APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1992, p. 3553–3560 Vol. 58, No. 11
0099-2240/92/113553-08$02.00/0

Investigation of Mitochondrial Transmission in Selected Matings between Homokaryons from Commercial and Wild-Collected Isolates of *Agaricus bisporus* (= *Agaricus brunnescens*)

TIANRU JIN,[1] ANTON S. M. SONNENBERG,[2] L. J. L. D. VAN GRIENSVEN,[2] AND PAUL A. HORGEN[1]*

Centre for Plant Biotechnology, Department of Botany, University of Toronto, Erindale Campus, Mississauga, Ontario, Canada L5L 1C6,[1] and Mushroom Experimental Station, Horst, The Netherlands[2]

Received 23 June 1992/Accepted 18 August 1992

Ten heterokaryons of *Agaricus bisporus* (= *Agaricus brunnescens*) were shown to carry four different mitochondrial (mt) genotypes by analysis of mt restriction fragment length polymorphisms (RFLPs). Fifteen homokaryons derived from these strains were used to investigate mt inheritance in *A. bisporus*. One hundred eighty-nine pairings were performed in 25 different combinations. Pairings in 15 different combinations produced heterokaryons on the basis of nuclear RFLP analyses and/or fruiting trials. The mt genotype of each new intraspecies hybrid was examined by using mt RFLPs as genetic markers. Our results suggest the following. (i) Recombination between the mt genomes was not a common event. (ii) From most individual pairings, all heterokaryons carried the same mt genotype. (iii) Heterokaryons carrying either of the two possible mt genotypes were observed in certain crosses after modification of the pairing procedure. A biparental transmission pattern was demonstrated for some crosses, but there appears to be a preference for one of the mt genotypes to predominate in any specific pairing.

Although the economically important edible fungus *Agaricus bisporus* Lange (Imbach) (= *Agaricus brunnescens* Peck) has been cultivated for three centuries, genetic improvement has been extremely difficult to achieve. This has been mainly due to the unusual secondarily homothallic life cycle (9, 27). Most basidia of *A. bisporus* are bisporic, with each basidiospore receiving two of the four meiotic nuclei. The mycelium originating from these spores is self-fertile, heterokaryotic, and able to fruit, carrying the life cycle into the next generation. Although a minority of basidia (0 to 20%) are tri- or tetrasporic (8, 20), the recovery of homokaryons through micromanipulation of spores from these basidia is extremely time consuming and difficult. Therefore, an easily recoverable, uninucleate, haploid propagule, which is a prerequisite for controlled breeding, is absent in the *A. bisporus* life cycle (15, 16). In many other basidiomycetes, the presence of clamp connections may indicate sexually compatible mating interactions. Clamp connections are absent in *A. bisporus*, making the verification of heterokaryon formation problematic (15, 16).

A simple, unambiguous breeding approach, which uses protoplast formation and regeneration for the isolation of self-sterile homokaryons from self-fertile heterokaryons, has been developed (5, 6). Nuclear restriction fragment length polymorphisms (RFLPs) have been successfully used as the genetic markers to identify the two unique nuclear types in a heterokaryon (6). The somatically produced homokaryons isolated in this way can be used in matings on petri plates or in compost. The formation of new heterokaryons can be identified with nuclear RFLPs (6, 15, 16). In the present study, we have utilized homokaryons from protoplast regenerates and from single-spore isolates to examine the mitochondrial (mt) inheritance in the commercially important button mushroom.

mt inheritance has been investigated in a number of basidiomycetes. Biparental transmission of mt genomes has been demonstrated in laboratory matings for *Coprinus cinereus* (2, 23) and in *Armillaria bulbosa* (30). Biparental mt inheritance was also observed in *Agaricus bitorquis*, the field mushroom with a heterothallic life cycle (11). However, the transmission was generally biased in favor of one or the other mt genomes (12).

The major objective of this study was to investigate the mt transmission pattern of *A. bisporus* in laboratory matings. Previously, it was established that a number of mt genetic polymorphisms existed in wild populations of *A. bisporus*, whereas fewer polymorphisms were observed in commercial strains (12–14, 18). In this study, we have utilized 15 homokaryons from commercial and wild-collected strains of *A. bisporus*, which carry four different mt genotypes for the mating experiments. Data from the Mushroom Research Group in the Centre for Plant Biotechnology at the University of Toronto, Ontario, Canada, were pooled with data from the Dutch Mushroom Experimental Station at Horst, The Netherlands. Our results suggest that mt inheritance can be biparental but with a bias in many crosses showing the inheritance of only one of the two possible mt genotypes.

MATERIALS AND METHODS

Materials. Unless specified, all the chemicals were of reagent grade. The nutrient components of the media were purchased from Difco Laboratories (Detroit, Mich.). Restriction endonucleases were the products of Bio/Can (Mississauga, Ontario, Canada) or Life Technologies, Bethesda Research Laboratories (Bethesda, Md.).

Mushroom strains. The homokaryotic and heterokaryotic

* Corresponding author.

TABLE 1. Canadian *A. bisporus* strains used in this study and verification of their nuclear and mt status

Mushroom strain	mt genotype[a]	Nuclear status of plasmid probe[b]			Fruiting body production	Source or reference
		p33n10	p4n6	p33n6		
Ag2 (heterokaryon, white commercial)	II	6.7, 6.3, 1.4	8.1, 5.8	6.3	Yes	ATCC 24558
Ag1-1 (homokaryon)[c]	II	6.3, 1.4	5.8	6.3	No	ATCC 24662
Ag2-20 (homokaryon of Ag2)	II	6.3, 1.4	8.1	6.3	No	6
Ag2-23 (homokaryon of Ag2)	II	6.7, 1.4	5.8	6.3	No	6
Ag89 (heterokaryon, wild collected)	I	6.7, 1.7/1.4	8.1, 6.9	6.3	Yes	21
Ag89-59 (homokaryon of Ag89)	I	6.7, 1.4	6.9	6.3	No	6
Ag89-65 (homokaryon of Ag89)	I	6.7, 1.4	6.9	6.3	No	6
Ag90-30 (homokaryon of Ag90)	I	6.7, 1.4	8.1	6.3	Yes	6
Ag85 (heterokaryon, wild collected)	IV	6.3, 1.7	6.9	8.4, 6.3	No	19
Ag85-51 (homokaryon of Ag85)	IV	6.3, 1.7	6.9	6.3	No	This study
Ag50 (heterokaryon U3 derived)	I	6.3, 1.7, 1.4	8.1, 6.9	6.3	Yes	This study
Ag50HA (homokaryon of Ag50)	I	6.3, 1.4	8.1	6.3	No	This study
Ag50HB (homokaryon of Ag50)	I	6.3, 1.7	6.9	6.3	No	This study

[a] The homokaryons derived from any given heterokaryon carry the mt genotype of the heterokaryon.
[b] Values refer to the sizes in kilobases of *Eco*RI fragments hybridized to probes described in Castle et al. (5).
[c] Ag1-1 is a homokaryotic single-spore isolate of Ag2.

strains of *A. bisporus* used in this study are listed in Tables 1 and 2.

(i) Canadian strains. All stock cultures are maintained under liquid N_2. The working cultures were grown on complete yeast medium (CYM) agar at 23°C (33). The nuclear DNA RFLPs of these strains, illustrated by three nucleus-specific probes, are listed in Table 1.

Dutch strains. Cultures (Table 2) were maintained on MPP agar media (1% malt extract, 0.5% mycological peptone, 10 mM 3-[*N*-morpholino]propanesulfonic acid [pH 7.0], 1.5% agar).

Plasmids. (i) Canadian. Plasmids p33n10, p33n6, p4n6, and p33n25 (5, 6) were used individually as the probes to investigate the nuclear status of the culture. Plasmids p50m1, p50m4b, p50m6, and p50m7, generated by Hintz et al. (12), were mixed at equal concentrations and used as the mt probe to investigate the mt RFLPs of the isolates.

(ii) Dutch. For verification of nuclear types, the plasmids p33n10 and p33n25, first described by Castle et al. (5, 6), were used. For determination of mt genotype, mtDNA was isolated from CsCl-bisbenzimide gradients (12) and digested with *Cfo*I. The identification of mt genotype after mating was done by probing total DNA with p50m2 (12).

Matings. (i) Canadian. Pairings between the homokaryotic cultures were performed in dried and preincubated petri dishes containing CYM agar as described by Castle et al. (6).

(ii) Dutch. Matings were done on MPP agar or in compost at 24°C. Before the compost was inoculated with *A. bisporus*, it was first sterilized and then inoculated with the thermophilic fungus *Scytalidium thermophyllum*. After 3 days of incubation at 45°C, *S. thermophyllum* was inactivated by heating to 100°C (34). This pretreatment stimulates the colonization of the compost by *A. bisporus*.

Protoplast formation and regeneration. The techniques used for protoplast formation and regeneration were those reported previously by Castle et al. (5), with the modification described by Royer et al. (28) or by Sonnenberg et al. (32).

Homokaryon isolation. Protoplasts were obtained from heterokaryotic cultures of *A. bisporus*, and the regenerated protoplasts were examined to establish whether a regenerate possessed only one or both nuclear types (6). RFLPs were used as the genetic markers to verify homokaryon or heterokaryon status (6).

DNA isolation. Total DNAs were isolated from 14- to 21-day-old mycelium. The mycelium was harvested onto cheesecloth, washed with distilled water, frozen in liquid nitrogen, and freeze-dried, or it was lifted on cellophane sheets from solid-agar plates, removed from the cellophane,

TABLE 2. Dutch *A. bisporus* strains used in this study and verification of their nuclear and mt status

Mushroom strain	mt genotype[a]	Nuclear status of plasmid probe[b]		Fruiting body production	Source
		p33n10	p33n25		
Somycel 53 (S53; heterokaryon)	II			Yes	Somycel
53-59 (homokaryon S53)	II	3.3,3.2	0.8	No	MES[c]
Somycel 9.2 (S9.2; heterokaryon)	I			Yes	Somycel
9.2-97 (homokaryon S9.2)	I	3.7,3.2	0.7	No	MES
Sinden A4 (A4; heterokaryon)	I			Yes	Penn State Collection
A4-337 (homokaryon A4)	I	3.7,3.2	0.7	No	MES
Horst-U3 (U3; heterokaryon)	I			Yes	MES
U3-PF52/108 (homokaryon U3)	I	3.3,3.2	0.7	No	This study
H33-C1 (heterokaryon)	III			Yes	Japan
H33-C1-418 (homokaryon H33-C1)	III	3.3,3.2	0.8	No	MES

[a] The homokaryons derived from any given heterokaryon carry the mt genotype of the heterokaryon.
[b] Values refer to the sizes in kilobases of *Cfo*I fragments hybridized to probes described by Castle et al. (5).
[c] MES, collection of Mushroom Experiment Station, Horst.

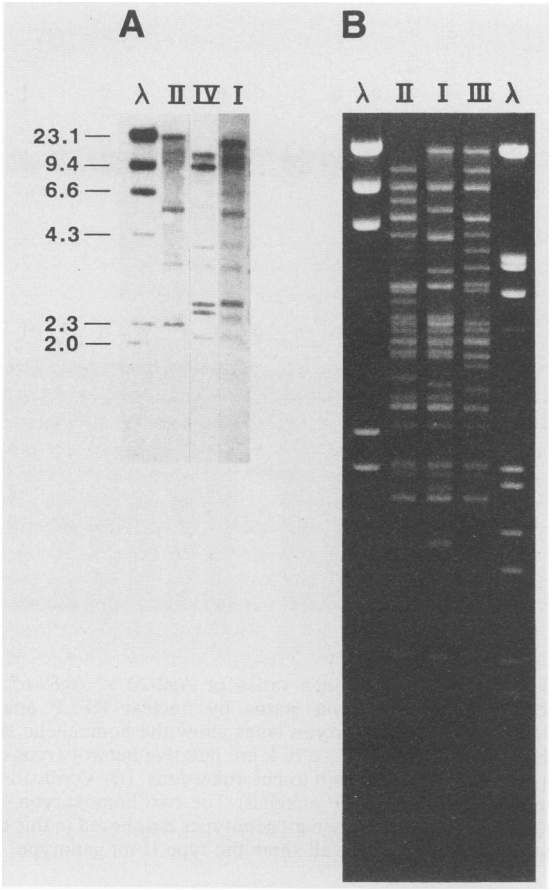

FIG. 1. (A) *Eco*RI-digested DNAs of *A. bisporus* hybridized to the mt-specific probes (the combination of four *Bam*HI fragments of Ag50 mtDNA cloned into pUC18, namely, B1, B4b, B6, and B7 [12]) differentiated three distinct mt genotypes (type I, Ag89; type II, Ag 2; type IV, Ag85). (B) mtDNAs were digested by *Cfo*I and separated into 1% agarose gels (mt genotypes I, II, and III are illustrated).

and freeze-dried. Total mycelial DNA was extracted by the method of Murray and Thompson (25), with the modification described by Zolan and Pukkila (35), or by the method of Raeder and Broda (26). Bacterial plasmid DNA was isolated by alkaline lysis and CsCl centrifugation (22).

Nick translation and Southern hybridization. Radiolabelling the DNA and hybridization were either by the methods described by Castle et al. (6) or by the PhotoGene Nucleic Acid Detection system (Life Technologies).

Fruiting trials. Fruiting trials were done by the cased-grain method of San Antonio (29), with the modification described by Castle et al. (6), or were performed by Sylvan Spawn Co. (Cabot, Pa.).

RESULTS

Identification of mt genotypes. mt genotypes were identified by two different approaches. One approach involved the use of a combination of four recombinant pUC18 plasmids carrying *Bam*HI fragments from the Ag50 mt genome (p50m1, p50m4b, p50m6, and p50m7 [12]). This combination was used as a hybridization probe in the survey for mt genomic polymorphism (Fig. 1A). Three mt genotypes were identified among the Canadian strains examined. Mushroom strains Ag50, Ag89, and Ag90 all possess a common mt genotype comparable to the Ag50 (U3) type mapped by Hintz et al. (12), which we arbitrarily call type I. Strains Ag2 and Ag85 were observed to have distinct mt genotypes differing from each other and from Ag50 (Fig. 1A). These are designated type II and type IV, respectively (Tables 1 and 2).

In some sets of experiments, mt types were identified by digestion of mtDNAs with restriction endonuclease *Cfo*I followed by separation in 1% agarose gels. Three mt genotypes were found in the strains used by the Dutch group. The banding patterns are shown in Fig. 1B. The strains Somycel 9.2, Sinden A4, and Horst U3 all possess a common mt genotype. This mt genotype was designated type I because comparison of *Eco*RI and *Bam*HI restriction fragments showed that this mt genotype was equivalent to the mt genotype found in strain Ag50. Strain Somycel 53 possesses the mt genotype II equivalent to the genotype found in Ag2. The mt genotype found in the Japanese isolate H33-C1 was designated type III.

Isolation of homokaryons. The source of the homokaryons of Ag50, Ag89, Ag90, and Ag2 are described in Table 1. Homokaryons of the wild-collected strain Ag85 were isolated by protoplast formation and regeneration (6). Seventy-one regenerated protoplasts were examined with nucleus-specific probe p33n6. Six isolates were found to be homokaryotic, carrying the same nuclear genome. Homokaryons which carried the other nuclear genome were not obtained in these experiments. The homokaryotic isolate Ag85-51 was chosen as one of the parental homokaryons for our mating experiments (Table 1). Strains 53-59, 9.2-97, A4-337, and H33-C1-418 are infertile single-spore isolates from the fertile heterokaryons described in Tables 1 and 2. Strain U3-PF52/108 is an infertile protoplast regenerate produced from heterokaryon U3 (Table 2).

Pairings. In an examination of the homokaryons of Ag2, we observed that Ag2-20 grew much more vigorously on CYM than Ag2-23 did. It also grew faster than the heterokaryon Ag2 itself on a number of solid media (data not shown). The ATCC homokaryotic strain Ag1-1, which shared one nuclear allele (p33n10) with Ag2-20 and another nuclear allele (p4n6) with Ag2-23, showed a radial growth similar to that of Ag2-20. We designate Ag2-20 and Ag1-1 as fast-growing homokaryons and Ag2-23 as a slow-growing homokaryon. Similar observations have been made on two homokaryons of Ag50. Ag50HB has been determined to be the fast-growing homokaryon, and Ag50HA has been determined to be the slow-growing homokaryon. All of the other four homokaryons (Ag89-59, Ag89-65, Ag90-30, and Ag85-51) were considered slow-growing homokaryons. They all grow less vigorously on CYM media than their respective parental heterokaryons.

These nine homokaryons described carrying three different mt genotypes were used in the laboratory matings. Nineteen combinations were designed for homokaryons carrying different mt genotypes. For each combination, 2 to 10 pairings were performed. With each pairing, 3 to 12 isolates were examined for heterokaryotic status with nuclear RFLPs (Table 3).

The new heterokaryons were generated from 10 different combinations of pairings (Table 3). No heterokaryons were confirmed in the other nine combinations (Table 3).

In most of the pairings, the formation of heterokaryons can be verified by using p33n10 and/or p4n6 as probes of *Eco*RI-digested genomic DNA (Tables 1 and 2). However, for the isolates from the pairings of $Ag2\text{-}20_{(II)}$ (subscript numeral = mt genotype) with $Ag50HA_{(I)}$, these probes were not informative (Table 1). In these cases, another plasmid,

TABLE 3. mt transmission in selected pairings of *A. bisporus* homokaryons

Homokaryon designation	No. of pairings	Successful crosses (*y*/*x*)[a]	mt genotype
Canadian			
1-1 × 89-65	6	35/42	II
1-1 × 89-65	2[b]	8/8	II
1-1 × 89-65	2[b]	5/7	I
1-1 × 89-59	2	15/24	II
2-20 × 89-59	2	15/19	II
2-20 × 89-65	10[c]	58/68	II
1-1 × 90-30	4	13/36	II
2-20 × 90-30	4	0/20	
2-23 × 90-30	4	0/20	
1-1 × 85-51	4	9/24	II
2-20 × 85-51	3	0/20	
2-23 × 85-51	4	0/20	
89-59 × 85-51	3	0/18	
89-65 × 85-51	3	0/18	
90-30 × 85-51	3	6/18	IV
2-20 × 50HA	4	0/20	
2-20 × 50HB	4	13/20	I
2-23 × 50HA	4	0/20	
2-23 × 50HB	4	0/20	
2-23 × 89-59	2	0/14	
2-23 × 89-65	1	3/6	I
2-23 × 89-65	1	2/4	II
Dutch			
53-39 × 9.2-97	24[d]	16/24	II
		8/24	I
53-39 × 9.2-97	20[e]	9/20	II
		11/20	I
53-39 × A4-337	20[d]	3/20	I
		1/20	II
53-39 × A4-337	18[e]	18/18	II
	20[e]	20/20	II
H33-C1-418 × U3-PF52/108	11[e]	11/11	III

[a] *y*, number of isolates investigated; *x*, number of isolates to be confirmed by nuclear RFLPs as new heterokaryons.

[b] In these pairings, the slow-growing parental homokaryon (Ag89-65) was allowed to grow for 7 days before being paired with the faster-growing homokaryon (Ag1-1).

[c] In six crosses, the slow-growing homokaryon Ag89-65 was allowed to grow for 7 days before being paired with Ag2-20. All 28 heterokaryons recovered in this cross carried the type II mt genotype.

[d] Pairings were done in petri dishes with MMP agar.

[e] Pairings were done in petri dishes on compost.

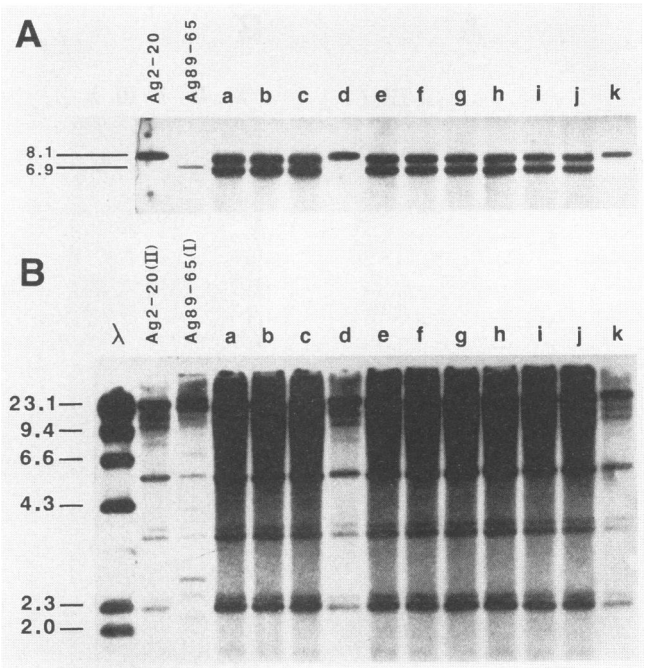

FIG. 2. mt inheritance in a cross of Ag2-20 × Ag89-65. (A) Verification of heterokaryon status by nuclear RFLP analysis (p33N10). The two homokaryon lanes show the homoallelic signal for this nuclear probe. Lanes a to k are putative heterokaryons. All but lanes d and k were shown to heterokaryons. (B) Verification of mt genotype utilizing RFLP analysis. The two homokaryon lanes show the RFLP profiles of two mt genotypes monitored in this cross (see panel A). Lanes a to k all show the type II mt genotype.

p33n25 (5), was utilized. Twenty isolates from four pairings between Ag2-20$_{(II)}$ and Ag50HA$_{(I)}$ were examined, and none was found to be heterokaryotic.

Pairings were done initially on MMP agar for the crosses of 53-39$_{(II)}$ × 9.2-97$_{(I)}$, 53-39$_{(II)}$ × A4-337$_{(I)}$, and H33-C1$_{(III)}$ × U3-PF52/108$_{(I)}$. Heterokaryon formation was verified by using p33n10 and p33n25 as probes for *Cfo*I-digested genomic DNA (Table 2). Only for the crosses of 53-39$_{(II)}$ × 9.2-97$_{(I)}$ were heterokaryons readily obtained on MMP agar. In previous experiments, it was shown that heterokaryon formation more easily occurred (Table 3) when pairings were done on compost. Therefore, we repeated the pairings on compost.

mt inheritance. No evidence for a mixture of mt genotypes in any of the initial heterokaryons examined was observed. No detectable recombination between the mt genomes of two donor homokaryons was observed by utilizing mt RFLPs. When the fast-growing homokaryons of Ag2 [i.e., Ag1-1$_{(II)}$ and Ag2-20$_{(II)}$] were mated with the slow-growing homokaryons Ag89-59$_{(I)}$ and Ag89-65$_{(I)}$, the heterokaryons recovered all were found to carry the type II mt genotype (Table 3). This uniparental transmission was observed in 15 heterokaryons recovered from 2 pairings of Ag1-1$_{(II)}$ and Ag89-59$_{(I)}$, for 15 heterokaryons recovered from 2 pairings of Ag2-20$_{(II)}$ and Ag89-59$_{(I)}$, for 43 heterokaryons recovered from 8 pairings of Ag1-1$_{(II)}$ and Ag89-65$_{(I)}$, and for 58 heterokaryons from 10 pairings of Ag2-20$_{(II)}$ and Ag89-65$_{(I)}$ (Table 3).

Since the homokaryons Ag1-1$_{(II)}$ and Ag2-20$_{(II)}$ exhibited very-vigorous aerial growth on CYM plates when compared with the slower nonaerial growth of Ag89-59$_{(I)}$ and Ag89-65$_{(I)}$, we were concerned that these differences in radial growth and morphology could bias our results. In an attempt to minimize this bias, we did the following. (i) In one cross between Ag2-20$_{(II)}$ and Ag89-65$_{(I)}$, five samples were taken from the region where the paired mycelia contacted one another but slightly toward the Ag89-65$_{(I)}$ parent. Analysis of these samples revealed either the type II mt genotype in heterokaryons or unmated type I. (ii) The slow-growing homokaryon Ag89-65$_{(I)}$ was inoculated and allowed to grow for 7 days before pairing with either Ag2-20$_{(II)}$ or Ag1-1$_{(II)}$. This resulted in recovery of heterokaryons carrying the mt genotype of Ag89-65$_{(I)}$ (Table 3). However, in six pairings of the fast-growing homokaryon Ag2-20$_{(II)}$ and the slow-growing homokaryon Ag89-65$_{(I)}$ by using the procedure described in (ii) above, all 28 heterokaryons recovered were also found to carry the type II mt genotype (Table 3).

Figure 2 shows a representative Southern blot used in the analysis of the data shown in Tables 1 to 3. The analysis of nuclear RFLPs is illustrated in Fig. 2A for a cross between

Ag2-20$_{(II)}$ and Ag89-65$_{(I)}$. Of the 11 isolates examined from the pairing, 2 (lanes d and k) were shown to be unmated Ag2-20$_{(II)}$ and carried the RFLP genotype of only Ag2-20$_{(II)}$ parent; the other 9 isolates carried the RFLP allele for both nuclear types and were confirmed as new intraspecies heterokaryons (Fig. 2A). All of the confirmed new heterokaryons were found to carry the type II mt genotype (Fig. 2B).

Four putative heterokaryons from the RFLP-confirmed crosses between Ag1-1$_{(II)}$ and Ag89-65$_{(I)}$ were examined for their ability to produce fruiting bodies. Three cultures of Ag1-1$_{(II)}$ × Ag89-65$_{(I)}$, and all nine cultures of Ag2-20$_{(II)}$ × Ag89-65$_{(I)}$ from fruiting trials, produced mushrooms. Total DNA was isolated from the fruiting bodies, and RFLP genetic markers were utilized to determine nuclear and mt genotypes. The data produced from the fruiting body analyses confirmed the results obtained from mycelial analyses, specifically that heterokaryon formation had indeed occurred.

Ag1-1$_{(II)}$ was also successfully mated with Ag90-30$_{(I)}$. The newly produced heterokaryons were all found to carry the type II mt genotype (Table 3).

When the slow-growing homokaryon Ag85-51$_{(IV)}$ was mated with Ag1-1$_{(II)}$, all nine heterokaryons recovered were found to carry the type II mt genotype. However, all of the heterokaryons generated in the crosses between Ag85-51$_{(IV)}$ and the slow-growing homokaryon Ag90-30$_{(I)}$ carried the type IV mt genome (Table 3).

In our attempts to mate Ag50HA$_{(I)}$, a slow-growing homokaryon of Ag50, with Ag2-20$_{(II)}$ and Ag2-23$_{(II)}$, no heterokaryons were recovered (Table 3). If, however, the fast-growing homokaryon Ag50HB$_{(I)}$ was paired with Ag2-20$_{(II)}$, 13 new heterokaryons were recovered which all carried the type I mt genotype (Table 3).

The slow-growing homokaryon Ag2-23$_{(II)}$ has been successfully mated with Ag89-65$_{(I)}$. In one successful cross between Ag2-23$_{(II)}$ and Ag89-65$_{(I)}$, three recovered heterokaryons carried the type I mt genotype, while in another successful cross, two heterokaryons were recovered that carried the type II mt genotype. In summary, 182 new heterokaryons were obtained in 41 successful matings performed on CYM plates (Table 3) by the Canadian group. In all of these matings, a biased mt transmission was observed.

In another set of experiments, pairings were done initially on MMP agar for crosses 53-39$_{(II)}$ × 9.2-97$_{(I)}$, 53-39$_{(II)}$ × A4-337$_{(I)}$, and H33-C1-418$_{(III)}$ × U3-PF52/108$_{(I)}$. The formation of new heterokaryons was verified by using p33n10 and p33n25 as probes of *Cfo*I-digested DNA (Table 2). mt genotypes were determined by probing *Cfo*I digests with the mt clone p50m2. From previous experiments in a breeding program, it was known that the homokaryons in these combinations were compatible and that the formation of a heterokaryon becomes visible as a fast-growing sector of fluffy mycelium. However, on MMP agar, only for the pairings for the cross 53-93$_{(II)}$ × 9.2-97$_{(I)}$ were heterokaryons readily obtained, visible as fast-growing sectors. Twenty-four heterokaryons were obtained from the cross between 53-39$_{(II)}$ × 9.2-97$_{(I)}$, 16 heterokaryons carrying mt genotype II and 8 heterokaryons carrying mt genotype I (Table 3). For the cross 53-39$_{(II)}$ × A4-337$_{(I)}$, only four heterokaryons were obtained, three heterokaryons carrying mt genotype I and one homokaryon carrying mt genotype II. From the matings between H33-C1-418$_{(III)}$ and 9.2-97$_{(I)}$, no heterokaryons were obtained on MMP agar.

Results from repeated pairings in the breeding programs at Horst suggested that heterokaryon formation more easily occurred when pairings were done on compost. We therefore repeated the above crosses on compost. For all pairings, fast-growing sectors were observed after 2 to 6 days of contact between the colonies of the paired homokaryons. For matings between homokaryons 53-93$_{(II)}$ and 9.2-97$_{(I)}$, 20 heterokaryons were obtained, 9 heterokaryons carrying mt genotype II and 11 heterokaryons carrying mt genotype I. This biparental inheritance of mt was also observed on MMP agar. In two pairings between 53-93$_{(II)}$ and A4-337$_{(I)}$, 38 intraspecies heterokaryons were obtained, all carrying mt genotype II. From matings between H33-C1-418$_{(III)}$ and 9.2-97$_{(I)}$ carrying mt, 11 heterokaryons were obtained, all carrying mt genotype III (Table 3).

DISCUSSION

To effectively study the mt transmission in *A. bisporus*, it was necessary to develop a methodology that allowed for the identification and differentiation of the mt genomes in parental stains and in the resultant new heterokaryons. We utilized a combination of four large *Bam*HI fragments (B1, B4b, B6, and B7) of the Ag50 mt genome (12) as the differentiating probes in this study. These four fragments constituted about 30% of the Ag50 mt genome (12) and gave informative RFLPs (Fig. 1A). In addition to determining mt genotype by using these specific cloned DNAs, it was also possible to distinguish mt genotypes with purified mtDNAs digested with *Cfo*I (Fig. 1B).

Our initial observation suggested that there was only one mt genotype in commercial strains (11). This genotype, designated type I in this study, is associated with a large number of commercial strains derived from the Dutch U3 strain. The type I genome was also found in the wild-collected strain Ag89 (21) and in the fungicide-resistant strain Ag90, a U3 derivative (7). The Dutch U1 hybrid and derivatives from this strain, as well as the older commercial strain Ag2, carried the mt type II genome. The Japanese strain H33C1 and the field-collected Ag85 (18, 19) each carried a distinct mt genotype (type III and type IV, respectively).

In an earlier study, Castle et al. (6) were unsuccessful in their attempt to isolate homokaryons from strain Ag85. In the current study, we suggest that the RFLPs they initially used were homoallelic for this particular strain. The nuclear probe p33n6 gave a heteroallelic RFLP in the Ag85 heterokaryon and allowed us to differentiate the homokaryons of Ag85 after protoplast regeneration (Table 1). These results suggest that it is necessary to examine a number of different cloned fragments to find an RFLP that differentiates between nuclear types of heterokaryons and facilitates the isolation of parental homokaryons.

The isolation of both pairs of parental homokaryons from any given heterokaryon is problematic. For some heterokaryons, there appears to be little difficulty in recovering both possible homokaryons; for other heterokaryons, it may be possible to isolate only one of the two parental homokaryons (6). In this study, we were unable to isolate both types of homokaryons from the heterokaryon Ag85. One possible explanation for this phenomenon is that some nuclear types have lost the ability to grow independently as homokaryons under the growth conditions examined. A homokaryotic or haploid structure is not a necessary part of the life cycle of *A. bisporus* (15). It is possible that some heterokaryons (pairs of nuclei) may have existed together in a common cytoplasm for many generations. During this period of time, one of the nuclear types may have experienced a mutation or a recombinational event that affected its ability to exist as an

independent homokaryon. Furthermore, under the conditions used for this study, we observed different growth characteristics between two pairs of isolated homokaryons. For example, homokaryons Ag2-$20_{(II)}$ and Ag50$HB_{(I)}$ were found to grow more vigorously than homokaryons Ag2-$23_{(II)}$ and Ag50$HA_{(I)}$.

In basidiomycetes, mt inheritance has been investigated in *C. cinereus* (23), *A. bitorquis* (11), and *A. bulbosa* (30). Biparental inheritance of mt in *C. cinereus* resulted from bidirectional nuclear migration, an event which occurs directly after hyphal anastomosis between pairs of compatible homokaryons (2). Donor nuclei usually migrated bilaterally through the established hyphae of the recipient mates, forming two discrete dikaryons having identical pairs of nuclei but unique cytoplasms. The formation of a dikaryon could easily be identified by the presence of clamp connections. May and Taylor (23) have investigated the mt transmission in *C. cinereus*. In one cross, nuclear migration did not occur, and the only dikaryons recovered from the junction zone showed a mixed mtDNA pattern. However, each fruiting body developed from these mixed dikaryons carried only one of the possible mt genomes (23). Hintz et al. (11) investigated mt transmission in *A. bitorquis*, an *Agaricus* species with a heterothallic life cycle. In a selected cross between two homokaryons, unidirectional nuclear migration was observed (1, 11). The dikaryon recovered from the recipient mate contained only one mt type. The dikaryons recovered from the zone of anastomosis contained both parental mt genotypes. After a number of transfers, one of the mt genotypes was lost by vegetative segregation (11). Dikaryons carrying different mt genotypes but identical nuclear genotypes were found to exhibit different growth characteristics (11). In *A. bulbosa*, transmission of mt genome in the field was shown to favor one mt genotype (30). Smith et al. (30), however, provide evidence for biparental inheritance of mt genotypes in laboratory matings.

The results presented in this study suggest that biparental transmission of the mt genotype only occurred in selected crosses in *A. bisporus*. A number of interesting differences were observed between mt transmission in *A. bisporus* and that in the other basidiomycetes studied (11, 23, 30). Nuclear migration has not been observed in *A. bisporus* (1, 27, 31). In the 189 pairings performed in this study, no evidence of nuclear migration was observed. Therefore, the formation of the heterokaryon, as the result of nuclear migration into the resident cytoplasm, is unlikely to be involved in the mt transmission of *A. bisporus*. In *A. bisporus*, the formation of heterokaryons only occurred at the junction zone by hyphal anastomosis between the parental homokaryons. The novel heterokaryon formed may contain nuclear and mt genomes from both parental homokaryons (heteroplasmon). Heteroplasmons have been considered to be formed in laboratory matings in *A. bitorquis* (11), but we were unable to confirm their formation in this study. The uniparental inheritance observed in most crosses of this study suggests a very strong bias toward the vegetative transmission of the mt genome from one of the parental homokaryons. *A. bisporus* is therefore distinct from other basidiomycetes examined (11, 23, 30) with respect to vegetative transmission of the mt genome.

The mt transmission pattern in eukaryotes is generally quite variable (3, 10). The uniparental inheritance of the mt genome in oogamous species has been attributed either to (i) the failure of mt to enter into the egg or (ii) the presence of comparatively few mt in the male gamete. These mechanisms, however, do not explain the uniparental, or biased, biparental mt inheritance in some isogamous species (4, 17, 24). In these species, nonrandom factors may play an important role in mt transmission. These factors, such as extra rounds of premating synthesis of mtDNA in one of the gametes, may affect the input ratio of the organelle genome. Furthermore, the selection for particular organelle-nucleus combinations may affect the output ratio. Uniparental inheritance of the mt genome has been observed in the acellular slime mold *Physarum polycephalum* (17). In *P. polycephalum*, fusion of two haploid amoebae with different mating types will generate a diploid zygote which develops into a macroscopic diploid plasmodium by repeated mitosis in the absence of cell division. Therefore, the random partitioning of mt by cell division does not occur. It has been suggested that the inheritance of the mt genome in *P. polycephalum* is due to preferential replication of one of the two mtDNAs (17). Mirfakhrai et al. (24) observed that the mt genome was inherited uniparentally (for most crosses) in the cellular slime mold *Polysphondylium pallidum*. They suggested that one mt type was selectively eliminated at a stage between zygote formation and zygote germination.

In the yeast *Saccharomyces cerevisiae*, the number of mtDNA per cell has been found to vary dramatically with genotype, ploidy, cell volume, and, in some strains, with the physiological state of the cell (3). It has been demonstrated that the bias in the input ratio of organelles from the parental homokaryons would affect the output ratio of transmission of mtDNA to the progeny. In this study, when Ag89-$65_{(I)}$ (slow-growing homokaryon) was mated with Ag2-$23_{(II)}$ (slow-growing homokaryon), the resultant heterokaryons carried either the type I or type II mt genome. However, when Ag89-$65_{(I)}$ was paired with the fast-growing homokaryon Ag1-$1_{(II)}$ or Ag2-$20_{(II)}$, all of the resultant heterokaryons carried the type II mt genotype. These data would suggest that the biased mt inheritance was directly related to the radial growth of the parental homokaryons. However, if Ag89-$65_{(I)}$ was allowed to grow for 7 days before it was paired with Ag1-$1_{(II)}$, it was possible to demonstrate transmission of the mt from the slow-growing homokaryon (Table 3). When two fast-growing homokaryons, Ag2-$20_{(II)}$ and Ag50$HB_{(I)}$, were paired, 13 heterokaryons were recovered and all carried the type I mt genome. Although both Ag50$HB_{(I)}$ and Ag2-$20_{(II)}$ were considered fast-growing homokaryons, Ag2-$20_{(II)}$ grows even more vigorously than Ag50$HB_{(I)}$ under the conditions used in this study. These data, therefore, would argue that the biased mt transmission in *A. bisporus* is not simply determined by the radial growth rates of the parental homokaryons. Since absolute uniparental inheritance patterns have been observed in a number of crosses such as Ag2-$20_{(II)}$ × Ag89-$65_{(I)}$, $Ag20_{(II)}$ × Ag50$HB_{(I)}$, 53-$39_{(II)}$ × A4-$337_{(I)}$, and H33-C1-$418_{(III)}$ × U3-PF52/$108_{(I)}$, it seems unlikely that the biased mt transmission observed in *A. bisporus* is due simply to the unequal contribution of mtDNA from the parental homokaryons, as observed in the yeast *S. cerevisiae* (3).

Our data could suggest that a nonrandom mechanism(s) was involved in the biased mt transmission observed for *A. bisporus*. One possible mechanism is the unequal distribution of nuclei in the newly formed heterokaryons. The positions of the paired parental nuclei relative to their respective cytoplasms in the heteroplasmon could favor the inheritance of one cytoplasmic type. To test this possibility, Sonnenberg et al. (31) constructed two pairs of homokaryons: $39_{(I)}$ and $39_{(II)}$, and $97_{(I)}$ and $97_{(II)}$. Each pair of homokaryons carried a common nuclear genome but different mt genomes (designated I and II as in this study). The

intraspecies heterokaryons generated from the crosses of $39_{(I)} \times 97_{(II)}$ and $39_{(II)} \times 97_{(I)}$ were all found to carry the type I mt genotype (31). These data do not support the concept that unequal distribution of nuclei occurs in *A. bisporus* (31).

Another possible explanation is that during the process of anastomosis, mtDNA from one of the parental homokaryons is selectively stimulated or inhibited from replication or that one of the mt genomes is selectively degraded. The selective degradation model has been previously proposed to explain uniparental organelle transmission in a number of protists (4, 17, 24), as discussed above.

In this study, we present for the first time data on mt transmission in the commercially important mushroom *A. bisporus*. These data will be utilized in designing and performing experiments which are directed at examining the effect of mt genotype on strain performance under commercial growth conditions. It is possible to construct heterokaryons with a common nuclear background but with different mt genotypes. Experiments of this type are currently under way in our research laboratories.

ACKNOWLEDGMENTS

Support for this study came from the Canadian Mushroom Growers Association, Sylvan Spawn Research, L. F. Lambert Spawn Co., Inc., Monterey Mushrooms Inc., J. B. Swayne Spawn Co., Highline Mushroom, The Horticultural Research and Development Corporation (Australia), and the University Research Incentive Fund of the Province of Ontario.

We thank J. B. Anderson, M. Smith, and C. Bowden for their helpful comments, and Jose' in't Zandt-Linders for technical assistance.

REFERENCES

1. **Anderson, J. B., D. M. Petsche, F. B. Herr, and P. A. Horgen.** 1984. Breeding relationships among several species of *Agaricus*. Can. J. Bot. **62**:1884–1889.
2. **Baptista-Ferreira, J. L. C., A. Economou, and L. A. Casselton.** 1983. Mitochondrial genetics of *Coprinus*: recombination of mitochondrial genomes. Curr. Genet. **7**:405–407.
3. **Birky, C. W., Jr.** 1978. Transmission genetics of mitochondria and chloroplasts. Annu. Rev. Genet. **12**:471–512.
4. **Boynton, J. E., E. H. Harris, B. D. Burkhart, P. M. Lamerson, and N. W. Gillham.** 1987. Transmission of mitochondrial and chloroplast genomes in crosses of *Chlamydomonas*. Proc. Natl. Acad. Sci. USA **84**:2391–2395.
5. **Castle, A. J., P. A. Horgen, and J. B. Anderson.** 1987. Restriction fragment length polymorphisms in the mushrooms *Agaricus brunnescens* and *Agaricus bitorquis*. Appl. Environ. Microbiol. **53**:816–822.
6. **Castle, A. J., P. A. Horgen, and J. B. Anderson.** 1988. Crosses among homokaryons from commercial and wild-collected strains of the mushroom *Agaricus brunnescens* (= *Agaricus bisporus*). Appl. Environ. Microbiol. **54**:1643–1648.
7. **Challen, M. P., and T. J. Elliott.** 1987. Production and evaluation of fungicide resistant mutants in the cultivated mushroom, *Agaricus bisporus*. Trans. Br. Mycol. Soc. **88**:433–439.
8. **Elliott, T. J., and M. P. Challen.** 1984. Effect of temperature on spore number in the cultivated mushroom, *Agaricus bisporus*. Trans. Br. Mycol. Soc. **82**:293–296.
9. **Evans, H. J.** 1959. Nuclear behaviour in the cultivated mushroom. Chromosoma **10**:115–135.
10. **Gray, M. W.** 1989. Origin and evolution of mitochondrial DNA. Annu. Rev. Cell Biol. **5**:25–50.
11. **Hintz, W. E. A., J. B. Anderson, and P. A. Horgen.** 1988. Nuclear migration and mitochondrial inheritance in the mushroom *Agaricus bitorquis*. Genetics **119**:35–41.
12. **Hintz, W. E. A., J. B. Anderson, and P. A. Horgen.** 1988. Physical mapping of the mitochondrial genome of the cultivated mushroom *Agaricus brunnescens* (= *A. bisporus*). Curr. Genet. **14**:43–49.
13. **Hintz, W. E. A., J. B. Anderson, and P. A. Horgen.** 1989. Relatedness of three species of *Agaricus* inferred from RFLP analysis of the ribosomal DNA repeat and mitochondrial DNA. Genome **32**:173–178.
14. **Hintz, W. E. A., M. Mohan, J. B. Anderson, and P. A. Horgen.** 1985. The mitochondrial DNAs of *Agaricus*: heterogeneity in *A. bitorquis* and homogeneity in *A. brunnescens*. Curr. Genet. **9**:127–132.
15. **Horgen, P. A., and J. B. Anderson.** 1992. Edible mushrooms, p. 447–462. *In* D. Finkelstein and C. Ball (ed.), Biotechnology of filamentous fungi. Butterworth-Heinemann, Boston.
16. **Horgen, P. A., T. Jin, and J. B. Anderson.** 1991. The use of protoplast production, protoplast regeneration and restriction fragment length polymorphisms in developing a systematic and highly reproducible breeding strategy for *Agaricus bisporus*, p. 67–72. *In* L. J. L. D. van Griensven (ed.), Genetics and breeding of *Agaricus*. Pudoc, Wageningen, The Netherlands.
17. **Kawano, S., R. W. Anderson, T. Nanba, and T. Kuroiwa.** 1987. Polymorphism and uniparental inheritance of mitochondrial DNA in *Physarum polycephalum*. J. Gen. Microbiol. **133**:3175–3182.
18. **Kerrigan, R. W.** 1990. Evidence of genetic divergence in two populations of *Agaricus bisporus*. Mycol. Res. **94**:721–733.
19. **Kerrigan, R. W., P. A. Horgen, and J. Anderson.** 1992. The California population of *Agaricus bisporus* comprises at least two ancestral elements. Syst. Bot., in press.
20. **Kerrigan, R. W., and I. K. Ross.** 1987. Dynamic aspects of basidiospore number in *Agaricus*. Mycologia **79**:204–215.
21. **Malloch, D., A. Castle, and W. Hintz.** 1988. Further evidence for *Agaricus brunnescens* (Peck) as the preferred name for the cultivated *Agaricus*. Mycologia **79**:839–846.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **May, G., and J. W. Taylor.** 1988. Patterns of mating and mitochondrial DNA inheritance in the agaric basidiomycete *Coprinus cinereus*. Genetics **118**:213–220.
24. **Mirfakhrai, M., Y. Tanaka, and K. Yanagisawa.** 1990. Evidence for mitochondrial DNA polymorphism and uniparental inheritance in the cellular slime mold *Polysphondylium pallidum*: effect of intraspecies mating on mitochondrial transmission. Genetics **124**:607–613.
25. **Murray, M. G., and W. F. Thompson.** 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. **8**:4321–4325.
26. **Raeder, U., and P. Broda.** 1985. Rapid preparation of DNA from filamentous fungi. Lett. Appl. Microbiol. **1**:17–20.
27. **Raper, C. A., J. R. Raper, and R. E. Miller.** 1972. Genetic analysis of the life cycle of *Agaricus bisporus*. Mycologia **64**:1088–1117.
28. **Royer, J. C., W. E. Hintz, and P. A. Horgen.** 1991. Efficient protoplast formation and regeneration and electrophoretic karyotype analysis of *Agaricus bisporus*, p. 52–56. *In* L. J. L. D. van Griensven (ed.), Genetics and breeding of *Agaricus*. Pudoc, Wageningen, The Netherlands.
29. **San Antonio, J. P.** 1971. A laboratory method to obtain fruit body from cased grain spawn of the cultivated mushroom, *Agaricus bisporus*. Mycologia **63**:16–21.
30. **Smith, M. L., L. C. Duchesne, J. N. Bruhn, and J. B. Anderson.** 1990. Mitochondrial genetics in a natural population of the plant pathogen *Armillaria*. Genetics **126**:575–582.
31. **Sonnenberg, A. S. M., P. C. C. Van Loon, and L. J. L. D. Van Griensven.** 1991. Mitochondrial genotypes and their inheritance in the cultivated mushroom *Agaricus bisporus*, p. 42–51. *In* ed. L. J. L. D. Van Griensven (ed.), Genetics and breeding of *Agaricus*. Pudoc, Wageningen, The Netherlands.
32. **Sonnenberg, A. S. M., J. G. H. Wessels, and L. J. L. D. Van**

Griensven. 1988. An efficient protoplasting/regeneration system for *Agaricus bisporus* and *Agaricus bitorquis*. Curr. Microbiol. **17:**285–291.
33. **Stevens, R. B.** 1981. *In* R. B. Stevens (ed.), Mycology guidebook. University of Washington Press, Seattle.
34. **Straatsma, G., J. P. G. Gerrits, M. P. A. M. Augustijn, H. J. M. Op Den Camp, G. D. Vogels, and L. J. L. D. Van Griensven.** 1989. Population dynamics of *Scytalidium thermophilum* in mushroom compost and stimulatory effects on growth rate and yield of *Agaricus bisporus*. J. Gen. Microbiol. **135:**751–759.
35. **Zolan, M., and P. J. Pukkila.** 1986. Inheritance of DNA methylation in *Coprinus cinereus*. Mol. Cell. Biol. **6:**195–200.