Use of Taxon-Specific Competitive-Priming PCR to Study Host Specificity, Hybridization, and Intergroup Gene Flow in Intersterility Groups of *Heterobasidion annosum*

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ABSTRACT

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Two intersterility groups (ISGs) of the forest pathogen *Heterobasidion annosum* are found in California: S and P. We devised a polymerase chain reaction (PCR) method called taxon-specific competitive-priming (TSCP) PCR to differentiate the two ISGs. Using TSCP-PCR, we typed 537 live isolates and dry basidiocarps from 204 trees and 114 stumps from 60 sites in eight California national forests. All isolates from fir and sequoia stumps or trees were S; isolates from pine stumps were 80% S and 20% P; isolates from pine, incense cedar, and western juniper trees were 23% S and 77% P. The recovery of a well-established hybrid "SP" genet in a pine center was confirmed by isozyme analysis. The PCR amplification of the mitochondrial ML5-ML6 region also was diagnostic for the two ISGs, but in areas where both fir and pine mortality centers were present, about 7% of S isolates yielded the P-specific fragment. These results indicate the possibility of gene flow in nature between the two ISGs. The presence of S isolates on trees previously regarded as exclusive P hosts broadens the potential host range of this ISG.

The basidiomycete *Heterobasidion annosum* (Fr.) Bref. is regarded as a complex species containing at least three intersterility groups (ISGs): F, P, and S (3,19). ISGs generally represent biological species among which gene flow is virtually nil (2). In the case of *H. annosum*, however, intersterility barriers are not complete and display a marked regional variation. In Europe, 4 to 10% interfertility between the S and the P ISGs has been reported (19, 30), and results from in vitro tests with North American isolates indicate that up to 18% between-ISG interfertility is possible (5,13). High levels of interfertility between two ISGs were recorded when pairing small numbers of isolates of the F ISG from Italy and S isolates from northern Europe (37% interfertility) and North America (100% interfertility) (3,30). This high interfertility is consistent with the hypothesis that in allopatric speciation abrupt intersterility barriers between conspecific populations are not present (1).

Intersterility barriers are often associated with the development of host specificity or preference (2). In the *H. annosum* complex, the P ISG is mostly associated with mortality of trees in the genera *Pinus*, *Juniperus*, and *Calocedrus*. The S ISG host range includes the genera *Picea*, *Abies*, *Pseudotsuga*, *Tsuga*, and *Sequoiadendron* (20,25). To date, the F ISG has only been recorded on *A. alba* in some regions of southern and central Europe (3,22).

Different host associations also are correlated with different pathologies caused by *H. annosum*. For instance, in North America and Europe, P isolates kill the roots of living pine trees, and S isolates either cause butt rot of spruce trees and hemlocks or sapwood and heartwood decay of roots and boles of true firs (25,26).

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Host specificity and different pathogenicity are important to understanding the role played by *H. annosum* in shaping forest structure and composition and in determining succession pathways. Such issues are also a major concern for silviculturists and forest pathologists who can use host specificity as a management tool to reduce losses caused by this pathogen. European P and S ISGs are characterized by a host preference that does not preclude infection of other hosts (20,29,30). In North America, extensive field surveys have found a very strong correlation between host species and ISG (23). The term "host specificity" is used to describe this strong correlation. Greenhouse inoculation tests confirmed the hypothesis of host specificity (34), although results showed the possibility of infection of pine trees by S isolates.

Larger, more detailed field experiments to confirm host-specificity hypotheses have been hindered by the length and complexity of performing mating tests and isozyme analysis. Until recently, these were the two main methods employed to determine the ISG of fungal isolates, and both require the availability of living fungal cultures (17,23,19,30). The need for live cultures made it impossible to determine the ISG of isolates in old infection centers, where the only signs left by the pathogen were decayed wood and dry basidiocarps.

Randomly amplified polymorphic DNA (RAPD) analysis has been used successfully to differentiate and compare ISGs (10,11). RAPDs also can be used as genetic markers to infer the relatedness or, at least, the similarity of isolates (32). Unfortunately, RAPD analysis requires relatively large amounts of template DNA, and results are highly affected by the presence of biological contaminants. Restriction fragment length polymorphisms (RFLPs) of the polymerase chain reaction (PCR)-amplified internal transcribed spacer (ITS) region have been reported to discriminate between North American S and P ISGs (6). This method includes several steps (e.g., PCR amplification; electrophoresis to check

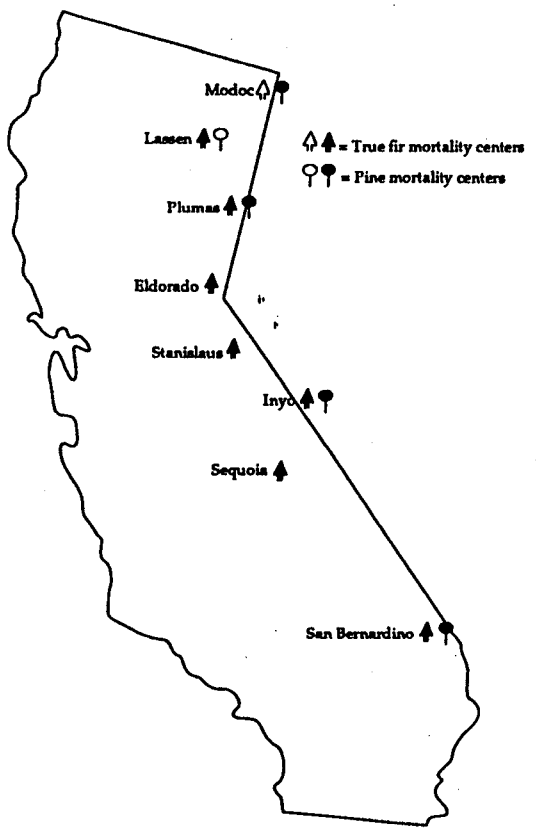

Fig. 1. Location of national forests in California in which mortality centers caused by *Heterobasidion annosum* were sampled. Shaded symbols indicate actual sampling of host species; unshaded symbols indicate host species were present in the area, but they were not sampled.

the PCR products; use of endonucleases; and electrophoresis to check the RFLPs) and is sensitive to the presence of biological contaminants whose ITS regions may be amplified as well. These drawbacks have prompted the development of a fast technique more suitable for ISG determination of old, dry basidiocarps, which generally contain small amounts of viable DNA and are consistently overgrown by secondary fungi and bacteria.

The objectives of this study were twofold: (i) to develop a faster and highly specific molecular technique for ISG determination that also could be employed on dry specimens and (ii) to field test the hypotheses of host specificity and of lack of nuclear and mitochondrial gene flow between the S and P ISGs in California coniferous forests. We used sequence differences in the ITS region of the nuclear ribosomal DNA to design taxon-specific PCR primers to differentiate the two California ISGs (18). The ML5-ML6 region of the large mitochondrial ribosomal DNA was amplified with the aid of the PCR (13) and was used to study divergence and sharing of a mitochondrial marker between ISGs.

MATERIALS AND METHODS

Sample collection. A total of 537 live isolates and dry basidiocarps was typed from 204 trees and 114 stumps from 60 sites in eight California national forests (Fig. 1; Table 1). Study sites were located in the Cascade, Sierra Nevada, and San Bernardino mountain ranges and could be assigned to one of two main ecological areas. Sites in the Eldorado, Stanislaus, and Sequoia national forests ("western" sites) were more mesic and were characterized by typical mixed conifer forest with a predominance of true firs or sequoias. Sites in the Modoc, Plumas, Lassen, Inyo ("eastern" sites), and San Bernardino national forests were typically drier and included both pine mortality centers, in the drier areas, and fir mortality centers, in the more mesic sites.

TABLE 1. Location, host, and intersterility group (ISG: S and P) of *Heterobasidion annosum* isolates determined by taxon-specific competitive-priming polymerase chain reaction

National forest	Climate	No. of sites sampled	No. and species of sample trees[a]	No. of isolates from trees[b]	% S	% P	No. and species of stumps sampled[a]	No. of isolates from stumps[c]	% S	% P	Collector
Fir mortality center											
Lassen	Dry	6	16 WF	54	10	0	17 WF	35	10	0	Garbelotto
Plumas	Dry	1	11 WF	10	10	0	6 WF	6	10	0	Garbelotto
Eldorado	Wet	4	66 WF; 1 IC	68	10	0	21 WF; 1 IC	21	10	0	Garbelotto
Stanislaus	Wet	4	34 WF; 1 PP	86	10	0	8 WF	12	10	0	Garbelotto
Sequoia	Wet	4	12 SG	20	10	0	4 WF	4	10	0	Workinger
Inyo	Dry	4	1 RF	1	10	0	3 WF; 1 RF	7	10	0	Garbelotto
San Bernardino	Dry	18	2 WF	2	10	0	26 WF	35	10	0	Garbelotto
Pine mortality center											
Modoc	Dry	7	21 WJ; 21 PP	58	22	73	9 PP	18	44	56	Ratcliff
Plumas	Dry	3	14 JP; 1 LP	29	3	10	7 JP	38	84	16	Ratcliff
Inyo	Dry	8	0	0	...	...	11 PP	19	95	5	Otrosina
San Bernardino	Dry	1	3 PP	3	0	10	0	0	...	...	Otrosina

[a] WF = white fir; IC = incense cedar; PP = ponderosa pine; SG = sequoia; RF = red fir; WJ = western juniper; JP = Jeffrey pine; and LP = lodgepole pine.
[b] Does not include 10 hybrid SP ISG isolates (one genet) found in pine mortality center M5 in the Modoc National Forest.
[c] Does not include one hybrid SP ISG isolate found in pine mortality center M5 in the Modoc National Forest.

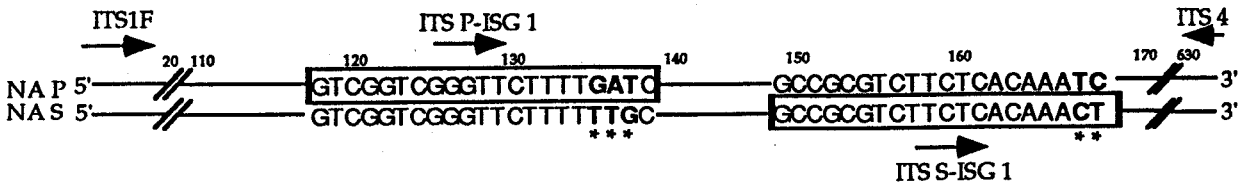

Fig. 2. Schematic representation of the internal transcribed spacer (ITS) sequences of the North American P (NA P) and S (NA S) intersterility groups (ISGs) of *Heterobasidion annosum*. Sequences in boxes were used to design the taxon-specific primers that differentiated the two North American ISGs. Asterisks indicate positions at which the nucleotides of the two ISGs differed.

At all sites, one or more isolates per sample tree were analyzed. Sample trees included dead, symptomatic, and asymptomatic trees. Isolations were performed either from basidiocarps or infected wood. Wood samples were incubated in moist chambers at room temperature for 6 to 12 days, and isolations were made by transferring infected wood or hyphae of the imperfect state of *H. annosum* (*Spiniger meineckellus* (Olson) Staplers) from the wood surface onto a petri dish previously filled with Fomes select medium (16). All isolates were subsequently grown on malt extract agar (MEA).

DNA extractions. DNA templates for PCR reactions were obtained in two ways. For ISG typing, a few hyphae from fungal colonies growing on MEA were collected and suspended in 100 µl of sterile water. The suspensions were frozen and thawed at least once to enhance the rupture of cell membranes. Hyphal suspensions were vortexed briefly, and small hyphal pellets were precipitated by pulse spinning in a microcentrifuge (33). For PCR amplification of the mitochondrial ML5-ML6 region, isolates were grown in liquid malt extract and harvested as described by Garbelotto et al. (11). DNA was extracted by the method of Rogers et al. (27), and 2 µl of each DNA extract was electrophoresed on 1% nonmodified agarose gel and stained with ethidium bromide. DNA concentrations were estimated by visual comparison of the intensity of DNA extraction products with known standards. Concentrations of DNA extracts ranged between 50 and 250 ng/µl. For PCR amplifications, DNA dilutions with a concentration of 2 ng/µl were used. Dry basidiocarps were ground, and DNA was extracted as described above, except that a 1:50 standard dilution was used for PCR reactions.

PCR primer designing and amplification conditions. ITS sequences of North American S and P isolates were provided by K. Mitchelson and T. Kasuga (Department of Molecular and Cell Biology, University of Aberdeen, Scotland). Base substitutions between the two ISGs in the 115- to 170-bp region were used to design primers (ITS S1 and P1) that would preferentially amplify the region from one of the two ISGs (Fig. 2). When used in combination with primer ITS 4 (13), the ITS P1 and S1 primers amplified a 518-bp P-specific fragment or a 486-bp S-specific fragment, respectively (Fig. 3).

Two types of PCR reactions were tested on 20 North American isolates of known ISG. Initially, the ITS P1 (0.5 µM/25-µl reaction) and S1 (0.5 µM/25-µl reaction) primers were used individually in combination with the ITS 4 (0.5 µM/25-µl reaction) primer. Subsequently, the four primers, ITS 1F (12), P1 and S1 (each 0.16 µM/25-µl reaction), and 4 (0.5 µM/25-µl reaction), were used concomitantly in the same reaction. We called this type of PCR reaction taxon-specific competitive-priming PCR (TSCP-PCR). All 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 (United States Biochemicals, Cleveland), 1 to 25 ng of genomic DNA (6.25 µl of the DNA dilutions), and 0.5 units of *Taq* polymerase (Boehringer GmbH, Mannheim, Germany). All amplifications were conducted in a Techne PHC-2 thermal cycler (Techne, Cambridge, England) programmed for an initial 1.5-min denaturation at 94°C, followed by 35 cycles of denaturation (1 min, 95°C), annealing (1 min, 58°C), and extension (1 to 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, a 2-min extension for the next 13 cycles, and a 3-min extension for the last 9 cycles. A final extension of 10 min followed the 35 cycles.

The complete ITS region of a subset of 37 isolates was amplified with the primer combination ITS 1F-ITS 4. Amplification conditions were as above, except for the annealing temperature, which was lowered to 53°C. A portion of the LrRNA was amplified with the ML5 and ML6 (each 0.5 µM/25-µl reaction) primers (13) for a subset of 162 isolates (Table 2). Amplification conditions were as described above for the ITS region amplification. Amplification products were analyzed by electrophoresis in 3% (ML5-ML6) or 4% (ITS) agarose gels (1% nonmodified and 2 or 3% NuSieve [FMC Bioproducts, Rockland, ME]) in Tris-acetate buffer (100 mM Tris, 12.5 mM sodium acetate, and 1 mM EDTA, pH 8.1) stained with ethidium bromide.

RFLPs of PCR products. Amplified ITS fragments were digested with the enzyme *Cfo*I (Boehringer). ML5-ML6 amplicons were digested with the following Boehringer enzymes: *Alu*I, *Cfo*I, *Hae*III, *Hin*fI, *Mbo*I, *Msp*I, *Rsa*I, and *Taq*I. Digest reactions were performed with 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 whole reaction was loaded onto a 3% agarose gel, electrophoresed, and stained with ethidium bromide as described above.

Isozyme analysis. To test the newly developed ISG typing technique and obtain preliminary information on the genetic variability of our sample populations, we performed an isozyme analysis on a subset of 37 isolates (Table 3) selected to represent the most

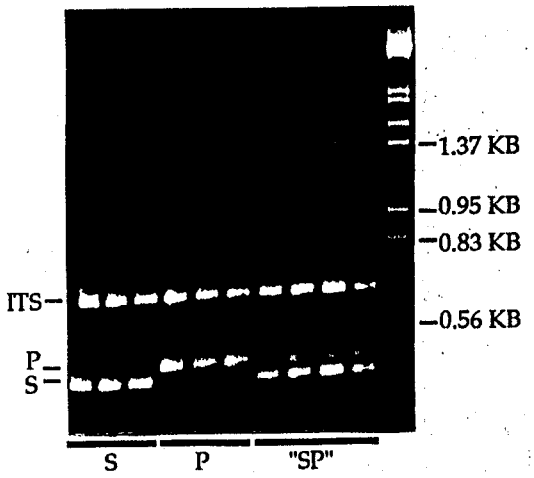

Fig. 3. A 3% agarose gel showing taxon-specific competitive-priming polymerase chain reaction results for 10 isolates of the fungus *Heterobasidion annosum*. Lanes 1 to 3, S intersterility (ISG) isolates; lanes 4 to 6, P ISG isolates; lane 7, hybrid SP isolate AWR 400; lanes 8 to 10, hyphal tip cultures from hybrid SP isolate AWR 400; and right lane, molecular standard lambda cut by *Eco*RI and *Hin*dIII. Larger amplified fragments (approximately 650-bp) represent the entire internal transcribed spacer (ITS) region; the intermediate amplified fragments (518 bp) represent the P-specific bands; and the smallest amplicons (486 bp) represent the S-specific bands.

TABLE 2. Results of polymerase chain reaction amplification of the ML5-ML6 region of the large ribosomal mitochondrial DNA of isolates of *Heterobasidion annosum*

National forest	Host[a]	ISG[b]	No. of isolates	ML5-ML6 amplicon (%)	
				1.75 kb	2.05 kb
Pine mortality center					
Modoc	PP/WJ	P	18	0	100
Plumas	JP	P	9	0	100
Modoc	PP/WJ	S	1	100	0
Modoc	PP/WJ	SP	1	100	0
Fir mortality center					
Lassen	WF	S	35	94	6
Plumas	WF	S	17	100	0
Eldorado	WF	S	5	100	0
Stanislaus	WF	S	56	100	0
Inyo	WF/RF	S	6	83	17
San Bernardino	WF	S	14	93	7

[a] PP = ponderosa pine; WJ = western juniper; JP = Jeffrey pine; WF = white fir; and RF = red fir.

[b] ISG = intersterility group.

important classes determined by TSCP-PCR. The classes were S isolates from firs; P isolates from pine trees and stumps; S isolates from live pines or junipers; S isolates from pine stumps; and hybrid "SP" isolates.

Mycelia were grown at room temperature for 3 weeks in 250-ml Erlenmeyer flasks containing 100 ml of potato dextrose broth. Extraction of mycelia for electrophoresis was performed as described by Otrosina et al. (23). Starch gel electrophoresis was performed on the extracts by four gel buffer systems designated A (Tris-borate, pH 8.3), B (Tris-citrate, pH 8.8), D (morpholine-citrate, pH 8.1), and E (morpholine-citrate, pH 6.1) (9). Enzyme and gel buffer systems are listed in Table 4. Electrophoresis, gel slicing and staining, data interpretation, and analysis were performed as described by Otrosina et al. (23). A total of seven enzyme systems with 28 allozymes was analyzed.

Somatic compatibility (sc) tests. To ascertain that different fungal isolates represented different genotypes, sc tests were performed on all live isolates from all study sites as described by Stenlid (28). Pairs of isolates were challenged on Hagem medium, and results were read after 2, 4, and 6 weeks. Most pairings were repeated at least twice. A barrage, a pseudosclerotial plate, or an evident interaction zone between the two isolates was considered a negative reaction. Absence of such reactions and free intermingling of hyphae from the two isolates were considered positive reactions. Isolates with positive reactions were considered to share the same sc alleles and to be in the same sc group. Isolates in the same sc group were assumed to represent the same genet. In theory, a genet would most likely be generated by a single genotype that, after having successfully infected and colonized a host tree, spreads through root contact to other trees (15,24). In this paper, we only discuss preliminary results for the M5 study site, in which a complex combination of S, P, and hybrid SP isolates were found.

RESULTS

ISG typing by TSCP-PCR. Taxon-specific primers were successfully used to determine the ISG of *H. annosum* isolates. The

TABLE 3. *Heterobasidion annosum* isolates used for isozyme and internal transcribed spacer (ITS) restriction fragment length polymorphism (RFLP) analyses

Isolate[a]	Origin[b]	Host[c]	Tree/Stump	Health[d]	ISG by isozyme	ISG by TSCP-PCR[e]	ISG by ITS RFLPs
S isolates from white fir trees or stumps							
ST1 1, R1	StNF	WF	Stump	...	S	S	S
SB 20	SBNF	WF	Stump	...	S	S	S
B1 2, R1	ENF	WF	Tree	3	S	S	S
PL1 73, R2	PNF	WF	Tree	2	S	S	S
L1 4.1	LNF	WF	Tree	1	S	S	S
S isolates from pine or juniper trees							
AWR 293	MNF	PP	Tree	1	S	S	S
AWR 365	MNF	PP	Tree	1	S	S	S
AWR 593	MNF	PP	Tree	1	S	S	S
AWR 948	MNF	PP	Tree	0	S	S	S
S isolates from pine stumps							
P1 47, 2D	PNF	JP	Stump	...	S	S	S
AWR 303	MNF	PP	Stump	...	S	S	S
ALS 463	...	...	...	...	S	S	S
SP hybrid isolates from juniper and pine trees							
AWR 319	MNF	WJ	Tree	1	SP	SP	SP
AWR 326	MNF	WJ	Tree	1	SP	SP	SP
AWR 327	MNF	WJ	Tree	1	SP	SP	SP
AWR 400	MNF	WJ	Tree	...	SP	SP	SP
AWR 402	MNF	WJ	Tree	...	SP	SP	SP
AWR 403	MNF	WJ	Tree	...	SP	SP	SP
AWR 409	MNF	PP	Tree	1	SP	SP	SP
AWR 410	MNF	PP	Tree	1	SP	SP	SP
AWR 412	MNF	PP	Tree	1	SP	SP	SP
AWR 413	MNF	PP	Tree	1	SP	SP	SP
P isolates from juniper and pine trees or stumps							
ALS 398	...	...	...	...	P	P	P
AWR 145	MNF	PP	Stump	...	P	P	P
AWR 150	MNF	PP	Stump	...	P	P	P
AWR 83	MNF	WJ	Tree	0	P	P	P
AWR 84	MNF	WJ	Tree	0	P	P	P
AWR 282	MNF	PP	Tree	3	P	P	P
AWR 297	MNF	WJ	Tree	1	P	P	P
AWR 315	MNF	PP	Tree	1	P	P	P
AWR 332	MNF	PP	Tree	3	P	P	P
AWR 345	MNF	WJ	Tree	2	P	P	P
P1 9, 5, 3	PNF	JP	Tree	2	P	P	P
P1 13, 3, 1	PNF	JP	Tree	0	P	P	P
P1 26, 1B	PNF	JP	Tree	0	P	P	P
P3 1, 6	PNF	JP	Stump	...	P	P	P

[a] S, P, and SP hybrid indicate intersterility group (ISG).
[b] StNF = Stanislaus National Forest; SBNF = San Bernardino National Forest; ENF = Eldorado National Forest; PNF = Plumas National Forest; and MNF = Modoc National Forest.
[c] WF = white fir; PP = ponderosa pine; JP = Jeffrey pine; and WJ = Western juniper.
[d] 0 = healthy; 1 = slightly symptomatic; 2 = symptomatic; and 3 = dead.
[e] TSCP-PCR = taxon-specific competitive-priming polymerase chain reaction.

success of PCR amplification was 100% for hyphal suspensions of live cultures and 95% for DNA extracts of dry basidiocarps (Table 1). In all cases, there was complete correspondence between results obtained with TSCP-PCR and results obtained with other techniques (Table 3).

The use of single pairs of primers had two major disadvantages. First, it allowed for occasional amplification of the wrong ITS fragment (e.g., the ITS S1-ITS 4 pair produced a 486-bp band for both S and P isolates). Second, it was not possible to distinguish between lack of amplified products due to absence of the correct DNA template or nonoptimal amplification conditions. Such conditions could be caused by the presence of *Taq* polymerase inhibitors and low DNA template concentrations in the PCR reaction.

Using the four primers together in TSCP-PCR eliminated both problems. The taxon-specific primer outcompeted the other primer, and only the appropriate taxon-specific band and the universal fragment were amplified. The ITS 1F-ITS 4 primer pair allowed unsuccessful amplification to be distinguished from the presence of a fungal contaminant in the PCR reaction. In the first case, no amplification product would be expected. In the second case, only the contaminant's ITS region would be amplified, while no ISG-specific band would be seen. Thus, successful amplification reactions from pure *H. annosum* cultures gave three possible results: (i) the S-specific 486-bp band; (ii) the P-specific 518-bp band; or (iii) both S- and P-specific bands for hybrid ISG (SP) isolates. In addition to the ISG-specific bands, the complete 640-bp ITS region amplified by the ITS 1F-ITS 4 primer combination was observable in most cases (Fig. 3).

The recovery of hybrid SP isolates was not expected. To further ascertain the nature of the hybrid isolates, TSCP-PCR was performed on 20 cultures originated from single hyphal tips of the SP isolate AWR 400. Hyphal tips were obtained by isolation of a few cells from single hyphae of cultures grown for 5 to 7 days on water agar overlaid with cellophane. All hyphal tips yielded both S- and P-specific bands. This result confirmed that in AWR 400 both ITS types were present in single hyphae.

When TSCP-PCR was performed on the DNA extracts from old, dry basidiocarps, either the S- or the P-specific band was amplified in high molarity, and the results could be clearly interpreted. Several other fragments were amplified as well and, in all likelihood, corresponded to the ITS regions of secondary fungi present on the *H. annosum* fruit bodies. The contaminants' ITS bands were amplified by the primer pair ITS 1F-ITS 4. Amplifications from basidiocarps were repeated with the omission of primer ITS 1F, and a single *H. annosum* ISG-specific band was obtained for each isolate.

RFLPs obtained by *Cfo*I digests of the PCR-amplified ITS region for the subset of 37 isolates employed in isozyme analyses were diagnostic for the ISG (Fig. 4). There was complete correspondence between results obtained with this method and with TSCP-PCR (Table 3).

Host association of *H. annosum* P and S ISGs. All 219 isolates from live or dead true firs and 119 isolates from true fir stumps had the TSCP-PCR pattern diagnostic of the S ISG. The same was true for the 20 isolates collected on sequoia. Isolates from stumps and standing trees of western junipers, Jeffrey or ponderosa pines, and incense cedars had TSCP-PCR patterns diagnostic of the S, P, and presumed hybrid SP ISGs. In all pine mortality centers, both S (44 to 95% of isolates analyzed) and P isolates (5 to 56% of isolates analyzed) were found in pine and juniper stumps (Table 1). S isolates were found on live trees in pine mortality center M5 in the Modoc National Forest (Table 5). Somatic compatibility tests indicated that many different genotypes had been employed in this study and that at all sites the vast majority of local genets was represented by the selected subsample of isolates.

In pine mortality center M5, 33% of isolates from trees were typed as S, 13% as hybrid SP, and 54% as P. A total of 18 sc groups were identified in M5: 6 (33%) sc groups were typed as S, 11 (61%) were typed as P, and 1 (6%) was typed as SP. Fifteen sc groups were present in more than one tree (ranged from 3 to 10). The number of trees or stumps per sc group ranged from one to nine for P isolates, one to eight for S isolates, and was four for the

TABLE 4. Enzyme and gel buffer systems used for allozyme analysis of *Heterobasidion annosum* isolates

Enzyme system	EC no.	Abbreviation	Gel buffer system[a]
Aconitase	4.2.1.3	ACO	D
Alcohol dehydrogenase	1.1.1.1	ADH	E
Catalase	1.11.1.6	CAT	B
Fluorescent esterase	3.1.1.1	f-EST	A
Glutamate oxaloacetate transaminase	2.6.1.1	GOT	B
Malate dehydrogenase	1.1.1.37	MDH	E
Sorbitol dehydrogenase	1.1.1.14	SRDH	B

[a] By Conkle et al. (9): A, Tris-borate; B, Tris-citrate; and D and E, morpholine citrate.

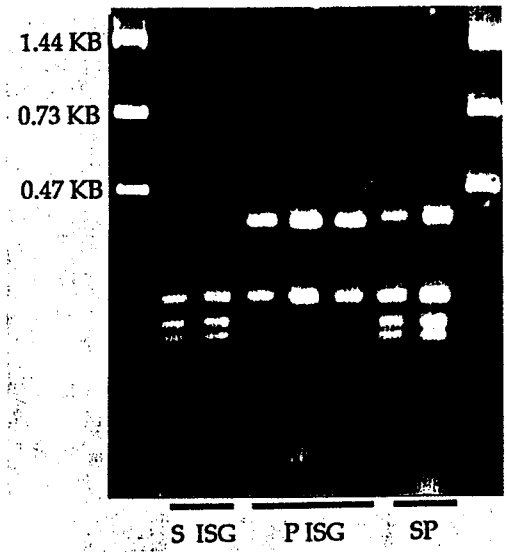

Fig. 4. Restriction fragment length polymorphisms obtained by *Cfo*I endonuclease treatment of the internal transcribed spacer (ITS) region of *Heterobasidion annosum*. ITS amplifications were obtained with the primer combination ITS 1F-ITS 4. Left and right lanes, molecular standards (pUC19 cut by *Taq*I); lanes 2 and 3, S intersterility group (ISG) isolates; lanes 4 to 6, P ISG isolates; and lanes 7 and 8, hybrid SP isolates.

TABLE 5. Summary of species and health of tree hosts, somatic compatibility (sc) groups, and intersterility groups (ISG) of 45 *Heterobasidion annosum* isolates from 26 trees and 3 stumps in the M5 (Modoc National Forest) pine mortality center

	S ISG[a]	P ISG[a]	SP hybrid group[a]
Number and percentage of sc groups	6 (36%)	10 (58%)	1 (6%)
Number and species of trees	5 WJ, 7 PP	9 WJ, 5 PP	2 WJ, 1 PP
Percentage of trees	46%	54%	12%
Number of class 0 trees[b]	1	...	1
Number of class 1 trees	4	3	2
Number of class 2 trees	1	2	...
Number of class 3 trees	3	9	...
Number and percentage of trees with a single ISG	9 (35%)	11 (42%)	2 (8%)
Number and species of stumps	2 PP	3 PP	1 PP

[a] WJ = western juniper and PP = ponderosa pine.
[b] Class 0 = healthy trees; class 1 = slightly symptomatic trees; class 2 = symptomatic trees; and class 3 = dead trees.

SP genet. S and P isolates were found in trees of all health conditions, from apparently vigorous to sick or dead (Table 5). Numbers and percents presented are partial estimates: more isolates have been collected in this site, but they have not been analyzed yet. The M5 study site occupied an area of 644 m^2, including 3 large stumps and 134 trees with diameters at breast height ranging from 2 to 37 cm (mean = 10.2 cm, SD = 6.3).

In the two "western" mixed conifer sites, B2 (Eldorado National Forest) and S4 (Stanislaus National Forest), a live incense cedar and a live Jeffrey pine, respectively, were infected by a S isolate. In all of the other true fir mortality centers in mixed conifer sites, no fungal isolates were obtained from cedars or pines.

Isozyme analysis. The seven enzyme systems analyzed gave clearly interpretable bands. Allozyme banding patterns associated with mobility of putative alleles are diagrammed in Figure 5. A total of 8 loci and 28 putative alleles was analyzed. Frequencies of putative alleles are given in Table 6. With the exception of malate dehydrogenase (MDH) and sorbital dehydrogenase (SRDH), all enzyme systems had single-banded phenotypes.

Isozymes have been used as a diagnostic tool to determine the ISG of *H. annosum* isolates (17,23). MDH-1, for instance, has two alleles that show complete association with the S and P ISGs, and it has been used as a diagnostic locus for ISG typing (23). For all 37 isolates employed in the isozyme analysis, the ISG determined by isozymes matched that determined by TSCP-PCR.

In six of seven systems, no alleles were shared by the two ISGs, therefore individual alleles could be assigned to either S or P. The only exception found was in the aconitase (ACO) system, in which the B allele was present in all 11 P isolates tested but was shared by 3 of the 13 S isolates. The three S isolates with the ACO B allele were from a fir and two pine trees in dry "eastern" sites.

In the MDH (locus 1), SRDH, glutamate oxaloacetate transaminase, and alcohol dehydrogenase systems, hybrid SP isolates were characterized by the presence of a S allele, a P allele, and a different heteromeric band whose mobility was exactly intermediate between the S and P alleles (Fig. 5). Dimeric patterns of allozymes from *H. annosum* have been reported by Otrosina et al. (23), and the multiple banding patterns observed in this study match patterns reported for dimeric enzymes in fungi (21). In the fluorescent esterase, catalase, and ACO systems, hybrid SP isolates had both a P and a S allele, but no new heteromeric band was seen (Fig. 5). Mean observed heterozygosity values (±SE) were 0.068 (0.027) for S, 0.147 (0.086) for P, and 0.727 (0.141) for hybrid SP isolates.

PCR amplification of the mitochondrial ML5-ML6 region. The PCR primer pair ML5-ML6 amplified either a 2.05- or a 1.75-kb fragment (Fig. 6). Sizes were approximately determined by visual comparisons. There was complete association of the 2.05-kb fragment with the 28 P isolates amplified. On the other hand, S ISG populations could be divided in two groups. The first group was composed of 61 S isolates from western true firs, and the second group was represented by 75 S isolates from true firs in eastern mortality centers. In populations within the first group, there was complete association between the S ISG and the 1.75 kb. In populations of the second group, 83 to 100% of the isolates yielded the 1.75-kb fragment, whereas 0 to 17% yielded a 2.05-kb band (Table 2).

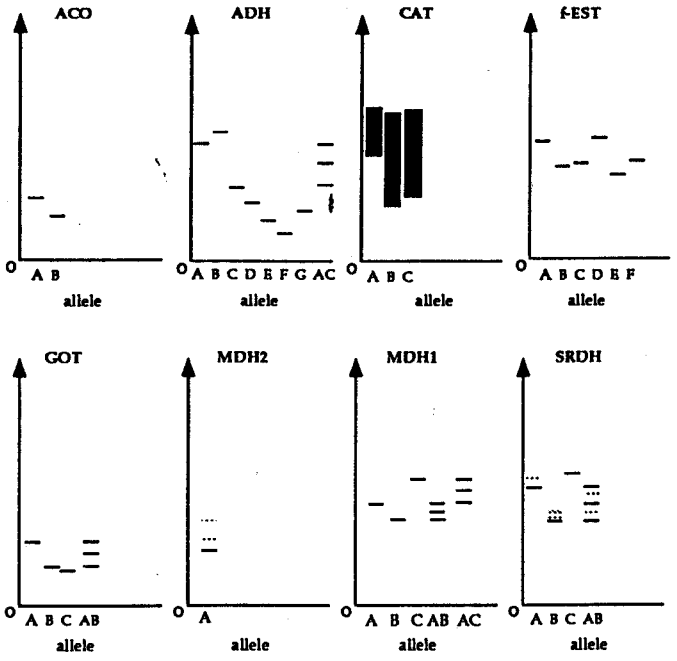

Fig. 5. Relative mobilities of putative alleles in eight allozyme loci from *Heterobasidion annosum* isolates. Lanes with double letters indicate presumed dimeric isozyme patterns. For easier visualization of dimeric enzymes, presumed heteromeric bands and their putative subunits are included in such lanes. Arrows indicate anodal direction of bands from origin 0. Table 4 lists the seven enzyme treatments used.

TABLE 6. Allele frequencies of eight putative allozyme loci in S, P, and hybrid SP intersterility group (ISG) isolates of *Heterobasidion annosum*[a]

Locus Allele	S ISG (N = 12)	P ISG (N = 14)	SP hybrid[b] (N = 10)
Aconitase			
A	0.83	0	0.5
B	0.17	1	0.5
Alcohol dehydrogenase			
A	0	0.96	0.5
B	0	0.04	0
C	0.08	0	0.5
D	0.08	0	0
E	0.42	0	0
F	0.29	0	0
G	0.125	0	0
Catalase			
A	0	1	0
B	1	0	0
C	0	0	1
Fluorescent esterase			
A	0	0.32	0.33
B	0.92	0	0.33
C	0	0.32	0
D	0	0.11	0
E	0	0.25	0.33
F	0.08	0	0
Glutamate oxaloacetate transaminase			
A	1	0	0.5
B	0	0.38	0.5
C	0	0.62	0
Malate dehydrogenase 1			
A	0	0.86	0.5
B	1	0	0.5
C	0	0.08	0
Null	...	0.06	...
Malate dehydrogenase 2			
A	1	1	1
Sorbitol dehydrogenase			
A	0	0.93	0.5
B	1	0	0.5
C	0	0.07	0

[a] All isolates were considered heterokaryotic.

[b] The 10 isolates were collected from different hosts but belonged to the same genet. In alcohol dehydrogenase, glutamate oxaloacetate transaminase, and malate dehydrogenase, a presumed heteromeric band also was present in the hybrid isolates.

The expected size of the exonic region of the LrRNA gene included by these two primers is about 400 bp. The balance is presumed to be accounted for by the presence of introns. Our preliminary sequencing data (data not shown) indicates that the two amplified fragments differ by at least two introns. The two introns interrupt the ML5-ML6 exonic sequence at base pair 219 in the 2.05-kb fragment and at base pair 230 in the 1.75-kb fragment. A total of 90 bp at the 5′ end of the introns was sequenced with a ABI Prism 377 DNA sequencer (Perkin-Elmer Co., Foster City, CA). No homologies were detected between introns found in the 1.75- and the 2.05-kb amplicons. On the other hand, introns in 2.05-kb amplicons from both P and S ISG isolates had identical 5′-end sequences.

RFLPs were generated by treating four 2.05-kb ML5-ML6 fragments (two from S and two from P ISG isolates) with *Alu*I, *Cfo*I, *Hae*III, *Hin*fI, *Mbo*I, *Msp*I, *Rsa*I, and *Taq*I endonucleases. No *Cfo*I restriction sites were found in any fragment. The other seven enzymes outlined a total of 16 restriction sites. RFLPs by six enzymes were identical for all four isolates. *Msp*I had a single restriction site that was present in the P isolates and absent in the S isolates (Fig. 7).

DISCUSSION

TSCP-PCR is a fast, highly reliable molecular method to determine the ISG of *H. annosum* isolates. It differentiates California S, P, and hybrid SP ISG isolates. TSCP-PCR has several advantages over RFLPs of the entire ITS-amplified region (6). First, because the ITS P1 and S1 primers are based on the *H. annosum* ITS sequence, the method is very specific and eliminates the problem of false results caused by amplification of the ITS of fungal contaminants; thus, contaminated field material can be used. Second, it is faster because there is no need to wait for the restriction digestion to occur or to check amplification efficiency prior to digestion. Third, it is less expensive because there is no need to use enzymes or buffers for restriction digests, and only one gel electrophoresis of the TSCP-PCR products is needed.

RAPDs also have been used to differentiate ISGs (10,11). RAPD results are not based on a single locus (e.g., the ITS region) but on several loci. For this reason, RAPDs can be used to differentiate the S and F ISGs, which do not have nucleotide differences in the ITS region (18) and are not reliably distinguished by isozyme analysis (24). RAPD analysis also allows for the determination of the geographic provenance of the isolates (11) and the calculation of similarity values based on number of shared bands, treated as dominant markers, among individuals (32). Nevertheless, TSCP-PCR has at least three advantages over RAPDs for diagnosis of North American isolates. First, DNA amplification reactions are more reliable and easier to reproduce; RAPD analysis can be very easily affected by changes in concentration and quality of DNA template or other ingredients. Second, TSCP-PCR yields positive results with an extremely wide range of DNA template concentrations. Whereas RAPD analysis requires larger amounts of DNA template (about 25 ng per reaction), no DNA extractions are needed for TSCP-PCR of pure fungal cultures because hyphal suspensions in water provide enough template. Third, TSCP-PCR is not affected by contaminants and can be used on dry specimens (e.g., old basidiocarps) and possibly even on infected wood. This greatly enhances diagnostic power because dry basidiocarps are abundant in California forests, are easy to collect, and do not require any specific care or treatment.

TSCP-PCR enabled us to process large numbers of isolates collected through intensive samplings and to further study the relationship between ISGs and tree host species. All isolates from sequoia and true fir trees or stumps were typed as S. This information confirms previous reports of host specificity of the S ISG (34). In the San Bernardino and Inyo national forests, several collections were made from stumps just a few hundred meters from active pine mortality centers caused by *H. annosum*. The appearance of these stumps indicated that most had been infected after being cut. In theory, freshly cut stumps of uninfected fir trees could provide a substrate amenable to infection by both ISGs. Lack of successful fir-stump colonization by P isolates in areas with high P inoculum could be ascribed either to fir-P ISG incompatibility reactions or to the failure of P isolates to outcompete S isolates. Even with particular stress conditions imposed on the hosts, such as overstocking and drought, only S isolates were found on true firs. The mechanisms underlying this presumed resistance are unknown, but the evidence collected in this and other field surveys (23,24) and greenhouse inoculations (8,34) indicates that the host is not merely escaping infection and that some kind of inhibitory reaction occurs when the host and pathogen come in contact.

Pines and junipers were susceptible to both ISGs in eastern mortality centers. In previous extensive surveys, S ISG isolates had been found in pine stumps but not in trees (23). In our study, up to 95% of the isolates from stumps were typed as S. This may reflect a predominance of S isolates in pine stumps, but many

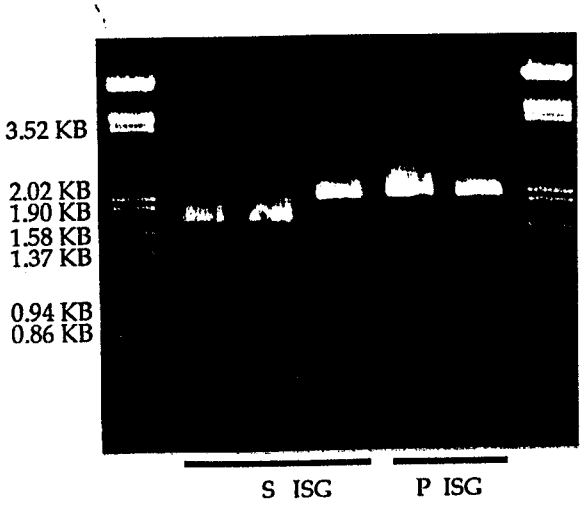

Fig. 6. Agarose gel electrophoresis of the polymerase chain reaction-amplified ML5-ML6 region of the large subunit of the mitochondrial ribosomal RNA gene from five isolates of *Heterobasidion annosum*. Left and right lanes, molecular standards (lambda cut by endonucleases *Eco*RI and *Hin*dIII); lanes 2 and 3, S intersterility group (ISG) isolates from the Eldorado National Forest; lane 4, a S ISG isolate from the Lassen National Forest; and lanes 5 and 6, P ISG isolates from the Modoc National Forest.

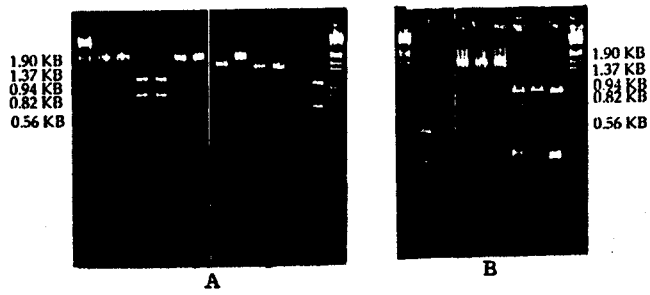

Fig. 7. Agarose gel showing restriction fragment length polymorphisms of the polymerase chain reaction-amplified 2.05-kb ML5-ML6 region of the large subunit of the mitochondrial ribosomal RNA gene from isolates of *Heterobasidion annosum*. **A**, Left and right lanes, molecular standards (lambda cut by *Eco*RI and *Hin*dIII); lanes 2 and 3, untreated S and P intersterility group (ISG) fragments; lanes 4 and 5, S and P fragments digested by *Rsa*I; lanes 6 and 7, S and P fragments treated with endonuclease *Cfo*I; lanes 8 and 9, S and P fragments treated with *Msp*I; lanes 10 and 11, S and P fragments digested by *Hin*fI; and lanes 12 and 13, S and P fragments digested by *Mbo*I. **B**, Left and right lanes, molecular standards (lambda cut by *Eco*RI and *Hin*dIII); lanes 2 and 3, S and P fragments digested by *Alu*I; lanes 4 to 6, two S and one P fragment digested by *Hae*III; and lanes 7 to 9, two S and one P fragment digested by *Taq*I.

stumps were sampled only by collecting basidiocarps. The high percentage of S isolates found also may have been determined by the more abundant fruiting of S isolates compared to P isolates.

In this study, intensive and extensive sampling schemes were combined. In five of six intensively sampled pine mortality centers, only P ISG isolates were obtained from live pine and juniper trees. In center M5, however, S ISG isolates also were collected from both pine and juniper trees. S genets represented a significant proportion of the *H. annosum* population within this center in terms of percentage of total isolates analyzed for this study (33%) and percentage of sc groups (36%). In some instances, S and P isolates coexisted on the same host, but in 9 of 26 positively sampled trees, only S isolates were found. Among these nine trees were healthy, sick, and dead trees, which suggests that S isolates were not simply secondary or saprobic invaders but were primary pathogens.

The presence of S isolates on trees regarded as exclusive P hosts broadens the potential host range of the S ISG and should be taken into account by forest managers. It is known that European S isolates not only cause butt rot of spruce but also kill young pines (20). The fact that adult pines do not become infected may be an indication of ontogenic resistance and potential pathogenicity in situations of lowered or undeveloped host resistance. The situation in California may be similar to the European situation if ecological and site characteristics are taken into account. Greenhouse inoculations have shown that in California S isolates are potentially pathogenic but are less virulent than P isolates on P hosts (8,34). In this study, S isolates were found in pines and junipers mostly in harsher eastern sites where precipitation is scarce and soils are shallow. In the more mesic western sites, pines are interspersed in fir mortality centers but rarely are infected by S isolates. This may be due to more favorable ecological conditions and to decreased root contact between pine trees and stumps in mixed conifer stands with a dominant component of true fir. The occurrence of S isolates on a Jeffrey pine and an incense cedar in two western stands may indicate lowered resistance due to the long drought occurring in California at the time of sampling. In both cases, the trees showed few disease symptoms and may have been infected only recently. The hypothesis of decreased host specificity determined by host stress warrants further study.

Differences in host-pathogen interactions between western and eastern areas also may be explained by the genetic variability of the pathogen. It is not known whether there is a significant difference between S populations from the two areas or whether that difference could be partially accounted for by the presence/absence of virulence genes. In this study, as well as in previous studies (23,24), isozyme analyses have shown a low percentage of alleles shared by both ISGs. This may suggest high levels of genetic isolation between the two ISGs, but it does not mean that gene flow between the two ISGs is precluded. In our isozyme analysis, for instance, the ACO allele associated with P isolates also was present in three S isolates, suggesting that gene flow may occur between ISGs; alternatively, these alleles may be a remnant of the ancestral shared origin. Interestingly, the three S isolates with the P allele in the ACO system were from areas in which both ISGs coexist. Genetic exchanges may be responsible for the transfer of P virulence genes to S isolates. Further research is needed to clarify the frequency of gene transfers between the two ISGs and the extent of genetic isolation or communication of populations from different geographic areas in California.

The retrieval of a hybrid SP genet in the M5 study site is an indication of interfertility in nature between the two ISGs. Between-group interfertility has already been observed in vitro (5, 14,31), and a genetic explanation has been formulated (7); however, hybrid isolates have never been reported in the field. The difficulty of finding hybrids in nature may be determined by their reduced vegetative or reproductive fitness, by the presence of genes that allow for recognition by both pines and firs, and by our inability to find hybrid zones in which hybridization may occur (4). PCR and isozyme analyses show that the SP heterokaryon is a true hybrid and that it is heterozygous for S and P alleles at many loci. The observed mean heterozygosity for the hybrid isolates was 0.727: this value matches the expected Hardy-Weinberg mean heterozygosity value for a true hybrid (0.724) and is significantly higher than the observed mean heterozygosity values of either the P or S ISG. A total of 11 hybrid SP isolates was characterized in this study; all belonged to the same compatibility group and, thus, were considered part of the same genet. The hybrid genet was found on two slightly symptomatic codominant junipers, on a slightly symptomatic codominant ponderosa pine, and on an adjacent ponderosa pine stump. The extent of secondary growth of this fungal genet and the symptoms associated with its hosts suggest this hybrid genotype is stable and virulent.

The hybrid genotype and both ISGs were found on a ponderosa pine stump. It seems highly plausible that stumps infected by isolates of both ISGs may provide the opportunity for hybridization. The frequency of hybridization events and the biological characteristics of hybrid isolates are unknown. All collections of the hybrid genotype were from infected wood, and it is not known whether this isolate can produce basidiocarps. When the ML5-ML6 region of the SP isolate was amplified with the aid of PCR, a 1.75-kb fragment was obtained. The RFLPs obtained by *Rsa*I endonuclease treatment of the PCR product were identical to those obtained from the 1.75-kb ML5-ML6 fragments of S isolates (data not shown). This indicates that the SP isolate was generated by a S homokaryon heterokaryotized by a P homokaryon or heterokaryon.

The polymorphism in the PCR-amplified ML5-ML6 mitochondrial region enabled further study of mitochondrial gene flow between and within ISGs. The 1.75- and 2.05-kb amplicons usually were diagnostic for the S and P ISGs, respectively. In some fir mortality centers of the Lassen, San Bernardino, and Inyo national forests, the 2.05-kb fragment was amplified at low frequencies (6 to 17%) in S populations (Table 2). Fir and pine mortality centers are present in these national forests (Fig. 1), and both ISGs can be found. In contrast, all S isolates from western sites, where fir mortality centers and S isolates predominate, yielded only the S-associated 1.75-kb fragment. Although RFLPs of the 2.05-kb amplicon from S isolates were not identical to RFLPs of the analogous fragments from P isolates, they displayed a high degree of similarity. Only 1 restriction site of 16 differed between S and P isolates, and loss of the *Msp*I restriction site in S isolates could have been caused by a single point mutation. Preliminary sequencing data also indicate that the similarly sized amplicons from the two ISGs are highly homologous. These results suggest there may be mitochondrial gene flow between the two ISGs in geographic areas where both ISGs are present. The 2.05-kb fragment from S isolates is limited to a few geographic areas, and this may indicate that gene flow between S populations from different California regions is not very efficient. If this is the case, *H. annosum* population structure in California may be represented as a mosaic of islands having varying degrees of gene flow between them.

Further studies implementing a wider range of molecular markers are needed to understand patterns and frequencies of gene flow within and between ISGs. Research on the genetic structure of *H. annosum* will provide important insights for forest managers on the epidemiology of this disease on a large regional scale and on possible shifts in pathogen host range. These shifts may be mediated by transfer of virulence genes and ecological and climatic changes.

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