Identification and characterization of *Aspergillus flavus* and aflatoxins

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Aspergillus flavus is the main producer of the well known carcinogenic aflatoxins. The presence of this fungus and aflatoxins is of huge concern in terms of food safety. The identification of *A. flavus* is not straightforward due to similarities with closely related species (e.g. *A. parasiticus* and *A. nomius*). Also, from the biochemical point of view the closely-related species are able to produce different mycotoxins. In order to clarify the differentiation between species the identification schemes is revisited. Selective media, data from mycotoxins production and molecular biology tools are discussed in order to clarify the concept of *A. flavus* species.

Keywords *Aspergillus flavus*; aflatoxins; cyclopiazonic acid; selective media; PCR primers

1. Introduction

Aspergillus is a large genus composed of more than 180 accepted anamorphic species [1], with teleomorphs described in nine different genera [2]. The genus is subdivided in 7 subgenera, which in turn are further divided into Sections [3].

As with fungi in general, *Aspergillus* taxonomy is complex and ever evolving. The genus is easily identified by its characteristic conidiophore, but species identification and differentiation is complex, for it is traditionally based on a range of morphological features. Macromorphological features which are considered include conidial and mycelial colour, colony diameter, colony reverse colour, production of exudates and soluble pigments, presence of sclerotia and cleistothecia. Micromorphology characterization is mainly dependent on seriation, shape and size of vesicle, conidia and stipe morphology, presence of Hülle cells, and morphology of cleistothecia and ascospores [3]. Furthermore, all these morphological features have to be determined under standardized laboratory conditions [4] by trained mycologists, in order to obtain an accurate identification. Several *Aspergillus* taxonomic keys and guides are available [3, 5].

Aspergillus Subgenus *Circumdati* Section *Flavi*, also refered to as the *Aspergillus flavus* group, has attracted worldwide attention for its industrial use and toxigenic potential. Section *Flavi* is divided in two groups of species. One includes the aflatoxigenic species *A. flavus*, *A. parasiticus* and *A. nomius*, which cause serious problems worldwide in agricultural commodities, and the other includes the non-aflatoxigenic species *A. oryzae*, *A. sojae* and *A. tamarii*, traditionally used for production of fermented foods in Asia [6]. This study is focused on the first group.

2. Morphological Studies of *A. flavus*, *A. parasiticus* and *A. nomius*

An important group of foodborne fungi are the aflatoxin producers: *A. flavus*, *A. parasiticus* and more recently *A. nomius*. Isolates of these are maintained in all the major world biological resource centres, which are used extensively for reference and as verified isolates for mycotoxin and other research. The veracity of isolate species names associated with such collections is rarely if ever questioned.

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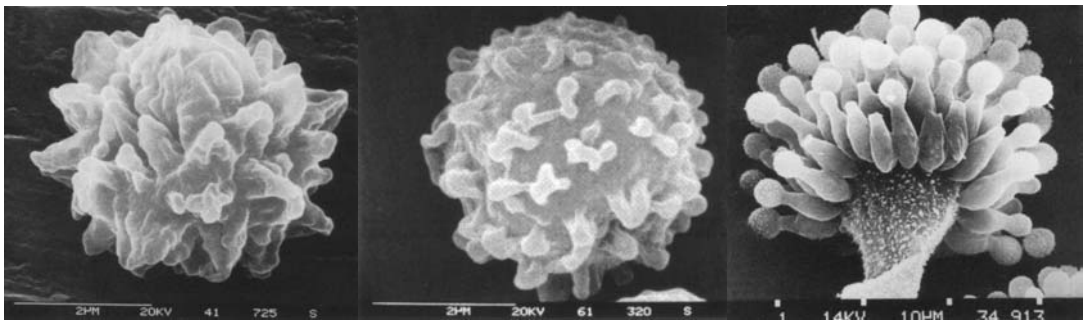
A detailed morphological study of ex-type and other isolates of *A. flavus* and *A. parasiticus* was undertaken [7]. *Aspergillus parasiticus* Speare originally isolated from material on sugar cane in Hawaii [8] was subsequently subcultured around the world. Light and scanning electron microscopy studies of all extant isolates disclosed conidia of two distinct ornamentations or morphs, but never mixtures of both. When these morphs were assigned to their respective cultures a sharp dichotomy was revealed. One form occurs in the isolate derived from the type of *A. parasiticus* (Fig 1a) and the other has been established as that of *A. flavus* (Fig 1b). Consequently cultures for the type of *A. parasiticus* held in three major world collections are in fact *A. flavus*. More worryingly, since the publication of Kozakiewicz [7] no other than the collection at CABI, Egham, UK (formerly IMI) which has re-disbursed the particular isolates, has confirmed that these mistakes have been rectified; implying that wrongly named material continues to be sold and distributed. Furthermore, in routine examinations of the IMI collection, ten additional isolates have been re-identified to date [9].



(a) (b) (c)

Fig. 1 Scanning Electron Micoroscopy pictures of (a) *A. parasiticus* and (b) *A. flavus* spores, where spore ornamentation differences are clearly seen; and of (c) *A. parasiticus* conidial head.

The situation is further complicated by the species *Aspergillus nomius* [10]. Morphologically, it resembles *A. flavus* but differs by the production of small bullet-shaped sclerotia; those in *A. flavus* being more globose. However, it is unclear whether fresh isolates of *A. nomius* always produce these distinctive sclerotia. In their absence only isoenzyme patterns and mycotoxin production provide reliable identification techniques. That is, for *A. nomius* the detection of aflatoxins B_1, B_2, G_1, G_2 (as does *A. parasiticus*), but without the detection of the secondary metabolite cyclopiazonic acid. *A. flavus* produces detectable aflatoxin B_1 and B_2 and cyclopiazonic acid only.

2.1 Separation of *A. flavus* and *A. parasiticus*

Contemporary diagnosis of the two species is based on the descriptions and keys of Raper and Fennell [5]. The primary separation being the presence of metulae and phialides (biseriate conidial head) for *A. flavus* and phialides only (uniseriate conidial head) for *A. parasiticus* (Fig 1c). Herein lies the problem. In the key for *A. parasiticus,* the words "strictly uniseriate" replace the former terms of "usually" or "mostly uniseriate" as used in previous keys [11, 12]. Examination of a large number of *A. parasiticus* isolates [13] has shown that up to 10% of conidial heads in an *A. parasiticus* colony can have metulae and phialides (biseriate). Furthermore, not all *A. flavus* isolates consistently produce metulae [14].

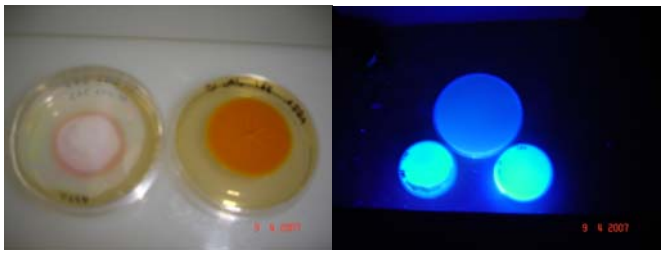
Conidial wall ornamentation is now regarded as the primary diagnostic character for separation of these two species. Conidia of *A. flavus* have relatively thin walls which are finely to moderately roughened. Their shape can vary from spherical to elliptical. Conidia of *A. parasiticus* are more spherical and noticeably echinulate or spinulose. Scanning Electron Micoroscopy (SEM) micrographs clearly show these ornamentation differences (Fig 1a and 1b). Furthermore, once SEM micrographs have been studied and compared, then with practice these differences become apparent using light microscopy.

Additionally, there are a few selected media, which may be employed to help less trained mycologist: (i) *Aspergillus* differentiation agar (AFPA); (ii) coconut cream agar (CCA) and (iii) Czapek Dox agar (CZ). AFPA is a selective identification medium for the detection of *A. flavus* group strains [15]. With this method is possible to distinguish these species from other *Aspergillus* based on the development of orange colour on the reverse of the plates (Fig 2a). The CCA is used to detect aflatoxin producer strains (Fig 2b). The production of aflatoxin is detected by a blue fluorescence when exposed to a UV-light [16]. When grown on CZ, colonies taxonomically between the two species can also be separated. Those of *A. flavus* being yellow-green and those of *A. parasiticus* a distinctly darker green, referred to as near Ivy green (Fig 3). Table 1 summarises the morphological differences between the two species.

Table 1 Morphological separation of *A. flavus* and *A. parasiticus*

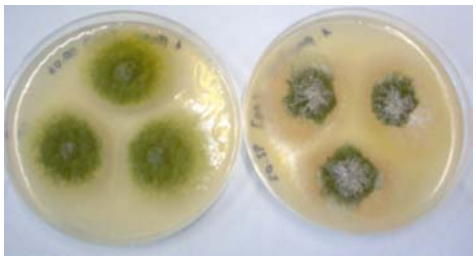
	Colony colour	Seriation[1]	Conidia	
			shape[2]	texture[3]
Aspergillus flavus	Yellow/green	b/u	gl/el	sm/fr
Aspergillus parasiticus	Ivy green	u/b	gl	r

[1]- u = uniseriate; b = biseriate
[2]- gl = globose; el = elliptical
[3]- sm = smooth; fr = finely roughened; r = rough



(a) (b)

Fig. 2 (a) *A. flavus* in AFPA, after 7 days incubation at 25ºC, with the characteristic orange colour on the reverse side of the plate; (b) aflatoxigenic *A. flavus* grown on small plates of CCA under long-wave UV light, after 7 days incubation (large plate = uninoculated CCA plate).



(a) (b)

Fig. 3 *A. flavus* (a) and *A. parasiticus* (b) strains growing on CZ.

3. Molecular methods for *Aspergillus* Section *Flavi* species differentiation

Molecular methods have been widely applied in the identification of a large number of *Aspergillus* species. DNA amplification followed by DNA sequence analysis is a powerful tool in taxonomy studies. In fact, *Aspergillus* are among the best studied fungi genetically. The complete genome of *A. flavus*

NRRL 3357 is now completely sequenced and has been released to the National Centre for Biotechnology Information (NCBI) in July 2005 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide&cmd=Search&term=AAIH01000001:AAIH01004860[PACC]), and numerous sequences from several strains of *A. flavus* group species are available.

The most widely used DNA target regions for discriminating *Aspergillus* species are the ones in the rDNA complex, mainly the internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) and the variable regions at the 5' end of the 28S rRNA gene (D1-D2 region) [*e.g*. 17-20].

Single-copy conserved genes can also be used as targets for taxonomic studies within the *A. flavus* group, when multi-copy segments from the rDNA complex lack variability. Universal β-tubulin, calmodulin and topoisomerase II genes have been used in fungal species identification but only within distantly related species, since variability is generaly low [21]. Genes involved in secondary metabolism are considered to be more variable within closely related species [22]. Several genes involved in aflatoxin biosynthesis have been identified, cloned and studied. They include a regulatory gene locus *aflR* from *A. flavus* and *A. parasiticus*, and several structural genes, *e.g. pks*A, *nor*-1, *ver*-1, *uvm*8 and *omt*A [23, 24].

However, *A. flavus* group species are difficult to differentiate even genetically. *A. flavus*, *A. parasiticus*, *A. oryzae* and *A. sojae* have shown to possess high degrees of DNA relatedness and similar genome size. Furthermore, (a) *A. flavus* and *A. oryzae*, and (b) *A. parasiticus* and *A. sojae,* were considered virtually impossible to discriminate, since their DNA relatedness was found to be of 100% and 91%, respectively [10, 25]. But, a Random Amplified Polymorphic DNA (RAPD) analysis was able to distinguish *A. parasiticus* and *A. sojae* [26].

Also mitochondrial DNA showed contrasting levels of variability. Moody et al [27] could not discriminate between similar species based on mtDNA studies, although Wang et al [28] reported a reliable tool for discriminating species based on cytochrome b sequence variability.

Genetic relatedness between *A. flavus* and *A. nomius* has also been studied, and showed contrasting results [10, 27], which means that larger numbers of *A. nomius* isolates have to be examined in order to determine relatedness with other *Aspergillus* species from the *A. flavus* group.

For studies within *A. flavus*, or for comparing *A. flavus* with other *Aspergillus* species, and even for differentiating aflatoxin producers from non-producers, several rDNA complex regions and structural aflatoxin genes have been tested for use as molecular markers, with different levels of success. Some of these studies are based on Polymerase Chain Reaction (PCR) amplification followed by sequencing for variability analysis [17, 18, 29]. But PCR amplification of known DNA target regions or genes followed by Restriction Fragment Length Polymorphisms (RFLP) [19, 30], Single-Strand Conformation Polymorphisms (SSCP) [19] or Heteroduplex Mobility Assay (HMA) [6] are easier to apply in most laboratories for the study of numerous test samples, and NCBI information can be used for generating primers and DNA probes. Kumeda and Asao [19] successfully applied PCR-SSCP and PCR-RFLP to differentiate *A. flavus* from other species (including *A. parasiticus*), based on a 600 bp fragment corresponding to the amplification of the ITS1/5.8S/ITS2 region with the primer pair ITS1-ITS4. These authors considered that, by using a fragment as big as 600 bp, they could eliminate the major problem associated to PCR-SSCP analysis, which is intraspecific variability. However, these analyses failed on differentiating between those strains where aflatoxin was detected or not detected. Chang et al [31] found the *afl*R gene to be virtually identical in *A. flavus* and *A. parasiticus*, but Somashekar et al [30], using a limited number of strains, were able to differentiate *A. parasiticus* from *A. flavus* based on the RFLP resulting from digesting an *afl*R gene fragment with the restriction enzyme *Pvu*II. Multiplex PCR, using several primer pairs for different target regions, is an alternative approach for species differentiation, and has been successfuly applied using *aflR*, *ver-1*, *omt-1* and *nor-1* genes [29, 32].

3.1 Molecular methods for differentiation of aflatoxin producers and non-producers

Aflatoxins may be produced but not detected because of the inherent detection limits of the analytical systems. Not surprisingly therefore, none of the previously described molecular methods have been able

to clearly differentiate aflatoxin producers from non-producers. Multiplex PCR with the aflatoxin pathway genes *aflR*, *ver-1*, *omt-1* and *nor-1* did not produce any clear pattern [29, 32].

Aflatoxin production and aflatoxigenic strains differentiation can be assessed by monitoring aflatoxin genes expression in the *A. flavus* group, using the reverse transcription PCR (RT-PCR) methodology. RT-PCR allows the detection of mRNAs transcribed by specific genes by PCR amplification of cDNA intermediates synthesised by reverse transcription. Such a system has been successfully applied to monitor aflatoxin production and aflatoxin gene expression based on various regulatory and structural aflatoxin pathway genes in *A. parasiticus* and/or *A. flavus* [33-35], and it was found to be very rapid and sensitive. Scherm et al [35] studied 13 strains of both species and found consistency of 3 genes (*aflD*, *aflO* [syn. *dmtA=omtB*] and *aflP* [syn. *omtA*]) in detecting aflatoxin production ability, further indicating them as potential markers.

One has to be aware that some genes are not exclusive of the aflatoxin biosynthetic pathway, one example being the *aflR* gene, which could create false-positives from sterigmatocystin producing fungi [36].

3.2 Molecular methods limitations

When using molecular methods, some cares should be taken. Systems chosen for the analysis of variance within species should be tested on a subset of the taxa of interest, using several isolates of all relevant species, and tests should include close relatives and more distant species [22]. Furthermore, internal amplification controls (IACs) are necessary, in order to detect false negative results [37]. These could arise from PCR reagents defaults, thermal cycling machines inconsistencies and presence of inhibitors from cultures or substrates [37, 38]. The latter are of major importance when studying toxigenic fungi, since secondary metabolites are strong inhibitors of PCR reactions [37], and on studies of food samples, because of inhibiting food components [38]. When contaminating DNA is thought to be present (mainly in food and environmental samples), nested PCR should be tried by using two sets of primers with different levels of specificity to eliminate contamination interference [17, 21].

3.3 Methods for differentiating *A. flavus* from *A. parasiticus* and aflatoxin producers from non-producers

3.3.1 PCR-based techniques

Growth conditions

For large scale DNA extraction (control strains and protocols optimization), a loop full of spores from a 7 day old culture grown on Malt Extract Agar (MEA: Malt 20 g L^{-1}, Glucose 20 g L^{-1}, Peptone 1 g L^{-1}, Agar 20 g L^{-1}) is inoculated into a 500 mL flask containing 200 mL of Malt Extract broth (ME: MEA w/o agar), and incubated at 25 ºC on a rotary shaker (100 rpm) for 72 hours. Mycelium is harvested by filtration through Whatman #2 filter paper, rinsed with NaCl 0.85% solution and centrifuged. Mycelium is stored in 1 g (fresh weight) aliquots at -80 ºC until further use.

For small-scale DNA extraction (test strains), the same procedure is followed in 1.5 mL tubes with 500 µL of ME. Mycelium is collected by centrifugation, washed twice with NaCl 0.85% solution and stored at -80 ºC until further use.

DNA extraction

Large-scale DNA extraction is as follows: an aliquot of 1 g (fresh weight) of mycelium is placed in a mortar pre-cooled at -80 ºC and is ground to a fine powder. The powder is suspended in 1.5 mL of lysis buffer (200 mM Tris-HCl pH 8.5; 250 mM NaCl; 25 mM EDTA; 0.5% [w/v] SDS) and heated at 68 ºC for 15 minutes, with occasional gentle mixing. After centrifugation at 13 000 rpm for 15 minutes (4 ºC), the supernatant is transferred to a new tube and polysaccharides and proteins are precipitated by adding

750 µL of cold 4 M sodium acetate, at pH 5.2. This solution is gently mixed by inversion, placed at -20 ºC for 20 minutes and centrifuged at 13 000 rpm for 15 minutes (4 ºC). Clean supernatant is transferred to a new tube and precipitated with one volume of cold isopropanol (-20 ºC). This is gently mixed by inversion for a few minutes, incubated at -20 ºC for at least 10 minutes and centrifuged at 13 000 rpm for 15 minutes (4 ºC). DNA pellet is washed with 1.0 mL of cold 70% ethanol, centrifuged at 13 000 rpm for 10 minutes (4 ºC) and air dried. DNA is resuspended in 100 to 200 µL of TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0), depending on the yield, and stored at -20 ºC. For small-scale DNA extraction, DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) is used according to manufacturer's instructions.

PCR amplification
PCR amplifications are performed on 50 µL of a reaction mixture containing $MgCl_2$-free reaction buffer, 3 mM $MgCl_2$, 2.5 U of Taq polymerase, 200 µM of each dNTP, 0.25 µM of each primer and template DNA (10 ng for multi-copy sequences, 100 ng for single-copy sequences) [22].

PCR is carried out as follows: 1) 1 step at 94 ºC for 3 min; 2) 35 cycles of the following three steps: 1 min 94 ºC, 1 min at annealing temp (specific for each primer pair, usually at or close to 55 ºC), 1 min 72 ºC; and 3) one final 10 min step at 72 ºC. PCR products are separated by electrophoresis on a 1.5% agarose gel with 0.5 % ethidium bromide in 1x TAE buffer (40 mM Tris base, 40 mM acetic acid, 1.0 mM EDTA, pH 8.0) and visualized under UV light. For multiplex-PCR, annealing temperatures are optimized depending on the primers.

Target regions and PCR primers
Fragments of the universal target region ITS1/5.8S/ITS4, as well as several single-copy genes, are amplified with specific primer pairs. The resulting fragments are used for RFLP analysis (PCR-RFLP). This approach aims at selecting a solid molecular marker for discriminating *Aspergillus flavus* species.

For the differentiation of aflatoxin-producers and non-producers, multiplex-PCR are tested with several genes related to the aflatoxin biosynthetic pathway. A 1500 bp fragment from the 28S rRNA region (primer pair D2R [5' TTG GTC CGT GTT TCA AGA CG 3'] -D7R [5' TTG GAG ACC TGC TGC GG 3']) or a 900 bp fragment corresponding to the ITS2-LSU region (primer pair CS3 [5' CGA ATC TTT GAA CGC ACA TTG 3'] - LR3 [5' CCG TGT TTC AAG ACG GG 3']) can be used as IAC. The IAC will be chosen depending on the size of the fragments obtained by the testing primer pairs amplification.

RFLP analysis
This approach aims at selecting a solid molecular marker for use as a rapid tool for discriminating *A. flavus* species. PCR products are submitted to digestion with various restriction enzymes, which are chosen based on the nucleotide sequences of various *A. flavus* isolates, including those deposited on GenBank.

3.3.2 Analysis of gene expression

Growth conditions
For aflatoxin induction experiments, each *Aspergillus* strain is grown in aflatoxin-inducing medium YES (yeast-extract-sucrose: 2% yeast extract, 15% sucrose) and non-inducing medium YEP (yeast-extract-peptone: 2% yeast extract, 15% peptone).

Total RNA extraction
Total RNA is extracted with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), according to manufacturer's instructions.

Reverse transcriptase PCR

RT-PCR may follow the protocol described by Scherm et al [35]: Reverse transcription is performed using the Qiagen OmniscriptR kit (Qiagen, Valencia, CA, USA) with 20 µL reaction mix containing: 1x reaction buffer, 0.5 mM each dNTP, 1 µM oligo dT primer, 10 U RNAse inhibitor, 4 U Omniscript Reverse Transcriptase, and 100 ng RNA. Reaction mixtures are briefly vortexed and incubated for 60 min at 37 ºC. Samples are inactivated by heating to 93 ºC for 5 min followed by rapid cooling on ice. RT-PCR is performed in a volume of 25 µL containing: 1x reaction buffer, 200 µM of each dNTP, 0.2 AM of each primer, 1 U of Red TaqR polymerase, and 1 µL of the cDNA mixture.

Cycling parameters are: 5 min at 94 ºC; 30 s at 94 ºC, 60 s at 55 ºC and 90 s at 72 ºC for 35 cycles; and a final extension at 72 ºC for 7 min. To check for the presence of genomic DNA contamination in the total RNA samples, PCR is carried out as described above, using the same set of primers designed for RT-PCR and 100 ng of total RNA as template.

RT-PCR primers

The housekeeping gene tub1 coding β-tubulin may be chosen as a system control for reverse transcription. The 3 genes *aflD*, *aflO* and *aflP* indicated by Scherm et al [35] are tested as markers for discriminating between aflatoxin producers and non-producers (Table 2).

Table 2 Details of the target genes, primer sequences, annealing temperatures and product length in base pairs (bp) for PCR and RT-PCR analysis (adapted from [35])

Primer pair	gene	Primers sequences (5′→3’)	Optimal Annealing Temp. (ºC)	PCR product legth (bp)	RT-PCR product size (bp)
Tub1-F Tub1-R	*tub1*	GCTTTCTGGCAAACCATCTC GGTCGTTCATGTTGCTCTCA	55	1498	1198
Nor1-F Nor1-R	*aflD*	ACGGATCACTTAGCCAGCAC CTACCAGGGGAGTTGAGATCC	55	990	812
OmtB(F)-F OmtB(F)-R	*aflO*	GCCTTGACATGGAAACCATC CCAAGATGGCCTGCTCTTTA	55	1333	1131
Omt1-F Omt1-R	*aflP*	GCCTTGCAAACACACTTTCA AGTTGTTGAACGCCCCAGT	55	1490	1210

4. Conclusion

The identification of *A. flavus* is not an easy task due to the similarities with *A. nomius* and *A. parasiticus*. As shown, by combining different methods (poliphasic approach), it is possible to achieve a reliable identification and a descrimination of putative aflatoxin producers.

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