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Population biology of the forest pathogen *Heterobasidion annosum:* Implications for forest management.

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Heterobasidion annosum ranks as one of the most destructive pathogens in North American coniferous forests. Understanding the population biology of' this fungus may help us to understand not only the basic biology of the organism, but also general patterns of disease development, modes of host-pathogen interactions, effect of management practices on the dynamics of dispersal, establishment, and evolution of the pathogen and/or the host. in turn, this information allows for a more profound understanding of the general health of an ecosystem leading to more refined and targeted management practices. In the case of *H. annosum*, previous research has elucidated aspects of the etiology and spread of disease for host species such as European pines and spruces. In these instances, primary stump infection and root-to-root secondary contagion appear to be major avenues of disease development. No information is available on the true fir/H. *annosum* pathosystem for Western North America, although the pathogen is increasingly affecting this tree species. Because of the different hosts involved, of the different biogeographic region and of the significant genetic divergence amongst groups of *H. annosum* characterized by different host preferences, it is not possible to extrapolate results from other regions of the world to Western North America, and in particular to Caiifomia. Two genetically distinct intersterility groups (ISGs) of the fungus are present in California: the S ISG mostly infects true firs, hemlocks, Douglas-firs and sequoias, while the P ISG is found mostly on pines, incense cedars, and junipers. These two ISGs are known to mate in the laboratory, but evidence of mating in nature has been gathered only recently. Still, there is no understanding of the frequency of mating and gene flow between the two groups.

In these last years our research effort has been to elucidate the dynamics of fungai establishment and spread in California mixed conifer forests with a predominance of true fir. The scale of our analyses has ranged from small scale studies designed to understand the genetic structure of pathogen populations in individual mortality centers to larger analysis at the broader regional level, designed to shed light on medium to long distance gene flow between demes of one ISG and even potentially between ISGs. One of our focuses has been to relate the genetic structure of this organism to forest stand characteristics in the attempt to understand the impact of forest management on the population biology of this organism, on the epidemiology of the disease, and on the severity of the mortality associated with this pathogen. Our regional scale analysis has also benefited from a parallel undergoing research program Alice Ratcliff (PSW Forest Service), on the genetic structure of H. *annosum* in pine mortality centers.

Materials and Methods

Sampling

Our main effort has been to intensively sample a significant number of individual mortality centers. In such areas, also known as root-disease centers, mortality and/or disease symp toms appear on a cluster of trees, which enlarge over time. We selected 15 sites (Table 1) in 12 different forest stands to be intensively sampled. Five sites were in two National Forests (Lassen and Piumas) in the Southern Cascades: the remaining ten were located in the Stanislaus and Eldorado National Forests in the Sierra Nevada mountain range. The boundaries of each study site were delineated either by discontinuity in the tree cover (e.g. a road or a

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meadow) or by a ring of seemingly asymptomatic trees, All stands were mixed in composition with a prevalence of white fir. Sampling included obtaining a full section of the stem at the root collar for all live, dying and recently dead (up to 5-7 years) trees and for all stumps. Underground sampling was performed by excavating the root systems of ail trees and stumps (from now on referred to collectively as "stems"), and by excising a complete section of a variable number of roots (1 to 11, the number of sampled roots increasing with increasing size of the stem) at about 0.5 to 1 m from the root collar. Colonies of *H. annosum* were visually scored after one week incubation in a plastic bag, and each discrete colony was isolated and cultured on MEA.

Sites were mapped and site characteristics such as density, species composition, tree health (based on indicators such as the live crown ratio, the crown shape index, the color of the foliage, and the reduction in terminal growth), degree of wood decay and time since death (based on amount and color of the retamed foliage). Particular attention was devoted to describe stumps in order to determine whether they had been colonized prior or post felling. A smooth stump surface was taken as indication that the stump had been infected after the tree had been logged: on the contrary the presence of decay pockets on the stump surfaces was considered to be a sign that the stump in fact had been infected as a tree prior to felling.

Genetic characterization of fungal isolates

Two different methods were employed to detine individual genotypes of the fungus:

1- Somatic compatibility tests, based on genetic mechanisms of self vs., non-self recognition, resulting in a line of demarcation between two fungal colonies growing next to each other on a culture medium. This technique was-employed for all sites.

2- Genetic fingerprinting, not too dissimilar from DNA fingerprinting used in forensics. aimed at visualizing a large number of markers (corresponding to genetic loci), whose combination will produce patterns specific and diagnostic for each genotype. We chose a method based on the PCR reaction called arbitrary primed (AP) PCR fingerprinting.

Although both methods allow for a distinction of even sib-related individuals, more resolution is given by the latter, which also has the benefit of providing the researcher with a tool to quantify genetic similarity among individuals or demes. This technique was employed for two sites (one in the Cascade and one in the Sierra Nevada mountain ranges)

Ploidy of isolates

Although in most Holobasidiomycetes (such as *H.* annosum) the haploid phase is assumed to be ephemeral and quickly followed by a long-lasted dikaryontic (n+n genotype), we ran different tests to independently test the ploidy of our field isolates. These tests included microscopic observation of hyphal septa (dikaryonts possess specific structure called clamp connections that are missing in haploid isolates; nature of somatic interactions between isolates (homokaryon-homokaryon, homokaryon-dikaryon, and dikaryon-dikaryon interactions are different) In two sites, we also separated (dedikaryotized) the two nuclei in our field dikaryonts through a series of single hyphal tipping isolations. The mating allele of each parental hapioid was determined through a series of mating tests with other field isolates and with known testers. *H. annosum* is a bipolar fungus and two different mating alleles are necessary for mating to occur. Reoccurring identical alleles are expected in a local population if a)- many sib-related individuals are present or b)- some mating alleles are present at high frequencies in the population. DNA fingerprints were run on haploids with identical mating alleles to check whether these haploids

were genetically identical or shared the same mating allele but represented different genotypes.

Data analyses

Distribution and shape of fungal genotypes was inferred by overlapping site maps 'with fungal genotypes maps. Size of fungal genotypes was determined by linking ail isolates representing the same genotype.

Distance between trees colonized by the same fungal genotype was regressed against the size of the trees involved to study the effect of tree size on secondary (vegetative) tree-to-tree spread of the pathogen.

Sites were divided in two groups: I-high chance of secondary tree-to-tree spread (more than two fungal genets has infected neighboring trees). II-low chance of secondary spread (O-1 secondarily spreading fungal genotypes). Stem densities were calculated for each group and compared with a Student's t-test

Rates of fungal spread were approximated for secondarily expanding genotypes by determining the time of onset of symptoms or of death in hosts known to have been colonized by the same genotype through root to root contagion.

Genetic structure between the two populations for which the DNA fingerprints had been produced was analyzed by calculating Wright's Fst values and by AMOVA (analysis of molecular variance).

Results

Results are summarized in Tables 1-3 and in Figures 1-3.

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. annosum.* 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 primary pathogen of white fir in California. All isolates belonged-p the S ISG, and 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.

Somatic compatibility (SC) tests and AP PCR fingerprinting indicated that many different genotypes were present in each discrete mortality center. Both homokaryons and heterokaryons were collected. Although somatic compatibility tests are based on interactions between heterokaryons 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. Genotypes defined by AP PCR fingerprints 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 as shown for *H. annosum,* and when individuals are randomly mating. Identical nuclei were shared in three cases by a homokaryotic and a heterokaryotic genet. 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 twd separate sectors of the homokaryotic mycelium. Di-mon mating has long been known to be a frequent event in laboratory pairings of Basidiomycetes, and has been suggested in field studies based on distribution of mating alleles and RFLPs of diploid isolates, but this is the first report to provide direct evidence of sharing of nuclei between spatially contiguous genets in nature.

Thi distribution of many discrete genets observed 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 multi-tree genets, it also appeared that initial infection had not necessarily occurred on stump tops. Our analysis, based on careful sites description combined with reconstructions of the development of mortality and decay in root disease centers, suggested that at least for 22 of 32 multi-tree genets, the fungus was already established in those areas by the time the stumps were created. Thus, stumps were infected by 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. Therefore, we conclude that live trees provide the most common infection courts for *H. annosum* in white fir stands of the Sierra Nevada and southern Cascades mountain ranges.

Only 14% of the fungal genets had colonized more than one host tree. 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. Nevertheless, the role of these expanding genets in shaping mortality centers was significant, and on the average they represented 33% of the colonized basal area. The area occupied by multi-tree genets was uninterrupted, except for some overlap with other genets colonizing the same trees. By taking into account stems from which positive isolations of *H. annosum* were obtained, and trees or stumps which did not yield *H. annosum* isolates but that displayed symptomatic wood decay, genet expansion could always be explained by direct tree to tree spread. No evident pattern of mitotic 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 and the average was 5.5 m. Estimates of fungal spread based upon onset of symptoms or tree mortality within individual multi-tree genets, ranged from 0.2 to 1 m/year. 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 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 most likely way of contagion. In fact, adjacent roots from different trees were colonized by the fungus in 23 of 32 multi-tree genets. The importance of such routes of contagion was also underlined by the fact that no free mycelium or evident root ectotrophic mycclium were encountered at our study sites.

The importance of size of tree root systems in the secondary spread of *H. annosum* 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 occur between two trees more than 6 m apart. 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. annosum* (e.g. Dark Canyon and Loggers, where trees are well spaced). 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. In the three sites Woodpile, Mountain View, and Huey though, secondarily expanding genets were prominent. Individual genets encompassed up to 11 trees in Woodpile and expanded up to 10 m by colonizing multiple trees in 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. annosum* genets in these cases.

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 a scale larger than that of individual mortality centers. Since the fungus is bipolar, the same mating allele would be expect&l in 50% of the meiotic progeny or even in un-related genotypes, if that particular mating allele occurred in high frequency in the general population of the fungus. At both Cougar and Ridge 2, many different mating alleles were identified. This result 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. 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 Ii. *annosum* in California is a highly outcrossing group with a corresponding high level of local genetic variation. However, a significant portion of the genetic variability was also detected between Cougar and Ridge 2 by AMOVA. The two populations are about 400 km apart, and are located in rather different ecological situations. The first is in a large area of uninterrupted mesic westside mixed-conifer forest, while the second is in the vicinity of drier sites where pines and other more draught-resistant species dominate. Ridge 2 is also located in the Lassen National Forest, in which introgression of genetic markers normally associated with the P ISG has been reported. In other words, although gene flow between the two S ISG populations is not improbable, there are genetic differences between the two populations, probably due to different selection pressure in the two areas, and maybe also due to significant levels of gene flow between S and P ISGs in the Lassen site population.

Implications for management

These considerations stem from results presented in this report and from other studies, in particular from 1)-a field inoculation study aimed at determining rates of secondary spread and effects of tree root architecture on such spread (Garbelotto et al 1997), 2)-a study on the host specificity and possibility of inter ISG flow of the S and P ISGs in California (Garbelotto et al. 1996), employing a wide range of nuclear and mitochondrial markers (PCR-based and isozyme), and 3)- a greenhouse inoculation study designed to assess the virulence of a hybrid SP isolate (Garbelotto 1996).

1- Stump creation may not be the most important contribution of man to the spread of Annosus root disease, but rather the creation of potential infection courts on live trees (e.g. wounding).

2-, Stand structure will highly influence the progression of the disease. Dense stands will favor the spread: controlling the density of a stand without inflicting a large number of wounds may be an effective way to reduce the

spread of the disease. Also large trees (or stumps) will allow the fungus to spread secondarily to larger distances through the root system. For the mountain ranges of Central and Northern California it is possible% use our graph showing the relationship between stem size and distance traveled by the fungus, to decide on the need for stump treatment or on the risk of selective cutting in any given stand.

3-Although not explicitly dealt with in this report, there is evidence indicating that a large amount of the inoculum may be resident, but latent, i.e. not causing disease. Further studies need to investigate the possibility that specific external ecological or physiological conditions may trigger the development of pathogenic effects.

4- Probably differently from the P ISG on pines, the S ISG is extremely abundant in true fir populations, if both the pathogenic and the latent stages are considered. Fire management has not only increased the density of true firs in west side sites. but has also determined a massive presence of true firs in dryer east-side type sites. Increased true fir presence signifies increased S ISG inoculum in dryer east-side sites.

5- *H. annosum* S ISG shows a high host specificity on mesic sites (e.g. it will *only* affect true firs and sequoias on the western slopes of the Sierra Nevada or in the moister sites of the Southern Cascades) but it will infect pines in dryer overstocked sites. Also pine and juniper stumps can harbor both ISGs.

6- Because of new increased coexistence of S and P ISGs in east-side type forests and because of the presence of new niches inhabitable by both ISGs (e.g. pine stumps or weakened pine trees), there is a potential for inter ISG contact and mating, with resulting hybrid populations and inter-ISG gene flow. We have shown that hybridization is possible and have already detected low levels of inter-ISG gene flow in east-side type sites.

7- Although we have demonstrated (with an inoculation experiment) that hybrid isolates may be less virulent than either parental types on their specific hosts, hybrids will thrive in stumps or weakened hosts. As potential habitat increases, so may the hybrid population and the gene flow between groups: this may allow for selection of new virulence traits in the two ISGs or even in hybrid isolates.

We believe we are witnessing profound changes in the population biology and maybe in the evolution of a native pathogen in forests that have been profoundly affected by man in the last 100 years or so. Because of anthropogenic effects, these forests may in fact represent novel environments in which old rules no longer apply. Because in turn, *H. annosum* may have profound effects on forest health and succession, we should be aware that changes in the fungus caused by changes in the habitat may determine further changes in the habitat itself.

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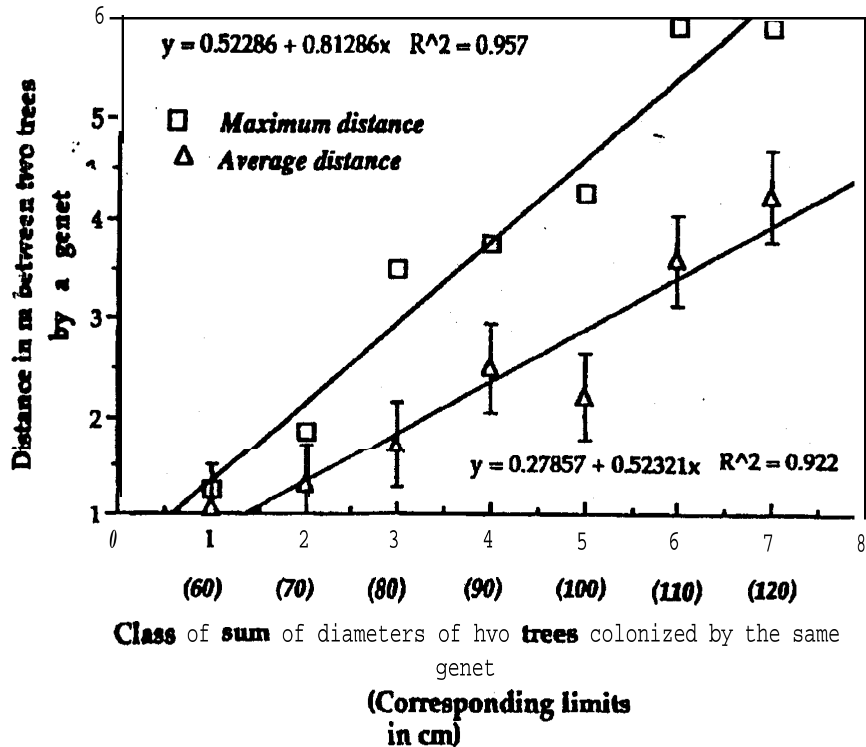

Figure 1. **Linear** regression of the sum of diameters of pairs of stems (trees or stumps) colonized by the same ***Heterobasidion** annosum* genet and the distance between the two stems in each pair. Position of the two stems in each pair suggests the fungus had spread directly from to the other through 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 thc regression line, contagion between the two stem may occur.

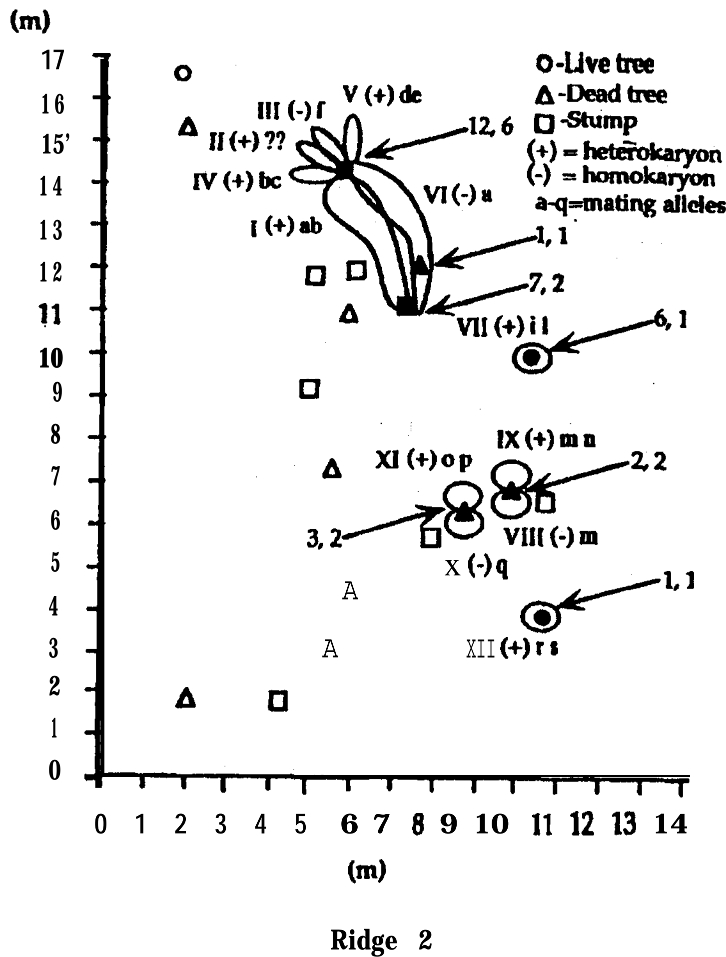

Figure 2. Distribution, size, and mating alleles of ***Heterobasidion annosum*** genets at Ridge 2 (Lassen National Forest). All trees and stumps in the area shown were sampled; shaded symbols indicate positive sample point. Arrows pointing at shaded symbols indicate number of isolations and genets obtained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

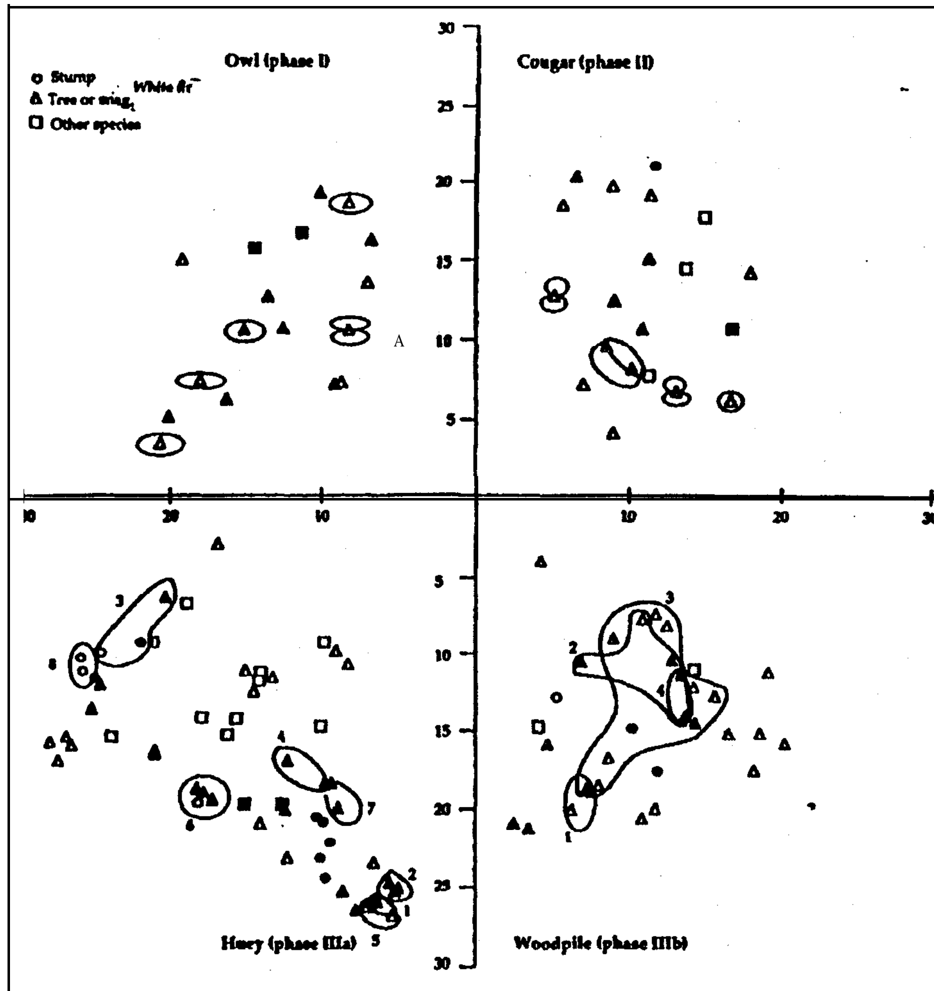

Figure 3. **Continuous solid lines visualize four progressive** patterns of distribution of larger ***Heterobasidion annosum*** genets in white fir at four study sites. Distances are in m, shaded triangles indicate dead trees or snags; shaded circles indicate stumps with evident decay pockets; other species include Douglas-fir, **black oak,** sugar pine and pondcrosa pines. In the lower two quadrants, genets are **identified** by a number corresponding to the number assigned during the study.

Table 1. White fir study sites, including some stand characters, number of trees or stumps which yielded *Heterobasidion annosum* isolates, total number of isolates collected, and number of genets determined by somatic compatibility tests.

Plot and National Forest[a]	Area m^2	Density trees/ha	Basal area /ha[b] (m^2)	Avg. dbh cm (SD)	% of basal area to yield isolates	Stems with *H. annosum*[b]	Isolates collected	No. of [c] genets	Genets found on 2 or more trees
Loggers, St.	960	438	120	53 (26)	31	10	53	30	1
cougar, St.	192	1042	102	31 (17)	19	5	25	13	2
Owl, St.	196	918	92	33 (15)	9	5	6	6	0
Woodpile, St.	193	1554	113	28 (12)	53	17	62	9	4
Lumberyard, Eld.	1050	962	119	34 (20)	25	17	49	29	3
Dark Cyn., Eld.	900	456	105	49 (23)	26	7	26	14	1
Meis Cabin. Eld.	300	1100	150	36 (22)	47	9	69	22	1
Huey, Eld.	512	1133	140	34 (20)	48	22	67	29	8
Duey, Eld.	400	1175	140	33 (20)	14	6	13	5	1
Luey, Eld	750	920	79	28 (19)	22	11	32	15	2
Mountain View, Pl.	864	1111	76	27 (11)	18	13	41	11	3
Ridge 1, Las.	304	658	74	36 (13)	54	12	45	19	3
Ridge 2, Las.	155	129	110	31 (11)	40	7	31	12	2
Obsidian 1, Las.	195	615	164	52 (27)	37	2	7	6	0
obsidian 2. Las.	192	469	279	82 (30)	22	3	26	8	1
Total or Average	7163	•	•	•	*31*	146	552	228	32

[a] St.=Stanislaus, Eld.=Eldorado, Pl. =Plumas, Las.=Lassen.
[b] Includes live and dead trees, stumps, and snags.
[c] Determined by somatic incompatibility tests.

Table 2. Effect of stand density on secondary spread.

Sites with >2 multi-stem genets n=8 density = 1084 stems/ha (SD=264)
Situ with 0- 1 multi-stem genets n-7 density= 739 stems/ha (SD-3 19)

Density was 1554 stems/ha in site with 8 multi-stem genets

Table 3. Analysis of molecular variance (AMOVA) from a Euclidian distance matrix generated by scoring 23 arbitrary primed (AP) PCR markers for 25 samples from two populations of *Heterobasidion annosum* 300 km apart in California.

comparison	Source	df	Sum of squared deviations	Mean squared deviations	Variance component	Percentage of tot. variance (%)	statistics	P-value[a]
Cougar vs.	Among pop.	I	*9.6*	*9.6*	*0.54*	*16*	*0.161*	<0.001
Ridge 2	Within pop.	23	*65.4*	*2.84*	*2.84*	*84*		

[a] Probability of obtaining equal or larger value determined by 1000 randomization of the treatments.