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ORIGINAL PAPER

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# Aflatoxin genes and the aflatoxigenic potential of Koji moulds

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**Abstract** Sixty-four *Aspergillus* isolates, 54 of which originated from food fermentations, and 18 *Aspergillus* reference strains were identified and screened for the presence of aflatoxin genes *afl*R and *omt*-1. Among the Koji moulds, not only *A. oryzae* but also *A. flavus* strains were found. Furthermore, 27% of *A. oryzae* and 93% of *A. flavus* strains lacked either *afl*R or both *afl*R- and *omt*-1. A selection of 29 strains was also checked for the presence of *pks*A and *nor*-1. This revealed large deletions in the aflatoxin gene cluster of some strains. The hybridisation patterns also suggested a polarity in the deletion events, originating in the vicinity of *pks*A and extending towards *omt*-1. Other strains exhibited *Bam*HI restriction fragment length polymorphisms (RFLPs) for either *afl*R or for *afl*R and *omt*-1. All *afl*R and/or *omt*-1 deletion strains turned out to be unable to produce aflatoxin. The RFLP-carrying strains either produced only traces of aflatoxin or none at all. In 73% of the *A. oryzae* strains, no apparent deletions were detected with the *afl*R and *omt*-1 probes. Nevertheless, after incubation in aflatoxin-inducing media, no aflatoxin $B_1$ production could be detected in those *A. oryzae* strains.

## Introduction

It is often stated that *Aspergillus oryzae* and *A. sojae* have been used for centuries in Oriental solid-state fermentations for the production of sake, miso and soy sauce (Baens-Arcega 1995; Klich et al. 1995). Unfortunately, it is rather difficult to distinguish these Koji moulds morphologically from the closely related and potentially aflatoxigenic species *A. flavus* and *A. parasiticus* (Raper and Fennel 1965). Consequently, considerable effort has been spent on molecular identification tools allowing unambiguous distinction between these species (Gomi et al. 1989; Kumeda and Asao 1996). However, it is quite common to find *A. flavus* strains in commercial and artisanal Koji starters (Bhumiratana et al. 1980). For example, both the ATCC and CCCCM collections contain *A. flavus* strains that have been isolated from soy sauce or sake fermentations. To complicate matters even further, the aflatoxin genes *afl*R, *nor*-1, *ver*-1 and *omt*-1 were detected in the Koji moulds *A. oryzae* and *A. sojae* despite the fact that they are not considered to be aflatoxigenic (Klich et al. 1995; Watson et al. 1999). The observation that no aflatoxin producers were found among the *A. oryzae* and *A. sojae* strains maintained at ATCC (Wei and Jong 1986) is consistent with the latter assumption. Additionally, Koji fermentations, and thus the fungi employed, have a well-documented history of safe use (Barbesgaard et al. 1992). One explanation could be that the aflatoxin genes in Koji moulds are simply not functionally expressed. For example, expression of the *afl*R gene product, required for the expression of several aflatoxin biosynthetic genes, has been detected in some *A. oryzae* strains at the mRNA (Watson et al. 1999) or even at the protein level. However, this *A. oryzae* AflR protein seems unable to stimulate transcription of aflatoxin genes (Liu and Chu 1998). Additionally, the short duration and/or the prevailing conditions in Koji fermentation could prevent aflatoxin production.

Aflatoxins are polyketide-derived secondary metabolites, whose production is attributed to three aspergilli from the section *Flavi*: *A. flavus*, *A. parasiticus* and *A. nomius*. In recent years, the biosynthetic pathway has been elucidated and many genes involved in aflatoxin biosynthesis have been cloned and characterised [for a review see (Woloshuk and Prieto 1998)]. Because of the clustering of most aflatoxin biosynthetic genes in a single genomic location (Yu et al. 1995), deletions and rearrangements in the aflatoxin genes are easily deduced from Southern blots. To better understand the safety of Koji fermentations with yellow-green aspergilli (section *Flavi*), we identified Koji mould isolates, assessed the integrity of the aflatoxin gene cluster and assayed their aflatoxigenic potential in an inducing synthetic medium.

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**Table 1** PCR oligonucleotides

| Primer | Sequence 5'- 3' | Target gene |
|---|---|---|
| 1485 | CCCCTACTTGCGCAAACGCAG | *A. flavus* EM162 *omt*–1 |
| 1486 | ATGGCACTACCGAGCAAAGCCG | (Yu et al. 1993) pos. 664–2153 |
| 1487 | ATGGATCCGGTATCCCTGCTGCATCGTC | *A. flavus* EM162 aflR |
| 1488 | AGAGGATCCAGGGCTCCCTGGAGC | (Woloshuk et al. 1994) Position 1–1613 |
| 2445 | GAATTCAACGGATCACTTAGC | *A. parasiticus* ATCC 15517 nor-1 |
| 2446 | GAATTCTAACGAAGTGCCCCG | (Trail et al. 1994) Position 272–1226 |
| A037 | CTCAGACAAGGCGTCCGTGG | *A. parasiticus* ATCC 15517 pksA |
| A038 | GGTGACTCCGTGGAAGACCCC | (Feng and Leonard 1995) Position 1141–2039 |

## Materials and methods

### Strains, identification and growth conditions

Strains were grown in 200 ml of minimal medium (MM) (Pontecorvo et al. 1953) for 21 h at 30°C in an orbital shaker at 200 rpm in 1 l conical flasks. $NaNO_3$ was routinely used as nitrogen source to prevent aflatoxin production by *A. flavus* and *A. parasiticus* (Chang et al. 1993). All strains were classified by a combination of *Sma*I restriction fragment length polymorphisms (RFLPs) in the rRNA gene cluster as described by Gomi et al. (1989) and by reverse pigmentation on Aspergillus differential medium (ADM) as described by Bothast and Fennel 1974). The reverse method, which is claimed to distinguish *A. oryzae* and *A. sojae* from *A. flavus* and *A. parasiticus*, is based on the secretion of aspergillic acid. On ADM containing iron(III)citrate, the aspergillic acid secreted by *A. flavus* and *A. parasiticus* complexes the $Fe^{3+}$ ions and stains the medium under and around the colony bright orange (Assante et al. 1981). Since *A. oryzae* and *A. sojae* do not secrete aspergillic acid, their colonies remain white on ADM. The potential of strains to produce aflatoxin was tested in 250 ml conical flasks containing 100 ml of YS or SLS medium as described by Reddy et al.(1971). For the solid state fermentation, 175 g of a mixture of defatted soybeans (75% w/w) and roasted wheat (25% w/w) were steam-cooked for 7 min at 120°C in an autoclave with closed lid, then allowed to cool to below 40°C. The cooked soybean-wheat mixture was inoculated with $10^9$ cfu of a conidiospore suspension in SP2 (20 mM $KH_2PO_4$-HCl pH 2.0, 0.9% NaCl). The inoculated mixture was transferred to a 3-l mushroom spawn bag (porosity 25 cc/min, filter width 160 mm, Van Leer, UK) and incubated at 30°C in a Lab-Term incubator (Kühner, Switzerland). The temperature inside the Koji bed was monitored not to exceed 34°C.

### Isolation of genomic DNA, Southern analysis and hybridisation

*Aspergillus* genomic DNA was isolated as described by Raeder and Broda (1985) and Southern analysis was performed as described by Sambrook et al. (1989). Gel purified restriction fragments were labelled by the random primed labelling method using a RediPrime kit (Amersham). The rDNA probes were recovered from *A. oryzae* ATCC 11494 and ATCC 17791 as 7.4 kb *Sal*I fragments and cloned into pBluescript $SK^+$. The rDNA positive clones pBO1, pBO2, pBF1 and pBF2 were identified by hybridisation at low stringency conditions (55°C, 4× SSPE, 1% SDS) with the 1.0 kb *Bam*HI fragment of pBMT105, which encompasses a part of the *A. nidulans* 26 S rRNA gene (Borsuk et al. 1982). pBMT105, containing the 1.4 kb *Eco*RI fragment from the *A. nidulans* ribosomal repeat was a generous gift from Dr. T. Goosen, Department of Genetics of the Agricultural University Wageningen, The Netherlands.

### Polymerase chain reaction

The *afl*R (Woloshuk et al. 1994) probe was obtained by PCR on *A. flavus* EM162 genomic DNA with oligonucleotide pair 1487 and 1488 (Table 1) and the *omt*-1 (Yu et al. 1993) probe with primers 1486 and 1485. The *pks*A (Feng and Leonard 1995) probe was amplified from *A. parasiticus* ATCC 15517 with primer pair A037 and A038, and the *nor*-1 (Trail et al. 1994) probe with primer pair 2445 and 2446. To amplify the *nor*-1, aflR, *omt*-1 and *pks*A probes, approximately 75 ng of chromosomal DNA, 25 ng of each oligonucleotide, and 100 µM dNTPs were dissolved in 50 µl PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatine and 0.1% Triton X-100). A pellet of Ampliwax (Roche) was added, the reaction mixtures heated to 80°C for 5 min and then cooled to room temperature. To each reaction 0.1 U of *Taq* polymerase (SuperTaq, Stehelin) was added in 50 µl PCR buffer. The reaction mixtures were subjected to one cycle of 2 min 94°C, 2 min 60°C and 3 min 74°C, followed by 30 cycles of 1 min 94°C, 1 min 60°C and 3 min 74°C and one cycle of 1 min 94°C, 1 min 60°C and 10 min 74°C. All PCR products were recovered from gels using a Qiaquick kit (Qiagen) and directly ligated into the pGEM-T vector (Promega) according to the instructions of the manufacturer. The identity of all amplification products was confirmed on a Licor model 4000 automatic sequencer with IRD800 labelled primers.

### Aflatoxin production and analysis

Approximately 100 ml of SLS medium was inoculated with $10^6$ conidiospores/ml and incubated for 10 days at 30°C with agitation at 200 rpm. The culture was macerated with 100 ml 80% methanol for 2 h, filtered through a filter paper and the filtrate evaporated. The crude extract was recovered with water and this solution was adjusted to pH 3 with 3 N HCl. The aqueous extract was extracted several times with ethyl acetate (v-v). The organic phase was dried with anhydrous $Na_2SO_4$ and evaporated in vacuum (30°C) to remove solvent. The residue was taken up in methanol. The methanolic extracts were analysed by isocratic high-performance liquid chromatography (HPLC) using postcolumn derivatisation with online electrochemically generated bromine and fluorescence detection. A reversed-phase ODS Hypersil column (3 µm, 125 mm ×4.6 mm i.d.) was used along with an ODS Hypersil guard column (3 µm, 25 mm ×4.6 mm i.d.), both from Metrohm-Bischoff (Leonberg, Germany). The mobile phase consisted of a mixture of water:acetonitrile:methanol (60:5:35, by vol.), containing 119 mg potassium bromide and 100 µl nitric acid (65%) per litre. Elution was carried out at a flow rate of 1.0 ml/min and at room temperature. The postcolumn derivatisation system consisted of a KOBRA cell with variable control current source (Rhône Diagnostics Technologies, Glasgow, Scotland). Current setting was adjusted to 100 µA. The individual aflatoxins were monitored with a Waters model 470 scanning fluorescence detector (excitation wavelength 365 nm, emission wavelength 428 nm). The presence of aflatoxins in selected culture supernatants was checked using a more selective method based on an immunoaffinity column clean-up, according to the procedure described by Truckses et al. (1991). Aliquots (1 ml) of methanolic extracts were diluted with 50 ml distilled water and applied to an AflaTest-P immunoaffinity column (Vicam, Watertown, Mass.) containing a monoclonal antibody specific for aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$. The toxins were isolated, purified, and concentrated on the column, removed from antibodies with

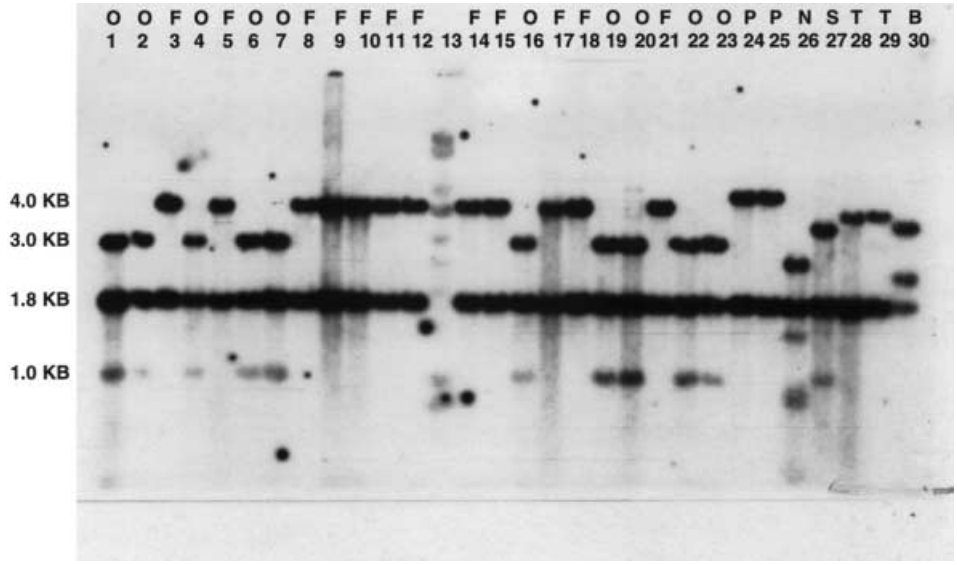


**Fig. 1** Identification of Koji moulds by *Sma*I rDNA restriction fragment length polymorphism (RFLP) analysis. A Southern blot of *Sma*I-digested genomic DNA was hybridised under stringent conditions (65°C; 0.2× SSC, 0.1% SDS) with the 3.4 and 3.9 kb *Pst*I-*Spe*I fragments from pBO1 encompassing the rRNA repeat from *A. oryzae* ATCC 13791. All aspergilli analysed in this study possess a common 1.8 kb rDNA hybridisation signal which, judging from its intensity, is probably composed of two fragments. Additionally, *A. flavus* (*F*) strains have a 4.0 kb rDNA hybridisation signal, while *A. oryzae* (*O*) strains exhibit an extra *Sma*I site splitting the signal into 3.0 and 1.0 kb bands. Similarly, *A. parasiticus* (*P*) strains possess a 4.5 kb rDNA hybridisation signal that in *A. sojae* (*S*) is split into 3.5 and 1.0 kb bands. Both *A. tamarii* (*T*) strains exhibit a 3.8 kb rDNA signal and *A. nomius* (*N*) shows 2.6, 1.6 and 0.8 kb *Sma*I rDNA fragments. The *A. niger* (*B*) *Sma*I digest shows 3.6 kb and 2.3 kb rDNA signals.Lanes: *1 A. oryzae* A1, *2 A. oryzae* A4, *3 A. flavus* A8, *4 A. oryzae* A12, *5 A. flavus* A56, *6 A. oryzae* TK3, *7 A. oryzae* TK4–1, *8 A. flavus* TK7, *9 A. flavus* EM120, *10 A. flavus* EM162, *11 A. flavus* EM176, *12 A. flavus* M56, *13* molecular weight marker (Eurogentec) 10–0.6 kb, *14 A. flavus* ATCC 11493, *15 A. flavus* ATCC 11494, *16 A. oryzae* ATCC 13791, *17 A. flavus* ATCC 20386, *18 A. flavus* ATCC 22787, *19 A. oryzae* ATCC 26666, *20 A. oryzae* ATCC 44193, *21 A. flavus* FRR 2807, *22 A. oryzae* NRRL 3488, *23 A. oryzae* CBS 570.65, *24 A. parasiticus* ATCC 15517, *25 A. parasiticus* FRR 2752, *26 A. nomius* ATCC 15546, *27 A. sojae* ATCC 42251, *28 A. tamarii* ATCC 16865, *29 A. tamarii* EM123, *30 A. niger* CBS 120.49

pure methanol, and quantified by reversed-phase HPLC with post-column derivatisation and fluorescence detection, under the conditions described above.

## Results

### Identification of Koji moulds

A total of 64 *Aspergillus* isolates were subjected to RFLP analysis of the rRNA gene cluster with the restriction enzyme *Sma*I. All strains were also tested for reverse pigmentation on ADM. The results of this analysis are summarised in Table 2. Sixty strains could be unambiguously assigned to either the *A. oryzae* or *A. flavus* group. Four strains were identified as *A. tamarii*. All Koji mould strains with a 4.0/1.8 kb rDNA hybridisation pattern stain orange on ADM, whereas all strains with a 3.0/1.8/1.0 kb rDNA pattern remain white on ADM. This 100% correlation between the two methods unambiguously identified these strains as *A. flavus* and *A. oryzae*, respectively (Fig. 1 and Table 3). As a control, 18 aspergilli from type strain collections were characterised with our classification key. *A. flavus* reference strains FRR 2807 and M 56 were identified as *A. flavus*, and the two *A. parasiticus* type strains FRR 2752 and M 66 as *A. parasiticus*. The classification of *A. oryzae* type strains ATCC 2666, ATCC 44193, NRRL 3485, NRRL 3488, and CBS 570.65 was also confirmed. The type strains *A. nomius* ATCC 15546, *A. sojae* ATCC 42251 and *A. tamarii* ATCC 16865 could easily be distinguished from each other and from the *A. flavus*, *A. oryzae*, *A. sojae* and *A. parasiticus* strains (Fig. 1). However, *A. oryzae* type-strains ATCC 11493, ATCC 11494, ATCC 20386 and ATCC 22787, do possess the *A. flavus* rDNA pattern, yet do not turn orange on ADM (Table 3). Conversely, strain ATCC 13791 has the *A. oryzae* pattern, but turns orange on ADM (Table 3). Therefore, on the basis of our classification key, these five strains cannot be unambiguously assigned to either the *A. oryzae* or the *A. flavus* group. In an attempt to accommodate the five ambiguous ATCC strains into our classification key, we searched for additional RFLPs. To facilitate this search, rDNA from *A. flavus* strain ATCC 11494 and *A. oryzae* strain ATCC 13791 was cloned as 7.4 kb *Sal*I fragments. Restriction analysis of these rDNA clones with *Bam*HI, *Eco*RI, *Eco*RV, *Hin*cII, *Hin*dIII, *Pst*I, *Sac*I, *Spe*I, *Xba*I and *Xho*I failed to reveal additional RFLPs (data not shown).

Most importantly, our data confirm that commercially available Koji starters can either be pure cultures of *A. oryzae* (e.g. strains A4 and A6, Table 2) or *A. flavus* (e.g. A31 and A35, Table 2) as well as a mixture of both (e.g. TK7 and TK8, Table 2).

**Table 2** Mould strains analysed

| Strain | Source | SmaI rDNA | Colour on ADM | Identification | *omt*-1 | *afl*R |
|---|---|---|---|---|---|---|
| A1 | Tane Koji | 3.0/1.0 | White | *A. oryzae* | + | + |
| A4 | Sake koji | 3.0/1.0 | White | *A. oryzae* | – | – |
| A6 | Sake koji | 3.0/1.0 | White | *A. oryzae* | + | + |
| A7 | Sake rice koji | 3.0/1.0 | White | *A. oryzae* | – | – |
| A8 | Sake rice koji | 4.0 | Orange | *A. flavus* | + | – |
| A10 | Tane koji Nikken foods D1 | 4.0 | Orange | *A. flavus* | + | – |
| A12 | Tane koji Nikken foods D2 | 3.0/1.0 | White | *A. oryzae* | + | + |
| A19 | Miso Tane Koji BF1 | 4.0 | Orange | *A. flavus* | + | – |
| A20 | Miso Tane Koji BF1 | 4.0 | Orange | *A. flavus* | + | – |
| A21 | Tane Koji Diamond C | 3.0/1.0 | White | *A. oryzae* | + | + |
| A27 | Tane Koji Diamond C | 3.0/1.0 | White | *A. oryzae* | + | + |
| A28 | Tane Koji Diamond C | 4.0 | Orange | *A. flavus* | + | – |
| A29 | Tane Koji Diamond C | 4.0 | Orange | *A. flavus* | + | – |
| A30 | Tane Koji Three Diamond | 4.0 | Orange | *A. flavus* | + | – |
| A31 | Tane Koji Ichimurasaki | 4.0 | Orange | *A. flavus* | + | – |
| A32 | Tane Koji Ichimurasaki | 4.0 | Orange | *A. flavus* | + | – |
| A34 | Contaminant | 3.0/1.0 | White | *A. oryzae* | + | + |
| A35 | Tane Koji Super Ichimurasaki | 4.0 | Orange | *A. flavus* | + | – |
| A36 | Tane Koji | 4.0 | Orange | *A. flavus* | + | – |
| A37 | Tane Koji | 4.0 | Orange | *A. flavus* | + | – |
| A38 | Tane Koji | 4.0 | Orange | *A. flavus* | + | – |
| A39 | Tane Koji | 3.0/1.0 | White | *A. oryzae* | + | + |
| A40 | Tane Koji | 3.0/1.0 | White | *A. oryzae* | + | + |
| A41 | Tane Koji | 4.0 | Orange | *A. flavus* | + | – |
| A42 | Tane Koji | 4.0 | Orange | *A. flavus* | + | – |
| A43 | Tane Koji | 3.0/1.0 | White | *A. oryzae* | – | – |
| A44 | Tane Koji | 4.0 | Orange | *A. flavus* | + | – |
| A45 | Tane Koji | 3.0/1.0 | White | *A. oryzae* | + | + |
| A46 | Tane Koji | 4.0 | Orange | *A. flavus* | + | – |
| A47 | Tane Koji | 4.0 | Orange | *A. flavus* | + | – |
| A48 | Tane Koji | 4.0 | Orange | *A. flavus* | + | – |
| A50 | Tane Koji Super Barley | 3.0/1.0 | White | *A. oryzae* | + | + |
| A51 | Tane Koji Super Barley | 3.0/1.0 | White | *A. oryzae* | – | – |
| A52 | Tane Koji Super Brilliant Miso | 3.0/1.0 | White | *A. oryzae* | + | + |
| A53 | Tane Koji Super Brilliant Miso | 3.0/1.0 | White | *A. oryzae* | – | – |
| A54 | Tane Koji Super Brilliant Miso | 4.0 | Orange | *A. flavus* | + | – |
| A55 | Tane Koji Super Brilliant Miso | 4.0 | Orange | *A. flavus* | + | – |
| A56 | Tane Koji Super Brilliant Miso | 4.0 | Orange | *A. flavus* | +RFLP | – |
| TK 3 | Miso | 3.0/1.0 | White | *A. oryzae* | – | – |
| TK 4–1 | Tane Koji | 3.0/1.0 | White | *A. oryzae* | + | + |
| TK 4–2 | Tane Koji | 3.0/1.0 | White | *A. oryzae* | + | + |
| TK 7 | Soy sauce Tane Koji Singapore | 4.0 | Orange | *A. flavus* | + | – |
| TK 8 | Soy sauce Tane Koji Singapore | 3.0/1.0 | White | *A. oryzae* | + | + |
| FJ 2 | Miso | 3.0/1.0 | White | *A. oryzae* | + | + |
| FJ 3 | Miso | 3.0/1.0 | White | *A. oryzae* | – | – |
| FJ 5 | Tane Koji | 3.0/1.0 | White | *A. oryzae* | + | + |
| EM 2 | Manna (Nepal) | 4.0 | Orange | *A. flavus* | + | + |
| EM 3 | Tane Koji | 4.0 | Orange | *A. flavus* | + | – |
| EM 4 | Tane Koji | 3.0/1.0 | White | *A. oryzae* | + | + |
| EM 5 | Tane Koji | 4.0 | Orange | *A. flavus* | + | – |
| EM 6 | Tapai pulut | 4.0 | Orange | *A. flavus* | – | – |
| EM 7 | Tapai pulut | 3.0/1.0 | White | *A. oryzae* | + | + |
| EM 10 | Laboratory contaminant | 4.0 | Orange | *A. flavus* | + | – |
| EM 20 | Manna (Nepal) | 3.0/1.0 | White | *A. oryzae* | + | + |
| EM 145 | Tane Koji | 4.0 | Orange | *A. flavus* | + | – |
| EM 258 | Tane Koji | 4.0 | Orange | *A. flavus* | + | – |
| EM 120 | Indonesian processed peanut presscake | 4.0 | Orange | *A. flavus* | + | – |
| EM 122 | Indonesian processed peanut presscake | 3.6 | White | *A. tamarii* | – | – |
| EM 123 | Indonesian processed peanut presscake | 3.6 | White | *A. tamarii* | – | – |
| EM 161 | Philippines soybean | 3.6 | Brown | *A. tamarii* | – | – |
| EM 162 | Philippines soybean | 4.0 | Orange | *A. flavus* | +RFLP | +RFLP |
| EM 176 | Sundried red oncom | 4.0 | Orange | *A. flavus* | + | – |
| EM 177 | Sundried red oncom | 4.0 | Orange | *A. flavus* | + | + |
| EM 184 | Fermenting groundnuts | 4.0 | Orange | *A. flavus* | + | – |
| EM 193 | Philippines soybean | 3.6 | Brown | *A. tamarii* | – | – |

**Table 3** Reference strains

| Strain | Name | SmaI rDNA | Colour on ADM | Name | *omt*-1 | *afl*R |
|---|---|---|---|---|---|---|
| ATCC 11493 | *A. oryzae* | 4.0 | White | *A. flavus*? | + | – |
| ATCC 11494 | *A. oryzae* | 4.0 | White | *A. flavus*? | + | + |
| ATCC 13791 | *A. oryzae* | 3.0/1.0 | Orange | *A. oryzae*? | + | – |
| ATCC 20386 | *A. oryzae* | 4.0 | White | *A. flavus*? | + | + |
| ATCC 22787 | *A. oryzae var. brunneus* | 4.0 | White | *A. flavus*? | +RFLP | +RFLP |
| ATCC 26666 | *A. oryzae* | 3.0/1.0 | White | *A. oryzae* | – | – |
| ATCC 44193 | *A. oryzae* | 3.0/1.0 | White | *A. oryzae* | + | + |
| ATCC 16865 | *A. tamarii* | 3.6 | White | *A. tamarii* | – | – |
| ATCC 44251 | *A. sojae* | 3.4/1.0 | White | *A. sojae* | + | + |
| NRRL 3485 | *A. oryzae* | 3.0/1.0 | White | *A.oryzae* | + | + |
| NRRL 3488 | *A. oryzae* | 3.0/1.0 | White | *A. oryzae* | + | + |
| CBS 570.65 | *A. oryzae* | 3.0/1.0 | White | *A. oryzae* | + | + |
| FRR 2807 | *A. flavus* | 4.0 | Orange | *A. flavus* | +RFLP | +RFLP |
| M 56 | *A. flavus* | 4.0 | Orange | *A. flavus* | +RFLP | + |
| M 66 | *A. parasiticus* | 4.4 | Orange | *A. parasiticus* | + | + |
| FRR 2757 | *A. parasiticus* | 4.4 | Orange | *A. parasiticus* | + | + |
| ATCC 15517 | *A. parasiticus* | 4.4 | Orange | *A. parasiticus* | + | + |
| ATCC 15546 | *A. nomius* | 2.6/1.5/0.8 | NT[a] | *A. nomius* | + | + |

[a] Not tested

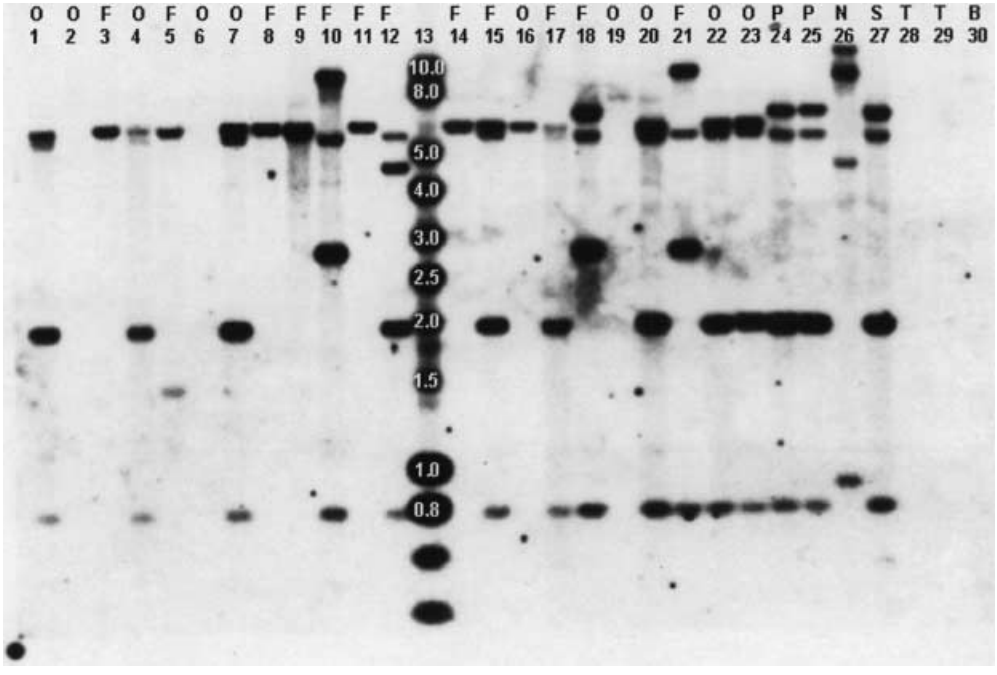


**Fig. 2** Southern blot analysis of the aflatoxin gene cluster in Koji moulds. A Southern blot of *Bam*HI-digested genomic DNA was simultaneously hybridised under non-stringent conditions (55°C; 5× SSC; 1.0% SDS) with *afl*R- *omt*-1- *nor*-1- and *pks*A-specific probes. As a control, blots were also hybridised with these probes individually (data not shown). Most *A. oryzae* (*O*), *A. flavus* (*F*), *A. parasiticus* (*P)* and *A. sojae* (*S*) strains exhibit *afl*R- *pks*A- and *nor*-1-hybridisation signals of 2.5; 0.8 and 5.8 kb, respectively(*lanes 1, 4, 7, 15, 17, 20, 22, 23, 24, 25 and 27*). Deviating hybridisation signals in *A. oryzae* and *A. flavus* were interpreted as RFLPs. In most *A. oryzae* and *A. flavus* strains a 6.0 kb *omt*-1 signal can be seen (*lanes 1, 3, 4, 5, 7, 8, 9, 11, 14, 15, 16, 17, 20, 22 and 23*), while both *A. para*siticus and *A. sojae* possess a 6.5 kb *omt*-1 signal (*lanes 24, 25 and 27*). *A. nomius* ATCC 16546 (*N*) (*lane 26*) gave a 0.9 kb *pks*A; a 4.5 kb *afl*R; a 9 *omt*-1 and an 11 kb *nor*-1 hybridisation signal. In *A. tamarii* (*T*) (*lanes 28 and 29*) no signals are detected. In *A. niger* (*B*) (*lane 30*), a very weak 9.0 kb *pks*A signal was obtained.Lanes: *1 A. oryzae* A1, *2 A. oryzae* A4, *3 A. flavus* A8, *4 A. oryzae* A12, *5 A. flavus* A56, *6 A. oryzae* TK3, *7 A. oryzae* TK4–1, *8 A. flavus* TK7, *9 A. flavus* EM120, *10 A. flavus* EM162, *11 A. flavus* EM176, *12 A. flavus* M56, *13* molecular weight marker (Eurogentec) 10–0.6 kb, *14 A. flavus* ATCC 11493, *15 A. flavus* ATCC 11494, *16 A. oryzae* ATCC 13791, *17 A. flavus* ATCC 20386, *18 A. flavus* ATCC 22787, *19 A. oryzae* ATCC 26666, *20 A. oryzae* ATCC 44193, *21 A. flavus* FRR 2807, *22 A. oryzae* NRRL 3488, *23 A. oryzae* CBS 570.65, *24 A. parasiticus* ATCC 15517, *25 A. parasiticus* FRR 2752, *26 A. nomius* ATCC 15546, *27 A. sojae* ATCC 42251, *28 A. tamarii* ATCC 16865, *29 A. tamarii* EM123, *30 A. niger* CBS 120.49

## Deletions in the aflatoxin gene cluster

To determine whether mutations in aflatoxin genes in Koji moulds are a widespread phenomenon, Southern blots of *Bam*HI-digested genomic DNA from all strains were probed with the *afl*R and *omt*-1 PCR fragments. Additionally, 29 strains, mostly showing abnormalities in the first screen, were checked for the presence of *pks*A and *nor*-1 (Fig. 2). For most *A. oryzae* and *A. flavus* strains, *pks*A, *afl*R and *nor*-1 hybridisation signals of 0.8, 2.5 and 5.8 kb, respectively, were obtained. Therefore, hybridisation signals in *A. oryzae* and *A. flavus* deviating from these sizes were interpreted as RFLPs. In most *A. oryzae* and *A. flavus* strains a 6.0 kb *omt*-1 signal was obtained, while in *A. parasiticus* and *A. sojae* a 6.5 kb *omt*-1 signal appeared. On the basis of these *omt*-1 signals, *A. oryzae* and *A. flavus* can be distinguished from *A. sojae* and *A. parasiticus*. *A. nomius* ATCC 16546 gave 0.9, 4.5, 11 and 9 kb hybridisation signals for *pks*A, *afl*R, *nor*-1 and *omt*-1, respectively. Therefore, *A. nomius* is easily distinguishable from all other species. In contrast, no signals at all were obtained in *A. tamarii*. Under these non-stringent conditions, only a faint 9 kb *pks*A signal is apparent in *A. niger*. However, this is probably cross-hybridisation to a polyketide synthase-encoding gene not involved in aflatoxin biosynthesis.

We can summarise the results of our screening as follows: in 93% of the *A. flavus* Koji mould strains, but in only 27% of the *A. oryzae* strains, the absence of *afl*R could be demonstrated. In 27 out of the 29 *A. flavus* strains lacking the *afl*R gene, we found a normal *omt*-1 signal. Of the two remaining *A. flavus afl*R deletion strains, one exhibited an *omt*-1 RFLP and the other completely lacked *omt*-1. In contrast, the *A. oryzae* strains either possessed both *afl*R and *omt*-1 or lacked both genes.

Furthermore, four *A. flavus* strains exhibited *Bam*HI RFLPs for either one or both aflatoxin genes (Tables 2, 3). In contrast, no *A. oryzae* strains were found that possessed *Bam*HI RFLPs for *afl*R and/or *omt*-1. From those that could not be identified as either *A. oryzae* or *A. flavus* (Table 3), two strains (ATCC 11494 and 20386) contained both *afl*R and *omt*-1, two strains (ATCC 11493 and 13791) lacked *afl*R, but possessed *omt*-1 and one (ATCC 22787) exhibited a *Bam*HI RFLP for both aflatoxin genes.

Among the selected 29 strains, all strains lacking *afl*R had also lost both *pks*A and *nor*-1, which suggests the presence of large continuous deletions in the aflatoxin gene cluster. Additionally, neither in *A. oryzae* nor in *A. flavus* were strains found that lacked *omt*-1 but retained the *afl*R gene. As reported by Kusumoto and co-workers (2000), this strongly suggests that the deletions in the aflatoxin gene cluster do not occur randomly, but originate in the vicinity of the *pks*A locus and progress towards the *omt*-1 locus.

## Deletions in the aflatoxin gene cluster lead to an inability to produce aflatoxin

To determine the effect of the *afl*R and *omt*-1 mutations on the aflatoxigenic potential of these moulds, we grew several strains and two positive control strains in the aflatoxin-inducing SLS medium (Reddy et al. 1971). After 10 days of incubation, the culture broth was analysed for the presence of the aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$. The data from this experiment (Table 4) show that a deletion of either or both of the aflatoxin genes results in absence of detectable levels of aflatoxin. The presence of a RFLP in either the *afl*R or in both the *afl*R and *omt*-1 genes leads to a million-fold drop in aflatoxin production or even the complete absence of detectable aflatoxin levels. If we extrapolate these results, we can conclude that 63% of the Koji isolates should be incapable of aflatoxin production, because of deletions in the relevant gene cluster.

## Can Koji moulds produce aflatoxin?

Since most *A. oryzae* strains possessed *afl*R and *omt*-1 hybridisation signals indicating integrity of the aflatoxin gene cluster, we tested 14 of these strains for aflatoxin production in SLS and YS medium. After 10 days, the culture broths of *A. oryzae* A1, A12, A21, A27, A34, A39, A40, A45, A50, A52, TK 4–1, TK8, FJ2 and FJ5

**Table 4** Aflatoxin production of strains with mutations in the aflatoxin gene cluster

| Strain[a] | Species | Genotype | B1[b] | B2 | G1 | G2 |
|---|---|---|---|---|---|---|
| EM 2 | *A. flavus* | *pks*A+, *nor*–1+, *afl*R+, *omt*–1+ | ND[c] | ND | ND | ND |
| TK 7 | *A. flavus* | *pks*A-, *nor*–1-, *afl*R-, *omt*–1+ | ND | ND | ND | ND |
| ATCC 26666 | A. oryzae | *pks*A-, *nor*–1-, *afl*R-, *omt*–1– | ND | ND | ND | ND |
| FRR 2807 | *A. flavus* | *pks*A+, *nor*–1+, *afl*R RFLP, *omt*–1RFLP | ND | ND | ND | ND |
| M 56 | *A. flavus* | *pks*A+, *nor*–1+, *afl*R+, *omt*–1RFLP- | 3.8 | 0.2 | 0.3 | <0.1 |
| ATCC 11493 | ? | *pks*A-, *nor*–1-, *afl*R-, *omt*–1+ | ND | ND | ND | ND |
| EM 176 | *A. flavus* | *pks*A-, *nor*–1-, *afl*R-, *omt*–1+ | ND | ND | ND | ND |
| EM 162 | *A. flavus* | *pks*A+ *nor*–1+, *afl*R RFLP, *omt*–1RFLP | 6.9 | 0.1 | ND | ND |
| FRR 2752 | *A. parasiticus* | *pks*A+, *nor*–1+, *afl*R+, *omt*–1+ | $3.393.10^3$ | $187.10^3$ | $21600.10^3$ | $1308.10^3$ |
| ATCC 15517 | *A. parasiticus* | *pks*A+, *nor*–1+, *afl*R+, *omt*–1+ | $6877.10^3$ | $577.10^3$ | $27726.10^3$ | $1840.10^3$ |

[a] All strains were grown in SLS medium and aflatoxin levels in the medium were determined as described in the Materials and Methods section

[b] The concentration of each aflatoxin is indicated in ng aflatoxin/ml medium

[c] Not detected

were extracted and analysed for the presence of the major aflatoxin $B_1$. Aflatoxins were not detected in any of these *A. oryzae* strains, either by HPLC or by affinity chromatography. In contrast, *A. parasiticus* ATCC 15517, serving as a positive control, produced 5.6 µg aflatoxin B1/ml SLS medium. This clearly demonstrates that in these *A. oryzae* strains, despite the absence of visible deletions, the aflatoxin gene cluster is not functional.

It has been suggested that Koji fermentation conditions are unfavourable for aflatoxin biosynthesis, thus providing inherent safety to the process. To test this hypothesis, we inoculated a mixture of soybeans and roasted wheat with $10^9$ cfu of *A. parasiticus* ATCC 15517. The ratio of soybeans to roasted wheat in this mixture (3:1 w/w) is very similar to the substrate for traditional soy sauce production. After 48 h, the standard duration of Koji fermentation (Baens-Arcega 1995), the Koji was extracted and analysed for aflatoxin $B_1$. Under these fermentation conditions, *A. parasiticus* produces 0.64 µg/g Koji of aflatoxin $B_1$. This suggests that either the 48 h incubation time is too short or that the conditions in solid state fermentation are unfavourable for aflatoxin production. Therefore, in a second solid state fermentation with *A. parasiticus* ATCC 15517 we extended the incubation time to 7 days. In this experiment, the aflatoxin $B_1$ level reached 5.45 µg/g Koji. From these data, we concluded that Koji fermentation conditions per se do not inhibit aflatoxin production, but that the short duration (24 h) of such fermentations prevents their accumulation.

## Discussion

Among 54 Koji mould isolates, we found 29 *A. flavus* strains, contradicting the assertion that only *A. oryzae* and *A. sojae* are used in food fermentations. This food application of both species renders taxonomists' attempts to distinguish the potential aflatoxin producer *A. flavus* from the "food-grade" *A. oryzae* (Gomi et al. 1989; Kumeda and Asao 1996) of limited practical use. In fact, prevention of regulatory confusion (i.e. preventing "generally regarded as safe" (GRAS) moulds from obtaining the same taxonomic status as aflatoxigenic moulds) was an important argument for maintaining *A. oryzae*, *A. flavus*, *A. sojae* and *A. parasiticus* as distinct species (Samson and Frisvad 1991). However, based on a multilocus RFLP analysis and sequence comparisons, Geiser and co-workers (1998) concluded that *A. oryzae* is part of a monophyletic *A. flavus* clade. The subtle morphological distinctions between *A. oryzae* and *A. flavus* were attributed to the strong selective pressure associated with domestication. Similar hypotheses for both the *A. flavus*/*A. oryzae* and the *A. parasiticus*/*A. sojae* group, though based on less conclusive data, have been previously put forward by Wicklow (1984), Kurtzman (1993) and Yamatoya et al. (1990). Our inability to unambiguously assign all strains to either group, the absence of additional rDNA RFLPs, and the similarities in the aflatoxin hybridisation patterns, are fully compatible with Geiser's domestication hypothesis. We therefore suggest that *A. oryzae* be reduced to a variant of *A. flavus* and that both be accepted as Koji moulds. The only remaining issue would then be absence of aflatoxin production.

Since 30–40% of *A. flavus* strains are aflatoxigenic (Wei and Jong 1986), the well-established safety of Koji fermentations requires some additional explanation. First of all, our data show that the short duration of Koji fermentation prevents the accumulation of high levels of toxin, even if an aflatoxigenic strain were used. Secondly, in all Koji moulds tested the aflatoxin gene cluster is not functional.

We found that 93% of the *A. flavus* strains isolated from Koji starters carry deletions in the aflatoxin gene cluster, rendering them incapable of aflatoxin production. Since *pks*A, *nor*-1 *afl*R and *omt*-1 belong to the same gene cluster (Yu et al. 1995), it is likely that the deletion of these genes would be the result of a single event. Therefore, we can assume that in quadruple deletion strains, like ATCC 26666 or TK 3, all the genes between *pks*A and *omt*-1 have also been lost. According to the physical map of the aflatoxin gene cluster (Woloshuk and Prieto 1998), this would mean that *pks*A/*omt*-1 deletion strains would have also lost the genes *nor*-1, *fas*-1, *fas*-2, *afl*R, *afl*J *adh*A, *nor*A, *ver*-1A, *afl*S, *avn*A, *ver*B and *ord*2, resulting in a genomic deletion of at least 50 kb.

Unfortunately, the interpretation of the effect of *afl*R and *omt*-1 RFLPs on aflatoxin biosynthesis is less straightforward. RFLPs can be caused by single bp substitutions or deletions, but can equally well signify the presence of more extensive rearrangements in the aflatoxin gene cluster. This could be reflected in the observation that a RFLP for both *afl*R and *omt*-1 in *A. flavus* FRR 2807 is linked to a complete absence of toxin production and that the *omt*-1 RFLP in *A. flavus* M 56 results in a million-fold reduction in aflatoxin production.

Surprisingly, for the majority of *A. oryzae* strains we obtained hybridisation patterns indicative of the absence of large deletions in the aflatoxin gene cluster. Nevertheless, even using sensitive detection methods, we were unable to detect any aflatoxin production. Obviously, our present analysis can neither detect small lesions (e.g. the amber mutation which truncates the *afl*R gene product in some *A. oryzae* and *A. sojae* strains (Watson et al. 1999), nor exclude mutations upstream of *omt*-1 or even outside the aflatoxin gene cluster. Northern or, even better, Western analysis would allow detection of aflatoxin gene expression, although would fail to detect functionality of the gene products. Consequently, for the majority of *A. oryzae* Koji moulds, the absence of aflatoxin production remains unexplained. Therefore, we suggest that the strain breeding of Koji moulds, irrespective of the species, should be limited to those strains containing deletions in the aflatoxin gene cluster. From a safety point of view, the use of *pks*A deletion strains would be very desirable. For such deletion strains not only aflatoxin production but also synthesis of its precursors [e.g. sterigmatocystin (Brown et al. 1996)] can be excluded. Fur-

thermore, our observation that deletions always seem to involve the same loci (e.g. *pks*A) would also prevent complementation of aflatoxin production in the course of strain breeding by protoplast fusion (Ushijima and Nakadai 1987) of two different strains with desirable characteristics.

Finally, the mutation frequency in the aflatoxin gene cluster of the *A. flavus* Koji moulds is significantly higher than expected from the 30–40% of *A. flavus* isolates reported to be aflatoxigenic (Wei and Jong 1986). This suggests that a bias towards the loss of these genes exists either in the Koji environment or during strain breeding.

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