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Crosses among Homokaryons from Commercial and Wild-Collected Strains of the Mushroom *Agaricus brunnescens* (=*A. bisporus*)

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A new technique for the production of hybrid strains of the cultivated mushroom *Agaricus brunnescens* is described. Homokaryons were recovered from regenerated protoplasts obtained from several heterokaryotic strains. A total of 16 novel hybrids were produced in 63 attempted crosses between paired homokaryons. Recovery of both homokaryons and hybrids was verified by analysis of restriction fragment length polymorphisms. Three of four hybrids fruited in small-scale tests, further confirming that the isolates were true hybrids. Colony morphology alone was found to be a poor indicator of hybrid status. In two instances, three homokaryons crossed successfully in all combinations, suggesting that there are at least three alleles at the putative mating-type locus. Crosses between homokaryons from commercial and wild-collected isolates indicated that these strains belong to the same biological species.

The ability to cross genetically nonidentical individuals is prerequisite to any program for the genetic improvement of agriculturally important organisms. Although the mushroom *Agaricus brunnescens* Peck [=*A. bisporus* Lange (Imbach)] has been cultivated for about three centuries and is now an economically important crop, there are few reports of controlled crosses that have been verified with unambiguous genetic markers (16, 19, 23). This deficiency is due in part to the fact that the main events in the life cycle of this organism became known only relatively recently (9, 11, 13, 19). Most basidia of *A. brunnescens* are bisporic, with each basidiospore receiving two of the four meiotic nuclei. Mitotic divisions follow to produce usually between four and eight nuclei within the developing spore (9, 11). Most mycelia originating from single spores are competent to fruit, presumably because they are heterozygous at the putative mating-type locus (6, 17, 19). This secondary homothallism, in addition to the absence of a uniformly uninucleate, haploid propagule at any stage of the life cycle, has made crossing and genetic manipulation problematic. Limited genetic analysis and breeding of *A. brunnescens* was made possible by the observation that a minority of basidia (0 to 20%) are tri- or tetrasporic, the frequency of which is strain specific and is at least partially dependent on environmental conditions (7, 12, 18). Spores from tetrasporic basidia receive only one meiotic nucleus and thus give rise to self-sterile homokaryons. These strains can be crossed to an isolate carrying another mating-type allele (6, 19). The recovery of self-sterile, presumably homokaryotic, strains by micromanipulation of spores from tetrasporic basidia (6) or by screening random single-spore isolates (10, 23), however, is time-consuming and difficult. Recently, another method for obtaining homokaryons was reported (1, 3). When protoplasts are produced from a heterokaryon and allowed to regenerate mycelia, approximately 1 in 10 is homokaryotic.

Previous analyses of crosses and meiotic segregations depended on the availability of auxotrophic or polymorphic isoenzyme loci as genetic markers (19, 23). Only a few auxotrophs have been recovered, and this has been done only with considerable effort. After intensive investigations, fewer than 10 isoenzymes have been identified as good genetic characters in *A. brunnescens* (20, 23). Recently, the suitability of restriction fragment length polymorphisms (RFLPs) as genetic markers in *A. brunnescens* was examined (3). Several genotypic classes were identified among commercial and wild-collected strains of *A. brunnescens*, and homokaryons among protoplast regenerates were distinguished from heterokaryons by RFLPs.

In this study we examined crosses among homokaryons obtained from several different heterokaryons by protoplast formation and regeneration. We used RFLPs (i) to identify homokaryons recovered from both wild-collected and commercial strains of *A. brunnescens* and (ii) to verify crosses among homokaryons. Two independent criteria, in addition to RFLPs, were used to establish the recovery of heterokaryons from the crosses: colony morphology and fruiting ability.

MATERIALS AND METHODS

Strains. Ten heterokaryotic strains of *A. brunnescens* were used in this study. Ag2 (ATCC 24558), Ag6 (Swayne 21), Ag33 (Amycel 220), Ag37 (Amycel U3), and Ag52 (Lambert 81) were commercial strains (3). Ag83 (RWK704), Ag84 (RWK1312), and Ag85 (RWK1291) were wild-collected strains obtained from R. Kerrigan, University of California, Santa Barbara (3). Ag89 was a wild-collected strain obtained from D. Malloch (15a). Ag90 was a carboxin-resistant derivative of the commercial strain C54 obtained from T. Elliott, Institute of Horticultural Research, Littlehampton, United Kingdom (4). Ag1-1 (ATCC 24662) and Ag1-2 (ATCC 24663) were homokaryons from the study of Raper et al. (19).

DNA analysis. DNAs were isolated from 20 to 50 mg of freeze-dried mycelium by the method of Zolan and Pukkila (24). Plasmids carrying cloned nuclear DNA segments from *A. brunnescens* or *A. bitorquis* were from the study of Castle et al. (3). The assay of restriction fragments by Southern hybridization was done as described by Castle et al. (3).

Homokaryon isolation. Protoplasts were obtained from heterokaryotic strains of *A. brunnescens*, and cultures from

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TABLE 1. Recovery of homokaryons among protoplast regenerates of *A. brunnescens*

Parent strain	No. of strains tested for RFLPs	No. of homokaryons	No. of RFLP genotypes
Ag2	13	3	2
Ag6	10	0	0
Ag33	2	0	0
Ag37	6	1	1
Ag52	41	0	0
Ag83	22	3	2
Ag84	11	1	1
Ag85	14	0	0
Ag89	12	4	1
Ag90	12	4	1

regenerated protoplasts were tested for homokaryosis by isolating DNA and assaying *Eco*RI fragments that hybridized to cloned probes as described previously (3).

Crosses. Pairings among homokaryotic strains were performed by placing 1-mm cubes of inoculum 1 cm apart in petri dishes containing complete yeast medium (CYM) (19). The plates were incubated for 2 to 4 weeks at room temperature (22 ± 2°C), until the two homokaryotic colonies grew together along a 1- to 3-cm front. Cubes of inoculum (1 mm^3) were removed from the confluent zone, as well as from outside of the confluent zone on each side of the paired cultures. The explants were incubated on CYM, and the morphologies of the colonies originating from the confluent zone were compared with those obtained from the area outside of the junction line or from unmated homokaryotic cultures of similar age. For each pairing, a second transfer was made from a colony originating from the confluent zone of the pairing. This inoculum was taken from sectors that were obviously more vigorous or "strandy" than the unmated controls whenever possible. When no morphological interaction was apparent, an inoculum was taken at random from the colony. One subculture was saved from each replicate mating for DNA isolation, assay of specific *Eco*RI fragments, and fruiting trials.

Fruiting trials. Fruiting trials were done by the cased-grain method of San Antonio (21), which was modified as follows. Plugs of agar and mycelium were inoculated into 200 g of autoclaved rye grain (ca. 50% moisture content) with 1% (dry weight) calcium carbonate in 500-ml flasks with morton closures. The cultures were incubated at room temperature and were shaken vigorously at weekly intervals to distribute the inoculum. When the rye substrate was fully colonized (12 to 24 days), about 100 g was placed in a 14-oz. (414-ml) clear plastic cup with several holes (diameter, 3 mm) in the bottom for drainage. About 100 g of nonsterile calcined earth (Turface; International Minerals and Chemical Corporation, Mundelein, Ill.) with 5% calcium carbonate was used to cover, or "case," the colonized grain. Initially, the casing layer was moistened with 80 ml of distilled H_2O. The cups were covered with aluminum foil and incubated at room temperature until the mycelium had colonized about 80% of the casing layer. The cultures were then incubated at 18°C with 90% humidity. The cultures were watered daily with an atomizer until primordia and fruiting bodies appeared.

RESULTS

Homokaryon isolation. Homokaryons were identified among protoplast regenerates by examining DNAs from selected colonies for RFLP patterns. In most intances, regenerates with a range of growth rates or colony morphologies were included in the samples assayed for RFLPs. Colonies which showed morphologies different from that of the parental heterokaryon, as well as those with similar morphologies, were selected. From a total of 143 regenerates assayed, 16 homokaryons were recovered (Table 1). Among the 16 isolates there were eight different genotypes, as determined by RFLPs. Southern hybridizations with plasmid clones revealed that DNAs from the presumptive homokaryons had fewer restriction fragments hybridizing to each probe than did the parental heterokaryons (Table 2). Each homokaryon within a genotypic class was morphologically similar to other isolates in that class and different from isolates in any other class. No homokaryons were recovered from four strains: Ag6, Ag33, Ag52, and Ag85.

Crosses. From one to three replicate crosses were conducted for each combination of homokaryons. Two homokaryons, Ag1-1 and Ag1-2, isolated previously (19) were included in this study. Sixty-three different paired combinations of homokaryons were examined. When DNA of a subculture from a pairing had the combination of all *Eco*RI fragments of the component homokaryotic strains, the cross was considered successful (Fig. 1). Sixteen crosses were compatible based on this criterion (Table 3).

One cross, Ag2-20 × Ag2-23, resulted in the resynthesis of the heterokaryon from which these two homokaryons were isolated by protoplast production. In two instances, three homokaryons crossed in all three pairwise combinations, a result which would require more than two mating-type alleles in a unifactorial, sexual incompatibility system. Ag1-1, Ag89-65, and Ag90-30 constituted one such group, while Ag2-20, Ag89-59, and Ag2-23 constituted another.

When a probe distinguishing different *Eco*RI fragment patterns in the paired homokaryons registered a failure to cross, all informative probes applied to that replicate indicated the presence of the same homokaryon. For example, in the pairing of Ag1-1 with Ag37-4, only the RFLP patterns

TABLE 2. Restriction fragments in DNAs from homokaryotic strains of *A. brunnescens*

Strain	Fragment size (kilobase pairs) of the following probes:			
	p33n10	p4n6	p4n27	p33n25
Ag1-1[a]	6.3/1.4	5.8	3.3	2.4
Ag1-2[a]	6.3/1.4	8.1	3.3	1.4
Ag2	6.7/6.3/1.4	8.1/5.8	3.3/2.2	2.4
Ag2-20	6.3/1.4	8.1	2.2	2.4
Ag2-22	6.3/1.4	8.1	2.2	2.4
Ag2-23	6.7/1.4	5.8	3.3	2.4
Ag37	6.3/1.7/1.4	8.1/6.9	3.5/2.2	1.4/1.0
Ag37-4[b]	6.3/1.4	8.1	3.5	1.0
Ag83	6.7/1.7/1.4	8.1/6.9	3.3	1.4/1.0
Ag83-15	6.7/1.4	8.1	3.3	NT[c]
Ag83-22	6.7/1.4	8.1	3.3	1.0
Ag83-28[b]	6.7/1.7	6.9	3.3	1.4
Ag84	6.7/6.3/1.7	10.8/8.1	3.3	1.4
Ag84-7[b]	6.3/1.7	10.8	3.3	1.4
Ag89	6.7/1.7/1.4	8.1/6.9	NT	NT
Ag89-59	6.7/1.4	6.9	NT	NT
Ag89-65	6.7/1.4	6.9	NT	NT
Ag90	6.7/6.3/1.7/1.4	NT	NT	NT
Ag90-30	6.7/1.4	8.1	NT	NT

[a] Isolates Ag1-1 and Ag1-2 were from Raper et al. (19). Single-number isolates are parental heterokaryons. All other isolates are homokaryons.
[b] Isolates were from Castle et al. (3).
[c] NT, Not tested.

TABLE 3. Crosses among homokaryons of *A. brunnescens*

Cross	RFLP pattern for the following plasmid probes[a]:				Morphological interaction[b]	Fruiting competence
	p33n10	p4n6	p4n27	p3n25		
Ag1-1 × Ag1-2	?	−b	?	−b	−	
Ag1-1 × Ag2-20	?	−a	−a	?	−	
	?	±	−a	?	−	
	?	−a			−	
Ag1-1 × Ag2-22	?	−a	−a	?	−	
Ag1-1 × Ag2-23	−a	?	?	?	−	−
	−b	?			+	
Ag1-1 × Ag37-4	?	−a	−a	−a	−	
	?	−b			−	
Ag1-1 × Ag83-15	−a	−a	?		−	
	−a	−a			−	
Ag1-1 × Ag83-22	−a	−a	?	−a	−	
Ag1-1 × Ag83-28	−a	−a	?	−a	−	
	−a	−a			−	
Ag1-1 × Ag84-7	−a	−a	?	−a	−	
	−b	−b				
Ag1-1 × Ag89-59[c]	+	+			+	−
Ag1-1 × 89-65[c]	+	+			+	+
Ag1-1 × Ag90-30[c]	+	+			+	
Ag1-2 × Ag2-20	?	?	−b	−b	−	
Ag1-2 × Ag2-22	?	?	−a	−a	−	
Ag1-2 × Ag2-23	−a	−a	?	−a	−	
Ag1-2 × Ag37-4	?	?	−a	−a	−	
	?	?	−a	−a	−	
Ag1-2 × Ag83-15	−a	?	?		+	
Ag1-2 × Ag83-22	−a	?	?	−a	+	
Ag1-2 × Ag83-28	±	±	?	?	+	
Ag2-20 × Ag2-22	?	?	?	?	−	
Ag2-20 × Ag2-23[c]	+	+	+	?	−	
	−a	−a			−	
Ag2-20 × Ag37-4	?	?	−a	−a	−	
	?	?			−	
Ag2-20 × Ag83-15		?			−	
	−a	?			−	
Ag2-20 × Ag83-22	−a	?	−a	−a	−	
Ag2-20 × Ag83-28	−b	±	−b	−b	−	
	−a	−a			−	
Ag2-20 × Ag84-7[c]	+	+	+	+	+	
	−a	−a			−	
Ag2-20 × Ag89-59[c]	+	+			+	
Ag2-20 × Ag89-65[c]	+	+			−	
Ag2-20 × Ag90-30	−a	?			−	
Ag2-22 × Ag2-23	−a	−a	−a	?	+	
Ag2-22 × Ag37-4	?	?	−a	−a	+	
Ag2-22 × Ag83-15	−a	?	−a		−	
Ag2-22 × Ag83-22	−a	?	−a	−a	−	
Ag2-22 × Ag83-28	−b	−b	−b	−b	−	
Ag2-22 × Ag84-7	−a	−a	−a	−a	−	
Ag2-23 × Ag37-4	−a	−a	−a	−a	+	
	−b	−b			−	
Ag2-23 × Ag83-15	?	±	?		+	
Ag2-23 × Ag83-22	?	−b	?			
Ag2-23 × Ag83-28	−a	−a			−	
Ag2-23 × Ag84-7	−b	−b	?		+	
	−b	−b			+	
Ag2-23 × Ag89-59[c]	?	+			+	
Ag2-23 × Ag89-65	?	−a			−	
Ag37-4 × Ag83-15		?			−	
	−a	?			−	
Ag37-4 × Ag83-22	−a	?	−a		−	
Ag37-4 × Ag83-28		±			−	
	−a	−a			−	
Ag37-4 × Ag84-7[c]	+	+	+	+	+	
	+	+			−	
Ag37-4 × Ag89-59	−a	−a			−	

Continued on following column

TABLE 3—*Continued*

Cross	RFLP pattern for the following plasmid probes[a]:				Morphological interaction[b]	Fruiting competence
	p33n10	p4n6	p4n27	p3n25		
Ag37-4 × Ag89-65[c]	+	+			−	
Ag83-15 × Ag83-28	−b	+	?		−	
Ag83-15 × Ag84-7	−b		?		+	
Ag83-15 × Ag89-59[c]	?	+			+	+
Ag83-15 × Ag89-65[c]	?	+			+	
Ag83-15 × Ag90-30	?	?			−	
Ag83-22 × Ag83-28	−b	±	?		−	
Ag83-22 × Ag84-7	−b	−b	?	−b	−	
Ag83-28 × Ag84-7	−a	−a	?	?		
	−b	−b			−	
Ag83-28 × Ag89-65	−a	?			−	
Ag83-28 × Ag90-30[c]	+	+			−	
Ag84-7 × Ag89-59[c]	+	+			+	+
Ag84-7 × Ag89-65	−a	−a			−	
Ag84-7 × Ag90-30[c]	+	+			−	
Ag89-59 × Ag90-30	?	−b			−	
Ag89-65 × Ag90-30[c]	?	+			+	

[a] Each replicate cross appears on a separate line. Abbreviations: ?, probe not informative, paired strains had identical restriction fragments that hybridized to that probe; +, probe informative, hybrid band pattern, 1:1 stoichiometry; −a, probe informative, no hybrid, restriction fragment(s) of first partner present; −b, probe informative, no hybrid, restriction fragment(s) of second partner present; ±, hybrid band pattern, stoichiometry markedly different from 1:1.

[b] Morphological interaction indicates the appearance of the pairing compared with that of the unpaired homokaryons. Often the unpaired homokaryons were slow growing, whereas a successful cross was fast growing and strandy.

[c] Denotes vegetatively stable hybrids confirmed on the basis of RFLPs.

of Ag1-1 were observed in DNA from the explant that was examined with three informative probes (Table 3). In six pairings, e.g. Ag1-2 with Ag83-28, probe pAg4n6 indicated a hybrid banding pattern, but the stoichiometry was very different from 1:1. These pairings were registered as unsuccessful. These cultures probably represented a mixture of two homokaryotic colonies rather than a heterokaryon. In only one pairing, Ag83-15 with Ag83-28, did a probe, pAg4n6, indicate a successful cross, while other informative probes indicated that the cross was unsuccessful.

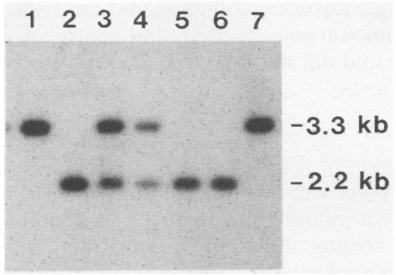

FIG. 1. *Eco*RI-digested DNAs from subcultures of crosses probed with ^{32}P-labeled pAg4n27 in Southern hybridizations. Lane 1, DNA from Ag2-20 × Ag83-28; lane 2, Ag2-20 × Ag2-22; lane 3, Ag2-20 × Ag84-7; lane 4, Ag2-20 × Ag2-23; lane 5, Ag2-20 × Ag83-22; lane 6, Ag2-22 × Ag83-15; lane 7, Ag83-22 × Ag84-7; lanes 3 and 4, successful crosses showing restriction fragments of both paired homokaryons; lanes 1, 5, and 6, unsuccessful crosses showing restriction fragments of one or the other paired homokaryon; lanes 2 and 7, crosses in which paired homokaryons had identical fragments. kb, Kilobase pairs.

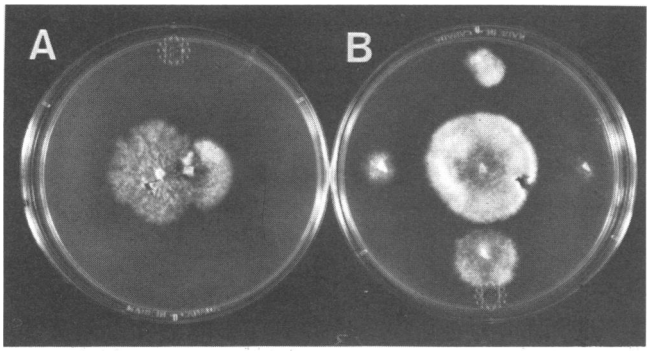

FIG. 2. Morphological interaction in a pairing of homokaryons. (A) Pairing of homokaryons Ag89-65 (left) and Ag90-30 (right). (B) Subcultures from pairings: left, one subculture from the Ag89-65 side of the pairing; middle, three subcultures from the confluent zone of the pairing; and right, one subculture from the Ag90-30 side of the pairing. Note that at least two of the subcultures from the confluent zone grew more vigorously than the subcultures taken from outside the confluent zone.

In terms of colony morphology, many pairings yielded subcultures which were considerably more vigorous than the component homokaryons (Fig. 2), whereas other pairings produced no morphologically distinct subcultures (Table 3). Of 16 compatible combinations, 11 showed evidence of morphological interaction. The subcultures from the junction line were either faster growing or more strandy than the unmated component homokaryons. In the combination Ag37-4 × Ag84-7, one replicate pairing yielded subcultures which were morphologically distinct from either homokaryon, while another replicate yielded no morphologically distinct subcultures. Both, however, were subsequently deemed successful based on RFLP patterns. Of 47 unsuccessful combinations, 37 showed no evidence of any morphological reactions. The remaining 10 combinations yielded subcultures that were morphologically distinct from either of their respective unmated homokaryons, while the RFLP pattern of only one of the component homokaryons was maintained.

Fruiting trials. Subcultures from five crosses, four successful and one unsuccessful, as determined by RFLP patterns, were tested for the ability to fruit. Of the successful crosses, three cultures fruited and one did not. The culture from the single unsuccessful cross did not fruit. None of the unmated homokaryons fruited, but many of these strains grew slowly and did not colonize the grain substrate used in the fruiting tests.

DISCUSSION

Homokaryons were found among protoplast regenerates in this study at about the same rate as reported previously (3). Several strains, Ag6, Ag33, Ag85, and Ag52, however, yielded no homokaryons. The samples of protoplast regenerates from these heterokaryons may not have included homokaryons by chance alone. Alternatively, these heterokaryons, especially Ag52, for which 41 protoplast regenerates were assayed, may harbor recessive alleles that are extremely deleterious to growth or viability in vegetative culture. Any homokaryon carrying such alleles would have been less likely to be selected in the sample assayed for RFLPs. There is evidence to suggest that recessive alleles with a negative effect on growth are common in cultivated and wild-collected heterokaryons of *A. brunnescens*. Most homokaryons grow considerably more slowly in culture than do their respective parent heterokaryons. Recessive alleles carried by heterokaryons such as Ag52 may be relatively more deleterious to growth or viability than those carried by heterokaryons from which homokaryons have been successfully recovered.

Several hybrid heterokaryons were recovered from this series of pairings, as indicated by RFLPs. These isolates appeared to be true hybrids rather than mixtures of homokaryons for several reasons. First, the putative hybrids were vegetatively stable. They were subcultured repeatedly with no apparent loss of vigor or changes in colony morphology. Second, the stoichiometry of the polymorphic restriction fragments observed in Southern hybridizations was 1:1, even after subculture, as is expected for a stably heterokaryotic, hybrid strain. Several commercial and wild-collected heterokaryotic strains also show a 1:1 stoichiometry in polymorphic DNA fragments (3). Third, three of four putative hybrids fruited, while one unsuccessful cross and the unmated homokaryons failed to fruit. Since the development of fruiting bodies is a complex phenomenon that is dependent on environmental and genetic factors and since homokaryotic fruiting is possible in *Homobasidiomycetes* (14), fruiting competency alone is not a sufficient criterion for determining sexual compatibility. We agree with Raper et al. (19) that the recovery of a genetically stable heterokaryon, verified whenever possible by fruiting competency, is the best criterion for determining sexual compatibility in *A. brunnescens*.

In most crosses, all probes capable of distinguishing *Eco*RI fragment polymorphisms in the paired homokaryons were consistent in indicating either a successful or an unsuccessful cross and, if unsuccessful, in indicating which homokaryotic type was present (Table 3). Two exceptions were in the crosses Ag1-1 × Ag2-20 and Ag2-20 × Ag83-28, in which pAg4n6 indicated a hybrid band pattern in which the stoichiometry of the RFLP components was markedly different from 1:1, while at least one other informative probe registered the cross as unsuccessful. This result might have been due to an unequal mixture of unmated, homokaryotic mycelia in the subcultures and a greater sensitivity in the hybridization assay with pAg4n6. Variation in any of several parameters, such as concentration and specific activity of probe DNA, can influence the sensitivity of a given hybridization assay. If this were the case, then pAg4n6 detected the lesser of the two RFLP patterns, while the other probe failed to do so. In the cross Ag83-15 × Ag83-28, however, pAg4n6 indicated a successful cross in which the intensity of the both bands was 1:1, while the other probe indicated an unsuccessful cross. We have no explanation for this discrepancy, except that it appears not to be an artifact of the hybridization assays and could, in principle, be due to somatic nuclear fusion, followed by any of several possible events that are known to cause mitotic segregation in fungi.

The importance of using unambiguous genetic criteria to establish the recovery of hybrid strains of *A. brunnescens* is emphasized by the less than complete correlation between hybrid formation and morphological interaction in vegetative cultures. Fully one half of the subcultures which showed morphologies different from that of either homokaryotic parent were not hybrids. It is possible that these pairings were sexually compatible, but heterokaryons were not included in subcultures from the respective pairings. Nuclear migration is limited or absent in *A. brunnescens*, and heterokaryons are expected to form only near the junction line of a

pairing (19). Inefficient recovery of heterokaryons, therefore, would not be surprising. Conversely, several hybrids determined by RFLP analysis were morphologically identical to one of the component homokaryons. The lack of a morphological interaction in true hybrids can be explained readily if one homokaryon carries morphological determinants which are dominant to the determinants of the homokaryotic partner. This explanation was supported by the observation that the hybrid from the cross between Ag2-20 and Ag89-65 was morphologically identical to Ag2-20, but was contradicted by the observation that the hybrid from the pairing of Ag2-20 with Ag89-59 was morphologically different from either homokaryon. On the basis of RFLP patterns (Table 2) and morphology, Ag89-59 and Ag89-65 were identical and should have given the same results when crossed with Ag2-20. A reasonable hypothesis is that these two hybrids, which have identical nuclear genotypes, carry different mitochondrial genotypes; this can be tested since mitochondrial RFLPs distinguishing Ag2-20 from Ag89-59 or Ag89-65 have recently been noted (Malloch et al., in press). Until there is more information on the determinants of colony morphology, however, this character is not a trustworthy indicator of heterokaryosis or homokaryosis (5).

In two instances three homokaryons were sexually compatible in all pairwise combinations. It was described previously (19) that mating in *A. brunnescens* is controlled by a single locus, and only two alleles were identified. The recovery of hybrids from all pairwise combinations of three homokaryons indicates that there are at least three alleles at the putative mating-type locus of this organism. There was insufficient information from these crosses to assign specific mating types to the homokaryons based on earlier mating-type assignments (6, 19). In fact, the existence of a mating-type locus itself in *A. brunnescens* has not been proven conclusively. Previous results (17, 19), as well as those presented here, might be explained by the complementation of recessive alleles which are deleterious to growth or fruiting rather than by the controlling effect of a mating-type locus (18).

Several of the hybrids provided evidence that has a bearing on the recent nomenclatural controversy over the cultivated mushroom. According to Singer (22), the correct name of the commercial mushroom is *A. bisporus*, while *A. brunnescens* is a closely related wild species. Others (15a) contend that there is only one bisporic *Agaricus* species: *A. brunnescens*. In this study homokaryons from commercial strains were sexually compatible with homokaryons from wild, bisporic collections identified by experts as *A. bisporus* (Ag83 and Ag84) and as *A. brunnescens* (Ag89). For example, homokaryons Ag89-59 and Ag89-65 crossed with several of the other homokaryons. In one instance, Ag1-1 × Ag89-65 (Table 3), the cross was confirmed by both RFLP analysis and fruiting body production. All of these homokaryons, therefore, belong to the same biological species, whatever the correct name may prove to be.

Our results indicate that RFLPs are suitable markers both for the confirmation of hybrid recovery in crosses and for distinguishing homokaryons from heterokaryons (3). In addition to existing auxotrophic (19) and isoenzyme loci (16, 20), RFLPs should provide another, almost endless source of genetic markers. One restriction enzyme which recognizes a 6-base-pair sequence should produce approximately 8,400 fragments of *A. brunnescens* DNA (genome size, 3.4×10^7 base pairs [2]). Based on a small sample (3), most of these fragments should reveal RFLPs when they are cloned. Coupled with the fact that over 100 different restriction enzymes are available commercially, the potential supply of genetic markers is virtually limitless.

By use of the techniques presented here, we have outlined a relatively straightforward method for producing new hybrid strains of the commercial mushroom for either proprietary purposes or for basic studies on the genetics of this organism. The isolation of homokaryons from protoplasts obtained from vegetative heterokaryons is faster and more direct than the isolation of homokaryons from meiotic material (6, 8). Homokaryons obtained from protoplasts, like homokaryons from single spores, can be crossed to yield novel hybrids. Hybrid status can be genetically verified with RFLPs, isoenzymes, or any other well-defined marker. After fruiting, single-spore isolates can be analyzed further for desirable commercial traits such as color, growth and fruiting temperature optima, and disease resistance. In addition, the segregation of genetic markers in well-defined crosses (23) should lead to clarification of the events of meiosis in this organism.

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