

Use of molecular markers to differentiate between commercial strains of the button mushroom *Agaricus bisporus*

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Abstract

Agaricus bisporus is an edible basidiomycete cultivated industrially for food production. Different spawn and mushroom producers use genetically related *A. bisporus* strains frequently marketed as different products. In this paper we show that the use of suitable molecular markers reveals the high level of genetic homology of commercial strains of *A. bisporus*, and allows, at the same time, to distinguish between them. In the course of this work, a molecular marker potentially linked to the agronomic character ‘mushroom weight’ has been identified by bulked segregant analysis. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Molecular marker; Button mushroom; Strain typing; Bulked segregant analysis; *Agaricus bisporus*

1. Introduction

Agaricus bisporus is a crop whose production has undergone an important increase during the last years. Most commercial strains show secondary homothallism resulting in the production of basidiospores containing two meiotic nuclei which give rise upon germination to self-fertile vegetative mycelia. Inbreeding is the direct consequence of secondary homothallism. Despite most *A. bisporus* commercial strains deriving from hybrids Horst U1 and Horst U3 [1], different spawn and mushroom producers have released a number of new commercial strains whose genetic identity has been frequently under debate.

Biochemical and molecular markers have been used to study the relationships between different *A. bisporus* strains and with other *Agaricus* species. Isozymes were used to discriminate among genotypic classes of *A. bisporus*, to confirm crosses between lines, and to identify new alleles and new genotypes in a wild population [2,3]. Recombinant DNA technology has been used to analyse breeding relationships among several species of *Agaricus*, to study relationships between *A. bisporus* and *Agaricus*

bitorquis, to monitor crosses among homokaryons from commercial and wild collected strains [4,5], to detect the presence of DNA polymorphisms in commercial and wild strains of *A. bisporus* [6], and to construct the first genetic linkage map in *Agaricus* [7]. Random amplified polymorphic DNA (RAPD) markers have been used to fingerprint commercial and wild strains of *A. bisporus*, while sequence-characterised amplified sequences ([8]) have been used to study the inheritance of cap colour in the course of *Agaricus* breeding programmes [9]. Recently, mobile genetic elements found in *Agaricus* have been used to determine the origin of present-day hybrids [10,11].

The objective of this work was to study the genetic variability present in commercial strains of the button mushroom *A. bisporus* and to set up a system for distinguishing between strains commonly used by spawn and mushroom producers. For both objectives, molecular markers based on polymerase chain reaction (PCR) approaches were used.

2. Materials and methods

2.1. Fungal strains and culture conditions

The commercial strains of *A. bisporus* and *A. bitorquis*

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used in this study are listed in Table 1. In addition, *A. bisporus* strains Darlington-521 and LeChampion-229 were used as reference for high and low ‘mushroom weight’, respectively. All *A. bisporus* strains with the exception of Gurelan 300 and Gurelan OX were hybrid, while the hybrid/non-hybrid nature of Darlington was unknown. The *A. bisporus* strains studied have two-spored basidia and present fruit bodies of small–large size while the *A. bitorquis* strains carry white four-spored basidia, and have large sized white pilei. Both species require between 85 and 90% humidity for growing and temperatures ranging from 15–19°C (*A. bisporus*) to 20–22°C (*A. bitorquis*).

Stock cultures of the different strains were made on Petri dishes containing 25 ml of solid malt agar medium [12] incubated at 24°C in darkness and stored at 4°C. Liquid cultures of vegetative mycelia were made in SMY (sucrose 1% w/v, malt extract 1% w/v, yeast extract 0.4% w/v [13]) and incubated for 25–30 days at 24°C without shaking. The mycelium was then harvested by filtration of the culture and disposal of the broth, and processed for DNA purification.

2.2. Molecular techniques

Genomic DNA was purified from liquid cultures of vegetative mycelium or from fruit bodies as described by Lassner et al. [14] and Ramírez et al. [15]. For the analysis of RAPD, different types of oligonucleotides were used as

primers (Table 1): decamers (10-mer) belonging to the S, L, and M Operon Series (Operon Technologies Inc. Alameda, CA, USA), oligonucleotides synthesised in our laboratory (4360 (GGACTTACAG), 4391 (CTGACTCATG), and 4392 (CTTCATTGCC)), and three DNA fingerprinting probes (DFPs) (core sequence of the phage M13, (GATA)₄ and (GTG)₅). RAPD markers were generated according to Williams et al. [16]. PCRs were performed with 10–100 ng of template DNA in a PTC-200 (Peltier Thermal Cycler, MJ Research, Watertown, MA, USA) using the following reaction programme: 4 min denaturation at 94°C, followed by 40 cycles of PCR reaction (1 min denaturation at 94°C, 1 min annealing, 1.5 min extension at 72°C, per cycle). When the 10-mer oligonucleotides were used as primers, the annealing temperature was 37°C, when the DFPs were used as primers, the annealing temperature was 50°C. The amplification products were analysed by electrophoresis in 1.3% (w/v) agarose gels in TAE buffer, and stained with ethidium bromide. As size markers, *Hind*III-digested Φ 29 DNA was used. With the purpose of generating RAPD markers linked to specific agronomic traits, pools of DNA containing equal amounts of DNA purified from the corresponding strains were used as templates [17]. All RAPD reactions were repeated at least three times to avoid problems derived from low reproducibility. Only those bands appearing consistently in all experiments were used.

Table 1
Molecular markers produced using different primer oligonucleotides in the strains of *A. bisporus* and *A. bitorquis* studied

Strain	Primer oligonucleotide														Σ^a	
	S-03	S-10	S-11	S-16	S-17	S-18	S-19	M-05	M-12	4360	4391	4392	M13	(GATA) ₄		(GTG) ₅
<i>A. bisporus</i>																
1 Gure-55	5	13	3	4	5	8	5	8	7	3	14	7	12	19	17	136
2 Pla-814	5	13	3	4	5	8	5	8	7	3	12	7	12	19	16	133
3 Gure-45	6	12	4	5	5	8	5	8	9	1	14	7	13	20	15	138
4 Gure-102	5	12	5	5	4	8	6	8	9	2	10	6	15	19	16	136
5 Gure-15	5	13	3	4	5	8	5	8	7	2	12	7	12	19	17	133
6 Gure-300	6	13	3	5	3	5	6	7	8	2	12	5	14	16	16	127
7 Gure-35	5	11	4	5	3	8	7	8	8	4	13	7	15	18	16	138
8 Som-91	4	13	5	5	6	7	3	8	8	3	13	6	12	18	16	133
9 Gure-50	6	11	4	6	5	8	5	1	9	1	11	5	13	19	17	127
10 Fung-25	5	13	3	4	5	8	5	8	7	3	12	7	12	19	15	132
11 Som-209	5	13	4	5	5	8	5	8	8	3	11	7	13	19	17	137
12 Som-512	5	12	4	6	5	8	5	8	9	2	11	8	11	19	17	136
13 Gure-OX	4	8	7	4	5	10	6	7	8	2	10	8	18	19	18	140
<i>A. bitorquis</i>																
14 <i>A. bitorq-01</i>	7	6	7	6	6	5	9	6	6	2	6	8	14	15	16	122
15 <i>A. bitorq-02</i>	7	7	5	6	6	6	7	10	5	2	5	7	12	16	18	122
16 <i>A. bitorq-03</i>	1	4	7	1	7	10	3	6	2	7	5	3	6	12	13	94
Polymorphic products ^b																
<i>A. bisporus</i>	7	10	6	7	6	6	12	8	9	4	10	8	13	14	12	132
<i>A. bitorquis</i>	10	12	15	8	15	18	15	17	12	7	9	13	17	21	28	217
Polymorphism (%) ^c	21.0	12.9	29.6	20.0	26.3	19.5	31.0	21.4	17.9	26.2	11.1	20.0	14.7	12.2	15.4	17.3

^aNumber of products amplified in each *Agaricus* strain or group of strains.

^bNumber of polymorphic marker bands generated by each different primer oligonucleotide.

^cRatio between the number of polymorphic products and the total number of bands appeared in each strain [21].

2.3. Analysis of the data

Photographs of agarose gels containing the different types of markers were digitised and analysed using the Fragment Analysis Program (Molecular Dynamics – Amersham Life Sciences, Amersham, Buckinghamshire, UK) to determine marker sizes. Markers produced by the same primer and present in different strains were assumed to be homologous if they had the same size and were equally amplified. Similarities between strains based on the presence/absence of molecular markers were calculated using the Squared Euclidean Distance and the resulting pair similarities were expressed in a distance matrix. The correlation between similarity matrices obtained for each marker was evaluated by means of the Mantel test [18]. Cluster analyses were performed using the UPGMA method and depicted as dendrograms, the closest two strains are those that show the highest similarity between them. All the statistical analysis was performed using the SPSS Base 8.0.1 Program (SSPS Inc., Chicago, IL, USA).

3. Results and discussion

A total of 66 10-mer oligonucleotides and three DFPs were used as primers to measure the diversity of different commercial varieties of *Agaricus*. Only 12 10-mer primer sequences and the three DFPs revealed some polymorphism between the *A. bisporus* strains (Table 1), although all of them were able to differentiate those of *A. bitorquis* (data not shown). The ratio of discriminant/non-discriminant 10-mer oligonucleotides (12/66, 18%) is the same as that obtained by Kerrigan et al. [7] in *A. bisporus* using RAPD markers (5/28, 18%). The number of amplified products generated with each primer varied among strains, and a total of 349 polymorphic bands were amplified (Table 1). DFPs were more efficient in the generation of discriminant bands (105 with three DFPs) than the 10-mer oligonucleotides (244 with 12 primers), and the number of discriminant markers detected in *A. bisporus* was lower than that obtained in *A. bitorquis* (132 and 217, respectively). The higher number of discriminant markers present in *A. bitorquis* and the overall higher variability of this species can be explained on the basis of its heterothallic life cycle whereas it is homothallic in *A. bisporus*. Weising et al. [19] reported that the level of polymorphism exhibited by a particular probe within a particular species depended on the reproductive strategy exhibited by (or forced upon) the species under investigation (selfing, outcrossing, vegetative propagation, inbreeding), and on the molecular properties (mutation rate) of the target sequence and its surroundings.

The relationship of similarity between the *Agaricus* strains and species studied in this work was analysed using the Quadratic Euclidean Distance Coefficient, which generates distance matrices useful as input to cluster the different strains on the basis of their similarity (Fig. 1).

A. bisporus strains clustered together in a group separated from the *A. bitorquis* strains analysed. It could be seen that strains Gure-15, Gure-55, Pla-814, and Fung-25 were differentiated despite they were described as the same strain by the spawners. Otherwise, the non-hybrid strains Gure-300 and Gure-OX were the most different *A. bisporus*, and clustered independently.

The close genetic relationships between commercial strains marketed as different products have also been described by other authors which showed that ‘new hybrid strains’ presented a low level of genetic polymorphism and a close similarity to the first hybrid strain released [6,10,11,20]. Our results show that strains with low weight such as Gure-15, Gure-55, Pla-814, and Fung-25 cluster together suggesting that they constitute re-isolates of the same hybrid commercial strain labelled as original products by different companies. The genetic differences detected in this study could be due to recombination or mutation produced during the propagation process which is the result of successive generations of intramixis [7].

To determine if the markers generated using either 10-mer oligonucleotides or DFPs as primers were equally powerful to visualise the genetic variability of *Agaricus*, strain similarity matrices were constructed based on either one of the two primer types. No significant differences were obtained between the two matrices (Mantel test $r=0.9425$, $P=0.0002$ [18]) suggesting that both primer types were equally discriminating tools.

The *Agaricus* strains studied in this paper can be classified into two groups according to the size of their fruit bodies: group A is formed by strains yielding mushrooms with 9 g average weight and includes the strains Pla-814,

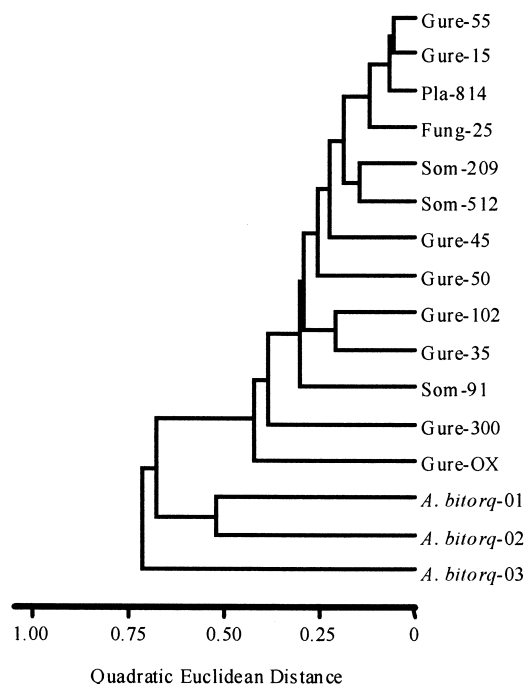


Fig. 1. UPGMA cluster of *Agaricus* strains based on the polymorphisms generated by the oligonucleotide primers described in Table 1.

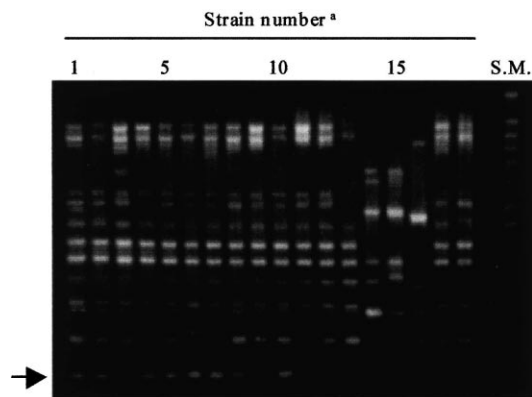


Fig. 2. RAPD markers generated using the oligonucleotide 4391 as primer. The strain numbers correspond to that indicated in Table 1. Strains 17 and 18 are Darlington-521 and LeChampion-229, respectively. S.M., size markers. The arrow indicates the band of 300 bp present in strains belonging to the group of low 'mushroom weight'.

Gure-15, Gure-35, Gure-102, Gure-300, Som-91, and Fung-25; whereas group B is constituted by strains with mushrooms of about 14–15 g average weight and includes the *A. bisporus* strains Gure-45, Gure-50, Som-209, Som-512, and Gure-OX, and the three *A. bitorquis* strains (*A. bitorq-01*, *A. bitorq-02* and *A. bitorq-03*). Two new strains (Darlington-521 and LeChampion-229) whose mushrooms had an average weight of 14–15 and 9 g, respectively, were used as control. With the purpose of finding molecular markers linked to the trait 'mushroom weight' the bulked segregant analysis strategy developed by Michelmore et al. [17] was applied. Pooled DNAs belonging to classes A and B, respectively, were used as templates for amplification reactions using 66 primers, and it was found that primer 4391 was able to differentiate both groups (Fig. 2) because it produced a fragment of about 300 bp present in pool A and absent in pool B. Furthermore, the critical band was present in all the strains grouped in class A and absent in all the strains belonging to group B. However, more studies should be done in other strains with different pilei sizes to corroborate this finding.

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