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Research Article

Aspergillus Associated with *Meju*, a Fermented Soybean Starting Material for Traditional Soy Sauce and Soybean Paste in Korea

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Abstract *Aspergillus* is an important fungal genus used for the fermentation of Asian foods; this genus is referred to as koji mold in Japan and China. *A. oryzae*, *A. sojae*, and *A. tamari* are used in the production of miso and shoyu in Japan, but a comprehensive taxonomic study of *Aspergillus* isolated from *Meju*, a fermented soybean starting material for traditional soy sauce and soybean paste in Korea, has not been conducted. In this study, various *Aspergillus* species were isolated during a study of the mycobiota of *Meju*, and the aspergilli were identified based on phenotypic characteristics and sequencing of the β-tubulin gene. Most strains of *Aspergillus* were found to belong to the following sections: *Aspergillus* (n = 220), *Flavi* (n = 213), and *Nigri* (n = 54). The most commonly identified species were *A. oryzae* (n = 183), *A. pseudoglaucus* (*Eurotium repens*) (n = 81), *A. chevalieri* (*E. chevalieri*) (n = 62), *A. montevidensis* (*E. amstelodami*) (n = 34), *A. niger* (n = 21), *A. tamari* (n = 15), *A. ruber* (*E. rubrum*) (n = 15), *A. proliferans* (n = 14), and *A. luchuensis* (n = 14); 25 species were identified from 533 *Aspergillus* strains. *Aspergillus* strains were mainly found during the high temperature fermentation period in the later steps of *Meju* fermentation.

Keywords *Aspergillus*, *Aspergillus oryzae*, *Aspergillus pseudoglaucus*, Fermentation, *Meju*

Traditional Korean *Meju* is a fermented soybean starting material for traditional doenjang (soybean paste) and ganjang (soy sauce), which are essential sauces in authentic Korean cuisine. *Meju* is naturally fermented in a process involving various microorganisms, such as bacteria, yeasts, and fungi. Fungi play an important role in *Meju* fermentation by degrading soybean macromolecules into small nutrient molecules [1, 2].

Aspergillus is an important fungal genus for the fermentation of Asian foods, and this genus are referred as "koji" molds in Japan and China [3]. Yellow koji molds, such as *A. oryzae*, *A. sojae*, and *A. tamari* are used in the production of shoyu and miso in Japan, and *A. oryzae* is also used in the production of Japanese sake [4]. Black koji molds, such as *A. luchuensis*, *A. niger*, and *A. tubingensis*, are used for shochu production in Japan and makgeolli production in Korea [5]. Species in section *Aspergillus*, such as *A. ruber* (*Eurotium rubrum*) and *A. pseudoglaucus* (*E. repens*), are used as starter cultures for Katsuobushi production [6]. Because of their importance in the fermentation industries, the taxonomy of *Aspergillus* strains isolated from Asian foods has been widely examined by mycologists [7-9].

However, a comprehensive study of *Aspergillus* isolated from *Meju* has not yet been published. *A. clavatus*, *A. flavus*, *A. flavus* var. *columnaris*, *A. fumigatus*, *A. melleus*, *A. niger*, *A. nidulans*, *A. oryzae*, *A. oryzae* var. *fulvus*, *A. parasiticus*, *A. phoenicus*, *A. sulpureus*, *A. terreus*, and *A. versicolor* were identified by Lee *et al.* [10], Lee [1], and Sakurai *et al.* [11], but the species were identified based only on morphological characteristics, and the isolates were not preserved in culture collections. Species of *Aspergillus* belonging to section *Aspergillus*, the teleomorph *Eurotium*, are very common in *Meju*, but were not reported in the studies mentioned above [1, 10, 11]. Recently, *Aspergillus* species on *Meju* in section *Aspergillus* [12] and section *Nigri* have been reported [13].

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In this study, we report all *Aspergillus* species isolated from *Meju* in Korea. We isolated 533 strains of *Aspergillus* from *Meju* and they were identified by morphological and molecular analyses based on partial sequences of the β-tubulin gene. The previously reported species in sections *Aspergillus* and *Nigri* were reviewed and improved, and *Aspergillus* species in the other sections have been newly added.

MATERIALS AND METHODS

We collected 98 *Meju* loaves from various regions in Korea

Table 1. Number of strains and the representative strains of *Aspergillus* species isolated from *Meju* in this study

Aspergillus section (No. of strains)	*Aspergillus* species	No. of strains	Representative strains	
			KACC No.	Isolation region
Candidi (4)	*A. candidus*	3	46481	Chilgok
			46482	Icheon
	A. tritici	1	46483	Yeoju
Circumdati (7)	*A. ochraceus*	6	46484	Haenam
			40486	Yongin
	A. westerdijkiae	1	46487	Sunchang
Aspergillus (220)	*A. brunneus* (*Eurotium echinulatum*)	5	46347	Damyang
			46349	Chilgok
	A. chevalieri (*Eurotium chevalieri*)	62	45344	Pocheon
			46344	Anseong
	A. cibarius	4	46346	Icheon
			46764	Yongin
	A. glaucus (*Eurotium herbariorum*)	2	45348	Yecheon
			M1061	Yecheon
	A. montevidensis (*Eurotium amstelodami*)	34	46370	Anseong
			46336	Yongin
	A. proliferans	14	45349	Pocheon
			46351	Pyeongchang
	A. pseudoglaucus (*Eurotium repens*)	81	45358	Anseong
			46359	Yongin
	A. ruber (*Eurotium rubrum*)	15	45362	Yeoju
			46365	Yongin
	A. tonophilus (*Eurotium tonophilum*)	3	45365	Yecheon
			46367	Yangpyeong
Flavi (213)	*A. flavus*	13	46449	Pocheon
			46454	Yongin
	A. oryzae	183	46471	Icheon
			46459	Yangju
	A. parasiticus	2	46475	Gangjin
			46037	Andong
	A. tamari	15	46478	Icheon
			46480	Icheon
Fumigati (13)	*A. fumigatus*	13	46488	Sunchang
			46489	Icheon
Nidulantes (9)	*Emericella nidulans*	9	46505	Goisan
			46506	Anseong
Nigri (54)	*A. luchuensis*	14	46491	Sunchang
			46490	Yongin
	A. niger	21	46495	Gimpo
			46497	Yangpyeong
	A. tubingensis	10	46498	Hoengseong
			46499	Sunchang
	A. welwitschiae	9	46492	Haenam
			46496	Goesan
Versicolors (13)	*A. sydowii*	7	46500	Cheongwon
			46501	Pocheon
	A. versicolor	6	46504	Gimcheon
			46503	Pocheon

from 2008 to 2011. We isolated *Aspergillus* from *Meju* using two isolation methods, including direct plating on malt extract agar (MEA) [50 g MEA (CM0059; Oxoid, Hampshire, UK), 1 L distilled water] and dichloran 18% glycerol agar (DG18) [31.5 g dichloran-glycerol agar base (CM0729; Oxoid), 220 g glycerol, 0.1 g chloramphenicol, 1 L distilled water] and dilution plating on DG18 and dichloran rose-bengal chloramphenicol agar (DRBC) [32 g rose-bengal chloramphenicol agar base (CM0549; Oxoid), 0.002 g dichloran, 0.1 g chloramphenicol, 1 L distilled water] [12, 14]. To determine which fungi grow during the *Meju* fermentation processes, we visited four *Meju* farms in Gyeonggi province each week, from molding process (late November, 2010) to submergence of the *Meju* in brine (mid-February, 2011). Additionally, we visited numerous *Meju* farms in the Chungnam, Chungbuk, Jeonnam, Jeonbuk, and Gyeongbuk provinces in mid-February 2011 to survey the *Meju* mycobiota present in the southern region of the Korean Peninsula. We isolated the fungi from in-process *Meju* by direct plating of fungi on MEA and DG18 [14].

A total of 533 strains of *Aspergillus* were isolated. For molecular identification, partial sequences of the β-tubulin gene (primers bt2a and bt2b) [15] were determined. The β-tubulin sequences of the strains isolated from *Meju* were compared with those published by Hong *et al.* [16], Hubka *et al.* [17], and Kim *et al.* [18] for section *Aspergillus*; with those published by Pildain *et al.* [19] for section *Flavi*; and with those published by Hong *et al.* [7] for section *Nigri*. The β-tubulin sequences of *Aspergillus* species in other sections were compared with those published by Peterson [20] and the type strains in the National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). Forty-six representative strains were selected based on their source and molecular and morphological characteristics (Table 1). To determine the taxonomic positions of the selected strains, DNA data were analyzed using the Tamura-Nei parameter distance calculation model with gamma-distributed substitution rates, which were then used to construct a neighbor-joining tree using MEGA ver. 5.1 [21]. To determine the support for each clade, bootstrap analysis was performed with 1,000 replications (Figs. 1~3). Identification of *Aspergillus* species based β-tubulin sequences was confirmed by examining their morphology with reference to Raper and Fennell [22] and Klich [3].

RESULTS AND DISCUSSION

Aspergillus strains were mainly found during the latter part of *Meju* fermentation. In particular, species of *Aspergillus* section *Aspergillus* (teleomorph *Eurotium*) were frequently isolated from finished *Meju* product. The 533 *Aspergillus* strains from *Meju* were identified as belonging to 25 species (Table 1). Most strains of *Aspergillus* were found to belong to sections *Aspergillus* (n = 220), *Flavi* (n = 213), and *Nigri* (n = 54) and the most commonly isolated species were *A. oryzae* (n = 183), *A. pseudoglaucus* (*Eurotium repens*) (n = 81), *A. chevalieri* (*E. chevalieri*) (n = 62), *A. montevidensis* (*E. amstelodami*) (n = 34), *A. niger* (n = 21), *A. tamari* (n = 15), *A. ruber* (*E. rubrum*) (n = 15), *A. proliferans* (n = 14), and *A. luchuensis* (n = 14).

***Aspergillus* section *Aspergillus* (teleomorph *Eurotium*).** Species of *Aspergillus* section *Aspergillus*, teleomorph *Eurotium*, comprise one of the fungal groups most frequently isolated from *Meju*. Members of this section germinate and grow at low moisture levels, conditions under which most other microorganisms do not grow. The moisture level of *Meju* is very low; in some cases, only strains belonging to section *Aspergillus* can grow on *Meju*. Of the 553 *Aspergillus* strains isolated from *Meju*, 220 were included in section *Aspergillus* (Table 1, Fig. 1). The predominant species was *A. pseudoglaucus* (*Eurotium repens*) (n = 81), followed by *A. chevalieri* (*E. chevalieri*) (n = 62), *A. montevidensis* (*E. amstelodami*) (n = 34), *A. ruber* (*E. rubrum*) (n = 15), *A. proliferans* (n = 14), *A. niveoglaucus* (*E. niveoglaucum*) (n = 5), *A. cibarius* (n = 4), *A. tonophilus* (*E. tonophilum*) (n = 3), and *A. glaucus* (*E. herbariorum*) (n = 2). *A. cibarius* was recently reported as a new species from *Meju* [16]. The name of the teleomorph genus, *Eurotium,* is typically used, but in this study we used the name *Aspergillus* based on Hubka *et al.* [17] and Samson *et al.* [23] to implement a single-name system in the nomenclature.

Strains belonging to section *Aspergillus* were primarily isolated from the surface of *Meju* loaves during the later stages of fermentation; however, in some instances, the strains occupied large areas inside the *Meju* loaves. The strains were also isolated from doenjang during the fermentation process. These species are xerophilic, show high proteolytic activity in meat, and are generally regarded as benign fungi that are free of mycotoxins [6]. Some species in the section *Aspergillus* have been used in food manufacturing, including as a starter culture for Katsuobushi and fish sauce [6]. Species belonging to section *Aspergillus* are frequently isolated from *Meju*, and their functions in *Meju* fermentation require further investigation.

***Aspergillus* section *Flavi*.** The species *A. oryzae* (n = 183), *A. tamari* (n = 15), *A. flavus* (n = 14), and *A. parasiticus* (n = 2), which belong to section *Flavi*, were isolated from *Meju* (Table 1, Fig. 2). Aflatoxin producibility was used to differentiate between *A. oryzae* and *A. flavus*, as the two species cannot be differentiated based on their β-tubulin and calmodulin gene sequences and phenotypic characteristics. The non-aflatoxigenic *Meju* strain was named as *A. oryzae*, and the *Meju* strain that could produce aflatoxin was named as *A. flavus* [24]. *A. parasiticus* was also distinguished from *A. sojae* based on the production of aflatoxins B and G, although they could not be differentiated based on the β-tubulin tree (Fig. 2). *A. tamari* was easily identified by its formation of unique brown colonies on MEA. Nearly all strains in section *Flavi* isolated from *Meju* were identified

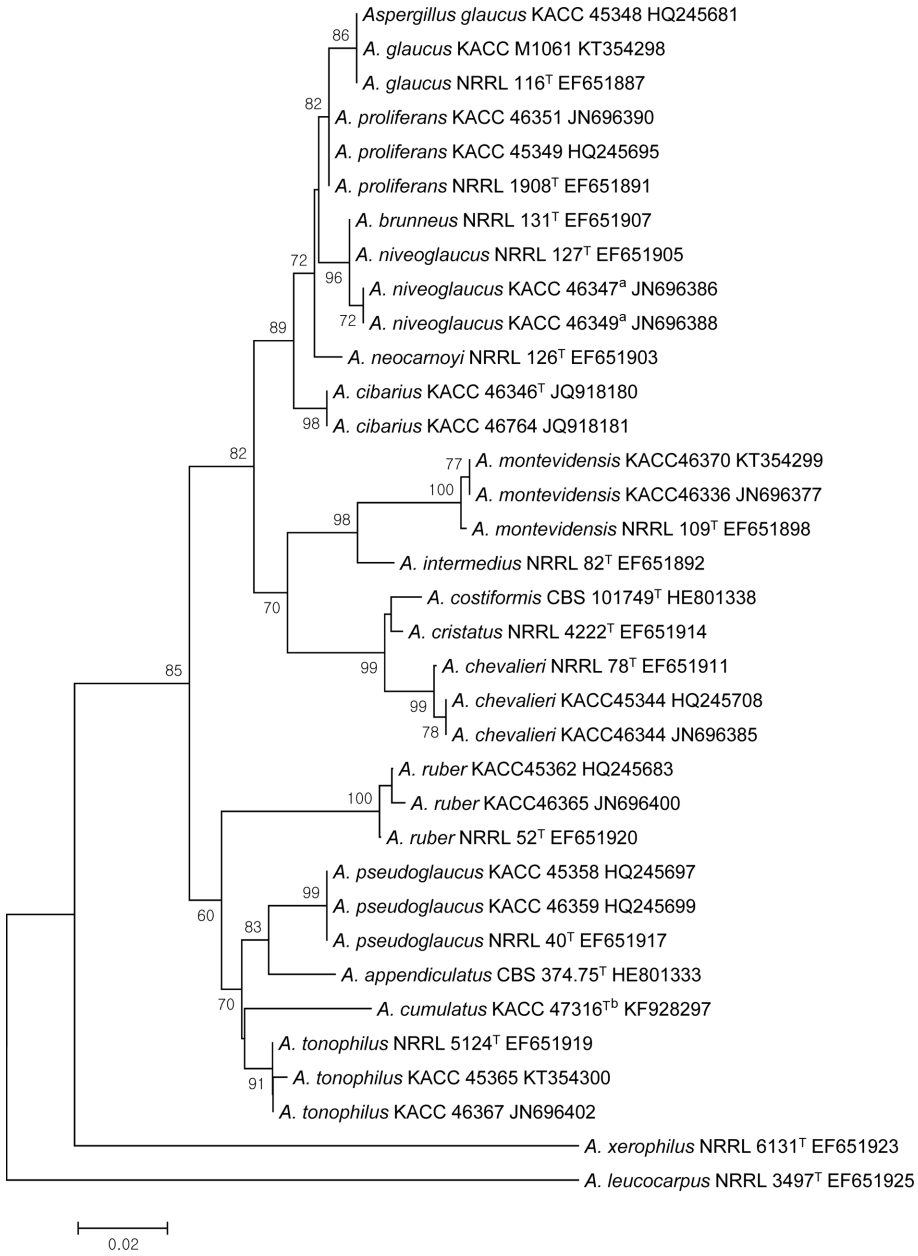

Fig. 1. Neighbor-joining tree showing taxonomic positions of *Aspergillus* section *Aspergillus* strains isolated from *Meju*. Strains that have KACC No. were isolated from *Meju*. [a]KACC 46347 and 46349 could not be differentiated between *A. brunneus* and *A. niveoglaucus*, but they clustered into *A. niveoglaucus* both in calmodulin and RPB2 gene trees. [b]Only, *A. cumulatus* KACC 47316[T] did not originate from *Meju*.

as the non-aflatoxigenic species *A. oryzae* and *A. tamari*. However, the aflatoxin-producible species *A. flavus* and *A. parasiticus* were also isolated from *Meju* or the *Meju* environment, but their rate of occurrence was very low. *A. parasiticus* was not isolated directly from *Meju*, but we isolated this strain using the dilution plate method from *Meju* powder supplied by another researcher. We could not determine whether this strain originated from *Meju* or from the surrounding environment, such as the air. *A. tamari* is used for brewing *Tamari*, a form of soy sauce produced in Japan. Fifteen strains of *A. tamari* were isolated from *Meju*, and determining the industrial uses of these strains requires further study.

Although *A. oryzae* was frequently isolated from *Meju*, *A. oryzae* is not considered to be the predominant species in traditional Korean *Meju*. *A. oryzae* did not grow on large portions of the *Meju* loaves when we observed *Meju* at the farms [24]. This species may have been isolated at a high frequency because of culture bias; *A. oryzae* grows rapidly on MEA, DG18, and DRBC during dilution plating. Another possible reason is that many *Meju* factories have used *A. oryzae* as a starter culture for improved *Meju*

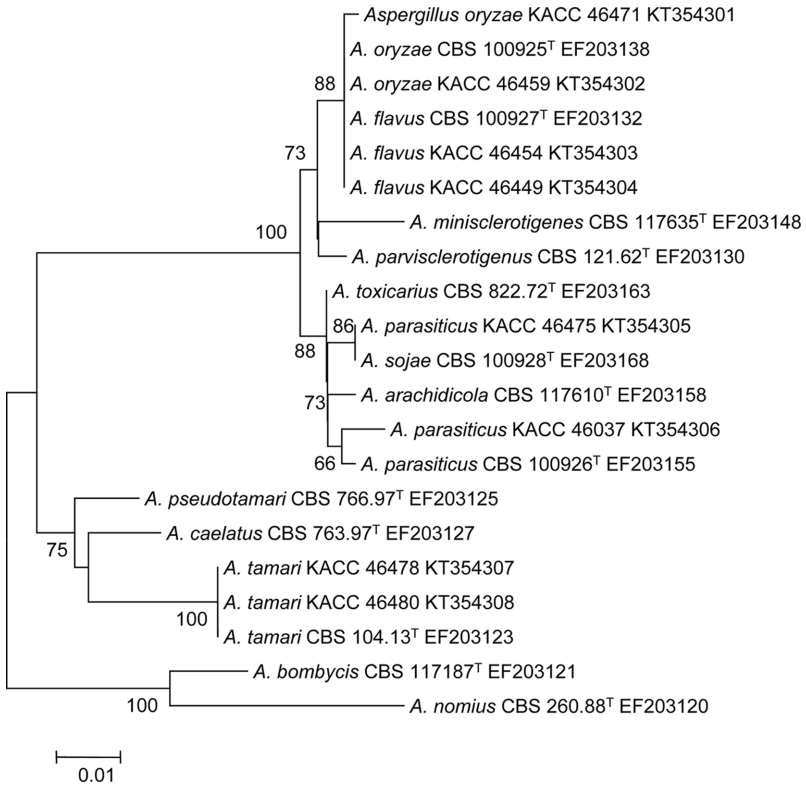

Fig. 2. Neighbor-joining tree showing taxonomic positions of *Aspergillus* section *Flavi* strains isolated from *Meju*. Strains with KACC No. were isolated from *Meju*.

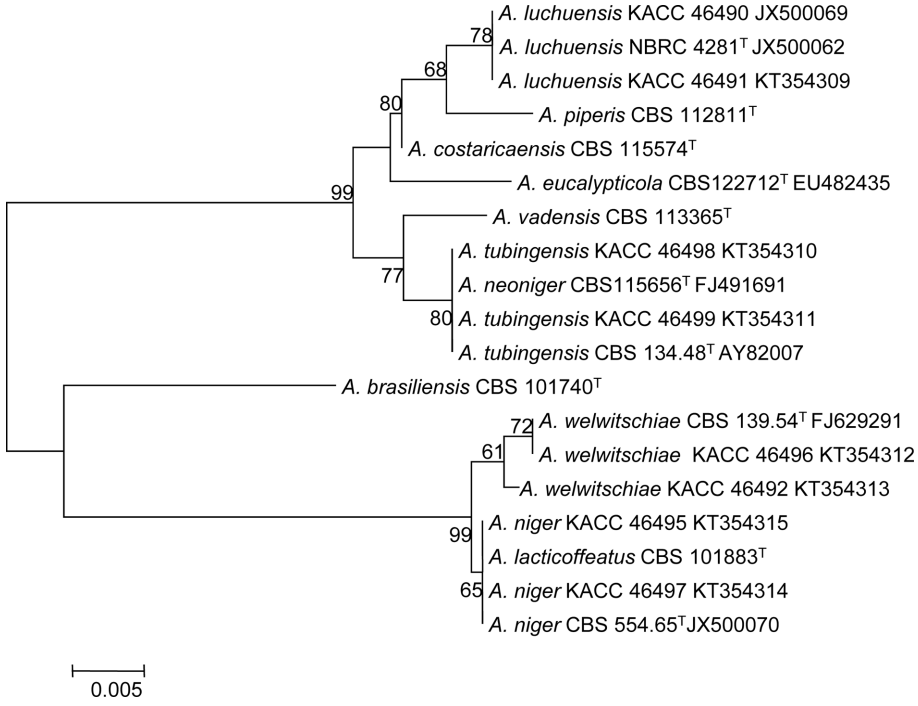

Fig. 3. Neighbor-joining tree showing taxonomic positions of *Aspergillus* section *Nigri* strains isolated from *Meju*. Strains with KACC No. were isolated from *Meju*.

production in Korea. Aflatoxin production is not thought to be a significant problem in *Meju* fermentation [24].

***Aspergillus* section *Nigri*.** *Aspergillus* species in the *A. niger* clade in section *Nigri* can be differentiated based on

their molecular characteristics, such as β-tubulin and calmodulin gene sequences, although they cannot be differentiated based on their morphological characteristics. *A. luchuensis*, *A. niger*, and *A. tubingensis* were clearly identifiable from their β-tubulin gene sequences, but for several strains, differentiation between *A. niger* and *A. welwitschiae* was equivocal [5, 7]. Of the 54 strains belonging to *Aspergillus* section *Nigri*, 21 were identified as *A. niger*, 14 as *A. luchuensis*, 10 as *A. tubingensis*, and 9 as *A. welwitschiae* (Table 1, Fig. 3) [13]. Almost black *Aspergillus* strains were found only on a small portion of the *Meju* loaf surface, but some black *Aspergillus* strains occupied large areas inside the *Meju* loaves. When growing on the surface of *Meju* loaves, black *Aspergillus* strains typically appear black because of their abundant conidiation, but they can appear white when growing inside the *Meju* loaves because they do not produce conidia when growing in airless environments [13]. Producers of *Meju* usually dislike black *Aspergillus* growing on *Meju,* because of the conidiation and color. However, *A. luchuensis* is one of the primary fungi employed in the production of Asian foods such as alcoholic beverages (shochu and makgeolli) and Puerh tea, and it does not produce any harmful mycotoxins [5]. This species also grows actively in *Meju*. Further studies are required to determine the utility of *A. luchuensis* on *Meju*.

Other *Aspergillus* sections. Forty-six of the 533 *Aspergillus* strains isolated from *Meju* were determined to belong to the other sections of *Aspergillus*. Section *Candidi* was easily identified by its white conidiation [22]. Four strains belonging to section *Candidi* were isolated from *Meju*; three were identified as *A. candidus* and one as *A. tritici* (Table 1). They were differentiated based on their growth at 37°C and their β-tubulin gene sequences. *A. candidus* is known to be a good lipase producer and a starter culture for processed meat manufacture [6, 14].

Section *Circumdati* was easily differentiated by its yellow-buff conidiation [22]. Six strains of *A. ochraceus* and one of *A. westerdijkiae* were isolated from *Meju* and its environment (Table 1). The two species were differentiated by growth at 37°C and their β-tubulin gene sequences. *A. ochraceus* did not grow at 37°C, but *A. westerdijkiae* did grow at this temperature. Few *A. ochraceus* strains are toxigenic, but *A. westerdijkiae* is known to produce ochratoxin A [6]. One strain of *A. westerdijkiae* was isolated from *Meju* powder using the dilution plating method. We were unable to determine whether this strain was isolated from the *Meju* or from the environment.

Seven strains of *A. sydowii* and six of *A. versicolor*, both in section *Versicolors*, were isolated (Table 1). *A. sydowii* could be differentiated from *A. versicolor* by its blue-green conidiation [14]. Thirteen strains of *A. fumigatus* and nine strains of *A. nidulans* (*Emericella nidulans)* were also identified from *Meju* (Table 1).

Aspergillus species belonging to sections *Aspergillus*, *Flavi*, *Nigri*, and *Candidi* have been used as starter cultures for the production of food sauces and alcoholic beverages. They are good producers of enzymes such as cellulase, amylase, and proteases. Large numbers of these strains were isolated from *Meju* and were observed to grow on large areas of the exterior and interior of the *Meju* loaves. Therefore, to develop these strains for use in starter cultures, their roles in *Meju* production must be determined. However, a few *Aspergillus* stains belonging to the other sections were isolated from *Meju*, and their habitat is typically the air. Therefore, these strains may be simple contaminants of *Meju*.

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