Multinucleate nature, and mating by use of isozyme analysis in *Poria cocos*

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Double staining study of nuclei and cell walls in *Poria cocos* indicated that the hyphal cells were multinucleate and had no clamp connections. Isozyme analysis of alcohol dehydrogenase (ADH) in 52 natural isolates revealed that there were three types of banding patterns: type I, five bands; type II, one slow band; type III, one fast band. Regenerants expressing type-II or type-III ADH-isozyme pattern were obtained from type-I isolates via protoplast manipulation. When the type-II regenerants were mated with the type-III regenerants, hyphae of type-I phenotype appeared. These data indicated that these type-II and type-III regenerants derived from protoplasts of the type-I isolates were primary hyphae. These primary hyphal cells were also multinucleate. Inter-strain mating of *P. cocos* was performed and confirmed by ADH-isozyme analysis. Confronting cultures of a type-III regenerant derived from protoplasts of a type-I isolate and a type-II regenerant derived from a type-II isolate resulted in type-I hyphae.

Key Words——ADH isozyme analysis; mating; multinucleate; *Poria cocos*; protoplast.

Poria cocos (Schw.) Wolf belongs to the Polyporaceae and forms sclerotia on the roots of several plants, such as cedar, citrus, corn, eucalyptus, fir, magnolia, oak, sumac, and pine (Weber, 1929). This fungus is distributed in China, Korea, Japan (Hino, 1937), Canada, the United States and Australia (Weber, 1929). The formation of sclerotia and/or fruitbodies in culture has been reported (Weber, 1929; Fritz, 1954; Tominaga, 1987; Tabata and Hiraoka, 1994), but there are no studies on the mating of *P. cocos*.

In basidiomycetes, it is usually possible to distinguish secondary hyphae from primary ones by the presence of clamp connections. In mating experiments, compatible mating is inferred from the presence of clamp connections on hyphae in the contact zone (Kobayasi and Tubaki, 1965). The number of nuclei per cell is another important criterion for distinguishing between secondary and primary hyphae: the former contain two nuclei per cell; the latter, one. It is also possible to discriminate between individual strains by isozyme analysis (Royse and May, 1982; Ohmasa and Furukawa, 1986), which is also used for confirmation in mating studies (Kirby and Mulley, 1982; Roux and Labarère, 1991; Addleman and Archibald, 1993).

The segregation of primary hyphae through protoplasts from secondary hyphae has been reported in several species of the basidiomycetes (Kropp and Fortin, 1985; Raper, 1985; Kawabata et al., 1992; Fox et al., 1994).

In this study, we have found that the hyphal cells of

P. cocos are multinucleate and have no clamp connections. Regenerants of protoplasts of *P. cocos* have been obtained, and distinguished from secondary hyphae by isozyme analysis of alcohol dehydrogenase (ADH). Mating study of these regenerants reveals that they are primary hyphae. We report the production of primary hyphae and confirmation of mating by ADH isozyme analysis in *P. cocos*.

Materials and Methods

Organisms and culture conditions Fifty-two isolates were used in this study (Table 1), which were isolated from the sclerotia in our laboratory except for Pc 11, Pc 12, and Pc 17-Pc 21. Pc 11 and Pc 12 were provided by the Canadian National Collection of Fungus Cultures, and Pc 17 to Pc 21 were from the United States Department of Agriculture. Stock cultures were maintained on a potato-dextrose agar (PDA, Difco) at 18°C, and subcultured at 4 to 6 month intervals.

Double staining of nuclei and cell walls The number of nuclei in each hyphal cell was counted according to the method of Iwase et al. (1988). The mycelia were incubated on PDA for 3 to 7 days at 30°C, fixed with ethanol: acetic acid (3 : 1) for 30 min at 5°C, and stained with 25 μg/ml propidium iodide (Sigma) for 10 min at room temperature. The excess stain was removed by washing with distilled water. Then the mycelia were stained again with 500 μg/ml Calcofluor white M2R (Polysciences, Inc.). A small piece of the mycelia was placed on a glass slide with a drop of 1 mg/ml RNase-A (Sigma) solution, then squashed under a cover slip. These speci-

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Table 1. Derivation and ADH isozyme banding patterns of isolates of *Poria cocos*.

Isolate	Locality	ADH isozyme[a]	Isolate	Locality	ADH isozyme
Pc 1	Ibaraki Pref.	I	Pc 27	Ibaraki Pref.	II
Pc 2	〃	I	Pc 28	〃	II
Pc 3	〃	I	Pc 29	China	II
Pc 4	〃	II	Pc 30	〃	II
Pc 5	Tokyo Metropolis	II	Pc 31	〃	II
Pc 6	〃	I	Pc 32	〃	II
Pc 7	〃	I	Pc 33	〃	II
Pc 8	〃	II	Pc 34	〃	II
Pc 9	Ibaraki Pref.	II	Pc 35	〃	III
Pc 10	Korea	I	Pc 36	〃	III
Pc 11	Canada[b]	III	Pc 37	Ibaraki Pref.	II
Pc 12	〃	II	Pc 38	〃	II
Pc 13	Shizuoka Pref.	II	Pc 39	〃	II
Pc 14	〃	I	Pc 40	〃	II
Pc 15	〃	II	Pc 41	〃	II
Pc 16	〃	I	Pc 42	〃	II
Pc 17	USA[c]	III	Pc 43	〃	II
Pc 18	〃	III	Pc 44	Chiba Pref.	I
Pc 19	〃	III	Pc 45	Ibaraki Pref.	II
Pc 20	〃	III	Pc 46	〃	II
Pc 21	〃	II	Pc 47	〃	II
Pc 22	Ibaraki Pref.	III	Pc 48	〃	II
Pc 23	〃	II	Pc 49	〃	II
Pc 24	〃	II	Pc 50	〃	II
Pc 25	〃	II	Pc 51	〃	II
Pc 26	〃	II	Pc 52	〃	II

a) Three types of banding patterns were observed: type I, five bands; type II, one slow band; type III, one fast band.
b) Obtained from the Canadian National Collection of Fungus Cultures.
c) Obtained from the United States Department of Agriculture.

mens were observed with a fluorescence microscope (Nikon Optiphot) equipped with a 100 W mercury lamp and a Nikon UV excitation apparatus (excitation filter EX330~380, absorbing filter BA420 and dichroic mirror DM400) or a Nikon G excitation apparatus (excitation filter EX510~560, absorbing filter BA590 and dichroic mirror DM580).

ADH isozyme analysis The ADH isozyme analysis was performed by the method of Soltis and Soltis (1989) with slight modifications. Each isolate was inoculated into 50 ml of potato-dextrose broth (PDB, Difco), which was adjusted pH 4.0 with 1.0 N HCl, and incubated for 6 days on a reciprocating shaker (115 rpm) at 30°C. The mycelia were harvested by filtration and frozen at −70°C. The frozen mycelia were lyophilized and ground to powder with mortar and pestle. Forty mg of the mycelial powder was mixed with 400 μl of extraction buffer, consisting of 0.1 M Tris-HCl, pH 7.5, 1.0 mM EDTA·4Na, 10 mM KCl, 10 mM MgCl$_2$·6H$_2$O, 40 mg/ml polyvinylpyrrolidone, and 1.0 μl/ml β-mercaptoethanol, and the mixture was centrifuged at 8000 × g for 10 min at 5°C. The supernatant was subjected to electrophoresis in an 11.1% (w/v) horizontal starch-gel for 5 h at 250 V, the temperature of the gel and buffer being maintained at 5°C throughout. The electrode buffer consisted of 223 mM Trizma-base and 69 mM citric acid, pH 7.2, and the gel buffer contained 8.0 mM Trizma-base and 2.0 mM citric acid, pH 7.2. After electrophoresis, the localized ADH activity was visualized by staining the enzyme activity. The gels were sliced horizontally, and incubated for 1 h at 30°C in 100 ml of the staining solution, which consisted of 0.1 M Tris-HCl, pH 8.0, 20 mg/100 ml NAD, 20 mg/100 ml 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyltetrazolium bromide (Sigma), 2.0 mg/100 ml phenazine methosulfate (Sigma) and 0.4 ml/100 ml ethanol.

Preparation and regeneration of protoplasts Each isolate was incubated statically in 20 ml of PDB for 4 days at 30°C. The mycelia were then transferred into 2 ml of the enzyme mixture containing 0.5% (w/v) MES, pH 5.5, 2.0% (w/v) Cellulase "Onozuka" RS (Yakult Co., Ltd.), 8.4 unit/ml chitinase (Sigma), and 0.5 M mannitol. They were incubated for 4 h at 25°C with gentle shaking (60 rpm). This suspension was filtered through nylon mesh (20 μm) to remove undigested cell debris. Suspension of protoplasts was diluted to 1 × 10^2 protoplasts/ml
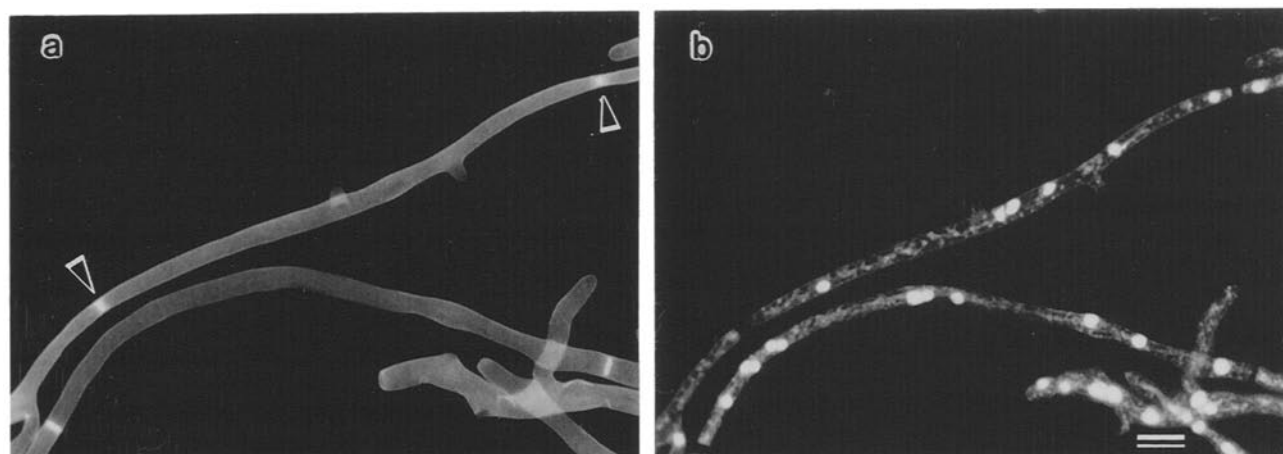
Fig. 1. Fluorescent micrographs of hyphae of *Poria cocos*, Pc 14. The hyphae were stained with propidium iodide and Calcofluor white M2R. **a** and **b** were observed with a fluorescence microscope in the same visual field. **a**, which was excited at 330~380 nm, shows hyphae have no clamp connections. Arrow heads indicate septa. **b**, which was excited at 510~560 nm, shows hyphae are multinucleate. Bar indicates 10 μm.

with PDA supplemented with 0.5 M mannitol and plated into a 9-cm Petri dish. After 4 days of incubation at 30°C, reversion colonies were picked up under a stereo microscope and transferred onto fresh slants. The regeneration percentage of protoplasts refers to the proportion of the number of regenerated colonies to the total number of protoplasts plated. This was calculated

Table 2. Number of nuclei in each hyphal cell of *Poria cocos*.

Isolate	Number of nuclei (Average ± S.D.)	Clamp connection
Pc 1	3–25 (11.9 ± 6.0)	—[a]
Pc 2	2–22 (8.9 ± 3.9)	—
Pc 3	2–30 (10.2 ± 5.9)	—
Pc 4	3–23 (10.3 ± 5.2)	—
Pc 6	6–33 (16.1 ± 6.4)	—
Pc 7	4–44 (17.6 ± 9.7)	—
Pc 8	2–29 (11.4 ± 6.5)	—
Pc 10	3–44 (15.1 ± 10.1)	—
Pc 11	3–37 (15.9 ± 8.1)	—
Pc 13	5–42 (16.7 ± 9.4)	—
Pc 14	3–39 (11.7 ± 8.1)	—
Pc 15	2–30 (12.6 ± 8.2)	—
Pc 16	3–25 (12.4 ± 4.9)	—
Pc 17	5–66 (18.3 ± 10.5)	—
Pc 18	2–67 (19.3 ± 12.2)	—
Pc 30	2–32 (13.0 ± 6.9)	—
Pc 31	3–32 (15.5 ± 8.1)	—
Pc 32	3–38 (13.7 ± 7.7)	—
Pc 34	2–23 (9.9 ± 5.1)	—
Pc 36	3–69 (22.3 ± 13.2)	—
Pc 44	3–32 (12.7 ± 6.9)	—

Each strain was cultured on PDA for 3–7 days at 30°C.
Fifty cells were observed for each isolate.
a) No clamp connections were observed.

to be 20.2% in Pc 14 and 8.6% in Pc 30.

Mating Mating experiments were performed between the protoplast regenerants expressing type-II ADH-isozyme pattern and those expressing type III. They were inoculated on a same PDA plate in a 9-cm Petri dish. The inoculation positions of each regenerant were separated by a distance of 2 cm. After 3 to 4 days of incubation at 30°C, mycelia came into contact with each other. After 7 days of incubation, small pieces of mycelia were picked up from sites between the inoculation position and the edge of the plate, and each was transferred to a new slant.

Results and Discussion

Double staining of nuclei and cell walls showed that the hyphal cells of *P. cocos* were multinucleate and had no clamp connections (Fig.1, Table 2). The number of nuclei was different in each cell of individual hyphae. As shown in Table 2, the number of nuclei per hyphal cell ranged from 3 to 25 in Pc 1.

Dikaryotization and the presence of clamp connections are common criteria used to distinguish secondary hyphae from primary ones, but these can not be applied to *P. cocos*. Isozyme analysis was thus adopted to distinguish between primary and secondary hyphae of *P. cocos*. The ADH isozyme analysis revealed three types of banding patterns in 52 isolates of *P. cocos* (Fig. 2). As shown in Table 1, 9 isolates showed five bands, designated as type I; 35 isolates one slow band, type II; and 8 isolates one fast band, type III. The ADH isozyme pattern was not correlated with the locality of the isolates.

It has been reported that a primary mycelium could be obtained from a secondary one by protoplast manipulation (Kropp and Fortin, 1985; Raper, 1985; Kawabata et al., 1992; Fox et al., 1994). Protplasts were prepared from Pc 14, a typical type-I isolate, and cultured in PDA supplemented with 0.5 M mannitol as described in
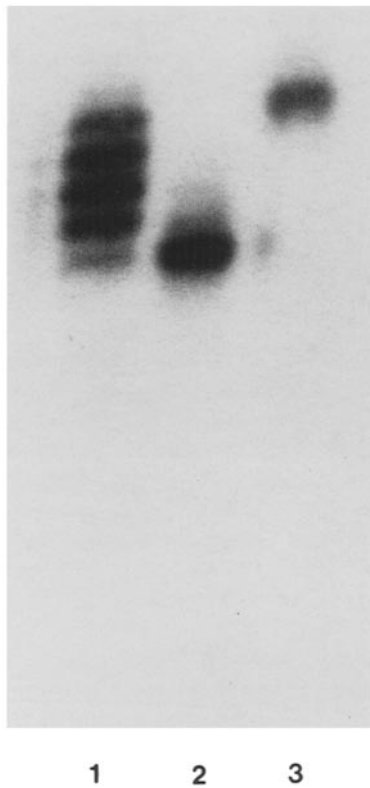
Fig. 2. Three types of ADH-isozyme banding patterns in *Poria cocos* isolates. The crude protein extracts were separated in an 11.1% starch gel by electrophoresis and localized ADH activity was visualized by staining as described in Materials and Methods. Lane 1 shows a typical pattern of five bands (type I). Lane 2, one slow band (type II). Lane 3, one fast band (type III).
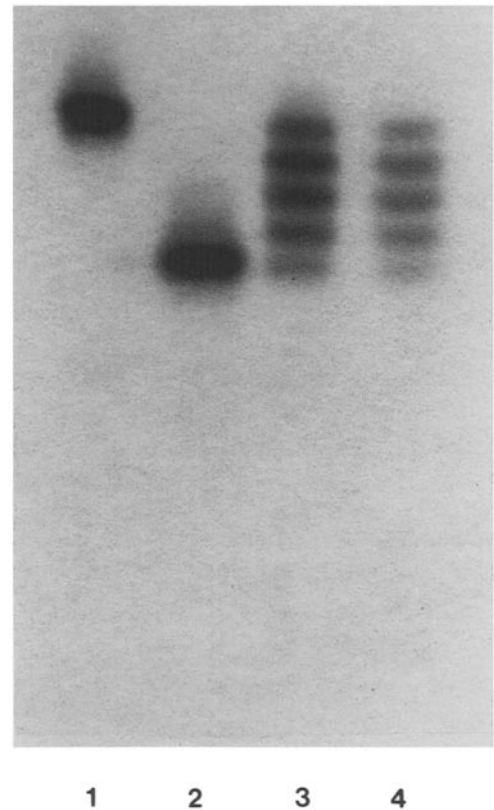

Fig. 3. ADH isozyme analysis of parents and mated hyphae. ADH isozyme analysis in two parents and two mated hyphae was performed as described in Materials and Methods. Two parents, Pc 14-P-16 and Pc 14-P-56, were regenerants from Pc 14 protoplasts. Two mated hyphae between Pc 14-P-16 and Pc 14-P-56 were isolated from sites between the inoculation position and edge of the plate. Lane 1: Pc 14-P-16; lane 2: Pc 14-P-56; lane 3: hyphae isolated from beside the inoculation site of Pc 14-P-16; lane 4: hyphae from beside that of Pc 14-P-56.

Materials and Methods. About 1000 colonies were regenerated, and we randomly isolated 61 regenerants (designated Pc 14-P-1 to Pc 14-P-61). The ADH-isozyme patterns of these regenerants showed not only type I, but also type II and type III. Fifty-two regenerants possessed type I, 2 regenerants type II, and 7 regenerants type III. It can be considered that the regenerants showing type-I phenotype were derived from heterokaryotic protoplasts, and those showing type II or type III were from monokaryotic or homokaryotic protoplasts. The ADH isozyme patterns of these regenerants were stable for more than 2 years.

Confronting culture of a type-III regenerant (Pc 14-P-16) and a type-II regenerant (Pc 14-P-56) was performed. Mycelia from both sides of the contact zone were isolated and examined by ADH isozyme analysis. Both showed type-I phenotype (Fig. 3). On the genetic basis of isozyme phenotypes, the ADH isozymes of *P. cocos* could be considered to be tetramers. The two types of primary hyphae produce two kinds of one homotetramer, and secondary hyphae produce five bands, consisting of two homotetramers and three heterotetramers (Micales et al., 1986). Thus we assumed that the hyphae of type-I phenotype obtained from the confronting culture

may be a mated mycelium. This result suggested that both type-II and type-III hyphae obtained from the type-I isolate via protoplasts were primary hyphae.

To support our assumption, protoplasts were prepared from eight other isolates carrying the type-I phenotype, Pc 1, Pc 2, Pc 3, Pc 6, Pc 7, Pc 10, Pc 16 and Pc 44, and were allowed to regenerate. The ADH-isozyme phenotypes of the regenerant are shown in Table 3. The results were coincided with those of Pc 14. Type-II and type-III regenerants were obtained from type-I isolates, Pc 2, Pc 3, Pc 7, Pc 10, Pc 16 and Pc 44. Confronting culture of a type-II regenerant (Pc 2-P-11) and a type-III regenerant (Pc 2-P-49) was carried out in the same way as for Pc 14. Hyphae isolated from sites between the inoculation position and the edge of the plate possessed type-I phenotype, as in the case of Pc 14. Other pairs of type-II and type-III regenerants derived from Pc 3, Pc 7, Pc 10, Pc 16 and Pc 44, respectively, gave the same results.

It is of interest to note that only type-III and type-I regenerants were obtained from Pc 1, and only type-II

Table 3. The ADH isozyme phenotypes of the regenerants derived from protoplasts of type-I isolates of *Poria cocos*.

Isolate	Number of regenerants			
	Type I	Type II	Type III	Total
Pc 1	113	0	9	122
Pc 2	51	1	1	53
Pc 3	68	3	1	72
Pc 6	140	5	0	145
Pc 7	46	4	1	51
Pc 10	73	2	2	77
Pc 14	52	2	7	61
Pc 16	41	1	1	43
Pc 44	85	1	1	87

and type-I regenerants were obtained from Pc 6. It has been reported that dedikaryotization via protoplasts gave asymmetric numbers of regenerants of the two nuclear types which deviated from a 1 : 1 ratio in some basidiomycetes (Raper, 1985; Kawabata et al., 1992; Fox et al., 1994). Kawabata et al. (1992) reported that

the ratio of segregation of nuclear types depended on the strain in *Flammulina velutipes* (Curt.: Fr.) Sing. They reported that a ratio of mating type of monokaryotic regenerants of 97 : 0 in one strain. The same phenomenon may be observed in our experiments, in the cases of Pc 1 and Pc 6.

The hyphal cells of Pc 14-P-16 and Pc 14-P-56, which were considered to be primary hyphae, were also multinucleate (Fig. 4), and the distribution of the number of nuclei in their hyphae was similar to that in Pc-14 hyphae (Fig. 5). Thus, in *P. cocos*, both of the primary and the secondary hyphal cells are multinucleate. Lindsey and Gilbertson (1977) reported that *Poria carnegiea* Baxter possessed multikaryotic primary hyphae and dikaryotic secondary ones. They described that the secondary hyphae carried clamp connections. It should be stressed that the secondary hyphae of *P. cocos* lack clamp connections and are multinucleate, in contrast to *P. carnegiea*. These characteristics seem to be species-specific in *Poria*. No morphological difference between the primary (Pc 14-P-16 and Pc 14-P-56) and secondary hyphae (Pc 14) was noted on microscopic observation.

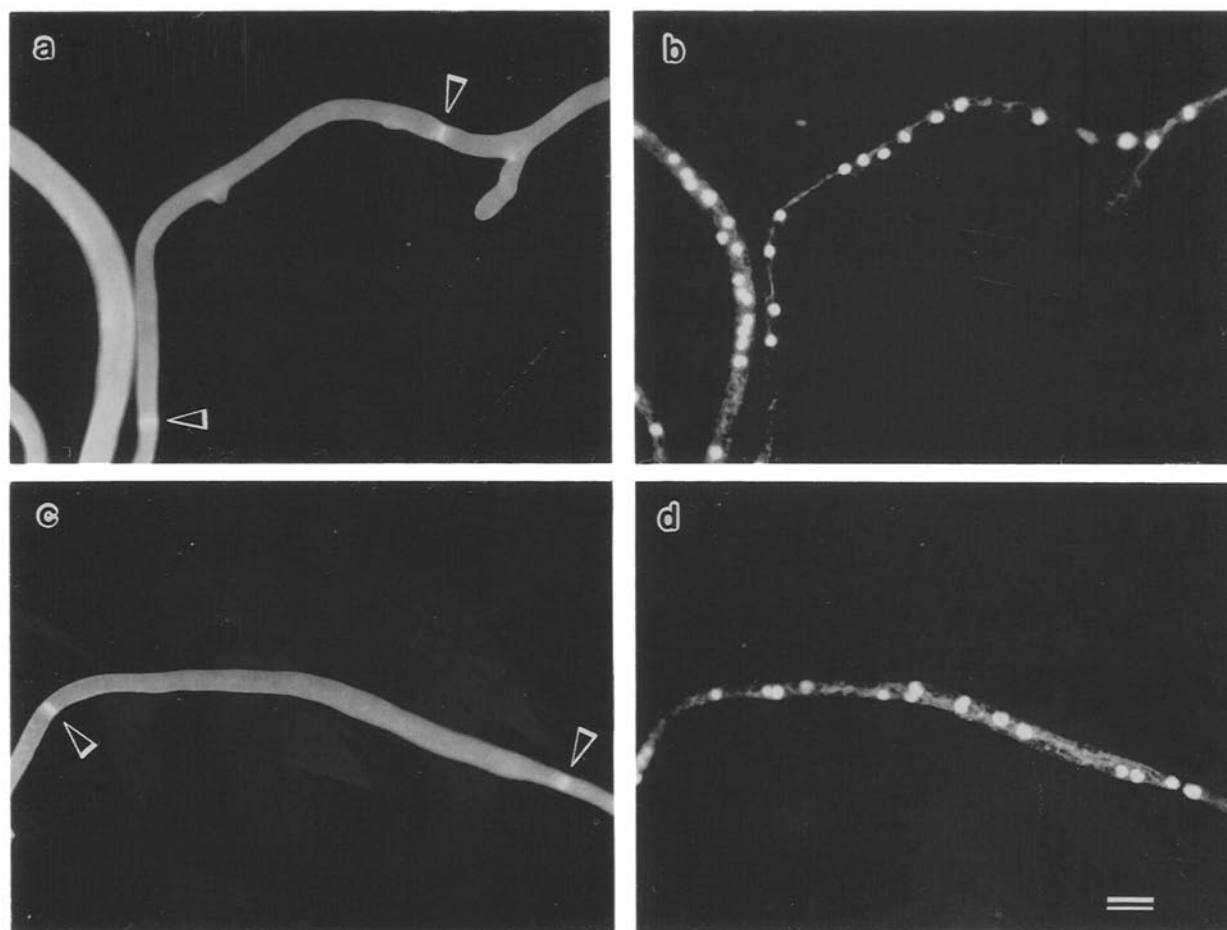Isolation of primary hyphae via protoplasts and the

Fig. 4. Fluorescent micrographs of primary hyphae, Pc 14-P-16 and Pc 1 4-P-56. a and b: hyphae of Pc 14-P-16 observed in the same visual field; c and d: hyphae of Pc 14-P-56. Excitation wave lengths were 330 ~ 380 nm for a and c, and 510 ~ 560 nm for b and d. Both hyphae are multinucleate. Arrow heads indicate septa. Bar indicates 10 μm.
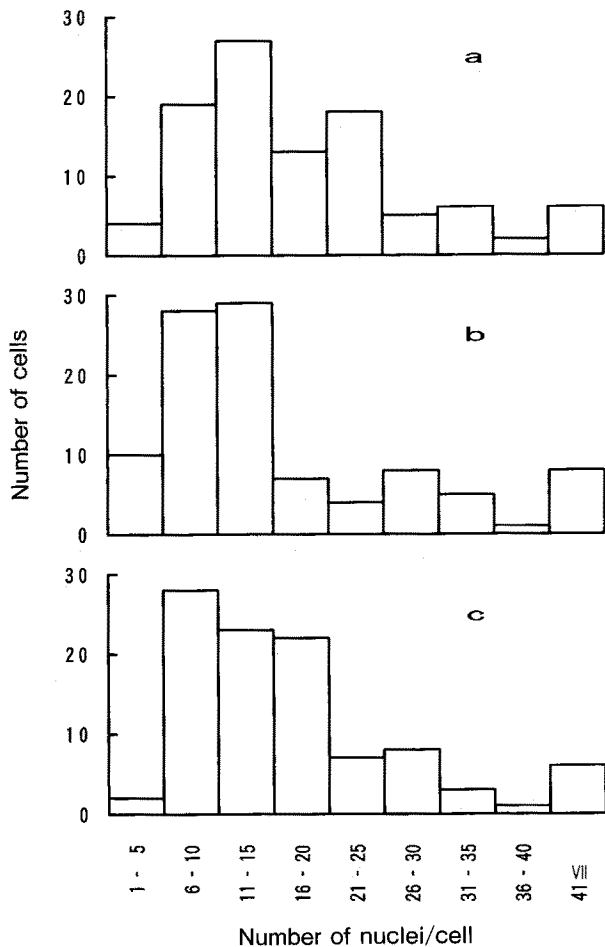
Fig. 5. Distribution patterns of the number of nuclei in each hyphal cell. Hyphae of each specimen were stained with propidium iodide and Calcofluor white M2R. a: Nuclei in each hyphal cell of natural isolate, Pc 14. b and c: Nuclei in hyphal cell of protoplast regenerants, Pc 14-P-16 and Pc 14-P-56, respectively. The number of nuclei in 100 cells of each specimen was counted under the fluorescence microscope.
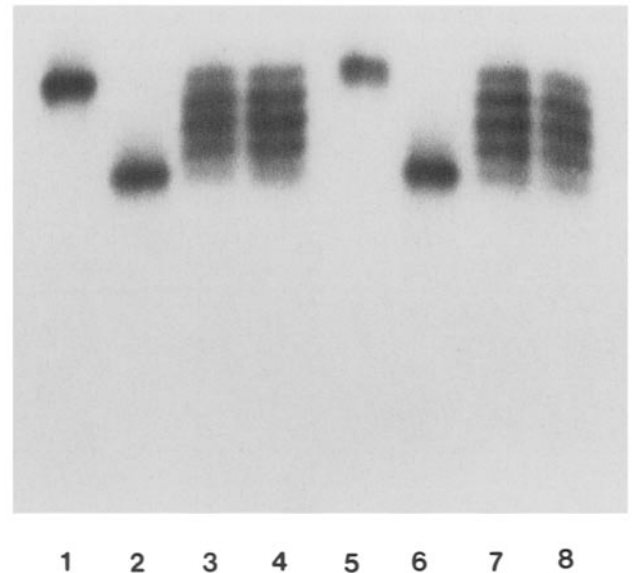

Fig. 6. Zymograms of mated hyphae. ADH isozyme analysis in three parents and four mated hyphae was performed as described in Materials and Methods. Two parents, Pc 30-P-39 and Pc 30-P-54, were regenerants from Pc 30 protoplasts. Lanes 1 and 5: Pc 14-P-16; lane 2: Pc 30-P-39; lane 6: Pc 30-P-54; lanes 3 and 4: mated hyphae obtained from Pc 14-P-16×Pc 30-P-39; lane 3: from Pc 14-P-16 side; lane 4: from Pc 30-P-39 side; lanes 7 and 8: mated hyphae from Pc 14-P-16×Pc 30-P-54; lane 7: from Pc 14-P-16 side; lane 8: from Pc 30-P-54 side.

confirmation of a secondary mycelium by the ADH-isozyme pattern might be a useful method in mating studies of *P. cocos*. The natures of the sclerotium of *P. cocos*, cultivability and color, depend on the strain. It is interesting to conduct inter-strain mating of *P. cocos*. Pc 14 and Pc 30, which were obtained from different localities (Table 1), were mated. Protoplast manipulation was performed in Pc 30, which possessed the type-II isozyme pattern (Table 1), and 61 regenerants were obtained (Pc 30-P-1~Pc 30-P-61), all of which showed the type-II ADH-isozyme pattern. Confronting cultures between these regenerants and Pc 14-P-16 (type-III) gave rise to mated hyphae carrying the type-I ADH-isozyme pattern in two combinations, Pc 14-P-16×Pc 30-P-39 and Pc 14-P-16×Pc 30-P-54 (Fig. 6). The other 59 combinations did not produce type-I mated hyphae. They showed the same ADH-isozyme patterns as the parental strains, and could be considered as secondary hyhae or incompatible primary ones. We have succeeded in mat-

ing two isolates of *P. cocos*, Pc 30 and Pc 14. The ADH-isozyme patterns of these mated hyphae were stable for more than 2 years. Further experiments are in progress to characterize these mated hyphae.

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