Mycobiology

Research Article

Analysis of the *MAT1-1* and *MAT1-2* Gene Ratio in Black Koji Molds Isolated from Meju

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Abstract *Aspergillus luchuensis* is known as an industrially important fungal species used for making fermented foods such as awamori and shochu in Japan, makgeolli and Meju in Korea, and Pu-erh tea in China. Nonetheless, this species has not yet been widely studied regarding mating-type genes. In this study, we examined the *MAT1-1* and *MAT1-2* gene ratio in black koji molds (*A. luchuensis*, *Aspergillus niger*, and *Aspergillus tubingensis*) and in *Aspergillus welwitschiae* isolated from Meju, a fermented soybean starting material for traditional soy sauce and soybean paste in Korea. The number of strains with the *MAT1-1* locus was 2 of 23 (*A. luchuensis*), 6 of 13 (*A. tubingensis*), 21 of 28 (*A. niger*), and 5 of 10 (*A. welwitschiae*). Fungal species *A. tubingensis* and *A. welwitschiae* showed a 1 : 1 ratio of *MAT1-1* and *MAT1-2* mating-type loci. In contrast, *A. luchuensis* revealed predominance of *MAT1-2* (91.3%) and *A. niger* of *MAT1-1* (75%). We isolated and identified 2 *A. luchuensis* MAT1-1 strains from Meju, although all strains for making shochu in Japan are of the MAT1-2 type. These strains may be a good resource for breeding of *A. luchuensis* to be used in the Asian fermented-food industry.

Keywords *Aspergillus luchuensis*, Mating type, Meju

Black aspergilli or *Aspergillus* section *Nigri* are an important and diverse group of fungi with several key characteristics including food spoilage, suitability as starters for fermentation, and production of metabolites for use in medicine. Black koji molds are major fungi belonging to this group and are associated with production of distilled alcoholic beverages awamori and shochu in Japan and makgeolli in Korea [1]. Three species *Aspergillus luchuensis*, *Aspergillus niger*, and *Aspergillus tubingensis* mainly constitute black koji molds among which *A. luchuensis* is an industrially important black aspergillus, especially in the food and fermentation industries of East Asia [2]. Fungus *A. luchuensis* has been well established as the chief fermentation agent in awamori-koji [3] but is also reported from nuruk and Meju, a Korean traditional fermentation starter for soybean paste and soy sauce [4, 5]. After *A. luchuensis*, *A. niger* is another important species of black koji mold that has been widely used in biotechnological processes and is the only one that has been granted the generally regarded as safe (GRAS) status by the U.S. Food and Drug Administration (FDA). With their potential applications, the members of black koji molds are a remarkable fungal resource for industrial research.

Almost all *Aspergillus* species from section *Nigri* exclusively propagate through asexual mode of reproduction. Nevertheless, there are reports on sexual reproduction of members of section *Nigri* that have the so-called mating-type (*MAT*) genes, which have been identified in other filamentous ascomycetes and found to play a major role in sexual reproduction [6, 7]. These *MAT* genes have been reported to encode several transcription factors that determine sexual identity and regulate later stages of sexual development. Two distinct classes of *MAT* genes, *MAT1-1* and *MAT1-2*, are recognized. The *MAT1-1* family is known to encode an α-domain protein, and the *MAT1-2* gene family codes for a high-mobility group (HMG) box protein. These genes are located either both in a single *MAT* locus or at 2 loci, called *MAT1-1* and *MAT1-2*, within the genome [8-11]. Homothallic aspergilli are known to possess both *MAT* genes in the same genome. In case of heterothallic species, only a single *MAT* locus is found to be present, which can

Mycobiology 2016 December, **44**(4): 269-276
https://doi.org/10.5941/MYCO.2016.44.4.269
pISSN 1229-8093 • eISSN 2092-9323

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Received October 31, 2016
Revised November 14, 2016
Accepted November 16, 2016

contain either *MAT1-1* or *MAT1-2* family genes.

Sequence analysis has indicated that these *MAT1-1* and *MAT1-2* loci are vastly divergent, and gene mutation studies in the fungus *Aspergillus fumigatus* have shown *MAT* genes to be essential for sexual development [12]. Cloning of *MAT* genes by conventional methods has largely been unsuccessful due to high divergence of the gene sequences among ascomycetes despite evolutionary conservation of the a box and HMG domains [13]. However, 2 methods, namely, the use of degenerate primers and whole-genome sequence mining, are now available for detection of the mating-type genes in *Aspergillus* species [9, 14-18].

MAT genes are therefore necessary for sexual reproduction among *Aspergillus* species. It has been reported that deletion of *MAT1* and *MAT2* genes leads to a loss of sexual reproduction in *Aspergillus nidulans*, whereas overexpression suppresses vegetative growth and induces sexual differentiation even under unfavorable conditions [19]. Thus, these genes may have an important function in the breeding of *Aspergillus* fungi. Apart from contributing to the evolution of new species, mating in aspergilli may aid survival during abiotic stress conditions and can improve industrially important strains via addition of traits of interest [20].

Reports of studies on *A. nidulans* showed the significance of mating-type genes in sexual development [9]. *MAT* genes have been reported in *A. niger*, *Aspergillus welwitschiae*, and *A. tubingensis* [21]. To date, however, there are no reports on the ratio of *MAT* genes in *A. luchuensis*, an industrially important species for fermentation in Asia. We planned to study the *MAT1-1* and *MAT1-2* gene ratio of *A. luchuensis* and extended our interest to black koji molds isolated from Meju using PCR analysis. The purpose is to expand existing knowledge about *MAT* genes in *Aspergillus* section *Nigri* and to use these data for future industrial breeding of *A. luchuensis*.

MATERIALS AND METHODS

Strains and media. Ex-types of *A. luchuensis*, *A. tubingensis*, *A. niger*, and *A. welwitschiae* were obtained from Korean Agricultural Culture Collection (KACC, Korea), and in addition, *Aspergillus* strains that were isolated from traditional Meju in South Korea were included in this study. Detailed information on the strains is given in Table 1. The strains were inoculated onto malt extract agar to revive the culture and for further experiments.

Genomic DNA extraction. Strains of section *Nigri* species used in this study (Table 1) were grown in 10 mL of malt extract broth at 200 rpm (25~28°C). The mycelium was harvested after 2~3 days of growth by filtration through Miracloth (Merck Millipore, Billerica, MA, USA) and was then placed loosely in microfuge tubes, frozen, and freeze dried. The freeze-dried biomass was ground to a fine powder,

Table 1. The list of black koji mold strains used in this study with their source and mating type

Species	KACC No.	Strain information	Source	Mating type
Aspergillus luchuensis	46772	NBRC 4281 = RIB 2642 = CBS 205.80 (extype of *A. luchuensis*)	Koji, Okinawa, Japan	MAT1-2
	46771	NBRC 4308 = CBS 117.80 = NRRL 4886 (extype of *A. kawachii*)	Koji, Kyusyu, Japan	MAT1-2
	46516	CF 1005 (industrial strain of *A. kawachiii* in Korea)	Koji, Korea	MAT1-2
	45131	NBRC 4121 = NRRL 4796 = CBS 564.65 (extype of *A. acidus*)	Koji, Okinawa, Japan	MAT1-2
	41731	CBS 119384 = NR 15-1 (extype of *A. coreanus*)	Nuruk, Korea	MAT1-2
	47234	JCM 2261 = IAM 2112 (extype of *A. awamori*)	Koji, Okinawa, Japan	MAT1-2
	47004	NBRC 4123 = NRRL 4797 = IAM 2397 = ATCC 16884 = CBS 565.65 = IMI 175963	Koji, Okinawa, Japan	MAT1-2
	47235	JCM 22302 = IAM 2255 = CBS 125.52 = R-0436 (extype of *A. inuii*)	Koji, Ryukyu, Japan	MAT1-2
	47005	K-4133 = NRRL 4750 = CBS 128.52	Unknown, Japan	MAT1-2
	M*29	This study	Meju, Yangpyeong, Korea	MAT1-2
	M532	This study	Meju, Hoengseong, Korea	MAT1-2
	M1003	This study	Meju, Yeoju, Korea	MAT1-2
	M2009	This study	Meju, Yongin, Korea	MAT1-2
	M2093	This study	Meju, Yongin, Korea	MAT1-2
	M2096	This study	Meju, Yongin, Korea	MAT1-2
	M2097	This study	Meju, Yongin, Korea	MAT1-2
	M2098	This study	Meju, Yongin, Korea	MAT1-2
	M2099	This study	Meju, Sunchang, Korea	MAT1-2
	46491	This study	Meju, Sunchang, Korea	MAT1-1
	M2103	This study	Meju, Yongin, Korea	MAT1-2
	M2104	This study	Meju, Anseong, Korea	MAT1-1
	46490	This study	Meju, Yongin, Korea	MAT1-2
	M2106	This study	Meju, Yongin, Korea	MAT1-2
	M2113	This study	Meju, Yangpyeong, Korea	MAT1-2

Table 1. Continued

Species	KACC No.	Strain information	Source	Mating type
A. tubingensis	M100	This study	Meju, Haenam, Korea	MAT1-2
	46498	This study	Meju, Hoengseong, Korea	MAT1-1
	M1001	This study	Meju, Yeoju, Korea	MAT1-1
	M1004	This study	Meju, Yeoju, Korea	MAT1-2
	M1005	This study	Meju, Pocheon, Korea	MAT1-2
	M1006	This study	Meju, Anseong, Korea	MAT1-2
	M2095	This study	Meju, Yongin, Korea	MAT1-1
	46499	This study	Meju, Sunchang, Korea	MAT1-1
	M2101	This study	Meju, Sunchang, Korea	MAT1-1
	46805	CBS 134.48 = R. Mosseray, No. 726 (type of *A. tubingensis*)	Unknown	MAT1-1
	46993	NRRL 4757 = IAM 2209 = CBS 136.52 = R6711 (type of *A. saitoi*)	Koji, Japan	MAT1-2
	46994	NRRL 4758 = IAM 2190 = CBS 137.52 = K-3931 (type of *A. saitoi* var. *kagoshimaensis*)	Koji, Japan	MAT1-2
	47137	This study	Meju, Korea	MAT1-2
A. niger	M44	This study	Meju, Yangpyeong, Korea	MAT1-2
	M224	This study	Meju, Cheongwon, Korea	MAT1-1
	M267	This study	Meju, Goisan, Korea	MAT1-2
	M347	This study	Meju, Korea	MAT1-2
	46493	This study	Meju, Icheon, Korea	MAT1-1
	M458	This study	Meju, Icheon, Korea	MAT1-2
	M478	This study	Meju, Yangju, Korea	MAT1-1
	46494	This study	Meju, Hoengseong, Korea	MAT1-1
	M561	This study	Meju, Hoengseong, Korea	MAT1-1
	46495	This study	Meju, Gimpo, Korea	MAT1-1
	M581	This study	Meju, Gimpo, Korea	MAT1-1
	M602	This study	Meju, Kimcheon, Korea	MAT1-1
	M619	This study	Meju, Iksan, Korea	MAT1-1
	M630	This study	Meju, Gimcheon, Korea	MAT1-1
	M639	This study	Meju, Jecheon, Korea	MAT1-1
	M654	This study	Meju, Sunchang, Korea	MAT1-1
	M701	This study	Meju, Sunchang, Korea	MAT1-2
	M826	This study	Meju, Andong, Korea	MAT1-1
	M2108	This study	Meju, Buan, Korea	MAT1-1
	M2110	This study	Meju, Gongju, Korea	MAT1-1
	M2111	This study	Meju, Gongju, Korea	MAT1-1
	46497	This study	Meju, Yangpyeong, Korea	MAT1-1
	45072	CBS 554.65 = ATCC 16888 = NBRC 33023 = IMI 050566 = NRRL 326 (neotype of *A. niger*)	Tannin-gallic acid fermentation, Connecticut, USA	MAT1-2
	46995	NRRL 4785 = CBS 115.34 = NBRC 4034 (type of *A. batatae*)	Unknown, Japan	MAT1-1
	47003	NBRC 4031 = RIB 2014 = NRRL 4784 = CBS 121.28 (type of *A. aureus*)	Koji, Japan	MAT1-1
	46998	NRRL 4859 = CBS 117.51 (type of *A. miyakoensis*)	Koji, Japan	MAT1-2
	47000	NBRC 4388 = RIB 2602 = NRRL 4760 = CBS 139.2 = IAM 2185 = R-0635 (type of *A. usami*)	Koji, Japan	MAT1-1
	47001	NRRL 4889 (type of *A. usami* mut. *shirousami*)	Unknown, Japan	MAT1-1
A. welwitschiae	46492	This study	Meju, Haenam, Korea	MAT1-1
	M268	This study	Meju, Goisan, Korea	MAT1-1
	M504	This study	Meju, Buan, Korea	MAT1-1
	M672	This study	Meju, Sunchang, Korea	MAT1-2
	M792	This study	Meju, Sunchang, Korea	MAT1-2
	M1002	This study	Meju, Yeoju, Korea	MAT1-2
	46496	This study	Meju, Goisan, Korea	MAT1-1
	M2109	This study	Meju, Buan, Korea	MAT1-1
	46882	This study	Meju, Haenam, Korea	MAT1-2
	46996	NRRL 4948 = CBS 557.65 = ATCC 16877 (neotype of *A. awamori*)	Unknown, Japan	MAT1-2

M*, Meju.

and total genomic DNA was extracted using the DNeasy Plant Mini-Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol.

Identification of mating types. The primers used to partially amplify the *MAT1-1* and *MAT1-2* genes in the 74 isolates are presented in Table 2. PCR amplification was conducted in 50-μL reactions, which included 5 μL of 10× PCR buffer, 3 μL of deoxyribonucleotide triphosphates (dNTP; 2.5 mM), 0.4 μL of each primer (100 pmol), 0.3 μL of Taq DNA polymerase (Inclone, Daejeon, Korea), 39.9 μL of sterile deionized water, and 1 μL (approximately 10 ng) of a DNA template. The PCR conditions for primer (MAT1-1F-An, MAT1-1R-An) were as follows: 5 min at 95°C; denaturation for 30 sec at 95°C and annealing and extension for 2 min at 72°C (25 cycles); and a final extension for 5 min at 72°C

Table 2. Primers used in this study

Primer	Sequence	Reference
MAT1-1F-An	5′-GCGGCCACTGAACAGTTTCATTGCT-3′	[23]
MAT1-1R-An	5′-TGATGGAGTATGCCTTGGCTACGATG-3′	[23]
MAT1-2F	5′-TTCCTCGTCCGCCAAATGCA-3′	This study
MAT1-2R	5′-GAGATGATGTTCGACGCTTC-3′	This study
M1F_Anig	5′-GGTCATCGCGAATGATGGAG-3′	[22]
M1R_Anig	5′-CAGCGTGCTTTCAACGCATTC-3′	[22]
MAT5-4	5′-AARRTICCIMGICCICCIAAYGC-3′	[10]
MAT3-2	5′-TTNCKIGGIGTRTAITGRTARTCNGG-3′	[10]

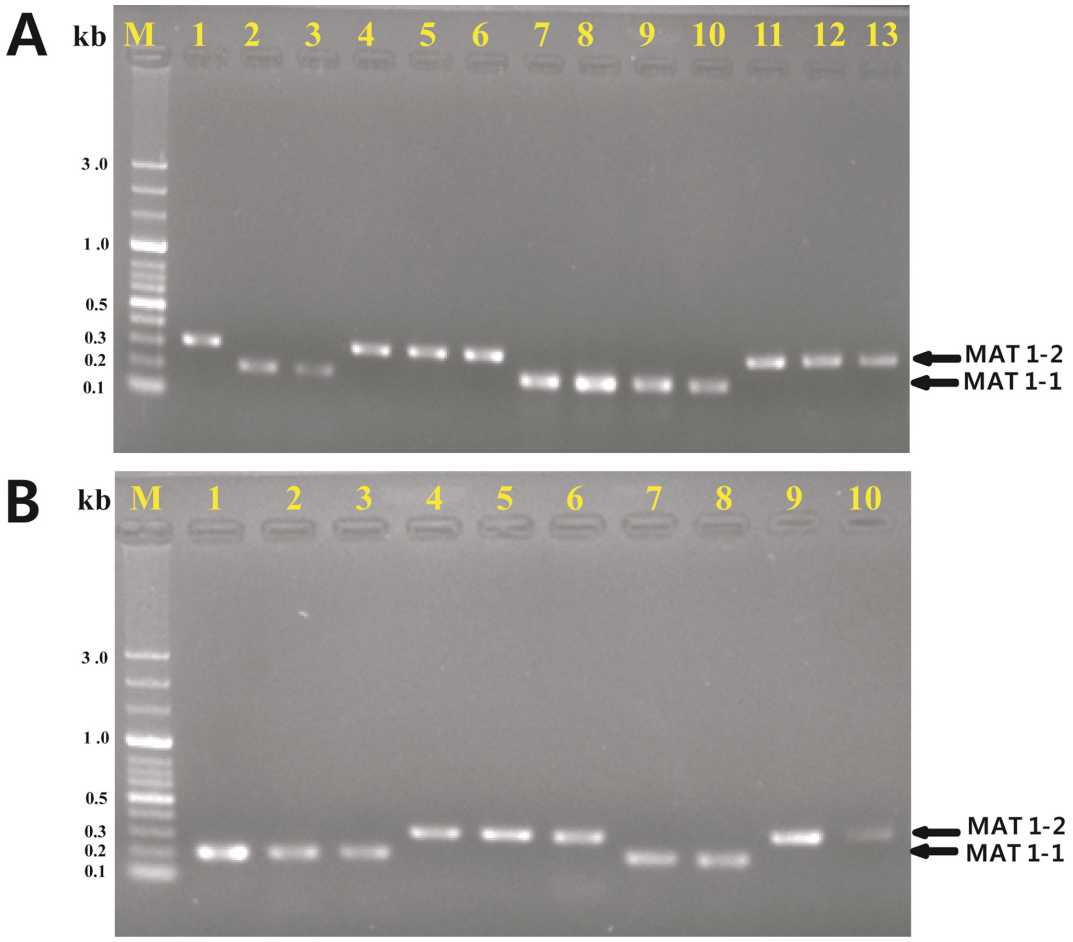

Fig. 1. *MAT* gene amplification from genomic DNA of *Aspergillus tubingensis* (A) and *A. welwitschiae* (B). A, Lane M, molecular weight markers; Lanes 1~9, amplicons from Meju isolates (M100, M554, M1001, M1004, M1005, M1006, M2095, M2100, and M2101); lanes 10~13, amplicons from KACC *A. tubingensis* (46805, 46993, 46994, and 47137); B, Lane M, molecular weight markers; lanes 1~8, amplicons from Meju isolates (M95, M268, M504, M672, M792, M1002, M2107, and M2109); lanes 9 and 10, amplicons from KACC *A. welwitschiae* (46882 and 46996).

for *MAT1-1*. For *MAT1-2* (MAT1-2F, MAT1-2R), we used the following conditions: 5 min at 95°C; denaturation for 1 min at 95°C, annealing for 1 min at 58°C, and extension for 30 sec at 72°C (25 cycles); and final extension for 5 min at 72°C. The PCR conditions for previously reported primers [10, 22] were as follows: Primer (M1F_Anig, M1R_Anig), the initial denaturation for 5 min at 95°C; followed by 25 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 60°C, and extension for 30 sec at 72°C; and a final extension for 5 min at 72°C for *MAT1-1*. In case of *MAT1-2* (MAT5-4, MAT3-2), we used denaturation for 5 min at 95°C; followed by 25 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and extension for 30 sec at 72°C; and a final extension for 5 min at 72°C. The reactions were run in a Peltier thermal cycler MJ Research PCT-200 gradient (Waltham, MA, USA). After the PCR, 5 mL of a PCR product was analyzed by electrophoresis and visualized by means of gel documentation equipment.

RESULTS AND DISCUSSION

During our experiments, we initially observed PCR amplification of both *MAT1-1* and *MAT1-2* in the same strains using the primers and protocol reported elsewhere [22]. After optimizing the PCR conditions as described above, we were able to rectify this problem and to obtain either *MAT1-1* or *MAT1-2* for a single strain.

After that, we used PCR to determine the ratio of *MAT* genes in 74 isolates of 4 species of *Aspergillus* section *Nigri* isolated from Meju, a traditional fermented food product of South Korea: namely, *A. luchuensis, A. niger, A. tubingensis*, and *A. welwitschiae*. PCR analysis revealed amplification of an approximately 200-bp fragment for *MAT1-1* and 300-bp fragment for *MAT1-2* with the primer set designed by Yamada (Figs. 1~3) and the primer set of Horn *et al.* [22] resulted in a ~500-bp amplicon for *MAT1-1* and a 300-bp amplicon for *MAT1-2* (data not shown). The present PCR results indicate that all isolates yielded either *MAT1-1* or *MAT1-2* but never both *MAT* genes during PCR amplification.

In this study, the *MAT1-1* locus was detected in 2 of 23 strains of *A. luchuensis*, 6 of 13 strains of *A. tubingensis*, 21 of 28 stains of *A. niger*, and 5 of 10 strains of *A. welwitschiae*. Furthermore, in case of *A. tubingensis* and *A. welwitschiae*, distributions of *MAT1-1* and *MAT1-2* were random approaching 50% for each mating type (Fig. 1). In contrast, the *MAT1-1* type is dominant in *A. niger* (75% of

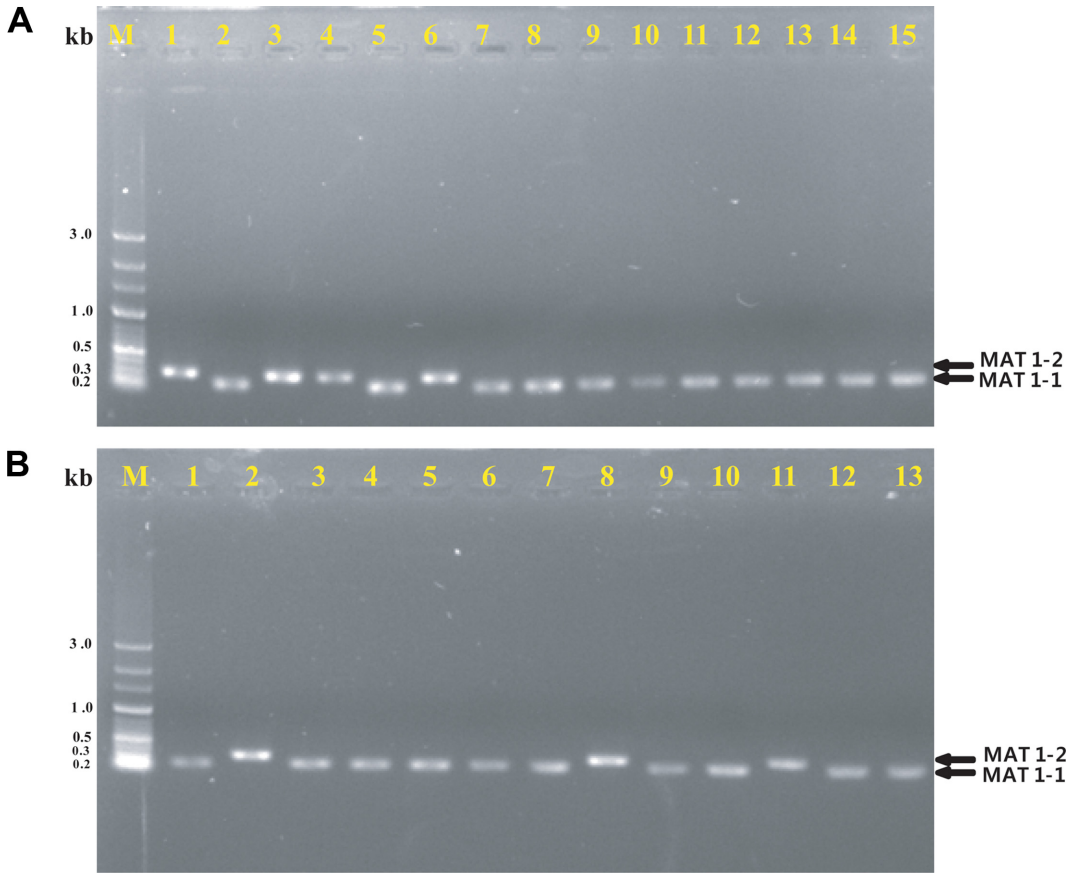

Fig. 2. *MAT1-1* and *MAT1-2* genes amplified from *Aspergillus niger* isolates. A, Lane M, molecular weight markers; lanes 1~15, amplicons from Meju isolates (M22, M224, M267, M347, M450, M458, M478, M557, M561, M579, M581, M602, M619, M630, and M639); B, Lane M, molecular weight markers; lanes, 1~7, amplicons from Meju isolates (M654, M701, M826, M2108, M2110, M2111, and M2112); lanes 8~13, amplicons from KACC *A. niger* (45072, 46995, 47003, 46998, 47000, and 47001).

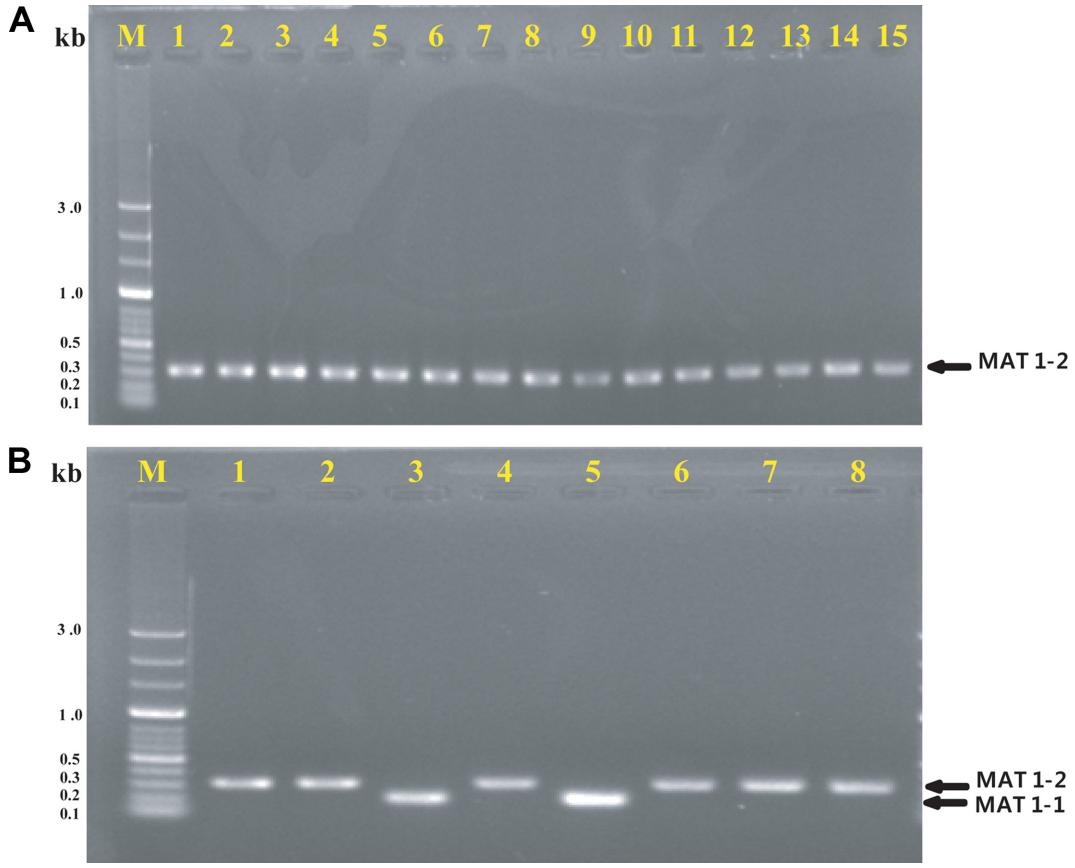

Fig. 3. PCR amplification of *MAT1-1* and *MAT1-2* genes from *Aspergillus luchuensis* isolates. A, Lane M, molecular weight markers; lanes 1~8, amplicons from KACC *A. luchuensis* (46772, 46771, 45131, 41731, 47234, 47004, 47235, and 47005); lanes 9~15, amplicons from Meju isolates (M29, M532, M1003, M2009, M2093, M2096, and M2097); B, Lane M, molecular weight markers; lanes 1~8, amplicons from Meju isolates (M2098, M2099, M2102, M2103, M2104, M2105, M2106, and M2113).

studied strains) (Fig. 2) and *MAT1-2* type is dominant in *A. luchuensis* (91.3% of strains under study) (Fig. 3).

Several research groups have reported the ratio of *MAT1-1* and *MAT1-2* genes in the species of *Aspergillus* [11, 19, 21, 22]. In the *Aspergillus* section *Flavi*, the *MAT1-1* to *MAT1-2* gene ratio showed mixed results but to some extent was found to be sample dependent. In *Aspergillus flavus* strains isolated from peanut field soil, this ratio is 1 : 1 [11]. Nonetheless, *A. flavus* from clinical samples carried only *MAT1-1* (100%) [21]. Among other species, e.g., in *Aspergillus oryzae* isolated from soy sauce, 72.5% of strains were found to be of the *MAT1-2* type. The preference for *MAT1-2* in *A. oryzae* from soybean was hypothesized to be due to its short aerial hyphal phenotype, which can both improve fermentation enzyme efficiency and block growth of contaminating fungi [19].

Other species of *Aspergillus* section *Nigri* except for *A. luchuensis* have been studied regarding the *MAT1-1* to *MAT1-2* gene ratio [21, 22]. Among 125 isolates from *A. tubingensis*, *A. niger*, and *A. welwitschiae*, the ratio of *MAT1-1* and *MAT1-2* was found to be 1 : 1 in isolates belonging to *A. tubingensis* and *A. niger*. Strains of *A. welwitschiae* showed a strong bias toward *MAT1-1* [21]. Our present results add to the existing knowledge on the ratio of *MAT* genes in the *Aspergillus* section *Nigri* with the emphasis on *A. luchuensis*, which has not been widely studied. Most of our strains were isolated from Meju. Although the strains of *A. tubingensis* and *A. welwitschiae* in our results indicated an approximately 1 : 1 distribution, the majority of *A. niger* strains indicated a bias toward the *MAT1-1* type, whereas the strains of *A. luchuensis* showed predominance of the *MAT1-2* type (91.3%). The remaining 8.7% of strains (2 strains) were found to be in the *MAT1-1* category. The presence of the *MAT1-1* mating type in *A. luchuensis* has not been reported until now, and hitherto various researchers have observed *MAT1-2* only among 28 strains of *A. luchuensis*, which are used for brewing Japanese traditional spirits such as awamori and shochu [23].

Mating experiments on fungi are useful tools for genetic analysis and are also a good strategy for strain improvement. The mating type in fungi has long been associated with breeding for production of industrially important strains with desired traits. In mushrooms, mating-type genes can be used as marker genes for breeding methods, and their functions that are required for formation of stable dikaryons are essential for breeding [24]. Strain improvement based

on the mating type in penicillin-producing fungus *Penicillium chrysogenum* has been reported to be biotechnologically advantageous because the fungus showed improved penicillin production and fermentor-friendly changes in the morphology of hyphae and conidia upon breeding [25]. Even in ascomycetes, the presence of mating-type genes and their biotechnological potential have been discussed in detail [26].

A. luchuensis MAT1-1 mating-type strains were proposed as a valuable source for breeding [23]. Our results therefore should advance the understanding of biology and evolution of *A. luchuensis*. Our findings also have industrial relevance because the sexual cycle can now be utilized for strain improvement for use in the brewing and fermented food industry. In general, natural mating of *A. luchuensis MAT1-1* and *MAT1-2* type strains can have advantages over genetic recombination technologies because recombination tends to occur throughout the genome resulting in excessive genetic variation for screening purposes. Mating is also an inexpensive technique for manipulation of several genes *in vitro* to produce an industrially preferable mutant strain. In addition, the stability of industrial strains remains unaffected when sexual reproduction is used for breeding because no harmful mutations are introduced. Therefore, our identification of *MAT1-1* type *A. luchuensis* can be used for selective breeding to produce commercially important strains that are cost-effective and beneficial for industries.

ACKNOWLEDGEMENTS

This study was carried out with the support (Project No. PJ01194902) of Rural Development Administration, Republic of Korea.

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