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# PCR-RFLP Analysis of Amplified 28S Ribosomal DNA for Identification of Rhizoctonia spp., the Causal Agents of Sheath Diseases of Rice Plants

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Isolates of five *Rhizoctonia* spp., the causal agents of sheath disease of rice plants, were characterized by the restriction fragment length polymorphisms (RFLPs) analysis of a 28S ribosomal DNA (rDNA) gene amplified by the polymerase chain reaction (PCR). PCR-amplified products specific to AG 1-IA and AG 2-2 of *R. solani*, WAG-O of *R. oryzae*, AG-Ba of *R. fumigata* and AG-Bb of *R. oryzae-sativae* were selected and used for RFLP analysis. RFLP profiles obtained after digestion of 28S rDNA with the restriction enzymes were different depending on the four enzymes, *Hpa*II, *Hha*I, *Sau*3AI and *Hae*III, used. The RFLP profiles by the digestion with *Hpa*II seemed to be useful for the benchmarks to discriminate *Rhizoctonia* spp.

## INTRODUCTION

In Japan, causal agents of rice sheath blight diseases and similar diseases have been identified as multinucleate *Rhixoctonia* spp. such as *R. solani* AG l-IA, AG 2-2 IIIB (Ogoshi, 1976; 1987) and *R. oryzae* WAG-O (Oniki et al., 1985) and binucleate *Rhixoctonia* spp. such as *R. fumigata* AG-Ba (Ogoshi *et al.*, 1979; 1983) and *R. oryzae-sativae* AG-Bb (Gunnell and Webster, 1987; Ogoshi *et al.*, 1979). The symptoms caused by these *Rhixoctonia* spp. on rice sheath are very similar, and reliable identification by visual observation is often difficult. Therefore, studies on discrimination and/or diagnosis of these *Rhixoctonia* spp. would increase understanding of the symptomatological complex (Cubeta *et al.*, 1991; Damaj *et al.*, 1993).

These five *Rhixoctonia* spp. have been previously classified according to their ecology, morphology and hyphal anastomosis (Watanabe and Matsuda, 1966; Ogoshi, 1976; Ogoshi *et al.*, 1979). Moreover, there have been many reports about molecular taxonomic studies on *Rhizoctonia* spp. (Duncan *et al.*, 1993; Jabaji-Hare *et al., 1990;* Vilgalys and Gonzalez, 1990). Analysis of nuclear-encoded ribosomal DNA (rDNA) by restriction fragment length polymorphism (RFLP) revealed that different AGs in *R. solani* and binucleate *Rhixoctonia* spp. are genetically divergent (Liu *et al.*, 1993; Liu and Sinclair, 1992; 1993; Cubeta *et al.*, 1991). In the present study, an attempt was made to investigate the appropriation of molecular benchmarks for the discrimination and diagnosis of these *Rhixoctonia* spp. based on the results of PCR for amplification, followed by RFLPs analysis of nuclear-encoded 28S rDNA.

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## MATERIALS AND METHODS

### Fungal isolates and maintenance

Thirty isolates of *Rhizoctonia* spp. used in this study are listed in Table 1. The isolates were maintained on potato-dextrose agar (PDA, Funakoshi Chem. Co.) slants amended with 50 mg/L streptomycin sulfate at 25°C in dark. To prepare DNA samples for restriction analysis, three thin mycelial disks of each isolate cultured on PDA, 4 mm in diameter, were gently floated on 50 ml of a liquid nutrient-broth medium (Difco) amended with 50 mg/L of streptomycin sulfate in 100-ml flask and incubated at 25°C for 7

**Table 1.** List of investigated isolates of *Rhizoctonia* spp.

| Species | Isolate | AG | Source") | Origin |
|---|---|---|---|---|
| *Rhizoctonia solani* | Cs-Ka | 1 IA | Rice | ATCC 76121 |
| | C-325 | 1 IA | Rice | IFO 30935 |
| | C-326 | 1 IA | Rice | IFO 30936 |
| | cs-2 | 1 IA | Rice | AHU |
| | Cs-Gi | 1 IA | Rice | AHU |
| | C-96 | 2-2 IIIB | Mat rush | ATCC 76124 |
| | c-100 | 2-2 IIIB | Mat rush | MAFF |
| | C-116 | 2-2 IIIB | Mat rush | MAFF |
| | C-328 | 2-2 IIIB | Rice | IFO 30944 |
| | c-354 | 2-2 IIIB | Rice | AKU |
| *R. oryzae* | Ro-0105 | WAG-O | Rice | AMU |
| | M23 | WAG-O | Rice | AMU |
| | c-505 | WAG-O | Rice | AKU |
| | R-l | WAG-O | Rice | AKU |
| | KAES-14 | WAG-O | Rice | AKU |
| *R. fumigata* | *TO-7* | Ba | Rice | AMU |
| | TM-2B | Ba | Rice | AMU |
| | KS-T1-3 | Ba | Rice | AKU |
| | K-T1 | Ba | Rice | AKU |
| | K-T4 | Ba | Rice | AKU |
| *R. oryzae-sativae* | 93Gi | Bb | Rice | AMLJ |
| | 94s | Bb | Rice | AMU |
| | KS-T2-4 | Bb | Rice | AKU |
| | KS-T2-5 | Bb | Rice | AKU |
| | K-T5 | Bb | Rice | AKU |

a) ATCC: American Type Culture Collection, USA.
IFO: Institution for Fermentation, Osaka, Japan.
AHU: Faculty of Agriculture, Hokkaido University, Hokkaido, Japan.
AKU: Faculty of Agriculture, Kyushu University, Fukuoka, Japan.
MAFF: Ministry of Agriculture, Forestry and Fisheries. Tsukuba, Japan.
AMU: Faculty of Agriculture, Meijyou University, Nagoya, Japan.

days. The mycelial mat was harvested, washed repeatedly with water, lyophilized and then stored at −20°C until use.

**Extraction of DNA and amplification with PCR**

Extraction of genomic DNA was done according to the procedure of Vilgalys and Gonzalez (1990) with slight modifications. The lyophilized and ground mycelia (30 mg) were homogenized in 450 μl of extraction buffer (50 mM Tris-HCl, pH 7.2, 50 mM EDTA, 1% sodium *N*-lauroyl sarcosinate, 1% 2-mercaptoethanol) and incubated at 65°C for 30 min. The homogenate was thoroughly shaken using 450 μl of chloroform/phenol/ isoamyl alcohol (24:25:1, v/v/v) mixture. After centrifugation for 15 min at 15,000 rpm, the water phase was collected and ammonium acetate was added so as to be 2.5 M. DNA was precipitated with 225 μl of isopropanol and then collected by the centrifugation at 13,000 rpm for 15 min. The pellet was washed with 70% aqueous ethanol solution, dried *in vacuo*, dissolved in 120 μ 1 of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and incubated at 37.5°C for 30 min for treatment with ribonuclease A (20 ng/ml, DNase-free; Sigma, USA).

**Polymerase chain reaction (PCR)**

Two primers were synthesized for the amplification of a portion of 28S rDNA repeat homologous to positions 17-1, 448 in the *Saccharomyces cerevisiae* 25S rRNA according to the procedure of Vilgalys and Hester (1990). Ten μl of 100-fold dilution of DNA solution prepared by the above procedure was added to reaction mixture as recommended by the manufacturer using *Tth* DNA polymerase (TOYOBO Biochemicals, Co. Japan) in 100 μ 1 volume. The thermal cycles were conducted 30 times, with parameter of 1 min at 94°C, 2 min at 50°C, 1 min at 50-72°C for gradual increase and 3 min at 72°C. After amplification, each sample was subjected to electrophoresis using a 1% agarose gel in TBE buffer (100 mM Tris-HCl, 20 mM EDTA, 100 mM boric acid, pH 8.3).

**Restriction fragment length polymorphism (RFLP) analysis**

After preparation of PCR-amplified rDNA, each PCR product was digested with 4 restriction enzymes (*Hpa*II, *Hae*III, *Hha*I and *Sau*3AI) and the sample was subjected to electrophoresis in a 3.5% agarose gel (NuSieve, FMC Bioproducts) with TBE buffer.

## RESULTS AND DISCUSSION

The 28S rDNA products, a single 1.4-kbp or 1.8-kbp fragment, were detected by agarose gel electrophoresis following amplification with PCR (Fig. IA). A single 1.4-kbp fragment was obtained from two test isolates (WAG-O and AG-Bb) and 1.8-kbp one was obtained from remaining isolates (AG l-IA, AG 2-2 IIIB and AG-Ba). Test isolates of AG 2-2 IIIB from mat rush and rice were used in this study, and the qualitative variation of PCR-amplified products of 28S rDNA was not observed (Data not shown).

RFLP profiles obtained by electrophoresis after digestion of PCR-amplified 28S rDNA represented specific fragment patterns (Figs. 1B-D, Table 2). After digestion of PCR-

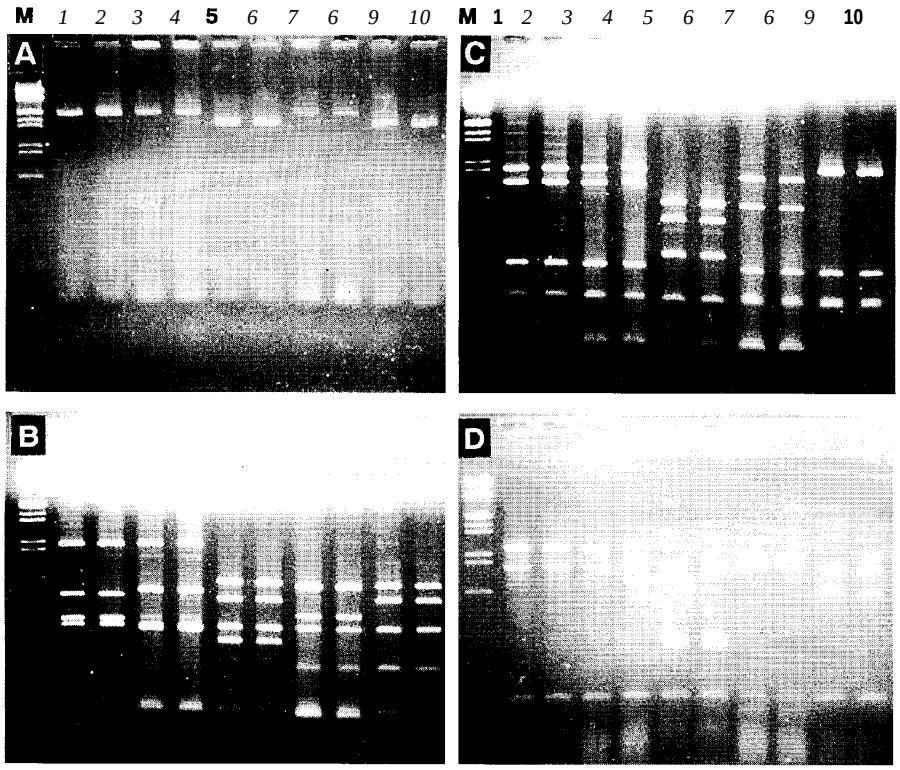


**Fig. 1.** The electrophoretic patterns of amplified 28S rDNA of Rhizoclonia sheath diseases of rice plant (A) and RFLP patterns of DNA fragments after digestion with *Hpa*II (B), *Hha*I *(C)* and *Sau*3AI (D). Lane 1, **Cs-** Ka (AG 1-IA); Lane 2, C-325 (AG l-IA); Lane 3, C-96 (AG 2-2 IIIB); Lane 4, C-354 (AG 2-2 IIIB); Lane 5, C-505 (WAG-O); Lane 6, Ro-0105 (WAG-O); Lane 7, TO-7 (AG-Ba); Lane 8, KS-Tl-3 (AG-Ba); Lane 9, 93Gi (AG-Bb); Lane 10, K-T5 (AG-Bb) and M, DNA size marker of J-DNA digested with *Eco*RI and *Hin*dIII (from top to bottom, 24756, co-migrating bands of 5148, 5148, 4937 and 4268, co-migrating bands of 2027 and 1904, 1584, 1375, 947, 831 and 564). Faint bands observed were non-specific products digested.

amplified 28S rDNA with *Hpa*II, all isolates produced a common 290-bp fragment (Fig. 1B). RFLP profiles were different among isolates of each five *Rhizoctonia* spp. (Fig. 1B). After digestion of PCR-amplified rDNA with the enzymes *Hha*I, *Sau*3AI and *Hae*III, all isolates produced a common 160-bp fragment (Fig. 1C), common 110- and 800-bp fragments (Fig. 1D) and common 150- and 220-bp fragments (Table 2), respectively. The isolates of AG 1-IA and AG 2-2 IIIB in *R. solani* showed identical RFLP profiles by digestion with each *Hha*I, *Sau*3AI and *Hae*III. However, the profiles from these isolates were obviously different from WAG-O, AG-Ba and AG-Bb. Moreover, the isolates of each WAG-O, AG-Ba and AG-Bb showed different RFLP profiles and produced unique sets of

**Table 2.** Sizes (bp) of DNA fragment generated after digestion of 28S rDNA from *Rhizoctonia* spp. with four restriction enzymes.

| | Restriction enzyme | | | |
|---|---|---|---|---|
| Anastomosis group[a] | *Hpa*II[b] | *Hha*I[c] | *Sau*3AI[d] | *Hae*III[e] |
| AG l-IA | 310, 450, 900 | 230,680, 990 | 950 | 200, 330, 450, 500 |
| AG 2-2 IIIB | 280, 500, 900 | 230, 680, 990 | 950 | 200, 330, 450, 500 |
| WAG 0 | 260, 400, 550 | 260, 390, 480 | 200,250 | 100,900 |
| AG Ba | 170, 330, 500 | 230, 480, 800 | 900 | 180, 250, 450 |
| AG Bb | 170, 400, 500 | 230,990 | 550 | 450, 550 |

a) Represent 12 AGs of *R.* solani, AGs of *R. oryzae* and 16 Japanese AGs of binucleate *Rhizoctonia* species

b) All isolates produced a common 290-bp fragment after digestion with *Hpa*II.

c) All isolates produced a common 160-bp fragment after digestion with *Hha*I.

d) All isolates produced common 1 10- and 800-bp fragment after digestion with *Sau*3AI.

*e)* All isolates produced common 150- and 220-bp fragment after digestion with HaeIII.

fragments by digestion with each *Hha*I, *Sau*3AI and HaeIII (Fig. 1C-D, Table 2).

RFLPs detected at the electrophoresis after digestion of amplified 28S rDNA with *Hpa*II, *Hha*I, *Sau*3AI and HaeIII revealed the possibility of distinction of five *Rhizoctonia* spp. by this method (Fig.lB-D, Table 2). The isolates of AG l-IA and AG 2-2 IIIB in *R. solani* showed identical RFLP profiles by the digestion with *Hha*I, *Sau*3AI and HaeIII, whereas the profiles by the digestion with *Hpa*II were obviously different. These results would suggest that RFLP profile by digestion with *Hpa*II will be available to use as molecular benchmarks to discriminate isolates of *Rhixoctonia* spp. Moreover, this method would involve the possibility of genetic diagnosis, and can be used for identification and characterization of isolates of *Rhixoctonia* spp. combining with anastomosis and the other properties.

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