Analysis of Inbreeding Depression in *Agaricus bisporus*

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ABSTRACT

Inbreeding depression was observed in the commercial button mushroom, *Agaricus bisporus*, by examining two laboratory populations. The outbred population consisted of 20 compatible pairings, 10 homokaryons with each of the homokaryons Ag1-1 and Ag89-65. The inbred population consisted of 104 backcrosses (among which 52 were expected to be sexually compatible) obtained from the pairings of two progenitor homokaryons, Ag1-1 and Ag89-65, with 52 progeny homokaryons derived from the mating between Ag1-1 and Ag89-65. The eight fitness components examined for these two populations were successful matings as identified by the analysis of restriction fragment length polymorphisms, positive mycelial interaction in these successful matings, heterokaryon growth rate, primordium formation by the successful matings, fertile fruiting body formation, time to first break, average number of fruiting bodies per square foot, and average weight per fruiting body. The outcrossed population showed a significant advantage over the inbred population in three of eight fitness components. Two pairs of traits were significantly correlated. The multiplicative fitness ratio of the inbred to the outcrossed population was 0.18. The relevance of inbreeding depression to the evolution of fungal mating systems and to mushroom breeding is discussed.

INBREEDING depression and its consequences for reproductive success in plants and animals have been the topic of intensive and diverse study, with subjects including laboratory (*e.g.*, LATTER and ROBERTSON 1962), cultivated (ALLARD 1960), captive (RALLS and BALLOU 1983), and natural (DOBZHANSKY *et al.* 1963; RITLAND and GANDERS 1987) populations. However, systematic studies of the consequences of inbreeding in fungal populations are rare (RAPER 1966; HANIFAH and GRAHAM 1986; CHARLESWORTH and CHARLESWORTH 1987). In contrast to inbreeding studies in plants and animals, where abundant literature shows that inbreeding depression occurs in almost all organisms studied, no genetic study of fungi has critically examined the effect of inbreeding depression on multiple life-stage fitness components, despite the number of studies that have uncovered genes that interfere with specific stages of sexual reproduction (NELSON 1959a-d, 1964, 1970; RAPER 1966; LESLIE and RAJU 1985). Linkage relationships and molecular properties of some of these genes are beginning to emerge (RAJU 1992). It is clear that quantitative studies must be done to investigate the extent to which inbreeding depression occurs in fungal populations.

Fungi use a great variety of sexual reproduction strategies, from homothallism (in which all sexual spores are self-fertile, with or without outcrossing capacity) to secondary homothallism (in which some sexual spores are self-fertile and others are self-sterile) and heterothallism (in which all sexual spores are self-sterile)

(RAPER 1966). The heterothallic systems can be subdivided into unifactorial, bifactorial and trifactorial systems according to the number of unlinked loci that control the mating process (RAPER 1966; JURAND and KEMP 1973). The general trend from lower fungi (*e.g.*, Phycomyces) to higher fungi (*e.g.*, Basidiomycetes) is of increasing levels of sexuality, with the strongest outcrossing capability being found in some Basidiomycetes (RAPER 1959, 1966). However, sets of closely related species exhibiting different mating systems are found in almost all groups of fungi, especially in the higher fungi (RAPER 1966). For example, putative homothallic and heterothallic sexual systems have been reported in genus *Agaricus* (ELLIOTT 1978; RAPER and KAYE 1978). One four-spored species, *A. silvicola* (ELLIOTT 1978), was determined to be homothallic by the absence of successful matings among basidiospores (sexual spores in Basidiomycetes) from the same fruiting body and by the ability of single-spore isolates to fruit. The common field mushroom, *A. campestris*, may include both homothallic (KLUSHNIKOVA 1939) and heterothallic (MAY and ROYSE 1982) forms. Unifactorial heterothallism was reported in *A. bitorquis* (RAPER 1976), *A. macrosporus* and *A. nivescens* (ELLIOTT 1978), and *A. vaporarius* (ANDERSON *et al.* 1984). The cultivated button mushroom, *A. bisporus* (= *A. brunnescens*) was reported to be secondarily homothallic with a unifactorial sexual incompatibility system (ELLIOTT 1972; MILLER 1971; RAPER *et al.* 1972). Recently, KERRIGAN and co-workers (1994) reported a natural *A. bisporus* population with isolates having a predominantly unifactorial heterothallic life cycle. Sets of closely related species or subspecies with diverse

mating systems could provide a good system to study fungal mating system evolution.

In most strains of *A. bisporus*, the majority of basidia (the sexual spore-bearing structures) produce two basidiospores, although low percentages of basidia bearing from one to seven spores have been reported (SONG *et al.* 1972). The average number of spores per basidium varies somewhat among different strains and may also vary slightly at different developmental stages in a given strain (ELLIOTT and CHALLEN 1984; KERRIGAN and ROSS 1987). Spores from bisporic basidia usually contain two of the four postmeiotic nuclei, and each pair of nuclei are usually nonsisters with respect to meiosis II (SUMMERBELL *et al.* 1989). Meiotic progeny analyses using isozyme or restriction fragment length polymorphism (RFLP) markers indicated that over 90% of the basidiospores were heterozygous at over 90% of the parental heterozygotic loci (ROYSE and MAY 1982; SUMMERBELL *et al.* 1989; ALLEN *et al.* 1992). Upon germination, these basidiospores form heterokaryons with cells having two genetically different haploid nuclei, and these heterokaryons are characteristically capable of fruiting. Other uncommon basidiospores, usually from rare three- or four-spored basidia, receive only one nucleus and form homokaryons upon germination. These homokaryons can mate with other homokaryons carrying compatible mating types to form stable heterokaryons. The mating type locus that controls the mating interaction in *A. bisporus* was found to be on the largest chromosome, linkage group I (XU *et al.* 1993a).

Because homokaryons are uncommon and morphologically indistinguishable from heterokaryons, the isolation of homokaryons from a basidiospore population is very laborious. The process generally involves screening monospore cultures for slow growth rates and then subjecting selected strains to multiple locus molecular marker analysis or fruiting tests. Basidiospores homoallelic at all loci that were heteroallelic in the parental strains are considered to be homokaryons (KERRIGAN *et al.* 1992). The more marker loci used, the more robust the results. In fruiting tests, self-sterile cross-fertile spores are considered to be homokaryons (FRITSCHÉ 1991a); however, this method is less robust than using molecular markers.

An advantage of the secondarily homothallic life cycle of *A. bisporus* is that most meiotic progenies are capable of fruiting without needing to find a mate before going through the next sexual cycle. This advantage is more obvious when the breeding population size is small and spores are widely and thinly dispersed. On the other hand, because the secondarily homothallic mating system tends to maintain heterozygosity because of the low frequency of crossing over during meiosis and the preferential association of nonsister nuclei in the basidiospores (SUMMERBELL *et al.* 1989), recessive and partially recessive mutations do not easily become homozygous, and one would therefore expect a large

genetic load. In breeding experiments, both improvement and reduction of several economically important traits have been found in monospore cultures compared with the parental strains (FRITSCHÉ 1972; WANG 1991). However, comparisons of multiple fitness components of inbred and outcrossed populations are not available. In this study, I examined whether there was an association between secondary homothallism and the accumulation of deleterious recessive genes in *A. bisporus*. One way to test this association is by comparing the fitness of inbred and outcrossed populations. Backcrossing is a type of inbreeding that allows maximum exposure of deleterious recessive mutations, with an inbreeding coefficient of 0.5. Homozygous deleterious recessive mutations should be expressed in the reduction of fitness of the inbred population.

Inbreeding depression has been observed in a number of edible mushrooms, including the phoenix mushroom, *Pleurotus sajor-caju* (HANIFAH and GRAHAM 1986); the oyster mushroom, *Pleurotus ostreatus* (EUGENIO and ANDERSON 1968) and the straw mushroom, *Volvariella volvacea* (CHANG *et al.* 1981). Most of the comparisons were made on yield and/or incubation period between parental strains and their inbred progenies. Multiple life-stage fitness components of inbred and outcrossed populations have not yet been critically compared. The second objective of this study was to examine multiple life-stage fitness components, their sensitivity in detecting inbreeding depression, and whether some of the fitness components are correlated.

Third, various environmental factors have been found to influence the expression of inbreeding depression in two very different species, *Sabatia angularis* (DUDASH 1990) and *Hydrophyllum appendiculatum* (WOLFE 1993). In a previous study, different media were found to greatly influence the mating interactions (the gross morphological and genetical changes at the junction zone where two cultures meet) of homokaryons of *A. bisporus* (XU *et al.* 1993b). Could different media also affect the expression of inbreeding depression in *A. bisporus*? To study these questions, I examined two populations of the secondarily homothallic fungus, *A. bisporus*, derived from laboratory outcrossing and backcrossing among homokaryons, for eight fitness components.

MATERIALS AND METHODS

Strains. Two homokaryons, Ag1-1 (ATCC 24662), a mutant carrying an unknown auxotrophic requirement, and Ag89-65, a homokaryon recovered by protoplast regeneration from strain Ag89, collected in an urban field site by Dr. D. MALLOCH (CASTLE *et al.* 1988), were used for both the outcrossing and inbreeding experiments. A spore print from a heterokaryon, Ag93b, derived from a laboratory cross between Ag1-1 and Ag89-65, was the source of the 52 homokaryotic offspring (KERRIGAN *et al.* 1992). These 52 progeny homokaryons were crossed to the two progenitor homokaryons, Ag1-1 and Ag89-65, forming the inbred population (inbreeding

A. Original pedigree (from KERRIGAN *et al.* 1992, 1993b): **B. Inbred population (104 pairings):**

Ag1-1 × 52 progeny homokaryons
 Ag89-65 × 52 progeny homokaryons

C. Outcrossed population (20 pairings):

Ag1-1 × 10 unrelated homokaryons
 Ag89-65 × 10 unrelated homokaryons

FIGURE 1.—Construction of the outcrossed and inbred populations in this study.

coefficient 0.5). Fifty-two of 104 crosses were expected to be compatible and able to complete their sexual life cycle. Thirteen homokaryons unrelated to the Ag93b pedigree were generated through either protoplasting heterokaryons or isolation of meiotic homokaryotic offsprings from cultivated and wild strains (CASTLE *et al.* 1988; JIN *et al.* 1992; M. LOFTUS, personal communication). Two sets of 10 of the 13 homokaryons were paired with the two homokaryons, Ag1-1 and Ag89-65, to form the outcrossed population (inbreeding coefficient 0). All 20 pairings were expected to be compatible based on the allelic analysis at the mating type locus (CASTLE *et al.* 1988; XU *et al.* 1993b; J. XU, unpublished data). Ag1-1 and Ag89-65 were used for both the outcrossed and inbred populations to minimize the differences in the genetic background between the two populations. Figure 1 shows the construction of the two populations. All cultures were stored in liquid nitrogen and cultured on complete yeast medium (CYM) (RAPER *et al.* 1972) before tests for mating and fruiting were conducted.

Fitness components: To assess the overall fitness of the inbred and outcrossed populations, eight fitness components were included for comparison. Mycelial interaction refers to the macroscopic interaction at the junction zone of two paired isolates. Three types of interaction were defined: positive, ambiguous and negative (XU *et al.* 1993b). Positive mycelial interaction was characterized by the presence of obviously vigorously (fluffy) growing mycelium along the junction zone. Ambiguous mycelial interaction was defined as showing a small amount of vigorously growing mycelium along the junction zone. Negative mycelial interaction was characterized as showing no vigorously growing mycelium along the junction zone. The ability of an isolate to interact with other isolates has been used as an indicator of mating capability in many fungi (NELSON 1959a; XU *et al.* 1993a,b; ANDERSON *et al.* 1984; KERRIGAN *et al.* 1994). However, successful mating in *A. bisporus* can only be confirmed unambiguously through genetic analysis of nuclear components, using either allozyme or RFLP markers (ROYSE and MAY 1982; CASTLE *et al.* 1988). The successful formation of a stable heterokaryon by two homokaryons is called heterokaryotization. The growth rate of a newly formed heterokaryon was measured as an indicator of the rate of colonization on natural substrate. Two other life stages assessed were the formation of mushroom primordia and the formation of complete fertile fruiting bodies with sexual propagules. The three fecundity correlates of the fruit-

ing bodies examined were the time to first break [the time it took for a fruiting body to appear after full colonization of the substrate and the spread of a layer of turf soil ("casing") on top of the colonized substrate to stimulate fruiting], the number of fruiting bodies per square foot and the average weight per fruiting body. The time to first break is inversely related to fecundity: the shorter the time required to form fruiting bodies, the better the chance for the sexual propagules to establish new colonies. The other seven components have positive correlations with fitness.

Pairings: In a previous study, a medium with diluted CYM supplemented with extracts of compost (CCE) allowed the expression of mycelial interaction and heterokaryotization in more pairings of homokaryons than did full strength CYM (XU *et al.* 1993b). All pairings in this study were made on both CYM, the traditional laboratory mating medium, and CCE to examine the influence of this environmental factor on the expression of inbreeding depression. Pairings were set up and scored as described by XU *et al.* (1993b). Each pairing was done at least once on each medium. Mycelial interactions for the same pairings repeated on the same medium were the same.

RFLP analysis and estimate of heterozygosity: Sample subculturings, growth and harvesting of mycelium and RFLP analysis were all done as described by XU *et al.* (1993a). Nine probes containing inserts of random *EcoRI* fragments of nuclear DNA from *A. bisporus* Ag33 (plasmids of p33n5, p33n10, p33n25 and p33n18) and Ag1-1 (plasmids of p1n17 and p1n38) or from *A. bisporus* Ag4 (plasmids of p4n6 and p4n14) were used to confirm heterokaryotization and to determine the heterozygosity of subcultures (CASTLE *et al.* 1987; KERRIGAN *et al.* 1993b). There were two segregating loci for plasmid p33n10 and one locus for each of the other eight probes with predominant signal in the Southern hybridization pattern. Only subcultures taken from pairings on CCE medium were used for RFLP analysis. Initially, DNA of one subculture from each of the 124 pairings was assayed with all nine plasmid probes. Those samples showing heteroallelism at informative loci were considered to be sexually compatible. For each sample showing no heteroallelism at informative loci, DNA of a second subculture was assayed for heteroallelism. For each sample showing no heteroallelism in the second round of tests, a third subculture was assayed. Pairings showing no heteroallelism after three tests were treated as incompatible or unsuccessful.

Estimates of heterozygosity for the outcrossed population are based on the 10 loci derived from the nine probes. Because 9 of 10 loci used here are informative for the Ag1-1 and Ag89-65 cross, the heterozygosity of their progeny heterokaryon, Ag93b, should be 0.9. In fact, the proportion of DNA restriction fragments that segregated in Ag93b was estimated to be 0.55, based on Southern hybridizations of 73 DNA probes (from KERRIGAN *et al.* 1993b). Without correcting this discrepancy, the expected average heterozygosity of the heterokaryons in the inbred population based on the 10 loci would be 0.45 (0.9/2), but given the Ag93b value it should be 0.275 (0.55/2). The bias correction factor of 0.62 (0.55/0.9) is applied to the heterozygosity calculations for all the heterokaryons in the inbred population. Even though only 10 loci were used to confirm the successful matings in both the inbred and outcrossed populations, the genotypes of Ag1-1, Ag89-65 and their 52 progeny homokaryons for 64 loci were available from a genetic mapping experiment using this cross (KERRIGAN *et al.* 1993b). The more robust estimates of heterozygosity from the 64 loci are used for the inbred population in this study.

Heterokaryon growth rate: Previous experiments conducted on 12 different media varying significantly in nutrient

content and concentration revealed no significant difference in radial growth rate for the same isolates among media (XU *et al.* 1993b). In this study, confirmed heterokaryons from both the outcrossed and inbred populations were therefore grown only on potato dextrose agar (PDA) (Difco, Detroit, MI) supplemented with 10% compost extract, at room temperature. Inocula of 2 mm² were placed in the center of each plate, with two replicate plates for each heterokaryon. Radial growth measurements were taken at arbitrarily placed right angles for each colony (4 measurements per colony), once every 7 days for 4 weeks. The mean weekly growth rates for each heterokaryon were calculated from these 32 measurements.

Fruiting and fruiting body fecundity correlates: Spawn was prepared according to the method of SAN ANTONIO (1971) with some modifications as indicated by XU *et al.* (1993a). Fruiting was conducted at Sylvan Spawn Laboratory Inc. (Kittanning, PA), Lambert Spawn Company Inc. (Coatesville, PA) and Erindale College (Mississauga, Ontario, Canada) according to standard commercial fruiting procedures (FLEGG and WOOD 1985). Three replicates were conducted for each pairing. The fruiting capacities in laboratory and industrial conditions have been shown to be consistent for the same pairing or heterokaryon (JIN *et al.* 1992; JIN and HORGAN 1994). A method for estimating fruiting ability in nature has not been established.

Data analysis: The outcrossed and inbred populations were compared statistically for each of the eight fitness components. Chi-squared tests were performed for the following four traits: percentage of pairings resulting in heterokaryotization as indicated by RFLP analysis over expected compatible pairings, percentage of heterokaryotized crosses showing positive mycelial interaction, percentage of newly formed heterokaryons producing mushroom primordia, and percentage of mushroom primordia developing into complete fruiting bodies. Student's *t*-tests were done using the program Systat 5.2 for the other four components.

The strength of inbreeding depression is usually summarized by $\delta = 1 - W_i/W_o$, where W_i and W_o are the fitnesses of the inbred and outcrossed populations respectively (ECKERT and BARRETT 1994). The individual δ value for each fitness component can be calculated directly from the above formula. However, to derive the multiplicative fitness ratio of the inbred and outcrossed populations, the independence of the fitness components needs to be examined. The correlations among the four continuous variables, heterokaryon growth rate and the three fruiting body fecundity correlates were derived from Pearson correlation tests. Mycelial interaction and heterokaryotization are two measures of one life stage, the mating of homokaryons, and their association was tested using a 2 × 3 contingency table. The mating of homokaryons, the formation of mushroom primordia by a heterokaryon, and the development of fertile fruiting bodies from promordia represent three different life stages. Only the pairings that successfully mate are able to form mushroom primordia (XU *et al.* 1993a). In turn, only those that form mushroom primordia can go on to form fertile fruiting bodies. Based on this logic, the three different life stages were treated independently here. Variables showing significant correlations were combined to give an average value that was combined with other independent variables to obtain the multiplicative fitness ratio for the two populations.

RESULTS

Media, the expression of mycelial interaction and inbreeding depression: Mycelial interactions were scored

for all pairings. Nineteen of 20 pairings in the outcrossed population and 43 of 52 expected compatible pairings in the inbred population successfully underwent heterokaryotization, as revealed by RFLP analysis. The effects of the two media, CYM and CCE, on the expression of mycelial interaction by the successful pairings are presented in Table 1. Significant differences were observed between the two different media with both populations. No significant difference was obtained between the two populations on CYM, but a significant difference was observed on CCE (Table 1). No significant difference was observed between the two media for the incompatible or unsuccessful pairings in the inbred population or the one unsuccessful pairing in the outcrossed population (data not shown).

Mycelial interaction and heterokaryotization on CCE medium: Because CCE medium was found to be more favorable than CYM in allowing heterokaryotization and the expression of mycelial interaction, only subcultures from pairings on CCE medium were taken for RFLP analysis. The association of mycelial interaction and heterokaryotization was examined only for pairings done on CCE medium. Significant association between these two variables was found ($\chi^2 = 68.77$, $df = 2$, $P < 0.001$) (Table 2). Specifically, positive mycelial interaction was associated with heterokaryotization and negative mycelial interaction with unsuccessful or incompatible pairings. Ambiguous mycelial interaction was not associated significantly with either successful or unsuccessful matings.

Heterokaryon growth rate and fruiting body fecundity components: Table 3 shows the Pearson correlation analyses among the three fruiting body fecundity components and heterokaryon growth rates. A significant association between the time to first break and the number of fruiting bodies was found. This indicates that the earlier mushrooms were produced, the greater the number of mushrooms produced. There was no other significant correlation among the four traits (Table 3).

Heterozygosity and fitness components: Correlation tests between heterozygosity and other fitness components were conducted for the inbred population only. There are two reasons for excluding the outcrossed population from this analysis. First, the estimate of heterozygosity for the inbred population was based on 64 loci, whereas the estimate for the outcrossed population was based on only 10 loci; hence, the estimate is more robust for the inbred population than for the outcrossed or combined population. Second, because there are significantly different levels of heterozygosity for the inbred and outcrossed populations (Table 4), combining these two populations would create a large variance and bias the estimates of correlations. The statistical test showed a significantly higher level of heterozygosity for the crosses that fruited than for the crosses that heterokaryotized but did not fruit in the inbred

TABLE 1
Effect of two media on the expression of mycelial interactions of the heterokaryotized crosses in the inbred and outcrossed populations

Medium	Inbred population			Outcrossed population		
	Positive	Ambiguous	Negative	Positive	Ambiguous	Negative
CYM	0	6	37	2	4	13
CCE	26	13	4	18	1	1

Statistical test results: 1. In the inbred population, the difference between the two media is significant, with $\chi^2 = 55.14$, d.f. = 2, $P < 0.001$. 2. In the outcrossed population, the difference between the two media is significant, with $\chi^2 = 27.6$, d.f. = 2, $P < 0.001$. 3. With CYM, the difference between the inbred and outcrossed populations is not significant, with $\chi^2 = 5.6$, d.f. = 2, $P > 0.05$. 4. with CCE, the difference between the inbred and outcrossed populations is significant (2×3 contingency test), with $\chi^2 = 7.52$, d.f. = 2, $P < 0.05$.

population ($t = 5.561$, df = 12, $P < 0.001$) (Table 5). However, no significant correlation was found between heterozygosity and the other fitness traits in the inbred population (data not shown).

Fitness comparison between the outcrossed and backcrossed populations: Data from the eight fitness components for the two populations are analyzed and presented in Table 4. Three of eight components showed statistically significant advantages of the outcrossed population over the inbred population. These three components were percentage of heterokaryotized crosses showing positive mycelial interaction ($\chi^2 = 6.87$, df = 1, $P < 0.01$), heterokaryon growth rate ($t = 2.441$, df = 60, $P < 0.05$) and percentage of heterokaryons forming fruiting body primordia ($\chi^2 = 10.56$, df = 1, $P < 0.005$). The δ values ranged from -0.036 to 0.607 for the eight traits (Table 4). The formation of fruiting body primordia by heterokaryons was the most sensitive of the eight traits in detecting inbreeding depression ($\delta = 0.607$). The multiplicative δ value based on the eight fitness traits was 0.82 . When only the three fitness components that showed significant advantages of the outcrossed over the inbred population were used for calculating the multiplicative δ value, $\delta = 0.77$. Overall, the results indicate that there was a significant fitness reduction in the inbred population.

DISCUSSION

The comparison of an inbred with an outcrossed population of *A. bisporus* shows that significant inbreeding depression occurred. Three of eight fitness compo-

TABLE 2
Association of mycelial interaction and heterokaryotization on CCE medium

Heterokaryotization	Mycelial interaction		
	Positive	Ambiguous	Negative
Positive	44	14	4
Negative	4	12	46

Result: $\chi^2 = 66.77$, d.f. = 2, $P < 0.001$.

nents showed significant advantages for the outcrossed population. CCE medium permitted the expression of greater numbers of positive mycelial interaction in both the inbred and outcrossed successful matings than did CYM and exposed inbreeding depression with respect to mycelial interaction whereas CYM did not. The traits of mycelial interaction and heterokaryotization were significantly associated. The time to first break and the number of fruiting bodies produced were also significantly correlated. The inbreds that fruited showed a significantly higher level of heterozygosity than did the successful inbred matings that did not fruit.

Mycelial interaction has been used as an indicator of mating for many fungal species, including *A. bisporus* (KERRIGAN *et al.* 1994; P. CALLAC, personal communication). However, in *A. bisporus*, the association of mycelial interaction and heterokaryotization has never been critically examined. In this study, I found a significant association between these two traits. Specifically, a positive mycelial interaction between homokaryons is likely to reflect heterokaryotization, whereas a negative mycelial interaction is likely indicative of an unsuccessful or incompatible pairing (Table 1). However, caution is required in using mycelial interaction as the sole indicator

TABLE 3
Matrices of Pearson correlations and probabilities among heterokaryon growth rate and three fruiting fecundity traits from the inbred and outcrossed populations combined

PCI	HRGR	TTFB	NOFB	WPFB
HRGR	—	0.175	0.159	0.423
FBRK	-0.419	—	0.001*	0.792
NOFB	0.434	-0.878*	—	0.762
WPFB	0.256	-0.085	-0.098	—

Pearson correlation index (PCI) is shown in the lower left half of the table and probability of associations is shown in the upper right half of the table. HRGR, heterokaryon radial growth rate; TTFB, time to first break; NOFB, number of fruiting bodies per unit area; WPFB, average weight per fruiting body. * Significant association was found between the time to first break and number of fruiting bodies per unit area, $P < 0.001$.

TABLE 4

Comparison of individual fitness components in the outcrossed and inbred populations of *Agaricus bisporus*

Fitness components	Outcrossed		Inbred		d.f.	t	χ^2	δ
	Value	n	Value	n				
Percent heterokaryotized pairings	95	20	83	52	1	—	1.83	0.126
Percent heterokaryotized pairing showing positive mycelial interaction on CCE	95	19	63	43	1	—	6.87*	0.337
Heterokaryon radial growth rate (mm/wk) ^a	6.73 ± 1.11	19	5.94 ± 1.96	43	60	2.441*	—	0.117
Percent of heterokaryons to form primordia	84	19	33	43	1	—	10.56*	0.607
Percent of primordia to form fertile fruit bodies	87	16	79	14	1	—	0.70	0.092
Time to first break (days) ^a	25.7 ± 11.36	14	29.0 ± 53.55	11	23	1.166	—	0.114
No. of fruit bodies per square foot ^a	2.42 ± 1.467	14	1.40 ± 2.674	11	23	1.698	—	0.421
Average weight per mushroom (g) ^a	8.88 ± 1.642	14	9.20 ± 17.23	11	23	0.247	—	-0.036
Heterozygosity ^a	0.604 ± 0.027	19	0.275 ± 0.005	43	60	7.204*	—	0.545

^a Values are means ± SD.

* Statistically significant difference was observed.

of mating and therefore in using the data from mycelial interaction in population genetic analysis. As was found in this study, not every positive mycelial interaction indicates a successful mating and not every negative mycelial interaction indicates an unsuccessful or incompatible mating (Tables 1 and 2).

Mycelial interaction has been found to vary under different environmental conditions (XU *et al.* 1993b). In this study, two media were found to differently affect the expression of inbreeding depression (Table 2). On CYM, a nutritious artificial medium, most mycelial interactions were negative and no significant difference between the outcrossed and inbred populations was found. On the other hand, among matings that occurred on CCE, a less nutritious medium supplemented with water extracts of compost, there was a significantly higher percentage of positive mycelial interactions than on CYM, and significant inbreeding depression was observed. This result might indicate that some components from the natural substrate permit the expression of inbreeding depression. It is possible that a different substrate could produce a different result, but the result might not be significantly different from what we observed here. The earlier study on variation in mating interactions on 12 media indicated that the most obvi-

ous difference in the expression of mycelial interaction was to be found between CYM and CCE (XU *et al.* 1993b). The mechanism for the variation in mycelial interaction and expression of inbreeding depression under different environmental conditions is not clear. A method of conducting mating test in nature has not been established. This study is congruent in some ways with those of DUDASH (1990) and WOLFE (1993) in which natural environments tended to allow the expression of inbreeding depression.

The significant reduction in three traits in the inbred population suggests that these traits are probably indicators of the expression of deleterious recessive genes in this fungus (Table 4). Two of three traits, mycelial interaction and heterokaryon growth rate, could be used as indicators in mushroom breeding programs as they are relatively easy to estimate. The third trait, the formation by heterokaryons of mushroom primordia (part of a fruiting test), has been the major indicator selected for in breeding (FRITSCH 1983). The small sample size used in this study probably contributed to the low power to detect differences of some of the fitness components, especially the fruiting body fecundity correlates. More markers and much larger sample sizes in both the inbred and outcrossed populations are

TABLE 5

Statistical test of difference in heterozygosity between heterokaryotized backcrosses that fruited and those that did not fruit

	n	Minimum heterozygosity	Maximum heterozygosity	Mean ± SD heterozygosity
Fruited	13	0.255	0.435	0.334 ± 0.055
Did not fruit	30	0.105	0.385	0.252 ± 0.062

Results: $t = 5.561$, d.f. = 12, $P < 0.001$.

needed to infer the mechanism(s) of inbreeding depression and possible linkage relationships among the fitness traits and with the marker genes. Because of the discovery of a natural *A. bisporus* population that produces predominantly homokaryotic basidiospores (CALLAC *et al.* 1993; KERRIGAN *et al.* 1994; R. KERRIGAN, personal communication), the isolation of a large number of homokaryons may become feasible. Presently, the isolation of homokaryons from the secondarily homothallic population is still a very laborious and time-consuming task (CASTLE *et al.* 1988; JIN *et al.* 1992; KERRIGAN *et al.* 1992).

There are currently three predominant breeding methods in *A. bisporus*: isolating and crossing homokaryons, multiple spore culturing, and monospore culturing (FRITSCHÉ 1991a,b). Improvement in yield of monospore cultures over parental strains has been observed in *A. bisporus* (FRITSCHÉ 1972; WANG 1991). It could be inferred from these studies that some deleterious mutations affecting yield are dominant or partially dominant. Two pairings from the inbred population of this study also showed improvements in time to first break and yield over the parental heterokaryon Ag93b (data not shown). However, the overall results from my study suggest that the expression of recessive and/or partially recessive mutations is mostly responsible for the observed inbreeding depression in *A. bisporus*.

Pleurotus sajor-caju, the phoenix mushroom, which has a bifactorial heterothallic mating system, showed significant inbreeding depression in a comparison between the parental strain and 20 spore-derived heterokaryons (inbreeding coefficient 0.25). The two traits examined, the incubation period (similar to time to first break) and the yield, both revealed inbreeding depression and were negatively correlated (HANIFAH and GRAHAM 1986). The longer it took to fruit, the lower the yield. My result of a negative correlation between the time to first break and the number of fruiting bodies produced parallels their study. The reduced yield of heterokaryons obtained by sib-mating compared with their parental heterokaryons was also observed in *P. ostreatus* (EUGENIO and ANDERSON 1968).

Fitness reduction due to expression of recessive or rarely dominant mutations has also been described in some ascomycetes (NELSON 1959a–d; LESLIE and RAJU 1985). In *Cochliobolus heterostrophus*, a unifactorial heterothallic fungus with two mating type alleles, certain genes with either recessive or rarely dominant effects were found to influence mating capability (NELSON 1959a), perithecia formation (NELSON 1959d), ascus formation (NELSON 1959c) and ascospore formation (NELSON 1959b). Variation in mating capability and formation of perithecia have also been documented in another heterothallic species, *Cochliobolus carbonum*. The genetic basis of these variations is unknown (NELSON 1964, 1970).

In another ascomycete species, *Neurospora crassa*, al-

though no significant inbreeding depression has been quantified, genes or gene complexes affecting fitness have been observed both in nature and in laboratory populations (RAJU and PERKINS 1978; LESLIE and RAJU 1985). The authors speculated that these recessive mutations may be important in determining the breeding structure of natural *Neurospora* populations and in the evolution of its heterothallic reproductive systems (LESLIE and RAJU 1985). However, if inbreeding depression is primarily responsible for the evolution and maintenance of the heterothallic life cycle of *N. crassa*, more mating type loci and alleles (or idiomorphs), rather than only one locus with two alleles, should be found in this species given the high mutation load found in natural populations (LESLIE and RAJU 1985). In basidiomycetes, mixed mating systems are widespread and all heterothallic species examined so far have been found to have multiple mating types in natural populations (RAPER 1966).

The finding of significant inbreeding depression in *A. bisporus* is consistent with the hypothesis that secondary homothallism could harbor a significant number of deleterious recessive genes. However, the inbreeding depression found in this study does not mean that the phenomenon is common in nature and that the secondary homothallism of *A. bisporus* is unstable. First, although backcrossing is an efficient method of exposing recessive genes, it is unlikely to occur because of the predominance of heterokaryons in nature. Second, sib-mating among homokaryons is another form of inbreeding that would result on average an inbreeding coefficient of 0.25. From the present study, we can hypothesize that inbreeding depression might also be observed in a sib-mating population. However, because <10% of meiotic progenies are homokaryotic, <0.5% ($10\% \times 10\% \times 0.5$) of the sib-matings would be compatible homokaryon–homokaryon matings (XU *et al.* 1993a; XU *et al.* 1995). Furthermore, it has been found that homokaryotic basidiospores have lower growth rates than do heterokaryotic basidiospores (KERRIGAN *et al.* 1992, 1994). Finally, if the number of mating types in a breeding population is high, the proportion of sib-mating would also be reduced (RAPER 1966). Overall, we can hypothesize that the chances of backcrossing and sib-mating are probably very low and the genetic contribution through these two types of mating would probably be minimal in nature.

Recently, CALLAC and co-workers (1993) discovered a natural *A. bisporus* population in desert California with predominantly four-spored basidia. This variety of the button mushroom, *A. bisporus* var. *burnettii*, has a unifactorial heterothallic life cycle and presumably is predominantly outcrossing in nature (KERRIGAN *et al.* 1994). It would be interesting to compare the consequences of inbreeding in the two varieties of *A. bisporus*, var. *bisporus* and var. *burnettii*. If genetic load is determined primarily through the mutation-selection bal-

ance, theoretically, one would expect stronger inbreeding depression in predominantly outcrossing species or populations (LANDE and SCHEMSKE 1985). On the other hand, *A. bisporus* var. *burnettii* may not allow the accumulation of as many deleterious recessive genes as the secondarily homothallic populations (var. *bisporus*). The comparison of the consequences of inbreeding in these two varieties should also provide us insight into the evolution and maintenance of mating systems in *Agaricus*.

This study shows significant inbreeding depression presumably due to mutation load in this secondarily homothallic fungus. However, an alternative but not mutually exclusive hypothesis for this inbreeding depression is that adaptive genetic units in the genome are disrupted by generating homokaryotic offsprings through meiosis. Persistent association of nuclei in the same cytoplasm might cause the establishment or accumulation of such adaptive gene complexes (KERRIGAN *et al.* 1993b). Furthermore, distorted segregation has been observed in the meiotic production of homokaryons (LEGG 1994). It is possible that the secondarily homothallic life cycle could be an effective way of eliminating deleterious genes through the production of homokaryotic offspring if the homokaryotic offspring contribute little to future reproduction. Information on the relative contribution of genes through homokaryotic and heterokaryotic offsprings and the existence of coadaptive genetic units in natural populations is needed to test these hypotheses. We are currently studying these two aspects.

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