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Interorder Hybridization between *Pleurotus ostreatus* and *Elfvingia applanata* by Protoplast Fusion

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原形質體 融合에 의한 느타리버섯과 잔나비걸상버섯의 異目間 交雜

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ABSTRACT: Interorder somatic hybrids were obtained by protoplast fusion between *Pleurotus ostreatus* in the order Agaricales and *Elfvingia applanata* in the order Aphyllophorales. The fusants were classified into stable heterokaryons and spontaneously segregated heterokaryons. Hyphae of all fusion products except two strains did not form clamp connections. Out of them, two clamped and three clampless fusants produced mature fruiting bodies by light-dark cycle on sawdust rice bran medium. All of these basidiocarps had clamp connections. Three fusants were analysed with the distribution of progenies and segregation of genetic characters by random spore analyses. The genetic markers were shown to segregate and recombine in the first generation of monospores isolated from basidiocarps. Phenotypes of a large number of auxotrophic progenies were not detected in the two clamped fusants. The aberration ratio of segregants indicated the gene interaction resulting from different genome structure between distantly related species. The polymerase chain reaction (PCR) was adopted for the detection of somatic hybrids nuclear DNA. Four fusants showed a positive results in three kinds of primers. The prominent reaction products are represented by new bands in primer # 87 and # 125. Out of four fusants, two somatic hybrids had non-parental mtDNA patterns when digested with *Eco*R1 and *Hind*III. Comparison of somatic hybrids, tissue culture isolates(TC) and multispore germination isolates(MS) were made using esterase isozyme analysis. It is apparent that somatic hybrids had a minor banding patterns which are quite different from those of parents.

KEYWORDS: Interorder protoplast fusion, Genetic analysis, Polymerase chain reaction, Restriction pattern, Esterase, *Pleurotus ostreatus*, *Elfvingia applanata*, *Basidiomycotina*.

Protoplast fusion is an efficient instrument for the study of genetics and breeding. Intergeneric somatic hybrids have been obtained through protoplast fusion in fungi(Provost *et al.*, 1978 ; Gunge and Sakaguchi, 1981 ; Minuth and Esser, 1983 ; Groves and Oliver, 1984 ; Tamaki, 1986 ; Pina *et al.*, 1986 ; Kirimura *et al.*, 1988). Furthermore, there are studies on interkingdom cell fusion(Ahkong *et al.*, 1975 ; Jones *et al.*, 1976 ; Lima De Faria *et al.*, 1977 ; Crane and Dvorak, 1980 ; Hadlaczky *et al.*, 1980 ; Mastrangelo and Mitra, 1981 ; Rassoulzadegan *et al.*, 1982 ; Wiegand *et al.*, 1987). These intergeneric somatic hybrids were generally unstable and spontaneously segregated into the original parental strains, although some of the fusants were stable and show intermediate characteristics between the parents. Genetic recombination between distantly related species provides the opportunity for a broadening of the gene pool. Intergeneric hybridization of protoplasts in edible

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mushrooms have been carried out between *Pleurotus sajor-caju* and *Schizophyllum commune*(Liang and Chang, 1989), and *Pleurotus cornucopiae* and *Lentinus edodes*(Ogawa, 1993).

In the previous paper, we reported the interorder protoplast fusion between *Pleurotus ostreatus* in the order Agaricales and *Ganoderma applanatum* in the order Aphyllophorales(Yoo *et al.*, 1989). This investigation described characterization of fusion products by classical and molecular genetic analysis of these interorder somatic hybrids between *P. ostreatus* and *E.applanata.*

Materials and Methods

Strains and Genetic Character Identification

All strains that were derived from somatic hybrids of protoplasts between *Pleurotus ostreatus* ASI 2-1-o 2018-83-arg(Yoo *et al.*, 1988) and *Elfvingia applanata*(syn.*Ganoderma applanatum*) ASI 7-18-a 07031-880(Park *et al.*, 1987) were used. Induction of carpophores was attempted using 570 g sawdust substrates containing poplar tree plus 20% rice bran in 1,000 m*l* glass bottle. For cultivation under sterile conditions, the media were autoclaved at 121℃ for 90 min. On cooling the media were inoculated with spawn. The bottle was plugged with cotton. The cultures were incubated at 27℃ for 25-50 days under low intensity of lights. When mycelia were grown completely on sawdust media, the bottle was transferred to a light room. In order to get primordia, the mycelia in a bottle with cotton plug were exposed to high intensity of white lights for 20-60 days at 5-15℃. Lighting illuminated for 14 hours per day. When primordia initiation was developed into primordia the bottle which removed cotton plug was transferred to a light-moisture room.

Basidiospore prints obtained from the basidiocarp of somatic hybrids were stored at 4℃ for the analysis of progeny. The procedure of genetic analysis was based on those of Yoo *et al.*(1986). The standard media used mushroom complete medium(CM) containing(per liter) $MgSO_4 \cdot 7H_2O$ 0.5 g, KH_2PO_4 0.46 g, K_2HPO_4 1.0 g, Peptone 2.0 g, Yeast extract 2.0 g, Glucose 20.0 g and agar 20.0 g ; mushroom minimal medium(MM) containg $MgSO_4 \cdot 7H_2O$ 0.5 g, KH_2PO_4 0.46 g, K_2HPO_4 1.0 g, Glucose 20.0 g and Bacto agar 20.0 g. Various supplements were added to the minimal medium as required. Basidiospores were spread on CM in petridish and incubated for 5-10 days at 27℃. Sporelings were individually transferred from the germination medium to CM and incubated for a week at 27℃. All colonies or sectors were transferred to minimal medium 12 colonies per plate. After 7-20 days incubation, prototrophs and auxotrophs could be distinguished, and the latter were identified by testing, again in replicate sets of 12 inocula on the appropriate screening media.

Isolation and Purification of DNAs

Mycelia were grown in 1 *l* flasks containing 400 m*l* CM solution. The flasks were incubated for 5-7 days at 27℃. Nuclear and mitochondrial DNAs were isolated by a modification of the method of Hudspeth *et al.*(1980). The mycelia were filtered through a nylon clots and rinsed with distilled water. And then the harvested mycelia were ground to a fine powder in liquid nitrozen with a mortar and pestle. It was incubated in 50 m*l* 2× lysis buffer(10 m*l* 20× TE, 16 m*l* 0.5 M EDTA, 10 m*l* 5 M NaCl, 20 m*l* 10% SDS and 44 m*l* distilled water) and 30 m*l* 10% SDS at 60℃ for 2 h. The suspension was then extracted with phenol : choroform : isoamyl alcohol(25 : 24 : 1) followed by centrifugation at 4℃ until supernatant was clear. The DNA in the supernatant was precipitated with 3 M NaAc and ethanol for 2 h and then centrifused. The pellet was resuspend in TE buffer. Total DNA was collected by centrifuged in a C_SCl gradient containing 600 μg/m*l* ethidium bromide at 65,000 rpm for 20 h. After removal of ethidium bromide by n-butanol extractions, nuclear and mitochondrial DNAs were further purified by repeated centrifugation in C_SCl gradient containing 150 μg/m*l* bisbenzimide at 100,000 rpm for 20 h. The two fluorescing DNA bands(the upper one containing the mtDNA, lower one nDNA) were collected from the gradient. Bisbenzimide was removed by extraction with C_SCl-saturated

isopropanol and the DNA solution dialysed prior to ethanol precipitation.

Random Amplified Polymorphic DNA(RAPD) Reactions

RAPD reaction was carried out in 50 μl containing 100 ng primer, 200 μM each of dATP, dCTP, dGTP and dTTP, 50 ng template DNA and 2.5 units of Tag DNA polymerase in 1× PCR buffer (10 mM Tris-HCl(pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.1% Triton X-100) basically as described by McPherson *et al.*(1991). The mixture was covered with 50 μl of mineral oil to keep evaporation for reaction. The HYBAID Thermal Cycler was programmed for 40 cycles of 1 min at 93 C, 1 min at 35 C and 2 min at 72 C. After the last cycle, the samples were kept at 72 C for an additional 5 min. DNA was detected after running on 2% agarose EtBr gels using TBE as running buffer. RAPD primers were random 10-mers purchased from Univ.of British Columbia, Canada. The three random primers used in this study were :

1. #87 5'd-GGG GGG AAG C-3' ;
2. #125 5'd-GCG GTT GAG G-3' and
3. #152 5'd-CGC ACC GCA C-3'.

Restriction Enzyme Analysis

mtDNAs were digested with the restriction endonucleases *Eco*RI and *Hind*III under conditions recommended by manufacturers. Digested mtDNA was analyzed by electrophoresis in horizontal gels containing 1% agarose in TE buffer. Gels were stained with ethidium bromide.

Preparation of Mycelial Extracts and Polyacrylamide Gel Isoelectric Focusing

Mycelia were grown in 250 m*l* Erlenmeyer flasks containing 100 m*l* CM solution. The flasks were incubated for 15 days at 27℃ . The harvested mycelia in liguid nitrogen were ground at 4℃ in a pre-cooled mortar for 20 min and then PVP 4000 was added to remove phenol substances. The mycelial fragments were removed by centrifugation at 13,000 g for 30 min. The homogenized mycelia were analysed by the isoelectric focusing(IEF). The IEF experiments were performed horizontally with gels 0.6 mm thick consisting of a mixture of 6% polyacrylamide gel, 3.67% ampholyte pH 3-10, 1.33% ampholyte pH 4-6.5, and 1.33% ammonium persulfate basically as described by Osterman(1984). IEF runs were carried out with a 100 V applied for 30 min prior to sample application. After the samples had application eletrofocusing was continued at 300 V for 1 h and then increased to 1200 V gradually for 2 h. The focusing plate was cooled with running circulator.

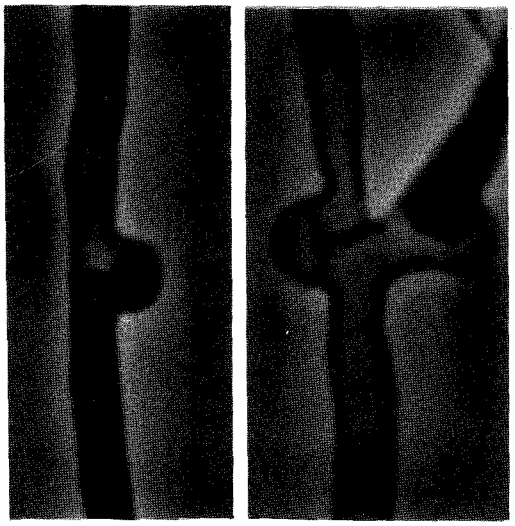

Fig. 1. Clamp connection hyphae produced by somatic hybrids between *P. ostreatus* and *E. applanata* following protoplast fusion.

Results and Discussion

Interorder fusion products were obtained between *Pleurotus ostreatus* in the order agaricales and *Elfvingia applanata* in the order aphyllophorales. Out of 36 fusants, 2 fusants formed clamp connections(Fig. 1). We have never obtained mature fruit bodies from fusion products as described by Yoo *et al.*(1989). However, primordia and fruit bodies from somatic hybrids were induced by light-dark cycle in a glass bottle. Among them, two clamped and three clampless fusants produced primordia and developed mature basidiocarps on sawdust rice bran substrates in a glass bottle. All hyphae or small tissues from basidiocarps of

Table 1. Characteristics of fusion products of protoplasts between *Pleurotus ostreatus* ASI 2-1-arg (A) and *Elfvingia applanata* ASI 7-18-cyn met (B).

Strain	Genetic background	Mycelial colony[1]		Fruiting body[2]		No. of isolate	% Isolate
		Morphology type	Clamp	Type	Clamp		
Fusant P386, P399	synkaryon	*P. ostreatus*	+	*P. ostreatus*	+	2	5.6
P384, P380	synkaryon	*P. osteratus*	−	*P. ostreatus*	+	2	5.6
		P. ostreatus	−	non-fertile		26	72.2
P382	synkaryon	non-parental	−	*P. ostreatus*	+	1	2.8
P389		non-parental	−	primordia	−	1	2.8
		non-parental	−	non-fertile		4	11.8
P. ostreatus 2-1-arg	monokaryon	parental A	−	non-fertile			
2018-wild	dikaryon	parental A wild	+	*P. ostreatus*	+		
E. applanata 7-18-cyn met	monokaryon	parental B	−	non-fertile			
7031-wild	dikaryon	parental B wild	+	*E. applanata*	+		

1, 2) +: present clamp connections, −: absent clamp connections.

clampless fusants presented true clamp connections, but primordia of fusant P389 lacked them (Table 1).

Strain *P. ostreatus* ASI 2018-wild was dikaryon that formed clamp connections and produced fruiting body. From this wild type, auxotroph ASI 2-1-arg was induced by irradiation of protoplast to ultraviolet lights. Strain 2-1-arg mutant was monokaryon and did not form basidiocarp on sawdust substrates. *E. applanata* ASI 07031-wild was also dikaryon and produced basidiocarp on oak tree log. Auxotrophic mutant was induced by irradiation of mycelial fragment to ultraviolet lights. Strain ASI 7-18-cyn met was monokaryon and did not form clamp connections. Wild type ASI 07031 and auxotroph ASI 7-18 did not produce primordia on various sawdust substrates. The clampless fusant did not produce fruit bodies on complete agar medium or complete liquid medium in flasks. Clamp connections did not form in the phase of vegetative mycelial growth on sawdust substrates but light and low temperature was required for development of clamped mycelia from clampless mycelial colonies in glass bottles. When clamped mycelia from clampless mycelial colonies were grown completely, mature fruiting bodies developed on sawdust substrates in glass bottles. It is capable of inducing clamp connections and fruiting development in certain somatic hybrids when sawdust spawn bottle of clampless fusants were exposed to lights at low temperatures as reported earlier(Yoo, 1992 ; Yoo and Cha, 1993). All hyphae or small tissues from these basidiocarps were present true clamp connections. When small tissues of stipe taken from basidiocarps cultured on agar plate, mycelial colonies grew more vigorously than original clampless fusants. Fruiting body character of somatic hybrids produced *P. ostreatus* type(Fig. 2). Parental *P. ostreatus* and *E. applanata* exhibited grey and greyish orange color on pileus in the young mushroom, respectively. Interorder somatic hybrids showed various mixed color of parental species(Table 2).

Three somatic hybrids of *P. ostreatus* ASI 2-1-arg and *E. applanata* ASI 7-18-cyn met were analysed with respect to the distribution of progenies and segregation of genetic markers by random spore analysis(Table 3). The genetic characters were shown to segregate and recombine abnormally in first segregation of monosporus isolates taken from the basidiocarps. The genetic characters did not detect in a large number of auxotrophic progenies. These progenies were aneurine-requiring, riboflavine-requiring, and aneurine and ribo-

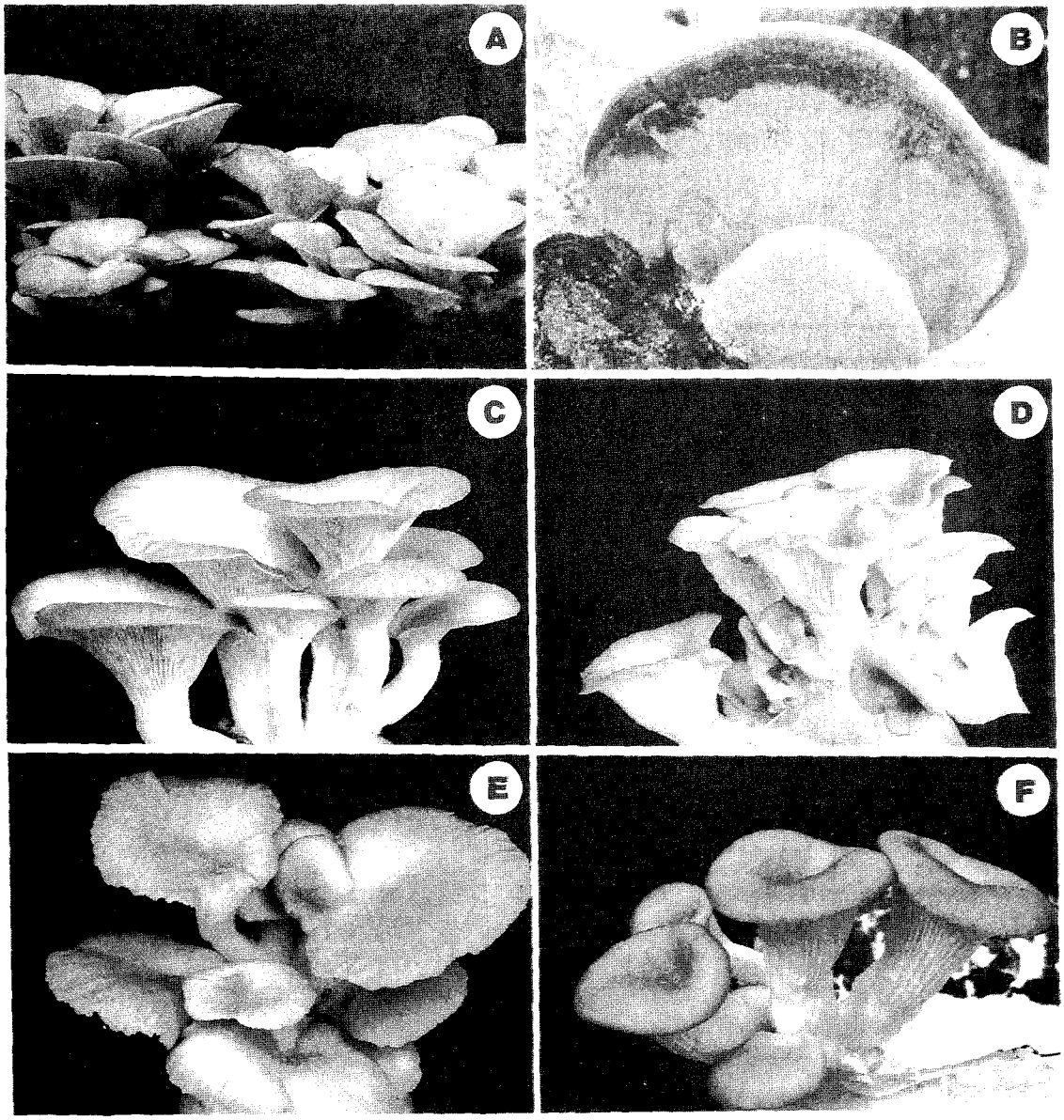

Fig. 2. Basidiocarps of somatic hybrids between *Pleurotus ostreatus* and *Elfvingia applanata* following protoplast fusion: (A) *P. ostreatus* ASI 2018-wild, (B) *E. applanata* ASI 07031-wild, (C) fusant P382, (D) fusant P384, (E) fusant P386, and (F) fusant P399.

flavine-requiring mutants. When parentals were transferred to MM plus aneurine, MM plus riboflavine, and MM plus aneurine and riboflavine, respectively, they were non-viable.

The polymerase chain reaction(PCR) was adopted for the molecular genetic analysis of somatic hybrids chromosomal DNA(Fig. 3). Figure shows that three primers of arbitrary sequence can be used to amplify chromosomal DNA segments, and that polymorphisms detected amplification products of four protoplast fusants and parents. In most cases, the DNA of *E. applanata* was not distinct, but somatic hybrids showed non-parental bands. The size of major products ranged between 0.5-

Table 2. Characteristics of basidiocarps of somatic hybrids between *Pleurotus ostreatus* and *Elfvingia applanata.*

Strain	Colour of pileus	
	Young	Mature
Fusion product P386	light brown	greyish brown
P399	greyish green	greyish yellow
P384	greysih green	greyish brown
P380	greyish green	greyish yellow
P382	grey	greyish brown
P. ostreatus ASI 2018-wild	grey	bluish grey
E. applanata ASI 07031-wild	greyish orange	orange grey

Table 3. Frequency of progenies of somatic hybrids between *Pleurotus ostreatus* ASI 2-1-arg(A) and *Elfvingia applanata* ASI 7-18-cyn met (B) following protoplast fusion

Phenotype[1)]		No. of individual		
		P382	P386	P399
prototroph		290	204	230
arg		13	1	5
met		0	0	4
arg cyn		0	0	3
arg cyn met		0	1	0
ane		0	1	1
rib		0	1	2
ane rib		0	88	55
Parentals	parental A	13	1	5
	parental B	0	1	0
Recombinant	prototroph	290	204	230
	auxotroph	0	91	62

1) Mutant symbols: ane(aneurine), arg(arginine), cyn (cystine) met(methionine), rib(riboflavine).

2.0 kbp. The prominent reaction products were represented by approximately 1.2 kbp and 0.6 kbp bands in primer #87 and #125 respectively.

Restriction patterns were obtained when the restriction endonuclease digests of the mtDNA were analyzed(Fig. 4). Two somatic hybrids had non-parental mtDNA patterns when digested with *Eco*RI and *Hind*III. Fusant P380 and P391 were recombination of mtDNA since restriction patterns were different from their parents. Comparison of somatic hybrids, tissue culture isolates(TC) and multis-

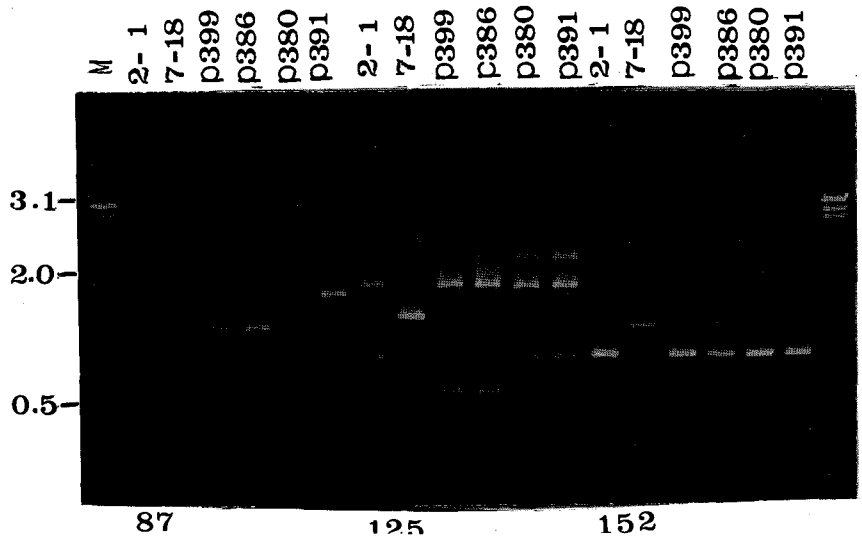

Fig. 3. 1.3% Agarose gel electrophoresis of PCR products of nuclear DNA from *P. ostreatus, E. applanata* and somatic hybrids stained with ethidium bromide: (2-1) *P. ostreatus* ASI 2-1-arg, (7-18) *E. applanata* ASI 7-18-cyn met, (P399, P386, P380, P391) fusion products and (M) phage DNA digested with *Hind*III was used as size marker.

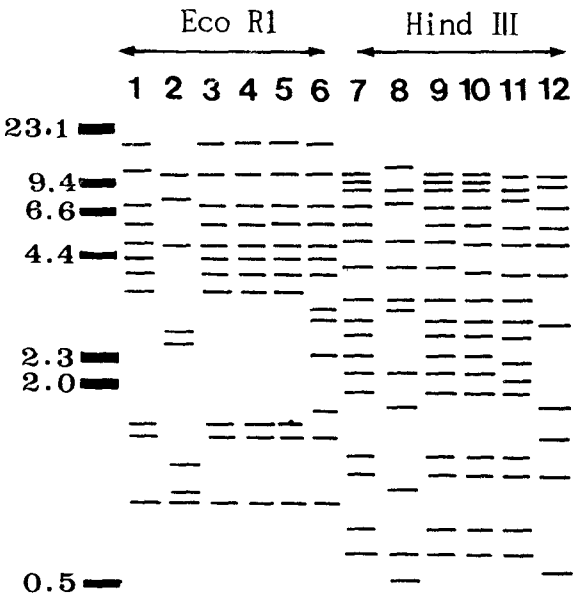

Fig. 4. Agarose gel electrophoresis of mt DNA of protoplast fusion products between *P. ostreatus* and *E. applanata*. The mtDNA were digested with *Eco*R1(lane 1-6) and *Hind*III(lane 7-12): (1,7) *P. ostreatus* ASI 2-1, (2,8) *E. applanata* ASI 7-18, (3,9) P399, (4,10) P386, (5,11) P380, and (6,12) P391.

pore germination isolates(MS) was made using isozyme analysis of esterase(Fig. 5). The major banding patterns of all fusion products were similar to those of the *P. ostreatus* parental strains except fusant P382. It is apparent that somatic hybrids had a minor abanding patterns quite a different from those of parents. A comparison of the somatic hybrids, tissue culture isolates, and F^2 strain(multispore germination) implied that banding patterns of both somatic hybrids and tissue culture isolates were very similar but different from those of F^2 strains.

Distribution of progenies and segregation of genetic characters by random basidiospore analysis were shown abnormally non-expected alleles. These results indicate that after fusion the nuclear and cytoplasmic genomes reassots in a cell. When the two protoplasts first fused a heterokaryon was produced which contained both nuclei and both cytoplasms. They did not stay together and eventually the mononuclear synkaryon cell was produced after fusion of the nuclear and cytoplasmic genomes. The genetic information from *Pleurotus ostreatus* genomes was predominant, but most of the somatic hybrids expressed genetic characters and gene functions from both parents.

No abnormal distribution and segregation were shown in the intraspecific-(Yanagi *et al.*, 1988) and closely related interspecific fusants(Yoo *et al.*, 19 86). The hyphae of interspecific fusants between vegetatively incompatible strains in pairings of *Pleurotus ostreatus*+*P. salmoneostramineus*, *P. ostreatus*+*P. sajor-caju*, *P. pulmonarius*+*P. sajor-caju* and *P. columbinus*+*P. sajor-caju* were revealed to be uninucleate in a cell(Toyomasu *et al.*, 1986 ; Toyomasu and Mori, 1989).

Intergeneric protoplast fusion between *Pleurotus sajor-caju* and *Schizophyllum commune* has been reported by Liang and Chang(1989). The most somatic hybrids did not produce fruiting body normally as in the control experiments of *P. sajor-caju*, while some fusants formed very small ugly callus tissue or normal fruit bodies similar to those of *P. sajor-caju*. They suggested that the fusion products were not real heterokaryons due to asynchronous physiological development of the two partners. Ogawa(1993) described the formation of intergeneric fusants between *Pleurotus cor-*

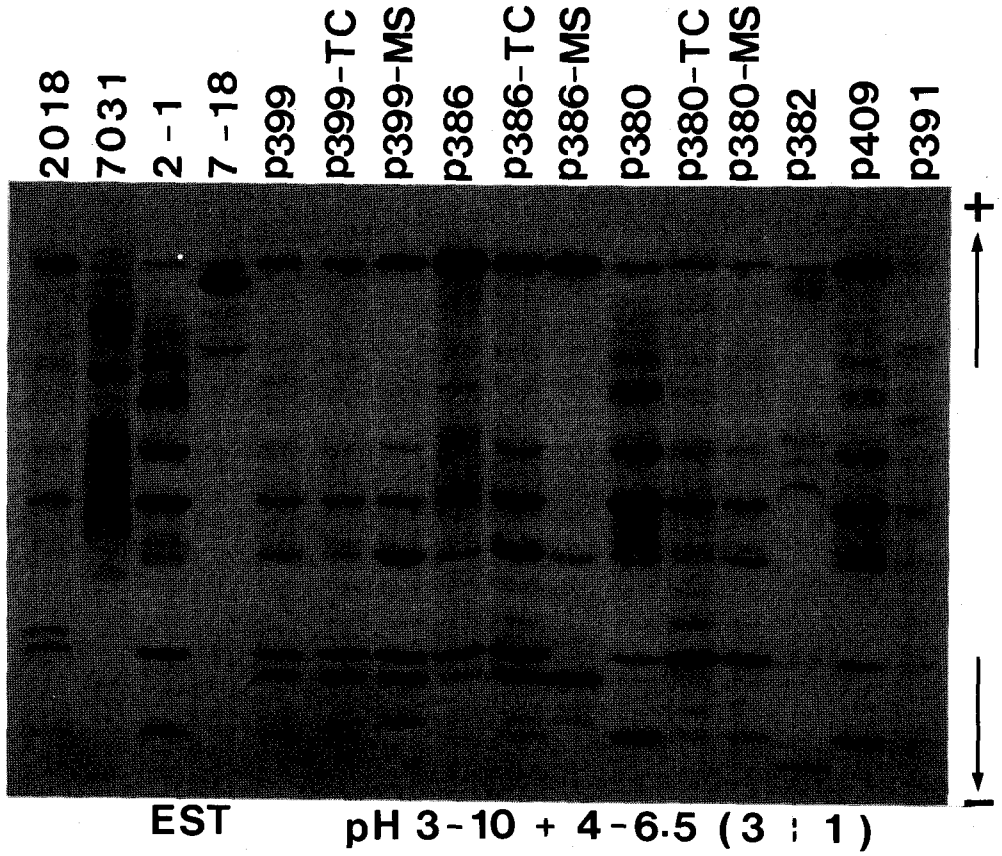

Fig. 5. Esterase zymograms of somatic hybrids between *P. ostreatus* and *E. applanata*: (2018) *P. ostreatus* ASI 2018-wild, (7031) *E. applanata* ASI 07031-wild, (2-1) *P. ostreatus* ASI 2-1-arg, (7-18) *E. applanata* ASI 7-18-cyn met, (P399) fusant P399, (P399-TC) P399-TC (tissue culture isolate), (P399-MS) P399-MS (multispore germination isolate), (P386) fusant P386, (P386-TC) P386-TC, (P386-MS) P386-MS, (P380) fusant P380,(P380-TC) P380-TC, (P380-MS) P380-MS, (P382) fusant P382,(P409) fusant P409, and (P391) fusant P391.

nucopiae and *Lentinus edodes*. The somatic hybrids produced same morphology of fruit bodies as those of *L. edodes*. In the interspecific hybridization of *Pleurotus cornucopia* and *Pleurotus florida* most of fruiting character of somatic hybrids were developed similar to those of *P. florida*(Yoo, 1992). The results of these somatic hybridization indicate most of basidiocarp characters express similar to those of one of the fusion parents except compatible fusion products between closely related species.

摘 要

원형질체 융합으로 주름버섯목 느타리버섯과 민주름버섯목 잔나비걸상버섯과의 이목간 체세포잡종을 36균주 얻어 꺽쇠연결체 clamp connections 있는 2균주와 꺽쇠연결체 없는 3균주의 자실체를 형성하였다. 꺽쇠연결체 없는 융합주는 한천배지나 액체배지에서는 자실체를 얻을 수 없었다. 그러나 톱밥배지에서 균사가 완전히 성장한 후 일정한 광과 온도를 유지한 결과 꺽쇠연결체가 있는 균사가 새로이 성장하였으며 거의 완전하게 다시 성장한 후 원기가 유도되고 자실체가 성숙하였다. 버섯자실체 특성은 느타리버섯과 유사하였으며, 갓 색택은 느타리와는 다소 다르게 나타났다.

3균주의 담자포자로 유전형질의 분리와 유전자재조합을 조사한 결과 꺽쇠연결체 있는 2균주는 비정상적인 분리 현상을 보였는데, 양친에 없는 ane, rib, ane rib 표지를 가진 것이 나타났다.

3종류의 random primer를 이용하여 핵산 연쇄 중합반응 polymerase chain reaction(PCR)에 의한 4개 체세포잡종의 염색체 DNA의 다형화현상을 조사한 결과 느타리친과 유사한 양상을 가졌으나 비양친의 밴드를 나타내었으며 primer #87, #125의 1.2 kbp, 0.6 kbp에서 각각 뚜렷하게 구분되었다. 4개 체세포잡종의 미토콘드리아 DNA의 제한효소 *Eco*RI과 *Hind*III의 절단결과 2균주는 느타리와 동일하였으며 2균주는 양친과 다른 양상을 나타내었다. 원형질체 융합주, 융합주 자실체로부터의 조직배양주, 융합주의 담자포자 발아주, 양친주를 포함하여 총 16균주를 등전점 전기영동으로 동위효소 esterase를 분석한 결과 융합주 6균주중 5균주는 느타리와 유사하였으며 1균주는 양친과는 전혀 다른 새로운 밴드양상으로 이들 모두 양친과는 뚜렷이 구분되었다. 융합주와 자실체 조직배양주는 밴드양상이 거의 유사하였으나 F^2는 다소 차이가 나타났다.

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