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# UNIVERSITÀ DEGLI STUDI DI TORINO

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

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**Abstract**

The effects of biological invasions by non-native species have been widely studied in terms of environmental, economic, and human health impacts. However, little is known on the consequences that non-native plant pathogens may determine on host plant symbionts, such as ectomycorrhizal (ECM) fungi. In this study, interactions between *Suillus luteus*, an ECM fungus of pine trees, and the allopatrically differentiated fungal pathogens of pines *Heterobasidion irregulare* and *H. annosum* were investigated in dual culture by morphological and gene expression analyses. Growth of *S. luteus* was inhibited by the both *Heterobasidion* species, but based on statistical analysis growth inhibition was due to the isolate rather than to the species. The expression analysis on genes related to cell wall hydrolytic enzymes and hydrophobins, putatively involved in the fungus-fungus interaction, allowed to identify significantly up- and down- regulated genes both in the symbiont and in the pathogens. Based on the transcript analysis, it was not possible to distinguish the impact of the two pathogenic species on the ECM fungus. The only exception was a *S. luteus* gene coding for a putative chitinase (*SIGH18\_8356*) that was found to be differentially regulated during interaction with *H. irregulare* compared to *H. annosum*.

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

**Introduction**

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. & Otrósina 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

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

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

## Material and methods

### Fungal isolates and culture maintenance

*S. luteus* LMSL8 was isolated from a basidiocarp, collected in Lommel Sahara, Limburg, Belgium. The fungus was grown in Petri dishes filled with Fries and Munzenberger medium (pH 4.8) (Fries 1978) and the cultures were kept in a dark room at 25°C. In one liter of distilled water, the medium contains: 10 g agar, 6 g glucose, 1 g di-ammonium tartrate, 30 mg KH<sub>2</sub>PO<sub>4</sub>, 0.1 g MgSO<sub>4</sub> 7H<sub>2</sub>O, 20 mg NaCl, 0.26 mg CaCl<sub>2</sub> 2H<sub>2</sub>O, 0.1 g KCl, 20 mg FeCl<sub>3</sub> 6H<sub>2</sub>O, 8.5 mg MnSO<sub>4</sub> H<sub>2</sub>O, 1.25 mg CuSO<sub>4</sub> 5H<sub>2</sub>O, 0.2 mg (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub> 4H<sub>2</sub>O, 5.75 mg ZnSO<sub>4</sub> 7H<sub>2</sub>O, 15 mg H<sub>3</sub>BO<sub>3</sub> and 10 ml of vitamin stock solution. Stock solution contains: 56 µM myo-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  $\mu$ M thiamine, 0.2  $\mu$ 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*.

At the end of the experiment, the final fungal growth was determined in mm<sup>2</sup> of the mycelium surface and the zone of inhibition was determined. Areas colonized by the fungi were measured with a planimeter, as previously described (Nicolotti et al. 1999). Final mycelium surfaces of *H. irregulare*, *H. annosum* and *S. luteus* isolates in dual cultures were compared to those of control cultures using the Wilcoxon test. For each isolate in dual culture, the inhibition growth rate (IGR in %) of average mycelium surface relative to the control was calculated as follows:

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

where: mm<sup>2</sup> control = average mycelium surface in control cultures, mm<sup>2</sup> dual = average mycelium surface in dual cultures.

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

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

each MCMC GLMM and the minimum DIC method was used to select the best model displaying at least one 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),  $\beta$ -N-acetylglucosaminidases (Glycoside Hydrolase family 20 - GH20), endo- $\beta$ -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-](http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.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-](http://genome.jgi-psf.org/Suilu1/Suilu1.home.html)  
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 $\alpha$  for *S. luteus*  
213 (*Sl\_699467\_EF1A*; Table S1A) and *Tryp Metab* (Protein ID: 43087) for *Heterobasidion* spp. (Raffaello and  
214 Asiegbu 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  $\mu$ l, containing  
221 1  $\mu$ l diluted cDNA (dilution 1:3), 5  $\mu$ l SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad  
222 Laboratories) and 2  $\mu$ l of each primer (3  $\mu$ 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

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

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

241

## 242 **Results**

243

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

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

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

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

The LIGRs of *S. luteus* and *Heterobasidion* spp. were normally distributed based on the Shapiro-Wilk test (*P* > 0.05). The DIC values observed in the MCMC GLMMs ranged from 79.043 to 112.547 for *S. luteus* LIGR and from 67.402 to 104.876 for *Heterobasidion* spp. LIGR, with the largest values achieved by M0 and M1 in both cases. M2 was the model displaying the lowest DIC and at least one significant coefficient (*P* < 0.05), other than the intercept, for both *S. luteus* and *Heterobasidion* spp. LIGRs. In the case of *S. luteus* LIGR, the 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  $\beta$  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.*,  $\beta$ -N-acetylglucosaminidases, as well as two coding for putative  
270 GH85, *i.e.*, endo- $\beta$ -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  $\beta$  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

category were up- and down- regulated (*HiGH85\_47693*, *HiGH20\_306181*, *HiGH20\_61259*, *HiGH18\_11431*, *HiHyd\_105914*, *HiHyd\_65822*). It has been already demonstrated in another biological system that gene expression is affected by the interaction between fungi (Iakovlev et al. 2004). Our results confirmed the rewiring of the transcriptional machinery of the ECM and the pathogenic fungi. In particular up- and down-regulation of genes encoding chitinolytic enzymes, such as putative N-acetylglucosaminidases (GH85 and GH20) and chitinases (GH18), might mirror a remodeling of cell wall chitin components. In the interface two GH85 genes, *SIGH85\_813330* and *HiGH85\_47693*, showed an up-regulation suggesting a role for their encoded enzymes. Enzymes classified as members of the GH85 family catalyze hydrolysis processes acting on asparagine-linked glycan of various glycoproteins and glycopeptides (Umekawa et al 2008) that could play a role in the cell-to-cell interaction (Bowman and Free 2006).

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

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

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

different trends in several considered conditions. By contrast, the GH18 gene family members of the *Heterobasidion* spp. showed a similar down-regulation trend during dual culture with the symbiotic fungus, mostly considering the interface area, suggesting that they were not involved in the inhibition of *S. luteus*. Statistical analysis on gene expression data showed that a *S. luteus* gene encoding a chitinase (*SIGH18\_8356*) was perceived differently by the two pathogenic species. As inferred by phylogenetic analysis, this chitinase belonged to subgroup B-V, together with its paralogous *SIGH18\_8357*. Members of the B cluster are proposed to be involved in interspecific fungal interactions (Karlsson and Stenlid 2008). *SIGH18\_8357* was generally significantly up-regulated both in the interface and in the outside area, irrespective to the pathogenic species present in the dual culture; on the contrary its paralogous *SIGH18\_8356* was generally down-regulated, specifically in the outside area during the interaction with *H. irregulare*. It could be hypothesized that the lack of co-evolution between the invasive *H. irregulare* and the symbiont might have affected the activity of *SIGH18\_8356*.

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

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

It might be speculated that the inhibition effect is probably due to other genes/proteins that we have not considered in this work (e.g., genes involved in nutrient assimilation). To clarify this point a non-target approach, such RNAseq, should be used as well as a metabolomics analysis. This study was carried out *in 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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**Supplementary material**

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

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

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

For each model (M0, M1, M2, M3), the intercept, the fixed and random factors (when included) are indicated. Under the factor “species”, the coding level associated to *H. irregulare* and *H. annosum* is reported. The  $\beta$  and  $Z$  coefficients, for fixed and random factors respectively, with the associated 95% confidence interval (CI<sub>95%</sub>) and  $p$ -value (P<sub>MCMC</sub>) are specified. The symbol \* is associated to significant coefficients ( $P < 0.05$ ).

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

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



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

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

*MUT Mycotheca Universitatis Taurinensis*

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 25<sup>th</sup> and 75<sup>th</sup> 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.

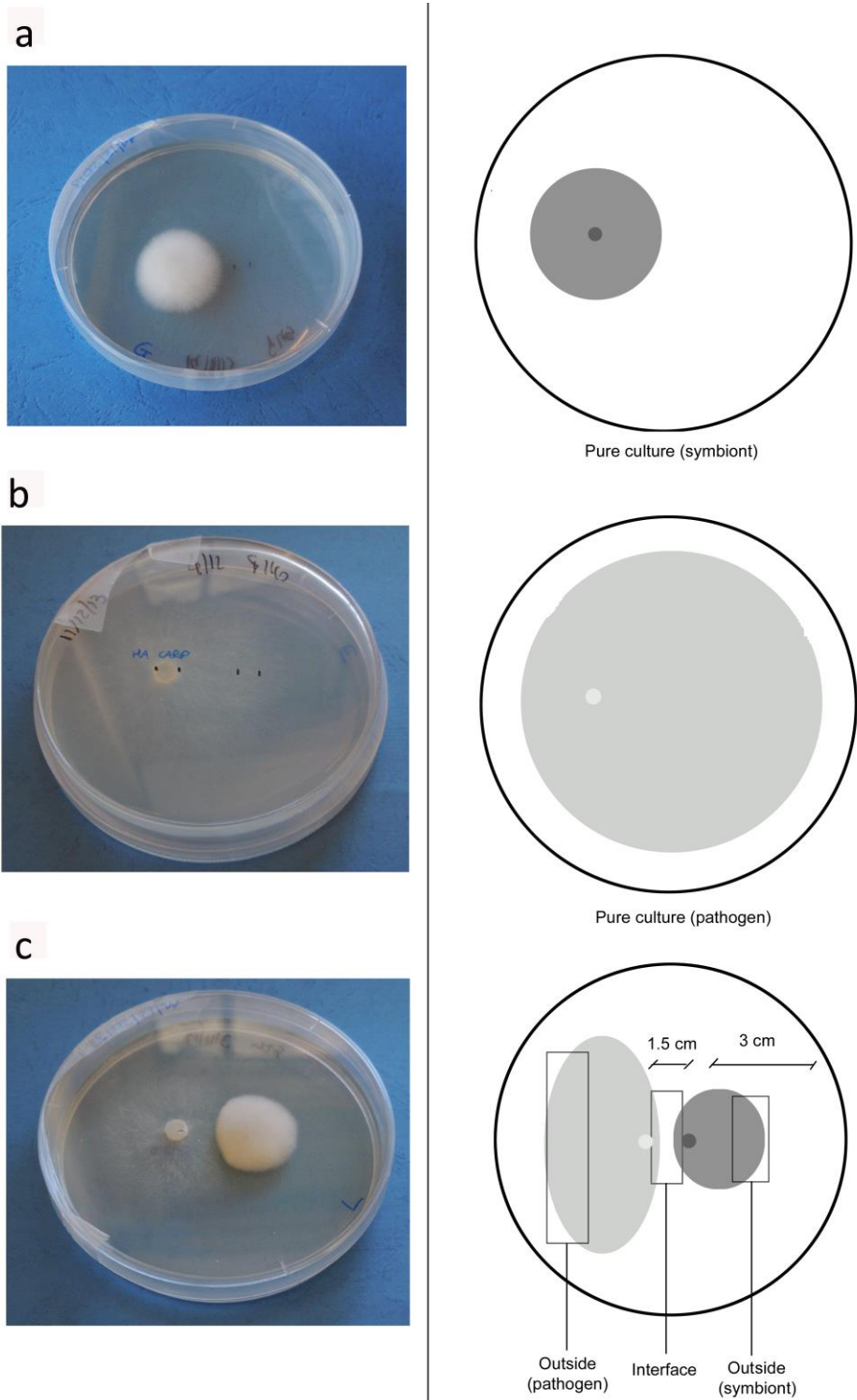
689 **Fig. 4** Phylogenetic relationships between filamentous fungi based on aminoacid deduced sequences for the  
690 representative hydrophobin family

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.

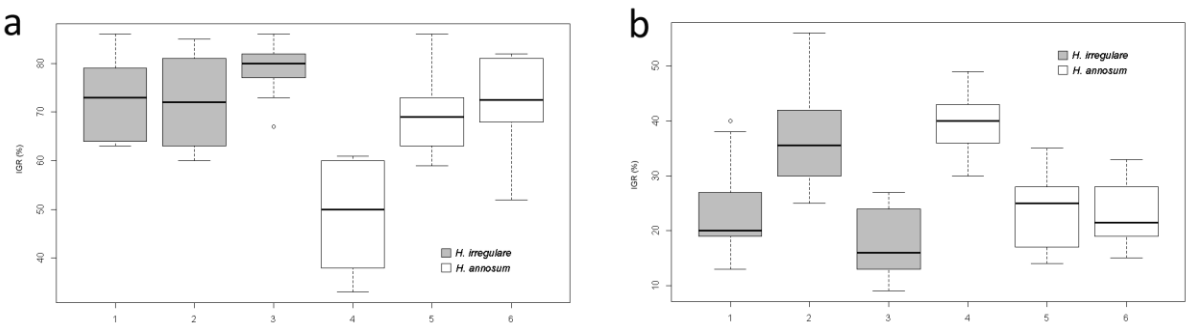
703 **Fig. 6** Principal component analysis (PCA) of gene expression results in different combinations performed  
704 with MeV  
705 The four plots considered the different sample areas for the gene expression analysis: *S. luteus* interface (a),  
706 *S. luteus* outside (b), *Heterobasidion* spp. 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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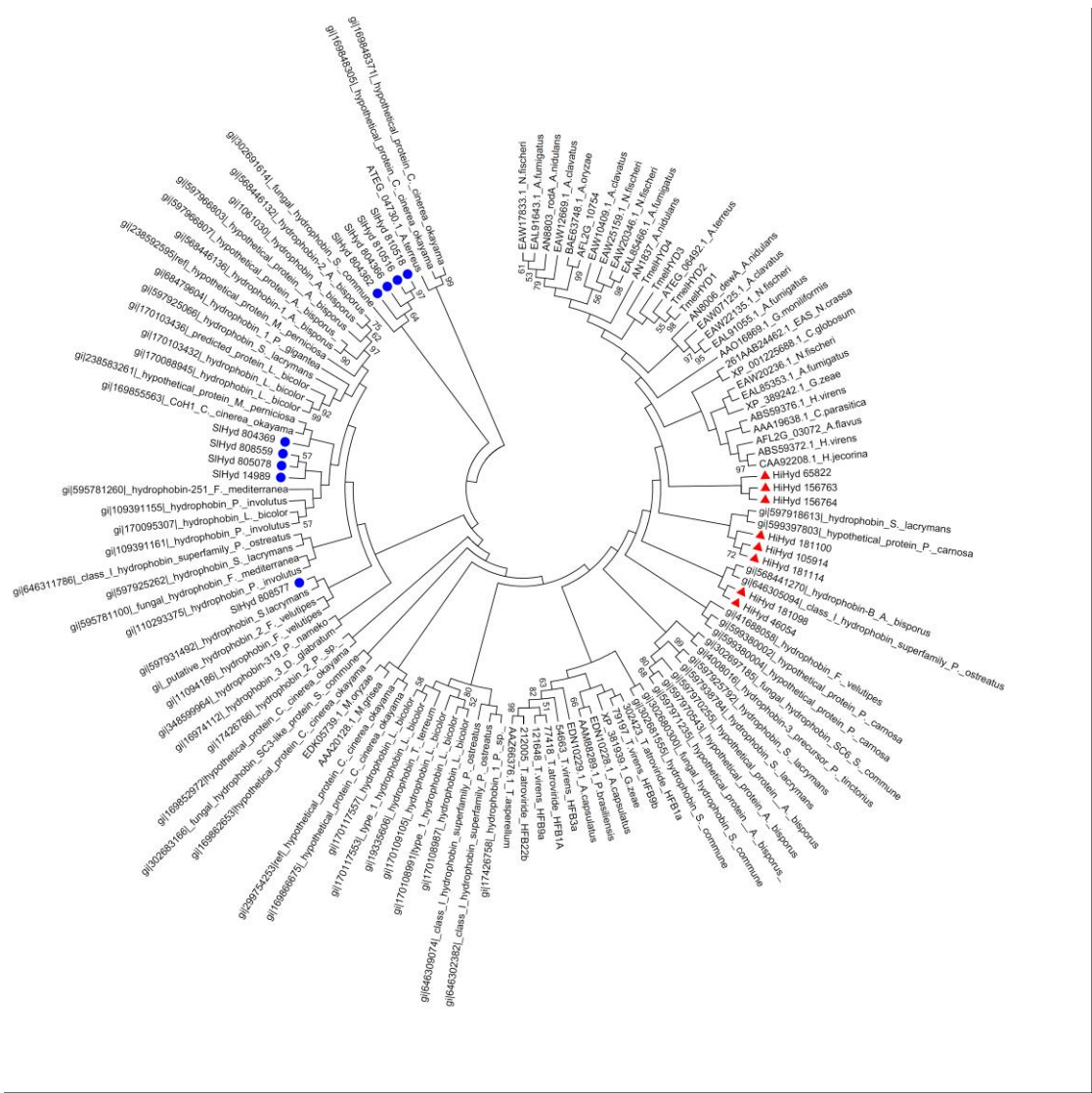
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712 **Fig. 2**



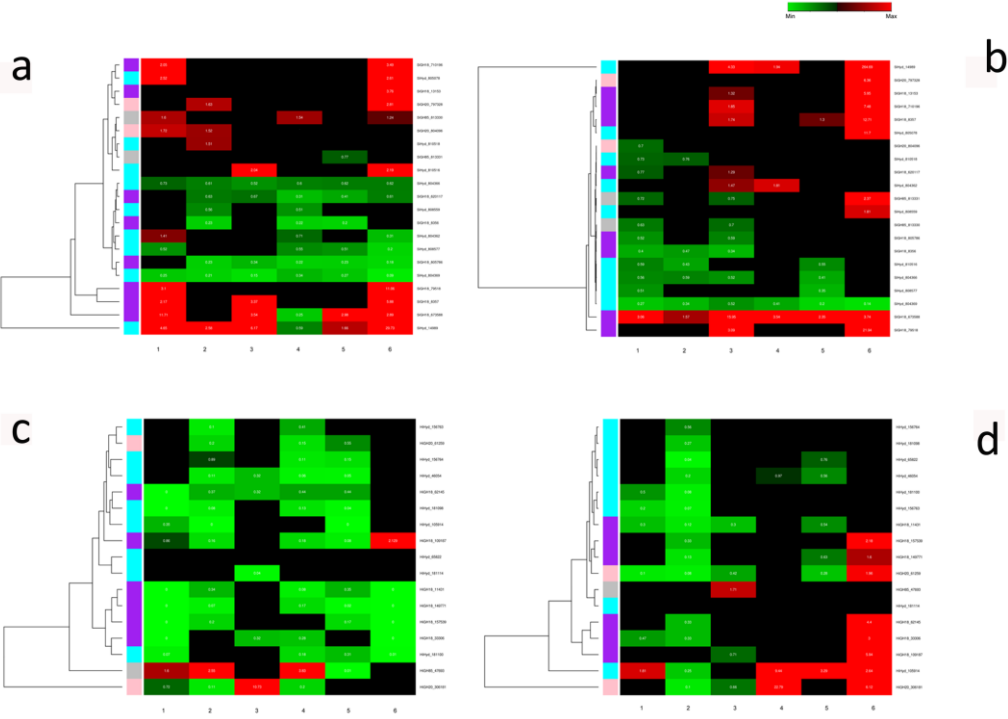




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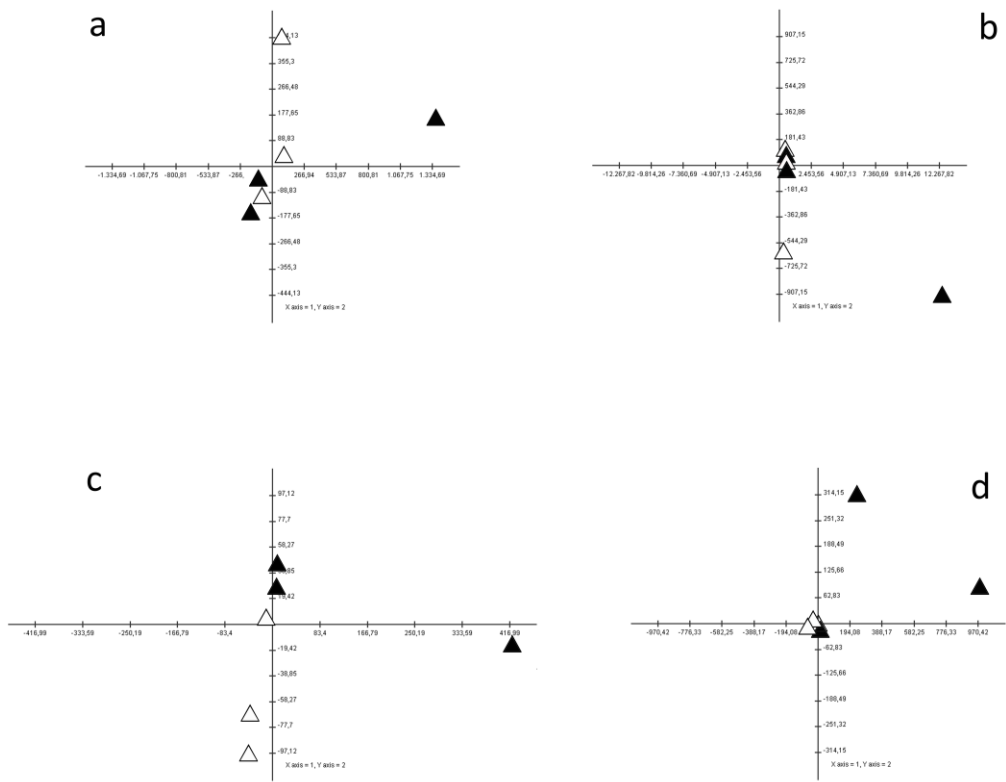
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