CONVENTIONAL AND MOLECULAR APPROACHES FOR BREEDING BUTTON MUSHROOM

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ABSTRACT

A total of 294 single spores were isolated from eleven-button mushroom strains and 132 were evaluated for their fertility of which 24 were found non-fruiting whereas eleven single spore isolates started fruiting after second flush of cropping and remaining were fertile. There were significant variations in the morphological traits like stipe length, gill size, average fruit body weight, etc. The colour of gills was also highly variable and it ranged from dark brown to off white and in some cases gills were almost absent. There was segregation for fruit body colour and SSIs of some of the varieties produced fruit bodies of white, off white, light brown and brown colour. The variability has been analyzed and selected SSIs were subjected to RAPD and gene sequencing of 5.8S rDNA for identifying markers related to fertility. The non-fruiting isolates also produced fruit bodies towards the end of the crop possibly due to natural hybridization in the cropping rooms. DNA from such natural hybrids and original SSIs have also been subjected to RAPD and ITS sequencing for confirmation of natural hybridization. The promising natural hybrids have also been cultured for comparing their yield performance and quality with selected SSIs.

Keywords: *Agaricus bisporus*, single spore isolates, homokaryon, RAPD, ITS sequences

INTRODUCTION

The life cycle and the sexuality pattern of only a few species of basidiomycetes are known, yet there seem to be the predominance of heterothallic sexual pattern in majority. Similarly the compatibility system governing the sexual behavior of these fungi are mostly bifactorial (tetra-polar) in nature, with only a few species showing uni-factorial control.

The breeding of *A. bisporus* is complicated proposition because of its unusual secondary homothallic sexual behavior where majority of the basidia produce two spores each containing two nuclei of opposite mating-type and only a few basidia are tri- or tetrasporic yielding homokaryotic spores. There is no way to identify the latter as *A. bisporus* mycelium unlike other basidiomycetes lacks clamp connections and is multinucleate. The only method to identify non-fertile isolates is the fruiting trial.

The diversity of gene pool is one of the most important prerequisite for any breeding program. During the present study, 294 number of single spore were isolated from eleven different strains (existing and wild), 132 of these were screened for their fertility and some of them were evaluated for morphological traits to analyze the diversity as well as the DNA profiling using RAPD and ITS 5.8S gene sequencing to identify a marker for the fertility so that cumbersome fruiting trial of a large SSI population may be avoided.

MATERIAL AND METHODS

Germplasm used. A total of 294 single spores were isolated from eleven strains, namely, S-11, U-3, A-4, A-6, A-16, A-2, A-15, A-94, S-465, S-130, and a wild strain W.I.-1. All the strains used in the study were obtained from culture collection of Directorate of Mushroom Research, Solan, and Indian Agriculture Research Institute, New Delhi.

Single spore isolation. All the strains were cultivated on pasteurized compost using standard cultivation practice to see the morphological variation and also to collect the spore prints for single spore isolation. SSIs were isolated using serial dilution technique on the standardized media containing dextrose – 5.0 g; $MgSO_4$ – 0.25 g; KH_2PO_4 – 0.95 g; succenic acid – 1.25g; Agar – 15.0 g; water – 500 ml and pH- 5.0. Placing *Agaricus* mycelium on the lid triggered the spore germination where the plates were placed in inverted position. The single germinating spores were marked under an inverted microscope and transferred to slants.

Cultivation of Single spore isolates. The spawn of a total of 132 isolated single spores was prepared on wheat grains following standard practice and was cultivated on pasteurized compost in 10 kg bags. Each SSI was replicated three times. The yield of the each bag was taken up to four weeks and then average for each SSI was calculated and subjected to statistical analysis using anova. The fruit bodies of selected SSIs were analyzed for stipe length, gill size & colour, average fruit body weight and colour of pileus.

DNA Isolation. Genomic DNA was extracted from liquid nitrogen dried mycelium grown on malt extract broth medium (malt extract 10 g and dextrose 5 g) according to the modified method described by Punja & Sun [1]. The DNA concentration was estimated by electrophoresis in 1% Agarose gel containing 0.05% ethidium bromide. The DNA concentration was further estimated spectrophotometrically by taking optical density of the DNA solution at 260 and 280nm. The final working concentration of DNA was standardized to 5ng μl^{-1}.

RAPD analysis. Multilocus genotyping by RAPD was performed using 30 primers supplied by Operon Technologies namely, OPA-01-10 and OPP-01-20. Amplification was performed in a total reaction mixture of 25µl following Singh *et al.* [2]. PCR amplification products were electrophoretically separated on 1.6% agarose gel (Sigma) prepared in 1x TAE. The gel was run for 3 h at 45 V. The staining was done with ethidium bromide and visualized under 300 nm UV light and photographed. The gel photographs were scored for presence and absence of scorable bands with the assumption of positional homology. To establish the genetic relationship among the isolates, similarity coefficients were calculated between isolates and dendrogarm drawn using UPGMA Algorithm (Unweighted Pair Group Method using Arithmetic Averages) of the NTSYS-pc, Version 2.02h programme [3,4].

PCR amplification of ITS regions of 5.8S ribosomal DNA gene. The PCR primer ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (5'- TCC TCC GCT TAT TGA TAT GC-3') developed by White et al [5] were used to amplify the ITS of ribosomal DNA, which encompasses the 5.8S gene and both ITS-1 and ITS-2 regions. Amplifications were done following Singh *et al.* [2]. The PCR products were visualized on 1% agarose gel in Tris-Acetic acid-EDTA (1x TAE) buffer at 80 V for 60 min. Agarose gels were stained with ethidium bromide and photographed under UV light for amplified ITS products.

Sequencing and sequence analysis of ITS region. PCR products were directly sequenced using automated sequencer of Applied Biosystems (3730 analyser) using ITS-1 and ITS-4 primers. Nucleotide sequence comparisons were performed by using the Basic Local Alignment Search Tool (BLAST) network services against the National Centre for Biotechnology Information databases. The multiple sequence alignment of ITS region of twenty samples (10 each of fertile and sterile monospore) was performed using the CLUSTAL X (1.8) and unrooted neighbour joining tree was plotted to determine grouping amongst the monospores.

RESULTS

A total of 294 single spores were isolated from eleven button mushroom strains and 132 were evaluated for their fertility. Out of 132 single spore isolates some of them were discarded due to very low yields or poor quality fruiting bodies and a total of 64 SSIs were selected for further studies. Twenty-four SSIs were found non-fruiting whereas eleven single spore isolates started fruiting after second flush of cropping that may be due to natural hybridization and remaining were fertile (Table 1). There were significant variations in the morphological traits like stipe length ranging between 1.2 to 4.6 cm, gill size between 0.5 to 4.0 mm, average fruit body weight between 6.86 to 14.9 g, and pileus dia between 1.8 to 4.7cm. The colour of gills was also highly variable and it ranged from dark brown to off white. During the evaluation of the single spore isolates, some of the isolates were found to have negligible gill and also the colour of the gills were very light. It has been noticed that the colour of the SSIs of white strains also varied from white to light brown, which may be due to segregation of traits for fruit body colour (Fig 1). The stipe and pileus size of the SSIs were found to be highly variable character, however, the pileus:stipe ratio was found to be quite constant ranging from 1.6 to 2.5.

The fertile isolates were used for selection of the high yielding and good quality mushroom fruit bodies whereas non-fertile isolates were subjected to hybridization experiments. In the hybridization experiments the isolates were mated in inter and intra-strainal combination so as to determine mating types and develop inter-strainal hybrids.

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. The clustering and similarity analysis of the single spore isolates was done using NTSys PC version 2.02. The dendrogram generated is presented in Figure 2. The dendrogram shows that the fertile and the non-fertile isolates could be clearly separated through the RAPD primers and are grouped in separate clades. Besides, the some of the RAPD primers was successful in identifying a marker amongst fertile single spore isolates and are marked in the Figure 3. Different primers generated fragments of different sizes in the fertile isolates whereas these fragments were not found in the non-fertile isolates. OPP-1 has generated a fragment of size 2100 bp in the fertile isolate whereas OPP-3 generated a fragment of 1500 bp size. OPP-4 and OPA-10 has generated fragments of size 1250 and 1350 bp, respectively. These bands are of quite large size hence primers against these fragments can be developed and tested to avoid one of the major demerit of the Random Amplified Polymorphic DNA technique i.e. the reproducibility of the fragments under varied conditions, which has always been a major issue while using RAPD markers in any genetic analysis.

Table 1: Number of fertile, non-fertile and natural hybrids from 6 strains of *A. bisporus*

Strain	Fertile SSI	Non-fertile SSIs	Natural Hybrid
A-94	20	2	--
A-6	9	--	--
A-4	5	--	--
U-3	36	22	11
A-15	20	--	--
WI-1	7	--	--

Figure 1. Variation fruit body colour, shape, gill size amongst Single Spore Isolates

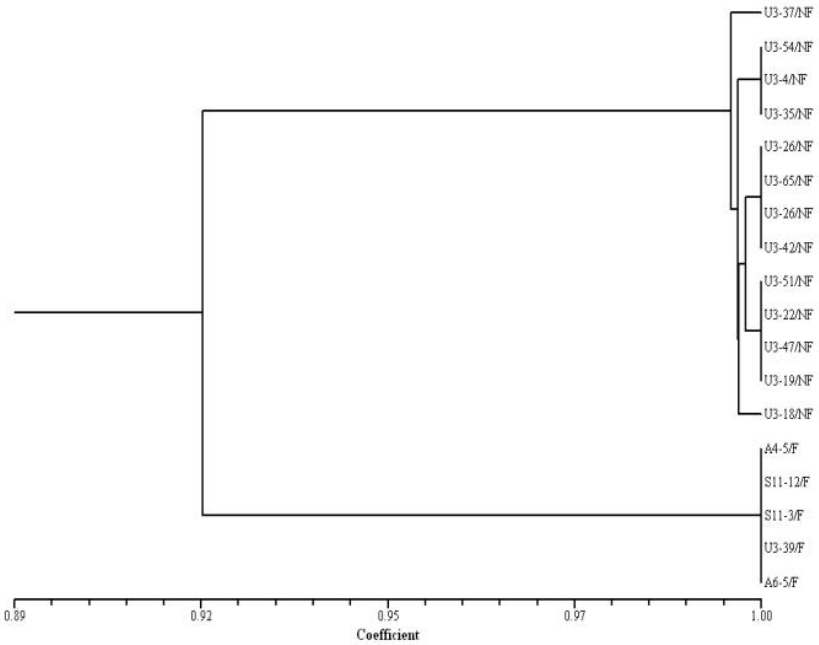

Figure 2. Dendrogram of 18 SSIs generated by NTSys pc based on the thirty primers

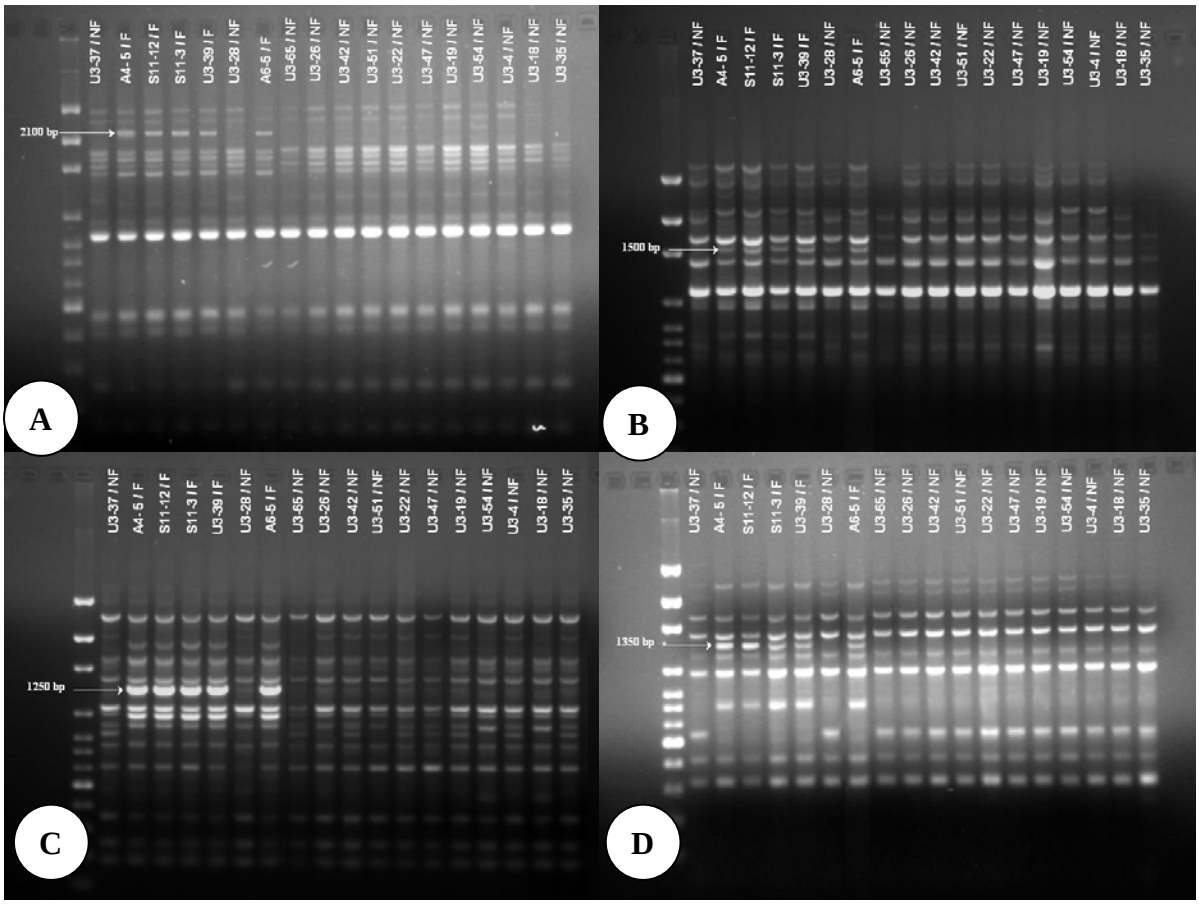

Figure 3. RAPD gel photographs showing marker band in fertile isolates of button mushroom (A = OPP-1; B = OPP-3; C = OPP-4; D = OPA-10).

For ITS sequencing a total of 18 SSIs were taken for the analysis and 10 of them were fertile isolates while the other 8 were non-fertile (table 2). The Electrophoretic separation of amplified ITS 5.8s rDNA region has shown the amplification of a fragment near about 750 bp. The PCR products have been sequenced for diversity analysis as well as to identify a marker for monokaryon identification. The multiple sequence alignment of SSIs exhibited a low level of polymorphism but the generated tree shown in Figure 4 clearly indicated three groups (i) the group of non-fruiting SSIs, (ii) Group of fruiting SSIs, and (iii) a group in which both types of SSIs are included.

Table 2: List of ITS sequenced Single Spore Isolates

Non-Fruiting isolates		**Fruiting Isolates**	
U-3-32/NF	U-3-4/NF	U-3-54/F	U-3-59/F
U-3-42/NF	U-3-18/NF	U-3-39/F	U-3-50/F
U-3-47/NF	U-3-23/NF	U-3-5/F	U-3-43/F
U-3-51/NF	A-94-10/NF	U-3-45/F	A-94-4/F
		U-3-31/F	A-94-9/F

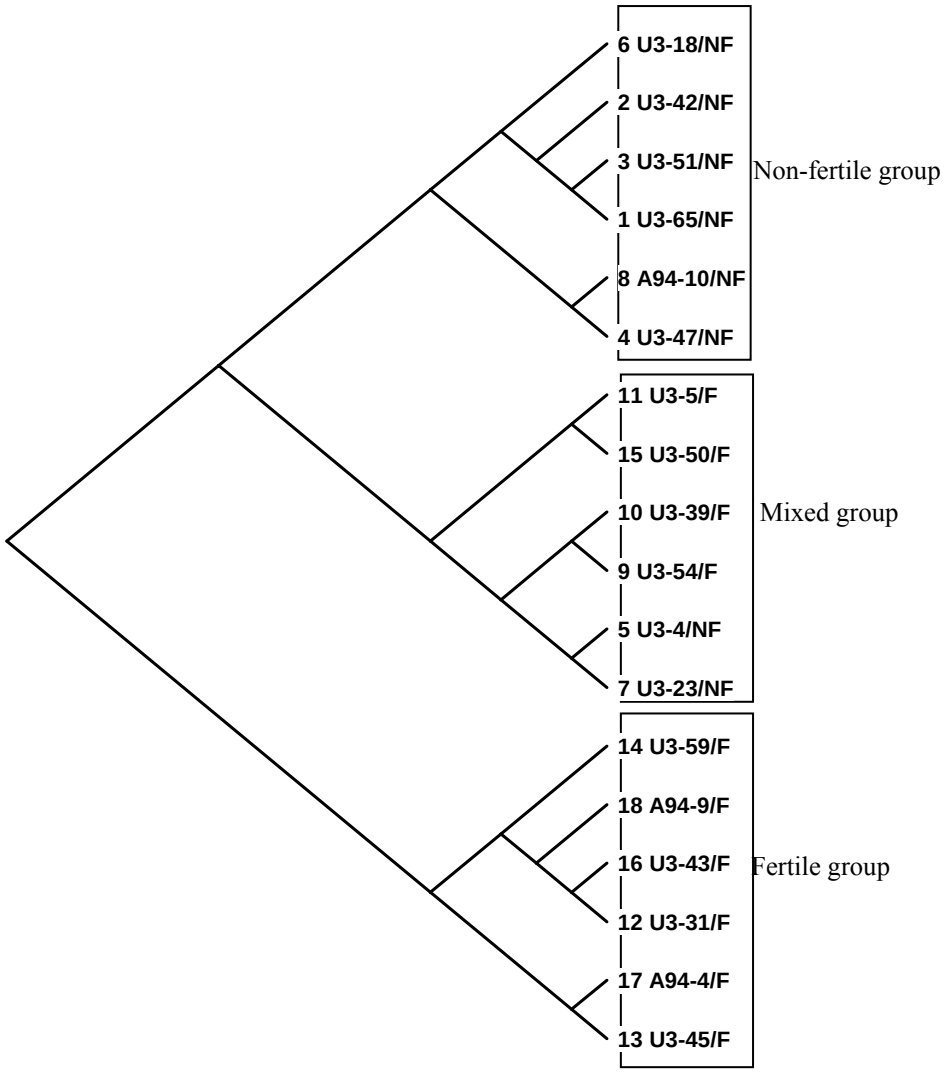

Figure 4. Sequence cladogram showing grouping of SSIs

DISCUSSION

Although button mushroom is an economically important crop, the breeding activity was based mainly only on selection until the late 1970s [6]. Fritsche in The Netherlands took the first systematic breeding approach, and this led to the introduction of the "hybrid" strains Horst U1 and Horst U3 [7]. By the late 1980s most of the cultivated strains in the world were identical to or derived from those strains [8], leaving the industry with a crop that was genetically very limited and had a high risk of sensitivity to disease.

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 [6]. 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. DNA restriction fragment length polymorphisms (RFLPs) have been used as genetic markers in a wide variety of organisms,

including humans [9], plants [10, 11, 12], fungi [13, 14, 15], and protozoa [16]. Nuclear ribosomal DNA (rDNA) is frequently used for taxonomic and phylogenetic studies of different species of edible fungi. The RAPD technique has been used both for studies on wild and cultivated strains of *A. bisporus* [1] or in delineation of its homokaryons [18].

In the present study, SSIs of eleven different strains of *A. bisporus* have been taken to identify marker for distinguishing between homo- and heterokaryon without going for a lengthy fruiting trials. Out of 294 a total of 132 SSIs were evaluated for their fertility first and then the molecular analysis was done using RAPDs and ITS sequences on some of them. Twenty-four non-fertile isolates could be identified during the present study and out of which 13 randomly selected SSIs along with five fertile isolates were tested for RAPD profiles for identification of markers. These studies could identify marker band of various molecular weight for fertile isolates by random primers OPA-10, OPP-1, OPP-3, OPP-4 and OPP15. These fragments could only be observed in the fertile isolates and not in the non-fertile ones. Hence this can be used in screening of a large number of populations of SSIs to restrict the number of SSIs to be taken in to the crossing for hybrid development. As the sample size in the study is not so large hence further analysis is under progress to confirm the findings in larger populations and across different strains of button mushroom.

On the other hand, the monokaryon and the heterokaryon differ in their ploidy level, the ITS 5.8S rDNA sequences may differ in the two. To take advantage of the differences in ploidy levels of the fertile and non-fertile isolates, the 5.8S rDNA was sequenced and analysed. However, the analysis of the sequences showed three groups of fertile, non-fertile and mixed group. The non-fruiting isolates, which are grouped completely separate may be the monokaryons and group of fertile SSIs are true heterokaryons but the mixed group contains the fruiting isolates along with the non-fruiting isolates. This may possibly due to four reasons (i) the non-fruiting isolates may be the bi-nucleate homokaryons (ii) isolates are heterozygous but non-fruiting is due to some genetic aberration (iii) wrongly identified as non-fruiting or (iv) error in sequencing. Further analysis of the sequences is under progress to specify the base pair changes, which may serve as a marker for fertility in button mushroom. The finding of the analysis is of immense importance in *Agaricus* breeding program but needs to be validated with larger number of populations across different strains.

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