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GENOME INSTABILITY IN FRUIT BODY DERIVED LINES GENERATED FROM FRUITING *PFLE* SOMATIC HYBRID LINES AND DEVELOPMENT OF HYBRID STRAIN SPECIFIC SCAR MARKER IN EDIBLE MUSHROOM

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ABSTRACT

Six fruit body derived lines (*pfle FB*) generated from six fruiting *pfle* somatic hybrid mushroom lines showed genetic diversity analysed by fruit body morphology and inter simple sequence repeat (ISSR) markers. Stipe length, pelius diameter and bioefficiency % (BE%) of all the strains showed variations between each other with respect to *Pleurotus florida* parent. Hybrid *pfle 1v* and *pfle 1q* showed the highest value of stipe length and pelius diameter, respectively, compared with parent *P. florida*. Four ISSR primers amplified a total of 47 reproducible fragments with 82.9% polymorphism in which primer ISSR-03 produced the highest number of amplicons. Unweighted pair group method with arithmetic mean (UPGMA) based dendrogram exhibited two major groups in which hybrids *pfle 1r* and *pfle 1q* showed genetical closeness to parents *P. florida* and *Lentinula edodes*, respectively. Tissue culture generated line from fruit body of *pfle 1r* hybrid showed maximum BE% compared with the other hybrids and *P. florida* parent. For identification of this line, a pair of hybrid strain-specific SCAR marker (RFB_2F and RFB_2R) was developed based on an unique 813 bp RAPD amplicon.

Key words: ISSR, Mushroom fruit body, Polymorphism, RAPD, SCAR

INTRODUCTION

Genetic diversity in the mushroom somatic hybrid progeny occurs due to genome recombination of two different parental strains (Peberdy & Fox 1993). In edible mushroom several reports had been published on intra-specific, inter-specific, inter-generic (Ogawa et al. 1988; Chakraborty & Sikdar 2010; Mallick & Sikdar 2014, 2015a) and even inter-order (Yoo et al. 2004) somatic hybridization and the hybrid fruit body morphology was characterized by various methods (Liang & Chang 1989; Ohmasa 1986). Such hybrid generations were also characterized by various DNA markers, which detected the polymorphisms between each other (Witte et al. 1989; Chakraborty & Sikdar 2008, Mallick & Sikdar 2014, 2015a). The important issue of such breeding programme is to establish stable generations at field level. Tissue culture-generated hybrids showed genomic instability in various crop plants including maize and rice (Yu et al. 2011; Wang et al. 2013). In *Brassica* hybrid the genomic instability was detected by ISSR primers (Leroy et al. 2000).

In recent years, a number of molecular markers such as, AFLP (Mignouna et al. 2002), ISSR (Nagaoka & Ogihara 1997; Shen et al. 2006), SSR (Zietkiewicz et al. 1994; Zhang et al. 2012), RAPD (Alam et al. 2009), DNA barcoding of rRNA-ITS (Oskiera et al. 2015) and RFLP of rRNA-ITS (Mallick & Sikdar 2015b) have been widely used to detect the genetic diversity, phylogenetic relationship, species identification and hybrid characterization in agricultural crops including fungi. ISSR technique

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has some specific advantages than the other molecular markers as follows: no need of prior sequence information, simple operation, high stability and low cost and thus it has been proposed as a more economical and reliable DNA marker system (Fang & Roose 1997; Prevost & Wilkinson 1999; Bornet & Branchard 2001). The RAPD-based SCAR marker was developed earlier through direct sequencing of PCR product in wheat cultivars (Hemandez et al. 1999). In the past, strain-specific SCAR marker was developed for identification of medicinal mushroom *Ganoderma lucidum* by ISSR marker (Su et al. 2008) and by RAPD marker in *Hypsizygus marmoreus* (Lee et al. 2012). In our laboratory we generated nine *pfle* somatic hybrid lines through protoplast fusion between *Pleurotus florida* and *Lentinula edodes* of which only six could produce fruit body (Mallick & Sikdar 2014). We also showed that the tissue culture-generated line from fruit body of one of such somatic hybrid lines, *pfle 1r* showed highest bioefficiency compared with other hybrids and *P. florida* parent (Mallick & Sikdar 2014). This hybrid is also having immune-enhancing properties of isolated glucan from fruit body (Maji et al. 2012).

In the first part of the study we analysed for the first time the genetic variation of fruit body of derived lines of fruiting six *pfle* hybrid mushrooms on the basis of fruit body morphology and ISSR markers. In the second part, we developed a pair of hybrid strain-specific SCAR marker for identification of *pfle 1r FB* keeping in mind its economic values from the commercial point of view.

MATERIALS AND METHODS

Strains and culture conditions

We have investigated a total of 14 mushroom samples in this study. Parents *P. florida* and *L. edodes* were collected from 'National Research Centre for Mushroom', Solan, Himachal Pradesh, India. Six *pfle* fruiting hybrids (viz. *pfle 1v*, *pfle 1q*, *pfle 1p*, *pfle 1r*, *pfle 1s* and *pfle 1o*) were developed through protoplast fusion between *P. florida* and *L. edodes* (Mallick & Sikdar 2014) and six fruit body (FB) tissue-derived cultures, that is., *pfle 1vFB, pfle 1qFB, pfle 1pFB, pfle 1rFB, pfle 1sFB* and *pfle 1oFB* of respective *pfle* hybrid were generated in laboratory condition. The surface area of 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 potato dextrose agar (PDA), pH 6.2 medium for further growth. All the strains were routinely maintained in PDA medium at 24 ± 1 °C. Mycelial tissue preparation for DNA isolation of all the strains under study was done by growing in liquid MYG medium (10 $g \cdot l^{-1}$ malt, 4 $g \cdot l^{-1}$ yeast extract and 10 $g \cdot l^{-1}$ glucose, pH 6.2) at 24 °C.

Production of fruit body

Hybrids *pfle* and *pfle FB* were maintained on PDA medium and used for spawn preparation on paddy grains. The spawn packets were prepared using autoclaved paddy grains of 200 g each. Matured spawns were used for fruit body production on overnight soaked and then autoclaved paddy straw substrate (100 g spawn/0.65 kg dry straw) in a cylindrical polypropylene bag. In fully colonized bag, several pores were made all over the surface for good aeration. Then the bags were placed in the mushroom cultivation room at 24±1 °C. After primordial initiation, high humidity (85-90%) was maintained. Data were recorded for all the hybrid strains and *P. florida* parent. Fruit body morphology and yield among the *pfle*, *pfle FB* hybrids were analysed by one-way ANOVA and the Tukey post hoc test was done for multiple comparisons of means and standard deviation (Zar 1998). The complete fruiting flushes (1st, 2nd and 3rd harvest) of each hybrid were collected repeatedly by five times for these analyses. Bioefficiency % was calculated on the basis of the fresh weight of fruit body in relation to the weight of dry substrate.

DNA extraction and PCR conditions

Genomic DNA was extracted from mycelial tissues of all mushroom strains using the modified CTAB method (Dellaporta et al. 1983). PCR for ISSR were conducted using modified protocol of Bornet & Branchard 2001, in 25 µl reaction mixture containing 1X Taq buffer with KCl salt (Thermo Scientific) of 2.5 µl, 75 ng template DNA of 3 µl (25 $ng \cdot \mu l^{-1}$), 2 mM dNTP mixture (four nucleotide triphosphate, Thermo Scientific) of 2.5 µl, 25 mM

of $MgCl_2$, 2.5 µl of 20 µM ISSR primer and 0.5 µl, 5U Taq DNA polymerase enzyme (Thermo Scientific). The final volume was made up by ultra-pure distilled water (Invitrogen). The ISSR amplification condition was: 5 min initial denaturation at 94 °C; 40 cycles consisting of 1 min denaturation at 94 °C, 1 min primer annealing ranged from 40º to 59 °C and 3 min extension at 72 °C, and a final extension for 10 min at 72 °C.

The concentration of ingredients in RAPD-PCR mixture was the same as in ISSR mixture, except the addition of 100 ng of DNA template. The amplification was conducted by preliminary denaturation of template DNA at 95 °C for 4 min followed by 35 cycles of DNA denaturation at 94 °C for 1 min, primer annealing at 37.5 °C for 45 seconds, initial primer extension at 72 °C for 1.5 min, followed by a final extension at 72 °C for 8 min. Ingredient of the PCR mixture with SCAR primers was prepared with the same concentrations as in RAPD and the amplification condition was optimized as: initial denaturation at 94 °C for 5 min followed by 35 cycles; denaturation at 94 °C for 1 min, primer annealing at 61 °C for 1 min and initial extension at 72 °C for 2 min followed by final extension at 72 °C for 10 min.

Gel electrophoresis

Amplified ISSR PCR products were separated on 1.8% (w/v) agarose gel whereas RAPD and SCAR PCR products were separated on 1.5% (w/v) agarose gel pre-stained with ethidium bromide solution (10 $mg \cdot ml^{-1}$). The gels were run at 10 $V \cdot cm^{-1}$ for ISSR and 8 $V \cdot cm^{-1}$ for RAPD and SCAR products. The DNA profiles were visualized under UV transilluminator and data were recorded with 'Molecular Analyst Gel Documentation System'. The sizes of the amplified ISSR, RAPD and SCAR fragments were determined by using DNA Ruler Plus (100-3000 bp) ladder (MBI, Thermo Scientific) as a standard molecular weight marker. The ISSR bands in gel profile were scored as either present (1) or absent (0) for each primer. The bivariate 0-1 data were analysed using the SPSS software (IBM, version. 19). Jaccard's proximity matrix (Jaccard 1901) was generated using this software and constructed a dendrogram employing the unweighted pair group method with mathematic average (UPGMA) algorithm. Polymorphism information content (PIC) value was calculated to refer the relative value of each RAPD and ISSR markers with respect to the amount of polymorphism exhibited. It was estimated using the formula: $PIC = [1 - (\Sigma P_i^2)]$ (Weir 1996), where 'i' is the total number of alleles detected from RAPD and ISSR marker, and 'Pi' is the frequency of the i^{th} plus allele in the set of *pfle* and *pfle FB* genotype.

Conversions of RAPD fragment into hybrid strain-specific SCAR marker

After successful screening of 30 RAPD primers (Operon Technologies), RAPD-07 was selected, which generated a unique polymorphic band (813 bp) in *pfle 1rFB* line. The RAPD repeatability tests were done by changing the primer concentration (20 and 25 µM), amount of template DNA (50, 75 and 100 ng) and Taq DNA polymerase enzyme (0.25 and 0.5 µl) and standardized for all the samples. The false and non-reproducible bands were eliminated using POPGENE software and checked the PIC values. Then, the excised band from agarose gel was purified using DNA Gel Extraction Kit (Qiagen) and ligated into pGME-T easy vector (Promega, U.S.A) following the supplier's instructions. Selected clones were sequenced using Big Dye Terminator v 3.1 method and nucleotide bases were read by an automated DNA sequencer (Applied Biosystems, model: 3500xL). Obtained sequence was submitted to the NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Based on the sequence a pair of SCAR primers was designed (Primer3Plus) and synthesized (Sigma Aldrich, Germany).

RESULTS

Fruit body morphology and yield

Stipe length, pelius diameter and bioefficiency % showed variation in all hybrids compared with *P. florida* parent (Table 1). Maximum (6.87 ± 0.75 cm) and minimum (2.07 ± 0.05 cm) stipe length was found in hybrid *pfle 1v* and *pfle 1vFB*, respectively compared with others and parent *P. florida* (6.38 ± 0.12 cm). Pelius diameter was found maximum (5.81 ± 0.52 cm) in hybrid *pfle 1q* and minimum (3.09 ± 0.11 cm) in *pfle 1oFB* when *P. florida* showed 4.98 ± 0.31 cm. The maximum

instability was found between hybrid *pfle 1v – pfle 1vFB* in stipe length and *pfle 1r – pfle 1rFB* in pelius diameter. Bioefficiency % was increased in hybrid *pfle 1pFB*, *pfle 1qFB* and *pfle 1rFB* compared with respective *pfle* strains while it was decreased in rest three hybrid *FB* lines compared with their *pfle* lines. Maximum bioefficiency % (113.50) was found in hybrid *pfle 1rFB* and minimum (24.16) in *pfle 1oFB* amongst all the strains including parent *P. florida* (110.23).

Table 1. Fruit body morphology and bioefficiency % of six *pfle* and respective *pfle FB* hybrid lines with *P. florida* parent. Tukey test is done at 5% of significance level to determine the specific differences between mean values ($p \leq 0.05$), where groups with the same letters are not significant

Lines	Stipe length in cm[a]	Pelius diameter in cm[a]	BE (%)[b]
P. florida	6.38 ± 0.12	4.98 ± 0.31	110.23
pfle 1p	2.56 ± 0.21	4.04 ± 0.25	51.75
pfle 1pFB	2.90 ± 0.18	5.53 ± 0.22	105.33
pfle 1q	3.08 ± 0.25	5.81 ± 0.52	51.75
pfle 1qFB	2.80 ± 0.22	4.40 ± 0.08	89.00
pfle 1v	6.87 ± 0.75	3.99 ± 0.88	78.32
pfle 1vFB	2.07 ± 0.05	3.48 ± 0.22	48.33
pfle 1s	2.97 ± 0.32	4.43 ± 0.21	84.91
pfle 1sFB	3.03 ± 0.78	4.14 ± 0.25	84.17
pfle 1o	3.09 ± 0.80	4.30 ± 0.92	36.77
pfle 1oFB	2.70 ± 0.55	3.09 ± 0.11	24.16
pfle 1r	2.24 ± 0.22	3.29 ± 0.07	46.35
pfle 1rFB	4.03 ± 0.52	5.60 ± 0.45	113.50
SE (Mean)	0.68	0.54	-
p-value (F statistics)	0.99 (3.69)	0.99 (3.69)	-
F-value	59.21	61.43	-

[a] Mean ± SD, data taken from five observations; [b] Biological efficiency.
The values of stipe length, pelius diameter and yield are means ± SD of five repeated experiments. Results of one-way ANOVA for each variable are shown as F values, $P < 0.05$.

Table 2 Details of RAPD, ISSR and SCAR primers used in this study

Primers	Sequence (5′-3′)	Annealing Temp. (°C)	Approx. fragment sizes (bp)	Maximum no. of scorable amplicons	PIC* value
RAPD-07	TCCCAGCAGA	37.5	600-2700	9	0.343
ISSR-02	CAGCAGCAGCAGCAG[$(CAG)_5$]	59	220-1500	10	0.466
ISSR-03	CAACAACAACAACAA[$(CAA)_5$]	44.7	480-2000	13	0.495
ISSR-11	CACCACCACGC[$(CAC)_3$GC]	45.5	380-2500	12	0.487
ISSR-12	GAGGAGGAGGC[$(GAG)_3$GC]	40	300-1700	12	0.489
RFB_2F	CTGACCCTGAGCACTCACAA	61	813	1	-
RFB_2R	TGTGGTATGGCGAAAATGAA	61	813	1	-

*Polymorphism Information Content

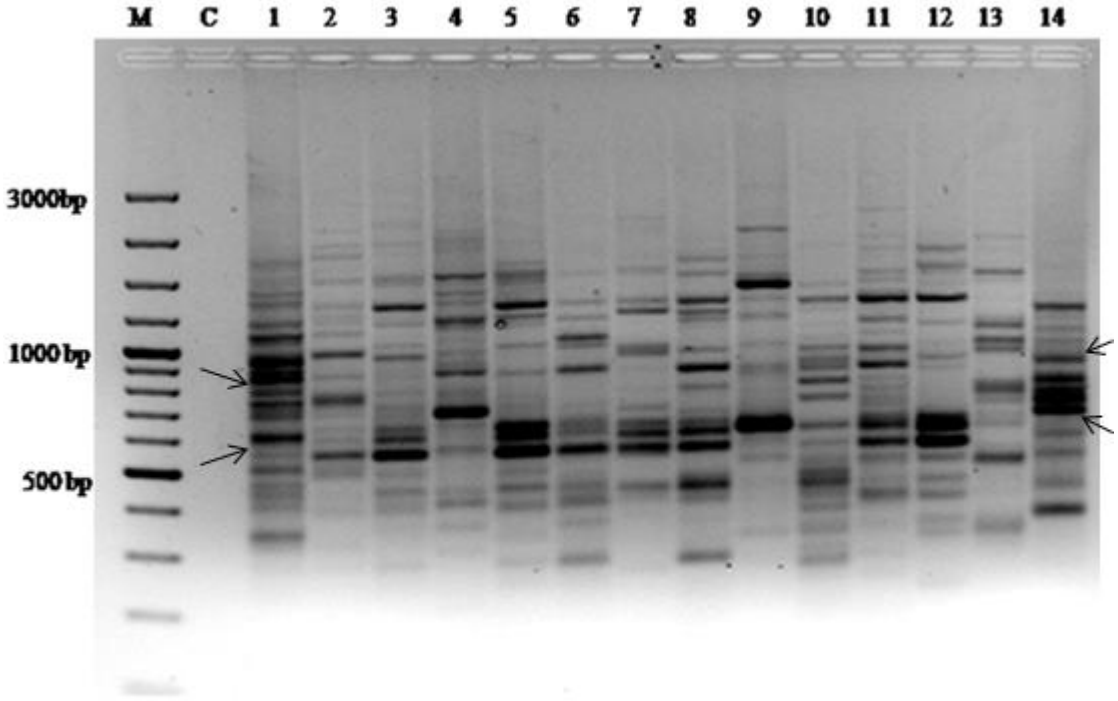

Fig. 1. Primer ISSR-03 amplified DNA profile of *pfle FB* hybrid lines, *pfle* hybrid lines and parental strains. Lane: M – marker (100 bp DNA ruler plus), Lane C – control (without template DNA), Lane 1 – *P. florida*, Lane 2 – *pfle 1v*, Lane 3 – *pfle 1vFB*, Lane 4 – *pfle 1q*, Lane 5 – *pfle 1qFB*, Lane 6 – *pfle 1p*, Lane 7 – *pfle 1pFB*, Lane 8 – *pfle 1r*, Lane 9 – *pfle 1rFB*, Lane 10 – *pfle 1s*, Lane 11 – *pfle 1sFB*, Lane 12 – *pfle 1o*, Lane 13 – *pfle 1oFB* and Lane 14 – *L. edodes*. Left arrows depict bands from *P. florida* and right arrows from *L. edodes* parent

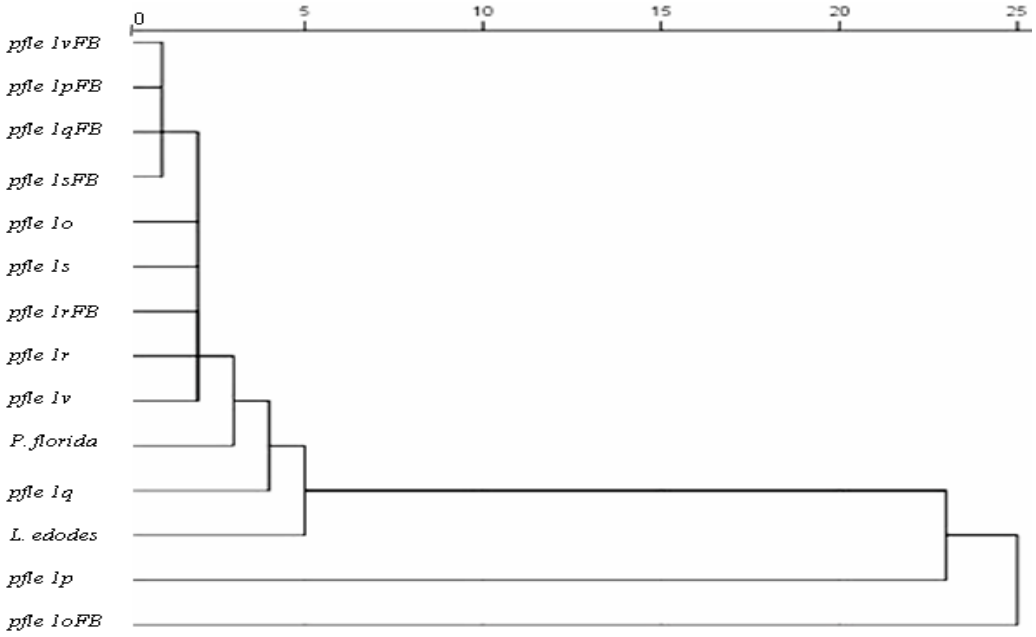

Fig. 2. Dendrogram showing the relationship amongst the *pfle FB* lines with their respective *pfle* lines compared with parents using genetic divergence obtained from DNA fingerprints of 4 ISSR primers and the UPGMA method of clustering and Euclidean distance. The horizontal scale indicates the Euclidean distance (dissimilarity) between the two clusters

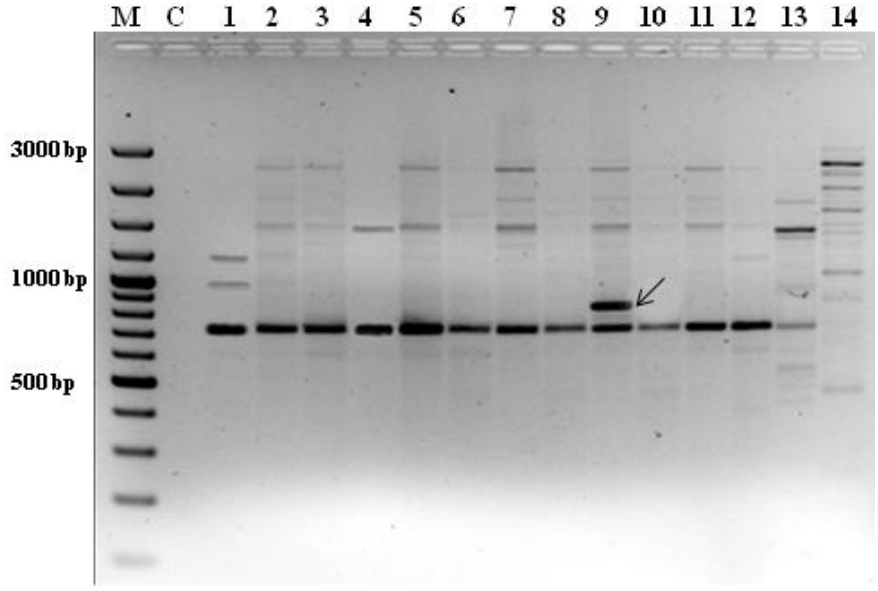

Fig. 3. PCR profile of the 14 mushroom strains amplified by primer RAPD-07. Lane: M - marker (100 bp DNA ruler plus), Lane C – control (without template DNA), Lane 1 – *P. florida*, Lane 2 – *pfle 1v*, Lane 3 – *pfle 1vFB*, Lane 4 – *pfle 1q*, Lane 5 – *pfle 1qFB*, Lane 6 – *pfle 1p*, Lane 7 – *pfle 1pFB*, Lane 8 – *pfle 1r*, Lane 9 – *pfle 1rFB*, Lane 10 – *pfle 1s*, Lane 11 – *pfle 1sFB*, Lane 12 – *pfle 1o*, Lane 13 – *pfle 1oFB* and Lane 14 – *L. edodes*. The arrow indicates the unique band present in Lane 9 (*pfle 1rFB*) that was converted into SCAR marker

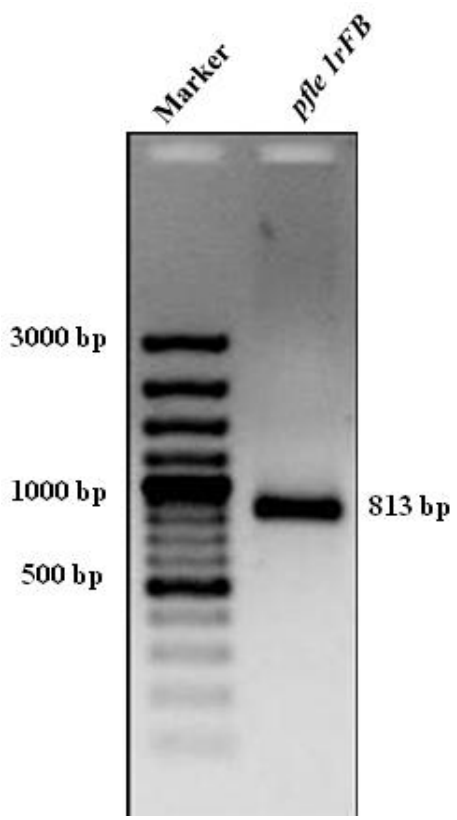

Fig. 4. Purified 813 bp fragment of hybrid *pfle 1rFB*. Lane: M - marker (100 bp DNA ruler plus)

ISSR analysis

Details including primer sequence, annealing temperature, number of amplified fragments with sizes and PIC values of ISSR primers are summarized in Table 2. Four ISSR primers generated a total of 47 clear and reproducible bands with 82.9% polymorphism amongst all the strains studied with an average of 11.75 bands per primer. The amplified fragments ranged from 220-2500 bp in size. Primer ISSR-03 $[(CAA)_5]$ and ISSR-02 $[(CAG)_5]$ generated the highest and lowest number of scorable bands ranged from 480-2000 bp and 220-1500 bp with mean PIC values of 0.495 and 0.466, respectively (Fig. 1).

Jaccard's proximity matrix was generated on the basis of amplified ISSR fragments of the *pfle*, *pfle FB* hybrids and parents where the genetic distances were recorded, ranged from 4.00 to 170.00. Based on this data, hybrids *pfle 1pFB* and *pfle 1vFB* were genetically most closely related, where most distantly related strain was found between *pfle 1p* and *pfle 1oFB*. Among the tissue culture-generated lines, *pfle 1sFB* and *pfle 1oFB* showed minimum and maximum genetic distances with the corresponding progenitor lines *pfle 1s* and *pfle 1o*, respectively.

Based on the dendrogram, two major clusters were developed in which *pfle 1vFB, pfle 1pFB* and *pfle 1sFB* were grouped in cluster I and *pfle 1qFB, pfle 1o, pfle 1s, pfle 1rFB, pfle 1v* in cluster II (Fig. 2). However, parents *P. florida* and *L. edodes* were very close to *pfle 1r* and *pfle 1q*, respectively.

Development of *pfle 1rFB* strain-specific SCAR marker

In order to obtain more specific and recognizable fragment for identification of *pfle 1rFB*, SCAR marker was developed from the sequence of RAPD amplicon. Primer RAPD-07 (Table 2) generated 813 bp unique polymorphic band in hybrid *pfle 1rFB* is shown in Fig. 3. The PCR-purified RAPD band showed the exact size in the gel (Fig. 4). Sequenced data was submitted at NCBI GenBank with an accession number of KJ196380. Designed SCAR primers (RFB_2F-forward and RFB_2R-reverse) (Table 2) were synthesized and used in PCR to check the performance of 14 DNA samples (six *pfle* hybrids, six *pfle FB* hybrids and two parents, *P. florida* and *L. edodes*). The result showed the presence of 813 bp band in strain *pfle 1rFB* only, while no such amplifications were observed in other hybrids and parents (Fig. 5).

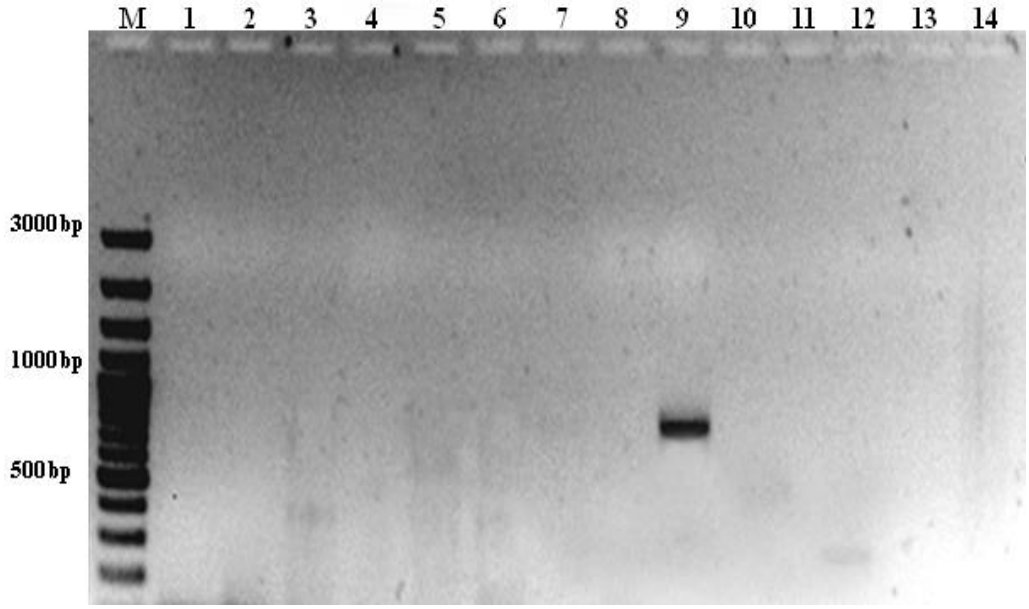

Fig. 5. SCAR profile of six *pfle FB* lines with respective *pfle* lines and two parents showing only single band (~813 bp) present in Lane 9 (*pfle 1rFB*). Lane: M – marker (100 bp DNA ruler plus). Lane 1 – *P. florida*, Lane 2 – *pfle 1v*, Lane 3 – *pfle 1vFB*, Lane 4 – *pfle 1q*, Lane 5 – *pfle 1qFB*, Lane 6 – *pfle 1p*, Lane 7 – *pfle 1pFB*, Lane 8 – *pfle 1r*, Lane 9 – *pfle 1rFB*, Lane 10 – *pfle 1s*, Lane 11 – *pfle 1sFB*, Lane 12 – *pfle 1o*, Lane 13 – *pfle 1oFB* and Lane 14 – *L. edodes*

DISCUSSION

Production of stable hybrid lines in agricultural crop is a complicated biological phenomenon. Generally, in sexually propagated crop plants, variation occurred due to genetic recombination during meiosis in the progeny lines. Whereas in case of micro-propagated plants raised through tissue culture, genetic fidelity may not be maintained due to somaclonal variation caused by DNA damage, change in ploidy level or chromosomal rearrangements and epigenetic alterations under various environmental conditions (Larkin & Scowcroft 1981; Yu et al. 2011; Leroy et al. 2000; Wang et al. 2013). In the first part of this study, it has been found that the genomic instability lies in the hybrid fruit body tissue and the possible occurrences were due to epigenetic influences during vegetative culturing of *pfle FB* lines. The phenotypic fruiting characteristics of all the *pfle* and *pfle FB* lines also showed variations between each other and hence declared its genomic instability supported by molecular

analyses. All the six *pfle* hybrid strains were independent recombinant somatic hybrid lines (Mallick & Sikdar 2014) and successive culture of mycelial tissue in the PDA medium did not cause any instability; but when mycelial tissue was generated from the fruit body part, the genetic variation occurred. Possibly, the heterogeneity is developed during the formation of basidiocarp or after formation of basidiospore in the basidiocarp of somatic hybrid strains, which might give rise to new mycelial tissue having altered genetic make-up in the *pfle FB* lines. Intra-genomic rearrangement might be there in the *pfle FB* lines due to cultivation practices also. In this case, the fruiting parameters of *pfle* generation were found to be similar with little changes by statistical analysis and it helped in comparison with *pfle FB* lines (Mallick and Sikdar 2014). In plant system, such phenomenon was reported in *Brassica* genome (Truco et al. 1996). The variations in ISSR profiles of the *pfle FB* lines compared with their corresponding progenitor *pfle* lines strongly supported this view (Supplementary Fig. 1). The dendrogram showed the closeness and Euclidean distances between two clusters indicating the genome dissimilarity of each hybrid in the group. The detailed mechanisms involved in this issue need to be explored in the future through molecular approaches by identifying new gene(s) responsible for fruit body development.

Development of new hybrid strain and its identification is an important issue in the mushroom industry from the commercial point of view. In the second part of this study, RAPD primer was used to develop a pair of SCAR marker for identification of high-yielding *pfle 1rFB* hybrid strain. The same technique was also used for fungal strain-specific SCAR development (Weber et al. 2002; Tanaka et al. 2004; Lee et al. 2012). However, there was no report of application of SCAR markers for identification of mushroom hybrid strain and we report it for the first time. Our results showed that PCR with the primers pair yielded *pfle 1rFB* strain-specific DNA bands, indicating that our strategy to develop SCAR marker is a reasonable approach.

CONCLUSIONS

In this study, we have reported that the mushroom genome is highly unstable when cultivated artificially on specific substrate and such instability showed the different degree of variation at phenotypic as well as genotypic level in this study. The actual reason behind it is still unclear to date. From previous reports, it can be said that mushroom genome is unstable and it reflects on several important economic traits to the commercial mushroom grower and researcher worldwide. In future, we have to identify the molecular mechanism responsible for such instability in this system. However, the developed SCAR marker will help in the identification of the particular hybrid mushroom at commercial approaches.

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