The European S and F intersterility groups of *Heterobasidion annosum* may represent sympatric protospecies

Matteo Garbelotto, William J. Otrosina, Fields W. Cobb, and Thomas D. Bruns

Abstract: In those regions of Europe where they coexist, the F and S intersterility groups (ISGs) of *Heterobasidion annosum* (Fr.) Bref. are primarily found on *Abies* spp. and *Picea abies* (L.) Karst., respectively. Eighty-three isolates of *H. annosum* were collected from *Abies alba* Mill. from 19 sites in Italy, including 10 *Abies-Picea* mixed conifer stands in the eastern Alps. The ISGs of a subsample of 34 isolates were determined by ISG-diagnostic arbitrary-primed (AP) PCR primers. For a subsample of 16 isolates, including two S isolates from Norway and one S isolate from California, nuclear markers generated by AP-PCR analysis, and mitochondrial markers generated by restriction fragment length polymorphisms and sequencing of the ML5–ML6 region of the mitochondrial large ribosomal RNA gene indicated that, in Europe, *(i)* the F and S ISGs can be found in the same forest stand but they are two genetically distinct units with restricted gene flow between them; *(ii)* each of the two ISGs is monophyletic and may lack strong genetic substructuring in subpopulations; and *(iii)* the two ISGs are closely related to each other and their nearest common close relative is the allopatric S ISG from North America. By combining these results with paleobotanical information and results from previous studies, we postulate a recent sympatric divergence of these two groups driven by differential host specificity and mating barriers.

Key words: species complex, protospecies, sympatric, mating barriers, host specificity.

Résumé: Dans les régions d'Europe où ils co-existent, les groupes interstériles (ISGs) F et *S* de l'*Heterobasidion annosum* (Fr.) Bref. se retrouvent surtout sur les *Abies* spp. et le *Picea* abies (L.) Karst., respectivement. Les auteurs ont récolté 83 isolats du *H. annosum* à partir de l'*Abies alba* Mill., sur 19 sites en Italie, incluant 10 stations conifériennes mixtes *Abies-Picea,* dans l'est des Alpes. Une d&termination sur un sous-échantillon de 34 isolats a été effectuée à l'aide du diagnostique ISG basé sur des amorces PCR, amorcées arbitrairement (AP). Dans un sous-échantillon de 16 isolats incluant deux isolats S provenant de Norvbge et un isolat S obtenu en Californie, les marqueurs nucléaires générés par l'analyse AP-PCR, et les marqueurs mitochondriaux générés par les polymorphismes des longueurs de restriction, et le séquençage de la région ML5–ML6 du grand gène ARN mitochondrial ribosomien indiquent qu'en Europe *(i)* les ISGs F et S peuvent se retrouver dans le m&me peuplement forestier, mais se cornportent comme deux unités génétiquement distinctes avec un flux génétique restreint entre les deux; *(ii)* chacun des deux ISGs est monophylétique et pourrait ne pas avoir de fortes sous-structures dans les sous-populations; et *(iii)* les deux ISGs sont Ctroitement reliés l'un à l'autre et leur parent commun le plus rapproché est le groupe S ISG de l'Amérique du Nord. En réunissant ces résultats aves les données paléobotaniques et les résultats d'études antécédentes, les auteurs proposent qu'il y a eu une divergence sympatrique récente de ces deux groupes sous l'influence de spécificité différentielle d'hôtes et de barrières aux croisements.

Mots clés : complexe d'espbces, espbces prototypiques, sympatrique, barrières aux croisements, spécificité d'hôtes.

[Traduit par la rédaction]

Introduction

Speciation is the consequence of the insurgence of reproductive barriers between conspecific populations. Geographic distance and physical barriers may efficiently block genetic flow between allopatric populations, thus leading to the independent evolution of isolated populations into different species (allopatric speciation). The potential for speciation without obvious extrinsic barriers to gene flow is still controversial, but it appears to be a viable hypothesis substantiated by theoretical models when two conspecific populations display a different habitat preference (Levine 19.53; Maynard Smith 1966). In this case, ecological adaptation would play a fundamental role in separating two populations in different niches. Maynard Smith (1966) has shown that genetic isolation leading to sympatric speciation is possible in the following scenario: two populations must be independently partitioned in two clearly separated niches; there must be a strong disruptive selection favoring a different population in each niche; and, finally, there must be strong assortative mating on the basis of niche.

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In this scenario, lack of gene flow may be determined by efficient mating barriers between two populations (reproductive character displacement), by negative selection on hybrids (reinforcement), or by a combination of both mechanisms (Butlin 1989). Diehl and Bush (1989) have shown that premating isolation based on habitat preference may lead to a subdivision of the population and to an enhanced reproductive isolation even in the presence of moderate fitness differences and weak habitat preferences. However, most theoretical models of sympatric processes (Levine 1953; Felsenstein 1981; O'Donald 1960; Endler 1977; Rice 1984) are based on the assumption that one or two loci may regulate habitat preference and mating. Such an assumption may be more appropriate for some species than for others.

Studies on a wide variety of organisms such as mice, amphibians, fishes, and insects have indicated that mating barriers between closely related species or between morpho- or biotypes within a species are much stronger among individuals from areas where both species (or biological species) coexist than among individuals from distant locations (Blair 1964; Hubbs 1961; Mecham 1961; McCarley 1964; Littlejohn 1965; Wasserman and Koepfer 1977; Zouros and D'Entremont 1980). This observation is used to support the idea that hybridization would result in a progeny less fit than either parental types. As a result, individuals that reject mates of the wrong population (or species) will be more fit, and barriers to mating between the two groups should be perfected over time (Levin 1970).

The issue of sympatric speciation has been studied only occasionally in fungi (Otrosina et al. 1993; Vilgalys and Sun 1994). Nevertheless, fungi represent ideal organisms to test the validity of sympatric speciation models for the following reasons: (i) there are several well-known species complexes including biological species with sympatric and allopatric geographic distributions; (ii) many fungal species are either pathogens or symbionts that display a marked host specificity (in these cases, the host clearly represents a specialized habitat); (iii) it is known that a single locus may determine the specific association between the host and a pathogenic fungus (Flor 1956), which enhances the validity of sympatric theoretical models based on one or two loci; and (iv) fungi provide the only known examples of intersterility genes among biological species that display a differential host preference (Chase and Ullrich 1990*a*, 1990b).

The present study examines the validity of the sympatric speciation hypothesis between two European host-specialized biological species of *Heterobasidion annosum* (Fr.) Bref. (Fungi: Basidiomycotina). *Heterobasidion annosum* is a species complex with a worldwide distribution, including the three intersterility groups (ISGs): S, F, and P (Korhonen 1978; Capretti et al. 1990). These groups, although defined on the basis of partial reproductive isolation (hence their definition as intersterility groups) (Korhonen 1978; Chase and Ullrich 1990*a*, 1990*b*), are also characterized by differences in pathogenicity on a range of hosts (Korhonen and Piri 1993; Worrall et al. 1983; Otrosina et al. 1992; Cobb et al. 1989; Capretti et al. 1990; Stenlid and Swedjemark 1988). At least five groups can be distinguished by molecular markers: the North American S and P ISGs (NA S and NA P), and the European S, P, and F ISGs (Eu S, Eu P, and Eu F). In the case of allopatric ISGs, the geographic distance can account for the lack of gene flow between populations, but in the case of sympatric ISGs, mechanisms other than geographic isolation must be considered.

Because of the specificity of ISGs on different hosts, host-mediated processes rather than geographic barriers may be responsible for the partitioning of sympatric *H. annosum* populations into genetically segregated and independently evolving groups (Otrosina et al. 1993). Isozyme and DNA analyses have indicated that some sympatric ISGs, for instance the NA S and NA P or the Eu S and Eu P ISGs, are genetically very different, and can thus be regarded as protospecies. In the case of the NA S and NA P groups, the importance of host-mediated processes has been supported by inoculation experiments, in which S-P hybrids were less fit than either parental type on their specific hosts (Garbelotto et al. 1996*a*, 19963). The large genetic distance between these groups though, makes it impossible to distinguish between a truly sympatric and an alloparapatric speciation process. A stronger case in favor of sympatric speciation is provided by the Eu F and Eu S ISGs. Based on isozyme analysis, Otrosina et al. (1993) have suggested that in Europe, the F ISG is closely related to the S ISG, and may be sympatrically derived from it. Studies based on a limited number of DNA markers have shown that the F and S ISGs are genetically distinguishable (Garbelotto et al. 1993; Fabritius and Karjalainen 1993; Stenlid et al. 1994; La Porta et al. 1993), thus supporting the recognition of this ISG based on "traditional" criteria such as host specificity and intersterility.

The F ISG mating compatibility is low (4-24%) with sympatric S individuals from southern Europe, but it is significantly higher (66-72%) with S individuals from northern Europe (Capretti et al. 1990; Stenlid and Karlsson 1991; Korhonen et al. 1992). Nevertheless, S individuals throughout Europe are highly intercompatible. This differential mating trait supports the general notion that genetic intersterility barriers are selected to maintain species borders only for sympatric populations.

The goals of this study were to verify that the Eu S and Eu F ISGs are genetically isolated in nature, presumably as the result of an ecologically mediated sympatric speciation process and that their phylogenetic positioning within the species complex is in agreement with the sympatric speciation model. The following hypotheses were tested: *(i)* the F and S are two genetically isolated ISGs in spite of their partial interfertility, and may be viewed as two monophyletic protospecies; *(ii)* the F and S ISGs are geographically sympatric and have coexisted as such for a significant period of time; and *(iii)* the sympatric F and S ISGs are the two closest relatives in a complex that includes more distant allopatric relatives.

Materials and methods

Study sites, sampling, and DNA extractions

Nineteen sites were sampled in Italy (Fig. 1). (Additional samples had been previously collected by us or other colleagues from Italy, Norway, and California; see Table 1). With the exception of one European larch (*Larix decidua* Mill.), European silver fir (*Abies alba* Mill.) was the only host species sampled. Our focus was on isolates from silver fir, because there is already a good record of S ISG isolates from Norway spruce (*Picea abies* (L.) Karst.) in all of the alpine areas studied by us (Capretti et al. 1990, 1993). Both stumps and sympto-

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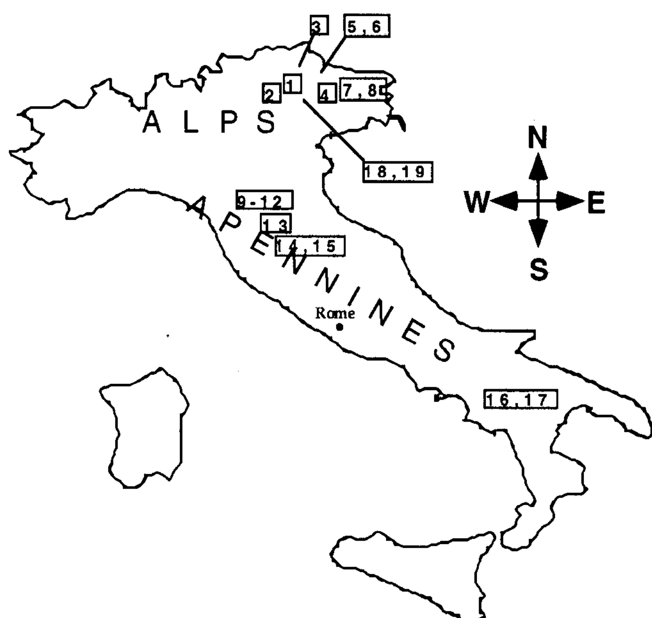
Fig. 1. Map of Italy showing study site locations.



matic or dead trees were sampled. Eighty-three fungal cultures were obtained by isolations on malt extract agar from the context of basidiocarps and from infected wood. Further molecular analysis was performed on a subset of 34 isolates (Table 1), selected to represent all study sites visited. For this subset of Italian isolates and for 12 other isolates from Italy, northern Europe, and North America, DNA extracts were obtained by the CTAB (cetyltrimethylammonium bromide) extraction method described by Gardes and Bruns (1993) modified as follows: (i) a 24:24:1 v/v/v phenol – chloroform – isoamyl alcohol solution was used instead of the 24:1 v/v chloroform – isoamyl alcohol solution; (ii) after the isopropanol precipitation step, the pellet was resuspended in 50 µL of high-salt TE buffer (10 mM Tris, pH 8; 1 mM EDTA, pH 8; 1 M NaCl); and (iii) 100 µL of 95% ethanol was added. DNA eluates were placed at -20°C for 10 min and pelleted at 12 000 x *g* in a microcentrifuge for 15 min. The ethanol DNA precipitation step was followed by an 80% ethanol wash. DNA pellets were resuspended in 25 µL of 0.1× TE (1 mM Tris, pH 8; 0.1 mM EDTA, pH 8). Two microlitres of each DNA extract were electrophoresed in a 1% agarose gel; extracts with known DNA concentrations were used as standards and run in the same gels. Agarose gels were stained with ethidium bromide, and DNA concentrations were determined by visual densitometric comparisons of the extracts with the standards. DNA solutions were diluted in double distilled water to a concentration of approximately 2 ng/µL.

Arbitrary primed (AP) PCR analysis

AP-PCR analysis (Williams et al. 1990; Welsh and McClelland 1990) was used to determine the ISG of the isolates and to generate genetic markers to assess the genetic diversity and the amount of gene flow between the F and the European and North American S ISGs. ISG-diagnostic amplicons were generated by the primer (ATG)5 (5′-ATCGATGATGATGATG-3′) (Garbelotto et al. 1993) under the conditions described by Garbelotto et al. (1993).

AP-PCR analysis was performed as in Garbelotto et al. (1997) on a subset of 16 samples. Isolates chosen for the subset came from different geographic areas and sites (including S isolates from California and Norway) and were selected from distant locations and different forest stands in an effort to maximize the sample of genetic diversity within each ISG studied. The following individual primers and combinations of two primers were used for the AP-PCR analysis on the 16 selected isolates: M13, NL13, ITS2, Ctb6, NS1, NS4, KimQ, NS6, KJ2, KJ2–Ctb6, NS2–NL13, ITS2–NS2, ITS4b–Mb2, ITS1F–Ctb6, NS3–Ctb6, and CNL2F–NS6. Primer sequences are shown in Table 2. Although all of these primers were originally designed to have specific targets in the rRNA genes (see references in Table 2), none were paired with appropriate primers, nor were they used with stringent annealing conditions. Thus, fragments amplified were arbitrary.

For each isolate, polymorphic amplified fragments were scored as present (1) or absent (0). The matrix generated (Table 3) was utilized to calculate a similarity matrix based on the formula

$$[1] \quad \text{Similarity} = F = 2n_{xy}/(n_x + n_y)$$

(Lynch 1990), in which n_x and n_y are the total number of bands in strains x and y, respectively, and n_{xy} is the number of bands common for both strains. The similarity matrix was employed to construct a dendrogram according to the neighbor-joining method using PHYLIP version 3.5c (Felsenstein 1992). Although only the NA S isolate L25R1 was employed in this analysis, it was one of 25 unique S genotypes collected in two different National Forests and extensively used in a previous study in California (Garbelotto 1996, pp. 142-192). Five primers and primer combinations (M13, ITS2–NS2, ITS4b–Mb2, ITS1F–Ctb6, NS3–Ctb6) were used in both studies (data not shown); thus, this AP-PCR data subset could be used to calculate within-ISG similarity for the California S population, and similarity between the isolate used in the neighbor-joining analysis and the other 24 isolates from California.

A discriminant analysis was performed on the three ISGs (European S, Italian F, and North American S) using the CANDISC procedure in the Statistical Analyses System (SAS Institute Inc. 1988) computer program. Seven loci (amplicons 3, 4, 13, 19, 36, 67, and 82 in Table 3) were selected for analysis by the STEPDISC procedure. The seven loci, which were selected for maximum discriminatory power among ISGs, were invariant within ISGs. Loci were scored 1 when a band was present and 0 when absent. The analysis was carried out using all characters with the assumption that only two alleles are present at each locus. Scores for canonical vectors were standardized to a mean of 0 and a within-group SD of 1.

Analysis of the PCR-amplified ML5–ML6 DNA region of the mitochondrial large ribosomal RNA (mt LrRNA)

For all 46 isolates in Table 1, a portion of the mt LrRNA gene was amplified using the MLin3 and ML6 primers (Table 2) (each 0.5 µM/25 µL reaction volume). Amplification reactions 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), 1-25 ng of genomic DNA (6.25 µL of the DNA dilutions) and 0.5 units of *Taq* polymerase (Boheringer Mannheim, Mannheim, Germany). Amplifications were conducted in a Techne PHC-2 thermal cycler programmed for an initial 1.5-min denaturation at 94°C followed by 35 cycles of denaturation (1 min, 95°C), annealing (1 min, 55°C), and extension (1-3 min, 72°C) with the shortest possible ramp time between annealing and extension. The extension time was increased over the course of the program; a 1-min extension was used for the first 13 cycles, 2 min for the next 13 cycles, and 3 min for the last 9 cycles. A final extension of 10 min followed the 35 cycles.

Amplification products were analyzed by electrophoresis in 3% agarose gels (1% nonmodified and 2% Nu-Sieve, FMC Bioproducts, Rockland, Maine) in Tris-acetate buffer (100 mM Tris, 12.5 mM sodium acetate, 1 mM EDTA, pH 8.1) stained with ethidium bromide. MLin3–ML6 amplicons were digested with the following Boheringer Mannheim enzymes: *Alu*I, *Hae*III, *Hin*fI, *Mbo*I, *Msp*I, *Rsa*I, and *Taq*I. Digest reactions were performed by using 7 µL of PCR products in a total volume of 15 µL. Reactions contained 1.5 µL of the restriction buffer recommended by the manufacturer and 3 units of DNA endonuclease. After an incubation of 2 h at 37°C (65°C for *Taq*I), the contents of each reaction were loaded onto a 3% agarose gel, electro-

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Table 1. Isolates analysed in this study, including provenance, symptoms at the sites, ISG determined by RAPDs, and size of the Mlin3–ML6 PCR-amplified mitochondrial large ribosomal RNA (LrRNA) gene.

Site	Symptoms[a]	Provenance[b]	No. on map[c]	Isolate	RAPD ISG	LrRNA[d] (kb)
Kraun (Mezzocorona)	+	TN	1	1000	F	1.84
Mount Misone (Fiave')	-	TN	2	1001	F	—
				1002	F	1.84
Madrutta (Salorno)	-	BZ	3	1010	F	2.05
Valdepena alta (Lorenzago)	-	BL	4	1012	F	2.05
				1014	F	1.84
Selvapiana (Comelico sup)		BL	5	1013	F	1.84
				1016	S	1.84
				1019	F	1.84
Valgrande (Comelico sup)	-	BL	6	1018	S	1.84
				1020	S	1.84
Vielia (Paularo)	-	UD	7	1015	F	1.84
						1.84
Zermula		UD	8	1021	S	1.84
Sestaione (Abetone)	+	PT	9	1036	F	1.84
Secchia	+	PT	10	1024	F	2.05
Mount Maiori (Abetone)	+	PT	11	1039	F	2.05
				1041	F	2.05
				1046	F	1.05
				1056	F	1.84
				1067	F	2.05
Le Regine (Abetone)	+	PT	12	1022	F	1.84
				1038	F	1.84
Bosco del Teso (Maresca)	+	PT	13	1030	F	1.84
Masso del Diavolo (Vallombrosa)	+	FI	14	1034	F	1.84
				1035	F	1.84
				1040	F	1.84
Metato (Vallombrosa)	+	FI	15	1029	F	1.84
Site 1	++	PZ	16	1047	F	—
				1043	F	—
Site 2	++	PZ	17	1064	F	1.84
(Marsico Nvo)				1028	F	1.84
Other isolates						
Norway (K. Venn legit)				211.3	S	1.84
Norway (K. Venn legit)				263.3	S	1.84
Italy (K. Korhonen legit)				429	S	1.84
Italy (K. Korhonen legit)				430	S	1.84
Germany (K. Korhonen legit)				435	S	1.84
Italy (K. Korhonen legit)				439	F	2.05
Italy (K. Korhonen legit)				443	F	1.84
Italy (K. Korhonen legit)				446	F	1.84
Italy (K. Korhonen legit)				447	F	1.84
Norway (K. Venn legit)				172.1	P	0.44
Norway (K. Venn legit)				340	P	0.44
Lassen National Forest, California				L2.5R1	S	1.80

[a]Symptoms at the site: –, only saprobic activity; +, moderate pathogenic activity; ++, extreme pathogenic activity.
[b]The following are Italian provinces: TN, Trento; BZ, Bolzano; UD, Udine; PT, Pistoia; FI, Firenze; PZ, Potenza. Names in parentheses are the townships in which the sites were located.
[c]Sites 1-8 are in the Alps (two more sites (18, S. Martino di Castrozza, TN, and 19, Mezzano, TN) were visited but no collections were made); sites 9-13 are in the northern Apennines; sites 14 and 15 are in the central Apennines; and sites 16 and 17 are in the southern Apennines. See Fig. 1.
[d]PCR amplified with primers Mlin3 and ML6 (see text). —, did not amplify.

phoresed, and stained with ethidium bromide as described above. Similarity between RFLPs were calculated only for amplicons of identical size, employing the same eq. 1 (Lynch 1990) used in the AP-PCR analysis.

Two P isolates (one from California and one from Norway), six S isolates (two from Norway, two from Italy, and two from California), and four F isolates were selected, and their *ML5–ML6* or *MLin3–ML6* DNA region was sequenced. For the intronless Eu P isolates the

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Table 2. Sequences of DNA oligonucleotide primers used for the arbitrary primed (AP) PCR analysis.

Primer	Sequence (S-3')	Reference
NS1	GTAGTCATATGCTTGTCTC	White et al. 1990
NS2	GGCTGCTGGCACCAGACTTGC	White et al. 1990
NS4	CTTCCGTCAATTCCTTTAAG	White et al. 1990
NS6	GCATCACAGACCTGTTATTGCCTC	White et al. 1990
ITS2	GCTGCGTTCTTCATCGATGC	White et al. 1990
ITS4b	CAGGAGACTTGTACACGGTCCAG	Gardes and Bruns 1993
M13	GAGGGTGGCGGTTCT	Stenlid et al. 1993
ML5	CTCGGCAAATTATCCTCATAAG	White et al. 1990
ML6	CAGTAGAAGCTGCATAGGTC	White et al. 1990
MLin3	CGACACAGGTTCGTAGGTAG	Li 1995
MLS	AAATTAGCCATATTTTAAAAG	This study
MLF	TAAAAATTTAAATTAGCCATAA	This study
Mito5	TAAGACCGCTATA(T/A)ACCAGAC	This study
KimQ	ACGCCTCTAAGTCAGAAT	Kim et al. 1992
KJ2	GCTTGAAATTGTCGGGAGGG	T. Bruns, unpublished data
CNS3.6	AATGAAGTCATCCTTGGCAG	T. Bruns, unpublished data
NL13	CAACGCAACTTTCATGCACG	T. Szaro, unpublished data
CNL2F	GTTTCCCTTTTAACAATTTCAC	White et al. 1990
Ctb6	GCATATCAATAAGCGGAGG	T. Bruns, unpublished data
Mb2	GTGAGTTTCCCCGTGTTGAG	M. Berbee, unpublished data

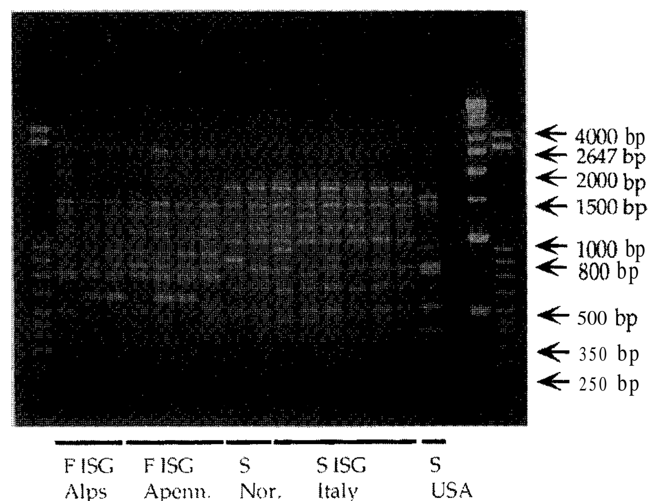
whole molecule was sequenced; for isolates with introns, sequences were obtained until the insertion points of the first intron. Variable portions of the 5′ ends of each intron were sequenced as well. Sequences were obtained with an ABI Prism 377 DNA Sequencer (Perkin-Elmer Co., Foster City, CA, 94404), following the manufacturer's instructions. Sequences were aligned using the program "Sequence Navigator" (Perkin-Elmer Co., Foster City, CA 94404).

Design of taxon-specific primers and conditions for taxon-specific competitive-priming (TSCP) PCR

Base substitutions between the two ISGs were used to design primers (MLS and MLF; Table 2, Fig. 5) that would preferentially amplify a portion of the ML5–ML6 mt LrRNA region from one of the two ISGs and could be used for diagnostic purposes in taxon-specific competitive-priming (TSCP) PCR (Garbelotto et al. 1996). The two primers overlapped partially, and their 3' ends were only nine base pairs apart. When used in combination with the primer ML6, the MLS and MLF primers would respectively amplify a 1.844-kilobase (kb) S-specific fragment or a 1.853-kb F-specific fragment. To enhance the detection of the small size difference between the two ISG-specific amplicons, a primer called Mito5 (Table 2) was designed based on a conserved part of the ML5–ML6 sequence which occurs just 5' the area of the first intron insertion site was found. When used in combination with Mito5, MLS and MLF would produce a 195 base pair (bp) F-specific fragment and a 185-bp S-specific fragment, respectively.

The three primers, MLS, MLF (each 0.16 μM/25 μL reaction volume), and Mito5 (0.5 μM/25 μL reaction volume) were concomitantly used in TSCP-PCR (Garbelotto et al. 1996*a*). All amplification reactions were performed as described above for the ML5 or MLin3–ML6 amplifications, except that the annealing temperature was lowered to 50°C. Amplification products were analyzed by electrophoresis in 5% agarose gels (2% non-modified and 3% Nu-Sieve, FMC Bioproducts, Rockland, Maine) as described above.

Fig. 2. A 1.5% agarose gel showing arbitrary-primed (AP) PCR results obtained with the primer NL13 for 16 isolates of *Heterobasidion* annosum. First and two last lanes are molecular standards from Sigma (St. Louis, Mo.) (first and last lanes are 50-bp ladders; second to last is a 1-kb ladder). Lanes 2-8 are F ISG isolates 1002, 1010, 1019, 1024, 1029, 1047, 1049; lanes 9-16 are European S ISG isolates 211, 263, 429, 430, 1016, 1018, 1020, 1021; lane 17 is North American S ISG isolate L25.R1; lane 18 is an empty lane where a water control was electrophoresed.



Results

Distribution, ISG, and pathogenicity of *H. annosum* on silver fir in Italy

The 34 Italian isolates used in this study were a subsample of 84 isolates obtained from silver fir and larch at eight sites in the Alps and at nine sites in the Apennines. At all sites, the fungus fruited abundantly on stump tops, on the root collar of trees and stumps, and in connection with fine roots in the duff layer. The type of decay observed in stumps and trees infected by *H. annosum* can be best described as a sapwood white rot. Effects of *H. annosum* at the study sites varied. Three types of effects were observed (Table 1): (i) *H. annosum* ap-

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Table 3. Presence (1) and absence (0) of AP-PCR bands for 16 F and S isolates of *Heterobasidion annosum* from Italy, Norway, and North America.

Isolate, provenance, and ISG[a]	AP-PCR fragments[b]
1002 IF	1 1 0 0 0 0 1 0 0 1 0 0 1 0 0 0 1 0 1 0 0 0 1 1 0 1 1 0 0 1 1 1 0 0 0 0 1 0 1 0 1 1 0 0 1 0
1010 I F	1 0 0 0 0 0 1 0 0 1 0 1 1 0 1 0 1 0 1 0 0 0 0 1 0 1 1 1 0 1 1 1 0 0 0 0 1 0 1 0 1 1 0 0 1 0
1019 IF	1 0 0 0 0 0 1 0 0 1 0 1 1 0 1 0 1 0 1 0 0 0 0 1 0 0 1 0 0 1 1 1 0 0 0 0 1 0 x 0 1 1 0 0 1 0
1024 IF	1 0 0 0 0 0 1 0 0 1 0 1 1 0 1 0 1 0 1 0 0 0 0 1 0 1 1 0 0 1 1 1 0 0 0 0 1 0 1 0 1 1 0 0 1 0
1029 IF	1 0 0 0 0 0 1 0 0 1 0 1 1 0 1 0 1 0 1 0 0 0 0 1 0 1 1 0 0 1 1 1 0 0 0 0 1 0 1 0 1 1 0 0 1 0
10471F	1 0 0 0 0 0 1 0 0 1 0 1 1 0 1 0 1 0 1 0 0 0 0 1 0 0 1 0 0 1 1 1 1 0 0 0 1 0 1 0 1 1 0 0 1 0
1049 IF	1 0 0 0 0 0 1 0 0 1 0 1 1 0 0 0 1 0 1 0 0 0 0 1 0 1 1 0 0 1 1 1 0 0 0 0 1 0 1 0 1 X X X 1 X
211 N S	0 0 0 1 0 1 0 1 0 0 1 0 0 1 0 0 1 1 1 1 1 1 0 1 0 1 0 0 0 0 1 0 0 0 1 0 0 1 0 0 1 1 1 1 1 1
263 N S	0 0 0 1 0 1 0 1 0 0 0 1 0 1 0 0 0 1 0 1 1 X 0 X 0 X 1 0 0 0 0 0 0 0 1 1 0 1 0 0 1 0 0 1 0 1
429 I S	0 0 0 1 0 1 0 1 1 0 0 1 0 1 0 0 1 1 0 1 1 1 0 1 0 1 1 0 0 0 1 0 0 0 1 0 0 x 0 0 1 0 0 0 1 1
430 I s	0 0 0 1 0 1 0 1 0 0 0 0 1 1 0 0 0 1 0 1 1 1 0 1 0 1 1 0 0 0 0 0 0 0 1 0 0 1 0 0 1 0 0 0 0 1
1016 IS	0 0 0 1 0 1 0 1 0 0 0 1 0 1 0 0 0 1 0 1 1 1 0 1 0 1 1 0 0 0 0 0 0 0 1 1 0 1 0 0 1 1 0 1 0 1
1018 IS	0 0 0 1 0 1 0 1 0 0 0 1 0 1 0 0 0 1 0 1 1 1 0 1 0 1 1 0 0 0 0 0 0 0 1 0 1 1 0 0 1 0 0 1 0 1
1020 I s	0 0 0 1 0 1 0 1 0 0 0 1 0 1 0 0 0 1 0 1 1 1 0 1 0 1 1 0 0 0 0 0 0 0 1 0 1 1 0 0 1 0 0 0 0 1
1021 IS	0 0 0 1 0 1 0 1 0 0 0 1 0 1 0 0 0 1 0 1 1 1 0 1 0 1 1 0 0 0 0 0 0 0 1 0 1 1 0 0 1 0 0 1 1 1
L2 NA S	0 0 1 0 1 0 0 0 1 0 0 1 0 0 0 1 1 0 1 0 1 0 1 1 1 1 1 1 1 0 0 0 0 1 1 0 1 0 0 1 0 X X X X X

[a]I, Italy; N, Norway; NA, North America; F, F ISG; S, S ISG.
[b]X, missing data.

peared to to be limited to saprobic activity on stumps and to cause no mortality (e.g., all alpine sites but one); (ii) *H. annosum* appeared to cause mortality and windthrows in individuals or small clusters of adjacent trees (e.g., central and northern Apennines and the Kraun alpine site); *(iii) H. annosum* appeared to cause large mortality centers, affecting seedlings and trees of all age-classes (e.g., southern Apennine sites).

DNAs from all isolates were extracted and successfully amplified. AP-PCR amplification patterns indicated that all 21 isolates from the Apennines belonged to the F ISG. Of 13 isolates collected in the Alps, 9 were typed as F and 4 as S. The four S isolates were found at three sites in the two easternmost provinces visited (Belluno and Udine). All were collected from silver fir stumps, and in one case, S and F isolates were retrieved from the same stump. In two areas, S and F isolates were found in sites just a few hundred metres from each other (Selvapiana and Valgrande and Vielia and Zermula, see Table 1), well within the dispersal range of basidiospores of *H. annosum.* Furthermore, S isolates are commonly found on spruce stumps in all the areas in which only F isolates were collected by us in this study; this was confirmed by further collections performed by us on spruce stumps in the eastern Alps (data not shown).

AP-PCR analyses

A total of 107 polymorphic fragments was produced by AP-PCR analysis (Fig. 2, Table 3). Within-ISG similarity values averaged 89 ± 12% (mean ± SD) for the F ISG, and 83 ± 9% for the Eu S ISG. The average similarity between the S and F ISGs was 35 ± 5.8%. A subset of 25 amplicons was used to determine the within-ISG similarity among the 25 NA S isolates from which the isolate L25R1 had been randomly chosen (data not shown). The average within-ISG similarity was 90 ± 4% among the California isolates, and the average similarity between L25R1 and the other 24 isolates was also 90 ± 3%. Neighbor-joining clustering based on the similarity matrix separated the European F and S ISG isolates into two distinct clades and placed the NA S isolate L25R1 in a third, more distant clade (Fig. 3). The two European ISGs appear to be two monophyletic groups, and Norwegian and Italian S isolates are clustered together.

The discriminant (canonical) analysis indicated that the three ISGs were distinct from one another, and that the Eu S and Eu F ISGs were closer to each other than to the NA S ISG (Fig. 4). All of the alleles selected by the STEPDISC procedure were invariant between Italian and Norwegian S isolates, suggesting a low level of genetic differentiation between populations from the two countries.

Analysis of the PCR-amplified ML5 (MLin3)–ML6 DNA region of the mt LrRNA

Fragments amplified with the MLin3–ML6 primer combination from the European isolates and from two NA S and one NA P isolates varied in size. The two Eu P isolates, 172.1 and 340, produced a 450-bp fragment; all other isolates produced larger fragments, presumably because of the presence of introns. The sizes of the fragments obtained were 1.80 and 2.05 kb for the two NA S isolates and 2.05 kb for the NA P isolate. All Eu S isolates amplified a 1.84-kb fragment, while the F isolates produced either a 1.84- or a 2.05-kb fragment.

Identical amplicons included 1.84-kb fragments from Eu S and F isolates, and 2.05-kb fragments from F and NA S and P isolates. RFLPs of 1.84-kb fragments from Eu S and F isolates were identical for all enzymes tested except for one fragment in the *AluI* digest (96% similarity). The difference was consistent for all 35 S and F isolates tested (Fig. 6) and was determined to be in the exonic sequence (see below). Thus, there was complete correlation between presence–absence of the *AluI* restriction site in this mitochondrial region and ISG as determined by presumed nuclear random amplified polymorphic DNA (RAPD) markers. RFLPs of the 2.05-kb fragment from F isolates displayed 73% similarity with the RFLPs of 2.05-kb fragment from one NA S isolate and 83% similarity with the similarly sized amplicon from a NA P isolate (Table 4).

The complete sequence of the MLin3–ML6 fragment of the

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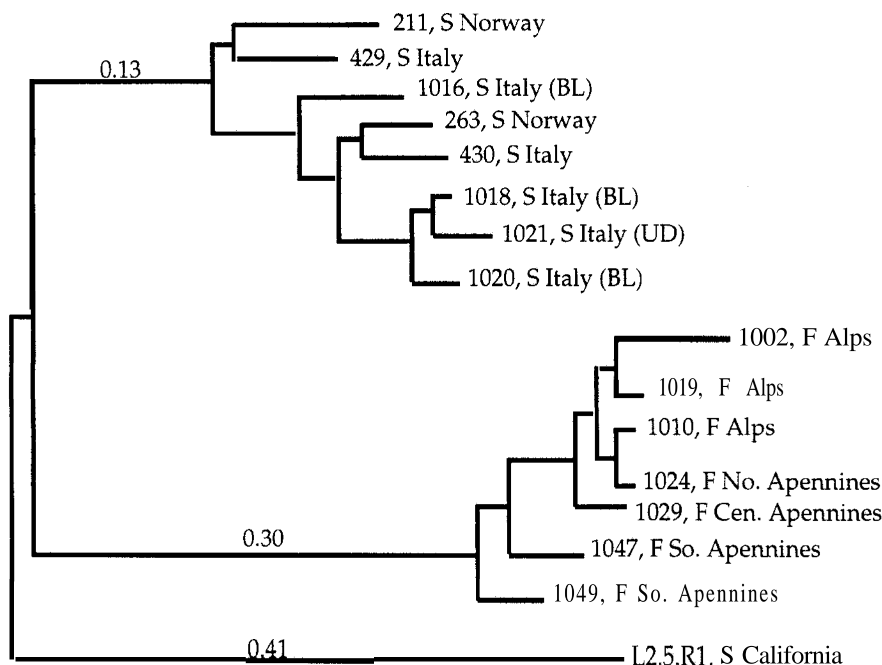
AP-PCR fragments[b]
1 0 0 1 0 0 0 1 0 0 0 1 0 1 0 0 1 0 1 0 0 0 1 0 1 0 0 0 1 0 0 1 0 0 0 1 0 0 1 0 1 0 0 1 0 0 1 0 0 0 0 0 0 0 0 0 0
1 0 0 0 0 0 0 1 0 0 0 1 0 1 0 0 1 0 1 0 0 0 1 0 1 0 0 0 1 1 1 0 0 0 0 1 0 0 1 0 1 0 0 1 0 0 1 0 0 0 0 0 0 0 0 1 0
1 0 0 0 0 0 0 1 0 0 0 1 0 1 0 0 1 0 1 0 0 0 1 0 1 0 0 0 0 1 1 0 0 0 0 1 0 0 1 0 1 0 0 1 0 0 1 0 0 0 0 0 0 0 0 x 0
1 0 0 1 0 0 0 1 0 0 0 1 0 1 0 0 1 0 1 0 0 0 1 0 1 0 0 0 1 1 1 0 0 0 0 1 0 0 1 0 1 0 0 1 0 0 1 0 0 0 0 0 0 0 0 x 0
1 0 0 0 0 0 0 1 0 1 0 1 0 1 0 0 1 0 1 0 0 0 1 0 1 0 0 0 1 0 1 0 0 0 0 1 0 0 1 0 1 0 1 1 0 0 1 0 0 0 0 0 0 0 0 x 0
1 0 0 1 0 0 0 1 0 0 0 1 0 1 0 0 1 0 1 0 0 0 1 0 1 0 0 0 1 1 1 0 0 0 0 1 1 0 1 0 1 0 0 1 0 0 1 0 0 0 0 0 0 0 0 0 0
1 X X X X X X X 0 X X X X X X X 0 1 0 1 0 0 0 1 0 1 0 0 0 1 1 1 0 0 0 0 1 0 0 1 0 1 0 0 1 0 0 1 0 0 0 0 0 0 0 0 1 0
0 1 1 0 0 0 0 1 0 0 0 1 1 0 1 0 1 0 0 0 1 1 0 1 0 1 0 0 1 0 0 0 1 1 0 0 0 1 1 0 1 0 0 0 1 0 0 0 0 1 0 1 1 1 0 0 x
0 0 1 0 0 1 0 0 0 0 0 1 0 1 0 0 1 0 0 0 1 1 0 1 0 1 0 1 0 0 0 0 1 1 0 0 0 1 1 0 1 0 0 0 1 0 0 0 0 1 0 1 1 1 0 0 x
1 1 0 0 0 0 0 1 0 0 0 1 1 0 0 0 1 0 0 0 1 1 0 1 0 1 0 0 1 0 0 1 1 0 0 1 0 1 1 0 1 0 0 0 1 0 0 0 0 1 0 1 1 1 0 0 1
0 1 x 0 0 0 0 0 0 0 0 1 0 1 0 0 1 0 0 0 1 1 0 1 0 1 0 1 0 0 0 0 1 1 0 0 0 1 1 0 1 0 0 0 0 0 0 0 0 1 0 1 1 1 0 0 0
1 1 1 0 0 1 0 0 0 0 0 1 1 0 1 0 1 0 0 0 0 1 0 1 0 1 0 1 0 0 0 0 1 1 0 1 0 1 1 0 1 0 0 0 1 0 0 0 0 1 0 1 1 1 0 1 x
0 0 0 0 0 1 0 1 0 0 0 1 0 1 0 0 1 0 0 0 1 1 0 1 0 1 0 1 0 0 0 0 1 0 0 1 0 1 1 0 1 0 0 1 0 0 0 0 0 1 0 1 1 1 0 1 0
0 0 0 0 0 1 0 1 0 1 0 1 0 1 0 0 1 0 0 0 0 1 0 1 0 1 0 1 0 0 0 0 1 0 0 0 0 1 1 0 1 0 0 0 0 0 0 0 0 1 0 1 1 1 0 1 0
0 0 0 0 0 1 0 1 0 1 0 1 0 1 0 0 1 0 0 1 1 1 0 1 0 1 0 1 0 0 0 0 1 0 0 1 x x x x x x x x x x x x x x x x x x x x x
X 1 0 0 1 0 1 0 1 0 1 0 0 1 0 1 0 1 0 0 0 0 0 1 0 0 1 0 0 0 0 0 0 0 0 1 0 0 1 0 1 0 1 0 0 0 1 1 1 1 1 1 1 1 1 1 0 1

Fig. 3. Neighbor-joining dendrogram of 16 isolates of *Heterobasidion annosum* based on a similarity matrix of AP-PCR data (see text). The ISG of the isolates if followed by the geographic provenance, including the province in parentheses when known. All F isolates are from Italy (No., northern; Cen., central; So., southern). Values are the length of the main branches.



mt LrRNA gene was obtained for the ELI P isolate 340. Partial sequences were obtained for representatives of all other groups (Fig. 5). North American isolates were sequenced using the ML5 primer, located about 50 bp downstream from MLin3. The MLin3–ML6 primer combination is preferable for the amplification of European isolates, because the ML5 primer sequence differs at two positions with the DNA sequence of European isolates (Fig. 5). Most of the sequenced portion of the exon showed no differences among Eu S, P, and F and NA S and P isolates; two nucleotide substitutions between Eu S and the other ISGs in the 5' region were the only exception. One of these two substitutions corresponded to an *Alu*I site (see previous paragraph). These differences were used to design the taxon-specific primers MLS and MLF (Table 2, Fig. 5).

Introns interrupted the exons of 1.80-kb NA S, 1.84-kb Eu S, and 1.84-kb Eu F fragments at the same site (Fig. 5). The 5' sequence of all introns was similar for at least the first 10 bp (Eu S and F had homologous intron sequences for at least 75 bp). Insertion points of introns in the 2.05-kb fragments were 15 bp downstream from the intron insertion point in the smaller fragment. The 2.05-kb NA S and P fragments and the 2.05-kb F fragments had introns inserted at the same point. The 5' end of these introns was homologous for the first 10 bp. Further intron sequences were not obtained, but low RFLPs similarity suggests there may be significant differences be-

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Fig. 4. Discriminant analysis of European F and S, and North American ISGs of ***Heterobasidion annosum***. Dots for the European S and F ISGs represent clusters of seven and eight isolates, respectively; the North American is represented by a single isolate.

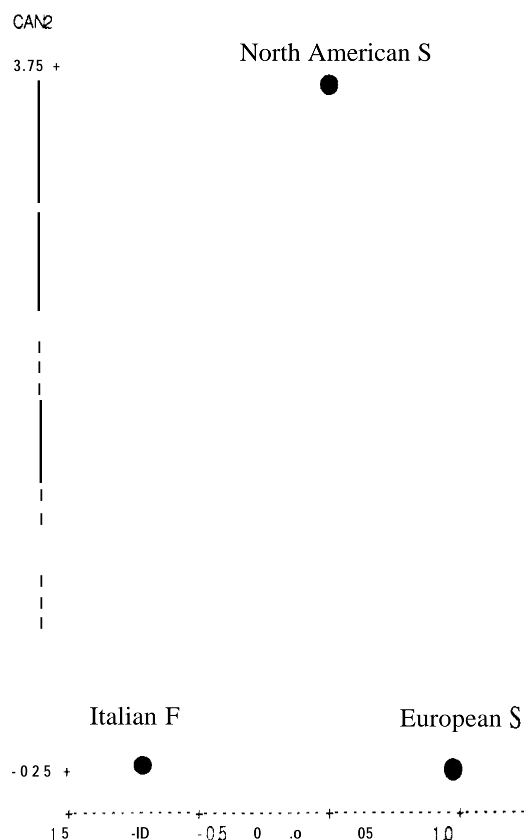

Table 4. RFLPs of the Mlin3–ML6 DNA region of the mitochondrial large ribosomal RNA for amplicons of similar size from isolates of ***Heterobasidion annosum.***

DNA endonuclease	ISG (size of amplicon) F (1.84 kb)	Eu S (1.84 kb)	NAS (2.05 kb)	NAP (2.05 kb)	F (2.05 kb)
*Alu*I	0.52	0.52	0.5	0.5	0.5
	0.43	0.43	0.3	0.3	0.3
	0.32	0.32	0.3	0.3	0.3
	0.25	0.21	0.25	0.25	0.25
	0.18	0.18	0.18	0.18	0.18
			0.15	0.15	0.15
Hue111	1.3	1.3	1.75	1.75	1.75
	0.28	0.28			
	0.14	0.14	0.14	0.14	0.14
	0.12	0.12			
*Hin*fI	0.98	0.98	1.65	1.65	1.6
	0.86	0.86	0.5	0.5	0.55
*Mbo*I	0.7	0.7	1.2	1.2	1.9
	0.47	0.47	0.7	0.7	
	0.32	0.32	0.15	0.15	0.15
	0.28	0.28			
*Msp*I	1.45	1.45	1.5	1.9	1.9
			0.55		
	0.38	0.38		0.15	0.15
*Rsa*I	1.15	1.15	1.15	1.15	1.15
	0.68	0.68	0.9	0.9	0.9
*Taq*I	0.7	0.7	0.95	0.95	0.95
	0.42	0.42	0.5	0.5	0.5
	0.42	0.42	0.38	0.38	0.38
	0.28	0.28	0.28	0.28	0.28

Note: Fragment size (in kb) approximately determined by visual comparison on an agarose gel; small size differences were confirmed with side-by-side comparisons. Eu, Europe; NA, North America.

tween European and North American 2.05-kb amplicons (Table 4). No homologies were found in the 5' portions sequence of introns from 2.05- and 1.80- or 1.84-kb amplicons (Fig. 5).

The concomitant use of the three primers MLS, MLF, and Mito5 resulted in the amplification of diagnostic TSCP-PCR fragments (Fig. 6). All 35 isolates were typed by this method and there was a complete correspondence with ISG typing obtained by AP PCR, which presumably targets nuclear DNA.

Discussion

Our molecular data indicate that although the Eu S and F ISGs are not easily differentiated by morphological and enzymatic characters (Otrosina et al. 1993); they represent two genetically distinct and isolated biological or protospecies. The AP-PCR neighbor-joining tree unequivocally places European S, Italian F, and one North American S isolates in three distinct branches. In the neighbor-joining tree, the Eu S and F ISGs appear as two monophyletic groups without strong genetic substructuring among geographically distinct populations, including Norwegian and Italian S demes. This is also indicated by the high levels (8347%) of intra-ISG AP-PCR average similarity values. While the Italian S and F populations were adequately represented by isolates from a wide range of geographic locations with varying ecological conditions, only two northern European isolates were employed in this study. Although a larger sample of northern European S isolates would have been necessary to obtain conclusive results in the neighbor-joining analysis regarding the genetic structure of the Eu S ISG, the fact that two randomly chosen Norwegian isolates were intermixed with Italian S isolates, rather than being clustered together, may suggest significant levels of intra-ISG gene flow between demes (e.g., between Scandinavian and southern European S populations). This hypothesis is supported by the reportedly large potential dispersal range of *H. annosum* in Europe (Rishbeth 1959; Kallio 1970). A study based on more comprehensive sampling has also shown only moderate levels of genetic substructuring associated with geographic variation in the P and S ISGs (Stenlid et al. 1994).

The presence of two different mitochondrial lineages, as determined by base substitutions in a DNA region of the mt LrRNA gene, also indicates the two ISGs should be considered as distinct genetic entities. This DNA region appears to include one or more variable introns, but most of the exonic sequence is extremely conserved in basidiomycetes, and it is normally used to resolve the taxonomic relationships at the family level (Bruns et al. 1991). Sequencing and TSCP PCR results indi-

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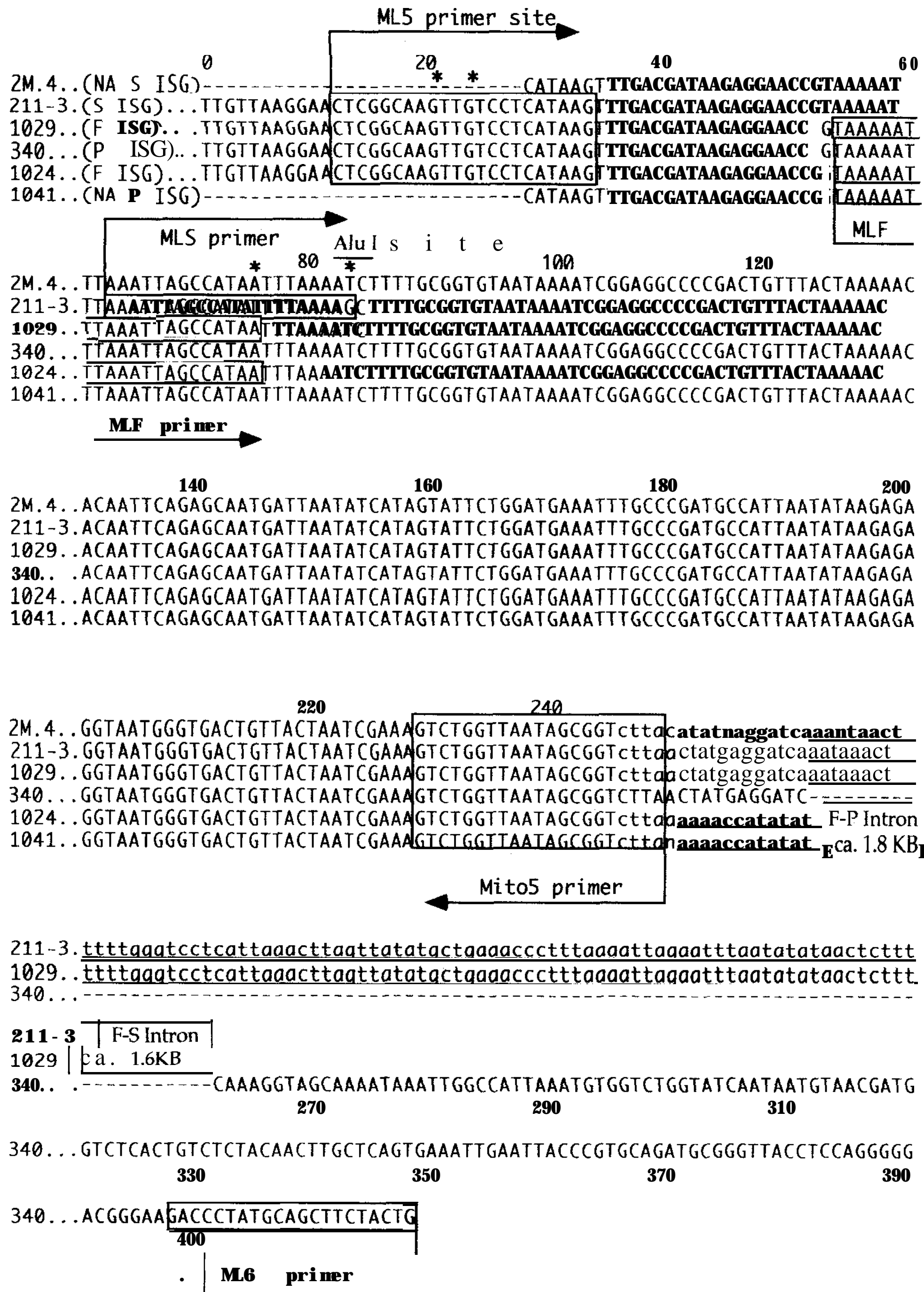
Fig. 5. ML5–ML6 and MLin3–ML6 DNA sequences for North American and European isolates, respectively. A complete sequence of the exonic region is shown only for the intron of the European P ISG isolate. The sequences of all other isolates were obtained only until the first intron insertion area. Variable portions of the 5' ends of the introns are shown as well. Boxed sequences correspond to primer sites; empty boxes correspond to undetermined intron sequences. Asterisks indicate base substitutions either within the previously designed ML5 primer sequence or among *H. annosum* ISGs. Underlined sequences are intronic; non-underlined sequences are exonic. Capitalized nucleotides were determined by sequencing of both DNA strands; lowercase nucleotides were determined by unidirectional sequencing.



cated that nucleotide substitutions in the exonic portion of the mt LrRNA DNA region were observed between the Eu S and F ISGs, and the differences appear to be fixed among isolates of the same ISG, suggesting the presence of two monophyletic mitochondrial lineages. Complete accordance of ISG, determined by nuclear AP-PCR fragments and mitochondrial DNA sequences, indicates that no hybridization and gene flow between the two ISGs is occurring. Based on these results we accept our first hypothesis that the Eu F and S are two genetically isolated ISGs and represent true monophyletic protospecies.

The distribution of the F ISG is still only partially known. Other studies have focused on the distribution of this ISG in

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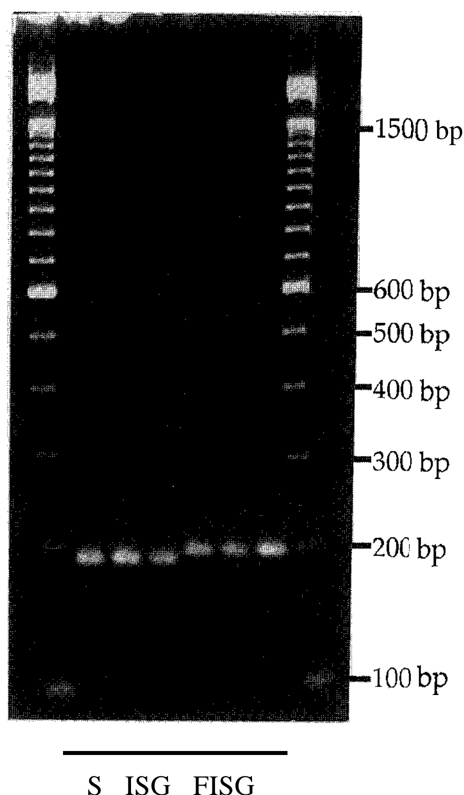
Fig. 6. Taxon-specific competitive-priming (TSCP) PCR diagnostic amplicons obtained with MLS, MLF, and Mito5 primers, and electrophoresed on a 5% agarose gel. First and last lanes are DNA molecular standards (100-bp ladder). Lanes 2-5 are S ISG isolates. Lanes 6-8 are F ISG isolates.



areas such as the Italian peninsula (Capretti et al. 1990) and the southern Balkans (Tsopelas and Korhonen 1996) where Norway spruce, the primary host of the S ISG in Europe, is absent or marginally present. In our study sites in the Alps, F isolates were collected from silver fir stumps in stands where S isolates can be often found on spruce stumps. For the three provinces of Bolzano, Belluno, and Udine, the report of *H. annosum* on silver fir is unprecedented. Furthermore, both the F and the S ISGs were found in silver fir stumps in the same or adjacent stands. As in North America (Otrosina et al. 1992), host specificity in stumps may not be as strict as on trees and may provide a suitable environment for both ISGs. The stands in which S isolates were found were typically mixed with a large component of Norway spruce, but only F isolates were found in the spruceless stands of the Apennines. This result suggests that S isolates found on silver fir stumps in the Alps may be part of S populations residing largely on the coexisting spruce trees. Without spruce as an inoculum source (e.g., in the Apennines), S isolates are absent or exist in low levels, perhaps because they are outcompeted by F isolates. The marginalization of S isolates in the Apennines may also be enhanced by the increasing importance of the pathogenic versus saprobic phase observed in a north to south gradient. S isolates, in fact, may be extremely unfit pathogens on live silver firs but good saprobes on silver fir stumps. A similar situation has been recorded in California, where P isolates represent 95% of the isolates found on live pine trees, but S isolates represent a significant portion (44-95%) of isolates collected from pine stumps (Garbelotto et al. 1996).

The finding of sympatric F and S populations in the southeastern Alps is relevant because radiocarbon dating has indicated that fir and spruce have coexisted in that area (a plant refuge during the last glaciation) for at least 9000 years (Lang 1994). Although the two ISGs and their hosts currently share only a portion of their total range, paleobotanical data from the beginning of the current postglacial era and from the Tertiary era indicate that silver fir was extremely abundant since the Pliocene in many areas where only spruce can be found at present times (Mai 1995; De Philippis 1985). Based on our results, we accept our second hypothesis that the S and F ISGs are now sympatric and may have been largely coexisting in the same geographic areas in past times.

Our third hypothesis states that the F and S ISGs are the two most closely related groups in a complex that includes more distant allopatric relatives. If most of the divergent evolution between S and F ISGs has occurred recently, then it must be accounted for by mechanisms other than geographic separation. These data allow for the distinction between allo-parapatric (i.e., speciation initially occurs in geographically isolated populations and ends in sympatry) and non-allopatric (without geographic isolation) speciation processes (Bush 1994). Alloparapatric populations in fact, have usually accumulated significant levels of genetic differences during their independent evolution in the allopatric phase. Isozyme analyses data (Otrosina et al. 1993) and Southern blot analysis of total genomic DNA RFLPs probed with anonymous DNA clones obtained from a NA S isolate (Garbelotto 1996) indicated that Eu S and F isolates are the two most closely related groups (isozyme Gs, = 0.08, RFLP similarity = 1) and that NA S isolates are the next closest relatives to Eu S and F isolates (isozyme Gs, =0.41/0.49, RFLP similarity = 0.66). Our AP-PCR data also suggest that the allopatric S ISG from North America is a more distant relative than the Eu S and F ISGs are to each other. The single NA S isolate employed in this analysis is a representative of 25 genotypes that displayed high values of similarity among themselves, according to a data set of 25 AP-PCR fragments. Therefore, this North American isolate can be considered as a good representative of the California S population. In the neighbor-joining (NJ) tree, the distances between the NA S isolate and the Eu S (NJ distance = 0.54) and F (NJ distance = 0.71) ISGs are larger than that between Eu S and F isolates (NJ distance = 0.43). Although the difference among neigbor joining distances may not be statistically significant, the discriminant analysis clearly places the NA S isolate as the most isolated group in the Eu S – Eu F – NA S triad.

The same conclusions can also be inferred by the analysis of presence-absence-type of introns in ML5 (MLin3)–ML6 amplicons of the mt LrRNA gene and by the sequence and RFLP analyses of 1.84-kb amplicons from Eu S and F isolates and of 2.05-kb amplicons from F, NA S, and NA P isolates. There is currently very little information on the distribution, function, and transfer mechanisms of introns in the LrRNA DNA region or in other regions of the genome (Lambowitz and Belfort 1993). Five introns can be found in the mt LrRNA DNA regions of *Suillus* spp. (Li 1995), and at least two different introns are reputed to be responsible for the different size of mt LrDNA amplicons (primers ML5–ML6) obtained from

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H. annosum individuals in California (Garbelotto et al. 1996*a*). Different amplicon sizes may be determined by introns of different sizes or by the presence of extra introns in the larger amplicons. There is a strong association between presence-absence or type of introns in most ISGs of *H. annosum,* as determined by the size, DNA sequences, and RFLPs of ML5 (MLin3)–ML6 amplicons. For instance, Eu P isolates are mostly intronless, NA P isolates mostly amplify a 2.05-kb fragment, and NA S isolates mostly amplify a 1.8-kb fragment. In contrast, RFLPs and sequencing data indicate that the same type of intron can be found at high frequencies in both Eu S and F ISGs. This result indicates that these two ISGs may have diverged only recently. Collectively, these results support the third hypothesis that the Eu S and F ISGs are the two most closely related ISGs in the *H. annosum* complex.

Three types of requirements are necessary to support the hypothesis of sympatric speciation (Lynch 1989): (i) two sister species must have arisen in the same geographic region and no extrinsic geographic barriers must exist between the two (Bush 1975); (ii) there must be permanent genetic isolation between the two sister species so that reversion to a single species will not occur (Grant and Grant 1989); and (iii) the two sister species must be genetically closely related to each other and less related to a third, allopatrically derived species (Lynch 1989). We have shown here that all three types of requirements are potentially met for the Eu S and F ISGs.

In the case of *H. annosum,* two possible mechanisms can be hypothesized to be contributing to the genetic isolation of sympatric populations: a genetic system regulating intersterility (Chase and Ullrich 1990*a*) and specialization of each ISG on different hosts (Korhonen 1978; Capretti et al. 1990; Otrosina et al. 1992). Because host preference and intersterility are strongly coupled, the loci determining both traits may be linked.

Mating compatibility between ISGs appears to be regulated by five loci (Chase and Ullrich 1990*a*, 1990b). Presence of the same positive allele at one of the five intersterility loci (S, P, V1, V2, V3) in both parents, allows for mating between two genotypes bearing different mating alleles. Most S isolates have a plus allele at the S locus and a minus allele at the P locus; P isolates have a plus allele at the P locus and a minus allele at the S locus. Thus, S and P isolates should not mate; nevertheless, presence of plus alleles at one of the three remaining V loci allows for "illegitimate" mating in some S-P combinations. Under this assumption, recombination or mutation of a plus allele at a single intersterility locus may determine the formation of new ISGs.

In agreement with the model of sympatric speciation in fungi proposed by Brasier (1987), Eu F-S mating incompatibility appears to have arisen only in sympatric F and S ISG populations (Capretti et al. 1990), but it is absent between northern European S and Italian F isolates. In this and previous studies (Otrosina et al. 1993; Garbelotto 1996) employing genetic markers such as isozymes and total genomic RFLPs, no evident genetic differentiation was detected between Norwegian and Italian S isolates. These results do not deny the potential for regional genetic differentiation among demes but substantiate the monophyletic nature of the European S ISG and indicate that the different mating compatibility of northern and southern Eu S populations is a trait under strong selection pressure in areas of sympatry with the F ISG (i.e., southern Europe). This sympatric mating barrier may be effectively keeping the gene pools of the two ISGs distinct (Brasier 1987); furthermore, if the S and F ISGs had been generated in an allopatric phase, they would not have developed such strong mating barriers, and they would show higher levels of interfertility such as those recorded for allopatric individuals from different species or biological species.

The specialization of ISGs on different hosts may act as a mechanism of reinforcement of the gene flow barrier between the F and S ISGs; this is particularly important because intersterility between ISGs is only partial. It has been shown that in many pathosystems, plant-pathogen compatibility can be regulated by a single locus (Flor 1956; Staskawicz et al. 1984). Mutation at a single locus may thus determine shifts in host specificity of pathogens and give rise to new populations with a different habitat preference. Although there is no understanding of the mechanisms regulating host specificity in *H. annosum,* host-pathogen recognition may be an important mechanism in sympatric speciation and may further restrict gene flow between ISGs by selecting against S-F hybrids of *H. annosum.* Garbelotto et al. (1996) and Garbelotto (1996) have shown with greenhouse inoculation trials that, in North America, an S-P hybrid isolate was less virulent than S isolates on S hosts and less virulent than P isolates on P hosts. Nevertheless, the hybrid isolate was as virulent as either S or P isolates on Sitka spruce (*Picea sitchensis* (Bong.) Carr.), a host that is susceptible to both ISGs in greenhouse inoculation trials. These results strongly suggest that mechanisms of host-pathogen recognition select against inter-ISG hybrids and can act as reinforcement of the sympatric speciation process in the *H. annosum* complex. Further research needs to assess mechanisms of potential selection against hybrids in European populations of *H. annosum* to understand the role played by habitat selection in the presumed sympatric evolution of Eu S and F ISGs.

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