Phytopathologia Mediterranea (2012) 51, 1, 198–206

Research Papers

Molecular characterization of an *Aspergillus flavus* population isolated from maize during the first outbreak of aflatoxin contamination in Italy

Antonia GALLO[1], Gaetano STEA[1], Paola BATTILANI[2], Antonio F. LOGRIECO[1] and Giancarlo PERRONE[1]

[1] Institute of Sciences of Food Production, ISPA-CNR, via Amendola 122/0, 70126, Bari, Italy
[2] Institute of Entomology and Plant Pathology, Università Cattolica del Sacro Cuore, via Emilia Parmense 84, 29100 Piacenza, Italy

Summary. An *Aspergillus* population (67 strains), isolated from maize in 2003, during the first outbreak of aflatoxin contamination documented in Northern Italy, was characterised according to gene sequencing data. All strains were identified as *A. flavus* by sequencing of β-tubulin and calmodulin gene fragments. Furthermore, the strains were analysed for the presence of seven aflatoxin biosynthesis genes in relation to their capability to produce aflatoxin B_1, targeting the regulatory genes *aflR* and *aflS*, and the structural genes *aflD*, *aflM*, *aflO*, *aflP*, and *aflQ*. The strains were placed into four groups based on their patterns of amplification products: group I (40 strains) characterised by presence of all seven amplicons; groups II (two strains) and III (nine strains), showing four (*AflM*, *aflP*, *aflO*, and *aflQ*) and three (*aflO*, *aflP*, *aflQ*) amplicons, respectively; and group IV (16 strains) characterised by total absence of PCR products. Only group I contained strains able to produce aflatoxin B_1 (37 out of 40), whereas the strains belonging to the other groups and lacking three, four or all seven PCR products were non-producers. The results obtained in this study pointed out that *A. flavus* was the only species responsible for aflatoxin contamination in Northern Italy in 2003, and that the aflatoxin gene cluster variability existing in populations can be useful for understanding the toxicological risk as well as the selection of biocontrol agents.

Key words: β-tubulin, calmodulin, aflatoxin B_1,aflatoxin gene cluster, PCR screening.

Introduction

Aflatoxin (AF) producing fungi belong to several *Aspergillus* species including *A. flavus* and *A. parasiticus*, the major species of concern for aflatoxin contamination, and other species like *A. nomius*, *A. pseudotamarii*, *A. bombycis*, *A. ochraceoroseus* (Varga *et al.*, 2003; Cary *et al.*, 2005; Frisvad *et al.*, 2005). Ability to produce AF is highly conserved in some species but is highly variable in others. Almost all *A. parasiticus* strains produce AFs, with only 3 to 6% non-aflatoxigenic strains (Horn *et al.*, 1996; Vaamonde *et al.*, 2003; Barros *et al.*, 2006), whereas toxigenicity in populations of *A. flavus* varies considerably with strain, substrate and geographic origin. Toxigenic *A. flavus* strains produce aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2) and often cyclopiazonic acid (CPA); with AFB_1 being the most relevant in food safety. Most *A. parasiticus* strains also produce aflatoxins G_1 (AFG_1) and G_2 (AFG_2) and never produce CPA (Horn and Dorner, 1999).

Aspergillus flavus populations have been studied extensively in order to find a correlation with diversity in aflatoxigenic ability. Their phenotypic variation and genetic diversity have been well documented (Horn *et al.*, 1996; Horn, 2007). A classification into two phenotypic groups is based on morphology of the sclerotia, which are either large (L) or small (S) with a diameter greater or less than 400 μm, respectively (Horn, 2005). Several studies report a positive correlation between high aflatoxin production and presence of small scle-

Corresponding author: A. Gallo
Fax: +39 080 5929374
E-mail: antonia.gallo@ispa.cnr.it

ISSN (print): 0031-9465
ISSN (online): 1593-2095
www.fupress.com/pm

rotia (Novas and Cabral, 2002; Pildain *et al*., 2004). Recently, one atypical S-type *A. flavus* producer of AFBs, AFGs and CPA has been described as the new species *A. parvisclerotigenus* by Frisvad *et al.* (2005). Geiser *et al.* (2000) subdivided *A. flavus* into Group I, which contains both L and S strains and produce AFB_1 and AFB_2, and Group II, which comprises only S strains producing AFBs and AFGs. Lately, some strains of Group II have been identified and described as an intermediate species between *A. flavus* and *A. parasiticus*, namely *A. minisclerotigenes* (Pildain *et al*., 2008).

The aflatoxin biosynthetic pathway involves approximately 25 genes clustered in a 70 kb DNA region (Yu *et al*., 2004). *A. flavus*, *A. parasiticus*, and other *Aspergillus* Sect. *Flavi* species share nearly identical sequences and conserved gene order in the cluster. In recent years PCR detection of aflatoxin biosynthetic gene presence or expression has been used as diagnostic tool for aflatoxigenic fungi in selected food commodities (Geisen, 2007). Sequence variability and deletions in various genes/regions of the aflatoxin biosynthetic cluster have also been used to determine the polyphyletic assemblage of *A. flavus* group/species (Chang *et al*., 2005; Chang *et al*., 2006).

In 2003, for the first time in Northern Italy, significant problems arose due to aflatoxin contamination of maize, because the summer was particularly dry and hot and maize crops were water-stressed, and consequently maize grain was highly contaminated (Battilani *et al*., 2005). A survey was performed by Giorni *et al*. (2007) aiming to characterize morphologically and ecologically the Italian *Aspergillus* Section *Flavi* population in key milk-producing regions of Northern Italy. In our work, a part of this population of *Aspergillus* Sect. *Flavi* was subjected to molecular identification to establish the *Aspergillus* species occurrence. Furthermore the strains were tested for presence in their genome of seven of the characterised aflatoxin biosynthetic genes in relation to aflatoxin production. To this aim primer pairs designed in this study and targeting the two regulatory genes *aflR* and *aflS* and the structural genes *aflD*, *aflM*, *aflO*, *aflP*, and *aflQ*, were used.

Materials and methods

Fungal strains, media and culture conditions

The 67 strains of *A. flavus* analysed in this study were isolated in 2003 from maize in several regions of Northern Italy, when an outbreak of aflatoxin contamination was documented in Italy for the first time. They were part of a larger survey study carried out by Giorni *et al.* (2007) and were included in the Agri-Food Toxigenic Fungi Culture Collection (ITEM) of the Institute of Sciences of Food Production, CNR, Bari, Italy (www.ispa.cnr.it/Collection). The strains, reported with ITEM accession numbers in Table 1, were routinely grown at 25°C on potato dextrose agar (PDA) (Oxoid Ltd., Basingstoke, UK) for 5 days. Conidia (final concentration of 10^6 spores mL^{-1}) were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of Wickerham medium (40 g L^{-1} glucose, 5 g L^{-1} peptone, 3 g L^{-1} yeast extract, 3 g L^{-1} malt extract). Incubation was carried out at 25°C under shaking conditions (150 rpm) for 2 days, and then mycelia were harvested by filtration and lyophilized prior to nucleic acid extraction.

For analysis of AFB_1 production, fungal isolates were inoculated into 50 mL of YES broth (20 g L^{-1} yeast extract, 150 g L^{-1} sucrose) and on coconut agar medium (200 g L^{-1} desiccated coconut powder, 15 g L^{-1} agar), and incubated 10 to 14 days at 28°C in the dark under stationary conditions.

DNA isolation

Genomic DNA was isolated using the NucleoMag Plant kit (Macherey-Nagel, GmbH & Co., Düren, DE) according to the manufacturer's instructions. Extraction was performed using the pipetting system workstation EpMotion 5075 (Eppendorf, Hamburg, DE) for automated liquid handling. DNA samples were resuspended in 100 μL of sterile distilled water at a concentration of 5 ng μL^{-1}.

PCR analysis

Amplification of a part of the β-tubulin gene (*benA*) and of a part of the calmodulin gene (*CaM*) was performed by using the primer pairs Bt2a/Bt2b (Glass and Donaldson, 1995) and CL1/CL2A (O'Donnel *et al*., 2000), respectively. Seven primer pairs were designed on the basis of the sequences of *A. flavus* aflatoxin biosynthetic genes *aflR*, *aflS*, *aflQ*, *aflP*, *aflD*, *aflM*, and *aflO* by using the Primer3Plus software (Untergasser *et al*., 2007). Oligonucleotides were synthesised by MWG Biotech AG (Ebersberg, Germany), dissolved to 100 μM final concentration with sterile water and stored at -20 °C. Sequences of primers are listed in Table 1.

Amplification was performed in 20 μL reaction

Table 1. Sequences of the nucleotide primers used in this study.

Primer code	Target gene	Primer sequences	PCR product size (bp)
AflR-1for	*aflR*	5′-AAGCTCCGGGATAGCTGTA-3′	1079
AflR-2rev		5′-AGGCCACTAAACCCGAGTA-3′	
AflS-1for	*aflS (aflJ)*[a]	5′-TGAATCCGTACCCTTTGAGG-3′	684
AflS-2rev		5′-GGAATGGGATGGAGATGAGA-3′	
AflD-1for	*aflD (nor-1)*	5′-CACTTAGCCATCACGGTCA-3′	852
AflD-2rev		5′-GAGTTGAGATCCATCCGTG-3′	
AflM-1for	*aflM (ver-1)*	5′-AAGTTAATGGCGGAGACG-3′	470
AflM-2rev		5′-TCTACCTGCTCATCGGTGA-3′	
AflO-1for	*aflO (omtB)*	5′-TCCAGAACAGACGATGTGG-3′	790
AflO-2rev		5′-CGTTGGCTAGAGTTTGAGG-3′	
AflP-1for	*aflP (omtA)*	5′-AGCCCCGAAGACCATAAAC-3′	870
AflP-2rev		5′-CCGAATGTCATGCTCCATC-3′	
AflQ-1for	*aflQ (ordA)*	5′-TCGTCCTTCCATCCTCTTG-3′	757
AflQ-2rev		5′-ATGTGAGTAGCATCGGCATTC-3′	

[a] Aflatoxin biosynthetic genes are named as proposed by Yu *et al*. (2004), old names are reported in brackets.

volume containing 2.5× Real Master Mix SYBR-ROX (5 PRIME GmbH, Hamburg, DE), 350 nM of each primer, and 20 ng of genomic DNA. Amplification parameters, after a denaturation step at 95°C for 10 min, consisted of 30 cycles of denaturation at 95°C (50 s), annealing at 58°C (50 s), and extension at 72°C (2 min). Amplification was performed in a 7000 Sequencer Detection System thermocycler (Applied Biosystem, Foster City, CA). A dissociation curve was performed after amplification by a gradual rise in temperature from 60°C to 95°C and the fluorescence signal was measured every 0.5°C to differentiate PCR products via dissociation curves. For each PCR experiment, corresponding positive (a lab strain tested for the presence of the genes) and negative (sterile water) controls were included. Each DNA sample was tested in triplicate to obtain positive/negative results with reference to aflatoxin biosynthetic genes.

DNA sequencing and multialignment analyses

Amplified products were separated on agarose gel, eluted by using the "Nucleo Spin Extract II" kit (Macherey-Nagel) and sequenced directly. All sequence data were obtained using the ABI Prism Big Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystem). Reactions were analysed using a model 3100 Genetic Analyser (Applied Biosystem). Alignment of the partial β-tubulin and calmodulin gene sequence data were performed using the software package BioNumerics 5.1 from Applied Maths and manual adjustment for improvement were made where necessary.

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura *et al*., 2007). Multialingment was perfomed by ClustalW (Thompson *et al*., 1994) using the sequences of several *Aspergillus* species belonging to Section *Flavi* (*A. minisclerotigenes*, *A. parvisclerotigenus*, *A. nomius*, *A. parasiticus*, *A. flavus*, *A. sojae*, *A. arachidicola*, *A. tamarii*, *A. pseudotamari*). Phylogenetic trees were prepared by the neighbor-joining method (Saitou and Nei, 1987). Evolutionary distances were computed using the Tamura-Nei method of the package and are in the units of the number of base substitutions per site (Tamura and Nei, 1993). All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Bootstrap values were calculated from 1000 replications

Table 2. Different PCR amplification patterns (groups) in the studied *A. flavus* population[a].

No. ITEM[b] *A. flavus* isolates	AFB_1[c]	*aflR*	*aflS*	*aflD*	*aflM*	*AflO*	*aflP*	*aflQ*	
8054	-	■	■	■	■	■	■	■	I[d]
8060	+	■	■	■	■	■	■	■	
8062	+	■	■	■	■	■	■	■	
8064	+	■	■	■	■	■	■	■	
8066	-	■	■	■	■	■	■	■	
8067	+	■	■	■	■	■	■	■	
8068	+	■	■	■	■	■	■	■	
8069	+	■	■	■	■	■	■	■	
8070	+	■	■	■	■	■	■	■	
8071	+	■	■	■	■	■	■	■	
8073	+	■	■	■	■	■	■	■	
8074	+	■	■	■	■	■	■	■	
8075	+	■	■	■	■	■	■	■	
8078	+	■	■	■	■	■	■	■	
8080	+	■	■	■	■	■	■	■	
8081	+	■	■	■	■	■	■	■	
8082	+	■	■	■	■	■	■	■	
8083	+	■	■	■	■	■	■	■	
8084	+	■	■	■	■	■	■	■	
8085	+	■	■	■	■	■	■	■	
8086	+	■	■	■	■	■	■	■	
8087	+	■	■	■	■	■	■	■	
8090	+	■	■	■	■	■	■	■	
8091	+	■	■	■	■	■	■	■	
8093	+	■	■	■	■	■	■	■	
8094	-	■	■	■	■	■	■	■	
8095	+	■	■	■	■	■	■	■	
8096	+	■	■	■	■	■	■	■	
8097	+	■	■	■	■	■	■	■	
8098	+	■	■	■	■	■	■	■	
8099	+	■	■	■	■	■	■	■	
8100	+	■	■	■	■	■	■	■	
8101	+	■	■	■	■	■	■	■	
8102	+	■	■	■	■	■	■	■	
8105	+	■	■	■	■	■	■	■	
8106	+	■	■	■	■	■	■	■	
8111	+	■	■	■	■	■	■	■	
8113	+	■	■	■	■	■	■	■	
8114	+	■	■	■	■	■	■	■	
8115	+	■	■	■	■	■	■	■	
8109	-	▒	▒	▒	■	■	■	■	II
8110	-	▒	▒	▒	■	■	■	■	
8052	-	▒	▒	▒	▒	■	■	■	III
8057	-	▒	▒	▒	▒	■	■	■	
8063	-	▒	▒	▒	▒	■	■	■	
8065	-	▒	▒	▒	▒	■	■	■	
8076	-	▒	▒	▒	▒	■	■	■	
8077	-	▒	▒	▒	▒	■	■	■	
8079	-	▒	▒	▒	▒	■	■	■	
8089	-	▒	▒	▒	▒	■	■	■	
8107	-	▒	▒	▒	▒	■	■	■	
8051	-	▒	▒	▒	▒	▒	▒	▒	IV
8053	-	▒	▒	▒	▒	▒	▒	▒	
8056	-	▒	▒	▒	▒	▒	▒	▒	
8058	-	▒	▒	▒	▒	▒	▒	▒	
8059	-	▒	▒	▒	▒	▒	▒	▒	
8061	-	▒	▒	▒	▒	▒	▒	▒	
8072	-	▒	▒	▒	▒	▒	▒	▒	
8088	-	▒	▒	▒	▒	▒	▒	▒	
8103	-	▒	▒	▒	▒	▒	▒	▒	
8104	-	▒	▒	▒	▒	▒	▒	▒	
8108	-	▒	▒	▒	▒	▒	▒	▒	
8112	-	▒	▒	▒	▒	▒	▒	▒	
8116	-	▒	▒	▒	▒	▒	▒	▒	
8117	-	▒	▒	▒	▒	▒	▒	▒	
8118	-	▒	▒	▒	▒	▒	▒	▒	
8119	-	▒	▒	▒	▒	▒	▒	▒	

[a] Presence (black box) or absence (grey box) of PCR products of the tested *afl* genes

[b] ITEM, Agri-Food Toxigenic Fungi Culture Collection, Institute of Sciences of Food Production, Bari, Italy.

[c] AFB_1, (+/-): aflatoxin and no aflatoxin production.

[d] Distribution of isolates in four amplification patterns (Roman numerals) is indicated.

of the bootstrap procedure using programs within MEGA 4 package which refers to tests of the reliability of an inferred tree (Felsenstein, 1985, 1995).

Sequence similarity searches were performed using BLAST programs at NCBI (National Center for Biotechnology Information).

Aflatoxin analysis

Nine circular plugs were cut uniformly across the whole surface of coconut medium, to be representative of each colony. Plugswere weighed in a plastic tube; then 3 mL of methanol were added and AFB_1 was extracted by shaking for 2 h at room temperature. The extraction solution was filtered through a syringe filter (RC 0.45 μm, Alltech, Deerfield, IL, USA). One mL of the filtered extract from coconut agar or 5 mL of YES broth were diluted with PBS (1:6 v/v and 1:1 v/v, respectively). The diluted extracts were applied to the immunoaffinity clean up column AflaTest© (VICAM, Milford, MA, USA). The column was then washed with 5 mL of distilled water (2 drops sec^{-1}) and aflatoxins were eluted with 2 mL of acetonitrile (1 drop sec^{-1}). The eluted extract was dried under gentle air stream and the residue redissolved with 0.25 mL of HPLC mobile phase. AFB_1 was determined by HPLC/fluorescence detector set at an excitation wavelength of 365 nm and emission wavelength of 435 nm (Agilent 1100 Series, Agilent Technology, Santa Clara, CA, USA) after post-column photochemical derivatisation (UVE™, LC Tech-GmbH, Dorfen, D). The analytical column was Luna PFP (2) (150×4.6 mm, 3 μm) (Phenomenex, Torrance, CA, USA) preceded by a SecurityGuard™ (Luna PFP, 4×3.0 mm, Phenomenex). The column was mantained at at 30°C. The mobile phase consisted of a mixture of water:acetonitrile (70:30) eluted at a flow rate of 1.0 mL min^{-1}. One hundred μL of filtered extract were injected into the chromatographic apparatus. The detection limit was 0.02 ng mL^{-1} for both media.

Recoveries of AFB_1 from coconut and YES media ranged from 60 to 80%, with relative standard deviations lesser than 20%.

Results

Molecular identification of *Aspergillus* isolates

All 67 isolates were identified as *A. flavus*, according to the sequence analyses of β-tubulin (*benA*) and calmodulin (*CaM*) partial genes (Figure 1). In particular, identity similarity of 100% was found in all the isolates, except two strains showing similarity of 99.7%, to *A. flavus* type β-tubulin sequence. A higher variability was observed for calmodulin sequences, with similarities to *A. flavus* type sequence ranging from 98.9 to 100%. Sequence diversity among *A. flavus* strains led us to identify three different groups (haplotypes) of sequences in *CaM* with genetic diversity of 0.2379, two in *benA* with genetic diversity of 0.0571; and a total of eight haplotypes when the analysis was made on the pooled data of the two loci with a genetic diversity of 0.5060. The evolutionary history inferred using the Neighbor-Joining method grouped all 67 analysed strains in the cluster of the *A. flavus* type strain (ITEM 7526) with a high supported bootstrap.

Aflatoxin B_1 production of the examined isolates

The chemical analyses established that 55% of the population investigated in this study, corresponding to 37 strains, was able to produce AFB_1 (Table 2).

Amplification patterns of aflatoxin biosynthesis genes

Primers pairs were designed for this study to target seven aflatoxin biosynthetic genes: the two regulatory genes *aflR* and *aflS*, and the structural genes *aflD*, *aflM*, *aflO*, *aflP*, *aflQ*.

As shown in Table 2 their presence in the genomes of *Aspergillus* isolates was monitored and the strains analysed could be separated into four groups on the basis of PCR products obtained. Group I of 40 strains was characterised by amplification of all seven biosynthetic genes. Of the strains in this group, only three (ITEM 8054, 8066 and 8094) were unable to produce AFB_1. Group II, consisting of only two strains, was characterised by amplification of four of seven aflatoxin biosynthetic genes, namely *aflM*, *aflO*, *aflP*, and *aflQ*. Group III showed amplification of three genes: *aflO*, *aflP*, and *aflQ* and included nine strains. Group IV consisted of 16 isolates that lacked all of the biosynthetic genes tested. All the strains with three or more genes missing failed to produce AFB_1.

Discussion

In this study *A. flavus* was the only species detected in connection with aflatoxin contamination of maize in Italy in 2003. Predominance of *A. flavus*

Figure 1. Phylogenetic trees produced from the combined sequence data of the two loci (*CaM, benA*) of 80 taxa belonging to *Aspergillus* Sect. *Flavi.* Numbers above branches are bootstrap values. Only values above 60% are indicated. The evolutionary history was inferred using the neighbor-joining method. Isolates are indicated with ITEM numbers and AFB_1 producing ability with +/-.

as an aflatoxigenic species in cultivated maize fields also has been reported in most other important maize-producing regions worldwide (Horn, 2007; Donner, 2009).

Aspergillus flavus is reported to be extremely diverse in terms of morphology and toxigenicity and incidence of non toxigenic-strains is dependent on geographic origin and substrate (Vaamonde *et al*., 2003; Pildain *et al*., 2004).

Molecular characterization also facilitated proper identification as *A. flavus* of four isolates which had been previously described as *A. parasiticus* on the basis of morphological parameters (Giorni *et al*., 2007). This result reinforces the fact that *A. parasiticus* has uncommonly been found as a contaminant in crops such as corn and cottonseed (Horn, 2005). Species identification based on morphological and biochemical characters is time consuming and not always precise; however, genetic similarity between species of *Aspergillus* sect. *Flavi*, as well as a high degree of intraspecific variability, also can prevent a clear differentiation of various species by molecular means.

In the last decade, development of molecular methods for distinction of aflatoxigenic and non-aflatoxigenic strains of *A. flavus* and *A. parasiticus* has been focused on aflatoxin biosynthesis genes.

In our work, a population of *A. flavus* including both aflatoxigenic and non-aflatoxigenic strains was screened for the presence of seven genes of the aflatoxin cluster. The result was the grouping of strains into four different amplification patterns, characterized by seven, four, three, or no DNA bands. All the aflatoxin-producing isolates exhibited the complete set of genes, whereas the non-aflatoxigenic isolates lacked the PCR products corresponding to three, four or even all seven genes. Interestingly, three strains were not able to produce AFB_1 even though they showed all seven amplicons.

To date, lack of amplification of aflatoxin biosynthetic genes has been consistently linked to non-aflatoxigenicity of strains. Chang *et al.* (2005) investigated and characterised deletions of a part or the entire gene cluster in non-aflatoxigenic *A. flavus* isolates supporting the hypothesis that the loss of aflatoxin-producing ability could be associated with deletions or mutations in the related genes. The results obtained in our study confirmed that lack of amplification of aflatoxin biosynthetic genes is correlated with non-aflatoxigenicity. Criseo *et al*. (2008) also reported that variable DNA banding patterns with one to four genes (*aflR*, *aflD*, *aflM*, *aflP*) missing was typical of the non-aflatoxigenic strains of *A. flavus*. These findings do not completely clarify if non-aflatoxigenicity is due to complete deletion of a gene, part of or the entire biosynthetic cluster, or to the presence of changes at the primer binding sites. These uncertainties illustrate the difficulties of biosynthetic gene amplification for diagnosis of aflatoxin production. Indeed, PCR systems based on one or several biosynthetic genes have so far failed to distinguish aflatoxigenic from non-aflatoxigenic strains. Moreover in this work we found that three strains, although showing all seven amplification products, are not aflatoxin producers or produced aflatoxins in quantities below the minimal detectable level. It is likely that one or more of the other genes involved in aflatoxin biosynthesis are lacking or carry some deletions in these three strains.

Recently, monitoring of the expression of aflatoxin genes has been applied for detection and differentiation of aflatoxigenic strains. To this aim, various regulatory and structural aflatoxin pathway genes in *A. parasiticus* and *A. flavus* have been targeted (Sweeney *et al*., 2000; Scherm *et al*., 2005; Degola *et al*., 2007). However, it must be considered that the pathway is highly complex and only some genes can be regarded as key genes directly coupled to aflatoxin biosynthesis.

Lack of amplification of biosynthetic genes has provided further evidence of the high level of genetic variability characterising *A. flavus* isolates. Selection not only of suitable primer pairs but also of the genes to be targeted is a basic step in the development of molecular detection methods. Consequences of a poorly designed PCR assay can be the presence of false negatives, with a possible underestimation of contamination risk, or false positives, as in the case of *aflO*, *aflP* and *aflQ* which, in our study, are the genes amplified for a high number of non-aflatoxigenic isolates.

It has been extensively reported that the probability of gene loss, recombination, DNA inversions, partial deletions, translocations, and other genomic rearrangements of the aflatoxin gene cluster are associated with proximity of the cluster to the telomeric region of the chromosome (Carbone *et al*., 2007). Comparative analyses of complete and partial aflatoxin clusters across *Aspergillus* species have been performed with the aim of clarifying the evolutionary biology of aflatoxigenesis and its link with spe-

cies adaptation and diversification (Ehrlich *et al*., 2003; Carbone *et al*., 2007; Moore, 2009).

The distinction in subgroups observed here in the population of *A. flavus* is worthy of further investigation to establish if they represent reproductively isolated subgroups.

The results may contribute to development of reliable molecular techniques for detection of aflatoxigenicity as well as illustrating the complexity of local fungal communities. At the moment the study of fungal populations isolated from maize in the same area in the following years is in progress, with the aim to analyse the extent of biodiversity and to monitor the mycotoxicological risk. In addition, the study of fungal variability at the molecular level is of interest for identification of atoxigenic strains potentially usable in biocontrol for limiting aflatoxin contamination. Utilization of atoxigenic strains of *A. flavus* which may displace aflatoxigenic strains in crop environments, is already a widespread practice directed at minimizing exposure of humans and domestic animals to aflatoxins (Cotty, 1994, Cotty *et al*., 2006; Dorner *et al*., 2007). Therefore, molecular characterization focused on biosynthetic genes may aid in selection of safe and effective atoxigenic strains to be used for biological control of aflatoxins.

Acknowledgements

This research was partially supported by EU-FPVII project MYCORED (KBBE-2007- 222690).

We thank Filomena Epifani and Giuseppe Panzarini for their technical assistance with microbiological work and chemical analyses, respectively.

Literature cited

Barros G., M.L. Chiotta, A. Torres and S. Chulze, 2006. Genetic diversity in *Aspergillus parasiticus* population from the peanut agroecosystem in Argentina. *Letters in Applied Microbiology* 42, 560–566.

Battilani P., A. Scandalora, C. Barbano, A. Pietri, T. Bertuzzi, A. Marocco, N. Berardo, G.P. Vannozzi, M. Balsini, S. Miele, E. Salera and T. Maggiore, 2005. Monitoraggio della contaminazione da micotossine in mais. *L'Informatore Agrario* 61, 47–49.

Carbone I., J.H. Ramirez-Prado, J.L. Jakobek and B.W. Horn, 2007. Gene duplication, modularity and adaptation in the evolution of the aflatoxin gene cluster. *BMC Evolutionary Biology* 7, 111. http://www.biomedcentral.com/1471-2148/7/111.

Cary J.W., M.A. Klich and S.B. Beltz, 2005. Characterization of aflatoxin-producing fungi outside of *Aspergillus* section *Flavi*. *Mycologia* 97, 425–432.

Chang P.K., B.W. Horn and J.W. Dorner, 2005. Sequence breakpoints in the aflatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic *Aspergillus flavus* isolates. *Fungal Genetics and Biology* 42, 914–923.

Chang P.K., K.C. Ehrlich and S.S.T. Hua, 2006. Cladal relatedness among *Aspergillus oryzae* isolates and *Aspergillus flavus* S and L morphotype isolates. *International Journal of Food Microbiology* 108, 172–177.

Cotty P.J., 1994. Influence of field application of an atoxigenic strain of *Aspergillus flavus* on the populations of *A. flavus* infecting cotton bolls and on the aflatoxin content of cottonseed. *Phytopathology* 84,1270–1277.

Cotty P.J., D. Barug, D. Bhatnagar, H.P. van Egmond, J.W. van der Kamp, W.A. van Osenbruggen and A. Visconti (ed.), 2006. Biocompetitive exclusion of toxigenic fungi. *The Mycotoxin Fatbook*, Academic Publishers, Wageningen, NL, 179–198.

Criseo G., C. Racco and O. Romeo, 2008. High genetic variability in non-aflatoxigenic *Aspergillus flavus* strains by using Quadruplex PCR-based assay. *International Journal of Food Microbiology* 125, 341–343.

Degola F., E. Berni, C. Dall'Asta, E. Spotti, R. Marchelli, I. Ferrero and F.M. Restivo, 2007. A multiplex RT-PCR approach to detect aflatoxigenic strains of *Aspergillus flavus*. *Journal of Applied Microbiology* 103, 409–417.

Donner M., J. Atehnkeng, R.A. Sikora, R. Bandyopadhyay and P.J. Cotty, 2009. Distribution of *Aspergillus* section Flavi in soils of maize fields in three agroecological zones of Nigeria. *Soil Biology & Biochemistry* 41, 37–44.

Dorner J.W. and B.W. Horn, 2007. Separate and combined applications of non toxigenic *Aspergillus flavus* and *A. parasiticus* for biocontrol of aflatoxin in peanuts. *Mycopathologia* 163, 215–223.

Ehrlich K.C., B.G. Montalbano, and P.J. Cotty, 2003. Sequence comparison of aflR from different *Aspergillus* species provides evidence for variability in regulation of aflatoxin production. *Fungal Genetics and Biology* 38, 63–74.

Felsenstein J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*: *International Journal of Organic Evolution* 39, 783–791.

Felsenstein J., 1995. *PHYLIP (Phylogeny Inference Package).* Version 3.57c. Distributed by the author. Department of Genetics, University of Washington, Seattle, USA.

Frisvad J.C., P. Skouboe and R.A. Samson, 2005. Taxonomic comparison of three different groups of aflatoxin producers and a new efficient producer of aflatoxin B1, sterigmatocystin and 3-O-methylsterigmatocystin, *Aspergillus rambellii* sp. nov. *Systematic and Applied Microbiology* 28, 442–453.

Geisen R., 2007. Molecular detection and monitoring of fungi. In: *Food Mycology: a Multifaceted Approach to Fungi and Food* (J. Dijksterhuis, R.A. Samson, ed.), CRC Press, Boca Raton, FL, USA, 255–278.

Geiser D.M., J.W. Dorner, B.W. Horn and J.W. Taylor, 2000. The phylogenetics of mycotoxin and sclerotium production in *Aspergillus flavus* and *Aspergillus oryzae*. *Fungal Genetics and Biology* 31, 169–179.

Giorni P., N. Magan, A. Pietri, T. Bertuzzi and P. Battilani, 2007.

Studies on *Aspergillus* section *Flavi* isolated from maize in northern Italy. *International Journal of Food Microbiology* 113, 330–338.

Glass N.L. and G.C. Donaldson, 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* 61, 1323–1330.

Horn B.W., 2005. Ecology and population biology of aflatoxigenic fungi in soil. In: *Aflatoxin and Food Safety Food Science and Technology,* 1st ed. (H.K. Abbas, ed.), CRC Press, Boca Raton, FL, USA, 95–116.

Horn B.W., 2007. Biodiversity of *Aspergillus* section *Flavi* in the United States: a review. *Food Additives & Contaminants* 24, 1088–1101.

Horn B.W. and J.W. Dorner, 1999. Regional differences in production of aflatoxin B1 and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. *Applied and Environmental Microbiology* 65, 1444–1449.

Horn B.W., R.L. Greene, V.S. Sobolev, J.W. Dorner, J.H. Powell and R.C. Layton, 1996. Association of morphology and mycotoxin production with vegetative compatibility groups in *Aspergillus flavus*, *A. parasiticus*, and *A. tamarii*. *Mycologia* 88, 574–587.

Moore G.G., R. Singh, B.W. Horn and I. Carbone, 2009. Recombination and lineage-specific gene loss in the aflatoxin gene cluster of *Aspergillus flavus*. *Molecular Ecology* 18, 4870–4887.

Novas M.V. and D. Cabral, 2002. Association of mycotoxin and sclerotia production with compatibility groups in *Aspergillus flavus* from peanut in Argentina. *Plant Disease* 86, 215–219.

O'Donnel K., H.I. Nirenberg, T. Aoki and E. Cilgenick, 2000. A multigene phylogeny of the *Gibberella fujikuroi* species complex: detection of additional phylogenetically distinct species. *Mycoscience* 41, 61–78.

Pildain M.B., G. Vaamonde and D. Cabral, 2004. Analysis of population structure of *Aspergillus flavus* from peanut based on vegetative compatibility, geographic origin, mycotoxin and sclerotia production. *International Journal of Food Microbiology* 93, 31–40.

Pildain M.B., J.C. Frisvad, G. Vaamonde, D. Cabral, J. Varga and R.A. Samson, 2008. Two novel aflatoxin-producing *Aspergillus* species from Argentinean peanuts. *International Journal of Systematic and Evolutionary Microbiology* 58, 725–735.

Saitou N. and M. Nei, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406–425.

Scherm B., M. Palomba, D. Serra, A. Marcello and Q. Migheli, 2005. Detection of transcripts of the aflatoxin genes *aflD*, *aflO*, and *aflP* by reverse transcription-polymerase chain reaction allows differentiation of aflatoxin-producing and non-producing isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. *International Journal of Food Microbiology* 98, 201–210.

Sweeney M.J., P. Pàmies and A.D.W. Dobson, 2000. The use of reverse transcription-polymerase chain reaction (RT-PCR) for monitoring aflatoxin production in *Aspergillus parasiticus* 439. *International Journal of Food Microbiology* 56, 97–103.

Tamura K. and M. Nei, 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* 10, 512–526.

Tamura K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24, 1596–1599.

Thompson J.D., D.G. Higgins and T.J. Gibson, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673–80.

Untergasse A., H. Nijveen, X. Rao, T. Bisseling, R. Geurts and A.M. Leunissen, 2007. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research* 35, W71–W74.

Vaamonde G., A. Patriarca, V.F. Pinto, R. Comerio and C. Degrossi, 2003. Variability of aflatoxin and cyclopiazonic acid production by *Aspergillus* section *Flavi* from different substrates in Argentina. *International Journal of Food Microbiology* 88, 79–84.

Varga J., K. Rigo, B. Toth, J. Teren and Z. Kozakeiwicz, 2003. Evolutionary relationships among *Aspergillus* species producing economically important mycotoxins. *Food Technology and Biotechnology* 41, 29–36.

Yu J., P.K. Chang, K.C. Ehrlich, J.W. Cary, D. Bhatnagar, T.E. Cleveland, G.A. Payne, J.E. Linz, C.P. Woloshuk and J.W. Bennett, 2004. Clustered pathway genes in aflatoxin biosynthesis. *Applied and Environmental Microbiology* 70, 1253–1262.

Accepted for publication: April 27, 2011