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Development of *Agaricus bisporus* hybrids and their evaluation for higher yield

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ABSTRACT

***Agaricus bisporus* (Lange) Imbach, the white button mushroom, is the major edible mushroom species cultivated throughout the world. Despite its economic potential, only a few genetic studies have been carried out concerning *A. bisporus*, due to its peculiar life cycle. Button mushroom is a secondary homothallic fungi and breeding is difficult as most of the spores are heterokaryotic in nature. Therefore, the present investigation as undertaken to develop the hybrid(s) of *A. bisporus* and their evaluation for higher yield. One thousand six hundred forty two single spore isolates were raised from parental strains, namely, A-15, S 11, S140 and U3 of *A. bisporus*. Out of these only 36 single spore isolates were homokaryons. ISSR studies revealed homokaryons lacking amplification products at multiple loci. Out of the 253 crosses, only seven crosses between the parents were compatible. The yield studies showed that that newly developed hybrid Hb4 produced highest average yield (26.94 kg/100 kg compost) and also exhibited better qualities as compared to other isolates. Percentage of polymorphism by RAPD markers were 60.00 per cent. All the isolates exhibited identical ITS length of approximately near about 650 bp. The ITS region showed single nucleotide polymorphisms (SNPs) at six nucleotide positions of 281, 290, 291,292, 293, 295 and 300 base pair in the ITS region of the best developed hybrids (Hb1, Hb4) and parent strains, namely, A-15 and U3 of *A. bisporus*.**

Key words: *Agaricus bisporus*, hybrids, molecular markers.

INTRODUCTION

Breeding of *Agaricus bisporus* is complicated because of its unusual secondary homothallic life cycle. Instead of forming basidia with four homokaryotic spores, the majority of the basidia produce two spores each containing two nuclei of opposite mating types. Such heterokaryotic offspring cannot be used directly in outcrossing experiments. Approximately 5 to 7 per cent of the *A. bisporus* basidia produce three or four spores (Summerbell *et al.*, 11) and most of these will form homokaryotic mycelia upon germination. Unfortunately, *A. bisporus* lacks clamp connections and until recently, successful matings could only be recognized by differences in growth rate of the vegetative mycelium and laborious fruiting trials. Other features of the *A. bisporus* are the multinuclear character of its mycelium and the absence of asexual spores, which make it difficult to generate mutants. The lack of genetic variation between the commercial mushroom strains forms a severe risk to the mushroom industry. Further, the industry is sensitive to crop losses caused by bacterial and fungal infections and losses caused by 'spontaneous' degeneration processes leading to the formation of ill shaped or malformed mushrooms (Sonnenberg *et al*., 9).

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During the last decade several major breakthroughs have been achieved in mushroom biotechnology, which greatly enhanced classical mushroom breeding. DNA markers are powerful method and good candidates for the assessment of variation and genetic relationship in the germplasm (Hospital, 3). The Internal Transcribed Spacer (ITS) regions evolve fastest and may vary among species with in a genus or among populations of the same species (Yadav *et al.*, 13). In the context of above, present investigation was undertaken with the objectives to isolate, identify and intermating compatible homokaryons to produce hybrid(s), evaluation of developed hybrid(s) in terms of production efficiency and molecular characterization of developed hybrid(s).

MATERIALS AND METHODS

Strains A-15, S 11, S-140 and U3 of *A. bisporus* were used for spore collection in the present study. Young and healthy fruit bodies of strains were selected for basidiospores collection. The sterilized glass beaker with disinfected fruit bodies were kept for 24-48 h in an incubator at 25°C. The spores were discharged and deposited onto the petri plates on the opening and rupturing of membrane in a thick mass known as spore print.

The homokaryons were isolated by using standard serial dilution technique. The single spores so isolated were screened *in vitro* for identification of homokaryons, based on their slow mycelial growth (≤ 2.0 mm/day) and appressed types of colony morphologies on malt extract agar medium. Mycelial culture of homokaryons along with parent strains A-15, S 11, S-140 and U3 were raised as stationary culture in 150 ml conical flask containing 50 ml malt extract broth media. The DNA was extracted using kit (Genei Pure* TM Plant Genomic DNA purification kit) following the protocol of the manufacturer. The integrity of the obtained genomic DNA was determined by electrophoresis in 1 per cent agarose gel stained with ethidium bromide. ISSRs were detected by the use of 24 repeat anchored primers. The micro-satellites primers pairs were synthesized from Operon Technologies Inc. The amplification reactions were carried out in 20 µl volume containing 50 ng DNA template, 35 ng primer, 0.20 mM dNTPs, 2 mM $MgCl_2$, 1 U *Taq* DNA polymerase and 1 X PCR buffer. The PCR condition was denaturation (94°C for 45 sec), annealing (50°C for 1 min.) and extension at 72°C for 5 min. with 42 cycles of amplification.

Possible crosses were made between compatible non fruiting homokaryons by placing 1 mm mycelial bits in petri plates opposite to each other at equal distance containing 2 per cent malt extract agar medium under optimum growing condition. In compatible cross, some raised growth was observed at the point of their contact, which was picked up with the help of sterilized needle and transferred to test tubes containing wheat extract agar medium for raising pure cultures. This growth was also observed under microscope to ascertain the fusion of mycelial strands and further confirmed by laying fruiting trials.

Fruiting trials of successful crosses or hybrid(s) were laid out on pasteurized compost under optimum growing conditions for primordial production and further confirmation of production of hybrid(s). Bags were spawned with the hybrids and parent strains. The average yield of four replications was calculated in kg/100 kg compost. Evaluation trial for yield and quality traits of developed hybrids on the basis of their mycelial growth rates and morphological characteristics of fruit bodies were laid out in completely randomized block design. The selected hybrids were evaluated along with parent strains as control for mushroom yield and quality traits. For molecular characterization of developed hybrids, RAPD and ITS were used. A total of 72 RAPD primers were used for molecular characterization of developed hybrids. The primers were synthesized from Operon Technologies Inc. The annealing temperature for PCR was 32°C for 1 min. with 45 cycles of amplification. ITS-1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS-4 (5' TCCTCCGCTTATTGATATGC 3') were employed to reveal variation, in the hybrids**.** The sequence data obtained from ITS-4 reverse primer was inversed using GENEDOC software and clubbed with sequence data of ITS-1 to obtain complete sequence of amplified ITS product. Nucleotide sequence comparisons were performed by using BLAST network services against NCBI database.

RESULTS AND DISCUSSION

Single spore colonies were raised from the collected spore prints by serial dilution method. Out of total 1,642 single spore isolates (SSIs), 36 SSIs from the parent strains were found slow growing (≤ 2.0 mm /day). Total genomic DNA of selected single spore isolates and parental strains were extracted. Inter-simple sequence repeats (ISSRs) are regions in the genome flanked by microsatellite sequences. PCR amplification of these regions using a single primer yields multiple amplification products that can be used as a dominant multi-locus marker system for the study of genetic variation in various organisms. Six ISSR

Table 1. ISSR analysis for selected homokaryotic single spore isolates of *Agaricus bisporus.*

Primer	sequence (5´-3´)	T_m (°C)	A-15				S11				S-140				U3				Size range (bp)
			Total bands	MB	PB	P	Total bands	MB	PB	P	Total bands	MB	PB	P	Total bands	MB	PB	P	
P3	$(GA)_8T$	52	9	5	4	44.44	8	4	4	50.00	9	4	5	55.56	9	5	4	44.44	250-2500
P8	$(CT)_8AGA$	55	9	5	4	44.44	9	6	3	33.33	8	4	4	50.00	10	5	5	50.00	125-2500
P22	$(AG)_8YC$	53	7	5	2	28.57	8	6	2	25.00	7	5	2	28.57	8	4	4	50.00	100-1500
P30	$(GA)_8C$	55	8	4	4	50.00	9	5	4	44.44	8	5	3	37.50	7	3	4	57.14	250-2500
P31	$(CT)_8RG$	56	9	5	4	44.44	9	4	5	55.56	8	3	5	62.50	8	4	4	50.00	200-1800
P39	$(CAAGG)_3$	54	10	6	4	40.00	10	5	5	50.00	9	6	3	33.33	9	5	4	44.44	250-2500
Total			52	30	22	42.30	53	30	23	43.40	49	27	22	44.90	51	26	25	49.02	

MB = Monomorphic bands; PB = Polymorphic bands; P = Polymorphism (%)

primers were used in this investigation (Table 1) and each primer gave rich and clear bands ranging from 100 to 2,500 bp and their combination was sufficient to differentiate all of the tested homokaryons.

The study revealed lack of ISSR polymorphic patterns in non-fertile homokaryotic single spore isolates, compared to the parent strains or fertile heterokaryotic single spore isolates. Primers P3 in A-15, P39 in S11, and P30 in S-140 and P8 in U3 strains were proved to be the best due to the banding patterns. These results also confirmed the identity of these single spore isolates to be homokaryons (Fig. 1A-D). Appressed colony morphology and slow mycelial growth used for the identification of homokaryons as the kind of morphological markers (Horgen and Anderson, 2). Yadav (12) identified 2,500 SSIs of *A. bisporus* based on their slow mycelial growth (<1.5 mm/day) and appressed types colony morphology on malt extract agar medium. Kavousi *et al*. (4) reported that RAPD markers could discriminate homokaryons from heterokaryons, based on number of bands generated. The number of bands in homokaryons were significantly less than the heterokaryons. Nazrul and Yinbing (6) used inter-simple sequence repeat (ISSR) analysis for confirmation of true homokaryons. ISSR fingerprinting detected 46.30 per cent polymorphic loci. All appressed homokaryons were not able to produce a fruiting body.

The spore of *A. bisporus* have high concentration of nicotinic acid which induces fusion. When two compatible colonies of *A. bisporus* grew on an agar medium in a petri dish, compatible colonies got fused with each other at the point of their contact**.** However, some raised growth was seen at their point of contact in compatible colonies and in case of incompatible colonies in petri plate, growth usually stopped as colonies approached near to each other. The gap or a distinct line observed between the incompatible colonies was usually some mm wide

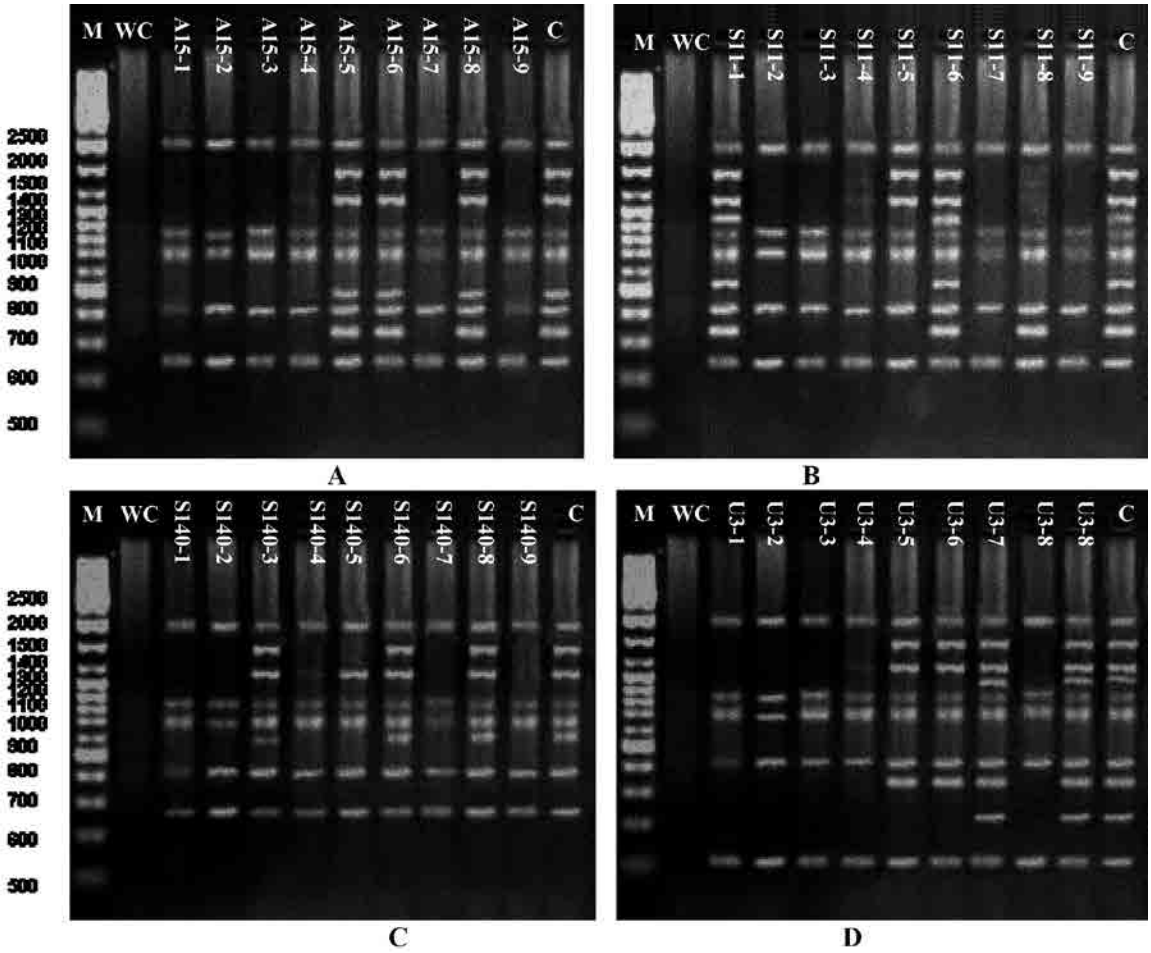

Fig. 1. ISSR fingerprints observed with primers P3, P39, P30 and P8 in A-15 (A), S-11 (B), S-140 (C) and U3 (D) strains of *Agaricus bisporus*. M = DNA ladder 2500 bp., WC = Water control; C = Parent control.

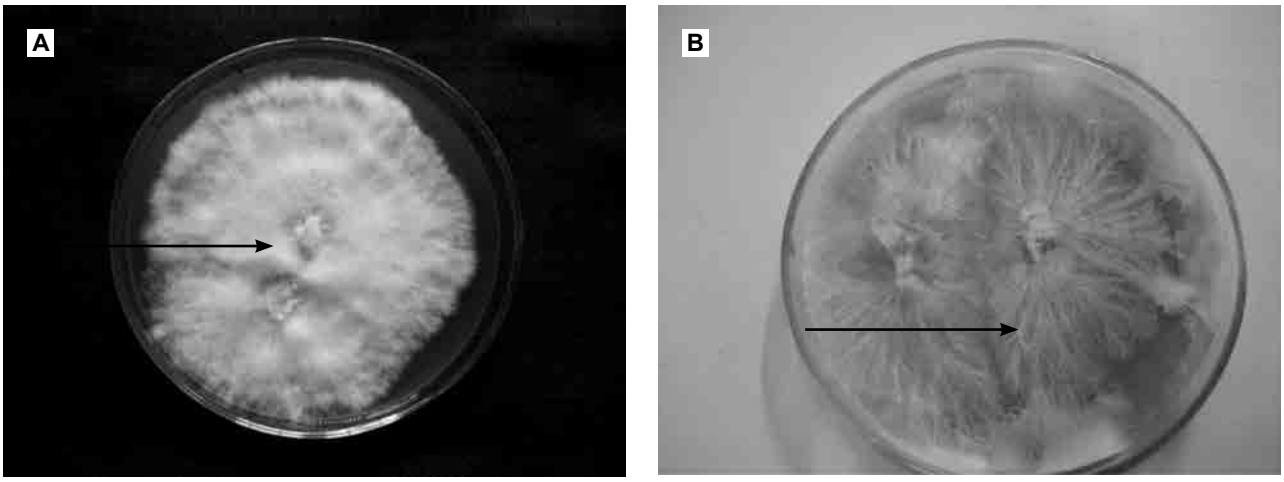

Fig. 2. Compatibility test of homokaryones of *Agaricus bisporus* (A: Compatible reaction; B: Incompatible reaction).

(Fig. 2). Score of successful crosses were obtained when non-fruiting homokaryons were grown in dual culture under optimum growing conditions. Out of these 253 crosses, only seven crosses between the parents were compatible, while the others were incompatible (Table 2). The low level of homokaryotic spores produced during the life cycle of *A. bisporus* (Raper *et al.*, 8) together with an apparently low frequency of recombination (Kerrigan *et al.*, 5) has hampered systematic breeding efforts. Earlier, Yadav (12) recorded a raised growth at the point of contact between two compatible homokaryons.

Table 2. Compatible crosses between different homokaryons of *Agaricus bisporus*.

Parent	Cross	Name of hybrid
A-15 × S11	A15-2 × S11-9	Hb1
A-15 × S140	A15-7 × S11-9	Hb 2
A-15 × S-140	A15-9 × S140-5	Hb3
A-15 × U3	A15-4 × U3-3	Hb 4
S11 × S-140	S11-8 × S140-1	Hb 5
S11 × U3	S11-2 × U3-8	Hb6
U3 × S-140	U3-3 × S140-7	Hb 7

The cropping yield data of four crops grown on compost is recorded in Table 3 and also shown in Fig. 3. The data revealed that hybrids Hb4 and Hb1 were superior to all and significantly differed from rest of the hybrids. Maximum (26.94 kg/100 kg compost) average yield was recorded in Hb4, followed by Hb1 (22.99 kg) and U3 (22.19 kg) and these three were highly significant among themselves. Moreover, when the interaction between hybrids and cropping patterns was studied, it was observed that hybrid (Hb4), recorded maximum (27.19 kg) average yield in 2nd crop, which was statistically at par with 3rd and 4th

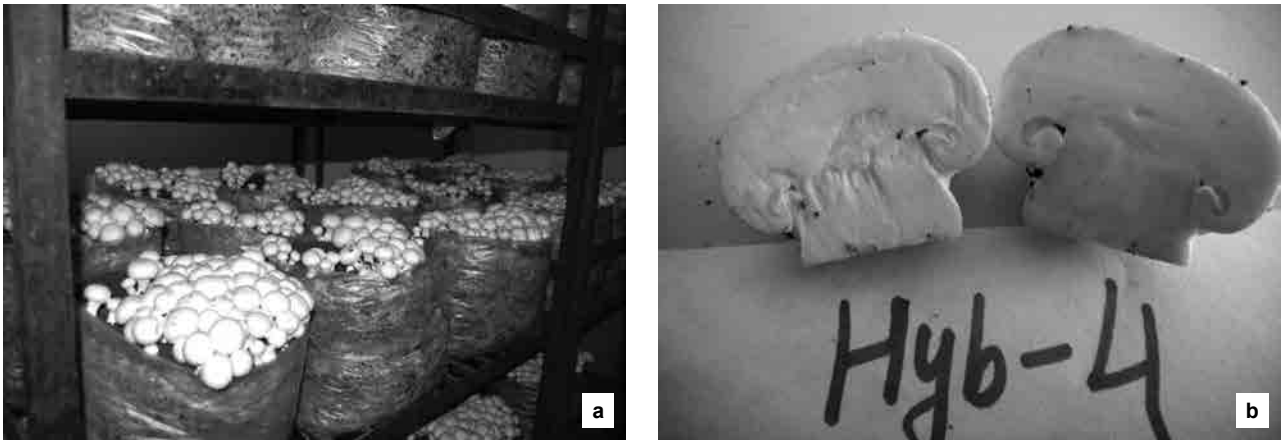

Fig. 3. Cropping trial (a) and fruiting body (b) of developed hybrid Hb4.

Table 3. Evaluation of developed hybrids in terms of production efficiency with parent strains of *Agaricus bisporus* on short method of composting (kg/100 kg compost).

Hybrid/ Parent strain	Av. yield in kg/100 kg compost in different cropping No. of crop				Mean yield (kg/100 kg compost)
	First	Second	Third	Fourth	
Hb1 (A-15 × S11)	22.96	23.12	22.96	22.91	22.99
Hb2 (A-15 × S-140)	11.42	11.40	11.38	11.34	11.38
Hb3 (A-15 × S-140)	12.18	12.24	12.26	12.18	12.22
Hb4 (A-15 × U3)	26.54	27.19	27.03	26.99	26.94
Hb5 (S11 × S-140)	17.50	17.46	17.49	17.39	17.46
Hb6 (S11 × U3)	15.58	15.68	15.55	15.50	15.58
Hb7 (U3 × S-140)	21.71	21.70	21.61	21.68	21.66
A-15 (P1)	9.67	9.72	9.62	9.62	9.66
S 11 (P2)	14.55	14.60	14.29	14.50	14.49
S 140 (P3)	17.3	17.33	17.35	17.36	17.34
U3 (P4)	21.51	22.38	22.21	22.68	22.19
Mean	17.36	17.53	17.43	17.46	
Effect		$CD_{0.05}$			
Hybrid/ Parent (Hb)		0.10			
No. of cropping (NC)		0.06			
Hb × NC		0.20			

crop. Minimum (9.62 kg) average yield was recorded in isolate A-15 in 3rd and 4th crop.

Following growing conditions was followed for all the four crops. Spawn dose was 0.8%, while compost parameters.

Particulars	1st crop	2nd crop	3rd crop	4th crop
Temperature of compost	25.00	25.00	25.00	25.00
Moisture content	70.34	69.40	69.86	69.98
pH	7.6	7.4	7.4	7.5

Out of 72 RAPD primers, 16 primers were found to be polymorphic. The size range of amplified products ranged from 250-3400 and total number of amplified bands were identified to be 130. With the total of 78 polymorphic bands, the polymorphism detected was 60.00 per cent. Maximum number of bands were produced by OPP-07 primer, while minimum was produced by OPA-01 decamer primer (Table 4). Among the eight genotypes, hybrid Hb4 produced the maximum number of amplicons (Fig. 4).

ITS PCR studies showed that all the isolates exhibited identical ITS length of approximately near about 650 bp. The nucleotide sequences of ITS region were compared between the hybrids and parent strains. A total of 4 strains were used for the generation of multiple alignment. The ITS region showed single nucleotide polymorphisms (SNPs) at six nucleotide position of 281, 290, 291,292, 293, 295 and 300 base pair in the ITS region of the selected hybrids (Hb1, Hb4) and parent strains, namely, A-15, U3 of *A. bisporus* (Fig. 5).

Ramirez *et al*. (7) revealed that the use of RAPD molecular markers showed the high level of genetic homology of commercial strains of *A. bisporus*, and allowed at the same time, to distinguish between them. Similarly, Staniaszek *et al.* (10) showed that there was great genetic similarity among 26 strains of *A. bisporus* and low polymorphism of the strains may be proof of a limited genetic pool used in the cultivation of those strains. Yadav (12) successfully used RAPD markers for selection of single spores isolates and two hybrids strain, which were superior than parent strain. Yadav *et al.* (13) revealed that twenty RAPD primers amplified 216 DNA fragments in nine germplasm strains and two newly developed hybrids of *A. bitorquis*, out of which 98.61% were polymorphic. Challen *et al.* (1) distinguished *A. bisporus* var. *burnettii* by a single transition mutation (G A) at 261 nucleotide position of ITS1 region. Yadav *et al*. (13) studied the molecular variation among 22 white cultivars of *A. bisporus* single nucleotide polymorphism at 522 and 563 nucleotide positions in the ITS region of *A. bisporus*.

Table 4. Primers used for detecting genotypic differential in developed hybrids and parent strains of *A. bisporus*.

Primer	Sequence (5´-3)	Total band(s)	Monomorphic bands	Polymorphic bands	DNA polymorphism (%)	Size range (bp)
OPA-01	CAG GCC CTT C	5	3	2	40.00	525-2600
OPA-04	AAT CGG GCT G	6	3	3	50.00	250-2500
OPD-12	CAC CGT ATC C	7	1	6	85.71	1000-3000
OPF-11	TTG GTA CCC C	6	4	2	33.33	500-1800
OPF-14	TGC TGC AGG T	7	3	4	57.14	600-2000
OPG-06	GTG CCT AAC C	8	6	2	25.00	650-2100
OPG-11	TGC CCG TCG T	9	5	4	44.44	650-1900
OPN-04	GAC CGA CCC A	9	3	6	66.67	700-2000
OPN-05	ACT GAA CGC C	8	2	6	75.00	800-2800
OPO-02	ACG TAG CGT C	7	1	6	85.71	700-3000
OPO-09	TCC CAC GCA A	9	7	2	22.22	875-2750
OPP-07	GTC CAT GCC A	14	4	10	71.43	500-3400
OPP-10	TCC CGC CTA C	11	4	7	63.64	500-3200
OPP-17	TGA CCC GCC T	6	1	5	83.33	600-2500
OPP-18	GGC TTG GCC T	9	3	6	66.67	500-2400
OPP-19	GGG AAG GAC A	9	2	7	77.78	600-2900
Total		130	52	78	60.00	

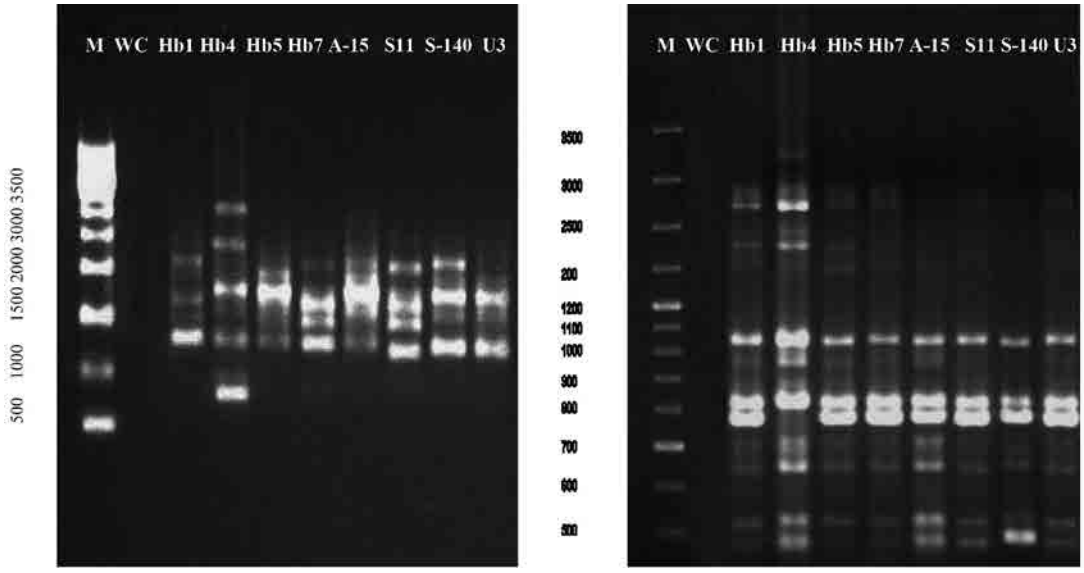

Fig. 4. RAPD profile of hybrids and parent strains of *Agaricus bisporus*. (M = DNA ladder 3,500 bp, WC = Water control).

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                 250        260        270        280        290        300
                  |          |          |          |          |          |
Hb1      ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTG
Hb4      ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTG
A-15     ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCTTTGGTATTCCG
U3       ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCTTTGGTATTCCG
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Fig. 5. Single nucleotide polymorphisms (SNPs) at six nucleotide positions in the ITS region among the two hybrids, Hb1, Hb4 and two parent strains A-15, U3 of *Agaricus bisporus*.

The present investigation were undertaken to develop the hybrid(s) of *A. bisporus.* Button mushroom is a secondary homothallic fungus, therefore, isolation of homokaryons is difficult as most of the spores are heterokaryotic in nature. Under this study we were successful to isolate the homokaryons from SSIs of *A. bisporus*. Seven hybrid(s) of button mushroom were developed by intermating compatible homokaryons from parental strains. The developed hybrids were variable in respect to rate of growth, type of growth and productivity. Among these, hybrid (Hb4) produced highest (26.94 kg/100 kg compost) average yield, followed by Hb1 (22.99 kg). The yield studies have been further supported by the results obtained from RAPD-PCR and ITS technology. According to this study, the maximum number of unique markers has been identified for the hybrid Hb4, which has shown highest average yield. Hence, this newly developed hybrid (Hb4) will prove to be a boon in the field of mushroom industry.

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