JARQ 33, 149-154 (1999)

Discrimination of Basidiomycete Species and Strains by Random Amplified Polymorphic DNA (RAPD) Analysis

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Abstract

To develop a method for the discrimination of basidiomycete species and strains with vegetative mycelia, DNAs isolated from the mycelia of *Coprinus* and *Tricholoma* strains, were subjected to random amplified polymorphic DNA (RAPD) analysis. Seven *Coprinus* species could be distinguished, clearly showing species-specific DNA patterns in the RAPD analysis. One specimen of unknown *Coprinus* strain was identified as *C. cinereus* by this method. Six strains of *C. cinereus* and 4 of *C. angulatus* could also be distinguished by the presence of strain-specific RAPD fragments. Five members of the *Tricholoma* family, *T. matsutake* and related 4 species, also showed species-specific DNA patterns in the RAPD analysis. The discrimination of *Tricholoma* species was confirmed by cluster analysis based on 192 RAPD fragments. The 5 species could be clearly divided into 5 groups in complete agreement with the taxonomic classification. RAPD analysis of mycelial DNA, as shown in these studies, is a suitable method for distinguishing basidiomycete species and strains.

Discipline: Genetic resources
Additional key words: *Coprinus*, *Tricholoma*

Introduction

Basidiomycetes are mainly classified based on the morphology of fruit bodies. However many strains of basidiomycete species do not form fruit bodies readily, if not at all, under experimental conditions. The development of a simple method for distinguishing species or strains with vegetative mycelia, therefore, is important.

Recently, molecular markers, such as isozymes, restriction fragment length polymorphisms (RFLP) and random amplified polymorphic DNA (RAPD) have been used to detect genetic differences in species and strains of basidiomycetes. Among these molecular markers, RAPD, which was introduced by the use of polymerase chain reaction (PCR) with arbitrary 10-mer primers[11], can express DNA variations for distinguishing basidiomycete species and strains with less labor and high reliability[4].

We used RAPD analysis for 2 basidiomycete groups, *Coprinus* species and *Tricholoma* species. *Coprinus* species, mainly *C. cinereus*, have been used as a model material for basic studies on basidiomycetes since they grow rapidly with a short life cycle span. We used *Coprinus* species to develop the RAPD method for the discrimination of basidiomycete species. Although *T. matsutake* (S. Ito and Imai) Singer is the most valuable edible mushroom in Japan, fruit body formation of *T. matsutake* under artificial conditions has not been achieved yet. Discrimination of *T. matsutake* based on the morphology of mycelia is almost impossible, because there are no clamps. This paper describes the successful application of the RAPD method.

Materials and methods

1) Strains and culture conditions

Coprinus and *Tricholoma* strains examined are listed in Table 1. To maintain the strains, MYG medium [1.0% (w/v) of malt extract (Difco, USA), 0.4% (w/v) of yeast extract (Difco, USA) and 0.4% (w/v) of glucose] with 1.5% (w/v) agar was used. For DNA extraction, mycelia of each strain were inoculated into 20 mL of liquid MYG medium each in 100 mL flasks, and cultured statically at 30°C for 2-3 days for the *Coprinus* strains, or at 20°C for 1-2 months for the *Tricholoma* strains. Harvested mycelia were stored at -20°C for DNA extraction.

2) DNA extraction and PCR reaction

Mycelial DNA of each *Coprinus* strain was extracted by the method of Doyle and Doyle (1987)[1]. DNA amplification was performed following a modification of the protocol of Williams et al. (1990)[11]. The reaction mixtures contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of dATP, dCTP, dTTP, and dGTP, 1 μM of primer, 0.4 U of recombinant *Taq* DNA polymerase (Takara, Japan), and 10 ng of genomic DNA in a total volume of 10 μL. Twelve decamer oligonucleotides were used as RAPD primers (Table 2). Amplification was carried out in a Program Temp Control System PC-800 (ASTEC, Japan) by the following procedure: initial denaturation at 94°C for 2 min; 45 cycles of 94°C for 30 s, 36°C for 1 min and 72°C for 2 min; and final extension at 72°C for 2 min.

For the *Tricholoma* strains, total DNA was extracted from mycelia as described by Draper et al. (1988)[2]. RAPD analysis was performed following a modification of the protocol of Williams et al. (1990)[11]. The reaction mixtures contained 10 mM

Table 1. Strains of *Coprinus* and *Tricholoma* species examined

Species	Strain	Source (collection sites)
Coprinus angulatus Peck	225	Okayama Univ.[a]
	516	Okayama Univ.[a]
	624	Okayama Univ.[a]
	711	Okayama Univ.[a]
C. atramentarius (Bull.: Fr.) Fr.	MAFF430151	G.B.[b]
C. cinereus (Schaeff.: Fr.) S. F. Gray	Wild	NFRI[c]
	5005	Okayama Univ.[a]
	5309	Okayama Univ.[a]
	5312	Okayama Univ.[a]
	5338	Okayama Univ.[a]
	5348	Okayama Univ.[a]
	FR1	Protoplast regenerant of Fis[c d]
C. comatus (Muller: Fr.)	7T	NFRI
	11T	NFRI
C. disseminatus (Pers.: Fr.)	E022	Forestry and Forest Products Research Institute
C. micaceus (Bull.: Fr.) Fr.	A342S	Forestry and Forest Products Research Institute
C. sp. (species unknown)	MAFF425079	G.B.
Tricholoma matsutake (S. Ito & Imai) Sing.	MAFF460031	G.B. (Hyogo, Japan)
	MAFF460037	G.B. (Aomori, Japan)
	MAFF460051	G.B. (Yamanashi, Japan)
	MAFF460052	G.B. (Hokkaido, Japan)
	MAFF460057	G.B. (China)
	MAFF460070	G.B. (Kumamoto, Japan)
	MAFF460101	G.B. (Korea)
T. caligatum (Viv.) Ricken	MAFF460044	G.B. (Algeria)
	MAFF460064	G.B. (Algeria)
T. magnivelare (Peck) Readhead comb. nov	MAFF460081	G.B. (U.S.A.)
T. bakamatsutake Hongo	MAFF460025	G.B. (Aomori, Japan)
	MAFF460087	G.B. (Hokkaido, Japan)
T. fulvocastaneum Hongo	MAFF460028	G.B. (Kohchi, Japan)
	MAFF460029	G.B. (Kyoto, Japan)
	MAFF460030	G.B. (Kyoto, Japan)

a): Maintained at National Food Research Institute for more than 10 years.
b): Genebank of Ministry of Agriculture, Forestry and Fisheries, Japan.
c): National Food Research Institute.
d): Fis[c] was supplied from the University of Tokyo, maintained at NFRI for more than 10 years.

Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM each of dATP, dCTP, dTTP and dGTP, 0.2 μM of RAPD primer (Table 2), 0.2 U of recombinant *Taq* DNA polymerase (Takara, Japan) and 1.0 ng of genomic DNA in a total volume of 10 μL. Amplification reactions were performed as follows: initial denaturation at 94°C for 2 min; 30 cycles of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min; and final extension at 72°C for 2 min.

Amplified DNA was electrophoresed in 1.6% (w/v) agarose gels, stained with 0.5 μg/L ethidium bromide, and photographed under UV light.

3) RAPD data analysis for Tricholoma *species*

Each polymorphic fragment was treated as a unit character, and compared between each pair of strains. The percentage of different fragments in each pair of strains was obtained by the following calculation: 100 × number of different fragments/number of total detected fragments between the 2 strains. Cluster analysis was performed, based on the percentage of similarity of RAPD fragments, using the unweighted-pair-group method[10].

Results and discussion

1) Discrimination of Coprinus *species*

Several DNA fragments from the genomic DNA of each *Coprinus* strain were successfully amplified with 12 primers. Two typical results of RAPD analysis are shown in Fig. 1-A and B. Strains of the same species gave similar RAPD patterns which included several DNA fragments of equivalent length (sample lanes 3-7, 8-11, and 12-13, respectively), making it possible to distinguish the species. Strains of different species, however, did not yield common fragments. RAPD patterns with other 10 primers gave similar results.

Table 2. RAPD primers used for discrimination of *Coprinus* strains and *Tricholoma* strains

	Sequence (5′ to 3′)		Sequence (5′ to 3′)
For *Coprinus* strains	GACTAGCCTC	For *Tricholoma* strains	GGCTCATGTG
	GTATCGCGGT		GGCACTGAGG
	TGGGCACTGA		AGGGCCGTCT
	TGGTCACCGA		ACGACCGACA
	TGCGTGCTTG		GTCAGGGCAA
	TTCGAGCCAG		GACCGCTTGT
	AGTGGAAGGT		GTGATCGCAG
	ATGCCTACAG		TGCCGAGCTG
	AGCGCCATTG		GTTGCGATCC
	CTCACCGTCC		GGTGACTGTG
	CACCGTATCC		
	CACCTAGTCC		

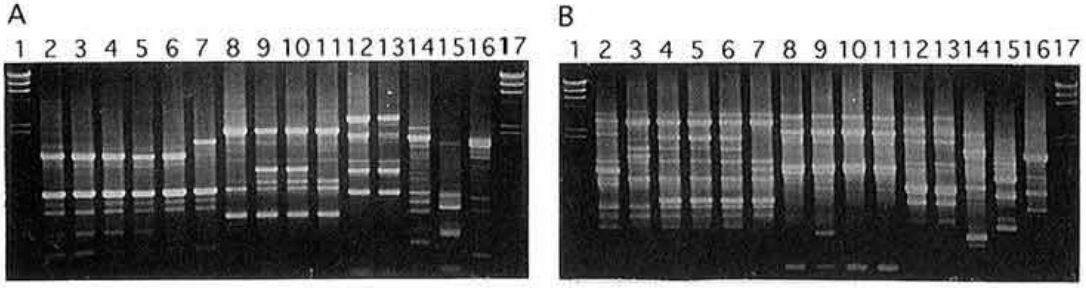

Fig. 1. RAPD patterns of 15 *Coprinus* strains in 6 species

A shows the RAPD patterns produced with the primer TGGTCACCGA, and B with AGCGCCATTG. Lanes 1 and 17, molecular size markers (λ DNA digested with Hind III); lane 2, unknown species of *C.* sp. 425079; lanes 3-7, *C. cinereus* wild, 5309, 5312, 5338 and 5348, respectively; lanes 8-11, *C. angulatus* 225, 516, 624 and 711, respectively; lanes 12, 13, *C. comatus* 7T and 11T, respectively; lane 14, *C. micaceus* A342S; lane 15, *C. disseminatus* E022; lane 16, *C. atramentarius* 430151.

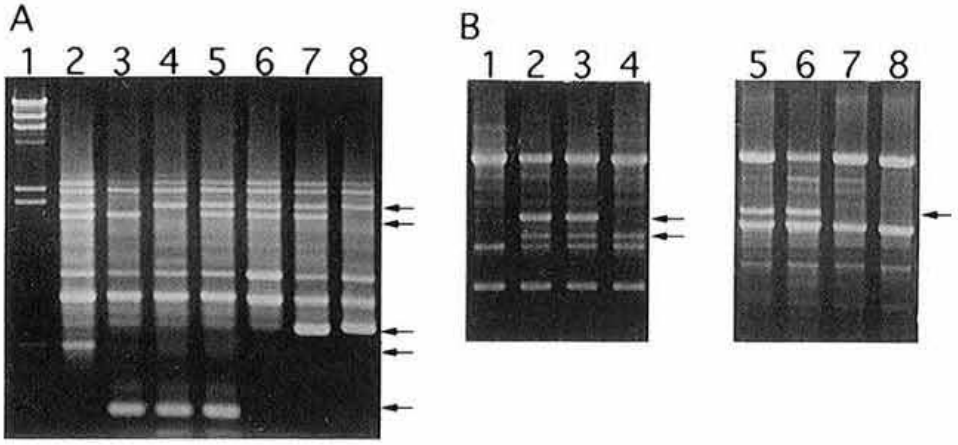

Fig. 2. Identification of basidiomycete strains by RAPD markers
A shows the RAPD patterns of 7 strains of *Coprinus cinereus* produced with the primer TTCGAGCCAG. Lane 1, molecular size marker (λ DNA digested with Hind III); lanes 2–8, *C. cinereus* wild, 5005, 5309, 5312, 5348, FR1, and *C.* sp. 425079 identified as *C. cinereus* in Fig. 1, respectively. B shows the RAPD patterns of 4 strains of *C. angulatus*. Lanes 1–4, *C. angulatus* 225, 516, 624, and 711, respectively, generated with the primer TGCGTGCTTG; and lanes 5–8, the same strains, respectively, generated with the primer GACTAGCCTC. Arrows indicate polymorphic fragments.

We attempted to identify the species to which a *Coprinus* strain stored as No. 425079 in the Genebank of MAFF belonged (Table 1). The RAPD patterns are presented in lanes No. 2 in Fig. 1–A and B. With all the primers used, the RAPD patterns of strain 425079 clearly showed fragments common to *C. cinereus* strains, and the absence of homology with strains of other species. Therefore, this strain was identified as *C. cinereus*. Compatibility tests of mating between strain 425079 and monokaryotic strains of *C. cinereus* also gave the same results in RAPD analysis.

Hopple Jr. and Vilgalys (1994)[3] detected differences in the restriction sites of rDNA between *Coprinus* and related species, and estimated their phylogenetic relationship. However in the RAPD patterns in this study, since different *Coprinus* species showed few similarities to each other, the RAPD patterns were not suitable for use for phylogenetic analyses. On the other hand, the low similarity of the patterns between species makes this technique suitable for the discrimination of *Coprinus* species, as demonstrated by the identification of the species to which an unknown *Coprinus* strain belonged.

2) Discrimination of strains of C. cinereus *and* C. angulatus

RAPD patterns of the *Coprinus* strains showed the presence of polymorphic DNA fragments among the strains in the same species. As shown in Fig. 2–A, 5 of such polymorphic fragments (indicated by arrows) were found simultaneously with 4 common fragments in the RAPD patterns of 7 *C. cinereus* strains using the primer TTCGAGCCAG. These polymorphic fragments were strain-specific, and the 7 strains could be distinguished by the presence or absence of these 5 polymorphic fragments which were generated with one primer only.

The RAPD patterns for 4 strains of *C. angulatus* are depicted in Fig. 2–B. One primer, TGCGTGCTTG, gave 2 polymorphic fragments shown in lanes 1–4. These 2 markers allowed the discrimination of strains 225 and 711, while strains 516 and 624 could not be distinguished. Another primer, GACTAGCCTC, amplified another polymorphic fragment, as shown in lanes 5–8. By the presence or absence of this fragment, strain 516 was clearly distinguished from strain 624. Thus, the combination of 2 primers, in this case, allowed a clear discrimination of the strains.

The clarity and simplicity of these examples in the discrimination of the strains of *C. cinereus* and *C. angulatus* indicated that the RAPD method is very efficient for strain discrimination. RAPD markers specific for strains could be obtained easily, and seemed to be useful as genetic markers.

Several papers on the identification of strains of *Coprinus* by DNA analysis have been published. Wu et al. (1983)[12] reported the presence of DNA polymorphisms caused by the insertion/deletion of DNA fragments or base pair substitutions by RFLP analysis of *C. cinereus*, and Laroche et al. (1995)[7] attempted to group and identify *C. psychromorbidus* by RAPD and RFLP analyses.

The method used to identify a strain is important for the protection of commercial mushroom species, like *Agaricus bisporus, Lentinus edodes, Pleurotus ostreatus*, and so on. It is easy to isolate the mycelia of commercially superior cultivars and to culture them without permission. To identify the cultivar, morphological characters of the fruit body can not be used, because they display wide variations with the environmental conditions. DNA fingerprinting techniques, such as RAPD, RFLP and AFLP, can describe specific DNA variations of the cultivar, and are useful to identify the cultivar.

3) *RAPD analysis for discrimination of* Tricholoma *species*

Several amplified DNA fragments were obtained from each of the 15 MAFF strains of the 5 *Tricholoma* species examined using 10 RAPD primers. Most of the RAPD primers gave several common fragments among the strains in the same species, while RAPD patterns of 5 *Tricholoma* species were species-specific (Fig. 3-A). However, *T. matsutake* showed similar RAPD patterns to those of *T. caligatum* or *T. magnivelare* in some cases (Fig. 3-B). To determine whether the RAPD analysis is suitable for the discrimination of *T. matsutake* from other *Tricholoma* species, 192 distinct RAPD fragments obtained by 10 primers were classified. Ten fragments were found only in *T. matsutake* strains to be species-specific, 4 were found in all the 7 *T. matsutake* strains and all the 2 *T. caligatum* strains, 7 in all the strains of *T. matsutake, T. caligatum*, and *T. magnivelare*, and 1 in all the strains of *T. matsutake* and *T. magnivelare*.

Based on the 192 RAPD fragments, cluster analysis was performed to determine the relationship between the 5 *Tricholoma* species. The 5 species were clearly divided into 5 groups in complete agreement with the taxonomic classification (Fig. 4). Intraspecific similarity between each strain was obviously higher than interspecific similarities among all the 5 species in *Tricholoma*. The highest similarity of 52.0% to *T. matsutake* was given by *T. caligatum*. *T. magnivelare, T. fulvocastaneum* and *T. bakamatsutake* gave 41.8, 38.7 and 24.6% similarities to *T. matsutake*, respectively. Since the intraspecific similarities among *T. matsutake* strains exceeded 85.0%, it appears that the RAPD patterns of the *T. matsutake* strains can be distinguished from those of the related species. Thus, RAPD analysis is a suitable method for distinguishing the 5 *Tricholoma* species as well as *T. matsutake* from the related species.

Six closely related species of *T. matsutake* have been reported, that is, the 4 species mentioned above and *T. robustum* and *T. zelleri*[8]. Among the 6 species, *T. matsutake, T. caligatum* and *T. magnivelare* have been considered to be closely related in terms of the morphology and fragrant aroma of the fruit body, the characters of cultured mycelia, the morphology of fungal colonies in soil called *shiro*, and mycotrophy with coniferous trees[8,9,13]. As described

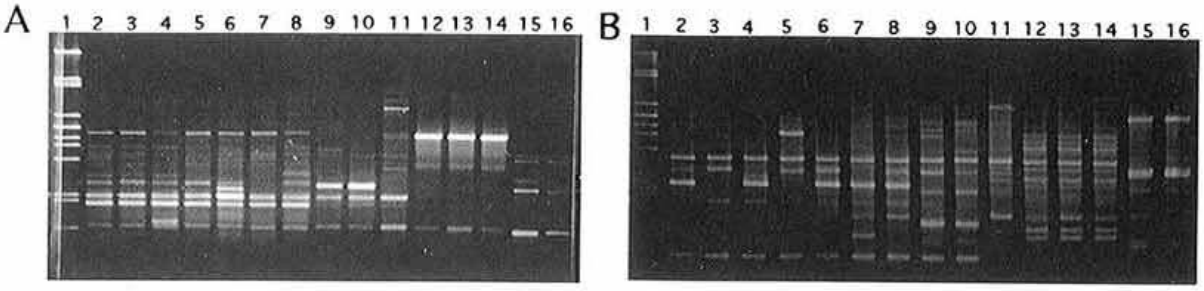

Fig. 3. RAPD patterns of strains of *T. matsutake* and related 4 species
A shows the RAPD patterns amplified with the primer of GACCGCTTGT, and B with TGCCGAGCTG. Lane 1, molecular size marker (λ DNA digested with *Pst* I); lanes 2-8, *T. matsutake* MAFF 460031, MAFF 460037, MAFF 460051, MAFF 460052, MAFF 460057, MAFF 460070 and MAFF 460101; lanes 9 and 10, *T. caligatum* MAFF 460064 and MAFF 460044; lane 11, *T. magnivelare* MAFF 460081; lanes 12-14, *T. fulvocastaneum* MAFF 460028, MAFF 460029 and MAFF 460030, lanes 15 and 16, *T. bakamatsutake* MAFF 460025 and MAFF 460087.

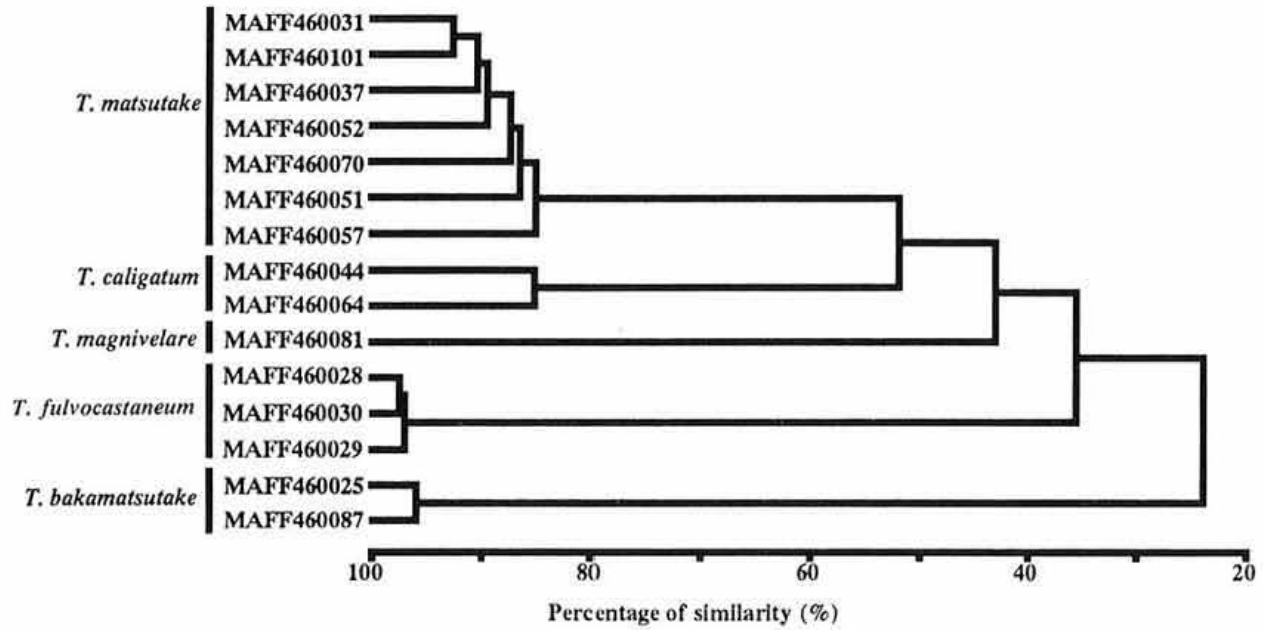

Fig. 4. Phylogenetic tree of *Tricholoma* species based on 192 RAPD fragments

above, our RAPD data corresponded to the morphological classification, confirming the close relationship of these 3 species at the genetic level.

The classification of *Tricholoma* species has not yet been completed, although *T. matsutake* and related species are widely distributed in the northern hemisphere. Several species were included in *T. caligatum* which is distributed in North and Central America, Europe and North Africa[5,6]. Some researchers consider that *T. matsutake* is an indigenous species in East Asia, but Kytövuori (1988)[6] stated that it was the synonym of *T. nauseosum* which is one of the species in the *T. caligatum* group distributed in Europe and North Africa. To define the species and elucidate the phylogenetic relationships of closely related *Tricholoma* species in more detail, it is suggested that DNA analyses, especially RAPD method as indicated in this study, may provide accurate genetic information.

References

1) Doyle, J. J. & Doyle, J. L. (1987): A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.*, **19**, 11-15.
2) Draper, J. et al. (1988): Plant genetic transformation and gene expression. A laboratory manual. Blackwell Scientific Publication, London, 212-214.
3) Hopple, Jr. J. S. & Vilgalys, R. (1994): Phylogenetic relationships among coprinoid taxa and allies based on data from restriction site mapping of nuclear rDNA. *Mycologia*, **86**, 96-107.
4) Ito, Y., Fushimi, T. & Yanagi, S. O. (1998): Discrimination of species and strains of basidiomycete genus *Coprinus* by random amplified polymorphic DNA (RAPD) analysis. *Mycoscience*, **39**, 361-365.
5) Iwase, K. (1996): Distribution of the Ectomycorrhizal fungus *Tricholoma matsutake* and the related species and some characteristics of their isolates. Soil microorganisms. Res. Counc. Secr. of MAFF and NIAR, 179-188.
6) Kytövuori, I. (1988): The *Tricholoma caligatum* group in Europe and North Africa. *Karstenia*, **28**, 65-77.
7) Laroche, A. et al. (1995): Grouping and identification of low temperature basidiomycetes using mating, RAPD and RFLP analyses. *Mycol. Res.*, **99**, 297-310.
8) Ogawa, M. (1978): Biology of matsutake mushroom. Tsukiji Shokan, Tokyo [In Japanese].
9) Ohara, H. & Ogawa, M. (1982): Microbial ecology of "shiro" in *Tricholoma matsutake* and its allied species XI. *Tricholoma caligatum* in *Cedrus libanotica* forest. *Trans. Mycol. Soc. Jpn.*, **23**, 365-377 [In Japanese with English summary].
10) Sneath, P. H. A. & Sokal, R. R. (1973): Numerical taxonomy. W. H. Freeman and Company, San Francisco.
11) Williams, J. G. K. et al. (1990): DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.*, **18**, 6531-6535.
12) Wu, M. M. J., Cassidy, J. R. & Pukkila, P. J. (1983): Polymorphisms in DNA of *Coprinus cinereus*. *Curr. Genet.*, **7**, 385-392.
13) Zeller, S. M. & Togashi, K. (1934): The American and Japanese matsu-takes. *Mycologia*, **26**, 544-558.

(Received for publication, January 11, 1999)