Relative abundance and potential dispersal range of intersterility groups of *Heterobasidion annosum* in pure and mixed forests

Paolo Gonthier, Matteo Garbelotto, Giovanna Cristina Varese, and Giovanni Nicolotti

Abstract: In Europe the forest pathogen *Heterobasidion annosum* (Fr.) Bref. includes the S, P, and F intersterility groups (ISGs), each displaying a preferential specialization on Norway spruce (*Picea abies* (L.) Karst.), pine, and silver fir (*Abies alba* Mill.), respectively. In this paper, we present data about (*i*) *H. annosum* ISGs frequency in different forest types, (*ii*) the degree of host specificity of each ISG, (*iii*) the significance of the potential movement of airborne spores among forests, and (*iv*) the occurrence of S–P chimeras in the northwestern Alps. Using woody spore traps, we sampled natural pure spruce and fir forests and a mixed spruce–fir forest. The ISG of 582 spores was determined by ISG-diagnostic taxon-specific competitive priming (TSCP) polymerase chain reaction (PCR) combined with PCR-mediated detection of ISG-specific introns in the ML5–ML6 DNA region of the mitochondrial large ribosomal RNA (mt LrRNA). All three ISGs were found, and a strong correlation was observed between the F ISG and fir and the S ISG and spruce. In the mixed forest, no clear relationship between tree host species and host-specialized ISGs was found. In spite of a relative dominance of fir in the overstory of the mixed stand, the fir-associated F ISG represented only 11% of the total number of spores collected. This discrepancy was explained by the recent establishment of firs at this site. No S–P nuclear-mitochondrial chimeras were found. This suggests limited gene flow between these ISGs.

Key words: *Heterobasidion annosum*, host specificity, ISGs, gene flow, PCR, Alps.

Résumé : L'*Heterobasidion annosum* (Fr.) Bref. est constitué en Europe par les groupes d'interstérilité (ISGs) S, P et F qui montrent une specialisation préférentielle respectivement sur l'épicéa commun (*Picea abies* (L.) Karst.), les pins et le sapin pectiné (*Abies alba* Mill.). Les auteurs ont étudié, dans les Alpes Nord-occidentales (*i*) la fréquence des ISGs dans différents types de forêt, (*ii*) leur degré de spécificité envers leurs hôtes, (*iii*) le mouvement potentiel de spores entre les forêts et (*iv*) l'existence de chimères nucléaires-mitochondriales S–P. Des spores ont été récoltées en disposant des rondelles-pièges dans des forêts pures d'épicéa et de sapin et dans une forêt mixte des deux espèces. L'ISG de 582 spores a été determiné au moyen du ISG diagnostique basé sur un amorçage compétitif avec amorces spécifiques pour chaque taxon (TSCP) par réaction de polymérisation en chaine (PCR) combiné avec la détection d'introns spécifiques effectués dans la région de l'ADN ML5–ML6 du grand gène ARN mitochondrial ribosomal (mt LrRNA). Les trois groupes ont été retrouvés et une forte spécificité a été observée entre le groupe F et le sapin, et entre le groupe S et l'épicéa. Dans la formation mixte, la fréquence des ISG n'était pas liée à celle de leur hôte principal : malgré la forte présence du sapin, les spores F représentaient seulement 11% des spores récoltées, probablement à cause de la récente implantation du sapin dans cette forêt. Aucune chimère nucléaire-mitochondriale S–P n'a été detectée et ceci indiquerait un flux génétique limité entre ces ISGs.

Mots clés : *Heterobasidion annosum*, spécificité d'hôte, ISGs, flux génétique, PCR, Alps.

Introduction

Heterobasidion annosum (Fr.) Bref. is the most serious forest pathogen on conifers in the north temperate zone (Woodward et al. 1998). The fungus infects its hosts by means of spores through fresh cut stumps or wounds and spreads through the root system to uninfected surrounding trees, causing root and (or) butt rot.

This basidiomycete is regarded as a species complex, including at least five allopatrically and sympatrically differentiated intersterility groups (ISGs) (Otrosina et al. 1993): the S, F, and P ISGs in Eurasia and the S and P ISGs in North America (Korhonen 1978; Chase and Ullrich 1988; Capretti et al. 1990). ISGs have recently been proposed as taxonomic species with the following names: *Heterobasidion parviporum*

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P. Gonthier and G. Nicolotti.[1] University of Torino, Department for the Exploitation and Protection of Agricultural and Forestry Resources (Di.Va.P.R.A.), Plant Pathology, Via L. da Vinci 44, I-10095, Grugliasco, Italy.
M. Garbelotto. University of California, Berkeley, Department of Environmental Science, Policy and Management, Ecosystem Sciences Division (ESPM-ES), Berkeley, CA 94720, U.S.A.
G.C. Varese. University of Torino, Department of Plant Biology, Viale Mattioli 25, I-10125, Torino, Italy.

[1]Corresponding author (e-mail: nicolotti@agraria.unito.it).

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Niemelä & Korhonen (ISG S), *Heterobasidion abietinum* Niemelä & Korhonen (ISG F), and *Heterobasidion annosum* (Fr.) Bref. (ISG P) (Niemelä and Korhonen 1998). In the *H. annosum* complex, interfertility is determined by at least five genes. Homozygosity for a "+" allele at any of the five loci is a prerequisite to the formation of a dikaryon (Chase and Ullrich 1990*b*).

ISGs are biological species among which gene flow is almost absent (Burnett 1983). Although in vitro experiments have shown that in *H. annosum* intersterility barriers are not complete (Chase and Ullrich 1990*a*; Korhonen et al. 1992), in nature sexual incompatibility seems to be quite strict (Otrosina et al. 1992; Stenlid et al. 1994). Several studies have shown absence of inter-ISG gene flow in Europe (Stenlid and Karlsson 1991; Garbelotto et al. 1998). Hybrids among ISGs (e.g., S–P hybrids) have been found only once in North America (Garbelotto et al. 1996*b*). Negative selection of hybrids is one of the mechanisms involved in sympatric speciation processes, since hybridization may result in progeny less fit than either parental types in their respective preferred niches (Butlin 1989; Garbelotto et al. 1996*a*).

Heterobasidion annosum has been reported from more than 200 species of woody plants, but it is most commonly found in Europe on Norway spruce (*Picea abies* (L.) Karst.), pines, and silver fir (*Abies alba* Mill.) trees. Intersterility barriers in a species complex are often associated with the development of host specificity or preference (Burnett 1983). In Europe *H. annosum* P type is typically associated with mortality of trees in the genus *Pinus* (i.e., mostly of *Pinus sylvestris* trees), but it can also be found on *Picea*, *Juniperus*, and even on some deciduous trees (i.e., *Betula*). ISG S is commonly associated with butt rot on spruce, but occasionally it may kill *P. sylvestris* saplings and attack exotics. The F type of *H. annosum* causes, according to the provenance, butt or root rot on trees of the genus *Abies* (i.e., mostly of *A. alba*), but it has also been reported from other coniferous and deciduous genera (Korhonen et al. 1998).

The European distribution of ISGs reflects that of the forests of their native main hosts. ISG S is found from northern parts of Finland to Bulgaria, France, and the southern Alps. ISG P is found from southern Italy to central Sweden, while ISG F has been found in fir forests of the Italian Apennines, the Alps, the Balkans, central France, and recently in Poland (Korhonen et al. 1998; Kowalski and Lakomy 1998). Where the host ranges overlap (i.e., in the Alps), all three ISGs can be found (Barzanti and Capretti 1997; La Porta and Ambrosi 1998; La Porta 1999). However, little is known about the degree of host specificity of each ISG in individual natural stands. The issue of host specificity is complex because often *H. annosum* ISGs can be found on hosts that are not considered to be the preferential ones, especially in areas outside the natural distribution of the host species (Korhonen et al. 1992, 1998; Capretti et al. 1994; Korhonen and Piri 1994; Barzanti and Capretti 1997; Vasiliauskas and Stenlid 1998). In general, it would be more appropriate to use the term host preference than the term host specificity in the case of the *H. annosum* complex.

Disease development is related to site history. Incidence of *H. annosum* is generally higher in planted stands than in natural forests (Graber 1994) and increases with successive rotations (Stenlid and Redfern 1998). Sometimes, ISGs are found in stands where their preferred hosts are absent. This observation is generally attributed to the presence of such hosts in previous rotations or to the adaptation of an ISG to a secondary host (Korhonen et al. 1998). This observation also underlines the fact that forests should not be considered as stable coenoses, since they are subject to slow and continuous evolutionary processes and to more rapid changes due to rare natural catastrophic events or to human activities. We define as dynamic those forests where stand structure and composition have been undergoing rapid changes in association with human forest management. There is still limited information on how forest dynamic processes may affect the population and ISG structure of *H. annosum*.

Studies on pathogen specificity in forest habitats have been executed mostly by sampling colonized wood and (or) fruit bodies. Our approach has been to determine ISG composition from the air spora, because of the essential role played by the spores in the biology of *H. annosum* (Rishbeth 1951; Swedjemark and Stenlid 1993; Vasiliauskas and Stenlid 1998; Garbelotto et al. 1999). One of the added benefits of this approach is the reduced bias due to nonsystematic and (or) incomplete sampling, which often occurs when results are based on collections from hosts.

The objectives of this study in the northwestern Alps were the following: (*i*) to investigate the presence of different *H. annosum* ISGs and the correlation between ISG and preferential host frequencies in stable natural pure spruce and fir forests (i.e., forests with a stable tree host composition); (*ii*) to compare ISG frequencies in adjacent forests that differ in tree composition; (*iii*) to assess the relationship between host composition and ISG structure of the pathogen in a mixed dynamic spruce–fir forest (i.e., a forest whose tree species composition is changing rapidly); and (*iv*) to study the potential for gene flow among ISGs present in the same forest by searching for fungal genotypes bearing the nucleus of one ISG and the mitochondrial genome of another ISG.

Materials and methods

Study sites and sample collection

The experiments were performed in 1998–1999 in four natural forests. A pure fir forest in Chiusa Pesio (Cuneo), a forest with about 95% fir (Jovençan, Aosta), a pure spruce forest (Charvensod, Aosta), and a mixed spruce–fir dynamic coenosis (Aymavilles, Aosta) were selected for this study. The last three stands were within 5 km of one another on the same mountain slope, thus minimizing variability due to distance or aspect. In the dynamic forest, fir has been replacing spruce over the last 40 years, turning a pure spruce stand into a mixed spruce–fir coenosis (Table 1).

In each forest, two permanent plots were established approximately 100 m from each other. Each plot was characterized by 3 transects, each including 3 collection points (Fig. 1). The transects crossed, according to the forest density, between 6 and 9 trees (with a diameter at breast height (1.3 m) >17.5 cm).

Spores of *H. annosum* were trapped using the wood-disk exposure method (James and Cobb 1984), modified as follows. Wood disks, 11–13 cm diameter and about 1.5 cm thick, cut the day before their exposure in the forest from living, healthy spruce (sites 3 and 4) and fir (sites 1, 2, and 4), were sprayed after bark removal with 65% ethanol. Disks were placed singly in Petri dishes (15-cm diam.) that also contained a sterile piece of filter paper dampened with 3.5 mL of sterile water to prevent drying during exposure.

Table 1. Main features of the forests investigated.

Location	Lat., long.	Exposure	Elevation (m a.s.l.)	Mean annual rainfall (mm)	Tree host		Incidence of disease (%)	Forest structure	Last thinning	Other species
					Species*	% comp.				
1. Chiusa Pesio, Cuneo	44°12′46.78″N, 4°47′36.63″W (Roma 40)	NW	1042	1445	*A. alba* (100)	100	50	Uneven-aged by groups	?	*Corylus, Sambucus, Laburnum, Sorbus*
2. Jovençan, Aosta	45°42′19.48″N, 7°17′1.77″W (Heyford ED50)	N	1090	700	*alba* (105) *P. abies* (110)	95 5	50	Irregular to uneven-aged by groups	1995	*Larix, Juniperus, Populus, Betula*
3. Charvensod, Aosta	45°41′40.93″N, 7°19′35.92″W (Heyford ED50)	NNW	1780	700	*P. abies* (100)	100	30	Irregular to uneven-aged by groups	1997	*Larix, Pinus, Alnus, Sorbus*
4. Aymavilles, Aosta	45°41′25.88″N, 7°15′30.48″W (Heyford ED50)	NNW	1475	700	*alba* (55) *P. abies (115)* *P. sylvestris (80)*	50 40 10	50	Irregular	?	*Larix, Juniperus, Populus, Betula*

*Oldest tree of this species is given in parentheses.

Fig. 1. Placement of spore traps in our permanent plot.

Sets of open Petri dishes, one dish per exposure point, were placed on the ground or on snow for 24 h starting at 8 a.m. One collection per season was performed (April, July, October, and February), during periods with no rain and no wind. Three closed Petri dishes were included as controls at each collection time to check for possible contamination caused by either *H. annosum* isolates already present in the wood disks used as traps or airborne spores that landed on the wood during trap preparation.

After exposure, filter papers were replaced and dampened with 3 mL of sterile water. Disks were then sprayed with a benomyl solution (0.010 g benomyl, 500 µL methanol, 1 L sterile water) and incubated at about 24°C for 10–12 days.

Isolations were made under a dissecting microscope (20× magnification) by transferring infected wood pieces or hyphae of *H. annosum* in its conidial stage (*Spiniger*) from distinct colonies on the wood surface onto Petri dishes filled with a PCNB-based selective medium for *H. annosum* (5 g bacto-peptone, 20 g agar, 0.25 g $MgSO_4$, 0.5 g KH_2PO_4, 190 ppm pentachloronitrobenzene, 100 ppm streptomycin, 2 mL lactic acid 50%, 20 mL ethyl alcohol 95%, 1 L distilled water) (Kuhlman and Hendrix 1962). When three or more colonies per disk were visible, three randomly chosen colonies were isolated. All isolates were subsequently grown at 24°C on 5-cm Petri dishes filled with MEA (20 g malt extract, 20 g glucose, 2 g peptone, 20 g agar, 1 L distilled water).

DNA extractions

DNA extractions were made by the CTAB (cetyltrimethylammonium bromide) extraction method described by Gardes and Bruns (1993) and modified as follows: (*i*) fungal material was prepared by microwaving Petri dishes for about 2 min; mycelia were then harvested with tweezers, blotted with sterile paper, and put into 1.5 mL Eppendorf tubes; and (*ii*) after the ethanol precipitation step, DNA pellets were resuspended in 50 µL of 0.1 TE buffer (1 mM Tris, pH 8; 0.1 mM EDTA, pH 8). A 1:100 standard dilution in PCR water, which had been filtered twice (i.e., 0.4 µM) and autoclaved, was used for each polymerase chain reaction (PCR).

PCR primers and DNA amplification conditions for the mitochondrial ISG-diagnostic TSCP-PCR

A two-step process was used to determine the ISG of 582 spores. First, to distinguish between S and non-S isolates, a taxon specific competitive priming (TSCP) PCR (Garbelotto et al. 1996*b*) was conducted in the ML5–ML6 DNA region of the mitochondrial large ribosomal RNA gene (mt LrRNA) using the primers MLS, MLF, and Mito 5 (Garbelotto et al. 1998) (Fig. 2).

Fig. 2. TSCP-PCR diagnosis in the ML5–ML6 DNA region of the mitochondrial LrRNA gene. Amplicons were electrophoresed on a 2.5% metaphor agarose gel. First and last lanes are DNA molecular standards (100-bp ladder); lanes 4, 10, 17, and 22 are S ISG isolates; the other lanes are non-S ISG isolates; lane 23 is the negative control.

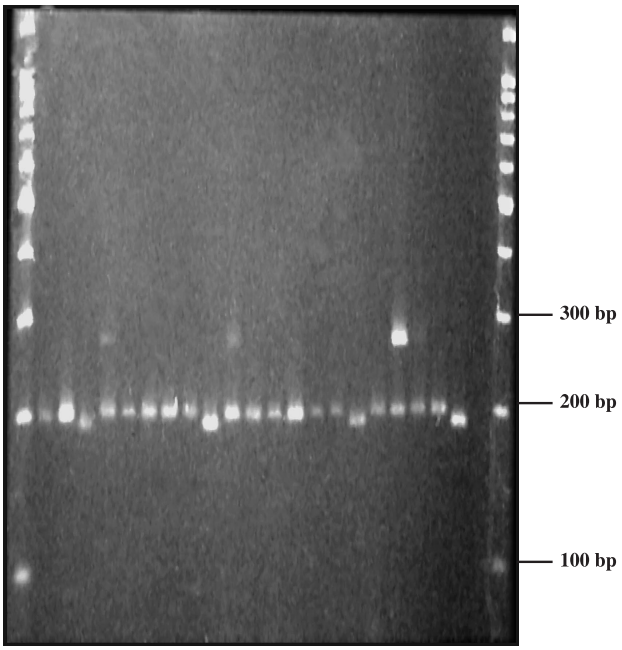
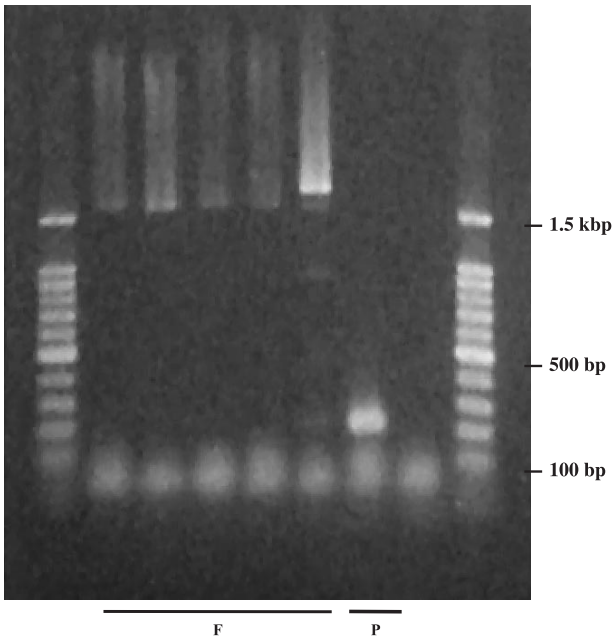


Fig. 3. PCR-mediated intron detection of ISG-specific introns in the ML5–ML6 DNA region of the mitochondrial LrRNA gene. Electrophoresis was performed in 1.5% agarose gels. First and last lanes are DNA molecular standards (100-bp ladder); lanes 2 to 6 are F ISG isolates; lane 7 is a P ISG isolate;: and lane 8 is the negative control.



Since ISG F can be distinguished from the intronless ISG P because of the presence of a 1.6- or 1.8-kb intron in the same region of the mitochondrial DNA (Garbelotto et al. 1998), the second step was to determine the presence of those specific introns. For this purpose, two primers named Mito 7 (5′-GCC AAT TTA TTT TGC TAC C-3′) and Mito 8 (5′-GCG GTG TAA TAA AAT CGG-3′) were designed based on conserved parts of the exon sequence flanking the intron termini (Fig. 3). Amplifications were performed in volumes of 25 μL containing 10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin, 200 μM of each dNTP (U.S. Biochemicals, Cleveland, Ohio), 0.5 units of *Taq* polymerase (Promega, Madison, Wisc.), 0.5 μM of each primer, and 1–25 ng of genomic DNA. Reactions were conducted in a Techne PHC-100 thermal cycler programmed for an initial 1.5 min denaturation at 94°C followed by 35 cycles of denaturation (1 min, 92°C), annealing (1 min, 53°C), and extension (3 min, 72°C). A final extension of 10 min followed the 35 cycles.

Amplification products were analyzed by electrophoresis as follows. For the first step they were run in 2.5% metaphor agarose gels (FMC Bioproducts, Rockland, Maine) in 1× Tris–borate buffer (TBE) at 3 $V \cdot cm^{-1}$ for 3 h, while for the second step they were run in 1.5% agarose gels (Nu-Sieve, FMC Bioproducts, Rockland, Maine) in 1× Tris–acetate buffer (TAE) at 3.4 $V \cdot cm^{-1}$ for 1 h. Both gels were stained with ethidium bromide.

To confirm the molecular typing, mating tests were performed by pairing 10% of isolates randomly chosen from each plot with homokaryotic testers (T4, T5, T6 (provided by Paolo Capretti), A2r, A27r, and A66r) as described by Stenlid and Karlsson (1991).

PCR primers and DNA amplification conditions for the PCR-restriction fragment length polymorphism (RFLP) on ITS

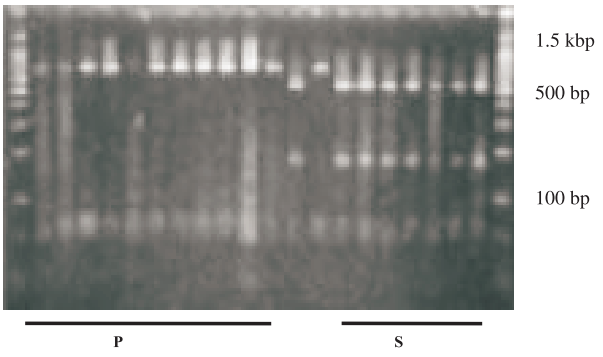
To check for the occurrence of inter-ISG hybridization, we searched for the presence of nuclear–mitochondrial chimeras. This analysis was performed by determining the ISG of all S and P isolates both from mitochondrial and nuclear markers and by comparing results of the two analyses. Although the European S and F ISGs are the two closest related taxa in the *Heterobasidion* complex (Korhonen et al. 1992; Otrosina et al. 1993), we decided to study inter-ISG gene flow between only the S and P ISGs for two reasons. First, it has been shown that there are very strong mating barriers between S and F isolates when the two ISGs are sympatric (Capretti et al. 1990; Garbelotto et al. 1998). Second, data presented in this study clearly indicates little host overlap between the S and the F ISG. In fact, S spores are almost absent in pure fir forests and F spores are absent in pure spruce stands.

The complete internal transcribed spacer (ITS) region of S and P isolates was amplified with the primer combination ITS 1F-ITS 4 (Gardes and Bruns 1993). Amplification conditions were as above. ITS amplicons were digested with the restriction enzyme *Ban*II (Roche Molecular Biochemicals, Indianapolis, Ind.) and loaded onto a 1.8% agarose gel, electrophoresed in 0.5× TAE at 3.4 $V \cdot cm^{-1}$ for 1 h 20 min, and stained with ethidium bromide as described above. While there are no *Ban*II restriction sites in the ITS region of P isolates, there is one restriction site in the ITS of S isolates, resulting in two fragments sized approximately 180 and 510 bp (Fig. 4).

Data interpretation and statistical analysis

Frequencies of ISGs in each study site were expressed both (*i*) as the percentage of spores of either ISG of the total number of spores collected and (*ii*) as the percentage of disks hit by at least one spore of either ISG of the total number of disks colonized by *H. annosum* spores. Data were used cumulatively for the four collection times to best express the yearly potential of each ISG to sporulate. We are currently investigating seasonal variations in the sporulation activity of each ISG.

Fig. 4. PCR RFLP in the ITS 1F-ITS 4 DNA region of the nuclear genome. Amplicons were digested with the restriction enzyme *Ban*II and electrophoresed in 1.8% agarose gels. First and last lanes are DNA molecular standards (100-bp ladder); lanes 2 to 11 and 13 are P ISG isolates; lanes 12 and 14 to 20 are S ISG isolates.



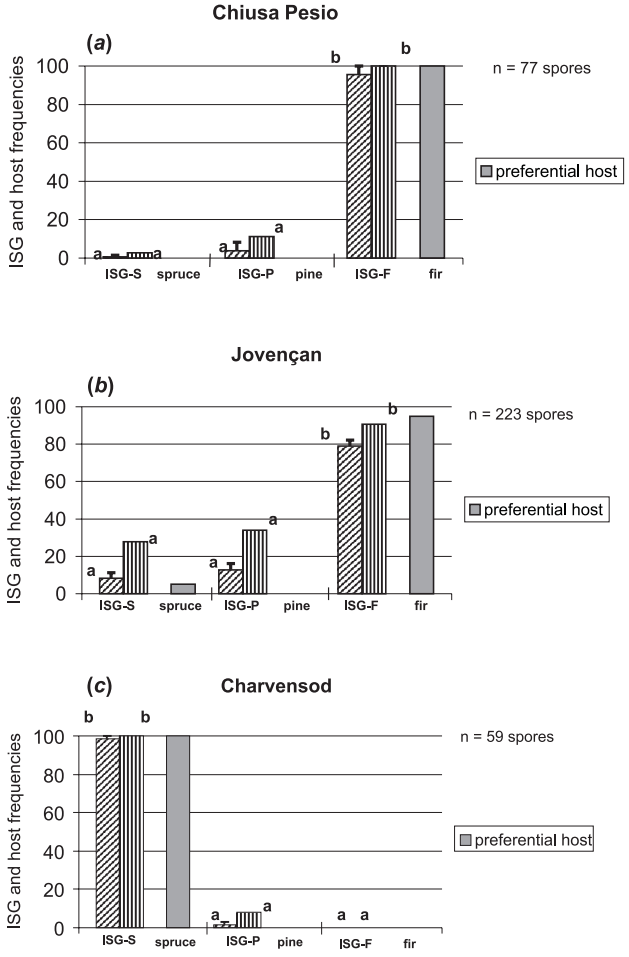
Statistics for the comparison among ISG frequencies within and for either ISG among the study sites were performed by transect and compared (*i*) by analysis of variance with the Tukey's honestly significant difference (HSD) test and (*ii*) by the nonparametric Mann–Whitney *U* test, using $P = 0.05$ and $P = 0.01$. The correlation between hosts and host-specific ISG frequencies for the evaluation of host specificity in stable and dynamic forests was assessed by the pairwise correlation test. The Spearman's rank-order correlation test and the Kendall's τ test were also performed to analyze host–pathogen correlation (data not shown). Statistical analyses were performed using StatSoft Inc. (1993) and SAS Institute Inc. (1997).

Results

Airborne spores of *H. annosum* were found at all four collection times in Chiusa Pesio, while no spores were trapped in February in the three sites in the Aosta Valley. The highest inoculum density was recorded in the sampling of July in all the forests; data from the two plots within the same forest show significant differences only in Charvensod ($P < 0.05$). Inoculum density ranged from 55 to 109 spores·m^{-2}·h^{-1} in Chiusa Pesio, from 42 to 442 spores·m^{-2}·h^{-1} in Jovençan, from 0 to 169 spores·m^{-2}·h^{-1} in Charvensod, and from 67 to 669 spores·m^{-2}·h^{-1} in Aymavilles. Seventy-seven of 2042 trapped spores were analyzed for the ISG typing in Chiusa Pesio, 223 of 3604 for Jovençan, 223 of 3235 for Aymavilles, and 59 of 714 for Charvensod. ISG frequencies of the yearly cumulative data were always concordant with data from each collection time. Moreover, the percentage of disks colonized by each ISG of the total number of disks exposed in periods when *H. annosum* spores were present generally did not differ significantly between the two plots of each forest. Significant differences ($P < 0.05$) have been recorded exclusively where the abundance of the ISGs was low (i.e., S ISG in Jovençan and F ISG in Aymavilles). No *H. annosum* colonies were observed on control disks.

In three of the four study sites, all three European ISGs were found. ISG frequencies were analogous whether expressed in terms of percentage of spores of each ISG of the total number of spores collected or in terms of disks hit by at least one spore of either ISG of the total number of disks

Fig. 5. Northwestern Alps. *Heterobasidion annosum* ISGs frequencies in pure fir and spruce forests based on spores collected in six transects per forest (see text). Diagonal and vertical line shading respectively show the yearly cumulative percentage of (*i*) spores of each one of the three ISGs and (*ii*) disks hit by at least one spore of each ISG. The variation bars correspond to SD. Columns with the same line shading were compared by ANOVA – Tukey HSD test for (*i*), and by the Mann–Whitney *U* test for (*ii*) ($P \leq 0.01$).



colonized by *H. annosum* spores (Spearman $\rho = 0.9983$, $P < 0.0001$; $\tau = 0.9924$, $P < 0.0001$; pairwise correlation = 0.9592, $P < 0.0001$).

In terms of percentage of spores of each ISG of the total number of spores collected, in almost pure and pure fir forests the frequency of the F ISG ranged from about 80% up to 96% (Figs. 5*a* and 5*b*). Where fir represented 100% of host species susceptible to *Heterobasidion* (Chiusa Pesio), the S and P ISGs had a combined frequency lower than 4%. In fir forests where spruce was sporadically present (Jovençan), S and P frequencies were 8% and 13%, respectively. In the pure spruce forest (Fig. 5*c*), 99% of the spores were S, and no F spores were trapped.

In terms of percentage of disks hit by at least one spore of either ISG of the total number of disks colonized by *H. annosum* spores, the F and S ISG frequency fully match that of their preferential hosts in pure fir and spruce forests (100% F vs. 100% fir in Chiusa Pesio, and 100% S vs.

Table 2. Statistical analysis for the assessment of the correlation between relative frequencies of hosts and host-specific ISGs of *Heterobasidion annosum* in stable and dynamic forests.

	Pairwise correlation test		
Forests	Value	Count (*n*)	*P*
Stable forests			
Chiusa Pesio	0.9996	3	0.018
Jovençan	0.9946	3	0.066
Charvensod	0.9999	3	0.008
Total	0.9922	9	0.001
Dynamic forests			
Aymavilles	0.0194	3	0.9877

100% spruce in Charvensod) (Figs. 5*a* and 5*c*). In the almost pure fir forest, the F ISG was present in 91% of the disks (vs. 95% fir), while the P and S ISGs were present in 34% and 28% of the disks, respectively (Fig. 5*b*).

Statistics for the comparison between frequencies of host and host-specific ISGs in each forest and, globally, between stable and dynamic forests are given in Table 2 (only host specificity is shown expressed in terms of percentage of spores of each ISG of the total number of spores collected). In the completely pure forests, significant positive correlations ($P < 0.05$) between ISG and preferential host frequencies were found. In the almost pure fir forest in Jovençan the correlation was significant at $P < 0.10$. In the mixed dynamic forest no significant relationship between tree host species and host-specialized ISGs was found. In spite of a relative dominance of fir, the F ISG represented only 11% of the total number of spores collected and 34% of disks colonized by *H. annosum* spores. These frequencies were significantly lower than S frequencies (64% spores, 81% disks) (Fig. 6).

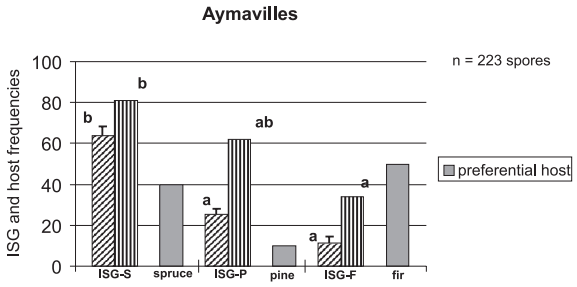
Frequency values of S and F ISGs differed significantly among adjacent forests in the Aosta Valley ($P < 0.01$), while the frequency of P spores showed significant differences only in the comparison between Charvensod and Aymavilles.

The P ISG was the only group present in all the sampled forests. Frequency of P spores, expressed either as a percentage of the total number of spores collected or as disks hit by at least one spore of the total number of disks colonized by *H. annosum* spores, was low in both pure spruce and fir coenoses, while it increased significantly ($P < 0.05$) in the mixed dynamic forest (Aymavilles) where Scots pine trees are present.

Nuclear PCR-RFLP typing on 243 S and P spores indicated the absence of nuclear–mitochondrial chimeras. In 100% of the cases, nuclear typing confirmed the mitochondrial characterization.

Isolates randomly chosen for sexual compatibility tests were all homokaryotic. Interfertility tests fully confirmed the molecular characterization. Using testers T4, T5, and T6 (not collected from the western Alps), results of pairings were difficult to evaluate because of the scant production of clamp connections. The use of testers collected in Aymavilles (A2r, A27r, and A66r) greatly facilitated the ISG typing. No inter-ISG matings were observed in any of the pairings. In about 30% of the cases, the subculturing of the mated isolates was necessary to observe clamp connections reliably.

Fig. 6. Northwestern Alps. *Heterobasidion annosum* ISGs frequencies in a mixed dynamic spruce–fir forest based on spores collected in six transects per forest (see text). Diagonal and vertical line shading respectively show the yearly cumulative percentage of (*i*) spores of each one of the three ISGs and (*ii*) disks hit by at least one spore of each ISG. The variation bars correspond to SD. Columns with the same line shading were compared by ANOVA – Tukey HSD test for (*i*), and by the Mann–Whitney *U* test for (*ii*) ($P \leq 0.01$).



Discussion

All three European ISGs of *H. annosum* have been found in a few countries of central and southern Europe (Korhonen et al. 1998; La Porta et al. 1998), including parts of the Alpine range (Barzanti and Capretti 1997). At the individual forest stand level, the concomitant presence of two ISGs has been reported both in southern and in northern Europe (Capretti et al. 1990; Munda 1994; Korhonen and Piri 1994). Recently, the first case of co-occurrence of all European *H. annosum* groups in a very large planted stand, encompassing different forest types, has been reported for the eastern Alps (La Porta 1999). In our study, all three ISGs have been found within a few square metres in the same forest. To our knowledge, this is the first evidence of co-occurrence of all ISGs in natural coenoses.

Heterobasidion annosum air spora was present year-round in Chiusa Pesio. Spores were absent in the winter sampling at all three study sites in the Aosta Valley. Lack of sporulation during the winter has been reported from the Scandinavian countries (Kallio and Hallaksela 1979; Brandtberg et al. 1996) and may be due to low winter temperatures (Morrison et al. 1986, in Redfern and Stenlid 1998). In contrast, winter sporulation in areas like Chiusa Pesio may be linked to higher temperatures and rainfalls.

In stable pure spruce and fir forests, host preference of the S and F ISGs was very strict: at least 96% of the trapped spores belonged to an ISG whose preferred host was present in each forest and there was a direct proportionality between the percentage of ISG and the percentage of each ISG preferential host in each forest. These results, obtained with a novel and systematic sampling approach, are in accordance with those obtained by direct sampling (Korhonen et al. 1992; Capretti et al. 1994; Munda 1994; Tsopelas and Korhonen 1996; La Porta et al. 1998). In the Alpine area, Barzanti and Capretti (1997) reported that 80.8% of the isolates from *P. abies* belonged to the S ISG and that 93.8% of the isolates from *A. alba* were typed as F.

Low frequencies of nonspecific ISGs (e.g., S and P) in the pure fir forest of Chiusa Pesio, where the host species present appears to be susceptible only to the F group, may be due to spores from local basidiomes on unusual hosts (i.e., *A. alba* and (or) deciduous species). These low frequencies can also be interpreted as background contamination from spores coming from other forests. Sometimes *Heterobasidion* spores have been reported to travel long distances (Kallio 1970; Stenlid 1994). It should be noted that such background levels are very low and do not appear to contribute significantly to the make up of local *Heterobasidion* populations. Such low levels of spore movement between different forests may be relevant only when dealing with the colonization of areas where *H. annosum* may not yet be present. Although the frequency of the P ISG is also low in Jovençan (13%) and Charvensod (1%), P establishment in these two locations can be linked directly to the presence of spruce, a well-documented alternate P host (Korhonen et al. 1992; Korhonen and Piri 1994; Hanso et al. 1994; Vasiliauskas and Stenlid 1998). Direct stump sampling in Charvensod (data not shown) has confirmed the significant presence of P isolates in spruce stumps at that site.

Although the presence of spruce as an alternative host for the P ISG allows for the establishment of P isolates in forests where the main P-host, Scots pine, does not grow, we measured a significantly higher frequency of the P ISG in Aymavilles, a forest where Scots pine is present. This finding is in agreement with reports from Fennoscandia (Korhonen et al. 1992). We should, however, note that in spite of the presence of a reportedly aggressive pine pathogen (the P ISG), Scots pines in the Aosta Valley are rarely symptomatic. The lack of symptoms may be explained by the partial resistance to the pathogen reported for Scots pine growing in the Alpine region (Anselmi and Minerbi 1989; Capretti et al. 1990; La Porta and Ambrosi 1998).

Establishment and survival of P isolates in the forests of the western Alps may be explained by a complex scenario, including the presence of not only a potential primary host (Scots pine) and of a proven secondary host (spruce), but also other lesser known or undocumented hosts such as *Betula* and other deciduous trees, *Juniperus*, *Larix*, or other pine species, which are present in our forests. For instance, in a recently completed study, we show that Swiss stone pine (*Pinus cembra*) is a host not only for the S group (Nicolotti et al. 1999) but also for the P group of *H. annosum* (data not shown).

While the ecological adaptation of the P ISG in the Alps is in need of further study, evidence of a more strict host specificity of S on spruce and F on fir is presented in this study. Pure fir and almost pure fir forests harbor an overwhelming majority of F inoculum, and the same is true for S inoculum in pure spruce forests. In our study this strong host–pathogen correlation held true even for two forests (Charvensod and Jovençan) that were different in tree composition, but only 3 km from each other. The apparent lack of spore flow between these two nearby sites and the "background" inoculum information obtained at the isolated pure fir forest of Chiusa Pesio suggest a geographically limited range of dispersal of basidiospores. This information is important from both an ecological and an epidemiological point of view and supports the belief that during thinning operations, the presence of basidiospore-producing fruit bodies increases the risk of stump infection within but not between forests (Stenlid 1994; Möykkynen et al. 1997).

In the mixed spruce–fir dynamic forest there was no direct correlation between the frequency of F and S groups and the frequency of their respective preferential hosts. In that forest, lack of thinning operations since the 1940s has led to the progressive and massive establishment of firs in a spruce stand. It appears that a corresponding "invasive" establishment of the F ISG (11% of all trapped spores) is following the "invasive" establishment of the host species. Nevertheless, there appears to be a lag between the rate of establishment of fir (50% of stand) and that of the F ISG (11%) spores. Primary infection and spread of *H. annosum* occurs by airborne spores (Rishbeth 1951; Otrosina and Cobb 1989; Stenlid 1994; Garbelotto et al. 1994). Large fruit body production and the subsequent massive release of air spora normally occur after colonization of large volumes of substrate. Furthermore, substrate colonization leading to fruit-body production may require several years and may be more likely to occur on larger trees. We expect that as fir trees grow larger and the overall fir biomass increases, so will the population of the fir-specific F ISG.

It has been suggested that the biogeographical history of the main hosts of *H. annosum* may have influenced the genetic structure (Stenlid et al. 1994) and the present distribution of the three European ISGs (Korhonen and Piri 1994). Our results indicate that within a shorter time frame, rapid changes in host composition and past and present silvicultural practices may have an effect on the population structure of this pathogen. For modeling purposes, new and regular samplings in the same plots would give us some important information about the rapidity of changes in the variation of population structure of the fungus and the extent to which that variation is influenced by host dynamic and growth.

Neither nuclear–mitochondrial chimeras (indirect evidence of hybridization) nor hybrids between ISGs S and P (in sampled stumps, data not shown) were found in this study. Nuclear molecular typing and sexual compatibility tests fully confirmed the mitochondrial characterization. Mitochondrial TSCP-PCR and PCR-mediated detection of ISG-specific introns are thus reliable and rapid methods for diagnostic purposes. Lack of hybridization and potential for gene flow among *H. annosum* ISGs in Europe has been discussed already (Stenlid and Karlsson 1991; Garbelotto et al. 1998). Generally, fungi from the same geographical area exhibit strong genetic barriers to interspecific hybridization (Brasier 2000). In vitro interfertility between European S and P groups of *H. annosum* is 4–10% (Korhonen 1978; Stenlid and Karlsson 1991). These mating barriers probably maintain the isolation of the gene pools of the two ISGs (Brasier 1987), conferring optimal adaptation to ecological niches (Dobzhansky 1937).

The potential for hybridization will also depend upon the frequency of niche contact and the ability of any resulting hybrids to compete with the parent species. In the western Alps different niches are in close proximity. In this study we show that spruce, fir, and Scots pine and the three host-associated ISGs are coexisting. While all the main hosts of the three European ISGs coexist in the western Alps, patho-

gen–host specificity or preference ensure that they do not each provide a favorable substrate for all three ISGs. The presence of both S and P isolates on spruce, and the creation of nonselective stumps through logging are examples of true niche overlaps and they increase the chance for hybridization (Garbelotto et al. 1996*b*, 1998). Lack of hybridization between the S and P groups in the Alps may be determined by efficient mating barriers between the two populations reinforced by negative selection on hybrids, as reported for the American S and P groups (Garbelotto et al. 1996*a*).

Results showing strong correspondence between hosts and host-associated ISGs in individual stands and lack of significant spore movement between adjacent coenoses have important implications in forest planning and management. This information, in fact, can be used to determine the usefulness of tree species substitution in areas infested by this pathogen.

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References

Anselmi, N., and Minerbi, S. 1989. Root rots involved in forest decline in Italy. *In* Proceedings of the 7th IUFRO Conference on Root and Butt Rots, Canada, August 1988. *Edited by* D.J. Morrison. Forestry Canada, Victoria, B.C. pp. 503–512.

Barzanti, G.P., and Capretti, P. 1997. Investigations into *Heterobasidion annosum* root rot in the Italian Alps: host trees and intersterility groups. Monti Boschi, **48**: 24–27.

Brandtberg, P.O., Johansson, M., and Seeger, P. 1996. Effects of season and urea treatment on infection of stumps of *Picea abies* by *Heterobasidion annosum* in stands on former arable land. Scand. J. For. Res. **11**: 261–268.

Brasier, C.M. 1987. The dynamics of fungal speciation. *In* Evolutionary biology of the fungi. *Edited by* A.D.M. Rayner, C.M. Brasier, and D. Moore. Cambridge University Press, Cambridge. pp. 231–260.

Brasier, C. 2000. The rise of the hybrid fungi. Nature (London), **405**: 134–135.

Burnett, J.H. 1983. Speciation in fungi. Trans. Br. Mycol. Soc. **81**: 1–14.

Butlin, R. 1989. Reinforcement of premating isolation. *In* Speciation and its consequences. *Edited by* D. Otte and J. Endler. Sinauer Associates, Inc., Sunderland, Mass. pp. 158–179.

Capretti, P., Korhonen, K., Mugnai, L., and Romagnoli, C. 1990. An intersterility group of *Heterobasidion annosum*, specialized to *Abies alba*. Eur. J. For. Pathol. **20**: 231–240.

Capretti, P., Goggioli, V., and Mugnai, L. 1994. Intersterility groups of *Heterobasidion annosum* in Italy: distribution, hosts and pathogenity tests. *In* Proceedings of the 8th IUFRO Conference on Root and Butt Rots, Wik, Sweden, and Haikko, Finland, 9–16 Aug. 1993. *Edited by* M. Johansson and J. Stenlid. Swedish University of Agricultural Sciences, Uppsala, Sweden. pp. 218–226.

Chase, T.E., and Ullrich, R.C. 1988. *Heterobasidion annosum*, root- and butt rot of trees. Adv. Plant Pathol. **6**: 501–510.

Chase, T.E., and Ullrich, R.C. 1990*a*. Genetic basis of biological species in *Heterobasidion annosum*: Mendelian determinants. Mycologia, **82**: 67–72.

Chase, T.E., and Ullrich, R.C. 1990*b*. Five genes determining intersterility in *Heterobasidion annosum*. Mycologia, **82**: 73–81.

Dobzhansky, T. 1937. Genetics and the origin of species. Columbia University Press, New York.

Garbelotto, M., Cobb, F., Bruns, T., Otrosina, W., Slaughter, G., and Popenuck, T. 1994. Preliminary results on the genetic structure of *Heterobasidion annosum* in white fir (*Abies concolor*) root decay fungi. *In* Proceedings of the 8th IUFRO Conference on Root and Butt Rots, Wik, Sweden, and Haikko, Finland, 9–16 Aug. 1993. *Edited by* M. Johansson and J. Stenlid. Swedish University of Agricultural Sciences, Uppsala, Sweden. pp. 227–232.

Garbelotto, M., Popenuck, T., Ratcliff, A., Cobb, F.W., and Bruns, T.D. 1996*a*. Host selection against SP hybrids of *Heterobasidion annosum*: implications for speciation. Phytopathology, **86**: S28-S29.

Garbelotto, M., Ratcliff, A., Bruns, T.D., Cobb, F.W., and Otrosina, W. 1996*b*. Use of taxon-specific competitive-priming PCR to study host specificity, hybridization, and intergroup gene flow in intersterility groups of *Heterobasidion annosum*. Phytopathology, **86**: 543–551.

Garbelotto, M., Otrosina, W., Cobb, F.W., and Bruns, T.D. 1998. The European S and F intersterility groups of *Heterobasidion annosum* may represent sympatric protospecies. Can. J. Bot. **76**: 397–409.

Garbelotto, M., Cobb, F.W., Bruns, T.D., Otrosina, W.J., Popenuck, T., and Slaughter, G. 1999. Genetic structure of *Heterobasidion annosum* in white fir mortality centers in California. Phytopathology, **89**: 546–554.

Gardes, M., and Bruns, T.D. 1993. ITS primers with enhanced specifity for fungi and Basidiomycetes: application to the identification of mycorrhizae and rusts. Mol. Ecol. **2**: 113–118.

Graber, D. 1994. Die Fichtenkernfäule in der Nordschweiz: Schadenausmass, ökologische Zusammenhänge und Waldbauliche Massnahmen. Schweiz. Z. Forstwes. **145**: 905–925.

Hanso, S., Korhonen, K., and Hanso, M. 1994. Attack of spruce and pine by S and P groups of *Heterobasidion annosum* on forest and former agricultural soils in Estonia. *In* Proceedings of the 8th IUFRO Conference on Root and Butt Rots, Wik, Sweden and Haikko, Finland, 9–16 Aug. 1993. *Edited by* M. Johansson and J. Stenlid. Swedish University of Agricultural Sciences, Uppsala, Sweden. pp. 254–259.

James, R.L., and Cobb, F.W. 1984. Spore deposition by *Heterobasidion annosum* in forests of Calif. Plant Dis. **68**: 246–248.

Kallio, T. 1970. Aerial distribution of the root-rot fungus *Fomes annosus* (Fr.) Cooke in Finland. Acta For. Fenn. **107**: 1–55.

Kallio, T., and Hallaksela, A.M. 1979. Biological control of *Heterobasidion annosum* (Fr.) Bref. (*Fomes annosus*) in Finland. Eur. J. For. Pathol. **9**: 298–308.

Korhonen, K. 1978. Intersterility groups of *Heterobasidion annosum*. Commun. Inst. For. Fenn. 94: 1–25.

Korhonen, K., Bobko, I., Hanso, S., Piri, T., and Vasiliauskas, A. 1992. Intersterility groups of *Heterobasidion annosum* in some spruce and pine stands in Byelorussia, Lithuania and Estonia. Eur. J. For. Pathol. **22**: 384–391.

Korhonen, K., and Piri, T. 1994. The main hosts and distribution of the S and P groups of sum. *In* Proceedings of the 8th IUFRO Conference on Root and Butt Rots, Wik, Sweden, and Haikko, Finland, 9–16 Aug. 1993. *Edited by* M. Johansson and J. Stenlid. Swedish University of Agricultural Sciences, Uppsala, Sweden. pp. 260–267.

Korhonen, K., Capretti, P., Karjalainen, R., and Stenlid, J. 1998. Distribution of *Heterobasidion annosum* intersterility groups in Europe. *In Heterobasidion annosum*, biology, ecology, impact and control. *Edited by* S. Woodward, J. Stenlid, R. Karjalainen, and A. Hüttermann. CAB International, Oxon, U.K. pp. 93–104.

Kowalski, T., and Lakomy, P. 1998. A new record of *Heterobasidion annosum* (Fr.) Bref. F group occurrence in Poland in connection with interesting mycological findings. Phytopathol. Pol. **15**: 49–55.

Kuhlman, E.G., and Hendrix, F.F., Jr. 1962. A selective medium for the isolation of *Fomes annosus*. Phytopathology, **52**: 1310–1312.

La Porta, N. 1999. Modelli di diffusione dei gruppi intersterili di *Heterobasidion annosum* in diversi soprassuoli forestali trentini. *In* Atti del 2° Congresso Nazionale di Selvicoltura, Venezia, 24–27 giugno 1998. Vol. IV. pp. 233–254.

La Porta, N., and Ambrosi, P. 1998. Co-occurrence of the intersterility groups of *Heterobasidion annosum* in mixed forests in Trentino – Eastern Italian Alps. *In* Proceedings of the 9th IUFRO Conference on Root and Butt Rots, Carcans-Maubuisson, France, 1–7 Sept. 1997. *Edited by* C. Delatour, J.J. Guillaumin, B. Lung-Escarmant, and B. Marçais. INRA Editions, Versailles CEDEX, France. pp 437–438 (abstract).

La Porta, N., Apostolov, K., and Korhonen, K. 1998. Intersterility groups of *Heterobasidion annosum* and their host specificity in Bulgaria. Eur. J. For. Pathol. **28**: 1–9.

Möykkynen, T., Von Weissenberg, K., and Pappinen, A. 1997. Estimation of dispersal gradients of S- and P-type basidiospores of *Heterobasidion annosum*. Eur. J. For. Pathol. **27**: 291–300.

Munda, A. 1994. Preliminary report on the distribution of *Heterobasidion annosum* intersterility group in Slovenia. *In* Proceedings of the 8th IUFRO Conference on Root and Butt Rots, Wik, Sweden, and Haikko, Finland, 9–16 Aug. 1993. *Edited by* M. Johansson and J. Stenlid. Swedish University of Agricultural Sciences, Uppsala, Sweden. pp. 272–275.

Nicolotti, G., Gonthier, P., and Varese, G.C. 1999. First report of *Heterobasidion annosum* on native European *Pinus cembra*. Plant Dis. **83**: 398 (Disease Note).

Niemelä, T., and Korhonen, K. 1998. Taxonomy of the Genus *Heterobasidion*. *In Heterobasidion annosum*, biology, ecology, impact and control. *Edited by* S. Woodward, J. Stenlid, R. Karjalainen, and A. Hüttermann. CAB International, Oxon, U.K. pp. 27–33.

Otrosina, W.J., and Cobb, FW. 1989. Biology, ecology and epidemiology of *Heterobasidion annosum*. *In* Proceedings of the Symposium on Research and Management of Annosus Root Disease (*Heterobasidion annosum*) in Western North America, Monterey, California, 18–21 April 1989. *Edited by* W.J. Otrosina and R.F. Scharpf. USDA Forest Service, Monterey, Calif. pp. 26–33.

Otrosina, W.J., Chase, T.E., and Cobb, F.W. 1992. Allozyme differentiation of intersterility groups of *Heterobasidion annosum* isolated from conifers in the western United States. Phytopathology, **82**: 540–545.

Otrosina, W.J., Chase, T.E., Cobb, F.W., and Korhonen, K. 1993. Population structure of *Heterobasidion annosum* from North America and Europe. Can. J. Bot. **71**: 1064–1071.

Redfern, D.B., and Stenlid, J. 1998. Spore dispersal and infection. *In Heterobasidion annosum*, biology, ecology, impact and control. *Edited by* S. Woodward, J. Stenlid, R. Karjalainen, and A. Hüttermann. CAB International, Oxon, U.K. pp. 105–124.

Rishbeth, J. 1951. Observations on the biology of *Fomes annosus*, with particular reference to East Anglian pine plantations. (II) Spore production, stump infection, and saprophytic activity in stumps. Ann. Bot. **15**: 1–21.

SAS Institute Inc. 1997. Jmp In 3.2.1. for Windows. SAS Institute Inc., Cary, N.C.

StatSoft Inc. 1993. Statistica 4.5 for Windows. StatSoft Inc., Tulsa, Okla.

Stenlid, J. 1994. Regional differentiation in *Heterobasidion annosum*. *In* Proceedings of the 8th IUFRO Conference on Root and Butt Rots, Wik, Sweden and Haikko, Finland, 9–16 Aug. 1993. *Edited by* M. Johansson and J. Stenlid. Swedish University of Agricultural Sciences, Uppsala, Sweden. pp. 243–248.

Stenlid, J., and Karlsson, J.O. 1991. Partial intersterility in *Heterobasidion annosum*. Mycol. Res. **95**: 1153–1159.

Stenlid, J., and Redfern, D.B. 1998. Spread within the tree and stand. *In Heterobasidion annosum*, biology, ecology, impact and control. *Edited by* S. Woodward, J. Stenlid, R. Karjalainen, and A. Hüttermann. CAB International, Oxon, U.K. pp. 125–141.

Stenlid, J., Karlsson, J.O., and Högberg, N. 1994. Intraspecific genetic variation in *Heterobasidion annosum* revealed by amplification of mini satellite DNA. Mycol. Res. **98**: 57–63.

Swedjemark, G., and Stenlid, J. 1993. Population dynamics of the root rot fungus *Heterobasidion annosum* following thinning of *Picea abies*. Oikos, **66**: 247–254.

Tsopelas, P., and Korhonen, K. 1996. Hosts and distribution of the intersterility groups of *Heterobasidion annosum* in the highlands of Greece. Eur. J. For. Pathol. **26**: 4–11.

Vasiliauskas, R., and Stenlid, J. 1998. Spread of S and P group isolates of *Heterobasidion annosum* within and among *Picea abies* trees in central Lithuania. Can. J. For. Res. **28**: 961–966.

Woodward, S., Stenlid, J., Karjalainen, R., and Hüttermann, A. 1998. Preface. *In Heterobasidion annosum*, biology, ecology, impact and control. *Edited by* S. Woodward, J. Stenlid, R. Karjalainen, and A. Hüttermann. CAB International, Oxon, U.K. pp. xi–xii.