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Genetic Analysis of the Life Cycle in Interspecific Hybrids of *Pleurotus ostreatus* and *Pleurotus florida* Following Protoplast Fusion

Young-Bok Yoo, Chang-Hyun You, Yong-Hwan Park and John F. Peberdy*

Applied Mycology and Mushrooms Division, Institute of Agricultural Sciences, Suweon 170, Korea and Department of Botany, University of Nottingham, Nottingham NG7 2RD, England*

원형질 체융합에 의한 느타리버섯과 사철느타리버섯 체세포 잡종의 유전 분석

劉 英 福·柳 昌 鉉·朴 容 煥·존 페버디

농촌진흥청 농업기술연구소 균이과·영국 노팅검대학 식물학과

Abstract: Interspecific hybrids of *Pleurotus ostreatus* and *Pleurotus florida* were formed by using protoplasts of complementing auxotrophs. The genetic markers were shown to segregate and recombine in the first generation of monosporus isolates from basidiocarp of seven fusion products. The analysis provides proof of heterokaryosis and strong evidence for haploidy of vegetative nuclei, a sexual cycle consisting of nuclear fusion and meiosis. In all the crosses there was no evidence of linkage between the genetic markers. Clamp connections were formed in monosporus mycelia from basidiocarp of fusion products.

Keywords: *Pleurotus ostreatus*, *Pleurotus florida*, Protoplast fusion, Genetic analysis, Recombination, Clamp connection.

The genus *Pleurotus*, saprophytic fungus, is wide spread all over the world. The cultivation of this mushroom on log was first described at the beginning of the 20th century (Falck, 1917; Passecker, 1959) and achieved on lignin- or cellulose-containing agricultural wastes (Block *et al.*, 1958, 1959; Bano and Srivastava, 1962; Herzig *et al.*, 1968). In the last decade, consumption of this fungus have grown at a rapid rate. Bifactorial heterothallism was detected in *P. ostreatus* by Vandendries (1933) and Terakawa (1957, 1960) and six species of *Pleurotus* were all proved to be tetrapolar (Whitehouse, 1949). The spore contained one nucleus but two nuclei may be found rarely in one spore. The hyphae produced from these two nuclei spores were monokaryotic (Terakawa, 1960). Cytological observation detected meiosis in the basidium, the subsequent formation of four uninucleate basidiospores, and the truly clamped dikaryotic characteristic of the fertile heterokaryon (Terakawa, 1957; Su, 1973). The prospect for breeding the edible mushroom to combine desired characteristics of different strains depend upon an understanding of the genetics. Genetic proof of meiosis was obtained by analysis of the segregation pattern for basidiocarp color (Arita, 1974). A true sexual process, however, has not yet been verified cleary by genetic analysis in *Pleurotus* species.

In a previous paper, we described the protop-

last fusion and selection of fusion products between *P. ostreatus* and *P. florida* (Yoo *et al.*, 1984). This experiment was undertaken to investigate life cycle, segregation and recombination of interspecific hybrid between *P. ostreatus* and *P. florida* after protoplast fusion.

Materials and Methods

Strains

All strains that were derived from the fusion products between *P. ostreatus* and *P. florida* after protoplast fusion were listed in Table. Stable heterokaryon were established and basidiospore prints obtained from the basidiocarp of fusion products were stored at 4°C for the analysis of progeny. The general scheme for carrying out genetic analysis is outlined in Fig. 1.

Media

The standard media used for *Agaricus* (Raper, 1972) have been used for *Pleurotus* without modification. These contain per liter of distilled water (g/l): mushroom complete medium (MCM)-$MgSO_4.7H_2O$ 0.05, KH_2PO_4 0.46, K_2HPO_4 1.0, peptone 2.0, yeast extract 2.0, glucose 20.0 Bactoagar 20.0; mushroom minimal medium (MMM)-$MgSO_4.7H_2O$ 0.5, KH_2PO_4 0.46, K_2HPO_4 1.0, thiamin-HCl 120 μg, DL-asparaine 2.0, glucose 20.0, Bacto agar 20.0, Various supplements were added to the minimal medium as required.

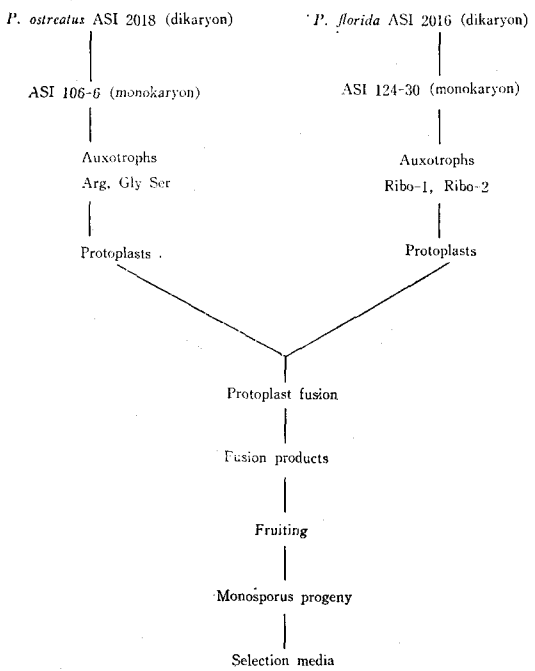

Fig. 1. Standard scheme for carrying out genetic analysis in *Pleurotus*.

Table I. List of strains.

Fusion mixture		No. of fusion products	Mycelial growth* on MMM	Basidiospore**
P. ostreatus	*P. florida*			
Arg	×Ribo-1	P1	M	LBS
		P2	F	LBS
		P3	S	LBS
		P5	F	LBS
		P6	F	LBS
		P7	F	LBS
		P10	M	LBS
Gly Ser	×Ribo-2	P12	M	SBS
		P15	M	LBS
		P17	M	LBS
		P20	F	LBS
Arg	×Ribo-2	P22	S	LBS
		P25	M	SBS
		P26	F	LBS
		P27	F	LBS
Gly Ser	×Ribo-1	P34	S	LBS
		P35	F	LBS
		P39	F	LBS

* F: Fast growing type
M: Moderate growing type
S: Slow growing type
** SBS: Small volume of basidiospore
LBS: Large volume of basidiospore

Basidiospore Germination and Genetic Character Identification

Basidiospores were spread on mushroom complete agar medium and incubated for 5~7 days at 25°C. Sporelings were individually transferred from the germination medium to complete med-

ium and incubated for abut a week at 25°C. All colonies or sectors were transferred to minimal medium 12 per plate. After 5~7 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.

Results

Segregation and Recombination of Genetic Markers

Seven fusion products in four crosses were analysed with respect to the distribution of progenies and segregation of markers by random spore analysis. Basidiospores could yield progeny of four genotypes in the cross Arg×Ribo−1 and Arg×Ribo−2 for prototrophs, auxotrophs of one parental type, auxotrophs of the other parental type and double auxotrophs, respectively. However, the three factor cross Gly Ser×Ribo−1 and Gly Ser×Ribo−2 were not detected segregants clearly. In such cross there are eight poss-

Table II-1. Frequency distribution of progenies in Arg×Ribo-1 crosses (P3).

Genotype	No. of individual
+ +	191
Arg +	0
+ Ribo	129
Arg Ribo	0

Table II-2. Allele ratio.

Locus	Mutant	Wild	X^2	P
Arg	0	320	320	<0.005
Ribo	129	191	12.01	<0.005

Table II-3. Genetic analysis of paired markers.

Parental	Recomb.	X^2	P
129	191	12.01	<0.005

Table III-1. Frequency distribution of progenies in Arg×Ribo-1 crosses (P5).

Genotype	No. of individual
+ +	212
Arg +	39
+ Ribo	178
Arg Ribo	33

Table III-2. Allele ratio.

Locus	Mutant	Wild	X^2	P
Arg	72	390	218.88	<0.005
Ribo	211	251	3.46	>0.05

Table III-3. Genetic analysis of paired markers.

Parental	Recomb.	X^2	P
217	245	1.70	<0.25

Table IV-1. Frequency distribution of progenies in Gly Ser ×Ribo-2 crosses (P12).

Genotype	No. of individual
+ + +	84
Gly Ser +	0
+ + Ribo	72
Gly + +	0
+ Ser +	0
Gly + Ribo	0
+ Ser Ribo	0
Gly Ser Ribo	0

Table IV-2. Allele ratio.

Locus	Mutant	Wild	X^2	P
Gly	0	156	156	<0.005
Ser	0	156	156	<0.005
Ribo	72	84	0.92	>0.25

Table IV-3. Genetic analysis of paired markers.

Parental	Recomb.	X^2	P
72	84	0.92	>0.25

Table V-1. Frequency distribution of progenies in Gly Ser×Ribo-2 crosses (P15).

Genotype	No. of individual
+ + +	240
Gly Ser +	0
+ + Ribo	227
Gly + +	0
+ Ser +	2
Gly + Ribo	3
+ Ser Ribo	2
Gly Ser Ribo	4

Table V-2. Allele ratio.

Locus	Mutant	Wild	X^2	P
Gly	7	471	450	<0.005
Ser	8	470	448	<0.005
Ribo	236	242	0.06	<0.90

Table V-3. Genetic analysis of paired markers.

Parental	Recomb.	X^2	P
227	251	1.31	>0.25

Table VI-1. Frequency distribution of progenies in Arg×Ribo-2 crosses (P22).

Genotype	No. of individual
+ +	94
Arg +	9
+ Ribo	98
Arg Ribo	43

Table VI-2. Allele ratio.

Locus	Mutant	Wild	X^2	P
Arg	52	192	80.32	<0.005
Ribo	141	103	5.92	>0.01

Table VI-3. Genetic analysis of paired markers.

Parental	Recomb.	X^2	P
107	137	3.68	>0.05

Table VII-1. Frequency distribution of progenies in Arg×Ribo-2 crosses (P25).

Genotype	No. of individual
+ +	62
Arg +	0
+ Ribo	46
Arg Ribo	34

Table VII-2. Allele ratio.

Locus	Mutant	Wild	X^2	P
Arg	34	108	38.56	<0.005
Ribo	80	62	0.28	>0.50

Table VII-3. Genetic analysis of paired markers.

Parental	Recomb.	X^2	P
46	96	17.60	<0.005

Table VIII-1. Frequency distribution of Ribo-1 cross (P34).

Genotype	No. of individual
+ + +	105
Gly Ser +	0
+ + Ribo	168
Gly + +	0
+ Ser +	0
Gly + Ribo	0
+ Ser Ribo	0
Gly Ser Ribo	1

Table VIII-2. Allele ratio.

Locus	Mutant	Wild	X^2	P
Gly	1	273	270	<0.005
Ser	1	273	270	<0.005
Ribo	169	105	16	<0.005

Table VIII-3. Gentic analysis of paired markers.

Parental	Recomb.	X^2.	P
168	106	14.02	<0.005

ible genotypes of which two are parental and the rest recombinant (Table II. 1 to Table VIII. 1). Strain P12 and P34, especially, only two or three genotypes of progeny could be selectable (Table IV. 1 and VIII. 1). The allele ratio of loci could be expected 1 : 1 from two crosses, Arg×Ribo−1 and Arg×Ribo−2, respectively. The ratio, however, would change toward 3 : 1 with increasing proportions of *P. florida* genotypes (Table II. 2, III. 2, VI. 2 and VII. 2). In the crosses Gly Ser×Ribo−1 and Gly Ser× Ribo−2 allele ratio of loci were different from epected 1 : 1 : 1 based on independent segregation, respectively (Table IV. 2, V. 2 and VIII. 2). Seven fusion products in four crosses were tested for linkage by random spore analysis. The results were shown in Table II. 3 to VIII. 3, In all the fusion products there was no evidence of linkage between the genetic markers. The parental genotypes were recovered with the recombinant progeny amounting to 38.7∼67.6%.

Formation of Clamp Connection in Monosporus Mycelia

In the all crosses, clamp connection could be found in prototrophs of monosporus mycelia from the basidiocarp of fusion products between *P. ostreatus* and *P. florida*. In the strain P 5, 78.5% of the prototrophic recombinants tested formed clamp connection (Table IX). Basidiocarps of monosporus strains which present clamp connection in the mycelia were normal morphology. The colour of the pilei were not different from fusion products between *P. ostreatus* and *P. florida* following protoplast fusion.

Table IX. Frequency distribution of clamp connections in monosporus mycelia of prototrophic recombinants from basidiocarb of fusion products.

Strain	No. of individual* +	No. of individual* −	No. of colonies examined	Clamp connection frequency (%)
P1	19	42	61	31.1
P2	26	29	55	47.3
P3	14	51	65	21.5
P5	51	14	65	78.5
P6	15	15	30	50.0
P7	18	32	50	36.0
P10	16	38	54	29.6
P15	5	76	81	6.2
P17	2	28	30	5.3
P20	8	47	55	14.5
P22	4	30	34	11.8
P26	10	48	58	17.2
P27	9	21	30	30.0
P34	2	52	54	3.7
P37	6	24	30	20.0
P39	8	34	42	19.0

*+: Presence of clamp connection
−: No clamp connection

Discussion

The major species of *Pleurotus* were all bifactorial heterothallism (Vandendries, 1933; Terakawa, 1957, 1960; Eugenio and Anderson, 1968; Roxon and Jong 1977; Kaufert, 1936). Seven fusion products in 4 crosses were detected meiotic segregation and recombination by random spore analysis. The results indicated that there was no obvious linkage beween the markers. Germination frequency of the spores in all case constitute a very significant feature of the results in the selection of particular genotypes (Raper, 1972; Santiago, 1981). Thus in this situation it is possible that the prototroph could have a selective advantage (Santiago, 1981).

Clamp connections were first described by Hoffmann (1856) and soon thereafter were noted by several authors in a characteristic of basidiomycetes (Hartig, 1866; de Bary, 1866). In the heterothallism the mycelium of monosporus isolate lacked clamp connections and was sterile. When monosporus strains were crossing, dikaryotic mycelia were formed clamp connections and

were fertile (Raper, 1966). The prototrophic colonies of single spore culture from basidiocarp of fusion products were formed clamp connections in the all crosses. These basidiospores that presence of clamp connection could be dinucleate or multinucleate and were heterokaryon form. But it is not known whether this is the case for fusion products of protoplast in this organism. The method of mushroom breeding have remained comparatively crude in the absence of genetic knowledge. This preliminary attempts to obtain knowledge of its genetic system in *Pleurotus* using protoplast fusion suggest that this experiment could be beneficial in an overall strain improvement programme and a better understanding of the life cycle in *Pleurotus*.

적 요

느타리버섯과 사철느타리버섯의 영양요구성 균주를 원형질체융합에 의하여 이종간융합균주를 선발하였다. 이 체세포잡종을 재배하여 자실체에서 담자포자를 분리하여 life cycle에 대한 유전분석을 한 결과를 요약하면 다음과 같다.

1. Arg×Ribo-1, Arg×Ribo-2, Gly Ser×Ribo-1 그리고 Gly Ser×Ribo-2 4개의 융합조합에서 7개의 융합균주를 분석한 결과 모두 유전형질이 분리되었으며 또한 유전자 조환이 일어났다.

Arg×Ribo-1과 Arg×Ribo-2에서는 4종류로 분리되었으며 Gly Ser×Ribo-1, Gly Ser×Ribo-2와 같이 대립유전자가 3인자 일때는 8종류로 기대되나 단지 2내지 6종류로만 분리되었다.

2. 대립유전자의 비에 있어서 Arg×Ribo-1과 Arg×Ribo-2에서 1:1로 기대되나 Ribo의 분리수가 많은 3:1으로 분리되었으며, Gly Ser×Ribo-1과 Gly Ser×Ribo-2에서는 1:1:1과는 아주 상이한 분리비를 나타내었다.

3. 4개의 융합조합 모두 연관에 속하지 않았으며 조환가는 38.7~67.6%였다.

4. 분리되는 prototrophic recombinants의 균사체에서 clamp connection이 형성되었는데 4개의 융합조합에서 모두 나타났으며 융합균주에 따라 차이가 났는데 Arg×Ribo-1의 p5균주는 검정된 수의 78.5%가 clamp connection을 형성하였다. 또한 이들 균주의 자실체는 정상이었으며 자실체의 색은 느타리버섯과 사철느타리버섯의 중간이었다.

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