Root and Butt Rots of Forest Trees
Carcans-Maubuisson (France), September 1-7, 1997
Ed. INRA, Paris, 1998 (Les Colloques, n°89)

Habitat preference and the evolution of sympatric Intersterility Groups 'in the *Heterobasidion annosum* species complex

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SUMMARY

Populations of the basidiomycete *Heterobasidion annosum* display varying degrees of intersterility and differential host specialization. At least three intersterility groups have been formally described, each characterized by a range of "preferred" hosts. It has been hypothesized that processes of host-pathogen compatibility may have been driving the evolution of sympatric populations of this fungus. Host specialization may also determine a selective disadvantage on inter-ISG hybrids, and keep the geene pools of these otherwise partially interfertile populations (intersterility in fact is only partial) separate. Molecular data generated in the past five years is indicating that in fact there are mom than three genetically distinct groups worldwide. The geographic distribution of these groups suggests that allopatric processes are involved in the evolution of distinct fungal populations.

We analyzed the relationships among European and North American populations of *H. annosum* with a range of nuclear and mitochondrial genetic markers. Results indicate that a) each population (intended here as all the individuals belonging to the same ISG and from the same world region) is genetically well distinct from the others, and, b) the European S and F ISGs as the two closest groups and have probably diverged more recently than the others. Because the European S and F ISGs are found preferentially on different hosts (*Picea* and *Abies*, respectively), it is plausible that host specificity may be driving the evolution of this organism. Furthermore, because 1)-both ISGs are present (sometimes even on the same stump) in European mixed conifer forests, and, 2)- paleobotanical data indicate *Picea* and *Abies* spp. shared in the past a largely overlapping geographic range, these results support the hypothesis

of sympatric speciation in the *H. annosum* complex. To provide evidence for possible mechanisms of sympatric speciation in *H. annosum* we have used North American S, P, and field SP hybrid isolates in greenhouse inoculation experiments. SP isolates were significantly less virulent than P isolates on P-hosts (*Pinus* spp.) and significantly less virulent than S isolates on S-hosts (*Abies, Tsuga*), but were as virulent as S or P isolates on the greenhouse "universal" host Sitka spruce. These results support the hypothesis that inter-ISG hybrids may be at a selective disadvantage in nature and provide evidence that mechanisms of host-pathogen interaction act as a driving force or as a reinforcement of the genetic isolation of the two North American ISG, which in the laboratory show moderate levels of interfertility.

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 (allopauic 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 1953, Maynard Smith 1966). In this case, ecological adaptation would play a fundamental role in separating two populations in different niches. 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 1987). Diehl and Bush (1987) 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.

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 bio-types within a species are much stronger among individuals from areas where both species (or biological species) coexist, than among individuals from distant locations (Blair 1955, Hubbs 1962, Mecham 1961, Mc Carley 1964, Littlejhon 1965, Wasserman and Koepfer 1977, Zouros and 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 first part of the present study examines the validity of the sympatric speciation hypothesis between the European Fand S biological species of *Heterobasidion annosum* (Fr.)

Bref. (Fungi: Basidiomycotina). 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 North American S and P or the European S and P ISGs, are genetically very different, and can thus be regarded as protospecies. In the case of the North American S and P groups, 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 European F and 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. The resolving power of isozyme analysis, though, has proven insufficient to differentiate the two ISGs and to address properly the issue of sympatric speciation. 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 & 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%) with sympatric S individuals from Southern Europe, but it is significantly higher (66%) with S individuals from Northern Europe (Capretti *et al.* 1990, Stenlid and Karlsson 1991, Korhonen et *al.* 1992), while 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 the first part of this study were to verify that, in nature, the European S and F ISGs are genetically isolated, 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: 1 H) The F and S are two genetically isolated ISGs and may be viewed as two monophyletic protospecies. 2 H) The F and S ISGs are geographically sympatric and have coexisted as such for a significant period of time. 3 H) The sympatric F and S ISGs are the two closest relatives in a complex that includes mom distant allopatric relatives.

The goal of the second part of the study was to elucidate the potential mechanisms underlying the sympatric speciation process. Because intersterility among ISGs is only partial we hypothesized that negative selection against ISG hybrids may be an important factor. The retrieval of an hybrid SP isolate in North America has allowed us to test the following hypotheses. I)- The SP hybrid is a functional and genetically fit isolate, and II) the hybrid is at an ecological disadvantage because it is recognized as a non-pathogen by hosts normally

infected by either parental ISG. According to these testable hypotheses, it would be the mechanisms of host-pathogen interaction (specificity) to be a major mechanism underlying the sympatric speciation process by restricting gene flow between two potentially interfertile ISGs.

MATERIALS AND METHODS

Study sites, sampling, and DNA extractions

Nineteen sites were sampled in Italy . (Additional samples had been previously collected by us or other colleagues from Italy, Norway, and California). Fungal cultures were obtained by isolations on malt extract agar (MEA) from the context of basidiocarps and from infected wood. DNA extracts were obtained by the CTAB extraction method described by Garbelotto et *al* (1997).

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 samples. Isolates chosen for the subsample set came from different geographic areas and sites (including S isolates from California and Norway, and P isolates from Norway), and were selected from distant locations and different forest stand 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: M13, NL13, ITS2, Ctb6, NS1, NS4, KimQ, *NS6, KJ2* , KJ2-Ctb6, NS2-NL13, ITS2-NS2, ITS4b-Mb2, ITS1F-Ctb6, NS3- Ctb6, 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 1), 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

Table 1. Sequences of DNA oligonucleotide primers used for the arbitrary primed (AP) PCR analysis.

Primer	Sequence (5'-3')	Reference
NS1	GTAGTCATATGCITGTCTC	White et al. 1990
NS2	GGCTGCTGGCACCAGACITGC	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 & Bruns 1993
M13	GAGGGTGGCGGTTCT	Stenlid et al. 1993
ML5	CTCGGCAAATTATCCTCATAAG	White et al. 1990
ML6	CAGTAGAAGCTGCATAGGTC	White et al. 1990
MLin3	CGACACAGGTTCGTAGGTAG	Y. Li 1995
MLS	AAATTAGCCATAAAAG	this study
MLF	TAAAAATTTAAATTAGCCATAA	this study
Mito 5	TAAGACCGCTATA(T/A)ACCAGAC	this study
KimQ	ACGCCTCTAAGTCAGAAT	Kim et al. 1992
KJ2	GCTTGAAATTGTCGGGAGGG	T. Bruns, unpublished
CNS3.6	AATGAAGTCATCCITGGCAG	T. Bruns, unpublished
NL13	CAACGCAACTTTCATGCACG	T. Szaro, unpublishhed
CNL2F	GTTTCCCTTTTAACAATTTCAC	White et al. 1990
Ctb6	GCATATCAATAAGCGGAGG	T. Bruns, unpublished
Mb2	GTGAGTTTCCCCGTGTTGAG	M. Berbee, unpublished

formula $Similarity=F=2n_{xy}/n_x+n_y$ (Lynch 1990). in which n_x and n_y ate 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 v 3.5c (Felsenstein 1992).

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 1988) computer program. Seven loci 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 standard deviation of 1.

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

For all 35 isolates, a portion of the mitochondrial LrRNA gene was amplified using the MLin3 and ML6 primers (Fig. 1) (each 0.5 µM/25 µL reaction, 55°C annealing temperature, 35

Table 2. Mortality data from greenhouse inoculation tests; combined results from 1995 and 1996 experiments.

ISG	S (n=2)[a]	P (n=1)	SP (n=1)			
Tree species				df	F-ratio	*P*
P. *ponderosa*						
seedlings inoc.	140	160	160			
Average mort.	0.29	0.69	0.25	31	13.9	0.0001
(SD)	(0.20)	(0.18)	*(0.12)*			
Homog. group	b	a	b			
A. concolor						
seedlings inoc.	140	160	160			
Average mort.	0.15	0.06	0.07	31	4.1	0.028
(SD)	(0.09)	(0.09)	*(0.05)*			
Homog. group	a	b	b			
P. sitchensis[b]						
seedlings inoc.	100	100	100			
Average mort.	0.85	0.97	0.94	16	0.98	0.39
(SD)	(0.23)	(0.04)	(0.06)			
Homog. group	a	a	a			

[a] **Two different isolates where used : in 1995 we used a field S heterocaryon unrelated to the hybrid; in 1996, we used a heterocaryon composed of one nucleus from a field homocatyon and the S nucleus from the hybrid isolate.**
[b] **Sitka spruce was inoculated only in 1996.**

cycles). 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 were sequenced. For the intron-less European P isolates the 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, and RFLPs were obtained with several endonucleases. 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 1, Fig. 1) that would preferentially amplify a portion of the ML5-ML6 mt LrRNA region

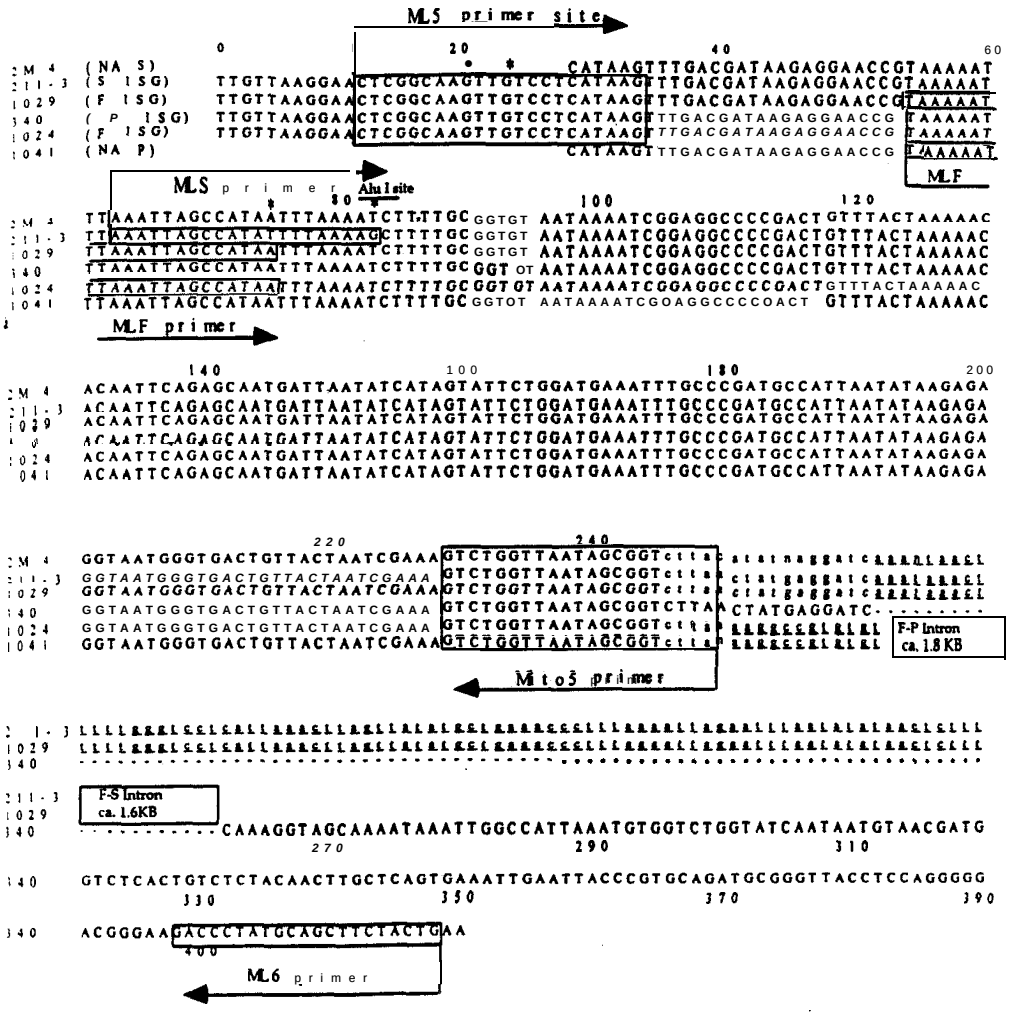


Figure 1. ML5-ML6 and MLin3-ML6 DNA sequences for North American and European isolates, respectively. A complete sequence of the exonic region is shown only for the intron-European P ISG isolate. The sequences of all other isolates were obtained only until the first intron insertion area. Variable portions of the Y-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 with the previous designed ML54 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; lower case nucleotides were determined by unidirectional sequencing.

from one of the two ISGs and could be used for diagnostic purposes in taxon-specific competitive-priming (TSCP) PCR (Garbelotto et *al.* 1996b). When used in combination with Mito5, MLS and MLF would produce a 195 bp F-specific fragment and a 185 bp S-specific fragment, respectively.

Isolate selection for the inoculation experiment

Two S ISG field isolate, one P ISG field isolate, and the SP hybrid isolate AWR400 were selected for the inoculation study. All four isolates were clamped and were presumed to be heterokaryotic. The P isolate AWR282 had been found in the Modoc National Forest: somatic compatibility tests indicated that AWR282 had successfully established itself in a pine root disease center and had vegetatively infected and caused mortality on several trees (Ratcliff *et* al. 1993). The S isolate 309Az200 had been obtained from a basidiocarp in a stump in the Lassen National Forest and had consistently caused fir seedlings mortality (10-20%) in preliminary inoculation tests in the greenhouse. The S isolate L2-400 was a heterokaryon obtained by heterokaryotization of a field homokaryon by the hybrid AWR400. The hybrid SP isolate AWR400 was also retrieved in the field (Modoc National Forest), where it had infected several trees, and caused pathogenic symptoms (Garbelotto et al. 1996b).

Inoculum preparation and experimental design

Pine wood wedges approximately 1 cm long were autoclaved for 30 minutes, immersed in malt extract broth for 2 hours and placed in molten malt extract agar in 10-cm diameter petri dishes. Once the MEA had solidified, plates were inoculated with *H. annosum* isolates and kept at room temperature for about 12 weeks. Wood wedges to be used for mock inoculations were inoculated with sterile MEA plugs.

Ponderosa pine (*Pinus ponderosa*), white fir (*Abies concolor* (Gord. & Glend.) Lindl. ex Hilderbr.), and Sitka spruce (*Picea sitchensis* (bong.) Carr.) seedlings were transplanted in 500 cc plastic cartridges and acclimated in the greenhouse on the Berkeley campus for approximately 3 months. Seedlings were 1-l (i.e. grown from seed for 1 year, and transplanted for 1 year). Inoculations were performed as described by Worrall et al. (1983).

A balanced, completely randomized block design was employed for the inoculation study; each of the three isolates and the sterile control wedges represented the four treatments in each of 5 blocks. Both tree species were represented in each block, and each treatment for both tree species consisted of 18 inoculated seedlings. Total numbers of seedlings inoculated with each isolate is shown in Table 2.

Sampling and data analysis

Health of the inoculated seedlings was monitored twice a week. General appearance of the seedlings and percentage of dead or dying foliage of each seedling were recorded. Seedlings that displayed at least 75% dead foliage were lifted and taken to the laboratory. The experiment was ended 5 months after each inoculation.

Sampled seedlings were inspected for presence. of xylem discoloration and for recent active root growth Stems and inoculation wedges from each sampled seedling were then plated separately in moist chambers, and left to incubate at room temperature for up to 2 weeks. Wedges and stems were periodically inspected under the dissecting scope for presence of hyphae and conidiophores of the ***H. annosum*** anamorph (***Spiniger meineckellus*** (Olson) Stalpers).

Pine, fir, and spruce mortality data were analyzed independently. An $arcsin\sqrt{}$ transformation was employed for the pine mortality data (actual data had a bimodal distribution), while a $\sqrt{(x+0.5)}$ transformation was used for the fir and spruce mortality data (actual data had an unimodal distribution). ANOVA was employed to compare differences in mortality among blocks. If no significant differences among blocks were found, all mortality data were pooled together and analyzed according the formulas for a completely randomized design. Tukey's multiple range comparison tests were used to compare individual isolates to one another.

RESULTS

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

The 35 ***H. annosum*** 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. Tree mortality increased in a North to South gradient. DNAs from all isolates were extracted and successfully amplified. RAPD amplification patterns indicated that all 23 isolates from the Apennines belonged to the F ISG. Of 12 isolates collected in the Alps, eight were typed as F and four 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; in one case S and F isolates were retrieved from the same stump. S and F isolates were found in the same or adjacent sites.

AP-PCR analyses

A total of 107 polymorphic fragments was produced by AP-PCR analysis. Within-ISG similarity values averaged 89% (SD=12) for the F ISG and 83% (SD=9) for the Eu S ISG, average similarity between the S and F ISGs was 35% (SD=5.8). Neighbor-joining clustering based on the similarity matrix separated the European F and S ISG isolates into two distinct clades and placed the North American S isolate in a third, more distant clade (Fig. 2). The two European ISGs appear to be two monophyletic groups, and Norwegian and Italian S isolates are clustered together.

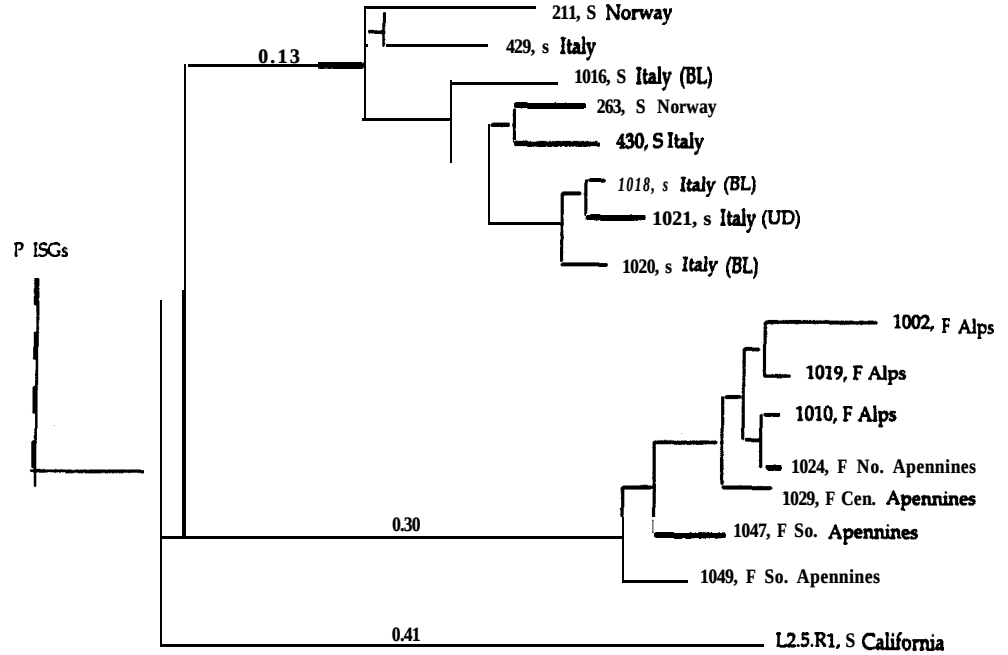


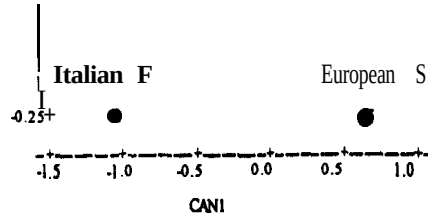
Figure 2. Neighbor-joining dendrogram of 16 isolates of *Heterobasidion annosum* based on a similarity matrix obtained by the formula $F=2n_{xy}/n_x+n_y$ (Lynch 1990), in which n_x and n_y are the total number of AP-PCR fragments in strains X and Y respectively, and n_{xy} is the number of fragments common for both strains. The ISG of the isolates is followed by the geographic provenance, including the province in parentheses when known. All F isolates are from Italy (No.=Northern, Cen.=Central, So.=Southem). Numbers indicate the length of the main branches. Dashed branch shows likely root, according to previous isozyme analysis of Otrosina et *al. (1992)* and to RFLPs of total genomic DNA digested with *Barn* HI.

The discriminant (canonical) analysis indicated that the three ISGs were at a significant distance from one another, and that the European S and F ISGs were closer to each other than to the North American S ISG (Fig. 3). 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 large ribosomal RNA (mt Lr-RNA)

Fragments amplified with the MLin3-ML6 primer combination from the European isolates, and from two North American S (NA S) and one North American P (NA P) isolates varied in size. The two European P (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 European S (Eu S) isolates amplified a 1.84 KB fragment; while the F isolates produced either a I .84 or a 2.05 KB fragment.

Figure 3. Discriminant analysis of European F and S. and North American S ISGs of *Heterobusidion annosum.* Dots for the European S and F ISGs represent clusters of seven and eight isolates respectively; the S dot includes Norwegian and Italian isolates.



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 Alu I digest (96% similarity), but the difference was consistent for all 35 S and F isolates tested, and was determined to be in the exonic sequence (see below). Thus, the Alu I RFLP pattern of the mitochondrial region was ISG-diagnostic, and perfectly matched ISG diagnosis based on the presumedly nuclear 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 North American P isolate.

The complete sequence of the MLin3-ML6 fragment of the mt LrRNA gene was obtained for the Eu P isolate 340. Partial sequences were obtained for representatives of all other groups (Fig. 1). Most of the sequenced portion of the exon showed no differences among European S , P, and F, and North American S and P isolates; two nucleotide substitutions between European 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 and Fig. 1).

Introns interrupted the exons of NA S 1.80 KB, Eu S 1.84 KB and F 1.84 KB fragments at the same site (Fig. 1). 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, and high RFLP similarity values). Insertion point of introns in the 2.05 KB fragments was 15 bp downstream from the intron insertion point in the smaller fragment. The NA 2.05 KB S and P fragments and the F 2.05 KB 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 between 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 1.80/1.84 KB amplicons (Fig. 1).

The concomitant use of the three primers MLS, MLF, and Mito5 resulted in the amplification of diagnostic TSCP-PCR fragments. 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.

Inoculation study.

See table 2.

DISCUSSION

Our molecular data indicate that although the European 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 proto-species. 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 (83-87%) of intra-ISG AP-PCR average similarity values. Although our sample size was limited, these results suggest that, while genetic differentiation of local population may be

occurring, intra-ISG gene flow is also occurring (or has occurred until recently) between demes. *H. annosum* has a large potential dispersal range in Europe (Rishbeth 1959, Kallio 1970), and a study based on more comprehensive sampling have shown only moderate levels of genetic substructuring associated with geographic variation in the P and S ISGs (Stenlid *et* al. 1994). These results substantiate the monophyletic nature of the European S ISG, and indicate that the different mating compatibility of Northern and Southern European 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 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 recoreded for allopatric individuals from different species or biological species.

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 indicated 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 European 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 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, though, both the F and 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 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 vs. the

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. 1996b).

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 em and from the tertiary em 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 were 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 allows 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). Alloparapanic 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 North American S isolate (Garbelotto 1996) indicated that European S and F isolates are the two most closely related groups (isozyme Gst=0.08, RFLP similarity=l) and that North American S isolates are the next closest relatives to European S and F isolates (NAS-EuS/F isozyme Gst=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 European S and F ISGs are to each other. In the neighbor-joining tree, the distances between the North American S isolate and the European S' (NJ distance=0.54) and F (NJ distance=0.71) ISGs are larger than mat between European 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- 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 *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 European S and F ISGs. This result indicates that these two ISGs may have diverged only recently. Collectively, these results support the hypothesis (3 H) that the European S and F ISGs are the two most closely related ISGs in the *H. unnosum* complex.

Three *types* of requirements are necessary to support the hypothesis of sympatric speciation (Lynch 1989): 1) two sister species must have arisen in the same geographic region and no extrinsic geographic barriers must exist between the two (Bush 1975); 2) 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, 3) 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 met for the European 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), 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. 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 hybrids of *H. annosum* . The greenhouse inoculation trials have shown 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, a host which in greenhouse inoculation trials is susceptible to both ISGs. 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,* in order to understand the role played

by habitat selection in the presumed sympattic evolution of European S and F ISGs. The retrieval of a hybrid in California and the introgression of genetic markers from one ISG into the other (Garbelotto *et* al. 1996b, Garbelotto and Gtrosina, unpublished) may indicate that massive modification of natural ecosystems caused by man, may favor secondary convergent evolution of two ISGs.

ACKNOWLEDGEMENTS

We are grateful for field assistance to: Dr. Paolo Capretti, Institute of Forest Pathology and Zoology, University of Florence; Prof. Nicola Luisi, Plant Pathology Institute, University of Bari; and Dr. Paolo Ambrosi, Institute for Agricultural Research of San Michele all'Adige, Trento. Employees of the Forest Service from the Provinces of Bolzano, Belluno, and Udine were instrumental to our field work in the Alps.

REFERENCES

BLAIR, W. F., 1964. Isolating mechanisms and interspecies interactions in anuran amphibians. *Quart. Rev. Biol.* 39: 333-344.

BRASIER, C. M., 1987. The dynamics of fungal speciation. Pp. 231-260. In : A. D. M. Rayner, C. M. Brasier. and D. Moore (Eds) *Evolutionary Biology of the fungi.*. Cambridge Univ. Press, Cambridge.

BRUNS, T. D., WHITE T. J., TAYLOR J. W., 1991. Fungal molecular systematics. Ann. *Rev. Ecol. Syst 22:* 525-564.

BUSH, G. L., 1975. Modes of animal speciation. *Annu. Rev. Ecol. Syst. 6: 339-364.*

BUSH. G. L., 1994. Sympatric speciation in animals: new wine in old bottles. Tree 9: 285-288.

BUTLIN, R., 1989. Reinforcement of premating isolation. *In* : D. Otte and J. A. Endler (Eds) *Speciation and Its Consequences.* Sinauer Associates, Inc., Sunderland, Mass. pp. 158-179.

CAPRETTI, P., KORHONEN, K., MUGNAI, L., ROMAGNOLI, C., 1990. An intersterility group of *Heterobasidion annosum* specialized to *Abies alba. Eur. J. For. Path.* 20: *231-240.*

CHASE, T. E., ULLRICH, R. C., 1990. Five genes determining intersterility in *Heterobasidion annosum. Mycologia* 82: 73-8 1.

DE PHILIPPIS, A., 1985. Abetine di abete bianco. *In : Lezioni di selvicultura speciale*. CUSL, Florence, Italy. 132-140.

DIEHL, S. R., BUSH G. L., 1989. The role of habitat preference in adaptation and speciation. *In :* D. Otte and J. A. Endler (Eds) *Speciation* and *Its* Consequences. Sinauer Associates, Inc., Sunderland. Mass. pp. 345-365.

FABRITIUS, A. L., KARJALAINEN, R., 1993. Variation in *Heterobasidion annosum* detected by random amplified polymorphic DNAs. *Eur. J. For. Pathol. 23:* 193-200.

FELSENSTEIN, J., 1992. PHYLIP - phylogenetic inference package, version 3.5. Computer programs distributed by the author, Dept. Genet. Univ. Wash. Seattle WA USA.

FLOR. H. H.. 1956. The complementary genetics systems of flax and flax *rust. Adv. Genet.* 8:29-54.

GARBELOTTO, M., 1996. The genetic structure of populations of *Heterobasidion annosum* (Fr.) Bref. from the global to the local scale: implications for the biology, the epidemiology, and the evolution of a forest pathogen. Dissertation, University of California, Berkeley.

GARBELOTTO, M., BRUNS, T. D., COBB, F. W., JR., OTROSINA, W. J., 1993. Differentiation of intersterility groups and geographic provenances among isolates *of Heterobasidion annosum detected* by random amplified polymorphic DNA assays. Can. *J. Bot.* 71: 565-569.

GARBELOTTO, M., LEE, K.K., SLAUGHTER, G., POPENUCK, T., COBB, F. W., BRUNS, T. D., 1997. Heterokaryosis is not required for virulence of *Heterobasidion annosum. Mycologia 89:* 92- 102.

GARBELOTTO, M., POPENUCK, T., RATCLIFF, A., COBB, F.W., BRUNS, T. D., 1996a. Host selection against SP hybrids of *Heterobasidion annosum:* implications for speciation. *Phytopathology* 86:S28-S29.

GARBELOTTO, M., RATCLIFF A., BRUN,S T.D.. COBB, F.W., OTROSINA, W.J., 1996b. Use of taxon specific competitive priming PCR to study host specificity, hybridization, and intergroup gene flow in intersterility groups of *Heterobasidion annosum. Phytopathology 86: 543-55* 1.

GRANT, P.R., GRANT, B.R., 1989. Sympatric speciation and Darwin's finches. *In :* D. Otte and J. A. Endler (Eds), *Speciation and Its Consequences.* Sinauer Associates, Inc., Sunderland, Mass., 433-457.

HUBBS, C.L., 1961. Isolating mechanisms in the speciation of fishes. *In* : W. F. Blair *(Ed) Vertebrate Speciation.* Univ. Texas Press, Austin, TX. pp. 5-23.

KALLIO, T.. 1970. Aerial distribution of the root-rot fungus *Fomes annosus* (Fr.) Cooke in Finland *Acta Forestalia Fennica* 107: l-55.

KORHONEN, K., 1978. Interfertility and clonal size in the *Armillariella mellea* complex. *Karstenia* 18: 31-42.

KORHONEN, K.. EOBKO I., HANSO, S.. PIRI, T., VASILIAUSKAS, A., 1992. Intersterility groups of *Heterobasidion* annosum in some spruce and pine stands in Byelorussia, Lithuania and Esthonia. Eur. *J. For. Path. 22:* 384-391.

LA PORTA, N., CAPRETTI, P., KAMMIOVIRTA, K., KORHONEN, K., KARJALAINEN. R., 1993. Genetic variation in F-group isolates of *Heterobasidion annosum* occurring in Italy. *In* : J. Stenlid and M. Johansson (Eds) *Proceedings of Eighth International Conference on Root and Butt Rots,* Wik, Sweden and Haikko, Finland. International Union of Forestry Research Organizations, 233-242.

LEVIN, D.A., 1970. Reinforcement of reproductive isolation: plants *versus* animals. *Amer. Natur.* 104, 571-581.

LEVINE, H., 1953. Genetic equilibrium when more than one ecological niche is available. *Am. Natur. 87:* 331-333.

LITTLEJHON, M. J., 1965. Premating isolation in the *Hyla ewingi* complex (Anura: hylidae). *Evolution* 19: 234 243.

LYNCH, J. D., 1989. The gauge of speciation: on the frequencies of modes of speciation. *In :* D. Otte and J. A. Endler (Eds) *Speciation and Its Consequences.* Sinauer Associates, Inc., Sunderland. Mass., 527-553.

LYNCH, M., 1990. The similarity index and DNA fingerprinting. *Molecular Biology and Evolution 7: 478-484.*

MAI, DIETER H., *1995. Tertiare Vegetationsgeschichte Europas : Methoden und Ergebnisse.* Gustav Fischer, Jena , New York.

MAYNARD SMITH, J., 1966. Sympatric speciation. *Am. Nat.* 100: 637-650.

MECHAM, J. S., 1961. Isolating mechanisms an uran amphibians. *In :* W. F. Blair *(Ed) Vertebrate Speciation..* Univ. Texas Press, Austin, TX. pp. 24-61.

OTROSINA , W. J., CHASE, T. E., COBB, F. W., JR., 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., JR., KORHONEN, K., 1993. Population structure of *Heterobasidion annosum* from North America and Europe. Con. *J. Bot.* 7 1: 1064- 1071.

RATCLIFF, A. W., COBB, F. W. JR., OTROSINA, W. J., 1993. Infection dynamics *of Heterobasidion annosum* (Fr.) Bref. in pine in northeastern California. Phytopathology 83 (12). 1363- 1364.

RISHBETH, J., 1959. Dispersal of *Fomes annosus* (Fr.) and *Peniophom gigantea* (Fr.) Masse. *Trans. Brit. Mycol. Soc*. 42: 243-260.

SAS Institute, Inc.. 1988. SAS/STAT™, Release 6.03 Edition. Cary, NC.

STASKAWICZ, B. J., DAHLBECK. D., KEEN, N. T., 1984. *Cloned* avirulence gene of *Pseudomonas syringae pv. glycinea* determines race-specific incompatibility on *Glycine max* (L) Merr. *Proc. Natl. Acad. Sci. USA* 81:6024-6028.

STENLID, J.. KARLSSON, J., 1991. Partial intersterility in *Heterobasidion unnosum. Mycologia 95:* 1153-l 159.

STENLID, J., KARLSSON, J.. HÖGBERG, N., 1994. Intraspecific genetic variation in *Heterobasidion annosum* revealed by amplification of minisatellite DNA. Mycol. *Res. 98: 57-63.*

TSOPELAS, P., KORHONEN, K., 1996. Hosts and distribution of the intersterility groups of *Heterobasidion unnosum* in the highlands of Greece. *Eur. J. For. Path. 26:* 4- *11.*

WASSERMAN, M., KOEPPER, H.R., 1977. Character displacement for sexual isolation between *Drosophila mojavensis* and *Drosophila arizonensis. Evolution 3 I : 8* 12-823.

WELSH, J., MCCLELLAND. M.. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* 18: 7213-7218.

WILLIAMS, J.G.K., KUBELIK, A.R.. LIVAK, K.J., RAFOLSKI, J.A., TINGEY, S.V., 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18: 653 1-6535.

WORRALL, J.J., PARMETER, J.R., JR., COBB, F.W., JR., 1983. Host specialization of *Heterobasidion annosum. Phytopathology 73: 304-307.*

ZOUROS, E., ENTREMONT, C.J.D., 1980. Sexual isolation among populations of *Drosophila mojuvensis:* response to pressure from a related species. *Evolution 34:* 421-430.

Les Colloques , n°89

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