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Short Communication

Rapid detection of *Rhizoctonia* species, causal agents of rice sheath diseases, by PCR-RFLP analysis using an alkaline DNA extraction method

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Restriction fragment length polymorphism (RFLP) analysis for DNA products amplified by the polymerase chain reaction (PCR) was used for the direct detection of *Rhizoctonia solani* AG 1 IA and AG 2-2 IIIB, *R. oryzae*, *R. oryzae-sativae* and *R. fumigata* from the diseased rice sheaths. A rapid DNA extraction method with a solution of sodium hydroxide was conducted to extract parasite DNA from diseased rice sheaths. 28S ribosomal DNA (rDNA) derived from fungal genomic DNA extracted by the alkaline method was specifically PCR-amplified. The results of PCR-RFLP analysis for DNA samples from artificially inoculated rice sheath tissues with each *Rhizoctonia* spp. and the corresponding culture on the medium using two restriction enzymes, *Hha*I and *Msp*I, showed identical polymorphisms. PCR-RFLP analysis using DNA samples from naturally infected rice sheath tissues also revealed the possibility of direct diagnosis of *R. solani* AG 1 IA, *R. oryzae* and *R. oryzae-sativae*.

Key Words——alkaline DNA extraction; PCR-RFLP analysis; rapid detection; *Rhizoctonia* spp.

Rhizoctonia solani Kühn (teleomorph, *Thanatephorus cucumeris* (Frank) Donk) anastomosis group (AG) 1 is a causal agent of sheath blight disease of the rice plant. The subgroup of *R. solani* responsible for sheath blight is AG 1 IA. This fungus is widespread in rice fields, forming sclerotia on diseased leaf sheaths. Several other fungi belonging to *Rhizoctonia* spp. have also been isolated from sheath blight-like lesions on rice plants. These are *R. oryzae-sativae* (Ogoshi et al., 1979; Gunnell and Webster, 1987), *R. solani* AG 2-2 IIIB (Ogoshi, 1976, 1987), *R. oryzae* (Oniki et al., 1985) and *R. fumigata* (Ogoshi et al., 1979, 1983). Since these fungi cause similar symptoms on rice sheaths, their identification and distinction by visual observation are often difficult. Therefore, a simple method for determining the *Rhizoctonia* diseases would assist in understanding their symptomatology.

Using an oligonucleotide primer obtained from the sequence of the 28S ribosomal DNA (rDNA) homologous to *Saccharomyces cerevisiae* (Vilgalys and Hester, 1990), polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analyses have been performed for differentiation and characterization of rice pathogens consisting of *Rhizoctonia* spp. (Cubeta et al., 1991; Matsumoto et al., 1996b). Two restriction endonucleases, *Hha*I and *Msp*I, provided specific digestion profiles for isolates of *R. solani* AG 1 IA and the other *Rhizoctonia* spp. (Matsumoto et al., 1996a). However, the PCR-RFLP analysis demonstrated in previous reports tends to require much time and labor for extraction of genomic DNA. An easy and rapid DNA extraction method could overcome this defect. Alkaline DNA extraction has been applied as a simple method for preparing plant DNA samples for PCR (Wang et al., 1993). The aim of this study is direct identification of the causal agents of rice sheath diseases in infected sheaths using PCR-RFLP analysis by a rapid DNA extraction method with NaOH solution.

Isolates of *Rhizoctonia* species used in this study are listed in Table 1. All isolates were initially grown on potato-dextrose agar (Difco) in a Petri dish. Plugs (4 mm in diam) taken from edge of the colonies were placed in 50 ml of a liquid nutrient-broth medium (Difco) amended with 5 mg of streptomycin sulfate in a 100-ml flask and cultured at 25°C for 7 d. The mycelium was harvested, washed repeatedly with distilled water, lyophilized and stored at −20°C until use.

Three isolates of each *Rhizoctonia* species were artificially inoculated on rice plants of cultivar Asominori. Inoculation was done by placing a 3 to 4-d-old PSA disk (7 mm in diam) inside or directly on a leaf sheath, and the inoculated sites were covered with aluminum foil for 7–10 d. Inoculated plants were then incubated in a growth chamber (16 h in the light at 28°C and 8 h in the dark at 20°C) under almost 100% relative humidity. Three plants per *Rhizoctonia* species (one plant per isolate) were inoculated. A total of 15 samples of artificially inoculated and 50 samples of naturally infected leaf sheath were harvested, washed with distilled water and stored at −20°C until use.

Table 1. Isolates of *Rhizoctonia* species used in this study.

Species	Isolate	AG	Origin
R. solani	C-326	1 IA	IFO[a] 30936
	Cs-2	1 IA	AHU[b]
	Cs-Gi	1 IA	AHU
	C-116	2 2 IIIB	NIAR[c]
	C-328	2 2 IIIB	IFO 30944
	C-354	2 2 IIIB	AKU[d]
R. oryzae	Ro-0105	WAG-O	AMU[e]
	M23	WAG-O	AMU
	C-505	WAG-O	AKU
R. fumigata	To-7	Ba	AMU
	TM-2B	Ba	AMU
	KS-T1-3	Ba	AKU
R. oryzae-sativae	93Gi	Bb	AMU
	94S	Bb	AMU
	KS-T2-4	Bb	AKU

a) IFO: Institute for Fermentation, Osaka, Japan.
b) AHU: Faculty of Agriculture, Hokkaido University, Hokkaido, Japan.
c) NIAR: National Institute of Agrobiological Resources, Ibaraki, Japan.
d) AKU: Faculty of Agriculture, Kyushu University, Fukuoka, Japan.
e) AMU: Faculty of Agriculture, Meijyo University, Nagoya, Japan.

PCR amplifications were conducted for pure fungal cultures, artificially and naturally infected, and mock-inoculated rice sheath tissues. Total genomic DNA was extracted by a quick alkaline DNA extraction method according to Wang et al. (1993) with slight modifications. Before DNA extraction, each sample was soaked for 5 min in 1 ml of sterile 0.05 M phosphate buffer, pH 7.0, containing 0.05% NaCl and 0.01% Tween 20 (phosphate buffered saline (PBS)). These samples were dissolved in 300 μl of 0.5 N NaOH with 0.5% (w/v) polyvinylpyrrolidone (PVP; Sigma Chem.). Five microliters of the lysate sample was transferred to a new tube containing 495 μl of 20 mM Tris-HCl, pH 8.0. Aliquots of 5 μl were used for individual PCR amplifications. Water was used as a negative control.

PCR amplification was performed in a reaction mixture containing 5 μl of DNA extract in 20 mM Tris-HCl, pH 8.0., 2.5 units of *Taq* DNA polymerase (Promega), 10 μl of 10$\times$ *Taq* PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100), 100 μM of each dNTP (dATP, dCTP, dGTP and dTTP), 50 pmol each of forward and reverse primers, 2.5 mM $MgCl_2$, and sterile water in a total volume of 100 μl in a 0.5-ml of microfuge tube. Fifty microliters of mineral oil (Katayama Chem.) were added to each tube to prevent evaporation. For identification of *Rhizoctonia* species, primer LR0R (5'-ACCCGCTGAACTTAAGC-3') was paired with primer LR7 (5'-TACTACCACCCAAGATCT-3'). These primers were synthesized for the amplification of a portion of the *R. solani* 28S rDNA repeat units. The program for amplification consisted of 30 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 2 min and DNA extension at 72°C for 3 min. Aliquots (10 μl of the amplification products) were subjected to electrophoresis using a 1.0% agarose gel with Tris-borate-EDTA buffer at 100 V for 1 h, and the gel was stained with ethidium bromide. Restriction endonucleases, *Hha*I and *Msp*I, were used for differentiation of the *Rhizoctonia* species by PCR-RFLP analysis. Isolates of each *Rhizoctonia* species obtained from naturally diseased rice sheath tissues were identified by morphological observations (data not shown).

Stringent PCR conditions allowed amplification of a single fragment of 28S rDNA with the same size of about 1.4 kbp in *R. solani* AG 1 IA, *R. oryzae* and *R. oryzae-sativae* and of one with a size of about 1.8 kbp in *R. solani* AG 2-2 IIIB and *R. fumigata* (data not shown). In particular, the PCR amplification fragment related to the fungal genomic DNA could be specifically obtained from diseased rice sheath tissues as well as from pure cultures. This method allowed for PCR-amplification of 28S rDNA obtained from fungal genomic DNA of *Rhizoctonia* species. To assess the direct identification of *Rhizoctonia* spp. in diseased rice sheaths, PCR-RFLP analysis was performed for DNA samples of *R. solani*, *R. oryzae*, *R. fumigata* and *R. oryzae-sativae* obtained from pure cultures and inoculated rice sheath tissues using an alkaline DNA extraction method (Fig. 1.). The digestion of PCR products with *Hha*I of *Msp*I provided unambiguous patterns for both DNA samples from pure cultures and inoculated rice sheath tissues, for all 15 isolates of four *Rhizoctonia* species tested.

PCR-RFLP analysis for direct identification of *Rhizoctonia* spp. was also performed for DNA samples extracted from sheath tissues of naturally diseased rice plants (Fig. 2.). Stringent PCR amplification of 28S rDNA yield-

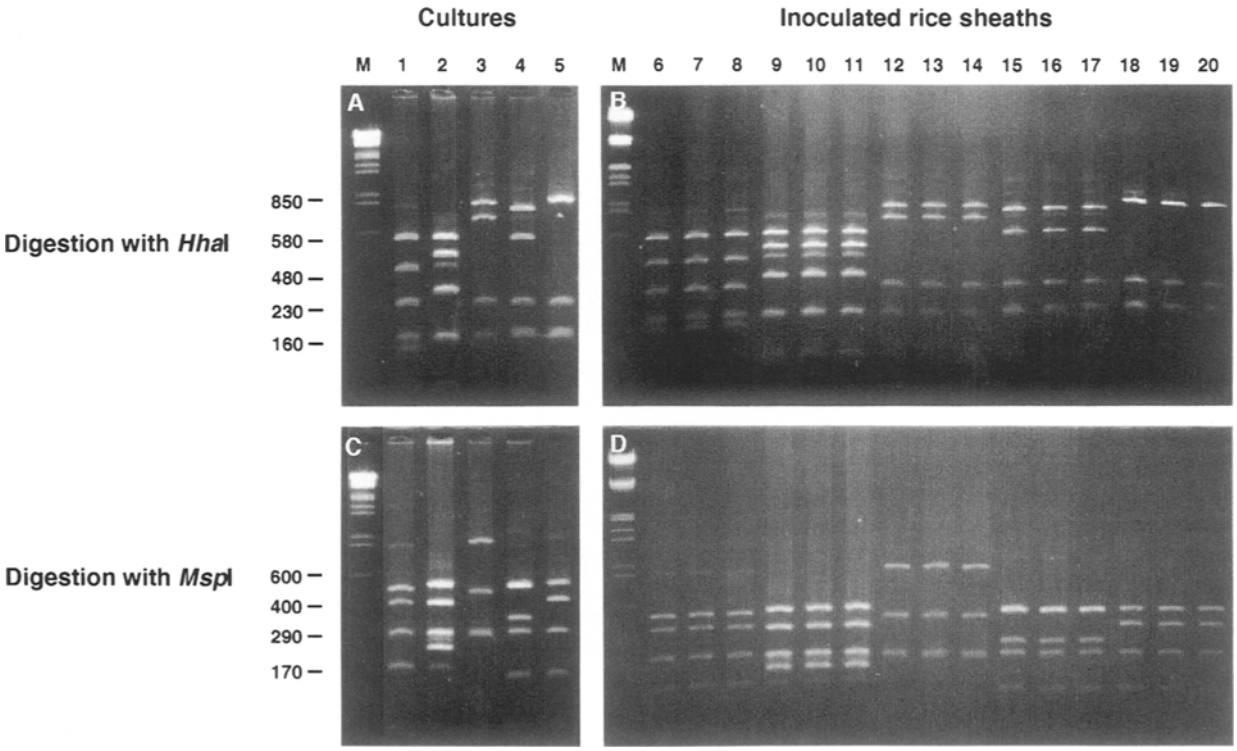

Fig. 1. Electrophoretic patterns of PCR-amplified rDNA of *Rhizoctonia* spp. digested with *Hha*I and *Msp*I. These RFLP profiles were obtained after digestion with *Hha*I using DNA samples extracted from pure cultures (A) and artificially inoculated rice sheath tissues (B), and with *Msp*I using DNA samples from pure cultures (C) and artificially inoculated rice sheath tissues (D). Lanes 1 and 6 to 8, *R. solani* AG 1 IA; lanes 2 and 9 to 11, *R. oryzae*; lanes 3 and 12 to 14, *R. solani* AG 2-2 IIIB; lanes 4 and 15 to 17, *R. fumigata*; lanes 5 and 18 to 20, *R. oryzae-sativae*; and M, molecular marker (λ-DNA digested with *Bam*HI and *Hin*dIII). Sizes of DNA fragments (bp) are indicated on the left.

ed a product of a single size of about 1.4 kbp or 1.8 kbp for 28S rDNA (data not shown). Of 50 samples, 47 were distinguishable and could be identified directly as a single species of the three *Rhizoctonia* species, while the remaining 3 could not be identified. The results showed that the three *Rhizoctonia* species, *R. solani* AG 1 IA, *R. oryzae* and *R. oryzae-sativae*, on naturally infected rice sheaths could be distinguished by analysis of the electrophoretic patterns of digestion products of amplified DNA fragments with *Hha*I or *Msp*I. *Rhizoctonia solani* AG 2-2 IIIB and *R. fumigata* were absent in the naturally infected samples collected. Analysis of the electrophoretic patterns of PCR-amplified 28S rDNA fragments with *Hha*I and *Msp*I showed compatible results between DNA samples from pure cultures and artificially inoculated rice sheaths, indicating that the pathogens *R. solani* AG 2-2 IIIB and *R. fumigata* can be detected and identified from naturally diseased rice sheaths.

The alkaline DNA extraction method allowed the easy and rapid preparation of DNA for a series of PCR amplifications and electrophoretic analyses by digestion of PCR-amplified products. This method will have significant advantages over the usual method of DNA sample preparation, because the time and effort required for DNA extraction is often a limiting step. In recent taxonomic studies, the detection and identification of *Rhizoctonia* species using oligonucleotide primers designed from their specific repetitive DNA segment for PCR amplification have contributed to enhanced diagnostic capabilities to differentiate *R. solani* AGs and *Rhizoctonia* species (Brisbane et al., 1995; Mazzola et al., 1996). However, studies on the direct detection of symptotaxonomically complex phytopathogens, like the causal agents of rice sheath diseases, are not known. Therefore, we conclude that PCR-RFLP analysis of 28S rDNA could become a convenient tool for direct detection and identification of such species on rice sheath tissues and in field soils infested with soilborne phytopathogenic fungi.

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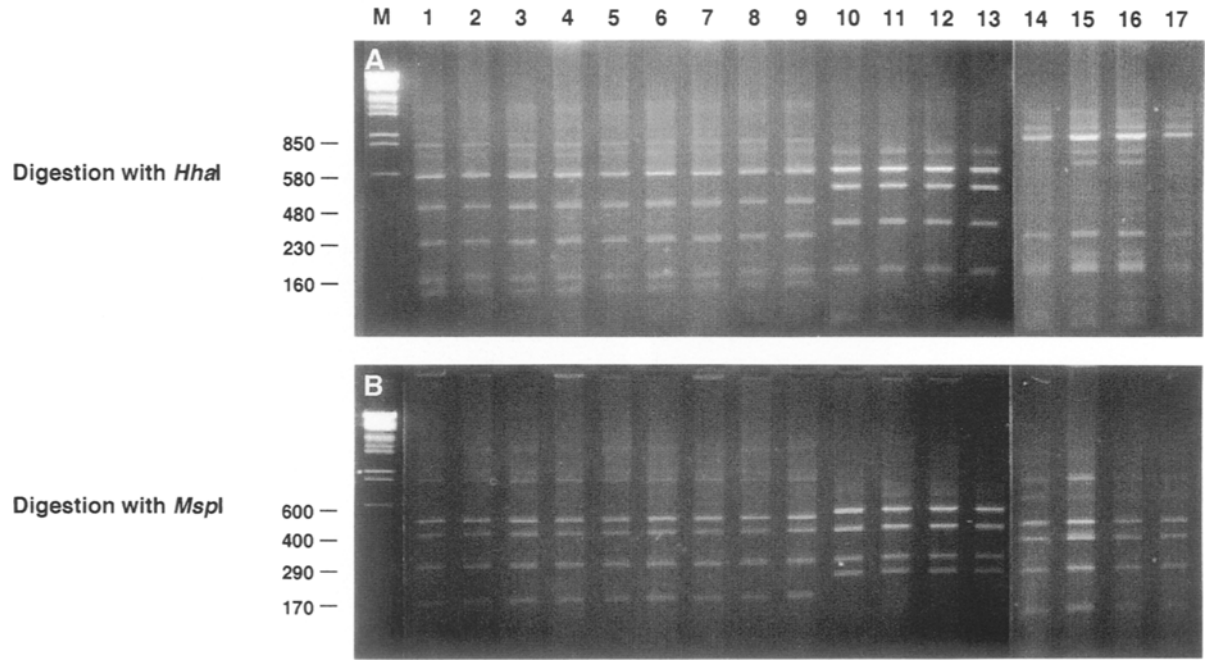

Fig. 2. Electrophoretic patterns of PCR-amplified rDNA of *Rhizoctonia* spp. digested with *Hha*I (A) and *Msp*I (B) using DNA samples extracted from naturally infected rice sheath tissues.
Lanes 1 to 9, *R. solani* AG 1 IA, causal agent of sheath blight disease; lanes 10 to 13, *R. oryzae*, causal agent of sheath spot disease; lanes 14 to 17, *R. oryzae-sativae*, causal agent of brown sclerotium disease; and M, molecular marker (λ-DNA digested with *Bam*HI and *Hin*dIII). Sizes of DNA fragments (bp) are indicated on the left.

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