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Microbial Population, Aflatoxin Contamination and Predominant *Aspergillus* Species in Korean Stored Rice

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We evaluated microbial populations and aflatoxin production in unhulled and white rice from rice processing complexes of the National Agricultural Cooperative Federation in five regions in Korea and identified three predominant *Aspergillus* species. Fungal and bacterial populations in rice samples were significantly different between regions in 2007. Aflatoxins were also detected and varied at the levels of 2.45 - 3.43 ng per g unhulled rice grain and 1.29 - 2.09 ng per g white rice grain. Unhulled rice generally detected higher level of aflatoxins than white rice regardless of sampling regions; however, no significant differences were found in Anseong and Cheonan in 2005 and Cheonan and Gimpo in 2007. Aflatoxin production between sampling regions was not different regardless of rice type and sampling year. Although the fungal diversity was highly distinct from region to region, three *Aspergillus* isolates were predominant in the rice samples; thus, representative isolates AC317, AF57, and AF8 were selected and identified based on their morphological and molecular characteristics. Consequently, isolates AC317, AF57, and AF8 were identified as *A. candidus*, *A. flavus*, and *A. fumigatus*, respectively. These fungi can produce mycotoxins that are harmful for consumers and thus it is important to detect and reduce the population of storage fungi in rice.

Keywords : aflatoxin, *Aspergillus candidus*, *Aspergillus flavus*, *Aspergillus fumigatus*, identification, microbial population, rice

Fungi can invade rice in the fields before harvest and survive even after harvest in dry grains through many years of storage (Williams et al., 1983). *Alternaria padwichii, Cochliobolus miyabeanus*, *Curvularia* spp., *Epicoccum* spp., *Fusarium graminearum*, *Gibberella fujikuroi*, *Heliocerus oryzae*, *Magnaporthe oryzae*, *Nigrospora* spp., *Phoma* spp., *Phyllosticta glumarum*, *Septoria oryzae*, *Tilletia barclayana*, and *Ustilaginoidea virens* are the representative field fungi affecting rice. These field fungi can cause grain discoloration, destruction, replacement, loss of viability, mycotoxin contamination, and subsequent seedling mortality (Wicklow, 1992). Some *Aspergillus* and *Penicillium* are plant pathogenic and are the predominant storage fungi in rice (Filtenborg et al., 1996; Oh et al., 2008a; Pitt, 2000).

Aspergillus species are ubiquitous and have a wide range of temperatures (–8~58°C) for optimal growth (Muir and White, 1998). Among more than 180 *Aspergillus* species, several *Aspergillus* species are known to be storage fungi (Wang et al., 2000). In particular, the *A. glaucus* group, which includes *A. amstelodami*, *A. candidus*, *A. chevalieri*, *A. flavus*, *A. ochraceus*, *A. repens*, *A. resticus*, and *A. rubber*, are responsible for dry grain spoilage (Miller et al., 1995; Muir and White, 1998). Some *Aspergillus* species such as *A. candidus*, *A. flavus*, *A. fumigatus*, and *A. ochraceus* are capable of causing infection in human. *A. candidus* can causes respiratory problems for storage barn workers, while *A. flavus* causes death in humans and animals (Pitt, 1994). In addition, the conidia produced by *A. fumigatus* can penetrate the lungs or respiratory tract of animals and humans, resulting in systemic mycoses (Pitt, 1994).

In our previous studies (Oh et al., 2007; 2008b), we evaluated fungal populations in stored rice from rice processing complexes (RPC) of the National Agricultural Cooperative Federation (NACF) and the indoor storage conditions. In 2005 and 2006, we sampled unhulled and white rice from RPC in 11 regions in Korea to compare the microbial populations and diversity between regions (Oh et al., 2007). We also sampled unhulled and brown rice stored in a room at Korea University, Seoul, Korea, to examine the temporal changes of microbial populations in relation to temperature and humidity under indoor storage conditions (Oh et al., 2008b). From these studies, we found that most unhulled, brown, and white rice was contaminated by *Aspergillus* species regardless of storage conditions (Oh et al., 2007; 2008b). In this study, we obtained rice samples from five regions, Anseong, Cheonan, Gimje, Gimpo, and Namwon, in which rice samples showed high fungal populations in our previous studies in 2005 and 2006 (Oh et

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al., 2007). Thus the current study was conducted to evaluate the population and diversity of fungi and bacteria and to identify the predominant *Aspergillus* species in stored rice samples from five regions of Korea in 2007. In addition, we attempted to detect the aflatoxins produced by *Aspergillus* species in the rice samples from the same regions in 2005 and 2007.

Materials and Methods

Fungal and bacterial populations. Unhulled and white rice samples were collected from RPC of the NACF in five regions, Anseong, Cheonan, Gimje, Gimpo, and Namwon in Korea in 2007. Samples were taken with a sampler (NAPQMS, Anyang, Korea) stabbing into ton bags from the RPC, and 3 g of the samples (a replication) were finely ground with an analytical mill (IKA A11 basic, IKA® Works, Inc. Wilmington, NC, USA). The ground rice was put in sterile distilled water (30 ml) and shaken at 120 rpm for 50 min at 28°C; the suspension was then smeared on different media at appropriate dilutions for microbial evaluations. The treated media were incubated in the dark at 28°C for 2 and 5 days for bacteria and fungi, respectively.

For assessing fungi, dichloran 18% glycerol agar (DG18) amended with 50 mg chlortetracycline per liter was used. Genus *Aspergillus* and *Penicillium* on the medium were identified under an optical microscope according to the descriptions by Pitt (2000) and Klich (2002). Three predominantly distinct *Aspergillus* colonies were also isolated for further morphological and molecular identification. All isolates were stored on malt extract agar (MEA) slants at 4°C. For isolating bacteria, nutrient agar amended with 50 mg NaCl and 50 mg cycloheximide per liter was used. The dominant and representative bacterial colonies on NA were isolated and incubated on tryptic soy agar (TSA, Difco, Detroit, MI, USA) at 28°C for 24 hr for bacterial identification. For identification of the representative bacterial strains, Biolog analysis was conducted with the Biolog GN Microplate System (Biolog Inc., Hayward, CA, USA) with Biolog software (Microlog 3 database, release 4.01A) according to the manufacturer's instructions. Total cellular FAME was analyzed for identification to the genus level by gas chromatography (Hewlett-Packard 5898A, GC system, Avondale, PA, USA) using the Microbial Identification System (MIDI, Newark, NJ, USA) according to the manufacturer's instructions. Experiments were conducted with three replications of three plates each. All data in this study were analyzed with SAS version 9.1.3 (SAS Institute, Cary, NC, USA).

Aflatoxin production. Unhulled and white rice from Anseong, Cheonan, Gimje, Gimpo, and Namwon in Korea in 2005 and 2007 were used in the analysis of aflatoxin production. Rice samples in plastic bags were stored at 4°C until used. Four grams (a replication) of rice samples were finely ground and suspended in 20 ml of 70% methanol, and shaken vigorously for 3 min. The suspensions were filtered through a Whatman #1 filter paper; then the filtraes were used for aflatoxin analysis. Aflatoxins including aflatoxin B_1, B_2, G_1, and G_2 were quantified three times per replication with a competitive direct enzyme-linked immunosorbent assay (CD-ELISA) using Veratox® HS (Veratox aflatoxin high sensitivity test kit; Neogene Co., Lansing, MI, USA) according to the manufacturer's instructions. The optical densities were read by Microplate spectrophotometer (Bio-Tek, Powerwave XS, Winooski, VT, USA) and results were calculated using Neogen's Veratox for Windows software supplied by the manufacturer. Aflatoxin production was determined as followings: aflatoxin (ng/g rice dry wt.) = aflatoxin concentration (ng/ml) in the sample extract × sample extract volume (ml) / sample weight (g). Experiments were conducted twice with three replications each.

Morphological identification. In this study, three representative isolates, AC317, AF57 and AF8, of the predominant *Aspergillus* species were selected for morphological and molecular identification. To observe microscopic characteristics, colonies were grown on MEA for 7 days; then, conidial heads, conidiophores, vesicles, conidium shapes, and roughness of conidial walls were observed under a microscope. These characteristics were compared with those of the reference isolates *A. candidus* KACC 41846, *A. flavus* KACC 40244, and *A. fumigatus* KACC 41390 obtained from the Korean Agricultural Culture Collection (KACC), Suwon, Korea and *Aspergillus* spp. described in the literature (Klich, 2002; Samson, 1979; Smith and Ross, 1991). Experiments were conducted twice with three replicate plates each.

Molecular identification. The DNA from mycelia of *Aspergillus* spp. was extracted from cultures grown in potato dextrose broth at 28°C for 7 days. DNA was extracted by the modified method of Boom et al. (1990). Species-specific oligonucleotide primers were designed for identification of three *Aspergillus* species. The primer pairs used for *A. candidus* amplified mitochondrial DNA for cytochrome b (Asp_mt_F: 5'-tatgagtgctataccttgaatcgg-3', Asp_mt_R: 5'-gtatcattcaggaacaatagcagg-3'). The primer pairs used for *A. flavus* and *A. fumigatus* amplified the β-tubulin gene (Bt2a: 5'-ggtaaccaaatcggtgctgctttc-3', Bt2b: 5'-accctcagtgtagtgacccttggc-3') (Glass and Donaldson, 1995). All sequence analyses to produce the oligonucleotide primers were carried out using the Oligos program version 9.2

(Institute of Biotechnology, Helsinki, Finland). Polymerase chain reaction (PCR) was performed in a final volume of 50 µl. PCR conditions for amplification of the mtDNA for cytochrome b from *A. candidus* and *β*-tubulin from *A. flavus* and *A. fumigatus* were as follows: each reaction mixture contained 5 µl (10 ng/µl) of template DNA, 5 µl (20 ng/µl) of forward primer, 5 µl (20 ng/µl) of reverse primer, 5 µl of 2.5 mM dNTP, 5 µl of 10x reaction buffer, 0.5 µl (5 unit/µl) of Taq, and 24.5 µl of distilled water. Cycling conditions were as follows: 95°C for 4 min for one cycle followed by 30 cycles of DNA denaturation at 95°C for 30 s, primer annealing at 58°C for 1 min, and DNA extension at 72°C for 1 min, and a final extension cycle at 72°C for 7 min. PCR products were electrophoresed on 1% agarose gel and product bands were excised from gel, extracted with MEGA-spin™ (iNtRON Biotechnology, Seoul, Korea), and ligated into T&A cloning vector (Cat. No. RC001, Real-Biotech Corp., Taipei County, Taiwan). Cloned plasmids were transformed into *E. coli* (DH5α) by chemical methods. The resulting DNA constructs were purified using DNA-spin™ (iNtRON Biotechnology, Seoul, Korea) and checked by digestion with the restriction endonuclease *Hind*III. The purified plasmid DNA was also sequenced with M13 universal primer (5'-gttttcccagtcacgac-3'). DNA sequences from each fungal isolates were compared to those of related *Aspergillus* species using BLAST network services at the National Center for Biotechnology Information (NCBI) of the U.S. National Library of Medicine, Bethesda, MD, USA. A phylogenetic tree was constructed with the neighbor-joining method using Molecular Evolutionary Genetics Analysis (MEGA) version 3.1 program (The Biodesign Institute, Tempe, AZ, USA), and bootstrap analysis was also conducted using the same program. The mtDNA sequences of *A. candidus* isolate AC317, *A. flavus* isolate AF57, and *A. fumigatus* isolate AF8 have been deposited in GenBank under accession numbers FJ436787, FJ436788, and FJ436789, respectively.

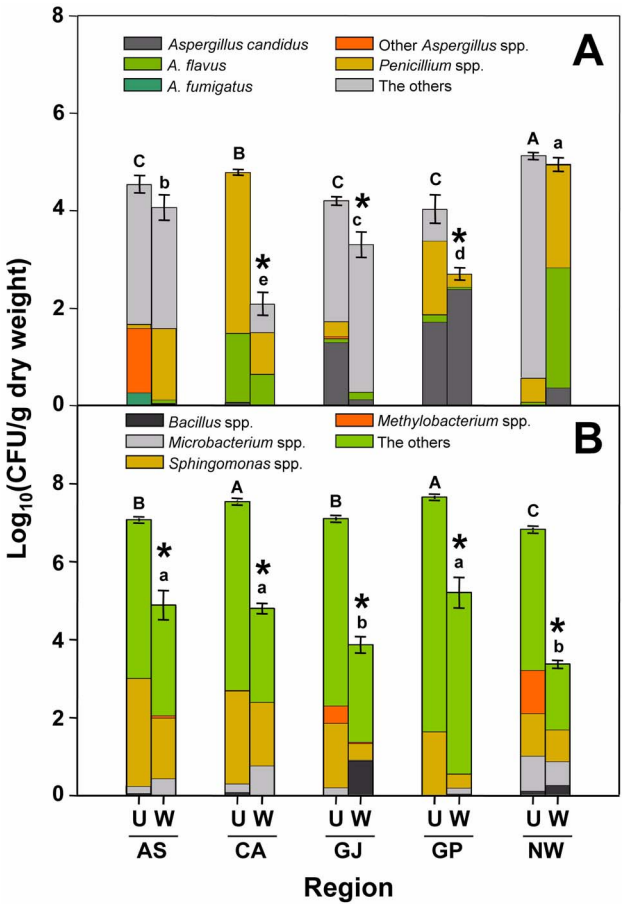

Fig. 1. Populations of (A) total fungi, *Aspergillus* spp., and *Penicillium* spp. and (B) total bacteria and several genera found in the stored rice samples collected from five regions of Korea in 2007. Asterisks on the bars of white rice indicate significantly ($P < 0.05$) different from unhulled rice in a region.
The different capital or small letters on the bars indicate that fungal or bacterial population in unhulled or white rice samples at each sampling region are significantly ($P<0.05$) different. Vertical bars indicate standard deviations of means from three replicates of three plates each. AS=Anseong, CA=Cheonan, GJ=Gimje, GP=Gimpo, NW=Namwon. U=unhulled rice, W=white rice.

Results

Fungal and bacterial populations. The population and diversity of fungi in unhulled and brown rice from five regions of Korea were distinct for each region (Fig. 1A). Total fungal populations in unhulled and white rice generally were $10^{4\text{-}5}$ and $10^{2\text{-}4}$ colony forming units per g dry weight, respectively. Total fungal populations of unhulled rice from Cheonan and Namwon were significantly ($P < 0.05$) different from those of Anseong, Gimje, and Gimpo; total populations in white rice were also significantly ($P < 0.05$) different from each other. In addition, total populations in white rice were significantly ($P < 0.05$) reduced from those in unhulled rice in Cheonan, Gimje, and Gimpo. Moreover, fungal diversity was highly distinct from region to region. *Aspergillus* and *Penicillium* species including *A. candidus*, *A. flavus*, and *A. fumigatus* were predominant in rice samples. Both unhulled and white rice samples from Cheonan were highly contaminated with *A. flavus* (ca. 30% of total population) and various species of *Penicillium*. From unhulled rice samples of Gimje, *A. candidus* was recovered, comprising 31% of the total fungal population and 92% of the total *Aspergillus* population. Unhulled and white rice from Gimpo were highly contaminated with *A. candidus*, which accounted for 43 and 88% of the total fungal population corresponding to 92 and 98% of the total *Aspergillus* population, respectively. White rice was contaminated with *A. flavus*, which accounted for 50% of the

total fungal population and 87% of the total *Aspergillus* population (Fig. 1A).

Although total bacterial populations in unhulled and white rice samples were significantly ($P < 0.05$) different between regions, the bacterial diversity was similar, in contrast to the fungal diversity (Fig. 1B). Total bacterial populations of white rice were reduced significantly ($P < 0.05$) compared to those of unhulled rice. The species of *Bacillus*, *Microbacterium*, *Sphingomonas*, and *Methylobacterium* were the predominant bacterial genera in the samples (Fig. 1B).

Aflatoxin production. Aflatoxins were detected from all unhulled and white rice of different regions in 2005 and 2007 (Table 1). Aflatoxin productions ranged from 2.45 to 3.32 ng per g rice grain in unhulled rice and from 1.29 to 1.80 ng per g rice grain in white rice in 2005; aflatoxin ranged from 2.65 to 3.43 ng per g rice grain in unhulled rice and from 1.55 to 2.16 ng per g rice grain in white rice in 2007. Unhulled rice generally produced more aflatoxins than white rice regardless of sampling regions; however, no significant ($P > 0.05$) differences between unhulled and white rice were found in Anseong and Cheonan in 2005 and Cheonan and Gimpo in 2007. Aflatoxin production between sampling regions also was not significantly ($P > 0.05$) different regardless of rice type and sampling year (Table 1).

Identification of fungal isolates. The microscopic characteristics of isolate AC317 were similar to those of isolate KACC 41846 and in the literature (Table 2). Conidiophores of isolate AC317 were borne from aerial hyphae, and the stipe was 300-400 μm in length, on average, with smooth walls. Stipe lengths of isolate KACC 41846 and in the literature were 300-400 and 200-500 μm with smooth walls, respectively. Conidial heads were radiate and metulae

Table 1. Aflatoxin production of unhulled and white rice from five regions by a competitive direct enzyme-linked immunosorbent assay (CD-ELISA)[a]

Region	Aflatoxin (ng/g rice dry wt.)[b]			
	Year 2005		Year 2007	
	Unhulled rice	White rice	Unhulled rice	White rice
Anseong	2.45 ± 0.72 aA[c]	1.80 ± 0.60 aA	3.43 ± 0.74 aA	2.09 ± 0.70 aB
Cheonan	2.56 ± 0.84 aA	1.66 ± 0.70 aA	2.65 ± 0.91 aA	1.55 ± 0.81 aA
Gimje	3.32 ± 1.37 aA	1.31 ± 0.79 aB	–[d]	–
Gimpo	2.96 ± 1.07 aA	1.53 ± 0.66 aB	2.93 ± 0.61 aA	2.16 ± 1.09 aA
Namwon	2.48 ± 0.70 aA	1.29 ± 0.80 aB	–	–

[a] The CD-ELISA test was performed using Veratox® HS (Veratox aflatoxin high sensitivity test kit), Neogene Co., Lansing, MI, USA.
[b] Aflatoxin (ng/g rice dry wt.) = aflatoxin concentration (ng/ml) in the sample extract × sample extract volume (ml) / sample weight (g).
[c] Means ± standard deviations followed by same small or capital letters do not differ significantly between regions at a given rice or between unhulled and white rice at a given region according to the LSD test at $P<0.05$. Values are means of six replications.
[d] –, not tested.

Table 2. Microscopic characteristics of *Aspergillus candidus* isolate AC317 and reference isolate KACC 41846 in comparison with characteristics described by Klich (2002)

Characteristics[a]	Present isolate AF317	Reference isolate KACC 41846	Klich (2002)
Stipe			
Length[b]	300-400 μm	300-400 μm	200-500 μm
Roughness	Smooth	Smooth	Smooth to finely roughened
Vesicle			
Shape	Globose	Globose to subglobose	Globose to elongate
Metulae coverage	Entire surface	Entire surface	Entire surface
Conidia			
Shape	Globose to subglobose	Globose to subglobose	Globose to slightly ovoid
Length	3-4 μm	3-4 μm	3-4 μm
Roughness	Smooth	Smooth	Smooth

[a] Features of stipe, vesicle, and conidia were determined using a microscope (×1,000).
[b] Lengths of stipe and conidia were determined with 40 and 100 observations, respectively.

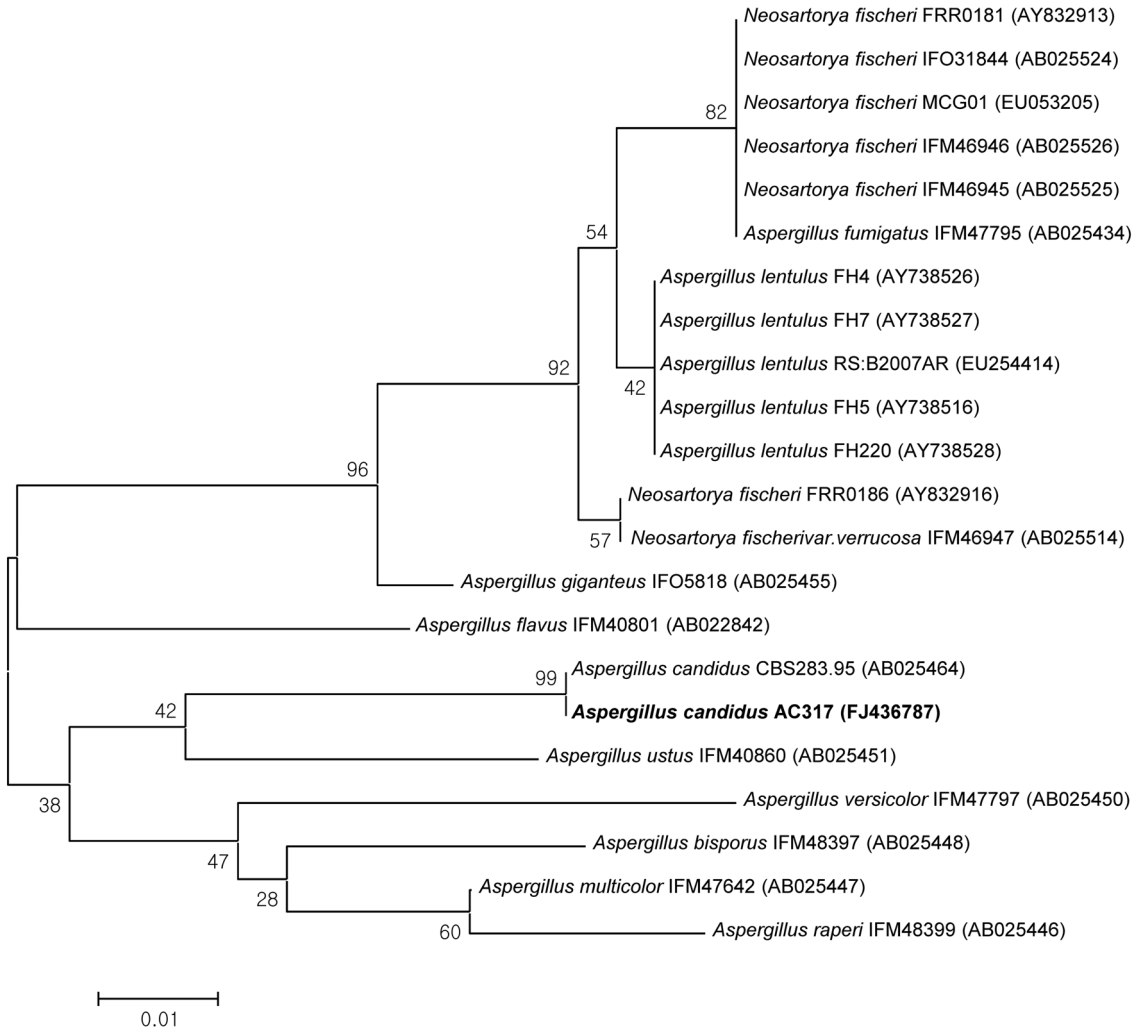

Fig. 2. A neighbor-joining tree of the *Aspergillus* species related to *A. candidus* isolate AC317 based on phylogenetic analysis of their mitochondrial DNA cytochrome b gene partial sequences. The type isolates of the species and accession numbers are indicated as "T" and in parentheses, respectively.

Table 3. Microscopic characteristics of *Aspergillus flavus* isolate AF57 and reference isolate KACC 40244 in comparison with characteristics described by Klich (2002)

Characteristics[a]	Present isolate AF57	Reference isolate KACC 40244	Klich (2002)
Stipe			
Length[b]	500-800 μm	500-700 μm	400-800 μm
Roughness	Finely roughened	Finely roughened	Finely roughened
Vesicle			
Shape	globose, subglobose to ellipsoidal	Globose to ellipsoidal	Spherical to elongate
Metulae coverage	3/4 surface	3/4 surface	3/4 to entire surface
Conidia			
Shape	globose to ellipsoidal	globose to ellipsoidal	globose to ellipsoidal
Length	3-5 μm	3-5 μm	3-6 μm
Roughness	Smooth to finely roughened	Smooth to finely roughened	Smooth to finely roughened

[a] Features of stipe, vesicle, and conidia were determined using a microscope (×1,000).
[b] Lengths of stipe and conidia were determined with 40 and 100 observations, respectively.

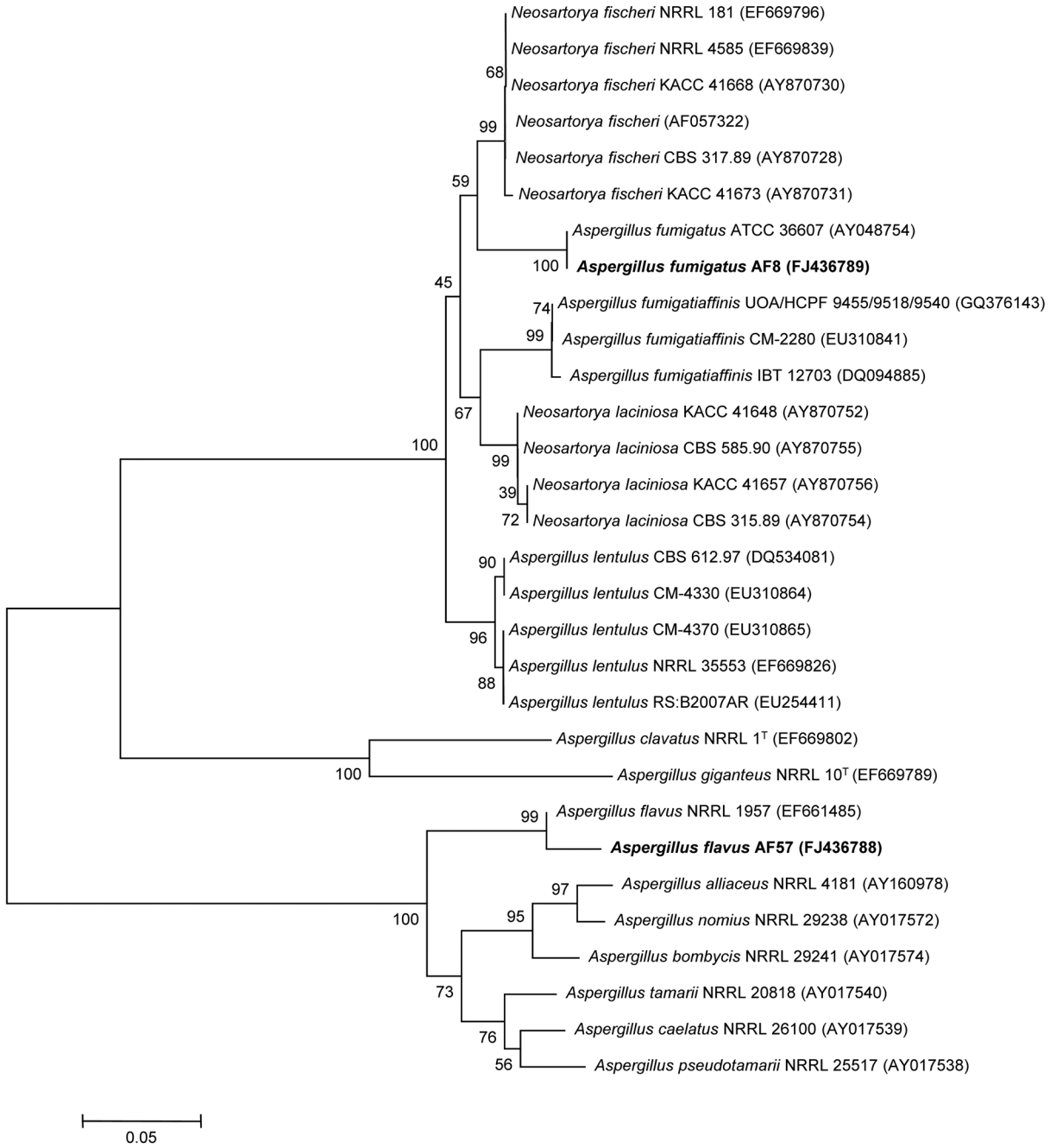

Fig. 3. A neighbor-joining tree of the *Aspergillus* species related to *A. flavus* isolate AF57 and *A. fumigatus* isolate AF8 based on phylogenetic analysis of β-tubulin gene sequences. The type isolates of the species and accession numbers are indicated as "T" and in parentheses, respectively.

covered the whole surface of the globose vesicle. Conidia were 3-4 μm, globose to subglobose with smooth walls, while those of isolate KACC 41846 and in the literature were 3-4 and 3-4 μm, respectively (Table 2). When the mtDNA cytochrome b gene sequence (385 bp) of isolate AC317 was compared to published sequences of related *Aspergillus* species, isolate AC317 exhibited 99% similarity to that of *A. candidus* (AB025464) (Fig. 2).

The microscopic characteristics of isolate AF57 were identical to those of isolate KACC 40244 and in the literature (Table 3). Stipe lengths of isolate AF57 were 500-800 μm with finely roughened walls while those of isolate KACC 40244 and in the literature were 500-700 and 400-800 μm, respectively. Conidial heads were radiate to columnar. Vesicles were globose, subglobose to ellipsoidal and metulae covered three-quarters of the entire surface of

Table 4. Microscopic characteristics of *Aspergillus fumigatus* isolate AF8 and reference isolate KACC 41390 in comparison with characteristics described by Klich (2002)

Characteristics[a]	Present isolate AF8	Reference isolate KACC 41390	Klich (2002)
Stipe			
Length[b]	300-400 μm	300-400 μm	200-400 μm
Roughness	Smooth	Smooth	Smooth
Vesicle			
Shape	Spatulate to pyriform	Spatulate to pyriform	Spatulate to pyriform
Metulae coverage	Upper 1/2 to 2/3 surface	Upper 1/2 to 2/3 surface	Upper 1/2 to 2/3 surface
Conidia			
Shape	Globose to ovoid	Globose to ovoid	Globose to ellipsoidal
Length	2-3 μm	2-3 μm	2-3 μm
Roughness	Smooth to finely roughened	Smooth to finely roughened	Smooth to finely roughened

[a] Features of stipe, vesicle, and conidia were determined using a microscope (×1,000).
[b] Lengths of stipe and conidia were determined with 40 and 100 observations, respectively.

the vesicle. Conidia formed globose or columnar conidial chain on vesicles. Conidia of isolate AF57 were, on average, 3-5 μm in diameter and globose to ellipsoidal with smooth to finely roughened walls. Diameters of isolate KACC 40244 and in the literature were 3-5 and 3-6 μm, respectively (Table 3). When the mtDNA *β*-tubulin gene sequence of isolate AF57 (545 bp) was compared to published sequences of related *Aspergillus* species, isolate AF57 exhibited 99% similarity to that of *A. flavus* (EF661485) (Fig. 3).

The microscopic characteristics of isolate AF8 were identical to those of isolate KACC 41390 and the literature (Table 4). Stipe of isolate AF8 were 300-400 mm in length and smooth-walled while those of isolate KACC 41390 and the literature were 300-400 and 200-400 μm, respectively. Conidial heads of isolate AF8 were predominantly columnar and vesicles were spatulate to pyriform. Metulae of isolate AF8 were parallel to each other covering the upper half to two-thirds of the vesicle. Conidia of isolate AF8 were, on average, 2-3 μm and globose to ovoid with smooth to finely roughened walls. Conidia of isolate KACC 41390 and in the literature were 2-3 and 2-3 μm, respectively (Table 4). When the mtDNA *β*-tubulin gene sequence of isolate AF8 (558 bp) was compared with published sequences of related *Aspergillus* species, isolate AF57 exhibited 99% similarity to that of *A. fumigatus* (AY048754) (Fig. 3).

Discussion

In our previous study (Oh et al., 2007), we found that *Aspergillus* species was the most predominant genus in storage rice; however, we were unable to evaluate the population of each fungal species. In this study, we evaluated fungal and bacterial populations at the species and genera levels, respectively, using unhulled and white rice samples from five regions in Korea. Consequently, we found that total fungi and total bacteria in stored rice from five regions in Korea were similar to those of our previous study (Oh et al., 2007); however, various fungal species and bacterial genera existed in the rice samples of the regions. For example, *Aspergillus* and *Penicillium* species including *A. candidus*, *A. flavus*, and *A. fumigatus* were predominant in the stored rice; The genera including *Bacillus*, *Microbacterium*, *Sphingomonas*, and *Methylobacterium* were the predominant bacterial genera. Even though the total fungal population in unhulled rice was higher than that in white rice, there was still a small fungal population with different predominant species in white rice. This indicates that fungi contaminating on unhulled rice were removed during milling, but in the drying process white rice was again contaminated with airborne and storage fungi from the rice storage systems (Jayaraman and Kalyanasundaram, 1990).

In a mycoflora study, Pitt et al. (1994) compared the fungal incidence in paddy and milled rice, and concluded that paddy rice contained field fungi, while milled rice contained storage fungi. Park et al. (2005) reported that *A. candidus* was the most prevalent fungal species, while *A. flavus* was discovered in 17% of polished rice samples in Korea. Previously, Mheen et al. (1982) also studied the fungal diversity of Korean rice in different types of storage structures. They concluded that *A. candidus*, *A. versicolor* and *A. glaucus* were the major storage fungi in brown and unhulled rice. Misra et al. (1995) reported that the percentage occurrence of *A. candidus*, *A. flavus*, and *A. fumigatus* in stored rice was 42, 80, and 70%, respectively. However, in our study, we found that the most predominant species were quite different depending on regions. This result indicates that different regional rice storage environments

such as storage and milling systems and storage bags could determine the fungal species and growth. The different rice cultivation in the fields could be another reason for the difference in predominant fungi between regions. In case of bacterial populations, Skyrme et al. (1998) reported that bacterial counts in rough rice and brown rice were higher than those in white rice. Lee et al. (2007) also reported the similar results, as observed in this study, that *Sphingomonas paucimobilis* (31%) and *Arthrobacter atrocyaneus* (22%) were the predominant bacteria in paddy rice.

When the characteristics of predominant *Aspergillus* isolates in stored rice were compared with their references isolates and the literature, *Aspergillus* isolates were highly similar to each reference isolate and descriptions of the literature. *A. candidus* belongs to *Aspergillus* subgenus *Circumdati* section *Candidi*, which can be distinguished by its unique white conidia (Klich, 2002). This fungus has been known to be present in soil, flour, fruit, food, and environment, but is found predominantly in stored grains and seeds (Klich, 2002). *A. candidus* grows slowly in neutral or slightly alkaline habitats at 50-55°C. Because this species is very competitive to low water potential, it can reside in dry substrates and may cause the self-heating of stored grains. *A. candidus* produces produce a number of toxins such as candidusins (Rahbæk et al., 2000), kojic acid (Hesseltine, 1974), and terphenyllins (Varga et al., 2007). *A. flavus*, which produces produce aflatoxin, belongs to subgenus *Circumdati* section *Flavi* and can easily be distinguished by its yellow-green colonies. It is closely related to *A. parasiticus* in the same section *Flavi* and difficult to differentiate since *A. parasiticus* has similar morphological characteristics and also produces aflatoxins. However, *A. flavus* can be distinguished by its smooth spores unlike *A. parasiticus* (Klich, 2002). *A. flavus* is ubiquitous, but is mostly reported as a food-borne fungus and as an insect and animal pathogen. This fungus appears in decaying vegetation, seeds, soils and environment and produces aflatoxin B, cyclopiazonic acid, and 3-nitropropionic acid. Previously, Begum and Samajpati (2000) found that *A. flavus* produced 555-10,416 μg of aflatoxin B_1 per kg rice. Liu et al. (2006) also reported that aflatoxin G_1 was a major type of aflatoxin in 92% of tested rice samples. This toxin is heat-tolerant and is the most carcinogenic toxin to animal and human. On the other hand, *A. fumigatus* belongs to subgenus *Fumigati* section *Fumigati* and is distinguished by rapidly growing, turquoise or dark green colonies (Klich, 2002). This fungus is widespread and inhabits a relatively wide range of temperatures in soil, plants, seeds, wood chips, cotton and environment. For instance, the fungus can grow optimally at 37°C and at 55°C for a long period of time, and it is abundant in high-temperature system such as grain storage and compost (Gugnani, 2003). *A. fumigatus* also produces mycotoxins, such as gliotoxin, verrucologen, and fumitremorgin A and B. Richard et al. (1989) detected 5.33 μg per g of gliotoxin in rice.

In this study, we detected aflatoxins from rice samples and also found some difference of aflatoxin production by *Aspergillus* species among rice samples according to sampling region and rice type. The aflatoxin production in the stored rice varied at the levels of 2.45-3.43 ng per g rice for unhulled rice and 1.29-2.09 ng per g rice for white rice. These aflatoxin levels were generally lower than the tolerance level (15 ng/g) of aflatoxins including aflatoxin B_1, B_2, G_1, and G_2 for grains suggested by the Korea Food and Drug Administration (2010). This implies that aflatoxin contamination by *Aspergillus* species in the stored rice in Korea might be not serious for consumers; however, the mycotoxin and fungi in the rice should be continuously monitored due to their potential hazard. Taken together, various bacteria and fungi including *Aspergillus* and *Penicillium* spp. existed in stored rice; *A. candidus*, *A. flavus*, and *A. fumigatus* were the predominant *Aspergillus* species. These fungi can produce mycotoxins that are harmful for consumers, and thus it is important to detect and reduce the population of storage fungi in rice.

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References

Begum, F. and Samajpati, N. 2000. Mycotoxin production on rice, pulses and oilseeds. *Naturwissenschaften* 87:275-277.

Boom, R., Sol, C. J. A., Salimans, M. M. M., Jansen, C. L., Wertheim-Van Dillen, P. M. E. and Van Der Noordaa, J. 1990. Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* 28:495-503.

Filtenborg, O., Frisvad, J. C. and Thrane, U. 1996. Moulds in food spoilage. *Int. J. Food Microbiol.* 33:85-102.

Glass, N. L. and Donaldson, G. C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl. Environ. Microbiol.* 61:1323-1330.

Gugnani, H. C. 2003. Ecology and taxonomy of pathogenic Aspergilli. *Front. Biosci.* 8:s346-357.

Hesseltine, C. W. 1974. Natural occurrence of mycotoxins in cereals. *Mycopathologia* 53:141-153.

Jayaraman, P. and Kalyanasundaram, I. 1990. Natural occurrence of toxigenic fungi and mycotoxins in rice bran. *Mycopatholo-*

gia 110:81-85.

Klich, M. A. 2002. Identification of Common *Aspergillus* Species. Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, 116 p.

Korea Food and Drug Administration. 2010. Food Code. Notice No. 2010-2. p.2-1-10. Seoul, Korea.

Lee, S.-Y., Kim, J.-H., Kim, K.-B.-W.-R., Song, E.-J., Kim, A.-R., Park, S.-M., Han, C.-S. and Ahn, D.-H. 2007. Antimicrobial activities of medicinal herbs and seaweeds extracts against microorganisms isolated from the rice warehouses. *J. Korean Soc. Food Sci. Nutr.* 36:476-480.

Liu, Z., Gao, J. and Yu, J. 2006. Aflatoxins in stored maize and rice grains in Liaoning Province, China. *J. Stored Products Res.* 42:468-479.

Mheen, T. I., Cheigh, H. S., Ragunathan, A. N. and Majumder, K. S. 1982. Studies on the fungi in stored rice. *Kor. J. Appl. Microbiol. Bioeng.* 10:191-196.

Miller, J. D. 1995. Fungi and mycotoxins in grain: Implications for stored product research. *J. Stored Products Res.* 31:1-16.

Misra, J. K., Gergon, E. B. and Mew, T. W. 1995. Storage fungi and seed health of rice: A study in the Philippines. *Mycopathologia* 131:13-24.

Muir, W. E. and White, N. D. G. 1998. Microorganisms in stored grain. In: *Grain Preservation Biosystems*, ed. by W. E. Muir, pp. 1-17. Department of Biosystems Engineering, University of Manitoba, Winnipeg, MB, Canada.

Oh, J. Y., Jee, S. N., Nam, Y., Lee, H., Ryoo, M. I. and Kim, K. D. 2007. Populations of fungi and bacteria associated with samples of stored rice in Korea. *Mycobiology* 35:36-38.

Oh, J. Y., Kim, E. N., Ryoo, M. I. and Kim, K. D. 2008a. Morphological and molecular identification of *Penicillium islandicum* isolate KU101 from stored rice. *Plant Pathol. J.* 24:469-473.

Oh, J. Y., Sang, M. K., Lee, S. Y., Ryoo, M. I. and Kim, K. D. 2008b. Temporal changes of fungal and bacterial populations in rice under indoor storage conditions. *Plant Pathol. J.* 24:74-79.

Park, J. W., Choi, S.-Y., Hwang, H.-J. and Kim, Y.-B. 2005. Fungal mycoflora and mycotoxins in Korean polished rice destined for humans. *Int. J. Food Microbiol.* 103:305-314.

Pitt, J. I., Hoching, A. D., Bhudhasamai, K., Miscamble, B. F., Wheeler, K. A. and Tanboon-Ek, P. 1994. The normal mycoflora of commodities from Thailand. 2 Beans, rice, small grains and other commodities. *Int. J. Food Microbiol.* 23:35-53.

Pitt, J. I. 1994. The current role of *Aspergillus* and *Penicillium* in human and animal health. *J. Med. Vet. Mycol.* 32 (Suppl. 1):17-32.

Pitt, J. I. 2000. A Laboratory Guide to Common *Penicillium* Species. Food Science Australia, North Ryde, Australia. 197 p.

Rahbæk, L., Frisvad, J. C. and Christophersen, C. 2000. An amendment of *Aspergillus* section *Candidi* based on chemotaxonomical evidence. *Phytochemistry* 53:581-586.

Richard, J. L., Lyon, R. L., Fichtner, R. E. and Ross, P. F. 1989. Use of thin layer chromatography for detection and high performance liquid chromatography for quantitating gliotoxin from rice cultures of *Aspergillus fumigatus* Fresenius. *Mycopathologia* 107:145-151.

Samson, R. A. 1979. A compilation of the Aspergilli described since 1965. *Stud. Mycol.* 18:1-38.

Skyrme, D. S., Marks, B. P., Johnson, M. G. and Siebenmorgen, T. J. 1998. Distribution of total aerobic and coliform bacterial counts among rice kernel components. *J. Food Sci.* 63:154-156.

Smith, J. E. and Ross, K. 1991. The toxigenic aspergilli. In: *Mycotoxins and Animal Foods*, ed. by J. E. Smith and R. S. Henderson, pp. 101-118. CRC Press, Boca Raton, FL, USA.

Varga, J., Frisvad, J. C. and Samson, R. A. 2007. Polyphasic taxonomy of *Aspergillus* section *Candidi* based on molecular, morphological and physiological data. *Stud. Mycol.* 59:75-88.

Wang, L., Yokoyama, K., Miyaji, M. and Nishimura, K. 2000. Mitochondrial cytochrome b gene analysis of *Aspergillus fumigatus* and related species. *J. Clin. Microbiol.* 38:1352-1358.

Wicklow, D. T. 1992. The Mycology of stored grain: An ecological perspective. In: *Stored-Grain Ecosystems*, ed. by D. S. Jayas, N. D. G. White and W. E. Muir, pp. 197-250. CRC Press, Boca Raton, FL, USA.

Williams, R. J. and McDonald, D. 1983. Grain molds in the tropics: Problems and importance. *Ann. Rev. Phytopathol.* 21:153-178.