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Article

Fungi Associated with the Traditional Starter Cultures Used for Rice Wine in Korea

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The '*Nuruk*' starter culture has been used for many years in the fermentation of rice wine in Korea. In present study, mycobiota in *Nuruk* cultures was identified by performing morphological, physiological, and phylogenetical analyses. Mucorales was the most common mycobiota in *Nuruk*, followed by yeast and *Aspergillus*. The composition of fungal species in *Nuruk* was different among samples and did not correlate with the geographical location from where the *Nuruk* culture was manufactured. For more detailed identification, 174 filamentous fungal strains were isolated from 39 *Nuruk* samples. Although the morphological and molecular analyses showed that the strains were identical at the genus level, some discordance was identified between species. Of the 174 strains, 160 showed thermotolerance, and the level of thermotolerance matched the clade generated by phylogenetic analysis. Six genera (*Lichtheimia*, *Aspergillus*, *Rhizopus*, *Rhizomucor*, *Mucor*, and *Syncephalastrum*) and 17 fungal species were identified. Among the genera, the genus *Syncephalastrum* had not been previously identified in *Nuruk* cultures, and the isolate was identified as *Syncephalastrum racemosum*. Two genera, *Lichtheimia* and *Aspergillus*, comprised approximately 84% of the filamentous fungal isolates from the *Nuruk* samples, and *Lichtheimia ramosa* and *Aspergillus oryzae* were the most commonly found species. The controversy regarding the presence of mycobiota in *Nuruk* starter cultures was addressed, and results showed that *Nuruk* contains unique mycobiota not yet found in other Asian starter cultures.

Key words: *Aspergillus*, mucorales, mycobiota, *Nuruk*, starter culture

In several regions of Asia, starter cultures have been used for many years to produce fermented alcoholic drinks or foods [Hesseltine, 1983; Thanh *et al.*, 2008]. These cultures are known by various names, depending on the country where they are used [Kozaki and Uchimura, 1990; Nikkuni *et al.*, 1996; Jeyaram *et al.*, 2008]. They are usually comprised of mixed cultures containing filamentous fungi, yeast, and bacteria grown on the dough of various cereals or on the surface of grains. *Nuruk* is a traditional starter culture for brewing alcoholic beverages in Korea. Many types of *Nuruks* exist, and most are made from the uncooked dough of coarsely ground grains and water. Other grains, such as rice, barley, millet, maize, soybean, rye, and oats are also used in different regions. *Nuruks* are commonly used as starter cultures in the fermentation of several rice wines in Korea, such as 'Takju' ('Makgeolli'), 'Cheongju', and 'Yakju' [Han *et al.*, 1997; Kim and Koh, 2004; Lee and Ahn, 2010]. These cultures provide amylolytic and proteolytic enzyme sources used in the fermentation of rice wine [Park *et al.*, 1995; Kim *et al.*, 1998; Lee *et al.*, 2009].

Various microorganisms, including fungi and bacteria, can be grown in *Nuruk*, because it is prepared from unsterilized and unheated grains. Previous reports have shown that Eurotiales and Mucorales members, including the genera *Aspergillus*, *Lichtheimia* (formerly *Absidia*), *Rhizopus*, *Rhizomucor*, and *Mucor*, are the most commonly isolated fungi from *Nuruk* [Uchimura *et al.*, 1990; Park *et al.*, 1995; Yu *et al.*, 1998]. Various yeasts

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(e.g., *Saccharomyces*, *Hansenula*, *Pichia*, *Candida*, *Schizosaccharomyces*, *Torulpsis*, and *Rhodotorula*) [Yu *et al.*, 1998; Kim *et al.*, 2006] and lactic acid bacteria (e.g., *Lactococcus*, *Enterococcus*, *Pediococcus*, *Leuconostoc*, and *Lactobacillus*) [Jo and Ha, 1995; Lee and Yu, 2000] have also been isolated from *Nuruk*.

The fungi in *Nuruk* produce amylolytic and proteolytic enzymes, which have important roles in starch saccharification and protein or peptide digestion, respectively [Park *et al*., 1995; Kim *et al*., 1997; 1998; Yu *et al*., 1998]. The yeasts in *Nuruk* use sugars for alcoholic fermentation [Ha *et al*., 1989; Kim *et al*., 2006]. Lactic acid bacteria appear to have a role in creating an acidic environment in the early stage of brewing [Bae *et al*., 2007]. In addition, each *Nuruk* microorganism can produce distinct organic acids or flavor compounds in the fermentation process that affects the taste of alcoholic products. Therefore, microorganisms in *Nuruk* are thought to be responsible for the deep and complex taste of traditional Korean alcoholic beverages [Yu *et al*., 1996; Kim *et al*., 1997; So, 1999].

Many attempts have been made to improve the quality of Koran rice wines using fungal strains from *Nuruk* or fermentation mash. *Aspergillus* has been the most popular species reported in screening fungal strains for rice wine brewing. A member of Mucorales, *Rhizopus*, also has been frequently used to investigate the development of new types of modified *Nuruk* [Lee *et al*., 1987; Shon *et al*., 1990; So, 1995; Lee *et al*., 2002; Yu *et al*., 2002]. Members of the order Mucorales are widely distributed in food, soil, and air [Guarro *et al.*, 1999; Ribes *et al.*, 2000]. Aspergilli, belonging to the order Eurotiales, are globally the most abundant and widely distributed organisms [Klich, 2002]. Identification of these fungi at the species level has traditionally been based on morphological observations. However, such approaches are laborious and often lead to identification errors, even for experienced researchers. Recently, several molecular markers have been used to identify fungal species using modern molecular techniques [Balajee *et al.*, 2009]. The ribosomal internal transcribed spacer (ITS) region is one useful molecular target for members of the Mucorales order and *Aspergillus* species [Henry *et al.*, 2000; Schwarz *et al.*, 2006; Alvarez *et al.*, 2009]. The calmodulin and β-tubulin gene sequences are also used for the accurate identification of species due to their prevalence in public databases, universality of application, and relative resolving power [Geiser *et al.*, 2007]. In addition to species identification, these sequences have been widely used for phylogenetic analysis in fungi, including the genera of Mucorales and *Aspergilli* [Hoffmann *et al.*, 2007].

Therefore, reconsideration of the microbial characteristics of *Nuruk* is necessary in order to improve the quality of Korean rice wines. Microbial diversity in *Nuruk* has been studied from the beginning of the 20th century, but no study has as yet taken an integrated approach for the taxonomical identification of fungi in *Nuruk*. In the present study, mycobiota present in *Nuruk* were examined, and isolates from *Nuruk* samples were identified using a combination of molecular analyses, physiological examinations, and morphological observations. Furthermore, phylogenetic studies were carried out to ascertain the relationships among the isolates.

Materials and Methods

***Nuruk* samples and fungal isolates.** *Nuruks* were collected from 39 markets in 13 Korean provinces and cities (Gyeonggi, Gangwon, Chungbuk, Chungnam, Gyeongbuk, Gyeongnam, Jeonbuk, Jeonnam, Jeju, Seoul, Busan, Daegu, and Ulsan) from 2003 to 2009. All *Nuruk* samples collected were made from wheat. Twenty-four fungal strains from the collection of the Korea Culture Center of Microorganisms (KCCM) were used as reference strains for the identification of fungal isolates obtained from *Nuruk* (Table S1).

Isolation of fungi from *Nuruk* samples. One gram of each *Nuruk* sample was suspended in 9 mL of sterilized, distilled water containing 0.85% sodium chloride, and 10-fold serial dilutions were made. Each diluent was plated in triplicate on Difco Cooke Rose Bengal Agar (BD Biosciences, Sparks, MD) containing chloramphenicol (100 μg/mL; Sigma-Aldrich, St. Louis, MO). After 5−7 days incubation at 25°C, fungal colonies were counted and characterized by observing the morphological characteristics, including the colony type and spore morphology. The fungal colonies were sub-cultured on potato dextrose agar (PDA; BD Biosciences) for single conidium isolation. All isolates were stored in 15% glycerol at −70°C.

Morphological and physiological identification. For each isolate, one inoculating loop of spores was suspended in 500 μL of 0.2% agar with 0.05% Tween 80. The suspension was used for one- or three-point inoculations on 9 cm diameter Petri dishes containing approximately 25 mL of media. Mucorales isolates were cultivated on PDA and malt extract agar (MEA; 2% malt extract, 2% glucose, 0.1% peptone, and 2% agar) at 25°C for 7−10 days. *Aspergillus* isolates were grown for 7 days on Czapek yeast agar (CYA) at either 25 or 37°C, CYA with 20% sucrose (CY20S) at 25°C or MEA at 25°C. For micromorphological observations, the fruiting bodies of fungi were observed with a SMZ1500 stereoscopic

microscope (Nikon, Tokyo, Japan), and the vegetative and asexual stages were observed with a DE/Axio Imager A1 microscope (Carl Zeiss, Oberkochen, Germany) after staining MEA colonies with lactophenol cotton blue (BD Biosciences). The morphological features of the isolates were characterized, and the species were identified according to the methods of previous studies on Mucorales [Ciesla *et al.*, 2000; Ribes *et al.*, 2000; Garcia-Hermoso *et al.*, 2009] and of *Aspergilli* [Klich, 2002]. All *Aspergillus* isolates thought to be of the *Aspergillus* section *Flavi* were cultured on *Aspergillus flavus* and *Aspergillus parasiticus* agar (AFPA) (Oxoid, Cambridge, UK) for 3-5 days at 25°C in the dark, and the colony color was then observed as previously described [Rodrigues *et al.*, 2009].

The thermotolerance of Mucorales and *Aspergillus* isolates was determined on MEA and Czapek-Dox agar (CZ) (BD Biosciences), respectively. Two microliters of each spore suspension were inoculated and incubated at 42 or 48°C. Colony diameters were measured after 5 (*Mucorales*, 48°C) or 7 (*Aspergillus*, 42°C) days of incubation.

DNA extraction. Mucorales and *Aspergillus* isolates used for the molecular studies were grown in 100 mL malt extract broth (Oxoid) and CZ broth, respectively, in 250 mL Erlenmeyer flasks. After 5 days of incubation at 25°C on an orbital shaker, the cultures were filtered using Whatman filter paper No. 4 and rinsed twice with 100 mL of distilled water. Genomic DNA was isolated using a cetyltrimethylammonium bromide (CTAB) procedure as previously described [Leslie and Summerell, 2006].

Polymerase chain reaction (PCR) amplification. PCR was performed in a PTC 200 Peltier thermal cycler (MJ Research, Waltham, MA). The 5.8S ribosomal DNA sequence flanked by ITS regions 1 and 2 was amplified using primers ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') for all fungal isolates [White *et al.*, 1990]. The β-tubulin gene was amplified using primers bt2a (5'-GGT AAC CAA ATC GGT GCT GCT TTC-3') and bt2b (5'-ACC CTC AGT GTA GTG ACC CTT GGC-3') [Glass and Donaldson, 1995] for the *Aspergillus* isolates. Oligonucleotides were synthesized by the Bioneer oligonucleotide synthesis facility (Daejon, Korea), dissolved to 100 μM in TE buffer (pH 8.0), and stored at −20°C.

The PCR reaction was performed in 20 μL of Maxime PCR PreMix Kit solution (Intron Biotechnology, Seongnam, Korea) comprised of 2.5 U of Taq DNA polymerase, 1 × gel loading buffer, 1 × reaction buffer, and 2.5 mM of each deoxynucleoside triphosphate. Subsequently, 10 pmol of each primer pair, and 20 ng of DNA template was then added to the PCR reaction. The following amplification steps were used: ITS-1/ITS-4, initial denaturation at 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 47–49°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min; bt2a/bt2b, initial denaturation at 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 53−60°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min.

DNA sequencing and phylogenetic analyses. All PCR amplicons were directly sequenced from both ends by the Solgent Co. (Daejon, Korea). The DNA sequence of the ITS-5.8S rDNA region from all isolates as well as the β-tubulin sequence from some *Aspergillus* isolates were edited with the BioEdit v.7.0.9.0 software (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and deposited in GenBank (Table S2). The nucleotide BLAST program (http://www.ncbi.nlm.nih.gov/blast) was used to search the database for sequence similarities.

The sequences of fungal isolates from *Nuruk* samples were aligned with the sequences of selected reference taxa from GenBank and KCCM reference strains using BioEdit, with the multiple sequence alignment option implemented in Clustal W [Thompson *et al.*, 1994]. The program was used with the default settings, and the alignment was further optimized manually. Phylogenetic analyses were conducted on DNA based on the ITS-5.8S rDNA and β-tubulin sequences using the Neighbor-joining method [Saitou and Nei, 1987] in MEGA v. 4.0 [Tamura *et al.*, 2007]. The evolutionary distances were computed using the Maximum Composite Likelihood method [Tamura *et al.*, 2004], and gaps were treated as pairwise deletions. To determine the support for each clade, bootstrap analysis was performed with 1000 replications.

Results and Discussion

Mycobiota in *Nuruk* samples. Twenty-one *Nuruk* samples were used to investigate mycobiota in *Nuruk* starter cultures. Mucorales were found in all of the *Nuruk* samples at a dilution of 10^4, whereas yeast and the *Aspergillus* species were not detected at the same dilution level in 6 and 7 of the samples, respectively (Table 1). Other fungal genera, such as *Penicillium, Cladosporium* and *Paecilomyces*, were also found, but showed very low incidence (data not shown). The colony forming unit (CFU) of Mucorales per gram ranged from 1.0×10^5 to 9.0×10^7, with the maximum value being observed in the Dalsung C sample. The CFUs of *Aspergillus* and yeast were highest in the Dalsung C and Seoul A samples, respectively. In addition, 14 out of the 21 samples had more filamentous fungi, including Mucorales and

Table 1. Total number of Mucorales, *Aspergillus,* and yeast isolates from 21 *Nuruk* samples

Sample	CFU/g[a]×10^4			
	Mucorales	Aspergillus	Yeast	Total
Andong A	10	ND[b]	37	47
Andong B	10	30	3,283	3,323
Ansung A	15	3	464	481
Busan A	120	67	533	720
Cheonan A	2,133	133	200	2,467
Dalsung A	17	3	183	203
Dalsung B	107	ND	13	120
Dalsung C	9,000	3,667	ND	12,667
Hansan A	367	267	ND	633
Icheon A	167	33	250	450
Kunsan A	157	ND	ND	157
Kunsan B	27	ND	7	33
Paju A	633	ND	567	1,200
Pyeongtaek A	103	ND	3	107
Pyeongtaek B	220	1	ND	221
Sangju A	1,667	133	100	1,900
Sangju B	433	ND	ND	433
Seongnam A	127	2	ND	129
Seoul A	700	133	4,333	5,167
Seoul B	49	3	41	94
Ulsan A	527	110	107	743
Average	790	218	482	1,490

[a]CFU/g = Colony Forming Units per gram of *Nuruk*.
[b]Not detected at dilution 10^4.

Aspergillus, than yeast. Moreover, only one sample (Andong B) had more *Aspergillus* than Mucorales, suggesting that Mucorales is the most common order present in *Nuruk*, followed by yeast and *Aspergillus* (Table 1).

The mycobiota in *Nuruk* was found to be much more diverse than that in the Japanese starter culture, Koji, which had uniform mycobiota and was comprised predominantly of the *Aspergillus* species. The difference may have resulted from different manufacturing processes between Koji and *Nuruk*. Koji is made with steamed rice or soybean and is inoculated with a pure, cultured inoculum [Thanh *et al.*, 2008], whereas *Nuruk* is manufactured with unsterilized grain and is naturally inoculated (Fig. 1.). A Chinese alcoholic starter culture, Qu, showed diverse mycobiota as found in *Nuruk*. The genus *Absidia* was predominant in Qu [Yokoyama *et al.*, 1994], and recent studies reported that representative fungal species associated with Qu were *Absidia corymbifera*, *Rhizopus oryzae*, *Rhizomucor pusillus*, *Aspergillus oryzae*, and *Emericella nidulans* [Xie *et al.*, 2007]. However, the proportion of *Aspergillus* species in Qu was very small compared with that in *Nuruk*.

Mycrobiota in *Nuruk* may be influenced by the regional location where *Nuruk* is manufactured, because it is naturally inoculated. However, our results showed that, although there were differences in mycobiota among samples, no significant relationship was found among geographical locations (Table 1). Therefore, the specific manufacturer or manufacturing conditions may affect the mycobiota in *Nuruk*, rather than the geographical location, because *Nuruk* is made at home or in traditionally operated small factories in Korea; thus, the manufacturing process can vary.

Morphological identification of fungal isolates. One hundred and seventy-four fungal isolates were isolated from 39 *Nuruk* samples. The micromorphologies of the sporangia, sporangiophores, chlamydospores, sporangiospores, and/or rhizoids of each Mucorales isolate from *Nuruk* were examined. A comparison to the identification key revealed that 61% of the filamentous fungal isolates from the *Nuruk* samples belonged to either *Lichtheimia* (*L. corymbifera* and *L. ramosa*), *Rhizopus* (*R. oryzae* and *R. microspores*), *Rhizomucor* (*Rm. pusillus* and *Rm. variabilis*), *Mucor (M. racemosus*) or *Syncephalastrum* (*S. racemosum*) [Ciesla *et al.*, 2000; Ribes *et al.*, 2000;

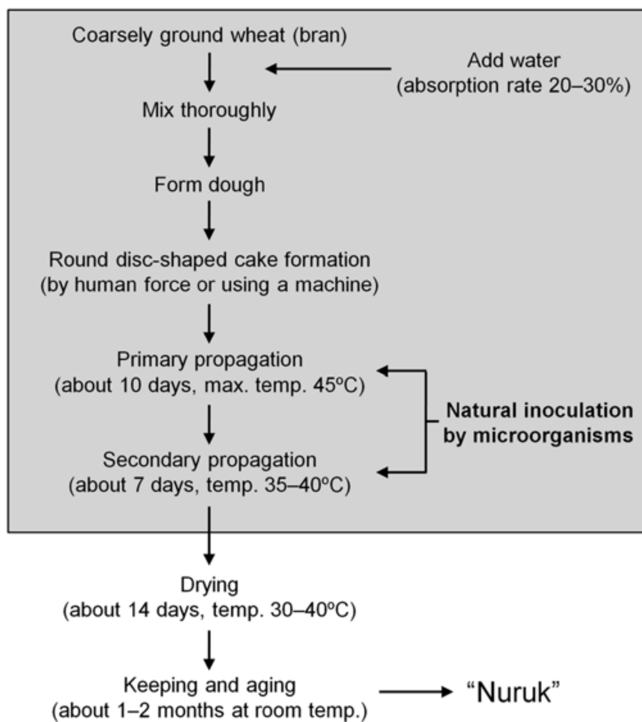

Fig. 1. Schematic outline of the *Nuruk* manufacturing process in a traditionally operated factory in Korea.

Garcia-Hermoso *et al.*, 2009]. The remaining 39% of the fungal isolates belonged to *Aspergillus* spp. Therefore, the micromorphologies of the conidial heads, conidiophores, and conidia were examined to determine the species of each isolate. A comparison with the identification key suggested by Klich [2002] revealed that *Nuruk* contained *A. oryzae*, *A. flavus*, *Aspergillus fumigates*, *Aspergillus niger*, *Aspergillus clavatus*, and *Emeircella nidulans*. Several genera such as *Lichthemia* (formerly *Absidia*), *Aspergillus,* and *Rhizopus* have been suggested as the most predominant fungi in *Nuruk* [Uchimura *et al.*, 1990; Park *et al.*, 1995; Yang and Lee, 1996; Yu *et al.*, 1996; Jo and Lee, 1997; Yu *et al.*, 1998]. Our results addressed the controversy on the mycobiota in *Nuruk* and showed that the genus *Lichthemia* is predominant in *Nuruk*.

Importantly, one isolate of *S. racemosum* obtained from the Hansan A sample had not been previously reported as being present in *Nuruk*. This isolate formed very abundant aerial mycelia of grey color on the PDA and MEA after 7 days incubation at 25°C. The sporangiophores that arose from rhizoids had irregular branches. In addition, the isolate produced vesicles bearing uniquely shaped merosporangia with merospores in a single row, which were spherical to ovoid in shape (Fig. 2). The morphological characteristics of this isolate were identical to those of *S. racemosum* [de Hoog and Guarro, 2000], and the ITS-5.8S rDNA sequence of the isolate supported this morphological-based identification.

Physiological identification of fungal isolates. Physiological tests were also performed to verify the

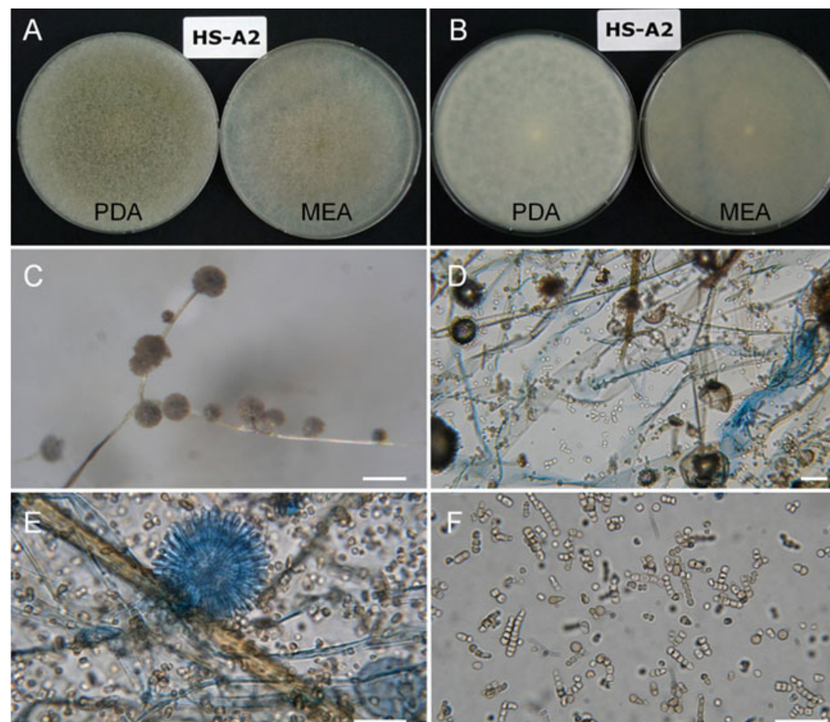

Fig. 2. Morphological characteristics of *Syncephalastrum racemosum* from *Nuruk*. Colony morphology obverse (A) and reverse (B). Micromorphologies of sporangia (C), sporangiophores (D), rhizoid (E), and spores (F). PDA, potato dextrose agar; MEA, malt extract agar. Scale bars: 100 μm (C), 25 μm (D-F).

Table 2. Thermotolerance of the isolates from *Nuruk* samples

Mucorales isolates	Colony diameter (d, mm)[a]						
	0[b]	0 < d < 5	5 ≤ d < 10	10 ≤ d < 15	15 ≤ d < 20	20 ≤ d < 25	25 ≤ d
Lichtheimia corymbifera		6	14	4			
Lichtheimia ramosa		2	18	22	8	3	
Rhizopus oryzae group	8						
Rhizopus microsporus						1	4
Rhizomucor pusillus							10
Rhizomucor variabilis	1						
Mucor circinelloides	1						
Mucor racemosus	1						
Syncephalastrum racemosum	1						
Aspergillus oryzae		1	7	9	7	18	9
Aspergillus flavus			1	1	3	2	2
Aspergillus fumigatus							1
Aspergillus niger							1
Aspergillus clavatus		1					
Emericella nidulans							1
Emericella sp.	2						

[a]*Lichtheimia*, *Rhizopus*, *Rhizomucor*, *Mucor*, and *Syncephalastrum* isolates were incubated on malt extract agar at 48°C for 5 days. *Aspergillus* and *Emericella* isolates were incubated on Czpaek Dox agar at 42°C for 7 days.
[b]No growth.

identification of each fungal isolate based on the morphological characteristics. The growing ability of the isolates at high temperature has been used as an important taxonomic key for identifying some Mucorales fungi [Schipper, 1984]. Moreover, thermotolerance has been reported in *Aspergillus* spp. [Rodrigues *et al.*, 2009]. It is believed that some fungal isolates from *Nuruk* samples are able to grow at high temperature, because relatively high temperature conditions are used for the proliferation of microorganisms during the *Nuruk* manufacturing process (Fig. 1). In order to obtain a higher accuracy of identification and to investigate the thermotolerance of each fungal isolate, the diameters of Mucorales and *Aspergillus* fungal colonies were measured after incubation at 48 and 42°C, respectively (Table 2). With the exception of *Mucor* spp., *Rm. variabilis,* and *S. racemosum*, most of the fungal isolates showed thermotolerance (Table 2). *Mucor* spp. and *Rm. variabilis* did not grow on a medium of steamed rice at 38°C; however, *S. racemosum* did grow, suggesting that the growing ability at this temperature is an important factor for differentiating *S. racemosum* from the *Mucor* and *Rhizomucor* species. In contrast to *Rm. variabilis*, *Rm. pusillus* showed the best thermotolerance among all of the isolates from *Nuruk*. Although all *Rhizopus* isolates grew at 38°C, *Rh. oryzae* was not able to grow at 48°C, whereas *Rh. microsporus* grew at this temperature (Table 2). With the exception of two isolates, all *Aspergillus* isolates grew at 42°C. Together, these results supported the morphological-based identification of the isolates and suggested that the level of thermotolerance is another important criterion for the classification of isolates from *Nuruk* samples. In addition, thermophilic fungi outgrow other airborne and waterborne fungi during the process of *Nuruk* manufacturing, which may be an important characteristic for this process.

An AFPA medium was used to differentiate *A. flavus* and *A. parasiticus* from other *Aspergillus* species [Bothast and Fennell, 1974; Pitt *et al.*, 1983; Rodrigues *et al.*, 2009]. Assante *et al*. [1981] suggested that ferric ions in medium react with aspergillic acid or neoaspergillic acid produced by *Aspergillus* species and form a yellow-orange colored complex. In the present study, 41 of 51 *A. oryzae* isolates from *Nuruk* samples showed strong positive reactions. Prolonged cultivation of *Aspergillus* isolates is known to produce aspergillic acid in isolates from shoyu, miso, and mirin [Wood, 1977]. This acid production may have prevented our group from obtaining definitive results in the present study. Therefore, we concluded that the AFPA medium is not suitable for differentiating *A. flavus* from *A. oryzae* in isolates from *Nuruk*. Moreover, the majority of *A. oryzae* isolates from *Nuruk* are able to produce aspergillic acid or neoaspergillic acid.

Phylogenetical identification of fungal isolates. Each fungal isolate was also identified based on sequence

Table 3. Classification of strains isolated from *Nuruk* samples

Species	No. of strains (%)	
	Morphological /physiological	Phylogenetic
Lichtheimia corymbifera	24 (14)	26 (15)
Lichtheimia ramosa	53 (30)	51 (29)
Aspergillus oryzae	51 (29)	54 (31)
Aspergillus flavus	9 (5)	6 (3)
Aspergillus fumigatus	4 (2)	4 (2)
Aspergillus niger	2 (1)	2 (1)
Aspergillus clavatus	1 (0.5)	1 (0.5)
Rhizopus oryzae	8 (5)	8 (5)
Rhizopus microsporus	5 (3)	5 (3)
Rhizomucor pusillus	10 (6)	8 (5)
Rhizomucor tauricus	0	2 (1)
Rhizomucor variabilis	1 (0.5)	1 (0.5)
Emericella nidulans	1 (0.5)	3 (2)
Emericella. spp.	2 (1)	0
Mucor circinelloides	1 (0.5)	1 (0.5)
Mucor racemosus	1 (0.5)	1 (0.5)
Syncephalastrum racemosum	1 (0.5)	1 (0.5)
Total	174	174

identity using sequences of the ITS-5.8S rDNA in the GenBank database (Table S1). The results of morphological identification and molecular analyses were identical at the genus level among isolates; however, slight discordance was found at the species level (Table 3). Most members of Mucorales showed good agreement between the two approaches; however, some isolates of the *Lichtheimia* species (AD-A4, HC-A3, JC-A2, and SEJ-A1) gave conflicting results from these methods (Table 3). Neighbor-joining tree analysis showed that the isolates belonging to *Lichtheimia,* based on morphological and physiological characteristics, were placed in two strongly supported subclades, *L. corymbifera* and *L. ramosa* (Fig. 3.). Recently, *Absidia* was reclassified based on the phylogenetic, physiological, and morphological characteristics of the genus, and three thermotolerant *Absidia* species (*Ab. corymbifera*, *Ab. blakesleeana*, and *Ab. hyalospora*) were integrated into the genus *Lichtheimia* [Hoffmann *et al.*, 2007; 2009]. In addition, Garcia-Hermoso *et al.* [2009] classified strains forming a separate clade distinct from that of *L. corymbifera* as *L. ramosa*. Our phylogenetic analysis supported these data, and showed that the genus *Lichtheimia* was distinct from the genus *Absidia* and was separated into two sub-clades (Fig. 3). In addition, the phylogenetic analysis in the present study clarified the misidentification based on morphological similarities between *L. corymbifea* and *L. ramose*.

Mucor, *Rhizopus*, *Rhizomucor*, and *Syncephalastrum* were separated into strongly supported clades (Fig. 3). All members that belonged to each clade showed the same level of thermotolerance, which suggested that a strong relationship exist between thermotolerance and phylogenic characteristics. Interestingly, the *Rm. variabilis* isolate is located in the clade of the *Mucor* species, and both *Rm. variabilis* and *Mucor* were sensitive to high temperature. These observations were consistent with previously reported results of Alvarez *et al*. [2009] in that *Rm. variabilis* var. *regularior* was synonymous with *M. circinelloides* in the *Mucor* clade. Our physiological and phylogenetic analyses support the conclusion that *Rm. variabilis* belongs to the genus *Mucor* rather than the genus *Rhizomucor*.

In the present, consistent data on *A. fumigatus, A. niger, and A. clavatus* isolates were obtained based on both molecular analyses and morphological observations. In contrast, members of Section *Flavi* (e.g., *A. oryzae*, *A. flavus*) and some isolates of the *Emericella* species showed disagreement (Table S3). To complement the results of the ITS-5.8S rDNA sequence analysis, the β-tubulin gene of *Aspergillus* isolates was sequenced. All isolates identified in the *Flavi* section based on morphological characteristics showed the most similarity to *A. oryzae*, and other *Aspergillus* isolates were in accordance with the sequencing results from the ITS-5.8S rDNA. Neighbor-joining tree analysis also showed that the *A. terreus, A. fumigatus, A. versicolor,* and *Emericella* species separated into strongly supported sub-clades; however, the isolates that belonged to the *Flavi* section formed one clade (Fig. 4). Topology of the β-tubulin tree was similar to that of the ITS-5.8S rDNA (Fig. 5). These results suggested that the *Aspergillus* species belonging to the *Flavi* section are not identifiable based on ITS-5.8S rDNA and β-tubulin sequence analyses.

In conclusion, our study has addressed the substantial controversy surrounding the presence of mycrobiota in *Nuruk* starter cultures by performing integrated morphological, physiological, and phylogenetic analyses on the fungal isolates from *Nuruk* samples. Various alcoholic starter cultures used in Asia are dominated by the genus *Rhizop1us, Mucor,* and *Amylomyces,* whereas the Japanese starter culture is mostly comprised of the genus *Aspergillus* [Wood, 1977; Thanh *et al.*, 2008]. However, our study has shown that 83% of the filamentous fungal isolates from *Nuruk* samples are of the *Lichtheimia* and *Aspergillus* genera, and that the predominant fungal species in *Nuruk* are *L. ramosa*, *L. corymbifera*, and *A. oryzae*. These results suggest that traditional Korean starter cultures contain unique mycobiota compared to other Asian starter cultures. It is clear that the tastes and flavors of the Korean traditional alcoholic

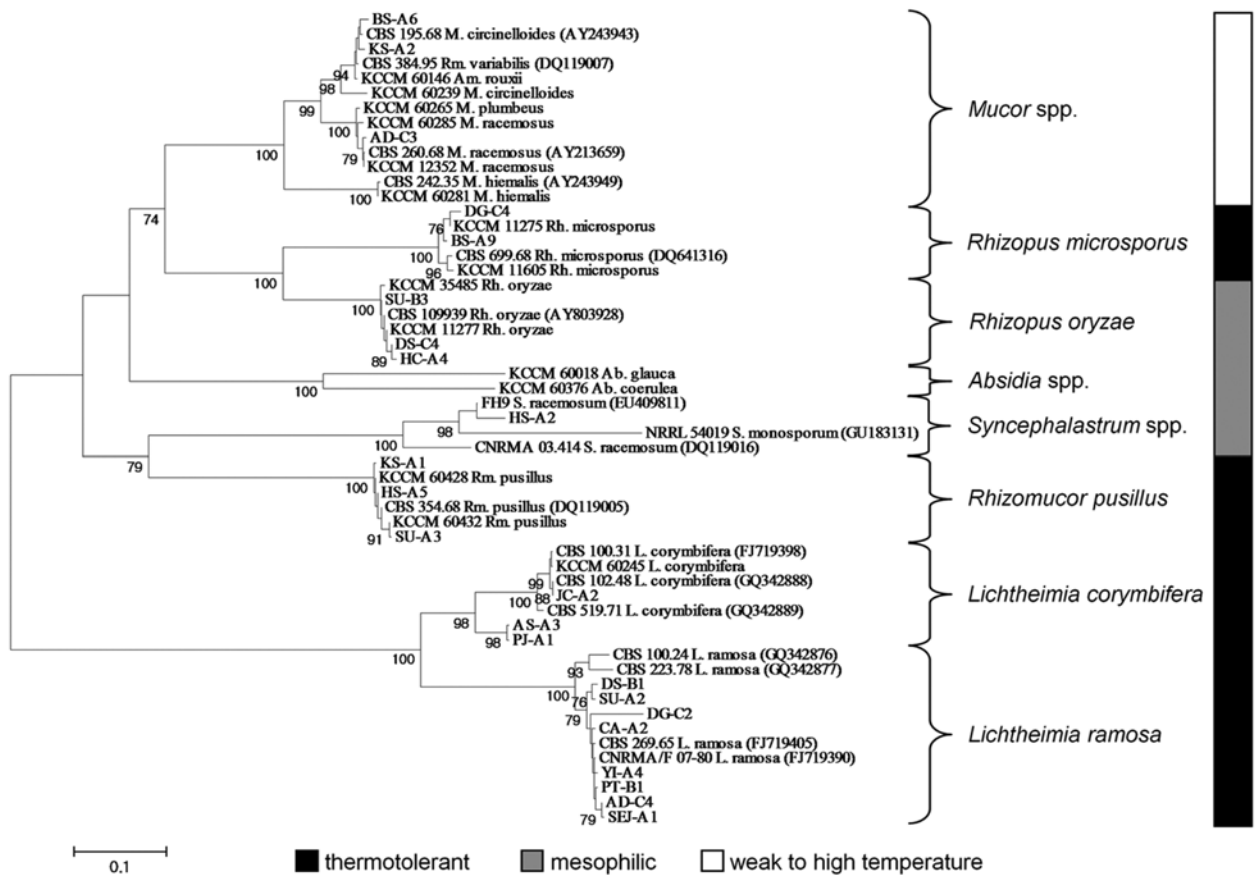

Fig. 3. A neighbor-joining analysis based on sequences from the ITS-5.8S rDNA region of representative Mucorales isolates from *Nuruk* samples. In the tree, the branch lengths are proportional to the distance. Bootstrap iteration frequencies (1,000 iterations) above 70% are indicated on the nodes. The graph indicates the thermotolerance of each fungal strain.

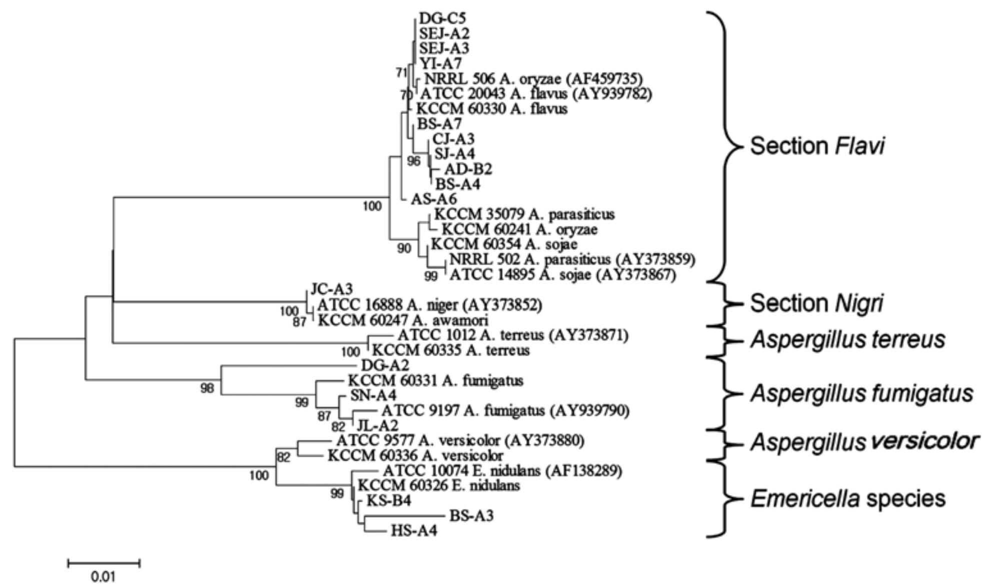

Fig. 4. A neighbor-joining analysis based on sequences from the ITS-5.8S rDNA of representative *Aspergillus* isolates from *Nuruk* samples. In the tree, the branch lengths are proportional to the distance. Bootstrap iteration frequencies (1,000 iterations) above 70% are indicated on the nodes.

beverages are greatly influenced by the microorganisms in *Nuruk*. In this respect, the roles of fungi, which are predominant in *Nuruk*, need to be characterized. Our results provide a better understanding of appropriate

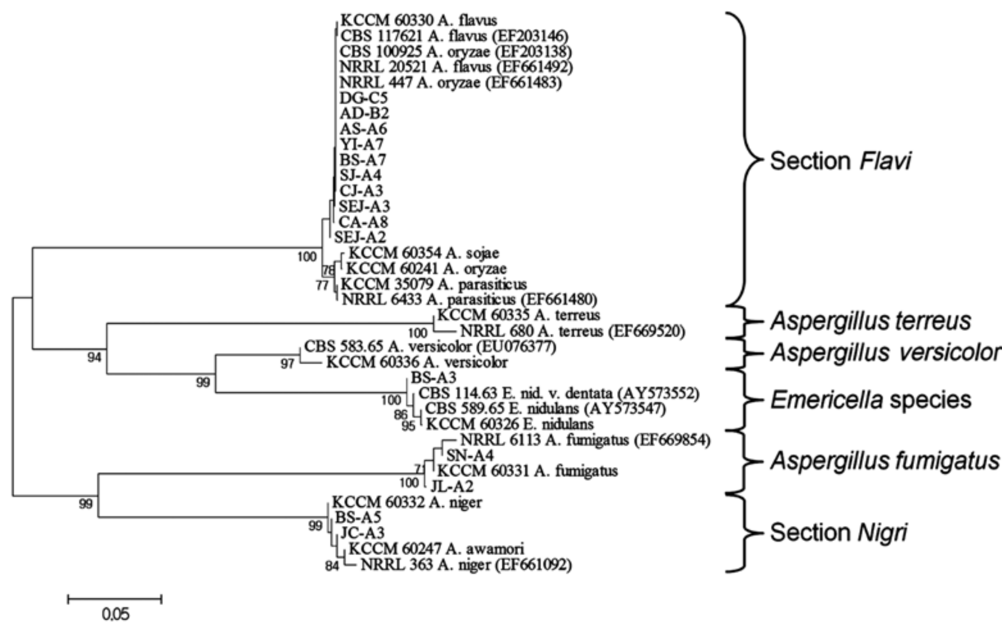

Fig. 5. A neighbor-joining analysis based on the sequences from the β-tubulin gene of *Aspergillus* isolates from *Nuruk* samples. In the tree, the branch lengths are proportional to the distance. Bootstrap iteration frequencies (1,000 iterations) above 70% are indicated on the nodes.

conditions for rice fermentation and for improving the quality of rice wine production. In addition, the fungal strains isolated in our study will be good sources for the further researches on the applications by the industries.

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References

Alvarez E, Sutton DA, Cano J, Fothergill AW, Stchigel A, Rinaldi MG, and Guarro J (2009) Spectrum of zygomycete species identified from clinically significant specimens in the United States. *J Clin Microbiol* **47**, 1650–1656.

Assante G, Camarda L, Locci R, Merlini L, Nasini G, and Papadopoulos E (1981) Isolation and structure of red pigments from *Aspergillus flavus* and related species, grown on a differential medium. *J Agric Food Chem* **29**, 785–787.

Bae KH, Shin KS, Ryu HY, Kwon CS, and Sohn HY (2007) Identification and fermentation characteristics of lactic acid bacteria isolated from the fermentation broth of Korean traditional liquor, Andong-Soju. *Korean J Microbiol Biotechnol* **35**, 310–315. (In Korean).

Balajee SA, Borman AM, Brandt ME, Cano J, Cuenca-Estrella M, Dannaoui E, Guarro J, Haase G, Kibbler CC, Meyer W, O'Donnell K, Petti CA, Rodriguez-Tudela JL, Sutton D, Velegraki A, and Wickes BL (2009) Sequence-based identification of *Aspergillus*, *Fusarium*, and *Mucorales* species in the clinical mycology laboratory: Where are we and where should we go from here? *J Clin Microbiol* **47**, 877–884.

Bothast RJ and Fennell DI (1974) A medium for rapid identification and enumeration of *Aspergillus flavus* and related organisms. *Mycologia* **66**, 365–369.

Ciesla MC, Kammeyer PL, Yeldandi V, Petruzzelli GJ, and Yong SL (2000) Identification of the asexual state of *Rhizopus* species on histologic tissue sections in a patient with rhinocerebral mucormycosis. *Arch Pathol Lab Med* **124**, 883–887.

de Hoog GS and Guarro J (2000) In *Atlas of Clinical Fungi*, Centraalbureauvoor Schimmelcultures, Baarn, The Netherlands.

Garcia-Hermoso D, Hoinard D, Gantier J-C, Grenouillet F, Dromer F, and Dannaoui E (2009) Molecular and phenotypic evaluation of *Lichtheimia corymbifera* (formerly *Absidia corymbifera*) complex isolates associated with human mucormycosis: Rehabilitation of *L. ramosa*. *J Clin Microbiol* **47**, 3862–3870.

Geiser DM, Klich MA, Frisvad JC, Peterson SW, Varga J, and Samson RA (2007) The current status of species recognition and identification in *Aspergillus*. *Stud Mycol* **59**, 1–10.

Glass NL and Donaldson GC (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microbiol* **61**, 1323–1330.

Guarro J, Gene J, and Stchigel AM (1999) Developments in fungal taxonomy. *Clin Microbiol Rev* **12**, 454–500.

Ha DM, Kim DC, Hong SM, and Lee CW (1989) Identification and properties of starch utilizing yeasts isolated from *Nuruk*. *J Korean Agric Chem Soc* **32**, 408–415. (In Korean).

Han EH, Lee TS, Noh BS, and Lee DS (1997) Quality characteristics in mash of takju prepared by using different *Nuruk* during fermentation. *Korean J Food Sci Technol* **29**, 555–562. (In Korean).

Henry T, Iwen PC, and Hinrichs SH (2000) Identification of *Aspergillus* species using internal transcribed spacer regions 1 and 2. *J Clin Microbiol* **38**, 1510–1515.

Hesseltine CW (1983) Microbiology of oriental fermented foods. *Ann Rev Microbiol* **37**, 575–601.

Hoffmann K, Discher S, and Voigt K (2007) Revision of the genus *Absidia* (*Mucorales*, *Zygomycetes*) based on physiological, phylogenetic, and morphological characters; thermotolerant *Absidia* spp. form a coherent group, *Mycocladiaceae* fam. nov. *Mycol Res* **111**, 1169–1183.

Hoffmann K, Walther G, and Voigt K (2009) *Mycocladus* vs. *Lichtheimia*: A correction (*Lichtheimiaceae* fam. nov., *Mucorales*, *Mucoromycotina*). *Mycol Res* **113**, 277–278.

Jeyaram K, Singh WM, Capece A, and Romano P (2008) Molecular identification of yeast species associated with 'Hamei' - A traditional starter used for rice wine production in Manipur, India. *Int J Food Microbiol* **124**, 115–125.

Jo GY and Lee CW (1997) Isolation and identification of the fungi from *Nuruk*. *J Korean Soc Food Sci Nutr* **26**, 759–766. (In Korean).

Jo KY and Ha DM (1995) Isolation and identification of the lactic acid bacteria from *Nuruk*. *Agr Chem Biotechnol* **38**, 95–99. (In Korean).

Kim HR, Baek SH, Seo MJ, and Ahn BH (2006) Feasibility of Cheonghju brewing with wild type yeast strains from *Nuruks*. *Korean J Microbiol Biotechnol* **34**, 244–249. (In Korean).

Kim HS, Hyun JS, Kim J, Ha HP, and Yu TS (1997) Characteristics of useful fungi isolated from traditional Korean *Nuruk*. *J Korean Soc Food Sci Nutr* **26**, 767–774. (In Korean).

Kim HS, Hyun JS, Kim J, Ha HP, and Yu TS (1998) Enzymological characteristics and identification of useful fungi isolated from traditional Korean *Nuruk*. *Korean J Microbiol Biotechnol* **26**, 456–464. (In Korean).

Kim JY and Koh JS (2004) Screening of brewing yeasts and saccharifying molds for foxtail millet-wine making. *J Korean Soc Appl Biol Chem* **47**, 78–84. (In Korean).

Klich MA (2002) In *Identification of Common Aspergillus Species*, Centraalbureauvoor Schimmelcultures, Utrecht, The Netherlands.

Kozaki M and Uchimura T (1990) Microorganisms in Chinese starter "Bubod" and rice wine "Tapuy" in the Philippines. *J Brew Soc Jpn* **85**, 818–824. (In Japanese).

Lee HH, Lee JH, Ko YJ, Park MH, Lee JO, and Ryu CH (2009) Changes in allergenicity and quality of *Nuruk* during fermentation. *J Korean Soc Food Sci Nutr* **38**, 76–82. (In Korean).

Lee JH and Yu TS (2000) Identification and characteristics of lactic acid bacteria isolated from *Nuruk*. *Korean J Biotechnol Bioeng* **15**, 359–365. (In Korean).

Lee SJ and Ahn BH (2010) Sensory profiling of rice wines made with *Nuruks* using different ingredients. *Korean J Food Sci Technol* **42**, 119–123. (In Korean).

Lee SS, Kim KS, Eom AH, Sung CK, and Hong IP (2002) Production of Korean traditional rice-wines made from cultures of the single fungal isolates under laboratory conditions. *Korean J Mycol* **30**, 61–65. (In Korean)

Lee WK, Kim JR, and Lee MW (1987) Studies on the changes in free amino acids and organic acids of Takju prepared with different Koji strains. *J Korean Agric Chem Soc* **30**, 323–327. (In Korean).

Leslie JF and Summerell BA (2006) In *The Fusarium* Laboratory Manual, Blackwell Pub., Ames, IA.

Nikkuni S, Karki TB, Terao T, and Suzuki C (1996) Microflora of Mana, a Nepalese rice koji. *J Ferment Bioeng* **81**, 168–170.

Park JW, Lee KH, and Lee CY (1995) Identification of filamentous molds isolated from Korean traditional *Nuruk* and their amylolytic activities. *Korean J Appl Microbiol Biotechnol* **23**, 737–746. (In Korean).

Pitt JI, Hocking AD, and Glenn DR (1983) An improved medium for the detection of *Aspergillus flavus* and *A. parasiticus*. *J Appl Microbiol* **54**, 109–114.

Ribes JA, Vanover-Sams CL, and Baker DJ (2000) Zygomycetes in human disease. *Clin Microbiol Rev* **13**, 236–301.

Rodrigues P, Venâncio A, Kozakiewicz Z, and Lima N (2009) A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* Section *Flavi* isolated from Portuguese almonds. *Int J Food Microbiol* **129**, 187–193.

Saitou N and Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Schipper MAA (1984) A revision of the genus *Rhizopus*. I. The *Rhizopus stolonifer*-group and *Rhizopus oryzae*. *Stud Mycol* **25**, 1–34.

Schwarz P, Bretagne S, Gantier J-C, Garcia-Hermoso D, Lortholary O, Dromer F, and Dannaoui E (2006) Molecular identification of zygomycetes from culture and experimentally infected tissues. *J Clin Microbiol* **44**, 340–349.

Shon SK, Rho YH, Kim HJ, and Bae SM (1990) Takju brewing of uncooked rice starch using *Rhizopus* koji. *Korean J Appl Microbiol Biotechnol* **18**, 506–510. (In Korean).

So MH (1995) Aptitudes for Takju brewing of wheat flour-*Nuluks* made with different mold species. *Korean J Food Nutr* **8**, 6–12. (In Korean).

So MH (1999) Characteristics of a modified *Nuruk* made by inoculation of traditional *Nuruk* microorganisms. *Korean J Food Sci Technol* **12**, 219–225. (In Korean).

Tamura K, Dudley J, Nei M, and Kumar S (2007) MEGA4:

Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Tamura K, Nei M, and Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc Natl Acad Sci USA* **101**, 11030–11035.

Thanh VN, Mai LT, and Tuan DA (2008) Microbial diversity of traditional Vietnamese alcohol fermentation starters (banh men) as determined by PCR-mediated DGGE. *Int J Food Microbiol* **128**, 268–273.

Thompson JD, Higgins DG, and Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Uchimura T, Takagi S, Watanabe K, and Kozaki M (1990) *Absidia* sp. in the Chinese starter (*Nuruk*) in Korea. *J Brew Soc Jpn* **85**, 888–894. (In Japanese).

White TJ, Bruns T, Lee S, and Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols a Guide to Methods and Applications*, Innis MA, Gelfand DH, Sninsky JJ, and White TJ (eds.), Academic Press, New York, NY.

Wood BJB (1977) Oriental food uses of *Aspergillus*. In *The British Mycological Symposium Series No 1. Genetics and Physiology of Aspergillus*, Academic Press, London, UK.

Xie G, Li W, Lu J, Cao Y, Fang H, Zou H, and Hu Z (2007) Isolation and identification of representative fungi from Shaoxing rice wine wheat Qu using a polyphasic approach of culture-based and molecular-based methods. *J Inst Brew* **113**, 272–279.

Yang JY and Lee KH (1996) Shelf-life and microbiological study of Sansung Takju. *Korean J Food Sci Technol* **28**, 779–785 (In Korean).

Yokoyama N, Tanaka K, Du L, Aramaki I, Kizaki Y, Kobayashi S, and Okazaki N (1994) Microflora and enzyme activities of Chinese Qu. *J Brew Soc Jpn* **89**, 72–76. (In Japanese).

Yu CH, Hong SY, and Koh JS (2002) Zymological properties of foxtail millet wine making by isolated strains from *Nuruk*. *J Korean Agric Chem Soc* **45**, 138–144. (In Korean).

Yu TS, Kim HS, Hong J, Ha HP, Kim TY, and Yoon IW (1996) Bibliographical study on microorganisms of *Nuruk* (Until 1945). *J Korean Soc Food Sci Nutr* **25**, 170–179. (In Korean).

Yu TS, Kim J, Kim HS, Hyun JS, Ha HP, and Park MG (1998) Bibliographical study on microorganisms of traditional Korean *Nuruk* (since 1945). *J Korean Soc Food Sci Nutr* **27**, 789–799. (In Korean).