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FULL PAPER

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Genetic analysis of nuclear ribosomal DNA of *Lentinula edodes*

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Abstract Genetic analysis of nuclear ribosomal DNA (rDNA) of *Lentinula edodes* was carried out using rDNA restriction fragment length polymorphisms (RFLPs) as genetic markers. Two compatible monokaryotic strains that differed in the endonuclease digestion patterns of their rDNA were used. The dikaryotic strain established by crossing them produced mixed RFLP patterns. Single-spore isolates derived from the dikaryotic strain showed three types of rDNA RFLP patterns: either one of the two parental types or a mixed type. From the frequency of the mixed type, the recombination value of rDNA tandem repeats was calculated to be 31.4%. Linkage analysis between rDNA and two incompatibility factors (*A* and *B*) revealed that rDNA was not linked to either factor. The rDNA genotypes did not affect mycelial growth among the single-spore isolates.

Key words Genetic analysis · *Lentinula edodes* · Ribosomal DNA · Incompatibility factors

Introduction

Shiitake, *Lentinula edodes* (Berk.) Pegler, is a commercially important edible mushroom cultivated widely in many countries, particularly in Japan and China. Although numerous cultivars are available, additional cultivars are necessary to meet changing agronomic and economic requirements. In this context, fundamental genetic research has an essential practical importance. So far, there are several genetic studies in *L. edodes* on mutations of the vegetative and generative phenotypes that are controlled by nuclear genes (e.g., Komatsu and Kimura 1968; Murakami et al. 1987; Hasebe 1991). In addition, Royse et al. (1983) have examined single-locus segregations for six isozyme loci. More recently, linkage analyses have been carried out using amplified fragment length polymorphism (AFLP) markers (Terashima et al. 2002) and randomly amplified polymorphic DNA (RAPD) markers (Kwan and Xu 2002; Miyazaki and Neda 2004). It is, however, desirable to obtain more information on the inheritance of genetically important characters of *L. edodes*.

Fukuda and Ono (1999) have characterized the sequence variation of nuclear ribosomal DNAs (rDNAs) in *L. edodes* by restriction endonuclease analysis. We carried out the present study to examine the inheritance pattern of rDNA tandem repeats in *L. edodes* by using the restriction fragment length polymorphisms (RFLPs) of the rDNA-internal transcribed spacer (ITS) region as genetic markers.

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Materials and methods

Strains of *L. edodes*

Two compatible monokaryotic strains of *L. edodes*, 1158a and 1485a, which also had been employed in a previous study (Fukuda and Fukumasa-Nakai 1996), were used. These strains had been produced through artificial dedikaryotization by the protoplast regeneration method (Fukumasa-Nakai et al. 1994) from two wild dikaryotic strains, TMIC-1158 from Japan and TMIC-1485 from Papua New Guinea, which were deposited in the culture collection of the Tottori Mycological Institute, Japan. Their rDNAs already had been shown to differ in RFLPs (Fukuda and Ono 1999). The strains were maintained on MYG agar (2% malt extract, 0.2% yeast extract, 2% glucose, 2% agar) medium.

Construction of dikaryotic strain and their single-spore isolates

By reciprocal mating, the dikaryotic strain 1158a × (1485a) was isolated from the periphery of the 1158a side of the mating colony (Fukuda et al. 1995). To produce fruiting

bodies under aseptic conditions, a mycelium of the dikaryotic strain was incubated on 200 ml peptone-glucose medium (Matsuo et al. 1992) containing 2% agar in a 500-ml Erlenmeyer flask. After 2 weeks incubation at 25°C in darkness, the flask was transferred into the light at 20°C to induce fruiting. Mature fruiting bodies were obtained after about 50 days of incubation under this condition.

Basidiospores were collected and allowed to germinate on MYG agar plates. Up to 300 germinating basidiospores were randomly isolated, transferred individually to new MYG agar slants, and incubated at 25°C for about 30 days before using the mating test.

Mating test

To determine the mating type of each single-spore isolate, mating tests were performed by placing small plugs of mycelia (2 mm in diameter) at a distance of 5 mm on MYG agar plates. Dikaryosis and common-*B* heterokaryosis of the paired monokaryons were confirmed by the appearance of clamp connections and pseudoclamps, respectively, under a microscope.

DNA extraction

To prepare mycelium for total DNA extraction, cultures were grown in MYG liquid medium at 25°C for 20 days and homogenized with a Waring blender. Then, 10 ml homogenate was added to a 500-ml Erlenmeyer flask containing 100 ml MYG liquid medium. The flask cultures were incubated in a stationary state at 25°C for 14 days, washed with distilled water, and lyophilized. Total DNA was extracted from the lyophilized mycelia using the procedure of Fukumasa-Nakai et al. (1992).

Polymerase chain reaction-RFLP analysis of ITS of nuclear rDNA

Polymerase chain reaction (PCR) amplification was performed by a thermal cycler (model 2400; Perkin-Elmer, Norwalk, CT, USA) in a 100-µl reaction mixture containing 50 ng total DNA, 0.2 µM primers ITS1 and ITS4 (White et al. 1990), 0.1 mM deoxyribonucleoside 5′-triphosphate (dNTP), 2.5 mM $MgCl_2$, 2.5 units *AmpliTaq* DNA polymerase (Perkin-Elmer), and 2.5 µl 10 × *AmpliTaq* PCR buffer (Perkin-Elmer). The thermal cycling program was as follows: 2.5 min initial denaturation at 94°C; 30 cycles consisting of 0.5 min denaturation at 94°C, 0.5 min primer annealing at 55°C, and 1.5 min extension at 72°C; and a final 8.5 min extension at 72°C. PCR products were electrophoresed in a 2% agarose (type S; Nippon Gene, Tokyo, Japan) slab gel in TAE [40 mM Tris/acetate, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0] at 5 V/cm for 3 h, before staining with ethidium bromide (0.5 µg/ml). DNA bands were photographed on a UV transilluminator.

RFLP analysis of PCR products was performed as described previously (Fukuda and Ono 1999). In this study, PCR products were digested with *Hin*fI (Nippon Gene), following the supplier's instruction. *Hae*III-digested Phi X174 DNA was used as a molecular size standard.

Growth test

For examination of mycelial growth, single-spore isolates were grown in 9-cm Petri dishes on MYG agar medium. Enlargement of the mycelial colonies on the medium at 25°C in darkness from the 5th to 9th day of incubation was used as the mycelial growth rate.

Results and discussion

More than 90% of basidiospores of the dikaryotic strain 1158a × (1485a) were germinated on MYG agar medium after 3 days of incubation at 25°C. Of about 300 single-spore isolates obtained, 175 were selected randomly and further analyzed.

A tetrapolar mating system has been demonstrated in *L. edodes* (Takemaru 1961). Mating types of the parental strains, 1158a and 1485a, were designated *A1B1* and *A2B2*, respectively. To obtain monokaryons of all four mating types, the parental strains and ten randomly selected single-spore isolates were paired in all possible combinations. Four mating-type testers [*A1B1* and *A2B2* (parental types) and *A1B2* and *A2B1* (nonparental types)] could be obtained and were used to determine the mating types of all single-spore isolates. As shown in Table 1, the four mating types appeared at equal proportion among the 175 single-spore isolates. Recombination types of the *B* factor, which had been obtained at 2.4%–9.2% in previous studies (Takemaru 1961; Tokimoto et al. 1973; Ratanatragooldacha et al. 2001), were not observed. The causes for this finding are not clear in this time.

The ITS region (including ITS1, ITS2, and 5.8S) was amplified by PCR and analyzed by electrophoresis; a single PCR product was detected for the two parental monokaryons, the dikaryotic strain, and the 175 single-spore isolates (Fig. 1). The size of the amplified ITS region was about 750 base pairs (bp). This value was similar to that reported by Molina et al. (1992), who amplified the ITS

Table 1. Mating types and rDNA genotypes of 175 single-spore isolates derived from the dikaryotic strain, 1158a × (1485a), of *Lentinula edodes*

Mating type*	rDNA genotype			Total
	1158a	1485a	Mixed	
A1B1	16	13	15	44
A2B2	14	15	13	42
A1B2	17	16	13	46
A2B1	14	15	14	43
total	61	59	55	175

* Recombinants of *A* and *B* factors were not found in this study

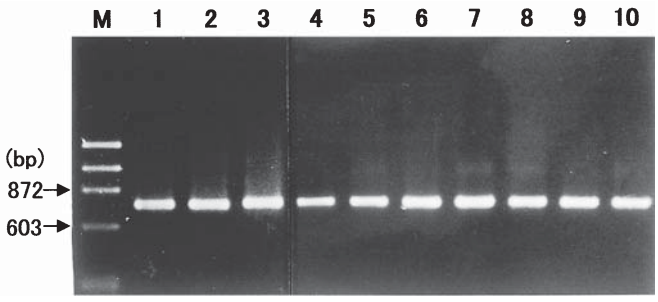

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction (PCR)-amplified internal spacer sequence (ITS) of 1158a (*lane 1*), 1485a (*lane 2*), 1158a × (1485a): dikaryotic strain isolated from the periphery of the 1158a side of reciprocal mating colony between 1158a and 1485a (*lane 3*), and single-spore isolates from 1158a × (1485a) (*lanes 4–10*). *Lane M* shows *Hae*III-digested Phi X174 DNA

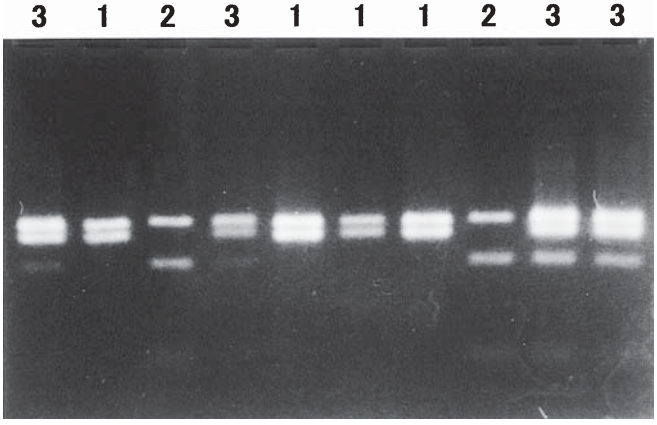

Fig. 3. *Hin*fI digests of PCR-amplified ITS of representative single-spore isolates from 1158a × (1485a). *Numbers at the top* indicate rDNA genotypes: (*1*) 1158a type, (*2*) 1485a type, (*3*) mixed type

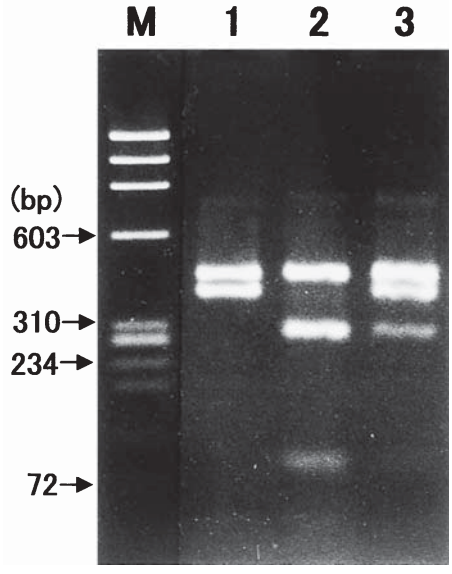

Fig. 2. *Hin*fI digests of PCR-amplified ITS of 1158a (*lane 1*), 1485a (*lane 2*), and 1158a × (1485a) (*lane 3*). *Lane M* shows *Hae*III-digested Phi X174 DNA

region of *L. edodes* using the same primers and estimated the amplified ITS region to include 700 bp.

The two parental monokaryons of *L. edodes* used in this study differed in the *Hin*fI digestion patterns of their ITS region (Fig. 2). In addition, the dikaryotic strain 1158a × (1485a) appeared to produce a mixture of the RFLP patterns of the parental monokaryons (Fig. 2).

Single-spore isolates showed three distinct rDNA RFLP patterns: a 1158a type, a 1485a type, and a mixed type of the two parental patterns (Fig. 3). Of 175 single-spore isolates, 61 were 1158a type, 59 were 1485a type, and the remaining 55 were mixed type (Table 1). Because the rDNA region is a repeat sequence on a chromosome (Hibbett 1992), the probability of a recombination of the rDNA tandem repeats is high. This explains the high proportion of mixed-type patterns among the single-spore isolates.

In a Bornean *L. edodes* wild strain, one of two nuclear types of its neohaplonts, which were derived through artificial dedikaryotization by the protoplast regeneration method, showed an RFLP pattern identical with that of Japanese wild strains whereas the other appeared to be identical with Papua New Guinean wild strains (Fukuda and Ono 1999). From this fact and the results of the genetic analysis of rDNA in this study, one may conclude that about one-third of the progeny derived from this Bornean wild strain (Fukuda and Ono 1999) would carry heterogeneous rDNA tandem repeats. Such strains differing in their rDNA tandem repeat structures possibly exist in the Bornean natural population of *L. edodes*.

The number of copies of the rDNA tandem repeats may be estimated from the recombination pattern of the rDNAs. Cytoplasmic ribosomes contain 5S, 5.8S, 18S, and 25S rRNAs consisting of about 120, 160, 1800, and 3400 bases, respectively (Hibbett 1992). The length of nuclear rDNA spacers of *L. edodes* is about 500 bases for the ITS (Hibbett et al. 1995) and about 3300 bases for the intergenic spacer (IGS) between the 25S and 18S rRNA genes (Saito et al. 2002). Therefore, the total length of one rDNA repeat in *L. edodes* can be estimated to be about 9300 bases. The total length of the rDNA tandem repeats appears to correspond to 31.4% of the length of the chromosome carrying them when considering simply from the recombination value of rDNA established in this study. The size of the chromosome carrying the rDNA has been estimated to be 7000 kilobases for a *L. edodes* strain (Arima and Morinaga 1993). Thus, the total length of the rDNA tandem repeats is about 2200 (7000 × 0.314) kilobases, and the number of copies can be estimated to be 237 (2200/9.3). This value is slightly larger than the estimated number of rDNA tandem repeats in other fungi, ranging from 60 in *Coprinus cinereus* (Schaeff.: Fr.) S.F. Gray (Cassidy et al. 1984) to 220 in *Neurospora crassa* Shear & Dodge (Russell et al. 1984).

The incompatibility factors *A* and *B* of *L. edodes* are located on different chromosomes (Takemaru 1961); consequently, they seemed to be unlinked in linkage tests (Table 2). Linkage analysis further revealed that rDNA was linked to neither of the incompatibility factors (Table 2), indicat-

Table 2. Linkage analysis between rDNA genotypes and incompatibility factors (*A* and *B*) for the cross between 1158a (*A1B1*) and 1485a (*A2B2*) of *Lentinula edodes*

Recombination between	Parental	Recombinant	χ^2 (1:1)	*P*
rDNA and *A*	63	57	0.21	0.75–0.50
rDNA and *B*	61	59	0.01	>0.90
A and *B*	86	89	0.02	0.90–0.75

ing that the rDNA region and the factors are located on different chromosomes.

RFLPs have been analyzed in Japanese and Papua New Guinean strains in which the 18S and 25S rDNA regions were digested with *Alu*I and *Nla*IV, respectively (Fukuda and Ono 1999). The two monokaryotic strains, 1158a and 1485a, were also distinct with respect to their RFLPs in the two rDNA regions (data not shown). This observation suggests that the structures of 18S and 25S rRNAs derived from the distinct rDNAs differ between 1158a and 1485a. It appeared to be of interest whether the heterogeneous structure of the rDNA tandem repeats of different single-spore isolates influences phenotypical properties of *L. edodes*. To examine the effects of rDNA genotypes on mycelial growth, growth tests were performed using the 175 single-spore isolates. The mycelial growth rate of the isolates incubated on MYG agar medium at 25°C for 4 days was 8.34 ± 3.46 mm (mean ± SD) for the 1158a type ($n = 61$), 8.54 ± 3.52 mm for the 1485a type ($n = 59$), and 8.40 ± 3.17 mm for the mixed type ($n = 55$); no significant differences were apparent, indicating that rDNA genotypes did not influence mycelial growth of the single-spore isolates. Therefore, it may not be necessary to consider the structure of the rDNA tandem repeats when choosing appropriate breeding material, as far as the growth performance is concerned. Hereafter, possible influences of the rDNA type on other properties of *L. edodes* (e.g., fruiting body productivity) must be studied in more detail.

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