MOLECULAR ECOLOGY

Molecular Ecology (2011) **20**, 2756–2770 doi: 10.1111/j.1365-294X.2011.05121.x

Amplified fragment length polymorphism and sequence analyses reveal massive gene introgression from the European fungal pathogen *Heterobasidion annosum* into its introduced congener *H. irregulare*

P. GONTHIER* and M. GARBELOTTO†

Department of Exploitation and Protection of the Agricultural and Forestry Resources (DIVAPRA), Plant Pathology, University of Turin, Via L. da Vinci 44, I-10095 Grugliasco, Italy, †Department of Environmental Science, Policy and Management, Ecosystem Sciences Division, University of California at Berkeley, 137 Mulford Hall, Berkeley 94720, CA, USA

Abstract

The paucity of fungal species known to be currently hybridizing has significantly hindered our understanding of the mechanisms driving gene introgression in these eukaryotic microbes. Here, we describe an area of hybridization and gene introgression between the invasive plant pathogen *Heterobasidion irregulare* (introduced from North America) and the native *H. annosum* in Italy. A STRUCTURE analysis of amplified fragment length polymorphism data for 267 individuals identified gene introgression in 8–42% of genotypes in the invasion area, depending on site. Data indicate that introgression is mostly occurring unilaterally from the native to the invasive species and is responsible for 5–45% of genomes in admixed individuals. Sequence analysis of 11 randomly selected and unlinked loci for 30 individuals identified introgression at every locus, thus confirming interspecific gene flow involves a large number of loci. In 37 cases, we documented movement of entire alleles between the two species, but in 7 cases, we also documented the creation of new alleles through intralocus recombination. Sequence analysis did not identify enrichment of either transcriptionally different nonsynonymous alleles or of transcriptionally identical synonymous alleles. These findings may suggest introgression is occurring randomly for extant alleles without an obvious enrichment process driven by selection. However, further studies are needed to ensure selection is not at work elsewhere in the genome.

Keywords: fungi, gene introgression, hybridization, intragenic recombination, selection

Received 13 January 2011; revision received 31 March 2011; accepted 31 March 2011

Introduction

Areas where interspecific hybridization occurs are generally regarded as ideal targets for the study of models of speciation, selection and the evolution of plant–pathogen interactions (Hewitt 1988; Boecklen & Spellenberg 1990; Harrison 1990). Additionally, hybridization between native and invasive species and any subsequent gene introgression are viewed as some of the most significant threats to local biodiversity and as such have been at the centre of many debates and studies in conservation genetics (Goodman *et al.* 1999). During the hybridization process, individuals from different species will mate thereby intermixing two distinct genomes. This admixing has obvious consequences from both an evolutionary and an ecological perspective. While there is an ample evidence that hybridization is often limited to transitional ecological conditions and has restricted evolutionary consequences (reviewed in Buggs 2007), increased fitness and significant evolutionary consequences may also be associated with hybridization either through the generation of new hybrid species (Rieseberg 2001) or with the introgression of adaptive traits under positive

Correspondence: Matteo Garbelotto, Fax: (510) 643 5098; E-mail: matteog@berkeley.edu

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selection (Clarke *et al.* 2002; Ballard & Whitlock 2004; Rieseberg *et al.* 2007). The literature supporting these ideas is very extensive and includes examples of introgression both between sympatric species and between exotic and native species during biological invasions (Petit *et al.* 2004; Whitney *et al.* 2006; Zayed & Whitfield 2008; Fonseca *et al.* 2009; Gomes *et al.* 2009).

However, selection may not be the only driving force behind the movement of alleles across species. Recent theories predict that in the presence of even modest interfertility between species, simple demographic expansion of a newly arrived species should result in a high frequency of introgressed alleles in the invasive species, irrespective of allelic function and the density of hybridizing populations (Currat *et al.* 2008). Conversely, genes of the invasive species are predicted to introgress into the native species only when they are under strong positive selection (Moran 1981; Buggs 2007; Currat *et al.* 2008). While asymmetric introgression has been widely reported for plants and animals (Barton & Hewitt 1985; Orive & Barton 2002), it has been difficult to define introgressed alleles as neutral or under selection, and thus, definitive support in favour of this 'demographic' theory is still lacking. Additionally, studies of any aspect of genetic introgression in fungi are surprisingly rare. The paucity of known fungal species currently hybridizing has significantly hindered advancements in this area, in spite of abundant indirect or historical evidence of hybridization in the fungal kingdom. Interspecific hybridization in the fungi is supported by the detection of admixture in fungal genomes (Tsai *et al.* 1994; Newcombe *et al.* 2000; Schardl & Craven 2003; Dunn & Sherlock 2008; Neafsey *et al.* 2010; Gladieux *et al.* 2011), by the incongruence of gene genealogies pointing to historical horizontal transfers of loci among fungal species (Linzer *et al.* 2008; Aguileta *et al.* 2009; Devier *et al.* 2010; Mallet *et al.* 2010; Thiery *et al.* 2010) and even by some examples of recent interspecific transfers of genes directly involved in pathogenicity or ecological fitness (Friesen *et al.* 2006; Paoletti *et al.* 2006; Slot & Rokas 2011). However, we are aware of only a single study reporting a large number of introgressed loci in admixed genomes between hybridizing species (Neafsey *et al.* 2010), as opposed to the large number of reports regarding massive introgression in plants and animals (Keim *et al.* 1989; Barilani *et al.* 2007; Plötner *et al.* 2008; Blair & Hufbauer 2009). In this study, we report on one of the first cases of broad current hybridization and massive gene introgression between two fungal species and use the data to test the validity of current introgression theories.

The introduction of the North American forest pathogen *Heterobasidion irregulare* Garbelotto & Otrosina (Otrosina & Garbelotto 2010) in Central Italy, presumably during World War II (Gonthier *et al.* 2004), has created a new area of sympatry with its native sister species *H. annosum* (Fr.) Bref. *sensu stricto*, hereafter referred as *H. annosum*. The two species are genetically clearly distinct and have attained reciprocal monophyly at several loci (Otrosina *et al.* 1993; Linzer *et al.* 2008; Dalman *et al.* 2010). The introduced species *H. irregulare* has become invasive and has occupied several discrete forests in a 100-km-long zone of infestation, resulting in the unprecedented mortality of groups of pine trees along the path of establishment (D'Amico *et al.* 2007; Gonthier *et al.* 2007). Although the native species is also known as an aggressive forest pathogen, it has not been causing significant mortality in the xeric Mediterranean pine forests, presumably because it is relatively rare in xeric conditions (Gonthier *et al.* 2007), and has a much lower reproductive potential than its North American counterpart (Garbelotto *et al.* 2010). In most forests, the overwhelming majority of genotypes belonged to *H. irregulare* and the few *H. annosum* genotypes did not display any spatial association. However, in the forest of the Circeo National Park, using a diagnostic assay based on two loci, a clear spatial segregation was identified. North American *H. irregulare* genotypes were found almost exclusively in the northern portion of the forest, while European *H. annosum* genotypes were found almost exclusively in the southern portion of the forest. In the central portion of the forest, both species were significantly present, and 4% of genotypes were found to be hybrid in a previous study (Gonthier *et al.* 2007); this number is assumed to be a significant underestimate because of the limited number of loci used in the analysis. For the purpose of this study, we define this central area a front of current sympatry. We interpret this distribution pattern as the result of the two species having come into contact only recently in the Circeo National Park as the invasive species moves south from the original point of introduction (Gonthier *et al.* 2007) and invades an area occupied by the native taxon.

The goals of this study were to further describe hybridization throughout the area of current sympatry between *H. annosum* and *H. irregulare*. Specific goals included (i) to examine the levels and the direction of gene introgression between the invasive and the native fungal species using a large number of anonymous amplified fragment length polymorphism (AFLP) markers and comparative analysis of 11 individual gene genealogies, (ii) to assess whether gene introgression is strongly affected by genetic structure of intermixing populations, density and/or by time since sympatry, (iii) to determine by manual annotation of allelic sequences whether introgression simply results in genotypes bearing alleles of both species or whether

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novel alleles may be generated by intralocus recombination and finally, (iv) to perform a preliminary analysis to identify the enrichment of either synonymous (polymorphic sequence-wise but transcriptionally identical) or nonsynonymous (transcriptionally different) alleles through introgression, which would indicate the action of selection.

Materials and methods

Samples, collection sites and species identification

All analyses were performed on 267 haploid colonies derived from germinated meiospores of *Heterobasidion* collected on wood discs used as selective traps (Table S1, Supporting information). Traps were exposed in 110 sampling points placed at least 350 m apart along transects in six forests located within the zone of infestation of *H. irregulare* in Italy and in 11 forests outside the zone of infestation (Table S2, Supporting information) as previously defined (Gonthier *et al.* 2007). Haploid colonies were initially identified at the genus level by microscopy and at the species level through a 2-loci DNA-based molecular assay as described in a previous study (Gonthier *et al.* 2007).

AFLP genotyping

True species determination and presence of admixtures and gene introgression between *H. irregulare* and *H. annosum* were investigated by AFLP analysis (Vos *et al.* 1995). Genomic DNA was extracted from 20 mg of lyophilized mycelium and eluted in 50 μL ultra-pure water using the Puregene DNA isolation kit (Gentra). AFLP reactions were performed by using the AFLP® core reagent kit (Invitrogen, Carlsbad, CA, USA) following a published protocol (Ivors *et al.* 2004). Six pairs of selective-base primers were used in the analysis: (E00-AA) + (M00-CA), (E00-AC) + (M00-CA), (E00-AC) + (M00-CC), (E00-AC) + (M00-CG), (E00-AC) + (M00-CT) and (E00-AT) + (M00-CG). Selective amplification products were diluted to 1:10 with deionized formamide, denatured for 5 min at 95 °C and placed on ice before being analysed. AFLP fragments were sized by capillary electrophoresis on an automated ABI 3100 Genetic Analyzer using the molecular standard GeneScan-500 ROX and GENESCAN 3.1.2. software (Applied Biosystems). Electropherograms were scored manually and side by side for the presence (1) or absence (0) of bands.

Furthermore, the R (CRAN) package RawGeno (Arrigo *et al.* 2009) was used for automating GenScan scoring and to normalize peak intensity. To test the repeatability of the method, 10% of genotypes were randomly chosen, and AFLP fragments were generated starting from the restriction/ligation step for each primer pairs. Outcomes of repeat runs were scored blindly and compared to those of original runs to calculate AFLP error rates.

Sequence analysis

To confirm and qualify the introgression documented and quantified by AFLP analysis, one mitochondrial and ten nuclear loci were amplified from eight putatively pure *H. annosum* genotypes and eight putatively pure *H. irregulare* genotypes. These 16 genotypes were randomly selected among genotypes characterized as pure by AFLP data as described in the data interpretation and statistical analysis section in the following paragraph. Additionally, 14 genotypes defined as admixed as they contained AFLP markers characteristic of both species were randomly selected and included in the sequence analysis. Primers and PCR conditions for three of these loci (nuclear elongation factor $1-\alpha$, EFA; nuclear glyceraldehyde 3-phosphate dehydrogenase, GPD; mitochondrial ATP synthase subunit 6, ATP) were described previously (Kretzer & Bruns 1999; Johannesson & Stenlid 2003; Gonthier *et al.* 2007). The recently released *H. irregulare* genome (http://genome.jgi-psf.org/Hetan1/Hetan1.home.html) was used to identify eight additional loci. Loci were selected to contain both intronic and exonic portions and to be on different scaffolds of the *H. irregulare* genome. The only exception was represented by the BTUB and GST1 loci (see below) that were located 956 947 bp apart on the same scaffold. Primers were designed using Primer 3 (http://www.embnet.sk/cgi-bin/primer3_www.cgi) on conserved regions flanking the loci, and annealing temperature was optimized with a gradient PCR (Table 1). The putative nature of each additional locus was inferred based on annotation in the EuKaryotic Orthologous Groups (KOG) browser in JGI and included: Atrazine Chlorohydrolase/Guanine Deaminase—Amidohydrolase (ACH), Beta-Tubulin (BTUB), putative Calmodulin (CAM), candidate Polygalacturonase Glycoside Hydrolase Family 28 protein (EPG), Glutathione-S-Transferase 1 (GST1), Internal Transcribed Spacer (ITS), Uridine 5′-Monophosphate Synthase/Orotate Phosphoribosyltransferase (OMP) and Transcription Factor (TF). The composition of the reaction mixture and the PCR conditions were the same as Linzer *et al.* (2008). PCR products were cleaned with the ExoSAP-IT clean-up kit (USB Corp., Cleveland, OH, USA). Both forward and reverse strands were sequenced using the above primers and BIGDYE Terminator (version 3.1) chemistry, according to the manufacturer's instructions. Reactions were precipitated, and products were visualized on an ABI Prism capillary sequencer, following

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Table 1 Putative biological function, primer sequences and annealing temperature for the amplification of the new loci investigated in this study

Locus	Putative biological function	Forward primer (5′-3′)	Reverse primer (5′-3′)	Annealing temperature (°C)
ACH	Atrazine Chlorohydrolase/ Guanine Deaminase—Amidohydrolase	CGGAGAAATACGCGTTGG	CCGCAGTTCCTTTATCAGG	52
BTUB	Beta-Tubulin	AGCGAGTGTGTGATCTGGAA	CTCTTCCGTCCGGACAACTT	59
CAM	Putative Calmodulin	AGACATGTCATGACGAACCTC	ACGAACCGTCGTAATTAATCTGG	57
EPG	Candidate Polygalacturonase Glycoside Hydrolase Family 28 protein	ATCAGGATGACTGCCTTGCT	ACCTGTCCCAGGAGTTCCAAG	52
GST1	Glutathione-S-Transferase 1 gene	AGTTCCCTATGAGTTCGTCTTTGTCG	GCATCAAGCTTCGCAATCTGATCAG	55
ITS	Internal Transcribed Spacer	TCCGTAGGTGAACCTGCGG	TCCTCCGCTTATTGATATGC	55
OMP	Uridine 5′-Monophosphate Synthase/Orotate Phosphoribosyltransferase	AGTATTGCCGCATCCCACTC	TCGACACTACAGCCCTATCAAG	57
TF	Transcription Factor	CCTCCTCGCGTACCAGTCAGTT	TGGTGACTCTGCTTCAAATGC	60

ABI protocols. Sequences were edited and aligned in Sequencer (Gene Coses Corp., Ann Arbor, MI, USA). Insertions and deletions of 2 bp or greater in length were coded as a single character and weighted as equal to one base substitution.

The nuclear loci selected were assumed to be unlinked given the magnitude of the minimum theoretical distance among them. Based on our manual annotation on the *H. irregulare* genome, and considering for each locus both the distance between the 5′ end of the scaffold and the forward primer and the distance between the 3′ end of the scaffold and the reverse primer, the smallest possible theoretical distance was between GST1 and ITS at 96 655 bp, followed by the distance between GST1 and CAM at 214 752 bp; the mean minimum theoretical distance considering all pairwise combinations of nuclear loci was 1 294 247 bp. BLAST searches performed against the *H. irregulare* genome confirmed that the sequences here analysed were present as a single locus.

Data interpretation and statistical analysis

The Bayesian clustering and assignment software STRUCTURE v. 2.3.3. (Pritchard *et al*. 2000; Falush *et al*. 2007; Hubisz *et al*. 2009) was used to determine whether the AFLP markers could distinguish *H. annosum* from *H. irregulare*, detect interspecific admixtures between the two species, and identify genetically distinct subpopulations within the sample analysed. We tested for up to nine clusters (K), with five repetitions for each K, to match the total number of study sites (Table 2; Table S2, Supporting information). A 10 000 run burn-in and 10 000 run length were used without assuming prior population information. Selection of K from this output data was carried out based on suggestions in the software documentation, i.e. using the maximum likelihood estimate. After selection of the most appropriate K, a new analysis was run with a 50 000 burn-in period and 100 000 repetitions. Two independent analyses of molecular variance (AMOVA; Excoffier *et al*. 1992) were also performed to study the presence of significant genetic structure within and among populations of *H. irregulare* and *H. annosum*. For these analyses, all admixed genotypes were excluded, leaving a total of 91 *H. irregulare* genotypes from six sites (Fregene, Coccia di Morto, Castelfusano, Anzio, Nettuno, and Circeo) and 104 *H. annosum* genotypes from six sites (Alps, Mesola, Feniglia, Castelfusano, Anzio, and Circeo). As a result of small sample size, the three contiguous *H. annosum* populations of Feniglia, Castelfusano and Anzio were pooled together, resulting in a total of four populations. For both fungal species, AMOVA and STRUCTURE results were also used to draw inferences on the frequency of migration among forests.

Genotypes were assigned to *H. irregulare* or *H. annosum* when the probability of membership of either group was at least 95% using STRUCTURE; otherwise,

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Table 2 Mean level and range of gene introgression between *H. annosum* and *H. irregulare* for each study site, including number of pure genotypes and all cases of heterospecific nuclear–mitochondrial genotypes

Collection site	No. of genotypes analysed	No. of pure *H. annosum* genotypes	No. of pure *H. irregulare* genotypes	No. of admixed genotypes	Mean percentage (range) of *H. annosum* genome introgressed in *H. irregulare*	Mean percentage (range) of *H. irregulare* genome introgressed in *H. annosum*	No. of *H. annosum* genotypes* with *H. irregulare* mitochondria	No. of *H. irregulare* genotypes* with *H. annosum* mitochondria
1. Alps	38	38	0	0	0 (—)	0 (—)	0	0
2. Mesola Forest	11	11	0	0	0 (—)	0 (—)	0	0
3. Feniglia Pinewood	3	3	0	0	0 (—)	0 (—)	0	0
4. Fregene Monumental Pinewood—5. Coccia di Morto Estate	24	0	18	6	12.4 (5.0–22.4)	17.3 (—)	1	0
6. Castelfusano Pinewood Urban Park	18	1	11	6	15.3 (6.8–27.1)	0	0	0
7. Gallinara Pine Plantation—Anzio	27	1	19	7	25.8 (5.3–44.8)	0	1	0
8. La Campana Pine Plantation—Nettuno	12	0	11	1	41.0 (—)	0	0	0
9. Forest of Sabaudia—Circeo National Park—northern area	57	4	24	29	18.8 (5.5–41.2)	44.6 (—)	2	2
9. Forest of Sabaudia—Circeo National Park—central area	48	22	14	12	19.7 (7.5–41.6)	26.4 (22.5–26.4)	1	6
9. Forest of Sabaudia—Circeo National Park—southern area	29	28	1	0	0 (—)	0 (—)	0	0

*Species assignment was based on the presence of at least 51% of species-specific markers.

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they were assumed to be admixtures between the two species. To determine the direction and amount of gene introgression, admixtures were classified as one of the two species if they carried at least 51% of AFLP fragments characteristic of that species, and the remainder of markers was regarded as introgressed from the other species. Genotypes missing over 80% of markers were omitted from the analysis; the mean percentage of missing markers in the other genotypes was 24%. The frequency of admixed genotypes was tabulated for each provenance. In the Circeo National Park, we further distinguished a central area representing the front of current sympatry, where both species are present in comparable frequency, and a northern and southern area characterized by a dominance of *H. irregulare* and *H. annosum* spores, respectively. To avoid biases because of small sample size, data from the Fregene Monumental Pinewood and the adjacent Coccia di Morto Estate were pooled together. The relative frequency of admixtures was compared among sites using the χ^2 test in contingency tables.

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 heuristic search with 10 random additions per replicate (Felsenstein 1985). Outgroup was either *Heterobasidion insulare* (Murrill) Ryvarden or *H. abietinum* Niemelä & Korhonen (Linzer *et al.* 2008). The presence of reciprocal monophyly at all 11 loci was determined by ensuring that in each gene genealogy, all isolates fell in one of two clades with significant bootstrap support for at least one of the two, and that all putative pure *H. annosum* genotypes from outside the zone of infestation always fell in the *H. annosum* clade. The fact that occasionally a genotype from the zone of infestation had an allele that clustered incongruently with the other species (as determined by AFLP and incidentally by analyses of most other loci) was taken as a sign of gene introgression.

Manual annotation of multiple sequence alignments was employed to determine whether such alleles were synonymous (i.e. changes occurred in intronic noncoding regions or in the third position of a codon) or nonsynonymous to those already found in the receiving species. Manual annotation of all alleles excluding putatively introgressed ones was used to define the overall ratio of synonymous vs. nonsynonymous alleles in between the two species in the area of sympatry. Chi-square analyses were employed to determine whether the ratio of synonymous vs. nonsynonymous introgressed alleles differed significantly from that expected based on the ratio of the two types of alleles between the two species. We used the Yates' corrected χ^2 in contingency tables to compare (i) the frequency of nonsynonymous and synonymous alleles between the two species, (ii) the frequency of synonymous vs. nonsynonymous alleles among *H. annosum* alleles introgressed into *H. irregulare* and among *H. irregulare* alleles introgressed into *H. annosum* and (iii) the proportion of synonymous and nonsynonymous introgressed alleles to the actual proportion of synonymous and nonsynonymous alleles between the two species (analysis without Yates' correction).

Finally, visual analysis of sequences was also used to differentiate between simple introgression of alleles from one species into the other (interlocus recombination) and the creation of new alleles through intralocus recombination.

Results

AFLP analysis

The six primer pairs used for the AFLP analysis on 267 genotypes produced 1245 polymorphic fragments (E00-AC+M00-CC: 251 loci, E00-AA+M00-CA: 249, E00-AC+M00-CA: 217, E00-AC+M00-CG: 212, E00-AT+M00-CG: 177, E00-AC+M00-CT: 139). A total of 564 loci were deemed consistent and repeatable and were used for all analyses (Table S1, Supporting information). In the repeat runs, 96% of bands had an identical size; hence, we estimate the error intrinsically linked to the technique to be <5%. When performing the analysis with STRUCTURE, the clustering level $K = 4$ yielded the highest log likelihood value (mean ln probability of data: $K = 2$, −11 131; $K = 3$, −10 837; $K = 4$; −10 597; $K = 5$, −10 724; $K = 6$, −10 860; $K = 7$, −11 026; $K = 8$, −11 206; $K = 9$, −11 620). At $K = 4$, all putative *H. annosum* genotypes were assigned to three clusters, while all putative *H. irregulare* were assigned to a fourth cluster (Fig. 1; Table S2, Supporting information). However, at $K = 2$, we obtained similar assignments (Fig. 1), with the three putative European clusters collapsing into a single one, suggesting that only two clusters may exist, one referring to *H. irregulare* and the other to *H. annosum*. Admixed genotypes were found only in the 100-km-long zone of infestation of *H. irregulare*, and they accounted for 25% of the total number of genotypes collected in that area. With the exception of the southernmost area of the Circeo National Park, where *H. irregulare* is still very infrequent, admixed genotypes were present at all sites, with a frequency ranging between 8% and 42% (Fig. 2). The highest frequency of introgression was recorded not at but rather behind the front of current sympatry, where the presence of both species is presumed to have occurred for a slightly

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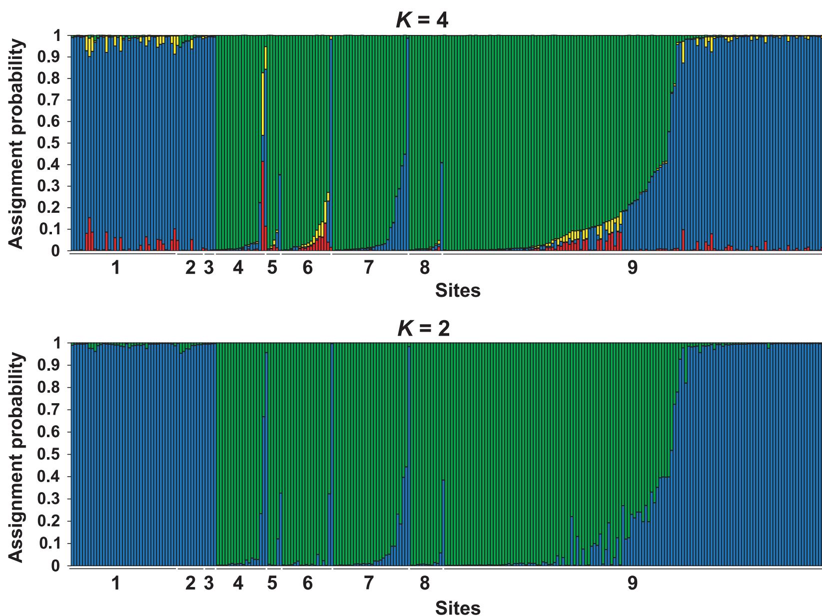

Fig. 1 Bar plots for K = 4 and K = 2 showing the assignment values for 267 *Heterobasidion irregulare* and *H. annosum* genotypes from nine Italian populations. For population codes, see Table 2. Green represents assignment to the North American *H. irregulare* and Blue assignment to the European native species *H. annosum*. Introgression of European genes (blue in a green background) is dominant, while introgression of North American genes (green) into the European background (blue) is more limited.

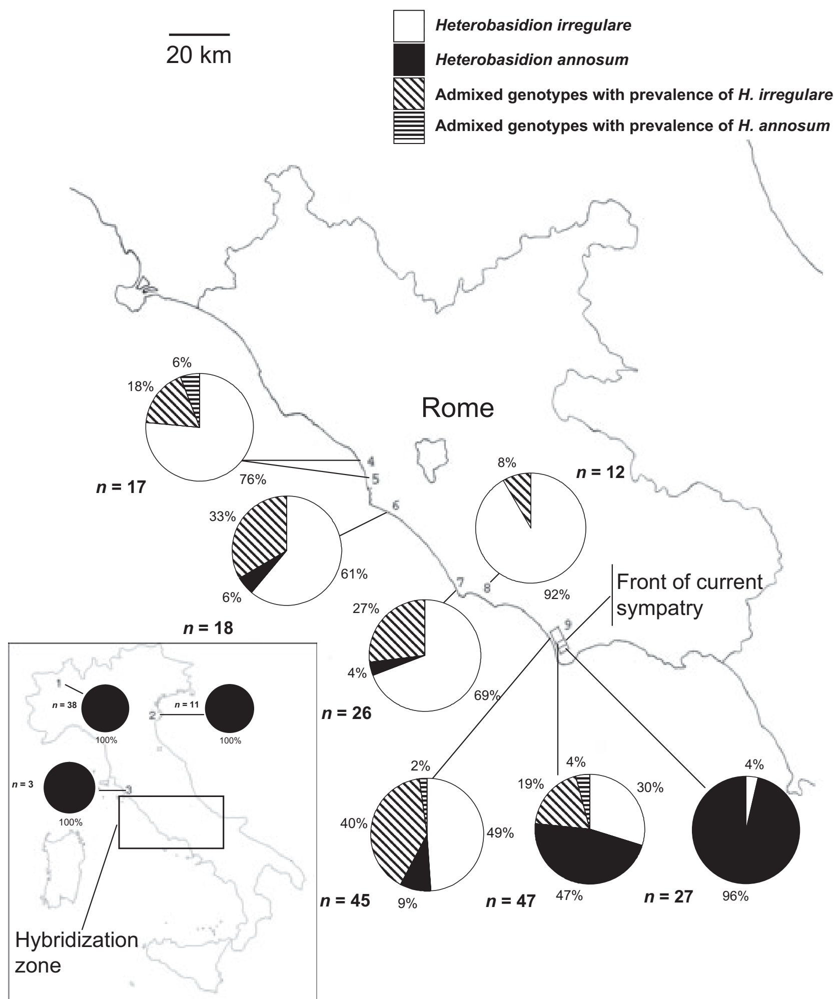

Fig. 2 Relative frequency of *Heterobasidion annosum*, *H. irregulare* and admixed genotypes between the two species in the zone of invasion of *H. irregulare* in Italy based on STRUCTURE analysis of AFLP data. For population codes, see Table 2. Population 9 (Forest of Sabaudia at the Circeo National Park) was further subdivided in northern, central and southern areas (see text). The location of the front of current sympatry is indicated. Populations 1–3, which are located outside the zone of invasion of *H. irregulare*, are shown in the smaller box.

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longer period of time than at the front itself. Although density of *H. annosum* differed significantly among the Circeo National Park and other sites (Gonthier *et al.* 2007), the frequency of admixed genotypes did not differ significantly among sites ($\chi^2 = 7.476$, d.f. = 5, $P = 0.188$). The large majority ($n = 44$) of all ($n = 48$) admixed genotypes (overall 92%; 75–100% depending on site) had a predominant genetic background of *H. irregulare*, indicating that gene introgression is occurring from the native to the invasive species. Introgression levels of *H. annosum* into *H. irregulare* ranged between 5% and 45% depending on genotype (Table 2), with an overall mean of 19%. While genotypes with *H. irregulare* mitochondria and predominant genetic background of *H. annosum* were found at a low frequency in most sites, genotypes with *H. annosum* mitochondria and predominant genetic background from *H. irregulare* were found exclusively in the northern (4% of 57 genotypes) and central areas (13% of 48 genotypes) of the Circeo National Park (Table 2).

AMOVA results on pure *H. irregulare* genotypes indicated no genetic structure within the exotic species, thus confirming the single cluster identified by the analysis with STRUCTURE. In pairwise comparisons, though, a marginally significant PHIst value was identified between Castelfusano and the Circeo National Park (PHIst 0.038, $P = 0.016$). The results for pure *H. annosum* genotypes indicated the presence of a small but significant portion of genetic variance among populations (3.26%, $P = 0.02$). However, in pairwise comparisons and after Bonferroni's correction for multiple tests, PHIst values between populations were never significant.

Sequence analysis

A total of 304 sequences and 4892 characters were analysed, 155 of which were parsimony informative. Representative sequences for each allele were deposited in NCBI GenBank (accession numbers JF411615–JF411739, Table S1, Supporting information). Putatively pure *H. annosum* genotypes and putatively pure *H. irregulare* consistently fell into two different clades, with significant bootstrap support (73–100) for at least one of them at all 11 loci. In the BTUB analysis, two *H. annosum* genotypes fell in a third clade, clearly distinct from the *H. irregulare* clade, and thus even at this locus, there was no overlap of alleles between the two species. Genotypes putatively defined as hybrid through AFLP analysis displayed incongruent placement in one or more individual gene genealogies, and in a few instances, sequence analysis identified introgression in genotypes that had been defined as pure by AFLP analysis (Fig. 3 and 4; TreeBASE Study accession n. S11259).

Using AFLP data to infer the overall genetic background of the genotypes analysed, and based on outcomes of the manual annotation of multiple sequence alignments, introgression occurred at all of the 11 loci studied (Table 3). Considering all 11 sequenced loci, the cumulative frequency of synonymous *H. annosum* alleles introgressed into *H. irregulare* was significantly higher than that of nonsynonymous alleles (20 vs. 9; Yates' corrected $\chi^2 = 6.90$, d.f. = 1, $P = 0.009$). Because of the magnitude of the minimum theoretical distance among loci, each introgression, even when occurring in the same genotype, can be regarded as independent. The actual frequency of synonymous alleles between the two species was also significantly higher than that of nonsynonymous ones (89 vs. 54; Yates' corrected $\chi^2 = 16.17$, d.f. = 1, $P < 0.001$). The proportion of synonymous and nonsynonymous introgressed alleles is not different than the actual proportion of synonymous and nonsynonymous alleles present between the two species ($\chi^2 = 0.54$, d.f. = 2, $P = 0.765$).

The number of synonymous *H. irregulare* alleles ($n = 9$) introgressed into *H. annosum* was slightly higher than that of nonsynonymous alleles ($n = 6$), but this difference was not significant (Yates' corrected $\chi^2 = 0.53$, d.f. = 1, $P = 0.465$); however, it should be noted that the low number of *H. annosum* genotypes involved ($n = 5$) makes these results at best preliminary.

While interlocus recombination was detected in 37 cases, intralocus recombination was exemplified by the presence of seven chimeric alleles in four loci (ACH, CAM, ITS and TF). Genotypes with intralocus recombined alleles were deposited at the *Mycotheca Universitatis Taurinensis* (MUT; accession numbers 4754–4756). Chimeric alleles were detected exclusively in *H. irregulare* genotypes introgressed by *H. annosum* alleles, and all were found to be synonymous to those already found in the receiving species. Most alleles showing evidence of intralocus recombination comprised a portion of sequence typical of one species, while the remaining portion was typical of the other species (Fig. 5a; Table 3). However, genotypes with more complicated recombination patterns were also identified. For instance, in two admixed genotypes, a CAM allele was characterized by several alternate portions of sequences from each of the two species (Fig. 5b; Table 3). Portions of recombined alleles always perfectly matched the equivalent portions of alleles from either species; this result suggests these novel alleles were generated by the recombination of alleles from the two species rather than by homoplasy. In eight cases, introgressed alleles were found either in two or more

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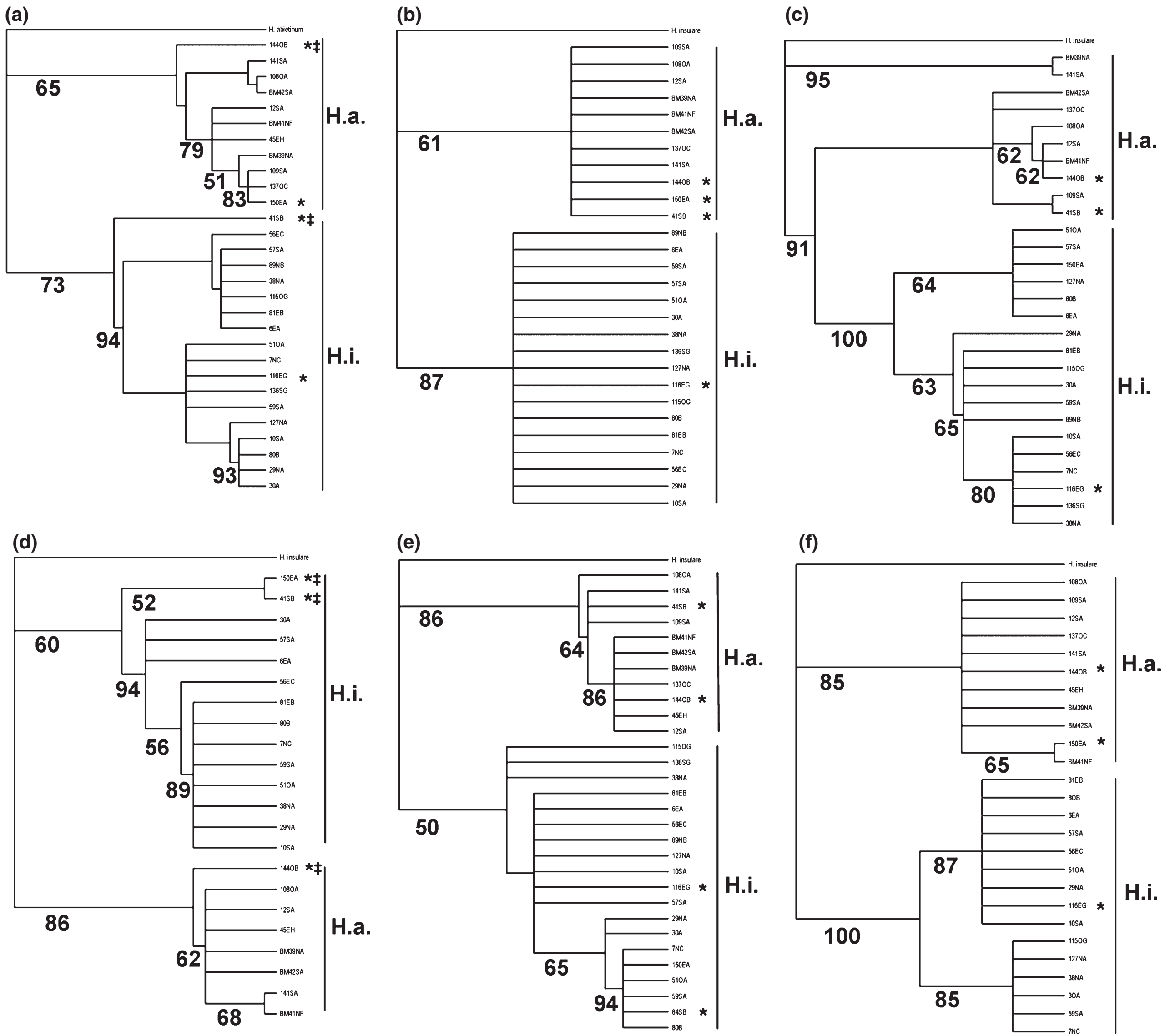

Fig. 3 Fifty percent majority-rule consensus trees obtained by maximum parsimony analysis of genotypes of *Heterobasidion annosum* (H.a.), *H. irregulare* (H.i.) and of admixed genotypes between the two species. Pure *H. annosum* genotypes: 12SA, 108OA, 109SA, BM39NA, BM41NF, BM42SA, 141SA, 137OC; pure *H. irregulare* genotypes: 3OA, 8OB, 10SA, 29NA, 38NA, 56EC, 59SA, 127NA; remaining genotypes are admixtures. (a) ACH, (b) ATP, (c) BTUB, (d) CAM, (e) EFA, (f) EPG. See text for codes of loci. Bootstrap values above 50% from 1000 replicates are shown below branches. *Incongruent placement of admixtures; †incongruent placement of putatively pure genotypes; and ‡intralocus recombination.

sampling points within the same forest or in two or more different forests.

Discussion

Results from AFLP analyses combined with those obtained from the sequence analysis of 11 loci clearly indicate that a massive, mostly unidirectional introgression of genes is occurring from the native into the invasive fungal species. This study represents one of the first describing ongoing admixing of large portions of genomes between two hybridizing fungal pathogens. Because the North American *H. irregulare* and the Eurasian *H. annosum* have attained reciprocal monophyly at all 11 loci studied, the frequency and levels of introgression reported in this study are not likely to be the result of retained ancestral polymorphisms misclassified as introgressions. Although introgression in the fungi is known (Friesen *et al.* 2006; Paoletti *et al.* 2006; Linzer *et al.* 2008; Aguileta *et al.*

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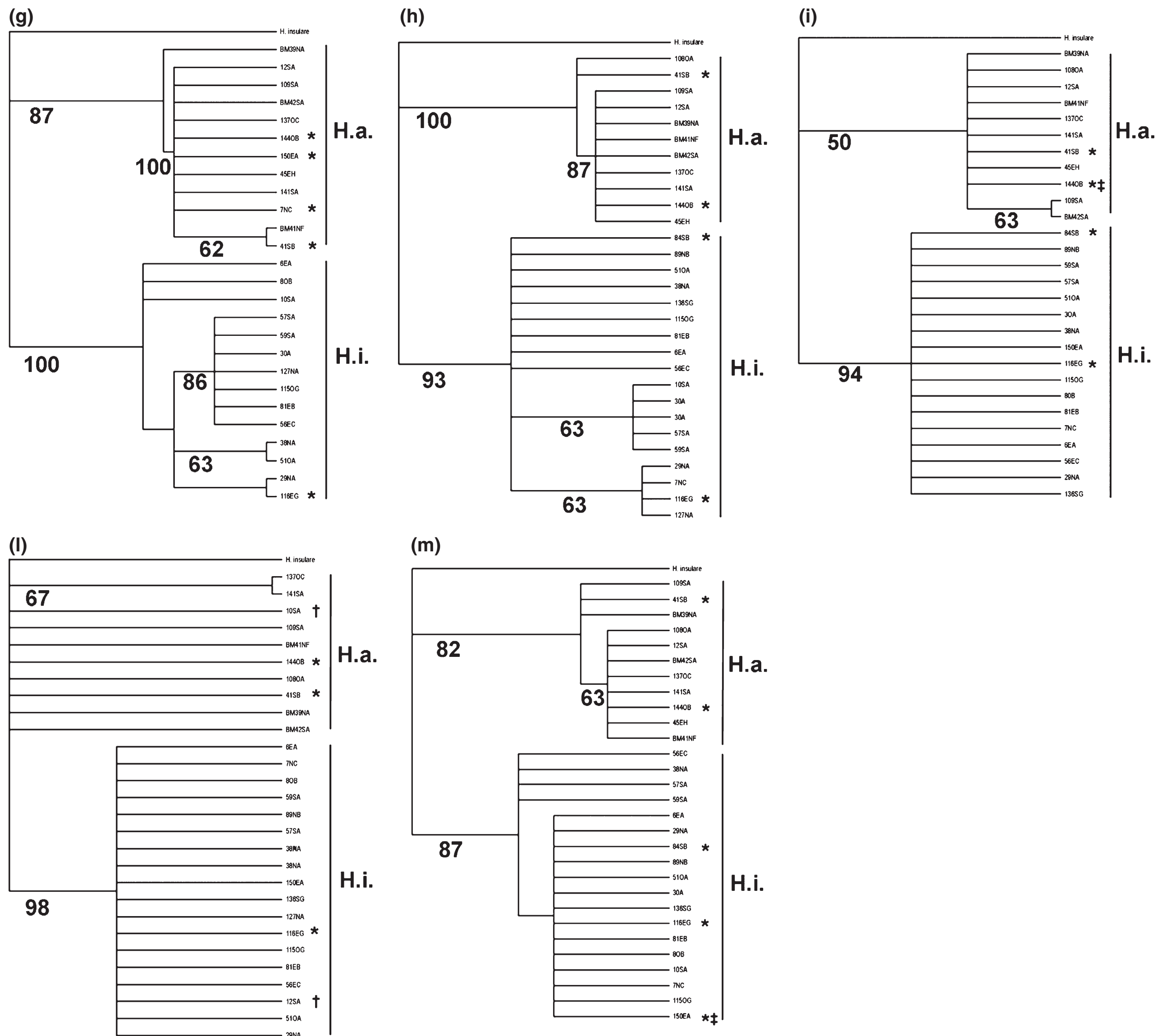

Fig. 4 Fifty percent majority-rule consensus trees obtained by maximum parsimony analysis of genotypes of *Heterobasidion annosum* (H.a.), *H. irregulare* (H.i.) and of admixed genotypes between the two species. Pure *H. annosum* genotypes: 12SA, 108OA, 109SA, BM39NA, BM41NF, BM42SA,141SA,137OC; pure *H. irregulare* genotypes: 3OA, 8OB, 10SA, 29NA, 38NA, 56EC, 59SA, 127NA; remaining genotypes are admixtures. (g) GPD, (h) GST1, (i) ITS, (l) OMP, (m) TF. See text for codes of loci. Bootstrap values above 50% from 1000 replicates are shown below branches. *Incongruent placement of admixtures; †incongruent placement of putatively pure genotypes; and ‡intralocus recombination.

2009; Mallet *et al.* 2010; Thiery *et al.* 2010), it has generally been reported to occur at low levels (i.e. the relative frequency of introgressed genes is low), presumably on loci under strong adaptive selection pressure (Paoletti *et al.* 2006) or balancing selection (Devier *et al.* 2010). Our study, instead, documents massive introgression between species in line with the report by Neafsey *et al.* (2010).

Asymmetric genic introgression from *H. annosum* into *H. irregulare* is supported by the fact that 92% of 48 admixed genotypes had a majority of *H. irregulare* AFLP markers and, with a few exceptions, an *H. irregulare* mitochondrion. Our results indicate that genotypes with admixed genomes are predominantly characterized by an *H. irregulare* mitochondrion. We interpret this finding as the result of backcrosses of first- and second-generation hybrids (carrying mitochondria of either species) into the invasive *H. irregulare*. Additionally, this pattern suggests a lack of a positive selection on the *H. annosum* mitochondrial genome, which, in

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Table 3 Patterns of introgression of synonymous and nonsynonymous alleles between the native *Heterobasidion annosum* and the invasive *H. irregulare* in Italy. The ID collection code of genotypes with intralocus recombined alleles is reported in the table footnote

Loci	Length (bp)	Genome position	Total no. of alleles including intragenically recombined ones	No. of *H. annosum*/ *H. irregulare* alleles, excluding intragenically recombined ones	No. of synonymous/ nonsynonymous pairwise combinations of alleles*	No. of *H. irregulare* genotypes with synonymous *H. annosum* alleles	No. of *H irregulare* genotypes with nonsynonymous *H. annosum* alleles	No. of *H. annosum* genotypes with synonymous *H. irregulare* alleles	No. of *H. annosum* genotypes with nonsynonymous *H. irregulare* alleles
ACH	724	Sc7: 864917–865640	17	8/6	20/28	2†‡	1	1	0
ATP	445	—	2	1/1	1/0	3	0	1	0
BTUB	297	Sc9: 986448–986744	12	8/4	32/0	2	0	1	0
CAM	137	Sc13: 1137600–1137736	7	2/3	6/0	3†§	0	0	0
EFA	321–324	Sc1: 2122529–2122840	7	3/4	0/12	0	2	0	2
EPG	392	Sc8: 470637–471028	4	2/2	4/0	2	0	1	0
GPD	613	Sc5: 1167993–1168605	10	4/5	20/0	4	0	1	0
GST1	380–382	Sc9: 29122–29501	5	2/3	0/6	0	2	0	2
ITS	536–539	Sc12: 1655545–1656080	6	4/1	4/0	1 + 1†¶	0	2	0
OMP	655	Sc10: 407651–408305	4	2/1	0/2	0	3	0	2
TF	330	Sc4: 1000926–1001255	7	4/2	2/6	1 + 1†**	1	2	0
Total					89/54	20	9	9	6

*Actual frequency of synonymous and nonsynonymous alleles between the two species, excluding introgression events and intragenically recombined alleles.
†Alleles characterized by intralocus recombination.
‡Position of intralocus recombination: in 41SB, Sc7: 865193–865411; in 144OB, Sc7: 865083–865101.
§Position of intralocus recombination: in 41SB and 150EA, Sc13: 1137603–1137662, 1137664–1137686, 1137688, 1137690–1137717, 1137727–1137735; in 144OB, Sc13: 1137688, 1137690–1137717.
¶Position of intralocus recombination: in 144OB, Sc12: 1655689–1655895, 1655897–1655936.
**Position of intralocus recombination: in 150EA, Sc4: 1001205.

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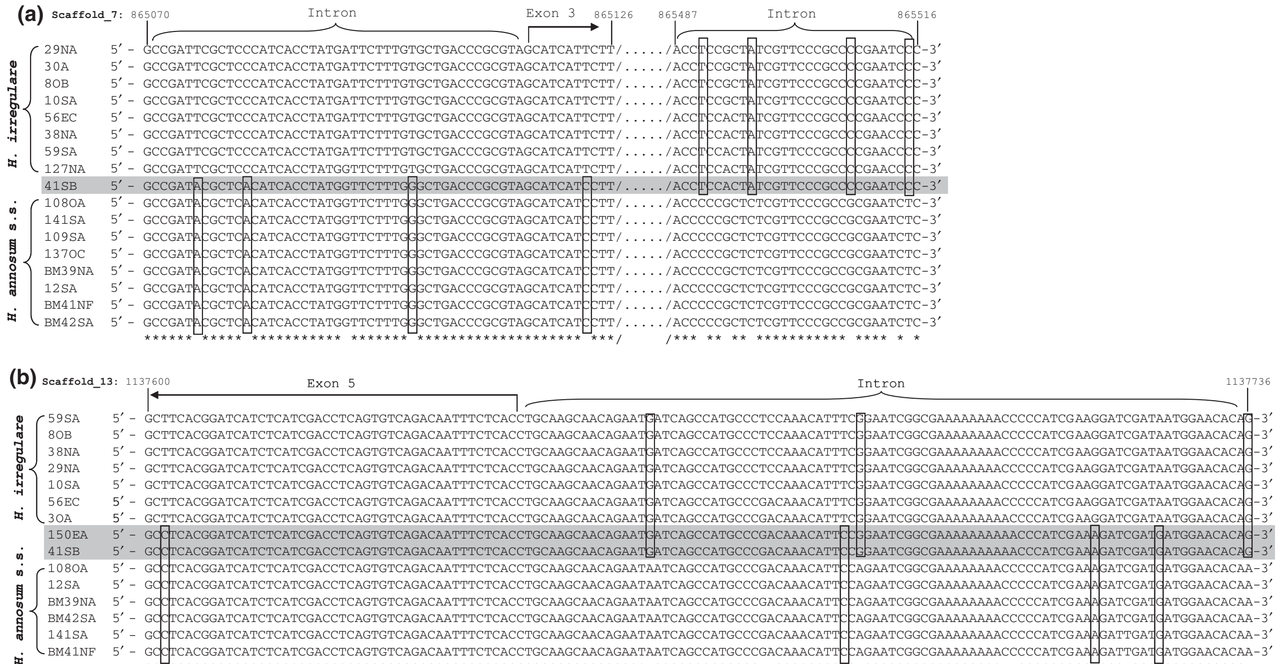

Fig. 5 Examples of intralocus recombination patterns. Multiple sequence alignment of representative *Heterobasidion annosum* and *H. irregulare* genotypes in two loci: (a) ACH and (b) CAM. Admixed genotypes highlighted in grey show either a simple (a) or a complex intralocus recombination pattern (b), consisting of several alternate portions of sequences from each species.

admixed genomes, seems to be spatially confined at and near the front of current sympatry. Density of native populations appears to have no effect on the frequency of introgression, but time since contact between the two species appears to be relevant. The highest frequency of introgression, in fact, was observed in the North Circeo, just behind the area of the expanding front of the exotic species. Both of these findings are in agreement with spatially explicit simulations predicting that, even in the presence of moderate interfertility, alleles would introgress into the exotic species at the front of sympatry, but, once acquired, their abundance would increase during the population growth phase of the invasive species in the area behind the front (Currat *et al.* 2008). Additionally, introgression is not likely to have been mediated by genetic differences among populations of either species, as they display no signs of significant genetic structure.

This study provides some of the first evidence of asymmetric introgression between two microbial species resulting in widespread genomic admixtures and in recombination both between loci (interlocus) and within alleles (intralocus). The latter phenomenon has been reported only once for fungi (Hughes & Petersen 2001). Furthermore, several alleles displayed complicated intralocus recombination patterns, i.e. alternate portions of sequences from each species; to the best of our knowledge, these patterns may be consistent with repeated meiotic, or less likely, mitotic crossing over at these loci.

The overall lack of within-species genetic structure evidenced by AMOVA and STRUCTURE analyses justifies the joint use of alleles from all sites to determine whether alleles being introgressed from *H. annosum* are already present or not in the receiving species. Despite the presence of significantly divergent alleles among *H. irregulare* genotypes, there is no geographical association of these alleles, rather they are probably the result of the introduction of genotypes with clearly distinct alleles, as can be inferred from the report of Linzer *et al.* (2008).

Our data from 11 sequenced loci indicate the majority of alleles introgressed from *H. annosum* into *H. irregulare* are synonymous with those already present in receiving populations. However, the proportion of nonsynonymous alleles being introgressed did not differ significantly from the proportion expected to be introgressed, based on the actual proportion of synonymous vs. nonsynonymous alleles between the two species. Lack of an enrichment of either category among introgressed alleles may indicate that selection is not driving movement of genes across species boundaries, or alternatively, that selective pressure during introgression is equally enriching both synonymous and nonsynonymous alleles in the receiving species. Only a genomic approach may adequately qualify the massive genic

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introgression between the two species (Neafsey *et al.* 2010). Selection may in fact be operating in loci other than those selected for this study and/or may be acting on loci not included in the analyses but linked to the ones analysed in this study.

Although alleles may be initially introgressed independent of function, it has been suggested that the frequency of beneficial alleles of heterospecific origin is likely to increase at a later stage because of selective pressures on adaptive alleles, and this process has been proposed as a key factor to the success of biological invaders (Ellstrand & Schierenbeck 2000; Hänfling & Kollmann 2002; Moody & Les 2002). It has also been suggested that variability in nucleotide sequences independent of transcriptional outcomes may be a key factor in the evolution of adaptive alleles (Forde *et al.* 2004); hence, the acquisition of new genetic information, even if transcriptionally identical to that already present in the receiving population, may be extremely important. The massive admixing of genomes here documented may provide a way for the invasive species to increase its genetic diversity after the bottleneck inevitably caused by the introduction process. The fact that some introgressed alleles (recombined internally or not) were found in multiple genotypes in different and clearly separate forests indicates that these are not dead ends but that they have arisen multiple times or, once arisen, are shared among genotypes even in distinct sites.

In summary, AFLPs data analysed by AMOVA and STRUCTURE have proven to be a powerful approach to quantify gene introgression between hybridizing species (Secondi *et al.* 2006; Bonin *et al.* 2007; Blair & Hufbauer 2009; Gaskin *et al.* 2009). AFLPs indicate a complete admixing of genomes with alleles being moved from the native into the exotic species throughout the genome. Sequence analysis has confirmed this massive introgression, has allowed the detection of intralocus recombined alleles and has shown a lack of enrichment of either synonymous or nonsynonymous alleles through hybridization. This last finding supports the notion of an introgression process that may be driven by population expansion, rather than by selection only. However, a genomic approach will be needed to confirm the suggestions put forth by the experiments here described using a limited number of genes.

Acknowledgements

The authors thank Silvia Mascheretti from UC Berkeley and Fabio Guglielmo from the University of Turin for technical assistance with AFLP and sequence analyses. This work was supported by the Italian Ministry of Education, University and Research, within the Project of National Interest (PRIN) program' (grant number 2008SBCC9S)'.

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P.G. is a forest pathology assistant professor working on population ecology and epidemiology of fungal forest pathogens. M.G. is an associate extension specialist and adjunct professor with long-standing interests in ecology, genetics and population biology of forest pathogens. Research interests of both authors include the invasion biology of exotic plant pathogens.

Data accessibility

AFLP data are provided in the Table S1 in Supporting information.

DNA sequences: Genbank accessions JF411615–JF411739.

Phylogenetic data: TreeBase Study accession n. S11259.

Genotypes deposited at the *Mycotheca Universitatis Taurinensis* (MUT): accession n. 4754–4756.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Collection sites, GenBank accession numbers and AFLP data of individuals analysed in this study. See text for codes of loci. Only representative sequences were deposited. AFLP data: 1 = fragment present, 0 = fragment absent, −9 = fragment not included in the analysis because its PCR amplification was inconsistent.

Table S2 Geographical location of study sites and Bayesian mean assignment probabilities per population (provenance) with K = 4 (STRUCTURE v. 2.3.3).

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