2014) was also deposited in
466 Mycocosm.

467

468 **Comparative genomic analysis**

469 Genome completeness with single copy orthologues was calculated using BUSCO v3.0.2 with
470 default parameters (Sima *et al.*, 2015). The coverage of transposable elements in genomes was
471 calculated using a custom pipeline Transposon Identification Nominative Genome Overview
472 (TINGO; Morin *et al.*, 2019). The counts for plant cell wall (PCW)-degrading enzymes, predicted
473 secreted auxiliary activity enzymes and fungal cell wall (FCW)-degrading enzymes were

combined and visualized with custom R scripts, Proteomic Information Navigated Genomic Outlook (PRINGO; available on request) incorporating R packages ggplot2, ggtree, and egg (Wickham, 2009; Auguie, 2017; Yu *et al.*, 2017).

Expert functional annotations

Genes from the *A* and *B* mating type loci were identified and manually curated as described in Kues *et al.*, 2015. CAZymes and auxiliary activity enzymes (AA) were annotated as in Lombard *et al.*, 2014. Gene models from AA2 Class II peroxidases, AA3_2 glucose-methanol-choline (GMC)-oxidoreductases and AA5 copper radical oxidases were further inspected by sequence-by-sequence exhaustive analysis and phylogenetic analysis. Peptidases were annotated using Blastx searches of gene models against InterPro and MEROPS databases (Mitchell *et al.*, 2015; Rawlings *et al.*, 2016) followed by manual curation. Glutathione transferases (GST)-coding genes were annotated with a combination of automated blastp using functionally characterized GST sequences from *P. chrysosporium* (Mathieu *et al.*, 2013; Meux *et al.*, 2013; Roret *et al.*, 2015), phylogenetic analysis and active site comparison. Genes coding for hydrophobins, laccases, the secretory pathway and carbon catabolism were also manually inspected. Proteins were predicted secreted if they fulfilled three conditions: 1) presence of a secretion signal peptide, 2) absence of endoplasmic reticulum retention motif, and 3) absence of transmembrane helix outside the signal peptide. Predicted Small Secreted Proteins (SSPs) were predicted secreted proteins <300 amino acids (Pellegrin *et al.*, 2015). Detailed analyses of expert annotations are provided in the Supp. Information file.

Cultures

497 Media for cultures on agar plates contained diammonium tartrate (1.84 g/l), yeast nitrogen base
498 (0.17 g/l), agar (15g/l) and were supplemented with either maltose (20g/l), Avicel PH 101 (Fluka)
499 (15g/l), ground and sifted wheat straw fragments < 2 mm (15g/l), *Pinus halepensis* pine wood
500 fragments < 2 mm (15g/l) or 1 mm *Populus tremuloides* Wiley-milled aspen (15g/l). The plates
501 were inoculated with one fungal disk (4 mm diameter) of 7-day-old mycelia and incubated at
502 30°C. Liquid cultures were maintained at 30°C in a rotary shaker at 120 rpm in 250-ml
503 Erlenmeyer flasks containing 100 ml of culture medium (Supp. Information) supplemented with
504 either maltose (20 g/l), Avicel (15 g/l), wheat straw fragments (15 g/l), *Pinus halepensis* wood
505 fragments (15 g/l) or *Populus tremuloides* fragments (15 g/l). Each culture was done in triplicate.
506 Inoculums of the liquid cultivations were prepared as described in Herpoël *et al.*, 2000.
507
508 **Integration of transcriptome and secretome profiles**
509 LC-MS/MS analysis of the secretomes was performed as described in Navarro et al., 2014.
510 Briefly, 10 µg of diafiltered proteins were loaded on SDS-PAGE gels and allowed to migrate on
511 a 0.5 cm length. Each lane was cut into two slices for in-gel digestion according to a standard
512 trypsinolysis protocol. On-line analysis of the peptides was performed with a Q-exactive mass
513 spectrometer (Thermo Fisher Scientific, United States), using a nanoelectrospray ion source.
514 Protein identification was performed by querying MS/MS data against the genome of *P.*
515 *cinnabarinus* BRFM 137, *P. coccineus* BRFM 310 or *P. sanguineus* BRFM 1264, together with
516 an in-house contaminant database, using the X!Tandem software (X!Tandem Cyclone, Jouy en
517 Josas, France). All peptides that matched with an E-value lower than 0.05 were parsed with
518 X!Tandem pipeline software. Proteins identified with at least two unique peptides and a log (E-
519 value) lower than -2.6 were validated.

520 Total RNA was extracted from 100 mg tissue ground with FastPrep Lysis Matrix A (MP
521 Biomedicals) in 1 ml TRIZOL (Ambion). Nucleic acids were precipitated with isopropanol,
522 resuspended in water and treated with RNase-Free DNase I (QIAGEN). Total RNA was
523 precipitated with LiCl and resuspended in DEPC-treated water. RNA quantity and quality were
524 determined using the Experion RNA StdSens kit (QIAGEN). The transcriptome response of *P.*
525 *sanguineus* to pine could not be analyzed because of poor quality of the extracted RNAs. Double
526 stranded cDNAs were synthesized from PolyA RNA and fragmented (200-300bp) before
527 construction of the sequencing libraries (Kapa Library Amplification Kit; Kapa Biosystems).
528 Sequencing was done on the Illumina HighSeq-2500 JGI platform generating paired end reads of
529 150bp each. Paired end 150 bp Illumina reads were trimmed for quality and aligned to the
530 corresponding genome using TopHat 2 with only unique mapping allowed (Kim *et al*., 2013).
531 Gene models for which the mean raw read counts were inferior to 5 were considered as not
532 transcribed and their read counts were changed to 0.

533 The counts of mapped Illumina reads from biological triplicates of each growth condition
534 (GEO accession number GSE82486) were normalized with the DESeq2 package and log2
535 transformed (Love et al., 2014). The normalized read counts of genes coding for CAZymes,
536 peptidases, hydrophobins and SSPs from *P. cinnabarinus* BRFM 137, *P. coccineus* BRFM 310
537 and *P. sanguineus* BRFM 1264 were retrieved and combined by conducting; (i) removal of batch
538 effects with Combat function in SVA package (Leek et al., 2012); and (ii) quantile normalization
539 with the preprocessCore package (Bolstad, 2019). We used Self-Organizing Map (SOM) to group
540 genes into nodes according to similar transcript patterns obtained from the different substrate
541 conditions. Self-organising maps were constructed with the R package kohonen (Wehrens &
542 Buydens, 2007). The genes showing similar transcription levels were sorted and grouped into
543 nodes of SOMs. It was empirically found that about 35 genes in a single node of the SOM gave

544 the best resolution of the gene clusters. In terms of the standard formula "X * sqrt (N)" to
545 calculate the number of map units, where N was the number of the rows/genes of the data, X was
546 1.5. The number of iterations (epochs) was 100 times more than the map units to minimise the
547 mean distance between the weights of the neighbouring nodes. The default initialisation, learning
548 rate, and radius were used. Hexagonal SOM models were constructed. The mean reads (>12 log2)
549 of the nodes (grouped genes) with the replicates combined were calculated for each substrate.
550 We integrated SOM with the experimentally detected secretomic information using Self-
551 organizing map Harboring Informative Nodes with Gene Ontology (SHIN+GO; Miyauchi *et al.*,
552 2016, 2017, 2018).
553

554 **Transcription regulation of co-orthologous genes**

555 One-to-one orthologous genes from *P. cinnabarinus* BRFM 137, *P. coccineus* BRFM 310 and *P.*
556 *sanguineus* BRFM 1264 were retrieved using orthoFinder v. 2.3.8 (Emms & Kelly, 2019).
557 Heatmaps were created on the log2 fold change of transcript read counts in each growth condition
558 as compared to growth on maltose after DESeq2 normalization using the "Heatmap" function
559 from the package "ComplexHeatmap" v1.10.1 in R. Pairwise comparisons of gene expression
560 based on Pearson correlation coefficients among all replicates was performed on CAZyme,
561 peptidase, hydrophobin and SSP co-orthologs after read count normalization by DESeq2, batch
562 effect removal and quantile normalization "cor" function in R and visualized as heatmap with R
563 package, ggplot2.
564

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577

578 **Author contribution**

579 All authors contributed the intellectual input and assistance to this study. MNR and IVG acquired
580 financial support and provided overall direction of the project. MNR, AF, JGB, LLM, DN
581 conceived the work. SM, HH, MNR, ED performed the work, analyzed the results and wrote the
582 manuscript. LLM, DC, SG, MH, FP, prepared the biological material. AL, SA, KB, KML, CD,
583 JM, CK, AL, JP, RR, MH, EM obtained and processed the sequence data. DC acquired the
584 proteomic data. DC, RPdV, AL, ACG, BH, KSH, UK, WL, MRM, ATM, MMR, AFJR, HABW,
585 FJRD, ER, IVG and MNR did the expert functional annotations of the genes and analyzed cross-
586 genome comparisons.

587

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834

835 **Figure legends**

836 **Fig. 1.** Gene counts for CAZyme domains of plant cell wall degrading enzymes (PCWDEs) and
837 fungal cell wall degrading enzymes (FCWDEs). The bar plots show the total count of genes
838 including PCWDE and FCWDE (left); and the ratio of PCWDE to FCWDE (right). The counts
839 for Auxiliary Activity enzymes that could contribute to PCW degradation include AA1_1
840 laccases and predicted secreted AA3, AA4 and AA5. The counts for PCW-active LPMOs include
841 AA9, AA13, AA14 and AA16. Enzymes active on cellulose, hemicellulose or pectin were
842 classified according to Figs. S15-S17.

843 **Fig. 2**. Phenotype polymorphism across three *Pycnoporus* species. **a.** Agar plates after 6 weeks
844 cultivation on ground wheat straw, pine or aspen. *P. cinnabarinus* BRFM 137 did not develop
845 mycelium on pine and aspen. The white dots formed by *P. sanguineus* on pine and aspen are
846 arthrospores indicating that the fungus stopped growing to form dormant structures. **b.**
847 Percentage of regulated genes after 3 Day growth in liquid cultures on Avicel (AVI), wheat straw
848 (WS), pine or aspen as compared to maltose (|fold change| ≥ 4) in the three *Pycnoporus* strains.
849 No RNASeq data was available for *P. sanguineus* grown on pine.

850 **Fig. 3.** Cross-species comparison of the early response of three *Pycnoporus* species to
851 lignocellulosic carbon sources.

852 **Fig. 4.** Global transcriptome similarity between co-orthologous genes from *Pycnoporus*
853 *cinnabarinus*, *Pycnoporus coccineus* and *Pycnoporus sanguineus*. **a.** Heatmap of changes in
854 transcript read counts (log2 fold change) after 3-day growth on each carbon source as compared
855 to maltose for 6524 groups of 1-to-1 co-ortholog genes. **b**. Pearson correlation coefficient for the

856 normalized transcript read counts in each growth condition for the 405 1-to-1 co-ortholog
857 CAZyme, peptidase, small secreted protein and hydrophobin genes identified in the genomes.
858 The comparisons of the response of each species to various substrates are highlighted in black
859 boxes. Cross-species comparisons on a same substrate are highlighted in blue boxes. (M: maltose;
860 Avi: Avicel; WS: wheat straw; Asp: aspen).

861 **Fig. 5.** Clustering of genes coding for predicted CAZymes, peptidases, small secreted proteins
862 and hydrophobins in three *Pycnoporus* strains according to their transcription profile on maltose
863 (M), Avicel (Avi), wheat straw (WS) and aspen (Asp). **a.** Self-organizing map (SOM) clustering
864 resulted in 72 nodes with average 31 genes per node. Nodes containing genes highly transcribed
865 or up-regulated on cellulose (blue), aspen (green) or wheat straw (orange) as compared to maltose
866 are highlighted. **b.** Hierarchical clustering of the nodes according to the averaged normalized
867 transcript read counts on each carbon source. **c.** Gene content and transcript profiles of nodes 57,
868 39 and 58. AA8-AA3-1: cellobiose dehydrogenase.

869 **Fig. 6.** Shared expression regulation of CAZyme genes across the three *Pycnoporus* species. **a.**
870 Numbers of genes with shared differential expression on cellulose and numbers of proteins
871 secreted during growth on cellulose. **b.** Numbers of genes with shared specific differential
872 expression on lignocellulosic substrates, not on cellulose. The numbers of orthologous groups of
873 genes with conserved transcription regulation and secretion across the three species are indicated.

874 **Fig. 7.** Regulation of Auxiliary Activity enzymes in response to various substrates. **a.** Numbers
875 up-regulated genes (left panel) and secreted enzymes (right panel) in response to wheat straw,
876 pine or aspen in each species. **b.** Hierarchical clustering of Class II peroxidase transcript profiles.
877 Groups of orthologous genes are indicated in brackets and labelled with colors. POD: Class II

878 Peroxidase, Aox: Alcohol Oxidase, Pox: Pyranose oxidase, Glox: Glyoxal oxidase, CCP: class I
879 peroxidase.

880 **Fig. 8.** Conserved co-regulations of CDH and AA9 LPMO genes in *P. cinnabarinus*, *P.*
881 *coccineus* and *P. sanguineus* and co-regulations of MnP and GLOX coding genes in *P.*
882 *coccineus*. Transcript levels are expressed as log2-transformed read counts after 3 day-growth on
883 maltose (M), Avicel (Avi), wheat straw (WS) or aspen (Asp). CBM: carbohydrate-binding
884 module.

885

886 **Supporting information**. Supporting materials and methods, expert gene annotations and
887 supporting data on cross-species comparative omics.

888 Table 1. Features of *Pycnoporus coccineus* BRFM 310, *Pycnoporus puniceus* BRFM 1868 and
889 *Pycnoporus sanguineus* BRFM 1264 genome assemblies and annotations. The reliability of gene
890 structural annotations was assessed using Universal Single-Copy Orthologs (BUSCO). The
891 genome of *Pycnoporus cinnabarinus* BRFM 137 (Levasseur *et al*., 2014) is indicated for
892 comparison.

	P. cinnabarinus	*P. coccineus*	*P. puniceus*	*P. sanguineus*
Genome size (Mbp)	33.67	32.76	30.26	36.04
Number of Contigs	2036	469	105	2046
Number of Scaffolds	784	222	105	657
Scaffold N50	54	20	12	35
Scaffold L50 (Mbp)	0.17	0.47	0.79	0.32
TE Coverage (%)	8.15	1.8	12.33	4.91
TE Coverage (Mbp)	2.74	0.59	3.73	1.77
Number of predicted proteins	10442	12690	10050	14165
BUSCO Complete protein sequences	1268	1321	1309	1293
BUSCO Fragmented protein sequences	32	7	14	21
BUSCO Missing protein sequences	35	7	12	21

893