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NOTE

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Preparation and crossing of mating-capable monokaryons via protoplasting of the dikaryotic mycelia of a mycorrhizal mushroom, *Lyophyllum shimeji*

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Abstract In order to develop a novel method of obtaining monokaryons for a mycorrhizal fungus, *Lyophyllum shimeji*, monokaryotization of dikaryotic stock culture via protoplast formation and regeneration was performed using 12 dikaryotic stocks. From 6 dikaryotic stocks, a total of 120 monokaryons were isolated, and their mating compatibility was tested. Mating-compatible monokaryons were successfully derived from a dikaryotic stock (NBRC 100325), and monokaryons of only 1 mating type relative to the parental dikaryons were isolated from another 3 strains (MH01710, OK2L-1, and HY7L-1). We successfully prepared monokaryotic stocks via protoplast monokaryotization, a technique that can be used to identify biological species of *L. shimeji*. This technique could be used for breeding various mycorrhizal mushrooms, including *Tricholoma matsutake*, for which the preparation of monospore cultures is extremely difficult.

Key words Biological species · *Lyophyllum shimeji* · Mushroom breeding · Mycorrhizal mushroom · Protoplast monokaryotization

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Introduction

The concept of the morphological species is dominant in fungal taxonomy: most fungi are initially classified with reference to their morphological characteristics. Another important taxonomic concept is that of the biological species. If two species are sexually intercompatible, they are grouped as one biological species. Given the broad acceptance of this idea, mating compatibility tests have been used to evaluate the final taxonomic identity of fungal species classified using morphological characters.[1–3]

Lyophyllum shimeji is a mycorrhizal fungus[4] and is known as a delicious wild mushroom in Japan. Generally speaking, *Tricholoma matsutake* is the best in terms of aroma, but in terms of taste, *L. shimeji* is the best. The ectomycorrhizal fungi such as *L. shimeji* and *T. matsutake* are difficult to cultivate artificially without the host plant. However, the fruiting bodies of *L. shimeji* have been recently cultivated on an artificial medium using barley grain without the host plant and the culture conditions for mass production of this mushroom were determined.[5–10] These results suggested that a quantity of sufficient starch used as a carbon source was able to supply the factor that allows successful fruit body formation without raising osmotic pressure in the medium. Amylases and starch-hydrolyzing ability of the *L. shimeji* strain are also very important for fruit-body formation.[10,11] Moreover, amylase production from *L. shimeji* varied considerably among the strains used, and production in the fruit-body-forming strains was higher than in the nonforming strains.[12] Therefore, selection and breeding of the commercial strains that have high starch-hydrolyzing ability are very important for mass production of *L. shimeji*. However, mass production of *L. shimeji* is only possible using limited strains isolated from nature. Therefore, an efficient method for deriving monokaryotic mycelia from various dikaryotic mycelia without monospore isolation is also required to breed new *L. shimeji* strains that give high yield and taste better. However, developing fruiting bodies from the majority of type strains of *L. shimeji* in stock culture centers such as the

NBRC (National Institute of Technology and Evaluation, Biological Resource Center, Japan) and collecting wild *L. shimeji* fruiting bodies for monospore isolation are both very difficult. Moreover, the relationships between the morphological and biological species of *L. shimeji* and related species remain poorly understood. Recently, fruiting bodies morphologically resembling *L. shimeji* were isolated from nature, and, according to mating compatibility tests, two biological species were identified from the strains.[13] Therefore, a morphological species of *L. shimeji* contains two biological species in nature and this is a very important problem for registration under Seed and Seedling Law**.**

Here, we describe the preparation of monokaryotic strains of *L. shimeji* from dikaryotic strains via protoplast formation and regeneration. Using this technique, mononucleate protoplasts can be isolated from monokaryotized cells on top of the hyphae of a mycelial colony. The present study is also a part of our attempt to develop a novel crossing procedure for breeding and determination of biological species of mycorrhizal fungi, for which it is difficult to prepare monospore cultures.

Materials and methods

Ten *Lyophyllum shimeji* strains were used in this study: NBRC100325; MH01709, MH01710, and MH01712 (Hokuto); Hy7L, IS1L, OK1L, and OK2L (Shiga Forest Research Center); 252 and YA (Faculty of Agriculture, Kin-ki University). Agar blocks ($5 \times 5 \times 5$ mm) from 7-day-old cultures of *L. shimeji* on GPYA plates (20 g/l glucose, 2 g/l peptone, 0.5 g/l yeast extract, 1 g/l KH_2PO_4, 0.3 g/l $MgSO_4 \cdot 7H_2O$, 15 g/l agar, pH 5.4) were inoculated into 10 ml of GPY liquid medium (20 g/l glucose, 2 g/l peptone, 0.5 g/l yeast extract, 1 g/l KH_2PO_4, 0.3 g/l $MgSO_4 \cdot 7H_2O$, pH 5.4) in an Erlenmeyer flask and incubated at 25°C. After 7 days, the mycelia were collected by centrifugation and washed with 50 mM MES buffer [2-(*N*-morpholino)ethane sulfonic acid, pH 5.5] supplemented with 0.6 M mannitol. The mycelial cake was suspended in MES buffer containing lytic enzymes [1% lysing enzyme (Sigma-Aldrich), 1% cellulase (ONOZUKA R-10, Yakult), 0.2% β-glucuronidase (Sigma-Aldrich), 0.1% zymolyase (Seikagaku), 0.5% yatalase Takara)], and incubated at 25°C. One milliliter of lytic enzyme solution was used for each 100 mg of wet mycelium. After incubation for 3 h, the suspension was filtered through a glass filter (3G1) to remove the mycelial debris. The number of protoplasts in the filtrate was counted using a Thoma blood corpuscle chamber (Erma Optical Workers). The protoplasts were washed with 50 mM MES–mannitol buffer before regeneration, then mixed with 3 ml SMY agar medium (0.6 M sucrose, 1% malt extract, 0.4% yeast extract, and 0.7% agar), and spread on SMY agar plates. Incubation was carried out at 25°C. Viable colonies originating from the regenerated protoplasts were picked up onto GPYA slants, and clamp cell formation was checked using a microscope. Monokaryotic stocks without clamp cells were subcultured on GPYA slants.

Results and discussion

For each strain, 2×10^7 protoplasts were plated on GPYA, and the rate of protoplast regeneration was about 0.0002%–0.03% (Table 1). The rate of protoplast regeneration in each strain was low. In particular, protoplasts did not regenerate in MH01709. In this study, we tried to isolate protoplast-regenerated strain under one condition that included combination of cell wall lytic enzymes. Therefore, in further studies, other conditions for protoplasting such as combination of cell wall lytic enzymes should be investigated to promote the rate of protoplast regeneration.

The 30 to 200 protoplast-regenerated colonies from each dikaryotic strain were isolated and clamp cell formation was checked. Nine, 22, 37, and 2 monokaryotic stocks were isolated from the NBRC100325, MH01710, OK2L, and Hy7L strains, respectively. The maximum ratio of monokaryotization (number of monokaryons per picked colony) was 100% for the OK2L strain. For the NBRC100325, MH01710, and Hy7L strains, 5%–20% of the isolated mycelial colonies were monokaryons (data not shown). However, all of the isolated colonies from the IS1L, MH01709, MH01721, YA, OK1L, and 252 strains were dikaryons. Monokaryotic mycelial stocks can be prepared by protoplasting from

Table 1. Rate of protoplast regeneration and monokaryotization in this study

Strain number	Rate of protoplast regeneration (%)	Rate of monokaryotization[a]	Source
NBRC100325	0.03	9/200	NBRC
MH01709	0	0/0	Hokuto Co.
MH01710	0.03	22/50	Hokuto Co.
MH01721	0.03	0/100	Hokuto Co.
Hy7L	0.0015	2/20	Kin-ki University
OK1L	0.001	0/30	SFRC
OK2L	0.015	37/37	SFRC
252	0.0002	0/10	Kin-ki University
YA	0.0002	0/10	Kin-ki University
IS1L	0.0002	0/10	Kin-ki University

NBRC, National Institute of Technology and Evaluation, Biological Resource Center, Japan; SFRC, Shiga Forest Research Center

[a] Number of monokaryons/number of picked colonies

parental dikaryons because of the mycelial monokaryotization that occurs in apical hyphal cells in various basidiomycetous mushrooms (e.g., *Flammulina velutipes*, *Lentinula edodes*, and *Pholiota nameko*).[14,15] However, the efficiency with which monokaryons can be prepared from dikaryotic mycelia by protoplasting depends on the characteristics of individual strains.[16]

In order to investigate the mating characteristics of monokaryons derived from dikaryotic mycelial stocks, monokaryons from each dikaryotic stock were crossed with tester stock monokaryons in the centers of GPYA plates. Five monokaryons (NBRC100325-18, NBRC100325-9, MH01710-6, OK2L-1, HY7L-1) were selected as tester stocks for these experiments. After the plates were incubated at 25°C for about 2 weeks, clamp connections were checked in mycelia growing in the contact zone between the two monokaryon stocks. The results of mating compatibility tests for monokaryons isolated from the same dikaryotic stocks are shown in Table 2. In general, dikaryotic hyphae carry two compatible nuclei, and two compatible monokaryotic stocks should be isolated from a dikaryotic strain. From dikaryotic stocks such as NBRC100325, two compatible monokaryons were isolated. However, the two mating types of monokaryons were not present in a 1:1 ratio, and the majority of mating types of protoplast-derived monokaryons from parental dikaryons was one of the mating types in the parental dikaryon. This phenomenon may be due to the isolation of dominant nuclei in the apical monokaryotized hyphal cells of dikaryotic mycelia, as reported for *P. nameko*.[14,16] We could not isolate two compatible monokaryons from the dikaryotic stocks MH01710-6, OK2L-1, and HY7L. However, it might be possible to obtain two mating types of monokaryons by increasing the number of isolates when preparing monokaryons via protoplasting basidiomycetous mushrooms.

Crosses were carried out between monokaryons derived from different dikaryotic stocks, (NBRC100325-18, NBRC100325-9, MH01710-6, OK2L-1, HY7L-1) to determine their mutual mating compatibilities. As shown in Table 3, all monokaryons produced dikaryotic mycelia by crossing with one of the other test monokaryons. In conclusion, monokaryotic stocks can be prepared via protoplast monokaryotization to permit cross breeding of mushrooms for which it is difficult to obtain monokaryons because of a very low basidiospore germination rate, as is the case for most mycorrhizal mushrooms.[15,17] In the future, we will

Table 2. Crosses among monokaryons isolated from the same dikaryotic stocks

Monokaryon	MH01710-6	Monokaryon	OK2L-1
MH01710		OK2L	
MH01710-1	–	OK2L-1	
MH01710-2	–	OK2L-2	–
MH01710-3	–	OK2L-3	–
MH01710-4	–	OK2L-4	–
MH01710-5	–	OK2L-5	–
MH01710-6		OK2L-6	–
MH01710-7	–	OK2L-7	–
MH01710-8	–	OK2L-8	–
MH01710-9	–	OK2L-9	–
MH01710-10	–	OK2L-10	–
MH01710-11	–	OK2L-11	–
MH01710-12	–	OK2L-12	–
MH01710-13	–	OK2L-13	–
MH01710-14	–	OK2L-14	–
MH01710-15	–	OK2L-15	–
MH01710-16	–	OK2L-16	–
MH01710-17	–	OK2L-17	–
MH01710-18	–	OK2L-18	–
MH01710-19	–	OK2L-19	–
MH01710-20	–	OK2L-20	–
MH01710-21	–	OK2L-21	–
MH01710-22	–	OK2L-22	–
NBRC100325	NBRC100325-18	OK2L-23	–
NBRC100325-1	–	OK2L-24	–
NBRC100325-2	–	OK2L-25	–
NBRC100325-3	–	OK2L-26	–
NBRC100325-4	–	OK2L-27	–
NBRC100325-5	–	OK2L-28	–
NBRC100325-6	–	OK2L-29	–
NBRC100325-8	–	OK2L-30	–
NBRC100325-9	+	OK2L-31	–
NBRC100325-18		OK2L-32	–
HY7L	HY7L–1	OK2L-33	–
HY7L-1		OK2L-34	–
HY7L-2	–	OK2L-35	–
		OK2L-36	–
		OK2L-37	–

Table 3. Crosses among monokaryons derived from different dikaryotic stocks

Strain	NBRC100325-9	NBRC100325-18	MH01710-6	OK21L-1	HY7L-1
NBRC100325-9		+	+	+	+
NBRC100325-18	+		+	+	+
MH01710-6	+	+		+	+
OK2L-1	+	+	+		
HY7L-1	+	+	+	+	

attempt to develop cross-breeding systems for other mycorrhizal fungi, including *Tricholoma matsutake*, for which monospore cultures are difficult to isolate.

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References

1. Bao D, Aimi T, Kitamoto Y (2005) Cladistic relationships among the *Pleurotus ostreatus* complex, the *Pleurotus pulmonarius* complex, and *Pleurotus eryngii* based on the mitochondrial small subunit ribosomal DNA sequence analysis. J Wood Sci 51:77–82
2. Bao D, Ishihara H, Mori N, Kitamoto Y (2004) Phylogenetic analysis of oyster mushrooms (*Pleurotus* spp.) based on restriction fragment length polymorphisms of the 5′ portion of 26S rDNA. J Wood Sci 50:169–176
3. Bao D, Kinugasa, S, Kitamoto, Y (2004) The biological species of oyster mushrooms (*Pleurotus* spp.) from Asia based on mating compatibility tests. J Wood Sci 50:162–168
4. Hongo T (1990) Mycorrhizal Agaricales (in Japanese). Trans Mycol Soc Jpn 31:281–286
5. Yoshida H, Fujimoto S (1994) A trial cultivation of *Lyophyllum shimeji* on solid media (in Japanese). Trans Mycol Soc Jpn 35: 192–195
6. Ohta A (1994) Some cultural characteristics of mycelia of a mycorrhizal fungus, *Lyophyllum shimeji*. Mycoscience 35:83–87
7. Ohta A (1994) Production of fruit-bodies of a mycorrhizal fungus, *Lyophyllum shimeji*, in pure culture. Mycoscience 35:147–151
8. Eto S (2001) Fruit-body production of a mycorrhizal fungus, *Lyophyllum shimeji*, on a medium consisting of cornmeal and sawdust (in Japanese). Mushroom Sci Biotech 9:171–174
9. Eto S (2005) Cultivation of *Lyophyllum shimeji* using rice bran (in Japanese). Mushroom Sci Biotech 13:29–33
10. Ohta A (1998) Culture conditions for commercial production of *Lyophyllum shimeji*. Trans Mycol Soc Jpn 39:13–20
11. Kusuda M, Ueda M, Konishi Y, Matsuzawa K, Shirasaka N, Nakazawa M, Miyatake K, Terashita T (2004) Characterization of extracellular glucoamylase from the ectomycorrhizal mushroom *Lyophyllum shimeji*. Mycoscience 45:383–389
12. Terashita T, KitaoT, Nagai M, Yoshikawa K, Sakai T (2000) Amylase productions during the vegetative mycelial growth of *Lyophyllum shimeji* (in Japanese). Mushroom Soc Biotechnol 8:61–69
13. Murakami S, Nagasawa E, Matsumoto T (2006) Biological species of *Lyophyllum shimeji* (in Japanese). Proceedings of the 10th Annual Meeting of the Japanese Society of Mushroom Science and Biotechnology, Akita, Japan, September 20–21, p 48
14. Cao H, Yamamoto H, Ohta T, Takeo K, Kitamoto Y (1999) Nuclear selection in monokaryotic oidium formation from dikaryotic mycelia in a basidiomycete, *Pholiota nameko*. Mycoscience 40:199–203
15. Ohta A (1986) Basidiospore germination of *Tricholoma matsutake* (II). Evaluation of germination condition and microscopic observations of germination stages. Trans Mycol Soc Jpn 27:473–480
16. Masuda P, Yamanaka K, Sato Y, Kitamoto Y (1995) Nuclear selection in monokaryotization of dikaryotic mycelia of *Pholiota nameko* as described by leading and following nuclei. Mycoscience 36:413–420
17. Ohta A (1986) Basidiospore germination of *Tricholoma matsutake* (I). Effects of organic acids on seedling and germination of the basidiospores. Trans Mycol Soc Jpn 27:167–173