JOURNAL OF PURE AND APPLIED MICROBIOLOGY, December 2012. Vol. **6**(4), p. 1841-1848

RAPD Marker Assisted Development of Improved Strains of *Agaricus bisporus* (Lange) Sing

Shivani Chauhan[1], Shammi Kapoor[2] and Saneel K. Thakur[3]

[1]Department of Pharmacology and Toxicology, College of Veterinary and Animal Sciences, CSKHPKV, Palampur - 176 062, India.
[2]Department of Microbiology, Punjab Agricultural University, Ludhiana, Punjab - 141 004, India.
[3]Department of Chemistry, Sri Sai University, Palampur - 176 062, India.

(Received: 18 October 2012; accepted: 30 November 2012)

Agaricus bisporus*, commonly known as the button mushroom, is the most widely cultivated species of edible fungi. Many polymorphic molecular markers have been exploited for strain identification in wild and commercial strains of this mushroom. The aim of this study was to develop hybrid strains using random amplified polymorphic DNA (RAPD) markers. For this purpose *A. bisporus* germplasm consisting of nineteen *Agaricus bisporus* parental strains (P-1, U-3, S-11, CM-1 to CM-16) were screened for yield, quality and morphological parameters. Out of these, ten parental strains were selected (P-1, U-3, S-11, CM-5, CM-7, CM-8, CM-10, CM-13, CM-14 and CM-16) which were found to be better than others. They were further analyzed for degree of divergence among them through RAPD analysis. They were found to possess similarity coefficient ranging between 0.73 to 0.98. These ten strains were used for spore collection. From spores germinated on Lambert's medium more than 500 single spore isolates (SSI's) were isolated. These SSI's were put to fruiting test out of which only 22 SSI's did not show fruiting and were selected for intra-crossing for development of hybrid. Out of 17 crosses made only 5 showed compatibility between monokaryons *i.e.* resulted in hybrid development. Five *A. bisporus* parental strains , their ten single spore isolates (SSIs) and five hybrid strains so obtained were further assessed for their similarity using 10 single 10bp primers, producing 252 scorable and 81 polymorphic bands. UPGMA clustering analysis showed three major phylogenetic clusters. First and second cluster comprised of 8 strains showing intra-cluster variation, third cluster had four genetically distinguishable strains. Germplasm strains mostly had strandy/dense colonies on Potato Dextrose Agar and the strains differ significantly in mycelium growth rate. However, these environmentally variable morphological traits could not be utilized as sole criteria for establishing strainal identities. Hence, the RAPD profile generated in this study along with morpho-physiological description could be used as a reference for identification of *A. bisporus* strains.

Key words: *Agaricus bisporus*, Genetic diversity, RAPD analysis, Molecular marker.

Agaricus bisporus (Lange) Sing. popularly known as white button mushroom, is a commercially cultivated mushroom with rich nutrition, high economic value and ecological significance. It belongs to the genus *Agaricus*, family *Agaricaceae*, order *Agaricales* and phylum *Basidomycota. A. bisporus* is one of the most widely cultivated mushrooms, with annual world-wide production of 2 million tonnes with a value of £3 billion (US$6.1 billion). The genome research benefits the growers and consumers through identification of improved quality traits (http://www.greencarcongress.com/2007/07/mushroom-genome.html). Hybrid development is one of the

* To whom all correspondence should be addressed.
Mob.: +91-9736501212;
E-mail: hod.as@srisaiuniversity.com

most efficient methods of creating variability by combining desirable traits of genetically diverse strains or isolates into a new heterokaryon. A well directed hybrid breeding programme essentially consists of

i) selection of genetically diverse parental lines
ii) isolation of homokaryons
iii) crossing of compatible homokaryons
iv) identification of hybrids
v) evaluation of hybrids.

With the availability and use of modern tools of molecular biology viz. allozyme[1], Restriction fragment length polymorphisms[2, 3] and Random amplified polymorphic DNA[4] for homokaryon isolation in *A. bisporus*, genetic improvement in button mushroom has gained momentum. RAPD markers are based on a modified form of the polymerase chain reaction which is used to amplify a specific region of the genomic DNA *in vitro*. Khush *et al.*[5] utilized RAPD markers for homokaryon identification and confirmation of hybrids generated by crossing two compatible homokaryons. RAPD markers were also used for separating cultivars of the button mushroom. Homokaryons carry a subset of the RAPD markers found in the parental heterokaryons whereas, Yadav *et al.,*[6] used RAPD markers for assessment of diversity of *A. bisporus* germplasm at DNA nucleotide level and identification of putative homokaryons among SSI's. Kavousi *et al.*[7] used RAPD markers for discriminating homokaryons from heterokaryons on the basis of number of bands generated. Although earlier improvement has been achieved on the basis of phenotypic selection from amongst the available cultivars but to sustain mushroom production on a long term basis productivity of strains needs to be improved with development of mushroom strains and hybrids showing better adaptation to climatic conditions. Therefore, the objective of the present study was to develop RAPD marker assisted improved strains in *Agaricus bisporus.*

MATERIALS AND METHODS

Source of cultures

Agaricus bisporus (Lange) Sing. Strains CM-1 to CM-16 and S-11, P-I, U3 were procured from different mushroom research laboratories of Northern India (Directorate of Mushroom Research, Solan and Department of Microbiology, PAU, Ludhiana, respectively). These cultures were grown on Potato dextrose agar (PDA) at 25±1°C for 15 days and then stored at 4°C.

Evaluation of strains

For the selection of potential parental lines with desirable traits, the strains were cultivated under the seasonal conditions. The data were recorded for the morphological characteristics of fruit bodies of all parental strains, including height of a fruit body (cm), pileus diameter (cm), stipe diameter (cm), stipe length (cm), pileus weight (g) and average weight of the fruiting bodies. For this study, 10 randomly selected fruit bodies from first flush of each strain were taken and recorded as mean of value.

Growth studies

Agaricus bisporus cultures were raised on potato dextrose agar (250g peeled and sliced potato boiled in 1 liter water for 30 minutes and filtered. To the filtrate 18g dextrose + 20g agar was added and volume made to 1 liter with water). Mycelial disc of standard size (5mm) cut from a culture slant was used to inoculate an agar plate. Five replicate plates were prepared per culture. The colony size was expressed as the linear diameters of the colony by measuring colony diameter at three lines perpendicular to each other. The mean of three lines served as diameter for measuring colony size.

Collection and germination of basidiospores

The spores were collected from strains, S-11, P-1, U-3, CM-1 to CM-16 in an indigenously constructed glass apparatus[8]. It consisted of inner half of a Petriplate (10cm diameter) with an inverted glass jar, cut out of a glucose bottle. A curved needle was fixed at its inner top to facilitate free hanging of the fruiting body. A round Whatman No. 1 filter paper was placed at the bottom for collecting spores as spore print. This apparatus was sterilized in an autoclave at 15psi for 20 min and then dried in hot air over at 60±5°C for 90 minutes before use. Normal, healthy, mature and unopened basidiocarp with intact veil were selected. The stipe was cut at the level of veil and the fruiting body was surface sterilized with absolute alcohol. The veil was cut open under sterile conditions and the hymenium was exposed. The fruiting body was then hanged in the spore

collection chamber from its pileus so that it did not touch any part of the apparatus or filter paper below. The whole set was incubated at 20±1$0^0$C until spores were shed. Thereafter, the glass jar was replaced with the sterilized lid of the Petriplate and the spore print was enclosed. All the spore prints were stored at 4-6$0^0$C in a refrigerator until germination trials were conducted.

Spore suspension preparation

Kumar's[9] technique was used for isolation of SSI's from the spore print of potential lines by serial dilution. For serial dilution, spore mass from the spore print was lifted on a sterile inoculation needle and serially diluted in 10ml sterile distilled water to obtain serial dilutions of 10^{-2}, 10^{-3} and 10^{-4}. For plating 0.5ml of the spore suspension was transferred to each Petri dish containing 8-10ml of solidified Lamberts medium and were spread using a sterilized glass spreader.

Isolation of single spore isolates

For single spore isolation, the germinated single spores were marked with ink marker under inverted microscope. These marked single spores were lifted with the help of a fine tip inoculation needle under the sterile environment of laminar air flow cabinet and transferred to PDA slants. These single spores were then incubated at 23±1$0^0$C in BOD incubator. After one week, the single spore isolates were observed for their growth.

Crossing of non-fertile SSI's

Different combinations between two monokaryons were made. The mycelial bits each of 4mm diameter of two different monokaryons were placed on sterilized PDA plate with the mycelium of the bits touching each other as well as agar medium of the Petri plate. These were incubated at 25±1$0^0$C for 10-15 days. Depending on their compatibility, the dikaryons were screened out by picking the part where the mycelium of the two showed fluffy growth at the junction. This cottony fluffy growth indicated possible dikaryon formation which was placed on fresh PDA slants and incubated at 25±1°C.

Molecular characterizaton using random primers

The following steps were used for RAPD-PCR technique for molecular characterization.

Raising of mycelial cultures

Pure mycelial cultures of all the monokaryons, hybrid dikaryons and their parent strains were maintained on PDA culture media in Petri plates. Further, each culture was raised in Malt extract Yeast extract Glucose broth for 4-5 week at 25±1°C. The mycelial mat was filtered and used for DNA extraction.

DNA extraction

Total genomic DNA was extracted as described by Graham *et al.*[10] Briefly, about 100mg fresh fungal mycelia were ground in 0.8mL Lysis buffer (200mM Tris-HCl, pH 8.0, 100mM NaCl, 25mM EDTA, and 0.5% Sodium dodecyl sulfate) and transferred into a 1.5mL tube. After incubation at 65$0^0$C for 1hr, 0.7mL 5% CTAB buffer (5% CTAB [w/v], 100 mM Tris-HCl , pH 8.0, 1.4M NaCl) was added, and the mixture was placed in a water bath at 65$0^0$C for 10 min. The solution was extracted with an equal volume of a mixture of phenol:chloroform:isoamyl alcohol (25:24:1, v/v), and then centrifuged at 12,000 rpm for 10 min, followed by extraction with chloroform:isoamyl alcohol (24:1, v/v) until the upper phase was clear. The upper phase was then transferred to a new tube and the DNA was precipitated with an equal volume of isopropanol. The precipitate was washed with 70% ethanol and pure ethanol, dried, and re-suspended in 50µL sterile water.

RAPD reactions

RAPD amplifications were performed as per Singh *et al.*[11] protocol. However, modifications were made to enhance the reproducibility and consistency in RAPD profiles. Amplifications were performed in 20µL of reaction volume with a thermal cycler (Biometra, model T -3000). The reaction components were: 2 µL 10X PCR buffer with 1.5mM $MgCl_2$, 4µL dNTP Mix (1mM each), 6µL 5µM each primer (Operon Biotechnologies, Germany), 2µL template DNA and 0.3µL Taq DNA polymerase (5U/ µL). The reaction mixtures were denatured at 94$0^0$C for 5 minutes and subjected to 45 cycles of 1 min at 94$0^0$C, 1 min at 37$0^0$C, 1 min at 72$0^0$C and a final extension step of 5 min at 72$0^0$C.

Gel analysis

Amplified DNA fragments were separated on a 1.5% agarose gel pre-stained with ethidium bromide solution using 0.5X TBE buffer (Tris base- 45 mM, Boric Acid- 45 mM and EDTA- 1mM). The gels were run for 3 hours at 5V/cm and RAPD products were visualized and recorded under UV light using photo documentation system.

Statistical analysis

The RAPD products were scored as

present (1) or absent (0) for each primer-genotype combinations. Data generated from 10 primers for 20 strains (including 5 parental strains, ten SSI's and five hybrids) were entered into a binomial matrix. The bivariate 0-1 data were analysed using SIMQUAL route to generate Jaccard's similarity coefficients using NTSYS-PC software version 2.02e.[12] These similarity coefficients were then used to construct dendrogram depicting the genetic relationship employing the Unweighted Paired Group Method of Arithmetic Averages (UPGMA) algorithm and SAHN clustering using NTSYS software.

RESULTS

Evaluation of parental strains

Nineteen *A. bisporus* parental strains (P-1, U-3, S-11, CM-1 to CM-16) were screened for yield, quality and morphological parameters. Out of these ten parental strains were selected (P-1, U-3, S-11, CM-5, CM-7, CM-8, CM-10, CM-13, CM-14 and CM-16) which were found to be better than others. They were further analysed for degree of divergence among them through RAPD analysis. They were found to possess similarity coefficient

Table 1. Summary of crossbreeding of single spore cultures

Parent strain	Monokaryon X Monokaryon	Compatible/ incompatible
P-1	A_{13} X A_{24}	Incompatible
	A_{13} X A_{52}	Compatible
	A_{24} X A_{52}	Incompatible
U-3	B_{12} X B_{20}	Incompatible
	B_{12} X B_{29}	Incompatible
	B_{20} X B_{29}	Incompatible
S-11	C_{19} X C_{20}	Compatible
CM-5	D_{21} X D_{38}	Incompatible
	D_{21} X D_{45}	Incompatible
	D_{38} X D_{45}	Incompatible
CM-7	E_{61} X E_{66}	Incompatible
CM-10	G_{19} X G_{57}	Compatible
CM-13	H_{11} X H_{36}	Compatible
CM-14	I_{42} X I_{59}	Incompatible
CM-16	J_{18} X J_{24}	Compatible
	J_{18} X J_{50}	Incompatible
	J_{24} X J_{50}	Incompatible

Table 2. Mycelial growth rate and colony morphology of parental strains of *A. bisporus*, their SSI's (2 each) and their respective hybrids

Strain No.	Growth rate (mm/day)	Colony morphology
P-1	1.96	Strandy mycelium
S-11	2.20	Thick strandy mycelium
CM-16	2.10	Strandy mycelium
CM-10	2.00	Normal dense mycelium
CM-13	2.00	Fine strand
A_{13} (SSI)	0.44	Dense mycelium
A_{52} (SSI)	0.50	Strandy mycelium
C_{19} (SSI)	0.88	Normal, dense mycelium
C_{20} (SSI)	0.94	Dense mycelium
J_{18} (SSI)	0.62	Strandy with aerial growth
J_{24} (SSI)	0.79	Dense mycelium
G_{19} (SSI)	0.55	Normal, dense mycelium
G_{57} (SSI)	0.43	Strandy with aerial growth
H_{11} (SSI)	0.96	Appressed mycelium
H_{36} (SSI)	1.01	Dense mycelium
SS1 (hybrid)	2.15	Fine strandy mycelium
SS2 (hybrid)	2.25	Normal dense mycelium
SS3 (hybrid)	2.20	Appressed mycelium
SS4 (hybrid)	2.10	Dense mycelium
SS5 (hybrid)	2.15	Appressed mycelium
CD (5%)	0.10	

ranging between 0.73 to 0.98. These ten strains were used for spore collection (unpublished data).

Collection and germination of Basidiospores

On the basis of the first experiment 8 parental strains (P-1, U-3, S-11, CM-8, CM-10, CM-13, CM-14 and CM-16) which performed better than the other strains on the basis of yield and 2 parental strains (CM-5 and CM-7) which performed better than the other strains on the basis of morphological characteristics (quality) of the fruit body were selected for single spore isolations. Basidiospores of these strains were collected on a sterile filter paper.

Isolation and characterization of single spore isolates

More than 500 SSI's were isolated from the selected parental lines viz. P-1, U-3, S-11, CM-5, CM-7, CM-8, CM-10, CM-13, CM-14 and CM-16. Out of these SSI's, only 100 SSI's were selected randomly and screened *in vitro* and characterized for mycelial growth rate and colony morphology on Potato dextrose agar medium. Colony morphology of different single spore isolates was observed after 10 days of growth at 23±1°C (unpublished data).

Fruiting test

The SSI's of 10 parental strains (CM-5, CM-7, CM-8, CM-10, CM-13, CM-14, CM-16, P-1, S-11 and U-3) were put to fruiting test. Out of these 100 SSI's only twenty-two SSI's (namely A_{13}, A_{24}, A_{52}, B_{12}, B_{20}, B_{29}, C_{19}, C_{20}, D_{21}, D_{38}, D_{45}, E_{61}, E_{66}, G_{19}, G_{57}, H_{11}, H_{36}, I_{42}, I_{59}, J_{18}, J_{24} and J_{50}) did not show fruiting. These SSI's were selected for intra-crossing for the development of hybrid.

Table 3. Primer sequence used for analysis of RAPD of *A. bisporus* along with number of fragments amplified

Primer	Primer sequence	Bands Amplified
OPA-1	5„-CAGGCCCTTC-3„	7
OPA-4	5„-AATCGGGCTG-3„	8
OPD-12	5„-CACCGTATCC-3„	11
OPP-6	5„-GTGGGCTGAC-3„	10
OPP-12	5„-AAGGGCGAGT-3„	9
OPP-13	5„-GGAGTGCCTC-3„	7
OPP-14	5„-CCAGCCGAAC-3„	6
OPP-15	5„-GGAAGCCAAC-3„	3
OPP-16	5„-CCAAGCTGCC-3„	7
OPP-19	5„-GGGAAGGACA-3„	13

Crossing of non-fertile SSI's

The non-fertile isolates were initially screened by their slow growth and finally confirmed by their inability to produce fruit bodies, 22 non-fertile SSI'S out of 100 SSI'S of *A. bisporus* were identified. These SSI'S were then crossed to develop hybrids. (Out of 17 crosses made only 5 crosses (A_{13} x A_{52}, C_{19} x C_{20}, G_{19} x G_{57}, H_{11} x H_{36} and J_{18} x J_{24}) showed compatibility between monokaryons *i.e* resulted in hybrid development (Table 1).

These hybrids were isolated, subcultured and characterized for mycelial growth rate and colony morphology along with their parents on Potato dextrose agar medium. Colony morphology of different hybrids was observed after 10 days of growth at 23±1°C. These hybrids were then put to cultivation trials.

Morphological characteristics

Differences in the mycelia characteristics of 20 germplasm including five parental strains, ten SSI's and five hybrids are summarized in Table 2. *A. bisporus* strains mostly produced strand/ dense or appressed mycelium growth. Significant differences were found in mycelium growth rate between strains. These differences may be attributed to the genetic constitution of individual strain. A perusal of Table 2 reveals that these twenty *A.bisporus* strains could be grouped into two categories- i) fast growers (e"2.0mm/day growth) that includes S-11, CM-16,CM-10, CM-13, SS1, SS2, SS3, SS4 and SS5 and ii) slow growers (<2.0mm/day growth) which includes P-1 and all the single spore isolates. In *A. bisporus* three types of mycelial growth pattern namely: fluffy, appressed and strandy have been reported.[13] Similarly, considerable differences in mycelial growth rates and morphological characteristics of SSI's of *A. bisporus* were also observed by other workers.[14, 15, 16, 17]

Molecular profiling

RAPD markers were used for molecular profiling of *A. bisporus* germplasm lines based on genetic relationship and molecular identity. The ten polymorphic primers from Operon Technologies Inc. were selected for PCR amplification on the basis of previous testing results of 16 primers with 4 strains of *A. bisporus*. RAPD reactions were performed twice to test the reproducibility of the profiles. All the primers amplified easily recognizable and consistent DNA

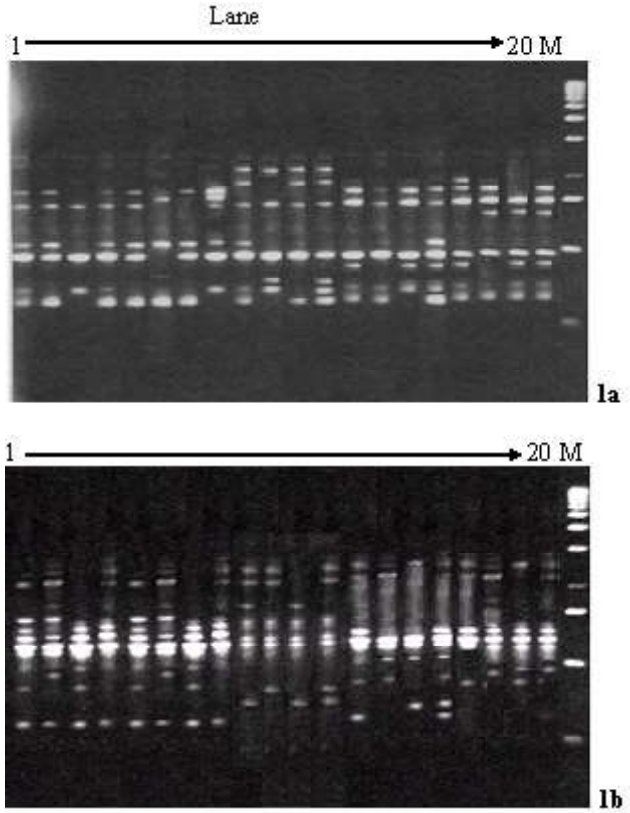
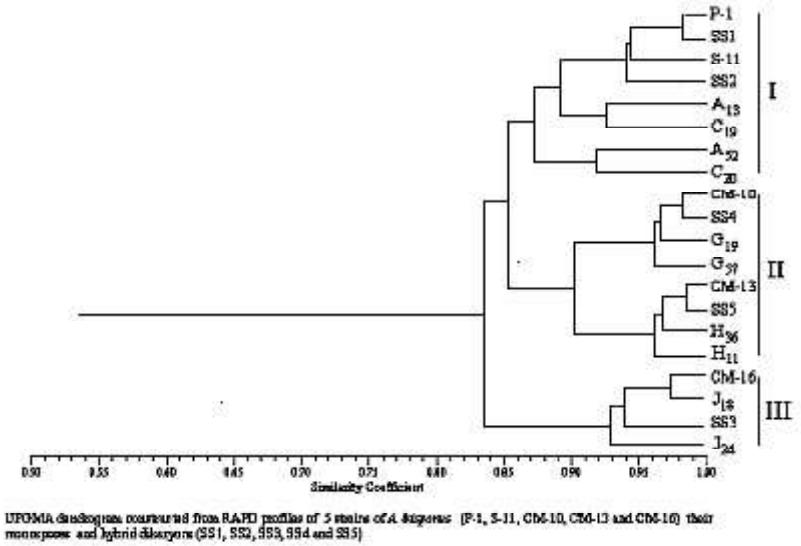


Plate 1a (primer OPA-1) and **Plate 1b** (primer OPA-4): RAPD Profile of hybrid dikaryons (SS1-SS5) in lane - 4, 8,12, 16 and 20, the parent strain (P-1, S-11, CM-16, CM-10 and CM-13) in lane - 1, 5, 9, 13 and 17) and their respective SSI's (A13, A52, C19, C20, G19, G57, H11, H36, J18 and J24) in lane - 2, 3, 6, 7, 10, 11, 14, 15, 18, and 19) where, M is 3 Kb DNA ladder



UPGMA dendrogram constructed from RAPD profiles of 5 strains of *A. bisporus* (P-1, S-11, CM-10, CM-13 and CM-16) their monospore and hybrid dikaryons (SS1, SS2, SS3, SS4 and SS5)

Fig. 1.

bands. A total of 81 RAPD markers were amplified (Table 3 and Plate 1a and 1b). The 10 primers amplified a total of 252 DNA fragments in 20 strains. Primer OPP-19 produced the most polymorphic electrophoretic pattern amplifying 13 marker bands, whereas primer OPP-15 amplified only three RAPD markers.

Genetic relationship

Genetic relationship of twenty *A. bisporus* accessions based on 81 RAPD markers are depicted in Figure 1. Cluster analysis using SAHN and UPGMA exhibited three major phylogenetic clusters. First cluster comprised of two genetically distinguishable strains, S-11 and P-1 their SSI's- A_{13}, A_{52}, C_{19}, C_{20} and respective hybrids-SS1 and SS2 showing intra-cluster variation. Second cluster also comprised of two strains, CM-10 and CM-13 their SSI's- G_{19}, G_{57}, H_{11}, H_{36} and respective hybrids-SS4 and SS5). They also showed intra-cluster variation. Third cluster had only one strain CM-16, its SSI's J_{18} and J_{24} and hybrid SS3. Cluster first and second consisted of white pileal strains whereas thirst cluster was that of brown pileal strain. DNA polymorphism in *A. bisporus* strains was also detected using RFLPs and RAPDs by earlier researchers.

DISCUSSION

The availability of uninucleate self-sterile homokaryons is a pre-requisite for producing hybrids in the conventional manner. However, homokaryons are difficult to obtain by conventional basidiospore isolation from *A. bisporus* strains because of secondary homothallic life cycle.[18] Similarly the lack of any morphological distinguishing features, such as a clamp connection between homokaryons and heterokaryons, remains a problem, as heterokaryosis can only be confirmed by time-consuming fruiting trials. Hence, a molecular-based method for distinguishing homokaryons from heterokaryons is required. Efforts to breed new strains of the cultivated mushroom *A. bisporus* have been hampered by the rarity of the genetic markers that are necessary for a controlled breeding program. The genetic diversity in the crop is very limited and also, the haploid, monokaryotic propagules are rare.

In the present study the genetic diversity among white and brown pileal strains was studied and were grouped into two categories- white and brown. A high degree of genetic diversity was found between white and brown strains with an average diversity of 46.1%.[6] Similar studies were also carried out earlier. [11, 19, 20, 21] Thus, the results of the present study on genetic relationship among *A. bisporus* strains are in agreement with this earlier finding. The variability amongst 18 randomly selected SSIs comprising 13 non-fertile and 5 fertile isolates has been analysed using 30 RAPD primers. The analysis exhibited a very low percent of polymorphism (9.0%) in single spore isolates of *A. bisporus* tested.[22]

The present study clearly showed that the morphological and molecular characterization of hybrids/single spore isolates of *A. bisporus* is a very efficient selection criteria and could be used for strain development programme of edible fungi by selection of genetically diverse parents as well as the hybrids/SSI's.

REFERENCES

1. Royse, D.J., and May, B. Use of isozyme variation to identify genotypic classes of *Agaricus brunnescens. Mycologia*, 1982; **74**: 93-102.
2. Castle, A.J., Horgen, P.A ., Anderson, J.B. Restriction fragment length polymorphism in the mushroom *Agaricus brunnescens* and *Agaricus bitorquis*. *Appl Environ Microbiol.*, 1987; **53**: 816-22.
3. Loftus, M.G., Lodder, S.C., Legg, E.J. Molecular mushroom breeding. *Mushroom Sci.*, 1995; **14**(1): 3-9.
4. Khush, R.S., Wach M.P., Horgen, P.A. Molecular strategies for breeding and karyotyping in *Agaricus*. In: Esser K, Lemke PA (eds) *Genetics and Biotechnology*. *The Mycota*, Vol II, Springer, Berlin Heidelberg, New York, 1995; pp 321-37.
5. Khush, R.S., Becher, E., Wach, M. DNA Amplification Polymorphisms of the Cultivated Mushroom *A. bisporus*. *Applied and Enironmental Microbiology*,1992; **58**(9): 2971-77.
6. Yadav, M.C., Mahfooz, S., Singh, S.K., Upadhyay, R.C. RAPD marker assisted selection of genetically diverse parents for hybridization in *Agaricus bisporus*. *Mushroom Res.*, 2003a; **12**(1): 19-26.
7. Kavousi, H.R., Fars, I.M., Shahriari, F. Comparison of RAPD markers and

morphological characters in indentification of homokaryon isolates of white button mushroom (*Agaricus bisporus*). *Pakistan J of Biol Sci.*, 2008; **11**(4): 1771-78.

8. Phutela, R.P. Strain selection and development in the cultivated mushroom *Agaricus bisporus*. Ph.D Thesis, Punjab Agricultural University, Ludhiana, India, 1986.
9. Kumar, S., Traditional techniques of breeding in mushrooms. In: *Advances in Mushroom Biology and Production,* Rai, R.D., Dhar, B.L., Verma, R.N. (Eds.) *Mush Soc of Ind.*, NRC for Mushroom, Chambaghat, Solan, 1997; 39-58.
10. Graham, G.C., Mayer, P., Henry, R. A simplified method for the preparation of fungal genomic DNA for PCR and RAPD analysis. *Biotechniquess*, 1994; **16**(1): 48-50.
11. Singh, S.K., Verma, R.N., Kamal, S., DNA fingerprinting of edible Mushroom using Random primers. In: *Current Vistas in Mushroom Biology and Production* (Eds. R.C. Upadhyay, S.K. Singh and R.D. Rai) *Mush Soc of Ind.*, 2003; 57-60.
12. Rholf, F.J. NTSYS-PC Numerical Taxinomy and Multivariate system-version 2.0. *Applied Biostatic Inc*., New York, 1998.
13. Fritsche, G., Untersuchungen des Naehrboden-einlusses auf verschiedene Myzelformen des Kulturchampignons. *Mush Sci.*, 1969; **7**: 515-29.
14. Lambert, E.B. Improving spawn cultures of cultivated mushrooms. *Mush Sci.*, 1959; **4**: 33-51.
15. Fritsche, G. Beispiel der Wikuing der Einstor Kultur ausleseals Zuchterische Methode beim Kulturchampignon. *Theor and Appl Gen.*, 1972; **42**: 62-64.
16. Kumar, S. and Munjal, R.L. Studies on the physiology of different single spore isolates of *A. bisporus* (Lange) Imbach. *Ind J Mush.*, 1980; **6**: 36-47.
17. Bhandal, M.S., Mehta, K.B. Evaluation and improvement of strains in *Agaricus bisporus*. *Mush Sci.*, 1989; **12**: 25-35.
18. Raper, C.A., Raper, J.R., Miller, R.E. Genetic analysis of the life cycle of *Agaricus bisporus. Mycologia* 1972; **64**: 1088-117.
19. Yadav, M.C., Molecular breeding for development of genetically improved strains and hybrids of Agaricus bisporus. In: *Curr Vistas in Mushroom Biology and Production* (Eds. R.C.Upadhyay, S.K. Singh and R.D. Rai) *Mush Soc of Ind.*, 2003; 261-74.
20. Yadav, M., Singh, S.K., Upadhyay, R.C., Mahfooz, S. Molecular profiling and morpho-physiological characterization of *Agaricus bitorquis* germplasm. *Mushroom Res.*, 2003b; **12**(2): 79-86.
21. Yadav, M.C., Challen, M.P., Singh, S.K., Elliott, T.J. DNA analysis reveals genomic homogeneity and single nucleotide polymorphism in 5.8S ribosomal RNA gene spacer region among commercial cultivars of the button mushroom *Agaricus bisporus* in India. *Current Sci.*, 2007; **93**(10): 1383-90.
22. Singh, M., Kamal, S. Conventional and molecular approaches for breeding button mushroom. Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products (ICMBMP7), 2011.