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# Discrimination of species and strains of basidiomycete genus *Coprinus* by random amplified polymorphic DNA (RAPD) analysis

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**All five examined strains of *Coprinus cinereus* could be clearly discriminated from the strains of five other *Coprinus* species by RAPD patterns with 12 of 13 primers. Also one specimen of unknown *Coprinus* strain was identified to be *C. cinereus* by this method. The RAPD patterns were similar among the strains in the same species; many common DNA fragments were recognized as well as some strain-specific DNA fragments. Thus all seven strains of *C. cinereus* and all four strains of *C. angulatus* examined could be distinguished individually. Dikaryotic strains showed the combined RAPD patterns of the two monokaryotic strains constituting the dikaryon. The combined RAPD markers observed in the dikaryons were segregated in their basidiospore progeny. All 18 randomly picked progeny showed different combinations of RAPD markers from the parental strains.**

Key Words——*Coprinus*; RAPD; strain identification.

Although basidiomycetes are mainly classified by the morphology of fruit bodies, many important basidiomycete species and strains do not readily, if at all, form fruit bodies under experimental conditions. A simple method to discriminate species or strains with vegetative mycelia will therefore be of great value.

Molecular markers, such as isozymes and restriction fragment length polymorphisms(RFLP), were investigated to detect genetic difference among species or strains of *Agaricus bisporus* (Lange) Imbach (Royse and May, 1982; Castle et al., 1987, 1988; Loftus et al., 1988), *Lentinula edodes* (Berk.) Pegler (Royse et al.; 1983; Kulkarni, 1991), *Pleurotus* (Magae et al., 1990; Sagawa et al., 1992), and *Coprinus* (Wu et al., 1983; Hopple and Vilgalys, 1994). These polymorphisms could also be used to discriminate strains as genetic finger-printing (Loftus et al., 1988).

Random amplified polymorphic DNA (RAPD), which is detected by use of the polymerase chain reaction (PCR) with arbitrary 10-mer primers, has been shown to be a useful molecular marker (Williams et al., 1990). RAPD analysis can detect DNA variations like other PCR methods and RFLP analyses, and it can be performed with less labor. This method has been utilized for taxonomy, systematic studies, hybrid identification, linkage analyses, and genetic mapping for wide a variety of organisms, in the case of basidiomycetes, reports include identification of *Agaricus brunnescens* Peck strains (Khush et al., 1992), genetic mapping of RAPD markers of *A. bisporus* (Kerrigan et al., 1993) and *Laccaria bicolor* (Maire) P. D. Orton (Doudrick et al., 1995), and phylogenic analyses of *Coprinus psychromorbidus* Redhead et Traguair (Laroche et al., 1995) and *Ganoderma lucidum* (W. Curtis: Fr.) Karsten (Hseu et al., 1996).

This paper describes an application of RAPD method for discrimination of *Coprinus* species and strains with DNAs isolated from mycelia.

## Materials and Methods

**Organisms** Species used for RAPD analyses were *Coprinus angulatus* Peck, *C. atramentarius* (Bull.: Fr.) Fr., *C. cinereus* (Schaeff.: Fr.) S. F. Gray, *C. comatus* (Muller: Fr.) S. F. Gray, *C. disseminatus* (Pers.: Fr.) S. F. Gray, and *C. micaceus* (Bull.: Fr.) Fr. A species-unkown *Coprinus* strain, *Coprinus* sp. maintained in the Gene Bank of Ministry of Agriculture, Forestry and Fisheries, Japan, was also analyzed. Source and mono/dikaryon type of each strain are listed in Table 1.

**Cultures** For mycelial cultures and experiments of spore germination, matings and fruiting were done on MYG medium (1% (w/v) malt extract, 0.4% (w/v) yeast extract, 0.4% (w/v) glucose, and 1.5% (w/v) agar) at 30°C under a cycle of 12 h dark and 12 h light. For DNA extraction, mycelia grown on the surface of an agar slant were inoculated into 20 ml of liquid MYG medium in 100-ml flasks and cultured statically at 30°C for 2–4 d. Harvested mycelia were stored at −20°C until the extraction of DNA.

**RAPD analysis** Whole mycelial DNA of each strain was extracted by the method of Doyle and Doyle (1987). Briefly, 200 mg of frozen mycelia were ground in liquid nitrogen, suspended in 1 ml of 2 × CTAB buffer (2% (w/v) hexadecyltrimethylammonium bromide, 100 mM Tris-

HCl, pH 8.0, 20 mM EDTA, pH 8.0, 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, and 1% (w/v) polyvinylpyrrolidone 40), and incubated at 65°C for 15 min. The suspensions were treated with chloroform : isoamylalcohol (24 : 1). After centrifugation for 5 min at 15,000×*g* in a microcentrifuge, DNAs in supernatants were precipitated with 2-propanol. DNA pellets were rinsed with 70% ethanol and dissolved in Tris-EDTA buffer.

DNA amplification was performed following a modified protocol of Williams et al. (1990). The reaction mixtures contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM concentrations of dATP, dCTP, dTTP, and dGTP, 1 μM primer, 0.4 U recombinant *Taq* DNA polymerase (Takara, Japan), and 10 ng genomic DNA in a total volume of 10 μl. Random primers were purchased from Toa Gousei. The primers used were GACTAGCCTC, GTATCGCGGT, TGGGCACTGA, TGGTCACCGA, TGCGTGCTTG, TTCGAGCCAG, AGTGGAAGGT, ATGCCTACAG, AGCGCCATTG, CTGTACATCT, CTCACCGTCC, CACCGTATCC, and CACCTAGTCC.

The procedure of amplification included: 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. These reactions were carried out in a Program Temp Control System PC-700 (ASTEC, Japan). Amplified DNAs were electrophoresed in 1.6% (w/v) agarose gels, stained with 0.5 μg/l ethidium bromide, and photographed under UV light.

## Results and Discussion

**Discrimination of species** Several DNA fragments from

Table 1. The strains used for the RAPD analyses.

| Species | Strain | Mono/dikaryon | Source |
|---|---|---|---|
| *Coprinus angulatus* | 225 | monokaryon | Okayama University, (maintained by NFRI[a] over 10 yr) |
| | 516 | monokaryon | Okayama University, (maintained by NFRI over 10 yr) |
| | 624 | monokaryon | Okayama University, (maintained by NFRI over 10 yr) |
| | 711 | monokaryon | Okayama University, (maintained by NFRI over 10 yr) |
| *C. atramentarius* | 430151 | unkown | Gene Bank of Ministry of Agriculture, Forestry and Fisheries, Japan |
| *C. cinereus* | wild | dikaryon | NFRI |
| | 5005 | monokaryon | Okayama University, (maintained by NFRI over 10 yr) |
| | 5309 | monokaryon | Okayama University, (maintained by NFRI over 10 yr) |
| | 5312 | monokaryon | Okayama University, (maintained by NFRI over 10 yr) |
| | 5338 | monokaryon | Okayama University, (maintained by NFRI over 10 yr) |
| | 5348 | monokaryon | Okayama University, (maintained by NFRI over 10 yr) |
| | FR1 | monokaryon | protoplast regenerant of Fis$^c$ [b] |
| *C. comatus* | 7T | dikaryon | NFRI (identified by Dr. T. Hongo) |
| | 11T | dikaryon | NFRI (identified by Dr. T. Hongo) |
| *C. disseminatus* | E022 | unkown | Forestry and Forest Products Research Institute |
| *C. micaceus* | A342S | unkown | Forestry and Forest Products Research Institute |
| *Coprinus* sp. | 425079 | dikaryon | Gene Bank of Ministry of Agriculture, Forestry and Fisheries, Japan |

a) National Food Research Institute. b) Fis$^c$ was supplied by the University of Tokyo, and maintained by NFRI over 10 yr.

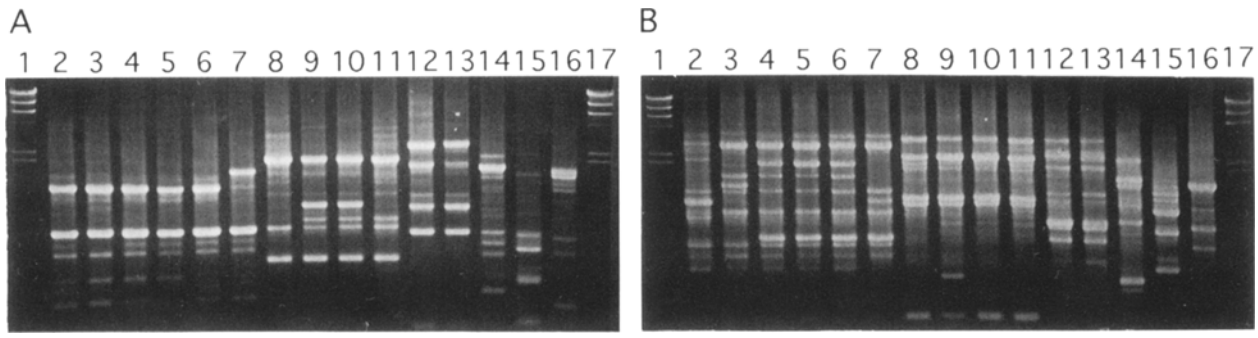


Fig. 1. RAPD patterns of 15 strains in six *Coprinus* species.
A shows RAPD patterns produced with the primer TGGTCACCGA, and B with AGCGCCATTG. Lanes 1 and 17, molecular size markers (lambda DNA digested with *Hind* III); lane 2, *Coprinus* sp. 425079; lanes 3–7, *C. cinereus* wild, 5309, 5312, 5338 and 5348, respectively; lanes 8–11, *C. angulatus* 225, 516, 624 and 711, respectively; lanes12, 13, *C. comatus* 7T and 11T, respectively; lane14, *C. micaceus* A342S; lane 15, *C. disseminatus* E022; lane16, *C. atramentarius* 430151.

the genomic DNAs of *Coprinus* strains (Table 1) were successfully amplified with 12 primers among 13 examined. One primer, CTGTACATCT, gave no amplified product in any species; the reason was not known. Two typical results of RAPD analyses are shown in Figs. 1 A, B. Strains of the same species revealed similar RAPD patterns that included several DNA fragments of equivalent length (sample lanes 3–7, 8–11, and 12–13, respectively), making it possible to discriminate species. Strains of different species, however, yielded no common fragments. RAPD patterns of other 10 primers gave similar results.

We tried to identify the species of a *Coprinus* strain which was stocked as No. 425079 in the gene bank of MAFF (Table 1). Its RAPD patterns are presented in lanes No. 2 in Figs. 1A, B. With all primers used, the RAPD patterns of strain 425079 clearly showed fragments common to *C. cinereus* strains and no homology with strains of other species. Therefore, this strain was identified as *C. cinereus*.

Compatibility tests between strain 425079 and *C. cinereus* monokaryotic strains were performed to confirm the results of RAPD analyses. Since strain 425079 is a dikaryon having clamp connections, dikaryon-monokaryon (di-mon) mating was performed between 425079 and *C. cinereus* monokaryotic strains FR1, 5005, and 5048, which have different A and B mating factors. Strains 5005 and 5048 formed dikaryotic hyphae with clamp connections on their monokaryotic colony margins, confirming that the strain 425079 is *C. cinereus*.

Hopple Jr. and Vilgalys (1994) detected differences in the restriction sites of rDNA between *Coprinus* and related species and estimated their phylogenetic relationship. Since different *Coprinus* species showed few similarities to each other in RAPD patterns in this work, these RAPD patterns would not be appropriate to use for phylogenetic analyses. However, the low similarity of patterns between species makes this technique suitable for discrimination of *Coprinus* species, as demonstrated by the identification of the species of an unknown *Coprinus* strain.

**Discrimination of strains** RAPD patterns of *Coprinus* strains of the same species showed the presence of polymorphic DNA fragments. As shown in Fig. 2A, five such polymorphic fragments (indicated by arrows) were found simultaneously with four common fragments in the RAPD patterns of seven strains of *C. cinereus* using the primer TTCGAGCCAG. These polymorphic fragments were strain-specific, and the seven strains could be discriminated by these five polymorphic fragments generated by only one primer (Table 2).

Data for four strains of *C. angulatus* are shown in Fig. 2B and Table 3. One primer, TGCGTGCTTG, gave two polymorphic fragments in the RAPD patterns shown

Table 2. Presence of RAPD markers represented in Fig. 2A in seven strains of *C. cinereus*.

| Strain | Wild | 5309 | 5312 | 5338 | 5348 | FR1 | *C.* sp |
|---|---|---|---|---|---|---|---|
| marker | | | | | | | |
| T4-a | + | + | + | − | + | + | + |
| T4-b | + | − | + | + | + | + | − |
| T4-c | − | − | − | − | − | + | + |
| T4-d | + | − | − | − | − | − | − |
| T4-e | − | + | + | + | − | − | − |

+ =presence; − =absence.

Table 3. Presence of RAPD markers represented in Fig. 2B in four strains of *C. angulatus*.

| Strain | 225 | 516 | 624 | 711 |
|---|---|---|---|---|
| marker | | | | |
| T2-a | − | + | + | − |
| T2-b | − | + | + | + |
| G1-a | + | + | − | − |

+ =presence; − =absence.

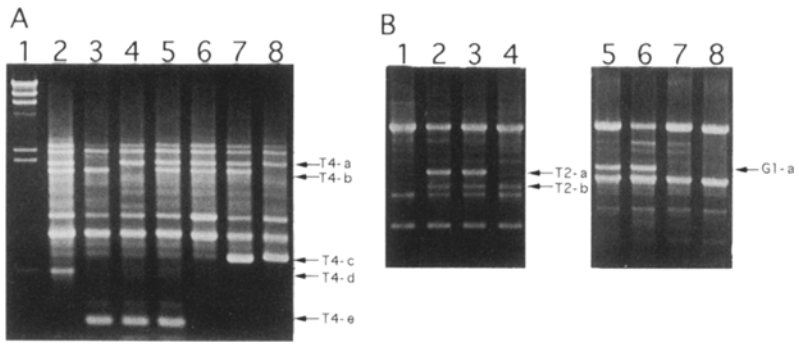


Fig. 2. Identification of basidiomycete strains by RAPD markers.
A shows RAPD patterns of seven strains of *Coprinus cinereus* produced with the primer TTCGAGCCAG. Lane 1, molecular size marker (lambda DNA digested with *Hind* III); lanes 2–8, *C. cinereus* wild, 5005, 5309, 5312, 5348, FR1 and *Coprinus*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

in lanes 1–4. These two markers allowed discrimination of strains 225 and 711, but strains 516 and 624 could not be separeted. 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 discriminated from strain 624. Thus, the combination of two primers, in this case, allowed the clear discrimination of strains.

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

Several papers have been published on the identification of strains of *Coprinus* by DNA analyses. Wu et al. (1983) reported DNA polymorphisms caused by the insertion/deletion of DNA fragments or base pair substitutions by RFLP analyses on *C. cinereus*, and Laroche et al. (1995) tried grouping and identification of *C. psychromorbidus* by RAPD and RFLP analyses.

**RAPD patterns together with mating and meiosis** The RAPD patterns of *C. cinereus* strains in relation to their life cycles are presented in Fig. 3. The dikaryon con-

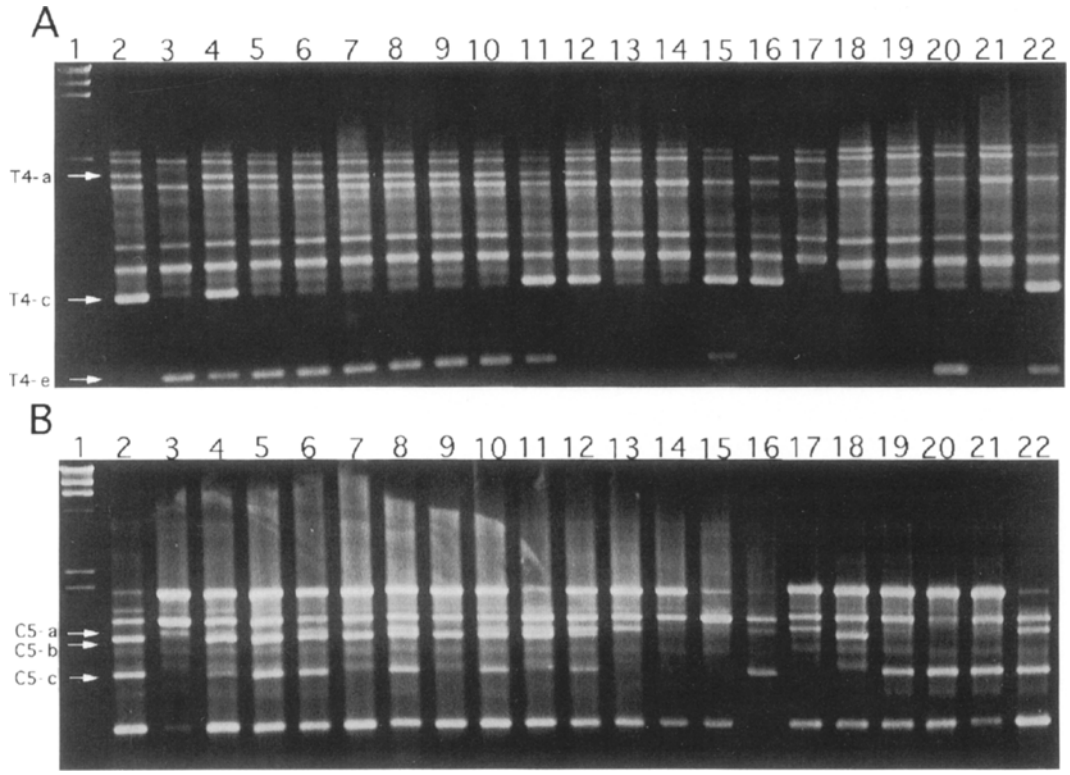


Fig. 3. Inheritance of RAPD markers in *C. cinereus* strains.
A shows RAPD patterns produced with the primer TTCGAGCCAG, and B with CTCACCGTCC. Lane 1, molecular size marker (lambda DNA digested with *Hind* III); lane 2, FR1; lane 3, 5005; lane 4, dikaryotic strain formed by the mating of FR1 and 5005; lanes 5–22, basidiospore-clones produced by the dikaryon. T4-a, c and e, and C5-a–c indicate polymorphic fragments.

Table 4. Presence of RAPD markers represented in Fig. 3.

| Lane | FR1 | 5005 | Hyb. | Progeny | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 |
| marker | | | | | | | | | | | | | | | | | | | | | |
| T4-a | + | − | + | + | + | + | + | + | + | + | + | − | − | − | − | − | − | − | − | − | − |
| T4-c | + | − | + | − | − | − | − | − | − | + | + | − | − | + | + | − | − | − | − | − | + |
| T4-e | − | + | + | + | + | + | + | + | + | + | − | − | − | + | − | − | − | − | + | − | + |
| C5-a | − | + | + | − | + | − | + | − | + | + | + | + | − | − | + | + | − | − | − | − | + |
| C5-b | + | − | + | + | + | + | + | + | + | + | + | − | − | − | − | − | + | − | − | − | − |
| C5-c | + | − | + | + | + | − | + | − | + | − | + | − | − | − | + | − | − | + | + | + | + |

+ = presence; − = absence.

structed by mating between FR1 and 5005 produced fruit bodies with germinable basidiospores. From the germinated basidiospore progeny, 18 were picked up randomly and analyzed by RAPD using two primers, TTCGAGCCAG and CTCACCGTCC.

The RAPD patterns of two parental strains in lanes 2 and 3 showed both common fragments and distinguishable polymorphic fragments, T4-a, -c, -e, and C5-a, -b, -c. The RAPD patterns of the constructed dikaryon in lanes 4 include both polymorphic fragments of FR1 (T4-a, -c and C5-b,-c) and 5005 (T4-e and C5-a) together with common fragments of the two strains, proving the formation of the hybrid. The additive appearance of both parental RAPD markers was also observed in the mating combination of strains 5338 and 5348 (data not shown).

Lanes 5–22 in Figs. 3A, B show RAPD patterns of the progenic basidiospore clones derived from the dikaryon (FR1 × 5005). Most of the polymorphic fragments in the parents were randomly segregated in the progeny, suggesting that the chromosome segregation happened randomly through meiosis in *C. cinereus* (Table 4). T4-a and C5-b, which were FR1-specific fragments, however, were co-segregated: the parental types occurred in 17 of 18 progenies. These two fragments, therefore, were suggested to be on the same chromosome and linked. The exceptional strain in lane 18 might represent recombination between these two markers.

In basidiomycetes, inheritance of molecular markers, including isozymes, RFLPs, PCR fragments, and RAPDs, has been monitored to study the crossing or behaviour on meiosis in *A. bisporus*=*A. brunnescens* (Castle et al., 1988; Loftus et al., 1988; Summerbell et al., 1989; Khush et al., 1992), *L. edodes* (Kulkarni, 1991), and *C. cinereus* (Freedman and Pukkila, 1997); and linkage maps were constructed for *A. bisporus* (Kerrigan et al., 1993), *L. edodes* (Bowden and Royse, 1991), and *L. bicolor* (Doudrick et al., 1995).

Here we showed in *C. cinereus* that the RAPD markers were inherited by basidiospore progeny through meiosis, and that they should be useful for genetic analyses in *Coprinus*.

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