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Intergeneric hybridization between *Pleurotus ostreatus* and *Schizophyllum commune* by PEG-induced protoplast fusion

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Intergeneric hybridization between *Pleurotus ostreatus* and *Schizophyllum commune* was studied using PEG-induced fusion. The fusion of protoplasts from auxotrophic mutant strains resulted in the formation of fusion hybrids in the frequencies of 3.6 to 7.3 $\times$ 10^{-5}. Most of these fusion hybrids were monokaryotic and sterile and no heterokaryosis occurred. Most fusants showed a significantly higher nuclear DNA content when compared to parental strains and no diploids (parent 1 genome plus parent 2 genome) were found. Some fusion hybrids revealed both parental fragments in nuclear and mitochondrial rDNA PCR profiles. AP-PCR (Arbitrarily-primed Polymerase Chain Reaction) fingerprints also indicated that most of the fusion products were recombinant hybrids.

Key words: Hybridization, PCR, *Pleurotus ostreatus*, protoplast fusion, *Schizophyllum commune*.

Crossing between selected compatible strains is a routine technique in mushroom breeding. With reference to its world-wide annual yield, *Pleurotus* is the second most important cultivated mushroom and strain improvement in this species would result in huge economic profits. *Schizophyllum commune* has been extensively studied and its genetic background is well known (Wessels 1993). Furthermore, Schizophyllan, a water-soluble polysaccharide which is marketed as a new anticancer medicine in Japan, is produced by this fungus. However, crossing between selected strains can only be done between individuals of the same species. In contrast, protoplast fusion can bypass the somatic incompatibility and may produce some desirable strains. When compared to lower fungi, however, protoplast fusion in higher fungi has not been very successful (Peberdy 1991). Though a few papers have been published in this field, many questions remain unanswered. One of these questions is whether heterokaryosis occurs after protoplast fusion in higher fungi. A few researchers claimed that they obtained some heterokaryotic fusants between various basidiomycetes species. However, complete identification of these fusants was not achieved (Yoo & Cha 1993). To resolve this controversial question, intergeneric fusion between *Pleurotus ostreatus* and *Schizophyllum commune* was carried out.

In this study, we fused isolates of *P. ostreatus* and *S. commune* in an attempt to study heterokaryosis of intergeneric fusants. After fusion, unambiguous identification methods, for example, the protoplasting technique (Zhao & Chang 1993), cytophotometric measurement of nuclear DNA content, polymerase chain reaction (PCR) and Arbitrarily-primed Polymerase Chain Reaction (AP-PCR) were used to distinguish fusion hybrids from their parents.

Materials and Methods

Strains and Media

The *P. ostreatus* auxotrophic mutant Po67 (ade$^-$, mating type: A_1B_1) was used as one of parental strains. The *S. commune* auxotrophic mutants Sc1 (pro$^-$, $A_{42}B_{42}$) or Sc3 (arg$^-$, $A_{30}B_{30}$) were used as another parental strain. Heterotrophic stains of *P. ostreatus* Po17 (A_2B_2) and *S. commune* Sc2 ($A_{30}B_{30}$) were used as tester strains to determine the mating type of the fusants. Media used in the experiments were as previously described (Zhao & Chang 1993, 1995).

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PEG-Induced Protoplast Fusion
Protocols of protoplast isolation and regeneration were described previously (Zhao & Chang 1993). Protoplasts were fused using a procedure similar to that described by Stasz *et al.* (1988). Protoplasts from the same strains were also fused as controls. Fusion frequency was determined as number of regenerated colonies in regeneration minimal medium (RMM)/number of regenerated colonies in regeneration complete medium. Each plate was sealed with parafilm to prevent contamination by other fungal strains.

Analyses of Fusion Products
Hyphal tips of regenerated colonies developing on RMM were transferred to minimal medium (MM). This procedure excluded the possibility of a dual culture. Only those progeny that continued to grow on MM were considered to be fusion hybrids. Mating types of potential fusants were determined by mating them with their monokaryotic tester strains. Clamp connections and nuclei number per cell were observed under a fluorescence microscope. A protoplasting technique was used to detect the occurrence of heterokaryosis (Zhao & Chang 1993).

Determination of Nuclear DNA Contents
Hyphal nuclei in all fusion hybrids and their parents were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) using the method described by Brensinsky *et al.* (1987). Spores of *S. commune* were used as internal standards to overcome the variation of values. The unpaired T-test was carried out to compare nuclear DNA contents in hybrids and their parents.

Analyses of Fusants Using PCR and AP-PCR Methods
Ribosomal DNA (rDNA) repeat units contain highly conserved DNA sequence and have been used to determine genetic variation between related fungal species (Kwan *et al.* 1992). Amplification of internal transcribed spacer (ITS) regions and mitochondrial-rDNA (mt-rDNA) regions has been used to produce specific DNA fragments for distinguishing different fungal species. In contrast, AP-PCR is a simple, fast and efficient method for detecting polymorphisms without time-consuming hybridization and cloning (Welsh & McClelland 1990). Therefore, both methods were used to characterize and confirm hybridization. Genomic DNA from fungi was isolated using the CsCl method (Yoon *et al.* 1991). Amplification of the ITS regions and mt-rDNA regions was done as described as Kwan *et al.* (1992). Six primers for amplification of fungal rDNA genes were ITS2, ITS3, ITS4, ITS5, MS1 and MS2. Four arbitrary primers were used in AP-PCR to distinguish between fusion hybrids and their parents (Welsh & McClelland 1990): Primer 1, M13 forward sequencing primer (5'-CGCCAGGGTTTTCCCAGTCACGAC); Primer 2, M13 reverse sequencing primer (5'-AGCGGATAACAATTTCACACAGGA); Primer 3, *Eco*RI-Ext primer (5'-TAGGCGTATCACGAGGCCCT); Primer 4, GalK primer (5'-TACGGTGGCGGAGCGCAGCA). Amplification products were analysed by electrophoresis in 2.0% agarose gels in 1 × TBE buffer and detected after staining with ethidium bromide.

Genomic Analysis Using Pulsed Field Gel Electrophoresis (PFGE)
Genomic DNA was compared between selected hybrids and their parents using PFGE (Witte *et al.* 1989). Protoplasts were suspended in SC buffer (1.0 M sorbitol, 10 mM Tris/HCl, pH 7.5) to give a concentration between 5×10^8 and 2×10^9 protoplast/ml. PFGE was carried out on a CHEF DRII system (Biorad) with 0.5 × TBE at 14°C, using 0.6% chromosomal grade agarose. Running conditions were set at 50 V with a pulse time of 1 hour for a running time of 166 hours.

Table 1. Genetic characteristics of fusion hybrids.

Fusant No.	Barrage with			Nuclei no. per cell	Mating type**	Nuclear DNA content	
	Po67	Sc1	CC*			No. of nuclei	Intensity Mean ± SD+
Fsp1	+	−	−	1	unknown	38	16 ± 3.5
Fsp2	+	−	−	1	$A_{42}B_{42}$	48	13.2 ± 4.2
Fsp3	−	−	−	2–10	unknown	46	19.1 ± 6.0
Fsp4	+	−	−	1	$A_{42}B_{42}$	72	18.9 ± 4.9
Fsp5	+	+	+	2	unknown	54	20.6 ± 3.5
Control							
Po67	−	+	−	1	A_1B_1	56	14.2 ± 6.3
Sc1	+	−	−	1	$A_{42}B_{42}$	50	12.3 ± 4.6

* Clamp connection. ** Mating types were determined by mating with tester strain (Po17 and Sc2). +—Standard deviation.

Fruiting Test
All hybrids were subjected to fruiting tests. The fruiting compost contained: 30% (w/v) sawdust, 2% (w/v) lime, 2% (w/v) cotton seed and 66% water. When mycelium colonized the substrate completely, the bags were opened to stimulate fruiting body formation. The temperature was then maintained at 25°C and relative humidity at 90%. If a strain did not form any primodia in all triplicate bags after 25 days, it was considered to be sterile.

Results

Selection of Fusion Products
The selection of fusants is based on the complementation of two auxotrophic mutants. After fusion, no fast growing colonies were found in the first two weeks. Some colonies appeared after 2–4 weeks. These colonies were subcultured several times in minimal medium. Only those colonies which maintained heterotrophic characteristics were considered potential fusion products. In the fusion between Po67 and Sc1, five potential fusants were obtained and the fusion frequencies were 3.6 to 7.3×10^{-5}. No hybrids were detected in the fusion between Po67 and Sc3. In control plates, no heterotrophic revertants were obtained in five repeats (data not shown). Fusants were designed with letters of Fsp and numbered according to the chronological order of production.

Analyses of Fusion Products
Large variations were found in all the fusants obtained. These variations included differences in morphology, auxotrophic markers, mating type, fertility, the presence or absence of clamp connections and number of nuclei per cell (Table 1). Monokaryotic strain Fsp1 grew fast in MM and

did not appear to form clamp connections. In the mating test, strain Fsp1 could mate with Sc1 and Sc2 and its mating type was, therefore, unknown. Monokaryotic Fsp2 was shown to be weakly prototrophic and easily lost prototrophy in complete medium. Its mating type was $A_{42}B_{42}$ and no clamp connections were found. Fsp3 was multinucleate without clamp connections and grew fast in MM. However, its mating type was unknown because of its inability to mate with Po17 and Sc2. The mating type of monokaryotic Fsp4 was $A_{42}B_{42}$ and it grew slowly in MM. After several transfers in complete medium, Fsp4 gradually lost its prototrophy and degenerated to the Sc1 phenotype (pro$^-$). In MM, Fsp4 prototrophic characteristics can be maintained even after 6 months of subculturing. Fsp5 was a dikaryon and possessed clamp connections. Its mating type could not be determined because of the failed mating reactions with Po17 and Sc2. In fruiting tests, no hybrids developed primodia even after 3 weeks of incubation.

Characterization of Fusants Using the Protoplasting Technique
All fusants were tested using a protoplasting technique (Table 2). After protoplast release and regeneration, between 50 and 80 colonies were chosen at random to check the auxotrophic markers and mating types. No segregants were detected in the regenerated progeny of Fsp1, Fsp2 and Fsp3. In 64 regenerated colonies of Fsp4, three maintained the heterotrophy of Fsp4. Others showed the same mating types and auxotrophic markers as Sc1 (pro$^-$). No *Pleurotus* segregants were recovered. Of 80 regenerated progeny of Fsp5, 42 were monokaryons. Though the morphology of the monokaryotic progeny resembled Sc1, the mating types of auxotrophic markers of Sc1 did not recover in the progeny. After 2–3 months subculture in complete medium, a few progeny segregated sectors. Most of the sectors recovered auxotrophic markers (pro $-$) and mating type of Sc1. However, no *Pleurotus*-like progeny were detected in protoplasted progeny and their sectors.

Analysis of Nuclear DNA Contents and PFGE
Fluorescence photometry was done to infer the ploidy level of selected fusants. The fluorescence intensities of nuclear DNA contents in Po67 and Sc1 were 14.2 and 12.3 respectively (Table 1). T-test results indicated that the DNA contents of Fsp3, Fsp4 and Fsp5 were significantly larger than both parents. Nuclear DNA content of Fsp1 was significantly larger than Sc1 but not than Po67. No significant difference was found between Fsp2 and both parents. PFGE results could not distinguish these hybrids from their parental strains (data not shown). A haploidization chemical, *p*-fluorophenylalanine, was also used to check the occurrence of diploid fusants and no diploid was found

Table 2. Protoplasting results of fusion hybrids.

Strain	No. of progeny	Parental type		Non-parental type
		Sc1 type	Po67 type	
Fsp1	50	0	0	50
Fsp2	50	50	0	0
Fsp3	50	0	0	50
Fsp4	64	61	0	3
Fsp5	80	0	0	80 (42)*
Control				
Po67	100	0	100	0
Sc1	100	100	0	0

* Monokaryotic colonies are listed in parentheses.

(data not shown). These results indicated that these fusants were not diploids.

Analyses of Fusion Hybrids by PCR and AP-PCR
Six primers were used to amplify the nuclear and mitochondrial rDNA genes (Figure 1A and B). Fragments amplified by primers Ms1 + MS2 were 0.66 kb for Po67 and 0.69 kb for Sc1 (Figure 1A). Fsp1 and Fsp2 showed the same fragment with Sc1. Fsp3 revealed a new size of fragment (0.59 kb), whereas Fsp4 had one fragment which was similar to Po67 (0.66 kb) but also showed two novel fragments (0.40 kb and 0.28 kb). One fragment of Fsp5 was the same size as Po67, but another fragment was new (0.82 kb). Amplified fragment sizes obtained using primers ITS 1 + 2 were 0.32 kb and 0.40 kb for Po67 and Sc1, respectively (Figure 1B). Fsp1 and Fsp2 showed the same fragment as Sc1. Fsp3 revealed a non-parental fragment (0.35 kb). Fsp4 and Fsp5 revealed a fragment similar to that of Po67 (0.32 kb). Using ITS 4 + 5, the hybrids showed a similar pattern of fragments to the parental strain Sc1 (data not shown). Four primers were used in AP-PCR experiments to distinguish hybrids (Figure 1C). The primers generated sets of products ranging from 0.20 to 1.30 kb. All the major fragments amplified were reproducible under identical conditions. Although the fusants shared fragments with the parental strains, large variations between fusants were detected.

Discussion

In intergeneric fusion between *P. ostreatus* and *S. commune*, fusion frequencies were very low (0.0036 to 0.0073%). Most fusion products appeared after 2–4 weeks. They were not a result of contamination, because wild-type strains could regenerate on RMM after 3 to 5 days. These fusion products were not back mutants, as no heterotrophic progeny were obtained in self-fusion controls. Based on the isolation method described, these fusion progeny were also not due to cross feeding or dual culture. From the results presented, we propose that these strains are the fusion

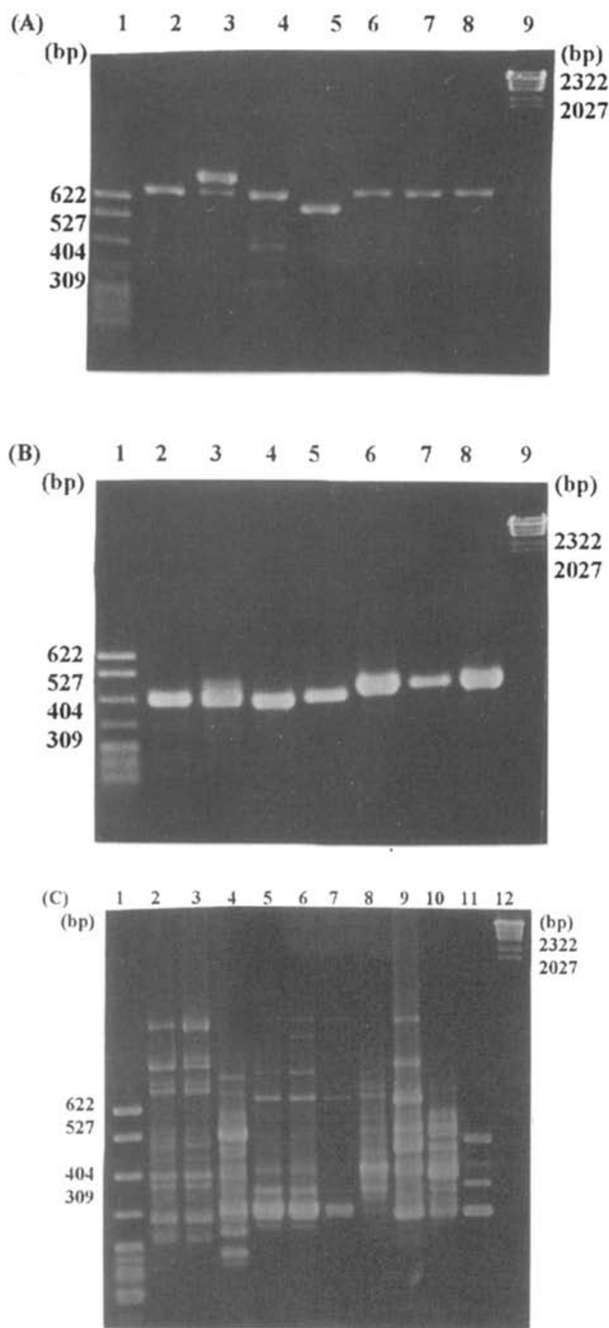

Figure 1. Analyses of fusion hybrids and their parents using rDNA-PCR and AP-PCR. (A) PCR amplification of mitochondrial rDNA using primers MS 1 + 2: Lane 1, pBR322 DNA-*Msp*I digest (Biolabs); Lane 2, Po67; Lane 3, Fsp5; Lane 4, Fsp4; Lane 5, Fsp3; Lane 6, Fsp2; Lane 7, Fsp1; Lane 8, Sc1; Lane 9, Lambda DNA-*Hind* III digest (Biolabs). (B) PCR amplification of nuclear rDNA using primers ITS 3 + 4: Lane 1, pBR322 DNA-*Msp*I digest (Biolabs); Lane 2, Po67; Lane 3, Fsp5; Lane 4, Fsp4; Lane 5, Fsp3; Lane 6, Fsp2; Lane 7, Fsp1; Lane 8, Sc1; Lane 9, Lambda DNA-*Hind* III digest (Biolabs). (C) AP-PCR fingerprint using primer 1. Lane 1, pBR322 DNA-*Msp*I digest; Lane 2, Po67 + Sc1; Lane 3, Po67; Lane 4, Fsp5; Lanes 5 to 7, Fsp4; Lane 8, Fsp3; Lane 9, Fsp2; Lane 10, Fsp1; Lane 11, Sc1; Lane 12, Lambda DNA-*Hind* III digest.

hybrids. Most of the fusants were monokaryotic, except for Fsp3 (multinuclear) and Fsp5 (binuclear). The protoplasting technique was carried out to recover parental types. However, only one type of parental strain was obtained from strains Fsp2, Fsp4 and Fsp5. Strains Fsp1 and Fsp3 did not reveal parental types in protoplasted progeny. Nuclear DNA contents were compared and no diploids (parent 1 genome plus parent 2 genome) were found. However, most fusion hybrids showed a significantly higher nuclear DNA content than the parental strain, which might be due to the occurrence of aneuploids or heteroploids. Some fusants revealed novel fragments in mt-rDNA profiles, which suggested that the recombination of mt-rDNA might have occurred after fusion. AP-PCR fingerprints indicated that many fusants shared some fragments with both parents, and some heterologous fragments were present in most of the fusion hybrids. The result is consistent with data presented by Durand *et al.* (1993) and indicates that new primer binding sites might have been created. Some of the *S. commune*-like hybrids did not show parental mating types. This unexpected result might be due to the insertion of heterogenous fragments into the A and B factor genes. Molecular analysis indicated that the mating type might be changed if a recombination or mutation occurred in the mating type genes (Wessels 1993).

Non-parental fusion products have been reported in many fungi. In intergeneric hybridization between *Yarrowia lipolytica* and *Kluyveromyces lactis* (Groves & Oliver 1984), the hybrids contained a double DNA content per cell. However, analytical CsCl density gradient centrifugation demonstrated that the nuclear DNA of the fusants was derived almost entirely from the *Y. lipolytica* parent. In intergeneric hybridization between *Saccharomyces cerevisiae* and *K. marxianus*, pulsed-field gel electrophoresis analysis indicated that most of the fusants showed the genome type of *K. marxianus* and no intact *S. cerevisiae* chromosome had been transmitted to the fusion products (Witte *et al.* 1989). Stasz & Harman (1990) reported non-parental progeny which resulted from protoplast fusion in *Trichoderma.* In interspecific fusion, fusion frequency was between 0.002% and 0.02%. Most fusants were weakly prototrophic and often segregated very strong heterotrophic sectors. Nearly all progeny were identical to one or the other parental isozyme phenotypes. Though many variations appeared in the morphology and nutritional requirement, no evidence of karyogamy was detected. Non-parental interspecific fusion products were also obtained in *Pleurotus* (Toyomasu *et al.* 1986; Go *et al.* 1989). The fusants from the compatible isolates produced normal fruiting bodies, while those from the incompatible isolates did not produce clamp connections and basidiocarps. These fusants were suggested to be heteroploids or aneuploids.

These reported results were consistent with our observations. Why were heterokaryotic fusion products not

detected in intergeneric fusion? Direct monitoring of the fusion process under a microscope revealed that the protoplast fusion yields were similar in intraspecific and intergeneric fusion experiments. About 20 to 40% of heterokaryons were produced from intraspecific fusion, and all of them were balanced dikaryons (Zhao & Chang 1995). Compared to intraspecific fusion, only 0.0036 to 0.0073% of fusion hybrids were produced in intergeneric fusion. Therefore, lack of detectable heterokaryosis in intergeneric fusion is not due to the failure of plasmogamy, but rather due to the failure of heterokaryosis to be maintained during subsequent growth (Stasz & Harman 1990).

If heterokaryosis does not explain the great variations of fusants obtained from intergeneric fusions, then other mechanisms of genetic variation must occur. One possibility is the occurrence of aneuploidy or heteroploidy. Our data on nuclear DNA contents partly supported this explanation. However, the formation of aneuploids or heteroploids require karyogamy. To verify this, a large number of fusion products that had undergone nuclear fusion would be necessary. Five fusants obtained in this study were, therefore, not sufficient to infer the occurrence of nuclear fusion. Another mechanism is nuclear-cytoplasmic interaction. Rearrangement of mitochondrial DNA after interspecific fusions were reported in many plant species (San *et al.* 1990; Temple *et al.* 1992; Xu *et al.* 1993). Our mt-rDNA PCR results strongly suggested that rearrangement of recombination had occurred in hybrids and these genetic variations led to the appearance of non-parental strains. Thirdly, a parasexual cycle suggested by Casselton (1965) was consistent with our results. Parasexuality is extremely rare in lower fungi, but occurs frequently in higher fungi (Leonard *et al.* 1978; Frankel 1979). This parasexual cycle in the absence of karyogamy might explain our results. Nuclei of strain Po67 may be degraded, and small parts of this genome may be incorporated into the Sc1 genome. Such incorporation would result in growth on minimal media and a change in morphology. Further studies are necessary to distinguish between the above possibilities.

In conclusion, this study showed that heterokaryosis did not occur in fusants of *P. ostreatus* and *S. commune*. However, genetic recombination between the nuclei of the different strains used in this study occurred, therefore confirming the usefulness of the protoplast fusants to improve mushroom strains. These improvements may result in increased production of *Pleurotus* mushrooms or the production of variant Schizophyllan molecules with increased activity. Further characterization of these fusants is in progress.

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