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14 **Identification of genes differentially expressed during the interaction between the plant symbiont**
15 ***Suillus luteus* and two plant pathogenic allopatric *Heterobasidion* species**
16

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27

28 **Abstract**

29 The effects of biological invasions by non-native species have been widely studied in terms of
30 environmental, economic, and human health impacts. However, little is known on the consequences that
31 non-native plant pathogens may determine on host plant symbionts, such as ectomycorrhizal (ECM) fungi. In
32 this study, interactions between *Suillus luteus*, an ECM fungus of pine trees, and the allopatrically
33 differentiated fungal pathogens of pines *Heterobasidion irregulare* and *H. annosum* were investigated in dual
34 culture by morphological and gene expression analyses. Growth of *S. luteus* was inhibited by the both
35 *Heterobasidion* species, but based on statistical analysis growth inhibition was due to the isolate rather than
36 to the species. The expression analysis on genes related to cell wall hydrolytic enzymes and hydrophobins,
37 putatively involved in the fungus-fungus interaction, allowed to identify significantly up- and down- regulated
38 genes both in the symbiont and in the pathogens. Based on the transcript analysis, it was not possible to
39 distinguish the impact of the two pathogenic species on the ECM fungus. The only exception was a *S. luteus*
40 gene coding for a putative chitinase (*SIGH18_8356*) that was found to be differentially regulated during
41 interaction with *H. irregulare* compared to *H. annosum*.

42

43 **Keywords:** ectomycorrhizal fungus, fungal pathogen, dual culture, gene expression, cell wall, phylogeny

44

45 **Introduction**

46

47 Non-native invasive organisms stand among the main elements of global change and are playing a role in
48 the biodiversity loss, ecosystem degradation, and impairment of ecosystem services (Pysek and Richardson
49 2010). In the worst case scenario, invasive organisms can also determine the extinction of native species
50 (Lövei 1997). The effects of biological invasions have been mostly studied in terms of environmental,
51 economic, and human health impacts (Keller et al. 2011).

52 *Heterobasidion irregulare* Garbel. & Otrrosina is an invasive fungal pathogen of pines, introduced from North
53 America to Italy during World War II (Gonthier et al. 2004). Once introduced, it has colonized pine and oak
54 stands along 103 km of coastline west of Rome (Gonthier et al. 2004; Gonthier et al. 2007; Gonthier et al.
55 2012; Garbelotto et al. 2013). Its sister species *H. annosum* (Fr.) Bref. is also present in the coastal pine
56 stands west of Rome, but only sporadically (Gonthier et al. 2007). *H. irregulare* and *H. annosum* have been
57 compared in terms of pathogenicity on a range of pine species (Garbelotto et al. 2010; Pollastrini et al.
58 2015). Transcriptomic approaches, including qRT-PCR and microarray experiments, have been carried out
59 on both species in order to better characterize the expression of several gene categories, focusing on
60 saprobic growth and substrate specificity (Yakovlev et al. 2012; Raffaello et al. 2014; Baccelli et al. 2015).
61 However, little is known on the possible and differential effects that these fungi may have on other
62 components of native ecosystems, including host plant symbionts.

63 It is known that some ectomycorrhizal (ECM) fungi grown in dual culture with saprotrophic fungi may affect
64 the physiology of the latter. *Laccaria laccata* (Scop.) Cooke, for example, has been reported to cause
65 protoplast release and to penetrate the hyphae of soil saprotrophic fungi such as *Mucor hiemalis* Wehmer
66 (Werner and Zadworny 2003), *Trichoderma harzianum* Rifai (Zadworny et al. 2004), *T. virens* (J.H. Mill.,
67 Giddens & A.A. Foster) Arx (Werner et al. 2002). Other ECM fungi showed antagonistic activities (*i.e.*,
68 *Amanita muscaria* (L.) Lam, *Suillus bovinus* (L.) Roussel, *S. luteus* (L.) Roussel) against the fungi listed
69 above (Mucha et al. 2006). Moreover, there is evidence that ECM fungi may display antibiotic activity both
70 against saprotrophic and pathogenic fungi (Mucha et al. 2009), the ability to inhibit the growth of root
71 pathogens (Cervinkova 1989; Lei et al. 1995; Mohan et al. 2015) and to modulate mycotoxin gene
72 expression (Ismail et al. 2011).

73 By using the dual culture technique, Adomas et al. (2006) were able to investigate, under a molecular and
74 physiological perspective, the interaction between *Heterobasidion parviporum* Niemelä & Korhonen and the
75 saprotrophic basidiomycete *Phlebiopsis gigantea* (Fr.) Jülich, and Yakovlev et al. (2004) identified genes

76 differentially expressed in the interaction between *H. annosum* and the basidiomycete *Physisporinus*
77 *sanguinolentus* (Alb. & Schwein.) Pilát.

78 During interaction in dual culture, a crucial role is played by the contact of the fungal cell walls. Fungal cell
79 wall is made up of proteins and polysaccharides, mainly glucan and chitin, and its formation and remodeling
80 requires the concerted action of several gene products that include several enzymes intimately associated
81 with the fungal cell wall (Bowman and Free 2006). However, the cell wall can be subjected to degradation
82 when exposed to hydrolytic enzymes produced by other organisms (Sivan and Chet 1989). In addition,
83 filamentous fungal cell wall contains proteins playing a role in the interaction with the environment and in
84 aggregation/adhesion events. Hydrophobins are such small secreted proteins that are highly surface-active
85 (Wösten and Scholtmeijer 2015). The dual culture approach has been employed to investigate the
86 interaction between native microbes or to determine the effects of mycorrhizal fungi on other fungi (see
87 references listed above). On the contrary, in this work, we tested the hypothesis that a non-native invasive
88 fungal pathogen may have greater effects on ECM fungi than native fungal pathogens using *H. irregulare*/*H.*
89 *annosum* and the ECM fungus *S. luteus* as a model system. Secondly, the effects of the symbiont on the
90 pathogens were also investigated. Our specific aims were: 1) to test if the interaction between a native
91 symbiont and a invasive/native pathogen might affect fungal growth and morphology; 2) to verify if changes
92 may occur in gene expression, focusing on genes encoding proteins related to cell wall
93 degradation/modification and cell-to-cell adhesion (*i.e.*, hydrophobins); and 3) to test if differential gene
94 expression might allow to distinguish the impact of the invasive *H. irregulare* compared to the native *H.*
95 *annosum* may have on the native symbiont *S. luteus*.

96

97 **Material and methods**

98

99 **Fungal isolates and culture maintenance**

100 *S. luteus* LMSL8 was isolated from a basidiocarp, collected in Lommel Sahara, Limburg, Belgium. The
101 fungus was grown in Petri dishes filled with Fries and Munzenberger medium (pH 4.8) (Fries 1978) and the
102 cultures were kept in a dark room at 25°C. In one liter of distilled water, the medium contains: 10 g agar, 6 g
103 glucose, 1 g di-ammonium tartrate, 30 mg KH₂PO₄, 0.1 g MgSO₄ 7H₂O, 20 mg NaCl, 0.26 mg CaCl₂ 2H₂O,
104 0.1 g KCl, 20 mg FeCl₃ 6H₂O, 8.5 mg MnSO₄ H₂O, 1.25 mg CuSO₄ 5H₂O, 0.2 mg (NH₄)₆Mo₇O₂₄ 4H₂O, 5.75
105 mg ZnSO₄ 7H₂O, 15 mg H₃BO₃ and 10 ml of vitamin stock solution. Stock solution contains: 56 µM myo-
106 inositol, 0.1 µM biotin, 0.5 µM pyridoxine, 0.3 µM riboflavin, 0.8 µM nicotinamide, 0.7 µM p-aminobenzoic

107 acid, 0.3 μ M thiamine, 0.2 μ M Ca-pantothenate. Each 7 days the culture was sub-cultured and refreshed
108 according to Kohler et al. 2015.

109 Three heterokaryotic (ploidy: n+n) isolates of *H. irregulare* and three heterokaryotic isolates of *H. annosum*
110 (Table 1), randomly selected among those available in the culture collection of the DISAFA at the University
111 of Turin were used. Isolates were deposited at the *Mycotheca Universitatis Taurinensis* (MUT) with the
112 accession numbers listed in the Table 1. All the isolates had been previously collected in Italy and identified
113 at the species level through taxon specific primers and AFLPs (Gonthier et al. 2007; Gonthier and Garbelotto
114 2011). The isolates were long term-stored at 4°C in mycological tubes (180 mm long x 18 mm diameter)
115 containing Malt Extract Agar (MEA: 31.3 g malt extract agar, 1L distilled water). Ten days before starting the
116 dual culture, all isolates were sub-cultured in new Petri dishes containing MEA. Mycelial plugs (0.5 mm
117 diameter) taken from the edge of actively growing colonies were used in the dual culture experiments
118 (Giordano et al. 2014).

119

120 **Comparative studies on antagonistic effects between *S. luteus* and *Heterobasidion* spp.**

121 Dual culture technique was used in comparative studies on antagonistic effects between *S. luteus* and
122 *Heterobasidion* spp. Mycelial plugs 5 mm in diameter were removed with a cork borer from actively growing
123 cultures (7 days old) and were paired, with the mycelial surface down, about 15 mm apart, 30 mm from the
124 edge of a 90 mm Petri dish containing Fries and Munzenberger agar. *S. luteus* and *Heterobasidion* spp.
125 isolates were paired in all possible combinations (dual cultures). For each *S. luteus-Heterobasidion* spp.
126 combination ten replicates were used and additionally ten replicates consisting of only one mycelial plug of
127 each fungal isolate were maintained as controls (pure cultures). All the Petri dishes were incubated at 25°C
128 in the dark.

129 Internal radial growth of the colonies in dual and pure cultures was measured every 48 hours under a
130 dissecting microscope and the growth rate of all the isolates, expressed in mm of colonization per day, was
131 calculated. Measurements were completed at the time that a corresponding control culture reached the
132 opposite edge of the Petri dish.

133 The mycelial interactions were scored using a system of classification according to Holdenrieder (1984): A)
134 the two colonies coexist side by side without interacting; B) formation of a mycelium-free inhibition zone
135 between the two mycelia, which stop expanding; C) *S. luteus* completely overgrows *Heterobasidion* spp.; D)
136 *S. luteus* partly overgrows *Heterobasidion* spp.; E) *Heterobasidion* spp. completely overgrows *S. luteus*; F)
137 *Heterobasidion* spp. partly overgrows *S. luteus*.

138 At the end of the experiment, the final fungal growth was determined in mm² of the mycelium surface and the
139 zone of inhibition was determined. Areas colonized by the fungi were measured with a planimeter, as
140 previously described (Nicolotti et al. 1999). Final mycelium surfaces of *H. irregulare*, *H. annosum* and *S.*
141 *luteus* isolates in dual cultures were compared to those of control cultures using the Wilcoxon test.
142 For each isolate in dual culture, the inhibition growth rate (IGR in %) of average mycelium surface relative to
143 the control was calculated as follows:

144

$$145 \quad \text{IGR}\% = \frac{\text{mm}^2 \text{ control} - \text{mm}^2 \text{ dual}}{\text{mm}^2 \text{ control}} \cdot 100$$

146

147 where: mm² control = average mycelium surface in control cultures, mm² dual = average mycelium surface in
148 dual cultures.

149 Since a viability test by using specific dye like FUN-1 (Millard et al. 1997; Lass-Flörl et al. 2001) was not
150 feasible in our model system because formation of clamps in *Heterobasidion* spp. is infrequent, thus
151 hampering to easily distinguish hyphae of *Heterobasidion* spp. from hyphae of the clampless *S. luteus*, a
152 growth recovery test was performed. After 3 weeks of incubation, in Petri dishes where *S. luteus* had been
153 over-grown by *Heterobasidion* spp. or *vice versa*, mycelial plugs were taken and transferred onto Fries and
154 Munzenberger agar to test growth recovery of the fungus. Lack of growth from these mycelial plugs after 2
155 weeks' incubation at 25°C in the dark were regarded as *S. luteus* had possibly been killed or had at least
156 been growth-arrested by *Heterobasidion* spp. or *vice versa*.

157 The IGRs in % of *S. luteus* and *Heterobasidion* spp. were logit-transformed (*i.e.*, logit inhibition growth rates -
158 LIGRs) and tested for normality with the Shapiro-Wilk test, with a cut-off value set to 0.05 (Crawley 2013).
159 Markov Chain Monte Carlo Generalized Linear Mixed Models (MCMC GLMMs) were fitted to test the effects
160 of *Heterobasidion* spp. (*i.e.*, fixed factor) and isolates (*i.e.*, nested random factor) on the observed LIGRs.
161 For both *S. luteus* and *Heterobasidion* spp. the MCMC GLMMs included the null model (M0), the two models
162 with either fixed (M1) or random factors (M2) and the model with both these factors (M3). All MCMC GLMMs
163 were fitted in R environment to estimate the β and Z coefficients, for fixed and random factors respectively,
164 with the associated 95% confidence interval (CI95%) and p value (PMCMC) as described by Giordano et al.
165 (2014), with the exception of the selected link function (*i.e.*, identity) and family distribution for the error term
166 (*i.e.*, Gaussian) (Hadfield 2010; Kéry 2010). The Deviance Information Criterion (DIC) was calculated for

167 each MCMC GLMM and the minimum DIC method was used to select the best model displaying at least one
168 significant coefficient other than the intercept (Berg et al. 2004; Crawley 2013).

169

170 **Primer design for gene expression analysis**

171 Taking advantage of the recently released genome sequence of *H. irregulare* (Olson et al. 2012) and *S.*
172 *luteus* (Kohler et al 2015), we have identified genes related to chitinases (Glycoside Hydrolase family 18 -
173 GH18), β -N-acetylglucosaminidases (Glycoside Hydrolase family 20 - GH20), endo- β -N-
174 acetylglucosaminidases (Glycoside Hydrolase family 85 - GH85) and hydrophobins.

175 For *S. luteus*, 22 qRT-PCR primers were designed using Primer3Plus ([http://www.bioinformatics.nl/cgi-
176 bin/primer3plus/primer3plus.cgi/](http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi)), considering the specific gene categories listed above. Full-length
177 sequences of these gene categories were obtained from the *S. luteus* genome ([http://genome.jgi-
178 psf.org/Suilu1/Suilu1.home.html](http://genome.jgi-psf.org/Suilu1/Suilu1.home.html)).

179 For *H. irregulare*, 17 primer pairs were designed by using Primer3Plus to target genes belonging to the same
180 categories as above (*i.e.*, GH18, GH20, GH85, hydrophobins). Full-length sequences of candidate genes
181 were obtained from the available *H. irregulare* genome (<http://genome.jgi-psf.org/Hetan2/Hetan2.home.html>).
182 Candidate genes of *H. irregulare* were selected on the basis of representativeness within each sub-class and
183 of their nucleotide identity to sequences of *H. annosum* (Sillo and Gonthier, unpublished). Before qRT-PCR,
184 all the primers were tested *in silico* on Primer-BLAST and *in vitro* in PCR reactions on genomic DNA
185 extracted from all the three species with the Dneasy Plant mini kit (Qiagen, Valencia, CA, USA) in order to
186 verify the absence of cross amplification.

187

188 **Sequence and bioinformatic analyses**

189 The protein sequences deduced from the gene sequences were used for extensive database searches for
190 both homologous sequences and sequences that were closely related phylogenetically, *i.e.*, pathogenic,
191 saprotrophic and mycorrhizal Basidiomycota and Ascomycota sequences. A multiple protein alignment was
192 built using the MUSCLE software inside Mega version 6.

193 Phylogenetic trees were constructed using the Neighbor joining (NJ) method, with the Mega software version
194 6 (Tamura et al. 2013). Bootstrap analyses were carried out on the basis of 1,000 re-samplings of the
195 sequence alignment.

196 Sequences were further analyzed using SignalP 4.1 (Peterson et al. 2011) to ensure that they also contain a
197 signal peptide.

198

199 **RNA extraction and cDNA conversion**

200 The same approach as above consisting of dual and pure cultures was used to harvest the mycelium for the
201 gene expression studies. The only differences were that each system was produced in triplicate and that
202 before fungal inoculation the agar surface was covered with a cellophane membrane in order to facilitate the
203 harvest of the mycelia. The cellophane membranes were cut to fit the size of Petri dishes and autoclaved for
204 20 minutes. The mycelium was harvested from pure cultures of both the symbiont and the pathogenic
205 isolates as well as from the interface and the outside of mycelia in dual culture as shown in Fig. 1. Outside
206 mycelia were considered as previously done by Adomas et al. (2006). The tubes containing the frozen
207 mycelium were put in a freeze dry machine overnight at -65°C before the RNA extraction. The RNA was
208 extracted using the RNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. After
209 extraction, the RNA was cleaned of DNA using Promega DNase (RQ1 RNase-Free DNase, Promega Corp.,
210 Madison, WI, USA) and measured using a NanoDrop (Thermo Scientific, Hudson, NH, USA). The absence
211 of genomic DNA was verified through one-step retrotranscription PCR (One-Step RT-PCR, Qiagen) using
212 the primers for the housekeeping gene, *i.e.*, respectively the elongation factor 1 α for *S. luteus*
213 (*Sl_699467_EF1A*; Table S1A) and *Tryp Metab* (Protein ID: 43087) for *Heterobasidion* spp. (Raffaello and
214 Asiebgü 2013, Table S1B).

215 Five hundred ng of total RNA was used for each sample to synthesize the cDNA, according to the
216 SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) procedure.

217

218 **Gene expression analysis**

219 Quantitative RT-PCR was carried out with the Connect™ Real-Time PCR Detection System (Bio-Rad
220 Laboratories, Hercules, CA, USA). Each PCR reaction was conducted on a total volume of 10 μ l, containing
221 1 μ l diluted cDNA (dilution 1:3), 5 μ l SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad
222 Laboratories) and 2 μ l of each primer (3 μ M), using a 96 well plate. Primer sequences with their optimal
223 annealing temperature are listed in Tables S1A-B. The following PCR program, which includes the
224 calculation of a melting curve, was used: 95°C for 30 s, 40 cycles of 95°C for 10 s, the optimal annealing
225 temperature for 30 s, ramp from 65°C to 93°C with a temperature increment of 0.5°C and a read plate every
226 2 s. All the reactions were performed for three biological and three technical replicates. The baseline range
227 and Ct values were automatically calculated using the Bio-Rad CFX Manager software. In order to compare

228 the data from different PCR runs or cDNA samples, the Ct values of all the genes were normalized to the Ct
229 value of the housekeeping gene.

230 The candidate gene expression was normalized to that of the housekeeping gene by subtracting the Ct value
231 of the housekeeping gene from the Ct value of the candidate gene efficiency correction, from equation $2^{-\Delta\Delta Ct}$
232 (Livak and Schmittgen 2001), where $\Delta\Delta Ct$ represents the ΔCt sample – ΔCt control (pure culture). Statistical
233 analyses were carried out using Rest 2009, version 2.0.13, considering 0.05 as the p value. Only significant
234 expression values were considered. A custom R script was used to visualize gene expression values as
235 HeatMaps. This representation of the transcript levels coupled to a hierarchical clustering was performed in
236 order to group genes with similar expression profiles. In addition, an unpaired t -test was carried out to
237 determine differentially regulated genes in *H. irregulare* compared to *H. annosum*, as well as *S. luteus* genes
238 differentially regulated during interaction with the invasive and the native *Heterobasidion* spp. Eventually,
239 principal component analysis (PCA) of the whole gene expression data set was run using MeV v4.9
240 (<http://www.tm4.org/mev.html>).

241

242 **Results**

243

244 **Comparative studies on antagonistic effects between *S. luteus* and *Heterobasidion* spp.**

245 Pure cultures of all *Heterobasidion* spp. isolates reached the opposite edge of Petri dish after 9 days of
246 incubation, while cultures of *S. luteus* did not.

247 The growth of *S. luteus* was significantly inhibited by all *Heterobasidion* spp. isolates (*S. luteus* IGR > 70%, P
248 < 0.05). The only exception was observed between *S. luteus* and *H. annosum* 137OC-142OH isolate (*S.*
249 *luteus* IGR 49%) (Fig. 2).

250 No inhibition zone was observed in the dual cultures. *Heterobasidion* spp. isolates always completely
251 overgrew *S. luteus*. In the growth recovery test, after 2 weeks' incubation, *S. luteus* growth was never
252 observed.

253 The LIGRs of *S. luteus* and *Heterobasidion* spp. were normally distributed based on the Shapiro-Wilk test (P
254 > 0.05). The DIC values observed in the MCMC GLMMs ranged from 79.043 to 112.547 for *S. luteus* LIGR
255 and from 67.402 to 104.876 for *Heterobasidion* spp. LIGR, with the largest values achieved by M0 and M1 in
256 both cases. M2 was the model displaying the lowest DIC and at least one significant coefficient (P < 0.05),
257 other than the intercept, for both *S. luteus* and *Heterobasidion* spp. LIGRs. In the case of *S. luteus* LIGR, the
258 Z coefficients were significant (P < 0.05) in M2 for the combination composed of *H. annosum* 137OC-142OH

259 and *S. luteus*, in the case of *Heterobasidion* spp. LIGR for combinations including *S. luteus* and *H. irregulare*
260 MUT00001151, *H. irregulare* MUT00003560 and *H. annosum* 137OC-142OH, respectively. The β coefficient
261 for the fixed factor was significant ($P < 0.05$) only in the M1 of *S. luteus* LIGR (Table S2).

262

263 **Sequence and bioinformatic analysis**

264 Eight GH18, *i.e.*, putative chitinases, were considered in *S. luteus*. As revealed by means of phylogenetic
265 analysis, three belonged to B subgroup, while the others to A subgroup, *sensu* Gruber et al. (2011). Six
266 GH18 were analyzed in *H. irregulare*: four belonged to the subgroup A, whereas the remaining belonged to
267 subgroup B (Fig. 3). All the sequences, except SIGH18_79518, HiGH18_11431 and HiGH18_3306, showed
268 the presence of a secretory signal peptide.

269 Two genes coding for putative GH20, *i.e.*, β -N-acetylglucosaminidases, as well as two coding for putative
270 GH85, *i.e.*, endo- β -N-acetylglucosaminidases, were found in the *S. luteus* genome. Members of the second
271 family in this ECM fungus did not have the signal peptide. In *H. irregulare* there were four genes coding for
272 putative GH20 and one for a putative GH85.

273 Nine hydrophobins, with a putative role in the adhesion and in the interaction with the environment, were
274 present in *S. luteus* and eight in *H. irregulare*, all belonging to class I hydrophobins. Multiple protein
275 sequence alignment revealed the presence of three main subgroups of hydrophobins in *S. luteus*. There was
276 also a clear distinction between the hydrophobins of the two fungi; the *Heterobasidion* spp. sequences were
277 divided in three clusters far from *S. luteus* sequences, as revealed by means of phylogenetic analysis (Fig.
278 4). Moreover, *S. luteus* sequences were grouped together with hydrophobins of other symbiont
279 basidiomycetes, such as *Laccaria bicolor* and *Paxillus involutus* (Batsch) Fr. All the putative *S. luteus*
280 hydrophobin sequences had a predicted signal peptide and a hydrophobin/HYDRO domain was recognized
281 by means of InterProScan, except in SIHyd_804366. All the putative *H. irregulare* hydrophobins harbored the
282 signal peptide and the HYDRO domain.

283

284 **Gene expression analysis**

285 Quantitative RT-PCR (qRT-PCR) was performed for 21 *S. luteus* and 17 *Heterobasidion* spp. genes. In
286 Tables S3A-B the fold change was shown for genes that were significantly and not significantly differentially
287 expressed.

288 Considering *S. luteus* interface, the most up-regulated gene was *SIGH18_673588* (fold 11.71) in dual culture
289 with *H. irregulare* MUT00001193, while the most down-regulated gene was *SIHyd_804369* (fold 0.09) in dual

290 culture with *H. annosum* MUT00001204. Hierarchical clustering analysis on *S. luteus* gene expression in the
291 interface areas allowed to identify three clusters (Fig. 5). The first cluster included genes not differently
292 expressed in the different combinations, the second comprised significantly down-regulated genes, while the
293 up-regulated genes were grouped in the last. Each cluster contained genes of different categories. In
294 particular, there was a trend for lower expression in hydrophobins and a higher expression for chitinases.
295 In the outside area of *S. luteus* the most up-regulated gene was *SlHyd_14989* (fold 264.69) in dual culture
296 with *H. annosum* MUT00001204, while the most down-regulated was *SlHyd_804369* (fold 0.14) in dual
297 culture with *H. annosum* MUT00001204. Moreover, also in these areas, the same trend described above for
298 the interface was observed.

299 Expression values of *Heterobasidion* spp. genes ranged from 0.0 to 10.73 in the interface area. In general
300 *Heterobasidion* spp. genes showed a wide down-regulation in all the categories.

301 In the outside area of *Heterobasidion* spp. the expression range was between 0.04 and 22.79, corresponding
302 to *HiHyd_65822* in *H. irregulare* MUT00001151 and to *HiGH20_306181* in *H. annosum* 137-OC 142-OH,
303 respectively. Based on the HeatMaps (Fig. 5), a trend was observed for the expression of some genes,
304 including three GH18, one GH20 and one hydrophobin, which showed a different regulation in the two
305 species of *Heterobasidion* spp.

306 PCA did not allow to discriminate the two pathogenic species based both on their overall gene expression
307 values during the interaction with the symbiont and on *S. luteus* expression values (Fig. 6).

308 However, in *S. luteus*, a gene coding for a chitinase (*SIGH18_8356*) was found to be significant differentially
309 regulated in the outside area during interaction with *H. irregulare* compared to *H. annosum* (df=4, p =
310 0.0012). A *t* test confirmed that only the *Heterobasidion* spp. chitinase encoding gene *HiGH18_11431* was
311 differentially regulated when the two pathogens were compared (df=4, p = 0.0211). In particular, a strong
312 down-regulation for this gene was inferred in *H. irregulare* compared to *H. annosum*.

313

314 **Discussion**

315

316 It has been previously shown that ECM fungi may interact with soil and pathogenic fungi (Werner et al. 2002;
317 Mucha et al. 2006; Mucha et al. 2009), but studies combining growth and molecular experiments to study the
318 effects of non-native and native fungal pathogens on ECM fungi were still lacking. In this work, we have not
319 only described the interaction between one isolate of *S. luteus* and three different isolates of the non-native
320 *H. irregulare* and of the native *H. annosum*, but we have also assessed the gene expression during the

321 interaction. The use of different isolates of *Heterobasidion* spp. was due to the previously documented intra-
322 specific genetic diversity (Werner and Lakomy 2002). The European isolate of *S. luteus* was employed not
323 only for its geographic origin, but also for the availability of its genome, which has been recently sequenced
324 by DOE Joint Genome Institute (JGI) in the frame of the Mycorrhizal Genomics Initiative (Kohler et al. 2015).
325 In addition, host range of this ECM fungus is similar to that of *Heterobasidion* spp., encompassing several
326 pine species (Dahlberg and Finlay 1999; Garbelotto and Gonthier 2013).

327 It is well known that the long term storage of pure cultures and sub-culturing may affect the genetic stability
328 of fungal isolates, thus introducing possible biases when comparative analyses are performed (Thomson et
329 al. 1993; Lalaymia et al. 2014). While we cannot exclude that such biases may have influenced our results,
330 the maintenance methods used in the study are well-established and are routinely employed for both growth
331 assays and gene expression analyses of *Heterobasidion* spp. and *S. luteus* (Giordano et al. 2014; Kohler et
332 al. 2015).

333 The morphological observations and measurements of the cultures showed that *S. luteus* was considerably
334 and significantly inhibited by all *Heterobasidion* spp. isolates, except by *H. annosum* 137OC-142OH.
335 Isolates of *H. annosum* were previously shown to overgrow *S. luteus* (Napierała-Filipiak and Werner 2000).
336 In this study we showed that the same occurred for the invasive *H. irregulare*. The fitted MCMC GLMMs
337 suggested that the LIGRs observed in *S. luteus* and *Heterobasidion* spp. depended on the isolates rather
338 than on the species of *Heterobasidion* spp. growing in dual cultures. In fact, the DIC values of M1s were
339 substantially similar to the M0s ones, indicating that the inclusion of the *Heterobasidion* spp. as fixed factor
340 could not improve the models performances, despite the significance achieved by the β coefficient in the M1
341 of *S. luteus*. On the contrary, a large gap between DICs was observed comparing M2s to either M0s or M1s.
342 The decrease of the DIC observed switching from M0s and M1s to M2s indicated that the isolate is an
343 adequate explicative factor to interpret *S. luteus* and *Heterobasidion* spp. IGRs. The overwhelming effect of
344 the isolate on the species in driving the LIGRs was also arguable from the visual inspection of the box plots
345 representing the IGRs of *S. luteus* and *Heterobasidion* species for each dual culture. Starting from the
346 results of the growth experiments obtained in this study, further experiments should be performed to
347 investigate the morphology of the hyphae in the interaction zone between *S. luteus* and the two
348 *Heterobasidion* species in dual culture at microscopic level.

349 Concerning gene expression analysis during dual culture, in *S. luteus* *SIGH85_813330*, chitinase
350 (*SIGH18_673588*, *SIGH18_8356* and *SIGH18_805786*) and hydrophobin (*SIHyd_14989* and *SIHyd_804369*)
351 genes were up- and down- regulated, while in *Heterobasidion* spp. one or two representatives for each

352 category were up- and down- regulated (*HiGH85_47693*, *HiGH20_306181*, *HiGH20_61259*, *HiGH18_11431*
353 *HiHyd_105914*, *HiHyd_65822*). It has been already demonstrated in another biological system that gene
354 expression is affected by the interaction between fungi (Iakovlev et al. 2004). Our results confirmed the
355 rewiring of the transcriptional machinery of the ECM and the pathogenic fungi. In particular up- and down-
356 regulation of genes encoding chitinolytic enzymes, such as putative N-acetylglucosaminidases (GH85 and
357 GH20) and chitinases (GH18), might mirror a remodeling of cell wall chitin components. In the interface two
358 GH85 genes, *SIGH85_813330* and *HiGH85_47693*, showed an up-regulation suggesting a role for their
359 encoded enzymes. Enzymes classified as members of the GH85 family catalyze hydrolysis processes acting
360 on asparagine-linked glycan of various glycoproteins and glycopeptides (Umekawa et al 2008) that could
361 play a role in the cell-to-cell interaction (Bowman and Free 2006).

362 The two genes coding for GH20 in *S. luteus* were not significantly different from the control and the two
363 GH20 in *Heterobasidion* spp. were down-regulated. The hydrolysis of terminal non-reducing N-
364 acetylglucosamine residues from chitin, generally due to these enzymes (Slámová et al 2010), was probably
365 a process not triggered by the dual culture.

366 On the other hand, the relative expression of GH18 genes displayed variable transcript profiles in the
367 different dual cultures. In general, in *S. luteus* there was not a common expression profile for all chitinase
368 genes, even when members within the same chitinase subgroup were considered. These differential
369 expression profiles indicate an absence of a common induction/repression expression pattern inside the *S.*
370 *luteus* GH18 family, suggesting that they may not have totally redundant roles, but rather they could have
371 different functions. Phylogenetic analyses, showing the presence of *S. luteus* sequences in almost all clades
372 of the two main subgroups, might support this scenario.

373 In the subgroup A-III the proliferation of *S. luteus* chitinases compared to the reduced presence of
374 *Heterobasidion* spp. members of this family (4 vs 2) is interesting. High and low abundance of saprotrophic
375 basidiomycetes, and pathogenic basidiomycetes, respectively in GH18 subgroup A-III may reflect different
376 life-styles of the species (Karlsson and Stenlid 2008). In addition, it has been speculated about the
377 involvement of A-III GH18s in fungal-fungal interactions in basidiomycetes (Karlsson and Stenlid 2008). In
378 *Trichoderma* species, it has been demonstrated that most of these enzymes show overlapping functions with
379 an involvement in both self- and non-self fungal cell wall degradation, suggesting that the activity of these
380 enzymes is regulated by the substrate accessibility rather than speciation of individual chitinases (Gruber
381 and Seidl-Seiboth 2012). Gene expression results obtained in this work suggest that *S. luteus* chitinases
382 cannot be grouped on the basis of a specific role. Members inside the same subgroup showed in fact

383 different trends in several considered conditions. By contrast, the GH18 gene family members of the
384 *Heterobasidion* spp. showed a similar down-regulation trend during dual culture with the symbiotic fungus,
385 mostly considering the interface area, suggesting that they were not involved in the inhibition of *S. luteus*.
386 Statistical analysis on gene expression data showed that a *S. luteus* gene encoding a chitinase
387 (*SIGH18_8356*) was perceived differently by the two pathogenic species. As inferred by phylogenetic
388 analysis, this chitinase belonged to subgroup B-V, together with its paralogous *SIGH18_8357*. Members of
389 the B cluster are proposed to be involved in interspecific fungal interactions (Karlsson and Stenlid 2008).
390 *SIGH18_8357* was generally significantly up-regulated both in the interface and in the outside area,
391 irrespective to the pathogenic species present in the dual culture; on the contrary its paralogous
392 *SIGH18_8356* was generally down-regulated, specifically in the outside area during the interaction with *H.*
393 *irregulare*. It could be hypothesized that the lack of co-evolution between the invasive *H. irregulare* and the
394 symbiont might have affected the activity of *SIGH18_8356*.

395 The genes down-regulated in the interface, but up-regulated in the outside zone may suggest that the
396 signals diffusing from the interface could lead to a regulated expression of some key genes in other regions
397 of the mycelium, during non-self interaction as reported by Adomas et al. (2006) in the dual culture *P.*
398 *gigantea* – *H. parviporum*. The non-self interaction could affect the synthesis of hydrophobic metabolites,
399 such as hydrophobins, as previously suggested (Rayner et al 1994). Hydrophobins secretion could lock the
400 hyphal boundaries at the interface zone or during the formation of the barrage zone (Rayner et al. 1994). In
401 addition, changes in hyphal hydrophobicity could also help to protect the fungus from hydrolytic enzymes of
402 the other organisms (Chaffin et al. 1998). It is particular worth noting that four *S. luteus* sequences belonged
403 to a group including the *Schizophyllum commune* Fr. Hyd 4, which might play a role during morphogenesis
404 of fruiting body in this model fungus (Ohm et al. 2010).

405 We can conclude that the pathogens can modulate the growth of the symbiont and that the gene expression
406 of target genes changes both in the symbiont and in the pathogens when the fungi coexist. Despite it was
407 not possible to distinguish the effects of the invasive pathogen from that of the native one on the ECM
408 fungus from a wide gene expression perspective, a single *S. luteus* gene encoding a putative chitinase was
409 found to differentially perceive the two pathogens, thus showing a diverse expression trend.

410 It might be speculated that the inhibition effect is probably due to other genes/proteins that we have not
411 considered in this work (e.g., genes involved in nutrient assimilation). To clarify this point a non-target
412 approach, such RNAseq, should be used as well as a metabolomics analysis. This study was carried out *in*
413 *vitro*, with the understanding that growth and gene expression in a host plant could be different; however, the

414 identification of regulated genes during the dual culture will help to better understand the interactions
415 occurring in the natural environment itself. Our findings elucidate the effects of a pathogen on growth and
416 expression of several candidate genes during the saprotrophic life phase of the symbiotic fungus. This
417 comparative study in dual culture will be pivotal to decipher the effects during the symbiotic life stage of *S.*
418 *luteus*. Large scale transcriptomic data of *S. luteus-Pinus sylvestris* ectomycorrhizae revealed that some cell
419 wall related genes considered in this work were up-regulated during the symbiotic stage (Kohler et al. 2015).
420 In our dual culture system, a regulation of these genes has been observed. Thus, it could be speculated that,
421 in the presence of the plant, expression pattern of cell wall related genes might change in a system including
422 fungal pathogens. Based on this hypothesis, an experiment including inoculation of several isolates of *H.*
423 *irregulare* and *H. annosum* on *Pinus* seedlings colonized by *S. luteus* is needed. While it has been reported
424 that ECM fungi may have a protective role against root pathogens, favoring the plant growth (Perrin 1990;
425 Branzanti et al. 1999), little it is known on the impact that pathogens, and especially invasive ones, have on a
426 mycorrhizal fungal species that coexist in the same environment.

427

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431

432 **Conflict of interest**

433 The authors declare that they have no conflict of interest.

434

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645

646 **Supplementary material**

647 **Table S1A** list of the *S. luteus* primers used in the current work and relative Temperature of annealing (Ta)

648

649 **Table S1B** list of the *Heterobasidion* spp. primers used in the current work and relative Temperature of
650 annealing (Ta)

651

652 **Table S2** MCMC GLMMs fitted on the inhibition growth rates of *S. luteus* and *Heterobasidion* spp. in logit
653 scale (LIGR)

654 For each model (M0, M1, M2, M3), the intercept, the fixed and random factors (when included) are indicated.

655 Under the factor “species”, the coding level associated to *H. irregulare* and *H. annosum* is reported. The β

656 and Z coefficients, for fixed and random factors respectively, with the associated 95% confidence interval

657 ($CI_{95\%}$) and p -value (P_{MCMC}) are specified. The symbol * is associated to significant coefficients ($P < 0.05$).

658

659 **Table S3A** expression values in qRT-PCR of *S. luteus* genes

660

661 **Table S3B** expression values in qRT-PCR of *Heterobasidion* spp. genes

662

663

664 **Table 1** Fungal isolates used in the present work

665

Isolate code	Isolation date	Geographic origin	Species	MUT accession N.
39NE	2005	Castelfusano, RM, Italy	<i>H. irregulare</i>	MUT00001193
45SE	2005	Sabaudia, LT, Italy	<i>H. irregulare</i>	MUT00001151
CP15	2002	Castelporziano, RM, Italy	<i>H. irregulare</i>	MUT00003560
137OC-142OH	2013	artificial heterokaryotic isolate	<i>H. annosum</i>	XXX
Ha. Carp.	2007	Sabaudia, LT, Italy	<i>H. annosum</i>	MUT00001143
43NA	2005	Sabaudia, LT, Italy	<i>H. annosum</i>	MUT00001204
LMSL8	2009	Lommel Sahara, Limburg, Belgium	<i>S. luteus</i>	XXX

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667 *MUT Mycotheca Universitatis Taurinensis*

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672 **Figure Legends**

673 **Fig. 1** Schematic illustration of the experimental design for sample collection

674 **a:** *S. luteus* pure culture, where the sampling point is in light grey. **b:** *H. annosum* MUT00001143 sampling
675 point in dark grey. **c:** dual culture of *S. luteus* and *H. annosum* MUT00001143; the plugs were placed at 3 cm
676 from the side of the Petri dish and 1.5 cm between them. Three sampling points were shown: the outside of
677 the pathogen, the interface and the outside of the symbiont

678 **Fig. 2** Box plots of the inhibition growth rate (IGR in %) of *S. luteus* (a) and *Heterobasidion* spp. (b) for each
679 dual culture (1: *H. irregulare* MUT00001193, 2: *H. irregulare* MUT00001151, 3: *H. irregulare* MUT00003560,
680 4: *H. annosum* 137OC-142OH, 5: *H. annosum* MUT00001143, 6: *H. annosum* MUT00001204)

681 The bounding rectangles of the box plots include the values lying between the 25th and 75th percentile, the
682 horizontal thick black line is the median IGR, the t-shaped lines outside the rectangles indicate the minimum
683 and maximum values, the circles are outliers.

684 **Fig. 3** Phylogenetic relationships between filamentous fungi based on aminoacid deduced sequences for the
685 representative chitinase family

686 The sequences were aligned using Muscle and the tree was constructed using the Neighbor Joining (NJ)
687 method. Numbers indicate bootstrap values, and are given only for >50%. Red triangles represented *H.*
688 *irregulare* sequences, while blue circles represented *S. luteus* sequences.

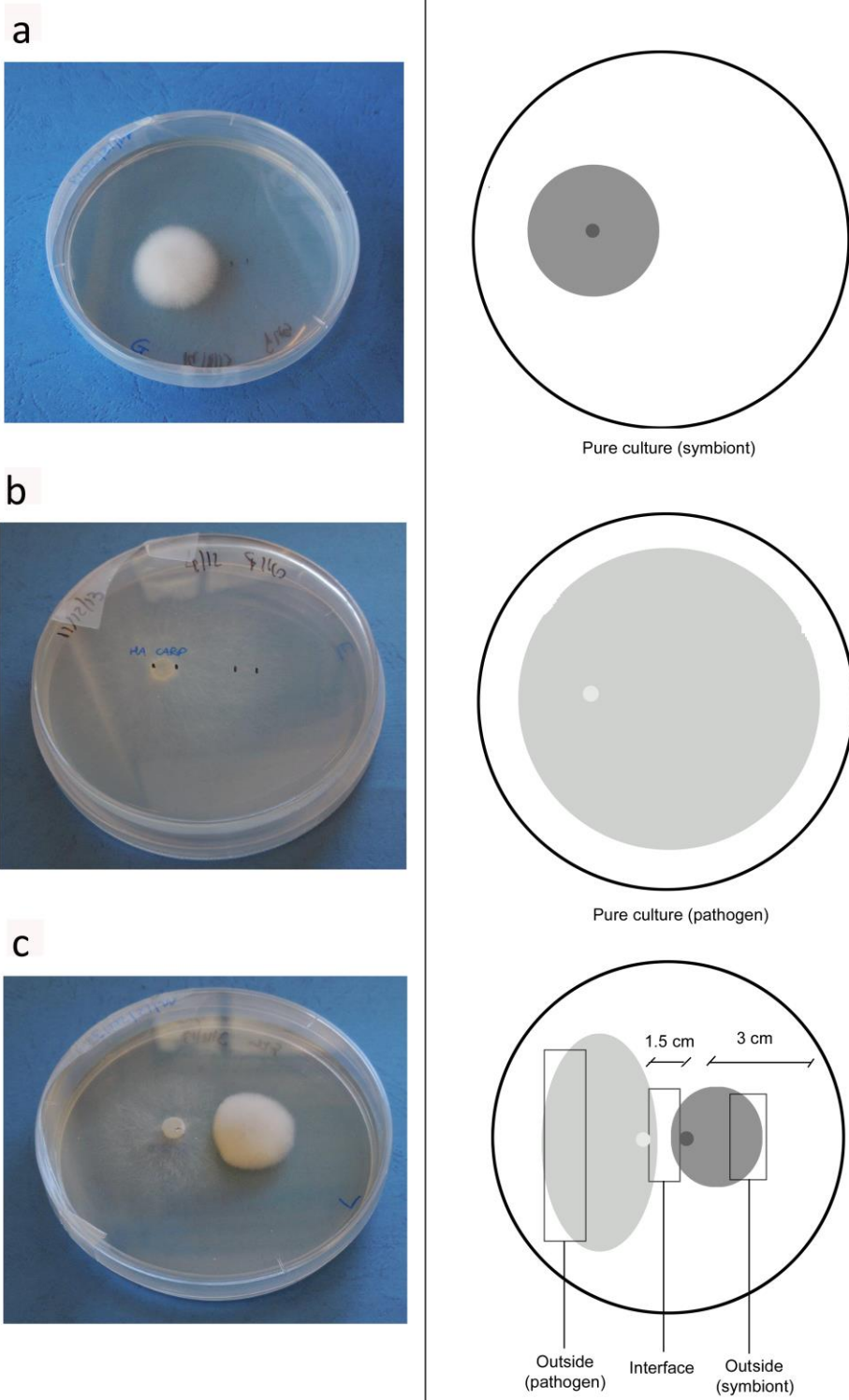
689 **Fig. 4** Phylogenetic relationships between filamentous fungi based on aminoacid deduced sequences for the
690 representative hydrophobin family

691 The sequences were aligned using Muscle and the tree was constructed using the Neighbor Joining (NJ)
692 method. Numbers indicate bootstrap values, and are given only for >50%. Red triangles represented *H.*
693 *irregulare* sequences, while blue circles represented *S. luteus* sequences.

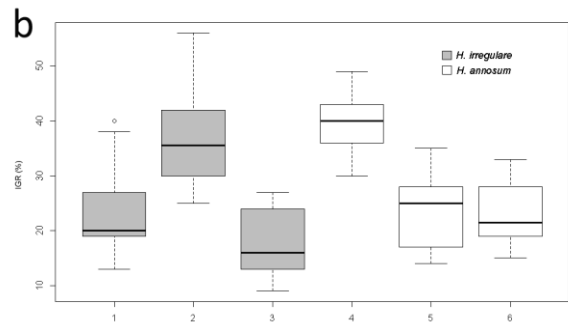
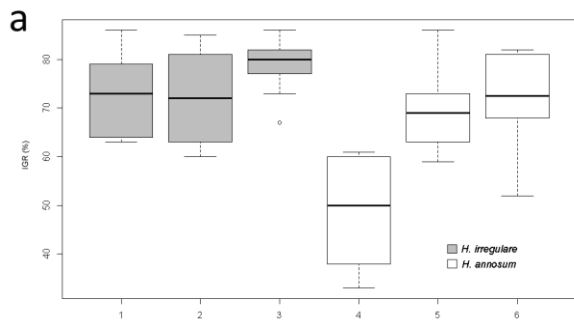
694 **Fig. 5** HeatMap representation of the transcript levels coupled to a hierarchical clustering

695 The four HeatMaps consider the different sample areas for the gene expression analysis: *S. luteus* interface
696 (a), *S. luteus* outside (b), *Heterobasidion* spp. interface (c), *Heterobasidion* spp. outside (d). Each column
697 represents a fungal isolate (1: *H. irregulare* MUT00001193, 2: *H. irregulare* MUT00001151, 3: *H. irregulare*
698 MUT00003560, 4: *H. annosum* 137OC-142OH, 5: *H. annosum* MUT00001143, 6: *H. annosum*
699 MUT00001204), while each row represents a single gene. Expression levels are colored green for low
700 intensities and red for high intensities (see scale at the top right corner). The black cells represent genes not
701 significantly different from the control. The colors in the hierarchical clustering are: violet for GH18, blue for
702 hydrophobins, pink for GH20 and grey for GH85.

703 **Fig. 6** Principal component analysis (PCA) of gene expression results in different combinations performed
704 with MeV
705 The four plots considered the different sample areas for the gene expression analysis: *S. luteus* interface (a),
706 *S. luteus* outside (b), *Heterobasidion* spp. interface (c), *Heterobasidion* spp. outside (d). White triangles
707 represent combination of *S. luteus* and *H. irregulare*, while black triangles *S. luteus* and *H. annosum*.
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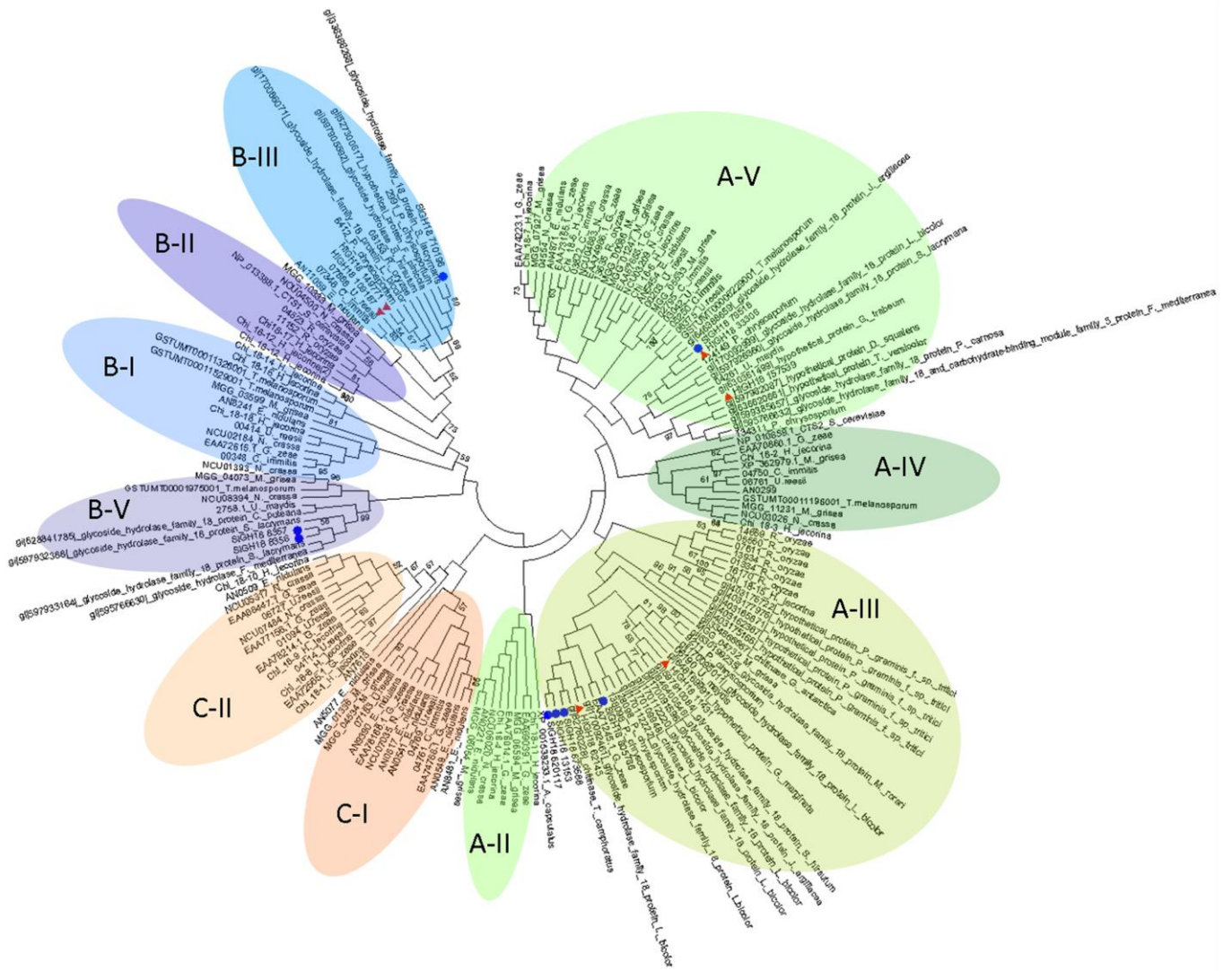


712 **Fig. 2**



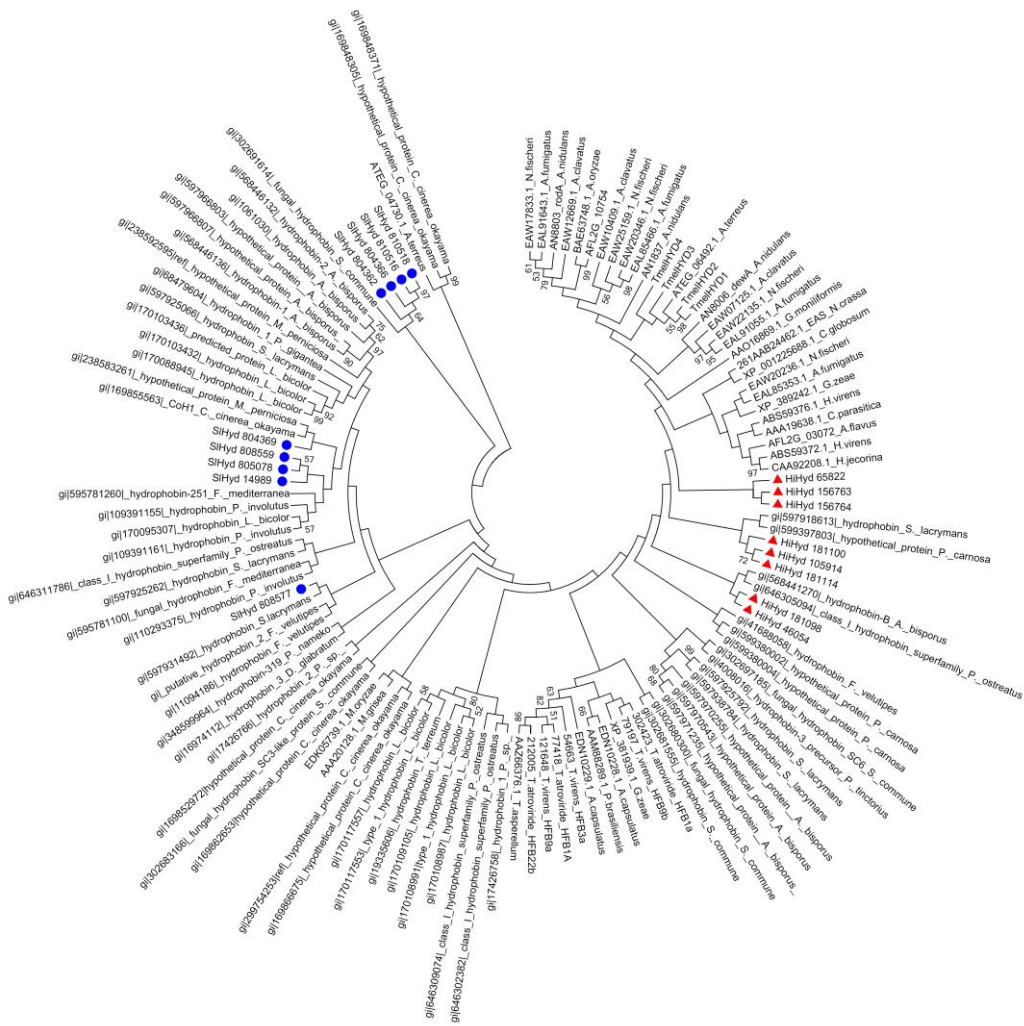
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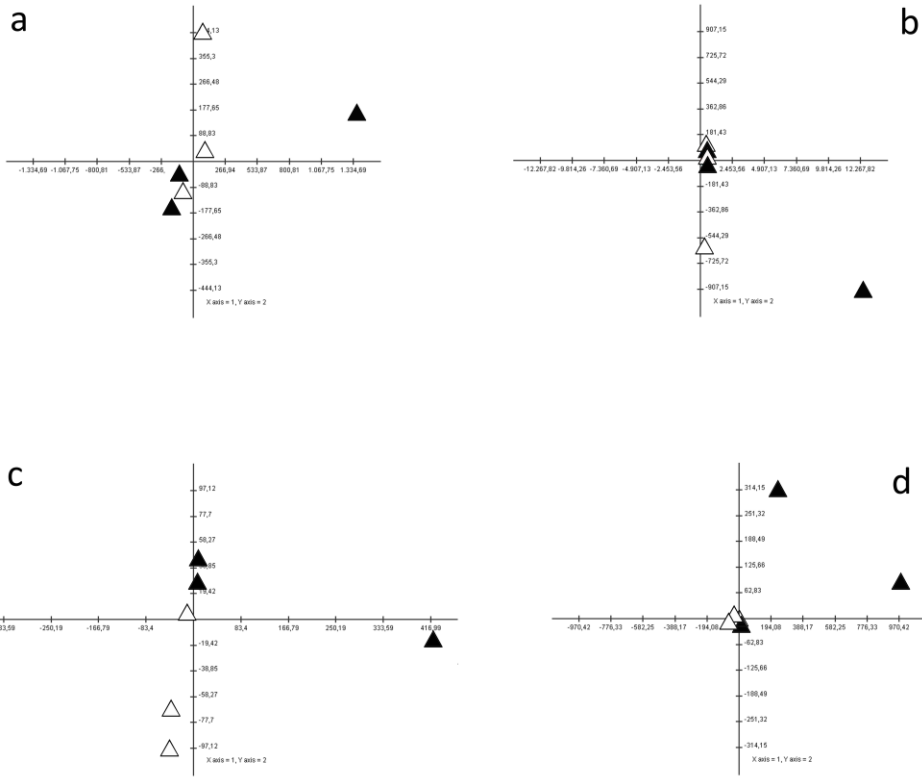
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