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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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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 formed in inoculated pine logs, hybrids with nuclei of both species and the *H. irregulare* mitochondrion were favoured, 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.

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 hinge 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 programmes 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; Newcombe et al., 2000; Brasier, 2001; 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, 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 favour 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 species are known to infect primarily freshly cut host stumps and logs by means of airborne meiospores (Garbelotto and Gonthier, 2013). Indeed, host stumps represent key substrates for both these plant pathogenic fungi not only because they act as major courts for their establishment in forest stands but also because they allow for tree-to-tree spread through root contacts, and because they are an important substrate for the production of fruiting bodies (Garbelotto and Gonthier, 2013).

This study aimed at improving our understanding of the outcome of direct ecological interactions between the native and the non-native species of *Heterobasidion*. Since the native *H. annosum* is largely widespread across Europe, whether the interaction with the non-native *H. irregulare* may be competitive, neutral or synergistic might substantially influence the spread of the latter. In this study, results published by Giordano et al. (2014) were amended with additional unpublished results and used in a completely new set of biological and statistical analyses, to test the following new hypotheses: (1) Did the two species display a competitive, neutral or synergistic interaction when growing on the same portions of a common substrate? (2) Did interspecific interactions alter the main macro-morphological traits of the fruiting bodies of either species? (3) Did the rate of hybridization suggest the presence of any pre and/or post-zygotic mating barriers resulting in hybrids with genomes biased in favour of specific nuclear and/or mitochondrial combinations.

In summary, while Giordano et al. (2014) assessed the fruiting ability of *H. irregulare* and *H. annosum* separately for each species, this study focused on fruiting potential and genomic admixing resulting from their interspecific interaction, providing data in a research area that is still poorly investigated for the fungi.

Methods

Inoculation experiment

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

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

This dual-inoculation experiment, replicated on 10 logs per each pair of genotypes, was performed on a total of 60 logs as detailed in the following. Beech dowels (4 cm in length and 0.8 cm in diameter) were sterilized three times for 20 min in malt extract broth (20 g malt extract, 1 L distilled water), and subsequently placed in Petri plates (15 cm in diameter) filled with Potato Dextrose Agar (PDA; 39 g potato dextrose agar, 200 mg streptomycin sulphate, 1 L distilled water). Beech dowels were inoculated by inserting in Petri plates mycelial plugs (0.8 cm in diameter) obtained from the edge of actively growing fungal cultures of the same genotype. Plates were incubated in the dark at 20°C for 4 weeks to allow the complete colonization of the dowels.

On each side of the logs, four holes (4 cm in depth) were drilled with a 0.8-cm diameter drill bit at ~2 cm from the edge of the section. Four dowels colonized by the same genotype were then inserted into the above holes. After inoculation, logs were individually sealed in a plastic bag and incubated horizontally in the dark for 11 months in a growth chamber set at a temperature of 19 ± 1°C and relative humidity of 80 ± 5 per cent.

Table 1 Six pairs of *Heterobasidion* genotypes displaying comparable *in vitro* growth rates used in the inoculation experiment (Giordano et al., 2014, modified).

Genotype combination	Geographical origin (isolation year)	*Heterobasidion* species	[1]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

[1]MUT, Mycotheca Universitatis Taurinensis.

At the end of the incubation period, fully developed fruiting bodies on each side of the log were counted. Each fruiting body was excised with a sterile scalpel under a laminar flow hood and a portion of the context of ~0.5 cm × 0.5 cm × 0.5 cm was removed, transferred into 2.5-mL Eppendorf™ tube and lyophilized. Subsequently, lyophilized samples were frozen in liquid nitrogen and immediately pulverized with a FastPrep FP 120 Cell Disrupter (Qbiogene, Carlsbad, CA, USA) running for 30 s at 4.5 $m \cdot s^{-1}$. DNA was extracted from samples of 100 mg per fungal fruiting body by using the E.Z.N.A.™ Stool DNA Isolation Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's instructions. The identification of *H. irregulare*, *H. annosum* or hybrid fruiting bodies between the two species was carried out by using an optimized PCR assay. Three sets of PCR primers targeting one nuclear and one mitochondrial locus of *Heterobasidion* were used, resulting in amplicons of different sizes depending on the species. Therefore, the assay allowed the identification of *H. irregulare*, *H. annosum* and their hybrids when a mismatch between the nuclear and the mitochondrial markers occurred. Further details about the molecular assays were described by Gonthier et al. (2007) and Giordano et al. (2014). Additionally, to determine if the fungal genotype inoculated on one side of the log had spread to the opposite half portion of the log, the central cross section of each log was cut into a slice of 6 cm in thickness and incubated for 1 week under moist conditions. After the incubation period, central cross sections were examined under a dissecting microscope (40× 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 × 0.2 cm × 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) following the manufacturer's instructions. Different areas were assigned to *H. irregulare*, *H. annosum* or to their hybrids using the abovementioned 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: (1) two capital letters identifying the nuclear composition of the *n*+*n* genotype, and, (2) 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).

Data interpretation and statistical analyses

The effect on the production of fruiting bodies associated with the interspecific direct interaction on a common substrate was assessed by calculating the ratio and its 95 per cent confidence interval (% and 95 per cent CI) (Blaker, 2000) between the number of fruiting bodies of a fungal species developed on the log side opposite to the one where that species was inoculated, and the total number of fruiting bodies observed on that same side. Such ratio will be hereafter referred to as Interaction Side Fruiting bodies Production (ISFP). Additionally, the number of fruiting bodies was cross-tabulated in a 2 × 2 contingency table based on the species (*H. irregulare vs H. annosum*) and log side (i.e. inoculation side *vs* opposite side). A χ^2 test with Yates' correction was carried out on the above contingency table to compare the ISFP between species. Finally, a χ^2 test was performed to compare the number of fruiting bodies of *H. irregulare* developed on the sides where it was inoculated, and on opposite sides (see results), expecting the development of an equal number of fruiting bodies on each side. Note that this calculation was not possible for *H. annosum*, due to the fact that this species failed to produce fruiting bodies on the side opposite to the one where it was inoculated.

Morphological features including the average surface of the pore-carrying tissue or hymenophore (mm^2), pore density (number of pores mm^{-2}) and diameter of pores (μm) of fruiting bodies (Giordano et al., 2014) were compared between inoculation sides with two-sample permutation tests (Carsey and Harden, 2014), which, again, could be performed only for *H. irregulare* (see Results).

The correlation between saprobic growth and fruiting bodies production of the inoculated fungal genotypes was tested by calculating the β coefficients and related *P*-values of negative binomial generalized linear regression models without intercept fitted, as described by Lione et al. (2016), for each *Heterobasidion* species and log inoculation side. The

saprobic growth potential of *H. irregulare* and *H. annosum* was gathered from Giordano et al. (2014) by ranking the performances of each of six genotypes per species in the saprobic colonization of wood logs. Ranks (hereafter referred to as genotype saprobic growth score) ranged 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 an 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 the 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 harbouring 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* (HiM = *IA,a* + *II,a*) mitochondrion. The expected frequencies of the four hybrid classes NH, MH, HiM and HaM

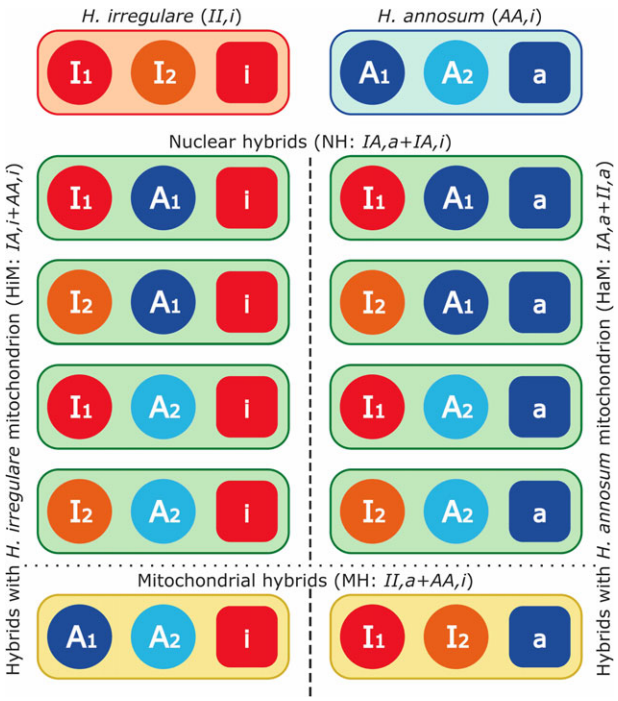

Figure 1 Theoretical combinations of nuclei and mitochondrion within all possible *Heterobasidion irregulare* × *H. annosum* heterokaryotic (*n*+*n*) hybrids. Heterokaryotic parental species are schematically represented at cellular level by their nuclear and mitochondrial composition, with letters I_1, 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 four 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).

were calculated accordingly. Based on an extensive body of the 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 post-zygotic barriers, nuclei migrate freely between parental cells, but mitochondria do not (Xu and Wang, 2015); thus, all 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 per cent 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 per cent Bayesian credible interval (highest posterior density method) using the non-informative Jeffreys' prior to test whether the above frequency was significantly different from 0 per cent (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

A total of 127 pure heterokaryotic (*n*+*n*) fruiting bodies were observed at the end of the experiment (Figure 2). Of these, 109 were identified as pure *H. irregulare* (*II,i*) and 18 as pure *H. annosum* (*AA,a*). While all *H. annosum* fruiting bodies developed only on its inoculation side, the fruiting bodies of *H. irregulare* were observed on both sides of the logs. The number of fruiting bodies developed by *H. irregulare* was 1.9-fold significantly larger on the log side opposite to the one where it was inoculated (χ^2=9.991, df = 1, $P = 1.573{\cdot}10^{-3}$). The ISFP values of *H. irregulare* and *H. annosum* fruiting bodies were significantly different (χ^2=24.013, df = 1, $P < 0.001$), confirming that the two species displayed a substantial quantitative diversity in the production of fruiting bodies depending on the log side (Table 2).

All permutation tests comparing the morphological features between fruiting bodies of *H. irregulare* observed on the inoculation side and on the opposite side showed no significant differences ($P > 0.05$) either for the average surface of the hymenophore (454.44 *vs* 329.78 mm^2), the average pores density (6.32 *vs* 6.43 pores mm^{-2}) or the average diameter of pores (181.05 *vs* 174.18 µm), respectively (Figure 3).

A positive and significant correlation between genotypic saprobic growth scores and number of fruiting bodies was detected for *H. irregulare*, with β values equal to 0.468 ($P = 1.15{\cdot}10^{-3}$) and to 0.663 ($P = 4.93{\cdot}10^{-5}$) on the inoculation and opposite side, respectively. On its inoculation side, *H. annosum* displayed a positive but statistically insignificant β value of

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).

Table 2 Number of fruiting bodies observed for *Heterobasidion irregulare* and *H. annosum* on their respective inoculation side and on the side opposite to this latter. For each species, the ISFP is reported along with the associated 95 per cent confidence interval ($CI_{95\ per\ cent}$). Different letters next to ISFP values indicate their significant 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[1]	0	0% **b** (0–17.8%, $CI_{95\%}$)

[1]13 observed in co-occurrence with fruiting bodies of *H. irregulare*.

0.242 ($P = 0.417$), while no β coefficients could be calculated for the opposite side because of the absence of fruiting 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 per cent 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 per cent 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* × *H. annosum* heterokaryotic hybrids were enumerated (Figure 1). Hybrid types *IA,i* and *IA,a* included four combinations of nuclei and mitochondrion (40 per cent) each, while hybrid types *II,a* and *AA,i* were represented by one combination (10 per cent) each. Hence, the expected frequencies for the different hybrid classes attained 8 (80 per cent) for NH and 2 (20 per cent) for MH, while the same frequencies were equally distributed among the classes HaM and HiM, achieving 5 (50 per cent) 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* × *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* (seven fruiting bodies representing the 33.3 per cent of the total number of hybrids, with a 15.2–55.1 per cent $CI_{95\ per\ cent}$), *IA,a* (nine fruiting bodies, 42.9 per cent, with a 22.7–64.9 per cent $CI_{95\ per\ cent}$), *II,a* (four fruiting bodies, 19.0 per cent, with a 6.8–40.1 per cent $CI_{95\ per\ cent}$) and *AA,i* (one fruiting body, 4.8 per cent with a 0.2–22.7 per cent $CI_{95\ per\ cent}$). Hence, 16 (76.2 per cent of the total number of hybrids, with a 54.5–90.1 per cent $CI_{95\ per\ cent}$) and 5 (23.8 per cent, with a 9.9–45.5 per cent $CI_{95\ per\ cent}$) fruiting bodies were classified as NH and MH, respectively, while 13 (61.9 per cent, with a 40.1–80.3 per cent $CI_{95\ per\ cent}$) and 8 (38.1 per cent, with a 19.7–59.9 per cent $CI_{95\ per\ cent}$) 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 per cent. 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 per cent, since its inferred range of variability was comprised between 6.6 per cent and 69.7 per cent with a 99.9 per cent 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 per cent, 26.0–18.4 per cent $CI_{95\ per\ cent}$) on an average wood surface of 47.79 cm^2, *IA,a* in 4 cross sections (30.8 per cent, 11.3–58.7 per cent $CI_{95\ per\ cent}$) on 32.86 cm^2, *II,a* in 1 cross section (7.7 per cent, 0.4–33.7 per cent $CI_{95\ per\ cent}$) on 22.6 cm^2 and *AA,i* in 1 cross section (7.7 per cent, 0.4–33.7 per cent $CI_{95\ per\ cent}$) on 59.63 cm^2. Hence, a total of 11 central cross sections (84.6 per cent, 56.6–97.2 per cent $CI_{95\ per\ cent}$) were colonized by NH, while only 2 sections (15.4 per cent, 2.8–43.4 per cent $CI_{95\ per\ cent}$) by MH mycelium. Similarly, five (38.5 per cent, 16.6–66.3 per cent $CI_{95\ per\ cent}$) and eight (61.5 per cent, 33.6–83.4 per cent $CI_{95\ per\ cent}$) 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

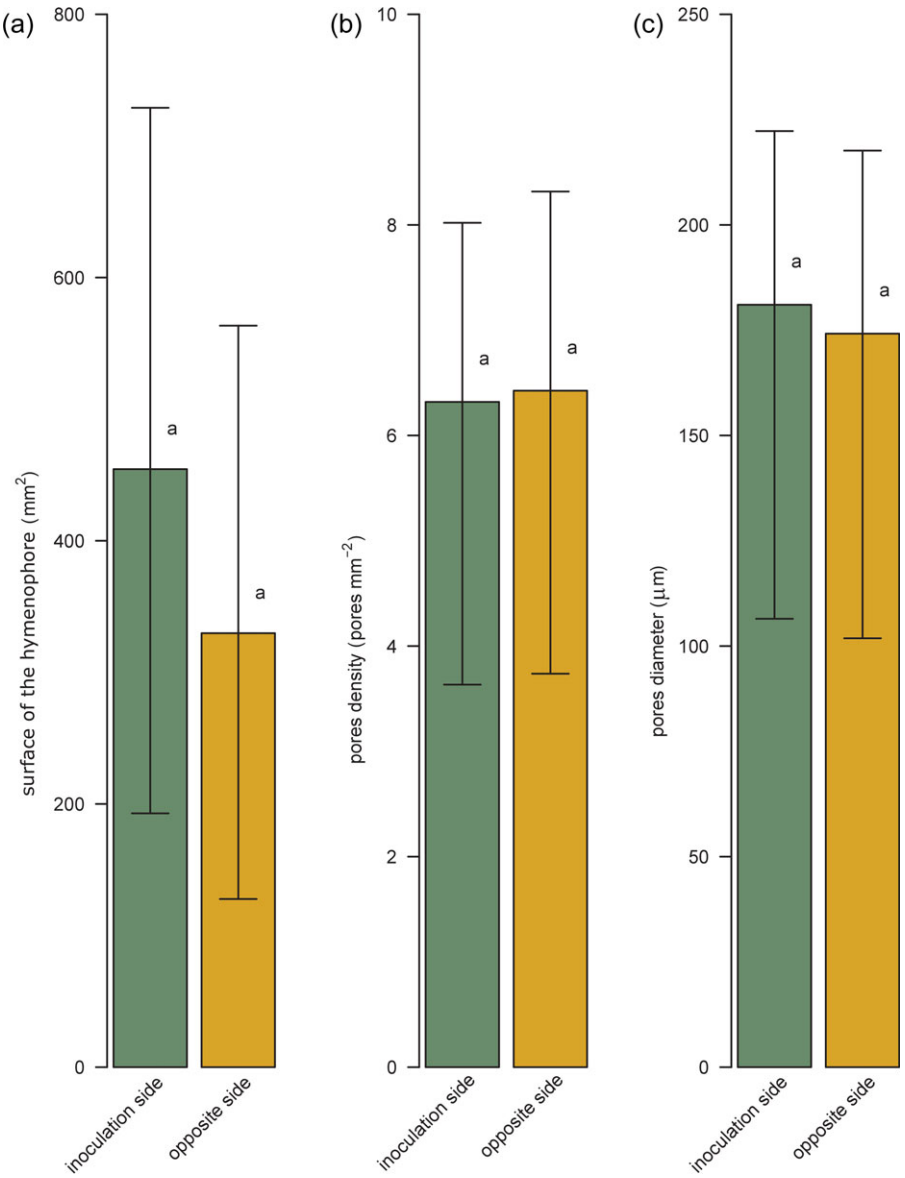

Figure 3 Comparisons of the morphological features between fruiting bodies of *Heterobasidion irregulare* observed on the inoculation side and on the opposite side. For both log sides, the barplots indicate the average attained by: (a) the surface of the hymenophore (mm^2), (b) the pores density (pores mm^{-2}) and (c) the diameter of pores (µm). Error bars refer to the associated 95 per cent 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 ($P > 0.05$).

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 the parts of central Italy where the Eurasian congener *H. annosum* is present provides an excellent model system to study such interaction. 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) have already shown that the fruiting potential of *H. irregulare* is greater than that of *H. annosum*. The main goal of this study was instead to determine whether fruiting of either species would be substantially increased or decreased when they both 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

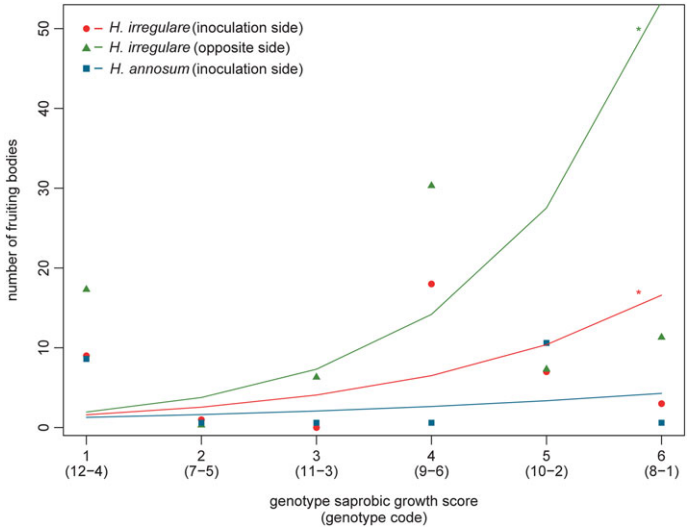

Figure 4 Correlation between saprobic growth and fruiting bodies production of *H. irregulare* and *H. annosum* genotypes. The genotype saprobic growth score attained by each fungal genotype within species is reported on the *x*-axis, indicating better saprobic abilities with increasing score values, 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 between the genotype saprobic growth score and the number of fruiting bodies, based on the outcomes of the negative binomial generalized linear regression models. Asterisks indicate significant correlations ($P < 0.05$). A vertical offset of 0.3 units was included to separate overlapping points.

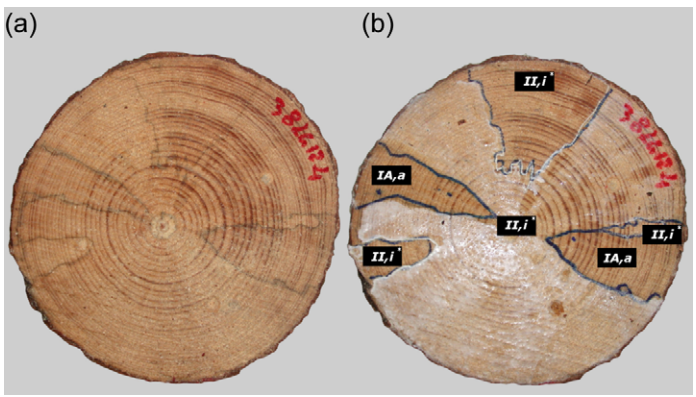

Figure 5 The central cross section of an inoculated log displaying: (a) areas delimited by distinguishable boundary lines after log cutting 11 months after inoculations, and (b) the same areas colonized by the mycelium of *Heterobasidion* spp. after 1 week of incubation under moist conditions of the central cross section. Labels indicate results of molecular analyses (*II,I*: *H. irregulare*; *IA,a*: hybrid mycelium). *May indicate either *II,i* or *I,i* because of the inability to distinguish between heterokaryotic and homokariotyc genotypes with the molecular assay used in this work.

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.

It is evident that the enhanced fruiting body production by *H. irregulare* occurred where the two *Heterobasidion* species coexist in the same side of the log and thus has to be the consequence of the interspecific interaction between the two. Even if our experimental design did not allow us to determine the possible mechanisms driving such process, it could be hypothesized that fruiting by *H. irregulare* might have been boosted due to physiological or ecological processes mediated by spatial niche sharing with the related congeneric taxon. As reported by Wardle et al. (1993), two fungal species sharing common natural substrates may unexpectedly display a highly unpaired reproductive success. However, the factors conferring an advantage to one species over the other are still poorly understood and deserve further investigations.

Based on our results, the interaction between the two fungal pathogens increased the number of fruiting bodies produced by *H. irregulare* but did not influence their morphology. In fact, *H. irregulare* fruiting bodies were fully comparable in terms of average surface of the hymenophore and in terms of pores density and diameter, regardless of the side of the log where they had been formed. Hence, larger airborne spore loads of *H. irregulare* might be expected in those stands where the two *Heterobasidion* species overlap due to increased fruiting bodies production. This hypothesis is corroborated by the results of a survey conducted across the current invasion area of *H. irregulare* in central Italy showing that the maximum spore load of *H. irregulare* was observed in the Circeo National Park, where both species are comparable in abundance and thus most likely to interact (Gonthier et al., 2007).

While *H. irregulare* genotypes often produced fruiting bodies on the log side opposite to the one where they had been inoculated, *H. annosum* genotypes never did because their mycelium was unable to grow along the entire length of the logs, as confirmed by the analysis of the logs central cross sections. These results clearly indicate that the presence of *H. annosum* as a competitor for trophic resources does not inhibit the higher efficiency of *H. irregulare* in utilizing the common growth substrate. Similarly, but using data from logs inoculated with a single genotype, Giordano et al. (2014) reported that *H. irregulare* displayed a saprobic ability superior than that of *H. annosum*. Our findings show additionally that the positive correlation between saprobic growth and fruiting bodies production was significant only for *H. irregulare* and that the magnitude of such correlation was larger on the side where *H. irregulare* grew in co-occurrence with *H. annosum*.

Since *H. irregulare* and *H. annosum* have been reported to hybridize in nature (Gonthier and Garbelotto, 2011), a further aim of this study was to investigate the outcome of interspecific mating. By deriving through combinatorics all possible nuclear and mitochondrial combinations of *H. irregulare* × *H. annosum* heterokaryotic hybrids, we obtained a theoretical model quantifying the proportion expected for each hybrid type (*IA,i - IA,a - II,a - AA,i*) and hybrid class (NH, MH and HaM, HiM). Since this

model assumes no constraining or prompting factors influencing the probability of formation of hybrids, it allows for the assessment of the presence of any intrinsic barriers to the formation of specific nuclear–mitochondrial combinations, at least in a controlled environment.

Our experiment proved that all four nuclear–mitochondrial combinations can be originated when *H. irregulare* and *H. annosum* co-occur in the same substrate with frequencies statistically equivalent to those expected according to our neutral theoretical model. This finding suggests that no nuclear–mitochondrial combinations are lethal and subjected to prezygotic negative selection. While our experimental design allowed for the detection of such combinations, the biological mechanisms leading to their formation can only be hypothesized. Each pair of genotypes inoculated was somatically incompatible to all other pairs based on *in vitro* pairing tests (not shown). However, nuclear reassortments between somatically incompatible mycelia resulting in the formation of novel heterokaryotic genotypes, in their turn somatically incompatible with either progenitor, have been reported in *Heterobasidion* spp. (Hansen et al., 1993). A similar mechanism may apply in the case of interfertile, yet somatically incompatible *H. irregulare* and *H. annosum* genotypes, leading to the formation of hybrid mycelia. This phenomenon might have occurred in the terminal portion of the inoculated logs, but a more complex and fluid scenario might have taken place as well. For instance, nuclei of *H. irregulare* could have migrated through the mycelium of *H. annosum* all the way to the opposite side of the log, forming hyphal mosaics with some mycelial segments bearing the original genotype and others harbouring new nuclear combinations. It is noteworthy to mention that different patterns of nuclear migration have been documented in other fungal model systems resulting in a variety of hyphal mosaics [see, for instance, Peabody et al. (2000) and the literature therein].

Our experimental design also allowed for a more insightful analysis of hybridization rates accounting for the effects of temporal and spatial dynamics of substrate colonization on hybridization outcomes. Assuming that mitochondria do not migrate (Xu and Wang, 2015), the mitochondrial type of hybrids should be determined by the fungal species first established in a portion of a substrate. Hence, in our experiment hybrid fruiting bodies developed on a given log side should harbour the mitochondrion of the parental species inoculated in that side. While the small number of hybrid fruiting bodies did not allow to test the above hypothesis on log ends inoculated with *H. irregulare*, one-third of the 18 hybrid fruiting bodies developed on the *H. annosum* side of inoculated logs harboured the *H. irregulare* mitochondrion (i.e. HiM hybrids). This HiM ratio is significantly different from the expected 0 per cent, with a probability of 99.9 per cent, suggesting that the mitochondrion of *H. irregulare* might provide a competitive advantage over the mitochondrion of *H. annosum* in hybrid genotypes carrying nuclei of both parental species. This finding is in agreement with 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 when considering the feasibility of eradication or control strategies targeting *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 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 outside the current zone of infestation. 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). Results of this study showing that the *H. irregulare* mitochondrion may be favoured where both species coexist implies that the use of mitochondrial markers may further increase the likelihood of detecting the presence of the invasive species.

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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