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EVOLUTIONARY SIGNIFICANCE OF IMBALANCED NUCLEAR RATIOS WITHIN HETEROKARYONS OF THE BASIDIOMYCETE FUNGUS *HETEROBASIDION PARVIPORUM*

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Many fungi have heterokaryotic life stages in which genetically different nuclei inhabit the same cell. In basidiomycetes, the heterokaryon is the product of mating and represents a genomic union very similar to a diploid thallus, yet the maintenance of unfused nuclei suggests a more complex association of the two genomes relative to diploidy. In species with variable numbers of nuclei per heterokaryotic cell, nuclear ratios within a mycelium may possibly become imbalanced (differ from 1:1) due to nuclear competition. In this study, heterokaryons of the basidiomycete *Heterobasidion parviporum* were examined to determine the effects of genotype and environment on nuclear ratios within vegetative mycelia. The data reveal that nuclear ratios are frequently imbalanced, generally stable over time, and genetically determined. The nuclear ratios were affected by environment, but the observed nuclear ratios did not follow the expectations of strong selection acting on a population of nuclei. Instead, these ratios were largely driven by genetic effects and epigenetic effects. Finally, the data suggest that nuclear ratio imbalance also affects both gene transcription and growth rate, implying that heterokaryotic basidiomycetes are not functionally equivalent to diploid individuals and have a higher potential for genotypic and phenotypic variation.

KEY WORDS: **Butt rot, conidia, genomic conflict, *Heterobasidion annosum*, heterokaryosis, multinucleate.**

A heterokaryon is a thallus or tissue type composed of cells containing multiple, genetically different nuclei. Heterokaryons are primarily restricted to fungi of two phyla, the Ascomycota and Basidiomycota. They may arise through mating or vegetative fusion of homokaryotic individuals of different nuclear types or through spontaneous mutation of one nucleus during somatic growth of a homokaryon. The heterokaryotic cell type resulting from mating stands in contrast to the predominant outcome of sexual reproduction in plants and animals in which karyogamy immediately follows plasmogamy, that is, nuclei fuse to become diploid.

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Heterokaryons are assumed to be functionally equivalent to diploids with respect to dominance and recessivity of traits and transacting gene regulation (Beadle and Coonradt 1944; Raper 1966; Day and Roberts 1969). Another possibility is that the heterokaryon represents a collective population of nuclei, and the actual "unit of selection" is each individual nucleus (Pontecorvo 1946; Lewontin 1970; Johannesson and Stenlid 2004).

In Basidiomycota, heterokaryosis is required to complete the life cycle and typically involves combining very different nuclear genomes in the same cell. The heterokaryotic phase in this group is the dominant growth phase in the life cycle and may display indeterminant growth. The existence of a heterokaryotic stage in the life cycle has a number of evolutionary implications. Heterokaryons are expected to have increased adaptive potential relative to homokaryons due to genetic variation between the two nuclei (Clark and Anderson 2004). Moreover, heterokaryons have potential for additional genetic variation relative to diploidy because the distance between the two nuclei within a cell may vary. This variation in spatial arrangement was shown to impact the phenotypes of heterokaryotic cells of *Schizophyllum commune* (Schuurs et al. 1998). Heterokaryons, nonetheless, may undergo somatic recombination of genomes during vegetative growth, allowing the shuffling of genes between nuclei without the commitment to and complete reassortment of meiosis (Pontecorvo et al. 1953; Anderson and Kohn 2007).

Most importantly, although heterokaryons retain the potential for genetic mixture and recombination during vegetative growth, they represent a much less stable union of nuclear genomes relative to diploidy (Anderson and Kohn 2007). The breakdown of the heterokaryotic state can occur through nuclear partitioning into asexually produced spores, such as conidia, as a normal part of the life cycle of many fungi. Conidia often contain only one nucleus of the heterokaryon allowing a homokaryon to reemerge following dispersal and germination. The heterokaryotic union may also be severed by remating. For example, when a heterokaryon encounters a homokaryon during vegetative spread, nuclei of the heterokaryon may emigrate into the homokaryotic individual, a process termed as the Buller phenomenon (Buller 1930; Raper 1966). Exchanging and shuffling of nuclei between interacting heterokaryons has also been observed (Hansen et al. 1993). All of these processes of reassortment allow an individual nucleus to interact with other genetically different nuclei without undergoing karyogamy, further suggesting that nuclei within heterokaryons have a degree of autonomy and could be considered distinct units of selection.

In most basidiomycete species, heterokaryons are termed dikaryons because each cell has two nuclei, one from each mating partner. Although the majority of outcrossing Agaricomycetes (mushroom-forming fungi) form dikaryons after mating, many (>30% based on a subsample of species; [Boidin 1971]) form heterokaryons with multinucleate cells. In these multinucleate heterokaryons, the number of nuclei per cell can also vary (Korhonen and Stenlid 1998; de Fine Licht et al. 2005), and this raises the question of whether nuclear ratio in such fungi is as tightly controlled as in the dikaryon.

A nuclear ratio that is not strictly genetically controlled raises the possibility that heterokaryons could possess an additional level of adaptive flexibility to a changing environment whereby the stoichiometry of select loci could be altered merely by changing nuclear ratios. Such changes could alter allele frequencies in units of genomes by merely greater cellular representation of one nucleus over another. In fact, in the ascomycete molds *Penicillium cyclopium* and *Neurospora crassa* nuclear ratios of heterokaryons changed depending on environmental condition in a manner that reflected the underlying relative fitness of the constituent homokaryons grown in isolation (Jinks 1952; Davis 1960). Experimental work on ascomycete heterokaryons is limited, however, by a heteroallelic vegetative incompatibility system that allows only closely related homokaryons to form heterokaryons.

It may be hypothesized that in multinucleate heterokaryons lacking strict control over numbers of nuclei per cell, nuclear ratios could differ from 1:1, thereby creating a situation in which genomic conflict may emerge. Genomic conflict arises because selection may act in opposition at different levels: a nucleus may be favored by selection within the heterokaryotic mycelium, but this nuclear ratio imbalance may have a negative effect on the fitness of the heterokaryotic mycelium. Competition among nuclei could ultimately lead to the dissolution of a heterokaryon or the takeover of the whole mycelium by a single nuclear type (Rayner 1991). Whether nuclear ratios within heterokaryotic mycelia of multinucleate Agaricomycete fungi are typically balanced or imbalanced is unknown. A few studies have demonstrated biased nuclear ratios in some Agaricomycetes using the recovery of nuclear types from germinated conidia (Ramsdale and Rayner 1994, 1996) or recovering protoplasts (Raper 1985). However, the significance of nuclear ratio variability and whether such variability reflects selection acting on individual nuclei (competition) or selection acting on nuclear ratios (cooperation) is uncertain. Furthermore, studies suggesting nuclear ratios are imbalanced have mostly relied on germinated conidia. These data may be misleading because conidia formation itself may be a bias-generating mechanism if one nucleus can cheat its partner by gaining increased inclusion into conidia (Buss 1982). The biased recovery of nuclei from conidia of even dikaryotic Agaricomycetes appears to be the norm rather than exception (Cao et al. 1999; Kitamoto et al. 2000).

This article examines the prevalence, stability, and adaptive significance of nuclear imbalance within mycelia of the multinucleate basidiomycete fungus *Heterobasidion parviporum*

(=*H. annosum* S-intersterility group). *Heterobasidion* is a model for the study of nuclear ratios in heterokaryons because the fungus has multinucleate cells, disseminates by both meiotically produced basidiospores and asexually formed conidia, and has a highly variable population structure (Stenlid 1985). Heterokaryons produce both homokaryotic and heterokaryotic conidia, and germinated homokaryotic conidia have been shown to have biased nuclear ratios suggesting the possibility of the same in mycelium (Ramsdale and Rayner 1994, 1996). To overcome the bias that conidial assays yield, we have developed and employed molecular markers to directly determine nuclear ratios of the heterokaryotic mycelia. The temporal stability of nuclear ratios was also tested by repeated subculturing of heterokaryotic isolates. We then tested whether the environment can influence the nuclear ratio by placing the same heterokaryotic strain in multiple environments. These data are used in combination with growth rate data to address whether changes in nuclear ratios can be adaptive or whether competition among nuclei can decrease the collective fitness of the mycelium. Finally, the ratios of allelic variants in mRNA messenger pools were investigated to determine the contribution of nuclear ratio to phenotypic determination.

Materials and Methods

STRAINS AND CULTURE CONDITIONS

Ten homokaryons of *H. parviporum*, originally isolated from nature, were used to construct "synthetic" heterokaryons (Table 1). Laboratory heterokaryons were used rather than field-isolated heterokaryons so that specific nuclei could be tracked using molecular markers. Eight isolates originated from Europe, and two were from North America. The North American population of *H. parviporum* is phylogenetically distinct from the European population (Johannesson and Stenlid 2003) but not sexually incompatible (Stenlid and Karlsson 1991). Strains were routinely cultured on weak malt extract agar (M/10: containing 0.2% malt extract and 2% agar), and long-term storage was at 4°C in screw-capped test tubes of M/10. Among the strains used in the experiments were two homokaryotic strains that adopted a "senescent" growth form before they were mated. Such strains have an altered growth pattern consisting of slow growth, sparse aerial hyphae, and abnormally branching mycelium (online Supplementary Fig. S1; see [Stenlid and Rayner 1989] for a complete description of the senescent phenomenon).

The following media were used for growth rate or nuclear ratio estimation, with values indicating amounts per liter: MEB (5 g malt extract), MEA (5 g malt extract, 20 g agar), ME (5 g malt extract, 8 g gellan gum, 1 g $MgSO_4 \cdot 7H_2O$), MMN (5 g glucose, 0.25 g $(NH_4)_2HPO_4$, 0.5 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 0.067 g $CaCl_2$, 0.025 g NaCl, 0.012 g $FeCl_3$, 0.1 mg thiamine-HCl, 8 g gellan gum), MMN + gly (same as MMN except replacing $(NH_4)_2HPO_4$ with 2 g glycine), MMN-pH3 (same as MMN except buffered with 10% pH 3.0 citric acid-phosphate buffer [0.1 M]), MMN-pH7 (same as MMN except buffered with 10% pH 7.0 citric acid-phosphate buffer [0.1 M]). Gellan gum (with $MgSO_4$) was used as a solidifying agent in the media (except MEA) so that the mycelium could be recovered directly by resolubilization through chelation of cations with sodium citrate (Doner and Bécard 1991) followed by filtration.

GENETIC CROSSES AND SYNTHESIS OF HETEROKARYONS

The homokaryotic strains were crossed in the combinations shown in Figure 1. Heterokaryons of the desired nuclear composition were synthesized by inoculating two homokaryons into the middle of Petri dishes containing 0.4% malt extract and 2% agar, approximately 1 cm apart. After 3 weeks of co-culture on these plates, putative heterokaryons were subcultured to fresh M/10 media. In matings of homokaryons of most Agaricomycetes nuclei

Table 1. Homokaryotic strains used in this study.

Strain	Origin	Date isolated	Collector	Source	Senescent[1]	Growth rate[2]
93026/1	Bayern, Germany	1993	O. Holdenrieder	basidiospore	no	0.29 (0.27–0.30)
A358r	Aymavilles, Italy	1990	P. Gonthier	airspora	no	0.25 (0.24–0.27)
ORE103c7	Oregon, USA	1982	T. Chase	conidiospore	no	0.25 (0.23–0.26)
B1142	Ajusco, Mexico	1996	D. Rizzo	conidiospore	no	0.25 (0.23–0.26)
RB_48-9	Ramsåsa, Sweden	1984	J. Stenlid	basidiospore	no	0.19 (0.18–0.21)
87074/1	Vicenza, Italy	1987	K. Korhonen	basidiospore	no	0.19 (0.18–0.21)
Br-518_c2	Brynge, Sweden	1984	J. Stenlid	conidiospore	no	0.18 (0.17–0.20)
95191	Ural, Russia	1995	K. Korhonen	conidiospore	no	0.14 (0.13–0.16)
HR32	Rhodge, Greece	2005	P. Tsopelas	conidiospore	yes	0.04 (0.02–0.07)
91132/1	Saaremaa, Estonia	1991	K. Korhonen	basidiospore	yes	0.04 (0.04–0.04)

[1]For a thorough description of this phenotype see Stenlid and Rayner (1989) and photograph in online Supplementary Figure S1.
[2]In mm/h on MEA (95% CI given in parentheses).

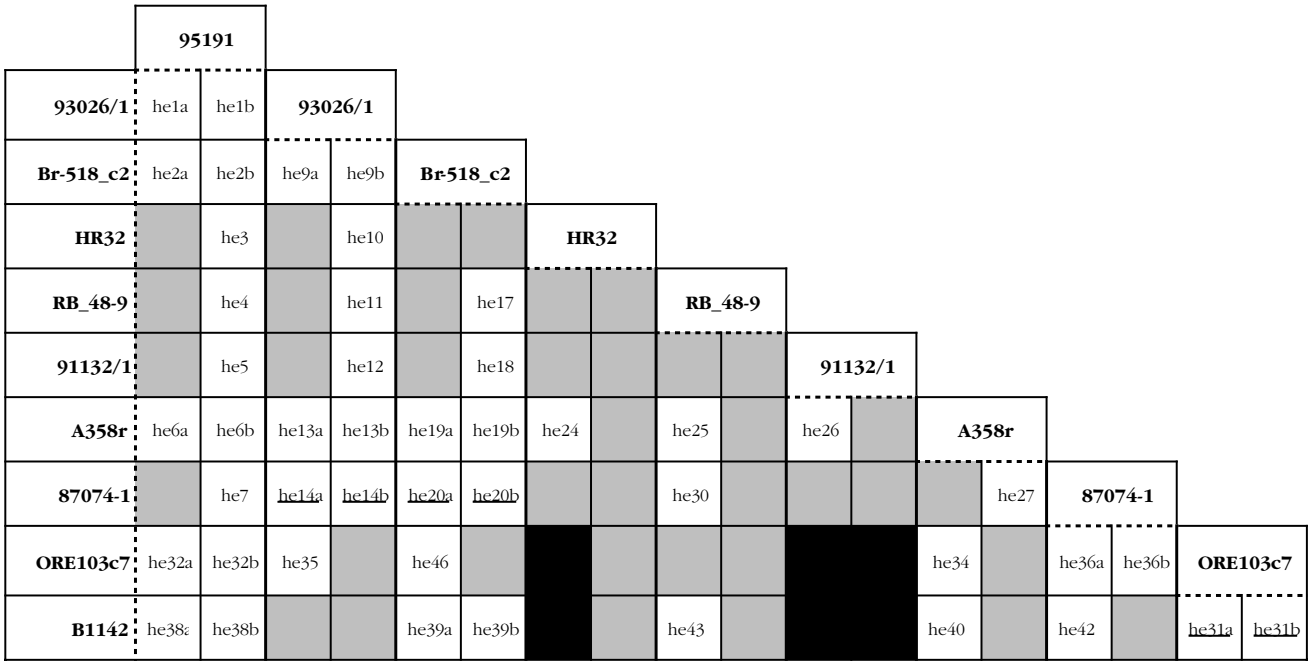

Figure 1. **Summary of crosses and resulting heterokaryons synthesized for the experiments. Homokaryotic strains along top row were crossed with the homokaryotic strains in the first column. In each pairing, heterokaryons of identical nuclear composition may be isolated in different cytoplasmic/mitochondrial backgrounds depending on which side of the interaction plate the heterokaryon is subcultured. Listed are the heterokaryons produced with the alternative cytoplasmic backgrounds listed as "a" and "b." For example, in the cross 95151 × 93026/1, two heterokaryons were isolated: he1a with cytoplasmic background of 93026/1 and he1b with cytoplasmic background of 95151. Strains that are underlined were not used in further analyses because fewer than three polymorphic loci were available. Gray squares indicate heterokaryotization was unsuccessful in that direction of nuclear migration. Black squares indicate cross not attempted.**

but not mitochondria undergo reciprocal migration and exchange to form two genetically distinct heterokaryons that differ only in their cytoplasm (May and Taylor 1988; Ihrmark et al. 2002). These reciprocal migrations establish the maternal nucleus as the nucleus resident in the mycelia into which a paternal nucleus migrates (Aanen et al. 2004). Hyphal samples were therefore taken from both sides of the interaction zone that forms between mating homokaryons and subcultured to establish heterokaryons of identical nuclear but different cytoplasmic constitution. Subcultures were confirmed as heterokaryotic by detecting the presence of the characteristic clamp connections found between neighboring cells. The synthesized heterokaryons were purified to single hyphal tips to ensure that a homogeneous mycelium was used in future experiments rather than a mixture of genetically different mycelia that could arise from bulk-hyphal transfer. Hyphal tips were isolated by growing heterokaryons on M/10 overlain with cellophane similar to the method described by (Hansen et al. 1993).

GROWTH RATE ESTIMATION

Linear growth rate on semisolid media was used as a proxy for fitness (Pringle and Taylor 2002). Both homo- and heterokaryons of *H. parviporum* grow in a radially symmetric manner. Three replicate dishes were used for each strain. Plates were inoculated with 5 mm diameter plugs taken from the margin of an actively growing strain on M/10 and transferred onto the center of a 90 mm Petri dish containing the media of interest. Strains were grown at 20°C in darkness. The amount of linear extension was recorded along one radius for each replicate dish over 3–4 time intervals (of typically 48 h) before the mycelium reached the side of the dish. Growth rate was estimated by linear regression and reported in units of mm per hour.

DNA TECHNIQUES AND MOLECULAR MARKERS

DNA was extracted from fungal tissue by homogenization in a CTAB buffer containing 2% hexadecyltrimethylammonium bromide, 1.4 M NaCl, 20 mM Na_2EDTA, 100 mM Tris-HCl (pH 8.0). Cells were disrupted by grinding with a microcentrifuge pestle or using glass beads (2 mm) in a FastPrep (Savant) homogenizer. Homogenates were extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform, and DNA was precipitated with two-thirds volume of isopropanol.

Two types of markers were used for genotyping and estimating nuclear ratios: microsatellites and pyrosequencing-SNPs. The microsatellite loci Ha-ms1, Ha-ms2, Ha-ms6, Ha-ms10 were used (Johannesson and Stenlid 2004). Reactions contained 1× PCR buffer, 200 μM dNTPs, 0.5 μM each primer, 3.0 mM $MgCl_2$, 0.035 U/μl Expand High Fidelity Polymerase (Roche, Stockholm,

Sweden), and ~1 ng/μl genomic DNA. The PCR amplification profile was an initial denaturation step of 3 min at 94°C followed by 25 cycles of 94°C for 30 sec, 52°C for 30 sec, 72°C for 45 sec and a final extension step of 72°C for 7 min. Genotypes were determined by electrophoresis on a MegaBACE 1000 (Amersham, Uppsala, Sweden) capillary sequencer by using fluorophore labeled reverse primers, and electropherograms were analyzed with the software Genetic Profiler (Amersham).

The pyrosequencing-SNP markers were developed for genes (laccase [LAC], glyceraldehyde-3-phosphate dehydrogenase [gpd], heat stress protein 80-1 [Hsp]) and anonymous target sequences (SWAPP1, SWAPP2) based on single-nucleotide polymorphisms found in published sequences (Ihrmark et al. 2002; Johannesson and Stenlid 2003; Asiegbu et al. 2004). The primers used for PCR and sequencing are shown in Table 2. All reverse PCR primers were 5′ end labeled with Biotin-TEG (Operon, Cologne, Germany). PCR reaction components for pyrosequencing-PCR were: 1X PCR buffer, 200 μM dNTPs, 0.2 μM each primer, 1.5 mM $MgCl_2$, 0.035 U/μl Expand High Fidelity Polymerase (Roche), and ~1 ng/μl genomic DNA. The amplification profile was similar to that used for microsatellite PCR, except the annealing temperature was increased to 60°C (54°C for LAC) and the number of cycles was increased to 40. Biotinylated strands were captured on streptavidin-coated Sepharose beads, purified, and sequenced using Pyro Gold Reagents on a PSQ 96MA pyrosequencer following the manufacturer's protocol (Biotage, Uppsala, Sweden).

NUCLEAR RATIO ESTIMATION

Nuclear ratios were estimated by quantitative analysis of DNA extracted from heterokaryotic mycelia. Two different and established marker systems, microsatellite peak heights (Meyer et al. 2006), and allele quantification by pyrosequencing (Wittkopp et al. 2004) were used to provide cross-validation of results. Microsatellite estimation of nuclear ratios was accomplished by analyzing log-phase microsatellite PCR amplicons of DNA isolated from heterokaryons. Peak heights were extracted for each

Table 2. Oligonucleotides used for pyrosequencing-SNP genotyping.

Locus	Primer	Sequence (5′ → 3′)	Coordinates	Usage
LAC	LAC-2F	CCTGCGACTCTAATTTCGTGTTT	463–485	forward PCR primer
	LAC-3R-B	AACGATCAAGGGGTTGACATT	531–551	biotinylated reverse PCR primer
	LAC-3F	AATTTCGTGTTTTCCAT	474–490	differentiates European (491T) from North American genotypes (491C)
	LAC-4F	CCATCATCGAAGCGG	508–522	differentiates A358r (524C) from other European genotypes (524T)
gpd	gpd-1F	TGCCCCGATGTTCGTCTG	109–126	forward PCR primer
	gpd-1R-B	TGCCGTACTTGTCGTGGAT	277–295	biotinylated reverse PCR primer
	gpd-2F	CCCGATGTTCGTCTG	112–126	differentiates ORE103c7 (127C) from B1142 (127T)
	gpd-3F	CTTGACGCTTACGACTC	137–153	differentiates European (154C) from North American genotypes (154T)
Hsp	Hsp-1F	AAGGTCATCCGCAAGCACAT	61–80	forward PCR primer
	Hsp-1R-B	GAGCTTGCTGCGGTTCTGA	192–210	biotinylated reverse PCR primer
	Hsp-2F	CCGCAAGCACATCGT	69–83	differentiates European (84T) from North American genotypes (84C)
	Hsp-3F	GAAGCTCGGCATCCA	168–182	differentiates ORE103c7 (183C) from B1142 (183T)
SWAPP1	SWAPP1-3F	AGAGGGCTTCCGATATCCCA	129–148	forward PCR primer
	SWAPP1-3R-B	CCCTGGTTGCCTGACATCAC	17–36	biotinylated reverse PCR primer
	SWAPP1-4F	TATCGAGCACCCGTT	94–108	differentiates 87074-1, HR32, 91132/1 (109C) from other European genotypes (109T)
SWAPP2	SWAPP2-2F	GGAACGTGAAGGTCGAAGCTAT	36–57	forward PCR primer
	SWAPP2-2R-B	CGTCCCTGCGAACAACAA	133–150	biotinylated reverse PCR primer
	SWAPP2-3F	TGTAACATGCCGTTGA	71–86	differentiates HR32, 95191, RB_48-9 (87A) from Br-518_c2, 91132/1, 93026/1 (87G)
	SWAPP2-4F	GGGAGTGTTTTTGAATGA	96–113	differentiates 95191 (117A) from RB_48-9 (117G)

The coordinates for each marker are given with respect to the following GenBank accession numbers: LAC, Y16951; gpd, AY273421; Hsp, AB255585; SWAPP1, AY273340; SWAPP2, EU491500.

allele from the electropherogram using Genetic Profiler, and nuclear ratios were inferred by the ratio of peak heights of the two alleles of a heterokaryotic strain. Nuclear ratios were estimated by pyrosequencing-SNP markers through quantitative analysis of pyrograms using the AQ mode of PSQ 96MA SNP Software (Biotage). The effectiveness of these markers was validated by pooling DNA from homokaryons to calculate a standard curve. Allele combinations were accepted if they showed a strong linear correlation ($r \geq 0.90$) between peak height ratio and DNA ratio (online Supplementary Fig. S2).

The baseline nuclear ratios were estimated from all synthesized heterokaryons using three replicate cultures grown in a common environment: stationary culture of 15 mL of MEB at 20°C in darkness. Mycelia were harvested after one week by filtration, DNA was extracted, and nuclear ratios were estimated using all markers that were polymorphic between the two nuclear types constituting a heterokaryon. Only strains that could be genotyped at three or more loci were included in the results.

To facilitate comparison with previous estimates of nuclear ratios in heterokaryons of *H. annosum* based on conidia (Ramsdale and Rayner 1994, 1996), nuclear ratios were also estimated for three strains (he1a, he4, he18) both by isolating and genotyping single conidia and by directing genotyping of vegetative mycelia. Single conidial isolates were obtained by scraping conidia from the surface of an active (2-week old) culture into 2 mL of sterile H_2O. Conidia were serially diluted, plated onto M/10 media, and after three days germlings were identified using a light microscope and sub-cultured onto fresh M/10 media. Single conidial isolates were grown in 5 mL of liquid M/10 and genotyped for the marker Ha-ms1 using the amplification protocol given above but with 30 amplification cycles.

ANALYSIS OF TEMPORAL AND ENVIRONMENTAL STABILITY OF NUCLEAR RATIOS

The temporal stability of nuclear ratio was estimated by genotyping five serially propagated heterokaryotic strains (he1a, he2b, he4, he13a, and he18). The heterokaryotic mycelia were grown on 90 mm Petri dishes containing ME medium. After 1 week of growth, the strains were investigated for the frequency of clamp connections by microscopic observation of 200 septa randomly sampled from around the periphery of the colony. The strain was subcultured by transferring a 5 mm diameter plug from the periphery onto fresh ME. Thereafter, the culture was harvested and the nuclear ratio of the strain was estimated. In this manner, each of the five heterokaryotic strains was transferred three times to assess the stability of nuclear ratio over four time points.

Environmentally induced variation in heterokaryotic nuclear ratio was tested by growing six synthesized heterokaryons (he4, he13a, he17, he27, he32a, he46) under five different conditions: ME, MMN, MMN+gly, MMN-pH3, and MMN-pH7. Three replicates of each treatment were used and grown at 20°C in darkness for 2–3 weeks. The growth rate of each replicate was measured, and then the mycelia were harvested by solubilization of the gellan gum composed media for nuclear ratio analysis.

GENE EXPRESSION IN HETEROKARYONS

Gene expression was analyzed for the heterokaryons he32a and he46 grown under varying conditions to ask if allele ratios of messenger RNA pools reflect allele ratios in genomic DNA and thus nuclear ratios. This experiment used the three replicate cultures from the environmental heterogeneity experiment. DNA and RNA were coextracted using CTAB buffer. Following removal of proteins with organic solvents, the extracts were divided into halves and total nucleic acids were harvested by precipitation with two-thirds of volume of isopropanol at −20°C overnight and RNA was selectively precipitated using one-half of volume of 8M LiCl at 4°C overnight. Contaminating DNA was eliminated from the RNA extracts using DNase I (Fermentas, Helsingborg, Sweden). cDNA was synthesized from mRNA using the enzyme SuperScript III Reverse Transcriptase (Invitrogen, Paisley, UK) following the manufacturer's instructions with 250 ng RNA in a 20 μl reaction. RT-PCR was then performed by using 1 μl of the cDNA to amplify the pyrosequencing markers gpd and Hsp. Negative controls for DNA contamination were samples treated in an identical fashion but without the reverse transcriptase enzyme. The RT-PCR products were then genotyped by pyrosequencing and ratios of allele expression in cDNA were quantified using methods for genomic DNA and further compared to ratios of alleles among DNA pools from the same mycelia.

DATA ANALYSES

Statistical analyses were performed using JMP 6.0.0 (SAS Institute). Because allele bias in PCR is well established (Mutter and Boynton 1995), and nuclear ratios estimated using the different marker loci were found to show slight, but consistent, differences, a conservative approach was used for rejecting the null hypotheses that nuclear ratios are 1:1 in MEB. This was done by using each locus mean (of three biological replicates) as an independent variable and testing the locus means for significant differences from 1:1 using the *t*-test and the paired *t*-test to test the effect of treatment (e.g., cytoplasmic background). For analysis of whether environment affected nuclear ratio, the biological replicates were treated as independent variables, because (1) a much higher level of variation among replicates was found than in the experiment with MEB, and (2) biases among loci should cancel out because the same alleles are being assayed for a given heterokaryon, regardless of environmental condition. When cytoplasmic background was shown to have no effect on nuclear ratio, values were averaged for the two backgrounds of a given heterokaryon before analysis.

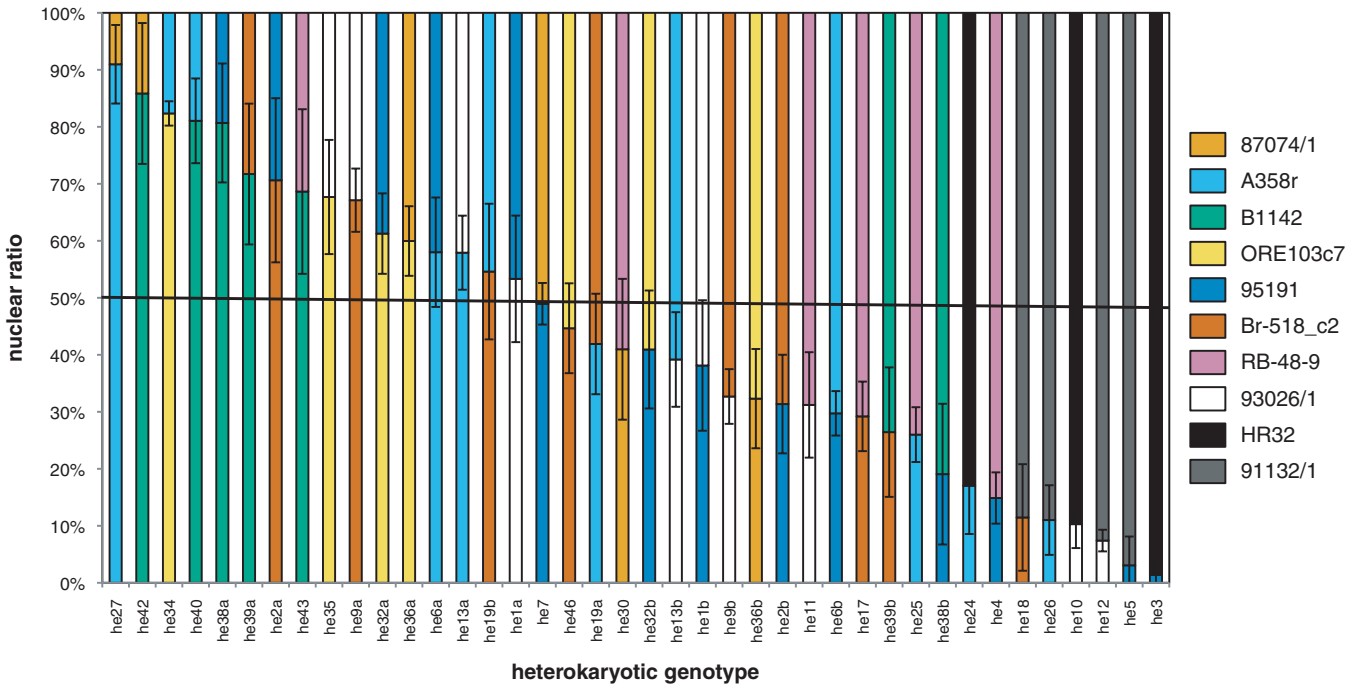

Figure 2. **Nuclear ratios among the 40 synthesized heterokaryons grown in MEB ranked in decreasing order of percent maternal nucleus (cytoplasmic parent). For each strain, the maternal nucleus is displayed as the lower portion of the bar, and the paternal nucleus is displayed on the upper portion. The means of three replicate cultures are shown for each heterokaryotic genotype and the error bars represent the 95% confidence interval as estimated among loci.**

Results

NUCLEAR RATIOS DIFFER FROM 1:1 WITHIN HETEROKARYONS

Nuclear ratios were investigated for 40 heterokaryons isolated from the crosses among 10 homokaryotic strains (Fig. 1). The components of variance of nuclear ratios were first estimated by performing ordinary least squares regression with random effects. This analysis showed significant effects of heterokaryotic strain ($F_{[39,138.2]} = 65.971$, $P < 0.0001$), locus values within a heterokaryotic strain ($F_{[127,252]} = 10.733$, $P < 0.0001$), and biological replicates within heterokaryotic strain ($F_{[80,252]} = 1.709$, $P = 0.0009$) on nuclear ratios. The variance components explained 93.1% by heterokaryotic strain, 5.1% by between locus estimates within heterokaryotic strain, and 0.3% by biological replicates within heterokaryons. Thus, these data demonstrate that nuclear ratios were very consistent among replicate cultures of the same strain because nuclear ratio estimates varied ~20 times more among individual locus estimates within a heterokaryotic strain than among replicate cultures of the strain.

The estimated nuclear ratios for the 40 heterokaryotic genotypes are presented in Figure 2 in descending order of maternal nucleus percentage. Of 40 heterokaryotic genotypes, 32 had ratios that significantly differed from 1:1 by *t*-test ($P < 0.05$). The average frequency of each nuclear type among the heterokaryons ranged from 92% to 29% mean relative frequency (Fig. 3). These values suggested that nuclear ratios were influenced by the genotypes of the participating nuclei, because some nuclear types tended to predominate a mycelium (e.g., HR32 and 91132/1), whereas others (e.g., 95191) were always the minority component.

STABILITY OF HETEROKARYONS OVER MULTIPLE SERIAL TRANSFERS

Nuclear ratios were found to be largely constant through the time series of four serial transfers for each of the five heterokaryons. In two of the five tests (he2b and he13a), the heterokaryons displayed 1:1 ratios of the two nuclear types throughout the time series (Fig. 4A, C). For heterokaryons he1a and he4, the percentage of the maternal nucleus averaged ~0.40 over the whole time series and were statistically different from 1:1 at two (he4, Fig. 4B) or one (he1a, results not shown) of the four times points. For he18, a heterokaryon containing a nucleus from the senescent homokaryon 91132/1 (Fig. 4D), the mycelium was highly biased toward the nucleus from 91132/1 at the beginning of the time series. The level of bias decreased steadily, and by the third time point, the nuclear ratio of the mycelium reached and stabilized at 1:1. Clamp connections were found on all heterokaryotic mycelia, but could be found in very low frequency (e.g., ~5% for strain he13a, Fig. 4C) or relatively high frequency (e.g., ~50% for strain he2b, Fig. 4A). In two strains, he2b and he18, clamp connection frequency increased over the course of the time series.

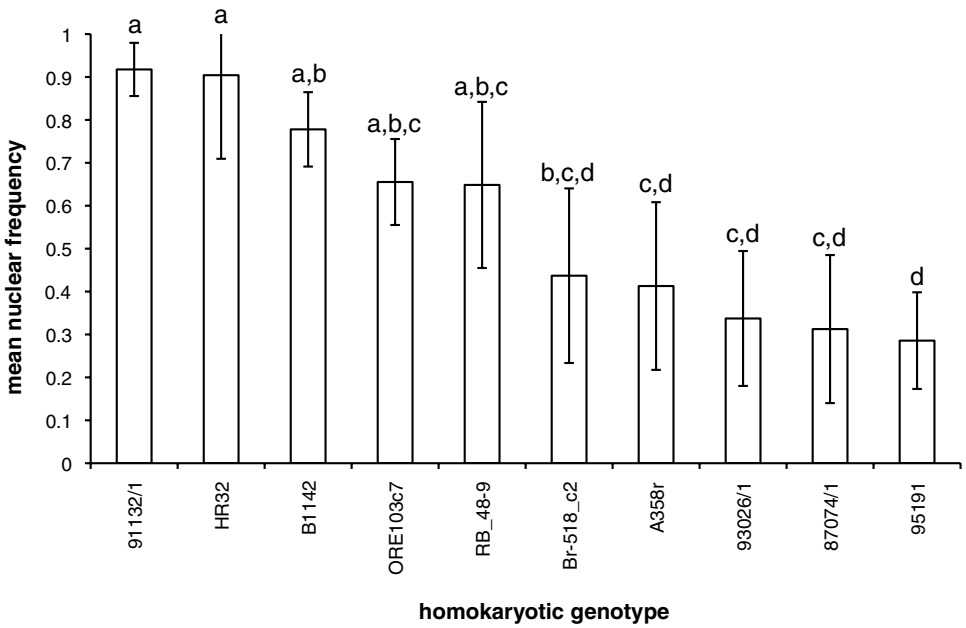

Figure 3. Frequencies of individual nuclei among the 40 heterokaryons grown in MEB. Shown are means and 95% confidence intervals. Letters indicate significantly different means as assessed by the Tukey-Kramer HSD test ($P < 0.05$).

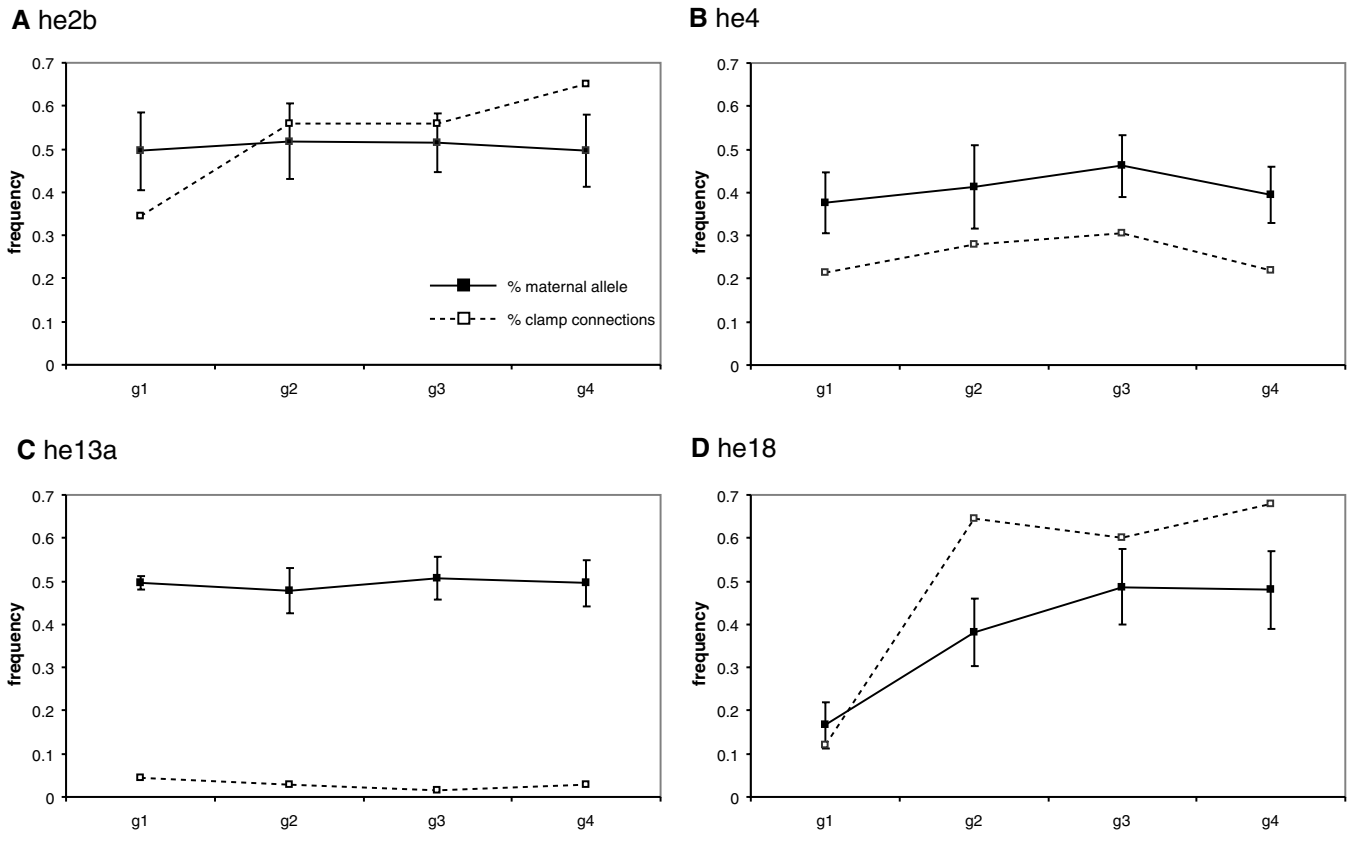

Figure 4. Nuclear ratios of four heterokaryotic strains through serial transfer on ME. Shown are four time points (g1–g4) and the estimated frequencies of the maternal nucleus in the mycelium and frequency of clamp connections per septa examined. The error bars indicate the 95% confidence interval based on the values among loci (minimum four loci employed). Strain he18 (panel D) contains a paternal nucleus from a senescent strain 91132/1 that decreased in frequency between the first and third subcultures.

Table 3. Nuclear ratios of heterokaryons subjected to several growth environments.

Heterokaryon	MEB	ME	MMN	MMN+gly	MMN-pH3	MMN-pH7	ANOVA
he4	0.15	0.39	0.24	0.12	0.06	0.13	F=10.3
	(0.12–0.18)	(0.35–0.42)	(0.13–0.39)	(0.09–0.15)	(0.04–0.08)	(0.07–0.19)	P=0.0014
he13a	0.58	0.54	0.55	0.50	0.54	0.57	F=18.0
	(0.58–0.58)	(0.53–0.55)	(0.54–0.55)	(0.48–0.52)	(0.53–0.54)	(0.57–0.58)	P=0.0001
he17	0.29	0.17	0.37	0.39	0.15	0.12	F=8.8
	(0.27–0.32)	(0.15–0.21)	(0.25–0.51)	(0.33–0.48)	(0.14–0.17)	(0.05–0.18)	P=0.0026
he27	0.91	0.56	0.53	0.84	0.51	0.63	F=35.6
	(0.90–0.92)	(0.55–0.57)	(0.52–0.55)	(0.83–0.84)	(0.49–0.53)	(0.56–0.72)	P<0.0001
he32a	0.61	0.52	0.53	0.67	0.55	0.52	F=31.9
	(0.56–0.62)	(0.50–0.56)	(0.51–0.54)	(0.65–0.70)	(0.55–0.55)	(0.52–0.54)	P<0.0001
he46	0.45	0.43	0.43	0.47	0.39	0.39	F=10.8
	(0.44–0.45)	(0.41–0.45)	(0.41–0.45)	(0.45–0.49)	(0.37–0.40)	(0.38–0.41)	P=0.0012

Reported under each media type are the frequencies of the maternal allele and the range of observed values of the three biological replicates. Shown in column MEB are the mean and range of the heterokaryons in liquid culture, as shown in Figure 2. The ANOVA results are for a test of growth environment effect on nuclear ratio within each heterokaryon. For the ANOVA results, the MEB data were not included and only the data from semisolid media were used.

NUCLEAR RATIOS VARY ACROSS ENVIRONMENTS

Six heterokaryotic strains were subjected to differing culture conditions to determine how environment affects nuclear ratio and the consequences for nuclear ratio on fitness (Table 3). Nuclear ratios in this experiment were again different from 1:1. There was a significant effect of environment on nuclear ratio for each of the six heterokaryons as assessed by ANOVA (Table 3). For all heterokaryons, the direction of bias always favored the same nucleus. Nonetheless, nuclear ratio bias could differ by almost an order of magnitude between treatments (e.g., ∼9:1 on MMN+gly and ∼1:1 on MMN for strain he27). For most of the strains the medium MMN+gly appeared to cause the greatest effect on nuclear ratio (Fig. 5). The data for nuclear ratio in liquid malt extract media are also included (column MEB in Table 3). These values were typically different from that on malt extract semi-solid medium (ME), but mostly fell within the range of values observed among treatments for a given heterokaryon, with the exception of strain he27.

RELATIONSHIP BETWEEN GROWTH RATE AND NUCLEAR RATIO

Ramsdale and Rayner (1994) suggested that dominant nuclei in heterokaryons were from homokaryotic strains that had the highest growth rates. This was also tested in the current experiment by estimating growth rates for homokaryons (Table 1). The growth rate estimates of two of the senescent strains (HR32 and 91132/1) were very low. These same isolates also showed unidirectional migration in matings (only donating nuclei) and tended to dominate the nuclear pools of the heterokaryons they formed (Figs. 1–3).

Based on these results, the nuclear prevalence in mycelia (excluding nuclei from the senescent homokaryons) could be roughly assigned to the following dominance hierarchy: B1142 > ORE103c7 > RB_48-9 > Br-518_c2 > A358r > 93026/1 > 87074/1 = 95191. The dominance hierarchy in nuclear frequency does not correspond to relative homokaryotic growth rates on MEA. Strain 95191, however, is the slowest growing of the homokaryotic strains and was also the minority nucleus in all heterokaryons of which it was part ($n = 8$). The data placing B1142 ahead of ORE103c7 in the hierarchy are also based on the results for he31a+b which were not included because only two polymorphic loci were available (data not shown).

The effect of homokaryotic genotype on heterokaryotic growth rate was calculated by a one-way ANOVA using growth rates among heterokaryons with senescent strains removed from the dataset. The mean homokaryotic growth rate (0.22 ± 0.03 mm/h) was lower than that of heterokaryons (0.26 ± 0.01 mm/h). Significant differences among heterokaryons in growth rate were detected ($F_{[25,73]} = 20.20$, $P < 0.0001$) with an estimated ratio of among heterokaryon variance to total variance (broad-sense heritability) of 0.87. The additive genetic variance in heterokaryotic growth rate was estimated by linear regression of midparent values (means of the two parental homokaryons) on mean offspring (heterokaryon) values. This correlation was not significant ($r^2 = 0.145$; $P = 0.0548$), but the slope of the regression (narrow sense heritability) was -0.48. These analyses suggest heterokaryotic growth rate is genetically determined, but it is not an additive trait between homokaryon and heterokaryon generations, meaning other factors such as dominance and epistasis are involved.

Next, the effect of nuclear ratio on growth rate of heterokaryons was tested by linear regression of squared deviation of nuclear ratios from 50% and growth rate for each strain, excluding

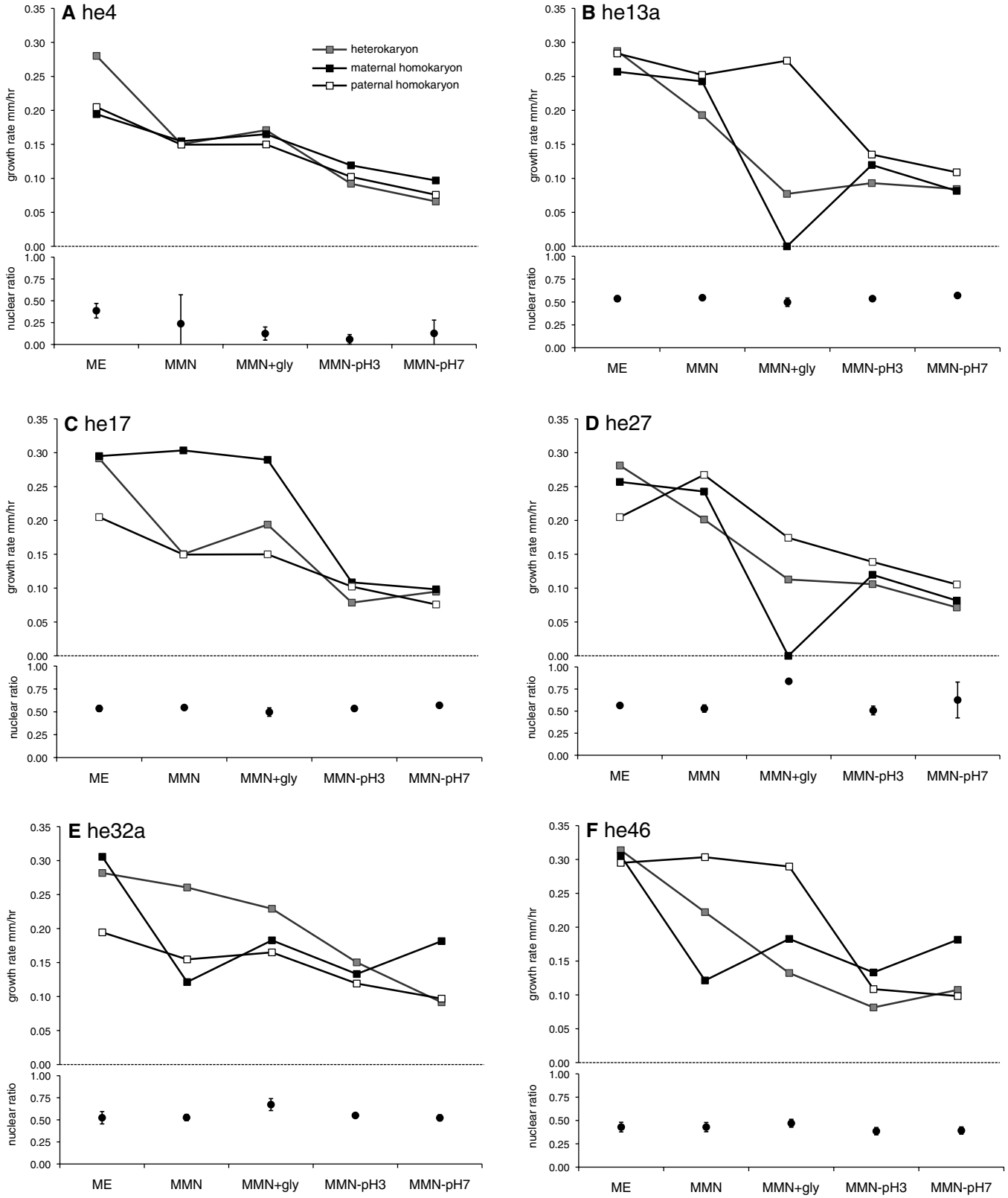

Figure 5. Influence of nuclear ratio and homokaryotic parental traits on growth rates of heterokaryons across heterogeneous environment. The growth rates in mm/h of six heterokaryons and their parental homokaryons are shown as the top portion of the panels A–F. The nuclear ratios expressed as frequency of maternal nucleus are included for each heterokaryotic strain as the bottom portion of each panel. Values shown are the mean over the three biological replicates. Error bars indicate 95% confidence intervals for mean among the three replicates.

strains with a senescent nuclear component. These data showed a strong negative correlation between level of bias in nuclear ratio and growth rate ($r^2 = 0.28$, $P = 0.0071$, slope $= -0.39$, mean $=$ 0.26 mm/h), meaning heterokaryons with imbalanced nuclear ratios tended toward slower growth rates. Heterokaryotic strains containing senescent nuclei showed a large range of growth rates, from 0.05 mm/h (he5) to 0.30 mm/h (he18).

When subjected to differing environmental conditions, the growth rate of heterokaryons relative to parental homokaryons changed depending on environmental condition but showed no consistent trend for any condition or strain (Fig. 5). Heterokaryons displayed higher growth rates than either parental homokaryon in 8 of 30 strain $\times$ environment combinations. Heterokaryons grew slower than either homokaryon in 11 of 30 combinations; and heterokaryons grew intermediate to parental values in 11 combinations. For each heterokaryotic strain, the nucleus that was in the majority could not be predicted based on relative growth rates of the two parental strains as would be predicted if selection was operating by promoting growth of the better adapted nucleus.

NUCLEAR RATIOS DIFFER BETWEEN CONIDIA AND VEGETATIVE MYCELIUM

Nuclear ratios within conidia versus mycelium were compared for three isolates (he1a, he4, and he18). These results showed that allele frequencies estimated from single-conidial isolates and from heterokaryotic mycelia differed from each other (Table 4). For isolate he4, the frequency of the 95191 nucleus in conidia was 22%, but in vegetative mycelia it was estimated to be 38% (95% C. I.: 31–45%). For isolate he1a, the predominant nucleus in conidia was from 93026/1 (71%), but the predominant nucleus in the mycelium was 95191 (61%, 95% C. I.: 54–68%). For he18, fewer single conidial isolates were used, and the difference between conidia and mycelium was not significant (Table 4).

EFFECT OF MITOCHONDRIAL GENOTYPE ON NUCLEAR RATIO

The crosses among the homokaryotic isolates did not always show reciprocal migration (Fig. 1). Some strains (HR32, 91132/1, RB_48-9) only donated but never accepted nuclei. Nuclei from those particular strains also tend to dominate the heterokaryotic mycelium in which they were present (right side of Fig. 2), creating an overall trend for the maternal nucleus of the recipient mycelium to be in the minority. The average frequency of the maternal nucleus when considering all 40 strains was 43.5%. After eliminating the values of strains HR32, 91132/1, and RB_48-9, the mean frequency of the maternal nucleus was 54.4%. In cases in which nuclear migration was bidirectional, the effect of mitochondrial genotype on nuclear ratio could be directly tested, and 10 such cases existed. Of the 10 cases, mitochondrial genotype was determined to have a significant effect on nuclear ratio in only three cases ($P < 0.05$, Table 5). In each of these cases, the dominant nucleus displayed a higher frequency as a paternal component than as a maternal component.

SYMPATRIC VERSUS ALLOPATRIC SYNTHESIZED HETEROKARYONS

A comparison of heterokaryotic nuclear ratios and growth rate was performed between allopatric and sympatric populations to test the hypotheses that nuclear ratio bias and outbreeding depression increase as the genetic distance between homokaryons increases (Ramsdale and Rayner 1996). In 9 of 10 heterokaryons formed between a North American and European strain, nuclear ratios were skewed in the direction of the North American component (Fig. 2). In the remaining strain (he46) the nuclear ratio was not distinguishable from 1:1. If outbreeding depression is greater in allopatric-synthesized heterokaryons, the linear growth rates of these heterokaryons would be expected to decrease as would overall mycelial fitness. The observed data are

Table 4. Frequencies of conidial genotypes recovered from three heterokaryotic isolates.

Heterokaryon	Nuclear Nuclear type	Number observed	Number expected	χ^2	*P*-value
he1a	93026/1	30	16.3	18.68	<0.0001*
	95191	12	25.7		
	95191+93026/1	2	–		
he4	95191	12	20.3	5.45	0.020*
	RB_48-9	42	33.7		
	95191+RB_48-9	3	–		
he18	Br-518_c2	0	3.0	3.60	0.058
	91132/1	18	15.0		
	Br-518_c2+91132/1	0	–		

χ^2 values test the comparison of observed and expected numbers of homokaryotic conidia. Assuming equal segregation of nuclei into conidiospores, the expected number of conidiospores of the given genotype is the frequency of the allele in the vegetative mycelium times the number of homokaryotic spores.

Table 5. Effect of cytoplasmic origin on nuclear ratio in pairs of heterokaryotic strains of identical nuclear constitution but differing mitochondrial genotypes.

Heterokaryon pair	Origin of cytoplasm	Dominant nucleus	Frequency of dominant nucleus	*P*-value
95191+93026/1 (*he1a&b*)	93026/1	93026/1	53.3%	0.002
	95191	93026/1	61.9%	
95191+Br-518_c2 (*he2a&b*)	Br-518_c2	Br-518_c2	70.6%	0.473
	95191	Br-518_c2	68.6%	
93026/1+Br-518_c2 (*he9a&b*)	Br-518_c2	Br-518_c2	67.1%	0.824
	93026/1	Br-518_c2	67.3%	
95191+A358r (*he6a&b*)	A358r	A358r	58.0%	0.007
	95191	A358r	70.3%	
93026/1+A358r (*he13a&b*)	A358r	A358r	57.9%	0.123
	93026/1	A358r	60.8%	
Br-518_c2+A358r (*he19a&b*)	A358r	Br-518_c2	58.1%	0.096
	Br-518_c2	Br-518_c2	54.6%	
ORE103c7+95191 (*he32a&b*)	ORE103c7	ORE103c7	61.3%	0.262
	95191	ORE103c7	59.1%	
B1142+95191 (*he38a&b*)	B1142	B1142	80.7%	0.625
	95191	B1142	80.9%	
B1142+Br-518_c2 (*he39a&b*)	B1142	B1142	71.7%	0.304
	Br-518_c2	B1142	73.6%	
ORE103c7+87074/1 (*he36a&b*)	ORE103c7	ORE103c7	60.0%	0.027
	87074/1	ORE103c7	67.7%	

***P*-value indicates significance level between means as assessed by a paired *t*-test.**

consistent with these predictions because the mean growth rate of allopatric heterokaryons (0.24 mm/h) was significantly less than that of sympatric heterokaryons on MEA (0.27 mm/h; *t*-test, $P = 0.028$).

ALLELE-SPECIFIC GENE EXPRESSION REFLECTS UNDERLYING NUCLEAR RATIOS

Even if nuclear ratios are imbalanced, the level of gene transcription may be uncoupled from actual genomic DNA ratios if one nucleus is transcriptionally repressed or if transcription levels of most genes are regulated in a complex manner. To test this hypothesis, simultaneous analysis of nuclear ratios and allelic ratios within mRNA pools were calculated using pyrosequencing-SNPs. Allele-specific gene expression was tightly correlated with the underlying nuclear ratio (gpd: $r^2 = 0.56$, $P < 0.0001$; Hsp: $r^2 = 0.79$; $P < 0.0001$). Mean allelic ratios between mRNA and genomic DNA were not significantly different for 3 out of the 4 strain × locus combinations, but the mRNA levels of he32a at Hsp were significantly more biased toward the ORE103c7 allele (56%) than the nuclear ratio estimated from genomic data (52%; paired *t*-test, $P = 0.0001$). These data confirm that the underlying nuclear ratios have an effect on gene transcription and are likely to influence phenotypes.

Discussion

In this study we used direct genotyping of heterokaryotic mycelia to demonstrate that nuclear ratios are often imbalanced in heterokaryons of *H. parviporum*. The nuclear ratios were largely influenced by the genotypes of the interacting nuclei and were mostly stable over repeated subculture. For a given heterokaryotic genotype in a given environment, replicate cultures showed very similar nuclear ratios and growth rates. In contrast, nuclear ratios were significantly influenced by the environment the heterokaryon experienced. These largely imbalanced ratios in the mycelium are consistent with the previous findings by Ramsdale and Rayner (1994, 1996) of imbalanced nuclear ratios in germinated conidia of *H. annosum*, yet the nuclear ratios in conidia and mycelia were not necessarily the same, suggesting an avenue by which nuclei could compete for reproduction.

Our data have major implications for the biology of basidiomycete heterokaryons with multinucleate hyphae. First, the data call into question whether the primary unit of selection is the heterokaryotic mycelium as a whole or each individual nucleus within the mycelium. Second, the data implicate a genetic mechanism by which some nuclei are able to overreplicate relative to their partner nucleus, often to the detriment of the mycelium as a whole. Thus, these data suggest that

multinucleate basidiomycetes may experience genomic conflict due to contradictory selection pressures acting at multiple levels.

THE HETEROKARYON AS A GENETIC INDIVIDUAL

The concept of the "individualistic mycelium" in basidiomycete fungi states that a heterokaryotic mycelium is analogous to a genetic individual, because it shows high resistance to genetic invasion or spatial encroachment when encountering heterokaryotic mycelia of another genotype (Rayner 1991). Inherent in this model is that the components of the heterokaryon cooperate with each other to form a single "unit of selection." This model of heterokaryon stability has received much empirical support through laboratory pairing studies showing a high frequency of somatic incompatibility among genetically distinct heterokaryons (Rayner 1991; Worrall 1997), including studies of *H. annosum* (Stenlid 1985; Swedjemark and Stenlid 1993; Piri 1996).

We propose that for multinucleate organisms, such as *H. parviporum*, the heterokaryotic mycelium is not equivalent to a genetic individual because nuclear ratios are environmentally plastic, and nuclei within the mycelium can compete with each other. *Heterobasidion parviporum* further violates the model of heterokaryon stability, because heterokaryons paired in the laboratory are still able to exchange nuclei as well as transmit viruses (Hansen et al. 1993; Ihrmark et al. 2002). Hansen et al. (1993) additionally provided evidence to suggest that heterokaryotic mycelia may be a mosaic of heterokaryotic hyphae and homokaryotic hyphae in which one nucleus has outgrown or outcompeted the other.

If heterokaryons are to be viewed as populations of nuclei, then both selection and genetic drift could influence nuclear ratios. For a long-lived species, such as *H. parviporum*, in which heterokaryotic genotypes may grow for decades and cover an area of hundreds of square meters (Stenlid 1985), nuclear ratios within the common mycelium may be heterogeneous among microhabitats due to varying selective forces. If, instead, genetic drift is the predominant force impacting nuclear ratio, in extreme cases heterokaryons could break down into their homokaryotic components due to lack of selective pressure maintaining the heterokaryon. Currently, there are no empirical data to support either hypothesis, but most older isolates of *H. parviporum* in nature are typically heterokaryotic and have maintained both nuclei (Swedjemark and Stenlid 1993). It is likely that nuclear ratios are required to converge to 1:1 during sexual fruitbody production and meiosis, meaning that a strong selective force operates to maintain heterokaryosis. Alternatively, older heterokaryotic isolates could be those for which selection has acted to minimize the negative effects of competition among nuclei (Ramsdale and Rayner 1996).

The production of homokaryotic conidia by heterokaryons is likely to foster nuclear competition. Imbalanced nuclear ratios in the vegetative mycelium should generally translate into imbalances in the asexual conidia. Selfish nuclei capable of dominating a heterokaryotic mycelium are likely to gain an advantage early in the infection cycle, when the number of conspecific interactions is high (Swedjemark and Stenlid 2001). For example, on a freshly cut stump surface, a nucleus capable of dominating a heterokaryotic mycelium would have many more opportunities for local spread through conidia or by remating with neighboring homokaryotic or heterokaryotic mycelia. The available data for nuclear distribution within a single excavated stump support frequent remating and shuffling of nuclei between mycelia (Johannesson and Stenlid 2004).

THE RELATIONSHIP BETWEEN NUCLEAR MIGRATION AND NUCLEAR RATIO

One of the most striking results of the present study was the numerical dominance of nuclei from senescent isolates over that of the maternal nuclei when paired as heterokaryons (Fig. 2). Following serial subculture of a heterokaryotic strain containing a senescent nucleus (he18, Fig. 4D), the nuclear ratio bias gradually diminished. The senescent homokaryotic phenotype was completely linked to unilateral nuclear donation, meaning senescent homokaryons never acted as a maternal nucleus. Unilateral nuclear donation was also observed in strain RB_48-9, a homokaryon without senescent morphology that also showed numerical dominance within most of the strains within which it was a component (Fig. 2). These observations suggest a plausible shared mechanism controlling unilateral nuclear migration (or invasion) and nuclear ratio domination. As nuclei migrate during mating, they appear to undergo an active level of nuclear replication as evidenced by the fact that both peripheral and intercalary cells of the maternal homokaryon become heterokaryotized (Ellingboe and Raper 1958; Raudaskoski 1998).

This association between nuclear migration and numerical dominance could also explain the differences in nuclear ratios of heterokaryons of identical nuclear constituency but differing cytoplasms (Table 5). In all instances in which the nuclear ratios were significantly different, the maternal nucleus was always underrepresented, suggesting the paternal nucleus has some replicative advantage. This hyper-replicability may be transient if it is epigenetic, as observed in strain he18 with a senescent nucleus, but it would persist into subsequent generations if it is genetically determined. One possible genetic factor that could regulate nuclear division is the *B* mating-type locus encoding for pheromone receptors and peptide pheromones that also regulates nuclear migration in compatible matings (Brown and Casselton 2001). Alleles of the *B* mating-type locus have been shown to influence nuclear division in recovering protoplasts of *S. commune* (Raper 1985). In a multinucleate basidiomycete, such as *H. parviporum*, mating type controlled differences in nuclear division could result

in nuclear ratio imbalance in the absence of equivalency enforcing mechanisms, such as clamp-cell partitioning. Unfortunately, very little is known about mating-type genes in *Heterobasidion*, except that the species has a bipolar mating system with multiple alleles (Korhonen 1978; Chase and Ullrich 1983).

NUCLEAR COMPETITION VERSUS COOPERATION AND THE ADAPTIVE SIGNIFICANCE OF NUCLEAR RATIOS

Ramsdale and Rayner (1994, 1996) attributed much of the imbalanced nuclear ratios in *Heterobasidion* to outbreeding depression between nuclear genomes. Among the evidence cited for outbreeding depression was that uninucleate conidia of allopatric heterokaryons germinated at a faster rate than binucleate, and potentially heterokaryotic, conidia. Their studies never explicitly tested the fitness of allopatric-derived heterokaryons, but their model predicts a negative effect on heterokaryon fitness with increasing genetic distance between nuclei. The present experiments allowed explicit testing of the fitness of allopatric and sympatric derived heterokaryons using growth rate on MEA and demonstrated that allopatric heterokaryons grew significantly worse than sympatric heterokaryons. The correlation between genomes derived from geographically distant locations and the reduced fitness of the resultant heterokaryons presumably due to absence of coevolution is not always observed among fungal taxa (Van Putten et al. 2003), but interestingly it has been reported in pathogenicity assays for *Heterobasidion* (Garbelotto et al. 2007).

By exposing a single heterokaryotic strain to multiple environments, these experiments tested whether nuclear ratio modulation could reflect cooperation or competition between nuclei. The following pairs of culture conditions may be considered paired conditions: complete versus synthetic media (ME vs. MMN), inorganic versus organic nitrogen source (MMN vs. MMN+gly), and low versus high pH (MMN-pH3 vs. MMN-pH7). By comparing the relative growth rates of a heterokaryon and its component homokaryons under these different conditions with nuclear ratios of the heterokaryons, the degree of competition versus cooperation could be assessed (Fig. 6). For example, under a scenario of competition, nuclear ratios may solely reflect the underlying ability of one nucleus to dominate over another regardless of the relative fitness of the component nuclei in differing environments (Fig. 6A). Alternatively, if nuclei cooperate to maximize fitness, the nuclear ratios could either change to favor the nucleus with the highest fitness in a given environment (Fig. 6E) as has been suggested for ascomycetous molds (Pontecorvo 1946; Rees and Jinks 1952), or nuclear ratios could remain the same, but nuclei could cooperate through coordination of metabolic processes within the cytoplasm (Fig. 6D).

To test these models, we imposed strong selection on nuclei within heterokaryons containing the A358r nucleus by growing them on medium with glycine, because the A358r homokaryotic strain was unable to grow on such media. If cooperative interactions between nuclei largely determine nuclear ratios, the relative frequency of the A358r nucleus is expected to be lower in media containing glycine than glycine-free environments. Two heterokaryons containing the A358r nucleus were tested, and two very different results were observed. Heterokaryon he13a displayed the lowest frequency of the A358r nucleus among all media types on MMN+gly as predicted by the cooperation hypothesis. In contrast, he27 displayed the highest frequency of the A358r nucleus (84%) specifically on MMN+gly (Figs. 5D and 6C). Nonetheless, both he13a and he27 had slow growth rates relative to the other heterokaryons on MMN+gly (Fig. 5B, D), implying the fitness of the mycelium was reduced by effects from the A358r nucleus. The shift in nuclear ratio of he27 is a clear example of genomic conflict in which a nucleus is over-replicated within the heterokaryotic mycelium to the detriment of the heterokaryon as a whole (Fig. 6C). This large replicative advantage is reminiscent of mitochondrial "petite" mutations in yeast. Petite mitochondrial genomes possess large DNA deletions and are able to replicate faster than wild type, however the fitness of the cell is also reduced due to respiratory defects (Zeyl and DeVisser 2001). Data from the additional *H. parviporum* heterokaryons did not generally fit into either competitive or cooperative patterns (Fig. 5). Six paired growth conditions suggested a preponderance of cooperation, two growth conditions suggested competition, and 10 growth conditions were difficult to interpret under the proposed models. On the whole, changing the environments to which heterokaryons were subjected did not change which nucleus dominated, despite underlying differences in the fitness of the component nuclei in these environments.

The present observations differ greatly with those observed for ascomycete molds such as *Penicillium* and *Neurospora* in which nuclear ratios can be directly modulated by artificial selection (Ryan and Lederberg 1946; Jinks 1952; Davis 1960). One major difference between the studies with outcrossing basidiomycetes and ascomycetes is that the heterokaryon represents the combination of genetically very different genomes in the former, but in the latter the genomes being combined are very similar, often differing by single-gene mutations, making predictions about the fitness advantage of each nucleus more apparent. When genetically distinct autonomously replicating genomes are brought together into the same cell there must be mechanisms to reduce genomic conflict and cheating (Partridge and Hurst 1998). Both enforced dikaryotization and diploidy help maintain a strict nuclear balance. When these mechanisms are not in place, selfish nuclear behavior may be observed. The difference in nuclear ratios of conidia versus vegetative mycelium of *H. parviporum* (Table 4) confirms the notion that nuclei may compete to be included into conidia. An alternative explanation for bias among

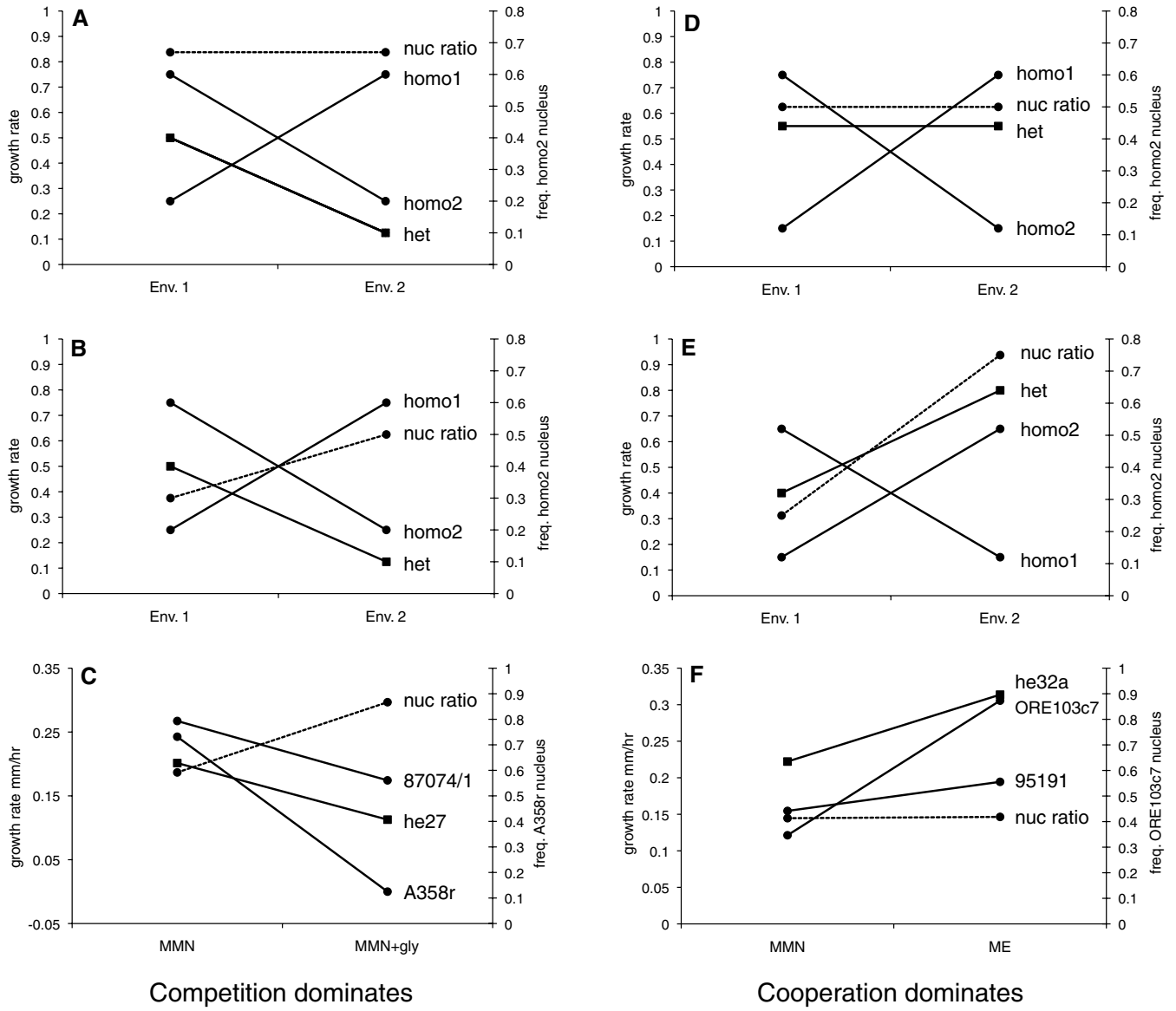

Figure 6. **Models of varying competition and cooperation among nuclei as a means of explaining nuclear ratios in heterokaryons subjected to differing environments. On the first y-axis the growth rate is plotted, and on the second y-axis the nuclear ratios are shown. (A–C) Possible outcomes predicted if nuclear competition negatively impacts the fitness of the heterokaryon. (A) Hypothetical example in which nuclear ratios do not change across environments and the heterokaryon is less fit than predicted by its component homokaryons. (B) Hypothetical example in which nuclear ratios do change across environments, but in a direction that confers a negative impact on heterokaryotic growth. (C) Observed data for heterokaryon he27 and its component homokaryons that fit the prediction of B. (D–F) Outcomes predicted under the hypothesis that nuclei cooperate to increase the fitness of the heterokaryon. (D) Hypothetical example in which nuclear ratio is constant across environments, and heterokaryon growth rate is better than the mean growth rate of the component homokaryons. (E) Hypothetical example in which nuclear ratios are altered in favor of the nucleus with the highest growth rate as a homokaryon, and heterokaryotic fitness is increased as a result. (F) Observed data for heterokaryon he32a fitting the prediction of D.**

conidial germlings could be the result of strong differences in growth characteristics, such as germination rate or linear growth rate, among nuclei. In fact, a high degree of postgermination mortality was noticed in the conidial sample that showed the greatest difference between mycelial and conidial nuclear ratios (he1a): 19 single conidial germlings of 64 did not continue to grow after transfer in contrast to 3 of 64 for isolate he4. To separate postgermination effects from actual nuclear inclusion bias, direct genotyping of conidia before germination should be attempted.

In Agaricomycetes, an additional source of genomic conflict arises because nuclei but not mitochondria migrate during mating (Aanen et al. 2004). Thus mitochondrial genomes will only

reproduce through female (nuclear receptivity) function of their resident mycelia and gain no benefit through male behavior (nuclear migration). In *H. parviporum*, many homokaryotic strains displayed unidirectional migration, but it was exclusively in the direction of unilateral donation (female sterility). Nuclear ratio was affected by cytoplasmic background (Table 5), but was always biased in the direction of the paternal nucleus, which we have suggested may be due to an epigenetic status of the invading nucleus. In heterokaryotic hybrids of *H. annosum*, a strong effect of mitochondrial genotype on pathogenicity has been demonstrated (Olson and Stenlid 2001), suggesting mitochondrial-nuclear epistasis. If mitochondrial genotype influences nuclear ratio, it was undetected in this study.

One confounding factor in the effort to detect adaptive changes in nuclear ratio was the absence of additive genetic variation when comparing homokaryotic and heterokaryotic growth rates. Interpreting the data in the context of cooperation versus competition assumes that growth rates are heritable between the homokaryotic and heterokaryotic generations and that radial growth rate is a good proxy for mycelial fitness. Whether these assumptions will prove valid requires further testing, and violation of these assumptions could explain the difficulty in determining the adaptive value of nuclear ratio variability. In *S. commune*, growth rate and morphology is inherently different between monokaryons and dikaryons (Clark and Anderson 2004), and it is possible that the developmental switch that occurs between the two life cycle stages includes changes in relative growth strategies even on identical media (Casselton 1978; Stenlid and Rayner 1989). Previous studies of genetic variation for growth rate in the Agaricomycetes *S. commune* and *Flammulina velutipes* found evidence for heritable genetic variation between monokaryon and dikaryon generations, but the authors concluded that growth rates of the two life stages were largely controlled by different sets of genes (Simchen and Jinks 1964; Simchen 1965). The nonadditivity of homokaryotic and heterokaryotic generations observed in this study could also be a result of nuclear competition. Competition could be manifested by the time spent by each nucleus in a transcriptional relative to a replicative mode. This was tested by analyzing the correlation of allele ratios of mRNA pools with nuclear ratios for two housekeeping genes (gpd and Hsp). The data revealed little indication of a difference in gene transcription relative to DNA replication, suggesting nuclei contribute to cellular metabolism according to their genomic representation. It must be mentioned that the number of genes surveyed was small, and other genes may show quite different patterns of allele-specific expression depending on *cis*-regulatory elements and other modifiers of gene expression. In dikaryotic basidiomycetes as opposed to multinucleate basidiomycetes, synchronous nuclear division is enforced by the *A* mating-type locus (Raper 1966). Incentives to cooperate rather than compete are expected to be higher in such a scenario, perhaps reducing negative epistasis between monokaryon and dikaryon generations.

Conclusion

These results demonstrated that nuclear ratios in heterokaryotic mycelia of *H. parviporum* are often imbalanced, and that these imbalances can be detrimental to the fitness of the mycelium. The nuclear ratios were shown to be largely temporally stable and predicted both by the genotype of the nuclei comprising the heterokaryon and their epigenetic state. Environmental factors were found to have a significant influence on the nuclear ratio and replicate cultures grown in identical conditions generally converged to very similar, typically imbalanced, nuclear ratios, and these imbalanced nuclear ratios were further shown to influence transcript levels within the mycelium. All of these results suggest that heterokaryotic Agaricomycete fungi are functionally different from diploids and possess the potential for biased nuclear ratios due to nuclear competition. The observation that nuclear ratios can become grossly imbalanced furthermore suggests that individual nuclei within a mycelium could actually be units of selection.

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Supporting Information

The following supporting information is available for this article:

Figure S1. Homokaryotic isolate showing senescent morphology on M/10 media.

Figure S2. Validation of the microsatellite markers for estimating nuclear ratios in heterokaryons.

Supporting information may be found in the online version of this article.