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Invasion of European pine stands by a North American forest pathogen and its hybridization with a native interfertile taxon

P. GONTHIER,* G. NICOLOTTI,* R. LINZER,† F. GUGLIELMO* and M. GARBELOTTO†
*Department of Exploitation and Protection of the Agricultural and Forestry Resources — Plant Pathology, University of Torino, Via L. da Vinci 44, I-10095 Grugliasco, Italy, †Department of Environmental Science, Policy and Management — Ecosystem Sciences Division, University of California at Berkeley, 151 Hilgard Hall, Berkeley, CA 94720, USA

Abstract

It was recently reported that North American (NA) individuals of the forest pathogen *Heterobasidion annosum* were found in a single pine stand near Rome, in association with the movement of US troops during World War II. Here, we report on some aspects of the invasion biology of this pathogen in Italian coastal pinewoods, and on its interaction with native (EU) *Heterobasidion* populations. Spores of *Heterobasidion* were sampled using woody traps in pine stands along 280 km of coast around Rome. DNA of single-spore colonies was characterized by two sets of nuclear and one set of mitochondrial taxon-specific polymerase chain reaction primers. NA spores were found not only in a single site, but in many locations over a wide geographic area. Invasion occurred at an estimated rate of 1.3 km/year through invasion corridors provided by single trees, and not necessarily by sizable patches of forests. Within the 100-km long range of expansion, the NA taxon was dominant in all pure pine stands. Because abundance of the EU taxon is low and identical among stands within and outside the area invaded by NA individuals, we infer that the exotic population has invaded habitats mostly unoccupied by the native species. Discrepancy between a mitochondrial and a nuclear marker occurred in 3.8% of spores from one site, a mixed oak-pine forest where both taxa were equally represented. Combined phylogenetic analyses on nuclear and mitochondrial loci confirmed these isolates were recombinant. The finding of hybrids indicates that genetic interaction between NA and EU *Heterobasidion* taxa is occurring as a result of their current sympatry.

Keywords: dispersal, exotic pathogen, gene introgression, *Heterobasidion*, replacement, root rot

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Introduction

Because of intensified globalization, biological invasions are on the rise. Exotic organisms are currently regarded as one of the main threats to survival of native ecosystems, they reduce biodiversity and can have adverse effects on human well-being (Lövei 1997; Pimentel 2002). After introduction, novel (non-native) organisms may become established and spread as invasive or noninvasive colonizers, depending on their impact on the new environment (Davis & Thompson 2000).

Correspondence: Matteo Garbelotto, Fax: +1 510 643-5098; E-mail: matteo@nature.berkeley.edu

The release from natural enemies or competitors is a frequent explanation for the success of introduced species (Torchin *et al*. 2003). One possible scenario of successful colonization is that of introduced species spreading undisturbed by exploiting niches unoccupied by native organisms. Often, however, the novel organism directly interacts with pre-existing native ones. It has been suggested that the presence of pathogens, predators, competitive species, and, in more general terms, of high levels of biodiversity, may be key in making native ecosystems more resilient to colonization (Kennedy *et al*. 2002).

The closer the ecological requirements of invasive and endemic individuals, the larger will be the impact of the interaction between invader and resident individuals on

the final outcome of the colonization process. In the presence of a native organism genetically or ecologically similar to the colonizer, possible outcomes may include (i) niche partitioning, with the colonizer exploiting a niche only marginally occupied by the indigenous organism; (ii) direct competition between the colonizer and native organisms potentially leading to significant hybridization between the two taxa; (iii) replacement of the native species by the colonizer, but transient or sporadic hybridization may cause varying levels of gene introgression between the two interacting taxa.

Replacement of native species by exotic, invasive species has been well documented for plants and animals (Abbott *et al*. 2003; Thomson 2004). Only one convincing example exists for microbes: the cultivated mushroom *Agaricus bisporus*, whose European type has been reported to outcompete the native type in California (Kerrigan *et al*. 1998). However, because of the artificial multiplication and human-aided transport of this cultivated mushroom within the colonized region, the potential for spread of this species under natural conditions is unknown.

Interaction between exotic and native species may also involve gene flow between them when these are interfertile. This phenomenon has been shown in plants and animals (Perry *et al*. 2002; Abbott *et al*. 2003). As a result of this interaction, while native species may be displaced by introduced ones, some genes of native taxa may persist as introgressed alleles in the genomes of the colonizing organisms. For instance, in America, Africanized bees have replaced the common European honeybees, but, in the process, they have acquired some alleles of the species they have displaced (Clarke *et al*. 2002). The only significant example of recent hybridization for the fungi is that of *Ophiostoma ulmi* with the related but more aggressive *Ophiostoma novo-ulmi*. It has been hypothesized that gene introgression between them may have been advantageous for the latter pathogenic agent (Brasier 2001), which has caused a second and more serious epidemic of Dutch elm disease in Europe and North America. The *Ophiostoma* example regards gene flow between two exotic fungal species, hence there is no information regarding either the genetic interaction or gene flow between a native and an exotic fungal species. Furthermore, because it has been suggested that hybridization or recombination between different plant pathogens may result in expanded host ranges (Brasier 2000; Slippers *et al*. 2005), novel interactions between fungal species due to recently attained sympatry may have serious effects on invaded plant ecosystems.

There are a number of cases of known introductions of fungi and fungus-like organisms (Wingfield *et al*. 2001; Diez 2005; Ivors *et al*. 2006). In the numerous cases of known introduced plant pathogens, considerable attention has been given to the effects on host populations and on the associated plant communities. On the other hand, there is no information on the effects of interactions between exotic pathogenic fungi and their native counterparts.

Heterobasidion annosum (Fr.) Bref. *sensu lato* (s.l.) is a species complex comprising five allopatrically and sympatrically differentiated intersterility groups (ISGs), all responsible for root and/or butt rot of forest trees. Three *H. annosum* ISGs, recently named *H. abietinum* Niemelä & Korhonen, *H. parviporum* Niemelä & Korhonen, and *H. annosum sensu stricto* (s.s.), are present in Europe, while two are reported in North America. Although the two North American taxa represent clearly distinct and distantly related clades within the complex (Otrosina *et al*. 1993; Harrington *et al*. 1998; Warner *et al*. 2005), they are still awaiting formal species description, and are currently referred to as ISG P (Am-P) and ISG S (Am-S). Each taxon within the *H. annosum* species complex is characterized by distinct host specialization: while *H. abietinum*, *H. parviporum*, and Am-S are mostly reported on spruce or fir trees (*Picea* spp. or *Abies* spp.), *H. annosum* s.s. and Am-P are typically associated with root rot and mortality of trees in the genus *Pinus* (Korhonen *et al*. 1998). Infection of new hosts and infestation of new forest stands occur by means of airborne haploid meiospores, generated by inconspicuous fruiting bodies normally found under the litter. These fungi are also capable of infecting adjacent trees by colonizing grafted roots that act as bridges of contagion among individual trees in the same stand (Redfern & Stenlid 1998; Stenlid & Redfern 1998).

In 2002, it was discovered that individuals of the North American *H. annosum* ISG P were present in a single Italian stone pine stand (*Pinus pinea* L.) in the forest of Castelporziano, near Rome (Gonthier *et al*. 2004). In the above paper, the introduction was linked to the movement of the US troops in 1944 during World War II (WWII).

Although other introduced fungal diseases have been extensively studied (chestnut blight, white pine blister rust, and Dutch elm disease, just to name a few), the one described in this study presents many unique traits: (i) the pathogen has a mixed infection biology: primary infection (short to long range) via airborne meiospores and secondary infection (short range) through pathogen growth from one tree to an adjacent one via root contacts; (ii) the time of introduction is almost certain; and (iii) so is the general area where the introduction occurred. Unlike all other currently studied exotic plant pathogens, the native Italian *H. annosum* s.s. is interfertile with the introduced organism (Stenlid & Karlsson 1991). Although morphologically indistinguishable, the North American *H. annosum* ISG P and the European *H. annosum* s.s. are likely to have been in allopatry for millions of years (Otrosina *et al*. 1993), resulting in two distinct phlylogenetic groups easily differentiated, based on a range of molecular markers (Otrosina *et al*. 1993; Harrington *et al*. 1998; Warner *et al*. 2005).

In this study, we investigate the invasion biology of this introduced North American pathogen in Italian coastal

pine woodlands on the central Tyrrhenian coast. We describe patterns of invasion, including patchiness or continuity of the distribution, geographic range of invasion, average rate of spread, correlation of invasion success with habitat type, estimated damage caused by the introduced pathogen, and presence and distribution of hybrids. Our main goal was to produce data that would allow us to discriminate among the following scenarios:

1 Invasion is not occurring, and the presence of the exotic taxon is limited to the area where it was first reported.
2 The exotic and the native taxa are currently coexisting in sympatry.
3 The exotic taxon is invading niches unoccupied by the native species.
4 The exotic taxon is replacing the native one.
5 Hybridization is occurring and resulting in
   a. a persistent hybrid swarm of progeny clearly distinguishable from both parents, or
   b. transient hybrids, potentially present in the zone of contact between the two taxa, resulting in gene introgression between them.

Materials and methods

Study area and sampling

Understanding patterns of invasion requires knowledge of the precise distribution of individuals of the exotic and the native *Heterobasidion* taxa. Large-scale surveys of root-rot fungi are difficult because they live underground, their hosts frequently do not show any evident symptoms, and their distribution is spatially aggregated in clusters (Anderson & Kohn 1985). However, it should be noted that *Heterobasidion* spp., besides colonizing underground roots, also produces haploid airborne meiospores responsible for infection of new hosts and infestations of new areas (Korhonen & Stenlid 1998). It has been shown that *Heterobasidion* spores germinate readily and form colonies on freshly cut wood, such as the stump surface of recently felled trees or wooden discs (Rishbeth 1959).

The wood-disc exposure method (Rishbeth 1959), as modified by Gonthier *et al*. (2001), was used to sample airborne spores in stands and patches of pine between southern Tuscany and northern Campania, along 280 km of coast approximately centred around Castelporziano. The size of stands ranged from 0.3 to 420 ha. With the exception of two *Pinus halepensis* Miller stands, all study sites were either pure *Pinus pinea* stands or mixed oak-*P. pinea* stands (Table 1).

Forests were sampled in one of two periods, either at the end of November 2005 or at the end of March 2006. In order to assess whether November and March data were comparable, we sampled three points in both seasons. A total of 104 sampling points were selected at regular intervals along transects dissecting the entire length of each forest stand. Intervals between sampling points along transects were either 350 m or 700 m, in smaller and larger stands, respectively, to allow for a complete coverage of each stand. Four Norway spruce (*Picea abies* (L.) Karst.) wood discs were exposed, for 24 h at each sampling point. Wood of Norway spruce, like that of other tree species, 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 all three European *Heterobasidion* species (Gonthier *et al*. 2001; Gonthier *et al*. 2005). The four discs were placed 5 m far from the centre of each sampling point along the four cardinal directions. If sampling occurred along the edge of a stand, each sampling point included only two wood discs. With the exception of Portella woods in Monte San Biagio, which comprised only one sampling point, the number of sampling points per forest stand ranged from 3 to 16.

Discs were incubated at room temperature as previously described (Gonthier *et al*. 2001), and inspected four times on a weekly basis under a dissecting microscope for the presence of colonies of *Heterobasidion*. Colonies were counted, and, depending on total number of observed colonies, 1–7 random isolations per disc were performed by transferring infected wood pieces onto 5-cm Petri dishes filled with a selective growth medium (Kuhlman & Hendrix 1962). All isolates were subsequently grown at room temperature on 5-cm Petri dishes filled with malt extract agar (20 g malt extract, 20 g glucose, 2 g peptone, 20 g agar, 1 L distilled water).

Taxon-specific polymerase chain reaction characterization of single-spore colonies

DNA from fungal colonies was extracted by a 'hyphal tipping' method (Schweigkofler *et al*. 2004), modified as follows. Fungal mycelium was collected with the tip of a micropipette and suspended in 100 μL of distilled water, frozen on dry ice for 3 min, thawed at 75 °C, vortexed for 1 min, and finally centrifuged for 5 min at 19 000 ***g*** Freezing and thawing were repeated three times, with the last thaw extended to 15 min. Samples were then centrifuged for 5 min at 19 000 ***g*** and the supernatant was used as template for polymerase chain reactions (PCR).

DNA was characterized by three sets of PCR primers (Table 2) specifically designed to target one nuclear locus (two sets) and one mitochondrial locus in *Heterobasidion*. Primers differentiate between North American (NA) and European (EU) isolates, belonging to *Heterobasidion annosum* ISG Am-P and *H. annosum* s.s., respectively, as follows: Mito 5, Mito 7 and Mito 8 amplify a 121-bp amplicon in the mitochondrial ribosomal operon for the NA isolates and one of 158 bp for EU isolates. EFaNAPFor and EFaEuPFor

Table 1 Forest locations and main stand characteristics

Code and sample locality	Lat., long. coordinates	Distance from Castelporziano (km)	Approx. forest size (ha)	No. of sampling points	Main species	Other genera	Attributes
1. Maremma Regional Park; Marina di Alberese, Alberese (GR)	42°38′58.30″N, 11°02′08.65″E	154 NW	300.0	3	*Pinus pinea* *Pinus pinaster*	*Juniperus*, *Mirtus*, *Pistacia*	naturalized, uneven-aged forest with Mediterranean undergrowth
2. Feniglia pinewood; Porto Ercole/Ansedonia (GR)	42°24′54.10″N, 11°12′54.66″E	125 NW	420.0	9	*P. pinea*	*Erica*, *Juniperus*, *Pistacia*	even-aged forest with Mediterranean undergrowth
3. Marina di Montalto urban pinewood; Montalto di Castro (VT)	42°19′45.68″N, 11°34′41.23″E	100 NW	16.0	3	*P. pinea*	—	urban park
4. Riva di Tarquinia pinewood plantation; Tarquinia (VT)	42°17′30.63″N, 11°38′42.69″E	90 NW	8.4	3	*P. pinea*	—	artificial plantation
5. San Agostino coastal pinewoods; San Agostino (VT)	42°08′53.49″N, 11°44′44.18″E	70 NW	15.0	5	*P. pinea*	—	artificial plantation
6. Fregene Monumental Pinewood; Fiumicino (RM)	41°51′25.40″N, 12°11′52.79″E	24 NW	18.7	6	*P. pinea* *Quercus ilex*	—	urban park
7. Fregene pine plantation; Fiumicino (RM)	41°50′27.74″N, 12°12′56.26″E	20 NW	3.0	3	*Pinus halepensis*	—	artificial plantation
8. Coccia di Morto Estate; Fiumicino (RM)	41°48′00.50″N, 12°13′40.44″E	17 NW	33.0	6	*P. pinea*	*Cistus*, *Mirtus*, *Phillyrea*, *Pistacia*	even-aged forest with Mediterranean undergrowth
9. Castelfusano Pinewood Urban Park; Rome (RM)	41°43′22.81″N, 12°18′34.10″E	7 NW	400.0	13	*P. pinea* *Q. ilex* *Quercus frainetto*	*Erica*, *Rhamnus*	even-aged forest with Mediterranean undergrowth
10. Edge of the Castelporizano Estate; Rome (RM)	41°42′07.58″N, 12°21′00.87″E	—	—	16	—	—	Mediterranean vegetation
11. Gallinara pine plantation; Anzio (RM)	41°32′00.89″N, 12°33′32.35″E	24 SE	124.0	11	*P. pinea*	—	artificial plantation
12. La Campana pine plantation; Nettuno (RM)	41°30′51.80″N, 12°40′19.95″E	32 SE	54.0	6	*P. pinea*	—	artificial plantation
13. Circeo National Park/the forest of Sabaudia; Sabaudia (LT)	41°19′32.96″N, 13°01′56.21″E	68 SE	183.0	9	*P. pinea*, *Quercus robur*, *Quercus cerris*, *Q. ilex*, *Q. frainetto*, *Quercus suber*	*Carpinus*, *Fraxinus*, *Hedera*	naturalized, even-aged, forest with rich and abundant undergrowth
14. Viale dei Pini pinewood; San Felice Circeo (LT)	41°15′06.68″N, 13°03′50.80″E	75 SE	7.0	3	*P. pinea*, *Q. ilex*, *Q. suber*	*Hedera*, *Carpinus*	naturalized, even-aged, forest with rich and abundant undergrowth
15. Via Terracina urban pinewood; San Felice Circeo (LT)	41°15′06.68″N, 13°20′13.17″E	79 SE	0.3	3	*P. pinea*	—	urban park
16. Portella woods; Monte San Biagio (LT)	41°20′37.62″N, 13°20′13.17″E	88 SE	15.9	1	*Cupressus* spp., *P. halepensis*, *Pinus* sp.	—	even-aged forest with Mediterranean undergrowth
17. Baia Domizia pinewood; Sessa Arunca (CE)	41°13′15.63″N, 13°43′59.60″E	127 SE	54.0	4	*P. pinea*, *Q. ilex*	*Erica*, *Juniperus*, *Eucalyptus*	even-aged forest with Mediterranean undergrowth

Table 2 Sequences of DNA oligonucleotide primers used in this study

Primer	Sequence (5′–3′)	Reference
Mito 5	TAAGACCGCTATA(T/A)ACCAGAC	Garbelotto *et al*. 1998
Mito 7	GCCAATTTATTTTGCTACC	Gonthier *et al*. 2001
Mito 8	GCGGTGTAATAAAATCGG	Gonthier *et al*. 2001
EFaHaFor	CTATGTCGCGGTACAGCTTG	This study
EFaHaRev	GCGAGGA(T/C)AAGAAGTAATCAGCA	This study
EFaNAPFor	GTACATGGTCACTGTACGTAGATGC	This study
EFaEuPFor	ATGGTCACTGTACGTAGATCATGC	This study
ATP6-2	TTATTCTAN(T/A)GCATCTTTAAT(G/A)TA	Kretzer & Bruns 1999
ATP6-3	TCTCCTTTAGAACAATTTGA	Kretzer & Bruns 1999
Elongation factor 1-α forward	TCAACGTGGTCGGTGAGCAGGTA	Johannesson & Stenlid 2003
Elongation factor 1-α reverse	AAGTCACGATGTCCAGGAGCATC	Johannesson & Stenlid 2003
GPD 1 forward	AGCCTCTGCCCA(T/C)TTGAA(G/A)G	R. Linzer, unpublished
Glyceraldehyde 3-phosphate dehydrogenase reverse	(G/A)TANCCCA(T/C)TC(G/A)TT(G/A)TC(G/A)TACCA	Johannesson *et al*. 2000

were designed on the nuclear elongation factor 1-α to exclusively amplify either NA or EU isolates, when each of them is used in combination with the two universal primers EFaHaFor and EFaHaRev. The use of both EFa primer sets allowed us to confirm results obtained by each primer set.

PCR amplifications of the mitochondrial locus were conducted as described by Gonthier *et al*. (2001), while amplifications of the nuclear locus were performed in 25-μL reactions containing 2 μL of template DNA and 23 μL of reaction mix [final concentrations: 0.2 mM dNTPs, 0.5 μM of each primer, 1× Go*Taq* reaction buffer (1.5 mM $MgCl_2$), and 0.625 U Go*Taq* DNA polymerase (Promega)] on a Biometra thermocycler. The PCR amplification programme was as follows: 5 min at 94 °C, followed by 35 cycles of 45 s at 95 °C, 45 s at 62 °C, 45 s at 72 °C, and a final extension step at 72 °C for 7 min before storage at 4 °C. PCR products were electrophoresed in a 0.5× Tris-borate buffer (TBE) gel containing 1% standard agarose and 1% Metaphor agarose gels (FMC Bioproducts) at 4 V/cm for 2 h.

Sequencing to assess hybridization between EU and NA genotypes

When a discrepancy occurred between the mitochondrial and nuclear markers, for example a genotype was characterized as belonging to one taxon based on the mitochondrial marker and to the other taxon based on the nuclear markers, that isolate was assumed to be a recombinant of the two taxa. In order to confirm the recombinant nature of putative recombinant isolates, three loci were sequenced for a subset of genotypes inclusive of three putative recombinants, three putative EU genotypes and three putative introduced NA genotypes. The three selected loci were namely the nuclear elongation factor 1-α (EFA, about 400 bp), the nuclear glyceraldehyde 3-phosphate dehydrogenase (GPD, 593 bp), and the mitochondrial ATP synthase subunit 6 (ATP, 639 bp). Phylogenetic analyses (see below) were performed on sequences from the above genotypes and a representative subset (Warner *et al*. 2005) of the worldwide *H. annosum* sequence data set (Table 3).

DNA was extracted by 'hyphal tipping', as described above. Primers used for subsequent PCR amplification (Table 2) included the following: elongation factor 1-α forward and reverse (EFA), GPD 1 forward and glyceraldehyde 3-phosphate dehydrogenase reverse (GPD), ATP6-2 and ATP6-3 (ATP). DNA was sequenced using an ABI 3100 automated sequencer following standard protocols provided by the manufacturer (ABI). Sequences were edited and aligned in SEQUENCHER (Gene Codes Corp.), and where necessary, alignments were manually refined using SE-AL (Rambaut 1996). Insertions and deletions of 2 bp or greater in length were coded as a single character and weighted as equal to one base substitution. Maximum parsimony phylogenetic trees were calculated in PAUP, version 4.0b10, using a heuristic search with tree-bisection–reconnection branch swapping and 10 replicates with random addition (Swofford 2000). Internal branch support was assessed by 1000 replicate bootstrap analyses using the FASTSTEP algorithm with 10 random additions per replicate (Felsenstein 1985). Previous studies have shown that phylogenetic analyses at these three loci place NA and EU isolates in separate and well-supported clades (Gonthier *et al*. 2004; Warner *et al*. 2005). Incongruent placement of the same genotype in different clades, when comparing genealogies of the three different loci, would indicate recombination has occurred.

Data interpretation and analysis

Because sampling intensity differed among stands, we standardized spore deposition rate (DR) data by dividing the total number of colonies by the cumulative area of wood disc used in each forest. Taxon assignment was performed

Table 3 Geographic origin and GenBank Accession nos of genotypes selected for the phylogenetic analysis

ID collection	Geographic origin	*Heterobasidion* taxon†	GenBank Accession nos		
			ATP‡	EFA‡	GPD‡
791200-1-2	Finland	EU	DQ916071	DQ916077	DQ916093
810928-1-1	Finland	EU	DQ916071	DQ916078	DQ916094
34na	Fiumicino, Italy	EU	DQ916071	DQ916078	DQ916093
73sa	S. Felice Circeo, Italy	EU	DQ916071	DQ916078	DQ916095
108oa	Porto Ercole, Italy	EU	DQ916071	DQ916079	DQ916093
CP3	Castelporziano, Italy	NA	DQ916072	DQ916083	DQ916098
CP9	Castelporziano, Italy	NA	DQ916072	DQ916084	DQ916098
CP15	Castelporziano, Italy	NA	DQ916072	DQ916085	DQ916098
98006	Cushing, Québec, Canada	NA	DQ916074	DQ916086	DQ916103
98012	Forêt Larose, Ontario, Canada	NA	DQ916072	DQ916086	DQ916104
1A	Nolan Hess, LA, US	NA	DQ916073	DQ916086	DQ916102
365	Savannah River, SC, US	NA	DQ916072	DQ916088	DQ916103
CL-3	Clear Lake, MO, US	NA	DQ916072	DQ916092	DQ916099
CL-5	Clear Lake, MO, US	NA	DQ916072	DQ916086	DQ916100
Conk1	De Soto National Forest, MS, US	NA	DQ916072	DQ916087	DQ916101
BBh	WA, US	NA	DQ916075	DQ916089	DQ916103
c6	Cleveland National Forest, CA, US	NA	DQ916075	DQ916089	DQ916103
305–4	Lassen National Forest, CA, US	NA	DQ916075	DQ916090	DQ916102
340–3	Modoc National Forest, OR, US	NA	DQ916075	DQ916089	DQ916102
47ec	Sabaudia, Italy	putative hybrid	DQ916071	DQ916080	DQ916097
47ob	Sabaudia, Italy	putative hybrid	DQ916072	DQ916082	DQ916098
49oe	Sabaudia, Italy	putative hybrid	DQ916071	DQ916081	DQ916096
26719	American Type Culture Collection	*Heterobasidion insulare*	DQ916076	DQ916091	DQ916105

†As determined by taxon-specific PCR; EU, European; NA, North American; ‡ATP, mitochondrial ATP synthase subunit 6; EFA, portion of the nuclear elongation factor 1-α; GPD, portion of the nuclear glyceraldehydes 3-phosphate dehydrogenase gene.

on a subset of the total number of observed colonies as described above. The total relative abundance of spores of each taxon 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. For each study site, we also calculated the percentage of discs infected by each taxon.

At each study site, dominance of one taxon over the other was assessed by performing Student's *t*-tests, with DR data from each sampling point used as a replicate; points where no spores were trapped were excluded. In sites where spores were trapped at three or less sampling points, a χ^2 test was used instead of the *t*-test.

One-way analysis of variance (ANOVA) and the Tukey's honestly significant difference (HSD) for unequal N (Spjotvoll & Stoline 1973) were used to compare DR of different stands for each of the two taxa, using values from each sampling point as replicates. For the NA taxon, comparisons were made exclusively among stands located within its current range of expansion. The Portella woods in Monte San Biagio comprising less than three sampling points were excluded from the analysis.

In order to determine the potential impact of the exotic taxon on the native one, we compared spore loads of the native taxon in areas occupied by the introduced taxon with spore loads observed in areas outside the range of expansion of the exotic taxon. Such comparison was performed by ANOVA on DR values of the native species within and outside the observed area of expansion of the exotic taxon. All statistical analyses were performed using the software STATISTICA (StatSoft Inc.).

Results

Spores of *Heterobasidion* were found in 12 out of 17 forests surveyed in this study (71%). November and March DR values at points sampled twice were comparable (data not shown); hence, a single analysis was performed, including sites sampled in November and sites sampled in March.

A total of 1052 *Heterobasidion* single-spore colonies were counted, 582 were isolated and characterized by PCR amplification. Of these, 490 (84%) were NA, 86 (15%) EU, and 6 (1%) putative recombinant. Sixty-three out of 104 points (61%) were positive for *Heterobasidion*: in 50 (48%), only NA individuals were found, in 6 (6%) only EU individual were found, in 7 (7%) individuals of both taxa were found (Fig. 1). All putative recombinants came from the Circeo National Park, near the southernmost edge of

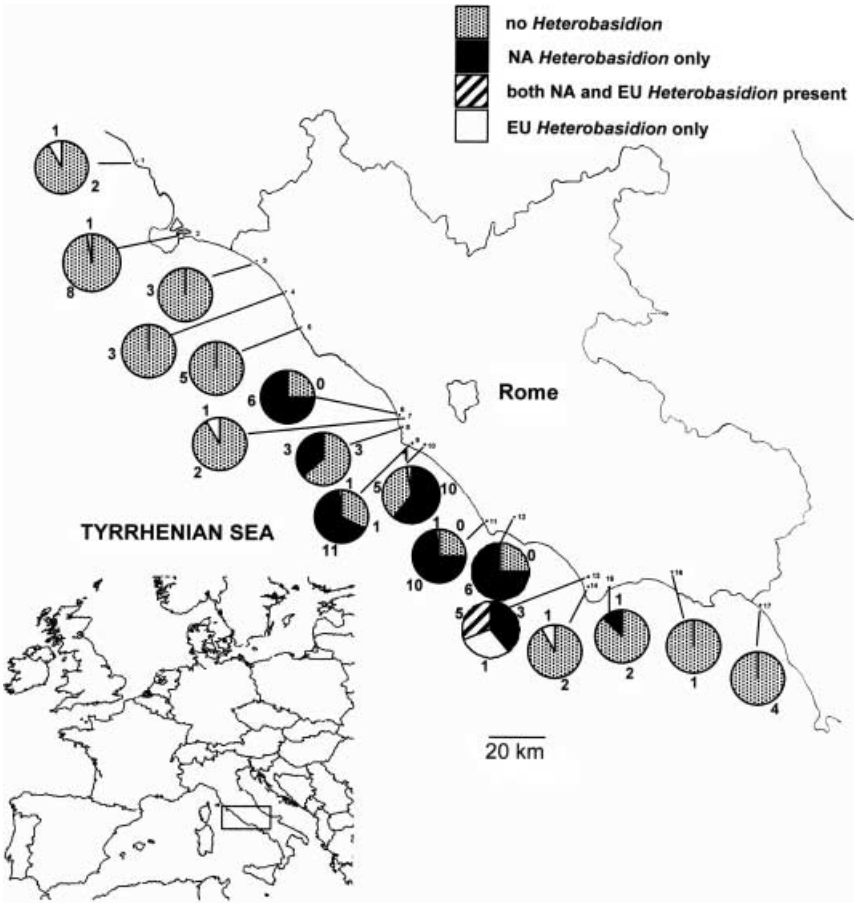

Fig. 1 Map showing the spatial distribution of localities where airspora of *Heterobasidion* spp. was sampled. Locality codes are the same as Table 1. Pie charts display the percentage of discs *Heterobasidion* free, or infected by the native (EU), the exotic (NA), or by both *Heterobasidion* taxa, as identified by taxon-specific PCR. Numbers in the pie charts refer to the number of sampling points associated to each category.

expansion of the NA population. Of these putative recombinant genotypes, five were typed as EU at the mitochondrial locus and as NA at the nuclear locus, while the opposite was true for one individual.

Spores of the exotic NA taxon were found in each Italian stone pine stand sampled between the Fregene Monumental Pinewood, about 24 km northwest of Castelporziano, and the small urban stand in San Felice Circeo, about 79 km southeast of Castelporziano. Within the 100-km range of expansion, the NA isolates were largely and significantly dominant, with two exceptions: (i) the small *Pinus halepensis* plantation in Fregene, where only EU spores were trapped; and (ii) the Circeo National Park, where EU and NA populations were equally represented (Table 4).

South of San Felice Circeo and north of Fregene, spores of *Heterobasidion* were either absent or present at low levels. All spores sampled in these areas were characterized as belonging to *Heterobasidion* EU populations. The abundance of spores of the European *Heterobasidion* differed significantly among stands ($F = 8.5660$, $P < 0.0000$). Tukey's test showed that only DR values from the Circeo National Park were significantly different from those obtained at all other study sites (Table 4). The abundance of spores of the North American taxon did not differ significantly among stands ($F = 1.8509$, $P = 0.0753$). DR of the European *Heterobasidion* species did not differ significantly between forests within and outside the range of expansion of the introduced species ($F = 2.4130$, $P = 0.1234$).

The putative characterization of genotypes as EU, NA, or recombinant based on congruence (or incongruence for recombinants) of PCR amplification using taxon-specific primers, was confirmed by combined phylogenetic analyses of three DNA sequences. Clade assignment of three putative recombinants selected for this analysis was incongruent between the mitochondrial ATP and the nuclear EFA analyses. In the EFA sequence analysis, isolates bearing EU mitochondria clustered together in a subclade within western North America and not within the European clade. Because divergence between eastern NA and western NA clades is limited to three SNPs, one in a noncoding portion of the gene, and two in the third position of a codon, we believe the affinity of EFA sequences from these isolates with western NA sequences may be due to rapid evolution upon introduction and convergence to a type similar to the western NA one. Finally, clade placement by GPD analysis, a nuclear locus not tested in the PCR screening, matched the mitochondrial type and was incongruent with EFA placement (Fig. 2).

Table 4 Deposition rates (DR), expressed as the mean number of spores per square metres per hour, and standard errors (SE), of the native (EU) and the exotic (NA) *Heterobasidion* taxa in 17 pine stands along 280 km of Tyrrhenian coast. The abundance of spores in the different stands was compared, for each taxon, by the ANOVA and the Tukey's honestly significant difference (HSD) for unequal N. Within each stand, the dominance of one taxon on the other was tested either by the *t*-test or by the χ^2 test (see text)

Forest stand	EU taxon mean DR	SE		NA taxon mean DR	SE		Test of dominance (t or χ^2 value, P value)
1. Maremma Regional Park	0.366	0.366	a*	0	0	—	—
2. Feniglia pinewood	0.122	0.122	a	0	0	—	—
3. Marina di Montalto urban pinewood	0	0	a	0	0	—	—
4. Riva di Tarquinia pinewood plantation	0	0	a	0	0	—	—
5. San Agostino coastal pinewoods	0	0	a	0	0	—	—
6. Fregene Monumental Pinewood	0	0	a	15.171	4.867	a*	$t = -3.1171$, $P = 0.0109$
7. Fregene pine plantation	0.731	0.731	a	0	0	a	—†
8. Coccia di Morto Estate	0	0	a	3.290	2.010	a	$\chi^2 = 6.6700$, $P = 0.0098$
9. Castelfusano Pinewood Urban Park	0.084	0.084	a	12.738	2.905	a	$t = -4.6601$, $P = 0.0001$
10. Castelporizano Estate (edge)	0.069	0.069	a	5.757	1.597	a	$t = -4.4987$, $P = 0.0002$
11. Gallinara pine plantation	0.100	0.100	a	13.060	5.119	a	$t = -2.5312$, $P = 0.0198$
12. La Campana pine plantation	0	0	a	14.439	3.068	a	$t = -4.7060$, $P = 0.0008$
13. Circeo National Park	21.357	5.822	b	42.125	22.053	a	$t = -0.9105$, $P = 0.3761$
14. Viale dei Pini pinewood, S. Felice C.	3.290	3.290	a	0	0	a	—†
15. Via Terracina pinewood, S. Felice C.	0	0	a	3.656	3.656	a	$\chi^2 = 6.6700$, $P = 0.0098$
16. Portella woods	0	—	—	0	—	—	—
17. Baia Domizia pinewood	0	0	a	0	0	—	—

*values with the same letter are not significantly different ($P > 0.05$); †not applicable; χ^2 assumptions not verified.

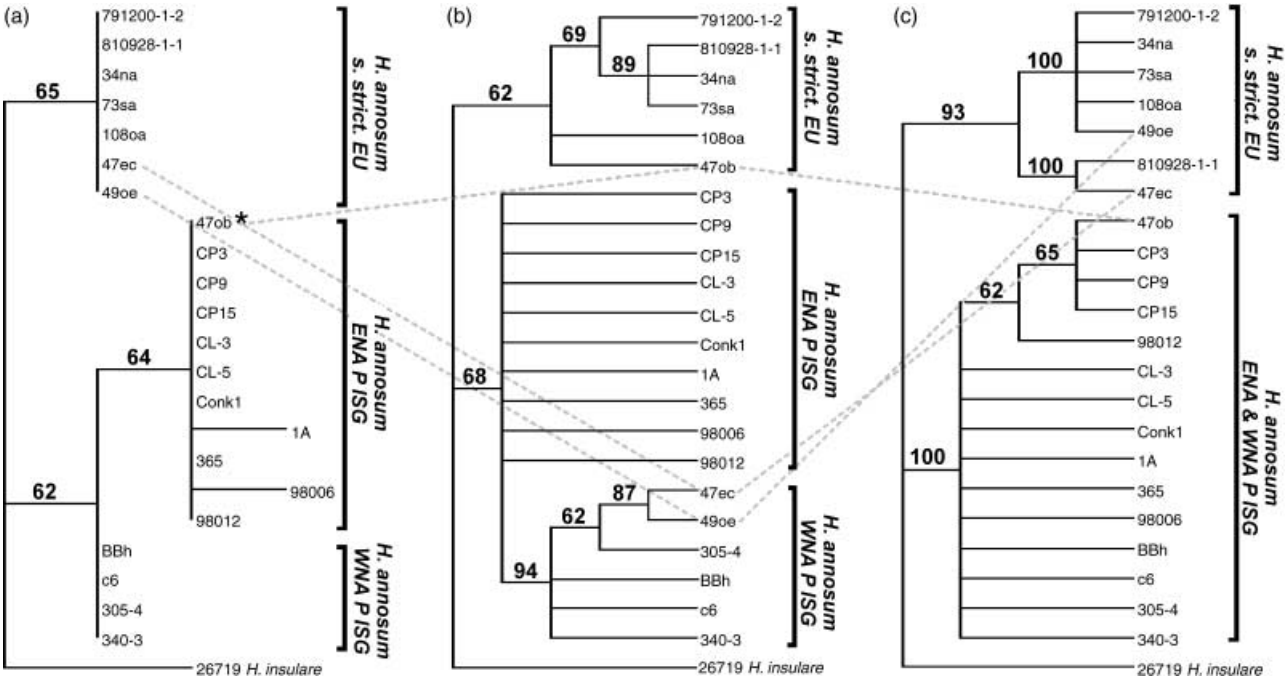

Fig. 2 Maximum parsimony phylogenetic analysis of representative worldwide *Heterobasidion annosum* genotypes, including putative North American (NA) × European (EU) recombinant individuals. (a) Single most-parsimonious tree for part of the mitochondrial ATP synthase subunit 6. (b) Strict consensus of 12 equally parsimonious trees for part of nuclear elongation factor 1-α. (c) Strict consensus of six equally parsimonious trees for part of nuclear glyceraldehyde 3-phosphate dehydrogenase gene. Bootstrap values above 50 percent from 1000 replicates shown above branches. Dashed lines connect the same putative recombinant genotype in different trees. WNA, western North America; ENA, eastern North America; EU, Europe. Outgroup for all analyses is *Heterobasidion insulare*. *Isolate 47ob was also of NA type in the mitochondrial ribosomal operon, as determined by taxon-specific PCR.

Discussion

The results of this study document the presence of spores of the exotic NA *Heterobasidion* taxon in a large area extending from Fregene, in the north, to San Felice Circeo, in the south.

While we cannot exclude that the success of this exotic taxon in Italy may be due both to its significant parasitic and saprotrophic abilities, we have gathered evidence regarding at least its pathogenic effects on Italian stone pine. This evidence includes isolation of the fungus from roots of dying trees, and the presence of fruiting bodies on recently created stumps at Castelporziano. Furthermore, in forests such as the Gallinara pine plantation at Anzio, the Campana pine plantation in Nettuno, and the forest of the Circeo National Park, the highest DR values of spores of the exotic *Heterobasidion* taxon were recorded in sampling points located within 50 m from canopy gaps. In light of the fact that upon death, trees are promptly removed from such gaps, the significant association between high spore counts and tree mortality supports a pathogenic role played in invaded ecosystem by the exotic pathogen.

Our field observations indicated the extent of mortality associated with the introduced pathogen varied among different forests. At Castelporziano, gaps included tens of trees, at Coccia di Morto (17 km northwest of Castelporziano) gaps included more than 10 dead trees, at Anzio and Nettuno (24 km and 32 km southeast of Castelporziano, respectively) gaps included 5–10 dead trees, and at the Circeo National Park (68 km southeast of Castelporziano) gaps included 1 or 2 dead trees. Based on the assumption that the extent of the damage should be positively correlated with the age of the infestation, Castelporziano was identified as the most likely point of introduction, as previously suggested (Gonthier *et al*. 2004). Conversely, based on the same logic, the Circeo forest should be the area most recently invaded. It should be noted that Castelporziano is one of the study sites located in the central area of the range currently invaded by the exotic taxon. In the absence of any obvious significant climatic and geographic barriers in the coastal area north and south of Rome, a central location for a putative introduction point would be expected and congruent with a symmetrical pattern of spread of the introduced organism. Based on a symmetrical model of spread, the introduction site could also be in the Anzio-Nettuno area.

Our data also allow for an estimation of the rate of spread of the exotic organism. Wood colonization by *Heterobasidion annosum* has been reported to occur at a rate of 0.2–1 m/year (Slaughter & Parmeter 1995; Garbelotto *et al*. 1999). *Heterobasidion* spores have been reported to travel long distances (Kallio 1970), but their dispersal range is generally limited to a few hundreds of metres (Stenlid 1994; Gonthier *et al*. 2001). Information on overall rates of spread, inclusive of (i) within stand tree-to-tree contagion via fungal growth along grafted roots, and (ii) spread via airborne spores, is currently lacking. In this study, this rate can be inferred by dividing the distance between Castelporziano and the small urban pinewood of San Felice Circeo by the time elapsed between 1944 and the present time, resulting in an estimate of 1.3 km/year.

It should be noted that there is no continuity among the sampled forests, which are surrounded by agricultural and urban areas. Thus, we suggest invasion corridors may be provided by single trees interspaced in the landscape, and not necessarily by sizable patches of forests. Italian stone pine is extremely frequent as a landscape species in the Roman countryside. Our ability to collect *Heterobasidion* spores around few pine trees in the Via Terracina urban pinewood at San Felice C. and in proximity of isolated pine trees along the edge of the Castelporziano Estate, supports this hypothesis.

Spores of the exotic *Heterobasidion* taxon were found in coastal pine stands over more than 100 km of Italian coasts. In addition to coastal pinewoods, spores of the NA taxon were also found in the forest plantation of Nettuno, located 7 km inland, thus demonstrating the ability of this introduced organism to establish itself in areas beyond the strict coastal ecosystem.

Spores of the native *Heterobasidion* taxon were absent or present at low frequencies in the sampled pine stands. This is mostly in agreement with previous reports stating that coastal forests south of Tuscany are only marginally affected by *H. annosum* s.s., possibly due to the long and dry summers characteristic of the coastal Mediterranean climate (Capretti *et al*. 1994; Capretti 1998). A single site (Circeo National Park) was characterized by high spore loads produced by the native species. This forest is strikingly different from the dry pine stands sampled everywhere else, being characterized by an extremely lush mixed oak-pine forest growing on marshes.

While DR values of the native taxon differed significantly between pure dry pine stands and the mixed oak-pine forest, DR values of the exotic taxon were equally high in both types of ecosystems. These results indicate that: (i) the introduced taxon is competitive with the native taxon even where the latter is well established; and (ii) the NA taxon has successfully invaded the dry pine stands of the region of Rome, a habitat in which the EU species is only marginally present. We cannot exclude that the success of the exotic taxon may have been enhanced by rapid evolution in response to novel abiotic and biotic conditions (reviewed in Sakai *et al*. 2001). Alternatively, high virulence of the introduced fungus on Italian stone pine may be explained by the lack of co-evolution between host and pathogen. In order to partially elucidate the mechanisms involved in the success of the introduced fungus, comparative controlled inoculation studies of EU and NA genotypes on both NA and EU pines may be necessary.

DR values of the native species were low and statistically undistinguishable both within and outside the boundaries of the area invaded by the NA taxon. These results indicate that replacement of the native species at the regional level has not occurred; instead, it appears that the exotic taxon has invaded habitats only marginally occupied by the native species.

Incongruent phylogenies at the three studied loci, provides evidence that genetic interaction between the two taxa is occurring. Incongruent phylogenetic placement of putative recombinant genotypes was observed not only between the mitochondrial ATP and the nuclear EFA, but also between the nuclear EFA and GPD sequence data sets. Thus, the recombinant genotypes here analysed should be at least the F2 meiotic progeny of F1 NA × EU hybrids.

Recombinant spores were found only in the Circeo National Park, where both taxa were significantly present. Six spores out of 157 from that site, that is 3.8%, were characterized as recombinant according to the two-locus PCR screening used in this study. We believe 3.8% to be an underestimation of the actual hybridization rate, as, inevitably, an assay based only on two loci will miss to detect some recombinants generated by backcrossing mating events.

The discovery of hybrid individuals in the only area where a seemingly recent interaction is ongoing between significant populations of both the exotic and the native taxa, may signify hybridization events may be on the rise. Considering that *in vitro* interfertility between the two taxa is almost complete (Stenlid & Karlsson 1991), it is likely hybridization may be occurring in other study sites where the EU taxon is only marginally present, but at frequencies too low to be detected at our current sampling intensity by using two or three loci only as genetic markers. It should be noted that, if the exotic organism reaches the north of Italy, where forests are significantly infested by *H. annosum* s.s., hybridization may occur more frequently.

Hybrids in the complex *Heterobasidion* have been found once only, between sympatric North American taxa (Garbelotto *et al*. 1996). Here we report the first occurrence of hybridization between naturally allopatric taxa in the complex, as a consequence of anthropogenic introduction. Hybridization between introduced and native interfertile species has been documented for plants and animals (Huxel 1999; Clarke *et al*. 2002; Perry *et al*. 2002) but such examples do not exist for fungi. In general, known hybridization reports describe the result of mating between two introduced species (Bates *et al*. 1993; Brasier *et al*. 1999) or between two native species (Tsai *et al*. 1994).

While further research focusing on multilocus genetic information is needed to describe in detail the outcomes of the ongoing genetic interaction between the two taxa, this study documents the extensive area invaded by NA *Heterobasidion* in Italy and describes the ability of this exotic organism to invade not only habitats where the endemic species is marginally present, but also those habitats where it is significantly present. A further and more subtle invasion, that of NA alleles moving into native EU populations, may also be occurring given the hybridization documented in this study. The successful dispersal of the introduced organism, its ability to invade a significant portion of native ecosystems, its interaction with native fungal populations, and, finally, the mortality of Italian stone pine observed in association with its presence indicate that the NA *Heterobasidion* taxon is a true invader *sensu* Davis & Thompson (2000).

The introduction of the NA *Heterobasidion* is most likely linked to US military activity during WWII (Gonthier *et al*. 2004). Human activity has thus brought together two clearly phylogenetically distinct groups of microbes, likely to have been separated for millions of years. We have shown that this newfound sympatry has lead to genetic exchanges between the two taxa and has potentially forever changed the evolutionary trajectory of this organism in Europe. Hence, movement of organisms due to globalization may result in undesirable or unpredictable outcomes both from an ecological and an evolutionary perspective.

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P. Gonthier is an assistant professor working on population ecology and epidemiology of fungal forest pathogens, with special emphasis on *Heterobasidion annosum*. G. Nicolotti is an associate professor working on epidemiology and control of *H. annosum* as well as on urban tree pathology. R. Linzer and F. Guglielmo are researchers interested in fungal phylogeography and molecular diagnostics of plant pathogens, respectively. M. Garbelotto is an associate extension specialist and adjunct professor with longstanding interests in ecology, genetics and population biology of forest pathogens.
