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

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Molecular variation was studied among 22 white pileus cultivars of *Agaricus bisporus* using random amplified polymorphic DNA markers and by sequence analysis of 5.8S rRNA gene along with ITS regions. Twenty-four primers amplified 175 RAPD markers, of which 53.7% were polymorphic. Genetic similarity index varied from 0.64 to 0.99, with an average of 0.81. The varieties exhibited 92.7% genetic similarity, while the hybrids showed 84.3% similarity amongst them. Both the UPGMA dendrogram and PCO plot grouped all the varieties into a single cluster, while the hybrids formed a separate cluster exhibiting DNA polymorphism. The length of ITS1, 5.8S rRNA gene and ITS2 was 290, 154 and 208 bases respectively, in all the genotypes of *A. bisporus* studied. We report two single nucleotide polymorphisms (SNPs) at 522 and 563 nucleotide positions in the ITS2 region which distinguished different strains within the species. This study demonstrates that the RAPD markers are useful and robust tools for identification of hybrids in the germ plasm and for detection of intraspecific molecular variation in the white button mushroom cultivars. In the present study we report the identification of SNPs in the ITS2 region of the 5.8S ribosomal RNA gene which could differentiate cultivars of *A. bisporus*.

Keywords: *Agaricus bisporus*, commercial cultivars, DNA polymorphism, ITS sequencing.

THE white button mushroom, *Agaricus bisporus* (Lange) Imbach, is a commercially important vegetable crop and significantly contributes to the economies of many countries[1], with a world value of ca. £2.5 billion p.a. This cultivated species accounts for 90 per cent of mushroom production in India and is a potential indoor crop for rural livelihood and health food. It is a secondary homothallic mushroom[2] with intramictic breeding behaviour and low levels of genetic recombination[3,4]. The release of genetic variation is further blocked due to the tissue-culture propagation of spawn (seed material) for mushroom growing. Reliable estimates of variation and genetic relationship are the pre-requisites for effective utilization of genetic resources in crop improvement programmes. The abundance, stage-independent expression and absence of influence of environmental factors on the expression make DNA markers invaluable tools for the assessment of variation and genetic relationship in the germ plasm.

DNA markers are quick and reliable for genetic differentiation of strains within the species. RAPD uses only a single short primer with an arbitrary sequence and binds at many complementary sites in the genomic DNA[5]. These markers are sensitive to nucleotide differences between the primer and DNA template. DNA polymorphisms are easier to identify with RAPD markers than with RFLP markers[6]. RAPD fingerprinting has been used for the assessment of molecular variation in the germ plasm of diverse species of higher plants[7–9] and fungi[10–12]. RAPD markers have also been utilized for the assessment of genetic diversity in wild and cultivated strains of *A. bisporus*[13–16].

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 fastest and may vary among species within a genus or among populations of the same species[17]. The ITS region comprising ITS1, 5.8S rRNA gene and ITS2, is an area of particular importance for fungal diagnosis at species level. The ITS sequences show more sequence divergence than their flanking coding regions[18] and they are often used to distinguish related fungal species[19–21] and to infer phylogenetic relationships from populations to families[22–24]. The purpose of this study was: (1) to assess molecular variation and DNA fingerprinting of *A. bisporus* germ plasm that includes white pileus commercial varieties and hybrids in India and (2) to identify single nucleotide polymorphisms (SNPs) in the ITS region of these genotypes. In this article we re-

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Table 1. White pileus genotypes of *Agaricus bisporus* used in the molecular analysis

Serial no.	Original code	Gene Bank code[a]	Source	Germ plasm description[b]
1.	S-11	A-1	NRCM, Solan	White pileus, commercial variety
2.	U-3	A-13	NRCM, Solan	White pileus, commercial hybrid
3.	MS-39	A-51	NRCM, Solan	White pileus, commercial variety
4.	S-44	A-2	NRCM, Solan	White pileus, commercial variety
5.	S-56	A-4	NRCM, Solan	White pileus, commercial variety
6.	S-791	A-6	NRCM, Solan	White pileus, commercial variety
7.	RRL-89	A-7	NRCM, Solan	White pileus, commercial variety
8.	S-310	A-10	NRCM, Solan	White pileus, commercial variety
9.	P-1	A-11	NRCM, Solan	White pileus, commercial variety
10.	ITCC-3708	A-44	ITCC, New Delhi	White pileus, commercial hybrid
11.	ITCC-3710	A-45	ITCC, New Delhi	White pileus, commercial hybrid
12.	ITCC-3709	A-46	ITCC, New Delhi	White pileus, commercial hybrid
13.	ITCC-1924	A-47	ITCC, New Delhi	White pileus, commercial variety
14.	ITCC-1933	A-48	ITCC, New Delhi	White pileus, commercial variety
15.	ITCC-3554	A-49	ITCC, New Delhi	White pileus, commercial variety
16.	ITCC-3609	A-50	ITCC, New Delhi	White pileus, commercial variety
17.	NCS-100	A-68	NRCM, Solan	White pileus, commercial variety
18.	NCS-101	A-69	NRCM, Solan	White pileus, commercial SSP from hybrid
19.	NCH-102	A-70	NRCM, Solan	White pileus, commercial hybrid
20.	Hybrid-1	A-98	NRCM, Solan	White pileus, new hybrid
21.	WI-1	–	Wild collection	White pileus, wild collection
22.	Chail strain	A-80	NRCM, Solan	White pileus, commercial hybrid

[a]Mushroom Gene Bank, National Research Centre for Mushroom (NRCM), Solan, India; SSP, Single spore progeny; ITCC, Indian Type Culture Collection, Mycology and Plant Pathology Division, IARI, New Delhi, India; [b]Grow out tests of these genotypes were conducted[15] in the mushroom growing facilities at NRCM during 2000–03.

port the genomic homogeneity of currently grown *A. bisporus* varieties in India using reproducible RAPD markers and also two SNPs in ITS2 region which can distinguish different cultivars within the species.

Materials and methods

Mushroom strains

Twenty-two strains that comprised of commercial white pileus varieties and hybrids of *A. bisporus* were used in this study (Table 1).

DNA extraction

Genomic DNA from the somatic tissues of young fruit bodies was isolated using the CTAB method[25] with some modifications[15]. DNA samples were purified with Ribonuclease I A (Amersham Pharmacia Biotech Inc, USA) and purified DNA was run on a 0.7% agarose gel with diluted uncut lambda DNA (25 ng/μl) as standard to assay its concentration and integrity by ethidium bromide fluorescence. DNA was also quantified with UV/VIS spectrophotometer (Hitachi model U-1500) by measuring OD_{260} and OD_{280}. 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.

RAPD analysis

RAPD reactions were performed according to the protocol of Williams *et al.*[5] with the following modifications to enhance reproducibility and consistency of RAPD profiles. RAPD amplifications were performed in 25 μl of reaction volume containing 1× PCR buffer {10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin}, 200 μM each dNTP (Sigma, USA), 20 pmol of 24 random decamer primers (Operon Technologies Inc, USA; Table 2), 0.6 unit of *Taq* DNA polymerase (Bangalore Genei, India) and 100 ng genomic DNA template. PCR amplifications were performed in a thermal cycler (M.J. Research Inc., model PTC-100) with heated lid technology for 45 cycles. The PCR conditions were: initial denaturation of genomic DNA at 95°C for 4 min followed by 45 cycles of DNA template denaturation at 94°C for 1 min, primer annealing at 32°C for 1 min, DNA amplification at 72°C for 2 min and final primer extension at 72°C for 8 min. Amplicons were separated on a 1.5% agarose gel pre-stained with ethidium bromide solution using 1× TAE buffer. The gels were run for 4 h at 45 V and the RAPD amplicon profiles were recorded using Syngene Gel Documentation System with GeneSnap software. The size of the amplified fragments was determined using 100 bp plus ladder (MBI Fermentas, Lithuania) and GeneTools software. All RAPD reactions were performed twice to test the reproducibility of the amplicon profiles.

Table 2. Efficacy of individual primers for detecting DNA polymorphism and genotypic differentiation in *A. bisporus*

Primer	Sequence (5′–3′)	Number of RAPD markers	Size range (bp)	DNA polymorphism (%)	PIC[a] value	Specific cultivars identified
OPF-11	TTGGTACCCC	6	825–2850	16.7	0.17	NCS-101, NCH-102
OPF-12	ACGGTACCAG	5	375–1125	40.0	0.93	Hybrids from varieties
OPF-13	GGCTGCAGAA	8	600–3500	75.0	2.12	Hybrids from varieties
OPF-14	TGCTGCAGGT	12	360–3200	58.3	1.72	U-3, ITCC-1924, ITCC-3554
OPF-16	GGAGTACTGG	4	950–2250	75.0	0.97	U-3, RRL-89
OPG-06	GTGCCTAACC	6	600–3500	50.0	0.98	Hybrid-1
OPG-11	TGCCCGTCGT	10	775–4000	80.0	2.07	S-11, U-3, MS-39, ITCC-3554
OPG-12	CAGCTCACGA	8	250–3100	37.5	0.93	Hybrids; NCS-101, NCH-102
OPG-14	GGATGAGACC	5	800–1800	80.0	1.36	Hybrids; NCS-101, NCH-102
OPN-01	CTCACGTTGG	6	1425–2850	50.0	1.16	Hybrids; ITCC-3710
OPN-02	ACCAGGGGCA	3	950–2200	00.0	0.00	None
OPN-04	GACCGACCCA	12	450–3300	25.0	0.95	Hybrids; NCH-102
OPN-05	ACTGAACGCC	9	525–2600	55.6	1.13	Hybrids; Chail strain
OPN-06	GAGACGCACA	10	480–2650	50.0	1.33	Hybrids; NCS-101, NCH-102
OPN-07	CAGCCCAGAG	5	350–2800	20.0	0.40	U-3 derivative hybrids
OPN-08	ACCTCAGCTC	3	375–1375	33.3	0.48	Hybrids from varieties
OPN-09	TGCCGGCTTG	10	675–2250	60.0	1.61	U-3, ITCC-1924, ITCC-3554, ITCC-3710, MS-39, NCS-100
OPN-10	ACAACTGGGG	7	425–1850	71.4	1.22	Hybrid-1, MS-39, ITCC-3709
OPO-02	ACGTAGCGTC	8	640–3200	62.5	1.34	S-11; hybrids from varieties
OPO-03	CTGTTGCTAC	14	425–2750	85.7	1.86	S-56, NCS-100, NCS-101, NCH-102, ITCC-3710, ITCC-1924
OPO-04	AAGTCCGCTC	7	475–2900	28.6	0.86	U-3 derivative hybrids
OPO-05	CCCAGTCACT	4	875–2750	50.0	0.17	WI-1
OPO-12	CAGTGCTGTG	4	1125–1925	75.0	0.79	Hybrids from varieties
OPO-13	GTCAGAGTCC	9	700–2600	44.4	1.18	S-11; hybrids from varieties

[a]Polymorphism information content.

ITS sequence analysis

The ITS regions (comprising ITS1, 5.8S rRNA gene and ITS2) were amplified using ITS1 ext B and ITS4 ext A forward and reverse primers[23] respectively. The sequences of these primers were: ITS1 ext B (forward primer): 5′-AACAAGGTTTCCGTAGGTGAACCTGC-3′ and ITS4 ext A (reverse primer): 5′-TTCTTTTCCTCCGCTTATT-GATATGC-3′. Hot start PCR was used for the ITS amplification in 50 µl reaction mix that consisted of two phases: (i) upper phase (25 µl): 1× PCR buffer, 400 µM each dNTP, 2 units of *Taq* DNA polymerase, and (ii) lower phase (25 µl): 1× PCR buffer, 20 pmol each of primers ITS1 ext B and ITS4 ext A, and 100 ng genomic DNA. These two phases were separated by a layer of Dyna wax. PCR conditions for ITS amplification were: 94°C for 5 min for initial genomic DNA denaturation; 30 cycles of 94°C (DNA denaturation) for 30 s, 55°C (primer annealing) for 30 s, 72°C (DNA amplification) for 30 s and final extension at 72°C for 5 min. ITS amplified products were purified using Qiagen columns following QIAquick PCR purification kit protocol. Purified ITS DNA was eluted in 1 mM Tris-HCl, pH 8.0. The purified ITS DNA was analysed to test its integrity and concentration on 1% agarose gel.

Cycle sequencing reactions were performed using ABI PRISM™ BigDye Terminator Sequencing ready reaction kits (Applied Biosystems, Perkin-Elmer Corp., UK) for sequencing the ITS PCR products. The cycle sequencing reaction consisted of the following components (10 µl): 2 µl BigDye reaction mix, 1.6 µl primer (1 pmol/µl), 1 µl PCR product DNA (15–20 ng/reaction) and 5.4 µl deionized water. Four sequencing primers ITS1, ITS2, ITS3 and ITS4 were used for cycle sequencing reactions[17]. The sequences of ITS2 and ITS3 primers were: 5′-GCTGCGT-TCTTCATCGATGC-3′ and 5′-GCATCGATGAAGAAC-GC AGC-3′ respectively. The PCR conditions for cycle sequencing were: 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. The contents of the sequencing reaction were spin down and processed according to the protocol of ABI Prism 3100 Genetic Analyzer from Applied Biosystems. The samples were electrophoresed at sequencing facilities of Warwick Horticulture Research International, Wellesbourne, UK and Delhi University, South Campus, New Delhi, India.

Statistical analysis

The RAPD amplification products were scored as present (1) or absent (0) for each primer–genotype combination. Molecular data were entered into a binomial matrix and were used to determine Jaccard's similarity coefficients[26] with the NTSYS-pc software[27] version 2.02 h. These

similarity coefficients were then used to construct dendrograms depicting the genetic relationship 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 software using the DCENTER and EIGEN procedures. The Jaccard's matrix of genetic similarity was also used to generate a 3D plot of principal coordinates to resolve the patterns of variation among the genotypes. Bootstrap analysis was carried out using the WINBOOT program[28] with 1000 replications. Most informative primers were selected based on high polymorphism information content (PIC) values of individual primers[29].

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 variation (GD) was measured using Jaccard's similarity coefficients according to the formula:

% GD = (1 – Average similarity coefficient) × 100.

The ITS nucleotide sequences were analysed after assembling the sequence traces generated by all the four sequencing primers into a contig using SeqManII module of Lasergene software package (DNAstar Inc., Wisconsin). All reads for each sequence were then manually inspected; the consensus sequences were generated and saved in EditSeq files. Multiple sequence alignments of consensus sequences were made via ClustalX (1.83) algorithm[30]. The multiple aligned sequences were then utilized to generate bootstrap (η = 1000) N–J phylogenetic tree[31]. The tree was viewed with the help of TREEVIEW software (http://taxonomy.gla.ac. uk/rod/treeview.html).

Results

RAPD fingerprinting

Twenty-four reproducible primers amplified a total of 175 RAPD markers, out of which 94 (53.7%) were polymorphic. The DNA fragment size varied from 250 to 4000 bp (Figure 1 *a–d*). A total of 2640 amplicons were produced in 22 genotypes, with an average of 5.0 amplicons per genotype per primer. The hybrid ITCC-3710 produced the maximum number of amplicons (134), whereas the minimum (115) was obtained in S-11, MS-39 and WI-1. The highest number of amplicons was produced by the primer OPN-04 (243) followed by OPO-13 (174), while the least amplicons (52) were obtained with primers OPG-14 and OPO-12. The capacity of individual primers in the discrimination and identification of genotypes is shown in Table 2. The PIC value for RAPD primers ranged from 0.00 to 2.12. Maximum number of polymorphic markers was obtained with primer OPO-03 (12) followed by OPG-11 (8).

DNA polymorphism and genetic relationship

Similarity indices estimated on the basis of all the 24 primers ranged from 0.64 to 0.99, with the average of 0.81. The varieties exhibited relatively more genetic similarity (average similarity 92.7%) in comparison to hybrids (average similarity 84.3%). The highest molecular variation (36%) was recorded between variety S-11 and hybrid ITCC-3710. Cluster analysis based on similarity values grouped all the varieties into the first cluster, whereas the hybrids formed the second cluster exhibiting relatively

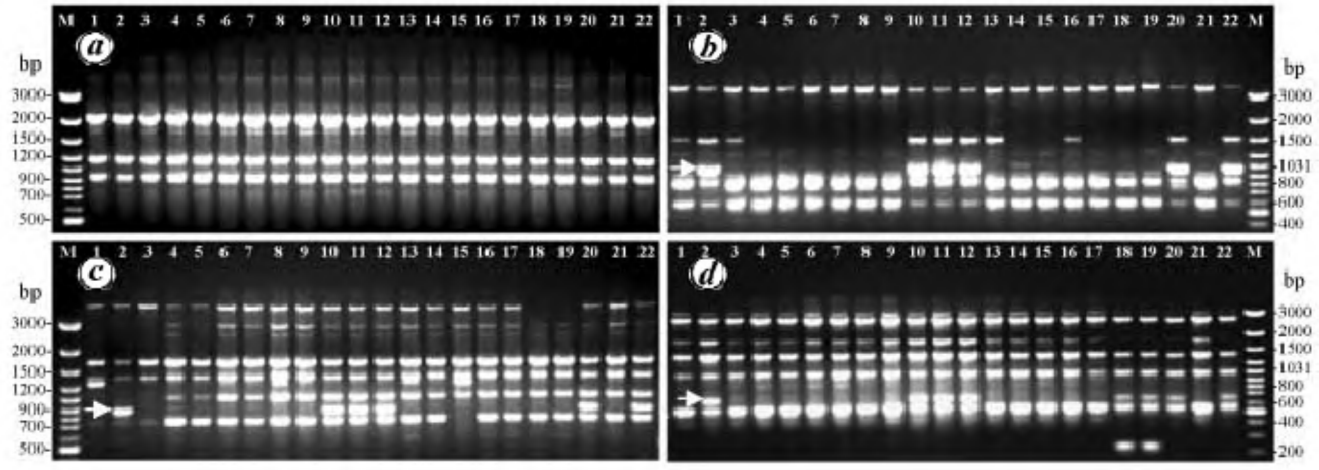

Figure 1. RAPD profiles of 22 cultivars of *Agaricus bisporus*. M, DNA ladder; lanes 1–22, Genotypes listed under serial nos 1–22 in Table 1. DNA polymorphism obtained with primers OPN-02 (***a***), OPG-06 (***b***), OPG-11 (***c***), and OPG-12 (***d***). Arrows indicate prominent bands present only in hybrid genotypes.

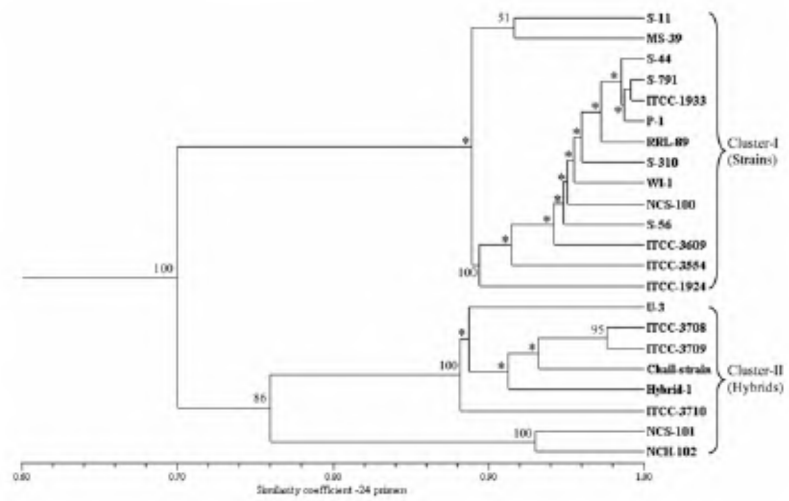

Figure 2. UPGMA dendrogram constructed from RAPD profiles of 22 cultivars of *A. bisporus*. The scale at the bottom represents Jaccard's coefficients of similarity. Numbers on the forks denote per cent bootstrap support to each node (branches showing less than 50% bootstrap values are indicated by asterisks). Major phylogenetic clusters are indicated at the right margin.

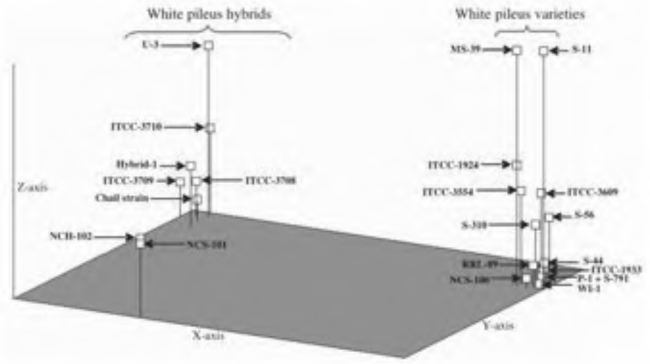

Figure 3. A 3D plot of the principal coordinates analysis of RAPD data showing molecular variation and relationships among white pileus cultivated varieties and hybrids of *A. bisporus*. Note: Hybrids are clearly separated from the varieties.

more DNA polymorphism (Figure 2). All the cultivars were distinguished using aggregated primer data. Individual primers were also informative in providing specific RAPD polymorphisms for genotypic differentiation (Table 2). However, none of the primers was able to differentiate all the 22 genotypes. High bootstrap values (100%) were recorded for the major nodes, which indicated the robustness of the clustering.

The 3D PCO plot clearly separated the varieties from the hybrids (Figure 3). Both the varieties and hybrids exhibited a comparatively narrow genetic base both in the UPGMA dendrogram and PCO plot. However, the NRCM hybrid NCH-102 and single spore selection from hybrid NCS-101 showed molecular variation from the varieties as well from the U-3-derived hybrids. The white pileus Indian collection (WI-1) from Himachal Pradesh was found to be clustered with commercial varieties, proving that it is not a real wild collection but a variety escaped from cultivation into the nearby forest area.

ITS sequence polymorphism

ITS1, 5.8S rRNA gene and ITS2 were amplified as a single unit from 22 cultivars of *A. bisporus*. In all cases, PCR yielded a single product without any length variations in the ITS profiles. The length of the ITS region was approximately 750 bases on gel in all genotypes. Multiple nucleotide sequence alignments of the ITS region were generated from 15 cultivars representing the major sub-clades of the dendrogram constructed based on RAPD markers. The full length ITS region was 756 bp in all the *A. bisporus* cultivars analysed. The 5.8S rRNA gene sequences were conserved within the species. The nucleotide length of ITS1 region, 5.8S rRNA gene and ITS2 region was 290, 154 and 208 bases respectively, in all the genotypes of *A. bisporus* studied. The 40 bases (3′–5′) of 18S rRNA gene and 64 bases (5′–3′) of 28S rRNA gene were also included in the full length ITS sequences.

The genetic relationships inferred from the nucleotide sequence polymorphism in the ITS region of different genotypes of *A. bisporus* are depicted in Figure 4. Two sub-clades were formed within the major phylogenetic clade of *A. bisporus*. All the hybrids grouped into one sub-clade. All the varieties, except ITCC-1924 and ITCC-3609 were clustered into a single sub-clade. The ITS1 region was found to be conserved in all the genotypes of *A. bisporus* analysed, while the ITS2 region showed SNPs at nucleotide positions of 522 and 563 (nucleotide positions are indicated from the beginning of the ITS1 sequence). The transition mutations (T→C) occurred at both the SNP positions (Figure 5).

Discussion

Assessment of genetic variation and discrimination of individual strains in the germ plasm are essentially required

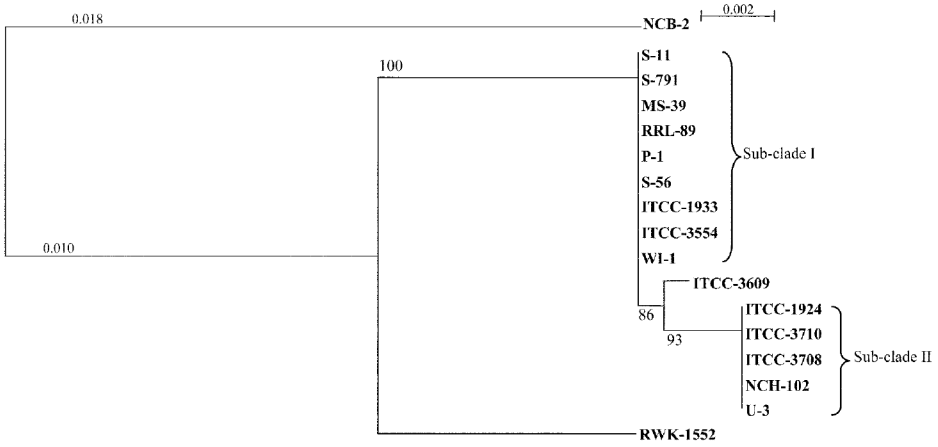

Figure 4. Neighbour-joining phylogenetic tree inferred from sequence polymorphisms in the ITS region of cultivated varieties and hybrids of *A. bisporus*. Numbers on the branches denote per cent bootstrap support to each node. The tree was rooted using two related species, *A. bitorquis* (NCB-2) and *A. subfloccosus* (RWK-1552). Sub-clades within the *A. bisporus* phylogenetic clade are indicated at the right margin.

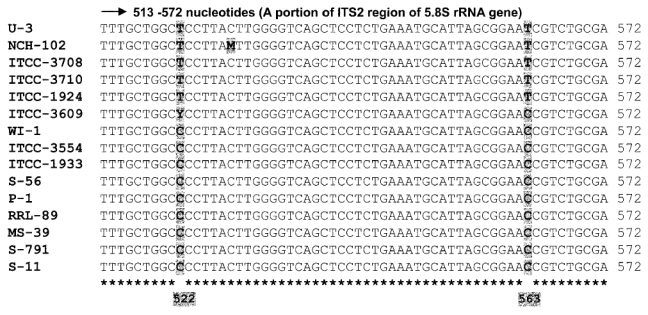

Figure 5. Single nucleotide polymorphisms at two nucleotide positions (522 and 563 bp) in the ITS2 region of the 5.8S ribosomal RNA gene among the cultivars of *A. bisporus*. Nucleotide positions are indicated from the beginning of the ITS1 sequence.

for the utilization of genetic resources in breeding programmes. We have demonstrated that RAPD can distinguish hybrids from the varieties of cultivated mushroom *A. bisporus*, and established genomic homogeneity among the varieties. Khush *et al.*[13] were the first to use the RAPD technique for DNA fingerprinting of *A. bisporus* strains and to establish a genetic relationship amongst wild and commercial strains. RAPD profiles exhibited unique fingerprints for a cultivated brown strain and five wild collections.

In our study, the RAPD fingerprinting revealed low levels of DNA polymorphism among white pileus commercial varieties and hybrids. The white pileus varieties showed only 7.3% molecular variation among them. Moore *et al.*[14] distinguished 26 strains of *A. bisporus* that included 24 commercial cultivars using aggregated data of 20 primers. The hybrids showed a high degree of genetic similarity and appear to be derivatives of two original hybrids. The overall similarity index of 0.81 in this study indicates that the cultivars of white button mushroom currently grown in India possess a narrow genetic base. The commercial cultivars of *A. bisporus* have been shown to be genetically homogeneous using isoenzymes[32,33] and nuclear DNA RFLPs[34,35]. The isoenzyme and RFLP analyses scan only a limited number of loci in the genome and are not suitable for assessment of genetic variation in the pedigree-related and genetically close strains.

Ribosomal RNA genes evolve cohesively within a single species[36] and exhibit only limited sequence divergence in the ITS regions of rRNA genes between individuals of a species[37,38]. Direct sequencing of PCR amplified 5.8S rRNA gene along with flanking ITS regions has been exploited for determining the genetic inter-relationships among the different species of the same or related genera of mushrooms[19,20,39]. Challen *et al.*[23] distinguished *A. bisporus* var. *burnettii* Kerrigan & Callac by a single transition mutation (G→A) at 261 nucleotide position in the ITS1 region, while *A. bisporus* var. *eurotetrasporus* had the characteristic deletion (a single base –T– at 118 nucleotide position) in the ITS1 region. Similarly, Callac *et al.*[24] observed ITS sequence polymorphisms at five nucleotide positions, two in the ITS1 region and three transition mutations in the ITS2 region, namely at 509 (T→C), 550 (T→C) and 628 (G→A) nucleotide positions in the wild populations of *A. bisporus*. In this work we have compared the ITS sequences from 15 cultivars of *A. bisporus* (differentiated based on RAPD markers) and found that the ITS1 region is conserved (does not have base substitutions or deletions). However, we have identified SNPs at two nucleotide positions, namely at 522 and 563 in the ITS2 region, which could differentiate the commercial hybrids from the varieties in *A. bisporus*.

Our results have demonstrated that the RAPD markers and ITS sequencing could be employed for discrimination of hybrids from the varieties in the *A. bisporus* germ plasm. The narrow genetic base of the germ plasm should be broadened by collecting wild strains from different agro-climatic regions of India and exploiting them for genetic improvement of this commercially important vegetable crop.

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Development of *barnase/barstar* transgenics for hybrid seed production in Indian oilseed mustard (*Brassica juncea* L. Czern & Coss) using a mutant acetolactate synthase gene conferring resistance to imidazolinone-based herbicide 'Pursuit'

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The use of herbicide-based field selection marker is an integral component in the development of hybrid seeds based on transgenic approach using *barnase* and *barstar* genes. It is an added advantage if the same marker can also be used efficiently as an *in vitro* selection marker. In the present study we report the development of male sterile transgenic lines and their restorers in *Brassica juncea* using the *barnase–barstar* system in conjunction with a selection marker gene ALS^{dm} conferring resistance to the imidazolinone, imazethapyr, the active ingredient of the herbicide 'Pursuit'. This herbicide is commercially available at low costs in India. It therefore provides a viable alternative to phosphinothricin/glufosinate herbicides used in the first-generation hybrids developed using the *barnase–barstar* system. Using constructs containing the ALS^{dm} gene we have developed stable male sterile *barnase* lines and homozygous *barstar* lines showing proper restoration, in appropriate combiners Varuna and EHII. We also successfully demonstrate the use of 'Pursuit' in field selections of male sterile lines in a segregating population.

Keywords: Acetolactate synthase, *barnase*, *barstar*, *Brassica juncea*, hybrid seed technology.

BRASSICA JUNCEA L. Czern & Coss (Indian mustard) is a major oilseed crop of the Indian subcontinent, being cultivated in around 6 million ha in the rainfed areas of northern India. Heterosis breeding has been successfully deployed for enhancing crop productivity in mustard, wherein hybrids between Indian and eastern European varieties lead to ~25–30% heterosis over the Indian check varieties[1]. As *B. juncea* is predominantly a self-pollinating crop, production of the hybrid seed is dependent on the development of a proper pollination control mechanism.

We have previously reported the development of a male sterility and restorer system in *B. juncea* based on transgenics with *barnase* and *barstar* genes respectively[2–4]. In this system male sterile lines were generated in one of the combiners by expressing the *barnase* gene from *Bacillus amyloliquefaciens* using a tapetum-specific TA29 promoter from tobacco. A major modification was made in the earlier *barnase* constructs to achieve more regulated expression of the tapetum-specific promoter[2]. Restoration of male fertility in the F_1 hybrids was brought about by an improved tapetum-specific expression[4] of the *barstar* gene, also from *B. amyloliquefaciens*, which was introduced into the other parental line.

Male sterile lines are maintained by backcrossing with an isogenic line without the *barnase* gene. The progeny thus obtained segregates for male sterility and fertility. In order to select for the male sterile lines in field, these lines are also made resistant to an herbicide by incorporating a gene conferring herbicide resistance in the *barnase* construct. Since for hybrid seed production both male sterile lines and *barstar* lines are grown together in the same plot and field selection of segregating male sterile population is concomitantly carried out by herbicide spray, the *barstar* transgenics are also made resistant to the same herbicide. Further, it is an advantage if the gene conferring resistance to herbicide can also be used for *in vitro* selection of initial transformants as it avoids the use of a second gene as a selection marker.

The commercial exploitation of this technology is dependent upon the availability and cost of the herbicide being used for field selections. The first generation *barnase* and *barstar* lines developed in our laboratory[2–4] used the *bar* gene (from *Streptomycin hygroscopicus*) for resistance against phosphinothricin/glufosinate, which is an active

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