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RESEARCH NOTE

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Use of Restriction Fragment Length Polymorphism Analysis to Differentiate Fungal Strains in Sunchang *Meju*

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Abstract Twenty-three fungal strains were isolated from *meju* that had originated from the Sunchang province, the famous location for making fermented soybean foods in Korea. The restriction fragment length polymorphism (RFLP) of the internal transcribed spacer (ITS) region of the rDNA (ITS-RFLP) was applied to differentiate the isolated fungal strains. First, the ITS region by polymerase chain reaction (PCR) with specific primers was amplified and then cleaved the products with different restriction enzymes. Cleavage of the amplified fragments with the restriction enzymes *Alu*I, *Hae*III, *Hha*I, and *Taq*I revealed extensive polymorphisms. The ITS-RFLP results highly correlated with ITS sequence analysis. All of the 23 fungal strains were classified into 5 groups by ITS-RFLP analysis. *Aspergillus oryzae* was the major fungal strain isolated from Sunchang *meju* (12 out of 23), while *Aspergillus fumigatus* was the next most frequently isolated strain (7 out of 23). In contrast, it was found that *Fusarium asiaticum, Aspergillus sydowii*, and *Arthrinium* sp. were the minor fungal strains in *meju*.

Keywords: *Aspergillus fumigatus, Aspergillus oryzae, meju,* internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP)

Introduction

In Asian countries, many traditional fermented foods are made from soybeans, such as *miso, sufu*, and *doenjang*. Among these foods, *doenjang* is one of the most important fermented foods in Korea. *Doenjang* not only contains high quality proteins in the form of amino acids, but also is rich in flavonoids, beneficial vitamins, and minerals. Recently, *doenjang* has received great attention due to its nutritional and medical functions. Many reports have claimed that *doenjang* lowers blood pressure, prevents dementia and osteoporosis, reduces the risk of heart diseases, and possesses anti-cancer properties (1).

The primary ingredient used to make *doenjang* is *meju*, a lump of fermented soybean. The enzyme activity in *meju* is one of the most important parameters to maintain good quality flavor, taste, and color in *doenjang*. This enzyme activity stems from the growth of fungal strains because fungi are major microorganisms involved in the fermentation of *meju*. Therefore, the fungal strains in *meju* are necessary for creating high quality *doenjang*.

A few research studies have reported on the fungal strains found in *meju*. Kim *et al.* (2) isolated 25 strains of true fungi from homemade *meju*. These strains were identified as *Aspergillus oryzae, Aspergillus flavus, Penicillum* sp., *Candida* sp., *Spicaria* sp., and *Rhizopus* sp. (2). Among the fungal strains found in the study by this group, the *Aspergillus* strains that were found in the homemade *meju* showed relatively strong amylolytic and proteolytic activities. Lee *et al.* (3) also isolated various *Aspergillus* strains from *meju* and analyzed these strains by a RAPD-DNA technique. From this work, Lee *et al.* (3) suggested that their isolated *Aspergillus* strains were closely related to *A. flavus*.

Traditionally, identification and characterization of fungi strains have been based on morphological traits and physiological abilities (4). Identification of fungal species based on morphological methods requires adequate growth time (5 days or more) for evaluation of colony characteristics and microscopic features. These characteristics are strongly influenced by cultural conditions and can yield uncertain results. Therefore, approximately 50-100 biochemical tests need to be conducted to reliably identify most fungi to the species level. In addition, morphology tests are usually labor intensive and require expert mycology personnel. In contrast, DNA-based molecular techniques can provide alternative tools to properly identify many fungal strains in nature. In particular, the internal transcribed spacer (ITS)-restriction fragment length polymorphism (RFLP) technique has been successfully used to analyze regions of ribosomal DNA from many fungi (5-7). This method uses two known procedures to detect polymorphisms in ITS regions that have been amplified by specific oligonucleotide primers and cut with different restriction endonucleases. It has significant advantages over other molecular taxonomic methods which involve time-consuming DNA extraction and DNA-DNA hybridization step.

In this study, the diversity of fungal strains was analyzed in *meju* originated from the Sunchang province, the famed

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Korean location for making fermented soybean foods. Twenty-three different fungal strains were isolated from Sunchang *meju* and these strains identified by ITS-RFLP and ITS sequence analyses.

Materials and Methods

Isolation of fungi from Sunchang *meju* Traditionally made *meju* was kindly provided by Moonokrae Foods in Sunchang, Korea. One g of *meju*, in which fungi growth was apparent, was collected using a sterile spatula in a petri dish. The fungal strains present in the sample were isolated using Rose Bengal agar (Difco Lab., Franklin Lakes, NJ, USA). All fungi that were considered to represent different species were isolated and transferred to agar plates for identification. The isolated strains were maintained on potato dextrose agar (PDA) or YEPD (2% D-glucose, 2% peptone, and 1% yeast extract). The fungi were grown at 30°C.

Fungal genomic DNA preparation After sporulation, 15 mL of sterile distilled water with 0.1%(v/v) Triton X-100 was added to the pre-grown agar plate. The plate was then shaken gently and the solution was collected in a sterile conical tube. This fungal spore solution was used to immediately inoculate liquid YEPD medium. After 5-day incubation on a shaker (200 rpm), the culture was filtered and mycelia were collected from the medium. Each sample was then frozen in liquid nitrogen and immediately ground into a fine powder. The freeze-dried ground mycelium was re-suspended in 500 μL of lysis buffer [200 mM Tris-HCl pH 8.5, 250 mM NaCl, 25 mM ethylenediamine tetraacetic acid (EDTA), and 0.5% sodium dodecylsulfate (SDS)]. Next, the slurry was mixed with 500 μL phenol: chloroform: isoamyl alcohol (25:24:1) and this suspension was centrifuged at 10,000×g for 30 min. After centrifugation, the upper phase was transferred to a tube and 0.1 volumes of 3 M sodium acetate and 0.6 volumes of isopropanol were added. The genomic DNA was collected by centrifugation and used in further experiments.

Polymerase chain reaction (PCR) amplification of the ITS region and restriction enzyme digestions The ITS region was amplified by PCR using 2 universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (8,9). PCR amplification reactions were performed with *PfuUltra*™ High-fidelity DNA polymerase (Stratagene, Garden Grove, CA, USA). The PCR amplification reactions consisted of an initial step at 94°C for 3 min, 35 cycles of 94°C for 40 sec, 53°C for 40 sec, and 72°C for 1 min, with a final extension of 3 min at 72°C. Amplified PCR products were visualized by 2%(w/v) agarose gel electrophoresis. PCR products were extracted from the gel using the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) and digested with the 4 restriction endonucleases *Alu*I, *Hae*III, *Hha*I, and *Taq*I (New England Biolabs, Ipswich, MA, USA). A 10 μL aliquots of the amplified DNA were digested with each enzyme at 37 or 65°C overnight. The restriction fragments were separated on 4%(w/v) agarose gels.

Sequencing analysis and phylogenetic tree The PCR product of the ITS region was cloned into the T-easy vector (Promega Co. Madison, WI, USA) and the nucleotide sequence of the PCR-generated gene was determined using the BigDye Terminator Cycle Sequencing kit for ABI377 PRISM (PerkinElmer Inc., Boston, MA, USA). The ITS region sequences were aligned using the multiple-sequence alignment program Clustal X (10), with gaps treated as missing data. Clustering was performed by using the neighbor-joining method (11). The neighbor-joining tree and the statistical confidence of a particular group of sequences in the tree, as evaluated by a bootstrap test (1,000 pseudoreplicates), were performed using the MEGA program, version 4.0 (12,13).

Results and Discussion

Isolation and ITS-RFLP analysis of fungal strains from Sunchang *meju* A total of 23 fungal strains were isolated from Sunchang *meju*. These strains were designated as SMF 1 to SMF 23. As an alternative to more laborious techniques that can be used for fungal strain identification and characterization, such as morphological and physiological analyses, a rapid and reliable DNA-based fingerprinting method like the ITS-RFLP was applied to classify isolated fungal strains. Esteve-Zarzoso *et al.* (14) proposed that the RFLP analysis of the 5.8S RNA gene and the 2 ribosomal internal transcribed spacers was a fast and simple method for species identification. Also, Rahman *et al.* (15) successfully employed an amplified ribosomal DNA restriction analysis (ARDRA) method to cluster highly thermophilic bacteria that were associated with cool soil environments. These reports imply that ITS-RFLP can be also used to classify the fungal strains isolated from *meju*.

PCR amplification of the ITS region with ITS1 and ITS4 primers resulted in clear single PCR products in all isolates (data not shown). However, the amplified PCR fragments showed variations in length, ranging from 540 to 600 bp (Table 1). To differentiate among the isolates, 4 restriction endonucleases (*Alu*I, *Hae*III, *Hha*I, and *Taq*I) were used for the restriction analyses. Among them, *Hae*III, *Hha*I, and *Taq*I produced the highest number of differentiating bands for the majority of the isolates. Fungal isolates which were indistinguishable with one restriction endonuclease analysis could be distinguished, however, by following digestion with the other restriction endonuclease. The typical restriction patterns are shown in Fig. 1. Each individual profile, as designated by the letters A-E, can be combined into specific restriction patterns (Table 1). Each of the 23 isolates analyzed was then assigned to a specific profile. Finally, the comparison of ITS restriction profiles that were obtained with *Alu*I, *Hae*III, *Hha*I, and *Taq*I digestions allowed us to differentiate 6 groups among the 23 fungal strains isolated from Sunchang *meju*.

Analysis of fungal strain isolates from Sunchang *meju* To verify the effectiveness of the ITS-RFLP method in identifying the fungal strains isolated from *meju*, DNA sequence analysis were performed on each ITS PCR fragment. The ITS sequences within the same group that was identified by ITS-RFLP analysis were matched completely. The restriction patterns expected from the ITS sequence perfectly correlated with the ITS-RFLP results.

Table 1. ITS-RFLP patterns and compositions exhibited by the fungal strains

Group	Species	Restriction pattern[1)]				Number of isolates	ITS region size (bp)
		*Alu*I	*Hae*III	*Hha*I	*Taq*I		
1	*Fusarium asiaticum*	A	A	A	A	2	547
2	*Aspergillus oryzae*	C	B	B	B	12	597
3	*Aspergillus fumigatus*	C	C	C	C	7	598
4	*Arthrinium* sp.	B	D	D	D	1	592
5	*Aspergillus sydowii*	C	E	E	E	1	569

[1)]A, B, C, D, and E designate different restriction patterns in each restriction enzyme digestion (refer to Fig. 1 in this study).

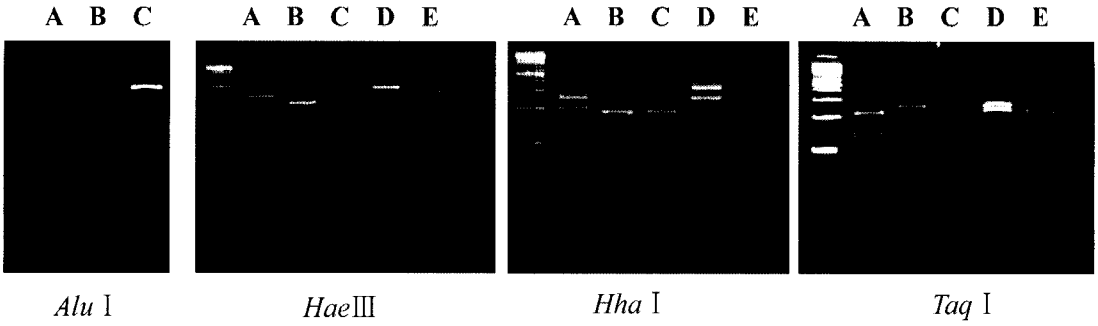

Fig. 1. Patterns appeared in ITS-RFLP analysis. *Alu*I, *Hae*III, *Hha*I, and *Taq*I restriction endonucleases were used and the electrophoresis was performed in 4% agarose gels.

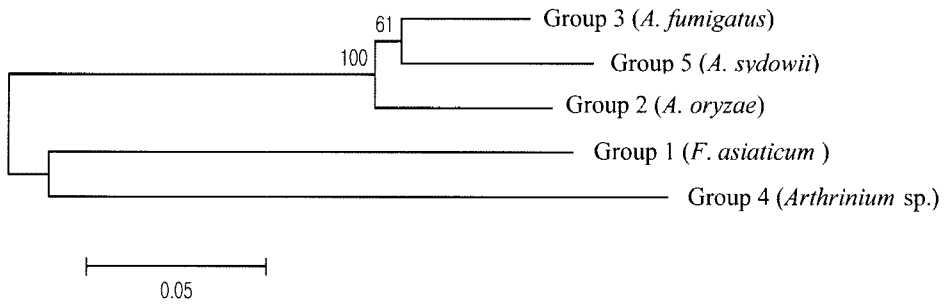

Fig. 2. A neighbor-joining phylogenetic tree based ITS sequence diversity in each group. The numbers on the nodes are the frequency (in percentages) in which a cluster appears in a bootstrap test of 1,000 runs.

The BLAST search of the ITS sequence indicated that the groups could be assigned to *A. oryzae, A. fumigatus, A. sydowii, Arthrinium* sp., and *Fusarium asiaticum*. The most prevalent isolate was Group 2, which represented 12 isolates out of the 23 total strains (52%). The ITS sequence alignment showed that this isolate corresponds to *A. oryzae*. *A. oryzae* is one of the most widely used filamentous fungi in fermentation industries. This fungus species is also used for large-scale production of hydrolytic enzymes and other organic compounds (16). It was anticipated that *A. oryzae* might be the most popular fungal strain in *meju* since it was used for hundreds of years to produce fermented soybean foods in Asian countries, including Korea.

Seven isolates (30%) were identified from Group 3. The ITS sequence from Group 3 matched with *A. fumigatus*. *A. fumigatus* is one of the most common *Aspergillus* species in the natural environment and is closely related to *A. oryzae*. Furthermore, *A. fumigatus* produces fumagillin, an inhibitor of endothelial cell proliferation and angiogenesis (17,18). ITS sequences from other Groups were also identified. ITS sequences of 2 fungal isolates belonging to Group 1 were equivalent to the sequences of *F. asiaticum*. Both Group 4 and Group 5 contained only 1 isolate corresponding to *Arthrinium* sp. and *A. sydowii*, respectively (Table 1). These fungal strains have never been reported to exist or be isolated from *meju*. Previous reports have displayed different patterns obtained using ITS-RFLP within the same species because of heterogeneous 5.8S-ITS rDNA regions, while in contrast, other research has shown that the RFLPs of 5.8S-ITS rDNA are identical between strains within the same species (14,19,20). Our results show that the ITS-RFLP patterns are identical within the same species.

In the phylogenetic tree based on the ITS region sequence, Group 5 constituted a cluster with Group 2 and Group 3 and had a high bootstrap value, whereas Group 1 and Group 4 were not closely related (Fig. 2). As mentioned above, the isolates from Group 2, Group 3, and Group 5 are all determined to be *Aspergillus* strains. In conclusion, the ITS-RFLP method was successfully applied to the identification of fungal strains isolated from Korean *meju*.

ITS-RFLP results are highly correlated with the sequence analysis of the ITS region. Furthermore, not only were the typical *Aspergillus* strains, including *A. oryzae, A. fumigatus,* and *A. sydowii,* found in *meju,* but also the *Fusarium* and *Arthrinium* strains were observed. The microbiological and biochemical characteristics of the major fungal strain, *A. oryzae,* need to be studied more intensively in order for this strain to be used as a starter strain for making modern *meju* in the near future.

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