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The alien invasive forest pathogen *Heterobasidion irregulare* is replacing the native *Heterobasidion annosum*

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Abstract Invasions by alien pathogens are a major threat to forest conservation. The North American fungal pathogen of conifers *Heterobasidion irregulare*, inadvertently introduced in Central Italy in the 1940s, has been spreading causing high mortality of Italian stone pine (*Pinus pinea*). While invading new-found niches, *H. irregulare* has established itself in the current range of the native congener *H. annosum*. The aims of this study were to determine whether in time: (I) *H. irregulare* populations may be increasing in size; (II) *H. irregulare* may be replacing *H. annosum*, rather than simply coexisting with it; and, (III) *H. annosum* may disappear in forests infested by *H. irregulare*. The presence, abundance and distribution of *H. annosum* and *H. irregulare* were assessed through an aerobiological assay replicated ten years apart in a forest in which both species have been coexisting. Replacement index (RI), Markov chains and geometric progressions were used to model the interspecific interaction between the two species and to assess the invasiveness of *H. irregulare*. Results showed that, in 10 years, the incidence of *H. annosum* dropped from 39.4 to 6.1%, while that of *H. irregulare* increased from 57.6 to 81.8%, with the alien pathogen replacing the native species (RI = 84.6%) and spreading at a maximum rate of 139 ha/year. Although our models show that the extinction of *H. annosum* may be unlikely, the ability of *H. irregulare* to replace it suggests the alien pathogen may also readily colonize those parts of Europe where *H. annosum* is more abundant than in Central Italy.

Keywords Biological invasion · Fungi · Modelling · Pine · Plant pathogens · Spores

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Introduction

The threat posed by alien forest pathogens to naïve forests is well recognized (Lovett et al. 2016). Once introduced and established, alien pathogens may become invasive by spreading at a rate that is affected by many factors, including, but not limited to, their reproductive potential, their ability to withstand disturbances, competition and/or predation, their dispersal ability, the density of susceptible and/or of infectious hosts, environmental and climatic factors,

and the permeability of the landscape (Garbelotto and Pautasso 2011). A frequent invasion scenario involves alien organisms exploiting niches unoccupied by native organisms. With regards to plant pathogenic fungi, this is the case, for instance, of the invasion by the Asian chestnut blight fungal pathogen *Cryphonectria parasitica* in both North America and Europe. In North America, where the native American chestnut (*Castanea dentata*) is highly susceptible, the spread rate has been estimated at more than 30 km per year, resulting in devastating effects throughout its native distribution range in a few decades (Evans and Finkral 2010; Rigling and Prospero 2017). However, introduced pathogens and pests frequently interact with pre-existing organisms, both native or themselves introduced. One of the most notable examples includes the causal agents of Dutch elm disease *Ophistoma novo-ulmi* introduced where the related *O. ulmi* was already well established (Brasier and Buck 2001). The fitness advantage exhibited by *O. novo-ulmi* compared to *O. ulmi* led to a very successful invasion of the former species and to a replacement of the less fit *O. ulmi* by *O. novo-ulmi,* possibly due to direct competitive antagonism between the two (Brasier and Buck 2001).

While it is acknowledged that asymmetry in fitness between two species with ecological overlap is responsible for the dominance of one over the other, the mechanisms underlying the dominance and maybe even the replacement of one species by another are multiple (see Reitz and Trumble 2002; Short and Petren 2011) and involve direct (e.g. antagonism) and indirect (e.g. better resource utilization) interactions, as well as demographic (e.g. reproductive and transmission potentials) and metapopulation level dynamics (e.g. migration rates of each species). It should be noted that not all interactions need to be antagonistic: for instance, the alien organism here studied, *Heterobasidion irregulare*, has been reported to increase its sporulation potential when sympatric with the native congeneric *H. annosum* (Giordano et al. 2019). Likewise, priority effects may favor less fit pathogens which arrive first on a substrate or a host (Hood 2003; Simpson et al. 2003; Al-Naimi et al. 2005; Laine 2011). Additionally, interfertility between interacting species further complicates the scenarios by allowing individuals belonging to two species to coexist in the same physical space where they can mate and exchange advantageous adaptive genes or deleterious

infectious agents (Paoletti et al. 2005; Hessenauer et al. 2020; Sillo et al. 2021).

The role played by each factor in determining the outcome of a biological invasion may be hard to assess when two or more species interact with one another. Normally, such role has been inferred based on data that simply document changes in population size of each interacting species in any given area. Unfortunately, those data often have limited geospatial detail and/or are obtained from different and differently designed studies (Short and Petren 2011; Downey and Richardson 2016). Repeat surveys over time across a predetermined set of sampling points may be one of the best ways to obtain a better understanding of whether alien invasive species may be: I) increasing in populations size or carrying capacity, II) increasing the proportion of their overall representation, III) broadening their geographic range and, IV) replacing native individuals. In a recent publication, Kozanitas et al. (2017) through a repeated sampling of individual trees across multiple transects and sites were able to show the alien pathogen *Phytophthora ramorum* was directly replacing the native pathogen *Phytophthora nemorosa*, at rates that increased with increasing rainfall. A similar study showed a similar replacement on Ash of the native and largely saprobic fungus *Hymenoscyphus albidus* by the alien and pathogenic *Hymenoscyphus fraxineus* (Mckinney et al. 2012).

Studies employing repeated samplings and spatially defined sampling schemes may also provide information useful to both predict the rate of future spread of the invasive organism and to reconstruct its past spread history. Although this information is necessarily site specific, it may provide invaluable baseline information that combined with other information such as the introduction date of the exotic pathogen, the original introduction location and data on landscape fragmentation at various geographic scales, may help refine our understanding of the expansion potential of the alien organism. This type of information is of pivotal importance to predict the outcomes of invasions and to make decisions on eradication or containment measures, but, unfortunately, it is often not readily available for introduced forest pathogens.

A relatively recently discovered biological invasion is that of the North American fungal plant pathogen *H. irregulare*, inadvertently introduced a few

miles Southwest of Rome, Italy, by the US army in 1944 (Gonthier et al. 2004; Garbelotto et al. 2013). This root rot agent is now associated with significant mortality of Italian stone pine (*Pinus pinea*) in pure planted pine stands, in mixed oak-pine natural forests and in urban parks located within and around the city of Rome (D'Amico et al. 2007; Gonthier et al. 2007, 2014). The current zone of infestation covers an area approximately 103 km long in a Northwest-Southeast direction along the Mediterranean Sea, West of Rome (Gonthier et al. 2014). Based on its current impact in Italy and on its potential impacts on pine stands at the continental scale, *H. irregulare* has been included in the A2 list of organisms recommended for regulation by the European and Mediterranean Plant Protection Organisation (EPPO). Many aspects of the introduction and invasion of *H. irregulare* in Italy have been well deciphered. All lines of evidence, including the results of population genetic analyses (Garbelotto et al. 2013) and estimates of the areas of the outbreaks (Gonthier et al. 2014), support a single introduction in the Castelporziano area, from a single source population located in the South-East of the USA (Linzer et al. 2008). Movement of untreated wood by US Army during WWII has been identified as the most likely pathway of introduction (Gonthier et al. 2004; Garbelotto et al. 2013). *Heterobasidion* spp. infest forests by means of spores landing on freshly cut stumps. Once a stump is colonized, the fungus may infect neighboring trees via mycelium growing through root contacts and grafts (Garbelotto and Gonthier 2013). Based on the distance between Castelporziano and the furthest invaded site and the time length since its introduction, the spread rate of the pathogen has been estimated at 1.3 km/year (Gonthier et al. 2007). However, this estimate is thought to be extremely conservative, given that available habitats are very fragmented in the invasion area, with gaps in between them as large as 20 km (Gonthier et al. 2007, 2014).

All published studies have simply reported that the overall abundance and the proportional representation of *H. irregulare* is overwhelmingly higher than that of the native species *H. annosum* (Gonthier et al. 2007, 2012). The overlap of the two congeners in the invasion area has led to their hybridization, resulting in interspecific allelic introgression, in the creation of new alleles deriving from intra-locus recombination, as well as in nuclear-mitochondrial reassortments

between the two species (D'Amico et al. 2007; Gonthier et al. 2007; Gonthier and Garbelotto 2011). However, no data has been provided to directly support actual replacement of the native species by the alien one. Replacement, though, is a distinct possibility, given the clear asymmetry in fitness identified in favor of the invasive species, which is characterized by a greater sporulation potential and by faster saprobic wood decay ability (Giordano et al. 2014). The first of these two traits is known to facilitate greater primary establishment in forests, while the second is believed to be associated with higher transmission rates of the pathogen via mycelial growth through root contacts and grafts. These two traits suggest that the alien species may be invasive and replacing the native one, but an alternative explanation of the predominance of *H. irregulare* may simply be that the incidence of the native species has always been low in the pine stands around Rome. Additionally, invasiveness of the alien pathogen could in part be driven by its host range, documented to be broader than that of the native one (Gonthier et al. 2012). If indeed *H. irregulare* is capable of replacing *H. annosum,* that would mean the alien species could potentially occupy *H. annosum* niches on top of additional niches that are unavailable to the native species: this aspect is not trivial in light of a possible spread of *H. irregulare* northwards, where the incidence of *H. annosum* is reportedly much higher than in central Italy (Gonthier et al. 2001, 2005, 2007). Given that *H. irregulare* has a faster saprobic wood decay rate and a higher sporulation rate than *H. annosum*, a replacement of *H. annosum* by *H. irregulare* would lead to overall higher secondary and primary tree infection rates, with subsequent higher tree mortality rates than the ones currently experienced because of tree infection by *H. annosum* alone.

While habitat fragmentation in the *H. irregulare* zone of invasion in Italy is significant, there is one sizeable unfragmented forest located in the southern range of the pathogen, i.e. the Sabaudia forest in the Circeo National Park. From a research perspective, this forest is invaluable not only because of its relevant size, but also because of three other reasons. First, one of the fronts of invasion of *H. irregulare* was identified in this forest (Gonthier et al. 2007, 2012), second, the overall population proportion of each pathogen was about 50% in 2006 and, third, population size measured

in terms of deposition rates of airborne propagules (i.e. basidiospores) was comparable between the two *Heterobasidion* species (Gonthier et al. 2012). Therefore, the Sabaudia forest appears ideal not only to study the invasiveness of *H. irregulare*, but also to test whether this fungus may or may not be replacing the native *H. annosum*.

In this study, we compared the results of two samplings performed in the same points across the Sabaudia forest ten years apart, the first in 2006 and the second in 2016. Samplings and analyses followed an identical methodology and were aimed at answering the following questions: I) Did the proportional representation of the alien species in the Sabaudia forest overall increase between 2006 and 2016? II) Was the abundance of the alien pathogen in the Sabaudia forest overall larger in 2016 than in 2006? III) In 2016, was the exotic species present where it was absent in 2006? IV) In how many of the resampled points, had the native pathogen been replaced by the alien one and vice versa, and was the overall replacement rate symmetrical or asymmetrical? Finally, the approach used in our study, by detailing the changes in pathogen status at each sampling point between 2006 and 2016, with possible status being: a) "no pathogen", b) "both pathogens", c) "*H. annosum* only", and, d) "*H. irregulare* only", allowed us to generate both a "forward" predictive model on future changes in pathogen incidence and pathogen composition and a "backward" historical reconstruction to determine the likely date of arrival of the exotic pathogen in the Sabaudia forest. That date was then used together with the introduction date of the pathogen in Italy to determine the regional spread rate of the pathogen in the presence of habitat fragmentation. The estimated arrival of the pathogen in the Sabaudia forest was also used together with total area occupied by the exotic pathogen by 2016 to determine the annual increase in number of hectares infested by the pathogen in a contiguous forest. Providing data corroborating the invasive nature of *H. irregulare*, documenting whether it is replacing the native *H. annosum*, and estimating its spread rate in invaded ecosystems are all essential prerequisites to better quantify the threat level this alien North American pathogen represents for Europe.

Materials and Methods

Study site

The study was conducted in the Sabaudia Forest, a 3030 ha mixed-species woodland with a dominance of *Quercus* and *Pinus* spp., located in the Circeo National Park, a protected area of the western Mediterranean coast of the Lazio Region, Italy (336,000 m E, 4,578,000 m N–WGS84/UTM – elevation 40 m a.s.l.). A detailed description of the site is reported in Gonthier et al. (2012). The Sabaudia Forest was deemed as the most suitable site to deploy our experimental design thanks to its set of rather unique conditions: I) the site is located within the invasion area of the alien fungal pathogen *Heterobasidion irregulare* in Central Italy (Gonthier et al. 2014); II) both the native *H. annosum* and the alien congener *H. irregulare* coexist and sporulate in the same stand, and were present a comparable incidences at the beginning of the study (Gonthier et al. 2007, 2012); and III) no anthropic disturbances such as thinnings, cuttings and fires have been reported in the area.

Spore samplings and laboratory analyses

Spore samplings at the Sabaudia Forest were conducted to assess and compare the presence, abundance and distribution of both *H. annosum* and *H. irregulare* both in 2006 and in 2016. In December 2006, 33 long-term sampling points were located across the entire forest following a random spatial pattern design. Such points were a subset of those used to investigate the ecological association between vegetation types, *H. annosum* and *H. irregulare* in Gonthier et al. (2012). The coordinates (m) of each sampling point were recorded with a GPS device (Magellan® MobileMapper – precision±1 m) in the WGS84/UTM projection system. Both in 2006 and 2016, the spore deposition rates (DR, in spores·m^{-2}·h^{-1}) of *H. annosum* and *H. irregulare* were assessed at each sampling point based on the wood disc exposure method described in Gonthier et al. (2001, 2005, 2007, 2012). The above method is currently recommended by the European and Mediterranean Plant Protection Organization (EPPO) to detect the presence and quantify the abundance of *H. irregulare* (see EPPO 2020$_{a,b}$ and Online Resource 1 – Sect. 1). To determine the proportion of DR

attributable to *H. annosum* or *H. irregulare*, up to 10 colonies per disc were isolated and identified at the species level as described in Gonthier et al. (2012) (see Online Resource 1 – Sect. 2). For each sampling point, the proportion of colonies of either fungal species (in %) was taken as a proxy to assign a specific DR to *H. annosum* and *H. irregulare* (Gonthier et al. 2012).

Statistical analyses and modelling

The spatial pattern of the sampling points located in the study site was assessed by computing and testing the Clark and Evans aggregation index (R) (Clark and Evans 1954) (Online Resource 1 – Sect. 3.1). The overall incidences of *H. annosum* and *H. irregulare* were calculated as the ratio (%) between the number of points where the fungal species were detected and the total number of sampling points. The incidence/average DR of *H. annosum* and *H. irregulare* were compared between and within 2006 and 2016 by using conditional inference tree models (ctree) (Hothorn et al. 2006; Hothorn and Zeileis 2015) as reported in Lione et al. (2020).

Points sampled in 2006 and 2016 were split among the four mutually exclusive categories below: points where only *H. annosum* was detected (coded as Ha), points that were positive only to *H. irregulare* (Hi), points where both fungal species coexisted (Ha+Hi), or points where both species were absent (Ø)(Online Resource 1 – Sect. 3.2). The frequencies of sampling points scored as either Ha, Hi, Ha+Hi, or Ø were compared within each sampling year, while the frequencies of points classified in the same category were compared between sampling years. In both cases, comparisons were carried out with a n-sample test for the equality of proportions with continuity and Bonferroni corrections (Crawley 2013).

To test if species replacement had occurred between 2006 and 2016, a replacement index was calculated for two possible scenarios: the first with *H. irregulare* replacing *H. annosum*, and the second with *H. annosum* replacing *H. irregulare*. For the first scenario, the replacement index (RI$_1$, in %) was calculated as the ratio between the number of points where in 2016 only *H. irregulare* was detected ($\sum_{2016}(\text{Hi})$) and the number of the same points where in 2006 *H. annosum* was present alone ($\sum_{2006}(\text{Ha})$) or in coexistence with *H. irregulare* ($\sum_{2006}(\text{Ha} + \text{Hi})$), namely

$\text{RI}_1 = \sum_{2016}(\text{Hi})/\left[\sum_{2006}(\text{Ha}) + \sum_{2006}(\text{Ha} + \text{Hi})\right]$. For the second scenario, the replacement index (RI$_2$) was calculated in a similar way as $\text{RI}_2 = \sum_{2016}(\text{Ha})/\left[\sum_{2006}(\text{Hi}) + \sum_{2006}(\text{Ha} + \text{Hi})\right]$, with $\sum_{2016}(\text{Ha})$ and $\sum_{2006}(\text{Hi})$ indicating the number of points where only *H. annosum* or *H. irregulare* were present in 2016 and 2006, respectively. The two replacement indexes were compared with an exact version of the two-sample Poisson test (Huffman 1984) verifying: I) whether $\text{RI}_1 > \text{RI}_2$ (i.e. scenario 1 was the most likely, with *H. irregulare* replacing *H. annosum*); or II) $\text{RI}_1 < \text{RI}_2$ (i.e. scenario 2 was the most likely, with *H. annosum* replacing *H. irregulare*); or finally III) $\text{RI}_1 = \text{RI}_2$ (i.e. no replacement of either fungal species is likely to have occurred between 2006 and 2016).

To predict the future trend in the incidence of *H. annosum* and *H. irregulare*, a Markov chain model was fitted based on Dobrow (2016). The Markov matrix (M) storing the transition probabilities was calculated for the four categories (i.e. states) Ha, Hi, Ha+Hi, and Ø (Online Resource 1 – Sect. 3.3). The long-term trend of the incidence was appraised through the product of the row vector v, representing the distribution of the sampling points among the four states, and the n-step Markov matrices M^n, with n being an integer ranging from 2 to k+1. The value of k was calculated by applying the numerical approximation algorithm reported in Dobrow (2016), until the convergence condition required for a stable equilibrium was achieved (Online Resource 1 – Sect. 3.4).

To infer what the incidence of *H. irregulare* may have been in past years and assess when the alien pathogen may have first become established in the study site, a geometric progression model (Calter and Calter 2011) with equation $I(\text{Hi})_{t-10} = c \cdot I(\text{Hi})_t$ was fit, with $I(\text{Hi})_t$ being the incidence of *H. irregulare* at a given time indicated by the subscript, t being an integer representing a 10-year backward step since 2016 to 1916, and c representing the common ratio of the geometric progression. The constant c was assessed from the observed data as $c = I(\text{Hi})_{2006}/I(\text{Hi})_{2016}$. Based on the sampling size, the detection threshold to assess the lowest incidence value of *H. irregulare* was estimated at 3% (1 out of 33 sampling points). The abscissa of the intersection between the graph of the geometric progression and the detection threshold was deemed as a proxy of the

year in which *H. irregulare* established in the study site. An overall incidence model for *H. irregulare* was generated by merging the results from the geometric progression (backward model, appraising the past incidence), the spore samplings (observed data, showing the real incidence), and the Markov chain (forward model, forecasting the future incidence), accounting for models uncertainty (Online Resource 1 – Sect. 3.5).

Based on the outcomes of the geometric progression model, updated estimates of the linear spread rate (km/year) of *H. irregulare* across the invasion area in Central Italy were calculated through the ratio between the distance invaded (i.e. distance separating the site of first introduction and the study site) and the time needed for the invasion (i.e. time-lapse between the year when the first introduction in Italy occurred and the estimated year in which *H. irregulare* established in the study site) (Online Resource 1 – Sect. 3.6). Estimates of the surface spread rate (ha/year) were assessed from the overall incidence model dividing the area of the study site invaded by *H. irregulare* since its establishment by the time-lapse between each model step (Online Resource 1 – Sect. 3.6).

Statistical analyses and modelling were conducted with R version 3.6.0 (R Core Team 2019) (Online Resource 1 – Sect. 3.7). The significance threshold was set to 0.05 for all tests, while confidence intervals were calculated as reported in Online Resource 1 – Sect. 3.8).

Results

The results of the Clark and Evans aggregation index ($R = 1.180$, $P > 0.05$) confirmed that our sampling pattern was spatially random. Our observations during field surveys and samplings confirmed that no significant anthropic disturbances such as thinnings, cuttings and fires had occurred in between the two samplings. Such observations were confirmed by personal interviews with the Circeo National Park staff. Spore samplings confirmed the presence of both fungal species, i.e. the native *H. annosum* and the alien invasive *H. irregulare*, in both 2006 and 2016. However, the incidence of *H. annosum* dropped from 39.4% (23.6–57.8% $CI_{95\%}$) to 6.1% (1.1–19.2% $CI_{95\%}$) between 2006 and 2016, while that of *H. irregulare*

increased from 57.6% (40.1–73.2% $CI_{95\%}$) to 81.8% (65.5–91.8% $CI_{95\%}$) in the same time period. Differences among incidences were significant ($P < 0.05$) (Fig. 1). In 2006, deposition rates DR of *H. annosum* (31.0 spores·m^{-2}·h^{-1}, 14.1–71.2 spores·m^{-2}·h^{-1} $CI_{95\%}$) and *H. irregulare* (32.4 spores·m^{-2}·h^{-1}, 15.8–75.0 spores·m^{-2}·h^{-1} $CI_{95\%}$) were comparable ($P > 0.05$) (Fig. 2). Conversely, 10 years later, the DR of *H. annosum* (0.79 spores·m^{-2}·h^{-1}, 0–2.2 spores·m^{-2}·h^{-1} $CI_{95\%}$) was significantly lower ($P < 0.05$) than the DR of *H. irregulare* (117.4 spores·m^{-2}·h^{-1}, 69.4–194.4 spores·m^{-2}·h^{-1} $CI_{95\%}$) (Fig. 2).

In 2006, sampling points where only one of the two fungal species could be detected were 12.1% of the total for *H. annosum* (Ha) and 27.3% for *H. irregulare*, while the remaining 60.6% of points was equally split in points where: a) either both pathogens were present (Ha + Hi), or, b) none were present (Ø) (Table 1). No significant differences ($P > 0.05$) were found when comparing the frequencies of sampling points among the four categories Ha, Hi, Ha + Hi and Ø in 2006 (Table 1). Conversely, in 2016, most of the points (75.7%) were included in the category Hi, since only spores of *H. irregulare* were detected in the corresponding spore traps. The remaining points were split with comparable frequencies ($P > 0.05$) among Ha (0%), Ha + Hi (6.1%), and Ø (18.2%), all significantly lower ($P < 0.05$) than the percentage of points occupied by Hi (Table 1). Significant changes ($P < 0.05$) were observed for Hi (+ 45.4% of sampling points) and for Ha + Hi (-21.2%) between 2006 and 2016, while the decrease in frequency of either Ha or Ø (-12.1% each) was not significant ($P > 0.05$) (Table 1).

The index RI_1 of the scenario envisaging the replacement of *H. annosum* by *H. irregulare* attained a value of 84.6% ($CI_{95\%} = 56.6$–97.2%), while the index RI_2 modelling the replacement of *H. irregulare* by *H. annosum* was significantly lower at 0% ($CI_{95\%} = 0$–16.9%) ($P < 0.05$) (Online Resource 2). Since $RI_1 > RI_2$, scenario 1 was the most likely to have occurred, with *H. irregulare* replacing *H. annosum*. The Markov matrix M showed that, between 2006 and 2016, no sampling points switched their state from Ha, Hi, Ha + Hi, or Ø to Ha. Conversely, most of the points that in 2006 were classified in either Ha, Hi, Ha + Hi, or Ø switched to the state Hi in 2016, with a transition probability
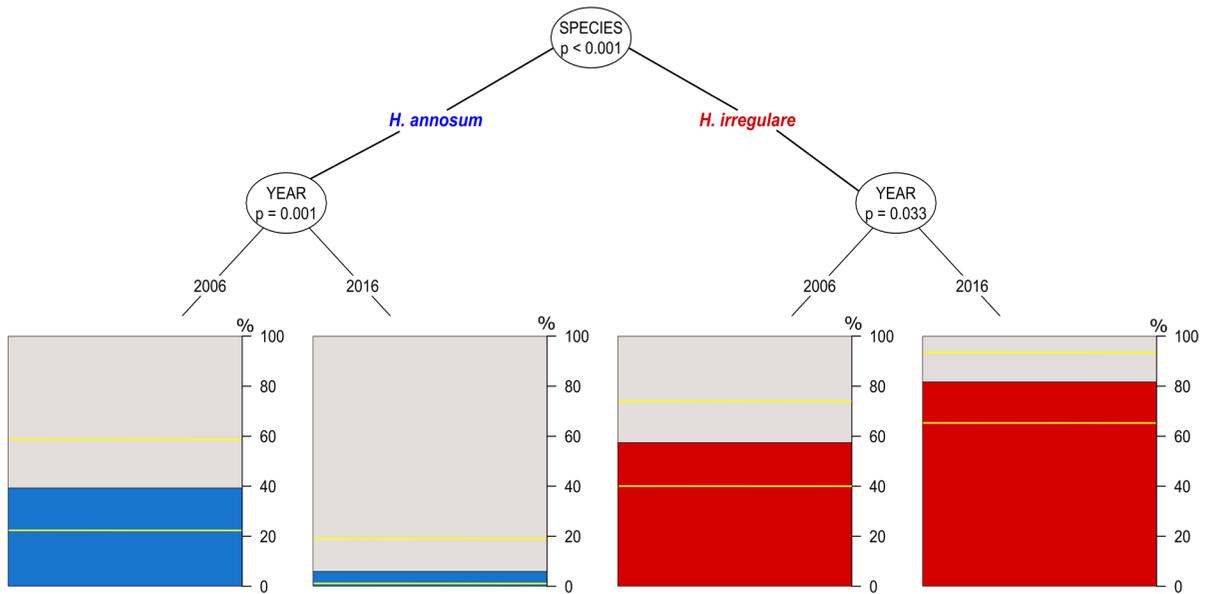
Fig. 1 Conditional inference tree model comparing the incidences of *Heterobasidion annosum* and *H. irregulare* between 2006 and 2016. Barcharts reported in the terminal nodes display the incidence of the two species along with the lower and upper bounds of their 95% confidence intervals. Upper nodes show that the differences observed for the incidence values are significant for both species and sampling years at *P* < 0.05
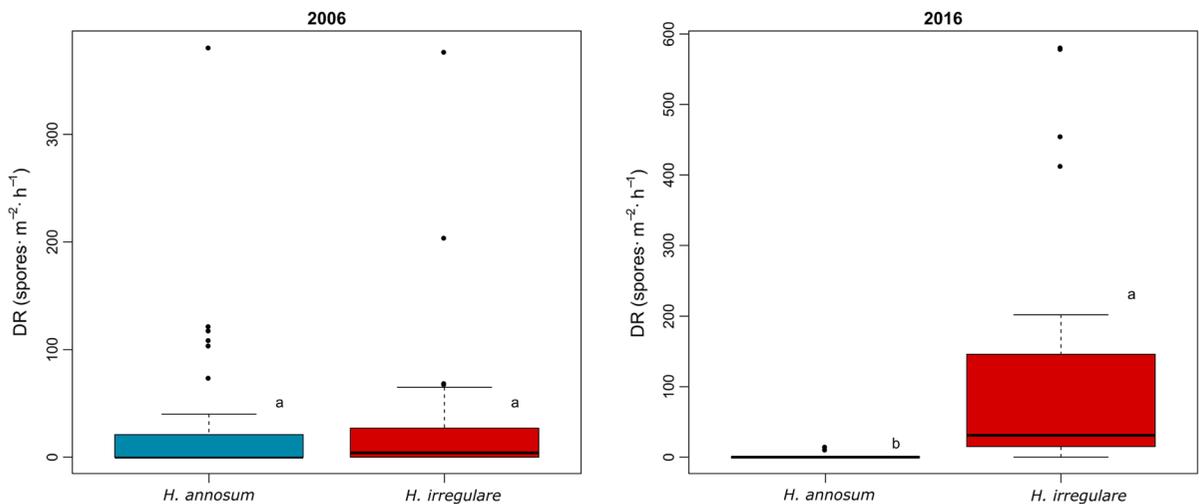
Fig. 2 Comparison between the spore deposition rates of *Heterobasidion annosum* and *H. irregulare* in 2006 and 2016. For each sampling year the boxplot associated with the deposition rate (DR, spores·m^{-2}·h^{-1}) of both fungal species is reported. The boxes display the values ranging between the 25th and 75th percentiles, the horizontal thick line in between marks the median DR, the t-shaped whiskers outside the boxes identify the minimum and maximum values, while points over the whiskers' bounds are outliers. Different letters indicate significant differences of the associated average values (*P* < 0.05)

ranging between 70 and 89% (Table 2). Only 11% of the sampling points where the two fungal species occurred in coexistence in 2006 maintained such a state in 2016 (Table 2). Finally, the probability of a transition from the state Ha + Hi to the state of absence Ø of the two species was 0% (Table 2).

Table 1 Number and proportion (%) of sampling points classified based on the presence of either, both or none of the two fungal species *Heterobasidion annosum* and *H. irregulare* in 2006 and in 2016

	2006	2016
Ha	4 (12.1%, 4.2–28.1% $CI_{95\%}$)a,A	0 (0%, 0–9.6% $CI_{95\%}$)b,A
Hi	10 (30.3%, 15.7–48.4% $CI_{95\%}$)a,B	25 (75.7%, 57.8–88.3% $CI_{95\%}$)a,A
Ha + Hi	9 (27.3%, 14.4–45.3% $CI_{95\%}$)a,A	2 (6.1%, 1.1–19.2% $CI_{95\%}$)b,B
Ø	10 (30.3%, 15.7–48.4% $CI_{95\%}$)a,A	6 (18.2%, 8.2–34.5%)b,A

Proportions are indicated along with their 95% confidence intervals. Lowercase letters refer to column-wise comparisons of sampling point frequencies among categories in the same sampling year, while uppercase letters are related to row-wise contrasts between sampling years within the same category. Different letters indicate significant differences of the associated values ($P < 0.05$). Ha: sampling points where only *H. annosum* was detected. Hi: sampling points where only *H. irregulare* was detected. Ha + Hi: sampling points where both *H. annosum* and *H. irregulare* were detected. Ø: sampling points where neither *H. annosum* nor *H. irregulare* were detected

Table 2 Markov matrix modelling the transition probabilities for the Markov chain model predicting the future trend in the incidence of *Heterobasidion annosum* and *H. irregulare*

	Ha_{2016}	Hi_{2016}	$Ha_{2016} + Hi_{2016}$	$Ø_{2016}$
Ha_{2006}	$Pr(Ha_{2006} \rightarrow Ha_{2016})$ 0% (0)	$Pr(Ha_{2006} \rightarrow Hi_{2016})$ 75% (3)	$Pr(Ha_{2006} \rightarrow Ha_{2016} + Hi_{2016})$ 0% (0)	$Pr(Ha_{2006} \rightarrow Ø_{2016})$ 25% (1)
Hi_{2006}	$Pr(Hi_{2006} \rightarrow Ha_{2016})$ 0% (0)	$Pr(Hi_{2006} \rightarrow Hi_{2016})$ 70% (7)	$Pr(Hi_{2006} \rightarrow Ha_{2016} + Hi_{2016})$ 10% (1)	$Pr(Hi_{2006} \rightarrow Ø_{2016})$ 20% (2)
$Ha_{2006} + Hi_{2006}$	$Pr(Ha_{2006} + Hi_{2006} \rightarrow Ha_{2016})$ 0% (0)	$Pr(Ha_{2006} + Hi_{2006} \rightarrow Hi_{2016})$ 89% (8)	$Pr(Ha_{2006} + Hi_{2006} \rightarrow Ha_{2016} + Hi_{2016})$ 11% (1)	$Pr(Ha_{2006} + Hi_{2006} \rightarrow Ø_{2016})$ 0% (0)
$Ø_{2006}$	$Pr(Ø_{2006} \rightarrow Ha_{2016})$ 0% (0)	$Pr(Ø_{2006} \rightarrow Hi_{2016})$ 70% (7)	$Pr(Ø_{2006} \rightarrow Ha_{2016} + Hi_{2016})$ 0% (0)	$Pr(Ø_{2006} \rightarrow Ø_{2016})$ 30% (3)

Each cell shows the transition probability (Pr, %) associated with any possible state transition ($\rightarrow$), as assessed from samplings conducted in 2006 and 2016. The corresponding number of sampling points is reported in brackets below the probability value. Ha: sampling points where only *H. annosum* was detected. Hi: sampling points where only *H. irregulare* was detected. Ha + Hi: sampling points where both *H. annosum* and *H. irregulare* were detected. Ø: sampling points where neither *H. annosum* nor *H. irregulare* were detected. $Pr(S1_{2006} \rightarrow S2_{2016})$: transition probability from the state S1 in 2006 (i.e. Ha_{2006}, Hi_{2006}, $Ha_{2006} + Hi_{2006}$, or $Ø_{2006}$) to the state S2 in 2016 (i.e. Ha_{2016}, Hi_{2016}, $Ha_{2016} + Hi_{2016}$, or $Ø_{2016}$)

The complete Markov matrix is reported in Table 2, along with the indication of the number of sampling points next to the canonical transition probabilities. The spatial distribution of the sampling points where *H. annosum* and *H. irregulare* were detected in 2006 and 2016 is shown with the corresponding transition state in Fig. 3. The row vector used for the Markov chain model was v = (0%, 75%, 6%, 18%), with the elements ordered as in the Markov matrix. The equilibrium was reached at k = 2, corresponding to the year 2026. The following probabilities were associated with the different states at the equilibrium: 0% for Ha, 72% for Hi, 8% for

Ha + Hi and 20% for Ø. The same probabilities were obtained up to k = 7, confirming the stability of k.

The common ratio c of the geometric progression modelling the past incidence of *H. irregulare* attained a value of 0.4. The intersection between the graph of the geometric progression and the detection threshold had its abscissa at year 1980, indicating the most likely estimate of the year when *H. irregulare* may have become established in the study site. The graphs of the lower and upper bounds of the $CI_{95\%}$ crossed the detection threshold in points with abscissae at years 1958 and 1992, respectively. The overall incidence model of *H. irregulare*, obtained by merging the results of the geometric progression, the spore

samplings, and the Markow chain, resulted in an S-shaped ascending graph shown in Fig. 4. The linear long-range spread rate of *H. irregulare* was estimated at 2 km/year by dividing the distance between the initial introduction point at Castelporziano and the northern border of the Sabaudia forest (66 km) by the number of years it took the pathogen to reach Sabaudia (from 1944 to 1980), with lower and upper estimates of 1.4 and 5 km/year. The surface spread rate attained a maximum value of 139 ha/year between 2006 and 2016, using 1980 as arrival date of *H. irregulare* at Sabaudia. At the beginning of its establishment the surface spread rate was estimated at 6–9 ha/year (Online Resource 3). All relevant data are provided in Online Resource 4.

Discussion

This study corroborates the invasiveness of the alien pathogen *Heterobasidion irregulare* by comparing its overall and relative incidence, its distribution across the sampling points, and its spore load measured through two identical samplings performed in the same forest ten years apart.

The use of spore counts on woody discs avoids both sampling constraints and the subjective bias present when attempting to identify trees or stumps infected by *Heterobasidion* species based on symptoms and/or the presence of fruiting bodies. In fact, although systematic sampling and processing of wood tissues from stumps or roots through microbial isolation assays or molecular analyses might have been used to assess the abundance of the two fungal species, such strategy would have been only partially effective due to the large extent of the study site, and because of field constraints limiting the accessibility and exploration of some forest patches. Additionally, airspora is the fungal life stage epidemiologically and biologically most relevant, being responsible for establishment in new sites of both *Heterobasidion* species (Garbelotto and Gonthier 2013). Likewise, propagule pressure has been identified as one of the best ways to predict the impact of biological invasions (Reaser et al. 2007). In this light, colony counts on woody traps provide a reliable proxy for the effective population size of the pathogen, given that: I) symptoms might be not visible, especially at the early stage of the stand infestation process, and,

II) fruiting bodies may never occur on some infected trees or stumps. Simple spore counts on substrates other than wood discs (e.g. filter papers or adhesive tapes) may be misleading because many spores may not germinate. Conversely, colony counts on wood discs represent the number of viable and germinating spores on a substrate that is similar to that of freshly-cut stumps. Consequently, spore trapping through wood discs mimics the mechanism underlying primary infections of *Heterobasidion* spp. in the field. It should be noted that, although *H. irregulare* is a better competitor than *H. annosum* with regards to its sporulation potential and saprobic wood decay ability, there is no evidence that such traits may be associated with differential germination rate of spores of the two species (Giordano et al. 2014). Finally, while we acknowledge the existence of hourly, daily and seasonal variations in spore deposition rates of fungi, we attempted to minimize the effects of such variation by exposing traps for an entire cycle of 24 h and by performing both samplings in December, when sporulation by both *Heterobasidion* species is peaking and comparable in central Italy (Garbelotto et al. 2010).

The surveys performed in this study indicated that in the 2006–2016 decade, the incidence of *H. irregulare* in the Sabaudia forest increased significantly, while that of *H. annosum* decreased in the same period. Additionally, the overall average *H. irregulare* spore load increased when comparing 2006 to 2016 data, while that of *H. annosum* decreased. Overall spore loads of the two species were undistinguishable in 2006, but the spore load of *H. irregulare* was significantly higher than that of *H. annosum* in 2016. The overall increase in number of sampling points where *H. irregulare* spores were detected for the first time ten years after the first sampling indicates this species is increasing its geographic range, thus confirming it is invasive. Likewise, the increase in the spore load of *H. irregulare* between 2006 and 2016 suggests the population size of this pathogen is also increasing. This is another trait normally associated with invasive species (Mack et al. 2000). Although the presence of daily variation in spore loads makes comparisons between 2006 and 2016 tenuous, we also note the average spore load by *H. irregulare* increased 3.7-fold between 2006 and 2016. This suggests that the carrying capacity of the alien species may be higher than that of the native one. Given that *H. irregulare* is dominant in the region around the study site
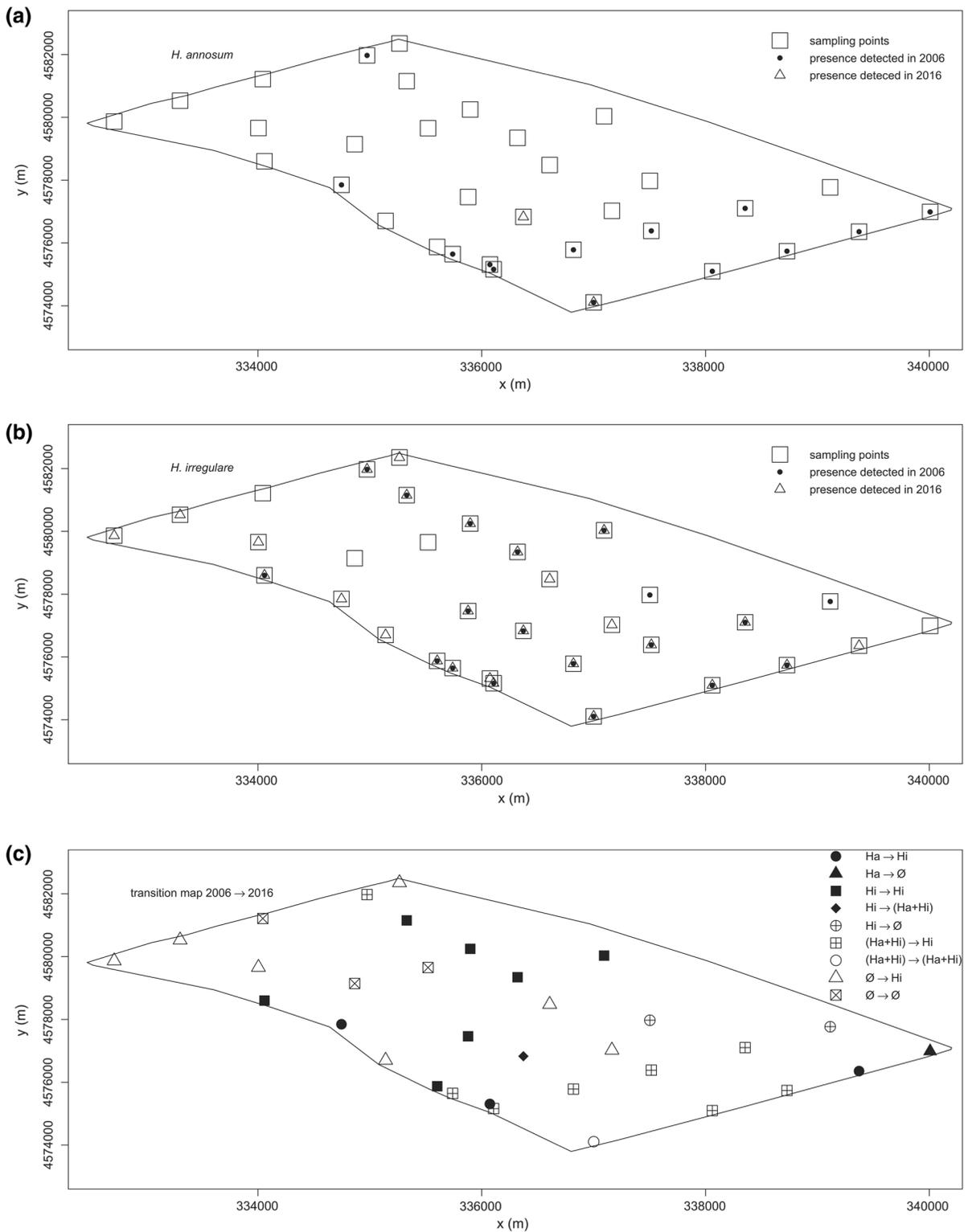
Fig. 3 Map of the study site (Sabaudia forest) showing the spatial distribution of the sampling points where *H. annosum* (panel **a**) and *H. irregulare* (panel **b**) were detected in 2006 and 2016. The transition map (panel **c**) shows the state transition of each sampling point between 2006 and 2016 as described by the Markov matrix. In the dot legend, the first state refers to 2006, the second to 2016. Acronyms of states included in the transition map are: Ha (sampling points where only *H. annosum* was detected), Hi (sampling points where only *H. irregulare* was detected), Ha+Hi (sampling points where both *H. annosum* and *H. irregulare* were detected), and Ø (sampling points where neither *H. annosum* nor *H. irregulare* were detected). Map coordinates reported along the x and y-axes are in WGS84/UTM projection system (m)

(Gonthier et al. 2007), regional-scale migration from surrounding forests into the Sabaudia's forest is likely to further contribute to an increase in *H. irregulare* population size.

It is noteworthy that while the distribution (i.e. number of sampling points with a positive *H. irregulare* detection), proportional representation (% of sampling points with a positive *H. irregulare* detection) and abundance (i.e. spore load) of *H. irregulare* increased when comparing 2006 to 2016 data, *H. annosum* displayed an inverse trend, with a significant decrease in its distribution, proportional representation and abundance. These results obtained using the entire Sabaudia's forest dataset strongly suggest a competitive interaction is ongoing between the two species, with an outcome in favor of the alien pathogen. Similar results have been shown for other dual interactions among pathogens, such as, for instance, the interaction between O*phiostoma novo-ulmi* and *O. ulmi* or between *Hymenoscyphus fraxineus* and *Hymenoscyphus albidus,* both ending in favor of the first species in each pair (Brasier and Bick 2001; McKinney et al. 2012).

Our analysis, like that of Kozanitas et al. (2017) was spatially-explicit, and included results from the same points resampled twice, ten years apart. The spatially explicit analysis allowed us to further state that species replacement occurred, with *H. irregulare* replacing *H. annosum* across most of its distribution (with a replacement index of 84.6%) in a 10-year period. Because *H. annosum* was present in these sampling points in 2006, the results also indicate that antagonistic a priori effects do not exist, meaning that the prior presence of *H. annosum* does not per se prevent the establishment of *H. irregulare*. This result is consistent with results presented by Giordano

et al. (2019) who experimentally determined that coexistence of the two pathogens on the same wood substrate enhances the fruiting potential of the alien species without affecting that of the native one. We believe this to be one of the few examples in which spatially explicit resampling at several locations was employed to demonstrate species replacement of a native fungal species by an alien one (for other examples see McKinney et al. 2012; Kozanitas et al. 2017).

Although our experiment proves an increase in the spatial range of *H. irregulare*, an increase in its populations size, lack of a priori effects favoring *H. annosum* and its replacement by *H. irregulare*, the mechanisms leading to such expansion range, population increase and species replacement could be multiple. First, by producing a larger number of spores, *H. irregulare* is more likely to colonize newly available substrates such as snags and freshly cut stumps, thus reducing substrates available for its competitor. Second, by being a faster wood colonizer, *H. irregulare* may better utilize the available substrate than its competitor. Third, given that wood decay basidiomycetes are notoriously territorial through a process called somatic or vegetative incompatibility (Leslie 1993), the two traits above may lead to a direct antagonistic exclusion of *H. annosum* by *H. irregulare*. Although our data show that *H. annosum* is outcompeted by *H. irregulare*, the results of this study should not be used to infer an increased competitive advantage of *H. irregulare* over other wood-inhabiting fungi. In fact, two recent papers (Poloni et al. 2021; Pellicciaro et al. 2021) show that the North American *Heterobasidion* species can be controlled by stump applications of the saprobic competitor *Phlebiopsis gigantea*, as already reported for European *Heterobasidion* species (Garbelotto and Gonthier 2013).

The forward and backward modelling analyses, although performed using different methodologies and analytical approaches, are both robust and commonly used for demographic modelling (Verhulst 1977; Calter and Calter 2011; Dobrow 2016). Combined, the models predict an increase in the proportional representation of *H. irregulare* across the total number of points sampled in the Sabaudia forest. While Markov chain simulations estimate that the two interacting fungal species are likely to reach equilibrium in 2026, ten years from the second sampling, actual changes in proportional representation of the two species should be minimal between 2016 and
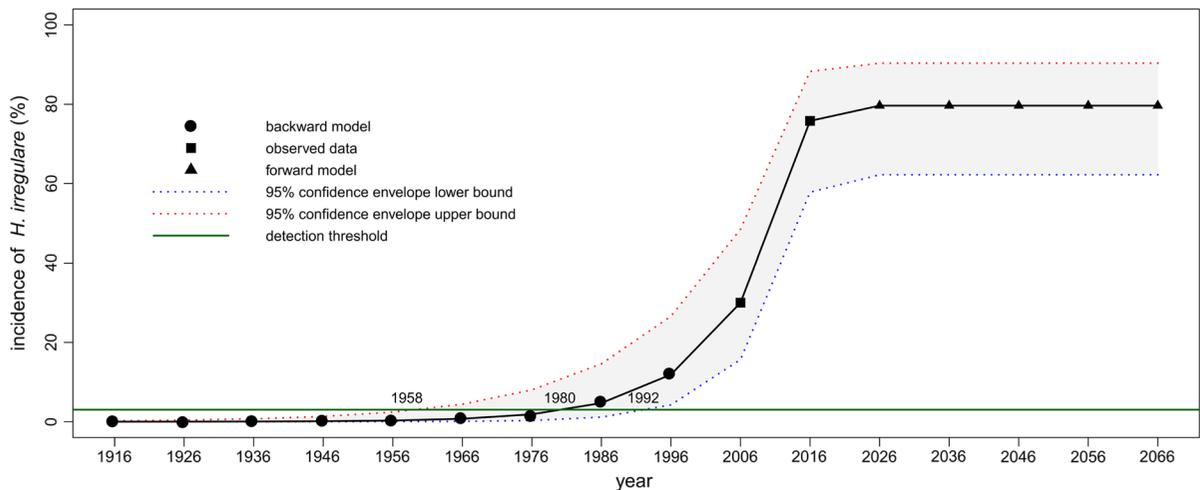
Fig. 4 Overall incidence model for *Heterobasidion irregulare* merging the results from the geometric progression (backward model), the spore samplings (observed data), and the Markov chain (forward model). The incidence (%) of the pathogen is shown on the y-axis as a function of the year, indicated on the x-axis with steps of 10 years. The uncertainty of the model is represented by the envelope of the 95% confidence intervals. The intersections of the detection threshold with the graph and with the envelope bounds are marked with the corresponding years

2026 and may be insignificant from a practical perspective. Forward simulations predict that by 2026 and then onward, 72% of the Sabaudia forest could be colonized by *H. irregulare*, 8% might be colonized by both *H. irregulare* and *H. annosum*, while 20% should be *Heterobasidion* free. The persistence of *H. annosum,* despite the asymmetrical fitness in favor of *H. irregulare*, is to be expected and has been reported previously for other biological invasions involving one native and one alien organism characterized by asymmetrical fitness (Gurevitch and Padilla 2004; Downey and Richardson 2016).

The backward model indicated that the most likely arrival year of *H. irregulare* at Sabaudia could have been 1980. That estimation of the arrival date may have a twofold application. First, it allows us to refine the regional scale prediction of spread rate in the highly fragmented and heterogeneous Roman countryside. Second, and more importantly, it allows to appraise the spatial spread in terms of number of hectares per year affected by pathogen in a single forest. Based on the data provided by this study and by the date and location of its original introduction, the regional scale spread rate of the pathogen in the Roman countryside should be 2 km/year, up from the previous estimate of 1.3 km/year. *Heterobasidion irregulare* in a mixed oak-pine forest characterized

by mild Mediterranean climate and good pine representation (Gonthier et al. 2012) is predicted to spread at an initial rate of 6–9 ha/year, reaching a maximum rate of 139 ha/year in the exponential phase of its spread. This is a significant spread rate, even at the beginning of the infestation, one that better exemplifies the actual threat that *H. irregulare* poses to European forests. It is worth noting that forward and backward modelling analyses both hinge on field data collected at two time-points (i.e. 2006 and 2016), while both the past and the future scenarios span over several decades. Hence, our reconstructed and predicted scenarios necessarily include a level of uncertainty relative to the limited data available and to the assumptions underlying the models. Such assumptions include the stability of the probabilities embedded in the Markov matrix and the constancy of the common ratio of the geometric progression (Calter and Calter 2011; Dobrow 2016). Nonetheless, these and other assumptions are the backbone of modelling approaches across all scientific fields including biology, plant pathology, ecology, medicine, and geology (Dobrow 2016 and literature therein), in spite of the uncertainty inextricably associated with any modelling approach.

Finally, the issue of the *H. irregulare*'s carrying capacity compared to that of *H. annosum* remains

open, mostly because the carrying capacity of *H. annosum* prior to the arrival of *H. irregulare* at the Sabaudia forest could not be modelled with our current propagule data, and because there are no other sites where both *Heterobasidion* species currently coexist at significant and comparable levels. However, it is not uncommon for invasive species to be characterized by higher carrying capacity than their native counterparts, resulting in a higher burden on the ecosystem. Specifically, when the invasive species is a plant pathogen, a higher carrying capacity would result in higher plant mortality. Based on the published literature reporting a higher sporulation potential of *H. irregulare* compared to that of *H. annosum* (Giordano et al. 2014), the expectation is that spore loads in a forest infested by *H. irregulare,* and thus incidence of infections, will be much higher than those in forests infested by *H. annosum*. We believe that our spore load data may indeed be used to predict a sizeable increase in *Heterobasidion* carrying capacity. In 2016, the average spore load of both species combined was 1.9 times that of 2006, and the 2016 value can be regarded as being comparable to that predicted for 2026, when the system will likely reach its equilibrium. This increase in spore load is obviously correlated to the population expansion of *H. irregulare* paralleled by the population shrinkage of *H. annosum* in the 2006–2016 decade, both shown to be occurring in this study. Hence, the establishment of *H. irregulare* in any forest originally inhabited only by *H. annosum* should be paralleled by a progressive increase in overall *Heterobasidion* spore loads and incidence of *Heterobasidion* root rot disease.

Conclusions

By comparing the presence and abundance of each of two *Heterobasidion* species in the same sampling points ten years apart, this study provides the first direct evidence that the alien North American forest pathogen *H. irregulare* is invasive in Italy and is increasing both its geographic range and its population size, while locally replacing the native *H. annosum*. Although *H. annosum* is not predicted to completely disappear, its final frequency in Mediterranean mixed oak-pine forests invaded by *H. irregulare* will be low and its presence is predicted to always be in sympatry with the alien congeneric species.

Replacement of *H. annosum* by *H. irregulare*, as documented by this study, suggests *H. irregulare* may become dominant even in pine stands, such as those of Central and Northern Europe, where incidence of *H. annosum* is already significant.

Data availability All data relevant to this work are provided as Online Resources.

Code availability Can be provided upon request by the corresponding author.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The funding sources of the research had no role in the study design, in the collection, analysis and interpretation of data, in the writing of the manuscript, and in the decision to submit the article for publication.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication All authors have approved the contents of this paper and have agreed to the submission policies of Biological Invasions.

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