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The saprobic and fruiting abilities of the exotic forest pathogen *Heterobasidion irregulare* may explain its invasiveness

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Abstract

The North American fungal pathogen *Heterobasidion irregulare* is invading and threatening pine stands in Italy and is freely hybridizing with the native species *H. annosum*. Susceptibility of native hosts has been excluded as a factor driving *H. irregulare* invasion. Here we tested whether *H. irregulare* and *H. annosum* differ in their ability to saprobically colonize pine wood, and whether saprobic growth is correlated to fruiting bodies production. When inoculated in pine logs, *H. irregulare* genotypes colonized a volume of wood significantly larger than *H. annosum* genotypes. *Heterobasidion irregulare* significantly exceeded *H. annosum* in all parameters used as metrics of fruiting body production, including number and size of fruiting bodies, and *PPI*, an index summarizing the amount of surface available for spore production. Number of fruiting bodies and *PPI* were significantly correlated with volume of wood colonized by *Heterobasidion* genotypes. Results may explain why *H. irregulare* has been reported to sporulate more abundantly than *H. annosum* and provide explanations for its high transmission potential in Italy. This knowledge implies that approaches to control the spread of *H. irregulare* should be aimed at limiting saprobic establishment of the fungus rather than focusing on identifying more tolerant tree species.

Keywords *Exotic pathogens, Fruiting body,* Heterobasidion*, Pines, Saprobic ability, Transmission potential*

Introduction

Invasions by exotic pathogens may have significant effects on native ecosystems, potentially culminating in the decimation or even the extinction of host species (Laurance et al. 1996; Wikelski et al. 2004; LoGiudice 2006; Fisher et al. 2012). Consequences of invasions may also include evolutionary effects if the invasive organism occupies an area where a sibling species already exists. In fact, allopatrically evolved sibling species often lack any significant mating barriers (Dobzhansky 1940), and the long distance movement of a species during an invasion process and its interaction with a native interfertile related taxon may result in the hybridization between the two. Several plant pathogens are known to either have been generated by, or having benefited from, hybridization events: e.g. the Dutch elm disease agents *Ophiostoma* spp., the poplar leaf rusts *Melampsora x columbiana* G. Newc. and *M. medusae-populina* Spiers, as well as a number of *Phytophthora* species, including *P. alni* Brasier & S. A. Kirk and *P. andina* Adler & Flier (Spiers and Hopcroft 1994; Man in't Veldt et al. 1998; Brasier 2000; Newcombe et al. 2000; Brasier 2001; Brasier et al. 2004; Goss et al. 2011).

Successful invasions by an exotic pathogen depend on complex and often interacting ecological and evolutionary factors (May and Anderson 1983; von Broembsen 1989; Keane and Crawley 2002; Torchin et al. 2003; Parker and Gilbert 2004). These factors may be grouped into two broad categories or scenarios (Gonthier and Garbelotto 2013). The first refers to the relative susceptibility of native hosts, i.e., the likelihood of host infection by a pathogen that did not co-evolve in the same region as the host, while the second focuses on a pathogen's ability to be transmitted from an infected to an uninfected host. Understanding which one of the above scenarios best explains a biological invasion allows for the implementation of the most effective strategies against the spread of the invasive organism (Gonthier and Garbelotto 2013). For instance, in the case of plant pathogens, if high host susceptibility is driving the invasion, a successful control strategy may include selective removal of more susceptible individuals, planting of more tolerant or resistant ones, and increasing landscape-level barriers among susceptible hosts by either intermixing them with resistant species or simply increasing the distance amongst them. On the other hand, if transmission is a major driver of the invasion, independent of high host susceptibility, the above strategies would have limited effects and a different approach may be needed to curtail establishment, survival, and production of infectious inoculum (Gonthier and Garbelotto 2013).

Heterobasidion irregulare (Underw.) Otrosina & Garbelotto is a major fungal pathogen of pines in North America (Otrosina and Garbelotto 2010; Garbelotto and Gonthier 2013) accidentally introduced into Italy during World War II

(Gonthier et al. 2004; Garbelotto et al. 2013). The fungus has become invasive and has been reported to cause pine mortality over a 100 km stretch of the Tyrrhenian coast around Rome (D'Amico et al. 2007; Gonthier et al. 2007; Scirè et al. 2008). Because the genus *Pinus* includes several dominant species within several ecosystems, the spread of this North American pathogen in Europe threatens a large number of habitats, ranging from coastal to montane, and spanning from subtropical to boreal latitudes.

Heterobasidion species are necrotrophic facultative parasites being able to saprobically colonize dead wood, on which their fruiting bodies later develop. While this is not an uncommon feature for many root and butt pathogens capable of continuing tree colonization after their host has been killed, *Heterobasidion* spp. have the additional unique trait of also being adapted to establish themselves as saprobes before they pathogenically infect living hosts. *Heterobasidion* spp. may infect their hosts through airborne meiospores landing on stumps (primary infection) and then proceed to infect adjacent trees via root contacts (secondary infection) (Redfern and Stenlid 1998; Stenlid and Redfern 1998). Stump to tree secondary infection pathways explain why the pathogen is so important in managed forests (Swedjemark and Stenlid 1993; Stenlid and Redfern 1998).

Heterobasidion irregulare and the European sibling species *H. annosum* (Fr.) Bref. have evolved and differentiated in allopatry for at least 34 millions of years (Otrosina et al. 1993; Linzer et al. 2008; Dalman et al. 2010), and hybrid swarms between the two species have been recently reported in the area west of Rome (Gonthier et al. 2007). Amplified Fragment Length Polymorphism (AFLP) markers and sequences analyses have indicated that about 25% of *Heterobasidion* genotypes collected in the invasion area bear alleles of both parental species, with 5–45% of genomes being admixed (Gonthier and Garbelotto 2011). The high level of allele introgression, along with the finding that 16% of alleles could not be assigned to either parental species, but were newly created as the result of intra-locus recombination (Gonthier and Garbelotto 2011), point to the fact that the interaction between the two species is significantly affecting the evolutionary trajectories of both species, with unpredictable consequences.

The presence of both *Heterobasidion* species in central Italy has allowed for comparative observations and experiments aimed at explaining the invasive ability of the exotic fungus in Italy. In its invasion area, *H. irregulare* is also able to colonize pure oak stands that are unavailable to its native congener (Gonthier et al. 2012). Despite the presence of the exotic fungus in pure oak stands, no obvious mortality of oaks has been observed, suggesting a saprobic rather than a parasitic interaction between pathogen and hosts in these ecosystems (Gonthier et al. 2012). Additionally, based on the results of common garden inoculation experiments, pathogenicity levels of *H. irregulare*

and *H. annosum* are comparable on several pine species, including *Pinus pinea* L. (Italian stone pine), *P. sylvestris* L. (Scots pine), and *P. taeda* L. (loblolly pine) (Garbelotto et al. 2010). However, based on spore trappings in the invasion area, sporulation appeared to be constantly high only for the exotic fungal species (Garbelotto et al. 2010), suggesting that differences in transmission potential rather than hyper-susceptibility of native hosts may be driving the invasion of the exotic pathogen in Italy.

The aims of this study were to determine: i) whether *H. irregulare* and *H. annosum* differ in their ability to saprobically colonize wood, and ii) whether saprobic growth is significantly correlated to the production of sexual fruiting bodies. Because sporulation is likely to be linked to fruiting body production, the results may provide an answer for the invasiveness of *H. irregulare* and for its ability to colonize Mediterranean pine stands independently from its virulence on native hosts. Additionally, results of this study may help to identify the most appropriate strategies to reduce the establishment of *H. irregulare*, thereby protecting native pines and limiting interspecific hybridization between the two congeneric pathogens.

Materials and methods

Two experiments were designed to compare the saprobic growth (experiment 1) and the production of fruiting bodies (experiment 2) of *H. irregulare* and *H. annosum*. Six heterokaryotic (ploidy = n + n) genotypes for each species were used in both experiments (Table 1). Genotypes were randomly selected among those available in the culture collection of the University of Turin. All genotypes had been previously isolated in Italy (Gonthier et al. 2004; Gonthier et al. 2007), and their identification at the species level was accomplished by using two taxon-specific PCR methods (Gonthier et al. 2001; Gonthier et al. 2007), targeting both the mitochondrial and the nuclear genome. The heterokaryotic state of genotypes was assessed under a microscope (X 200 magnification) by checking the presence of clamp connections on hyphae (Hansen et al. 1992). Additionally, in order to avoid using genotypes with admixed genomes, genotypes were selected among those identified as pure (> 95% membership to a single species) by STRUCTURE analysis of over 500 AFLP markers (Gonthier and Garbelotto 2011). Genotypes used in the experiments were deposited at the Mycotheca Universitatis Taurinensis (MUT) (Table 1).

In vitro growth rate of each genotype was assessed on agar medium. Petri dishes (9 cm diameter) filled with Potato Dextrose Agar (PDA; 39 g potato dextrose agar, 200 mg streptomycin sulphate, 1 L distilled water) were inoculated in

the center with a single mycelial plug (8 mm diameter) obtained from the edges of actively growing fungal cultures. Ten replicates for each genotype were incubated in the dark at 20°C. The radial growth of fungal cultures was measured after ten days along two perpendicular lines.

Experiment 1: saprobic growth on wood

In the first experiment, *Heterobasidion* genotypes were inoculated in one of the two extremities of freshly cut *Pinus sylvestris* logs (30 cm length, about 20 cm diameter). *Pinus sylvestris* was selected for this experiment because it is the most largely distributed host species for *Heterobasidion* spp. in Europe (Korhonen et al. 1998) and comparative data of pathogenicity on this species have already been published (Garbelotto et al. 2010). The ecological and economic importance of *P. sylvestris* in central and northern Europe was a further determinant for selecting this tree species. Logs were obtained from branches of healthy trees in Gressan, Italy (45°42´56.46˝ N, 7°18´25.78˝ E), and were transferred to the laboratory in plastic bags and stored at 4°C before inoculations. Beech dowels 4 cm in length and 0.8 cm in diameter were autoclaved three times for 20 minutes in malt extract broth (20 g malt extract agar, 1 L distilled water) before being placed in 15 cm-diameter Petri dishes filled with PDA. Each plate was inoculated as described above. Plates were incubated in the dark at 20°C for 4 weeks to allow for colonization of dowels.

On the extremity of each log, two perpendicular diameters were drawn. For each diameter two holes, about 4 cm in depth, were drilled with a 0.8 cm diameter drill bit at approximately 2 cm from the edge of the section. After drilling each log, the drill bit was carefully wiped with 60% (wv^{-1}) ethanol before being used again. Drill holes were inoculated by inserting a fungus-colonized dowel in each one. Each fungal genotype was inoculated in 5 logs. Inoculated logs were individually sealed in a plastic bag and stored horizontally in the dark for 60 days in a growth chamber set at a temperature of 19 ± 1°C and at a relative humidity of 80 ± 5%. After incubation, logs were cut into transversal sections 1.5 cm in thickness. The bark was removed and sections were incubated for six additional days in plastic bags containing filter paper dampened with sterile water to promote the development of the fungus on their surfaces (Nicolotti et al. 1999). Both filter paper and water were sterilized in the autoclave at 121°C for 20 minutes. A dissecting microscope (X 40 magnification) was used to identify the area of each section colonized by the fungus based on the presence of *Heterobasidion* conidiophores, i.e., asexual reproductive structures that are diagnostic for the genus. Areas colonized by the fungus were measured with a planimeter as previously described (Nicolotti et al. 1999), and the volume colonized by a fungal genotype in each log was calculated as follows:

$$V_c = h \cdot \sum_{1}^{n} s_i$$

Where:

V_c: wood volume colonized by the fungus (in cm^3)

h: wood sections height (in cm)

s_i: surface colonized by the fungus on the i^{th} slice (in cm^2)

n: number of sections per log

Experiment 2: development of fruiting bodies

Based on preliminary evidence that the production of *Heterobasidion* fruiting bodies is enhanced when genotypes of both species interact with one another, the ability to develop fruiting bodies was tested through dual inoculation experiments on pine logs. Materials and methods of inoculation were the same as in experiment 1, with the exception that both extremities of a pine log were inoculated, each with a genotype belonging to a different species. Genotype combinations were selected on the basis of comparable *in vitro* growth. Final combinations were as follows: 2 (*H. annosum*) – 12 (*H. irregulare*), 6 – 11, 1 – 10, 4 – 8, 3 – 9, 5 – 7 (see Table 1). Ten pine logs per each genotype combination were inoculated and incubated for 11 months in a growth chamber as described above.

At the end of the trial, fruiting bodies displaying a fully developed pore layer or hymenophore (pores are the structures where meiospores are produced) were counted and removed from each extremity. Areas of pore layers were measured using a planimeter, and pore density (i.e., number of pores per mm^2) was assessed under a dissecting microscope by counting the number of pores at least in 3 1-cm^2 sub-areas per sample. The mean diameter of pores was also calculated by measuring the diameter of at least 10 pores per fruiting body. Isolations were attempted from all fruiting bodies by transferring small pieces (2 x 2 x 2 mm) of their inner tissue onto 6-cm diameter Petri dishes filled with a *Heterobasidion*-selective growth medium (Kuhlman and Hendrix 1962). Genotypes isolated from fruiting bodies were assigned to *H. irregulare* or to *H. annosum*, or were identified as hybrids, by using the DNA extraction and the DNA-based diagnostic molecular methods described by Gonthier et al. (2007). The PCR-based method hinges on DNA

primers that will specifically amplify only one of the two species, resulting in differently-sized amplicons. When isolations failed, PCR was conducted on fungal DNA extracted directly from the fruiting bodies by using the E.Z.N.A.™ Stool DNA Isolation Kit (Omega Bio-Tek, Norcross, GA) following the manufacturer's instructions.

Data interpretation and statistical analyses

In vitro growth rates of *H. irregulare* genotypes expressed in mm of colonization per day were compared to those of *H. annosum* genotypes using the Mann-Whitney U test (Marques de Sà 2007). Since data on the wood volume colonized by *Heterobasidion* genotypes were not normally distributed (Shapiro-Wilk, $P < 0.05$), two Poisson regression models named M1 and M2 were fitted to test the effects of species and the genotype (Crawley 2013). The fitting was performed in R 2.15.2 (R Core Team 2012) with the MCMC GLMMs (Markov Chain Monte Carlo Generalized Linear Mixed Models) (see Hadfield 2010 for the complete model description). In M1, only "species" was included as fixed effect. In M2, both "species" and "genotype" were included: the first one as fixed effect and the second one as a nested random effect (Crawley 2013). After selecting the default prior for the *MCMCglmm()* R function, both fixed and random effect coefficients (β and Z, respectively) were estimated with their related 95% credible intervals ($CI_{0.95}$) and P_{MCMC} values (Bolker et al. 2009; Hadfield 2010). In both M1 and M2 "species" was coded assigning the value 1 to *H. irregulare* and 0 to *H. annosum*.

The ability of *H. irregulare* and *H. annosum* to develop fruiting bodies was evaluated in terms of i) total number of fruiting bodies (N), ii) mean surface of hymenophore (S expressed in mm^2), iii) density of pores (D expressed as number of pores mm^{-2} of hymenophore), and iv) mean diameter of pores (d expressed in µm). The ability to develop fruiting bodies was also evaluated in terms of Pores Perimeter Index (*PPI*), calculated as follows:

$$PPI = 10^{-6} \pi NSDd$$

Where:

PPI: Pores Perimeter Index in m

N: number of fruiting bodies

S: mean surface of the hymenophore in mm^2

D: mean pores density expressed as number of pores mm^{-2}

d: mean diameter of pores in µm

Comparisons between *H. irregulare* and *H. annosum* were performed through the χ^2 test with exact significance for *N*, and with the Mann-Whitney U test for *S*, *D*, *d* and *PPI*, using values of genotypes as replicates. Hybrid fruiting bodies were excluded from statistical analyses.

The relationship between saprobic growth and production of fruiting bodies was explored by correlating through the Spearman rank order correlation test the mean volume colonized in pine logs by each genotype as measured in experiment 1 with the fruiting parameters *N* and *PPI* of the same genotype as assessed in experiment 2. The relationship between mean volume colonized and fruiting parameters was investigated cumulatively for both species and individually for each species. A 0.05 threshold was used as cut-off value for all tests. Statistical analyses were performed with R 2.15.2 (R Core Team 2012).

Results

Average *in vitro* growth rate of *H. irregulare* genotypes was slightly lower than that of *H. annosum* genotypes [5.92 (SD 0.97) vs 6.15 (SD 1.83) mm day^{-1}]. However, this difference was not significant (U = 17.000, P = 0.873). The mean volume of wood colonized in two months by *H. irregulare* and *H. annosum* ranged from 598.66 cm^3 to 2613.69 cm^3, and from 183.82 cm^3 to 472.49 cm^3, respectively, depending on genotype (Fig. 1). On average, the volume colonized by *H. irregulare* genotypes was 1439.74 cm^3 (SD 1682.85 cm^3) while that colonized by *H. annosum* genotypes was 301.44 cm^3 (SD 380.61 cm^3). In both M1 and M2, the Poisson regression β coefficients were significantly different from 0 for the species ($P_{MCMC} < 0.05$), while the genotype Z coefficients estimated in M2 were not ($P_{MCMC} > 0.05$) (Table 2). Moreover all the significant β coefficients were positive.

Eleven months after inoculation, 148 fruiting bodies were counted. Of these, 109 were of *H. irregulare*, 18 of *H. annosum*, and 21 were hybrids between the two species (Table 3). The number of fruiting bodies (*N*) of *H. irregulare* was significantly higher than that of *H. annosum* (χ^2 = 65.205; $P_{exact} < 0.001$). The mean surface of hymenophore (*S*)

and the density of pores (*D*) of *H. irregulare* were also significantly higher than those of *H. annosum* (*S* = 536.98 mm^2 vs 209.70 mm^2; *D* = 7.93 vs 5.26 pores mm^{-2}, for *H. irregulare* and *H. annosum*, respectively). The mean diameter of pores was lower for *H. irregulare* compared to *H. annosum* (*d* = 211.64 vs 246.68 µm), however differences were not significant. *Heterobasidion irregulare* also displayed a *PPI* index significantly higher than *H. annosum* (*PPI* = 43.06 vs 2.45 m; *U* = 2.000, P_{exact} = 0.009).

The relationship between the mean volume of wood colonized by *Heterobasidion* genotypes and fruiting parameters is shown in Fig. 2. Cumulatively, the mean volume of wood colonized by *Heterobasidion* genotypes in pine logs was correlated with both *N* (ρ = 0.620, *P* = 0.031) and *PPI* (ρ = 0.637, *P* = 0.026). However, no significant correlations were observed when data were analyzed separately for each species (*N*: ρ = 0.116, *P* = 0.823 for *H. irregulare* and ρ = -0.068, *P* = 0.899 for *H. annosum*; *PPI*: ρ = -0.200, *P* = 0.714 for *H. irregulare* and ρ = -0.338, *P* = 0.512 for *H. annosum*).

All genotypes combinations yielded hybrid fruiting bodies, with a frequency ranging from 1 to 9 depending on the genotype combination. The *H. irregulare* genotype which colonized the largest volume of wood yielded an intermediate number (4) of hybrid fruiting bodies, while the *H. annosum* genotype which occupied the widest volume of wood produced the smallest number (1) of hybrid fruiting bodies.

Discussion

Results of the two trials performed in this study clearly indicate the North American *H. irregulare* outperforms the native *H. annosum* both in its ability to saprobically colonize wood of *P. sylvestris* and in its ability to produce fruiting bodies. Additionally, genotypes displaying greater saprobic growth had a greater fruiting ability. Based on these results, differences in the transmission potential between the two species appear to be playing a key role in the documented different ability of the two species to become established in Mediterranean pine stands.

Choice and number of genotypes is obviously critical when designing these comparative studies. In order to minimize any bias while providing sufficient replication, we randomly selected genotypes of *H. irregulare* and *H. annosum* isolated in Italy, where both species have recently become sympatric. Because the two species are known to be hybridizing (Gonthier and Garbelotto 2011) we used over 500 genetic (AFLP) markers as described in Gonthier and Garbelotto (2011) to ensure admixed genotypes were not employed. Lack of correlation between *in vitro* growth and growth on natural substrates or *in planta* is a known phenomenon (Bajo et al. 2008). Our *in vitro* trial was meant to

avoid the use of genotypes that may be impaired due either to senescence (Stenlid and Rayner 1989; Korhonen and Stenlid 1998) or to unusual gene expression patterns (Kasuga et al. 2012). Additionally, using isolates with comparable rates of *in vitro* growth ensures avoiding biases that may occur if selecting disproportionate numbers of "minus" or "plus" individuals (e.g., individuals with reduced or enhanced growth rates) for each species. While no significant differences between growth rates of the two species were detected *in vitro*, the Poisson regression coefficients indicate that the difference in the volume of wood colonized by the two species in experiment 1 is due to the species effect rather than to genotype effect. This is clearly showed by the fact that the β coefficients remain significant when the categorical variable genotype is included in M2. Furthermore, coefficient values indicate that *H. irregulare* colonized a wood volume significantly higher than that colonized by its congener. Based on data of volume colonized, the exotic *H. irregulare* genotypes occupied on average a volume of wood 4.8 times larger than genotypes of *H. annosum*. Although the assessment of direct competition between the two fungal species in the consumption of trophic resources was not among the aims of this study, and still need to be proved, the above results may suggest that *H. irregulare* can outcompete *H. annosum* when establishing as a saprobe on wood substrates. Because saprobic stump colonization is known as the most common first step of establishment by this fungus in pine stands (Rishbeth 1951), this difference could result in a much greater establishment of *H. irregulare* compared to that of *H. annosum*.

Based on results of experiment 2, although *H. irregulare* produced smaller pores than its native congener, it exceeded *H. annosum* in all of the parameters used as metrics of fruiting body production, i.e., number of fruiting bodies, mean surface of hymenophores, density of pores, and the *PPI* index that includes all of the above parameters. Indeed, such an index seems appropriate to describe the potential ability of spore production of fungi characterized by fruiting bodies with porous hymenophores. In fact, spores are produced attached to the inner vertical surface of the pores, and the *PPI* index well summarizes the total amount of such a pore surface available to each genotype and each species for production of spores. It has been previously demonstrated that an increasing number of pores implies a reduced diameter of each of them and an increased global perimeter (Nurmela and Östergård 1997), and this is consistent with our data. By assuming a correlation between size of fertile tissues of fruiting bodies and total amount of spores produced, our findings are consistent with the observed higher inoculum density of *H. irregulare* compared to *H. annosum* in the invasion area in Italy (Gonthier et al. 2007; Garbelotto et al. 2010; Gonthier et al. 2012). Although the experiments in this study were exclusively aimed at comparing traits of pure *H. irregulare* and *H. annosum*, dual inoculations of pine logs with combination of genotypes of both species resulted in the development of 21 (14%) hybrid fruiting bodies from all tested combinations, providing experimental evidence that hybridization

between the two species can freely occur on a substrate relatively comparable to that occupied by these organisms in nature. Hybridization has been reported to occur at extremely high rates in nature where *H. irregulare* and *H. annosum* are sympatric in Italy (Gonthier and Garbelotto 2011). Additionally, because heterokaryotic genotypes of *H. irregulare* and *H. annosum* were used in both trials, our results further suggest that hybridization between the two species may occur through processes of nuclear reassortments between two heterokaryotic individuals. *Heterobasidion* genotypes are not 2n but n + n, i.e., nuclei from two compatible parents are associated but not fused and the organism is functionally a diploid. Because of this looser association between parental nuclei, disassociation of the two and reassortments have been previously documented both in the laboratory (Hansen et al. 1992) and in nature (Garbelotto et al. 1999; Johannesson and Stenlid 2004). This is the first time however that such reassortments are documented between genotypes belonging to two different species. The lack of a clear relationship between saprobic growth in wood and the number of hybrid fruiting bodies for both *H. irregulare* and *H. annosum* genotypes may suggest that factors other than the ones related to growth could play a role in the hybridization process. It should be noted, however, that this study was not designed to investigate hybridization and hence the above observation must be considered at best as preliminary.

Sporulation is essential for the spread of all *Heterobasidion* species (Garbelotto and Gonthier 2013), and is the end result of a process that includes the ability both to infect and colonize a substrate and to produce fruiting bodies (Korhonen and Stenlid 1998). No significant correlations were detected between volume of wood colonized and fruiting parameters when data were analyzed individually for each fungal species, possibly due to the small sample size. However, the analysis performed on the cumulative dataset clearly show that the ability of an *Heterobasidion* genotype to produce fruiting bodies is significantly and positively correlated to its ability to colonize wood saprobically. This observation provides a plausible explanation of why *H. irregulare* has been reported to sporulate more abundantly than *H. annosum*, resulting in significantly higher transmission and establishment rates than its native counterpart (Garbelotto et al. 2010). These findings also support the notion that the invasive ability of *H. irregulare* is due to mechanisms other than increased pathogenicity levels on native hosts. As stated in Garbelotto et al. (2010), plant pathologists have traditionally overemphasized lack of co-evolution and the high susceptibility of native hosts to introduced pathogens as a major mechanism explaining the success of exotic diseases: here we provide one of the first solid examples of an exotic disease whose success is explained by factors other than high virulence of an introduced pathogen on novel hosts. Only a comparison between introduced *H. irregulare* populations in Italy and native *H. irregulare* populations in North America may help to understand whether the high transmission

potential of the fungus in Italy is a pre-existing trait or has emerged only after the introduction in Europe. However, whether pre-existing or recently acquired, the understanding that an unusually high saprobic growth on stumps or dead wood followed by a comparably high production of fruiting bodies may be driving the invasion of Italy by *H. irregulare* is pivotal for the implementation of effective strategies against its further spread. Rather than focusing on identifying more tolerant tree species, the focus should be on identifying those infection courts where saprobic establishment may occur. Based on widely accepted literature (Rishbeth 1951; Swedjemark and Stenlid 1993; Redfern and Stenlid 1998) and on novel recent results (Gonthier et al. 2012), stumps of both conifers and broadleaf species may provide such a substrate. Treatment of such stumps with either chemical compounds (Pratt et al. 1998) or biological control agents (Holdenrieder and Greig 1998), the elimination of dead standing trees, and the removal from the forest floor of dead and down wood – all potential substrates for saprobic establishment and production of fruiting bodies – may be successful strategies to implement. At stake here are both the health of Eurasian pines and the productivity of forests used for timber production. *Heterobasidion irregulare* is currently causing significant pine mortality in areas that were previously unaffected by the native *H. annosum* (Gonthier et al. 2007). Were *H. irregulare* to reach the Alpine mountain range, or central and northern Europe, its effects will be added to those significant ones already caused by *H. annosum* (Dobbertin et al. 2001; Gibbs et al. 2002; Rönnberg et al. 2006), potentially increasing losses and reducing the ecological role played by pines in those sites that may be conducive to the establishment of *Heterobasidion* spp. (reviewed in Garbelotto and Gonthier 2013).

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Figure legend

Fig. 1 Box plots of volume (cm^3) colonized by each *Heterobasidion* genotype in *Pinus sylvestris* logs. The rectangles represent the values lying between the 25th and 75th percentiles, the horizontal black line in between these percentiles is the median volume, the t-shaped lines outside the rectangles identify the minimum and maximum values, the asterisks are outlayers. See Table 1 for genotype codes

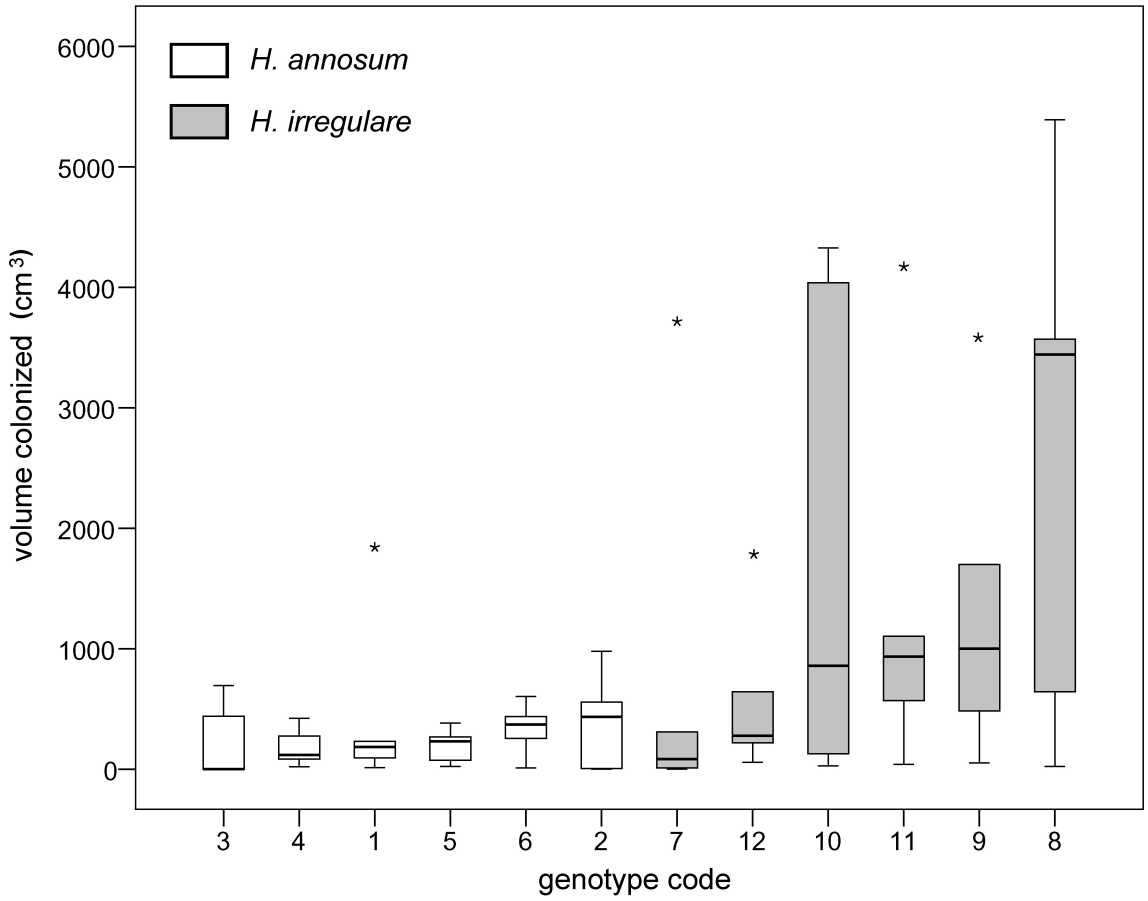

Fig. 2 Scatterplot showing the relationship between the mean volume of *Pinus sylestris* logs colonized by *Heterobasidion* genotypes and the fruiting parameters number of fruiting bodies (*N*) and Pores Perimeter Index (*PPI*)

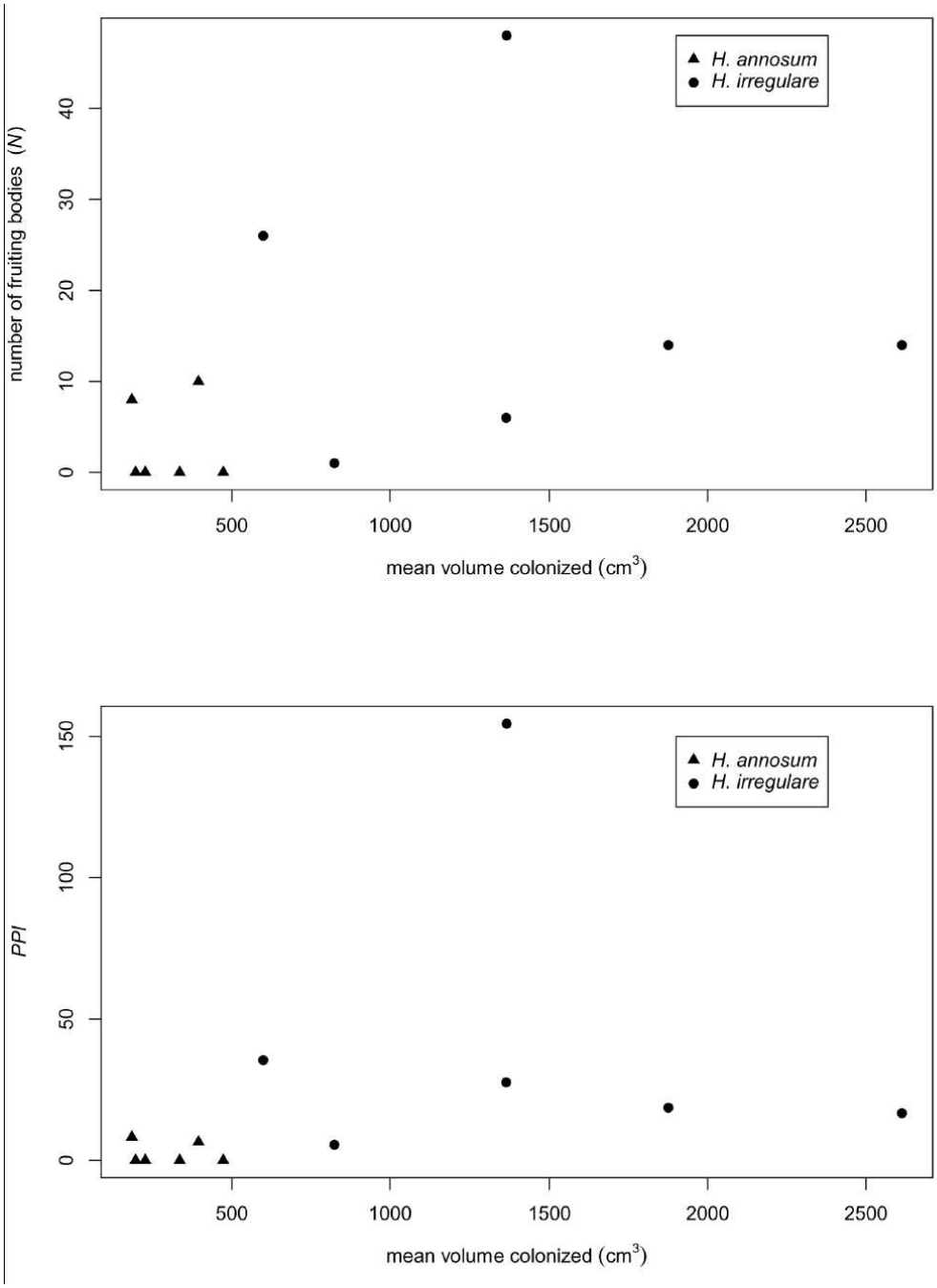

Table titles

Table 1 *Heterobasidion* genotypes used in this study

Genotype code	Isolate	Isolation date	Geographic origin	*Heterobasidion* species	Collector [a]	MUT [b] accession N.
1	CAL1	1995	Taverna, CZ, Italy	*H. annosum*	NL	1215
2	Pd3	1999	Meugliano, TO, Italy	*H. annosum*	GN	1149
3	J7	2009	Brusson, AO, Italy	*H. annosum*	PG	1208
4	LG12	2005	Morgex, AO, Italy	*H. annosum*	PG	1216
5	43NA	2005	Sabaudia, LT, Italy	*H. annosum*	PG	1204
6	Ha. Carp.	2007	Sabaudia, LT, Italy	*H. annosum*	PG & MG	1143
7	38NA	2006	Castelfusano, RM, Italy	*H. irregulare*	PG & MG	1161
8	39NE	2005	Castelfusano, RM, Italy	*H. irregulare*	PG & MG	1193
9	45SE	2005	Sabaudia, LT, Italy	*H. irregulare*	PG & MG	1151
10	49SA	2005	Sabaudia, LT, Italy	*H. irregulare*	PG & MG	1197
11	CP8	2002	Castelporziano, RM, Italy	*H. irregulare*	NA	3563
12	CP15	2002	Castelporziano, RM, Italy	*H. irregulare*	NA	3560

[a] Collectors: NA Naldo Anselmi, PG Paolo Gonthier, MG Matteo Garbelotto, GN Giovanni Nicolotti, NL Nicola Luisi.

[b] MUT: Mycotheca Universitatis Taurinensis

Table 2 MCMC GLMMs Poisson regressions coefficients for species (β) and genotype (Z) in models M1 and M2. For details on the models, see the text. For each coefficient, estimations of 95% credible intervals ($CI_{0.95}$) and P_{MCMC} value are provided. Asterisks indicate significant coefficients. For genotypes code see Table 1

Fixed and random effects	Model M1	Model M2
Species (*H. irregulare* = 1; *H. annosum* = 0)	β = 1.664*	β = 1.664*
	$CI_{0.95}$ (0.432; 2.834)	$CI_{0.95}$ (0.431; 2.834)
	P_{MCMC} = 0.010	P_{MCMC} = 0.010
Intercept	β = 4.379*	β = 4.378*
	$CI_{0.95}$ (3.564; 5.253)	$CI_{0.95}$ (3.522; 5.246)
	$P_{MCMC} < 10^{-4}$	$P_{MCMC} < 10^{-4}$
Genotype 1	-	Z = 0.014
		$CI_{0.95}$ (-0.200; 0.280)
		P_{MCMC} = 0.975
Genotype 2	-	Z = -0.005
		$CI_{0.95}$ (-0.225; 0.232)
		P_{MCMC} = 0.996
Genotype 3	-	Z = -0.045
		$CI_{0.95}$ (-0.432; 0.154)
		P_{MCMC} = 0.936
Genotype 4	-	Z = 0.006
		$CI_{0.95}$ (-0.228; 0.255)
		P_{MCMC} = 0.981

Genotype 5	-	$Z = 0.011$ $CI_{0.95}$ (-0.219; 0.261) P_{MCMC} = 0.971
Genotype 6	-	$Z = 0.019$ $CI_{0.95}$ (-0.184; 0.332) P_{MCMC} = 0.964
Genotype 7	-	$Z = -0.037$ $CI_{0.95}$ (-0.361; 0.170) P_{MCMC} = 0.935
Genotype 8	-	$Z = 0.017$ $CI_{0.95}$ (-0.213; 0.275) P_{MCMC} = 0.960
Genotype 9	-	$Z = 0.008$ $CI_{0.95}$ (-0.246; 0.239) P_{MCMC} = 0.975
Genotype 10	-	$Z = 0.005$ $CI_{0.95}$ (-0.200; 0.286) P_{MCMC} = 0.996
Genotype 11	-	$Z = 0.007$ $CI_{0.95}$ (-0.246; 0.244) P_{MCMC} = 0.984

Genotype 12	-	Z = -0.005 $CI_{0.95}$ (-0.228; 0.264) P_{MCMC} = 0.995

Table 3 Number and characteristics of fruiting bodies of *Heterobasidion* spp. developed 11 months after inoculations of pine logs with 6 genotypes of *H. irregulare* and 6 genotypes of *H. annosum*

Parameter	*H. irregulare*	*H. annosum*	Hybrids
Number of fruiting bodies (*N*)	109	18	21
Mean surface of hymenophore (*S*), mm^2	536.98	209.70	132.71
	(SD 331.69)	(SD 82.45)	(SD 165.50)
Mean density of pores (*D*), number of pores mm^{-2}	7.93	5.26	6.02
	(SD 0.99)	(SD 0.04)	(SD 1.90)
Mean diameter of pores (*d*), µm	211.64	246.68	217.14
	(SD 12.14)	(SD 17.76)	(SD 47.58)
Pores Perimeter Index (*PPI*), m	43.06	2.45	11.51
	(SD 55.52)	(SD 3.83)	(SD 0.40)