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FULL PAPER

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Isolation and characterization of a sporeless mutant in *Pleurotus eryngii*

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Abstract A sporeless mutant dikaryon, completely defective in sporulation, was isolated from mycelial protoplasts of *Pleurotus eryngii* mutagenized by UV irradiation. Newly established dikaryons between one component monokaryon from the mutant, and 12 different wild type monokaryons from 3 other wild type dikaryons, all exhibited the sporeless phenotype, whereas those between the other monokaryon and the same wild type monokaryons all produced normal fruiting bodies. These results indicated that the sporeless mutation was induced in one of two nuclei of the mutant and was dominant. In the wild type basidia, the pattern of nuclear behavior during sporulation corresponded to the pattern C nuclear behavior as defined by Duncan and Galbraith. Cytological observation revealed that in the sporeless mutant meiosis was blocked at the meta-anaphase I in most basidia and hence basidiospores and sterigmata were not produced. Although fruiting bodies of the sporeless mutant showed a somewhat leaning growth, their gross morphology and its fruiting body productivity were comparable to that of the original wild type strain. Based on these results, it was considered that the sporeless mutant could serve as a potential material in breeding of sporeless *P. eryngii* commercial strains.

Key words Nuclear behavior · *Pleurotus eryngii* · Sporeless mutation

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Introduction

Basidiospore formation following meiosis is an essential and important process for sexual reproduction in the basidiomycetes. However, the enormous spores produced by the fruiting bodies cause many serious problems in mushroom cultivation, the most serious being the occurrence of respiratory allergic reactions among people who are working in mushroom farms. These symptoms, called the mushroom workers lung, are provoked by the inhalation of spores. Spreading spores also give rise to other problems such as increased populations of infesting bacteria and mold in the cultivation facilities, reduced commercial value because of spores deposited on mushrooms, and erosion of genetic variation in natural populations of the mushroom species. In avoiding these problems, sporeless strains, which fail to produce basidiospores, may be useful.

So far, spontaneously occurring sporeless mutants have been found from natural populations in some basidiomycetes including *Coprinus cinereus* (Schaeff.: Fr.) S.F. Gray [= *C. lagopus* Fr.; = *C. macrorhizus* (Pers.: Fr.) Rea] (Day 1954; Gibbins and Lu 1982), *Pleurotus* spp. (Egar et al. 1976; Ohira 1979), *Schizophyllum commune* Fr. (Bromberg and Schwalb 1977), and *Lentinula edodes* (Berk.) Pegler [= *Lentinus edodes* (Berk.) Sing.] (Hasebe et al. 1991). Furthermore, mutagenesis by chemical treatment and UV irradiation has been applied for induction of sporeless mutants in *Coprinus cinereus* (Takemaru and Kamada 1972; Kanda et al. 1989), *Pleurotus ostreatus* (Jacq.: Fr.) Kummer and *P. pulmonarius* (Fr.) Quel (Imbernon and Labarere 1989), and *Agrocybe cylindracea* (DC.: Fr.) Maire [= *A. aegerita* (Bringanti.) Singer] (Murakami 1993; Kiuchi 1998). It has been reported that the mutations responsible for those sporulation defects were recessive or dominant and that most of the sporulation blockages are caused by aberration of the meiotic process or sterigma formation for sexual reproduction (Tani et al. 1977; Kanda et al. 1989; Murakami 1998). Futhermore, molecular genetic approaches have been applied for construction and characterization of

sporulation-defective mutants (= meiotic mutants) in *C. cinereus* (Cummings et al. 1999).

Pleurotus eryngii (DC:Fr.) Quél. is a tetrapolar basidiomycete that belongs to Pleurotaceae (Agaricales) and is distributed widely in areas of southern Europe, central Asia, and North Africa. This fungus is also well known to be weakly parasitic on the roots of *Eryngium* species (Hilber 1982; Lewinshon et al. 2001). In Japan, *P. eryngii* has recently become popular as a new edible mushroom because of its good taste, and its production has increased rapidly in recent years. However, as *P. eryngii* cultivation was extended, the major drawbacks in relation to spores as mentioned earlier became intensified, because the fruiting body produces a huge number of spores during its growth period. Accordingly, research on the development of sporeless strains has practical importance. This article describes the first isolation of a sporeless mutant in *P. eryngii* and its cytological and genetic characteristics.

Materials and methods

Strains

A wild-type dikaryon, NPO010 (*A1B1* + *A2B2*), of *Pleurotus eryngii* was used for an original strain for mutagenesis treatment. Three other wild type dikaryotic strains from Germany (*A3B3* + *A4B4*), WC827 (*A9B9* + *A10B10*) and ATCC90212 (*A25B25* + *A26B26*), were used as the control. They were maintained on MA plates (2% malt extract and 1.5% agar).

Preparation and culture of protoplasts

Protoplast production was carried out following the procedure of Fukumasa-Nakai et al. (1994). The dikaryon was grown on liquid MYG broth (2% malt extract, 0.2% yeast extract, and 2% glucose) at 25°C for 2 weeks. Mycelia were fragmented with a Waring blender at 10000 rpm for 15 s. Portions of 2 ml of the mycelial suspension were inoculated into 100-ml Erlenmyer flasks containing 15 ml MYG broth. The flasks were incubated statically at 25°C for 3 days. After the incubation, the mycelium was harvested, washed once with sterilized water and 0.6 M mannitol solution by centrifugation at 800 *g* for 10 min, then incubated in the enzyme mixture of 1.0% lysing enzyme (Sigma, St. Louis, MO, USA) and 0.3% chitinase (Wako, Osaka, Japan) dissolved in the 0.6 M mannitol solution at 28°C for 2 h with shaking (90 strokes/min). The mixture of protoplasts and undigested hyphae was passed through a 3G2 glass filter to remove hyphal debris. The protoplasts collected were washed twice with the 0.6 M mannitol solution by centrifugation at 600 *g* for 6 min. The resulting protoplasts (approximately 1×10^8 protoplasts/100 mg fresh weight mycelium) were suspended in the 0.6 M mannitol solution at a concentration of 5×10^6 protoplasts/ml.

Induction of sporeless mutants

One hundred microliters of the protoplast suspension was spread out onto Petri dishes (90 mm in diameter) containing 15 ml semisynthetic medium (casamino acid, 2 g; glucose, 20 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 1.0 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $FeSO_4 \cdot 7H_2O$, 1 mg; $ZnSO_4 \cdot 7H_2O$, 0.9 mg; $MnSO_4 \cdot 4H_2O$, 0.8 mg; H_3BO_3, 1 mg; $CuSO_4 \cdot 5H_2O$, 0.15 mg; $Co(NO_3)_2$, 0.1 mg; $(NH_4)_6Mo7O_{24} \cdot 6H_2O$, 0.2 mg; thiamine hydrochloride, 0.1 mg; adenine hydrochloride, 5 mg; and agar, 15 g per liter of distilled water) containing 0.5 M sucrose as osmotic stabilizer. Ultraviolet (UV) light was used as a mutagen. The plates were irradiated with a Toshiba 10-W germicidal lamp 10 cm apart for 17–22 s in the darkroom. The survival rate of protoplasts after this treatment was 1%–3%. The plates were then incubated at 25°C in the dark for 10 days. Regenerated colonies from protoplasts were examined microscopically for the formation of clamp connections indicative of dikaryons. Dikaryotic regenerates with clamp connections, namely protoclones, were randomly isolated and maintained on MA slants.

Fructification test

To examine morphological variation of the fruiting bodies, protoclones were cultured on a sawdust basal substrate [sawdust of Sugi (*Cryptomeria japonica*):corn cob:wheat bran:rice bran = 3:1:1:1 in dry weight; moisture content, 65%] in test tubes (length, 98 mm; inner diameter, 22 mm). About 20 g (in fresh weight) of the substrate was packed into a test tube. The test tubes were capped by aluminum foil and sterilized for 30 min at 121°C. After cooling, the medium was inoculated with mycelial plugs from the MA slant culture. The cultures were incubated for 30–40 days at 23°–25°C, 75%–85% relative humidity (RH). Then the surface of the culture was removed to induce fruiting body formation. The cultures were transferred to a fruiting room maintained at 15°–17°C, 90% RH, and continuously illuminated (200 lx) by fluorescent lamps. Sporeless fruiting bodies were readily distinguished from those of the wild type by the absence of white spore prints on the black paper that was set under the fruiting bodies. Morphological differences between the wild type and the mutant fruiting bodies were examined by the conventional bottle cultivation using an 800-ml polypropylene bottle with sawdust basal medium for the condition as described earlier. To investigate temperature sensitivity of the mutant, some cultures forming fruiting body primordia were transferred to 10° or 25°C growth conditions.

Light and electron microscopy

For light microscopy, the HCl-Giemsa staining method after Aist (1969) was employed as described below. Small pieces of lamella were fixed with ethanol:acetic acid (3:1, v:v) for 5 h at 5°C, and then transferred to 35% ethanol solution for 15 min. They were rinsed by tap water for 15 min, followed by hydrolysis for 6–8 min in 1N HCl at

60°C, then washed in cool tap water for 15 min. The samples were soaked in 1/15 M phosphate buffer (pH 7.0) for a few minutes, and stained for more than 2 h in Giemsa solution (Sigma, St. Louis, MO, USA) at 5°C. After staining, the sample was mounted on a slide glass and squashed under a cover slip.

The procedures for fixation and dehydration of materials for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were the same as those described by Tsuneda et al. (1986) and Nakai and Ushiyama (1974), respectively. For SEM, small pieces of lamella were fixed with 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 4 h at 5°C. The fixed samples were washed in buffer and postfixed in 2% O_SO_4 for 2 h. They were then dehydrated in an ethanol series followed by washes in a graded series of amyl acetate solutions in ethanol. The samples in absolute amyl acetate were critical-point dried in a Hitachi HCP-2 unit using carbon dioxide. All materials were coated with gold and examined with a Hitachi S-2250N. For TEM, small pieces of lamella were fixed with 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 2 h at 5°C. The fixed samples were washed in buffer and postfixed in 2% O_SO_4 for 2 h. They were then dehydrated in a graded ethanol series, transferred to propylene oxide, and embedded in Epon mixture resin. Thin sections were cut on an LKB ultratome with a glass knife, and the sections were stained with 2% uranyl acetate for 3 min followed by 3% lead citrate for 1 min. The samples were observed in a JEM-100CX electron microscope.

Results

Isolation of sporeless mutants

Of a total 7977 dikaryotic protoclones of the NPO010 original strain mutagenized with UV irradiation, only two protoclones were found to fail to sporulate (Fig. 1). Although many other morphological variants in fruiting body development were also detected at a rate of 35% frequency, we concentrated on one of the sporeless mutants, U2553 strain in this study.

To examine the stability of the sporeless mutation of the U2553 strain, dikaryotic tissue isolates from the mutant fruiting bodies were serially transferred at 1-month intervals on sawdust basal medium. For each transfer, a part of the mycelial colony grown in sawdust basal medium was cultured for fruiting to check the stability of the sporeless phenotype. As a result, it was found that the sporeless mutant phenotype was mitotically stable and maintained over 1 year.

Genetic characteristics of the sporeless mutation

The U2553 sporeless mutant was tested for dominance in dikaryons with the compatible wild type monokaryons. Two component monokaryons (neohaplonts) from the sporeless mutant and those from its parental wild type strain were

Fig. 1. Fruiting bodies produced by the sporulating protoclone (*left three*), the U2553 sporeless mutant (*middle three*), and the parental wild type (*right three*) of *Pleurotus eryngii*. White spore prints were not detected on the black papers under the pileus of the U2553 sporeless mutant (*middle three*). *Bar* 20 mm

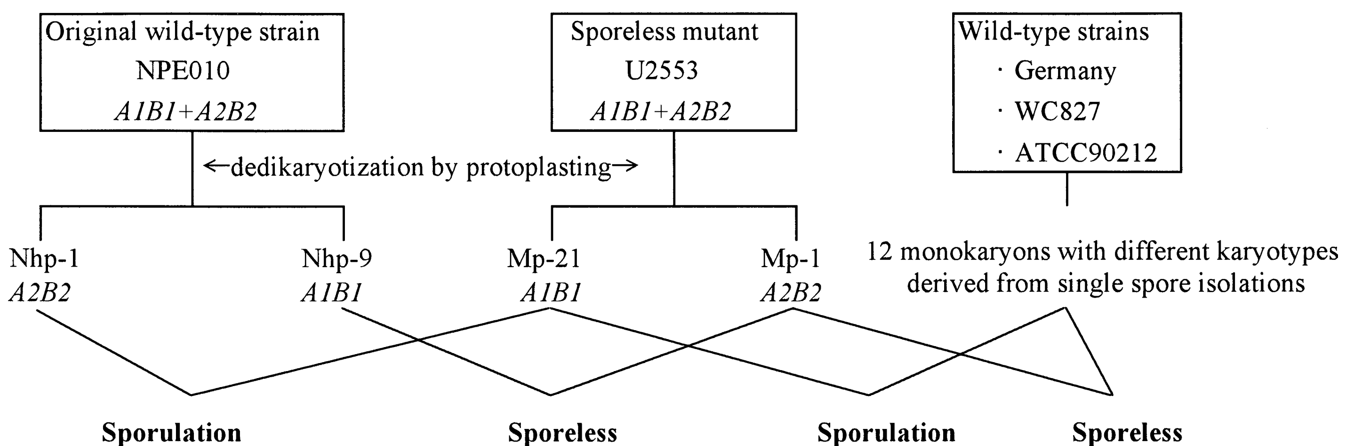

Fig. 2. Mating tests for confirming the dominant character of the sporeless mutation of *Pleurotus eryngii* obtained in this study

obtained by dedikaryotization using the protoplast regeneration method as previously described. The two component monokaryons (Mp21; *A1B1* and Mp1; *A2B2*) from the mutant were mated to those (Nhp9; *A1B1* and Nhp1; *A2B2*) from the parental wild type strain in compatible combinations (Fig. 2). The dikaryon between Mp1 and Nhp9 formed sporeless fruiting bodies. The dikaryon between Mp21 and Nhp1 formed normal fruiting bodies bearing numerous basidiospores. In addition, the Mp1 and Mp21 monokaryons were respectively mated to 12 compatible, basidiospore derivative monokaryons from three other wild type dikaryons: Germany (*A3B3* + *A4B4*), WC827 (*A9B9* + *A10B10*), and ATCC90212 (*A25B25* + *A26B26*). Of 24 dikaryons thus formed, all 12 that have the Mp1 monokaryon as a parent were shown to produce sporeless fruiting bodies. These results indicated that the sporeless trait of this mutant was the result of a dominant mutation induced in the Mp1 nucleus.

Fig. 3. Morphology of fruiting bodies produced by the parental wild-type strain (*left*) and the U2553 sporeless mutant of *Pleurotus eryngii* (*right*). *Bar* 50 mm

Morphological and cytological characterization of the sporeless mutant

The gross morphology of the mutant fruiting bodies was somewhat different from those of the original wild type strain, as they showed a leaning growth pattern (Fig. 3a,b). However, the mutant readily produced fruiting bodies at a timing similar to that of the parental strain, and its fruiting body productivity was indistinguishable from that of the original strain (Table 1).

In the hymenium of wild type fruiting bodies, many basidia at different developmental stages were observed by scanning electron microscopy (Fig. 4a). The basidia usually formed four sterigmata each whose apices swelled and developed into a basidiospore. In contrast, the hymenium of mature fruiting bodies of the sporeless mutant was composed of basidia of different sizes that had no sterigmata or basidiospores (Fig. 4b). Most of those basidia seemed to be arrested at a certain stage during development. Similar hymenial structures were observed on the mutant fruiting bodies grown at 10° or 25°C, suggesting that the sporeless mutation was not temperature sensitive.

To further examine cytological sporeless characteristics of the mutant, nuclear behavior following basidial development was studied in comparison with the wild type strain by light microscopy using the HCl-Giemsa staining method. Normal sexual sporulation in the wild type strains was observed to progress as follows. A conjugate mitotic division occurred at the base of basidial initials (Fig. 5a). The newly formed young basidia contained two haploid nuclei and were cylindrical in shape, two haploid nuclei fused at the middle portion of the basidial cell. After nuclear fusion, the resulting diploid nucleus moved nearly to the apex region of a clavate basidium (Fig. 5b,c), and its first meiotic division occurred (Fig. 5d). Successively, the second meiotic division occurred, and four daughter nuclei appeared (Fig. 5e). The tetrad nuclei at once moved back nearly to the middle portion of a basidium. Then, four sublate-shaped sterigmata developed at the apex of a basidium, and their tip parts swelled to form basidiospores (Fig. 5f,g). When the

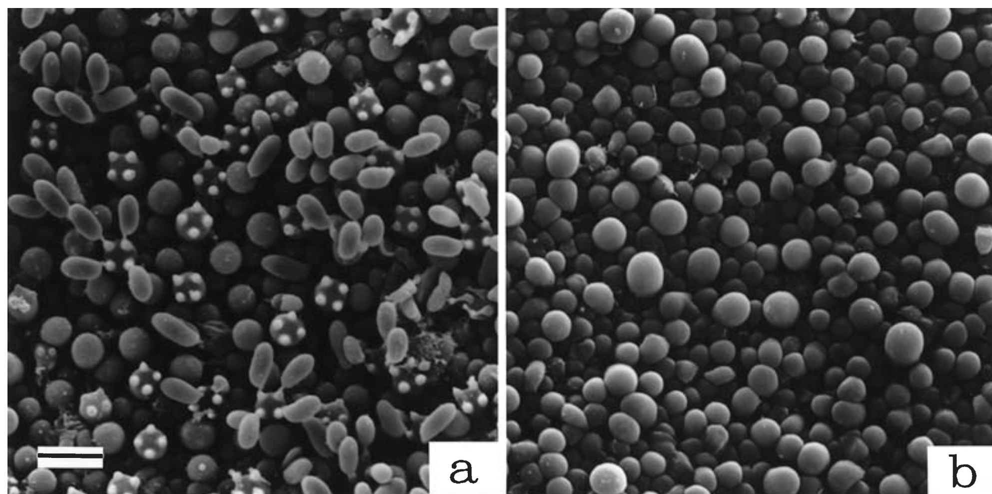

Fig. 4. Scanning electron micrographs of gill surfaces of the parental wild type (**a**) and the U2553 sporeless mutant (**b**) of *Pleurotus eryngii*. *Bar* 10 µm

Table 1. Fruiting body productivity of the wild type and the sporeless mutant in *Pleurotus eryngii*

Strain	Number of fruiting bodies per bottle		Yeilds of fruiting bodies per bottle (fresh weight, g)	Total cultivation period (days)
	Pileus diam ≧ 5 cm	Pileus diam < 5 cm		
Parental wild type	2.9 ± 1.1[a]	1.9 ± 1.1[a]	131.6 ± 14.5[a]	56.6 ± 0.5[a]
Sporeless mutant	1.9 ± 1.0	4.0 ± 2.4	134.9 ± 17.0	59.9 ± 1.7

Fruiting body productivity was examined by the conventional bottle cultivation using 800-ml polypropylene bottle with sawdust basal medium
[a] Average ± standard deviation, $n = 32$

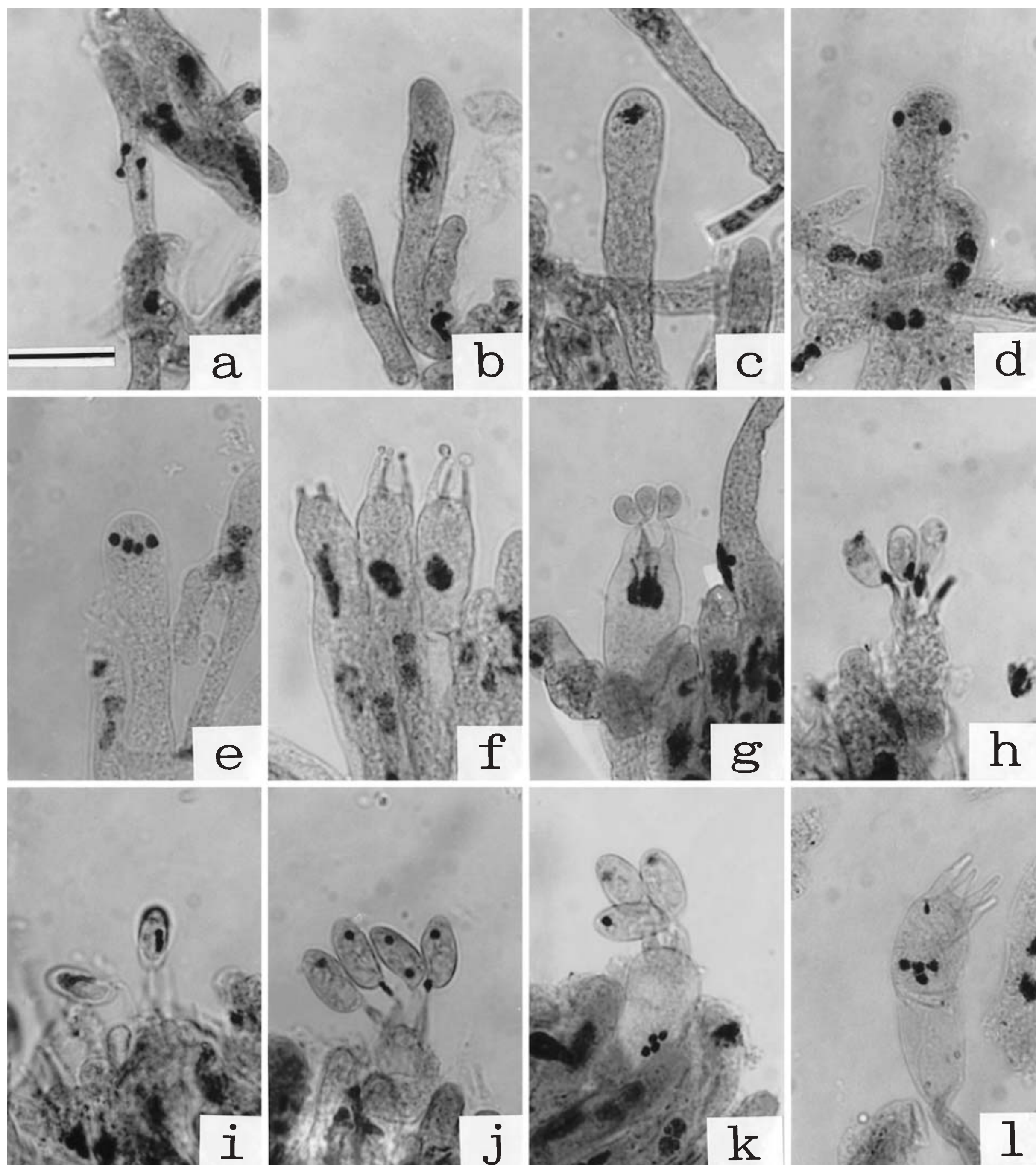

Fig. 5. Light micrographs of meiosis and basidiospore development in the wild type of *Pleurotus eryngii*. Nuclei were stained by HCl-Giemsa staining. A conjugate miotic division occurs at the base of basidial initials (**a**). Prefusion nuclei in young basidia (**b**). Prophase I (**b**). Anaphase I (**c**). Interphase (**d**). Nuclear tetrad (**e**). Basidium with meiotic tetrad and developing sterigmata and spores (**f**, **g**). Nuclei entering spores (**h**). Postmeiotic mitosis occurring in basidiospores (**i**). One nucleus remained in the spore, but one returned to the basidium (**j**). One nucleus in each basidiospore and four nuclei in the basidium (**k**). Basidium containing four nuclei after basidiospore discharge (**l**). All figures are same magnification. *Bar* 10 μm

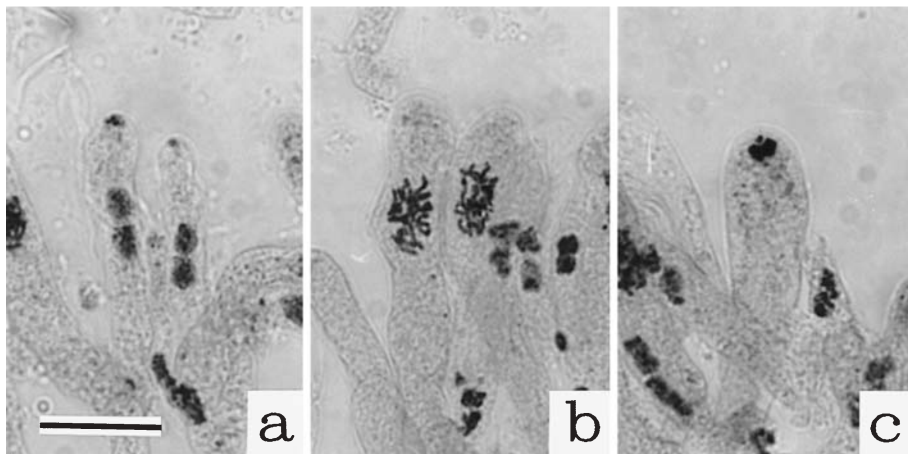

Fig. 6. Light micrographs showing uncompleted meiosis in basidia of the U2553 sporeless mutant of *Pleurotus eryngii*. **a** Prefusion nuclei in young basidia. **b** Prophase I. **c** Meta-anaphase I. *Bar* 10 μm

basidiospores were nearly mature, each of four daughter nuclei initiated movement into their respective basidiospores through sterigmata (Fig. 5h). The migrating nucleus underwent mitosis when entering the basidiospore (Fig. 5i). One nucleus remained in the basidiospore and the other returned to the basidium (Fig. 5j,k). After the basidiospore discharged, each basidium had four nuclei (Fig. 5l).

In the sporeless mutant, the sexual reproduction processes from initiation of basidial cell formation to the meta-anaphase I stage of meiosis occurred normally and were comparable with those of the wild type (Figs. 6a,b,c). Electron microscopy of thin sections of the synaptonemal complexes showed no recognizable difference in their fine structures between the sporeless mutant and the wild type strains (Fig. 7). However, in the mutant fruiting bodies no basidia were observed to proceed to advanced stages beyond the meta-anaphase I of meiosis.

Discussion

The cytological features of basidial development followed by meiosis during basidiosporogenesis in *P. eryngii* were similar in general to those of many other basidiomycetes of Agaricales (Mueller et al. 1993; Hibbett et al. 1994). The pattern of nuclear behavior during sporulation of *P. eryngii* corresponded to the pattern C nuclear behavior as defined by Duncan and Galbraith (1972): a single postmeiotic nuclear division occurs in each basidiospore while it is still attached to the sterigma, and then one of the two daughter nuclei moves back to the basidium. The present observation did not agree with the previous report describing the three species of the *P. eryngii* complex to have pattern A nuclear behavior (Slézec 1984).

In the present study, only two sporeless mutants could be induced by artificial mutagenesis using UV irradiation (2/7977, 0.03%). Takemaru and Kamada (1972) attempted the induction of morphological mutants of fruiting body development in *Coprinus cinereus* by UV, *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (NG), or 5-bromo-uracil (BU) treatments for the dikaryotic hyphal fragments; they reported that UV and NG were very efficient in inducing the mutants and that sporeless mutants were induced at a rate of 4.4% and 12.8%, respectively. For this extremely low rate of induction of sporeless mutants in *P. eryngii*, however, the reason remains unexplained.

The sporeless mutant U2553 of *P. eryngii* isolated in this study was shown not to be able to form any sterigmata or basidiospore structures. The present cytological observations showed that the blockage of meiosis at the meta-anaphase I stage is the cause of this sporulation deficiency. Kanda et al. (1989) and Murakami (1998) performed cytological investigations on sporeless mutants of *C. cinereus* and *A. cylindracea* induced by UV irradiation, respectively, and noticed that the mutants were generally classified into the following four types according to the stage of the blockage: (1) meiosis stopped at meta-anaphase I(MA-I type); (2) meiosis completed, but further basidial development did not occur (tetrad, Te type); (3) basidial development stopped at the sterigma stage (sterigma, St type); and (4) basidial development stopped at the prespore stage (prespore, PS type). They have also reported that most of the sporeless mutants were of the MA-I or St type. The type of the present U2553 mutant of *P. eryngii* was seen to correspond cytologically to the MA-I type.

So far, sporeless mutants have been obtained in many basidiomycetes including commercially important mushrooms, and the mutations responsible for these sporulation defects have been reported to be dominant or recessive to the wild type (Takemaru and Kamada 1972; Egar et al. 1976; Bromberg and Schwalb 1977; Tani et al. 1977; Ohira 1979; Gibbins and Lu 1982; Imbernon and Labarere 1989; Hasebe et al. 1991; Kanda et al. 1989; Murakami 1998). Further, the recessive mutants have been known to have single gene mutations. On the other hand, it has been suggested that the dominant mutants may represent regulatory gene mutations because almost all the dominant sporeless mutants of *C. cinereus* showed temperature sensitivity; they showed normal sporulation at low temperature (Tani et al. 1977; Kanda et al. 1989). Of the U2553 sporeless mutant of *P. eryngii*, the mutation trait was revealed to be dominant

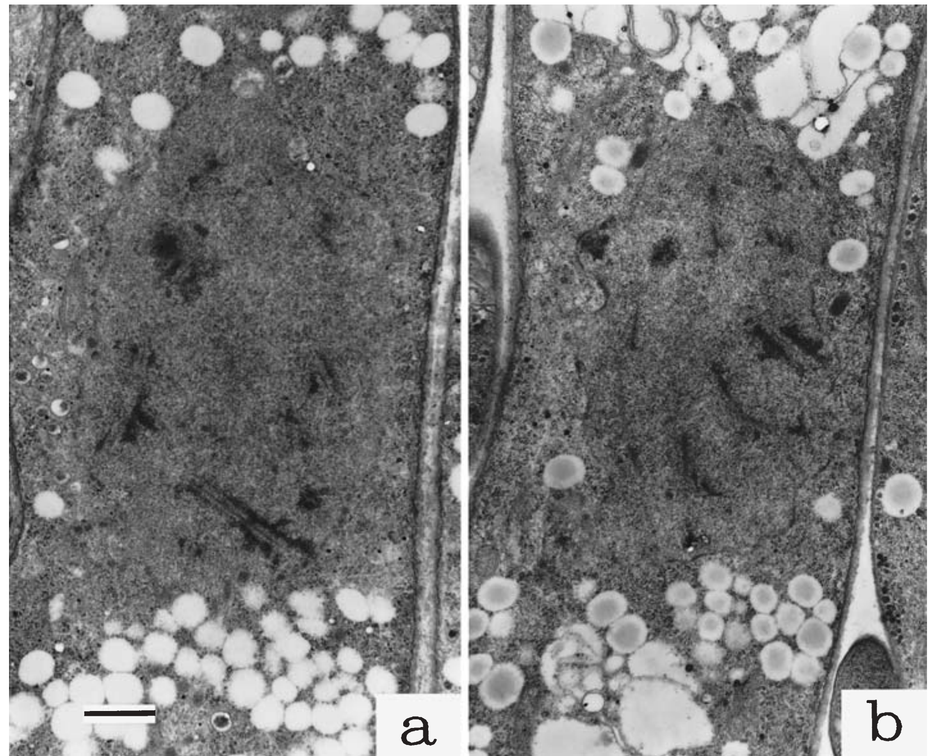

Fig. 7. Transmission electron micrographs of longitudinal basidial sections of the original wild type (**a**) and the U2553 sporeless mutants (**b**) of *Pleurotus eryngii*. Some synaptonemal complexes are similarly observed in both sections. *Bar* 1 μm

and not to be temperature sensitive. However, in this study we could not perform further genetic analysis to clarify how many gene defects are involved in the dominant sporeless phenotype of the U2553 strain because of the stable dominant sporeless phenotype.

The dominant sporeless mutation of *P. eryngii* could be inheritable to any strains by conventional crossings and did not exert a deleterious influence on the fruiting body morphology and productivity in the trait-transferred strains. Therefore, it was considered that the U2553 sporeless mutant might be a potential genetic resource for breeding of sporeless commercial strains of *P. eryngii*. Moreover, as this fungus is not originally living in Japan, the use of sporeless strains in commercial cultivation would be also significant in avoiding the risks of aggression of alien species against domestic fungal populations and the possibility of attacks of such species on plants of Apiaceae inhabiting or cultivated in Japan.

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