http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 11 (14): 1771-1778, 2008
ISSN 1028-8880

Comparison of Random Amplified Polymorphic DNA Markers and Morphological Characters in Identification of Homokaryon Isolates of White Button Mushroom (*Agaricus bisporus*)

H.R. Kavousi, M. Farsi and F. Shahriari
Department of Biotechnology and Plant Breeding, College of Agriculture,
Ferdowsi University of Mashhad, Iran

Abstract: The secondarily homothallic life cycle of the white button mushroom that results in scarcity of uninucleate basidiospores (homokaryons) in its progeny, is the most important impediment for genetic improvement of the commercial strains. Identification of homokaryons for breeding programs of *Agaricus bisporus* (button mushroom) is, therefore, crucial. Verifying homokaryons through fruiting trial is time consuming and unreliable. In this study, ability of RAPD markers, compared to morphological characters for identification of homokaryon isolates, was investigated. Based on morphological characters, 42 isolates were screened and exposed to RAPD markers. The results showed that RAPD markers could discriminate homokaryons from heterokaryons, based on number of bands generated. The numbers of band in homokaryons were significantly less than those of heterokaryons. Results also showed that cluster analysis, based on average of band number generated, could separate homokaryon from heterokaryon isolates. It is suggested that RAPDs could be used to identify hyomokaryons from heterokaryons for breeding program of *A. bisporus*.

Key words: Secondarily homothallic, homokaryon, RAPD markers, white button mushroom

INTRODUCTION

Agaricus bisporus (Lange) Imbach, is a cultivated mushroom, produced on a large commercial scale with an annual value of about US $2 billion worldwide (Xu *et al*., 2002). Despite the economic importance of *A. bisporus*, in comparison with other vegetable crops and to other commercially important filamentous fungi, little effort has been made to improve genetics of the strains used for commercial production (Horgen and Anderson, 1992). One of the major reasons for the lack of a traditional genetic improvement program for *A. bisporus* is the secondarily homothallic nature of its life cycle (Khush *et al*., 1995). The majority of basidiomycetous species have four spored basidia, with each basidiospore receiving only one of the four post meiotic nuclei. However, due to secondarily homothallic life cycle of *A. bisporus*, 90% or more of the basidia lining the gills at fruit bodies are predominantly two-spored, with each spore receiving two of the four post meiotic nuclei. This cause most of spores to be heterokaryotic and self-fertile and retain the heterokaryotic multilocus parental genotype (Kerrigan *et al*., 1993; Song *et al*., 2000; Xu *et al*., 2002; Callac *et al*., 2003). Thus uninucleate basidiospores (homokaryons) as a prerequisite for genetic analysis and selective breeding of cultivated mushroom are uncommon in its life cycle (Callac *et al*., 1993). In addition, both homokaryons and heterokaryons are multinucleate and there is no direct observable criterion such as the presence or absence of clamp connection that would permit the reliable sorting of single spore isolates (SSIs) by their effective ploidy level (n vs. n+n) (Khush *et al*., 1995).

Although small portions of basidiospores in *A. bisporus* are uninucleate, the isolation of haploid homokaryon from SSIs is an extremely laborious task. Traditional method to verify a homokaryon through the fruiting trial is unreliable and time-consuming (Horgen and Anderson, 1992).

Other methods for screening homokaryons have relied upon the morphology type and growth rate. In general, although colony morphology has been identified as a reliable indicator of the nuclear status of a strain and sorting diverse meiotic offspring, the mycelium of homokaryotic strain is commonly less vigorous and grows more slowly than that of heterokaryotic strain (Song *et al*., 2000). Breeding approaches involving auxothrophy and dominant fungicide resistance markers have been used to distinguish homokaryons from heterokaryons of *A. bisporus*. However, auxothrophes of *A. bisporus*, as a

Corresponding Author: H.R. Kavousi, Department of Biotechnology and Plant Breeding, College of Agriculture, Ferdowsi University of Mashhad, Iran Tel: +985117686549 Fax: +985118788805

result of its multinucleate hyphae, are difficult to isolate and fungicide resistant lines of *A. bisporus* have not yet resulted in new, improved strains (Khush *et al*., 1995).

The first genetic test for homokaryon that become available to the breeder was isozyme analysis (Khush *et al*., 1995). However, isozyme analysis in *A. bisporus* was restricted to about ten useful loci (Khush *et al*., 1995). In the mid 1980s more powerful DNA-based markers, restriction fragment length polymorphism (RFLP), were introduced (Botstein *et al*., 1980). Use of RAPD method in *A. bisporus* was firstly reported by Khush *et al*. (1992). In this experiment, homokaryon displayed a subset of the parental bands and lacked amplification products at multiple loci, but heterokaryotic isolates were heteroallelic at all of the parental loci (Khush *et al*., 1992). Use of RAPD markers in screening homokaryons have been reported by several workers. (Kerrigan *et al*., 1993; Loftus *et al*., 1995; Song *et al*., 2000; Moore *et al*., 2001). The main aim of this study was identification and isolation of homokaryon isolates for breeding programs of white button mushroom in IRAN. The ultimate goal in this program is the identification of parental homokaryons through RAPD markers for hybridization programs to produce high yielding strains.

MATERIALS AND METHODS

Mushroom strains: Two local strains of *A. bisporus* (130 and A15) and two strains received kindly from California State University (MC370 (U3) and MC378 (U1)) were used in this study at Ferdowsi University of Mashhad.

Germination of basidiospores: Basidiospores were suspended in sterile distilled water and counted with a haemocytometer. Spore concentrations were adjusted to 10^5 spores mL^{-1}. About 20000 spores were inoculated onto the PDA medium and incubated at 25°C. Germination routinely occurred in the presence of 25 μg mL^{-1} streptomycin. Basidiospores germination was controlled with microscopic observation and the colony of each SSIs transferred onto new PDA medium in sterile condition and incubated at 25°C.

Selection of isolates: After 30 days of SSIs incubation, colony diameter and morphology type of each isolate was determined. Based on growth rate, isolates were divided into four classes including: very slow (<30 mm), slow (31-45 mm), fast (45-60 mm) and very fast (>60 mm) growing. For morphology type, five classes were determined: strandy, cottony, fluffy, appressed and spotty (very slow growth). Based on these criteria 62 isolates were selected for further experiments.

Fruiting trial: Pure SSIs cultures were transferred to 80 mL volumes of sterile cooked wheat grain, buffered with 5% $CaCO_3$ (dry weight). Colonized grains (40 g) were used to inoculate plastic bags containing 2 kg of commercial compost, a complex, biologically modified, straw-based substrate. Pasteurized soil was used as the casing material. Case-run, initiation and maturation all occurred in controlled conditions (Callac *et al*., 1998). Two replications were used for each isolate. The isolate MC395-21 used as control.

Preparation of genomic DNA: Genomic DNA for RAPD reactions was isolated using a modified method of Dellaporta *et al*. (1983) from mycelial culture growing in solid PDA medium (Song *et al*., 2000). DNA concentration was determined by spectrophotometer (Genova MK3), following the procedures supplied by the manufacturer. Agarose gel electrophoresis also was carried out for DNA quantity and quality analysis. For use in PCR, the DNA was diluted with TE buffer (10 mM Tris-HCl, pH, 8.0, 0.1 mM EDTA) to 50 ng μL^{-1} and stored at 4°C until used for RAPD analysis.

RAPD reaction: RAPD reaction conditions were essentially as described by Williames *et al*. (1990). PCR cycles consisted of initial denaturation at 94°C for 4 min, followed by 35 cycles of amplification, each having denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 2 min. A final extension step at 72°C for 10 min was followed by termination of the cycle at 4°C. Following amplification, PCR products (10 μL) were loaded in 1.2% agarose gel and separated by electrophoresis at 75 V for about 3 h. RAPD fragments were stained with ethidium bromide and photographed on UV photo documentation system.

Data analysis: Stained gels analyzed by Labwork and number of bands and their molecular weight of all isolates were calculated. Cluster analysis based on morphological characters and RAPD markers carried out by STATISTICA ver.5.5 using UPGMA method.

RESULTS

Fruiting trial: Out of 62 SSIs, 42 of them fruited in both replication and the rest isolates did not fruit and considered as putative homokaryons. However, among the 20 putative homokaryons, the isolate 130-7 produced a mature fruit in one replication that was morphologically abnormal and had a loose stipe. The A15-8 isolate produced a few pinheads (primordia), but none of them grew to mature fruit (Fig. 1).

Use of morphological characters in screening homokaryon:

- Based on growth rate, 75% of putative homokaryon isolates laid in very slow and slow growth classes and none of them had more than 60 mm colony diameter after four weeks (Fig. 2). On the other hand, 88% of fertile isolates laid in fast and very fast growth classes and the colony diameter of all fertile isolates were more than 30 mm (Fig. 2). Although there was an obvious difference in the distribution of growth rate classes among heterokaryon and homokaryon SSIs on PDA medium, however, there was an overlap in the classes of growth rate, such that both putative homokaryon and heterokaryon isolates were found in slow and mid growth class.
- Based on morphology type, 95% of putative homokaryons were appressed and spotty (very slow growth type), but one isolate was strandy (Fig. 3). Among heterokaryon isolates more than 65% were allocated in strandy class, however, a number of these isolates had appressed and spotty (very slow growth type) morphology type (Fig. 3). Thus, based on this criterion, an overlap between isolates was clearly observed.

Grouping of isolates based on morphological characters: Results of cluster analysis showed that based on morphological characters putative homokaryon and heterokaryon isolates could not be separated from each other (Fig. 4).

Results of RAPD markers: In each strain RAPD profiles of heterokaryons and putative homokaryons, generated by 6 primers, were compared. For each primer, heterokaryon isolates nearly displayed identical amplification products and the putative homokaryotic isolates from each strain displayed a subset of parental bands. This is illustrated for amplification products produced by primer OPM-02 in strain MC370 (Fig. 5). It is evident that this primer amplified nine heteroallelic loci in parental strain. Table 1 summarizes the segregation results for these nine loci among 14 SSIs of this strain.

Three SSIs, 1, 20 and 100 did not generate any band at multiple loci and these isolates had also showed slow growth and therefore probably represent either homokaryon arising from uninucleate basidiospores or heterokaryons composed of post meiotic sister nuclei.

Fig. 1: Difference between homokaryon (A15-5) and heterokaryon (A15-16) fruiting trial

Fig. 2: Colony diameter frequency in heterokaryon (■) and homokaryon (□)

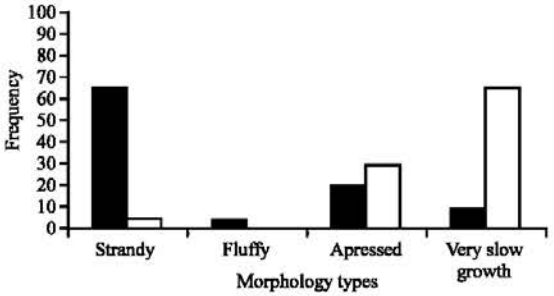

Fig. 3: Morphology type frequency in heterokaryon (■) and homokaryon (□)

As described by Summerbell *et al*. (1989) a single crossover between any heteroallelic locus in *A. bisporus* heterokaryon and its centeromer will result in a homoallelic genotype in half of the progeny that receive post meiotic nonsister nuclei. Since RAPDs are dominant genetic markers, however, only 50% of these homoallelic individuals-those are homoallelic for the absence of an amplification product- will be recognizable. An example of a crossover proximal to a RAPD locus was observed in some fast grower isolates, which lacked one or two amplification product (s). The rest of the SSIs, similar as parental strain displayed amplification products at all of

Table 1: The loci amplified by primer OPM-02 in isolates of MC370 strain

Amplification locus size (bp)	Presence of amplification products in isolates														Parental strain
	1	2	20	27	34	37	42	43	48	53	56	57	77	100	
OPM-02-1	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+
OPM-02-2	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+
OPM-02-3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPM-02-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPM-02-5	-	-	-	-	+	-	-	-	+	-	+	+	+	-	+
OPM-02-6	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+
OPM-02-7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OPM-02-8	-	+	-	+	+	+	+	+	+	+	+	+	+	-	+
OPM-02-9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth rate class	1	3	1	3	3	3	3	3	4	4	3	4	4	1	4
Fertility	NF	F	NF	F	F	F	F	F	F	F	F	F	F	NF	F

1: Very slow, 2: Slow, 3: Fast, 4: Very fast, + = Presence of band, - = Absence of band, F: Fertile, NF: Non-Fertile

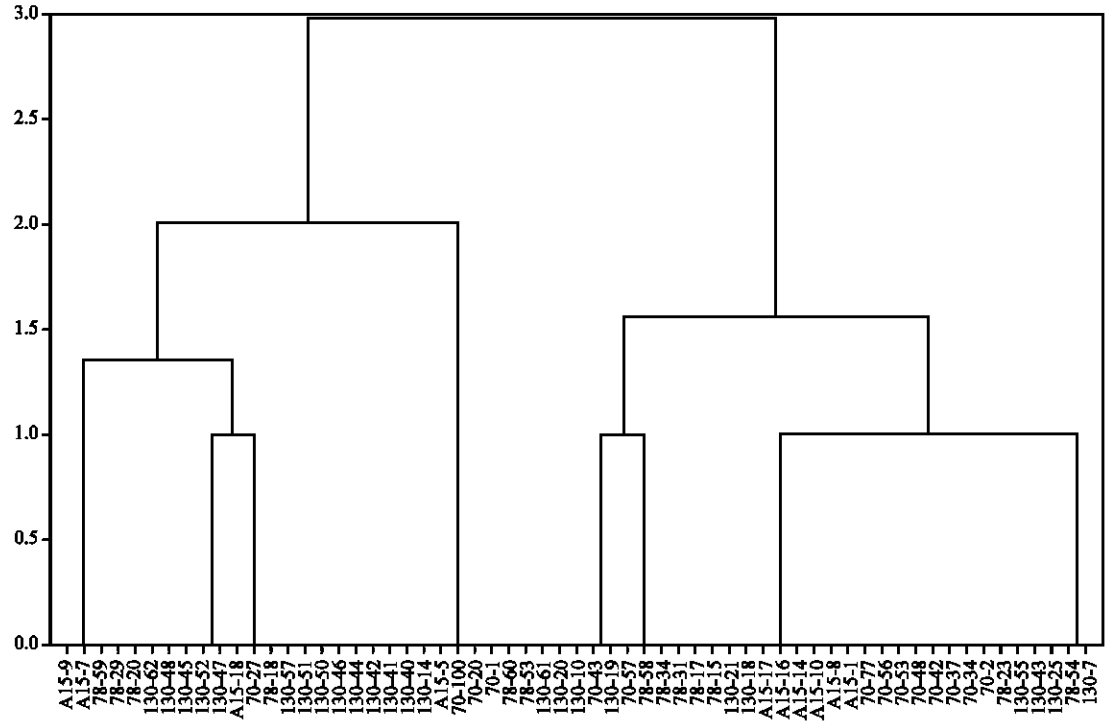

Fig. 4: Cluster of 62 isolates of *A. bisporus* based on morphological characters using Euclidean distance

nine loci. However in amplification products produced by using OPM-02 primer in strain MC378, no crossover observed and all of heterokaryon isolates were heteroallelic at all parental loci (Fig. 6).

In RAPD markers for each primer, the number of bands in putative homokaryons significantly reduced compared to those of heterokaryons. Average of band number per primer in putative homokaryon isolates were less than 6 and in heterokaryon isolates were more than 6 bands.

Cumulative frequency graph of average of band number showed that 90% of putative homokaryons possessed average band of 5.5 or less (Fig. 7), but 90% of heterokaryon had an average of six bands or more (Fig. 8).

Grouping of SSIs by RAPD markers: Cluster analysis based on average of band number could separate homokaryon and heterokaryon isolates in two distinct groups. Clustering showed homokaryon isolate 130-7 and heterokaryon isolate 130-14 possessed the greatest distance and heterokaryon isolate 130-62 and heterokaryon isolate 130-18 had the least distance (Fig. 9).

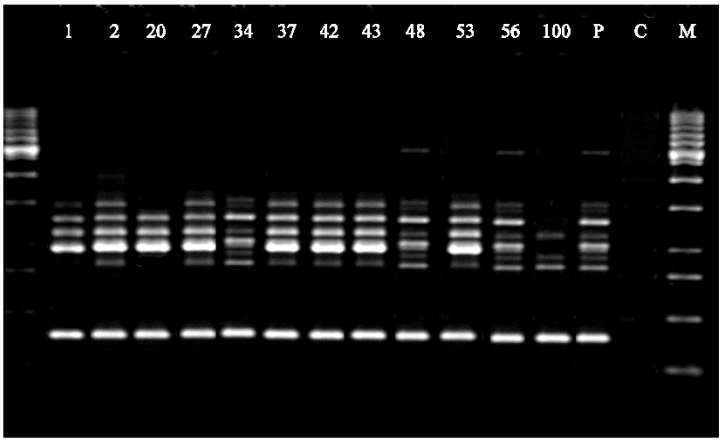

Fig. 5: RAPD pattern of MC370 isolates by OPM-02 primer. M = 1 kb ladder (MBI Fermentas), P = Parental strain, C = Negative control

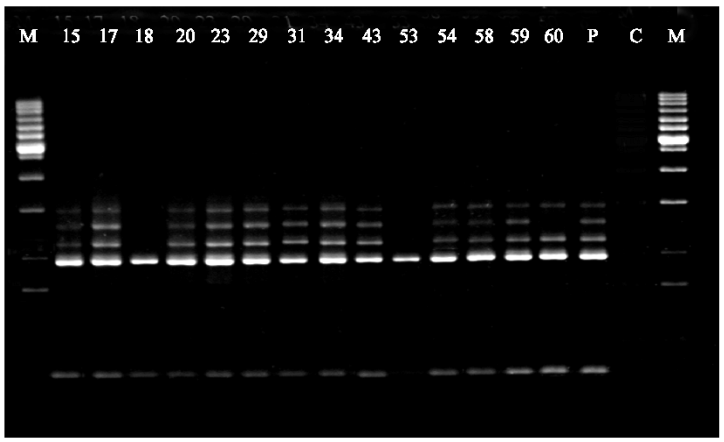

Fig. 6: RAPD pattern of MC378 isolates by OPM-02 primer. M = 1 kb ladder (MBI, Fermentas), P = Parental strain, C = Negative control

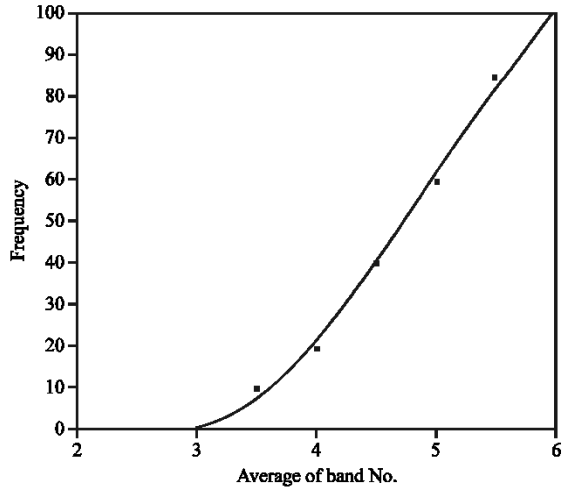

Fig. 7: Cumulative frequency graph of average band number in homokaryon isolates

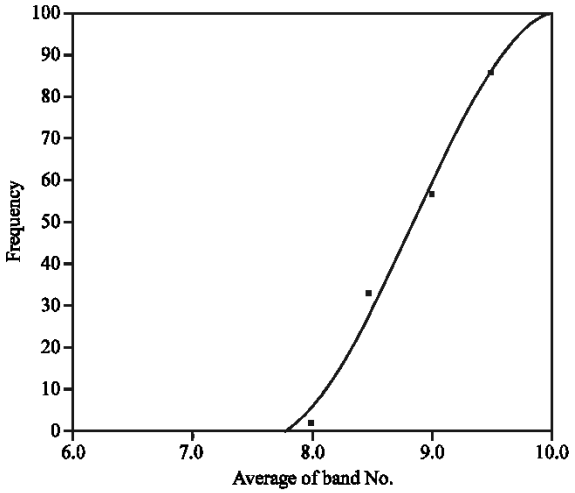

Fig. 8: Cumulative frequency graph of average band number in heterokaryon isolates

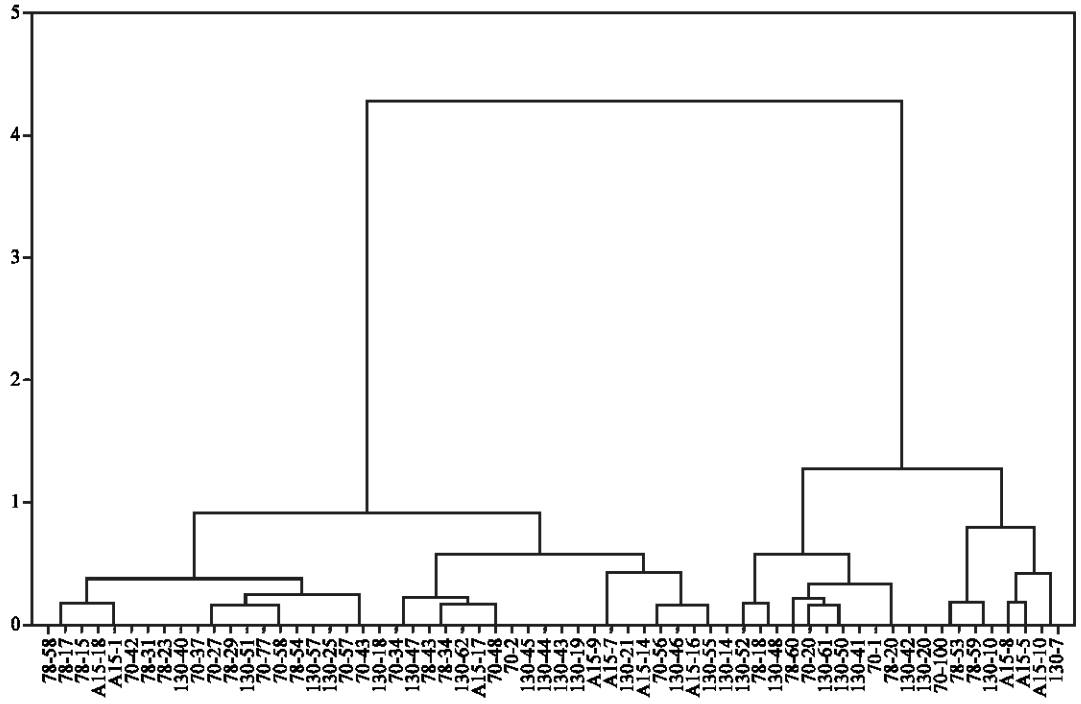

Fig. 9: Cluster of *A. bisporus* isolates based on average of RAPD band numbers using Euclidean distance

DISCUSSION

A distinctive characteristic of *Agaricus bisporus* that so far has defined the species is that virtually all known strains predominantly produce only two spores on each basidium. Following meiosis in a typical two-spored basidium of *A. bisporus*, each spore receives two nuclei, both are necessary for fertility. As a result, most spores of this species of mushroom produce fertile heterokaryotic progenies (Callac *et al*., 1993).

Genetic analysis and selective breeding of cultivated mushrooms however require the isolation of homokaryon from heterokaryotic stocks. In this study, it was assessed the potential of RAPD markers and the results showed that two ploidy classes of SSI offspring were indistinguishable with regard to colony morphology and growth rate. However, with elimination members of growth rate and morphology type classes that unlikely to contain homokaryons from further, screening is more efficient. This may result that process of isolation bias to homokaryon isolates. As a result, approximately one third of isolates that selected on the basis of morphological characters (20 out of 62 isolates), re-confirmed as homokaryons by RAPD markers. These results showed the relative efficiency of morphological markers in increasing of homokaryon isolation. However, colony morphology and growth rate has been identified as reliable indicators of nuclear status of a strain, although, the mycelium of homokaryotic isolates is commonly less vigorous and grows more slowly than of those heterokaryotic isolates (Horgen and Anderson, 1992; Kerrigan, 1990; Khush *et al*., 1995).

The limited genetic manipulation of *A. bisporus* to date and ambiguity surrounding its sexual life cycle underscore the necessity of stable and simple markers for search a commercially important organism. As previously shown for a range of species from bacteria to human (Welsh and McClelland, 1990; Williams *et al*., 1990) single, arbitrary 10 bp oligonucleotids are capable of amplifying multiple fragments of *A. bisporus* genome and variations in primer binding sites between isolates result in amplification polymorphisms that function as genetic markers (Khush *et al*., 1992).

In the present results, few RAPD band that were present in parents and all offspring (homokaryons and heterokaryons) and other RAPD bands which only were found in parents and were absent in some offspring. When many of these bands were missing, the offspring considered as a homokaryon. When none or a few band were missing, the offspring considered as a heterokaryon.

The existence of monomorphism among the isolates reflects the lack of genetic diversity. Available evidence also indicates a narrow genetic base for this species

(Khush *et al*., 1995). This is in part due to the inbreeding nature of commercial *A. bisporus* secondarily homothallic life cycle (Kerrigan *et al*., 1993; Summerbell *et al*., 1989) termed intramixis by Kerrigan (1990). This life cycle, characterized by reduced meiotic recombination accompanied by packaging of complementary post meiotic nuclei into spores, result in maintenance of parental genotype in spore progenies (Calvo-Bado *et al*., 2001). Present results showed that most of heterokaryon isolates were heteroallelic in all of parental loci and crossover events were rare.

Recessive, deleterious alleles are likely to accumulate and mask under the perpetual heterokaryosis of the secondarily homothallic life cycle. The slower growth rate of homokaryons may be, in part, a manifestation of this genetic load (Kerrigan *et al*., 1993).

Based on our study, the degree of polymorphism of RAPD pattern decrease in non-fertile homokaryotic isolates compared to that of either their corresponding parental strains or their heterokaryons. Similar phenomenon had been found in those studies using isozyme or RFLPs (Castel *et al*., 1987). Fewer band for homokaryons seems in agreement with their reduced genomic complexity. In general, distinct RAPD profiles were visible in our *A. bisporus* progenies and such profile could be used to differentiate between homokaryons and heterokaryons.

The average number of spores per basidium in *A. bisporus* varies somewhat among different strains and may also vary slightly at different developmental stage of a given strain (Callac *et al*., 1998; Kerrigan, 2000). The number of spores per basidium is primarily determined by the BSN locus linked to the mating type locus (MAT) on chromosome I. The Bsn-t allele (tetrasporic basidia) is dominant with respect to the Bsn-b allele (bisporic basidia) with variable penetrance. By introducing Bsn-t allele into heterokaryons, highly recombined haploid progeny could be obtained from crosses between bisporic and tetrasporic strains (Kerrigan, 2000). A few years ago, tetrasporic strains from desert population in California, *Agaricus bisporus* var. *burnatti* and from French field strains of *A. bisporus* were identified. In these strains more than 90% of basidia produce homokaryotic basidiospores with gametic function (Callac *et al*., 1998; Kerrigan *et al*., 1993). The discovery of these strains and the demonstration of interfertility between bisporic and tetrasporic lineage expand breeding programs on this important species and improve the obtaining homokaryons from the strains of *A. bisporus* in much greater proportion than they are typically found (Callac *et al*., 1996).

REFERENCES

Botstein, D., R.L. White, M. Skolnick and R.W. Davis, 1980. Construction of a genetic linkage map using restriction fragment length polymorphisms. Am. J. Hum. Genet., 32: 314-331.

Callac, P., M. Imbernon and R.W. Kerrigan, 1996. The Two Life Cycles of *Agaricus bisporus*. In: Mushroom Biology and Mushroom Products, Royse, D.J. (Ed.). University Park, Pennsylvania, USA, ISBN: 1-883956-01-3, pp: 57-66.

Callac, P., S. Hocquart, M. Imbernon, C. Desmerger and J.M. Oliver, 1998. Bsn-t alleles from french field strains of *Agaricus bisporus*. Applied Environ. Microbiol., 64: 2105-2110.

Callac, P., I.J. de Haut, M. Imbernon, J. Guinberteau, C. Desmerger and I. Theochari, 2003. A novel homothallic variety of *Agaricus bisporus* comprises rare tetrasporic isolates from Europe. Mycologia, 95: 222-231.

Calvo-Bado, L.A., M.P. Challen, C.F. Thurston and T.J. Elliott, 2001. RAPD characterisation of heterogeneity in spore progenies and sexuality in the genus *Agaricus*. Mycol. Res., 105: 370-376.

Castel, A.J., P.A. Horgen and J.B. Anderson, 1987. Restriction fragment length polymorphism in the mushroom *Agaricus brunnescens* and *Agaricus bitorquis*. Applied Environ. Microbiol., 53: 816-822.

Dellaporta, S.L., J. Wood and J.B. Hicks, 1983. A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep., 1: 19-21.

Horgen, P.A., and J.B. Anderson, 1992. Biotechnology and Edible Mushrooms. In: Biotechnology and Filamentous Fungi, Finkelestein, D. and C. Ball (Eds.). Butter Worth, Boston, USA., ISBN: 0750691158, pp: 447-462.

Kerrigan, R.W., 1990. Evidence fore genetic in two population of *Agaricus bisporus*. Mycol. Res., 94: 721-733.

Kerrigan, R.W., 2000. A Brief History of Marker Assisted Selection in *Agaricus bisporus*. In: Science and Cultivation of Edible Fungi, Van Griensven, L.J.L.D. (Ed.). Balkema, Roterdam, The Netherlands, ISBN: 90 5809 143 0, pp: 183-189.

Kerrigan, R.W., D. Carvalho, P.A. Horgen and J.B. Anderson, 1993. Meiotic behaviour and linkage relationship in the secondarily homothallic fungus *Agaricus bisporus*. Genetics, 133: 225-236.

Kerrigan, R.W., P. Callac, M. Imbernon, C. Billet and J.M. Oliver, 1994. The heterothallic life cycle of *Agaricus bisporus* var. *burnettii* and inheritance of its tetrasporic triat. Exp. Mycol., 18: 193-210.

Khush, R.S., E. Becker and M. Wach, 1992. DNA amplification polymorphisms of the cultivated mushroom *Agaricus bisporus*. Applied Environ. Microbiol., 58: 2971-2977.

Khush, R.S., M.P. Watch and P.A. Horgen, 1995. Molecular Strategy for *Agaricus* Breeding. In: The Mycota, Vol. II. Genetics and Biotechnology, Kuck, U. (Ed.). Springer- Verlag, Berlin- Heidellberg, Germany, ISBN: 3540427708, pp: 321-337.

Loftus, M.G., S.C. Lodder and E.J. Legy, 1995. Molecular Mushroom Breeding. In: Science and Cultivation of Edible Fungi, Elliott, T.J. (Ed.). Balkama, Roterdam, The Netherlands, ISBN: 90 5410 570 4, pp: 3-9.

Moore, A.J., M.P. Challen, P.J. Warner and T.J. Elliott, 2001. RAPD discrimination of *Agaricus bisporus* mushroom cultivars. Applied Microbiol. Biotechnol., 55: 742-749.

Song, S., W. Su, W. Zeng and Z. Wang, 2000. RAPD Analysis of Species Diversity and Genomic Differences in *Agaricus bisporus*. In: Science and Cultivation of Edible fungi, Van Griensven, L.J.L.D. (Ed.). Balkema, Roterdam, The Netherlands, ISBN: 90 5809 143 0, pp: 191-200.

Summerbell, R.C., A.J. Castle, P.A. Horgen and J.B. Anderson, 1989. Inheritance of restriction fragment length polymorphisms in *Agaricus brunnescens*. Genetics, 123: 293-300.

Welsh, J. and M. McClelland, 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res., 18: 7213-7218.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 6531-6535.

Xu, J., C. Desmerger and P. Callac, 2002. Fine-scale genetic analyses reveal unexpected spatial-temporal heterogeneity in two natural populations of the commercial mushroom *Agaricus bisporus*. Microbiology, 148: 1253-1262.