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Alkaline Extraction of DNA from Pathogenic Fungi for PCR-RFLP Analysis

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For the preparation of DNA samples from fungal mycelia alkaline extraction method was applied and assessed its usefulness for PCR-RFLP analysis. Using alkaline treatment protocols, 18S ribosomal DNAs (rDNA) derived from fungal genomic DNA of *Pyricularia oryzae, P. zingiberi, Rhizoctonia solani* and *R. oryzae* were PCR-amplified and digested with *Hha* I, *Msp* I and *Hae* III. RFLP analysis with *Hha*I showed the divergent polymorphism between genus *Pyricularia* and *Rhizoctonia*. The alkaline DNA extraction method saves the time and labor required for DNA sample preparation and seemed to be useful for rapid identification of specific fungal species by PCR-RFLP analysis.

INTRODUCTION

The polymerase chain reaction (PCR) has been used for amplifying specific DNA sequences in isolates of *Rhizoctonia* species and restriction fragment length polymorphism (RFLP) analysis of these amplified products has revealed the possibility of rapid identification of *Rhizoctonia* species (Matsumoto *et al.*, 1996a; 1996b). However, DNA sample preparation from fungal mycelia is often time-consuming and several protocols for DNA preparation involve multiple steps. Since PCR requires only a minute quantity of template DNA for successful amplification and a crude DNA preparation is enough for PCR, it might be possible to extract sufficient DNA by a simplified method.

An alkaline DNA extraction method with NaOH was successfully introduced to prepare DNA samples in many plant species for PCR assaying (Wang *et al.*, 1993). This method allowed sufficient DNA extraction without any inhibition for the amplification. Improvement of a rapid DNA extraction method will lead to the development of ability of PCR-RFLP analysis for large number of isolates. The aim of this study is to assess the value of an alkaline DNA extraction method for rapid detection and identification of specific phytopahogenic fungi by PCR-RFLP analysis.

MATERIALS AND METHODS

For effective trials, an 18S ribosomal RNA (rRNA) gene from *Pyricularia oryzae, P. zingiberi, Rhizoctonia solani* and *R. oryzae* was chosen as the sequence to be amplified. The isolates used in this study were listed in Table 1. All isolates were maintained on a slant culture of potato-dextrose agar (Funakoshi Chem. Co.). Primers NS 1 (5'-GTAGTCATATGCTTGTCTC-3') and NS 2 (5'-GGCTGCTGGCACCAGACTTGC-3') amplified partial length of 18S rRNA genes (White *et al.*, 1990). DNA samples for PCR

Table 1. List of isolates of *Pyricularia* spp. and *Rhizoctonia* spp. used.

Species	Isolates	Origin	Source
P. oryzae	TH 67-22	Rice	AKU[a]
	TH 68-86	Rice	AKU
	KEN 53-33	Rice	AKU
	KEN 60-19	Rice	AKU
P. zingiberi	NNZiM 1-1-1	Mioga	AKU
	NNZiM 2-2-1	Mioga	AKU
	FOZiM 1-2-1	Mioga	AKU
	YNZiM 1-1-1	Mioga	AKU
R. oryzae	Ro-0105	Rice	AKU[b]
	M23	Rice	AKU
	C-505	Rice	AKU
	R-1	Rice	AKU
R. solani	Cs-Ka	Rice	ATCC[c] 76121
	C-325	Rice	IFO[d] 30935
	C-326	Rice	IFO 30936
	Cs-2	Rice	AHU[e]

a) AKU: Faculty of Agriculture, Kyushu University, Fukuoka, Japan.
b) AMU: Faculty of Agriculture Meijyou University, Nagoya, Japan.
c) ATCC: American Type Culture Collection, USA.
d) IFO: Institute for Fermentation, Osaka, Japan.
e) AHU: Faculty of Agriculture, Hokkaido University, Hokkaido, Japan.

were prepared from fungal mycelia according to following protocols. Lyophilized mycelia, which were grown in a liquid nutrient-broth medium at 28 °C for 7 days, were placed into 1.5 ml of Eppendorf tube, and roughly ground with 10 μl of 0.5N NaOH until no large pieces of mycelia are left. Five μl of NaOH solution were transferred quickly to a new Eppendorf tube containing 495 μl of 20mM Tris-HCl (pH 8.0), mixed well and the 5 μl of this DNA extract was used for PCR assaying. PCRs were carried out in a 100 μl of reaction volume. A 5 μl of DNA extract was added to 95 μl of reaction mixture as recommended by the manufacturer using *Tth* DNA polymerase (Toyobo Biochem. Co.). The thermal cycles were conducted 30 times, with parameters of 1 min at 94 °C, 2 min at 50 °C and 3 min at 72 °C.

The enzymes, *Hha* I, *Msp* I and *Hae* III, were used for restriction fragment analysis to differentiate *Pyricularia* species and *Rhizoctonia* species. Restricted DNA was analyzed by electrophoresis in 3% agarose gel (Katayama Chem. Co.) in TBE buffer (100 mM Tris-HCl, 20 mM EDTA, 100 mM boric acid, pH 8.3). Gels were stained with ethidium bromide (Sigma Chem. Co.) and photographed under UV light (312 nm).

RESULTS AND DISCUSSION

Using the alkaline DNA extraction protocols, specific PCR-amplified products, a single

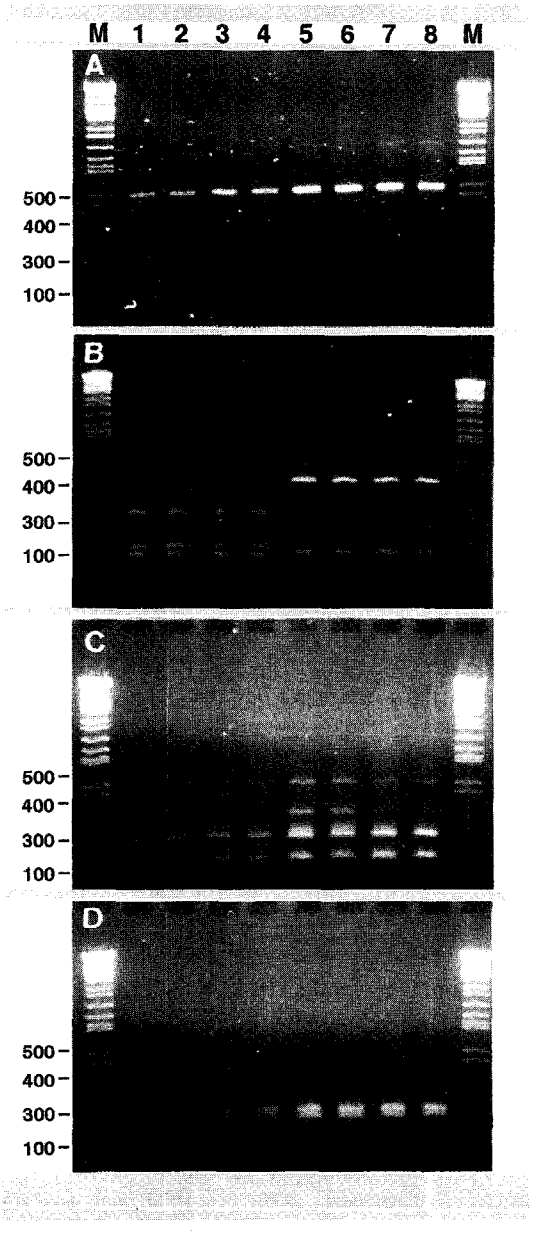

Fig. 1. Electrophoretic patterns of PCR-amplified 18S rDNA of *Pyricularia* spp. and *Rhizoctonia* spp. (A) and restriction fragments patterns of amplified products after digestion with *Hha* I (B), *Msp* I (C) and *Hae*III (D). *P. oryzae*, Lane 1 and 2; *P. zingiberi*, Lane 3 and 4; *R. solani*, Lane 5 and 6; *R. oryzae*, Lane 7 and 8; M; DNA size marker of λ -DNA digested with *Eco*RI and *Hin*d III.

Table 2. Sizes (bp) of DNA fragment generated after digestion of 18S rDNA from *Pyricularia* spp. and *Rhizoctonia* spp. with three restriction enzymes.

	Restriction enzymes		
Strain	*Hha* I	*Msp* I	*Hae* III
P. oryzae	120, 140, 340	180, 360	210, 340
P. zingiberi	120, 140, 340	180, 360	210, 340
R. oryzae	110, 490	180, 360	210, 340
R. solani	110, 490	180, 360	210, 340

size of putative 600-bp fragment, were obtained in all isolates of *P. oryzae, P. zingiberi, R. solani* and *R. oryzae* (Fig. 1A). Specific digestion profiles of PCR-amplified 18S rDNA with *Hha* I, *Msp* I and *Hae* III were presented by electrophoresis (Fig. 1B-1D; Table 2). Weak bands observed in digestion products of *P. oryzae* and *P. zingiberi* could be responsible for the low concentration of DNA sample. Digestion with *Msp* I and *Hae* III did not show any polymorphisms (Fig. 1C and 1D). After digestion of PCR-amplified 18S rDNA products with *Msp*I, all tested isolates showed equal sets of 180- and 360-bp fragments (Fig. 1C; Table 2). In the cases of *R. solani* and *R. oryzae*, bands other than these two bands were also detected (Fig. 1C) and this could be responsible for the high concentration of the sample. Digestion with *Hae* III presented equal sets of 210- and 340-bp fragments for all isolates (Fig. 1D; Table 2). The digestion with *Hha* I resulted distinct polymorphisms and produced unique sets of 120-, 140- and 340-bp fragments for isolates of *Pyricularia* spp., and 110- and 490-bp fragments for isolates of *Rhizoctonia* species, respectively (Fig. 1B; Table 2).

The alkaline DNA extraction method was successfully applied for DNA sample preparation from fungal mycelia at PCR and following RFLP analysis. By alkaline (NaOH) extraction, multiple steps required previously for DNA sample preparation was avoided. This rapid and easy method will be useful for PCR assaying of large numbers of isolates. Because of its instability of the prepared DNA samples at −20 °C as alkaline solution, a series of their experimental operation has to be done quickly and DNA samples should be supplied for PCR right away after the extraction.

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