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Comparison of *Aspergillus flavus* and *Aspergillus oryzae* by amplified fragment length polymorphism

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Abstract. The aflatoxin producer *Aspergillus flavus* and the koji mold *Aspergillus oryzae* are morphologically similar species that belong to the *Aspergillus* section *Flavi*. We used amplified fragment length polymorphism (AFLP) to differentiate these two species. In this study, we tested thirteen *A. flavus*, nine *A. oryzae*, and three *A. flavus* var. *columnaris* strains. DNA fragment profiles amplified with each of three selective primer pairs displayed similar patterns for the various *A. flavus* strains. Different patterns were observed with these primer pairs for the *A. oryzae* strains. We combined these data to increase the grouping of the various strains within each species and to distinguish between *A. flavus* and *A. oryzae*. By unweighted pair group method with arithmetic average (UPGMA) analysis, the AFLP data obtained from the three selective primer pairs, *Eco*RI-AC/*Mse*I-CAT, *Eco*RI-TA/*Mse*I-CAT, and *Eco*RI-TA/*Mse*I-CTT, differentiated *A. flavus* from *A. oryzae* successfully. Three strains of *A. flavus* received from ATCC grouped outside of the other *A. flavus* strains we examined. The morphology of these isolates and our results indicated those strains were originally misidentified and they should be classified as *A. parasiticus*. We found that the AFLP technique is a reliable and reproducible tool with the ability to differentiate *A. flavus* from *A. oryzae* and should be generally useful in distinguishing between closely related species or strains.

Keywords: AFLP; *Aspergillus flavus*; *Aspergillus oryzae*.

Introduction

The aflatoxigenic species *Aspergillus flavus* Link and *A. parasiticus* Speare can produce the potent carcinogen aflatoxin and can pose a significant human health threat. The nonaflatoxigenic species *A. oryzae* (Ahlburg) Cohn and *A. sojae* Sakaguchi & Yamada are widely used for the production of food-grade amylase and the fermentation of sake, miso and soy sauce. All four species belong to the *Aspergillus* section *Flavi* and have many phenotypic similarities. Current methods for the identification of these species still depend primarily on cultural and morphological characteristics. However, it is often difficult to differentiate these species because the phenotypic differences are not distinct and are easily affected by the environment and are also confused by the high degree of intra- and interspecies variation.

Because of the economic value of members of the *Aspergillus* section *Flavi* and the importance of differentiating them, several molecular methods have been developed to distinguish these strains. DNA relatedness of these species in *Aspergillus* section *Flavi* was studied by Kurtzman et al. (1986) with the DNA reassociation method. The Cot value calculation results showed 100% relatedness between *A. flavus* and *A. oryzae*. Similarly, *A. parasiticus* and *A. sojae* were 91% related. The homology between these two groups was 70%. Therefore Kurtzman et al. suggested the four taxa be regarded as varieties of *A. flavus*. A medium containing bleomycin has been used to distinguish *A. parasiticus* from *A. sojae* (Klich and Mullaney, 1989; Yuan et al., 1995). The random amplified polymorphic DNA (RAPD) method has been used to differentiate *A. parasiticus* from *A. sojae* with three screened decamer primers (Yuan et al., 1995), but the same primers could not separate *A. flavus* from *A. oryzae* (Yuan et al., unpubl.). The sequence of a regulatory gene for the aflatoxin biosynthetic pathway was analyzed, and distinct DNA sequences within the 5' untranslated region and the zinc- finger domain were found in limited strains of *A. flavus*, *A. oryzae*, *A. parasiticus*, and *A. sojae* (Chang et al., 1995). PCR-amplified regions of ribosomal internal transcribed spacers have also been used to differentiate species of *Aspergillus* section *Flavi* with the single-strand conformation polymorphism (SSCP) method (Kumeda and Asao, 1996). The SSCP results indicated that 67 of the 68 test strains could be classified into four groups as *A. flavus*/*A. oryzae*, *A. parasiticus*/*A. sojae*, *A. tamari*, and *A. nomius*. Sixteen strains of *A. flavus* or *A. oryzae* could not be clearly separated with the sequence of ITS1-5.8S-ITS2 region of ribosomal DNA (Lin et al., 1995). Restriction site polymorphism and DNA sequences of protein-encoding genes were analyzed, and 31 *A. flavus* strains could be separated into two distinct groups. Five

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of the 31 strains belonged to industrial fungus *A. oryzae*, and they all clustered together in one of the two groups (Geiser et al., 1998).

Previous research has indicated the difficulty of differentiating *A. flavus* from *A. oryzae*. In this study, we have used amplified fragment length polymorphism (AFLP) to differentiate strains of these two groups. The AFLP method is a relatively new molecular technique for DNA fingerprinting. The method combines the principle of restriction fragment length polymorphism (RFLP) analysis with highly specific PCR amplification (Bears et al., 1998; Vos et al., 1995; Zabeau and Vos, 1993). Total genomic DNA is digested with one or more restriction enzymes, and then double-stranded nucleotide adaptors are ligated to the DNA fragments to serve as a primer binding site for PCR amplification. Complementary primers to the adaptor and restriction site sequence with additional nucleotides at the 3'end are used to amplify a subset of the ligated fragments. In addition to fingerprinting DNA from human, animals, and plants, the AFLP technique has been used to differentiate intra- and interspecies strains of several bacteria, such as *Aeromonas* and *Acinetobacter* (Huys et al., 1996; Janssen et al., 1997), and fungi, such as *Colletotrichum*, *Cylindrocladium*, *Puccinia*, and *Phytophthora* (Paul et al., 1996; Neill et al., 1997; Henricot and Culham, 2002; Villareal et al., 2002; Ochwo et al., 2002).

The purpose of this study was to apply the AFLP technique to strains of *A. flavus* and *A. oryzae* and evaluate the ability of this technique to differentiate between them. A total of 25 strains—including thirteen *A. flavus* strains, nine *A. oryzae* strains, and three *A. flavus* var. *columnaris* strains—were investigated in this study.

Materials and Methods

Fungal Strains and Mycelia Collection

All the fungal strains used in this study (Table 1) were originally obtained from different culture collection centers or were isolated from fermented materials and preserved in the Biosource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute. BCRC 30165 (=ATCC 16883) and BCRC 30433 (=ATCC 16870) have been deposited as type strains of *A. flavus* and *A. flavus* var. *columnaris*, respectively. BCRC 30174 (=ATCC 16868) was deposited as a reference strain of *A. oryzae*.

Table 1. Strains used in this study and their related information.

Name & BCRC No.	Geographical origin	Source	Notes
Aspergillus oryzae (Ahlburg) Cohn			
30174 (ATCC 16868)	China	Soy sauce	Reference strain (ref. 1)
30229 (ATCC 20423)	Japan	Soil	
30428 (ATCC 22788)	Japan	Rice koji for sake	
30429 (ATCC 10196)	Virginia, U.S.A.	Painted pine panel	
31646	Taiwan	Fermented black bean	
31658	Taiwan	Soy source starter	
31659	Taiwan	Miso starter	
32268	Japan	Soy sauce	
32269	Japan	Miso	
Aspergillus flavus Link			
30010 (ATCC 10124)	—[a]	—	Non-toxigenic (ref. 5)
30018 (ATCC 26771)	Poland	Dry cracower sausage	Aflatoxin B1, B2, G1, G2 (ref. 2)
30019 (ATCC 28539)	Japan	Buck wheat flour	Aflatoxin B1, B2 (ref. 2)
30110 (ATCC 26768)	Poland	Dry cracower sausage	Aflatoxin B1, B2, G1, G2 (ref. 2)
30144 (ATCC 9643)	Australia	Shoe sole	
30165 (ATCC 16883)	South Pacific	Cellophane	Neotype (ref. 3)
30173 (ATCC 26946)	Texas, U.S.A.	Peanut kernel	Aflatoxin B1, B2 (ref. 2)
30202 (ATCC 22546)	Iowa, U.S.A.	Moldy corn	Aflatoxin B1, B2 (ref. 2)
30203 (ATCC 24457)	Canada	Air	
30291 (ATCC 11498)	Venezuela	Soil	Aflatoxin B1, B2, G1, G2 (ref. 2)
31654	Taiwan	Kaoliang liquor starter	
31737	Philippines	Rice wine	
32242	Taiwan	Stored paddy rice	
Aspergillus flavus var. *columnaris* Raper & Fennell			
30209 (ATCC 18170)	Canada	Bottled orange juice	Aflatoxin B2 (ref. 2)
30433 (ATCC 16870)	Japan	Butter	Type strain (ref. 3)
30435 (ATCC 18168)	—	Instant chocolate milk	Aflatoxin B2 (ref. 4)

[a]Information unavailable.

ref. 1: Raper and Fennell, 1965; ref. 2: Wei and Jong, 1986; ref. 3: Samson and Gams, 1985; ref. 4: Walbeek et al., 1968; ref. 5: Schmidt et al., 1977.

Fungal cultures for genomic DNA isolation were grown as follows. Conidia were harvested from 7- day-old potato dextrose agar slant cultures growing at 28°C and inoculated into 50 ml potato dextrose broth in 250 ml flask and then incubated at 25°C for 48 to 72 h without shaking. Mycelia were collected by vacuum filtration and lyophilized.

Preparation of Fungal Genomic DNA

Frozen mycelial mats were ground with a mortar and pestle to fine powder in liquid nitrogen. Approximately 20 mg of homogenized cell powder was mixed with 600 µl of lysis buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM EDTA and 2% SDS) and incubated at 60°C for 10 min. The DNA was extracted sequentially with an equal volume of phenol/chloroform (1:1), and chloroform/ isoamyl alcohol (24:1). Total nucleic acids were precipitated with 0.7 volume of isopropanol and vacuum dried. Finally, the pellets were re-dissolved in water and treated with RNase A at 37°C for 1.5 h to remove RNA, and the RNase was inactivated at 60°C for 1 h. Final DNA concentrations were determined by measuring with a Model U spectrophotometer (Hitachi, Tokyo, Japan), and the integrity of each DNA sample was examined with an agarose gel.

AFLP Analysis

AFLP assays were carried out with an AFLP™ Microbial Fingerprinting kit (Perkin Elmer Corp., Norwalk, CT, USA) according to the manufacturer's instructions. Genomic DNA (0.01 µg) was digested with *Eco*RI and *Mse*I. *Eco*RI, and *Mse*I adapters were ligated to the fragments to form the primary templates. Primers with complimentary sequences to the adapters and restriction site were used in the first step PCR reaction under the following cycle profile: holding at 72°C for 2 min, 20 cycles consisting of 94°C for 20 sec, 56°C for 30 sec and 72°C for 2 min. Then, the same primers, except for an additional two or three nucleotides at the 3'end as shown in Table 2, were used in the second PCR reaction with previous PCR products as a template to amplify a subset of fragments. The cycling profiles were as follows: (1) 10 cycles of 20 sec at 94 °C, 30 sec at an annealing temperature (66 to 57°C, a step-down procedure consisting of 1°C lower than that of each previous cycle), and 2 min at 72°C; (2) 20 cycles at 94°C for 20 sec, 56°C for 30 sec, and 72°C for 2 min; (3) holding for 30 min at 72°C. The PCR reactions were performed on a Perkin Elmer 9600 thermal cycler (Norwalk, CT, USA). The selective amplification products were loaded on a 6% denaturing polyacrylamide gel to analyze band patterns with an ABI 373 sequencer (Perkin Elmer Corp., Norwalk, CT, USA).

Data Analysis

The electrophoresis data of AFLP reactions were converted to MS-DOS format to make them compatible with BioNumerics Software, Version 2.5 (Applied Maths, Inc. Austin, TX, USA). The similarity between each pair of AFLP patterns was calculated by using the band-based Dice similarity coefficient, S_D. For pattern comparison, a band position tolerance value of 0.1% was optimized to compensate for misalignment of homologous bands. The strains investigated were grouped by the unweighted-pair group method with arithmetic average (UPGMA). Branch support of the tree was assessed by bootstrapping (Felsenstein, 1985). The bootstrap analysis was performed with 10,000 replicates.

Morphological Observation

The taxonomic systems of *Aspergillus* (Raper and Fennell, 1965; Klich and Pitt, 1988a, b) were followed for observation and identification. The isolates were cultured on CYA (Czapek Yeast Extract Agar: sucrose 3%, yeast extract 0.5%, $NaNO_3$ 0.3%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001% and agar 1.5%) and CzA (Czapek's Agar: sucrose 3%, $NaNO_3$ 0.3%, K_2HPO_4 0.1%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001% and agar 1.5%) at 25°C. Both macroscopical and microscopical characters were assessed when they were 3, 7, and 14 days old. The colony color was observed by naked eye with the aid of color charts in the Methuen Handbook of Colour (Kornerup and Wanscher, 1978). The material on the slide mounts was examined under light microscope (Axiophot, Zeiss, Germany). The metulae, conidial diameter and conidial rough were determined under 1,000x magnification.

For scanning electron microscopic observations, the fungi were cultured on CYA medium at 25°C for 10 days. Agar blocks containing spores were gradually dehydrated with increasing concentrations of ethyl alcohol (25 to 100%) and then placed in a solution of 100% acetone for more than 1 h (Tzean and Estey, 1978). Finally, these blocks were treated in a critical point drying apparatus (HITACHI HCP-2). Dried specimens were coated with evaporated gold (Eiko Engineering, IB-2 ion coater) and examined under scanning electron microscope (Leo 980, Leo Electron Microscopy Ltd) (Kusaka and Asano, 1987).

Results

Preliminary AFLP experiment results showed that the selective primers with only one additional nucleotide yielded band patterns too complicated to analyze. Therefore we chose selective primers with two or three additional nucleotides at the 3' end for the AFLP reaction, and the fragment length for analysis was limited to the range of 50 to 500 base pairs. Eight *Eco*RI and six *Mse*I selective primers were random paired in a selective PCR reaction (Table 2).

The UPGMA analysis of the AFLP results indicated that three pairs of selective primers, *Eco*RI-AC/*Mse*I-CAT, *Eco*RI-TA/*Mse*I-CAT, and *Eco*RI-TA/*Mse*I-CTT, could distinguish *A. flavus* from *A. oryzae* successfully. The combined data of the three primer pairs were analyzed and generated the dendrogram shown in Figure 1. None of the other primer combinations examined in this study could clearly separate *A. flavus* from *A. oryzae* with AFLP. With these multiple fungal strains, repetitive AFLP reactions

using the three primer-sets we studied have consistently yielded similar polymorphic band numbers and patterns. Different DNA preparations yielded similar results, and although certain bands may not appear occasionally, the structure of dendrogram clustering *A. flavus* and *A. oryzae* is maintained. The dendrogram clustered our test strains into four groups with high bootstrap values (89-95), including *A. flavus* in group I and II, *A. flavus* var. *columnaris* in group III, and *A. oryzae* in group IV.

Table 2. Selective primer pairs used in this study.

*Mse*I \ *Eco*RI	-AA	-AG	-AT	-AC	-TA	-A	-C	-G
-CAG								
-CAC								
-CAT				*	*			
-CTT					*			
-CA								
-CC								

: Primer pairs used in this study.
* : Primer pairs used to differentiate *A. flavus* from *A. oryzae* successfully.

It was found that three aflatoxin-producing *A. flavus* strains, BCRC 30110, 30018 and 30291, clustered together in group I and remained distinct from the other *A. flavus* strains in the dendrograms of AFLP data obtained with the three primer pairs. Two of them (BCRC 30018 and 30110) were originally isolated from sausage in Poland, and the other was isolated from the soil of Venezuela. We reordered the corresponding cultures from ATCC and compared their morphology with those cultures previously maintained in BCRC/FIRDI. No significant difference was found in the previous or new ordered cultures based on the light microscopic or scanning electron microscopic observation. However, the distinctly rough-walled conidia of previous and new ordered cultures (Figure 2) suggested that the three strains were originally misidentified and should be correctly identified as *A. parasiticus* instead.

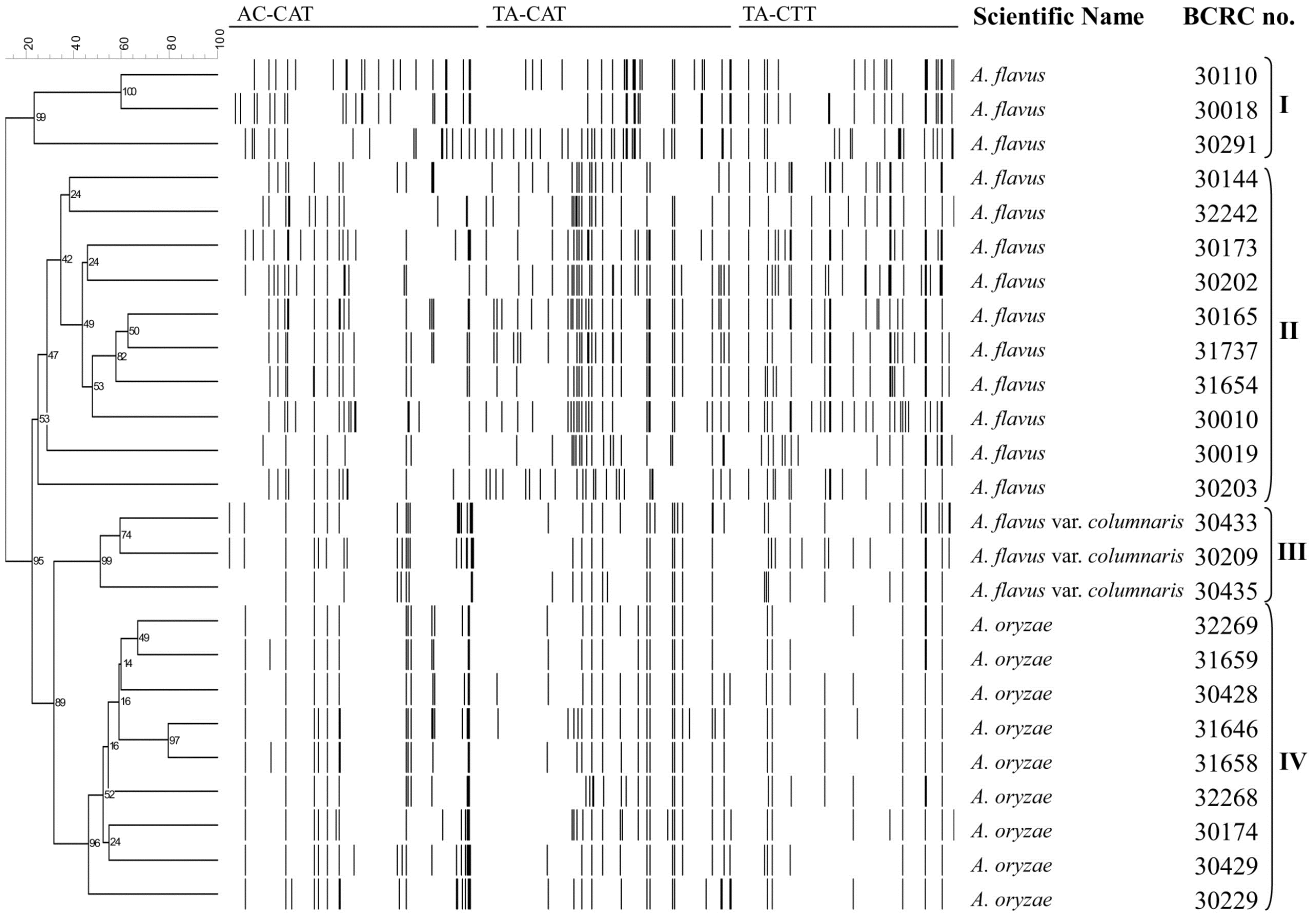

Figure 1. Combination of amplified fragment length polymorphism analysis of thirteen *A. flavus* strains, nine *A. oryzae* strains, and three *A. flavus* var. *columnaris* strains, using *Eco*RI-AC/ *Mse*I-CAT, *Eco*RI-TA/ *Mse*I-CAT, and *Eco*RI-TA/ *Mse*I-CTT selective primer pairs. UPGMA tree was constructed by comparing polymorphic bands generated from the three selective primer pairs. Values at branch nodes indicate bootstrap support obtained from bootstrap analysis with 10,000 replicates.

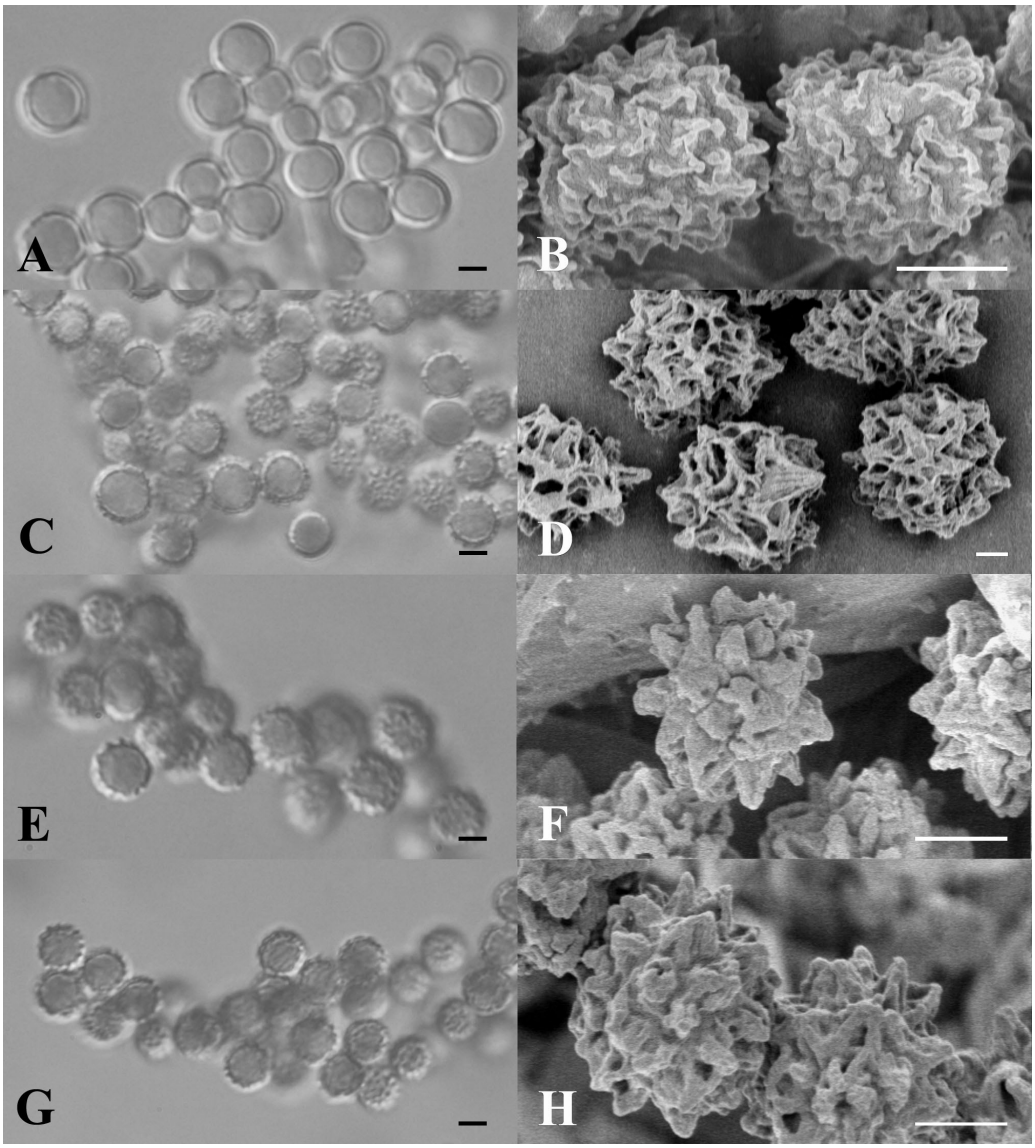

Figure 2. Conidia ornamentation of *A. flavus* strains. A, C, E, G: Light microscope micrographs, scale bar = 3 µm; B, D, F, H: Scanning electron micrographs, scale bar = 2 µm; A, B: BCRC 30165 (ATCC 16883), type strain of *Aspergillus flavus*; C, D: BCRC 30291 (ATCC 11498); E, F: BCRC 30110 (ATCC 26768); G, H: BCRC 30018 (ATCC 26771).

Discussion

Many strains have been isolated and named *A. flavus* or *A. oryzae*. In order to use strains with more diversity, we selected them according to their typification, geographical origin, isolation source, and aflatoxin productivity in this study. Type strains of *A. flavus* and *A. flavus* var. *columnaris* were recruited. Based on the study of Kurtzman et al. (1986), we used BCRC 30174 (= ATCC 16868 = NRRL 451) as a reference strain for *A. oryzae* (Table 1).

The food fermentation and industrial importance of *A. oryzae* makes it essential to differentiate this species from the aflatoxigenic species, *A. flavus*. Whole genomic DNAs were compared in both the AFLP reactions and the DNA hybridization test. The 100% similarity between *A. flavus* and *A. oryzae* of Kurtzman's (1986) hybridization results indicated the close relationship of the two species. It was found in this study, that except with the three primer pairs described above, no primer combinations tested could clearly separate *A. flavus* from *A. oryzae* via AFLP. These results are consistent with the high relatedness of the two species.

Aspergillus oryzae is generally recognized as a safe (GRAS) microorganism. Previous research and our results indicate that the difference between *A. flavus* and *A. oryzae* is intraspecific level (Kurtzman et al., 1986; Yuan et al., 1995; Kumeda and Asao, 1996; Geiser et al., 1998). Therefore, it is worthwhile to reconsider the safety of *A. oryzae*. In our results, all the *A. oryzae* strains were tightly clustered in group IV (Figure 1), and no aflatoxin production was recorded by these strains. Thus, we suggest recognizing these strains as safe. Two *A. flavus* strains, BCRC 31654 and 31737, originally isolated from fermentation starter or products, were grouped with the other aflatoxigenic *A.*

Table 3. Distinguishing features of *Aspergillus flavus* and *A. parasiticus*.

Strains	*A. flavus* BCRC 30165[a]	*A. parasiticus* BCRC 33603[a]	*A. parasiticus* BCRC 30018	*A. parasiticus* BCRC 30110	*A. parasiticus* BCRC 30291
Colonies on CYA					
7 days	Yellowish green to dark green	Yellowish green to dark green	Greyish green to dark green	Greyish green to dull green	Olive
14 days	Olive brown	Olive	Olive	Olive	Olive
Colonies on CZA					
7 days	Yellow to olive yellow	Light yellow to deep yellow	Olive yellow	Yellow to olive	Yellow to olive
14 days	Olive yellow to olive	Yellow to olive	Brown yellow to olive	Brown yellow to olive	Dark yellow to olive
Microscopic characteristics					
Metulae	+/-	+/-	+/-	+/-	+/-
Conidial diam. (μm)	(3-)4-5	4-6	4-5	4-5	4-5
Conidia rough	-	+	+	+	+

[a]Type strain.
+: character present; -: character absent.

flavus strains in group II. These results indicate the importance of confirming the safety of strains used to prevent contamination during fermentation.

The variety *Aspergillus flavus* var. *columnaris*, with a columnar pattern of its conidial head, is morphologically different from *A. flavus* and *A. oryzae* (Raper and Fennell, 1965). In the dendrogram of Figure 1, strains of *A. flavus* var. *columnaris* clustered together in group III, and they displayed molecular information closer to *A. oryzae* than to *A. flavus*. These results suggest the existence of variance strains between *A. flavus* and *A. oryzae* and provide indirect support for the previous proposal that *A. oryzae* has been domesticated from the naturally occurring *A. flavus* (Kurtzman et al., 1986).

The three misidentified strains in group I have been studied for many years (Strzelecki and Badura, 1972), but the previous research did not focus on taxonomy. According to the description of Klich and Pitt (1988a, b), conidial color and microsopic characteristics strongly indicate that the three strains are *A. parasiticus* (Table 3). The significantly different polymorphic patterns of these three strains from the *A. flavus* or *A. oryzae* strains suggest that AFLP technique can also be applied to differentiate *A. parasiticus* from *A. oryzae*/*A. flavus*. On the other hand, the difference between *A. parasiticus* and *A. sojae* is more clear than between *A. oryzae* and *A. flavus* (Yuan et al., 1995). We therefore expect the AFLP method to be useful in differentiating *A. parasiticus* from *A. sojae.*

The high relatedness between *A. flavus* and *A. oryzae* suggests that the process of differentiating them requires an under-species classification accomplished by a number of different approaches (Olive and Bean, 1999). AFLP is a method for classifying strains, and we have demonstrated in this study its high differentiation power and reproducibility for differentiating *A. flavus* from *A. oryzae*. AFLP should be valuable in distinguishing other closely related species or strains.

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利用 AFLP 指紋分析技術比較 *Aspergillus flavus* 與 *Aspergillus oryzae* 間之差異

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Aspergillus flavus 及 *Aspergillus oryzae* 兩者形態相似且皆屬於 *Aspergillus* section *Flavi* 菌群中，然而 *Aspergillus flavus* 會產生高度致癌的黃麴毒素，*Aspergillus oryzae* 卻是發酵用菌種。在本研究中，我們選擇 13 株 *Aspergillus flavus*，9 株 *Aspergillus oryzae* 及 3 株 *Aspergillus flavus* var. *columnaris*，共 25 株 *Aspergillus* 菌株，利用 AFLP指紋分析技術，來比較這些菌株間的差異性。我們篩選出 3組選擇性引子：*Eco*RI-AC/*Mse*I-CAT, *Eco*RI-TA/ *Mse*I-CAT, *Eco*RI-TA/ *Mse*I-CTT，其所產生的 AFLP 指紋樣式，經由 unweighted pair group method with arithmetic mean（UPGMA）分析後，能夠明確區分出 *A. flavus* 與 *A. oryzae*。從 ATCC 獲得的三株 *A. flavus*，其 AFLP 指紋樣式及形態觀察有別於其它 *A. flavus*，原始命名可能誤判，建議應該更名為 *A. parasiticus*。從本研究結果得知，AFLP 指紋分析技術能夠成功區分出 *A. flavus* 與 *A. oryzae*，是區別類緣關係相近物種的一項利器。

關鍵詞：*Aspergillus flavus*；*Aspergillus oryzae*；AFLP 指紋分析；黃麴菌；米麴菌。