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FULL PAPER

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Analysis of mitochondrial DNA restriction fragment length polymorphisms for examining genetic variability among isolates of *Phellinus linteus*

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Abstract Restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNAs (mtDNAs) from nine Japanese wild isolates of *Phellinus linteus* was carried out to examine their genetic variability. *Bam*HI and *Eco*RI digests of mtDNAs from these isolates produced four and five distinct RFLP patterns, respectively. By combining the RFLP patterns obtained with the two endonucleases, mtDNAs from the nine isolates could be assigned to five different genotypes, but no mtDNA variation was detected among the isolates collected from a small area. Distance values calculated among all pairs of mtDNA genotypes, based on the presence or absence of comigrating restriction fragments, were clearly smaller than those among the mtDNA genotypes of *Lentinula edodes* and *Pleurotus ostreatus* samples collected worldwide, suggesting the necessity of collecting *P. linteus* wild isolates for genetic resources from geographically wider areas.

Key words Genetic variability · Mitochondrial DNA · *Phellinus linteus* · Restriction fragment length polymorphism

Introduction

Phellinus linteus (Berk. et Curt.) Teng is a polypore of the hymenomycetes family and a heart-rot fungus occurring especially in groves of *Morus bombycis* Koidz. (Imazeki and Hongo 1989). In Japan, this fungus has been named Meshimakobu (Imazeki and Hongo 1989) because it was mainly found in Meshima of the Danjo Islands in Nagasaki Prefecture. In Chinese medicine, it has been called Souou and used as a medicinal ingredient (Ryu 1982). About 30 years ago, certain pharmacological effects of this fungus were examined, and it was revealed that the fungus had the highest antitumor activity among the Hymenomycetes such as polypores and edible mushrooms used (Ikekawa et al. 1968). Recently, the authors found that some pharmacological effects varied from strain to strain of *P. linteus* (Nakamura et al., unpublished data). To utilize this fungus effectively, it is desirable to obtain information on the genetic variability among *P. linteus* strains. However, little is known about genetic differences among the wild strains of this fungus.

Restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNAs (mtDNAs) has been a promising method for differentiating the strains of several fungi (Specht et al. 1983; Hintz et al. 1985; Fukuda et al. 1994; Matsumoto and Fukumasa-Nakai 1995). The purpose of the present study was to examine genetic variability among Japanese isolates of *P. linteus* by using RFLP analysis of mtDNAs.

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Materials and methods

Phellinus linteus wild isolates and culture conditions

Nine wild dikaryotic isolates of *P. linteus* were used in this study (Table 1). These nine isolates were somatically incompatible to one another as shown by a somatic incompatibility test performed by the procedure of Fukuda et al. (2000). To prepare mycelia for mtDNA isolation, cultures were grown in MYG liquid medium (2% malt extract, 0.2% yeast extract, 2% glucose) at 25°C for 14 days and fragmented with a Waring blender; 10 ml was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of the MYG liquid medium. The flask cultures were incubated in a stationary state at 25°C for 10–14 days, harvested, washed with distilled water, and lyophilized.

Table 1. mtDNA genotypes among *Phellinus linteus* strains used in this study

Strain no.	mtDNA genotype	RFLP pattern[a]		Size (kb)[b]	Geographic origin	Year isolated
		*Bam*HI	*Eco*RI			
IFO6989	I	A	A	74.0	?	1961
MAFF420302	II	B	B	73.0	Tokyo	1950
PL-02	III	C	C	81.5	Shiga	1999
PL-03	IV	D	D	73.0	Miyazaki	1998
PL-04	V	A	E	74.0	Miyazaki	1998
PL-05	III	C	C	81.5	Shiga	1999
PL-06	III	C	C	81.5	Shiga	1999
PL-07	III	C	C	81.5	Shiga	1999
PL-08	V	A	E	74.0	Miyazaki	1998

[a] See Fig. 1 (RFLP pattern of *Bam*HI and *Eco*RI)
[b] The average size estimated by summation of *Bam*HI and *Eco*RI fragment sizes

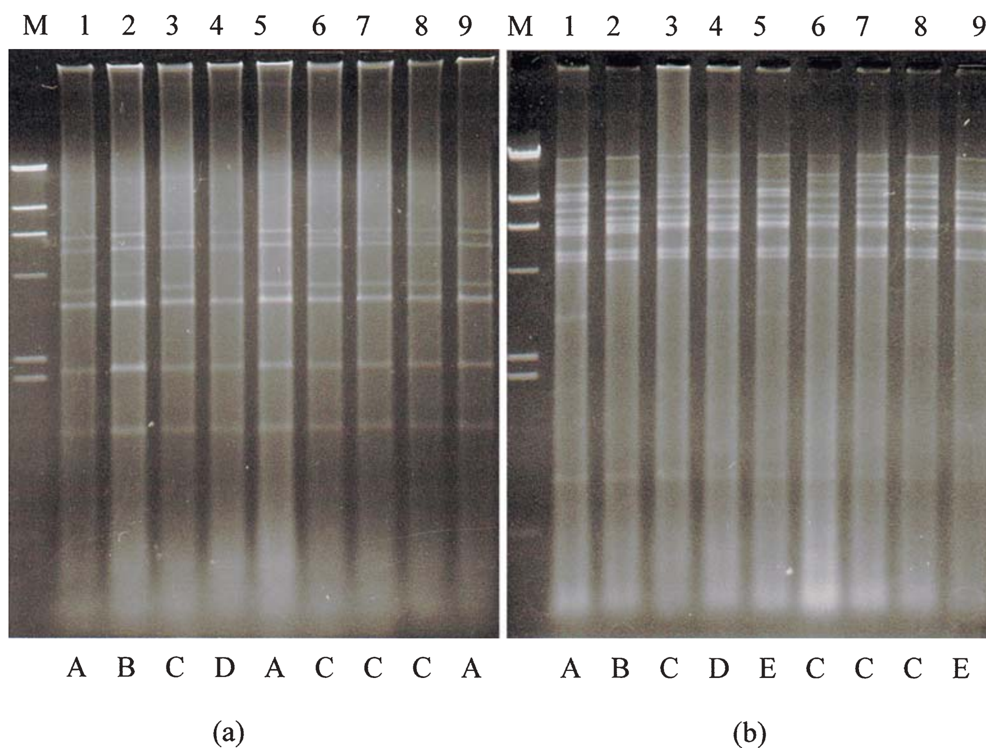

Fig. 1. *Bam*HI (**a**) and *Eco*RI (**b**) RFLP patterns of mtDNAs isolated from nine wild isolates of *Phellinus linteus*. *Lane 1*, IFO6989; *lane 2*, MAFF420302; *lane 3*, PL-02; *lane 4*, PL-03; *lane 5*, PL-04; *lane 6*, PL-05; *lane 7*, PL-06; *lane 8*, PL-07; *lane 9*, PL-08; *lane M*, *Hin*dIII-digested lambda phage DNA with sizes (from *top* to *bottom*): 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6 kb. *Capital letters at the bottom of each gel track*, RFLP pattern produced by *Bam*HI (**a**, A–D) and *Eco*RI (**b**, A–E)

mtDNA isolation and restriction analysis

mtDNA was isolated by the procedure of Fukumasa-Nakai et al. (1992). mtDNA isolated from each of the nine isolates was digested separately with two endonucleases, *Bam*HI and *Eco*RI (Nippon Gene, Tokyo, Japan), following the supplier's specifications. Electrophoresis of all digests was carried out on 0.8% agarose (Nippon Gene, Type S) slab gels in TAE (40 mM Tris/acetate, 10 mM EDTA, pH 8.0) at 5 V/cm for 4 h, and the gels were stained with ethidium bromide (0.5 μg/ml). Restriction patterns were recorded by photographing the gels on a UV transilluminator. For determination of molecular sizes of restriction fragments, lambda phage DNA digested with *Hin*dIII was used as a molecular size standard.

Numerical analysis

The presence or absence of individual restriction fragments derived from *Bam*HI and *Eco*RI digests was scored, and a distance value based on the scoring data was calculated between pairs of different mtDNA genotypes using the Dice coefficient as $1 - 2(Nxy)/(Nx + Ny)$, in which Nxy is the number of restriction fragments shared between a pair of the genotypes and Nx and Ny are the total numbers of restriction fragments in all digests in genotype x and y, respectively.

Results and discussion

By digestion with *Bam*HI and *Eco*RI, *P. linteus* mtDNA was found to be polymorphic, as described previously for

Table 2. Distance matrix based on mtDNA genotypes of *P. linteus* wild isolates

mtDNA genotype	I	II	III	IV	V
I	0.000				
II	0.128	0.000			
III	0.150	0.179	0.000		
IV	0.158	0.135	0.158	0.000	
V	0.077	0.158	0.128	0.081	0.000

other basidiomycetes, including *Schizophyllum commune* Fr.: Fr. (Specht et al. 1983), *Agaricus bitorquis* (J. Lange) Imbach (Hintz et al. 1985), *Lentinula edodes* (Berk.) Pegler (Fukuda et al. 1994), and *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (Matsumoto and Fukumasa-Nakai 1995). *Bam*HI and *Eco*RI digests of mtDNAs from the nine isolates produced four and five distinct RFLP patterns, respectively (Fig. 1: *Bam*HI patterns A to D; *Eco*RI patterns A to E). By combining the RFLP patterns obtained with these two endonucleases, mtDNAs from the nine isolates could be assigned to five different mtDNA genotypes (I–V), although these same assignments could be made based on the *Eco*RI RFLP patterns alone. Based on the summation of restriction fragment sizes from the *Bam*HI and *Eco*RI digests, the molecular sizes of *Phellinus linteus* mtDNAs were estimated to range from 72.8 kb for genotype IV to 81.4 kb for genotype III. The average estimated molecular size of *P. linteus* mtDNA was 77.1 kb. The mtDNA size of *P. linteus* obtained in this study falls within the range of mtDNA sizes described previously in the other basidiomycetes, from 36 kb for *Boletinus cavipes* (Opat.) Kalchbr. (Bruns et al. 1988) to 178 kb for *A. bitorquis* (Hintz et al. 1985), and similar to those for *L. edodes* (Fukuda et al. 1994) and *Pleurotus ostreatus* (Matsumoto and Fukumasa-Nakai 1995).

In *Flammulina velutipes* (Curt.: Fr.) Sing., four mtDNA genotypes have been detected among 12 wild isolates collected within about 5 m of a fallen tree trunk (Fukuda et al. 2000). In contrast, no mtDNA variation was detected among the *Phellinus linteus* isolates collected from one small area, that is, all 4 isolates from Shiga Prefecture (PL-02, -05, -06, and -07), which were individually collected from four different host trees within about 5 m of each other in a forest, showed the same mtDNA genotype (III). In addition, two isolates from Miyazaki Prefecture (PL-04 and -08), which were collected from two host trees about 1 m apart, also showed the same type (V), although another Miyazaki isolate (PL-03), collected about 200 m away, showed a different genotype (IV). Within such small areas, mtDNA variation among strains may be low in natural populations of *P. linteus*. If mtDNA variation correlates well with nuclear level variation, as reported in *L. edodes* (Fukuda et al. 1994; Hibbett et al. 1995), it will be difficult to obtain *P. linteus* strains whose genetic compositions differ greatly within a small area.

Distance values calculated between all pairs of five mtDNA genotypes are shown in Table 2. The distance values among the five mtDNA genotypes ranged from 0.077 to 0.179, a smaller range than those for 38 Japanese *L. edodes* (0.026–0.300) and 18 Japanese *P. ostreatus* (0.171–0.543) wild isolates (Fukuda et al. 1994; Matsumoto and Fukumasa-Nakai 1995). In addition, the maximum distance value among samples of *L. edodes* and *P. ostreatus* collected worldwide is 1.000 and 0.909, respectively. The mtDNA variation of Japanese *P. linteus* wild strains could be inherently small, because the major factor in this finding is that the isolates used for this study were gathered from a limited area.

According to Imazeki and Hongo (1989), *P. linteus* is distributed in Japan, the Philippine Islands, Australia, and North America. To obtain isolates of wide genetic diversity, it is important to collect *P. linteus* wild isolates from the geographically distant regions, which would make it possible to select strains with greater pharmacological effects than existing ones. It is further important to examine the relationship between the genetic divergence of each isolate and its pharmacological effect to use this fungus as a medicinal material.

In this study, mtDNA RFLP analysis was effective for examining the genetic difference among *P. linteus* isolates. However, it would be necessary to also use other indicators such as nuclear DNA fingerprinting to examine the differences in detail.

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