STUDIES ON THE ECOLOGY AND GENETICS OF HYBRIDIZATION IN *HETEROBASDION*

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The *Heterobasidion* taxonomic complex comprises two of the most important fungal tree pathogens in North America (Otrosina 1989), and includes at least three other taxa in Eurasia (Niemela 1998). These taxa were initially identified as intersterility groups (ISGs) (Korhonen 1978) within the species *H. annosum*, but the current trend is to assign them the species rank based on molecular, morphological, and host-association traits. European ISGs have already been accepted as different species (Niemela 1998). Although yet to be defined as species by taxonomists, the North American *Heterobasidion* ISGs have a much wider genetic divergence and isolation than their European counterparts (Otrosina 1993), and are also ecologically specialized on different hosts (Otrosina 1989; Worrall 1983). In this paper we will use the terms "species" and "ISGs" interchangeably when referring to the two North American taxa of Heterobasidion.

In California, host specificity effectively strengthens the genetic isolation between the two sympatric *Heterobasidion* ISGs. Isolates of the "S" ISG infect mostly true firs, hemlocks, and sequoias; the "P" ISG infects mostly pines, junipers, and incense cedars (Otrosina 1989). Nevertheless, there is significant interfertility among S and P isolates in laboratory mating tests (Harrington 1989), where host-specific discrimination is artificially removed.

We have recently gathered evidence for hybridization between the North American S and P ISGs. In an analysis of 7 loci of 51 S and 34 P isolates from California (data not shown), allelic frequencies ranging between 0.01-0.11 in one ISG and 0.86-1 in the other were suggestive of inter-ISG introgression in areas where both ISGs coexist (Garbelotto et al., in prep.). Outside the areas of sympatry, frequencies of putatively introgressed alleles are close to zero. Moreover, a first generation hybrid genotype was found in a mixed ponderosa pine-Western juniper stand in Northeastern California (Garbelotto 1996). The hybrid had colonized at least three neighboring trees and one stump, and time since initial colonization was estimated to be 5-25 years. This is the first stable natural *Heterobasidion* hybrid reported in the literature.

The potential ecological and evolutionary consequences of hybridization are widely recognized. Recent studies have provided evidence of past hybridization events in fungi (Tsai 1994; O'Donnell 1997; Brasier 1998, 1999), and have indicated that this process may be a viable mode of fungal speciation. Hybridization can be repressed either by the incompatibility of different mating systems (prezygotic isolation) or, at the cellular level, by low hybrid vigor due to the presence of two incompatible non co-adapted genomes in the same individual (unconditional postzygotic isolation) (Brasier 1995; Orr 1995).

These hypotheses do not entirely explain the repression of hybridization in the *Heterobasidion* complex, considering the significant compatibility between the North American taxa in the laboratory (Harrington 1989) and the stable nature of artificial and natural hybrids (Harrington 1989; Garbelotto 1996). A significant correlation has been shown between pathogenicity and type of mitochondrial genome, by means of inoculation of laboratory hybrids on pine germlings (Stenlid 2001), nevertheless, no information is available on the overall fitness of hybrids.

In this paper, we present experiments designed to test the following hypotheses:

A) Hybrids are genetically stable, in spite of the known potential for genomic instability within the species belonging to the complex

B) Hybrid host range is not larger than that of parental types, nonetheless hybrids are as competitive as parental types and are capable of surviving in nature

C) Presence of stumps and changes in forest composition increase sympatry of the North American *Heterobasidion* species.

MATERIALS AND METHODS

Studies on the genetic stability of hybrids

It has been shown that in *Heterobasidion* heterokaryons, each parental nuclear genome can be found by itself in a homokaryotic hyphae within the thallus (Hansen 1994). Individual parental genomes can also be recovered by subculturing unicellular conidia. It has also been shown that the more genetically unrelated the parental nuclei are, the greater the tendency of a heterokaryon genotype to produce uninucleate conidia. This observation has been interpreted in terms of "genomic conflict" between two genetically different parental nuclei (Ramsdale 1994).

The presence of genomes belonging to two different species in a hybrid genotype, should result in maximum levels of instability due to genomic conflict. In order to investigate the genetic stability of hybrids, we studied the nuclear composition of a) hyphae in the thallus and b) of conidia produced by the natural hybrid genotype retrieved in California. Subcultures were grown on cellophane overlaid on standard malt extract agar (MEA) and a total of 100 individual hyphal tips were subcultured. All subcultures were analyzed for presence of clamps and typed as S, P or hybrid SP, by Taxon-Specific Competitive-Priming (TSCP) PCR (Garbelotto 1996).

Conidia of three isolates (Table 1) were stained with DAPI and analyzed at 300X magnification under fluorescent lighting. For each isolate, the number of uni-, bi- and multi-nucleate conidia was tabulated.

Table 1. Recovery of uninucleate, binucleate, and multinucleate conidia from three isolates of *Heterobasidion annosum*.

				Uninucleate conidia		Binucleate conidia		Multinucleate conidia[c]	
Fungal isolate	**Nuclear status**	**ISG[a]**	**N[b]**	**No.**	**%**	**No.**	**%**	**No.**	**%**
L2.8.R1.a	Homokaryon	S	188	61	32	104	56	23	12
L2.7.R5	Heterokaryon	S	187	108	58	74	39	5	3
AWR400	Heterokaryon	S-P	194	135	70	55	28	4	2
Chi-square	*P<0.001*[d]	*d.f=2*							

[a] ISG = Intersterility group.

[b] N = total number of conidia sampled.

[c] Multinucleate = 3 or more nuclei per cell.

[d] Distribution of nuclei are significantly different among the three isolates. Pairwise comparisons of uninucleate conidia distribution (Z-tests):

L2.8.R1.a-L2.7R5, Z = 4.92

L2.8R1.a-AWR400, Z = 7.26

L27R5-AWR400, Z = 2.4

Z values for all comparisons are >1.645, and therefore significant at P=0.05.

Finally, thirty individual conidiophores were isolated, and 10 individual conidia from each conidiophore were subcultured. The resulting 300 single-conidium isolates were analyzed for clamps and their ISG was determined by TSCP PCR.

Studies on the pathogenicity and virulence of hybrids: inoculation experiments

We performed two greenhouse experiments and one field inoculation experiment. In greenhouse trials each ISG has shown higher virulence on its corresponding natural hosts (Worrall 1983) (Otrosina et al., in prep.). True firs, sequoias, hemlocks and Douglas-firs ("S-hosts") were more susceptible to S isolates, while pines ("P-hosts") were more susceptible to P isolates. Sitka spruce seedlings were always susceptible to both ISGs, and thus this tree species was identified as a "universal" host.

In greenhouse experiment 1 (Fig. 1), we compared virulence of an S, a P, and the natural SP-hybrid isolate. Although these isolates were genetically unrelated, they each infected several trees and stumps, demonstrating their viability in the field. All three isolates were dikaryotic. Isolates were inoculated on seedlings of white fir (S-host), ponderosa pine (P-host) and Sitka spruce (S and P "universal" host) and virulence cwas mesaured in terms of seedling mortality. In greenhouse experiment 2, ponderosa pine seedlings were inoculated with one S and one P homokaryon and with an SP dikaryon obtained by mating the S and P homokaryons in the laboratory, allowing a direct comparison of virulence. Differences in ploidy may be irrelevant in this species, as both haploids and dikaryons of *H. annosum* can be virulent and are commonly found in nature (Garbelotto 1997).

Finally, we performed a field inoculation experiment (experiment 3, Fig. 1), in which fungus-colonized wood dowels were inserted into holes drilled in white fir (S-host) tree roots. In this experiment, virulence was expressed as the extent of longitudinal colonization of inoculated roots. We used the same S, P, and SP hybrid isolates as in experiment 1. However, in this case holes were drilled beyond the cambium and the outer layer of the xylem; this bypassed the pathogen-specific host defense responses. It has been shown that sapwood inoculations do not normally discriminate between *Heterobasidion* species (Swedjemark 1999). To further verify this assumption, host reaction was also studied through high pressure layer chromatography (HPLC) analysis of colonized root xylem extracted twice with methanol (Bonello 1993).

Stumps as a potential hybridization zone

The question arises whether conditions exist in nature that may be conducive to hybridization. Two main requirements exist for successful hybridization: (a) both species must be present in an area, and (b) there must be colonization courts where both species can come into close contact, mate, and their hybrid offspring thrive. Fresh stumps may provide a non-selective colonization court in which the S and P taxa can mate (Otrosina 1992; Garbelotto 1996).

To quantify the impact of stump availability on the composition of *Heterobasidion* populations, we studied the genetic structure of this fungus in stumps, trees and the air-spora in three California National Forests (NFs) dominated by the P-selective hosts, pine and juniper. Airborne spores provide a measure of the overall population structure in a site. Spores are also essential for the persistence of *Heterobasidion* in nature (Otrosina 1989). Spores were collected by using the exposed wood-disk method described by James and Cobb (1982). Individual colonies were isolated from the wood-disks and their ISG was determined by TSCP-PCR. Isolates from wood and stumps were obtained by isolations both from infected wood and from the context of basidiocarps. ISG was determined by isozyme analysis or by TSCP PCR.

Pathogen species are tracking their specific host(s)

While a common habitat is required for physical contact and mating between the two *Heterobasidion* species, it is also necessary that both species be present in the same geographic location. We analyzed the presence of S isolates in live pine/juniper trees and stumps in relationship to the geographic distance from the two most important hosts for this group: hemlock and true fir (*Tsuga* and *Abies* spp.). In this analysis, we included data from three National Forests in which the disease is known to affect both S- and P-hosts (Inyo, Plumas, Modoc), as well as data from NFs where the disease is known only on S-hosts (Stanislaus, Eldorado) or on P-hosts (Cleveland, data not shown). Regression analyses were performed to verify the presence of a correlation between distance from the specific host and frequency of retrieval of its adapted pathogen.

RESULTS AND DISCUSSION

On the genetic stability of the natural hybrid

All of the hyphal tip subcultures were clamped and heterozygous for the P- and S- specific markers (i.e. they were putative hybrids). This result is explainable by hypothesizing a complete lack of homokaryotic hyphae in the thallus. This feature would differentiate the hybrid isolate from S and P heterokaryons, in which homokaryotic hyphae are always present. This characteristic would also imply a significant stability of the hybrid thallus. Both laboratory experiments (Hansen 1994) and field surveys (Garbelotto 1998) have indicated that natural isolates may be mosaics of the two homokaryotic parents and of the resulting heterokaryon. The stability of the hybrid thallus was also confirmed by multiple isolations (over 20) of the same hybrid genotype from at least four different stems (stumps and trees).

The analysis of the nuclear condition of the conidia provides us with a further understanding of the ploidy modifications associated with the natural hybrid. The number of uninucleate conidia, as determined by microscopic observation after DAPI-staining, was significantly higher in the hybrid than in two S isolates (Table 1, chi, P). Despite a majority of uninucleate conidia in the hybrid, all isolates obtained by subculturing of individual mitospores were S-P hybrids as per TSCP PCR determination.

These results can be explained by hypothesizing the diploid or polyploid nature of the natural hybrid. Changes in ploidy are commonly reported for hybrids in other groups of organisms as well as in fungi (Kuldau 1999). While further studies are needed to determine the exact ploidy of hybrids, it should be noted that we could not differentiate the size of DAPI-stained nuclei between the hybrid and the two other isolates. This may indicate that hybrid nuclei may be diploid, rather than polyploid. As ploidy increases over 2, a significant and detectable increase in nuclear size should be evident.

Pathogenicity and virulence of hybrids assessed through inoculation trials

In experiment 1 and 2 (Fig. 1), the P isolate killed significantly more pines than the S isolate (ANOVA $P<0.001$). However, no significantly different levels of white fir mortality were detected among different ISGs. Furthermore, the hybrid caused as much mortality as the S and P isolates ($P=0.5$) on the universal host, Sitka spruce, indicating its pathogenic potential when the mechanisms of host-specificity are absent.

It should be noted that hybrids from experiments 1 and 2 were characterized by the presence of an intron, that has been associated with the mitochondrial genome of the S ISG (Garbelotto 1996). Our results confirm that virulence on pines is correlated with mitochondrial type (Stenlid 2001), but further indicate the fitness of hybrids by showing they can be virulent on additional species.

We also found that the SP hybrid colonized the inoculated roots at the same rate as that of S or P isolates ($P=0.6$, Fig. 2) and HPLC profiles of SP-inoculated roots were indistinguishable from those of S- and P-inoculated roots (data not shown). This demonstrates that bypassing the host's specific defense barriers can effectively remove the fitness handicap of a hybrid.

Potential for hybridization: the ecological and geographic picture

In California, large-scale logging is a relatively recent event, beginning in the late 1800s. Stumps, an obvious by-product of logging, are ideal colonization courts for *Heterobasidion* spores (Rishbeth 1952; Otrosina 1989). Only 7-17% of the isolates from standing pines and junipers were S. The percentage of S isolates in stumps ranged between 25 and 91%. The increase of the S ISG in stumps ranged from twofold (Modoc NF) to twelve-fold (Inyo NF) (Fig. 2a). Even in the Modoc, the proportion of S isolates in stumps was significantly higher than that in trees (stump S mean=0.3, n=5, SD=0.13; tree S mean=0.07, n=5, SD=0.1. Student's $t=3.23$, $DF=8$, $P=0.012$). Both ISGs can be isolated from the same stumps in these areas.

In all NFs, air-borne spores displayed a proportion of genotypes significantly skewed in favor of the S ISG (χ^2 test, $P<0.0001$, df=2). The proportions of S isolates from stumps and the air-spora were virtually indistinguishable in the Inyo and Eastern Plumas NFs (χ^2 test, $P=0.46$, df=1; Fig. 2a), suggesting that the availability of human-made stumps is driving the present-day genetic structure of *Heterobasidion* populations in these forests.

Under the assumption that a 1:1 ratio of isolates of the two species provides the most favorable condition for hybridization, it would be predicted that the Modoc NF is the area most conducive to hybridization (Fig. 2a). It is interesting to note that: a) the natural hybrid was found in this NF, and that b) this NF is characterized by the highest level of introgressed alleles (data not shown).

In California, there appears to be a clear geographic/ecological partitioning in the distribution of *Heterobasidion* species. In mesic mixed conifer sites there is an overwhelmingly dominance of the S ISG. This dominance decreases as we move further into drier pine-juniper sites. Only the P ISG is retrieved from pine stumps in areas where no significant true fir/hemlock populations are in the vicinity (Fig. 2b).

While spatial isolation between stumps and sources of S inoculum (i.e. S host tree species) would theoretically slow down the shift in population structure of this pathogen, the practice of fire suppression over the last 50 years has enabled the encroachment of true firs (S host) in pine/juniper forests (Rundel 1977), progressively reducing such geographic barrier to *Heterobasidion* hybridization.

The combined effects of logging and fire suppression in California have significantly altered the population structure of *Heterobasidion*, and potentially increased the chances for episodic selection (Brasier 1995) and novel evolutionary developments. The effects may be long reaching, as root pathogens not only cause tree mortality but also influence forest composition and succession (Holah 1993).

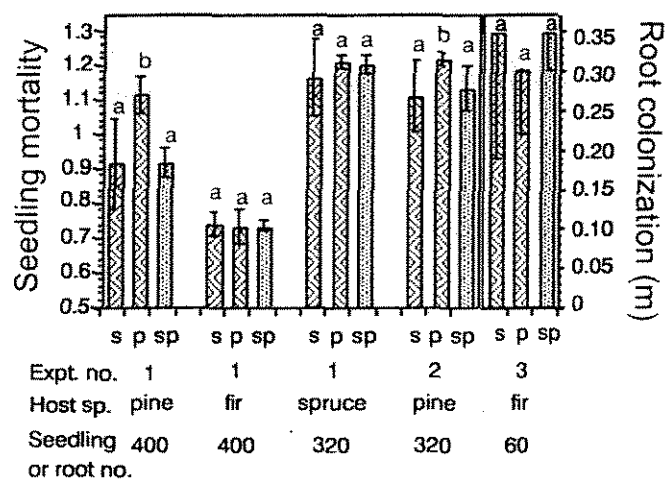
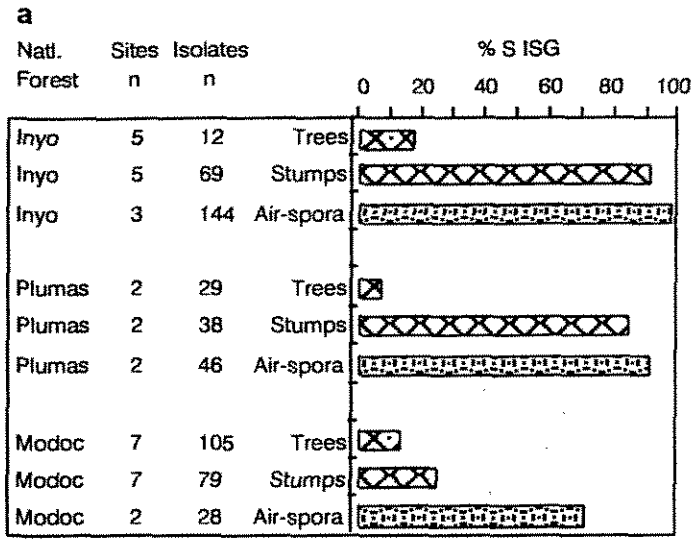


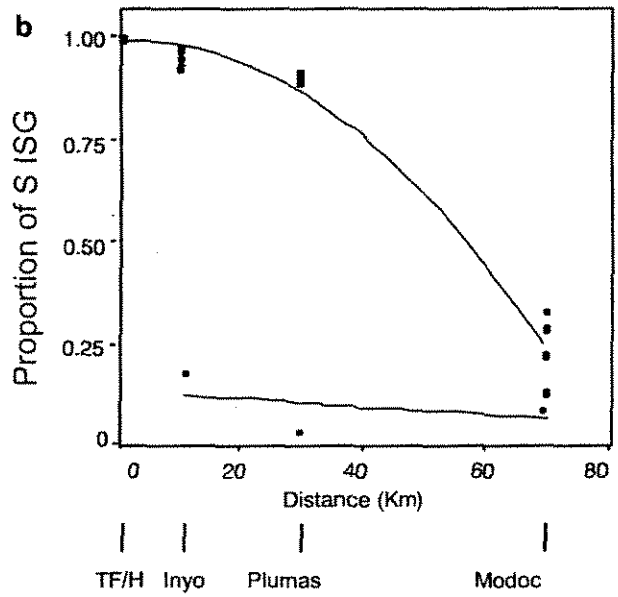
Figure 1. Results from three inoculation experiments: experiments 1 and 2 were onto seedlings in the greenhouse at U.C. Berkeley, and experiment 3 was into roots of white fir trees at Blodgett Forest (Eldorado NF). Seedlings were inoculated in completely randomized blocks with *Heterobasidion* isolates and mortality was scored cumulatively in a 12-month period (Worrall, 1983 #29). On the Y axis, mortality is expressed as $\sqrt{(0.5}$ +proportional seedling mortality). In experiment 3, 60 roots were inoculated in four randomized blocks as described in Garbelotto et al. (Garbelotto 1997). Extent of fungal colonization in the inoculated roots was measured after six months (data shown with one SD). ANOVA and Tukey Kramer HSD multiple range tests were performed separately for each species (letters indicate homogeneous groups at alpha=0.05).

Figure 2.

(a) Proportions of *Heterobasidion* ISGs from trees (T), stumps (S), and air-spora (A). Pooled data from all study sites within each NF are presented, however distributions were compared either with t-tests using individual sites as replicates, or alternatively with χ^2 tests (H^o= % S ISG in stumps= % S ISG in trees= % S ISG in air-spora).



(b) Results of regression analyses correlating the proportion of S isolates in stumps (filled squares) and trees (filled circles) and the distance from stands with a co-dominance of true fir or hemlocks (TF/H) for at least two miles. Data were from four TF/H sites (only stumps), three sites in the Inyo NF, two sites in the Plumas NF, four sites in the Modoc NF, and two sites in the Cleveland National Forest (only stumps). Regression analyses showed a statistically significant positive correlation ($Y=1.05-0.011X$, $R^2=0.94$, $P<0.0001$) between proportion of S isolates from stumps and proximity to TF/H stands, but no significant correlation was found for S isolates from trees ($R^2=0.056$).



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