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NOTE

PCR-DGGE and PCR-RFLP Analyses of the Internal Transcribed Spacer (ITS) of Ribosomal DNA in the Genus *Rhizopus*

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To estimate genetic relationships within the genus *Rhizopus*, genetic variations in 20 strains were investigated by DGGE and PCR-RFLP of rDNA ITS region (ITS1, ITS2, 5.8S). The size of the amplified products showed the interspecific polymorphisms, 650 bp, 700 bp, and 900 bp. The DGGE approach allowed the separation of PCR amplicons of the same length according to their sequence variations. When the rDNA ITS region was digested with six restriction enzymes, 20 strains were classified into five RFLP haplotypes. The range of similarity between the 20 strains by PCR-RFLP was 42.3-100%. Based on the results of DGGE and PCR-RFLP, the 20 strains were divided into four groups, *R. oryzae, R. stolonifer, R. microsporus* and *R. homothallicus*. Further study of *R. homothallicus* is required.

Key word: DGGE, Internal transcribed spacer (ITS), PCR-RFLPs, *Rhizopus*

Rhizopus, a filamentous fungus belonging to the Zygomycetes, is used in brewing and for the production of various fermented foods in Korea, Japan and China. The lack of definitive information on the numerous described species of *Rhizopus* has led to considerable difficulty in determining the extent of variability within a species and in the identification of species (Ellis, 1985). rDNA polymorphisms have been used for identification in systematic and phylogenetic studies of plants, animals and fungi (Lessa, 1992). The methods used for the molecular identification of fungi to the species level have been mainly based on the use of the internal transcribed spacer (ITS) region and results have been highly variable (Paul, 2002). Recently, molecular techniques like restriction fragment length polymorphisms (RFLP), sequencing and denaturing gradient gel electrophoresis (DGGE) have been used to classify genetic diversity among fungi with the aim of determining genetic relatedness. The DGGE procedure is well-suited for the detection of DNA polymorphisms in situations where RFLP is low but DNA sequence variations are expected. PCR fragment lengths and RFLPs within the ITS region have been successfully used to differentiate species in a small number of fungal genera (Siboe *et al.*, 2000).

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To further examine the rationality of *Rhizopus* classification based on morphology, the ITS region of the rDNAs from several disputed species was analyzed and compared by DGGE and PCR-amplified RFLP. Twenty strains of *Rhizopus* were used in this study (Table 1). Genomic DNA for PCR was extracted as described by Lee *et al.* (2000).

PCR-DGGE analysis

For GC-clamp evaluation, 20 μl PCR reactions were performed with pITS1-GC (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGTCCGTAGGTGAACCTGCGG-3') [the GC clamp sequence is underlined] (Kowalchuk *et al.*, 1997 and White *et al.*, 1990), and pITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). The PCR thermocycle was: 95°C for 3 min, then 45 cycles of 95°C for 40 sec, 47°C for 70 sec, and 72°C for 3 min, followed by a final extension at 72°C for 10 min. Electrophoresis was conducted using a gradient of 35 to 43% denaturant and a running time of 5 h at 100 V. These conditions gave optimal band separation. The profiles produced by DGGE were grouped into four groups, i.e., *R. oryzae, R. stolonifer, R. micro-sporus* and *R. homothallicus*. DNA fragments subjected to DGGE migrate on the basis of size and on fragment melting properties (Myers *et al.*, 1985a; 1985b). Consequently, this modality allows the simultaneous resolution of DNA fragment length polymorphisms and of DNA sequence

Table 1. List of *Rhizopus* species used in this study

Strains	Isolate number	Source*	Synonym
Rhizopus. oryzae Went et prinsen-Geerligs	20133	ATCC	*R. acetorinus*
R. oryzae Went et prinses-Geerligs	11910	ATCC	*R. acidus*
R. oryzae Went et prinses-Geerligs	K1	Korea	*R. acidus*
R. oryzae Went et prinses-Geerligs	11145	ATCC	*R. arrhizus*
R. oryzae Went et prinses-Geerligs	4772	IFO	*R. chinensis*
R. oryzae Went et prinses-Geerligs	4858	ATCC	*R. delemar*
R. oryzae Went et prinses-Geerligs	4732	IFO	*R. formosaensis*
R. oryzae Went et prinses-Geerligs	K2	Korea	*R. formosaensis*
R. oryzae Went et prinses-Geerligs	22580	ATCC	*R. javanicus*
R. oryzae Went et prinses-Geerligs	24794	ATCC	*R. japonicus*
R. oryzae Went et prinses-Geerligs	K3	Korea	*R. japonicus*
R. oryzae Went et prinses-Geerligs	10329	ATCC	*R. shanghaiensis*
R. oryzae Went et prinses-Geerligs	K4	Korea	
R. oryzae Went et prinses-Geerligs	K5	Korea	
R. sexualis var. *sexualis* (G. Smith) Callen	42542	ATCC	
R. homothallicus Hesseltine et Ellis	42221	ATCC	
R. microsporus var. *oligosporus*	22959	ATCC	*R. oligosporus*
R. microsporus var. *oligosporus*	48011	ATCC	*R. oligosporus*
R. stolonifer (Ehrenberg: Fries) Lind	6227b	ATCC	*R. nigricans*
R. stolonifer. Vuillemin var. *stolonifer*	4781	IFO	*R. nigricans*

*Source : ATCC (American Type Culture Collection)
IFO (Institute for Fermentation, Osaka)

variations. Accordingly, DGGE has proven especially useful in the monitoring of allelic variants, permitting the detection of even single base pair substitutions, pedigree assessments in highly selected populations comprised of closely related individuals, and gene mapping in species that are low in DNA fragment length polymorphisms by conventional methodologies (Dwikat *et al.,* 1994).

PCR-RFLP analysis

Using the PCR primers ITS1 (5'-TCCGTTGGTGAACCAGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.,* 1990), the ITS region including 5.8S was amplified from 20 strains of the *Rhizopus* species. The PCR product lengths were; 700 bp for *R. oryzae* IFO 4772, *R. microsporus* var. *oligosporus* ATCC 22959 and ATCC 48011, 900 bp for *R. stolonifer* ATCC 6227b and *R. stolonifer* IFO 4781, and 650 bp for *R. sexualis* ATCC 42542, *R. homothallicus* ATCC 42221 and the remaining 13 *R. oryzae* strains. Of the 13 restriction enzymes tested, *Xho*I, *Kpn*I, *Sac*I, *Sma*I, *Sal*I, *Eco*RV and *Pst*I had no restriction sites. According to the restriction patterns obstained using the six enzymes, *Alu*I, *Hinf*I, *Eco*RI, *Hae*III, *Rsa*I, and *Taq*I, five RFLP haplotypes were defined (data not shown). Digestion of the ITS amplification products with *Alu*I resulted in four banding patterns, 350/300, 380/270/430/260, and no recognition site. Digestion with *Hinf*I showed four banding patterns with two cutting sites in all of the strains, giving rise to fragment of 315/210/115, 400/250/250, 250/210/200, and 340/250/130. Digestion with *Eco*RI caused ITS region to be divided into three patterns, one restriction site and no recognition site showing different size. Digestion with *Hae*III divided ITS region into four patterns: one for *R. oryzae* IFO 4772 and *R. microsporus*, two for *R. stolonifer* and *R. homothallicus* with different sizes, and none for the remaining 14 strains. Digestion with *Rsa*I resulted in five patterns with one, two, or three cleavage sites. *R. oryzae*-group (except for *R. oryzae* IFO 4772) was divided into two haplotypes by *Rsa*I. *R. stolonifer* ATCC 6227b and *R. stolonifer* IFO 4781 had one restriction site. Banding patterns of the strains belonging to the *R. oryzae* groups could be clearly grouped into two RFLP haplotypes designated as I and II. Digestion with *Taq*I resulted in three banding patterns with two or three cleavage sites. The restriction patterns with the most variation in terms of both fragment sizes and restriction sites were generated by *Hae*III and *Rsa*I. Based on restriction fragment size and pattern analyses, the genetic similarity matrix was obtained (data not shown) and a dendrogram constructed. Similarity among the 20 strains ranged from a high of 100% to a low of 42.3% by the UPGMA method. Cluster analysis separated the 20 species into four major groups on the basis of a 63% similarity cut off (Fig 2). The similarity between the *R. microsporus* group and the *R. oryzae* group (except for *R.*

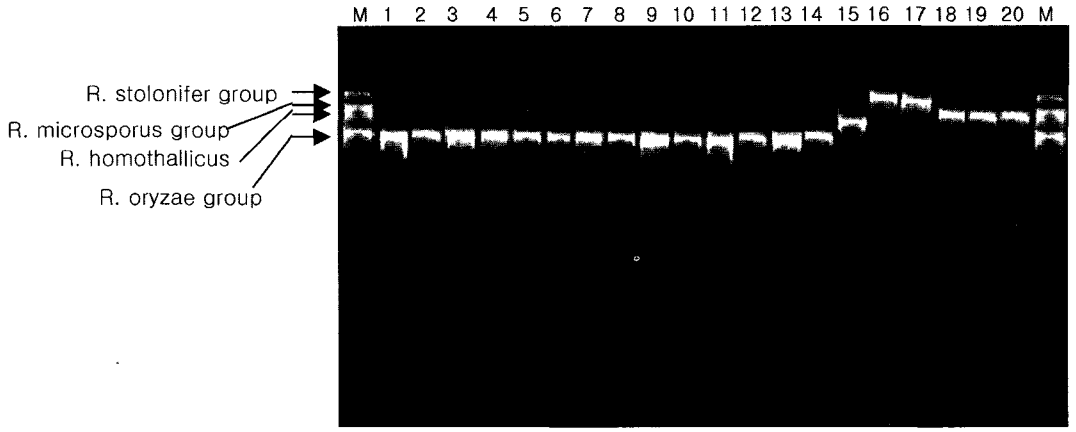

Fig. 1. DGGE analysis of ITS region from *Rhizopus* spp. Lanel, *R. oryzae* ATCC 20133; 2, *R. oryzae* KS1; 3, *R. oryzae* KS2; 4, *R. oryzae* KS3; 5, *R. oryzae* KS4; 6, *R. oryzae* KS5; 7, *R. oryzae* ATCC 10329; 8, *R. oryzae* ATCC 24794; 9, *R. oryzae* IFO 4732; 10, *R. oryzae* ATCC 22580; 11, *R. oryzae* ATCC 11910; 12, *R. oryzae* ATCC 4858; 13, *R. oryzae* ATCC 11145; 14, *R. sexualis* ATCC 42542; 15, *R. homothallicus* ATCC 42221; 16, *R. stolonifer* ATCC 6227b; 17, *R. stolonifer* IFO 4781; 18, *R. oryzae IFO* 4772; 19, *R. microsporus* var. *oligosporus* ATCC 22959; 20, *R. microsporus* var. *oligosporus* ATCC 48011 ; M, a sample obtained after PCR amplification of the mixed *Rhizopus* spp. genomic DNAs.

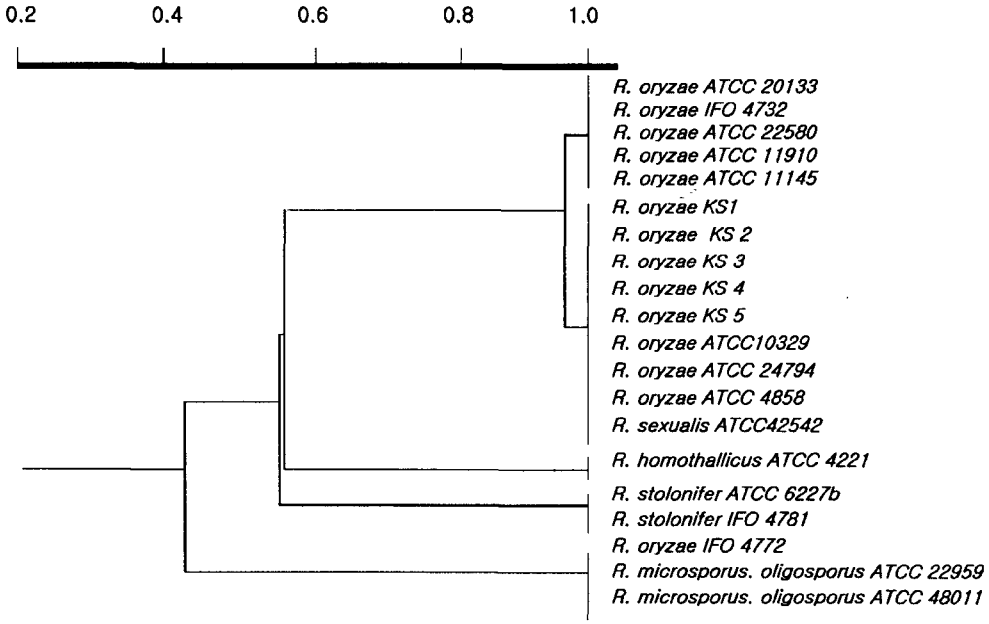

Fig. 2. Similarity based on cluster analysis with data obtained from ITS-RFLP of PCR product among 20 strains of *Rhizopus* spp.

oryzae IFO 4772) was 42%. These PCR-RFLP groupings coincided with those of DGGE. Two strains of *R. microsporus* were found to be distantly related to other species. These results coincide with those of Ellis (1985), who found that the extent of DNA relatedness of *R. microsporus* with other strains, *R. arrhizus* (a presumed type of *R. oryzae*) and two strains of *R. oryzae*, is quite low, no pair showed more than 26% complementarity. Also the G+C content from *R. arrhizus* and two strains of *R. orzyae* fall within 36.1-36.7 mol % range, and that the only obvious distinction was that of *R. microsporus*, 43.9 mol %. In the genus *Rhizopus* Ehrenb. ex Corda as many as 120 species and varieties have been described (Hesseltime and Ellis, 1973). The historical background of the genus has been reviewed in detail by Inui *et al.* (1965) who proposed a new system of classification, based on 13 species and a new variety. Schipper and Staplers (1984) divided the genus *Rhizopus* into three-groups; the *stolonifer*, *oryzae* and *microsprous* groups based on the general morphology and mating experiments. However, the morphological and physiological characteristics of these species tend to overlap species lines. None of the criteria so far employed appear sufficient to clearly delineate all species within this genus (Ellis, 1981) and little is known about the genus *Rhizopus* at the molecular level for species classification purpose.

The results of PCR-DGGE and PCR-RFLP, in general, are consistent in the taxonomy of Shippers and Staplers

(1984) with the exception of *R. homothallicus*. In case of *R. homothallicus*, first described by Hesseltine and Ellis (1965), Scholer (1970) described differences in sporagiophore length, width and in sporangial diameter versus *R. microsporus,* both are larger than *R. homothallicus*. Judging from their general morphologies and maximum growth temperatures, *R. homothallicus* and *R. microsporus* are closely related (Schipper and Staplers, 1984). However, in *Rhizopus* ITS study, *R. homothallicus* wasn't included in the same group. PCR-RFLP and DGGE analyses showed that *R. oryzae* IFO 4772 has the same RCR-RFLP pattern as *R. microsporus*. These results show that *R. oryzae* IFO 4772 is more closely related to the *R. microsporus* group than to the *R. oryzae* group. *R. sexualis* was included in the *R. oryzae* group rather than the *R. stolonifer* group (Schipper and Staplers, 1984). The accurate genetic relationship in *R. oryzae* IFO 4772, *R. sexualis* and *R. homothallicus* remains questionable and comparison with other region of rDNA are needed.

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