DNA MARKERS REVEAL GENOME-WIDE VARIATIONS IN THE STRAINS OF BUTTON MUSHROOM (*AGARICUS BISPORUS*)

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ABSTRACT

The wild strains of a cultivated species constitute an important breeding material for the genetic enhancement of commercial lines. Cultivated strains of button mushroom, *Agaricus bisporus* have narrow genetic base due to their origin from few high-yielding hybrids and single spore progenies through tissue culture propagation. The genetic identities of wild and cultivated strains of *A. bisporus* were established by DNA sequencing of internal transcribed spacer (ITS) regions of 5.8S rRNA gene. Molecular variation among the different strains was assessed using RAPD and AFLP markers. ITS1, 5.8S rRNA gene and ITS2 were 290, 154 and 208 bases, respectively in length in all the strains of *A. bisporus* studied. SNPs in ITS2 region were identified, which distinguished different strains within the species. RAPD and AFLP markers differentiated all the germplasm strains. White pileus cultivated strains were separated from the wild brown strains by a phylogenetic branch. The wild strains possessed a broad range of genetic variation (53.4%) and exhibited 53.7% genetic distance from the cultivated strains and hybrids, indicating a high level of DNA polymorphism in the germplasm. However, the cultivated strains showed less DNA polymorphism (16.8% genetic variation) as compared to the hybrid cultivars (26.8% variation). The cultivated strains showed a genetic distance of 33.6% from the hybrids and were clearly separated in the UPGMA dendrogram and PCO plot. These results have practical implications for the future breeding programmes as these molecularly diverse germplasm strains could be selected for genetic improvement of this commercially important button mushroom in India.

Keywords: *Agaricus bisporus*,AFLP, ITS sequencing, RAPD, wild strains

INTRODUCTION

The button mushroom, *Agaricus bisporus* (Lange) Imbach is a commercially important vegetable crop and significantly contributes to the economies of many countries [1]. This species has a secondarily homothallic life cycle [2] with low levels of crossing over and normal segregation [3-4]. Now, with the acquisition of a large collection of wild types through the *Agaricus* Resource Program (ARP) [5-8], the *A. bisporus* species encompass three different life cycles *viz.*, primary homothallism in *A. bisporus* var. *eurotetrasporus* Callac et Guinberteau [9], pseudohomothallism (= secondary homothallism) in traditional variety, *A. bisporus* var. *bisporus*, and heterothallism in *A. bisporus* var. *burnettii* Kerrigan & Callac [10-11].The wild relatives of a cultivated species represent a valuable genetic resource and provide the foundation for the genetic improvement. Thus, the reliable assessment of genetic variation and identity of the wild strains and their relationship with the cultivated germplasm becomes the prerequisite for the planning of a new breeding programme. These wild genetic resources can also provide useful information on the evolution of the species. The use of DNA markers is considered best for the above purpose as the phenotypic traits are stage specific and their expressions are considerably influenced by ecological factors.

DNA sequencing provides the ultimate fine scale measurement of genetic polymorphism directly at the nucleotide level. Nuclear ribosomal RNA genes (18S, 5.8S and 28S) evolve relatively slowly and are useful for studying distantly related organisms. The internal transcribed spacer (ITS) regions evolve faster and may vary among species within a genus or among strains of the same species [12]. The ITS region is an area of particular importance to fungal diagnostics. ITS regions show more sequence divergence than flanking regions [13] and are often used to distinguish related mushroom species and to infer phylogenetic relationships [9, 14-16]. Molecular characterization based on random amplified polymorphic DNA (RAPD) markers has been used for the assessment of genetic variation and strain identification in a wide variety of

fungal species [17-20]. DNA polymorphisms are easier to identify with RAPD markers than with restriction fragment length polymorphism (RFLP) markers [21]. In button mushroom *A. bisporus*, RFLPs [22-23] and RAPDs [24-28] were used for the assessment of genetic diversity in wild and cultivated strains.

The more robust and highly polymorphic amplified fragment length polymorphism (AFLP) markers have been utilized to study the genetic relationship and DNA polymorphism in plants [29-31] and fungi [32-33] including mushrooms [34-36]. Thus, the objectives of present study were i) to DNA fingerprint and assess molecular variation in *A. bisporus* germplasm that includes quondom cultivars, currently cultivated strains and hybrids, and the wild strains from Russia, France, USA and India using RAPD and AFLP markers, and ii) to determine the extent of intra-specific molecular variation among the wild strains and their genetic relationship with the commercially cultivated white strains and hybrids. In this paper we report the genetic identities of ARP wild strains of *A. bisporus* using ITS sequence data and the presence of a high degree of DNA polymorphism in ARP strains using reproducible RAPD and AFLP markers.

MATERIALS AND METHODS

Mushroom strains

The mushroom strains included 42 germplasm accessions of *A. bisporus* that comprised of wild collections from geographically isolated regions of the world, quondom cultivars, currently cultivated strains and hybrids in India, and a strain of closely related species, *A. subfloccosus* (Table 1).

DNA extraction

Genomic DNA from the somatic tissues of young fruitbodies of individual strains was isolated using the CTAB method [37] with modifications as previously described [38]. DNA samples were purified with RNase and purified DNA was run on a 0.7% agarose gel with diluted uncut lambda DNA (25 ng/ml) as standard to assay its concentration and integrity. DNA was also quantified with UV/VIS spectrophotometer of Hitachi model U-1500 by measuring OD_{260} and OD_{280}. The DNA samples showing OD_{260}/OD_{280} ratio of 1.8 to 1.9 were used for RAPD analysis. The quantified DNA samples were diluted in TE buffer to make a final concentration of 50 ng/µl for PCR reactions.

Molecular analysis

The ITS regions, comprising ITS1+5.8S rRNA gene + ITS2, were amplified using ITS1 ext B and ITS4 ext A forward and reverse primers, respectively [16]. The PCR amplifications were carried out in 50 µl reaction volume following the protocol detailed in Yadav *et al*. [28]. ITS amplified products were purified using Qiagen columns following the QIAquickPCR purification kit protocol. Purified ITS DNA was eluted in 1 mMTris-HCl pH 8.0. Cycle sequencing reactions were performed using ABIPRISM™BigDye Terminator Sequencing ready reaction kits (Applied Biosystems, UK) for sequencing the ITS products. The cycle sequencing reaction consisted of the following components (10 µl): 2 µl Big Dye reaction mix, 1.6 µl Primer (1pmole/ml), 1ml PCR product DNA (15-20 ng/reaction) and 5.4 ml deionized water. The sequencing primers ITS1 and ITS4 [12] were used for cycle sequencing reactions. The PCR conditions for sequencing were: 25 cycles of 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min. The contents of the sequencing reaction were spin down and processed as per the protocol of ABI Prism 3100 Genetic Analyser from Applied Biosystems. The samples were electrophoresed at sequencing facilities of Delhi University, South Campus, India.

RAPD reactions were performed in 22 strains of *A. bisporus* as per Williams *et al*. [39] protocol with minor modifications to enhance reproducibility and consistency of RAPD profiles [38]. PCR amplicons were separated on a 1.5% agarose gel pre-stained with ethidium bromide solution using 1X TAE buffer. The gels were run for 4 hours at 45 V and the RAPD amplicon profiles were recorded using Syngene Gel Documentation System using GeneSnap software. The size of the amplified fragments was determined using 100 bp plus ladder (MBI Fermentas, Lithuania) and Gene Tools software. All RAPD reactions were performed twice to test the reproducibility of the amplicon profiles.

Table 1. List of commercial strains, hybrids and wild strains of *Agaricus bisporus* and a wild strain of *A. subfloccosus* used in the molecular analysis

Sl. No.	Original code	Gene bank code[1]	Source/origin	Germplasm description[2]
1.	S-11	A-1	NRCM, Solan, India	White pileus, commercial strain
2.	U-3	A-13	NRCM, Solan, India	White pileus, commercial hybrid
3.	U-3	A-97	Wang Nan, EFI, China	White pileus, commercial hybrid
4.	MS-39	A-51	NRCM, Solan, India	White pileus, commercial strain
5.	S-44	A-2	NRCM, Solan, India	White pileus, commercial strain
6.	S-56	A-4	NRCM, Solan, India	White pileus, commercial strain
7.	S-791	A-6	NRCM, Solan, India	White pileus, commercial strain
8.	RRL-89	A-7	NRCM, Solan, India	White pileus, commercial strain
9.	S-310	A-10	NRCM, Solan, India	White pileus, commercial strain
10.	P-1	A-11	NRCM, Solan, India	White pileus, commercial strain
11.	ITCC-3708	A-44	ITCC, New Delhi, India	White pileus, commercial hybrid
12.	ITCC-3710	A-45	ITCC, New Delhi, India	White pileus, commercial hybrid
13.	ITCC-3709	A-46	ITCC, New Delhi, India	White pileus, commercial hybrid
14.	ITCC-1924	A-47	ITCC, New Delhi, India	White pileus, commercial strain
15.	ITCC-1933	A-48	ITCC, New Delhi, India	White pileus, commercial strain
16.	ITCC-3554	A-49	ITCC, New Delhi, India	White pileus, commercial strain
17.	ITCC-3609	A-50	ITCC, New Delhi, India	White pileus, commercial strain
18.	NCS-100	A-68	NRCM, Solan, India	White pileus, commercial strain
19.	NCS-101	A-69	NRCM, Solan, India	White pileus, SSP from hybrid
20.	NCH-102	A-70	NRCM, Solan, India	White pileus, commercial hybrid
21.	ARP-247	A-73	ARP collection, USA	White pileus, strain
22.	Hybrid-1	A-98	NRCM, Solan, India	White pileus, New hybrid
23.	WI-1	-	Wild collection, India	White pileus, wild strain
24.	Chail-1	A-80	NRCM, Solan, India	White pileus, commercial hybrid
25.	SM-110	A-82	SMF, New Delhi, India	White pileus, commercial hybrid
26.	SM-170	A-84	SMF, New Delhi, India	White pileus, commercial hybrid
27.	SM-210	A-85	SMF, New Delhi, India	White pileus, commercial hybrid
28.	SM-270	A-86	SMF, New Delhi, India	White pileus, commercial strain
29.	ARP-209	A-15	ARP collection, USA	Brown pileus, wild strain
30.	ARP-210	A-16	ARP collection, USA	Brown pileus, wild strain
31.	ARP-214	A-18	ARP collection, Russia	Brown pileus, wild strain
32.	ARP-216	A-20	ARP collection, Russia	Light brown pileus, wild strain
33.	ARP-217	A-21	ARP collection, Russia	Brown pileus, wild strain
34.	ARP-224	A-28	ARP collection, Russia	Brown pileus, wild strain
35.	ARP-243	A-67	ARP collection, USA	Brown pileus, commercial strain
36.	ARP-246	A-72	ARP collection, France	Brown pileus, wild strain
37.	ARP-249	A-75	ARP collection, USA	Brown pileus, wild strain
38.	ARP-250	A-76	ARP collection, USA	Brown pileus, wild strain
39.	ARP-251	A-77	ARP collection, USA	*A. subfloccosus*
40.	ARP-256	A-91	ARP collection, USA	Brown pileus, wild strain
41.	ARP-257	A-92	ARP collection, USA	Light brown pileus, wild strain
42.	ARP-259	A-94	ARP collection, USA	Brown pileus, wild strain
43.	ARP-261	A-96	ARP collection, USA	Brown pileus, wild strain

[1]Mushroom Gene Bank, DMR, Solan-173 213, India; EFI = Edible Fungi Institute, China; SSP = single spore progeny; ITCC = Indian Type Culture Collection, Mycology and Plant Pathology Division, IARI, New Delhi 110 012, India; SMF = Swadeshi Mushroom Farm, New Delhi, India; *Agaricus* Resource Programme, USA; [2]Grow out tests of these genotypes were conducted at DMR during 2000-2005.

AFLP fingerprints were generated based on the protocol of Zabeau and Vos [40] with slight modifications enumerated in Yadav *et al*. [36] using PCR reagents from AFLP analysis system kits of Life Technologies (Invitrogen), California, USA. AFLP fingerprints were obtained using six AFLP primer-pairs; five with three selective nucleotides in each of *EcoR*I and *Mse*I primers (AFLP Analysis System I) and one with only two selective nucleotides in *EcoR*I and three selective nucleotides in *Mse*I (AFLP small genome kit). All the PCR reactions were carried out in a PE-9600 thermocycler (Perkin Elmer Corp., Norwalk Conn., USA). AFLP reaction products were mixed with an equal volume of Formamide dye (98% Formamide, 10 mM EDTA, 0.005% each of Bromophenol blue and Xylene Cyanol). Three µl of each sample were loaded on a 5% denaturing polyacrylamide gel, run in 1X TBE electrophoresis buffer. The gel was dried for 2 h before subjecting it to autoradiography for 1-3 days at –70 °C depending on the signal intensity. The size of the fragments was estimated using 20 bp size standards (BioWhittakar Molecular Applications, USA).

Statistical analysis

The ITS nucleotide sequences were analyzed after generating the complementary and reversed sequences of ITS4 primer and then comparing with the ITS1 sequences using GeneDoc software [41]. Multiple sequence alignments of consensus sequences were made via ClustalX (1.83) algorithm [42]. The multiple aligned sequences were then utilized to generate a bootstrap (η = 1000) N-J phylogenetic tree [43]. The tree was viewed with the help of TREEVIEW software (http://taxonomy.gla.ac.uk/rod/treeview.html). Sequence divergence (d) and base substitutions were calculated based on Kimura's two-parameter model [44] and are presented as per cent value (d × 100).

The RAPD and AFLP amplification products (amplicons) were scored as present (1) or absent (0) for each primer-genotype combinations. Molecular data were entered into a binomial matrix and were used to determine Jacquard's similarity coefficients [45] using NTSYS-PC software version 2.02h [46]. The dendrograms depicting the genetic relationship were constructed employing the Unweighted Paired Group Method of Arithmetic Averages (UPGMA) algorithm and Sequential, Agglomerative, Hierarchial and Nested (SAHN) clustering. The principal coordinate analysis (PCO) was carried out with NTSYS-pc using DCENTER and EIGEN procedures. The Jaccard's similarity matrix was also used to generate a 3-D plot of principal coordinates to resolve the patterns of variation among the strains. The bootstrap analysis was carried out using the WINBOOT program [47] with 1000 replications. Most informative primers were selected based on high polymorphism information content (PIC) values of individual primers. The PIC was calculated using the formula [48]:

$$PIC = \sum_{i=1}^{n} \{2 \times F(1-F)\}$$, where, F is the frequency of presence of a maker band, i.

The per cent genetic distance (GD) was measured using the Jaccard's similarity coefficients as: % GD = (1- average similarity coefficient) × 100. Similarity matrices from high PIC primers were compared with that of all the primers using MXCOMP sub-program of NTSYSpc. Discrimination power (DP) was estimated to test the efficacy of individual primers in distinguishing the strains, employing the formula DP = number of pairs of strains differentiated /total number of pairs of strains × 100.

RESULTS

Genetic identity and ITS sequence polymorphism

Genetic identities of ARP wild strains were established by amplifying and sequencing the 5.8S rRNA gene along with flanking ITS1 and ITS2 spacer regions. The ITS regions comprising ITS1 + 5.8S rRNA gene + ITS2 were amplified as a single unit from 21 strains of *A. bisporus* along with a strain of related species *A. subfloccosus*. In all cases, the PCR yielded a single product without any visible length variations in the ITS profiles. The length of the PCR product was approximately 755 bases on gel. Multiple nucleotide sequences of the ITS region were generated for a subset of 14 strains. The full length ITS region was 756 bp in all the *A. bisporus* strains analyzed. The 5.8S rRNA gene sequences were found to be conserved

within the species as well as among the species *A. bisporus*, *A. bitorquis* and *A. subfloccosus*. The ITS1 region, 5.8S rRNA gene and ITS2 region were 290, 154 and 208 nucleotides long, respectively in all the genotypes of *A. bisporus* studied. Within the species *A. bisporus*, nucleotide sequence divergence ranged from 0 to 0.43% with the average value of 0.21%. The genotypes of *A. bisporus* exhibited 1.6 and 3.8% sequence divergence from *A. subfloccosus* and *A. bitorquis*, respectively. The ITS2 region had single nucleotide polymorphisms (SNPs) at four base pair positions of 509, 515, 550 and 590. The transition mutations (T→C) occurred at 509, 550 and 590 SNP positions, whereas the SNP at 515 bp exhibited a transversion mutation (C→A) in the wild strains ARP-224 (from Russia) and ARP-261 (from USA). The presence of informative indels in ITS1 (a triple base TGT deletion in *A. bisporus* and *A. subfloccosus*) and in ITS2 region (a double base TG deletion in *A. bisporus*) were used to delimit the wild strains into the species *A. bisporus*.

RAPD fingerprinting

Twenty-eight decamer primers amplified identifiable and consistent PCRamplicons that ranged from 275 bp to 3500 bp in length, and 283 RAPD marker bands, out of which 256 (90.5%) were polymorphic. A total of 2401 amplicons were produced in 22 genotypes with an average of 3.9 amplicons per genotype per primer. The wild strain ARP-224 from Russia exhibited the maximum number of amplicons (120), whereas the *A. subfloccosus* strain ARP-251 produced the minimum (93). The highest number of amplicons was produced by the primer OPN-04 (166), while the least amplicons were obtained with primer OPO-18 (31). Twelve most informative primers showing high PIC values were identified as OPN-02, 05, 06, 08, 09; OPO-01, 02, 03, 12, 13, 14 and OPO-20 (Fig. 1a-d).

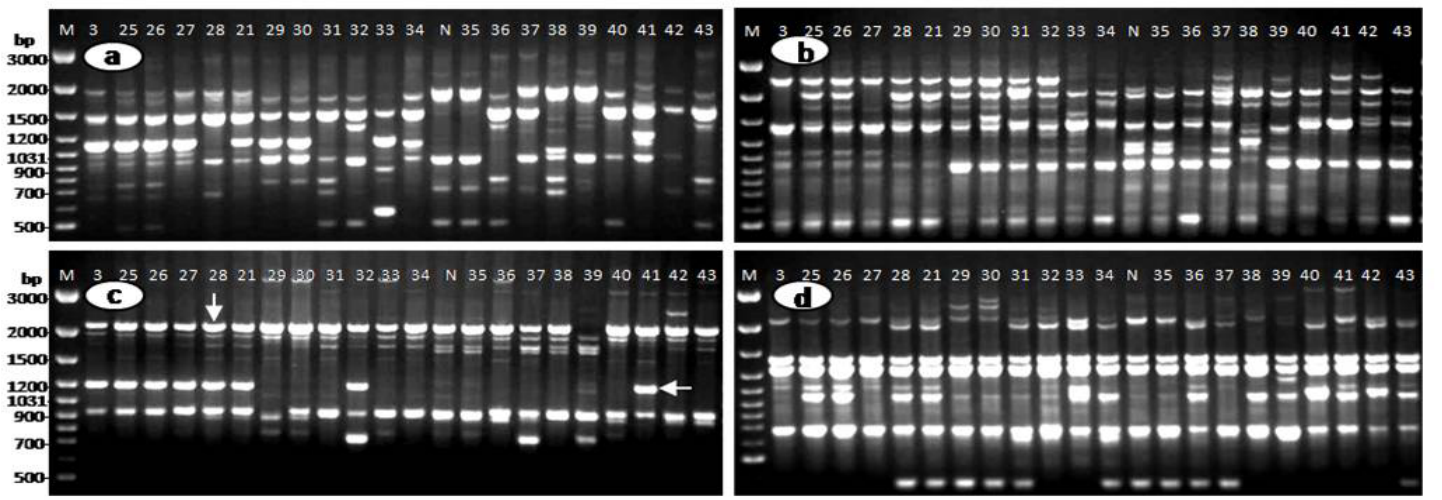

Figure 1. RAPD profiles of 21 germplasm strains of *A. bisporus* and one strain of *A. subfloccosus*. M: DNA ladder, lanes 2-23: correspond to germplasm strains listed under serial No. 3, 25-28, 21, 25-43 in Table 1 and N = ARP-234. DNA polymorphism obtained with primer OPO-12 (a), primer OPN-05 (b), primer OPN-02: Horizontal arrow indicates an OPN-02_{1200} band which is present in white and off-white pileus strains and hybrids; Vertical arrow indicates an OPN-02_{2200} band which is present only in *A. bisporus* strains (c),and primer OPN-08 (d)

These primers amplified a total of 168 bands (59.4% of the bands amplified by all the 28 primers) of which 160 (95.2%) were polymorphic. The PIC value for RAPD primers ranged from 0.25 to 4.64. The maximum number of polymorphic markers (18) was obtained with primer OPO-14. The DP values were more useful in the discrimination of strains and were varied from 25.5 – 98.7% with the average being 86.4 (Table 2). Primer OPN-02 proved useful for distinguishing the pileuscolor and for species identification. An RAPD band of approximately 1200 bp (OPN-02_{1200}) was present in all the white and off-white (light brown) pileus strains, while OPN-02_{2200} band which was present only in *A. bisporus* strains (Fig. 2c). Similarity indices estimated on the basis of all the 28 RAPD primers in a subset of 22 strains ranged from 0.30 to 0.94. The white pileus strains and hybrids exhibited relatively more genetic similarity (0.710) in comparison to brown and off-white pileus wild strains (0.488). The brown and off-white wild strains showed a high degree of genetic variation (51.2%) amongst them. The highest genetic variation (69.7%) was recorded between brown strains ARP-251 (*A. subfloccosus*) and ARP-246 (*A. bisporus*); while, the white pileus hybrids SM-110 and SM-170 exhibited the least (6.2% genetic variation).

Table 2. Efficacy of individual primers for detecting DNA polymorphism and discrimination between 21 strains of button mushrooms *A. bisporus* and one strain of *A. subfloccosus*

Primer	Sequence (5'-3')	No. of RAPD bands	Size range (approx. bp)	PIC* value	Discrimination power (DP) (%)	Number of pairs of strains not distinguished (Total No. of pairs = 231)
OPN-01	CTCACGTTGG	7	400-2850	1.96	91.3	20
OPN-02	ACCAGGGGCA	10	775-3500	2.94	92.6	17
OPN-04	GACCGACCCA	13	450-2600	2.84	96.1	9
OPN-05	ACTGAACGCC	16	500-2500	4.19	95.7	10
OPN-06	GAGACGCACA	14	600-2700	4.42	98.3	4
OPN-07	CAGCCCAGAG	8	350-3200	1.63	90.5	22
OPN-08	ACCTCAGCTC	14	375-3100	3.46	98.3	4
OPN-09	TGCCGGCTTG	17	750-2300	4.36	97.8	5
OPN-10	ACAACTGGGG	10	750-3000	2.73	93.5	15
OPO-01	GGCACGTAAG	11	490-3200	3.76	98.7	3
OPO-02	ACGTAGCGTC	15	400-1900	3.14	96.1	9
OPO-03	CTGTTGCTAC	15	400-2900	4.64	95.7	10
OPO-04	AAGTCCGCTC	6	475-2100	1.23	77.9	51
OPO-05	CCCAGTCACT	7	650-2900	1.74	78.8	49
OPO-06	CCACGGGAAG	6	350-1350	1.47	87.9	28
OPO-07	CAGCACTGAC	8	275-2700	1.54	87	30
OPO-08	CCTCCAGTGT	7	500-3000	1.34	86.6	31
OPO-09	TCCCACGCAA	2	650-875	0.25	25.5	172
OPO-10	TCAGAGCGCC	11	650-3000	2.34	90	23
OPO-11	GACAGGAGGT	10	700-3200	2.22	87.4	29
OPO-12	CAGTGCTGTG	14	475-1900	4.39	97.8	5
OPO-13	GTCAGAGTCC	12	550-2400	3.43	96.1	9
OPO-14	AGCATGGCTC	18	425-2700	4.42	96.1	9
OPO-15	TGGCGTCCTT	10	550-2800	2.41	88.3	27
OPO-16	TCGGCGGTTC	4	550-1150	1.6	71	67
OPO-18	CTCGCTATCC	2	700-850	0.48	50.6	114
OPO-19	GGTGCACGTT	4	625-1800	0.66	59.3	94
OPO-20	ACACACGCTG	12	325-2700	3.34	95.2	11

*Polymorphism information content

AFLP profiling

Six AFLP primer-pairs amplified a total of 467 marker bands in 42 strains of *A. bisporus* and a strain of *A. subfloccosus* with an average of 77.8 bands per primer. Out of 467 AFLP markers, 431 (92.3%) were polymorphic and the rest were monomorphic (7.7%). Out of six primer-pairs tested, two (E-ACT + M-CAC and E-ACT + M-CAG) were able to differentiate all the genotypes studied and had the discrimination power of 1.00 (Table 3).

Wild brown strains were differentiated by all the primer-pairs studied. Pair-wise Jaccard's similarity values varied from 0.282 to 0.936 with average being 0.567. The highest genetically similar strains were S-44 and S-56 (0.936 similarity), while strains S-791 *(A. bisporus*) and ARP-251 (*A. subfloccosus*) were found to be the least similar ones (0.28.2 similarity). The average genetic distance between white pileus cultivars (strains and hybrids) and brown pileus wild collections was found to be 53.7%, indicating a high level of DNA polymorphism amongst the germplasm of *A. bisporus.* However, the white pileus strains had less DNA polymorphism (av. similarity 0.832). The white pileus hybrids exhibited relatively

Table 3. Primer-pairs used for selective amplification in AFLP analysis and their utility in molecular differentiation of white pileus cultivars and wild brown strains of *A. bisporus*

Primer- pair	No. of AFLP markers	Polymorphic AFLP markers (%)	Discrimination power	Number of pairs of strains not distinguished (total number of strain pairs = 903)
E-AC + M-CAT	68	89.71	0.97	**24** (pedigree related white pileus strains and hybrids were not differentiated)
E-AAG + M-CAT	38	86.84	0.95	**43** (pedigree related white strains and hybrids were not discriminated)
E-ACA + M-CTT	56	91.07	0.97	**25** (pedigree related white strains and hybrids were not differentiated)
E-ACG + M-CAA	70	94.29	0.99	**1** (pedigree related white strains S-44 and RRL-89 were not discriminated)
E-ACT + M-CAC	117	92.31	1.00	**0**
E-ACT + M-CAG	118	94.92	1.00	**0**

more molecular variation between them (av. similarity 0.732) and had an average genetic distance of 33.6% from the white strains.

Genetic relationship and DNA polymorphism

The genetic relationships inferred from the nucleotide sequence polymorphism in the ITS region of different genotypes of *A. bisporus* are depicted in Fig. 2. Three sub-clusters were formed within the major cluster of *A. bisporus*.

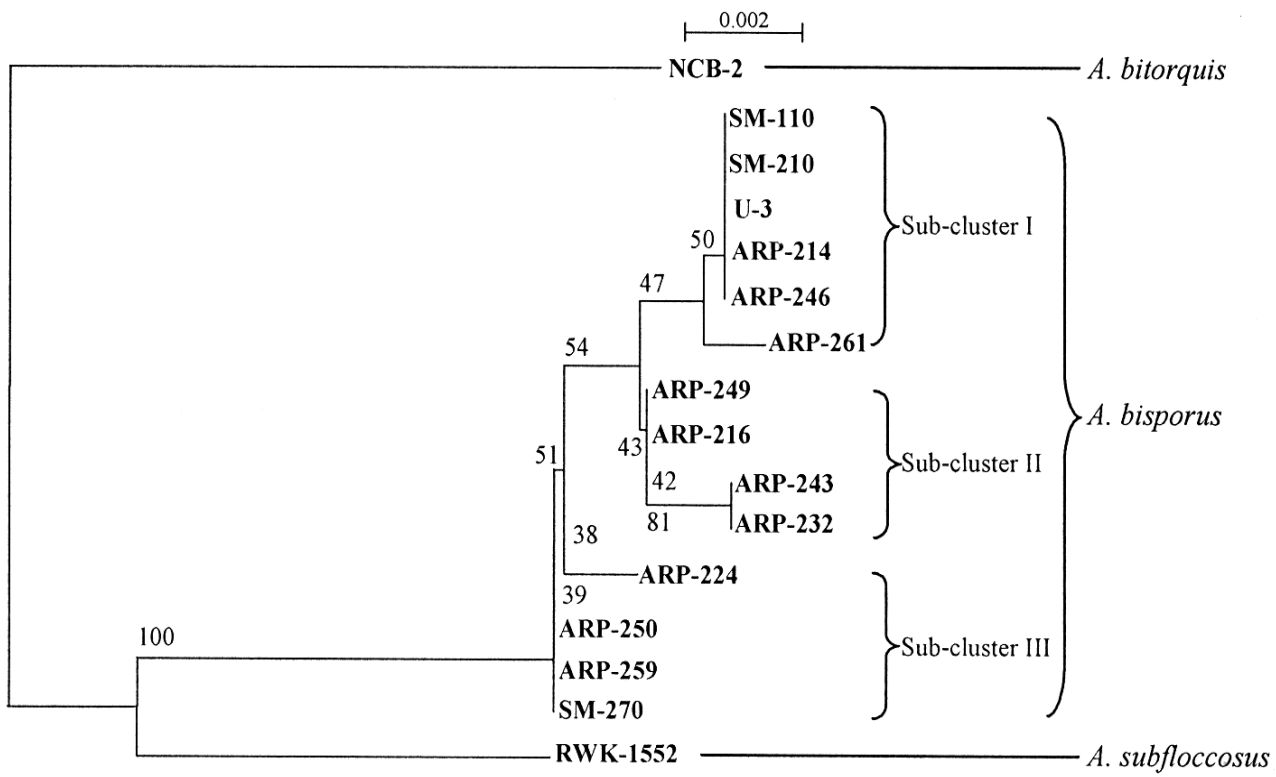

Figure 2. Neighbour-joining tree inferred from sequence polymorphisms in the ITS region of wild and cultivated strains of *Agaricus bisporus*. Numbers on the branches denote per cent bootstrap support to each node. The tree was rooted using two related species *A. bitorquis* (NCB-2) and *A. subfloccosus* (RWK-1552)

The wild strains from Russia showed high sequence polymorphism and were placed in all the three sub-clusters. The wild strain ARP-232 (from Canada) was clustered with ARP-243 (a brown strain from USA) within the sub-cluster II that also included the wild strains ARP-216 (from Russia) and ARP-249 (from USA).ARP-224, a wild collection from Russia along with wild strains ARP-250, ARP-259 (both from USA) and SM-270 (white strain) constituted the third sub-cluster. Cluster analysis based on RAPD markers grouped all the white pileus strains and hybrids into one cluster with 86% bootstrap value, whereas the wild brown ARP strains formed several sub-clusters exhibiting a high level of genetic polymorphism (Fig. 3).

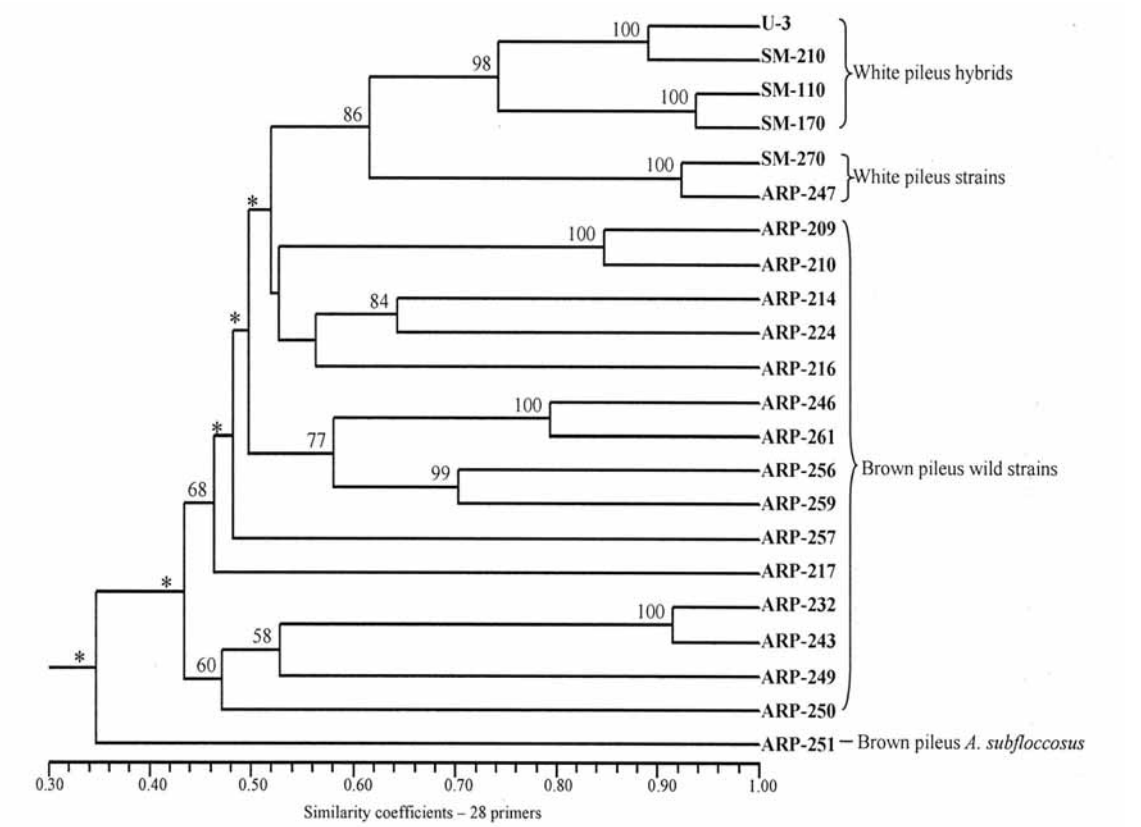

Figure 3. UPGMAdendrogram constructed from the RAPD profiles of 21 germplasm strains of *A. bisporus* and one strain of *A. subfloccosus*. The numbers on the forks denote per cent bootstrap support to each node (branches showing less than 50% bootstrap values are indicated by asterisks). Major clusters are indicated at the right margin

All the strains were distinguished using aggregated primer data. Individual primers were also informative in providing specific RAPD polymorphisms for strain differentiation (Table 2). However, none of the primers was able to differentiate all the 22 strains. Primer OPO-01 with 11 RAPD markers differentiated all the genotypes except three pairs namely U-3 and SM-110, SM-170 and ARP-210, and ARP-246 and ARP-261. Wild brown pileus strains exhibited a high degree of genetic variation and were clearly separated from cultivated white pileus strains and hybrids. Genetic relationships among 42 strains based on AFLP profiles are depicted in Fig. 4. Dendrogram based on UPGMA algorithm and SAHN clustering clearly distinguished the strains from the hybrids and the wild strains. *Agaricus subfloccosus* strain ARP-251 showed average genetic distance of 68.3% from *A. bisporus* strains and clustered with wild strains from USA namely ARP-249 (42.2% similarity) and ARP-250 (40.7% similarity) in the dendrogram constructed based on all the six AFLP primer-pairs (Fig. 4).

Within the *A. bisporus* accessions, all the white pileus strains and hybrids were separated from the brown pileus wild collections by a phylogenetic branch with 70% bootstrap value. The wild brown strains exhibited a wide range of genetic variation (av. genetic variation 53.4%). Within the wild brown strains, collections from Penza region of Russia were clearly

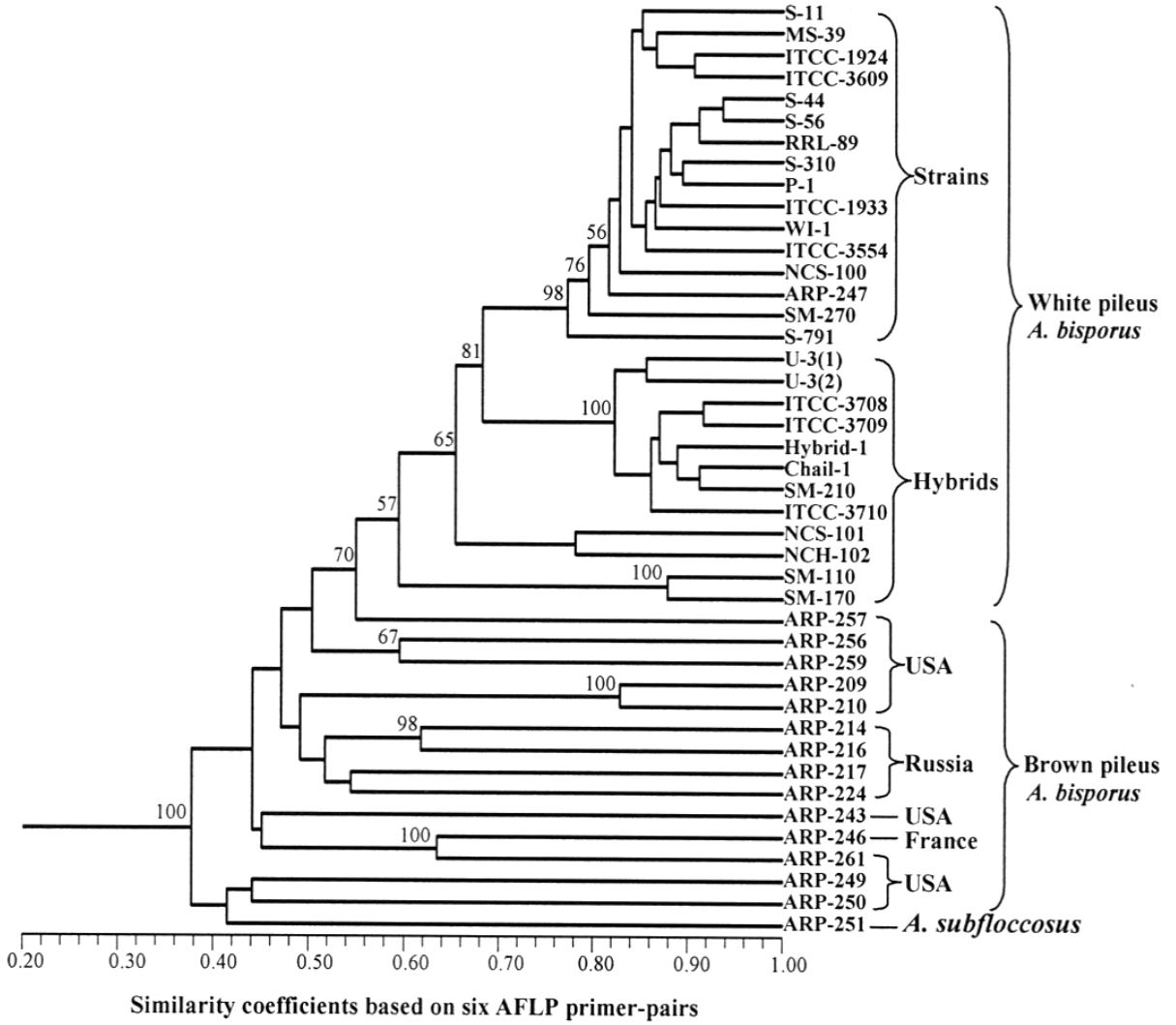

Figure 4. UPGMAdendrogram constructed from 467 AFPL markers showing genetic relationships amongst 42 strains of *Agaricus bisporus* and a strain of *A. subfloccosus*. The scale at the bottom represents Jaccard's coefficients of similarity. The numbers on the forks denote per cent bootstrap support to each node. Major clusters are indicated at the right margin

separated, whereas, the collections from different parts of USA exhibited wide genetic variation and formed divergent sub-clades. The wild strain ARP-246 from France clustered with wild strains ARP-261 and ARP-243 of USA. White pileus cultivated strains and hybrids exhibited a narrow genetic base and collapsed in the 3-D PCO plot (Fig.5). The Jaccard's similarity matrices obtained with the individual AFLP primer-pairs were compared with the matrix of all the six AFLP primer-pairs. The matrix correlation (r) values ranged from 0.80 - 0.96 indicating good to very good fit of the two matrices compared. The dendrogram based on AFLP data from primer-pair E-ACT + M-CAC was the most similar to the dendrogram based on all the six primer-pairs with matrix correlation of r = 0.96 (p= 1.00).

DISCUSSION

Ribosomal RNA genes evolve cohesively within a single species [49] and exhibit only limited sequence polymorphism in the ITS regions between individuals of a species [50]. Nucleotide sequences polymorphisms of the ITS region have been utilized in establishing the phylogenetic relationships among species of the same or related genera of mushrooms [14-15, 51]. Four SNPs were identified at 509, 515, 550 and 590 base pair positions in ITS2 region which could distinguish the wild strains of *A. bisporus* collected from geographically isolated regions. The presence of informative indels in the ITS1 region (a triple base – TGT – deletion in ITS1 of both *A. bisporus* and *A. subfloccosus*) and in ITS2 region (a double base – TG – deletion in *A. bisporus* only) unequivocally demonstrates that the genetically diverse strains studied in this work belong to

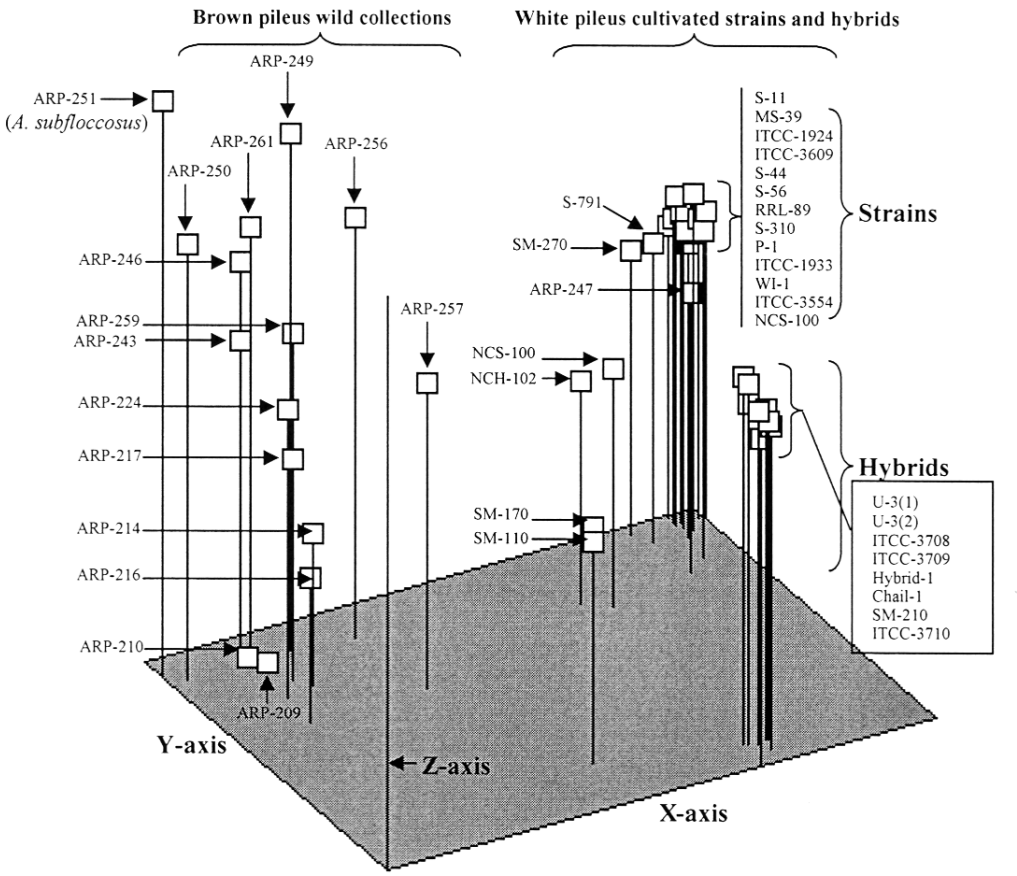

Figure 5. A 3-D plot of the Principal Coordinates Analysis of AFLP data showing genetic relationships among brown pileus wild collections from geographically different regions of the world and white pileus cultivated strains and hybrids of *A. bisporus*. White pileus cultivated strains and hybrids exhibited a narrow genetic base and coalesced in the 3-D plot

the species *A. bisporus*. In the previous finding [16] a tetrasporic *A. bisporus* var. *burnettii* [10] was distinguished by a single transition mutation (G→A) at 261 bp in ITS1, while the homothallic *A. bisporus* var. *eurotetrasporus* had the characteristic deletion (a single base –T– at 118 bp). Similarly, ITS sequence polymorphisms at five base pair positions, two in ITS1 region and three transition mutations in ITS2 region at 509 (T→C), 550 (T→C) and 628 (G→A) bp positions were reported in the sexually diverse wild specimens of *A. bisporus* [9]. In the present work, we have compared the ITS sequences from 14 strains (differentiated based on RAPD markers) of *A. bisporus* and found that the ITS1 region is conserved (does not have base substitutions or deletion) perhapsdue to the absence of both the *A. bisporus* var. *burnettii* and *A. bisporus* var. *eurotetrasporus* cultures in the germplasmanalyzed. In our study, we have found a transversion mutation (C→A) at 515 bp position in the wild strains ARP-224 (from Russia) and ARP-261 (from USA) and an additional transition mutation (T→C) at 590 bp position in the ITS2 region. The ITS sequence data of this work clearly validate the presence of European strains in USA as observed by Kerrigan *et al*. [10] using nuclear and mitochondrial markers.

RAPD fingerprinting was found efficient to reveal usable levels of DNA polymorphism among white pileus strains and a very high amount of molecular variation among wild strains of *A. bisporus*. A high degree of genetic homogeneity among hybrids has been reported using RAPD markers [24, 26]. Wild strains analyzed in this study are from the geographically isolated regions (Russia, France, Canada and USA) and thus exhibited a high degree of DNA polymorphism with RAPD. The wild strains ARP-217 (from Russia) and ARP-257 (from USA) exhibited unique bands in some RAPD profiles and consequently formed a solitary cluster in the dendrogram. AFLP markers revealed the overall similarity index of 0.57, which indicates the broad genetic base of germplasm. The similarity value of 0.83 between white pileus strains suggests that the level of genetic variation (16.8%) is low among the cultivated lines; while the white pileus hybrids exhibited relatively more genetic variation (av. similarity 73.2%) and had an average genetic distance of 33.6% from the white pileus strains. However, AFLP markers have differentiated pedigree related and genetically close white pileus strains and grouped them

into a phylogenetic cluster with 98% bootstrap value and form the strong point of the present investigation. The wild brown strains analysed with AFLP markers showed a high degree of DNA polymorphism with genetic variation of 53.4 per cent.

In earlier studies, the wild collections of *A. bisporus* exhibited a high level of genetic variation using allozymes [53] and nuclear DNA RFLPs [22, 23]. The wild strains of the *Agaricus* Resource Program were characterized using nuclear and mitochondrial DNA RFLP probes [5-8, 52-54] and repetitive DNA sequences [55] and were found to be genetically highly variable. However, allozyme and RFLP analyses scan only a limited number of loci in the genome, while the utility of RAPD and AFLP markers for the germplasm characterization and assessment of genetic variation has been well documented in various fungal species [17-20, 32,33] including mushrooms [24-28, 34-36].

CONCLUSION

The availability of genetically highly variable germplasm from ARP has opened new vistas for research in molecular genetics and breeding of *A. bisporus*. This research represents one of the most comprehensive analyses of molecular variation among the wild and cultivated strains of *A. bisporus*, and demonstrates the effective use of RAPD and AFLP markers for multilocus genotyping and assessment of genetic variation in the germplasm and for establishing the genetic relationships between wild and commercial strains of *A. bisporus*. The ITS sequence analysis unequivocally delimited these genetically highly variable wild strains into a single taxon of *A. bisporus*. These findings have an immediate practical application by the breeders to involve the genetically diverse strains in hybrid breeding programmes.

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