# A shift in nuclear state as the result of natural interspecific hybridization between two North American taxa of the basidiomycete complex *Heterobasidion*

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## Abstract

A natural first generation hybrid fungus shows interspecific heterozygosity. The nuclear condition of a rare natural hybrid between two taxa of the *Heterobasidion* complex is investigated. *Heterobasidion* species are known to be either homokaryotic (haploid) or heterokaryotic ($n + n$), but heterokaryons are made up of both homokaryotic and heterokaryotic sectors. The natural hybrid appears to be either a heterokaryon undergoing a primary homothallic phase or a diploid with limited ability to exchange nuclei when mated with homokaryons. The natural hybrid is stable and long lived, suggesting hybridization may play an important role in the evolutionary history of this fungal complex.
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## 1. Introduction

Changes in ploidy are common in interspecific hybrids across all taxonomic groups. The list of polyploid and aneuploid hybrids is extensive, including examples from the *Mycota* (Kuldau et al., 1999; Suzuki et al., 1986) and the *Oomycota* (Brasier et al., 1999). Often, unusual ploidy results in infertility or genomic instability.

The first natural hybrid holobasidiomycete reported in the literature was a cross between two species of the *Heterobasidion annosum* species complex (Garbelotto et al., 1996). *H. annosum sensu lato* is a widespread tree pathogen comprising three species in Eurasia (Niemelä and Korhonen, 1998) and two species in North America. Although the two North American taxa are await-

ing formal description at the species level, this taxonomic classification is strongly supported by some morphological traits, mating compatibility, differential host specificity, and numerous biochemical and DNA studies (Chase and Ullrich, 1983; Garbelotto et al., 1993; Johannesson and Stenlid, 2003; Otrosina et al., 1993; Worrall et al., 1983). In the meantime, the two taxa are still referred to as S and P intersterility groups (ISGs)[1] based on the hosts in which these two taxa were first described: spruce and pine (Korhonen, 1978). Hybrids between the two North American taxa can be easily obtained (20% success rate) in the laboratory (Harrington et al., 1989), but they appear to be rare in nature. A single hybrid SP genotype has been discovered in northeastern California (Garbelotto et al., 1996).

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[1] *Abbreviations used:* ISG, intersterility group; TSCP-PCR, taxon-specific competitive priming polymerase chain reaction; DAPI, 4′,6-diamidino-2-phenylindole.

Based on the spatial distribution of 16 isolations of the hybrid and published spread rate of the fungus (Garbelotto et al., 1997; Hodges, 1969), its longevity was estimated to be 5–25 years.

Because of the importance of this single natural hybrid genotype, we decided to look at its nuclear status in hyphae and conidia and at its ability to exchange nuclei with homokaryotic isolates. Nuclear migration between heterokaryons ($n + n$) or between a homokaryon ($n$) and a heterokaryon ("Buller phenomenon") is a potential mechanism for the exchange of genetic information among individuals of a population. Such migration may enhance the establishment of novel genotypes and genomes (Garbelotto et al., 1999; Johannesson and Stenlid, 2004).

Previous studies have highlighted some aspects of the nuclear make-up of holobasidiomycete hybrids obtained in the laboratory. In particular, Lanquetin and Boidin (1983) were able to show that parental nuclei of stable hybrids between *Dichostereum durum* and *Dichostereum sordulentum* behaved differently. While only *D. sordulentum* nuclei were found in conidia, only *D. durum* nuclei would migrate into *D. durum* homokaryons paired with the hybrid. No *D. sordulentum* migration occurred when the hybrid was paired with *D. sordulentum* homokaryons. It was also observed that cultures derived from monosporous conidia and basidiospores would eventually all form clamp connections, indicating primary homothallism between parental genomes both carried in the uninucleate propagules.

Korhonen (1978) showed that conidia of laboratory hybrids of *H. annosum* always bore the nucleus of one parental species, suggesting a tendency of hybrid genotypes to segregate nuclei and revert to the individual species. Ramsdale and Rayner (1994) report a similar phenomenon of nuclear segregation in intraspecific *H. annosum* heterokaryons comprised of two distantly related genotypes. The tendency of unrelated nuclei to segregate individually, defined as "genomic conflict," was evaluated by looking at the nuclear component of conidia and hyphae. A strong positive correlation was found between the proportion of uninucleate conidia produced by a heterokaryon and the genetic distance of the parental nuclei.

The existence of a natural *H. annosum* hybrid raises a number of questions. Will genomic conflict be strongly expressed in this interspecific hybrid, as is suggested by experiments on laboratory hybrids? If so, how can *H. annosum* hybrids survive? How will hybrid hyphae interact with homokaryotic hyphae in somatic interactions? Because the hybrid genotype has functional genomes of both North American species, will that coexistence result in enhanced (nuclei will migrate into homokaryotic genotypes of both species), restricted (no migration into either species), or intermediate nuclear transfer?

To provide the basis for answering these questions, we conducted several studies to address: (1) the frequency of S, P, and hybrid SP hyphae in the thallus of the natural hybrid; (2) the frequency of uninucleate conidia in the natural hybrid; (3) the frequency of recovery of S, P, or of hybrid genomes from conidia; and (4) the ability of the natural hybrid to heterokaryotize S and P homokaryons through di-mon mating.

## 2. Materials and methods

### 2.1. Characterization of hyphae in the thallus

Hybrid isolate SP400 was grown on cellophane overlaid on malt extract agar (0.125% malt, 1.5% agar) at room temperature. After 9–12 days, individual hyphae were subcultured from hyphal tips viewed at 60× magnification. Twenty hyphal tips were collected from each of five subcultures. All 100 resulting cultures were analyzed for presence/absence of clamps by direct observation of hyphae on the underside of plates at 320× magnification. The ISG of each culture was also determined by taxon-specific competitive priming polymerase chain reaction (TSCP-PCR) (Garbelotto et al., 1996).

### 2.2. Nuclear condition of conidia from hybrid and non-hybrid isolates

Three sets of approximately 200 conidia each, were harvested from the hybrid SP isolate, one homokaryotic S isolate, and one heterokaryotic S isolate. The harvest was conducted by pouring water over a 10-day-old colony growing on malt extract agar. The two S isolates L2.8.R1 and L2.7.R5 (Lassen National Forest) were chosen for comparison because they had been collected in the same region of northeastern California as the hybrid isolate and had been characterized by mating tests and RAPD analyses (Garbelotto et al., 1999). The suspension was filtered through cheesecloth and a droplet was then placed on a microscope slide previously overlaid with polylysine-D (mol. wt. = 450,000). The suspension was then air-dried, rehydrated, and stained with 4′,6-diamidino-2-phenylindole (DAPI (1 µg/ml)) for 10 min. After rinsing once with distilled water, the slides were examined under 400× magnification using an Olympus Axiophot microscope and a fluorescent light source, and frequency of uninucleate conidia for each isolate was quantified and compared using $Z$ tests. Distributions of conidial nuclear numbers were compared using a $\chi^2$ test.

### 2.3. ISG and nuclear condition of single conidial isolates

Thirty individual conidiophores were selected from the hybrid genotype for isolations of single conidia.

Conidia were collected by a sterile needle from the club-like top of each conidiophore and put into an Eppendorf tube containing 1 ml of sterile water in which a drop of Tween-20 viscous liquid (Sigma, St Louis, MO) was previously added. Tubes were immediately vortexed, and the suspension was added to malt extract agar in Petri dishes (5 cm diameter) once the temperature of the medium reached about 45 °C, but before solidification. Thus, conidia were buried in the media at different depths and germinated at different times, allowing for easy isolation of single conidia at 320× magnification. Ten germinating conidia per conidiophore were selected and isolated. Presence or absence of clamps was assessed for each of the 300 resulting cultures. ISG determination was obtained through TSCP-PCR and by RFLPs of the ITS fragment by the endonuclease *Cfo* I as described in Garbelotto et al. (1996).

For further analyses, we chose one S isolate (230-3 Plumas National Forest), one P isolate (T338 Modoc National Forest), the hybrid genotype, and 15 single conidial isolates. All selected isolates came from northeastern California, and single conidial isolates were randomly selected from different conidiophores of the hybrid isolate. We performed RFLP analyses of two additional nuclear loci and sequence analysis of one mitochondrial locus as follows. Segments of the nuclear elongation factor 1-α and the heat stress protein 80-1 loci were amplified using the primer sets elongation factor 1-α forward/reverse (Johannesson and Stenlid, 2003) and heat stress protein 80-1 forward/reverse (Johannesson et al., 2000), respectively. RFLPs were obtained by double digestion of each amplicon; each enzyme made one unique cut of either the S- or P- type fragment. *Sna*BI and *Bsi*EI were used to cut the elongation factor 1-α amplicon, while *Hind*III and *Bgl*II were used to cut the heat stress protein 80-1 amplicon. All enzymes were manufactured by New England BioLabs (Beverly, MA) and used according to manufacturer's instructions. Digests were electrophoresed at 50 V/cm on 3% MetaPhor agarose (Cambrex, East Rutherford, NJ). As S- and P-type mitochondrial ATP synthase subunit 6 (ATPase-6) sequences were too similar to design an RFLP strategy as above, we amplified this region using the primer set ATP6-2/3 (Kretzer and Bruns, 1999). PCR products were cleaned, then sequenced on an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, CA) following standard protocols provided by the manufacturer. Sequences from each of the 16 hybrid isolates, from the S ISG isolate 230-3, and from the P ISG isolate T338 were aligned and chromatograms for each were studied for the presence of ambiguous or double peaks as indicators of sequence heterozygosity.

RAPD fingerprints of the same subset of S, P, hybrid, and hybrid single conidial isolates were obtained using primers ATG5 (Garbelotto et al., 1993) and M13 (Stenlid et al., 1994). RAPD profiles were electrophoresed at 50 V/cm on 1.7% MetaPhor agarose (Cambrex, East Rutherford, NJ).

### 2.4. Nuclear migration in di-mon mating with the hybrid isolate

Four homokaryotic S and six homokaryotic P testers were selected for mating tests. All testers and the hybrid isolate were from northern California. Hybrid isolate SP400 was paired with all testers in duplicate. Mycelial plugs were placed on malt extract agar 1 cm apart and incubated in the dark. After 9–12 days, presence/absence of clamps was determined as described for the original mating plates, above. Furthermore, a subculture was taken from the testers' colonies at 1 cm from the interaction zone and analyzed after 9–12 days for the presence of clamps. One S and one P heterokaryon were also mated with the tester homokaryons as a control for the ability of the testers to be heterokaryotized.

## 3. Results

Hybrid SP400 and single conidial isolates derived from it displayed the following features in this study:

(1) All 100 hyphal tip subcultures were clamped and heterozygous for S- and P-specific markers based on TSCP-PCR results: all had the ITS type of both species.
(2) Nuclei in conidia stained with DAPI were clearly discernible and could be easily counted. The majority of conidia produced by the SP hybrid and S heterokaryons were uninucleate, while the homokaryotic S isolate had a nuclear distribution skewed towards binucleate conidia (Table 1). Distribution patterns of number of conidia from the three isolates were significantly different from one another. Z values for the three possible comparisons ranged between 2.4 and 7.26 and indicated significant differences at $P = 0.05$. $\chi_4^2$ was 64.59, indicating proportions of uni-, bi- and multinucleate conidia between the three isolates assessed were significantly different at $P = 0.001$.
(3) All 300 conidia were heterozygous for the S- and P-linked markers as determined by TSCP-PCR results and ITS RFLPs. In RFLP analysis of the two additional and unrelated nuclear loci, elongation factor 1-α and heat stress protein 80-1, the hybrid genotype and all 15 randomly selected single conidial isolates were heterozygous for S and P markers (Fig. 1; Table 2). Additionally, a total of 21 fragments (500–1600 bp in size, data not shown) were reliably produced by the M13 (13 bands) and the ATG5 (8 bands) RAPD primers. The SP400 isolate and its 15 single conidial isolates had identical profiles with

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

| Fungal Isolate | Putative nuclear status | ISG[a] | N[b] | Uninucleate conidia | | Binucleate conidia | | Multinucleate conidia | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | N | % | N | % | N | % |
| L2.8.R1 | Homokaryon | S | 188 | 61 | 32 | 104 | 56 | 23 | 12 |
| L2.7.R5 | Heterokaryon | S | 187 | 108 | 58 | 74 | 39 | 5 | 3 |
| SP400 | Heterokaryon | SP | 194 | 135 | 70 | 55 | 28 | 4 | 2 |

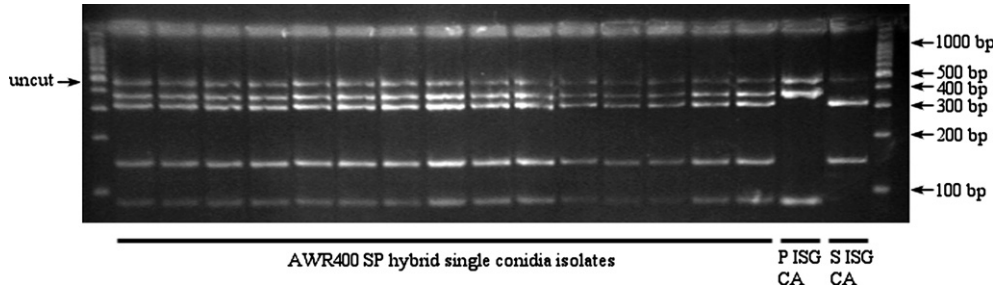[a] ISG, Intersterility group.
[b] N, Total number of conidia sample.



Fig. 1. A 3% MetaPhor agarose (FMC, Rockland, ME) gel showing RFLP fragments of the PCR product generated with elongation factor 1-α forward and reverse primers for SP hybrid, S ISG, and P ISG isolates of *Heterobasidion annosum* from California. PCR amplicon was digested with *Sna*BI and *Bsi*EI restriction enzymes. First and last lanes are molecular standards (100-bp ladder). Lanes 2–16 are randomly chosen single conidia isolates of the SP400 hybrid genet—isolate here designated AWR400 SP. Lane 17 is a Californian P ISG isolate, and lane 18 is a Californian S ISG isolate. The 448 bp uncut product appears in each lane. The P ISG digest yielded 85 and 363 bp fragments, and the S ISG digest yielded 135 and 313, while digests of all SP400 single conidia isolates show all four S and P ISG RFLP fragments.

Table 2
RFLPs of two unlinked DNA regions amplified by PCR differentiate between the S and the P intersterility groups of the pathogen *Heterobasidion annosum* from northeastern California

| DNA region | | P ISG | S ISG |
|---|---|---|---|
| Elongation factor 1-α | Enzyme | *Sna*BI | *Bsi*EI |
| | Fragment 1 size (bp) | 363 | 313 |
| | Fragment 2 size (bp) | 85 | 135 |
| | Total size uncut (bp) | 448 | 448 |
| Heat stress protein 80-1 | Enzyme | *Hin*dIII | *Bgl*II |
| | Fragment 1 size (bp) | 199 | 415 |
| | Fragment 2 size (bp) | 298 | 82 |
| | Total size uncut (bp) | 497 | 497 |

All hybrid conidia tested had heterozygous RFLPs at both loci.

both primers tested. The two unrelated S and P isolates could be easily differentiated from one another and from isolate SP400 and its conidia.

Sequence alignment and chromatogram analyses of a 581 bp portion of the mitochondrial ATPase-6 locus identified nine base substitutions between the S and the P isolate employed in the study. The hybrid isolate and all of the hybrid single conidial isolates randomly selected for this analysis had unambiguous S sequences of the ATPase-6 locus, without any trace of heterozygotic pattern. All sequences are deposited in GenBank with Accession Nos. AY560330–AY560346.

(4) Homokaryotic testers were not heterokaryotized by the SP hybrid, but heterokaryotization of the testers was successful when they were mated with S and P heterokaryons.

## 4. Discussion

The thallus of hybrid isolate SP400 comprised only hyphae that were heterozygotic for S- and P-linked markers (e.g., alternatively fixed polymorphisms in the ITS region). This result is in contrast with findings from natural *Heterobasidion* heterokaryons of two European taxa, in which thalli are always comprised of both homokaryotic and heterokaryotic hyphal compartments (Hansen et al., 1993). The data suggest that all hyphae in the hybrid thallus must bear nuclei of both species. The absence of homokaryotic hyphae was also supported by the observation that all subcultures from single hyphal tips were clamped. Artificial *Dichostereum* hybrids also have been reported to be always clamped (Lanquetin and Boidin, 1983). Obligate heterokaryosis, diploidy, or polyploidy of hyphal cells must be invoked to explain the results of the analysis.

The number of uninucleate conidia was lowest (32%) in the homokaryotic S isolate, intermediate (58%) in the S heterokaryon, and highest (70%) for the SP hybrid.

Nuclear distribution patterns in conidia of the S homokaryon and heterokaryon fell within the range of values published by Ramsdale and Rayner (1994) for homokaryons and heterokaryons, respectively. The hybrid isolate showed a percentage of uninucleate conidia greater than that obtained from either the S homokaryon or heterokaryon and larger than that reported for any isolate in previous studies (Ramsdale and Rayner, 1994). These data could be interpreted as the result of varying levels of genomic conflict (Ramsdale and Rayner, 1994). Our further analysis allows us to reject this hypothesis for the hybrid thallus (see below).

Although the majority of DAPI stained conidia from the hybrid were uninucleate, all cultures generated from them were clamped SP hybrids as determined per TSCP-PCR and ITS RFLPs. These results were confirmed by RFLP analyses of the nuclear elongation factor 1-α and the heat stress protein 80-1. For both loci, the entire subset of 15 single conidial isolates randomly selected from the hybrid culture displayed heterozygous (e.g., S-P) RFLP patterns. In contrast, only the mitochondrial ATPase-6 sequence associated with the S taxon was detected in all hybrid isolates. This is in agreement with the reported uniparental migration of the mitochondrion during hybridization (Olson and Stenlid, 2001). RAPD profiles also showed that single conidial isolates were undistinguishable both from one another and from the parental hybrid isolate. Thus, in the case of the hybrid isolate, high numbers of uninucleate conidia cannot be explained by the presence of extreme genomic conflict, but rather by a diploid or a polyploid state of the uninucleate conidial cells. Although we did not try to determine genome size of DAPI stained conidia, no obvious difference in size of nuclei could be detected when comparing nuclei from an S homokaryon, an S heterokaryon, and the hybrid SP400. We suggest that our inability to visually discriminate nuclear size may be indicative of simple diploidy.

The unique nuclear condition of the natural hybrid may affect nuclear migration during di-mon mating. Homokaryotic testers were not heterokaryotized by the hybrid isolate SP400, suggesting a new type of interaction between hybrid and haploid mycelia. In *Heterobasidion*, two isolates can mate if (a) they have the same positive allele at one of five intersterility (IS) genes (homogenic interaction), and (b) they have different mating alleles at a single mating locus (heterogenic interaction) (Chase and Ullrich, 1990). *Heterobasidion* is a highly outcrossing organism, characterized by a bipolar mating system and an extremely large number of mating alleles (Chase and Ullrich, 1983; Garbelotto et al., 1999). These traits explain the high success rate of heterokaryon–homokaryon pairings when isolates come from the same region. It should be noted, however, that the percentage of failed matings increases when isolates from distant locations are paired (Gonthier et al.,

2001). In order to explain the lack of success of di-mon matings between the hybrid SP400 and homokaryons from the same region, we surmise that nuclear migration is significantly reduced because of the nuclear ploidy of the hybrid isolate. Although diploid nuclei are known to invade haploid mycelia and replace haploid nuclei in *Armillaria* spp., this process is initially slow and less efficient than dikaryotization in di-mon matings (Rizzo and Harrington, 1992). The absence of independent nuclear migration in di-mon mating makes a good argument in favor of either a diploid, polyploid, or aneuploid nature or the natural *Heterobasidion* hybrid isolates. An alternative hypothesis may be that of primary homothallism resulting in a modification via recombination at the IS loci. A similar hypothesis has been suggested for *Dichostereum* artificial hybrids (Lanquetin and Boidin, 1983). Flow cytometric studies will be necessary to differentiate between these two viable hypotheses regarding the nuclear condition of the hybrid *Heterobasidion* thallus. Nonetheless, the second hypothesis is somewhat negated by the presence of identical RAPD profiles among all isolates generated by single conidia.

Results from this study identify a novel nuclear condition for *Heterobasidion*. Our findings also suggests interspecific hybridization may be a possible evolutionary mechanism through which Basidiomycetes may attain diploidy. Finally, this study indicates the natural *Heterobasidion* hybrid is genetically stable, and the entirety of its hyphae and mitospores are hybrid in nature as well. Instability or tendency for segregation of parental nuclei has been previously reported when studying hybrids obtained in the laboratory. The natural hybrid studied here is proof that stable hybrids also exist. These hybrids may be the only ones surviving in nature. The genetic stability described here suggests hybridization and interspecific gene flow may play an important role in the evolutionary history of this species complex.

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