Comparison of the *aflR* gene sequences of strains in *Aspergillus* section *Flavi*

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Aflatoxins are polyketide-derived secondary metabolites produced by *Aspergillus parasiticus*, *Aspergillus flavus*, *Aspergillus nomius* and a few other species. The toxic effects of aflatoxins have adverse consequences for human health and agricultural economics. The *aflR* gene, a regulatory gene for aflatoxin biosynthesis, encodes a protein containing a zinc-finger DNA-binding motif. Although *Aspergillus oryzae* and *Aspergillus sojae*, which are used in fermented foods and in ingredient manufacture, have no record of producing aflatoxin, they have been shown to possess an *aflR* gene. This study examined 34 strains of *Aspergillus* section *Flavi*. The *aflR* gene of 23 of these strains was successfully amplified and sequenced. No *aflR* PCR products were found in five *A. sojae* strains or six strains of *A. oryzae*. These PCR results suggested that the *aflR* gene is absent or significantly different in some *A. sojae* and *A. oryzae* strains. The sequenced *aflR* genes from the 23 positive strains had greater than 96.6% similarity, which was particularly conserved in the zinc-finger DNA-binding domain. The *aflR* gene of *A. sojae* has two obvious characteristics: an extra CTCATG sequence fragment and a C to T transition that causes premature termination of AFLR protein synthesis. Differences between *A. parasiticus*/*A. sojae* and *A. flavus*/*A. oryzae* *aflR* genes were also identified. Some strains of *A. flavus* as well as *A. flavus* var. *viridis*, *A. oryzae* var. *viridis* and *A. oryzae* var. *effuses* have an *A. oryzae*-type *aflR* gene. For all strains with the *A. oryzae*-type *aflR* gene, there was no evidence of aflatoxin production. It is suggested that for safety reasons, the *aflR* gene could be examined to assess possible aflatoxin production by *Aspergillus* section *Flavi* strains.

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INTRODUCTION

Aflatoxins are potent carcinogenic compounds that occur naturally as secondary metabolites produced by some strains of *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nomius* and *Aspergillus tamaraii*. These aflatoxin-producing species, and the non-aflatoxigenic species *Aspergillus oryzae* and *Aspergillus sojae*, which are widely used in industry, especially for oriental food fermentation, belong to *Aspergillus* section *Flavi*. In particular, *A. flavus* and *A. parasiticus* have numerous phenotypic similarities to *A. oryzae* and *A. sojae*, respectively (Kurtzman *et al.*, 1986; Klich & Pitt, 1988). The primary procedure employed to identify isolates of *Aspergillus* section *Flavi* depends on their morphologies (Murakami *et al.*, 1982); various molecular-based methods (Yuan *et al.*, 1995; Kumeda & Asao, 1996; Geiser *et al.*, 1998; Lee *et al.*, 2004) have also been used to differentiate closely related

Aspergillus section *Flavi* strains. However, it is still difficult to identify these species.

Aflatoxin contamination of agricultural commodities, such as maize, peanuts and cottonseed, is a serious risk to human and animal health, and has a significant economic impact. Over 30 years of investigations into the aflatoxin biosynthetic pathway has identified more than 20 enzymes involved. Most of the aflatoxin-related genes are clustered within a 75 kb region of the genome (Trail *et al.*, 1995; Yu *et al.*, 1995; Woloshuk & Prieto, 1998). The *aflR* gene, which regulates these clustered genes, has been identified in *A. flavus*, *A. parasiticus*, *A. sojae* and *A. oryzae*. The predicted AFLR protein contains a GAL4-type zinc-finger motif that transcriptionally activates most of the structural pathway genes, such as *ver-1* and *nor-1* (Woloshuk *et al.*, 1994; Trail *et al.*, 1995; Ehrlich *et al.*, 1999a). Notably, although there is no evidence of aflatoxin production by the non-aflatoxin-producing fungi *A. oryzae* and *A. sojae*, some genes (*nor-1*, *ver-1*, *omt-A* and *aflR*) needed for aflatoxin biosynthesis are present, but not expressed, in these fungi (Woloshuk *et al.*, 1994; Klich *et al.*, 1995, 1997; Kusumoto *et al.*, 1998a; Watson *et al.*, 1999). The sequence variability of a region of the *aflR* gene has been studied in a few strains of *A. flavus*, *A.*

Abbreviations: BCRC, Bioresource Collection and Research Center; ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the *Aspergillus* section *Flavi* *aflR* gene sequences reported in this paper are AY650922–AY650944.

parasiticus, *A. oryzae* and *A. sojae*; however, these studies have not achieved a high degree of differentiation for *Aspergillus* section *Flavi* strains (Chang *et al.*, 1995a).

The goal of this investigation was to elucidate whether a common but specific sequence variability occurs within the entire *aflR* gene of the non-aflatoxin-producing species *A. oryzae* and *A. sojae* of *Aspergillus* section *Flavi*. This study performs a sequence comparison of the entire *aflR* gene for strains of *Aspergillus* section *Flavi* and examines the potential significance of the differences in the *aflR* sequences and the encoded AFLR protein. It was found that a particular sequence variability differentiates some species in *Aspergillus* section *Flavi* and can be used to identify non-functionality of the AFLR protein.

METHODS

Fungal strains and culture conditions. The fungal strains employed in this investigation (Table 1) were collected at the Bio-resource Collection and Research Center (BCRC) at the Food Industry Research and Development Institute (FIRDI), Taiwan. The mycelial mats were prepared for DNA extraction by harvesting spore suspensions from approximately 7-day-old slants and inoculating these suspensions into 50 ml potato dextrose broth (PDB) in 250 ml flasks, which were incubated at 25 °C for 48–72 h. Mycelial masses were collected by suction filtration, ground to fine powder in liquid nitrogen and stored at –80 °C.

Preparation of fungal genomic DNA. Each 20 mg of mycelial powder in a 1.5 ml centrifuge tube was resuspended in 600 µl extraction buffer (100 mM Tris/HCl, pH 8.0, 100 mM NaCl, 20 mM EDTA, 2 % SDS) and incubated at 60 °C for 15 min. Mycelial lysates were extracted from mycelial masses and combined with an equal volume of phenol/chloroform (1:1). After mixing and centrifugation for 5 min at 12 500 g, the aqueous phase was collected. After a chloroform/isoamyl alcohol (24:1) extraction, the sample was centrifuged as previously described. Nucleic acids were precipitated using 0.7 vol. 2-propanol and redissolved in sterilized distilled water after drying. The RNA was removed by incubation with DNase-free RNase at 37 °C for 1 h. Agarose gel electrophoresis (0.7 % agarose in 1 × Tris/borate/EDTA) was used to evaluate DNA integrity. The DNA concentration was measured with a spectrophotometer (model U-2001, Hitachi).

PCR amplification of *aflR* gene fragments. To sequence each *aflR* gene, roughly 1700 bp with the ORF, 200 bp upstream and 160 bp downstream was amplified from genomic DNA using four primer pairs: F1/R1, F2/R2, F3/R3 and F4/R4 (MD Bio Inc., Taipei, Taiwan or DNAFax Inc., Taipei, Taiwan) (Table 2, Fig. 1). The primer sequences correspond to those of *A. parasiticus aflR* cDNA (GenBank accession no. L26222). The PCR reactions (50 µl) contained 0.2 mg genomic DNA as template, deoxynucleoside triphosphates at 0.2 mM each, primers at 100 pmol each, 1.1 U DNA polymerase and 1 × reaction buffer. The PCR reactions were performed with a model 9600 DNA thermal cycler (Applied Biosystems) programmed as follows: initial denaturation at 94 °C for 2 min followed by 30 cycles at 94 °C for 10 s, 60 °C for 30 s and 72 °C for 2 min. A final extension at 72 °C for 5 min was performed at the end of amplification. Alternatively, primer pairs F5/R5 and F6/R6 (Table 2) were employed to amplify the *aflR* gene at the lower annealing temperature of 55 °C. The internal transcribed spacer (ITS) fragment of the rRNA gene was amplified using primers ITS3 and ITS4 (White *et al.*, 1990) as a control for the PCR reaction.

Each PCR product was analysed by electrophoresis on a 1.5 % agarose gel in 1 × Tris/borate/EDTA to measure the amplified fragment size.

DNA sequencing and analysis. The PCR products were purified with a High Pure PCR Product Purification kit (Boehringer Mannheim). Sequence reactions were conducted with an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and analysed by electrophoresis on an ABI 373A DNA sequencer (Applied Biosystems). Sequence ambiguities were identified by comparison with their opposite strand sequences. Sequences of the four fragments (F1–R1, F2–R2, F3–R3 and F4–R4) were assembled to yield the entire *aflR* gene sequence; the *aflR* sequences of each strain were aligned using CLUSTAL X 1.83 (Thompson *et al.*, 1997). The alignment of all sequences was visually assessed and optimized when necessary. Phylogenetic evaluation was accomplished by applying a neighbour-joining program from the PHYLIP 3.63 package (Felsenstein, 2004). Phylogenetic trees were rooted with *Aspergillus pseudotamarii* (Ehrlich *et al.*, 2003). Finally, a bootstrap analysis with 1000 replications was performed. Trees were viewed using TreeView (Page, 1996).

RESULTS AND DISCUSSION

Although *A. oryzae* and *A. sojae*, used traditionally in the production of fermented foods such as rice wine and soy sauce, have no record of producing aflatoxin, aflatoxin-synthesis genes are nevertheless present in many strains of these moulds (Woloshuk *et al.*, 1994; Chang *et al.*, 1995a; Klich *et al.*, 1995, 1997; Kusumoto *et al.*, 1998a; Watson *et al.*, 1999). Therefore, there is concern regarding the potential expression of aflatoxin genes from *A. oryzae* and *A. sojae* under certain conditions, since such expression could cause severe health problems. The food industry requires strong evidence to prove the safety of the *A. oryzae* and *A. sojae* strains used in food production. The *aflR* gene is the principal regulator of the aflatoxin production pathway (Chang *et al.*, 1993; Payne *et al.*, 1993; Woloshuk *et al.*, 1994). Previous research has demonstrated that the AFLR protein can bind the promoter region of each aflatoxin synthesis gene and activate gene expression (Woloshuk *et al.*, 1994; Trail *et al.*, 1995; Ehrlich *et al.*, 1999b). In addition, the *aflR* gene has an auto-regulation function (Chang *et al.*, 1995b). Absence of the *aflR* gene or the presence of an abnormal *aflR* gene would be a strong indicator that a strain cannot produce aflatoxin. In this study, 34 strains of *Aspergillus* section *Flavi* were selected based upon diversity of source and origin, and aflatoxigenic capability (Wei & Jong, 1986); the type strain for each species was also included. The identification of each strain was confirmed by morphological observations.

Some strains of *A. oryzae* and *A. sojae* have no *aflR* gene

The *aflR* gene was amplified successfully for 23 out of the 34 strains of *Aspergillus* section *Flavi* (Table 1) tested. The obtained sequences were deposited at NCBI under accession nos AY650922–AY650944. However, five strains of *A. sojae* (BCRC 30419, 30431, 31200, 32265 and 38021) and six strains of *A. oryzae* (BCRC 32268, 32269, 30237, 31646, 31659 and 31658) did not generate any PCR products for

Table 1. Strains of *Aspergillus* section *Flavi* tested in this study

Species	BCRC no. (alternative strain no.)	Existence of <i>aflR</i> PCR fragment†	Source	Production	Aflatoxin production‡
<i>A. parasiticus</i>	33603* (IMI 015957)	+			—
	30150 (ATCC 16869)	—			+
	30164 (ATCC 26691)	+	Peanut, Uganda		+
	30110 (ATCC 26768)	+	Dry Cracower sausage, Poland		+
<i>A. sojae</i>	32869* (ATCC 42251)	+	Soy sauce, Japan		—
	30103 (ATCC 9362)	+		Protease, soy sauce	—
	30227 (ATCC 11906)	+			Trace
	30442 (ATCC 20245)	+	Koji, Japan	(US patent)	—
	33642 (IFO 32074)	+	Tamari soya		NT
	33643 (IFO 4403)	+	Forest soil		NT
	33644 (IFO 5241)	+	Miso brewing		NT
	30419 (ATCC 42249)	—	Koji for soy sauce		NT
	30431 (ATCC 20235)	—		(US patent) pectin, esterase	NT
	31200	—			NT
	32265	—		Soy sauce	NT
	38021	—	Soy sauce		NT
<i>A. flavus</i>	30165* (ATCC 16883)	+	Cellophane	Amylase	—
	31737 (UPCC 3712)	+	Rice wine		NT
	30010 (ATCC 10124)	+		Kojic acid	—
	30019 (ATCC 28539)	+	Buckwheat flour, Japan		+
	30202 (ATCC 22546)	+	Mouldy corn, USA		+
	30173 (ATCC 26946)	+	Peanut kernel, USA		+
<i>A. oryzae</i>	30289* (ATCC 1011)	+	Tane-koji	Amylase	—
	30174 (ATCC 16868)	+	Soy sauce, China		NT
	30229 (ATCC 20423)	+	Soil, Japan	(US patent) β -galactosidase	NT
	32268	—		Soy sauce	NT
	32269	—		Miso	NT
	30237 (ATCC 34704)	—	Freshwater pond, USA		NT
	31646	—	Fermented black bean, Taiwan		NT
	31659	—	Miso starter, Taiwan	Miso	NT
	31658	—	Soy sauce starter, Taiwan	Soy sauce	NT
<i>A. flavus</i> var. <i>columnaris</i>	30433* (ATCC 16870)	+	Butter, Japan		—
<i>A. oryzae</i> var. <i>viridis</i>	30428* (ATCC 22788)	+	Rice koji for making sake, Japan	Kojic acid, amylase	—
<i>A. oryzae</i> var. <i>effusus</i>	31859* (ATCC 1010)	+	<i>Zea mays</i> , USA	Kojic acid	—

*Type strain.

†+, Positive; —, negative.

‡+, Aflatoxin producer; —, non-aflatoxin producer; NT, not tested (Wei & Jong, 1986).

the *aflR* gene (Table 1). Although PCR conditions were improved by using other highly conserved primer pairs (F5/R5, F6/R6) and a low annealing temperature (55 °C), the 11 strains originally lacking a PCR product for an *aflR* gene

still did not yield any PCR products typical of the *aflR* gene. All of the 23 positive strains generated the expected PCR products and all 34 strains tested produced the ITS fragment of the rDNA gene (ITS3–ITS4) as the control.

Table 2. Primers used to amplify *aflR* gene fragments

Primer*	Sequence	Position†
F1	5'-TCGGTACGTAAACAAGGAAC	−232 to −206 (20-mer)
F2	5'-CCGATTCTTGGCTGAGT	581–598 (18-mer)
F3	5'-GCTGTCTGACGGAAGAGCG	1034–1052 (19-mer)
F4	5'-GCAATCCGCGCGCTCCCAGT	197–216 (20-mer)
F5	5'-GGCCTGTGCTCGGTGTAT	132–149 (18-mer)
F6	5'-CAATGGTAGCAGTAGCGTCT	408–427 (20-mer)
R1	3'-AGTTGAGCCGCTGGTAGTCT	245–226 (20-mer)
R2	3'-CCTAACACACCTACTCCT	1110–1083 (18-mer)
R3	3'-CCTAGGCAGAAACAGTACCA	1527–1508 (20-mer)
R4	3'-GCTGGGCAAGGAGCTCAGCC	667–648 (20-mer)
R5	3'-GAAAAGAGTGGTCTCAGG	452–435 (18-mer)
R6	3'-TACCGATGAACGACTCGTAC	1320–1301 (20-mer)

*F, forward primer; R, reverse primer.

†The sites corresponding to those of the *A. parasiticus aflR* cDNA (GenBank accession no. L26222).

Earlier studies did not identify an *aflR* transcript in *A. oryzae* or *A. sojae* (Klich *et al.*, 1997; Kusumoto *et al.*, 1998b; Matsushima *et al.*, 2001a). Our inability to amplify *aflR* implies that the *aflR* gene is missing or highly abnormal in some strains of these two species. Kusumoto *et al.* (1998b) also noted that *A. oryzae* IFO 30104 lacks the *aflR* gene. Some filamentous fungi traits can be unexpectedly lost, especially in cultures maintained in the laboratory for long periods (Bennett *et al.*, 1997). In this study, some of the test strains were found to lack an *aflR* gene. A degree of genetic variability was found to exist in other isolates that did contain an *aflR* gene homologue. Numerous methods have been used to differentiate *A. parasiticus* from *A. sojae*, and *A. flavus* from *A. oryzae* (Yuan *et al.*, 1995; Kumeda & Asao, 1996; Lee *et al.*, 2004). The putative absence of *aflR* in these *A. sojae* and *A. oryzae* strains offers a practical solution for selecting a completely safe industrial strain that cannot produce aflatoxin.

Level of *aflR* sequence similarity

Nucleotide analysis showed greater than 96.6% similarity for the entire *aflR* gene sequence from 23 strains of *Aspergillus*. The sequence similarity for the seven strains of *A. sojae*

was nearly 100%. These findings are consistent with the results obtained by Kurtzman *et al.* (1986), indicating a high degree of DNA similarity among species in *Aspergillus* section *Flavi*; their *Cot* value calculation results identified 100% relatedness between *A. flavus* and *A. oryzae*. A previous study also showed that 16 strains of *A. flavus* or *A. oryzae* could not be clearly differentiated by the sequence in the ITS1–5.8S–ITS2 region of rDNA (Lin *et al.*, 1995).

Variability of promoter and zinc-finger motif of *aflR*

Chang *et al.* (1995a) and Ehrlich *et al.* (1999b) have studied *aflR* sequences in the region −130 to −91, which may be important for *aflR* function, and have found that they are all the same, except at position −115, where *A. parasiticus*/*A. sojae* and *A. flavus*/*A. oryzae* have C and T, respectively (Table 3). The region +85 to +168 is putatively translated into the CX₂CX₆CX₆CX₂CX₆C binuclear-type zinc-finger motif (amino acid positions 29–56). The six cysteine residues in the motif were all conserved at the amino acid level in the 23 strains examined; however, some variation occurred in nucleotide residues (Table 3). The T↔C transition at position 102, T in *A. parasiticus*/*A. sojae*, C

Fig. 1. Positions of primers used to amplify segments of the *aflR* gene. Dotted bar, *aflR* ORF; black bar within dotted bar, zinc-cluster motif; black bar outside dotted bar, AFLR recognition site.

Position	-132	-115	-90	-89	-72	-79	-61	-43	-16	8	22	57	59	102	133	160	262	287	303	318	335	336	337	338	339	340	347	349	414	441	450	459
Majority	G	C	C	C	G	A	G	A	-	C	C	C	T	G	T	G	C	G	T	G	C											
BCRC no.																																
33603*									C	T					T																	
30150									C	T	T				C												T	T		A		C
30164									C	C	T				C					A							T	T		A		
30110									C		T				C	C				A						T	T				A	
32869*	A								C	C																						C
30103	A								C	C																						C
30227	A								C	C																						C
30442	A								C	C																						C
33642	A								C	C																						C
33643	A								C	C																						C
33644	A								C	C																						C
									C	A	C	C																				C
30165*	T	T																														G
31737		T																														G
30010	T																															G
30019	T																															G
30202	T																															G
30173	T																															G
30289*	T	T																														G
30174	T	T																														G
30229	T	T																														G
30433*	T	T																														G
30428*	T	T																														G
31859*	T	T																														G

Position	467	468	485	509	558	600	615	639	681	704	712	736	737	750	768	771	784	785	800	810	842	938	939	985
Majority	C	C	T	A	T	C	T	G	G	G	T	C	A	C	G	G	G	G	G	G	A	G	G	G
BCRC no.																								
33603*	T	T		C		T	C			A		T		T			A	A		A				
30150		T	A			T			C	A		T		T										
30164	T		A			T			C															
30110			A			T			C															
32869*	T			C		G						T	G											
30103	T			C		G						T	G											
30227	T			C		G						T	G											
30442	T			C		G						T	G											
33642	T			C		G						T	G											
33643	T			C		G						T	G											
33644	T			C		G						T	G											
30165*				G																				
31737				G				A	A							A	A		A	A	G	C	A	A
30010								A	A							A	A		A	A	G	C	A	A
30019								A	A							A	A		A	A	G	C	A	A
30202								A	A							A	A		A	A	G	C	A	A
30173								A	A							A	A		A	A	G	C	A	A
30289*				G				A	A							A	A		A	A	G	C	A	A
30174				G				A	A							A	A		A	A	G	C	A	A
30229				G				A	A							A	A		A	A	G	C	A	A
30433*				G				A	A							A	A		A	A	G	C	A	A
30428*				G				A	A							A	A		A	A	G	C	A	A
31859*				G				A	A							A	A		A	A	G	C	A	A

Table 3. Variation of *afIR* gene sequences in strains of *Aspergillus* section *Flavi*

Position	1030	1037	1059	1070	1081	1143	1153	1158	1206	1237	1260	1320	1321	1366	1377	1393	1395	1399	1411	1422	1441
Majority	G	G	C	T	G	C	C	C	C	G	G	T	G	G	T	G	A	G	A	G	G
BCRC no.																					
33603*																					
30150	A	C	T																		
30164	A	C	T																		
30110	A	C																			
32869*																					
30103	A																				
30227	A																				
30442	A																				
33642	A																				
33643	A																				
33644	A																				
30165*																					
31737																					
30010																					
30019																					
30202																					
30173																					
30289*																					
30174																					
30229																					
30433*																					
30428*																					
31859*																					

*Type strain.

in *A. flavus*/*A. oryzae*, did not alter the predicted amino acid residue, Ser, at position 34. These sequencing results indicated that the promoter and DNA-binding motif regions of the *aflR* gene are highly conserved. However, individual alterations still occur, e.g. *A. parasiticus* BCRC 33603 has a G→T substitution at position 133, changing the expected amino acid residue from Ala to Ser, and *A. flavus* BCRC 30173 has a different predicted amino acid residue (Leu→Phe), owing to a C→T substitution at position 160.

***aflR* sequences of *A. sojae* are significantly different from those of other strains**

Of the 12 strains of *A. sojae* studied, *aflR* could be amplified from seven strains. The *aflR* genes of these *A. sojae* strains are readily distinguished from those of the other species by the six-base insertion (CTCATG) at nucleotides 335–340, and their distinctive nucleotide changes (A, C, G, A, T, G, T, G) at the following positions: –132, –72, 737, 1070, 1153, 1158, 1260 and 1395 (Table 3). These differences produced two extra amino acids, Ala and His, at residues 114 and 115, and a stop codon at an arginine codon at position 385, as well as two distinct amino acid substitutions, Cys-246 and His-357 (Table 4). The premature stop codon in the *aflR* of *A. sojae* caused truncation by 62 residues of the C-terminal region. These effects have been found by other workers (Matsushima *et al.*, 2001b; Takahashi *et al.*, 2002). Although the HAHA motif is not important, the missing C terminal of the AFLR protein is essential for aflatoxin production (Matsushima *et al.*, 2001a; Takahashi *et al.*, 2002). It is believed that an acidic amino acid region of GAL4, AFLR and some other proteins constitutes the activation function of these DNA-binding regulators. The activation function of the AFLR protein is eliminated by the C-terminal deletion that results from the stop codon within the *aflR* gene (Chang

et al., 1999). A recently published report confirms that the truncated *A. sojae* AFLR cannot activate transcription of aflatoxin biosynthetic genes and does not interact with *aflJ*, a co-activator gene of the aflatoxin biosynthesis pathway (Chang, 2004). No aflatoxin production has been identified for *A. sojae*, a commonly used industrial organism. In this study, all the *aflR* genes in the seven *A. sojae* strains from which they were amplified showed 100 % identity, suggesting the existence of a very small possibility of aflatoxin production, perhaps through the activity of an alternative allele. Some studies have identified a duplicated *aflR* gene in *A. parasiticus*; however, the second *aflR* gene does not completely replace the first *aflR* gene (Chang & Yu, 2002; Cary *et al.*, 2002). We suggest that the *aflR* gene of these *A. sojae* strains cannot function normally and, therefore, cannot produce aflatoxin. It is very likely that these *A. sojae* strains are as safe as strains that lack an *aflR* gene.

Using *aflR* to distinguish *A. parasiticus*/*A. sojae* from *A. flavus*/*A. oryzae*

A detailed comparison of the *aflR* gene sequences demonstrated that certain base variations can be used to differentiate *A. parasiticus*/*A. sojae* from *A. flavus*/*A. oryzae*. These differences comprise ten transitions (at positions –115, 102, 262, 639, 681, 768, 771, 842, 939 and 1393), three transversions (at positions 459, 938 and 1377) and one deletion (at position –43, C→–). Some of these variations result in amino acid changes (Table 4). Phylogenetic trees were generated based on the *aflR* gene and the predicted AFLR protein sequences, and these separated *A. parasiticus*/*A. sojae* and *A. flavus*/*A. oryzae* into two groups (Fig. 2). The topology of the neighbour-joining tree of the *aflR* gene sequence is the same as that constructed from the amino acid sequence (Fig. 2). *A. parasiticus*/*A. sojae* can be differentiated from *A.*

Table 4. Sequence variation of *aflR* and the predicted AFLR protein in *Aspergillus* section *Flavi*

Nucleotides subject to variation are shown in bold, underlined type.

Sequence	Position	Species			
		<i>A. parasiticus</i>	<i>A. sojae</i>	<i>A. flavus</i>	<i>A. oryzae</i>
DNA	262	<u>G</u> GG	<u>G</u> GG	<u>A</u> GG	<u>A</u> GG
Amino acid	88	Gly	Gly	Arg	Arg
DNA	335–340	GCT	<u>GCTCATG</u> CT	GCT	GCT
Amino acid	114–115	A	AHA	A	A
DNA	736–737	<u>TAC/CAC</u>	<u>TGC</u>	<u>CAC</u>	<u>CAC</u>
Amino acid	246	Tyr/His	Cys	His	His
DNA	842	<u>AAG</u>	<u>AAG</u>	<u>AGG</u>	<u>AGG</u>
Amino acid	281	Lys	Lys	Arg	Arg
DNA	938–939	<u>G</u> GG	<u>G</u> GG	<u>GCA</u>	<u>GCA</u>
Amino acid	313	Gly	Gly	Ala	Ala
DNA	1070	<u>C</u> TC	<u>CAC</u>	<u>CTC</u>	<u>CTC</u>
Amino acid	357	Leu	His	Leu	Leu
DNA	1153	<u>CGA</u>	<u>TGA</u>	<u>CGA</u>	<u>CGA</u>
Amino acid	385	Arg	Terminate	Arg	Arg

Fig. 2. Neighbour-joining trees of (A) *aflR* gene sequences and (B) predicted AFLR protein sequences of *Aspergillus* section *Flavi* strains. Topologies were rooted with *Aspergillus pseudotamarii* (AF441427 and AF441428) (Ehrlich *et al.*, 2003). Numbers given on branches indicate the confidence level from a 1000-replicate bootstrap sampling (frequencies below 50% are not indicated). Aflatoxin production: +, positive; –, negative; NT, not tested.

Table 5. Comparison of the *aflR* sequence of strains of *A. flavus* with other strains of *Aspergillus* section *Flavi*

Nucleotides subject to variation are shown in bold, underlined type. Amino acids subject to variation are shown in bold type.

Sequence	Position	Species or strain				
		<i>A. parasiticus</i>	<i>A. sojae</i>	<i>A. flavus</i>	<i>A. flavus</i> 30165, 31737	<i>A. oryzae</i>
DNA	–90	C	C	C	T	T
DNA	414	CCC	CCC	CCC	CCG	CCG
Amino acid	138	Pro	Pro	Pro	Pro	Pro
DNA	558	TCT	TCT	TCT	TCG	TCG
Amino acid	186	Ser	Ser	Ser	Ser	Ser
DNA	800	TGC	TGC	TGC	TAC	TAC
Amino acid	267	Cys	Cys	Cys	Tyr	Tyr
DNA	985	GGT	GGT	GGT	AGT	AGT
Amino acid	329	Gly	Gly	Gly	Ser	Ser

flavus/A. *oryzae* by morphological observation by an experienced mycologist. This study presents a simple technique, by amplifying and sequencing the *aflR* gene, for differentiating *A. parasiticus*/A. *sojae* from A. *flavus*/A. *oryzae*. This method can be compared with the single-strand conformation polymorphism (SSCP) method studied by Kumeda & Asao (1996).

A. *oryzae*-type *aflR* may lose function in aflatoxin production

Although *aflR* could not be amplified from six strains of A. *oryzae*, *aflR* was still found to exist in three of the A. *oryzae* strains tested. The sequences of the amplified *aflR* genes from A. *flavus* and A. *oryzae* did not show consistent variations. However, two strains of A. *flavus*, BCRC 31737 and 30165, showed consistent variations, together with A. *flavus* var. *viridis*, A. *oryzae* var. *viridis*, A. *oryzae* and A. *oryzae* var. *effuses*, at positions -90, 414, 558, 800 and 985, and can be distinguished from the other strains of A. *flavus*. These variations cause Cys and Gly to change to Tyr and Ser, respectively, at amino acid residues 267 and 329 (Table 5). We call this type of *aflR* gene the 'A. *oryzae* type'. The A. *oryzae*-type *aflR* gene has T instead of C in the promoter region (position -90). Further studies are required to determine whether this change has an effect on the DNA-binding activity of the AFLR protein or that of other regulators. On the other hand, the amino acid substitutions at positions 267 and 329 of AFLR may significantly change protein activity and eliminate the normal function of the *aflR* protein. The A. *oryzae*-type *aflR* is quite similar to the A. *flavus* *aflR*, but can be distinguished from the *aflR* gene of A. *sojae*/A. *parasiticus* (Table 3, Fig. 2). Two A. *flavus* strains (BCRC 30165 and 31737) have the A. *oryzae*-type *aflR* gene. Neither strain has a record of aflatoxin production.

The study of Geiser *et al.* (1998) separated A. *flavus* isolates into two groups and showed that all A. *oryzae* strains are related to group I. We suspect that all group I isolates carry the A. *oryzae*-type *aflR* gene; however, further comparison studies are required. It has been postulated that A. *oryzae* was domesticated from A. *flavus* (Geiser *et al.*, 1998, 2000; Kurtzman *et al.*, 1986). Further, based on our results, we postulate that when A. *flavus* strains have the A. *oryzae*-type *aflR*, they are non-toxicogenic. Kusumoto *et al.* (1998b) have shown that some strains of A. *oryzae* have an *aflR* gene but do not produce detectable transcript. Since it is known that the AFLR protein possesses an autoregulation function (Chang *et al.*, 1995b), it is an appealing hypothesis to suggest that the changed amino acid residues of the A. *oryzae*-type *aflR* affect the autoregulation function and are responsible for the fact that *aflR* expression is non-detectable in these strains. We are undertaking further studies to test this hypothesis and/or to discover whether there is another regulatory mechanism responsible for blocking *aflR* gene expression in A. *oryzae*.

Additionally, A. *flavus* BCRC 30010 has A. *flavus*-type *aflR*, but does not produce aflatoxin (Wei & Jong, 1986). A

transversion of C to A occurs in the *aflR* gene of strain 30010 at position 22, but does not change the expected amino acid residue Arg-8. Since aflatoxin production requires the normal function of the complete aflatoxin biosynthesis gene cluster, we suspect that genes other than *aflR* are not functioning properly and inhibit aflatoxin production in BCRC 30010.

aflR of A. *parasiticus* BCRC 33603 is different from that of other strains

Variations were found in the *aflR* gene of A. *parasiticus*. In particular, the *aflR* gene of the type strain A. *parasiticus* BCRC 33603 had different nucleotides from the other A. *parasiticus* strains (BCRC 30150, 30164 and 30110) at positions -16, 59, 303, 347, 349, 441, 485, 704, 1030, 1037, 1206, 1237, 1422 and 1441 (Table 4). Some of these variations

Table 6. Variation of the *aflR* gene and AFLR in A. *parasiticus*

Nucleotides subject to variation are shown in underlined type. Amino acids subject to variation are shown in bold type.

Sequence	Position	Species	
		A. <i>parasiticus</i> 33603*	A. <i>parasiticus</i> 30150, 30164, 30110
DNA	-16	C	T
Amino acid	—	—	—
DNA	59	<u>CGC</u>	<u>CCC</u>
Amino acid	20	Arg	Pro
DNA	303	<u>ACG</u>	<u>ACA</u>
Amino acid	101	Thr	Thr
DNA	347	<u>TCT</u>	<u>TTT</u>
Amino acid	116	Ser	Phe
DNA	349	<u>CAT</u>	<u>TAT</u>
Amino acid	117	His	Tyr
DNA	441	<u>ATC</u>	<u>ATA</u>
Amino acid	147	Iso	Iso
DNA	485	<u>CTT</u>	<u>CAT</u>
Amino acid	162	Leu	His
DNA	704	<u>TGC</u>	<u>TCC</u>
Amino acid	235	Cys	Ser
DNA	1030	<u>GTG</u>	<u>ATG</u>
Amino acid	344	Val	Met
DNA	1037	<u>AGT</u>	<u>ACT</u>
Amino acid	346	Ser	Thr
DNA	1206	<u>GAC</u>	<u>GAT</u>
Amino acid	402	Asp	Asp
DNA	1237	<u>GCG</u>	<u>ACG</u>
Amino acid	413	Ala	Thr
DNA	1422	G	A
Amino acid	—	—	—
DNA	1441	C	T
Amino acid	—	—	—

*Type strain.

changed amino acids: at residues 20, 116, 117, 162, 235, 344, 346 and 413 (Table 6). Some studies have identified a duplication of the *aflR* gene in *A. parasiticus*, present as *aflR*-1 and *aflR*-2 (Chang & Yu, 2002; Cary *et al.*, 2002). Compared with the *aflR* gene sequence at GenBank, the *aflR* gene of BCRC 33603 amplified in this study is closer to *aflR*-1, and the *aflR* genes of BCRC 33110, 30164 and 30150 are closer still to *aflR*-2. Whether *aflR*-1 or *aflR*-2 is used, *A. parasiticus* can be differentiated from other species in *Aspergillus* section *Flavi* by using the *aflR* gene (Table 4, Fig. 2).

In conclusion, by using amplification and sequencing of the *aflR* gene, we have demonstrated that the *aflR* gene cannot be amplified from some strains of *A. oryzae* and *A. sojae*, and therefore is likely to be missing or highly abnormal. In the strains of *A. sojae* from which the *aflR* gene can be amplified, the nucleotide sequence is altered such that 62 amino acids are truncated in the AFLR protein. In strains of *A. oryzae* from which the *aflR* gene can be amplified, the gene shows differences from *aflR* of toxigenic *A. flavus*. Further studies are required to prove whether the strains with an *A. oryzae*-type *aflR* gene have a loss of function in aflatoxin production or whether other regulation mechanisms exist.

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