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The invasiveness of a non-native fungal forest pathogen is boosted by the presence of a congeneric native species

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Abstract

The North American introduced fungal plant pathogen *Heterobasidion irregulare* has become invasive in pine stands of central Italy and has broadly hybridized with the native congeneric species *H. annosum*. In this study, by genotyping *Heterobasidion* fruiting bodies and mycelia in pine logs inoculated with both fungal species, we showed that *H. irregulare* developed fruiting bodies at a 1.9-fold higher frequency when spatially overlapping

with *H. annosum* than when by itself. In spite of different fruiting rates, all fruiting bodies were morphologically identical, independently of where they were formed, indicating that increased fruiting rate is likely to increase production of spores. Although all possible nuclear-mitochondrial combinations were identified in hybrids present in inoculated pine logs, hybrids with nuclei of both species and the *H. irregulare* mitochondrion were favored, while hybrids with both nuclei of one species and mitochondria of the other species were less frequent. Based on these results, predictions on the wider invasion of *H. irregulare* in Europe and recommendations for its containment are formulated.

Key words: native and non-native pathogens; *Heterobasidion irregulare*; *Heterobasidion annosum*; interspecific interactions; competition; fitness; fruiting bodies; hybridization; pines; root rot fungi.

Introduction

The ongoing intensification of global trade has increased the introduction rate of non-native plant pathogens, often leading to microbial invasions resulting in detrimental alterations of native ecosystems (Parker and Gilbert, 2004; Desprez-Loustau *et al*., 2007; Santini *et al*., 2013). Not unlike other biological invasions, microbial invasions are complex processes involving multiple ecological and evolutionary factors (Garbelotto *et al*., 2010), and the successful establishment and spread of non-native microbial pathogens often hinges on the outcome of their interactions with native communities (Holle and Simberloff, 2005; McCallum, 2008). Such interactions may either boost or hinder the invasion process of microbial plant pathogens by modulating their transmission rates, or, alternatively, their outcome may be neutral (Klironomos, 2002; Wardle, 2002). Traits affecting transmission rates of invasive plant pathogens encompass pathogenicity, host specificity, phenotypic plasticity, and hybridization potential of the pathogens themselves (Garbelotto *et al*., 2015). An increasing effort has been devoted to disentangle the role played by each of the above traits in order to predict the patterns and rates of invasion, as well as to design effective monitoring programs and management strategies (Gonthier and Garbelotto, 2013).

To date, few studies have attempted to elucidate the role played by the interaction between native and invasive fungal plant pathogens on the invasion dynamics of the latter (Kozanitas *et al*., 2017). Mainstream ecological and evolutionary theory suggests that the strongest competition should be expected between closely related species (Darwin, 1859). Indeed, when a native and a non-native species share similar ecological traits, their ecological niches are likely to overlap, resulting in a competitive interaction for the same resources that may

hinder the invasion process. However, the varied outcomes of interspecific interactions between a native and a non-native plant pathogen have not been studied in depth, in particular if the two are interfertile and can successfully mate. Successful mating between sexually compatible congeneric fungal taxa can trigger the rapid emergence of new or modified pathogens via interspecific hybridization and reproductive interference, as largely reported for plants and animals (Perry *et al*., 2002; Abbott *et al*., 2003). Consequently, hybridization and introgression of individual loci (Brasier and Buck, 2001; Brasier *et al*., 2004) can significantly affect the dynamics and outcomes of biological invasions (Ellstrand and Schierenbeck, 2000; Perry *et al*., 2002). When occurring in pathogenic fungi and fungal-like organisms, interspecific hybridization and gene introgression may lead to unpredictable and varied consequences including a different morphology, new ecological adaptations, and modified host range (Brasier *et al*., 1999; Brasier, 2001; Newcombe *et al*., 2000; Olson and Stenlid, 2002). To our knowledge, no study other than the one on the hybridization between *Ophiostoma ulmi* (Buisman) Nannf. and *Ophiostoma novo-ulmi* Brasier (Brasier, 2001) has investigated whether hybridization between a native and a non-native fungal plant pathogen may change the dynamics of their interaction, promoting the establishment rather than the inhibition of the non-native species. This information may be critical when trying to contain an invasive plant pathogen.

A recent and relevant biological invasion of forest ecosystems is that of the North American root rot agent of conifers *Heterobasidion irregulare* Garbel. & Otrosina in Europe. *Heterobasidion irregulare* was accidentally introduced in central Italy in 1944, within the natural range of the Eurasian congeneric species *Heterobasidion annosum* (Fr.) Bref. (Garbelotto and Gonthier, 2013). After its introduction, *H. irregulare* has become invasive, colonizing pine and oak stands along 103 km of coastline west of Rome (Gonthier *et al*., 2007,

2012; Garbelotto *et al*., 2013; Gonthier *et al*., 2014). Comparative studies contrasting the biology and the epidemiology of the non-native and the native *Heterobasidion* species have proven that: 1) both species display similar pathogenicity levels on several Eurasian and North American pine species (Garbelotto *et al*., 2010; Pollastrini *et al*., 2015), 2) the saprobic and sporulation potentials of *H. irregulare* are significantly higher than those of *H. annosum* resulting in a substantially higher transmission rate of the invasive species (Garbelotto *et al*., 2010; Giordano *et al*., 2014), and 3) *H. irregulare* may colonize habitats unavailable to its native congener as a result of the adaptation to its new geographical range (Gonthier *et al*., 2012, 2014).

Heterobasidion irregulare and *H. annosum* evolved through an allopatric process started 34-41 million years ago (Dalman *et al*., 2010) and are characterized by clearly differentiated genomes (Sillo *et al*., 2015), however their mating systems have remained almost fully compatible (Stenlid and Karlsson, 1991). After the introduction of *H. irregulare* in Italy, the two species have started admixing their genomes through massive hybridization events, resulting in the generation of hybrid swarms (Gonthier *et al*., 2007; Gonthier and Garbelotto, 2011). Interestingly, the majority of hybrids retrieved during field studies were characterized by the *H. irregulare* mitochondrion (Gonthier and Garbelotto, 2011), suggesting a selective advantage in favor of the mitochondrion of the invasive species.

A few comparative studies have elucidated key traits of the biology and epidemiology of *H. irregulare* in Italy including one focusing on its impact and interaction with native microbes symbiotic to host plants (Zampieri *et al*., 2017). However, very little is known about the potential effects of the direct interaction between the two fungal pathogens on their respective fitness and hybridization potentials. It should be noted that where the two *Heterobasidion* species coexist, direct interactions between the two are likely to occur as both

143 species are known to infect primarily freshly cut host stumps and logs by means of airborne
144 meiospores (Garbelotto and Gonthier, 2013). Indeed, host stumps represent key substrates
145 for both these plant pathogenic fungi not only because they act as major courts for their
146 establishment in forest stands, but also because they allow for tree-to-tree spread through
147 root contacts, and because they are an important substrate for the production of fruiting
148 bodies (Garbelotto and Gonthier, 2013).
149 This study aimed at improving our understanding of the outcome of direct ecological
150 interactions between the native and the non-native species of *Heterobasidion*. Since the
151 native *H. annosum* is largely widespread across Europe, whether the interaction with the non-
152 native *H. irregulare* may be competitive, neutral, or synergistic might substantially influence
153 the spread of the latter. In this study, results published in Giordano *et al*. (2014) were
154 amended with additional unpublished results and used in a completely new set of biological
155 and statistical analyses, to test the following new hypotheses: I) Did the two species display a
156 competitive, neutral or synergistic interaction when growing on the same portions of a
157 common substrate? II) Did interspecific interactions alter the main macro-morphological traits
158 of the fruiting bodies of either species? III) Did the rate of hybridization suggest the presence
159 of any pre- and/or postzygotic mating barriers resulting in hybrids with genomes biased in
160 favor of specific nuclear and/or mitochondrial combinations.
161 In summary, while Giordano *et al*. (2014) assessed the fruiting ability of *H. irregulare* and *H.*
162 *annosum* separately for each species, this study focused on fruiting potential and genomic
163 admixing resulting from their interspecific interaction, providing data in a research area that
164 is still poorly investigated for the fungi.
165
166 **Methods**

167 *Inoculation experiment*

168 Results from the dual inoculation experiment of pine logs described in Giordano *et al*. (2014)
169 were re-analyzed. The absence of fruiting bodies in logs inoculated with a single fungal species
170 suggested that reproduction may be enhanced when genotypes of both species interact with
171 one another, at least under the experimental conditions described in Giordano *et al*. (2014).
172 However, the magnitude of this putative enhancement was not studied in a comparative way
173 for the two *Heterobasidion* species.

174 Six pure *H. irregulare* genotypes and six pure *H. annosum* genotypes were selected and
175 coupled in order to identify six pairs of fungal isolates displaying comparable *in vitro* growth
176 rates (Gonthier and Garbelotto, 2011; Giordano *et al*., 2014; Table 1). The *H. irregulare*
177 genotype of each pair was inoculated on one side (cut end) of a freshly cut log of *Pinus*
178 *sylvestris* L. (30 cm length, about 20 cm diameter), while the matched *H. annosum* genotype
179 was inoculated on the opposite cut end of the same log. This inoculation approach was chosen
180 because it could reasonably mimic the co-infection of stumps or logs by both pathogen
181 species, taking into account that, most often, spores of the two species will land on different
182 portions of the same large woody substrate.

183 This dual inoculation experiment, replicated on 10 logs per each pair of genotypes, was
184 performed on a total of 60 logs as detailed below. Beech dowels (4 cm in length and 0.8 cm in
185 diameter) were sterilized three times for 20 min in malt extract broth (20 g malt extract, 1 L
186 distilled water), and subsequently placed in Petri plates (15 cm in diameter) filled with Potato
187 Dextrose Agar (PDA; 39 g potato dextrose agar, 200 mg streptomycin sulphate, 1 L distilled
188 water). Beech dowels were inoculated by inserting in Petri plates mycelial plugs (0.8 cm in
189 diameter) obtained from the edge of actively growing fungal cultures of the same genotype.

190 Plates were incubated in the dark at 20°C for 4 weeks to allow the complete colonization of
191 the dowels.
192 On each side of the logs, four holes (4 cm in depth) were drilled with a 0.8 cm diameter drill
193 bit at approximately 2 cm from the edge of the section. Four dowels colonized by the same
194 genotype were then inserted into the above holes. After inoculation, logs were individually
195 sealed in a plastic bag and incubated horizontally in the dark for 11 months in a growth
196 chamber set at a temperature of 19±1°C and relative humidity of 80±5%.
197 At the end of the incubation period, fully developed fruiting bodies on each side of the log
198 were counted. Each fruiting body was excised with a sterile scalpel under a laminar flow hood
199 and a portion of the context of approximately 0.5x0.5x0.5 cm was removed, transferred into
200 2.5 ml Eppendorf™ tube and lyophilized. Subsequently, lyophilized samples were frozen in
201 liquid nitrogen and immediately pulverized with a FastPrep FP 120 Cell Disrupter (Qbiogene,
202 Carlsbad, CA, USA) running for 30 s at 4.5 $m \cdot s^{-1}$. DNA was extracted from samples of 100 mg
203 per fungal fruiting body by using the E.Z.N.A.™ Stool DNA Isolation Kit (Omega Bio-Tek,
204 Norcross, GA, USA) following the manufacturer's instructions. The identification of *H.*
205 *irregulare*, *H. annosum*, or hybrids between the two species was carried out by using an
206 optimized PCR assay. Three sets of PCR primers targeting one nuclear and one mitochondrial
207 locus of *Heterobasidion* were used, resulting in amplicons of different size depending on the
208 species. Therefore, the assay allowed the identification of *H. irregulare*, *H. annosum*, and their
209 hybrids when a mismatch between the nuclear and the mitochondrial markers occurred.
210 Further details about the molecular assays were described by Gonthier *et al.* (2007) and
211 Giordano *et al.* (2014). Additionally, to determine if the fungal genotype inoculated on one
212 side of the log had spread to the opposite half portion of the log, the central cross-section of
213 each log was cut in a slice of 6 cm in thickness and incubated for one week in moist conditions.

After the incubation period, central cross-sections were examined under a dissecting microscope (40X magnification) for the presence of the asexual stage of *Heterobasidion* (i.e. conidiophores). Areas characterized by the presence of conidiophores and delimited by distinguishable boundary lines on the surface of each cross-section were assumed to have been generated by different genotypes displaying some level of somatic incompatibility (Boddy, 2000; Swedjemark and Stenlid, 2001). Each discrete area was marked, numbered, and measured with a planimeter. Subsequently, small fragments of wood (0.2 cm x 0.2 cm x 0.2 cm) from each of the discrete areas described above were sampled with a sterile scalpel and stored at -20°C. Approximately 100 mg of each wood sample were lyophilized for 24 h, homogenized, and finally DNA was extracted by using the E.Z.N.A.™ Stool DNA Isolation Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's instructions. Different areas were assigned to *H. irregulare*, *H. annosum*, or to their hybrids using the above-mentioned DNA-based diagnostic molecular assay (Gonthier *et al*., 2007).

Based on the results of the molecular assays, an acronym was generated to describe the different nuclear-mitochondrial combinations present in each heterokaryotic (n+n) fungal genotype, whether from fruiting bodies at the end of each log or from wood in the central section of each log. The acronym consisted of: a) two capital letters identifying the nuclear composition of the n+n genotype, and, b) a lowercase letter separated by a comma to identify the mitochondrial genome (i.e. *I* and *A* for a *H. irregulare* and a *H. annosum* nucleus, *i* and *a* for the *H. irregulare* and *H. annosum* mitochondrion, respectively).

[Table 1]

Data interpretation and statistical analyses

238 The effect on the production of fruiting bodies associated with the interspecific direct
239 interaction on a common substrate was assessed by calculating the ratio and its 95%
240 confidence interval (% and 95% CI) (Blaker, 2000) between the number of fruiting bodies of a
241 fungal species developed on the log side opposite to the one where that species was
242 inoculated, and the total number of fruiting bodies observed on that same side. Such ratio will
243 be hereafter referred to as ISFP (Interaction Side Fruiting bodies Production). Additionally, the
244 number of fruiting bodies was cross-tabulated in a 2 x 2 contingency table based on the
245 species (*H. irregulare* vs. *H. annosum*) and log side (i.e. inoculation side vs. opposite side). A χ^2
246 test with Yates' correction was carried out on the above contingency table to compare the
247 ISFP between species. Finally, a χ^2 test was performed to compare the number of fruiting
248 bodies of *H. irregulare* developed on the sides where it was inoculated, and on opposite sides
249 (see results), expecting the development of an equal number of fruiting bodies on each side.
250 Note that this calculation was not possible for *H. annosum*, due to the fact that this species
251 failed to produce fruiting bodies on the side opposite to the one where it was inoculated.
252 Morphological features including the average surface of the pore-carrying tissue or
253 hymenophore (mm^2), pore density (number of pores mm^{-2}), and diameter of pores (μm) of
254 fruiting bodies (Giordano *et al*., 2014) were compared between inoculation sides with two-
255 samples permutation tests (Carsey and Harden, 2014), which, again, could be performed only
256 for *H. irregulare* (see results).
257 The correlation between saprobic growth and fruiting bodies production of the inoculated
258 fungal genotypes was tested by calculating the β coefficients and related *P*-values of negative
259 binomial generalized linear regression models without intercept fitted, as described in Lione
260 *et al.* (2016), for each *Heterobasidion* species and log inoculation side. The saprobic growth of
261 *H. irregulare* and *H. annosum* genotypes was gathered from Giordano *et al*. (2014) by ranking,

for each species, the performances in the saprobic colonization of wood logs. On the six genotypes per species used in this experiment, the above rank (hereafter referred to as genotype saprobic growth score) ranges from 1 to 6, with the higher values indicating better saprobic abilities. Models were fitted on the number of fruiting bodies developed on each log side, using the genotype saprobic growth score as independent variable.

The absolute frequencies of central cross-sections colonized by the mycelium of either *H. irregulare* or *H. annosum* were compared between species with a χ^2 test with Yates' correction.

Based on combinatorics (Figure 1), a basic theoretical model predicting all the possible outcomes of *H. irregulare* (*II,i*) x *H. annosum* (*AA,a*) heterokaryotic hybridization was outlined by listing all possible combinations of nuclei and mitochondrion at the cellular level. Combinations leading to the same nuclear-mitochondrial composition (i.e. hybrid type *IA,i - IA,a - II,a - AA,i*) were enumerated to calculate the expected absolute and relative frequencies for each hybrid type. In addition, hybrid types were classified as "nuclear" when harboring nuclei from different parental species (NH=*IA,i*+*IA,a*), otherwise they were considered as "mitochondrial" if both nuclei were inherited from the same parental species but the mitochondrial genome was discordant (MH=*II,a*+*AA,i*). Additionally, hybrids were split depending on the presence of a *H. irregulare* (HiM=*IA,i*+*AA,i*) vs. a *H. annosum* (HaM=*IA,a*+*II,a*) mitochondrion. The expected frequencies of the four hybrid classes NH, MH, HiM and HaM were calculated accordingly. Based on an extensive body of literature, three expectations had to be met: 1) in the absence of prezygotic mating barriers, hybrids with all possible nuclear-mitochondrial combinations should be identified in frequencies expected based on combinatorics; 2) in the absence of any pre- or postzygotic barriers, nuclei migrate freely between parental cells, but mitochondria do not (Xu and Wang, 2015); thus, all or a large

majority of hybrid fruiting bodies produced on a log side should bear the mitochondrion of the fungal species inoculated on that same side; 3) in the absence of mitochondrial migration, a genotype carrying two identical nuclei of the same species, and the mitochondrion of the other species would require a two-step process in which each of the two parental nuclei would be replaced by a nucleus of the other species; by contrast, hybrids with mismatched nuclei would only require a one-step process with one of the parental nuclei being replaced by a single nuclear genome of the other species.

The observed absolute and relative frequencies of hybrid types and hybrid classes were gathered from the results of molecular identifications. Such frequencies were calculated both for hybrid fruiting bodies and for genotypes found in central cross-sections. For the observed relative frequencies, the associated 95% CI was also calculated (Blaker, 2000). Observed frequencies of fruiting bodies and central cross-sections of logs associated with each hybrid type and class were compared with the frequencies expected according to the model above through χ^2 tests, computing *P*-values by Monte Carlo simulations based on 10^5 replicates when asymptotic approximation was not recommended (Hope, 1968).

For hybrid fruiting bodies observed on the log side where *H. annosum* genotypes had been inoculated, the relative frequencies of HiM were calculated along with its 99.9% Bayesian credible interval (highest posterior density method) using the non-informative Jeffreys' prior to test whether the above frequency was significantly different from 0% (Jeffreys, 1961; Kéry, 2010). All statistical analyses were performed in R 3.2.3. (R Core Team, 2015), with a significance cut-off set to 0.05.

Results

309 A total of 127 pure heterokaryotic (n+n) fruiting bodies were observed at the end of the
310 experiment (Figure 2). Of these, 109 were identified as pure *H. irregulare* (*II,i*) and 18 as pure
311 *H. annosum* (*AA,a*). While all *H. annosum* fruiting bodies developed only on its inoculation
312 side, the fruiting bodies of *H. irregulare* were observed on both sides of the logs. The number
313 of fruiting bodies developed by *H. irregulare* was 1.9-fold significantly larger on the log side
314 opposite to the one where it was inoculated (χ^2=9.991, df=1, P=1.573·10^{-3}). The ISFP values of
315 *H. irregulare* and *H. annosum* fruiting bodies were significantly different (χ^2=24.013, df=1,
316 P<0.001), confirming that the two species displayed a substantial quantitative diversity in the
317 production of fruiting bodies depending on the log side (Table 2).
318
319 [Table 2]
320
321 All permutation tests comparing the morphological features between fruiting bodies of *H.*
322 *irregulare* observed on the inoculation side and on the opposite side showed no significant
323 differences (P>0.05) either for the average surface of the hymenophore (454.44 vs. 329.78
324 mm^2), the average pores density (6.32 vs. 6.43 pores mm^{-2}) or the average diameter of pores
325 (181.05 vs. 174.18 µm), respectively (Figure 3).
326 A positive and significant correlation between genotype saprobic growth scores and number
327 of fruiting bodies was detected for *H. irregulare*, which attained β values of 0.468 (P=1.15·10^{-}
328 ^{3}) and 0.663 (P=4.93·10^{-5}) on the inoculation and opposite side, respectively. On its inoculation
329 side, *H. annosum* displayed a positive, yet not significant β value of 0.242 (P=0.417), while no
330 β coefficients could be calculated for the opposite side because of the absence of fruiting
331 bodies on that log extremity (Figure 4).

The molecular analyses carried out on the central cross-sections of logs showed that the mycelium of *H. irregulare* was present in 51 out of 60 logs (85% of the total number of central cross-sections; Figure 5), and *H. irregulare* colonized wood surfaces with areas ranging between 45.91 and 142.05 cm^2, depending on the combination of genotypes. In contrast, the mycelium of *H. annosum* was present only on a single central cross-section out of 60 (1.6% of the total number of central cross-sections), with a colonized surface amounting to 78.52 cm^2. The above absolute frequencies of central log cross-sections colonized by the mycelium of either species were significantly different (χ^2=81.482, df=1, P<0.001).

A total of 10 theoretical combinations of nuclei and mitochondria within possible *H. irregulare* x *H. annosum* heterokaryotic hybrids were enumerated (Figure 1). Hybrid types *IA,i* and *IA,a* included 4 combinations of nuclei and mitochondrion (40%) each, while hybrid types *II,a* and *AA,i* were represented by one combination (10%) each. Hence, the expected frequencies for the different hybrid classes attained 8 (80%) for NH and 2 (20%) for MH, while the same frequencies were equally distributed among the classes HaM and HiM, achieving 5 (50%) each. The dual inoculation experiment showed that all the six pairs of inoculated genotypes had hybridized, and a total of 21 heterokaryotic (n+n) *H. irregulare* x *H. annosum* fruiting bodies were observed. Each of the four possible nuclear-mitochondrial combinations was detected at least once, with the following absolute and relative frequencies: *IA,i* (7 fruiting bodies representing the 33.3% of the total number of hybrids, with a 15.2-55.1% $CI_{95\%}$), *IA,a* (9 fruiting bodies, 42.9%, with a 22.7-64.9% $CI_{95\%}$), *II,a* (4 fruiting bodies, 19.0%, with a 6.8-40.1% $CI_{95\%}$), and *AA,i* (1 fruiting body, 4.8% with a 0.2-22.7% $CI_{95\%}$). Hence, 16 (76.2% of the total number of hybrids, with a 54.5-90.1% $CI_{95\%}$) and 5 (23.8%, with a 9.9-45.5% $CI_{95\%}$) fruiting bodies were classified as NH and MH, respectively, while 13 (61.9%, with a 40.1-80.3% $CI_{95\%}$) and 8 (38.1%, with a 19.7-59.9% $CI_{95\%}$) fruiting bodies were split between HaM and HiM. The outcomes of χ^2

tests showed no significant differences (P>0.05) between the frequencies of hybrid fruiting bodies observed as a result of the inoculation trial and expected according to the theory. Not significant P-values were obtained both for hybrid types (χ^2=2.571, P=0.493) and for hybrid classes NH and MH (χ^2=0.190, P=0.784), HaM and HiM (χ^2=1.190, P=0.275).

On the log side where *H. annosum* genotypes had been inoculated, 18 out of 21 total hybrid fruiting bodies were observed, with the relative frequency of HiM (i.e. hybrids with a *H. irregulare* mitochondrion) attaining 33.3%. All hybrids on the *H. annosum* inoculation side were expected to bear the *H. annosum* mitochondrion, and consequently the frequency of HiM hybrids should have been zero. However, Bayesian credible intervals showed instead that the HiM relative frequency was significantly different from 0%, since its inferred range of variability was comprised between 6.6% and 69.7% with a 99.9% probability.

The presence of hybrid mycelium belonging to all hybrid types was detected with varying frequencies on 13 central log cross-sections. In detail, *IA,i* was detected in 7 cross-sections out of 13 (53.8%, 26.0-18.4% $CI_{95\%}$) on an average wood surface of 47.79 cm^2, *IA,a* in 4 cross-sections (30.8%, 11.3-58.7% $CI_{95\%}$) on 32.86 cm^2, *II,a* in 1 cross-section (7.7%, 0.4-33.7% $CI_{95\%}$) on 22.6 cm^2, and *AA,i* in 1 cross-section (7.7%, 0.4-33.7% $CI_{95\%}$) on 59.63 cm^2. Hence, a total of 11 central cross-sections (84.6%, 56.6-97.2% $CI_{95\%}$) were colonized by NH, while only 2 sections (15.4%, 2.8-43.4% $CI_{95\%}$) by MH mycelium. Similarly, 5 (38.5%, 16.6-66.3% $CI_{95\%}$) and 8 (61.5%, 33.6-83.4% $CI_{95\%}$) central cross-sections were colonized by HaM and by HiM mycelium, respectively. The outcomes of the comparisons between observed and expected frequencies in central log cross-sections were statistically equivalent to those previously illustrated for fruiting bodies of hybrids types (χ^2=1.038, P=0.818) and hybrid classes NH and MH (χ^2=0.173, P=0.753), HaM and HiM (χ^2=0.692, P=0.582).

Discussion

To date the outcomes of the direct interaction between native and non-native pathogens have not been thoroughly studied. The invasion by the North American species *H. irregulare* in parts of central Italy where the Eurasian congener *H. annosum* is present provides an excellent model system to study such interactions between two interfertile species characterized by overlapping ecological niches. In this study, we simulated interspecific interactions between these two fungal pathogens by mimicking natural environmental conditions (i.e. stump or log co-infections) through a dual inoculation experiment on a woody substrate (pine logs). Giordano *et al*. (2014) showed that the fruiting potential of *H. irregulare* is greater than that of *H. annosum*, while the main goal of this study was to determine whether fruiting of either species would be substantially increased or decreased when they co-occur and interact on the same portion of a natural substrate.

Results showed that *H. irregulare* formed a significantly larger number (i.e. 1.9-fold) of fruiting bodies when spatially overlapping with *H. annosum*. The analyses carried out on the central cross-sections of the logs further confirmed that the mycelium of *H. irregulare* had grown uninterruptedly from the side where it had been inoculated to the opposite side. The central cross-sections displayed boundaries visible not only among contiguous patches colonized by different fungal genotypes, but occasionally also between patches colonized by the same genotype. This unexpected incompatibility reaction might be the result of the interaction between heterokaryons and homokaryons sharing a common nucleus, as documented in other basidiomycetes (Worrall, 1997 and references therein). It should be noted that the molecular diagnostic assay we used does not allow to discriminate between conspecific homokaryotic and heterokaryotic mycelia.

403 It is evident that the enhanced fruiting body production by *H. irregulare* occurred where the
404 two *Heterobasidion* species co-exist in the same side of the log, and thus has to be the
405 consequence of the interspecific interaction between the two. However, our experimental
406 design did not allow us to determine the possible mechanisms underlying such a process.
407 Nonetheless, it could be hypothesized that fruiting bodies production of *H. irregulare* might
408 have been boosted by physiological or ecological processes mediated by spatial niche sharing
409 with the related congener. As reported by Wardle *et al.* (1993), two fungal species sharing
410 common natural substrates may unexpectedly display a highly unpaired reproductive success.
411 However, the factors conferring to one of the two species an advantage over the other, as
412 well as the causes of this outcome are still poorly understood, hence deserving further
413 investigations.
414 Based on our results, the interaction between the two fungal pathogens increased the number
415 of fruiting bodies differentiated by *H. irregulare*, but did not influence their morphology. In
416 fact, *H. irregulare* fruiting bodies were fully comparable in terms of average surface of the
417 hymenophore, pores density, and diameter, regardless of the side of the log where they had
418 been formed. Hence, larger airborne spore loads of *H. irregulare* due to increased fruiting
419 bodies production might be expected in those stands where the two *Heterobasidion* species
420 overlap. This hypothesis is corroborated by evidence from a survey conducted across all the
421 current invasion area of *H. irregulare* in central Italy showing that the maximum spore load of
422 *H. irregulare* was observed in the Circeo National Park, where both species are comparable in
423 abundance and thus are most likely to interact (Gonthier *et al*., 2007).
424 While *H. irregulare* genotypes often produced fruiting bodies on the log side opposite to the
425 one where they had been inoculated, *H. annosum* genotypes never did because their
426 mycelium was unable to grow up to that side, as confirmed by the analysis of the logs central

427 cross-sections. These results clearly indicate that the presence of *H. annosum* as a competitor
428 for trophic resources does not inhibit the efficiency of *H. irregulare* in utilizing the common
429 growth substrate. Giordano *et al.* (2014) pointed out that in single inoculation trial *H.*
430 *irregulare* displayed superior saprobic abilities than *H. annosum.* Not surprisingly, our findings
431 show that the positive correlation between saprobic growth and fruiting bodies production
432 was significant only for *H. irregulare* and the magnitude of such a correlation was larger on
433 the side where the interaction with *H. annosum* occurred.
434 Since *H. irregulare* and *H. annosum* have been reported to hybridize in nature (Gonthier and
435 Garbelotto, 2011), a further aim of this study was to investigate the outcome of interspecific
436 mating. By deriving through combinatorics the enumeration of all possible nuclear and
437 mitochondrial combinations in *H. irregulare* x *H. annosum* heterokaryotic hybrids, we
438 obtained a theoretical model quantifying the proportion expected for each hybrid type (*IA,i* -
439 *IA,a* - *II,a* - *AA,i*) and hybrid class (NH, MH and HaM, HiM). Since this model assumes no
440 constraining or prompting factors influencing the probability of formation of hybrids, it allows
441 for the assessment of the presence of any intrinsic barriers to the formation of specific
442 nuclear-mitochondrial combinations, at least in a controlled environment.
443 Our experiment proved that all four nuclear-mitochondrial combinations can be originated
444 when *H. irregulare* and *H. annosum* co-occur in the same substrate with frequencies
445 statistically equivalent to those expected according to our neutral theoretical model. This
446 finding suggests that no nuclear-mitochondrial combinations are lethal and subjected to
447 prezygotic negative selection. While our experimental design allowed for the detection of such
448 combinations, the biological mechanisms leading to their formation can only be hypothesized.
449 Each pair of genotypes inoculated was somatically incompatible based on *in vitro* pairing tests
450 (not shown). However, nuclear reassortments between somatically incompatible mycelia

451 resulting in the formation of novel heterokaryotic genotypes, in their turn somatically
452 incompatible with either progenitor, have been reported in *Heterobasidion* spp. (Hansen *et*
453 *al*., 1993). A similar mechanism may apply in the case of interfertile, yet somatically
454 incompatible *H. irregulare* and *H. annosum* genotypes, leading to the formation of hybrid
455 mycelia. This phenomenon might have occurred in the terminal portion of the inoculated logs,
456 but a more complex and fluid scenario might have taken place as well. For instance, nuclei of
457 *H. irregulare* could have migrated through the mycelium of *H. annosum* up to the opposite
458 side of the log, forming hyphal mosaics with some mycelial segments bearing the original
459 genotype and others harboring new nuclear combinations. Noteworthy, different patterns of
460 nuclear migration have been documented in other fungal model systems resulting in a variety
461 of hyphal mosaics [see, for instance, Peabody *et al*. (2000) and the literature therein].
462 Our experimental design also allowed for a more insightful analysis accounting for the effects
463 of temporal and spatial dynamics of substrate colonization on the hybridization outcomes.
464 Assuming that mitochondria do not migrate (Xu and Wang, 2015), the mitochondrial type of
465 hybrids should be determined by the fungal species first established. Hence, in our experiment
466 hybrid fruiting bodies developed on a given log side should harbor the mitochondrion of the
467 parental species inoculated in that side. While the small number of hybrid fruiting bodies did
468 not allow to test the above hypothesis on log ends inoculated with *H. irregulare*, one third of
469 the 18 hybrid fruiting bodies developed on the *H. annosum* side of inoculated logs harbored
470 the *H. irregulare* mitochondrion (i.e. HiM hybrids). This HiM ratio is significantly different from
471 the expected 0%, with a probability of 99.9%, suggesting that the mitochondrion of *H.*
472 *irregulare* might provide a competitive advantage over the mitochondrion of *H. annosum* in
473 hybrid genotypes carrying nuclei of both parental species. This finding is in agreement with
474 both field and experimental observations (Gonthier and Garbelotto, 2011; Giordano *et al*.,

2018) and may have important evolutionary consequences, as it could possibly lead to a species-wide substitution of the *H. annosum* mitochondrial genome by the *H. irregulare* one as a result of horizontal gene transfer through hybridization and interspecific gene introgression.

Our findings are extremely relevant for considering the feasibility of eradication or control strategies for *H. irregulare* in Europe. One of the main differences between the current zone of infestation in central Italy and the potential future range of *H. irregulare* in Europe lies in the much higher frequency of *H. annosum* in many central and northern European pine forests (Korhonen *et al*., 1998; Asiegbu *et al*., 2005). Our data indicate that significant levels *H. annosum* are likely to stimulate increased fruiting of *H. irregulare* and to increase hybridization rates. The first phenomenon will result in a faster establishment of *H. irregulare*, while the second will result in: 1) an acceleration of adaptation by generating genetically more varied populations characterized by genotypes with admixed genomes; and in 2) a possible introgression of nuclear genes and mitochondrial genomes from the invasive into the native species, possibly increasing the virulence of the latter (Gonthier and Garbelotto, 2011). Hence, priority for surveys, detection, and eradication of *H. irregulare* should be given to areas where *H. annosum* is well established. Because the complete eradication of *H. irregulare* in the current zone of infestation is unrealistic (Gonthier *et al*., 2014), the only possible way to effectively manage the disease is to intercept its expansion by promptly eliminating new outbreaks. A fast and specific detection method based on Loop-mediated isothermal AMPlification (LAMP) of nuclear markers has been recently developed for *H. irregulare* and is recommended for that purpose (Sillo *et al*., 2018), although the use of mitochondrial markers is also advisable to complement the detection.

Finally, our data may provide useful information to assess, and eventually model, the potential impact of the non-native species based on the actual or likely distribution of the native one. In conclusion, this study shows that the presence of a competitor can enhance the transmission of a non-native invasive microbe, rather than counteracting its spread.

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Conflict of interest statement

None declared.

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Figure captions

Figure 1 Theoretical combinations of nuclei and mitochondrion within all possible *Heterobasidion irregulare* x *H. annosum* heterokaryotic (n+n) hybrids. Heterokaryotic parental species are schematically represented at cellular level by their nuclear and mitochondrial composition, with letters I_1, I_2 and *i*, and A_1, A_2 and *a* indicating the two nuclei and

mitochondrion of *H. irregulare* and *H. annosum*, respectively. Nuclei and mitochondria are combined within the 4 possible hybrid types (*IA,i* - *IA,a* - *II,a* - *AA,i*) and grouped within nuclear hybrids (NH) and mitochondrial hybrids (upper and lower part of the figure, respectively, delimited by a dotted line) or within hybrids with a *H. irregulare* (HiM) or a *H. annosum* (HaM) mitochondrion (left and right part of the figure, respectively, delimited by a dashed line).

Figure 2 a) Fruiting bodies on one side of an inoculated log; b) detail of a fruiting body displaying a fully developed hymenophore (pore layer).

Figure 3 Comparisons of the morphological features between fruiting bodies of *Heterobasidion irregulare* observed on the inoculation side and on the opposite side. For both log sides the barplots indicate the average attained by: a) the surface of the hymenophore (mm^2), b) the pores density (pores mm^{-2}), and c) the diameter of pores (µm). Error bars refer to the associated 95% bootstrap confidence intervals (based on 10^5 iterations) associated with the averages. The same letters next to the bars indicate no significant differences between averages (P>0.05).

Figure 4 Correlation between saprobic growth and fruiting bodies production of *H. irregulare* and *H. annosum* genotypes. The genotype saprobic growth score attained by each fungal genotype within species is reported on the *x*-axis, indicating better saprobic abilities with increasing score values, based on Giordano *et al.* 2014. Beneath the score, the corresponding genotype codes are reported in brackets, referring to *H. irregulare* and *H. annosum*, respectively (see Table 1). The number of fruiting bodies associated with each fungal genotype is reported per species and inoculation side on the *y*-axis. The curves display the correlation

690 between the genotype saprobic growth score and the number of fruiting bodies, based on the
691 outcomes of the negative binomial generalized linear regression models. Asterisks indicate
692 significant correlations (P<0.05). A vertical offset of 0.3 units was included to separate
693 overlapping points.
694
695 **Figure 5** The central cross-section of an inoculated log displaying: a) areas delimited by
696 distinguishable boundary lines after log cutting 11 months after inoculations, and b) the same
697 areas colonized by the mycelium of *Heterobasidion* spp. after one week of incubation in moist
698 conditions of the central cross-section. Labels indicate results of molecular analyses (*II,I*: *H.*
699 *irregulare*; *IA,a*: hybrid mycelium).
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703 FIG. 1

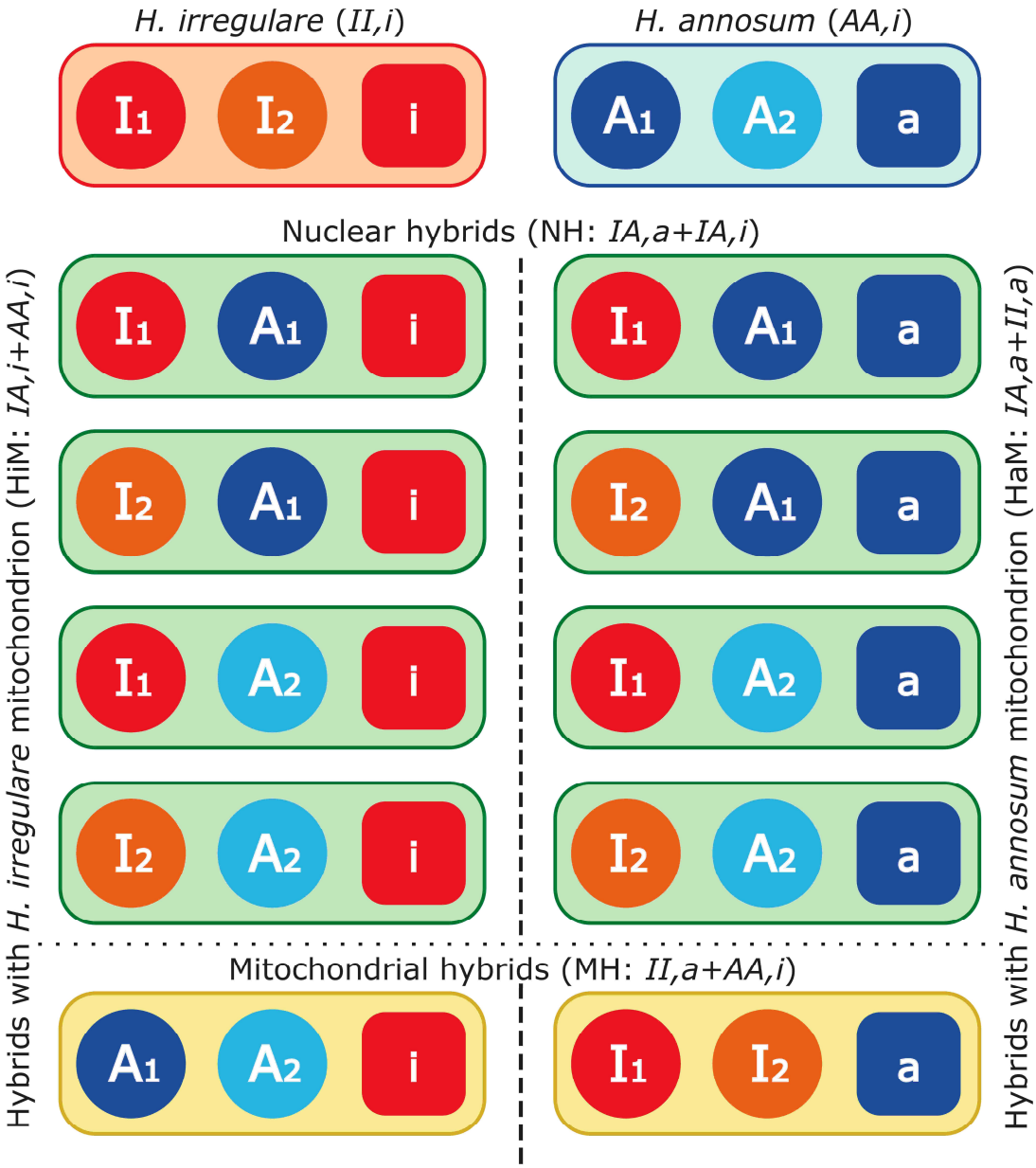

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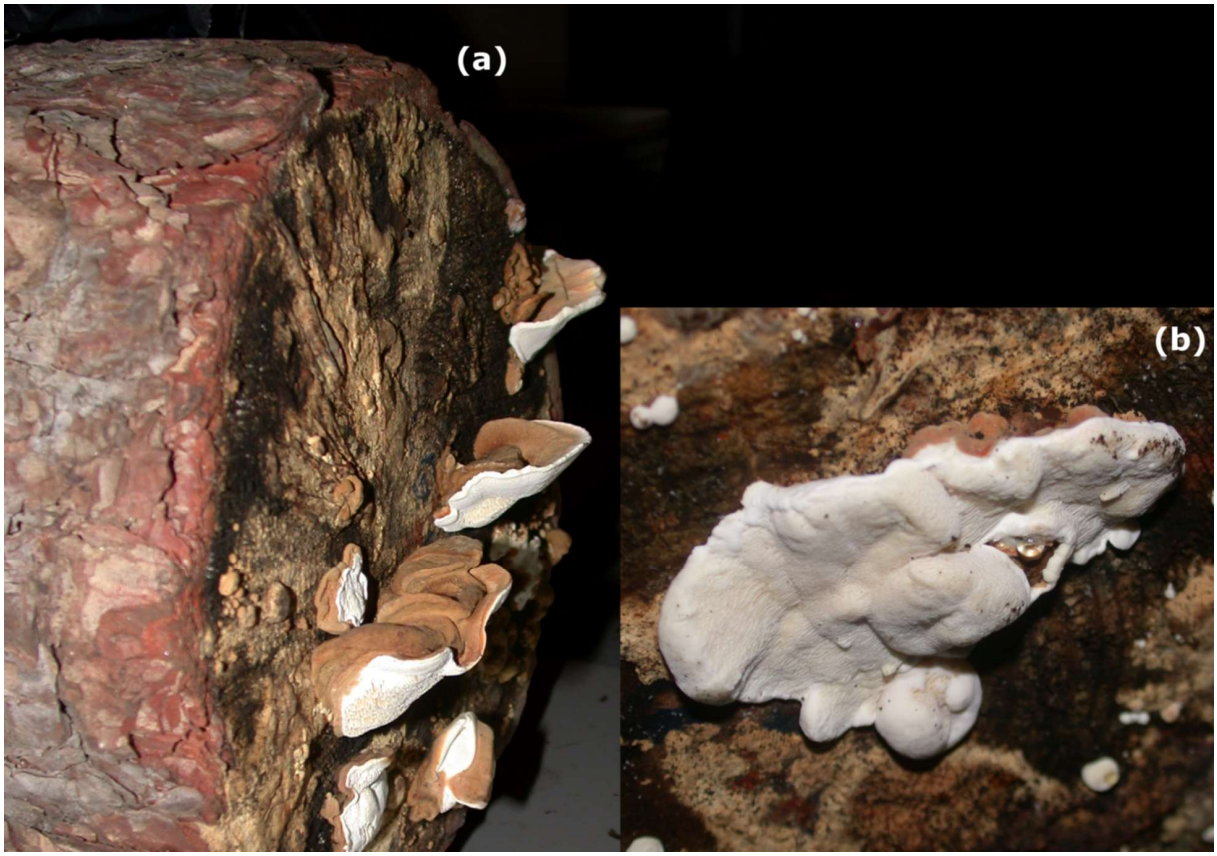
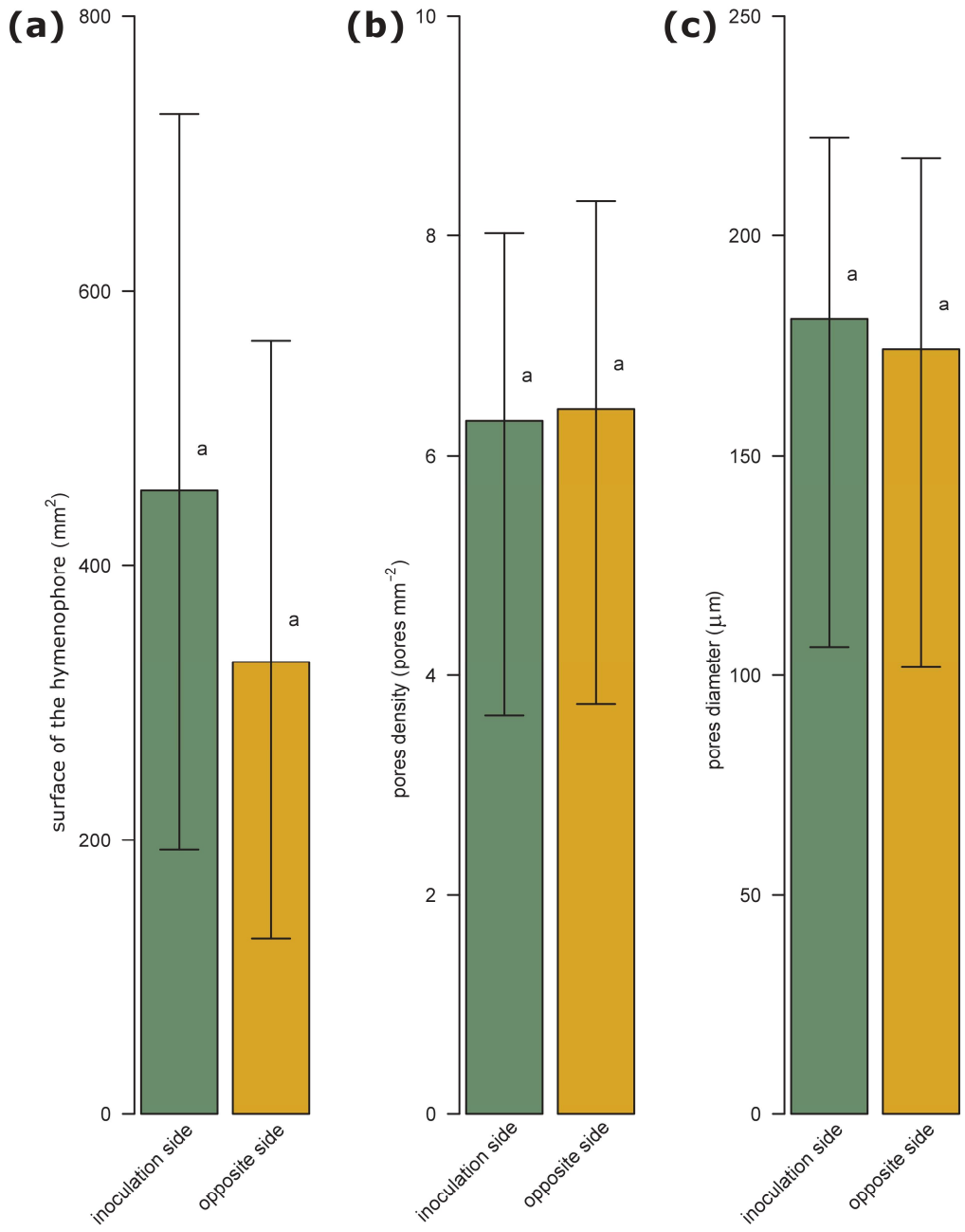
FIG. 2



711 FIG. 3



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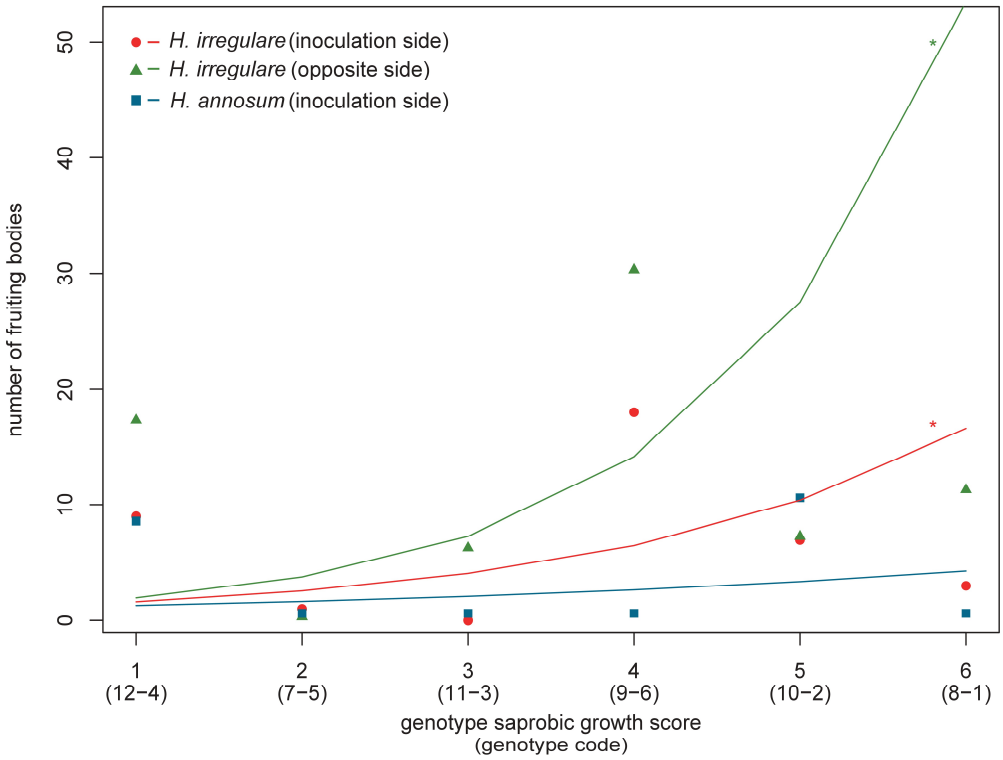
714 FIG. 4



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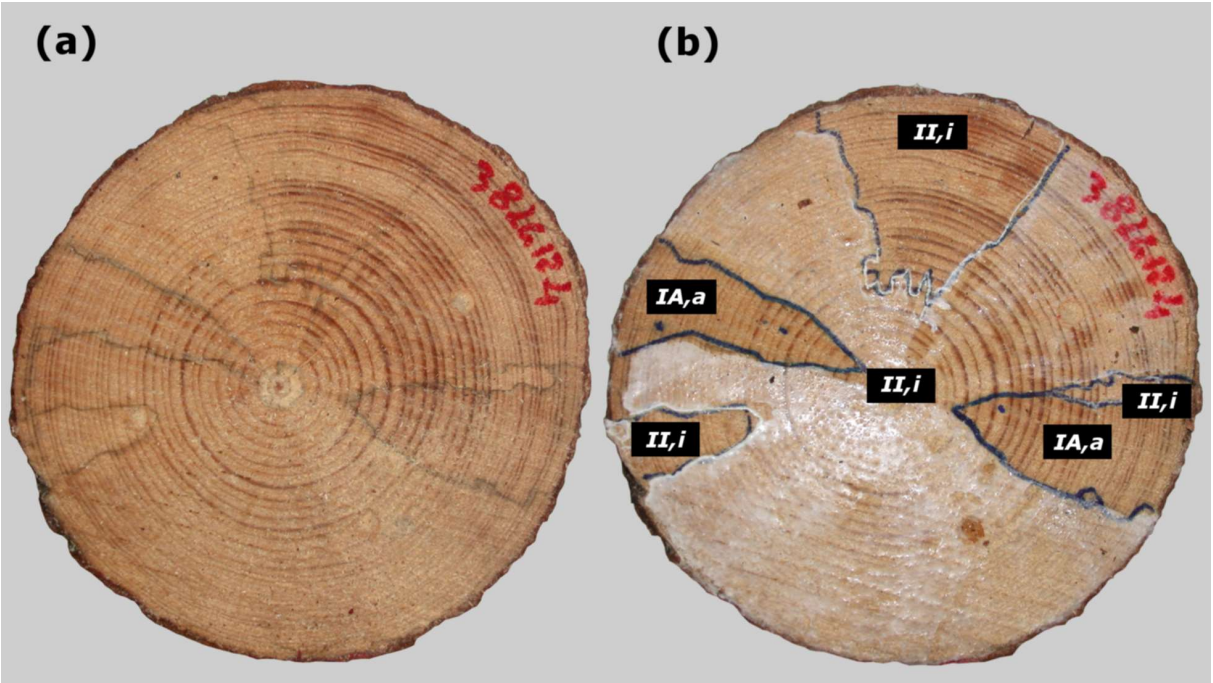
718 FIG. 5



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732 **Tables**

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734 **Table 1** Six pairs of *Heterobasidion* genotypes displaying comparable *in vitro* growth rates used

735 in the inoculation experiment (Giordano *et al*., 2014, modified).

Genotype combination	Geographical origin (isolation year)	*Heterobasidion* species	*MUT accession N.
1-10	Sabaudia, LT (2005)	*H. irregulare*	MUT00001197
	Taverna, CZ (1995)	*H. annosum*	MUT00001215
2-12	Castelporziano, RM (2002)	*H. irregulare*	MUT00003560
	Meugliano, TO (1999)	*H. annosum*	MUT00001149
3-9	Sabaudia, LT (2005)	*H. irregulare*	MUT00001151
	Brusson, AO (2009)	*H. annosum*	MUT00001208
4-8	Castelfusano, RM (2005)	*H. irregulare*	MUT00001193
	Morgex, AO (2005)	*H. annosum*	MUT00001216
5-7	Castelfusano, RM (2006)	*H. irregulare*	MUT00001161
	Sabaudia, LT (2005)	*H. annosum*	MUT00001204
6-11	Castelporziano, RM (2002)	*H. irregulare*	MUT00003563
	Sabaudia, LT (2007)	*H. annosum*	MUT00001143

736 **MUT Mycotheca Universitatis Taurinensis*

737

738 **Table 2** Number of fruiting bodies observed for *Heterobasidion irregulare* and *H. annosum* on
739 their respective inoculation side and on the side opposite to this latter. For each species, the
740 ISFP (Interaction Side Fruiting bodies Production) is reported along with the associated 95%
741 confidence interval ($CI_{95\%}$). Different letters next to ISFP values indicate their significant
742 difference according to the χ^2 test.

	Number of fruiting bodies observed on the inoculation side	Number of fruiting bodies observed on the opposite side	ISFP
H. irregulare	38	71	65% **a** (55.5-73.7%, $CI_{95\%}$)
H. annosum	18[§]	0	0% **b** (0-17.8%, $CI_{95\%}$)

743 [§] 13 observed in co-occurrence with fruiting bodies of *H. irregulare*

744