Running head: GIS identifies new host-pathogen association

The American forest pathogen *Heterobasidion irregulare* colonizes unexpected habitats after its introduction in Italy

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ABSTRACT

Habitat preference of an invasive fungal tree pathogen is here compared with that of a sympatric native closely related congener to test the hypothesis that the invasive ability of the exotic organism may be linked to its capacity to colonize substrates unavailable to the indigenous relative. We compared the distribution of infectious airspora of the North American *Heterobasidion irregulare* introduced into Italy with that of the native *H. annosum,* both regarded to be able to become established only in the presence of conifers. Geostatistical and statistical analyses were employed to test for association between either species and five Mediterranean vegetation types. Results show that *H. annosum* is positively associated with pines and negatively associated with deciduous oaks. The probability of finding its spores decreases to almost 0 at distances over 500 m from pines, and this species is virtually absent in pure oak forests. Spores of *H. irregulare* are present irrespective of vegetation type, and this species can be found not only where pines are present, but also in pure oak forests. This knowledge implies that spread of *H. irregulare* is not limited by the fragmented distribution of pine woodlands in central Italy and this information is essential to both predict and hinder its progress in Europe.

Key words: biological invasion; exotic pathogen; GIS; habitat association; Heterobasidion irregulare*; novel pathosystems; oaks; pines; root-rot fungi.*

INTRODUCTION

Invasiveness of exotic plant pathogens may be related to their ability to colonize unexpected habitats in their new range. In this paper, we combine field surveys with molecular, geostatistical, and statistical analyses to show that an exotic invasive pathogen of conifers is also found in broadleaf forests, an habitat unreported both for this organism in its native region and for its sister taxon present in the invaded area. Such a finding may be pivotal for modeling the invasion of this exotic pathogen and to slow down its progress through adequate forest management and control practices.

Successful invasion by an exotic pathogen depends on complex and often interacting ecological and evolutionary factors. The enemy release hypothesis, the lack of a long-established host-pathogen coevolutionary relationship, the possibility of a host-shift, the degree of adaptability of a introduced pathogen to the new environmental conditions and its ability to exploit the newly-available trophic resources have all been broadly cited as key elements driving a successful invasion (May and Anderson 1983, von Broembsen 1989, Keane and Crawley 2002, Torchin et al. 2003, Parker and Gilbert 2004). Understanding the factors underlying a successful invasion allow prediction of spread patterns and identification of habitats that may be at risk of pathogen colonization. Consequently, efforts to mitigate the spread of the invasive organism can be focused on relevant habitats, for instance by creating a buffer where available hosts for the invasive species are eliminated around the edges of the zone of infestation.

In the case of an invasive infectious pathogen, the success of the invasion may be determined by the ecological amplitude of the pathogen, intended as range of available hosts (von Broembsen 1989, Huyse et al. 2005), combined with factors such as the aggressiveness on each infected host, the reproductive rate, and the dispersal potential of infectious propagules (Williamson and Fitter 1996, Heger and Trepl 2003, Parker and Gilbert 2004). A thorough assessment of the invasive potential of a pathogen must thus consider each of the above factors.

Normally, in the initial phases following the discovery of the introduction of a known pathogen, most assessments are based on studies performed in the region of pathogen origin, where hosts and ecological conditions are likely to be different than those in the introduction area (Parker and Gilbert 2004). Occasionally, and only for organisms perceived as threats, data generated in laboratory experiments may also be available: for instance, host ranges may have been or may be quickly determined through artificial inoculation studies (De Vienne et al. 2009).

When an exotic organism shares a habitat with a closely related native species, the expectation is that its spread may be hampered by the competitive interaction between the two species, especially if their ecological amplitude is similar (Daehler 2001). Unfortunately, a solid comparative evaluation of the ecological amplitude and of other phenotypic traits between two related species is possible only when they are sympatric, hence predictions may be erroneous prior to the actual introduction of an organism into a new region (Heger and Trepl 2003). In any case, when an introduced pathogen becomes invasive in spite of the presence of a similar native organism competing for the same resources, a difference must be present to justify the selective advantage of the invasive species (Heger and Trepl 2003, Colautti et al. 2004, Parker and Gilbert 2004).

Heterobasidion annosum (Fr.) Bref. in Eurasia and *Heterobasidion irregulare* (Underw.) Otrosina & Garbelotto in North America, and now in Italy, are among the major causes of root rot on the genus *Pinus*, and as such are two of the best-studied fungal pathogens worldwide (Otrosina and Garbelotto 2010). These two species have evolved and differentiated in allopatry for at least 20 millions of years (Otrosina et al. 1993, Dalman et al. 2010). *Heterobasidion irregulare* has been recently found in several *Pinus pinea* L. (Italian stone pine) forests around Rome, probably accidentally introduced by the USA Army through infected lumber during World War II (Gonthier et al. 2004, Gonthier et al. 2007). The majority of pine species is reported to be susceptible to *H. irregulare* (Filip and Morrison 1998, Otrosina and Garbelotto

2010), and in fact this pathogen has already been reported to kill both *P. pinea* and *P. halepensis* Mill. (Aleppo pine) trees in Italy (Scirè et al. 2008). Because *Pinus* is a dominant genus of a large number of ecosystems, the spread of this North American pathogen in Europe threatens a large number of habitats, ranging from coastal to montane, spanning from subtropical to boreal latitudes. Although *H. annosum* and *H. irregulare* are now broadly sympatric and hybridizing at a significant rate around Rome (Gonthier and Garbelotto 2011), the exotic taxon is clearly invasive and its incidence is much greater than that of its native congener in most forests (Gonthier et al. 2007). The reason for this difference is only partially understood: while virulence between the two pathogens on pines is comparable, sporulation appears to be constantly high only for the exotic species (Garbelotto et al. 2010). Factors other than virulence on pines and spore production have not been thoroughly compared.

Heterobasidion irregulare is significantly more abundant than *H. annosum* in all known sites with the exception of the site at Sabaudia in the Circeo National Park, where both species coexist at significant levels (Gonthier et al. 2007) in a forest characterized by five distinct vegetation types. The habitat association of the two *Heterobasidion* species was investigated by determining the presence and abundance of either species across all five vegetation types. Associations between the two fungal species and vegetation types were studied by means of statistical and geostatistical analyses in Sabaudia. In order to confirm results obtained from this complex and vegetationally heterogeneous forest, we sampled five additional forests characterized each by the presence of single vegetation types.

This study was aimed at elucidating the mechanisms driving the invasion of an exotic fungal pathogen in a novel pathosystem (*H. irregulare*-Mediterranean ecosystem) in the attempt to improve our ability to predict its spread into the European continent and to design appropriate countermeasures.

METHODS

Study sites

The main study site was the 3030 ha Sabaudia forest in the Circeo National Park, on the west coast of central Italy (Table 1). This site was selected because: a) both *H. annosum* and *H. irregulare* were found to be present there in comparable frequencies (Gonthier et al. 2007), and, b) the forest is known for its high levels of biodiversity and includes five main typical Mediterranean vegetation types (Table 2) (Blasi and Carranza 1998, Filesi et al. 1998). Supplementary surveys were performed in five additional sites. Three were Mediterranean deciduous oak forests in the zone of invasion near Anzio and Nettuno, and two were a mixed oak-Italian stone pine forest and a deciduous oak forest located near Mesola, in north-eastern Italy, well outside the zone of invasion by *H. irregulare* (Table 1).

Characterization of vegetation types

Vegetation types were inferred on the basis of a raster map of vegetation of the Circeo National Park based on Landsat 5 TM remote sensing imaging (Manes et al. 2005). Raster vegetation type layers with spatial resolution of 30 x 30 m (Jones and Vaughan 2010) were converted into vector format, georeferred in UTM WGS 1984 coordinates, and merged into the five main vegetation types described for the Sabaudia forest (Table 2) (Padula 1985, Filesi et al. 1998, Milanese et al. 1998, Pignatti 1998, Manes et al. 2005). Vegetation types were confirmed on the ground at all sampling points.

Sampling method and laboratory analyses

The habitat association of the two *Heterobasidion* species was investigated by determining the presence and abundance of both species by quantifying viable spores of each using a systematic sampling scheme representing all vegetation types present in each forest. Deposition rates (DRs) of *H. annosum* and *H. irregulare* was determined by the wood-disc exposure method, described in several studies (Gonthier et al. 2001, Gonthier et al. 2005, Gonthier et al. 2007) and summarized as follows. Four wood discs, approximately 12 cm in diameter and 0.5 cm in thickness, were obtained from freshly-cut healthy *Picea abies* (L.) Karst. (Norway spruce) trees and were exposed inside open Petri dishes for 24 h at each sampling point. Wood of Norway spruce has been shown to be unselective for the saprotrophic growth of *Heterobasidion* spp. spores (Gonthier 2001), and has extensively been used to sample spores of several *Heterobasidion* species (Gonthier et al. 2001, Gonthier et al. 2005). The four discs were placed 5 m from the centre of each sampling point along the four cardinal directions. Coordinates of all sampling points were recorded with Global Positioning System (GPS) instrumentation with a precision of ± 1 m in the UTM WGS 1984 coordinate system. Samplings were performed in December 2006 in the Sabaudia forest, in November 2006 in Mesola and in May 2010 in Anzio and Nettuno. Late fall and spring are periods equally conducive to sporulation of both species in Mediterranean habitats (Garbelotto et al. 2010). The number of sampling points per forest ranged between 5 and 44 depending on size of the forest (Table 1). Distance between points averaged 700 m in the Sabaudia forest and 350 m in the other forests. At the end of the exposure time in the field, discs were treated as previously described (Gonthier et al. 2007) and incubated at 24°C for 15 days. At the end of the incubation period, discs were inspected twice weekly for a period of three weeks to identify individual colonies of *Heterobasidion* spp. that may be growing at different rates. These colonies can be easily identified at 40X magnification under the dissecting microscope thanks to the presence of the unequivocally diagnostic asexual stage of the fungus (Greig 1998). All visible colonies were traced on each disc using a marker to ensure they were not counted twice, and it was assumed that each clearly distinct colony had originated from a single germinated spore (Rishbeth 1959). DRs were calculated as number of viable spores per square meter per hour (spores m^{-2} h^{-1}).

Species identification

Up to 10 colonies were isolated from each disc by transferring fungal colonies to the *Heterobasidion* selective growth medium described by Kuhlman and Hendrix (1962). A section of each fungal colony was collected with the tip of a micropipette and suspended in 100 µL of distilled water before being processed as described by Gonthier et al. (2007). Fungal DNA was extracted from fungal suspensions following the method of Schweigkofler et al. (2004).

DNA was characterized by three sets of PCR primers that target one nuclear locus and one mitochondrial locus and differentiate *H. irregulare* from *H. annosum* (Gonthier et al. 2007). Based on PCR results, spore-generated colonies were assigned to each one of the two species and the total relative abundance of spores of each species at any given sampling point was assumed to be the same as that determined for the subset analysed by PCR assays at that same point.

Statistical and GIS analyses

In the Sabaudia forest, the association between vegetation types and species of *Heterobasidion* was assessed by statistical and geostatistical analyses performed on the basis of incidence of positive trappings of each species (number of sampling points positive to each species) and on their DRs (calculated by adding spores from the four wood discs and considering the cumulative surface of all four discs at any given sampling point). Comparisons of DRs of the two *Heterobasidion* species in the Sabaudia forest were performed with the Mann-Whitney U test. The pattern of distribution of sampling points was tested with the average nearest neighbour index test, while the spatial pattern of DRs for each *Heterobasidion* species was assessed with the global Moran's I test and the general G-statistic (Mitchell 2009). A 100 m radius circular buffer was generated around all sampling points to verify the presence/absence of each vegetation type. This distance threshold was chosen based on the knowledge that the vast majority of *Heterobasidion* spores lands within 100 m from the fruit body that generates them (Stenlid 1994, Möykkynen et al. 1997, Gonthier et al. 2001). The incidence of positive trappings of either fungal species and the frequency of sampling points whose buffer included each vegetation type were cross-tabulated and odds ratio values with their 95% confidence interval were employed as measures of association (Agresti 2002).

Since the odds ratio values indicated an association between Italian stone pine and *H. annosum* (see results), the distance in meters (d) between each sampling point and the edges of the nearest pine stand was also calculated. A binary logistic regression model was used to describe the functional relationship (1) between the probability of finding *H. annosum* spores ($\pi_{(d)}$) and d, as follows:

$$\pi_{(d)} = \frac{e^{\beta_1 d + \beta_0}}{1 + e^{\beta_1 d + \beta_0}} \qquad (1)$$

where β_1 is the parameter estimate and β_0 is the intercept.

Overall model significance was tested with the likelihood ratio test, while the significance of β_1 was evaluated using the Wald test (Hosmer and Lemeshow 1989). The odds ratio of the variable d and its relative 95% confidence interval were calculated as described by Hosmer and Lemeshow (1989). Goodness of fit of the model was assessed with the Hosmer-Lemeshow test (Hosmer and Lemeshow 1989). Model discrimination was evaluated using a 2 x 2 classification table (Hosmer and Lemeshow 1989, Peng et al. 2002) and subsequently tested by examining the significance of the AUC (Area Under the Curve) of the ROC (Relative Operating Characteristic) curve (Metz 1978, Sweets 1988). Model validity was assessed through the count of the EPV (Events Per Variable) (Peduzzi et al. 1996). When an association, positive or negative, was found between a *Heterobasidion* species and a vegetation type, the Mann-Whitney U test was used to compare DR values between points with the vegetation type associated with that species and points without the same associated vegetation type.

A supervised classification of Landsat 7 ETM+multi-spectral images (Jones and Vaughan

2010, Global Land Cover Facility 2010) was run to ensure no sizeable clusters of pine trees (i.e. more than one pixel) were present within 100 m from any given sampling point in the pure oak stands of Anzio, Nettuno, and Mesola. Training pixels for the supervised classification were derived from existing vegetation maps (Manes et al. 2005). Visual surveys during samplings were performed to validate the results of the supervised classifications.

GIS analyses were performed on ArcMap 9.1 (ESRI, 2006) (Mitchell 2009) and statistical analyses were run on PASW Statistics 18.0.0 (IBM, 2009) and StatXact-9 (Cytel, 2010). Significance cut-off levels were set at 0.05 in all analyses.

RESULTS

The percentage of sampling points whose buffer areas included each of the five vegetation types (Fig. 1a) were: 83% for Mediterranean maquis, 80% for deciduous oak, 54% for pines, 49% for both evergreen oaks and anthropic or agricultural areas.

In the Sabaudia forest, *Heterobasidion annosum* and *H. irregulare* were found in 36% and 66% of 44 sampling points, respectively (Fig. 1b). The average *H. irregulare* DR (44 spores m^{-2} h^{-1}) was higher than that of *H. annosum* (33 spores m^{-2} h^{-1}) (U = 72.000; $P = 0.093$).

The distribution of sampling points in the Sabaudia forest was significantly dispersed (nearest neighbour ratio = 1.162; $P = 0.047$). Based on DR values, *H. annosum* showed a spatial pattern of significant clustering (Moran's I = 0.179; $P < 0.001$), while *H. irregulare* was randomly distributed throughout the sampling area (Moran's I = -0.004; $P = 0.547$). General G-statistics results also indicated clustering of high *H. annosum* DRs (general G Index = 0.001; $P < 0.001$).

The buffer analysis and odds ratio values both indicated that the incidence of positive trappings of *H. annosum* spores was positively associated with pines and negatively associated with deciduous oaks. No significant association was found between spores of *H. annosum* and the remaining three vegetation types or between spores of the exotic *H. irregulare* and any of the five vegetation types (Table 3).

The significant associations identified for *H. annosum* were also confirmed by analyses performed on DR values. Sampling points whose buffers included pines had a significantly higher DR level (on average 49 spores m^{-2} h^{-1}) than those without pines (on average 2 spores m^{-2} h^{-1}) (U = 104.000; $P = 0.002$). Similarly, the mean level of DR of *H. annosum* spores in buffers with Mediterranean deciduous oak forests (14 spores m^{-2} h^{-1}) was significantly lower than the value recorded in points without deciduous oaks (80 spores m^{-2} h^{-1}) (U = 91.000; $P = 0.007$).

The binary logistic regression model defining the functional relationship between the probability of finding *H. annosum* spores and the distance from the nearest cluster of pines was significant (likelihood ratio $\chi^2 = 12.372$; $P < 0.001$). The $\beta_1 = –0.011$ parameter estimate was significant (Wald $\chi^2 = 5.514$; $P = 0.019$), and its odds ratio value was 0.989 (95%; confidence interval = 0.980–0.998), indicating a negative association between the distance d from pines and the probability $\pi_{(d)}$ of finding *H. annosum* spores (Fig. 2). The β_0 intercept value was 0.779. The goodness of fit of the model was confirmed (Hosmer and Lemeshow $\chi^2 = 9.853$; $P = 0.275$), and the 2 x 2 classification table showed an overall success rate of 68%, a specificity (true negatives) of 72%, a sensitivity (true positives) of 63%, a false positive rate of 28%, and a false negative rate of 37%. The ROC curve analysis showed a good discrimination power (AUC = 0.780; $P = 0.003$), and EPV > 10 confirmed the model was reliable.

In the three pure Mediterranean deciduous oak forests of Anzio and Nettuno, spores of *H. irregulare* were found in 68% of all sampling points with a mean DR of 18 spores m^{-2} h^{-1}. Spores of *H. annosum* were absent in all three forests.

In Mesola, *H. annosum* was present in all sampling points of the mixed oak-Italian stone pine forest (mean DR = 6 spores m^{-2} h^{-1}), while the pathogen was detected only in one out of 37 sampling points in the pure deciduous oak forest. In that single point, DR of *H. annosum* spores was only 2 spores m^{-2} h^{-1}. As expected, spores of the exotic *H. irregulare* were not found in either of the two Mesola forests, located hundreds of kilometers north of the zone of infestation.

In all four Mediterranean deciduous oak forests, 78% of sampling points that yielded spores were at least 350 m from pines. The remaining 22% was at least 100 m far from sizeable clusters of pine trees according to both ground verification during field surveys and the photointepretation of Landsat 7 ETM+multi-spectral images.

DISCUSSION

Results of this study indicate that, when in sympatry, the native *H. annosum* and the invasive *H. irregulare* are characterized by distinct habitat associations. Clustering of *H. annosum* DR values is in contrast with the random distribution of *H. irregulare* DRs and is a likely indicator of a tighter association of the native species with specific vegetation types. Overall spore DRs were not significantly different between the two *Heterobasidion* species in the Sabaudia forest at the time of sampling, hence, differences in aggregation and distribution of spores are not likely to be determined by different sporulation levels between the two species. Moreover, all of the five available vegetation types in the Sabaudia forest were sampled with an adequate intensity (either vegetation type was present in approximately 50% of sampling points or over). It should be noted that spores of *Heterobasidion* spp. are viable almost exclusively at a short distance from a fruit body (Stenlid 1994, Möykkynen et al. 1997, Gonthier et al. 2001). Fruit bodies are only produced on or adjacent to infected trees, and thus the presence and the density of spores in the air can be used as proxies to quantify distribution and abundance of these fungi on the forest floor (Gonthier et al. 2001, 2007).

Statistical tests identified a positive association between pines and *H. annosum,* and a negative association between the same fungal species and deciduous oaks. No association was found between the invasive *H. irregulare* and any of the vegetation types, suggesting this exotic fungus may be more of a generalist in terms of the substrates it can colonize.

Buffer analysis and odds ratios statistics demonstrated that *H. annosum* was positively associated with *Pinus pinea* and negatively associated with Mediterranean deciduous oak forests. These results were validated by the findings that *H. annosum* was present in the mixed oak-Italian stone pine stand at Mesola, while it was virtually absent in the pure oak forest in the same area. Additionally, the binary logistic regression model performed on data from Sabaudia showed that the probability of finding *H. annosum* spores sharply decreased with distance and approached 0 at 500 m. *Heterobasidion irregulare* was present in more sampling points than *H. annosum*, but this species was not associated with any vegetation type. The contrasting distribution pattern between the two species is not likely to be determined by differences in sporulation, survival, or dispersal rates, given that the two species are regarded as being extremely similar in all of the above aspects (Redfern and Stenlid 1998), and that the overall inoculum density was not statistically different between the two at Sabaudia. We infer the patterns of vegetation type association and pathogen species are directly determined by the two species ability to colonize and sporulate in different habitats. Although associations with five vegetation types were analyzed in this study, pine stands and Mediterranean deciduous oak forests are among the two most representative forest types in central Italy and in most of Europe. Based on our analysis of DRs distribution at Sabaudia, we conclude that the native species requires the presence of pines for its successful establishment and transmission, while the exotic species fares equally well both in pine and oak stands. This was regarded as highly improbable, given the presence of the North American species in absence of pines has never been mentioned in a body of literature comprising hundreds of papers (see literature in Filip and Morrison 1998, Otrosina and Garbelotto 2010, Worrall et al. 2010). However, our findings indicate the invasive fungus is present in pure oak stands as evidenced by the survey data from the pure oak forests in Anzio and Nettuno. Those results suggest *H. irregulare* is probably widespread in pure oak habitats, while *H. annosum* is absent. Several angiosperms, and in particular ericaceous hosts commonly intermixed with pines, have been occasionally reported as hosts for *H. irregulare* (Farr and Rossman 2012); however, this is the first report of a significant presence of this pathogen in forests exclusively composed of angiosperms. Given the significant distance between sampling points in the pure oak stands and rare isolated pines outside each stand, it is unlikely that *H. irregulare* in these stands resulted from spore drift from pine forests.

Although the description and quantification of the effects of colonization of *Heterobasidion* species on different vegetation types was not a goal of this study, mortality associated with the presence of either *Heterobasidion* species was evident only in pine stands, while oaks showed no symptoms of decline. Presence of *H. irregulare* in oak stands in the absence of symptoms may suggest the organism has a saprotrophic relationship with these angiosperms. All *Heterobasidion* species are known to alternate between pathogenic and saprotrophic phases (Asiegbu et al. 2005).

Although DRs of *H. irregulare* were twice as large in the presence (Sabaudia) rather than in the absence (Anzio and Nettuno) of pines, values from oak stands in Anzio, Nettuno and Sabaudia are well within the range of those reported for *H. irregulare* in coastal pine stands of central Italy (Gonthier et al. 2007, Garbelotto et al. 2010). The higher DRs in Sabaudia may be due to the unique characteristics that set apart this forest from all other study sites, namely its significantly larger size, its mesic rather than xeric conditions, and its cover including a significant component of both pine and oak trees. Further study is warranted to disentangle the role, if any, played by each one of the three conditions above and their effects on spore dispersal.

Given the random distribution pattern of *H. irregulare* and its lack of association with any the vegetation types at Sabaudia, it was not possible to calculate the probability of finding *H. irregulare* spores at variable distances from a source. However, spores of the two *Heterobasidion* species are identical in size, shape, and density (see references in Otrosina and Garbelotto 2010), and results of this and other studies suggesting most *H. annosum* spores fall within 500 m from a fruit body (Stenlid 1994, Möykkynen et al. 1997, Gonthier et al. 2001) are probably applicable to *H. irregulare*. The estimation of the dispersal potential and the identification of habitat type effects are important epidemiological parameters needed to model invasions by exotic pathogens (Heger and Trepl 2003, Parker and Gilbert 2004).

We do not yet know if the invasive species is replacing the native one, but another study on *H. irregulare* spread in this region shows that the invasive species is dominant in most sites within the invasion zone (Gonthier et al. 2007). Inoculation experiments have indicated that both species are equally aggressive on some European and North American pines, suggesting that the dominance of the invasive species is not determined by its greater pathogenicity on naïve hosts (Garbelotto et al. 2010). However, sporulation by the invasive species has been found to be significantly higher than that of its native congener during the summer, thus increasing the transmission potential of the exotic pathogen (Garbelotto et al. 2010). Sporulation is essential for the spread of all *Heterobasidion* spp., and is the end result of a process that includes traits such as the ability to infect and colonize a substrate, as well as to produce the fruit bodies that will generate the airborne meiospores (Korhonen and Stenlid 1998).

Based on the patterns of spore catches here described, the invasive species is thus capable of colonizing Mediterranean deciduous oak stands that are apparently unavailable to its congener and this advantageous trait may further explain the successful spread of the exotic fungus in the highly fragmented landscapes around Rome. Although the discovery of spores of *H. irregulare* in oak stands was unexpected based on the published literature, only a comparison between native *H. irregulare* populations in North America and introduced *H. irregulare* populations in Italy may further help to understand whether the ability to colonize oak woodlands is a trait that predates its introduction in Europe or whether it may have emerged after that event.

Whether recently acquired or pre-existing, the ability to colonize oak woodlands by *H. irregulare* is an unexpected novel result of this study that increases our understanding of the invasion biology of this exotic pathogen and will be pivotal in the formulation of accurate predictive models of its spread in Italy and Europe, where it may represent a significant threat to the health of native conifer forests. The discovery that oak forests are involved as an additional habitat for *H. irregulare*, points to the need for a comprehensive control approach encompassing both coniferous and broadleaf forests. Besides host removal, it is well known that stump treatment will greatly diminish the establishment of this pathogen in new stands (Nicolotti and Gonthier 2005). It is also known that infection is greatly enhanced by mechanical wounding of standing trees (Stenlid and Redfern 1998). Based on our results, and considering that complete removal of habitats is often not possible or desirable, we strongly recommend that at least stump treatment and a prescription to minimize wounding may be included in the management plans of all pine stands within 500 m from infested oak or pine forests.

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Tables

TABLE 1. Summary of the characteristics of the study sites, including vegetation types present and number of sampling points for *Heterobasidion* spp. spore trapping.

Study sites	Location	Area (ha)	Vegetation types	Sampling points (n.)
Sabaudia forest	Lat: 41°20'30" Long: 13°02'42"	3030	Mediterranean maquis Mediterranean evergreen oak forests Mediterranean deciduous oak forests Italian stone pine stands Anthropic or agricultural areas	44
Anzio	Lat: 41°30'59" Long: 12°36'37"	16	Mediterranean deciduous oak forest	13
Nettuno 1	Lat: 41°28'44" Long: 12°42'07"	13	Mediterranean deciduous oak forest	5
Nettuno 2	Lat: 41°28'12" Long: 12°43'23"	282	Mediterranean deciduous oak forest	7
Mesola 1	Lat: 44°54'28" Long: 12°13'41"	65	Mixed oak-Italian stone pine forest	6

Mesola 2	Lat: 44°50'44" Long: 12°15'09"	1063	Mediterranean deciduous oak forest	37

TABLE 2. Description and relative surface covered by vegetation types in the Sabaudia forest of the Circeo National Park.

Vegetation type and its relative abundance	Main species and description
Mediterranean maquis (7%)	Xeric angiosperm plant communities often dominated by either *Ampelodesmus mauritanicus*, *Cistus monspeliensis* or *Erica arborea* and characterized by the presence of one or more of the following species: *Arbutus unedo, Brachypodium ramosum, Elaeoselinum asclepium, Erica multiflora, Rosmarinus officinalis, Ligustrum vulgare, Myrtus communis, Phillyrea latifolia*, *Pistacia lentiscus, Rhamnus alaternus.*
Mediterranean evergreen oak forests (4%)	Forest stands with a variable dominance of *Quercus ilex* and *Quercus suber* associated with one or more of the following species: *Carpinus betulus, Erica arborea, Fraxinus excelsior, Fraxinus ornus, Quercus cerris, Quercus frainetto, Quercus pubescens, Quercus robur, Phillyrea latifolia*.
Mediterranean deciduous oak forests (74%)	Deciduous oak forests dominated by *Quercus cerris* and *Quercus frainetto* with the occasional presence of one or more of the following species: *Quercus robur*, *Fraxinus ornus, Carpinus betulus, Quercus pubescens*.

Italian stone pine stands (12%)	Forest stands dominated by even-aged *Pinus pinea* at times associated with a range of broadleaf species.
Anthropic or agricultural areas (3%)	Agricultural lands or areas with no vegetation.

TABLE 3. Association between vegetation types, *H. annosum* and *H. irregulare* expressed through odds ratios and tested with the odds ratios 95% confidence interval. A significant odds ratio > 1 indicates a positive association, while an odds ratio comprised between 0 and 1 indicates a negative association.

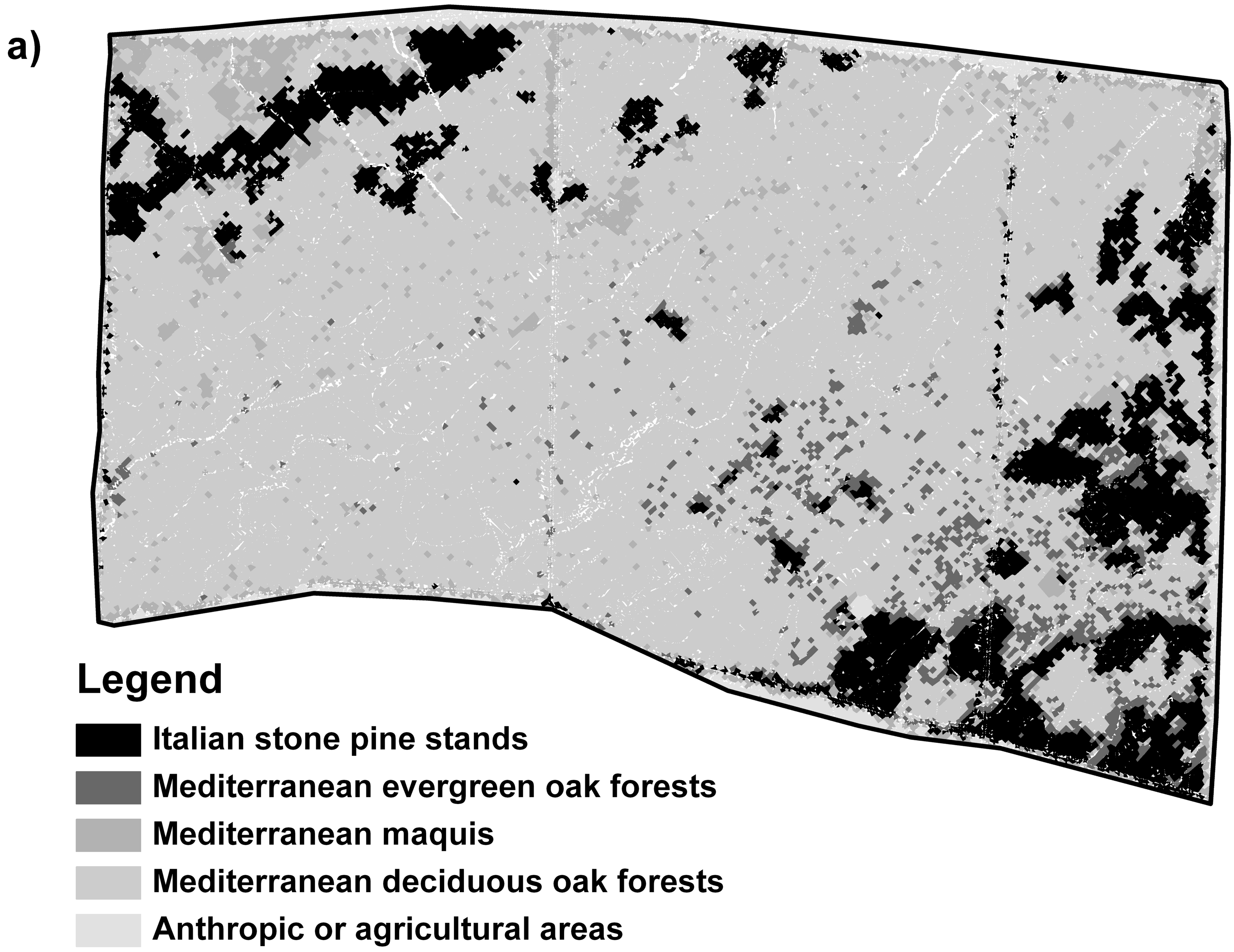
Vegetation type	*Heterobasidion annosum* Odds ratio and 95% confidence interval (lower bound; upper bound)	*Heterobasidion irregulare* Odds ratio and 95% confidence interval (lower bound; upper bound)
Mediterranean maquis	0.825 (0.159; 4.294)	1.800 (0.339; 9.551)
Mediterranean evergreen oak forests	1.636 (0.462; 5.795)	0.469 (0.122; 1.799)
Mediterranean deciduous oak forests	0.145* (0.250; 0.846)	0.667 (0.115; 3.861)
Italian stone pine stands	7.704* (1.723; 34.444)	1.556 (0.414; 5.838)
Anthropic or agricultural areas	1.636 (0.462; 5.795)	0.743 (0.199; 2.779)

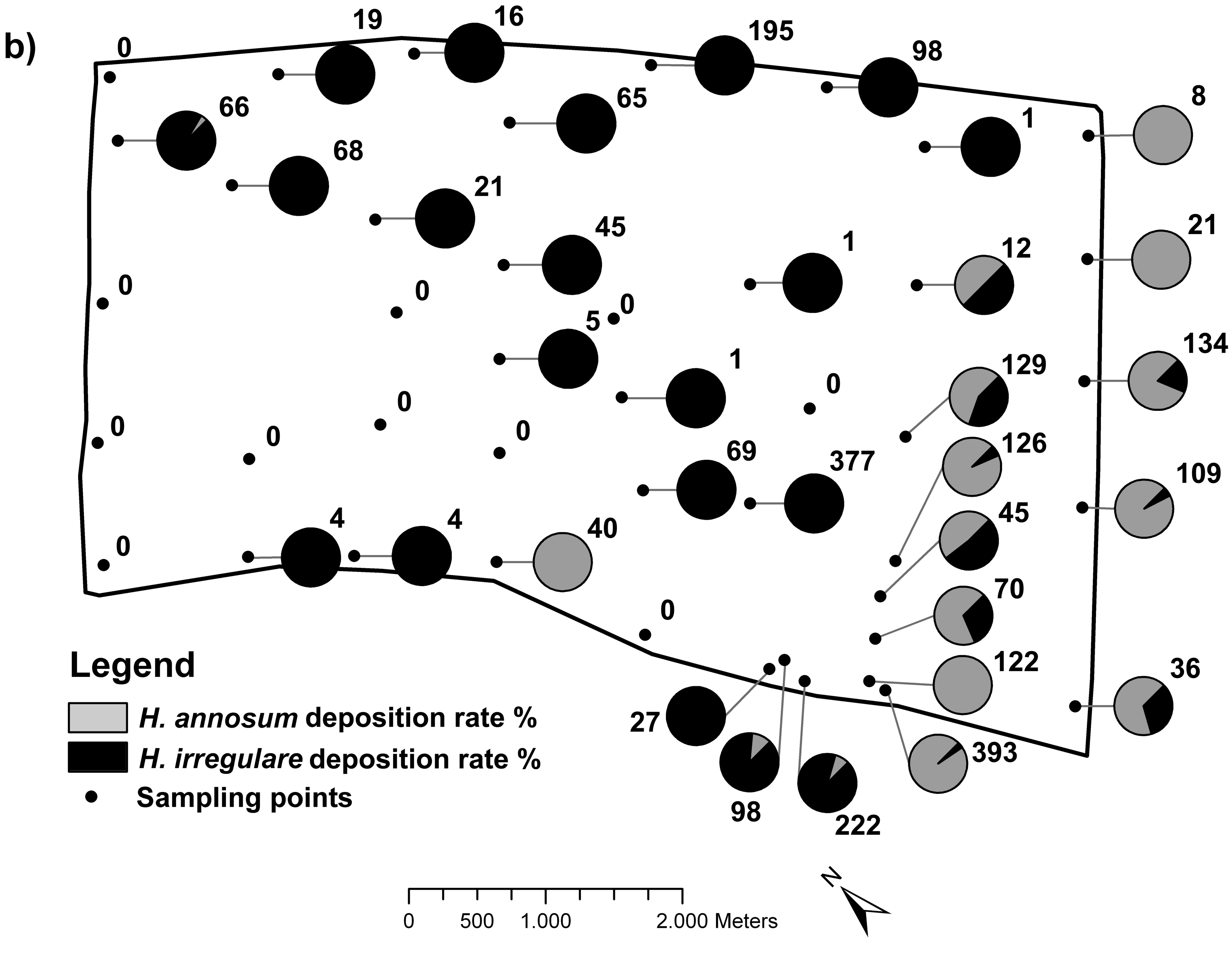
* Significant odds ratios.

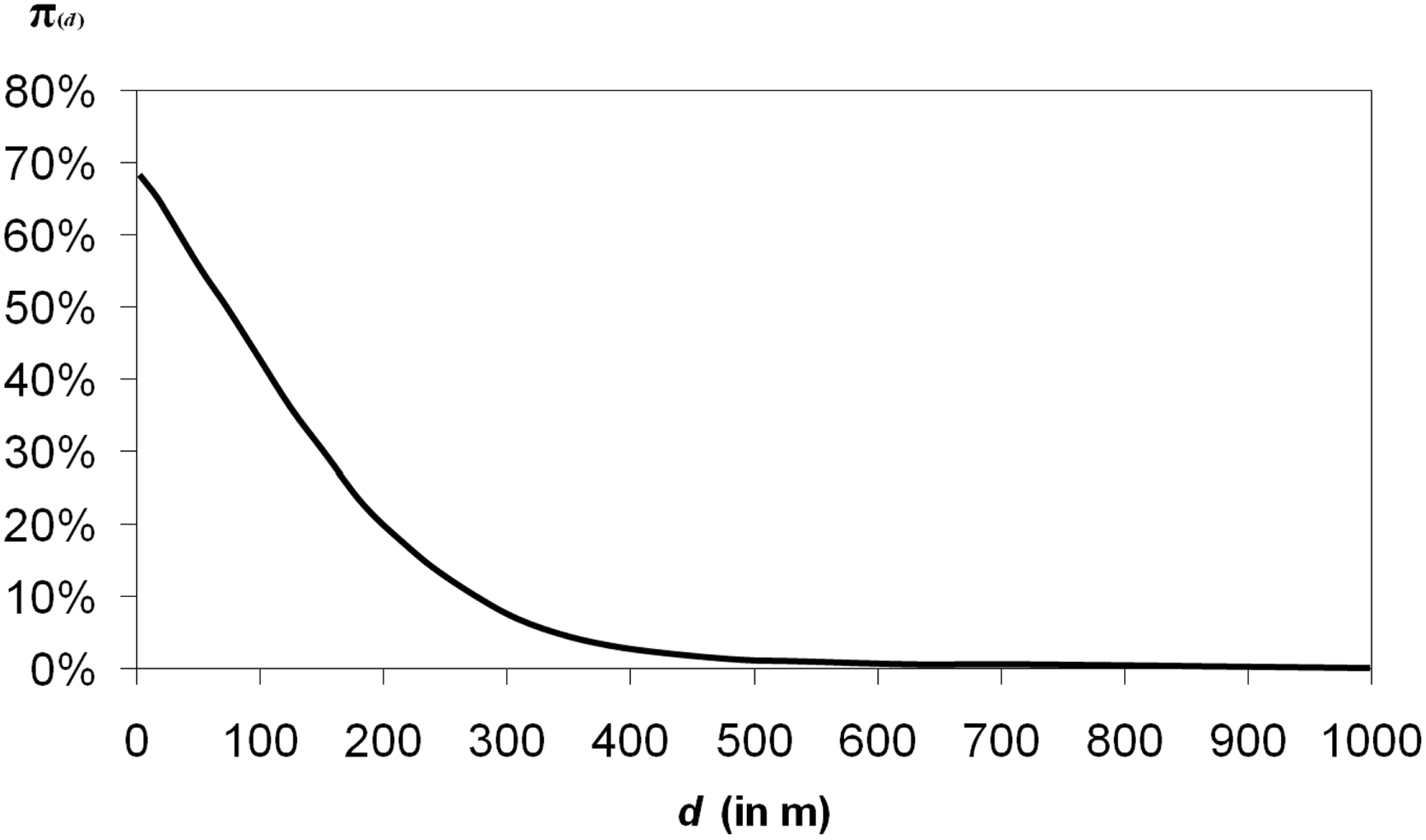
Figure legends

FIG. 1. Distribution of the five vegetation types in the study area of Sabaudia (a), and distribution of the 44 spore trapping sampling points (b). Pie charts refer to the percentage of spore colonies of *H. annosum* and *H. irregulare*. Numbers represent the deposition rates (DRs) of both species expressed as spores m^{-2} h^{-1}.

FIG. 2. The logistic regression shows a negative association between the probability of finding *Heterobasidion annosum* spores ($\pi_{(d)}$) and the distance (d) from the nearest Italian stone pine vegetation type.


a)
Legend
Italian stone pine stands
Mediterranean evergreen oak forests
Mediterranean maquis
Mediterranean deciduous oak forests
Anthropic or agricultural areas


b)
0
19
16
195
98
66
65
68
1
8
21
45
21
0
0
1
12
5
0
1
0
129
134
0
0
0
0
126
69
377
109
0
4
4
40
45
70
0
122
36
27
393
98
222
Legend
H. annosum deposition rate %
H. irregulare deposition rate %
Sampling points
0
500
1.000
2.000 Meters
N


$\pi_{(d)}$
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
100
200
300
400
500
600
700
800
900
1000
d (in m)