Int J Pharm Bio Sci 2015 July; 6(3): (B) 301 - 314

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

FRUIT BODY PRODUCTION AND CHARACTERIZATION OF HYBRID EDIBLE MUSHROOM STRAINS DEVELOPED BY PROTOPLAST FUSION BETWEEN *PLEUROTUS FLORIDA* AND *LENTINUS SQUARROSULUS*

P MALLICK AND SR SIKDAR*

Division of Plant Biology, Bose Institute, P-1/12, C.I.T. Scheme VII M, Kolkata 700 054, India.

ABSTRACT

Polyethylene glycol (PEG)-mediated protoplast fusion between *Pleurotus florida* and *Lentinus squarrosulus* successfully developed twelve *pfls* somatic hybrids using double selection screening method. Hybridity was proved by the colony morphology, mycelial growth, hyphal traits, fruit body parameters and RFLP profiling of rRNA-ITS region. ANOVA is used for phenotypic data analysis of the *pfls* hybrids and parents. Out of twelve, only six *pfls* hybrid could produce basidiocarp on paddy straw substrate in sub-tropical climate and showed phenotypic resemblance to the *P. florida* parent. PCA analysis showed maximum positive correlation between each phenotypic variable for all strains examined in which the highest influential variables were found between cell width and yield. The amplified rRNA-ITS bands were ranged from ~ 620 bp to 700 bp in size in all the samples tested. Restriction enzymes *Alu*I, *Hin*fI, *Hpa*II and *Hae*III were found to be highly polymorphic after digestion of rRNA-ITS products for all the *pfls* hybrids and parents. A total of 96 RFLP fragments were was produced for all in which *Hae*III showed the maximum (34) and *Hpa*II showed the minimum (16) restriction fragments in gel. The digested fragments were ranged from ~ 160 bp to 680 bp in size.

KEYWORDS: **Basidiocarp, PCA, Polymorphism, RFLP, rRNA-ITS, Somatic hybrid.**

*Corresponding author

SR SIKDAR
Division of Plant Biology, Bose Institute, P-1/12,
C.I.T. Scheme VII M, Kolkata 700 054, India.

INTRODUCTION

Development of new mushroom hybrids through para-sexual mating is now well established. The evaluation of genetic diversity would promote the efficient use of genetic variations in the breeding programme for crop improvement[1]. Fungal protoplast technology has been widely used in genetic transformation, fusion programme, selection of asexual clones with desired properties and physiological analysis[2-5]. Protoplasts can be used as a starting material for industrial applications as well as for basic research[4]. Protoplast fusion is an important approach for somatic hybridization and in the past, such attempt generated hybrid strains in mushrooms[2,6]. Generation of somatic hybrid strains in edible mushrooms through protoplast fusion had been attempted in the past in intra-specific, inter-specific, inter-generic and even inter-order combinations where basidiocarp was not successfully developed[7-9]. But in several cases, hybrid basidiocarp was successfully developed and characterized by morphological and yield assessment[10-15]. In the past, mushroom somatic hybrids were characterized by various molecular DNA markers which successfully proved their hybridity[12-14,16]. Restriction fragment length polymorphism (RFLP) analysis of rRNA-ITS region containing internal transcribe spacers (ITS1 & 2) and 5.8S rRNA genes are useful in determining the relationships between fungal genera and species[17-22]. The PCR-based RFLP technique helped to detect polymorphism of the the ribosomal RNA region (ITS1-5.8S-ITS2) of ectomycorrhizal fungi[23-28]. Somatic hybrids of albino *Cucumis melo* L. mutant and *Cucumis myriocarpus* Naud were was characterized by restriction fragment length polymorphism in the rRNA-ITS regions and showed genetic variation in the nuclear ribosomal DNA sequence[29]. The oyster mushroom *Pleurotus florida* is cultivated in the sub-tropical (22–26ºC) climate and they are popular not only for its taste but simple production technology for its immense nutritive values also. Quantitative compositional analyses of this cultivated species revealed to be highly nutritious, rich in protein, carbohydrate, fibre and essential fatty acids[30]. *Lentinus squarrosulus* (Mont.) singer is a white rot fungus which generally survives saprophytically on dead woods in the forest[31]. It is an *Agaricaceous* fungus with tough fruit bodies and rich in proteins, sugars, lipids, amino acids, vitamin-B, C, D and minerals[32]. This mushroom is very popular in Nigeria and has been highly recommended for commercialization[33]. The aim of the present study was to develop somatic hybrids through protoplast fusion between *Pleurotus florida* and *Lentinus squarrosulus* and characterization of the hybrids. We have successfully produced some somatic hybrid lines. The present communication deals with the details description of somatic hybrid production and characterization of the hybrid lines through morphological and molecular approaches.

MATERIALS AND METHODS

(i) Strains and culture condition

The *Pleurotus florida* strain was obtained from 'National Research Centre for Mushroom' Solan, Himachal Pradesh, India. Fruit body of *Lentinus squarrosulus* was collected from dead wood stock at Falta Experimental Farm, by Bose Institute, 24 Parganas (South), West Bengal, India and prepared the vegetative culture in the laboratory. Vegetative culture of two parental strains was maintained in PDA (Potato dextrose agar, pH 6.2) medium routinely at 24 ± 1°C. For protoplast preparation mycelial tissue was grown in liquid MYG medium (10 g/l malt, 4 g/l yeast extract and 10 g/l glucose, pH 6.2) for 4–5 d and 3–4 d for *P. florida* and *L. squarrosulus*, respectively.

(ii) Protoplast preparation

Mycelial tissues of parental strains grown on MYG medium were washed with sterile water for three times and twice with osmotic stabilizer (0.6 M mannitol), chopped into

pieces for enzymatic digestion. A combination of mycolytic enzymes (2% lysing enzymes, Sigma-Aldrich and Cellulase Onozuka R–10, Yakult) were dissolved in 0.6 M mannitol with 0.2% $CaCl_2$. $2H_2O$ (pH 5.5) and filter sterilized. Digestion of mycelial tissue in the enzyme mixture was done for 12 h for *P. florida* and 10 h for *L. squarrosulus* with shaking at 70 rpm at 24 ± 1ºC. Liberated protoplasts were purified by sieving through cotton wool pad and centrifuged at 3000 rpm for 5 min to collect the pellet. The pellet was then washed thrice with osmotic stabilizer and finally suspended with 200 µl of osmotic stabilizer. The total yield was measured using a hemocytometer and viability percent was calculated by FDA staining[34].

(iii) Hybrid selection strategy

In this fusion experiment hybrids were identified by double selection strategy. Protoplasts of *P. florida* was inactivated (cytoplasmic gene function) with 10 mM iodoacetamide (IOA) treatment in the dark for 5 min and *L. squarrosulus* was heat treated at 70°C for 10 min. The fused protoplasts were plated on MYG medium supplemented with 0.6 M mannitol and 1.5% agar, pH 6.2. As both the parental protoplasts were inactivated, they could not grow individually in the fusion medium. Only due to complementation of either both the parental genome or nucleo-cytoplasmic interaction, the hybrids could regenerate and develop colonies.

(iv) Protoplast fusion and culture

Protoplast fusion was carried out based on the protocol of Anne and Peberdy[35] with some modifications. Equal volume (50 µl) of inactivated *P. florida* and *L. squarrosulus* protoplasts were taken in a centrifuge tube at a density of 10^6–10^7 protoplasts/ml, mixed well and kept at 24°C for 5 min. To that, equal volume of 30% PEG (MW-3350, 0.05 M Glycine-NaOH, 50 mM $CaCl_2$. $2H_2O$, pH 7.5) was added, mixed well and incubated for 5 min with occasional shaking. Final suspension was diluted 6 times with MYG medium (pH 6.2) and each 100 µl of aliquot was plated onto MYG medium supplemented with 0.6 M mannitol and 1.5% agar, pH 6.2. Normal and treated protoplasts of the parental strains were plated on the same medium as positive and negative control, respectively. Fusion plates were maintained in the incubator at 24°C temperature. The presumptive somatic hybrids were transferred into PDA medium after macro-colony formation. The mycelial growth of putative somatic hybrids was tested through slide culture on PDA slab in aseptic condition at 24°C.

(v) Spawning and fruit-body development

Hybrid lines grown on PDA medium were used to inoculate the paddy grain packets for spawn preparation. Each paddy grain packets waswas made using 200 gm of autoclaved paddy grains. 10–18 days old mature spawn was finally inoculated for fruit body production on overnight soaked and autoclaved (20 lbs for 1 h) paddy straw substrate in a cylindrical polypropylene bag (100 g spawn/0.65 kg dry straw). After complete colonization bags were made porous for aeration and kept in the cultivation room maintaining high humidity at 24 ± 1ºC for fruiting.

(vi) Statistical analyses

All the morphological parameters including mycelium growth, cell width, colony diameter, stipe length, pelius diameter and fruit-body yield of all hybrids and parental strains were analyzed by One-way ANOVA and the Tukey Post-hoc test was done for multiple comparisons of means, standard deviation and level of significance[36]. Bivariate correlation between all the phenotypic data of hybrids and parents were analyzed by using IBM SPSS software. To understand the relationships between each phenotypic variable, Principal Component Analysis (PCA) was done using the extracted component matrices data (from SPSS) and a scatter plot was constructed in Microsoft excel.

(vii) Analysis of ribosomal RNA-ITS region

Genomic DNA was isolated from actively growing mycelial tissue using modified CTAB method[37]. Based on the protocol of White et al.[38], rRNA-ITS region was amplified from nuclear DNA of both the somatic hybrid strains and their parents. The PCR reaction was performed in a total volume of 25 µl reaction mixture containing 10 ng of template DNA, 10X Taq buffer (+KCl), 25 mM $MgCl_2$, 2 mM dNTPs mixture, primer ITS1 (5´ TCCGTAGGTGAACCTGCGG 3´) and ITS4 (5´ TCCTCCGCTTATTGATATGC 3´), Taq DNA polymerase (5 U/µl). The amplifications were conducted in a DNA thermal cycler (Applied Bio-systems 2027) by preliminary denaturation of DNA at 95°C for 4 minutes consisting of 40 cycles; DNA template denaturation at 94°C for 1 minute, primer annealing at 55°C for 1 min, initial extension at 72°C for 1 min 30 sec, followed by a final extension at 72°C for 8 min.

(viii)Restriction digestion & gel electrophoresis

PCR products were purified by sodium acetate precipitation. Each 10 µl of purified PCR product was used for restriction digestion according to the supplier's specification with each of the four restriction enzymes used in this experiment viz. *Alu*I, *Hin*fI, *Hpa*II and *Hae*III (Fermentas). The digested DNA fragments were size-fractionated in 1.5% agarose (w/v) gel (run in 1X TAE buffer at 80 V for 3 h) pre-stained with ethidium bromide (EtBr). The gel profiles were visualized under UV transilluminator and data were recorded in a Molecular Analyst Gel Documentation System. The sizes of the digested fragments were determined by gene ruler 100 bp plus DNA ladder (MBI, Fermentas) used as a standard molecular weight marker.

RESULTS

(i) Protoplast isolation, inactivation and generation of hybrid lines

Cell wall degrading enzymes successfully produced protoplasts in *P. florida* (1.5×10^7 protoplast / g tissue) and in *L. squarrosulus* (9.6×10^6 protoplast / g tissue) with 12 h and 10 h incubation, respectively (Figure 1a). Viability of the protoplasts was observed after FDA staining and recorded as 95.45 ± 3.25 % in *P. florida* and 98.35 ± 1.94 % in *L. squarrosulus* (Figure 1b). Regeneration percentage of *P. florida* protoplasts was 33.45 ± 9.02 while in case of *L. squarrosulus* it was 58.25 ± 9.30. Calculated plating efficiency for *P. florida* was recorded as 29.15 ± 3.40 % and for *L. squarrosulus* it was 46.75 ± 4.05. Protoplast regeneration was found in the fusion plates after 9–10 h of PEG treatment (Figure 1c). Macro-colonies were developed in the positive control plate of *P. florida* and *L. squarrosulus* after 7 d and 4 d, respectively. No regeneration was found in the negative control plates of *P. florida* and *L. squarrosulus* as these protoplasts were inactivated. Only due to complementation hybrid protoplasts could regenerate in the fusion plates. A total of 36 putative hybrid colonies were regenerated from the two fusion experiments involving 4.2×10^5 protoplasts (plating efficiency 0.000363%). Micro-colonies were scored in two spells i.e., after 5 d and 17 d from the fusion plates. All the 36 putative hybrid lines were transferred to PDA medium for further growth at 24ºC. Finally, only 12 hybrid lines (named as: *pfls 1k, 1i, 1s, 1m, 1g, 1j, 1q, 1n, 1h, 1o, 1e* and *1p*) could be maintained (Figure 1d) routinely in PDA medium for further characterizations.

Protoplasts account and vegetative cultures of pfls hybrid and parental strains

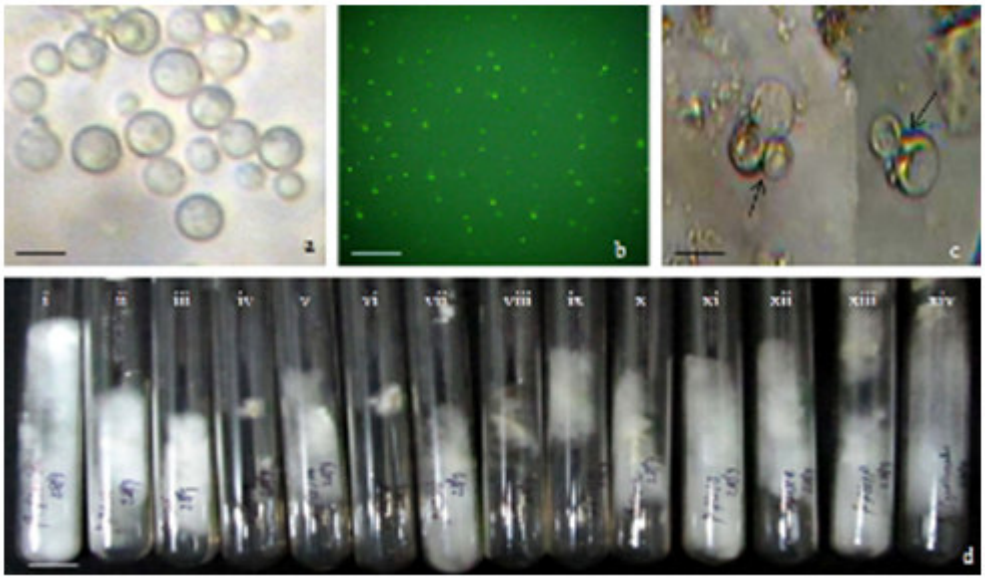

Figure 1

(a) Freshly isolated L. squarrosulus protoplasts. (b) FDA stained viable protoplasts of L. squarrosulus. (c) Microscopic view of protoplast fusion (marked by arrows) of two parental strains, after PEG treatment. (d) Two weeks old vegetative cultures of parental and hybrid strains (initial inoculum density was same for all the cultures). The cultures are (from left to right) P. florida (left), pfls 1k , pfls 1i, pfls 1s, pfls 1m, pfls 1g, pfls 1j, pfls 1q, pfls 1n, pfls 1h, pfls 1o, pfls 1e, pfls 1p and L. squarrosulus (right) respectively. Size bar correspond to 10 µ in (a) and (c), 100 µ in (b), 1.25 cm in (d).

(ii) Morphological assessment

Mycelial growth (4 days after inoculation), cell width on slide culture and colony diameter on PDA medium (7 days after inoculation) showed variations which are shown in Table 1. Growth of the *pfls* hybrid lines on slide culture ranged from 7.27 ± 0.30 cm (*pfls 1h*) to 1.41 ± 0.40 cm (*pfls 1m*) compared to 5.55 ± 0.08 cm in *P. florida* and 4.01 ± 0.22 cm in *L. squarrosulus* parents. Out of twelve hybrid strains, five (*pfls 1n*, *1j*, *1h*, *1e* and *1k*) were very close to *P. florida*, six (*pfls 1g*, *1p*, *1s*, *1m*, *1o* and *1i*) were close to *L. squarrosulus* and rest one (*pfls 1q*) was completely intermediate type of mycelial nature on morphological observations. Colony diameter of the hybrid lines (7 days old) ranged between 3.68 ± 0.15 cm (*pfls 1s*) to 1.66 ± 0.14 cm (*pfls 1i*) compared to 3.82 ± 0.11 cm in *P. florida* and 3.73 ± 0.29 cm in *L. squarrosulus*. Hyphal cell width also varied among the *pfls* hybrid lines and it was minimum in *pfls 1h* (11.6 ± 0.10 µm) and maximum in *pfls 1i* (36.5 ± 0.36 µm) compared to parents *L. squarrosulus* (33.7 ± 0.70 µm) and *P. florida* (25.37 ± 1.05 µm). No clamp connection was observed in any of the *pfls* hybrid lines and *L. squarrosulus* though it is a characteristic feature of *P. florida* parent.

Table 1
Morphological features of mycelia of the pfls hybrids and their parents

Strain	Growth after 4d in cm[a] (*F* = 11.51)	Colony diameter after 7d in cm[b] (*F* = 39.28)	Cell width in µm[c] (*F* = 47.66)	Mycelial nature
L. squamosulus	4.01±0.22	3.73±0.29	33.7±0.70	Fast growing, fluffy, brownish, leathery-type mycelia
pfls 1q	4.63±0.08	1.78±0.22	20.0±0.17	Off-white, compact, fast growing mycelia
pfls 1e	4.66±0.40	2.51±0.12	13.03±0.77	White, thread-like, fast growing mycelia radiated from the centre
pfls 1g	2.82±0.20	1.74±0.06	19.6±0.78	White, fluffy, slow growing mycelia with small colonies in liquid medium
pfls 1p	1.94±0.06	2.42±0.40	16.76±0.63	Off-white, very slow growth rate with small colonies in liquid and solid medium
pfls 1h	7.27±0.30	3.49±0.50	11.6±0.10	White, fluffy, dense, very fast growth rate with thread-like mycelia at mature stage
pfls 1n	5.46±0.14	2.44±0.04	35.73±0.23	Light-brown, fluffy with fast growing mycelia
pfls 1s	2.64±0.10	3.68±0.15	32.7±0.60	Brownish mycellial mat with slow growth rate, small colonies in liquid medium
pfls 1m	1.41±0.40	1.66±0.15	19.46±0.64	White, wooly, very slow growing mycelia
pfls 1k	6.58±0.27	2.72±0.10	33.86±1.93	White, fluffy, dense mycelia radiated from the centre with very fast growth rate
pfls 1o	3.14±0.17	1.76±0.08	25.1±0.36	White, fluffy, slow growing, scattered mycelia
pfls 1i	2.18±0.21	1.66±0.14	36.5±0.36	White, fluffy, slow growing, thread-like mycelia
pfls 1j	6.74±0.18	2.71±0.10	14.33±0.25	Off-white, dense, fluffy mycelia radiated from the centre with fast growing tip
P. florida	4.53±0.11	2.84±0.05	25.38±1.05	White, fluffy growth radiated from the centre
SE (Mean)	0.50	0.19	2.39	-
p- value	0.98	0.99	0.99	
CD (at 5 %)	0.88	0.74	0.69	-

The value of hyphal growth, colony diameter and cell width are means ± SD of five repeated experiments. Results of one-way ANOVA for each parameter are represented as F values, P<0.05 (considering the F value 3.18 for this experiment, the tabulated are greater). The difference between any two mean values (of any two different strains) of same parameter (i.e. within a column) is to be considered as significant at 5 % level if the said difference between the means exceeds the calculated CD (critical difference) values provided at bottom of the same column.
All the cultures were maintained at 24±1ºC.
[a,c] Mean ± SD, data taken from five slide culture.
[b] Mean ± SD, data taken from five PDA slab culture.

(iii) Fruit-body development and productivity

All the *pfls* hybrids were used for spawn preparation followed by fruit body production following the cultivation practice of *P. florida* at 24 ± 1ºC temperature. Out of twelve, only six hybrids (*pfls 1p, pfls 1h, pfls 1n, pfls 1m, pfls 1k* and *pfls 1j*) could ultimately produced fruit bodies. Details of *pfls* hybrid cultivation and yield were shown in Table 2. Hybrid *pfls 1k* and *pfls 1p* required comparatively less time (10 d) for spawn maturation like *P. florida* parent while hybrid *pfls 1m* required maximum time (18 d) for the same. Hybrid *pfls 1k* required minimum time for substrate colonization (Figure 2a) and primordial initiation (18–20 d). Hybrid *pfls 1p* required maximum time (36–38 d) for primordial initiation while parent *P. florida* required 20–22 d for it. Stipe length of all hybrid lines showed variation and it ranged from 1.72 ± 0.18 cm (*pfls 1j*) to 2.54 ± 0.15 cm (*pfls 1h*) while parent *P. florida* showed the highest value (6.38 ± 0.12 cm). The lowest and highest pelius diameter were found in hybrid *pfls 1m* (3.71 ± 0.12 cm) and *pfls 1n* (7.27 ± 0.21 cm), respectively while in *P. florida* parent it was found 4.98 ± 0.31 cm. Pileate, oyster-shaped and brownish basidiocarp of hybrid *pfls 1k* (Figure 2b) showed the highest fruit body yield

(385.5 ± 4.39 g) with 59.31 % bioefficiency and the small fruit body of hybrid *pfls 1m* (Figure 2c) showed the lowest yield (109 ± 8.83 g) with 16.77 % bioefficiency while parent *P. florida* showed comparatively maximum yield (716.5 ± 40.31) with 110.23 % bioefficiency. White, oyster-shaped hybrid *pfls 1p* (Figure 2d) also showed comparatively good yield (289 ± 13.35) with 44.46 % bioefficiency.

Table 2

Fruit-body characters of the fruit body generating pfls somatic hybrid lines

Strain	Spawn production Time (d)[a]	Primordial initiation Time (d)[a]	Stipe length in cm[b] (*F* = 61.22)	Pelius diameter in cm[b] (*F* = 71.52)	Wt of 3 flushes in gm[c]/0.65 kg of dry substrate (*F* = 62.27)	BE (%)[d]
P. florida	10	15-20	6.38±0.12	4.98±0.31	716.5±40.31	110.23
pfls 1p	10	36-38	2.36±0.04	4.67±0.08	289±13.35	44.46
pfls 1h	14	24-26	2.54±0.15	4.14±0.07	256±14.39	39.38
pfls 1n	15	30-32	2.53±0.04	7.27±0.21	167±18.83	25.7
pfls 1m	18	20-22	2.12±0.03	3.71±0.12	109±8.83	16.77
pfls 1k	10	18-20	2.45±0.09	4.35±0.37	385.5±27.32	59.31
pfls 1j	15	30-32	1.72±0.18	4.50±0.20	243.3±39.05	37.43
SE (Mean)	-	-	0.59	0.43	21.57	-
p-value	-	-	0.99	0.99	0.99	-

The values of stipe length, pelius diameter and yield are means ± SD of ten repeated experiments. Results of one-way ANOVA for each variable are shown as F values, P <0.05(considering the F value 3.18 for this experiment, the tabulated are greater).

[a] Data taken at 24±2°C temperature.

[b] Mean ± SD, data taken from ten observations.

[c] Mean ± SD, data taken from five bags.

[d] BE = Biological Efficiency.

Morphological characteristics of the fruit bodies generated from the somatic hybrid colonies

Figure 2

(a) Fully colonized pfls 1k bed after 12 days. (b) Bunch of oyster shaped and brownish basidiocarps produced by pfls 1k hybrid strain. (c) White, small sized fruit bodies produced by pfls 1m hybrid strain. (d) White fruit bodies produced by pfls 1p hybrid strain. Size bars correspond to 5 cm in (a), (c) and (d), 3.5 cm in (b).

(iv) Principal component analysis of pfls hybrids

Phenotypic traits of *pfls* hybrid lines were analyzed and correlated using IBM SPSS software. Six phenotypic variables such as mycelial growth on slide culture, colony diameter, cell width, stipe length, pelius diameter and fruit body yield of *pfls* hybrid lines were used to check the dimension of each trait between each variable through scatter diagram. Using PCA method, two component matrices were extracted and plotted on Microsoft Excel sheet and constructed a scatter plot (Figure 3). Data of principal component 1 (PC1) were plotted on X-axis and principal component 2 (PC2) on Y-axis. The variables like mycelial growth, colony diameter and stipe length were on the first quadrant and rest three i.e., cell width, pelius diameter and yield were on the fourth quadrant. The greater influential variables were found between cell width and yield where the lesser were found between colony diameter and yield. Based on the PCA results it can be conclude that cell width and yield could be use as morphological markers for hybrid selection. Bivariate correlation between six phenotypic variables of *pfls* hybrid lines was expressed in correlation matrix (Table 3). Calculated phenotypic variables were positively correlated but cell width was negatively correlated with growth (# -0.119), colony diameter (# -0.054) and pelius diameter (# -0.062). The yield also negatively correlated with growth (# -0.78) and colony diameter (# -0.030).

Scatter plot of Principal Component matrices for six phenotypic variables analyzed of pfls hybrid lines

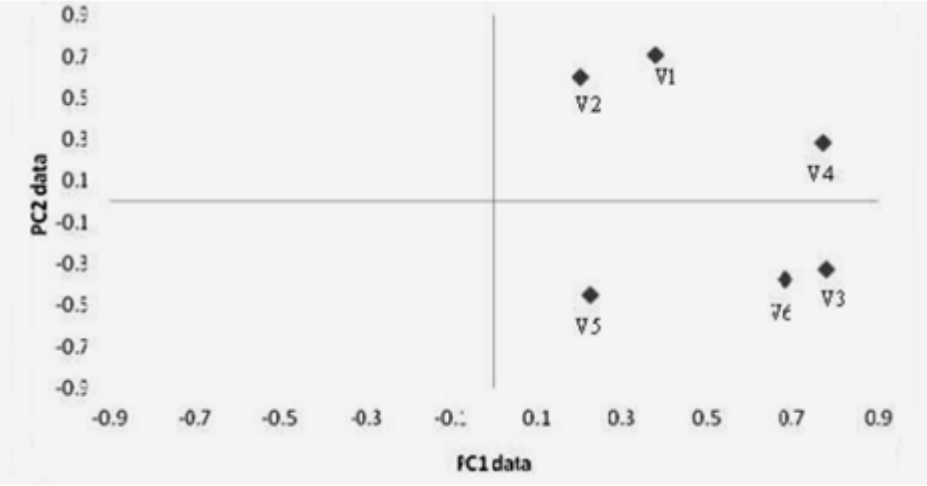

Figure 3

The geometric steps for finding the major influential variables among the extracted component data are represented by PC1 on X-axis (horizontal) and PC2 on Y-axis (vertical). The variable components are denoted by: V1 - growth, V2 - colony diameter, V3 - cell width, V4 - stipe length, V5 - pelius diameter and V6 - yield. Out of six, three variables are in the first quadrant and rest three in the fourth quadrant, indicating different influential concept between each other.

Table 3
Correlations using Principle Component Matrices between six variable phenotypes of pfls hybrids

	Growth	Colony diameter	Cell width	Stipe length	Pelius diameter	Yield
Growth	1					
Colony diameter	.412	1				
Cell width	-.119	-.054	1			
Stipe length	.330	.118	.119	1		
Pelius diameter	.081	.091	-.062	.131	1	
Yield	-.078	-.030	.485	.334	.029	1

(Using SPSS version.19, bivariate correlation)

(v) RFLP analysis of rRNA-ITS region of pfls somatic hybrids and parents

The primer set comprised of forward primer from ITS1 region and reverse primer from ITS4 region amplified the ITS1-5.8S rRNA-ITS2 region of nuclear DNA of the twelve *pfls* hybrid and two parental strains. The generated band size ranged from approx. 620 bp to 700 bp in agarose gel (Figure 4). Within the *pfls* hybrid population polymorphism was observed either in the size of PCR product or in the restriction patterns yielded by restriction digestion. Among the restriction enzymes tested *Alu*I, *Hin*fI, *Hpa*II and *Hae*III were found to be highly polymorphic for all the *pfls* hybrids and parents. Different restriction pattern had been recorded and analyzed. The approximate size of the digested product for all the strains are summarized in Table 4. A total of 96 fragments were produced for all the *pfls* hybrids and parents in which *Hae*III showed the maximum (34) restriction fragments and *Hpa*II showed the least (16) (Figure 5). *Hpa*II produced only single restriction fragment in all the sample except in hybrid *pfls 1p* and *pfls 1o*. The digested fragment size ranged from approx. 160 bp to 680 bp. All the four restriction enzyme i.e., *Alu*I*, Hin*fI*, Hpa*II and *Hae*III produced 8 and 6 restriction fragments in parent *P. florida* and *L. squarrosulus,* respectively. Hybrid *pfls 1m*, *pfls 1k* and *pfls 1i* showed same restriction pattern with respect to length and number (660 bp) by *Alu*I, where hybrid *pfls 1e* and *pfls 1g* showed similar type of restriction pattern (590 bp and 500 bp) by this same enzyme. Enzyme *Hae*III found similar restriction pattern with respect to length and numbers (340 bp, 200 bp and 160 bp) in hybrid *pfls 1e* and *pfls 1g* when hybrid *pfls 1s* and *pfls 1j* showed completely similar restriction pattern (380 bp, 200 bp and 180 bp) with parent *P. florida* by this same enzyme. Enzyme *Alu*I and *Hpa*II produced single restriction fragment in maximum hybrids and two parents except in hybrid *pfls 1e*, *pfls 1g*, *pfls 1o* and parent *P. florida* by enzyme *Alu*I and except *pfle 1p* and *pfle 1o* by *Hpa*II.

Profile of amplified rRNA-ITS product by primers ITS1 (forward) & ITS4 (reverse) of pfls hybrids and their parents

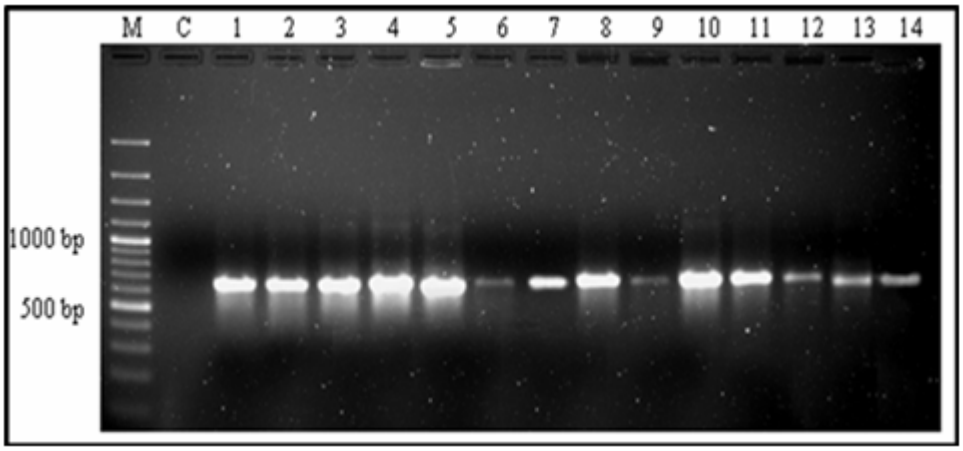

Figure 4

Lanes: M - marker, 100 bp DNA ruler plus., C - control, Lane 1 - L. squarrosulus, Lane 2 - pfls 1q, Lane 3 - pfls 1e, Lane 4 - pfls 1g, Lane 5 - pfls 1p, Lane 6 - pfls 1h, Lane 7 - pfls 1n, Lane 8 - pfls 1s, Lane 9 - pfls 1m, Lane 10 - pfls 1k, Lane 11 - pfls 1o, Lane 12 - pfls 1i, Lane 13 - pfls 1j and Lane 14 - P. florida.

Table 4

Size of restriction fragments obtained after digestion of rRNA-ITS products of different pfls hybrid and the parental strains with four different restriction enzymes

Line	*Alu*I Fragments (bp)	*Hin*fI Fragments (bp)	*Hpa*II Fragments (bp)	*Hae*III Fragments (bp)
L. squarrosulus	530	300, 180	640	340, 160
pfls 1q	590	330, 210	640	200, 160
pfls 1e	590, 500	330, 300, 210	640	340, 200, 160
pfls 1g	590, 500	330, 300, 210	640	340, 200, 160
pfls 1p	450	300, 280	430, 180	540, 200
pfls 1h	550	300	640	340, 200
pfls 1n	650	380, 240	640	380, 200
pfls 1s	650	390, 240	640	400, 200, 180
pfls 1m	660	380	640	200, 180
pfls 1k	660	380, 240	640	380, 200, 180
pfls 1i	660	380, 240	640	200, 180
pfls 1j	660	380, 240	640	380, 200, 180
pfls 1o	550, 430	300, 240	640, 430	540, 200
P. florida	560, 380	380, 240	680	380, 200, 180

Gel profiles of restriction enzymes digested rRNA-ITS region of pfls hybrids and parents

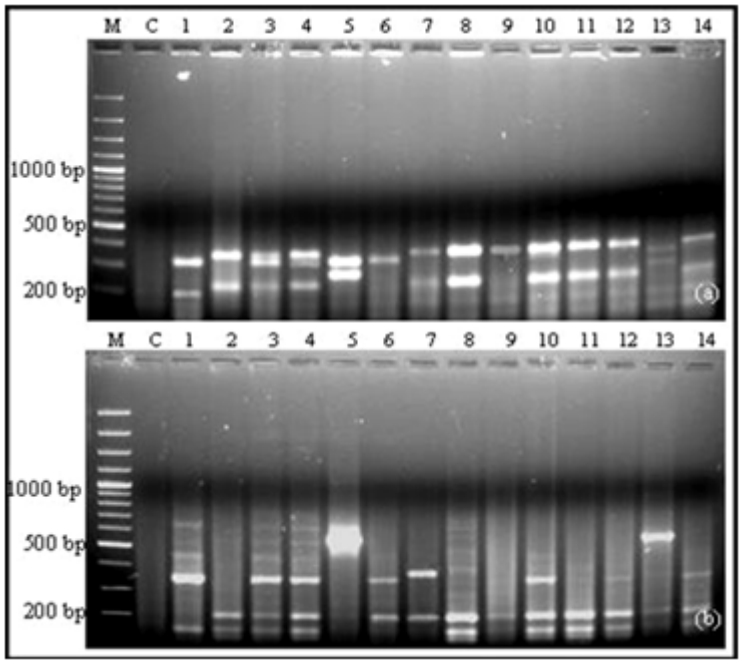

Figure 5

Digestion of rRNA-ITS region by a) restriction enzyme Hin*fI *and b) restriction enzyme Hae*III*. Lanes: M - marker, 100 bp DNA ruler plus., C - control, Lane 1 - L. squarrosulus, Lane 2 - pfls 1q, Lane 3 - pfls 1e, Lane 4 - pfls 1g, Lane 5 - pfls 1p, Lane 6 - pfls 1h, Lane 7 - pfls 1n, Lane 8 - pfls 1s, Lane 9 - pfls 1m, Lane 10 - pfls 1k, Lane 11 - pfls 1o, Lane 12- pfls 1i, Lane 13 - pfls 1j and Lane 14 - P. florida.

DISCUSSION

Morphological variations among the hybrid lines strongly proved that the genome constitution of the independent hybrid lines is not equal and it is evident from polymorphic nature of molecular data. Parent *P. florida* is generally cultivated on paddy straw substrate and could produce fruit body whereas the other parent *L. squarrosulus* did not produce any fruit body on paddy straw substrate following *P. florida* cultivation protocol. The non-production of *L. squarrosulus* fruit body might be due to its substrate specificity. All the six *pfls* hybrid fruit bodies showed *P. florida* type phenotypic morphology. Parent *P. florida* type fruit body of the six *pfls* fruiting lines may be either due to the dominance nature of *P. florida* genome over *L. squarrosulus* genome in expressing basidiocarp phenotype or due to elimination of the *L. squarrosulus* chromosome responsible for fruit body phenotype the hybrid environment. Based on the PCA analysis it can be concluded d that cell width and yield could be consider as morphological markers for hybrid selection. DNA sequence and RFLP study of rRNA-ITS region revealed that all the *pfls* hybrids were recombinant in nature compared to their parents. Polymorphism in RFLP profile could be observed in all the *pfls* hybrids though some common bands of both the parents were also observed in some of the hybrid lines. This type of variation (in number and sizes of restriction fragments) might be occurred due to availability of complementary sequences over the target region in respect to the primers used. Independent genome recombination or random conversion or deletion of genetic materials might be responsible for this

phenomenon also. But the actual mechanism is yet to be explored. The RFLP pattern of the *pfls* hybrid lines strongly proved their hybridity and support genome contribution from both the parents. All the *pfls* hybrids in this study showed morphological and molecular variations compared to their parents. However, in this experiment we developed twelve inter-generic somatic hybrids by protoplast fusion between two heterothallic genera, *P. florida* and *L. squarrosulus*. In the present report only *Pleurotus* character was expressed in the *pfls* hybrid strains and this kind of phenomena is already been reported[12-14]. Though the *pfls* hybrids looked like *P. florida* but the RFLP profile of rRNA-ITS strongly proved their recombinant nature. Immunological attribute of hybrid *pfls 1h* polysachharide (β-glucan) was extracted and structural properties have been studied[39-40] which showed that the polysaccharides isolated from *P. florida*[41] and *L. squarrosulus*[42] were completely different in structure and thus it strongly proved the hybridity of this particular hybrid strain.

CONCLUSION

So, the six *pfls* somatic hybrid strains of *P. florida* and *L. squarrosulus* could successfully produce fruit body on simple paddy straw substrate in the sub-tropical climate and based on the morphological, yield and molecular parameters it is evident that all are independent hybrid lines.

ACKNOWLEDGEMENT

We acknowledge the fellowship support to the first author from Bose Institute, Kolkata, India.

REFERENCES

1. Peterson AH, Tanksley SD, Sorrells ME, DNA markers in crop improvement. Advanced Agronomy, 46: 39–90, (1991).
2. Peberdy JF, Fungal protoplasts. In: Bennett JW, Lasure LL (eds.), *More gene Manipulation in Fungi*, Academic Press,California, ,pp 307–318, 1991.
3. Shishido R, Apisitwanichi S, Ohmido N, Okinaka Y, Mori K, Fukui K, Detection of specific chromosome reduction in rice somatic hybrids with the A, B and C genomes by multi-color genomic in situ hybridization. Theoretical and Applied Genetics, 97: 1013–1018, (1998).
4. Woestemeyer A, Woestemeyer J, Fungal protoplasts: Relics or modern objects of molecular research? Microbiological Research, 153: 97–104, (1998).
5. Eichlerova I, Homolka L, Nerud F, Zadrazil F, Baldrian P, Gabriel J, Screening of *Pleurotus ostreatus* isolates for their ligninolytic properties during cultivation on natural substrates. Biodegradation, 11: 279–287, (2000).
6. Bok JW, Choi EC, Kim BK, Studies on protoplast fusion between *Lentinula edodes* and *Ganoderma lucidum*. Archives of Pharmaceutical Research, 17: 492–496, (1994).
7. Ogawa K, Interspecific and intergeneric hybridization of edible mushrooms by protoplast fusion. In: Chang ST, Buswell JA, Miles PG. (eds.), *Genetics and breeding of edible mushrooms*, Gordon and Breach Science Publishers, The Netherlands ,pp 193–205, 1999.
8. Yoo YB, Lee KH, Kim BG, Characterization of somatic hybrids with compatible and incompatible species by protoplast fusion in genera *Pleurotus* (Fr.) P. Karst. and *Ganoderma* P. Karst by RAPD-PCR analysis. International Journal of Medicinal Mushrooms, 4: 147–157, (2002).
9. Yoo YB, Characterization and molecular genetic analysis of somatic hybrids by protoplast fusion in mushrooms. In: The Mushworld Newsletters, 32nd issue Available via DIALOG

http://www.mushworld.com: 1508/characterization and molecular genetic analysis of somatic hybrids by protoplast fusion in mushrooms, (10th Oct, 2004).
10. Ohmasa M, Intraspecific protoplast fusion of *Pleurotus ostreatus* using auxotrophic mutants. Japanese Journal of Breeding, 36: 429–433, (1986).
11. Liang ZR, Chang ST, A study on intergeneric hybridization between *Pleurotus sajor-caju* and *Schizophyllum commune* by protoplast fusion. Mushroom Science, 12: 125–137, (1989).
12. Chakraborty U, Sikdar SR, Production and characterization of somatic hybrids raised through protoplast fusion between edible mushroom strains *Volvariella volvacea* and *Pleurotus florida*. World Journal of Microbiology and Biotechnology, 24: 1481–1492, (2008).
13. Chakraborty U, Sikdar SR, Intergeneric protoplast fusion between *Calocybe indica* (milky mushroom) and *Pleurotus florida* aids in the qualitative and quantitative improvement of sporophore of the milky mushroom. World Journal of Microbiology and Biotechnology, 26: 213–225, (2010).
14. Mallick P**,** Sikdar SR, Production and molecular characterization of somatic hybrids between *Pleurotus florida* and *Lentinula edodes*. World Journal of Microbiology and Biotechnology, 30(8): 2283–2293, (2014).
15. Kaur L, Kapoor S, Protoplast electrofusion for development of somatic hybrids between *Pleurotus florida* and *Pleurotus* s*ajor-caju*. International Journal of Pharma and Bio Sciences, 5(4): 507-519, (2014).
16. Witte V, Grossmann B, Emeis CC, Molecular probes for the detection of *Kluyveromyces marxianus* chromosomal DNA in electrophoretic karyotypes of intergeneric protoplast fusion products. Archives of Microbiology, 152: 441–446, (1989).
17. Martin FN, Kistler HC, Species-specific banding patterns of restriction endonuclease digested mitochondrial DNA from the genus *Pythium*. Experimental Mycology, 14: 32-46, (1990).
18. Carder JH, Barbara D, Molecular variation and restriction fragment length polymorphisms (RFLPs) within and between six species of *Verticillium*. Mycological Research, 95: 935-942, (1991).
19. Chen W, Restriction fragment length polymorphisms in enzymatically amplified ribosomal DNAs of three heterothallic *Pythium* species. Phytopathology, 82: 1467-1472, (1992).
20. Chen W, Hoy JW, Schneider RW, Species-specific polymorphism in transcribed ribosomal DNA of five *Pythium* species. Experimental Mycology, 16: 22-34, (1992).
21. Manicom BQ, Baayen RP, Restriction fragment length polymorphisms in *Fusarium oxysporum* sp. *dianthi* and other fusaria from *Dianthus* species. Plant Pathology, 42: 851-857, (1994).
22. Matsumoto T, Fukumasa-Nakai Y, Mitochondrial DNA restriction fragment length polymorphisms and phenetic relationship in natural populations of the oyster mushroom, *Pleurotus ostreatus*. Mycological Research, 99: 562-566, (1995).
23. Gardes M, White TJ, Fortin JA, Bruns TD, Taylor JW, Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA. Canadian Journal of Botany, 69: 180-190, (1991).
24. Erland S, Henrion B, Martin F, Glover LA, Alexander IJ, Identification of the ectomycorrhizal basidiomycete *Tylospora fibrillose* Donk by RFLP analysis of the PCR-amplified ITS and IGS regions of ribosomal DNA. New Phytology, 126: 525-532, (1994).
25. Henrion B, Chevalier G, Martin F, Typing truffle by PCR amplification of the ribosomal DNA spacers. Mycological Research, 98: 37-43, (1994).
26. Farmer DJ, Sylvia DM, Variation in the ribosomal DNA internal transcribed spacer of a diverse collection of ectomycorrhizal fungi. Mycological Research, 102: 859-865, (1998).

27. Gomes EA, Barros EG, Kasuya MCM, Araujo EF, Molecular characterization of *Pisolithus spp*. isolates by rDNA PCR-RFLP. Mycorrhiza, 8: 197-202, (1999).
28. Glen M, Tommerup IC, Bougher NL, O'Brien PA, Interspecific and intraspecific variation of ectomycorrhizal fungi associated with Eucalyptus ecosystems as revealed by ribosomal DNA PCR-RFLP. Mycological Research, 105: 843-858, (2001).
29. Bordas M, Gonzáles-Candelas L, Dabauza M, Ramón D, Moreno V, Somatic hybridization between an albino *Cucumis melo* L. mutant and *Cucumis myriocarpus* Naud. Plant Science, 132: 179-190, (1998).
30. Crisan EV, Sands A, Nutritional value. In: Chang ST, Hayes WA. (eds.), *The biology and cultivation of edible mushrooms*, New York Academic Press,New York, pp 137–165, 1978.
31. Wuyep PA, Khan AU, Nok AJ, Production and regulation of lignin degrading enzymes from *Lentinus squarrosulus* (mont.) Singer and *Psathyrella atroumbonata* Pegler. African Journal of Biotechnology, 2(11): 444–447, (2003).
32. Royse DJ, Bahler BD, Bahler CC, Enhanced yield of Shiitake by saccharide amendment of the synthetic substrate. Applied and Environmental Microbiology, 56: 448–479, (1990).
33. Okhuoya JA, Mushroom cultivation: The Nigerian experience. In: Dirar A (eds.), *Food processing technologies for Africa-emerging technologies series*, UNIDO, pp 153–168, 1997.
34. Widholm JM, The use of fluorescein diacetate and phenosafranine for determining viability of cultured cells. Stain Technology, 47: 189–194, (1972).
35. Anne J, Peberdy JF, Induced fusion of fungal protoplasts following treatment with polyethylene glycol. Journal of General Microbiology, 92: 413–417, (1976).
36. Zar JH, Biostatistical Analysis, 4th Edn, Prentice Hall: ISBN-13: 9780130815422, (1998).
37. Dellaporta SL, Wood J, Hicks JB, A plant DNA minipreparation: version II. Plant Molecular Biology Reporter, 1: 19–21, (1983).
38. White TJ, Bruns T, Lee S, Taylor J, Amplification and direct sequencing of fungi ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds.), *PCR protocols: A guide to methods and applications*, Academic Press,San Diego,1990,pp 315-322.
39. Sen IK, Maji PK, Behera B, Maiti TK, Mallick P, Sikdar SR, Islam SS, Glucan of a somatic hybrid mushroom, *pfls1h*: structural characterization and study of immunological activities. International Journal of Biological Macromolecules, 53: 127– 132, (2013a).
40. Sen IK, Maji PK, Behera B, Mallick P, Maiti TK, Sikdar SR, Islam SS, Structural characterization of an immunoenhancing heteroglycan of a hybrid mushroom (*pfls1h*) of *Pleurotus florida* and *Lentinus squarrosulus* (Mont.) Singer. Carbohydrate Research, 371: 45–51, (2013b).
41. Rout D, Mondal S, Chakraborty I, Islam SS, The structure of a polysaccharide from Fraction-II of an edible mushroom, *Pleurotus florida*. Carbohydrate Research, 341(8): 995–1002, (2006).
42. Bhunia SK, Dey B, Maity KK, Patra S, Mandal S, Maiti S, Maiti TK, Sikdar SR, Islam SS, Structural characterization of an immunoenhancing heteroglycan isolated from an aqueous extract of an edible mushroom, *Lentinus squarrosulus* (Mont.) Singer. Carbohydrate Research, 345(17): 2542–2549, (2010).