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DNA PEDIGREE TRACKING TO IDENTIFY COMPATIBLE MATING PARTNERS OF *PLEUROTUS PULMONARIUS*

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ABSTRACT

The problems in precise discrimination of monokaryotic isolates of edible fungi hindered the breeders to enhance the improvement cycle of mushroom strains in an effective manner. In current study, 5′ end of intergenic spacer 2 (IGS2) region was amplified and used to assist categorizing the selected monokaryotic isolates of *Pleurotus pulmonarius* into two distinct groups (type A and B). Moreover, the dikaryotic isolates were able to produce double-banded PCR product and the monokaryons create only one unique amplicon. Candidate region approach was used in this study and enabled pedigree history to be recorded, and thus breeders are able to make informative decisions. Hence, this approach promotes time saving and breeding space to be allocated for strain improvement programmes. Meanwhile, this highly variable DNA marker can be used for the confirmation of successfully crossed strains. Eventually, this methodology can assist towards detecting efficient mating partners of *P. pulmonarius* in any breeding programmes including selective breeding, production of high yield hybrids and studies on structure mating types.

Keywords: Mating type, monokaryon, dikaryon, molecular marker, oyster mushroom.

INTRODUCTION

Pleurotus genera (Pleurotaceae, Agaricales, Basidiomycetes), also known as oyster mushrooms, are world-wide distributed macro fungi that comprise various kinds of highly priced edible mushrooms (Wang *et al.*, 2008). Among the various species of this genus, *P. pulmonarius* (Fries) Quélet has gained the most popularity due to its numerous properties and advantages, especially in Malaysia (Choi *et al.*, 2007; Ro *et al.*, 2007).

Domestication and commercial cultivation of *P. pulmonarius* and other mushroom species will face loss of genetic diversity and inbreeding effects that force farmers to introduce new varieties in their farms. Therefore, genetically improved varieties of the cultivated mushrooms can increase the quality of marketing, reduce the costs, and increase the farmers' revenue (Avin *et al.*, 2012). To generate the new high performance varieties, a range of breeding methods have hitherto been established, such as domestication of new wild resources (Raeesi *et al.*, 2013), programmed mutations (Lin Zhi *et al.*, 2013) and cross-breeding (Adebayo *et al.*, 2013). However, as far as mushrooms are concerned, cross-breeding and generation of improved strains have always been more promising and purposeful, and gained a great deal of success in the last few years (Fan *et al.*, 2006).

The life cycle of *P. pulmonarius*, in addition to those of many other higher Basidiomycetes, involves monokaryotic (n) and dikaryotic (n+n) phases. It is possible to cross two compatible and genetically distinctive monokaryotic hyphae (plasmogamy) and give rise to a fertile dikaryotic mycelia (Larraya *et al.*, 1999). In fact, a fertile dikaryotic hyphae is generated only when two heteroallelic basidiospore at both mating type loci (*AxBx, AyBy*) are crossed (Li *et al.*, 2012; Murata *et al.*, 1998).

The DNA pedigree tracking to identify compatible mating partners of *P. pulmonarius* is more essential and promising when a cross-breeding programme is being established. In fact, hybridisation of randomly selected spores can be time consuming and costly. Hence, identification of the spores' mating type prior to establishment of a mushroom cross-breeding programme will help breeders to detect desirable crosses in a shorter period.

Another significance of DNA pedigree tracking is to identify monokaryons and dikaryons in a short period. The latter identification has been performed through the fruiting trial (Nazrul & Yin Bing, 2011), estimating the vegetative growth rate (Song *et al.*, 2000), the presence or absence of clamp connections (Skrede *et al.*, 2013), and the mycelia morphology (Kavousi *et al.*, 2008) all of which are unreliable and time consuming.

The previous and recent studies on *P. pulmonarius* were mostly concentrated on composition of nutritional values (Khan & Tania, 2012; Smiderle *et al.*,

2012) and medicinal properties (Atri *et al.*, 2013; Patel *et al.*, 2012), mushroom cultivation techniques (Abdullah *et al.*, 2013) and strain improvement programmes using conventional hybridisation (Adebayo *et al.*, 2013). However, in spite of being prerequisite for establishment of strain improvement projects, little studies concentrated on identification of mono- and dikaryons of mushrooms. Therefore, there is a need for mycologists to create a simple molecular tool to detect compatible mating spores.

Identification of compatible mating partners using DNA markers results in a reliable and effective tool that can be implemented at any stage of a breeding programme. A range of molecular markers, including ISSR (Nazrul & Yin Bing, 2010), RAPD (Kavousi *et al.*, 2008; Larraya *et al.*, 2001), isozyme analysis (Khush *et al.*, 1995), and RFLP (Li *et al.*, 2012) have been employed to identify mushroom monokaryon isolates. These investigations have assisted in mushroom mating type identification. However, there is no study established to track DNA pedigree of mono- and dikaryons of *P. pulmonarius*.

The IGS regions are the two non-coding and highly variable DNA units that are located between conserved sequences of 25S, 5S and 18S within the nuclear rRNA gene (Babasaki *et al.*, 2007). The ability and superiority of IGS regions as a highly variable marker for detecting compatible mating spores of mushroom species have been demonstrated in *Ferula sinkiangensis* (Zhang *et al.*, 2006), *Schizophyllum commune* (James *et al.*, 2001), *Hebeloma cylindrosporum* (Guidot *et al.*, 1999) and *Pleurotus cornucopiae* (Iracabal & Labarere, 1994). Hence, the present study attempts to: (i) establish a hybridisation programme among selected single spore isolates of *P. pulmonarius*; (ii) investigate the potential of the 5′ end of IGS2 region to identify the compatible mating partners of *P. pulmonarius*; and (iii) compare the molecular based results to conventional mating compatibility and productivity tests.

MATERIALS AND METHODS

Fungal strains and preparation of dikaryotic cultures: The experimental strains of *P. pulmonarius* (PL27 and KUM61119) were obtained from the Fungal Biotechnology Laboratory, Institute of Biological Sciences, Faculty of Science, University of Malaya. Axenic dikaryon cultures were prepared by transferring a piece of tissue from the context of the fruit body to a potato dextrose agar (PDA) plate. The Petri dishes were incubated at 28±2˚C for 14 days.

Preparation of single spore isolates (SSIs): Fresh and mature pilei were selected and detached with a sterile knife (Bao *et al.*, 2004). A piece of tissue from spore bearing portion was attached, using Vaseline, to the inside cover of a sterile Petri dish containing potato dextrose agar (PDA) and 100 mg/l streptomycin sulphate. The tissues were then removed from the Petri dish when a spore print became barely visible on the agar surface. The Petri dishes were regularly checked for spore germination by using a dissecting microscope with sub-stage illumination (Kavousi *et al.*, 2008).

After the spores had germinated, a fine sterile needle was used to excise single germlings (Choi *et al.*, 1999) which were then planted on a fresh PDA plate (Gharehaghaji *et al.*, 2007). Single spore isolates were examined for the absence of clamp connections prior to the cross experiments to eliminate possible contamination with dikaryon (presence of clamp connection) isolates (Capelari & Fungaro, 2003; Miyazaki *et al.*, 2000).

Mating tests: Crosses between monokaryotic isolates were made by placing two mycelial plugs (Mahmud *et al.*, 2007), 35 mm apart on fresh PDA plates (Fig. 1, a). The pieces of monokaryotic mycelium were allowed to grow until the two mycelia met and formed a conspicuous contact zone (Gharehaghaji *et al.*, 2007; Miyazaki *et al.*, 2000). A strip of mycelium was picked up from the junction zone and transferred to a fresh PDA plate (Eichlerova & Homolka, 1999; Mahmud *et al.*, 2007) (Fig. 1, a & b). The mycelia in the Petri dishes were then allowed to grow at 27±2˚C for few days and examined by microscopic observation. Compatible (crossed) and incompatible mating isolates were decided by the presence and absence of clamp connections, respectively (Fig. 2).

Productivity examination: Productivity of the successfully crossed cultures was examined by cultivation of the new hybrid strains. The general formulation of making mushroom substrate was 89% of sawdust, 10% of rice bran, and 1% of $CaCO_3$. The mixture was moistened with distilled water till the moisture content reached around 70%. This moisture content was attained when no water oozed through the fingers and when a sample of the mixture was squeezed by hand; only some dampness could be felt. The moist mixture (1000 grams) was compacted into mushroom bags. After plugging of the bags with caps and cotton, the mushroom bags were sterilized at 121˚C for 30 minutes at 15 psi of pressure (Kavousi *et al.*, 2008). Finally, the sterilized bags were inoculated by approximately 2-3 scoops of colonized wheat grains (spawn).

DNA extraction: Mycelia were directly scraped from the surface of the pure and axenic PDA plate (Avin *et al.*, 2013). Briefly, sufficient SDS buffer (100mM Tris-HCL PH 8.0, 10mM EDTA, 2% SDS) was added to the samples and incubated for 30 minutes at 65˚C. After centrifugation at 13000×g for 5 minutes, the top layer was carefully extracted with phenol: Chloroform: Isoamyl alcohol (25:24:1). The DNA was then precipitated with isopropanol, and pelleted by

centrifuging at 13000×g for 15 minutes at 4˚C. Finally, the DNA pellets were dissolved in double sterilized H_2O and maintained at -20˚C for further analysis.

PCR amplification: The 5′ end of IGS2 of ribosomal DNA was amplified using a pair of previously reported primer 5SRNAR (5′- ACQ GCA TCC CGT CTG AT -3′) / IGS2R4 (5′- CAG TAC TAA CAG TCC TTG GTA -3′)(James *et al.*, 2001). The amplification reaction was in a volume of 25µl containing 0.25mM each of dNTPs, 5x green *Taq* buffer, 300ηM of each primer, 1.5mM of $MgCl_2$, 25ng of genomic DNA, and 2.0 units of Go*Taq*® Flexi DNA Polymerase (Promega). The thermal cycler was programmed to pre-denature the DNA at 94˚C for 5 minutes, followed by 35 cycles consisting of 1min denaturation at 94˚C, annealing at 57.9˚C, and extension at 72˚C; and a final extension at 72˚C for 10 minutes. The PCR products were analyzed by electrophoresis on 1.0% agarose gel and stained with ethidium bromide (EtBr), and were detected by Gel imaging system.

Purification of PCR product and phylogenetic analysis: The PCR products were directly cut from gel under UV light and purified using Nucleospin Extract II Kit (Chemopharm). The amplicons were sequenced in both directions by ABI-3730 XL Automated DNA Sequencer (Xu *et al.*, 2011). Chromas ver. 2.33 software (Technelysium Pty Ltd) was used to revise the chromatograms which were subsequently aligned by MEGA 4.0 software (Tamura *et al.*, 2007). The DNA sequences were used to perform the BLAST search at the National Centre for Biotechnology Information (NCBI) and deposited in the GenBank under accession No. from KC571204 to KC571219. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) phylogenetic tree was constructed and statistically tested again by bootstrap phylogeny analysis with 1000 replications (Tamura *et al.*, 2007). Haplotype data file, conserved regions and information of sites were calculated using DNAsp ver. 5.10.01 software (Librado & Rozas, 2009).

RESULTS

DNA pedigree tracking of SSIs: Amplification of 5′ end of IGS2 region revealed high variation in length. Two distinct amplicons detected by amplification of different monokaryotic cultures are shown in Fig. 3, a. Moreover, amplification of the above mentioned region produced double-banded products for hybrid and dikaryotic isolates (Fig. 3, b). However, an unexpected outcome was observed in lane AB what was a conventionally identified as a monokaryotic isolate, produced double-banded PCR product.

The length of sequences obtained in the present study consists of 5′ end of IGS2 region, varying from 1164bp for type A to 1301bp for type B. After excluding the sites with gaps, 23 variable and parsimony informative sites were found. Two conserved DNA regions were detected at region 1 (770-992) and region 2 (994-1301). A haplotype data file was also generated with only two haplotype groups being identified (type A and B).

According to the UPGMA tree, selected SSIs of strains PL27 and KUM61119 were categorized into two distinct clades (Fig. 4). In fact, the dendrogram could clearly separate two types of monokaryotic isolates. The phylogenetic tree was statistically tested again by bootstraping and it completely supported the clusters by presenting 100% value. Homology BLAST search analysis was performed at NCBI for the sequences and no significant similarity was found. What this signifies is that the sequences obtained in the current study had been deposited to the GenBank for the first time.

Mating tests: The information obtained from the DNA pedigree tacking of SSIs was employed to detect hypothetically efficient mating partners. Twelve SSIs from strain PL27 and thirteen from strain KUM61119 were obtained. Accordingly, 16 monokaryotic isolates (four SSIs from each mating type) were paired for all in all combinations for mating compatibility tests (Table 1). From the total number of crosses tested (112), only 10 pairs KA1xKB3, KA2xPB2, KA3xPB3, KA4xKB3, KB2xKB3, KB3xPB2 KB3x PB3, KB4xPA3, PA1xPB2 and PA3xPB1 (K refers to KUM61119 and P refers to PL27 strains) were compatible [distinguished by presence of clamp connections (Fig. 2)]. All compatible crosses were cultivated with 10 replications in a mushroom house to estimate major characteristics (yield, successfully fruited bags and growth rate). According to these results, the majority of the compatible crosses belonged to the combinations of mating types A and B (5.47% out of 7.81%). However, the crosses were totally incompatible (0% / 7.81%) when two similar mating types of A were crossed. Accordingly, a very low compatibility (2.34% / 7.81%) was recorded between the members of mating types B (Table 2).

Productivity examination: The productivity of the hybrids was examined by a fruiting trial. All successfully crossed strains were subjected to cultivation with 10 replications in a suitable mushroom house. The results obtained demonstrated that from the total number of bags inoculated, 62.5% successfully fruited when SSIs from different mating types of A and B were fused. The percentage was observed to be extremely lower (16.5%) when two mating partners from similar type (B) were crossed. Similar results were achieved for the mean yield of bags as well as the growth rate (Table 2).

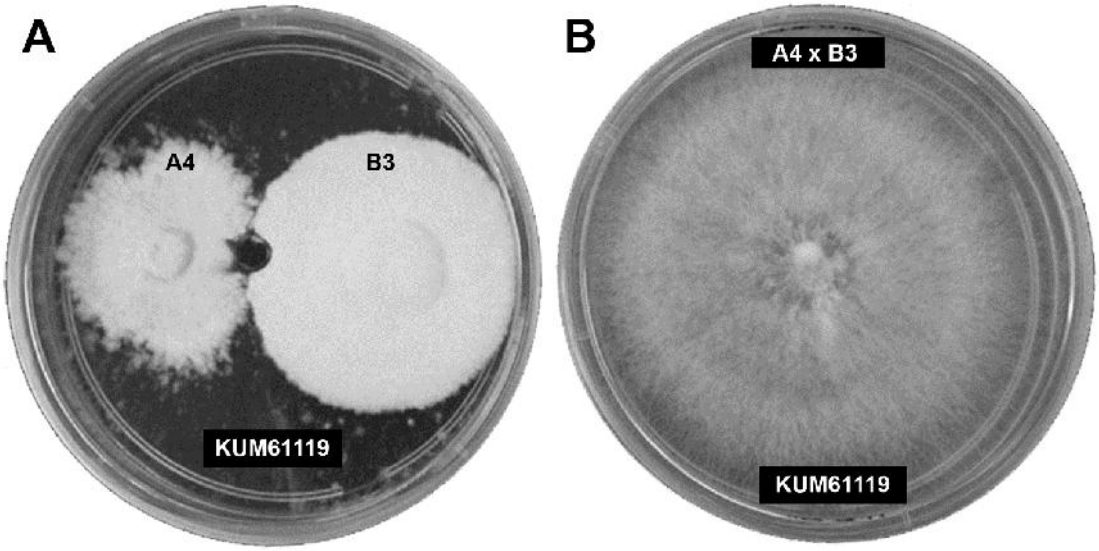

Fig. 1: Cross compatibility test. (A) A compatible mating between two selected monokaryotic isolates of A4 and B3 (obtained from KUM61119 strain). (B) Successfully crossed strain (dikaryotic).

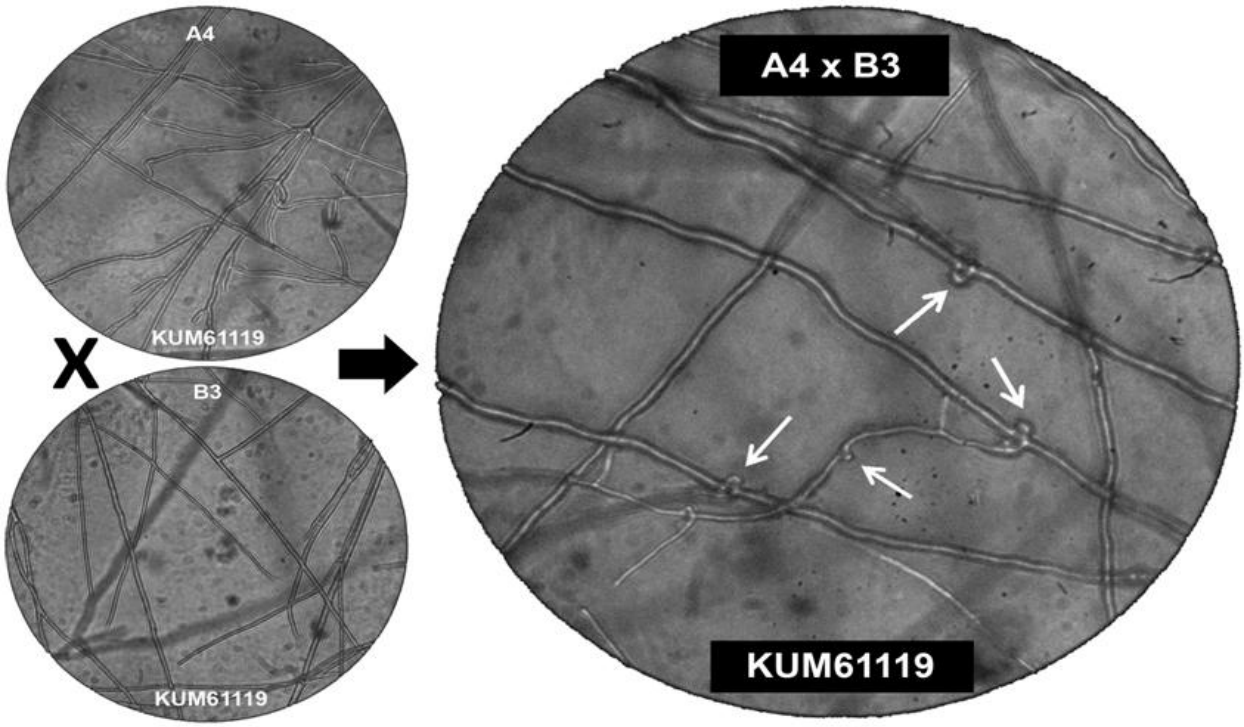

Fig. 2: Detection of compatibility by mating test. The crosses were scored as compatible if clamp connections observed.

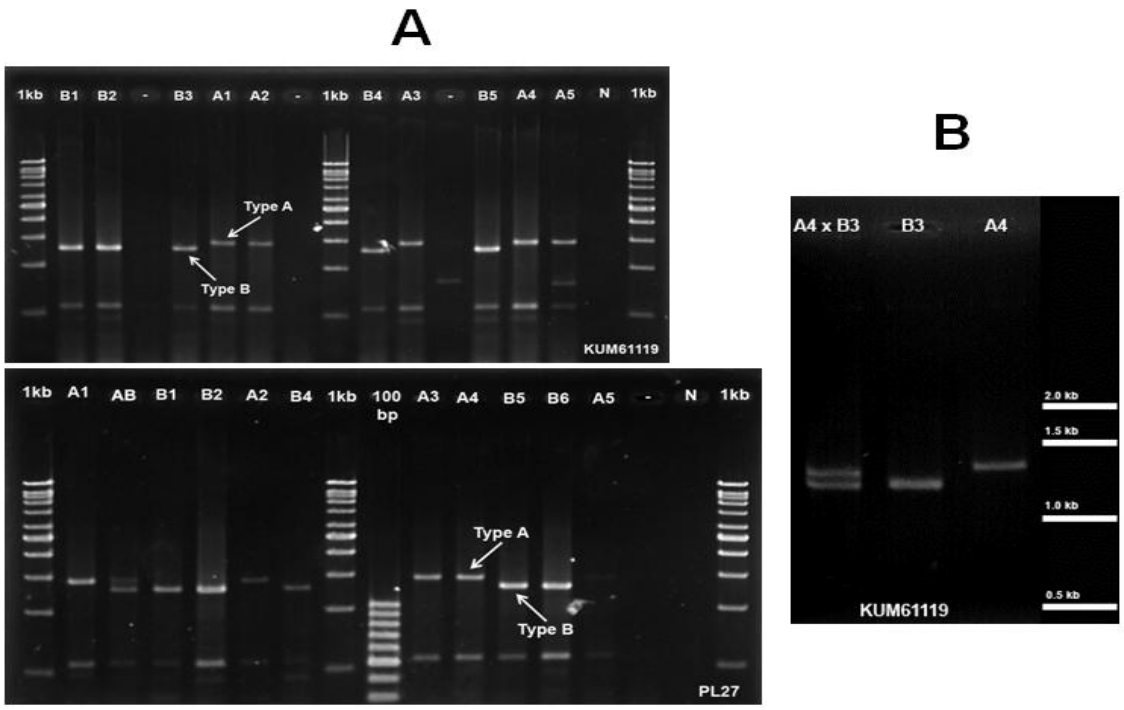

Fig. 3: Amplification of the 5′ end of IGS2 region of ribosomal rDNA on 1% agarose gel. (A) monokaryotic strains to have only a single band (two distinct amplicons are distinguishable that vary in length). Lane AB which was conventionally identified as monokaryotic isolate, but produced double-banded. (B) hybrid strain produced double-banded product.

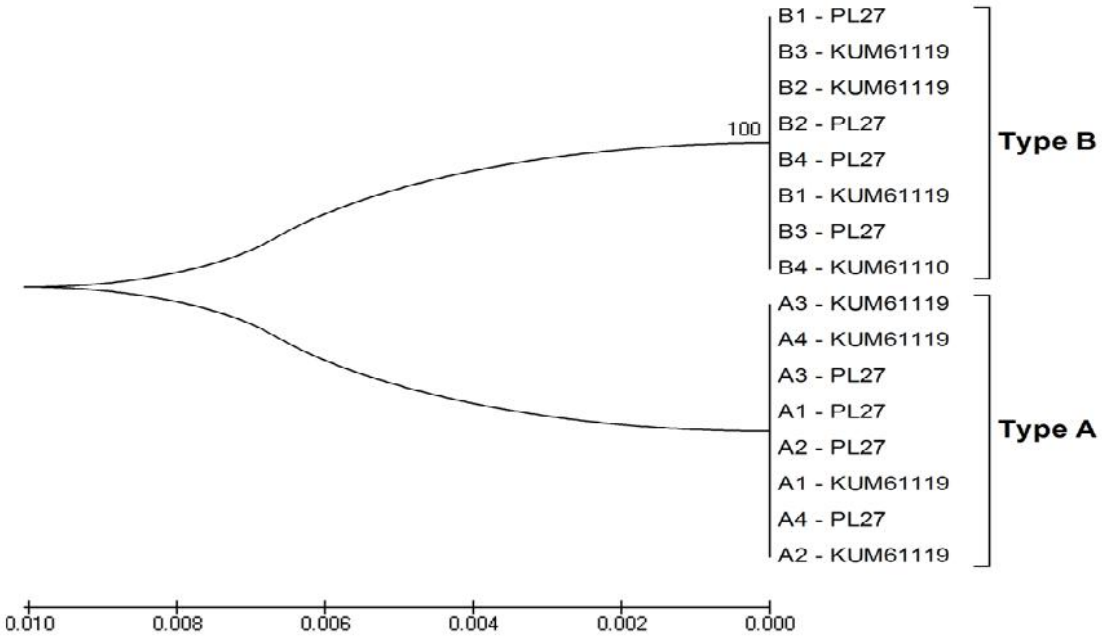

Fig. 4: Phylogenetic tree constructed based on the 5′ end of IGS2 region sequences of selected SSIs using UPGMA method. Number close to branches indicates 1000 replication of bootstrap test and the codes refer to sample ID.

Table 1: Mating compatibility test between selected SSIs
+ indicates a compatible cross (presence of clamp connections), - indicates an incompatible cross (absence of clamp connections)

			KUM61119								**PL27**							
			Type A				**Type B**				**Type A**				**Type B**			
			1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
KUM61119	Type A	1	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
		3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
		4	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	Type B	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
		3	+	-	-	+	-	+	-	-	-	-	+	-	-	+	+	-
		4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PL27	Type A	1	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
		2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		3	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-
		4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Type B	1	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
		2	-	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-
		3	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-
		4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2: The productivity examination of the obtained hybrids from different mating types

Crosses	**Compatibility percentage**	**Successfully Fruited Bags percentage**	**Yield grams**	**Growth Rate mm/day**
Type A x Type A	0%	0%	0	0
Type A x Type B	5.47%	62.5%	69.7g	3.55
Type B x Type B	2.34%	16.5%	46.3g	3.03
Total	7.81%	-	-	-

DISCUSSION

Tracking and identification of hyphal fusion and compatible monokaryon type are essential when breeding programmes are being employed such as development of new varieties as well as establishment of newly designated mushroom cross-breeding. The biggest concern would be the detection of mating partners that can be efficiently used prior to establishment of a mushroom hybridisation programme.

Moreover, identification of monokaryons and dikaryons has hitherto been carried out mainly through estimating the vegetative growth rate (Song *et al.*, 2000), the fruiting trial (Nazrul & Yin Bing, 2011), the presence or absence of clamp connections (Skrede *et al.*, 2013), and mycelia morphology (Kavousi *et al.*, 2008), all of which are unreliable and time consuming.

To date, many researchers have attempted to identify compatible and efficient mating partners of mushrooms. With the appearance of DNA based molecular methods, several molecular techniques including inter simple sequence repeat (ISSR), random amplified polymorphic DNA (RAPD) (Yan & Jiang, 2005), amplified fragment length polymorphism (AFLP) (Mahmud *et al.*, 2007), restriction fragment length polymorphism (RFLP) (Larraya *et al.*, 1999), and microsatellite markers (Larraya *et al.*, 1999) have been used to study mushroom mating type compatibility. The existing investigations have no doubt assisted in mushroom hybridisation as well as mating type identification. However, as the previously established methods required excessive work, there is need for mushroom breeders to initiate a simple and inexpensive method for identification of efficient mating partners.

In the present study, the potential of 5′ end of IGS2 region to identify the two mating types of *Pleurotus pulmonarius* was demonstrated by various analyses and examinations. The high variation among the sequences of IGS had previously been reported by Zhang *et al.* (2006), Guidot *et al.* (1999), Iracabal and Labarere (1994), and James *et al.* (2001).

The amplification of 5′ end of IGS2 region could clearly identify two types of monokaryons (A & B) and was able to separate the dikaryotic cultures from monokaryotic which were wrongly isolated as

monokaryon. Moreover, the pedigree tracking of a successfully crossed strain of A4 x B3 from two compatible mating partners has been demonstrated. Similar methodology had been employed by James *et al.* (2001) to discriminate the mono- and dikaryotic strains of the widespread mushroom *Schizophyllum commune*. In another study, Kavousi *et al.* (2008) could demonstrate that the homo- and heterokaryotic isolates are distinguishable by RAPD markers.

The high degree of polymorphism of rDNA region is a result of variations in both length and restriction sites that affect the IGS1 and 2 and the 5.8S gene (Guidot *et al.*, 1999; Iracabal & Labarere, 1994). In this study, the phylogenetic tree and haplotype data file which were constructed based on the sequences of 5′ end of IGS2 region clearly detected two different mating types. According to Iracabal and Labarere (1994)'s study, each homokaryotic isolate possesses only one of the two rDNA parental unit and that each type rDNA unit is specific to each nuclear type.

The present study yielded an unexpected outcome in that numbers of SSIs from similar mating types (B) were found to be compatible. However, it was demonstrated that the percentage of compatibility as well as the total number of successfully fruited bags were very much higher if the two mating partners were picked from different mating types. Moreover, higher values in yield and growth rate of hybrids were recorded when two genetically different monospores were crossed. However, amplification of the 5′ end of IGS2 could only partially assist towards tracking of the monokaryons of the evaluated mushroom species. Hence, development of newly designed primers that can easily work on other species and also disclose higher variability is recommended.

In conclusion, this study clearly demonstrated the potential of 5′ end of IGS2 region for identification of different mating types of SSIs. A range of examinations and analyses that were carried out in this study such as mating compatibility and productivity tests, phylogenetic tree, haplotyping and sites information provided useful data for establishment of a mushroom breeding programme. Another distinct outcome of the present study is a method for discrimination of mono- and dikaryotic strains of *P. pulmorarius* which cannot always be distinguished precisely by conventional methods. Moreover, this highly variable molecular marker can be utilized for the confirmation of successful crosses. To sum up, this methodology can be of assistance in detecting efficient mating partners of *P. pulmonarius* in breeding programmes including selective breeding, production of high yield hybrids and studies on structure mating types.

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