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**Specific detection of *Aspergillus parasiticus* in wheat flour by a highly sensitive PCR assay**

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6 **sensitive PCR assay**  
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**Abstract**

*Aspergillus parasiticus* is considered one of the most important aflatoxin producing species which contaminates foodstuffs and beverages for human consumption.

In this work, a specific and highly sensitive PCR protocol was developed to detect *A. parasiticus* using primers designed on the multicopy internal transcribed region of the rDNA unit (ITS1-5.8S-ITS2 rDNA). The assay proved to be highly specific to *A. parasiticus* when it was tested in a wide sample of related species and other fungal species commonly found in commodities, and it allowed discrimination from the closely related *A. flavus*. Accuracy of detection and quantification by conventional PCR were tested with genomic DNA obtained from wheat flour artificially contaminated with spore suspensions of known concentrations. Spore concentrations equal or higher than  $10^6$  spore/g could be detected by the assay directly without prior incubation of the samples. The assay described might be incorporated in routine analyses at critical points of the food chain within HACCP strategies.

**Keywords:** *Aspergillus parasiticus*, aflatoxins, polymerase chain reaction (PCR), detection, wheat flour, internal transcribed spacer (ITS)

**Running head** PCR detection method for *Aspergillus parasiticus* in wheat flour

## Introduction

Aflatoxins are among the most important mycotoxins in food safety causing both acute and chronic diseases and because of it, they are considered as the most carcinogenic natural secondary metabolites known. They are also recognized as a possible human carcinogen (group 1A) by International Agency of Research of Cancer (IARC, 1993). Currently, the upper limits of aflatoxins allowed by the European legislation in foodstuff are under a very stringent regulation (European Commission Regulation, 2006) obliging to destruction of contaminated agricultural products which results in significant economic losses.

Section *Flavi* consists of two groups of species; one includes *Aspergillus flavus*, *A. parasiticus*, *A. nomius* and the recently described species *A. pseudotamarii* and *A. bombycis* (Cary and Ehrlich 2006; Ehrlich et al. 2006), all of these strains produce aflatoxins. The other group includes the aflatoxin non-producing species *A. oryzae*, *A. sojae*, and *A. tamarii*, which have been used for production of traditional fermented foods in Asia (Kumeda and Asao 2001). *A. flavus* and *A. parasiticus* are the two major species that produce aflatoxins (Bennett and Klich 2003; Horn 2007). These species have two different toxicogenic profiles. *A. flavus* produces aflatoxin B<sub>1</sub> (M<sub>1</sub>), B<sub>2</sub>, cyclopiazonic acid, aflatrem, 3-nitropropionic acid, sterigmatocystin, versicolorin A and aspertoxin, whereas *A. parasiticus* produced Aflatoxin B<sub>1</sub> (M<sub>1</sub>), B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub> and versicolorin A (Wilson et al. 2002).

*Aspergillus parasiticus* can contaminate several food commodities including cereals (Pittet 1998, Giray et al. 2007), pistachios, nuts and peanuts (Jelinek et al. 1989), spices (Bartine and Tantaoui-Elaraki 1997) and figs (Doster et al. 1996; Färber et al. 1997).

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3 Foods and seeds are especially susceptible of colonization by aflatoxigenic *Aspergillus*  
4 species in warm climates where they may produce aflatoxins along several stages of the  
5 food chain: either at preharvest, processing, transportation or storage (Ellis et al. 1991).  
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10 The level of mold infestation and the identification of governing species are important  
11 indicators of the quality of the raw material and predict the potential risk for the  
12 presence of mycotoxins (Shapira et al. 1996).  
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20 The traditional methods for identification and detection of these mycotoxigenic fungi in  
21 foods include culture in different media and morphological studies. However, this  
22 approach is very time-consuming, laborious, and requires facilities and mycological  
23 expertise (Edwards et al. 2002). PCR-based methods that target DNA are considered a  
24 good alternative for rapid diagnosis because of their high specificity and sensitivity, and  
25 have been used for the detection of aflatoxigenic strains of *A. flavus* and *A. parasiticus*  
26 (Shapira et al. 1996; Färber et al. 1997; Sweeney et al. 2000; Criseo et al. 2001; Chen et  
27 al. 2002; Mayer et al. 2003; Zachová et al. 2003; Somashekar et al. 2004; González-  
28 Salgado et al. 2008). However, none of these methods has been yet able to reliably  
29 detect and discriminate only *A. parasiticus* from the other species of the Section *Flavi*  
30 by conventional PCR- assays.  
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48 Most of the PCR protocols are based on aflatoxin biosynthetic genes which are single  
49 copy and, therefore, less sensitive than multicopy genes (Bluhm et al. 2002; Jurado et  
50 al. 2006). Both ITS1 and ITS2 regions (Internal Transcribed Spacers or the rDNA) are  
51 highly variable allowing to discriminate among closely related species (Edwards et al.  
52 2002) and they have been successfully used to detect and identify other mycotoxigenic  
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3 *Aspergillus* species (Henry et al. 2000; Parenicová et al. 2001; Zhao et al. 2001; Varga  
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6 et al. 2004; Patiño et al. 2005; González-Salgado et al. 2005).  
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10 In this study, we have developed a PCR protocol based on multicopy sequences (ITS)  
11  
12 specific to *A. parasiticus* which allows distinction from other aflatoxigenic molds, in  
13  
14 particular from *A. flavus*, and organisms commonly found on grains. The method  
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16 proved to be very efficient even at short incubation times and at low spores  
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18 concentration of *A. parasiticus* in wheat flour samples. Cereals, and particularly wheat,  
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20 are important dietary products; therefore, aflatoxin risk might become relevant problem.  
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22 Early detection of aflatoxigenic-producing species is crucial to prevent aflatoxins  
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24 entering the food chain.  
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## 31 **Materials and methods**

### 32 *Fungal isolates and culture conditions*

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34 A detailed description of the fungal strains used in this study is given in Table 1. All  
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36 fungal cultures were maintained on potato dextrose-agar (PDA) (Pronadisa, Madrid,  
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38 Spain) at  $25\pm 1^\circ\text{C}$  for 4-5 days and then stored at  $4^\circ\text{C}$  until required and as spore  
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40 suspension in 15% glycerol at  $-80^\circ\text{C}$ . The isolates were cultured in 100 mL Erlenmeyer  
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42 flasks containing 20 mL liquid medium Sabouraud Broth (Pronadisa, Madrid, Spain) at  
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44  $28\pm 1^\circ\text{C}$  on orbital shaker (120 rpm). Mycelia from 3-day-old cultures were harvested by  
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46 filtration through Whatman paper n° 1 and kept at  $-80^\circ\text{C}$  for DNA isolation.  
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### 55 *DNA extraction*

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57 Genomic DNA extraction from fungal pure cultures was carried out with the protocol  
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59 described elsewhere (Querol et al. 1992). However to obtain genomic DNA from wheat  
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3 flour DNeasy Plant Mini Kit (Qiagen, Valencia, Spain) was used according to  
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5 manufacturer's instructions starting from 20 mg of flour sample frozen with liquid  
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7 nitrogen and grinded using a mortar and pestle. DNA concentrations were determined  
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9 using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington,  
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11 USA).

### 12 13 14 15 16 17 18 *Primer design and PCR amplification*

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20 All genomic DNAs used in this work were tested for suitability for PCR amplification  
21  
22 using primers ITS1 and ITS4 (Henry et al. 2000), which amplify the ITS region in  
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24 *Aspergillus*. ITS1-5.8S-ITS2 amplification products of a number of strains of  
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26 *Aspergillus* section *Flavi* species were purified by the UltraClean PCR Clean-up kit  
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28 (MO-BIO, Carlsbad, USA) and sequenced using the ABI PRISM 3730 DNA Sequencer  
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30 (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions in  
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32 the Genomic and Proteomic Unit of the University Complutense of Madrid (Spain). All  
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34 the amplification products were sequenced in both directions.

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41 Specific primer sets were designed on the basis of sequence alignments of the ITS1-  
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43 5.8S-ITS2 region of more than fifty *Aspergillus* strains from different origins, and other  
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45 related species and genus obtained in this and previous works carried out in our  
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47 laboratory, or retrieved from data bases. Sequences were edited and aligned by  
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49 ClustalW method using the MegAlign program of DNASTAR software (Lasergene,  
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51 Wisconsin, USA).

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57 Specific PCR assays were carried out using primers PAR1 (5'  
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59 GTCATGGCCGCCGGGGCGTC 3') and PAR2 (5' CCTGGAAAAAATGGTTG  
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TTTTGCG 3') for *A. parasiticus*. PCR reactions were performed in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). The PCR amplification protocol used for *A. parasiticus* was as follows: 1 cycle of 5 min at 95°C, 26 cycles of 30s at 95°C (denaturalization), 30s at 69.3°C (annealing), 30 s at 72°C (extension) and finally 1 cycle of 5 min at 72°C.

Amplification reactions were carried out in volumes of 25 µL containing 2 µL (10-80 ng) of template DNA, 1 µL of each primer (20 µM), 2.5 µL of 10x PCR buffer, 1 µL of MgCl<sub>2</sub> (50 mM), 0.2 µL of dNTPs (100 mM) and 0.15 µL of Taq DNA polymerase (5 U/µL) supplied by the manufacturer (Biotools Madrid, Spain). PCR products were detected in 2% agarose ethidium bromide gels in TAE 1X buffer (Tris-acetate 40 mM and EDTA 1.0 mM). The 100 bp DNA ladder (MBI Fermentas, Vilnius, Lithuania) was used as molecular size marker.

#### *Detection of fungal spores in wheat flour*

To test the ability of the PCR to detect *A. parasiticus* in food, different lots of commercially available ground wheat flour (1g each) were inoculated separately with 10<sup>2</sup>, 10<sup>4</sup> or 10<sup>6</sup> spores per g (low, medium and high concentration, respectively) from two strains of *A. parasiticus*, CECT 2681 and CECT 2680.

Fungal conidia suspensions were prepared from a sporulating culture (7 day-old) on Rose Bengale Chloramphenicol Agar (Pronadisa, Madrid, Spain) and filtered through Whatman N°1 paper. Concentrations were measured by microscopy using a Thoma counting chamber and spore suspensions were diluted when necessary.

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3 Inoculated samples were incubated at 28 °C for 8, 16, 24 and 48 h respectively before  
4 DNA extraction. Non-inoculated flour samples were used as a negative control. After  
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7 incubation, the samples were frozen in liquid nitrogen. Total DNA was extracted as was  
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10 described above. All the experiments were made by triplicate.  
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15 PCR amplifications were performed as indicated previously, with a DNA template  
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17 concentration of 100 ng/μl  
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## 22 **Results**

### 23 *PCR specificity with PAR1/PAR2*

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26 The specificity of the primers PAR1 and PAR2 was studied by conventional PCR in a  
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28 number of *A. parasiticus* strains from different sources as well as in closely related  
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30 species or which frequently occur in the same food matrices (Table 1). A single  
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32 fragment of about 430 bp was amplified when genomic DNA from *A. parasiticus* was  
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34 used (Figure 1). No product was observed with genomic DNA from isolates of the other  
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36 species tested including *Aspergillus* section *Flavi* species (*A. tamarii*, *A. nomius*, *A.*  
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38 *bombycis* and *A. flavus*).  
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43 Control amplifications of the genomic DNA with primers ITS1 and ITS4 were positive  
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45 for all the strains analysed.  
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### 50 *PCR studies with inoculated wheat flour*

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52 In order to test the functionality of the method under practical conditions we have  
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54 artificially inoculated wheat flour with different spore concentrations. The results are  
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56 shown in Figure 2 (2.a and 2.b).  
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3 Detection of target DNA was achieved with the highest spore concentration tested ( $10^6$   
4 spores) without previous incubation. Increasing incubation times improved detection at  
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6 low spore concentration (8 hours with a spore concentration of  $10^4$  and 16 hours with a  
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8 spore concentration of  $10^2$ ). No amplification products were obtained when DNA was  
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10 extracted from flour control (non-inoculated samples). The two different fungal strains  
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12 (CECT 2681 and CECT 2680) and the triplicates of the experiments showed the  
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14 reproducibility of the assays.  
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## 22 Discussion

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24 In this work, we have developed a specific PCR protocol for the detection of *A.*  
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26 *parasiticus* to be used between others, in a food matrix frequently contaminated with  
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28 aflatoxins and other related *Aspergillus* species. Furthermore, that is important because  
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30 provide knowledge about these species in relation with ecophysiological factors,  
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32 distribution or host/matrix preferences in order to improve strategies to prevent and  
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34 control fungal colonization and aflatoxins risk in commodities. The protocol described  
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36 is a useful tool for accurate discrimination among the most relevant aflatoxigenic  
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38 species within section *Flavi* what is important because they produce different secondary  
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40 metabolite profiles (Wilson et al. 2002). Moreover, *A. parasiticus* produces B and G  
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42 aflatoxins at generally high concentration and non-aflatoxigenic isolates are uncommon  
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44 (Horn et al 1996).  
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53 Although several PCR-based methods have been developed to detect aflatoxigenic fungi  
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55 using aflatoxin biosynthetic genes they do not detect specifically *A. parasiticus* and the  
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57 amplification signal obtained it is not very strong (Geisen 1996; Shapira et al. 1996;  
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59 Zachová et al. 2003). This could be due to the similarity of the aflatoxin biosynthesis  
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3 genes used as targets for specific primers in these two species (Yu et al. 1995; Scherm  
4 et al. 2005). The existing variability in ITS region we have used seemed to be sufficient  
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6 to discriminate among these closely related species. On the other hand, the use of ITS  
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8 regions to develop specific primers instead of single copy genes (either constitutive or  
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10 toxin biosynthetic genes) enhances the sensitivity of the assay due to its multi-copy  
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12 character (Suarez et al. 2005). Other approaches have been followed to discriminate  
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14 both species, but some are more laborious (Somashekar et al. 2004). Recently a protocol  
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16 of Real- Time PCR has been published to quantify *A. flavus* and *A. parasiticus* in soil  
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18 (Luo et al. 2009), however the authors point out that their protocol might be specific  
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20 only to the isolates in California, since they tested no isolates collected from other  
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22 geographical areas or from Culture Collections. In addition, quantitative PCR is more  
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24 expensive to be used in routine analyses and requires specific equipment.  
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34 Specific diagnosis in pure cultures is generally easier than directly on commodities  
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36 where a complex mycobiota is present, several compounds from food matrices may  
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38 affect the efficiency and sensitivity of the assay (Rossen et al. 1992; Färber et al. 1997)  
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40 and, in some cases, additional incubation of the food samples for several days is  
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42 required (Shapira et al. 1996; Chen et al. 2002; Gonzalez Salgado et al. 2008). In our  
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44 case, we could detect the presence of *A. parasiticus* when the highest spore suspension  
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46 concentration ( $10^6$  spores/ml) in wheat flour, even without incubation. DNA extraction  
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48 from spores is difficult because of their strong cell wall. However, contaminated food  
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50 also contains hyphal structures and this might improve detection levels.  
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58 In conclusion, the PCR based protocol reported in this work was rapid, specific and the  
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60 sensitivity achieved higher than previously reported protocols, providing a useful tool

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3 for early detection *A. parasiticus* in wheat flour and in other food systems. The PCR  
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5 assay and the sample preparation protocol were optimised to avoid the occurrence of  
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7 false negative reactions. The assay can be valuable in research, epidemiology studies, as  
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9 well as to control industrial quality aiming to reduce the levels of aflatoxins in food  
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11 commodities.  
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**Figure caption**

Figure 1. PCR amplification using primers PAR1/PAR2 and DNA from: Lanes 1-3: *Aspergillus parasiticus* CECT 2680, CECT 2681, Cab5dch6, respectively. Lane 4: *A. flavus* M1hip6. Lane 5: *A. tamarii* IHEM 16077. Lane 6: *A. bombycis* IHEM 19289. Lane 7: *A. nomius* IHEM 18041. Lane 8: *A. carbonarius* CECT 2086. Lane 8: *A. niger* CECT 2091. Lane 9: *Penicillium verrucosum* CECT 2906. Lane 10: *Cladosporium* sp. CL.1. Lane 11 : non- template control. M : DNA molecular size marker.

Figure 2. PCR amplification of *Aspergillus parasiticus* using PAR1/PAR2 primers in wheat flour samples inoculated with different spore concentrations at different incubation times (hours, showed on the top of the figures). (a) Lanes 1, 5 and 9:  $10^2$  spores per g. Lanes 2, 6 and 10:  $10^4$  spores per g. Lanes 3, 7 and 11:  $10^6$  spores per g. Lanes 4, 8 and 12 non- inoculated control. M: DNA molecular size marker.

(b) Lanes 13 and 17:  $10^2$  spores per g. Lanes 14 and 18:  $10^4$  spores per g. Lanes 15 and 19:  $10^6$  spores per g. Lanes 16 and 20 non- inoculated control. Lane 21: positive control Lane 22: non- template control M: DNA molecular size marker.



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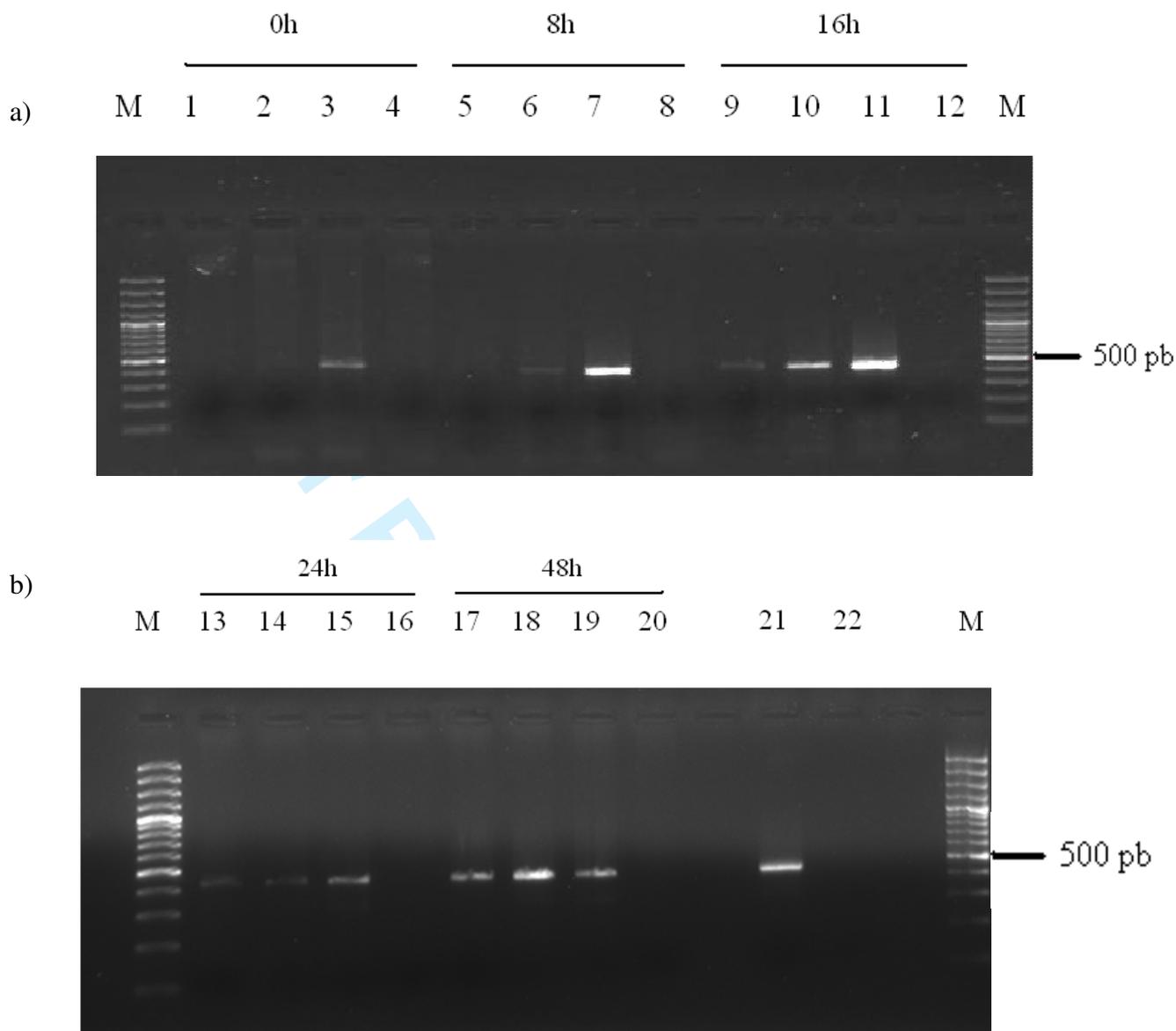


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(b) Lanes 13 and 17:  $10^2$  spores per g. Lanes 14 and 18:  $10^4$  spores per g. Lanes 15 and 19:  $10^6$  spores per g. Lanes 16 and 20 non- inoculated control. Lane 21: positive control Lane 22: non- template control M: DNA molecular size marker.

Table I. Fungal strains analysed indicating, origin, species, host and the occurrence of PCR amplification product with primers PAR1 and PAR2. (\*) Strains supplied by Dr. Sanchis (University of Lleida, Spain) and by (\*\*) Dr. Moretti (CNR, Bari, Italy)

STRAIN	ORIGIN	SPECIES	HOST	PAR1-PAR2
NRRL 502 <sup>1</sup>		<i>A. parasiticus</i>		+
NRRL 465		<i>A. parasiticus</i>		+
ATCC 22789		<i>A. parasiticus</i>		+
AP-H21		<i>A. parasiticus</i>	Barley	+
C31	Valladolid (Sp)	<i>A. parasiticus</i>	Barley	+
C32	Valladolid (Sp)	<i>A. parasiticus</i>	Barley	+
CECT 2680		<i>A. parasiticus</i>		+
CECT 2681		<i>A. parasiticus</i>		+
Cab5dch6	Albacete (Sp)	<i>A. parasiticus</i>	Barley	+
M1hip6	Madrid (Sp)	<i>A. flavus</i>	Barley	-
M12hip10		<i>A. flavus</i>	Barley	-
M2hip10	Sevilla (Sp)	<i>A. flavus</i>	Barley	-
ITEM 5135	Italy	<i>A. flavus</i>	Flour	-
ITEM 4592	France	<i>A. flavus</i>	Wheat	-
ITEM 4591	France	<i>A. flavus</i>	Wheat	-
ATCC 20043		<i>A. flavus</i>	Soil	-
NRRL 453 <sup>1</sup>		<i>A. flavus</i>	Brazil nut	-
NRRL 1957		<i>A. flavus</i>	Cellophane diaphragm	-
JMC 12721		<i>A. flavus</i>	Cottonseed	-
IHEM 16077	Belgium	<i>A. tamarii</i>	Bread	-
M6hip1	Albacete (Sp)	<i>A. tamarii</i>	Barley	-
IHEM 19289	Japon	<i>A. bombycis</i>	Silkworm disease	-
IHEM 18041	French Guiana	<i>A. nomius</i>	Mango tree	-
T.T.A.13	Zamora (Sp)	<i>A. fumigatus</i>	Grapes	-
R.T.A.16	Valladolid (Sp)	<i>A. fumigatus</i>	Grapes	-
CECT 2808		<i>A. terreus</i>		-
CECT 2907		<i>A. awamori</i>		-
CECT 2903		<i>A. versicolor</i>		-
CECT 2546		<i>A. sclerotiorum</i>		-
CECT 2091	Canada	<i>A. niger</i>		-
CECT 2574		<i>A. niger</i>		-
TR 20 1	Valladolid (Sp)	<i>A. tubingensis</i>	Wheat	-
TB 2 1	Soria (Sp)	<i>A. tubingensis</i>	Wheat	-
TB 5 2	Valladolid (Sp)	<i>A. tubingensis</i>	Wheat	-
350*	Spain	<i>A. carbonarius</i>	Grapes	-
190*	Spain	<i>A. carbonarius</i>	Grapes	-
CECT 2086		<i>A. carbonarius</i>		-
ITEM 4158**	Italy	<i>A. japonicus</i>	Grapes	-
ITEM 4685**	Portugal	<i>A. japonicus</i>	Grapes	-
CECT 2092		<i>A. ochraceus</i>		-
CECT 2948		<i>A. ochraceus</i>		-
CECT 2093		<i>A. ochraceus</i>		-
CL.1	Valladolid (Sp)	<i>Cladosporium sp</i>	Grapes	-
CECT 2906		<i>Penicillium verrucosum</i>		-

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