Chapter 3

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3.1 Mushroom strains:
Vegetative cultures of the parental strains, *Lentinula edodes* (Berk.) Pegler and *Pleurotus florida* (Eger.) were obtained from the National Research Centre for Mushroom, Solan, Himachal Pradesh, India. Another parental strain, *Lentinus squarrosulus* (Mont.) Singer fruit body was collected from dead wood stock at Falta Experimental Farm of Bose Institute, 24 Parganas (South), West Bengal, India and prepared its mycelial culture in the laboratory.

3.2 Culture generation from basidiocarp:
Immature basidiocarp of *Lentinus squarrosulus* and first generation somatic hybrid strains were used for this study. The surface area of the fruit body was wiped carefully with 90% ethyl alcohol and the outermost layer of a portion of pileus was removed with a sterile blade and then pieces of tissue from the exposed area were transferred aseptically onto PDA medium for new mycelial tissue generation in tissue culture.

3.3 Culture media and culture maintenance:
Vegetative parental cultures were maintained on PDA (Potato-Dextrose-Agar, pH 6.2) medium through sub-culturing at 15-20 days interval. Initially sub-culturing was done at frequent intervals in PDA medium. Later on continuous growing phase of the strains was maintained by routine sub-culturing of the tissue at an interval of 15 days for *P. florida* and *L. squarrosulus* and 20 days for *L. edodes* in PDA medium. The mycelial culture was maintained at 24±1°C for *L. squarrosulus, L. edodes, P. florida* and for all the developed somatic hybrid strains. Liquid MYG (10 g/l Malt, 4 g/l Yeast extract and 10 g/l Glucose, pH 6.2) medium was used to prepare fresh mycelial tissue which served as the source material for the isolation of protoplasts. Regeneration medium used for the growth of *Lentinula edodes* protoplasts is MYG + 0.6 M mannitol and for *P. florida* and *Lentinus squarrosulus* protoplasts it was MYG + 0.7 M NaCl, pH 6.2 and 1.5% agar was added in both the medium (Chakraborty and Sikdar 2008).

3.4 Sterilization of media and reagents:
The fungal tissue culture media were sterilized by autoclaving at 121°C at 15 lbs pressure for 20 min. All the osmotic solutions were also sterilized under similar conditions. But the enzymes, being heat labile, were filter sterilized through 0.2 µm membrane filter (Sartorius).
3.5 Preparation of mycelial tissue for protoplast isolation:

The most important key factor for the isolation of good protoplast is physiological status of mycelial tissues. Fast growing mycelium of parental strains was used for enzyme digestion. Pre or over matured tissue did not produced healthy and good amount of protoplasts. The mycelial tissue of *Pleurotus florida*, *Lentinula edodes* and *Lentinus squarrosulus* was grown in the liquid MYG medium under static condition. The incubation period in the MYG medium was 4-5 days at 24±1°C for *Pleurotus florida*, 10-12 days at 24±1°C for *Lentinula edodes* and 3-4 days at 24±1°C for *Lentinus squarrosulus*. After optimum growth was achieved fresh mycelial mat of each strain was washed thrice with sterile double distilled water and twice with osmotic stabilizer (0.6 M mannitol), chopped into pieces and put into enzyme mixture separately for protoplast liberation.

3.6 Enzyme mixture and mycelial tissue digestion:

Two cell wall degrading enzymes, Cellulase Onozuka R-10 (Yakult) and Lysing enzyme (Sigma-Aldrich) both in 2% concentration were dissolved in 0.6 M mannitol solution with 0.2% CaCl₂, 2H₂O, pH 5.5, filter sterilized and used for digestion of mycelial tissue for protoplast liberation. Approximately 500 mg of chopped mycelial tissue was incubated in 2 ml of enzyme mixture. The incubation periods were 9-12 h for *P. florida*, 16-18 h for *L. edodes* and 8-10 h for *L. squarrosulus* with shaking at 70 rpm. Incubation temperatures were kept at 24±1°C for all the strains.

3.7 Protocol for protoplast isolation and purification:

a) After digestion of mycelial tissue with enzyme mixture it was filtered through absorbent cotton mat placed on a steel mesh.

b) The filtered protoplast suspension was then centrifuged at 3000 rpm using a table top microcentrifuge for 5 min. The pelleted protoplasts were collected after removal of supernatant and were resuspended in 500 µl of the respective osmotic buffer, i.e. 0.6 M mannitol in case of *L. edodes* and *L. squarrosulus* and 0.7 M NaCl in case of *P. florida* protoplasts.

c) The protoplast suspension was centrifuged again at 5000 rpm for 5 min.

d) The pellet was collected and washed again with osmotic buffers and repeated the procedure b) and c).
e) The final pellet was suspended in 200 µl osmoticum.
f) The yield of protoplast was measured using a haemocytometer.

3.8 Protoplast viability testing:

Isolated and purified protoplasts were used for viability testing prior to regeneration and fusion experiments. The viability of the fresh protoplasts was examined by the Fluorescein diacetate (FDA) test (Widholm 1972). The viable intact protoplasts fluoresced yellow-green while the damaged protoplasts fluoresced red.

Procedure:

a) 5 mg of Fluorescein diacetate was dissolved in 1 ml of acetone in a sterile container.
b) 10 µl of concentrated protoplast suspension was taken in an eppendorf tube and diluted by adding 88 µl of osmotic buffer (0.6 M mannitol) and to that 2 µl of freshly prepared FDA solution was added (final FDA concentration 0.01% w/v).
c) The tube was incubated in the dark for 5-7 min.
d) After incubation the FDA stained protoplasts were observed under a fluorescent microscope. The number of viable yellow-green fluoresced protoplasts was counted with the help of a haemocytometer.
e) Percentage of viability was calculated by the following formula:

\[
\frac{\text{Average number of viable protoplasts} \times 100}{\text{Average number of total protoplasts counted}}
\]

3.9 Protoplast regeneration:

Once the high yield of protoplasts is achieved, the next factor to consider was the optimization of artificial conditions for protoplast regeneration, which is also empirical with factors such as the use of agar concentration, osmotic stabilizer substituted in regeneration medium and sometimes specific medium ingredients being taken into account (Peberdy and Fox 1993). The biological process of new cell wall synthesis on the protoplast surface and regeneration as the normal cell form key events in the application of protoplasts in genetic manipulation. Determination of regeneration
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frequency through the colony forming units was assessed on the appropriate regeneration media. Regeneration medium optimized for growth of *L. squarrosulus* and *P. florida* in MYG medium substituted with 0.7 M NaCl and 1.5% agar, pH 6.2, where *L. edodes* required MYG medium substituted with 0.6 M mannitol and 1.5% agar. Incubation temperature for regeneration in all parental protoplasts was 24±1°C.

### 3.10 Hybrid selection strategies:

In these two fusion experiments, no auxotrophic markers were used in any of the fusion partners. Somatic hybrids have been selected using the double selection method, in a combination where only the hybrids will grow in the selection medium due to complementation. Based on the hybrid selection strategy the parental protoplasts were treated as follows.

**Testing of salt (NaCl) tolerance level of parental strains:**

Mycelial tissues of three parental strains i.e., *Pleurotus florida*, *Lentinus squarrosulus* and *Lentinula edodes* were cultured in MYG medium supplemented with 0.1-1.0 M NaCl (ten concentrations of NaCl were used with the increment of 0.1 M) to test salt tolerance level of individual parental strain. The aim of this experiment was to develop a strategy for hybrid selection.

**Protoplast inactivation by iodoacetamide treatment:**

For both the fusion experiments protoplasts of *Pleurotus florida* were inactivated before fusion based on the protocol of Zhao and Chang (1997). According to this protocol protoplasts were treated with 10 mM iodoacetamide (IOA) solution in the dark for 5 min. The treated protoplasts were then washed twice in 0.7 M NaCl. Protoplast density after inactivation was kept at 10⁶ protoplasts/ml. In each fusion experiment IOA treated protoplasts of *P. florida* was plated separately onto regeneration medium as negative control.

**Protoplast inactivation by heat treatment:**

*Lentinus squarrosulus* protoplasts were inactivated with heat treatment. Freshly isolated and purified *L. squarrosulus* protoplasts suspension was treated in hot water bath at 70°C temperature for 10 min. After treatment, protoplast suspension was washed once with 0.6
M mannitol and part of it was plated onto regeneration medium (MYG + 0.6 M mannitol) as negative control and rest was used for fusion experiment.

3.11 Protoplast fusion:

Protoplast fusion for both the fusion experiments was carried out with slight modification of protocol of Chakraborty and Sikdar (2008). A combination of 30% PEG solution (MW-3350) supplemented with 0.05 M Glycine-NaOH, 50 mM CaCl$_2$.2H$_2$O, pH 7.5, was the standard concentration for fusion. For both the fusion experiment (P. florida + L. edodes and P. florida + L. squarrosulus) PEG added protoplasts suspension was incubated for 5 min. After incubation, the fused protoplast suspension was diluted by 6 times with respective osmoticum.

Reagents:

I. PEG fusion solution:

- 30% polyethylene glycol (Molecular Weight 3350): 30 gm of PEG was dissolved in 100 ml of distilled water.
- 0.05 M Glycine-NaOH: 7.507 gm of Glycine-NaOH buffer was added into the same solution.
- 50 mM CaCl$_2$.2H$_2$O: Finally, 14.7 gm of calcium chloride (dihydrated) was added into the same solution.
- Adjusted the pH upto 7.5 with NaOH.

[The PEG solution was mixed carefully till the complete dissolve of all the ingredients and filters sterilized. Distributed in aliquots in eppendorf tube and stored at -20°C for long term use].

II. Osmoticum 0.7 M NaCl and 0.6 M mannitol:

- 4.09 g sodium chloride was added in 100 ml of distilled water for 0.7 M NaCl solution, pH 7.5.
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- 10.93 g mannitol and 0.2 g CaCl$_2$.2H$_2$O dissolved in 100 ml distilled water by vortex.

Protocol for protoplast fusion:

After checking the viability, protoplasts of two parental strains were used for protoplast fusion as per following protocol:

a) For *P. florida* and *L. edodes* fusion, IOA treated *P. florida* protoplasts and normal *L. edodes* protoplasts (50 µl each having density of $10^6$ protoplasts / ml) were mixed in 1:1 ratio.

b) For *P. florida* and *L. squarrosulus* fusion, IOA treated *P. florida* protoplasts and heat treated *L. squarrosulus* protoplasts (50 µl each having density of $10^6$ protoplasts / ml) were mixed in 1:1 ratio.

c) Mixed protoplast suspension was centrifuged at 3000 rpm for 5 min.

d) The supernatant was discarded and suspended the protoplasts in remaining solution. To that equal volume of 30% PEG (MW 3350, Sigma-Aldrich) was added and mixed well.

e) The tubes were incubated at room temperature for 5 min with occasional shaking of the tube time to time.

f) The fused protoplast suspension was diluted 6 times with liquid selection medium.

g) 100 µl of diluted fused protoplast suspension was plated on 1 ml selection medium (MYG medium supplemented with 0.7 M NaCl, pH 6.2 and solidified with 1.5% agar) for the first fusion experiment and for the second fusion experiment it was MYG medium supplemented with 0.6 M mannitol and 1.5% agar, pH 6.2 in petri-dish (35 mm diameter).

3.12 Spawn production:

Spawn of parental and hybrid mushroom strains were prepared on rice grain. For spawn preparation rice grains were prepared by boiling and mixing of calcium carbonate and calcium sulphate at 4% and 2%, respectively. Double layered polypropylene grain
packets (5"×7") each containing 200 g above prepared rice grains were autoclaved at 20 psi constant pressure for 2 hours. Pure PDA culture of parental strains and hybrid lines were inoculated in these sterilized grain packets aseptically and incubated under different temperatures as required. Fully matured spawns were then used as mushroom seed and cultivated on substrate for basidiocarp development.

Cultivation of parental and hybrid strains:

Machine-chopped paddy straw was soaked overnight in water containing 0.2% lime. After drain out of water wet paddy straw was sun-dried keeping sufficient 65-70% moisture. The cultivation beds were prepared by using 16" × 20" sized polypropylene bags that were filled with this straw bits (650 g straw on dry weight basis/16" × 20" polypropylene packet) and autoclaved at 20 lbs pressure for 45 min. After autoclaving the straw bags were cooled down and then inoculated with spawn (100 g spawn/650 g dry weight of straw) of individual parent and hybrid strains. Spawn were placed in alternate layer with straw so that the entire bed contained 5-6 layers of paddy straw alternate with spawn. Care was taken by making small pores in the bags for good aeration which would also allow the mushroom primordia to emerge out from the bags. Data were recorded by time to time observation for spawn run and pin head initiation, yield per bag, colour of fruit bodies, spore shedding, brittleness, margin of the fruit body, pelius diameter, stipe length, texture and maturation period. All the prepared beds were incubated at 24±1°C for substrate colonization. When primordial initiation started 90-100% RH was maintained in the cultivated room.

3.13 Development of tissue culture generated line from fruit body generating hybrid lines:

Fruit body generating hybrid strains produced through protoplast fusion between *P. florida* + *L. edodes* and *P. florida* + *L. squarrosulus* were used to raise tissue culture generated lines. From the matured fruit body of the hybrid strains tissues were excised and cultured on PDA medium as per protocol described in section 3.2. Genome stability of the hybrids generation was studied by morphological and molecular analysis.
3.14 Morphological study of the mycelium and basidiocarps:

Morphological features including mycelium growth, width, clamp connection, colony diameter and colony morphology etc were analysed for all hybrid lines and parents. Data for mycelial growth and width were taken from five repeated slide cultures and colony diameter data was taken from five repeated PDA slab cultures. Clamp connection was studied by microscopic observation of mycelium on slide cultures. Morphology of the basidiocarp generated from hybrid strains from both the fusion experiments was studied. The stipe length, pelius diameter and total yield of the hybrid sporophores were measured along with parent. Stipe length and pelius diameter data were taken from ten observations and yield data was taken from five bags.

3.15 Barrage reaction:

Hybrid mycelia from both the fusion experiments were used for barrage reaction with respective parental mycelia. On PDA slab, mycelia were inoculated at 2 cm distance, three in each 90 mm (diameter) petri plate (i.e. the two parent cultures and a single hybrid line). Plates were incubated at 24±1ºC for 5 days. After this incubation, the point of contact zone was observed both micro and macro morphologically.

3.16 Slide culture:

Slide cultures were prepared individually for three parental strains and 21 hybrid strains from two different fusion experiments. A small piece of mycelial culture of each strain was inoculated on one end of sterile slide and placed the slides on wet Whatman filter paper in a sterilized petri plate. Then all the plates were sealed with parrafilm and incubated for 3-6 days at 24±1ºC. The mycelium on the slide was stained with Lacto-phenol cotton blue stain. Mycelial growth, hyphal width, clamp connection etc. were studied under the microscope. Five replications were studied for each strain.

3.17 Extraction of genomic DNA content from mushroom tissues:

Isolation of genomic DNA was carried out from young fruit bodies and actively growing mycelia of the three parental strains and their presumptive somatic hybrids using the modified CTAB (N-cetyl-NNN-trimethyl ammonium bromide) method (Dellaporta et al. 1983). Preserved frozen tissue (preserved at -20ºC for short term storage and at -80ºC for long term storage) was used for extraction of DNA.
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Equipments required:

- Weighing balance
- Mortar and pestle
- Refrigerated centrifuge / microcentrifuge
- Microcentrifuge tubes
- Micropipettes and tips
- Incubator (for 65°C temperature) or water bath
- Ice bucket
- 4°C chamber or refrigerator

Materials and reagents:

- Mushroom fruit body tissues / mycelial tissues - 2 gm (approx.).

Stock Buffers:

a) 1 M Tris-HCl, pH 8.0
b) 0.5 M EDTA, pH 8.0
c) 5 M NaCl
d) 3 M sodium acetate, pH 5.2
e) TE Buffer (10 mM Tris-HCl, 1 mM EDTA), pH 8.8

CTAB Extraction Buffer:

a) 100 mM Tris-HCl
b) 20 mM EDTA
c) 1.4 M NaCl
d) 2% CTAB (w/v)
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e) 0.2% β- mercaptoethanol, to be added just prior to use.

(All these components to be taken from the stock buffers and mixed and autoclaved)

- Chilled isopropyl alcohol
- Chilled 70% ethanol
- Chilled absolute alcohol
- Phenol: Chloroform (1:1) mixture

Preparation of phenol : chloroform (1:1) mixture:

100 gm of solidified phenol (distilled and free from RNAse, DNAse, SRL) was first melted in hot water bath. In a sterile measuring cylinder 50 ml of this molten phenol was collected carefully and immediately mixed with 50 ml of chloroform in a sterile conical flask. This phenol : chloroform mix was then equilibrated with 100 ml of Tris buffer (0.1 M, pH 8.0). To this solution, 50 mg of hydroxyquinoline was added for colour which turned the solution into yellow. The solution was kept for overnight stirring. For the phenol to be properly equilibrated / saturated, Tris buffer was given two changes, stirred and finally stored under refrigeration.

- Chloroform: Isoamyl alcohol (24:1) mixture:

- 50 X TAE (Tris acetate) buffer (Tris base 242 gm, Glacial acetic acid 57.1 ml, 0.5 M EDTA, pH 8.0 per litre of buffer)
- 6 X DNA gel loading buffer (0.25% Bromophenol blue, 0.25% Xylene cyanol FF, 30% glycerol in water, working solution to be 1X).

Procedure:

- 2 gm of preserved fruit body tissue or mycelial tissue samples were chopped and grounded into fine powder in liquid nitrogen with a mortar and pestle.

- The fine powdered samples were quickly mixed with 15 ml of CTAB buffer (100 mM TrisHCl, pH 8.0; 20 mM EDTA, pH 8.0; 1.4 M NaCl; 2% w/v CTAB). 0.2% of β-mercaptoethanol was added into the sample immediately.
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- All the samples were incubated in the pre-heated extraction buffer at 65°C for about 1 hour (The extraction buffer was already preheated at 65°C for 30 min). Gentle shaking was required in between incubation.

- After incubation the samples were extracted with equal volume of chloroform : isoamyl alcohol (24:1) by centrifugation at 10,000 rpm for 20 min at room temperature (24±1°C).

- The upper aqueous phase was collected separately in another set of eppendorf tube and extracted with equal volume of phenol : chloroform (1:1) mixture by centrifugation the solution at 10,000 rpm for 20 min.

- After centrifugation the upper aqueous layer was collected carefully (not to disturb the separation layer) and again extracted with equal volume of chloroform : isoamyl alcohol (24:1) at 10,000 rpm for 20 min at RT.

- Finally, the clear phase was taken in eppendorf tube and DNA was precipitated with 0.6 volume of pre-chilled isopropanol (thick white slurry was observed soon after isopropanol was added to it).

- The tubes were kept at -20°C for 30 minutes and then centrifuged at 10,000 rpm for 20 min at RT.

- The DNA pellets were washed thrice in 70% chilled ethanol by centrifugation at 8,000 rpm for 15 min at 24±1°C and air dried or vacuum dried completely leaving no traces of alcohol and finally re-suspended in the desired amount of TE buffer and stored at -20°C.

DNA purification:

DNA purification was done by removing the other bio-molecules like, RNA, carbohydrate, protein etc. by the following steps. TE dissolved DNA was treated with RNAse A followed by phenol extraction and ethanol precipitation.

- In 40 µl of DNA sample, 4 µl of RNAseA (10 mg / ml) was added and incubated at 37°C for RNA degradation.
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- After incubation, the DNA solution was first extracted with equal amount of 25:24:1:: phenol : chloroform : isoamyl mixture, to remove the protein contaminants.

- Solution was centrifuged at 10,000 rpm for 20 min at 24±1°C.

- The upper aqueous layer was collected separately and precipitated with 1/10th volume of 3 M sodium acetate and double volume of chilled absolute alcohol.

- After proper mixing of the components, DNA was incubated in ice for half an hour.

- Again the solution was centrifuged at 10,000 rpm for 20 min at 24±1°C.

- The DNA was finally pelleted after precipitation step, washed with 70% ethanol to remove the salts and other organic contaminants, dried completely and re-suspended in TE buffer at a concentration suitable for further experimentation.

[Note: Preparation of DNAsse free RNAs A: Pancreatic RNAs A (Bangalore Genei) was dissolved at a concentration of 10 mg / ml in 10 mM Tris, pH 7.5 and 15 mM NaCl. It was then heated at 100°C for 15 min and mixed properly. After cooling to room temperature, RNAs A solution was dispensed into aliquots and stored at -20°C].

Estimation of DNA concentration and its purity:

The purified DNA was quantified using spectrophotometric measurement of UV absorption at 230 nm, 260 nm and 280 nm. Genomic DNA was quantitated with the UV/Vis spectrophotometer of Hitachi Model by measuring the OD at 260 nm and 280 nm. Measurement was calculated in the installed software at A_{260}:A_{280} and A_{260}:A_{230} ratios. These ratios indicate the content of protein, RNA and poly-phenol, carbohydrate contaminations etc. The concentration of DNA purity and other contaminations are graphically presents by peak formation at respective ratios. The A_{260} value provides a measure of concentration (OD of 1.0 at 260 nm is approx. equivalent to 50 µg / ml). A pure DNA solution should have an A_{260} : A_{280} ratio of 1.8.

DNA purity was further checked by gel running. 0.8% of agarose gel was used to assay DNA concentration and integrity by ethidium bromide fluorescence under UV light. Aliquots of DNA mix were loaded (2 µl of DNA + 1 µl 1X gel loading dye + 3 µl water)
in the wells of a horizontal gel system. Electrophoresis was done in 1X TAE buffer at a constant voltage of 65 V as long as clear resolution was obtained. In the gel, 100 bp DNA ladder plus was run in separate lane simultaneously with the DNA sample and checked its approx. size. Purity testing data was recorded and DNA samples were diluted for further experiment at required concentrations.

3.18 RAPD reaction:

Hybrid lines of both the fusion experiment and three parental strains were analysed by ‘Random amplified polymorphic DNA’ (RAPD) marker. A detail procedure of this RAPD-PCR is mentioned below.

Equipment required:

- Thermocycler (Perkin-Elmer Cetus)
- Horizontal gel electrophoresis apparatus
- Power supply unit (with output at least 200 V and 150 mA)
- UV- Transilluminator
- Spectrophotometer
- Unit for taking photographs of ethidium bromide stained agarose gels or a digital image capture system.

Reagent required:

- Reaction buffers (Fermentas)
- 10X Taq buffer with KCl
- 25 mM MgCl₂
- dNTPs mixture (2 mM used and it was diluted from 10 mM stock of four nucleotides mixture of dNTP)
- 20 µM of RAPD primers (Operon technologies); Table 4.6 & 4.18.
- Taq polymerase enzyme (5 Unit from Fermentas)
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- Primer water (HPLC water from MERCK)
- Genomic DNA (template DNA from 25 ng / µl stock)

Reagents for agarose gel electrophoresis:

- Agarose powder
- Gene Ruler 100 bp plus DNA ladder, used as a standard molecular marker (Fermentas)
- Ethidium bromide (molecular probe)
- 1X TAE buffer (from 50X stock)
- 6X DNA loading buffer/dye (0.25 % Bromophenol blue, 0.25 % Xylene cyanol FF, 30 % Glycerol in sterile water and working solution to be 1X).

Methodology:

Based on the protocol of Williams et al. 1990, the RAPD-PCR amplifications were carried out. However, modifications were made to enhance or increase the reproducibility and consistency in RAPD profiles. The PCR reaction amplifications were performed in 25 µl of reaction volume in a DNA thermal cycler (Applied Bio-systems 2027). The reaction components were 100 ng template DNA 4 µl, 10X Taq buffer (+KCl) 2.5 µl, 25 mM MgCl₂ 2.5 µl, 2 mM dNTPs 2.5 µl, RAPD primer 2 µl, Taq polymerase (5 U / µl) 0.25 µl. Volume was made up with sterile primer water. All the reagents like, Taq buffer, dNTPs, MgCl₂ etc. taken from -20°C were first thawed completely and then used for master mix preparation. Assembled reactions were given a brief pulse for complete mixing and then placed in the Thermocycler for DNA amplification.

The polymerase chain reactions were conducted by preliminary denaturation of genomic DNA at 95°C for 4 minutes followed by 35 cycles of DNA template denaturation at 94°C for 1 minute, primer annealing at 37.5°C for 45 seconds, initial primer extension at 72°C for 1.5 minutes, followed by a final extension at 72°C for 8 minutes and finally stored at 4°C for 15 minutes or more. Annealing temperature was calculated from the $T_m$
values of the random primers used here and calculated according to $T_m = 4(G+C) + 2(A+T)$.

[Note: The annealing temperature was varied in special cases when primer did not melt at respective temperature and then the formula used to calculate the required annealing temperature].

After PCR amplification, product of DNA fragments were separated on a 1.5% agarose gel pre-stained with ethidium bromide solution using 1X TAE buffer. The gels were run for 2 hours at 80 volts and the RAPD products were visualized under UV light. RAPD fingerprint profiles were recorded with Molecular Analyst Gel Documentation System. The size of the amplified fragments was determined by used Gene Ruler 100 bp plus DNA ladder (MBI, Fermentas) as a standard molecular weight marker.

The PCR based RAPD technique was also used for characterization of first fusion generated hybrid’s fruit body generating tissue culture lines.

3.19 ISSR reaction:

Inter single sequence repeat (ISSR) marker is a highly reproducible molecular marker used for DNA fingerprinting. All hybrids and parental DNA was analyzed through this PCR based ISSR technology. The detail of ISSR-PCR technique is mentioned below.

**Equipments:**

Same as RAPD reaction.

**Reagents:**

Same as RAPD reaction. Only the quantity of ingredients was varied. Details of the ISSR primers are listed in Table 4.6 & 4.18.

**Reagents for agarose gel electrophoresis:**

Reagents kept same as RAPD reaction.

**Methodology:**

ISSR PCR reaction was done with little modification of Bornet and Branchard (2001) protocol. Modifications were made to increase the profile consistency and
reproducibility. The PCR reaction amplifications were performed in 25 µl of reaction volume in a DNA thermal cycler (Applied Bio-systems 2027). The reaction components were 25 ng template DNA 1 µl, 10X Taq buffer (+KCl) 2.5 µl, 25 mM MgCl₂ 3.5 µl, 2 mM dNTPs 3.5 µl, ISSR primer 2 µl, Taq polymerase (5 U / µl) 0.5 µl and final volume was made up with sterile primer water. Before starting the master mixture preparation all the reagents like, Taq buffer, dNTPs, MgCl₂, primers etc. taken from -20°C were first thawed completely and then used. Assembled reactions in PCR tube were given a brief pulse for 30 seconds for complete mixing and then placed in the thermo-cycler for DNA amplification.

The ISSR amplification was made in an automated PCR machine (Applied Biosystems 2027). The initial denaturation was done at 94°C for 5 min, followed by 40 cycles consisting of 1 min denaturation at 94°C, 1 min primer annealing ranged from 37°C to 61°C and 3 min extension at 72°C and a final extension for 10 min at 72°C.

Amplified ISSR fragments were separated in a horizontal gel electrophoresis system on 1.8% agarose gel with pre-stained ethidium bromide solution using 1X TAE buffer. The gels were run at 80 volts for 3 hours. Separated band profiles were visualized under UV light using a transilluminator system. All the fingerprints were recorded with Molecular Analyst Gel Documentation System. The sizes of the amplified fragments were determined by Gene Ruler 100 bp plus DNA ladder, (MBI, Fermentas) as a standard molecular weight marker.

**Lentinula edodes genome-rich ISSR:**

Hybrids of the first fusion experiment (P. florida + L. edodes) were analyzed by a L. edodes genome-rich ISSR marker to examine whether L. edodes or the percentage of L. edodes genome is present in their presumptive somatic hybrid. L. edodes specific sequence-rich ISSR [(aaagag)₃] marker was developed by Qin et al. (2006) to develop strain-specific SCAR markers for Lentinula edodes. The same ISSR protocol was followed in this experiment.

**3.20 Polymorphic Information Content (PIC) of molecular marker:**

PIC value is used to refer to the relative value of each marker with respect to the amount of polymorphism exhibited. It was estimated according to the protocol of Weir (1996)
and using this formula: PIC = \[1 – (\sum P_i^2)\], where ‘i’ is used as the total number of alleles detected from RAPD and ISSR marker, and ‘Pi’ is the frequency of the i\textsuperscript{th} plus allele in the set of the hybrid populations.

3.21 SCAR marker development:

Sequence characterized amplified region (SCAR) marker is generally used to develop for commercial strain identification of agricultural crops. Here, hybrid fruit body generating (from first fusion experiment) tissue culture generated one hybrid line showed maximum fruit body yield (bioefficiency\%) on paddy straw substrate. For this reason, a hybrid strain specific SCAR is developed. Thirty decemer RAPD primers (Operon technologies) were preliminary screened and used for DNA-typing of all first fusion generated hybrids and fruit body generating tissue culture generated hybrid lines.

The morphological traits, fruit body parameters and yield, RAPD reaction of tissue culture generated line from fruit body generating hybrid lines were done based on the protocol as described above.

Conversion of RAPD band to hybrid strain-specific SCAR marker:

RAPD generated unique polymorphic band was excised with a sterile scalpel from agarose gel and purified using a DNA Gel Extraction kit (Qiagen). The purified RAPD fragment was multiplied by PCR with the same primer and again purified by PCR purification kit (Qiagen). The purified fragment was further confirmed by gel running (1.5\% agarose) at 80 volts for 2 hours along with Gene Ruler 100 bp plus DNA ladder.

The fragment was then ligated into pGME-T easy vector (Promega, USA) for overnight at 4\°C. The vector molar ratio was 1:3 which showed the best result. Transformation was carried out in *E. coli* (*DH5α* strain) host cell at 42\°C incubation for 45 second. Transformed host cell were plated in X-gal-IPTG mixed LB agar at 37\°C for overnight. TA clones were screened by blue-white selection. Selected at least three white colonies were taken; plasmid extracted and sequenced using an automated DNA sequencer, Applied Bio-systems.

The RAPD sequence was submitted to NCBI genbank. Sequence similarity was checked at NCBI using blast tool to see the nearest matching. Three sequences were multiple aligned for further confirmation. Using primer designing software, the SCAR primer pair
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(forward and reverse) was designed. Finally, this primer pair was used for SCAR-PCR in all the hybrid lines with parent to check whether it can amplify only the desired hybrid genome or not. Genomic DNA of all hybrids, tissue culture generated hybrid lines from fruit body generating hybrid lines and parental strains were amplified using the SCAR primer and PCR cycling conditions for amplification was optimized as: 94°C for 5 min; followed by 35 cycles; at 94°C for 1 min, 61°C for 1 min and 72°C for 2 min; final extension was given at 72°C for 10 min. The PCR products were analyzed followed by RAPD gel analysis protocol.

3.22 Analysis of ribosomal RNA-ITS region:

Ribosomal RNA gene and internal transcribe spacer (rRNA-ITS) region was amplified from nuclear DNA of both the somatic hybrids and their respective parental strains. This experiment was carried out to study about the nature and degree of sequence polymorphism in this particular portion of hybrid’s genome.

Reagents required:

PCR-based amplification was followed for rRNA-ITS region.

- Reaction buffer (Fermentas)
- 10X Taq buffer with KCl
- 25 mM MgCl$_2$
- dNTPs (2 mM diluted from 10 mM stock of four nucleotides mixture of dNTP)
- 20 µM of ITS1 (5´ TCCGTAGGTGAACCTGCGG 3´,forward) and ITS4 (5´ TCCTCCGCTTATTGATATGC 3´,reverse) primers (synthesized form Sigma-Aldrich)
- Taq DNA polymerase enzyme (5 Unit from Fermentas)
- Primer water (HPLC water from MERCK)
- Nuclear DNA (from 25 ng / µl stock).
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Methodology:

Based on the protocol of White et al. (1990), the rRNA-ITS PCR amplification was done. This repeat sequence of rRNA was amplified by slight modification of this PCR protocol. The PCR reaction amplifications were performed in 25 µl of reaction volume and it contained 10-12 ng of nuclear DNA (0.5 µl), 10X Taq buffer (+KCl) 2.5 µl, 25 mM MgCl₂ 2.5 µl, 2 mM dNTPs 2.5 µl, primer ITS1 (F) 2 µl, primer ITS4 (R) 2 µl, Taq polymerase enzyme (5 U / µl) 0.25 µl and final volume was made up with sterile primer water. All the reagents including Taq buffer, dNTPs, MgCl₂, primers etc. from -20°C were first thawed completely and then used for PCR master mix preparation. In a PCR tube, reactions mixtures were given a brief pulse for complete mixing and then placed in the thermo cycler for rRNA-ITS region amplification.

The amplification reactions were conducted in a DNA thermal cycler (Applied Biosystems 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.

PCR amplified rRNA-ITS products were separated on a 1.5% agarose gel pre-stained with ethidium bromide solution using 1X TAE buffer. The gels were run for 2 hours at 80 volts and visualized under UV light. This gel profile was recorded in a Molecular Analyst Gel Documentation System. The size of the amplified fragments was determined by Gene Ruler 100 bp plus DNA ladder (MBI, Fermentas) used as a standard molecular weight marker.

[Note: The amplified rRNA-ITS regions were used for two different experiments; half part was for restriction fragment length polymorphism (RFLP) analysis and another half for nucleotide sequencing].

3.23 Purification of rRNA-ITS PCR products:

After PCR amplification the rRNA-ITS products were purified for further experiments. The following steps were followed for purification.

- Amplified PCR products were taken in another set of eppendorf tube.
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- Added double volume of absolute alcohol and 1/10th volume of 3 M sodium acetate.
- Mixed the solution carefully and kept at -20°C for 2 h.
- Then centrifuged at 12,000 rpm for 30 min at RT (24±1°C).
- Supernatant was discarded and pellet (generally invisible in naked eye) was washed in 70% chilled ethanol by centrifugation at 8000 rpm for 5 min.
- Allowed to dry the pellet and diluted in 10 µl (approx.) of ion-free sterile water.

3.24 RFLP analysis of rRNA-ITS region:

10 µl of purified PCR products were used for each restriction digestion. For this study, four restriction enzymes were used following the supplier’s specification.

Enzymes and reagents:

- **Alu I:**
  
  5’…AGCT…3’
  3’…TCTAGA…5’

- **Hinf I:**
  
  5’…GANTC…3’
  3’…CTNA3…5’

- **Hpa II:**
  
  5’…CGGG…3’
  3’…GGCC…5’

- **Hae III (Bsu RI):**
  
  5’…GGCC…3’
  3’…CCGG…5’

- rRNA-ITS PCR product
- 10X Tango buffer (for Alu I and Hpa II)
- 10X buffer R (for Hinf I and Hae III)
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- Nuclease free water.

Digestion:

Restriction digestion of rRNA-ITS PCR product was conducted in 30 µl of reaction volume. The digestion reaction contained 10 µl of PCR product, 2 µl of 10X Tango buffer or 10X buffer R, 1 µl of restriction enzyme and 18 µl of nuclease free water. The components were mixed gently and pulsed for few seconds. Incubation was given at 37°C for 1-16 hours (according to supplier’s instruction). The standardization of digestion period was varied for each restriction enzyme.

The fragmented PCR products were size-fractionated in 1.5% agarose (w/v) gel (run in 1X TAE buffer at 80 volts for 3 hours) stained with ethidium bromide (0.5 µg / ml). Photographs were taken under UV, using a gel-doc system unit and data were recorded. A Gene Ruler 100 bp plus DNA ladder was used as standard molecular marker.

3.25 Sequencing of ITS1-5.8S rRNA-ITS2 region:

The purified PCR products were sequenced using Big Dye Terminator v 3.1 method and nucleotide bases were read by an automated sequencer (Applied Biosystems).

Reagents required:

- Ready Reaction (RR) mixture
- 5X Sequencing buffer
- Primers (1 pmol / µl): ITS1 (F) and ITS4 (R)
- Template DNA (purified rRNA-ITS product)
- Ultrapure water

Sequencing PCR:

Sequencing PCR was conducted in a normal PCR tube of 10 µl total reaction volume. The PCR components were RR mix 1µl, 5X sequencing buffer 2µl, primers (pmol / µl) ITS1 (F) 1 µl, ITS4 (R) 1 µl, PCR product (50 ng, depending upon the purified PCR
concentration) and the final volume was made up with sterile water. Mixed the components gently and pulsed for few seconds before enter into the PCR machine.

The PCR amplification reactions were conducted in a DNA thermal cycler (Applied Biosystems 2027) by preliminary denaturation at 96°C for 1 min consisting of 25 cycles; template denaturation at 96°C for 10 sec, primer annealing at 37°C for 5 sec, extension at 60°C for 4 min.

After sequencing PCR, the amplified products were purified by following steps:

- Added 10 µl of ultrapure water into PCR tube
- Samples were taken in another set of 500 µl eppendorf tube
- Again added 2 µl of 125 mM EDTA, 2 µl of 3 M sodium acetate (pH 5.2) and 50 µl of absolute alcohol.
- Mixed gently and kept in dark for 20 min
- Centrifuged at 13,000 rpm for 20 min
- Discarded the supernatant and pellet was washed twice in 70% alcohol (200 µl) by centrifugation at 12,000 rpm for 15 min

The dried pellet was forwarded for next step:

- Added 12 µl of ‘High Dye’ formamide solution (injection buffer) in the tube and dissolved DNA by gentle mixing and incubated in dark condition for 20 min
- The solutions were taken in PCR tube and kept in thermal cycler for denaturation at 95°C for 5 min
- Immediately after denaturation, samples were dipped in ice for 10 min
- Then, finally loaded the sample of 10 µl for sequencing.

[After sequencing, all the read sequenced data were collected and used for analysis].
Submission of sequenced data at NCBI genbank:

The primer pair (ITS1 & ITS4) generated rRNA-ITS sequences of all hybrids and parents were deposited at NCBI genbank using BankIt tool. Firstly, all the sequenced data were confirmed with its replication data. The length of ribosomal gene unit (ITS1-5.8S-ITS2) was calculated by sequence data analysis software. Blast was done for all the sequences to check the nearest matching available in the genbank. Multiple sequence alignment was done for two different hybrid populations along with their respective parents and the sequence polymorphism was marked with ‘boxshade’ technique. Ribosomal RNA sequence based dendrogram was also constructed using UPGMA method in each case.

3.26 Statistical method and software used for data analysis:

Experimental data including morphological traits, DNA marker profiles, rRNA-ITS sequences etc. of all hybrids and parents were scientifically analyzed by different statistical methods and softwares. Variations in morphological traits (mycelial growth, colony diameter, hyphal width, pelius diameter, stipe length, yield etc.) among the hybrids from the two fusion experiments were analyzed by one-way-ANOVA and the Tukey Post-hoc test for multiple comparisons of means, standard deviation and level of significance (Zar 1998). A bivariate correlation was made among the hybrids with their parents using IBM SPSS version 19 software. Principal component analysis (PCA) was used for multivariate phenotypic data analysis and component matrices were generated by IBM SPSS software. Through PCA, the extracted component matrices were plotted in Microsoft Excel sheet. Scatter diagram was constructed from that component matrix for graphical presentation and influential variable analysis of phenotypic traits in each hybrid lines.

RAPD and ISSR primer generated DNA profiles of hybrid and parental strains were analyzed by scoring the band number present in the gel. Clear and reproducible bands were scored as present (1) or absent (0) for each primer genotype combinations. Data generated from RAPD and ISSR primers for each hybrid and parental strains were entered into a binomial matrix. The bivariate ‘0-1’ data were analyzed using IBM SPSS software, version 19. Jaccard’s proximity matrix was generated using this software and
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dendrogram was constructed employing the Un-weighted Pair Group method with Mathematic Average (UPGMA) algorithm (Jaccard 1901).

Based on the sequenced RAPD fragment, one SCAR primer pair was designed using PRIMER-3 PLUS software. All the sequenced data were submitted to NCBI genbank using BankIt tool. Sequenced data of all samples were taken and aligned in CLUSTALW software. The multiple aligned sequences of hybrids were compared with their parents by BOXSHADE 3.21 software and shaded. Based on the rRNA-ITS sequence, an UPGMA cluster was generated and found the dissimilarity in the rRNA-ITS region between each hybrid group and parents. All the sequenced data (FASTA format) were aligned in CLUSTAL 2.1 Multiple Sequence Alignments software.