Molecular characterizations of somatic hybrids developed between *Pleurotus florida* and *Lentinus squarrosulus* through inter-simple sequence repeat markers and sequencing of ribosomal RNA-ITS gene

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Abstract The 12 *pfls* somatic hybrids and 2 parents of *Pleurotus florida* and *Lentinus squarrosulus* were characterized by ISSR and sequencing of rRNA-ITS genes. Five ISSR primers were used and amplified a total of 54 reproducible fragments with 98.14% polymorphism among all the *pfls* hybrid populations and parental strains. UPGMA-based cluster exhibited a dendrogram with three major groups between the parents and *pfls* hybrids. Parent *P. florida* and *L. squarrosulus* showed different degrees of genetic distance with all the hybrid lines and they showed closeness to hybrid *pfls 1m* and *pfls 1h*, respectively. ITS1(F) and ITS4(R) amplified the rRNA-ITS gene with 611–867 bp sequence length. The nucleotide polymorphisms were found in the ITS1, ITS2 and 5.8S rRNA region with different number of bases. Based on rRNA-ITS sequence, UPGMA cluster exhibited three distinct groups between *L. squarrosulus* and *pfls 1p*, *pfls 1m* and *pfls 1s*, and *pfls 1e* and *P. florida*.

Keywords ISSR · Polymorphism · rRNA-ITS gene · Mushroom somatic hybrid

Introduction

Production of somatic hybrids in edible mushrooms through protoplast fusion had been attempted in the past and basidiocarp was successfully developed (Ohmasa 1986; Liang and Chang 1989; Mallick and Sikdar 2014, 2015). Characterizations of hybrids were also done through mycelial morphology, fruit body yield, and molecular DNA markers which successfully proved the hybridity (Witte et al. 1989; Mallick and Sikdar 2014, 2015). Commonly used molecular markers that showed polymorphism and genetic diversity in mushroom hybrids, fungi and plants are amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), inter-simple sequence repeats (ISSR), isozyme, randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), etc. (Nagaoka and Ogiwara 1997; Mignouna et al. 2002; Shen et al. 2006; Chakraborty and Sikdar 2008; Shao et al. 2011; Yüzbaşıoğlu et al. 2011; Staniaszek et al. 2002; Aylward et al. 2014; Mallick and Sikdar 2014, 2015, 2016; Naik et al. 2017). There are no such reports on chromosome mapping by fluorescent dye (FISH or GISH) in fungal somatic hybrids, where the approach is available in plant system (Rokka et al. 1998).

However, in the past, the internal transcribed spacer (ITS) of ribosomal RNA was used for interspecies strain identification (Jorgenson and Cluster 1988; Cullings et al. 1996; Vogler and Bruns 1998; Fan et al. 2015), intraspecies strain relationships (Baura et al. 1992), and study of phylogeny analysis and hybrid origin (Wang et al. 2007). Sequencing of nuclear ribosomal DNA of ITS1–5.8S rRNA-ITS2 regions is also used for species identification in fungi (Chen et al. 2001; Park et al. 2004; Tuchwell et al. 2005; Zhao et al. 2012). It has been reported that the ITS region showed maximum sequence divergence than their

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flanking coding regions (Hillis and Dixon 1991), where they often used to distinguish between related fungal species (Mitchell and Bresinsky 1999; Calvo-Bado et al. 2000; Manian et al. 2001). The rRNA-ITS sequencing is used for the characterization of somatic hybrids in plant (Babiychuk et al. 1992; Lelivelt et al. 1993; Sakata and Monma 1993; Saito et al. 1994; Zanke et al. 1995) and in edible mushroom (Mallick and Sikdar 2015, 2016). *Lentinula edodes* mushroom strain identification was also reported based on sequence polymorphism of 5.8S rRNA gene (Kwan et al. 1991). Though the rRNA-ITS region is highly conserved, sequence variation was observed in different species level during taxonomic studies (Bruns et al. 1991; Hillis and Dixon 1991).

In our laboratory, we successfully developed 12 *pfls* somatic hybrids between mushroom strain *Pleurotus florida* and *Lentinus squarrosulus*, which were successfully characterized by mycelial morphology, fruit body yield, and RFLP pattern of rRNA-ITS region (Mallick and Sikdar 2015). However, in this communication we will report about the hybrid genetic diversity through PCR-based ISSR and sequencing of rRNA-ITS region of *pfls* hybrids and parents.

Materials and methods

Strains and culture conditions

The strain of *P. florida* was obtained from 'National Research Centre for Mushroom' Solan, Himachal Pradesh, India. The *L. squarrosulus* fruit body was collected from dead wood stock at Falta Experimental Farm, Bose Institute, 24 Parganas (South), West Bengal, India and its vegetative culture was prepared in the laboratory. Twelve *pfls* somatic hybrids were developed in our laboratory through PEG-mediated protoplast fusion between these two parental strains (Mallick and Sikdar 2015). All the cultures were maintained in PDA (potato–dextrose–agar, pH 6.2) medium at 24 °C. For DNA extraction, cultures were grown in liquid MYG (10 g/l malt, 4 g/l yeast, and 10 g/l glucose, pH 6.2) medium for 14 days (*P. florida* and *pfls* hybrids) and 10 days (*L. squarrosulus*) at 24 °C. Fully grown mycelial mats were washed with sterile water and freeze-dried for next step.

DNA extraction, PCR, and gel run

Freeze-dried mycelial tissue was used for genomic DNA extraction using modified CTAB method (Dellaporta et al. 1983) and purified DNA was used for ISSR and rRNA-ITS amplification. The ISSR reaction was carried out with slight modification of the protocol of Bornet and Branchard (2001)

and previously described by Mallick and Sikdar (2014). The ITS1-5.8S-ITS2 region was amplified by primer ITS1 (forward 5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (reverse 5' TCCTCCGCTTATTGATATGC 3') by White et al. (1990). The PCR amplification was done in an automated thermo-cycler (Applied Biosystems) by following the protocol of Mallick and Sikdar (2016).

Amplified ISSR and rRNA-ITS products were run on 1.8 and 1.5% agarose gel pre-stained with ethidium bromide solution using 1× TAE buffer, respectively. The gels were run at 80 V for 3 h for each. The banding profiles were visualized under UV transilluminator and data were recorded in a Molecular Analyst Gel Documentation System (Bio-rad, Hercules, CA). DNA Ruler Plus (100–3000 bp) ladder (MBI, Fermentas) was used as a standard molecular weight marker.

PIC value and statistical analysis

Polymorphic information content (PIC) is used to identify and refer the relative value of each marker with respect to the amount of polymorphism exhibited in a population. Here, PIC value is calculated according to Weir (1996) equation $[PIC = \{1 - (\sum P_i^2)\}]$, where 'i' is the total number of alleles detected for each ISSR marker, and 'Pi' is the frequency of ith plus allele in the *pfls* hybrid genotype. The ISSR products were scored as present (1) or absent (0) for each primer genotype combination. The bivariate '0–1' data were analyzed into a binomial matrix using the SPSS software (IBM, version.19). Based on Jaccard's proximity matrix (Jaccard 1901) and unweighted pair group method with mathematic average (UPGMA) algorithm, a dendrogram was generated, where hybrids and parents were grouped by genetic distances.

Sequence alignment and clustering

The rRNA-ITS PCR products were purified by sodium acetate precipitation followed by ethanol wash. The purified fragments were sequenced in our institutional core facility using Big Dye Terminator 3.1 method and nucleotide bases were read in an automated sequencer system (Applied Biosystems). Sequenced data were submitted to NCBI GenBank and aligned in CLUSTALW software (Thompson et al. 1994).

Results

Molecular genetic analysis by ISSR

The five ISSR primers showed clear and reproducible banding pattern in all the strains. The details of used ISSR

primers are shown in Table 1. A total of 54 unambiguous and reproducible amplicons were scored for all the samples against all primers. The DNA banding profiles generated by primer ISSR-02 and ISSR-03 are shown in Fig. 1a, b. The five ISSR primers amplified the fragments with 98.14% polymorphism across the 14 strains studied and the size ranged from 250 to 3000 bp. Primer ISSR-3 [(CAA)₅] and ISSR-11 [(CAC)₃GC] produced maximum bands (13) ranged from 520 to 3000 bp and 300 to 2600 bp with PIC value 0.491 and 0.498, respectively. The PIC value of five ISSR primers was ranged from 0.433 (ISSR-12) to 0.498 (ISSR-11) with a mean of 0.478.

Jaccard's proximity matrix showed the genetic distances between the *pfls* hybrids and parents, and the dissimilarity coefficient ranged from 0.00 to 5.916 (Table 2). The most identical relationship was found between hybrid *pfls1i*–*pfls1l* (0.00) and distant relationship between parent *L. squarrosulus*–hybrid *pfls1n* (5.916). Among the hybrid strains, maximum genetic distances were found in between hybrid *pfls1e*–*pfls1p* and hybrid *pfls1h*–*pfls1n* (5.477). Another parent *P. florida* showed minimum genetic distance to hybrid *pfls1m* (3.742). UPGMA-based dendrogram produced three major clusters in which hybrids and parents were grouped between each other (Fig. 2). Hybrid *pfls1l*, *pfls1i*, and *pfls1k* were grouped in cluster-I, hybrid *pfls1q*, *pfls1s*, and *pfls1e* were grouped in cluster-II, and hybrid *pfls1p*, *pfls1n*, and *pfls1j* were grouped in cluster-III. No parental strains were directly grouped with any of the *pfls* hybrids. Parent *P. florida* showed closeness to hybrid *pfls1m* and another parent *L. squarrosulus* showed closeness to hybrid *pfls1h* in this dendrogram.

The rRNA-ITS sequence polymorphism

The primers ITS1 and ITS4 amplified the ITS1-5.8S rRNA-ITS2 region with a short portion of the small and large ribosomal subunits. PCR product showed a single band in gel in different sizes and the sequenced data ranged from 611 bp in hybrid *pfls1o* to 867 bp in hybrid *pfls1s*, while the parents *P. florida* and *L. Squarrosulus* were 672 and 668 bp, respectively. The submitted NCBI GenBank

Fig. 1 ISSR profiles of *pfls* hybrids and parents. **a** Primer ISSR-02 **b** Primer ISSR-03. Lanes: *M*—marker, 100 bp DNA ruler plus., *C*—control, Lane 1—*L. squarrosulus*, Lane 2—*pfls1q*, Lane 3—*pfls1e*, Lane 4—*pfls1g*, Lane 5—*pfls1p*, Lane 6—*pfls1h*, Lane 7—*pfls1n*, Lane 8—*pfls1s*, Lane 9—*pfls1m*, Lane 10—*pfls1k*, Lane 11—*pfls1o*, Lane 12—*pfls1i*, Lane 13—*pfls1j* and Lane 14—*P. florida*. Left arrows depict bands from parent *L. squarrosulus* and right arrows from *P. florida*

accession numbers are: KF150316 (*L. squarrosulus*), KF150319 (*pfls1q*), KF150329 (*pfls1e*), KF150328 (*pfls1g*), KF150320 (*pfls1p*), KF150327 (*pfls1h*), KF150322 (*pfls1n*), KF150318 (*pfls1s*), KF150323 (*pfls1m*), KF150324 (*pfls1k*), KF150321 (*pfls1o*), KF150326 (*pfls1i*), KF150325 (*pfls1j*), and KF150306 (*P. florida*).

Maximum sequence polymorphism is found in the ITS1 and ITS2 regions of *pfls* hybrids, where two parental strains showed huge nucleotide variation between each other. Hybrid *pfls1h*, *pfls1p*, *pfls1o*, and *pfls1e* showed maximum sequence variation compared to other hybrids in the whole ITS1-5.8S-ITS2 region. Hybrids *pfls1e* and *pfls1p*, and *pfls1o* showed maximum sequence similarity with

Table 1 Details of ISSR primers used in this study

Primer	Sequence (5'-3') [repeat motif]	No. of scored bands	Size range (approx in bp)	PIC value
ISSR-01	GACAGACAGACAGACA, [(GACA) ₄]	9	800–3000	0.493
ISSR-02	CAGCAGCAGCAGCAG, [(CAG) ₅]	12	250–2800	0.474
ISSR-03	CAACAACAACAACAA, [(CAA) ₅]	13	520–3000	0.491
ISSR-11	CACCACCACGC, [(CAC) ₃ GC]	13	300–2600	0.498
ISSR-12	GAGGAGGAGGC, [(GAG) ₃ GC]	7	500–1700	0.433

Table 2 Proximity matrix generated from five ISSR profiles of *pfls* hybrids and parents

	<i>L. squarrosulus</i>	<i>Pfls1q</i>	<i>Pfls1e</i>	<i>Pfls1g</i>	<i>Pfls1p</i>	<i>Pfls1h</i>	<i>pfls1n</i>	<i>Pfls1s</i>	<i>Pfls1m</i>	<i>Pfls1k</i>	<i>Pfls1l</i>	<i>Pfls1i</i>	<i>Pfls1j</i>	<i>P. florida</i>
<i>L. squarrosulus</i>	0.000													
<i>pfls1q</i>	5.000	0.000												
<i>pfls1e</i>	4.000	3.000	0.000											
<i>pfls1g</i>	4.583	2.449	2.236	0.000										
<i>pfls1p</i>	5.477	5.000	5.477	5.000	0.000									
<i>pfls1h</i>	3.000	4.243	3.606	3.742	5.196	0.000								
<i>pfls1n</i>	5.916	4.243	5.000	4.472	3.317	5.477	0.000							
<i>pfls1s</i>	5.099	2.236	3.162	2.646	4.899	4.123	4.359	0.000						
<i>pfls1m</i>	5.831	5.000	5.099	4.796	4.243	5.385	3.606	4.899	.000					
<i>pfls1k</i>	5.385	3.464	3.873	3.162	4.796	4.472	4.243	3.317	4.796	0.000				
<i>pfls1l</i>	5.292	3.317	3.742	3.000	4.899	4.359	4.359	3.162	4.899	1.000	.000			
<i>pfls1i</i>	5.292	3.317	3.742	3.000	4.899	4.359	4.359	3.162	4.899	1.000	0.000	0.000		
<i>pfls1j</i>	5.568	5.099	5.196	4.899	3.606	5.292	3.742	4.796	4.123	4.472	4.583	4.583	0.000	
<i>P. florida</i>	4.690	4.583	4.243	4.123	4.000	4.583	4.123	4.690	3.742	4.123	4.243	4.243	4.123	0.000

Fig. 2 Dendrogram showing the relationship amongst the *pfls* somatic hybrid lines and their parents using genetic divergence obtained by ISSR primers and UPGMA method of clustering of Euclidean distance

parents *P. florida* and *L. squarrosulus*, respectively. However, in the most conserved 5.8S region, sequence dissimilarities in between each hybrid were also seen (Fig. 3). The nucleotide polymorphism is found from single base 'A/T/G/C' (*pfls 1i*, *pfls 1k*, *pfls 1n*, *pfls 1p*, *pfls 1g*, and *pfls 1h*) to four bases 'GTTC' (only in *pfls 1k*). The polymorphic base(s) matched with neither any hybrid nor any parent except few single. Hybrids *pfls 1p* and *pfls 1e* showed maximum similarity with parents *L. squarrosulus* and *P. florida*, respectively. Throughout the entire sequence of *pfls* hybrids, few bases are found at different position which did not match with any of the parental sequence. The inserted new bases may be considered as recombinant bases occurred during random recombination

Fig. 3 Multiple sequence alignment of 5.8S rRNA gene of *pfls* hybrids and their parents. Black box shaded regions showing similarities. Gaps are denoted by dashes

of two parental nuclei or it might happen due to intra-genomic alteration in these particular hybrids.

Cluster analysis of *pfls* hybrids with their parents based on rRNA-ITS sequence

All the hybrids showed maximum sequence similarity with parent *L. squarrosulus* than *P. florida*. The rooted tree is developed using UPGMA clustering method based on the whole ITS1-5.8S rRNA-ITS2 sequence of all *pfls* hybrids and parents (Fig. 4). Three distinct groups were developed between *L. squarrosulus* and *pfls 1p*, *pfls 1m*, and *pfls 1s*,

Fig. 4 The rRNA-ITS sequence-based dendrogram generated by UPGMA method of clustering of *pfls* hybrids and parents

pfls 1e and *P. florida*. Rest of the *pfls* hybrids did not group directly with any of the parents or hybrids. They remain in the out-group or intra-cluster region.

Discussion

In the present study, genetic recombination between the two parental genera was successfully established by ISSR and rRNA-ITS sequence analysis. Recombination between the two parental genomes during fusion is random and there are no such techniques particularly in this event which can detect the exact mechanism. In hybrid genome, the independent genetic materials from both the nuclei are constructed by recombination, deletion or intra-genomic rearrangement. Our study revealed that the *pfls* hybrids are recombinant of the two parents, *L. squarrosulus* and *P. florida*.

The five ISSR profiles showed that the banding patterns of the *pfls* somatic hybrids neither represent the sum of parental bands nor resemble each other among the hybrid population. This type of phenomenon strongly proved that all the *pfls* hybrid strains were not heterokaryons of the parental nuclei rather after protoplast fusion between the two different mushroom genera, synkaryon was formed which was followed by chromosome elimination or deletion and somatic recombination. Similar observations were reported by previous works (Primrose 1987; Chakraborty and Sikdar 2008; Mallick and Sikdar 2014). Excluding the common band, there were little recombinant type of bands present in the gel which did not match with any of the parents and this strongly proved the hybridity due to recombination. However, the genetic distances between each *pfls* hybrid with parents also showed the nature of polymorphism generated by ISSR markers with maximum PIC values. The results proved that the ISSR is one of the reproducible molecular markers for hybrid characterization and analysis of genetic distance.

The ITS1-5.8S-ITS2 region is one of the most popular loci used in molecular phylogenetic analysis (Alvarez and

Wendel 2003). Previous studies on fungal rRNA-ITS region in both molecular systematics and ecological aspects resulted in the deposition of about two hundred thousand Sanger-derived sequences in the international nucleotide sequence database (Karsch-Mizrachi et al. 2012). However, RFLP and sequence analysis of rRNA-ITS region had been carried out earlier in mushroom somatic hybrids (Mallick and Sikdar 2016). In the natural hybrid of *Flammulina* fungi, gene recombination or gene conversion in the rRNA-ITS region was reported by Hughes and Petersen (2001). In the present work, the nature of polymorphism in the rRNA-ITS region of *pfls* somatic hybrids was identified and it might have happened due to the occurrence of non-functional genes present in the progeny population. However, other possibilities are the divergence of rRNA-ITS region which may contain pseudogene in the hybrid genome or the existence of inter-genetic recombination between different repeat types with complex DNA organization (Ko and Jung 2002).

Polymorphism in the ITS1-5.8S-ITS2 region was expressed in both the *pfls* hybrid and parents. The most conserved region 5.8S rRNA also showed sequence dissimilarity. Parent *L. squarrosulus* showed maximum sequence similarity with the *pfls* hybrids than *P. florida* parent. In spite of sequence polymorphism, sequence similarity in ITS region was also observed in between hybrids and between hybrids and parents. This result largely collaborates with earlier observations that fungal communities show structural similarity in the ITS1 and ITS2 regions (Jumpponen 2004; Arfi et al. 2011; Mello et al. 2011). Huge polymorphism in ITS2 region compared to ITS1 may be explained by the fact that after hybridization the hybrids retained or inherited the sequence of one of the two parents without or with little recombination in the ITS1 site. Whereas in the ITS2 region of the *pfls* somatic hybrids, the nucleotide sequence was combined to different degrees from the two parental strains. The advantage of the ITS locus in this study of hybrid characterization is its occurrence in the genome as tandem repeat arrays, most likely on several chromosomes, and is sensitive to concerted evolution over time, which bears the signature of both the parents that will emerge either through mitotic crossover or through meiotic recombination (Bertier et al. 2013).

From the present study, it has been proved that the *pfls* somatic hybrids are not genetically identical, though they show morphological similarity between each other. The results indicate that the *pfls* hybrids were truly formed by random recombination of parental genome and they individually showed similarity either with *P. florida* or with *L. squarrosulus*. Somatic hybridization through protoplast fusion generates recombinant type of genome in hybrids which comes from both the parents. In higher eukaryotes,

hybrids can be easily characterized through karyotyping, whereas in fungal system it is very tough due to the lack of sufficient chromosomal information. However, in this situation DNA markers are helpful to study about the genetic makeup, nature of genome recombination, etc., in fungal somatic hybrids. Among all other DNA markers, only ISSR and rRNA-ITS are used in this study. ISSR is widely used repeated sequences of few nucleotide bases, which can work over the whole genome during polymerase chain reaction, and thus produces maximum number of amplicon (see “[Introduction](#)”). The nuclear rRNA-ITS gene is known to be highly conserved and hence can be used to detect the polymorphism in the hybrid genome if present so that it can claim in strong support to hybridity. Use of rRNA-ITS genes as DNA marker in plant or animal system is common, but characterization of fungal somatic hybrid with this marker was first established in our laboratory, where we successfully showed its application in this field (Mallick and Sikdar 2016). So, we can conclude that these DNA markers are very useful tools for genetic analysis of somatic hybrids in fungal system.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

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