# The distribution of genets and their nuclear composition reveal the dynamics of establishment and survival of *Heterobasidion annosum* in white fir stands

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SUMMARY

The structure of *Heterobasidion* annosum populations in white fir was studied in 15 mixed conifer sites of central and northern California. Areas selected for the study displayed mortality of white fir trees in enlarging discrete patches (mortality centers). At each site, fungal genotypes were defined by somatic compatibility tests. In two sites, further genetic and molecular analyses were performed on field genotypes and on homokaryons obtained by de-dikaryotization of field heterokaryons. Isolates were found to be colonizing mostly the roots and the bole sapwood of white fir trees. No significant infections of other tree species were encountered, and the majority of white firs displayed disease symptoms associated with the presence of the pathogen. Each mortality center was characterized by the presence of several fungal genotypes, all belonging to the S ISG. Both homokaryotic and heterokaryotic strains were present in all sites. Eighty-six % of fungal genotypes were found only within a single tree or stump, while 14 % had spread to adjacent trees. The two largest genotypes had diameters of 9-10 m and had colonized 5-9 trees and stumps. The maximum distance between two adjacent trees colonized by the same genotype was 6 m, and a highly significant correlation was found between tree diameter and distance of fungal "vegetative" spread. The largest clones were found in areas characterized by high tree and stump densities. In most cases, original infection courts of existing genotypes could be traced to standing trees and not to stumps that became colonized after felling. The genetic analysis performed in two mortality centers revealed that most local

genotypes had different mating alleles, and thus originated from unrelated basidiospores. In a few cases, the same mating allele was shared by different genotypes. Molecular analysis showed that nuclei bearing the same mating allele were identical, providing evidence that the two nuclei forming heterokruyons can act independently in the field and can be shared among isolates, presumably via di-mon mating or by separate matings of different portions of widespread homokaryons.

## INTRODUCTION

*Heterobasidion annosum* (Fr.) Bref. is a basidiomycete affecting conifers worldwide (Capretti *et al.* 1990, Hodges 1969, Korhonen 1978, Sinclair 1964). By switching from a saprobic to a pathogenic existence, this fungus can effectively infest forest stands and be a persistent component of forest ecosytems (Rishbeth 195 1, Otrosina and Cobb 1989). Studies on the etiology of this pathogen in *Pinus* spp. (Rishbeth 195 1, Hunt et *al. 1976)* and *Picea* spp. (Morrison and Johnson 1978, Morrison et al. 1986, Swedjemark and Stenlid 1993) have indicated that stumps are important sites for primary infections, presumably by basidiospores. Root to root grafts and contacts allow for the secondary infection of trees adjacent to the initially infected stumps by mycelii colonization (Rishbeth 1950, Hodges 1969, Stenlid 1985). Conversely, there is no evidence that the fungus may be able to grow freely in the soil (Curl and Arnold 1964, Srago 1973, Srago and Cobb 1974), although it has been shown that basidiospores can percolate through the soil and germinate on root surfaces (Hodges 1969 and references therein). Once *H. annosum* is established in a pine or spruce stand, its pathogenic effects can be detected as patches of mortality, known as mortality or infection centers, enlarging in time to encompass adjacent susceptible host trees (Otrosina and Cobb 1989).

Although mortality centers resembling those in pine and spruce stands can also be observed in *Abies* spp., the population structure of *H. annosum* in such centers has yet to be determined. We studied the population structure of *H. annosum* in 15 white fir mortality centers in California, in order to answer the following questions: (1) Is one or are there several fungal genotypes involved in individual mortality centers, as would be expected were they the result of several primary infection events via basidiospores? (2) To what extent do individual genotypes spread secondarily from tree to tree through root contacts, and if so, how far will they spread, and what stand characters favor such secondary spread? (3) Ate stumps important infection sites, i.e. how often is it possible to trace the original infection site of a genotype to a stump? (4) Is the spatial distribution of individual genotypes continuous (as expected of undisturbed, secondarily spreading isolates) or fragmented (as expected of genotypes whose original distribution has been disturbed, and/or whose dispersal is enhanced by the movement of mitotic propagules)? (5) Are the different genotypes in a mortality center sibs, and arc there

mechanisms (e.g. di-mon mating) operating in nature to increase the diversity of S ISG genets *of H. annosum ?*

## MATERIALS AND METHODS

### Study sites, sampling, and isolations

We studied 15 white fir mortality centers in 12 mixed conifer stands with a predominace of white fir. Study sites corresponded to visually defined discrete mortality centers, in which signs of both old and recent *H. annosum*-related mortality were present. In each study site, all trees and stumps with diameter over four cm were mapped and their health condition described. Time of death was estimated for dead trees and snags, based on the amount and the color of the retained foliage. Time since felling was estimated for stumps, based on the state of decay of the stump wood. Presence and location of decay pockets on the stump tops were noted and used as an indication that the tree had been infected and colonized by *H. annosum* prior to being felled.

Above-ground sampling of all trees and stumps in each plot was performed by felling trees and snags or by cutting off the top portion of stumps, to obtain a 7-9 cm thick transversal hole section ("disk") near the root collar. Basidiocarps, usually found in stump decay pockets, were also collected and stored at 10° C.

Below-ground sampling was performed by excavating the root systems of most sample trees and stumps, and by excising complete transversal root sections. Root disks were quickly sealed, and incubated as described above for bole disks.

At the end of the incubation period, isolations were made by transferring infected wood or hyphae of the imperfect *state* of *H. annosum* (*Spiniger nzeineckellus* (Olson) Stapler) from the wood surface onto malt extract agar (MEA) medium. Isolates were also obtained from the context of all collected basidiocarps.

### Nuclear and genotypic characterization of fungal isolates

Isolates from seven white fir mortality centers were classified as homokaryons or heterokaryons as in Garbelotto et *al.* (1997a).

The intersterility group (ISG) of the isolates was determined by taxon-specific competitive-priming (TSCP) PCR (Garbelotto et *al.* 1996).

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

| Primer | Sequence (S-3') | Reference |
|---|---|---|
| NS1.5R | TCTAGAGCTAATACATGC(T/C) G | White et al. 1990 |
| NS2 | GGCTGCTGGCACCAGACTTGC | White et al. 1990 |
| NS3 | GCAAGTCTGGTGCCAGCAGCC | White et al. 1990 |
| NS5 | AACTTAAAGGAATTGACGGAA G | White et al. 1990 |
| NS6 | GCATCACAGACCTG'ITATTGCCTC | White et 1990 |
| ITS2 | GCTGCGTTCTTCATCGATGC | White et al. 1990 |
| ITS4b | CAGGAGACTTGTACACGGTCCAG | Gardes & Bruns 1993 |
| Ml3 | GAGGGTGGCGGTTCT | Steniid et al. 1993 |
| ML5 | CTCGGCAAATTATCCTCATAG | White et al. 1990 |
| ML6 | CAGTAGAAGCTGCATAGGTC | White et al. 1990 |
| MLin3 | CGACACAGGTTCGTAGGTAG | Y. Li 1995 |
| KimQ | ACGCCTCTAAGTCAGAAT | Kim et al. 1992 |
| KJ2 | GCTTGAAATTGTCGGGAGGG | T. Bruns, unpublished |
| CNS3.6 | AATGAAGTCATCCTTGGCAG | T. Bruns, unpublished |
| NL13 | CAACGCAACTTTCATGCACG | T. Szaro, unpublishhed |
| CNL2F | GTTTCCCTTTTAACAATTTCAC | White et al. 1990 |
| Ctb6 | GCATATCAATAAGCGGAGG | T. Bruns, unpublished |
| Mb2 | GTGAGTTTCCCCGTGTTGAG | M. Berbee, unpublished |

Two methods were employed independently to determine the genotype of each isolate. Somatic incompatibility groups (SIGs) were defined by somatic compatibility (C) tests (Stenlid 1985). Although SC tests supposedly only allow for the distinction of heterokaryons (Rayner 1991). we also included homokaryons, and attempted scoring of homokaryon-homokaryon and homokaryon-heterokaryon tests in a way comparable to the scoring of heterokaryon-heterokaryon tests.

In the centers Ridge 2 (Lassen) and Cougar (Stanislaus), molecular characterization and fingerprinting of individual fungal genotypes was also obtained with the aid of arbitrary primed (AP) PCR technique (Welsh and McLelland 1990). The genotype of each isolate was determined by scoring presence or absence of PCR-amplified bands. The PCR primers used individually or in pairs are shown in Table 1. Conditions described in Stenlid et al.( 1994) were used for the primers Ml3 and GTG5, conditions for all other primers were as in Garbelotto et al. (1997a). PCR products were electrophoresed on 1.5% agarose gel at 100 mV for 1 hour in O.5X Tris-acetate buffer (100mM Tris, 12.5 mM sodium acetate, 1mM EDTA, pH 8. 1), stained with ethidium bromide, and photographed under W light.

**De-dykaryotization of heterokaryons, mating allele study, and fingerprinting of derived haploid isolates**

Heterokaryotic mycelia of *H.* annosum include not only heterokaryotic hyphae, but also the two parental homokaryotic hyphae (Hansen *et* al. 1993). Hyphal tips were obtained from selected colonies; clamped cultures were discarded, and clampless cultures were retained as putative homokaryons. One of the putative homokaryons was randomly selected and paired with nine other putative homokaryons obtained from the same heterokaryons. All ten putative homokaryons were also paired with a standard tester homokaryon, to verify their ability to mate and form clamps. Matings were performed by transferring plugs of MEA colonized by the fungus on sterile MEA plates. Each mating was repeated at least twice. Plugs from two putative homokaryotic cultures were placed 1 cm apart in the center of a 9 cm diameter petri dish. Then, the plate was incubated for 7-14 d and analyzed for presence of clamps under the light microscope. Clampless plates were analyzed again at approximately 2 1 d. If at 21 d the matings were still clampless, a plug of MEA was transferred from the mating plate to a new MEA plate, subcultured for 2-3 weeks, and analyzed once again for the presence of clamps. When two putative homokaryons successfully mated (i.e. they formed a clamped colony), they were considered to bear different mating alleles and to be the two parental homokaryons of the heterokaryotic isolate from which they had been obtained.

Complete mating allele surveys for the Ridge2 and Cougar infection centers were performed by pairing parental homokaryons from each center in all possible combinations. All homokaryons were also mated with a standard homokaryotic tester to verify their ability to mate and form clamps. Isolates that were retrieved from the field as homokaryons were included. Matings were performed and analyzed as described above. Based on the results of the experiment and on the bipolar nature of *H. annosum* (Korhonen 1978, Chase and Ullrich 1990). arbitrarily named mating alleles were assigned to each isolate.

When the same mating allele occurred in two or more homokaryotic isolates, AP-PCR haplotypes were obtained as described in the previous section. The molecular analysis was used to determine whether isolates bearing the same mating allele represented the same genotype or not.

**Genetic substructuring of S ISG populations from white fir in California**

Dominant genetic markers (AP-PCR amplicons) were generated for isolates collected from white firs in the two mortality centers Ridge 2 (Lassen) and Cougar (Stanislaus). The two centers are in different mountain ranges, approximately 400 km apart, and provided us with an

opportunity to study potential genetic differentiation between two geographically separated . populations of *H. annosum.* For each genotype, polymorphic amplified fragments by AP-PCR were scored as present (1) or absent (0). The *Fst* (Wright 1951) between the two sampled populations was calculated according to the formulas by Lynch and Milligan (1994) using twenty putative loci. Although the Ridge 2 and Cougar populations included 31 and 25 isolates respectively, individuals representing the same genet were eliminated, resulting in final population sizes of 13 (Cougar) and 12 (Ridge 2).

Twenty-three AP-PCR markers were scored for the 25 unique genotypes as putative loci with two alleles. Markers were considered to be dominant (Williams et al. 1990), and the absence of comigration was also assumed. Co-migration, in fact, has been reported to be a rare phenomenon with RAPD markers (Grattapaglia and Sederoff 1994). An analysis of molecular variance (AMOVA version 1.55) was performed using a Euclidian distance matrix between all pairs of AP-PCR haplotypes (Excoffier at al. 1992). The AMOVA was used to partition the total variance into within population and between populations. The variance components were tested statistically by non-parametric randomization tests using 1000 repetitions.

## Frequency and distribution of fungal genotypes; effects of tree diameter and stand density on secondary spread of *H. annosum*

Isolates that were somatically compatible (and had the same AP-PCR haplotype in the centers Ridge 2 and Cougar) were considered to be part of the same genotype. Approximate boundaries of the area occupied by each genotype were determined by enclosing points in which the same genotype was retrieved with a continuous line. Boundaries of such areas are influenced by the intensity of sampling and by the size of the sample area; thus they should be considered only as approximations. Fungal individuals belonging to the same genotype were considered to represent the individual unit of *H. annosum* populations and were defined as representatives of the same genet (Rayner and Todd 1977). We use the broader definition of genet by Anderson and Kohn (1995) to include both homokaryotic and heterokaryotic genotypes. The area occupied by each genet was superimposed on the general map of each study site. This allowed for the determination of the number of trees and stumps colonized by each genet and of the number of genets present in each tree or stump. The basal area colonized by each genet was calculated by adding up the basal areas of trees colonized by a genet; when more than one genotype was found in a tree, only a portion of the basal area proportional to the abundance of that genotype on that tree, was assigned to each genotype. Estimates of the location of initial infection courts, and the rate and direction of spread of the symptoms caused by each genet, were based on intensity of symptoms, time incurred since tree death, and degree of wood decay displayed by trees and/or stumps colonized by a genet.

Distances between all pairs of immediately adjacent trees or stumps (both are referred to as stems) colonized by the same genet were calculated. Only pairs for which infection from a third stem could be unequivocally dismissed were taken into account. Our hypothesis was that larger sized stems, with larger roots and more extensive radical systems, should facilitate the fungus to move further away. Regression analyses were employed to study the relationship between the distance covered by the fungus between each stem pair and the size of the stems involved. Pair of trees were assigned to a dbh class. For each class, the average distance between stem-pairs and the maximum distance between stem-pairs were determined. Average and maximum distances were then regressed against the diameter of the largest stem in the pair, the diameter of the smallest stem in the pair, and the sum of the diameters of both stems.

An additional hypothesis was that higher stem densities, by increasing frequencies of root contacts and grafts among trees and/or stumps, should also facilitate the secondary spread of *H. annosum.* To test this hypothesis, we calculated host stem densities of areas in which at least two genets had secondarily spread to more than one stem, and compared them (Student t-test) to stem densities where one or less genets had spread vegetatively.

## RESULTS

### Isolate collection

A total of 552 isolates was collected in the 15 study sites. Number of isolates obtained in each plot ranged from 6 to 53. A total of 37 stumps, 42 dead trees, and 67 live trees yielded isolates. Fifty-four % of the live trees were symptomatic, 33% were asymptomatic, and 13% were of undetermined health. Symptoms included thin crown, reduced height growth, off-color foliage, low crown ratio (calculated as portion of the tree height occupied by the live crown), presence of pathological **wetwood,** and **sapwood** and/or heartwood decay. The asymptomatic trees displayed extensive **fungal** colonies in slightly discolored portions of the bole **sapwood.** Seventy % of the "positive" stumps displayed typical decay pockets on the tops, an indication that the tree had been infected by *H. annosum* before being felled; the rest had smooth tops. suggesting that either they had been infected after the tree was felled or that, at the time of cutting, the **fungal** colonization was only in the roots or in the lower part of the bole. The combination of bole and root sampling proved effective since in many instances live isolates were collected from either **part** of the tree, but not from both parts. A total of 233 isolates were obtained from roots, and 3 19 from boles. Ninety-seven % of the bole isolates came from the **sapwood,** and 3 % were found in the heartwood. No consistent association *between infected* trees and stumps was observed.

## ISG determination, ploidy, and genet identification

Taxon-specific competitive-priming (TSCP) PCR indicated all isolates belonged to the S ISG. Somatic compatibility tests outlined 228 different genets in 15 sites; six % of crosses were undetermined. Up to eight different genets were retrieved in individual trees or stumps. Averages of 2.07 and 2.1 genets were found per tree and stump, respectively. Genotypes from the heartwood were often also colonizing the sapwood. Both homokaryotic and heterokaryotic genets were found in the study sites (Garbelotto et al. 1997a). In Cougar and Ridge 2, 64 and 23 % of the isolates were homokaryotic, respectively. AP-PCR haplotypes could be used to differentiate isolates. Thirteen genotypes were identified in Cougar and 12 *in* Ridge 2. Results obtained by SC tests and AP-PCR fingerprinting were identical in these two plots, regardless of the ploidy of the isolates.

## Patterns of genet establishment and spread

At all sites many fungal genotypes were retrieved. Number of genets per plot averaged 15 (SD=8.8) and ranged from six to thirty. At each site, most genets was confined to a single tree or stump. A total of 32 genets was found to have spread secondarily to more than one tree or/and stump. Although this represents only 14% of all genets, the relative importance in terms of basal area occupied by these "multi-tree" genets (we use this expression even if genets include stumps because in the majority of cases the stumps were infected as trees before felling) averaged 35%. Twentythree multi-tree genets were obtained from adjacent roots of different trees. There was no compelling evidence that any of the 32 multi-tree genets had been initiated by infections on stump tops. Careful site observations allowed the classification of genets in one of three classes. Class 1 (22 genets): no stumps infected after being cut were present in the site. or, if present, their location and distance from the area occupied by the genet made it extremely unlikely that they were initial infection courts. Class 2 (4 genets): although stumps with a smooth top were included in the area occupied by the genet or were in the vicinity of such area, there were indications of *H. annosum* mortality preceding the creation of such stumps. This suggested the genets were already established in those areas, and the stumps were infected by these preexisting genets, either as trees prior to being felled (in this case fungal colonization had not reached the bole at the time of felling), or as stumps, but through root contact . Class 3 (6 genets): stumps with a smooth top were included in the area occupied by the genet or were in the immediate vicinity; nevertheless, mortality and *H. annosum* symptoms and signs (e.g. laminated white decay and dry fruit-bodies) could be dated to the same time the stumps were created (e.g. genets 6 and 8 *in* Huey). Although stump infections were not strongly supported

in any of the three classes (but could not be totally excluded either), genets in class 3 seem to have the highest chance of having being initiated through stumps.

There were considerable differences among sites regarding the incidence of multi-tree genets. In two plots (Obsidian 1 and Owl), no multi-tree genets were found. Conversely, in Woodpile, Huey, and Mountain View, four, eight, and three multi-tree genets were found, representing 72, 62, and 57 % of the infected basal area of each plot. These multi-tree genets represented an average of 33% of the infected basal area (range 13-56%), and were found on 2 to 7 stems (average=3.6). Average tree density in eight plots with two or more multi-tree genets (1083 stems/ha, SD=264) was significantly higher (P=0.039) than in seven plots with O-l multi-tree genets (739 stemd/ha, SD=319). Estimates of genet expansion based on distance of trees infected by a genet and onset of disease symptoms or death, were obtained in seven mortality centers, the estimates are: 0.5-0.75 m/yr in Ridge 2, 0.5 m/yr in Mountain View, 1 m/yr in Cougar, 0.25-0.7 m/yr in Woodpile, 0.4 m/yr in Meis Cabin, 0.7 m/yr in Huey, and 0.5-0.9 m/yr in Luey.

The maximum distance between trees infected by the same genet ranged between 1.5 and IO m, depending on the study site: the average of maximum distance values from 13 plots was 5.5 (SD=2.4). Regression analyses of distances between paired stems infected by the same genet, and dbh classes, yielded highly significant R2 values. The largest R2 values were obtained when regressing the maximum ($R^2$=0.957) and the average ($R^2$=0.922) distance between stem pairs against the sum of the two diameters (Figure 1).

## Mating alleles and AP-PCR fingerprinting of individual nuclei (parental homokaryons)

In Cougar, two heterokaryotic and twelve homokaryotic genets were isolated. *H. annosum* is a bipolar basidlomycete (Korhonen 1978, Chase and Ulhich 1990), and a total of 14 mating alleles are possible for the Cougar site. Two alleles from a heterokaryon and one from a homokaryon were not determined. A total of 10 mating alleles were identified. Heterokaryotic and homokaryotic genets were found in the same two trees, and shared an identical mating allele.
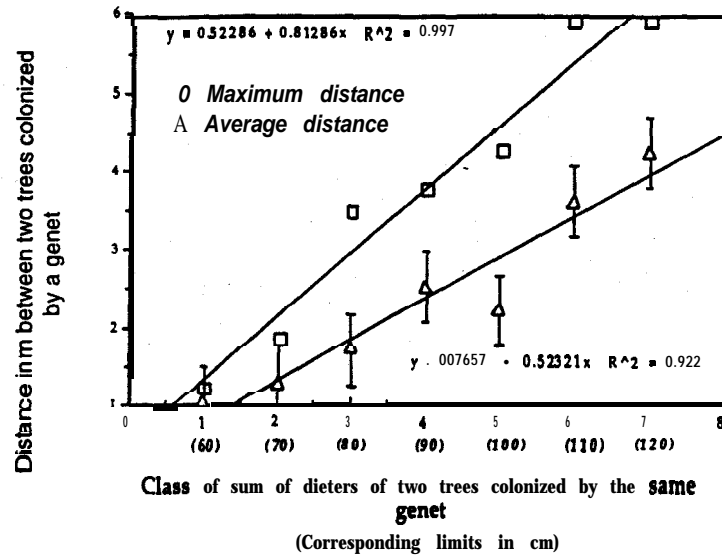
**Figure** 1. Linear regression of the sum of diameters of pairs of stems (trees or stumps) colonized by the same *Heterobasidion annosum* genet and the distance between the two stems in each pair. Position of the two stems in each pair suggests the fungus had spread directly from one to the other through contact points or grafts in the two root systems. Stem pairs were divided into classes according to the sum of their diameters at root collar (for stumps) or at breast height (for trees), and for each class the maximum and average between-stems distance were calculated. Two independent regression analyses are shown for the average (including standard error bars) and the maximum distance between trees within each class. To determine whether trees are at risk of being infected by adjacent trees or stumps already colonized by *H . annosum,* add the diameters of the infected and the healthy trees and determine the distance between them. If the two values define a point in the diagram under the regression line, contagion between the two stems may occur.

In Ridge 2, eight heterokaryotic and four homokatyotic genets were isolated (Fig. 2). Thus, a maximum of 20 alleles could be found in Ridge 2. The two alleles from heterokaryon II were not determined. A total of 15 mating alleles were identified. Identical mating alleles were shared by the heterokaryotic genets I and IV (b), the heterokaryon I and the homokaryon VI (a). and by the heterokaryon IX and the homokaryon VIII (m). In all cases, genets that shared mating alleles were spatially adjacent to each other.

Genotypes sharing a mating allele were found to be sharing a common nucleus. In fact. AP-PCR patterns of homokaryotic isolates hearing the same allele were indistinguishable from each other.
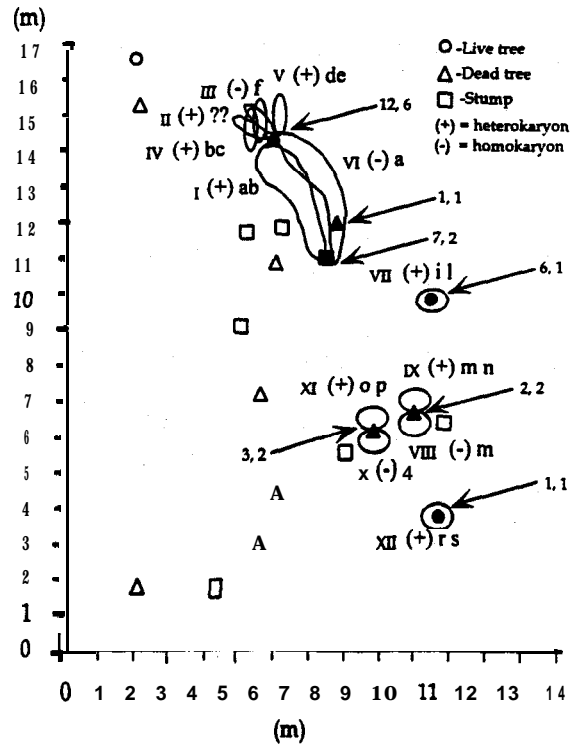
Ridge 2

Figure 2. Distribution, size, and mating alleles of *Heterobasidion annosum* genets at Ridge 2 (Lassen National Forest). All trees and stumps in the area shown were sampled; shaded symbols indicate positive sample point. Arrows pointing at shaded symbols indicate number of isolations und genets obtained from each tree or stump. Roman numerals indicate the different genotypes, which are visualized by the solid lines encircling the trees from which they were isolated.

## Population    subdivision

The *Fst* between Cougar and Ridge 2 was 0.22. This suggests a moderate level of genetic differentiation between the two populations. Hierarchical partitioning of the genetic diversity by AMOVA indicated that while most of the variance was due to sampling within populations (84%), a significant proportion of genetic diversity was found between the two populations *(16%* of the variance, $f_{st}=0.16, P<0.001$).

## DISCUSSION

*Heterobasidion annosum* isolates were obtained mostly from the **sapwood** of white fir stumps, and of dead and live white fir trees. Roots were also often extensively colonized by *H.* unnosum. The frequent occurrence of *H. annosum* in the **sapwood** rather than in the heartwood, and the symptoms observed in the host **trees** suggest that this fungus is a serious pathogen of white fir in California. The effects of *H.* unnosum on white fir may vary, depending on the amount of **sapwood** in the tree, the defense mechanisms triggered in the host, and by the general ecological and climatic conditions at the site (Otrosina and Cobb 1989, third chapter in this dissertation).

The isolation of *H. annosum* from the sound inner **sapwood** of asymptomatic trees may indicate that the fungus undergoes a latent phase between infection and pathogenic wood colonization. The modified moisture/aeration regime in our cut wood samples may have triggered the extensive growth and sporulation of *H. annosum,* and, in a parallel way, altered moisture/aeration regimes (e.g. because of a wound or of water stress) may trigger the growth of the fungus in standing trees (Boddy and Rayner 1983). This has been well documented for other decay fungi (Boddy and Rayner 1984, Chapela and Boddy 1988, Etheridge and Craig 1976, Webber and Gibbs 1984), but further evidence is required to validate our observations for *H. annosum* in white fir.

All isolates from the 15 study sites belonged to the S ISG. With two exceptions, the only species affected by *H. annosum* was white fir; *H* . annosum ISG S thus displayed a high level of host specificity in ecologically **mesic,** mixed conifer stands with a predominance of white fir (Worrall *et al.* 1983).

Several **fungal** genotypes were found at each site, most genotypes were limited to a single tree *or* stump and in many cases, several genotypes could be found on the same tree or stump. Some **fungal** isolates retrieved were homokaryotic (Garbelotto *et al.* 1997a). Although somatic compatibility tests are based on interactions between **heterokaryons** (Rayner 1991), we were able to differentiate homokatyons by scoring the type of interaction on a petri dish in a fashion equivalent to that of sc tests. In a few cases. "negative" homokaryon-homokaryon crosses were validated by observing newly-formed hyphal clamps in the cross plates. AP-PCR genotypes perfectly matched our sc classifications in Cougar and Ridge 2; a good agreement of the two techniques is expected when sc systems are regulated by a large number of alleles at several loci and when individuals are randomly mating (Smith et al 1994). The distribution of many discrete genets in white fir mortality centers indicates that primary infections, presumably via basidiospores, are extremely frequent and unlinked to the presence of stumps. This conclusion

was evident for small genets confined to single trees, particularly when the fungus was isolated only from the stem but not from the roots. In the case of genets that had expanded to include two or more trees, the analysis was more complex. Nevertheless, in most cases (22 of 32 multi-tree genets), through a careful description of the site and through a reconstruction of the development of mortality centers, we could strongly support the view that these genets had not been initiated on stump tops. Thus, live trees appear to provide the most common infection courts in white fir stands of the Sierra Nevada and southern Cascades mountain ranges. The fact that several genotypes could be found in individual standing trees differs from results obtained by studies in *Picea abies* (Karst.) stands (Stenlid 1985), and may be related to the fact that true fir trees become infected directly through aerial inoculum and not through mycelial contact with stump roots. This is similar to what is reported for *H.* annosum in *Tsuga* spp (Rhoads and Wright 1946), and it is in contrast with the known dynamics of establishment of *H. annosum* in *Pinus* and *Picea* spp, in which stump tops appear to be important infection courts (Rishbeth 195 1, Morrison and Johnson 1978, Swedjemark and Stenlid 1993). Wounds may provide suitable infection courts for *H.* unnosum on true firs (Aho 1983) but further research is needed to address the issue.

Only 14% of the fungal genets had colonized more than one host tree. Nevertheless, the role of these expanding genets in shaping mortality centers was significant, and on the average they represented 35% of the colonized basal area. The low frequency of tree to tree spread suggests that expansion beyond the initial infection site may be a rare and critical phase in the development of genets, as suggested for four *Armillaria* spp. (Worrall 1994). The area occupied by multi-tree genets was uninterrupted, except for some overlap with other genets colonizing the same trees. If trees included in the area occupied by a genet from which no isolates were obtained, but that displayed typical symptoms of *H. unnosum* decay, were taken into account, genet expansion could always he explained by direct tree to tree spread. No evident pattern of rnitotic spore transmission was found within a mortality center (e.g. the presence of spatially isolated ramets), but very short dispersal could not be excluded. The longest distance covered by a single genet in all sites was 10 m; the average was 5.5 m. This is a rather small value compared to those of other root pathogens such *as Armillaria* spp (Smith et *al.* 1992). but it is in agreement with values reported for *H. annosum* genets in Norway spruce in Europe (Stenlid 1985, Swedjemark and Stenlid 1993). Estimates of fungal spread ranged from 0.2 to 1 m/year. These values match results obtained in a field inoculation study of white fir (Garbelotto *et al.* 1997a and b) and are comparable to values reported by others (Hodges 1969, Slaughter and Parmeter 1995). By combining estimates of spread and size of genets, we can assume the life span of genets ranged from 10 to 50 years. This range, although an underestimation because it assumes unimpeded fungal growth, indicates that fungal genets have expanded in a period much shorter than the age of the stands in which they were found. The age of the dominant and co-dominant trees in the stands sampled in fact, ranged from 80 to 120 years, and in all likelihood

the trees had preceded the fungus. Thus, the data is best interpreted by excluding the possibility of carry-over of fungal genets from old-growth white fir trees that had occupied the sites prior to the younger growth, and by considering the existing genets as originated by primary infections on the present stand.

Tree to tree spread through root contacts or grafts appeared to be the the most likely way of contagion. In fact, adjacent roots from different trees were colonized by the fungus in 23 of 32 multi-tree genets. Root contacts or even grafts may be necessary for contagion because at our study sites we never observed ectotrophic mycelial growth on or away from roots. The lack of ectotrophic growth may also explain why tree to tree spread of fungal genets was not frequent. In fact, without ectotrophic growth, the fungus is unable to by-pass portions of the roots unsuitable for its growth or already occupied by competing microorganisms (Rayner and Boddy 1988, Garrett 1970). Furthermore, in a field inoculation study, Garbelotto et al. (1997b) have shown that small root size may be a limiting factor for the endotrophic growth of *H.* unnosum.

The importance of root size in the secondary spread of *H.* unnosum is also confirmed by the results of the regression analyses relating tree diameter with distance between trees colonized by the same genet. With increasing tree diameter, the distance covered by a genet between trees increased as well. In no case, did contagion occurred between two trees more than 6 m apart. The $R^2$ obtained by regressing the sum of diameters of tree pairs (or stumps) colonized by the same genet against the distance between them, was highly significant. The relationships between tree dbh and distance of contagion can have practical applications with respect to timing of thinning operations in *H. annosum* infested white fir stands .

In 12 of 15 study sites multi-tree genets only colonized a few trees. This could be due either to the recent establishment of the pathogen in a site (this may be the case for Owl and Obsidian 1, where only a few isolates were obtained), or to the sites characteristics being unfavorable to the secondary spread of *H. unnosum (e.g.* Dark Canyon and Loggers, where trees are well spaced). For instance, tree size and density appear to have an effect on the advance of other root pathogens such *as Phellinus weirii* (Murr.) Gilbn. in Douglas-fir plantations (Bloomberg and Reynolds 1982). In the three sites Woodpile, Mountain View, and Huey though, secondarily expanding genets were prominent. In the first two sites, individual genets encompassed up to 11 trees (Woodpile) or expanded up to 10 m by colomizing multiple trees(Mountain View). Tree densities in the two areas colonized by these relatively large genets were extremely high, and were the likely cause for the successful expansion of *H.* unnosum genets in these cases. In contrast, the documented inability of *H.* unnosum to spread between two adjacent trees further than 6 m apart (this study), the need for root contacts or grafts because of the lack of free growing mycelium (Curl and Arnold 1964, Srago 1973, Srago and Cobb 1974), the lack of ectotrophyc mycelium on the roots (this study), and the reduced growth

in smaller roots (Garbelotto *et* al. 1997b) are probably some of the factors determining the irregular enlargement and the arrest of *H. annosum* genets in white fir mortality centers.

The distribution and frequency of mating alleles and the molecular analysis provide further insights on the population dynamics of *H. annosum* in white fir. At both Cougar and Ridge 2, many different mating alleles were identified. This result is similar to results obtained by Chase and Ullrich (1983) in a red pine plantation and is a clear indication that: 1) independent basidiospore infections have occurred in the two sites and, 2) the source of the local inoculum is not a preestablished genet or a local prolific "fruiter", but rather a broader population. Broad breeding populations have also been reported for *Armillaria* spp. (Smith et al. 1994, Rizzo et al 1995, Saville et al 1996). According to the *Fst* value, and to the results of the AMOVA, most of the genetic variability is found within populations, indicating that the S ISG of *H. annosum in* California is a highly outcrossing group with a corresponding level of local genetic variation. However, a significant portion of the genetic variability was also detected between Cougar and Ridge2. The two popuations are about 400 km apart, and are located in rather different ecological situations. The first, in fact, is in a large area of uninterrupted mesic west-side mixed-conifer forest; the second is in the vicinity of drier sites where pines and other more draught-resistant species dominate. Ridge2 is also located in the Lassen National Forest, in which introgression of genetic markers normally associated with the P ISG has been reported (Garbelotto *et al.* 1996). In other words, although gene flow between the two populations is not improbable (Kallio 1970, Rishbeth 1950). there are genetic differences between the two populations, probably due to different selection pressure in the two areas. A moderate level of genetic substructuring is in agreement with results on the population structure of the S ISG of *H. annosum* in Europe (Stenlid *et al.* 1994).

Identical nuclei were shared in three cases by a homokaryotic and a heterokaryotic genet, and in one case, one allele was shared by two heterokaryotic genets. Genotypes sharing nuclei were adjacent to each other. These results indicate that either these homokaryons were derived from the heterokaryon or that they represent portions of the thallus that were not heterokaryotized. The independence of homokaryotic components of heterokaryons has also been shown in the laboratory by Hansen *et al.* (1993). By hyphal tipping they showed that heterokaryons maintain parental homokaryotic hyphae intermingled with heterokaryotic hyphae in the thallus. Furthermore, field and greenhouse experiments (Garbelotto et *al.* 1997a, Cobb *et al.* 1989) have demonstrated that homokaryons can survive in nature and be virulent.

Two adjacent heterokaryons in Ridge 2 shared an identical nucleus. This situation could have arisen by di-mon mating or by heterokaryotization by different homokaryons of two separate sectors of the homokaryotic mycelium. Di-mon mating has long been known to be a frequent event in laboratory pairings of basidiomycetes (Buller 1930 and 1931). and has been

suggested in field studies based on distribution of mating alleles (Kay and Vilgalys 1992) and *RFLPs* of diploid isolates (Rizzo *et al.* 1996), but this is the first report to provide proof of sharing of nuclei between spatially contiguous genets in nature.

The reason why *H.* annosum mortality appears in discrete infection centers composed of many trees is not completely explainable based on our results. While in some cases fungal secondary tree-to-tree spread does affect several contiguous trees, in most infection centers, only a small number of trees appears to be secondarily colonized by the same genet. A possible explanation of this mortality pattern may be that the death of one or a few trees affects the health of other adjacent trees. Environmental conditions, physiological status (e.g. transpiration rates may increase, modifying the oxygen/water ratio in the wood), attractiveness to other pathogens or pests which may. predispose the trees to colonization by *H. annosum,* may be profoundly affected in trees sorrounding dead trees. A more direct relationship to the pathogenic activity of *H. annosum* can be envisioned as follows. The pathogen is present, but inactive in a large number of host trees. A particular physiological condition in a tree, triggers the pathogenic activity of the fungus, which colonizes the lower portion of the bole and the roots. As roots from neighboring trees in contact with roots of the "symptomatic" tree, become physiologicall! dysfunctional because of colonization by the expanding genet, activation of resident genotypes of the pathogen occurs. The newly-activated genotypes will start expanding and consequently they will both activate more genotypes and block the expansion of the genotypes previously activated. The final outcome will be that of a group of adjacent symptomatic or dead trees colonized by different genotypes.

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