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Characterization of *Aspergillus* section *Flavi* isolated from fresh chestnuts and along the chestnut flour process

Simona Prencipe[1,2], Ilenia Siciliano[2], Cecilia Contessa[1], Roberto Botta[1], Angelo Garibaldi[2], Maria Lodovica Gullino[1,2], Davide Spadaro[1,2]

[1]DISAFA – Dipartimento di Scienze Agrarie, Forestali ed Alimentari, Università degli Studi di Torino, Largo P. Braccini 2, 10095 Grugliasco (TO), Italy.

[2] Centro di competenza per l'Innovazione in campo agro-ambientale (AGROINNOVA), Università degli Studi di Torino, Largo P. Braccini 2, 10095 Grugliasco (TO), Italy.

Abstract

An extensive sampling of *Aspergillus* section *Flavi* considered to be the main agent responsible for aflatoxin contamination, was carried out in the field and along the processing phases of chestnut flour production in 2015. Fifty-eight isolates were characterized by means of biological, molecular and chemical assays. The highest incidence of *Aspergillus* section *Flavi* was found in the field. The identification of the isolates was based on β-tubulin and calmodulin gene sequences. *A. flavus* was found to be the dominant species, and this was followed by *A. oryzae* var *effusus, A. tamarii, A. parasiticus* and *A. toxicarius.* Nineteen percent of the strains produced aflatoxins *in vitro* and forty percent *in vivo*. The pathogenicity assay on chestnut showed 56 virulent strains out of 58. The molecular, morphological, chemical and biological analyses of *A. flavus* strains showed an intraspecific variability. These results confirm that a polyphasic approach is necessary to discriminate the species inside the *Aspergillus* section *Flavi*. The present research is the first monitoring and characterization of aflatoxigenic fungi from fresh chestnut and the chestnut flour process, and it highlights the risk of a potential contamination along the whole chestnut production chain.

Keywords: aflatoxins, *Aspergillus flavus*, calmodulin gene, *Castanea sativa*, Ehrlich test, pathogenicity.

1. Introduction

European sweet chestnut (*Castanea sativa* Mill.) is mainly grown in the temperate regions of continental Europe, and it has a considerable economic value, with an annual production of about 170,000 tons in Europe. According to FAO, Italy is the main European producing country, with a cultivated area of 46,000 ha and a production of 43,000 tons/year, followed by Portugal and Spain (20,000 tons), Greece and France (10,000 tons) (Livre Blanc Châtaigne, 2014). Almost 20% of the total production is used to make chestnut flour, dried chestnuts and the marrons glacé sweet.

Several studies have reported the presence of spoilage fungi, such as *Penicillium* spp. and *Aspergillus* spp., and the contamination of chestnuts by mycotoxins, particularly aflatoxins (AFs) (Bertuzzi et al., 2015; Overy et al., 2003; Pietri et al., 2012; Rodrigues et al., 2013). *A. flavus* is the main contaminant and the most frequent species of *Aspergillus* in chestnut (Klich, 2007; Wells and Payne, 1975).

AFs are secondary metabolites, which have toxic effects, including mutagenic, teratogenic and immunosuppressive activities. These molecules are mainly produced by *Aspergillus* section *Flavi*, and the most frequently studied species are *A. flavus*, which is able to produce aflatoxins B1 (AFB_1) and B2 (AFB_2), and *A. parasiticus*, which can also produce aflatoxins G1 (AFG_1) and G2 (AFG_2) (Bennett and Klich, 2003). The incidence of toxigenic *A. flavus* strains is about 40%, while most strains of *A. parasiticus* are aflatoxigenic (Frisvad et al., 2006). The maximum permissible levels in Europe of AFB_1 in food and the maximum sum of aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) in nuts are specified in European Commission Regulation (EU) No 165/2010. Exceeding the AFB_1 and total AF thresholds in chestnuts and the related products, which have been established as 2 and 4 µg/kg, respectively, leads to product withdrawal from the market and is subjected to criminal procedures. The European Rapid Alert System for Food and Feed (RASFF, 2011) has in fact reported several cases of aflatoxin contamination in chestnut products in Italy. The above reported limits, which are lower than those of other nuts, are leading to a reduction in the commercialization of chestnut products. Therefore, national institutions in Europe have proposed modifying the

thresholds. Moreover, they are funding research with the aim of understanding how and when these mycotoxins are produced in chestnuts, and at preventing and controlling their occurrence.

Aflatoxigenic fungi have been reported as frequent contaminants of food and feed, and their population tends to increase during storage, with *A. flavus* being the main species that has been found, while *A. parasiticus* seems to be less prevalent (Essono et al., 2009).

As reported by the US Council for Agricultural Science and Technology (CAST, 2003), the environmental conditions where tree nuts are grown, stored and processed, are known to promote the growth of fungi and AF production, and expose nuts to a moderate risk of aflatoxin contamination.

The members of the *Aspergillus* section *Flavi* show a high level of genetic variability, and this makes it difficult to use traditional identification methods, based on the analysis of micro and macromorphological parameters, such as the colony diameter, the color and the texture of the conidia, and the growth rate (Klich and Pitt, 1988; Kumeda and Asao, 1996). Rokas and colleagues (2007) highlighted the problem of species identification, due to the high degree of DNA similarity of some species of the section *Flavi*, such as *A. flavus/A. oryzae* and *A. parasiticus/A. sojae*. The use of a single molecular approach for sequencing, combined with a comparison with unverified sequences deposited in public databases, contributes to creating an ambiguous identification of the species (Samson et al., 2014).

Furthermore, intraspecific variability has also been shown for chemical characterizations, as not all isolates belonging to the same species produce the expected metabolites, particularly AFs (Giorni et al., 2007; Rodrigues et al., 2009; Vaamonde et al., 2003). As reported by Samson et al. (2014), a polyphasic approach is required, in which morphology, molecular traits, and extrolite analysis are combined. A molecular method, based on multi locus sequence analysis with at least two conserved regions, and the analysis of extrolites, such as AFBs and AFGs, cyclopiazonic acid and aspergillic acid, are necessary (Rodrigues et al., 2011).

Knowledge about the presence of aflatoxigenic fungi and their aflatoxigenic potential is crucial to guarantee consumer safety, and to elaborate standard operational procedures for the production of chestnut flour and dried chestnut. This present study has been aimed at characterizing the biological, molecular and chemical properties of several strains of *Aspergillus* section *Flavi* isolated in the field or along the chestnut flour production chain.

2. Material and methods

2.1 Fungal strain

Aspergillus section *Flavi* strains were collected from field chestnuts and during chestnut flour production. The field strains were isolated from chestnuts harvested in October 2015, in the Piedmont region (Ormea, Perlo and Viola, in the Cuneo province - Italy). Moreover, the study considered three chestnuts lots of different origin to isolate the strains along the chestnut production chain: one from Italy (Parenti, Calabria), one from Albania (Tropojë, Scutari) and one from Spain (Ourense, Galicia). For each chestnut lot, the following phases were considered: dried chestnuts (phase 1); sorted dried chestnuts (phase 2); chestnut granulate (phase 3); roasted chestnut granulate (phase 4); roasted chestnut flour (phase 5). An additional sample of commercial chestnut flour from Italy (Lisio, Piedmont), which had been stored for 6 months, was also included.

Fifty-eight isolates, selected on the basis of the source of isolation and morphotype (Table 1), were maintained, as monoconidial cultures, in tubes of Potato Dextrose Agar (PDA, Merck, Germany) and characterized throughout the experiments.

2.2 Sampling and aspergillic acid production

Aspergillus Flavus and Parasiticus Agar (AFPA) (Fluka, Germany), supplied with Chloramphenicol Selective Supplement (Fluka) and PDA (Merck), were used for the sampling.

Sampling was carried out randomly on 30 fresh chestnuts harvested in field per replicate, with five replicates. The samples were surface-disinfected with 1% sodium hypochlorite and washed in

sterile deionized water. Four fragments of each fruit were cut and plated onto the two media. The Petri dishes were regularly inspected, every 2 days, for 10 days of incubation, and all the colonies of *Aspergillus* section *Flavi* were collected and transferred to AFPA medium. The material from the processing phases (from 1 to 4) was surface-disinfected and plated, as previously described. Each sample was composed by 3 replicates of 10 chestnuts (phases 1 and 2) or 4 replicates of 10 granulate pieces (phases 3 and 4). Chestnut flour (phase 5 or commercial sample; 20 g each replicate and 3 replicates) was previously homogenized with a Stomacher in sterile deionized water, and homogenates were taken and plated, after serial dilution (from 1:10 to 1:10,000), onto AFPA medium (Corry et al., 2003). Sampling was performed according to Pitt et al. (1983). Briefly, after 2 days of incubation at 30°C in the dark, the re-isolates were controlled for the presence of orange-yellow pigmentation on the reverse side of the colony, formed as a result of the reaction between ferric citrate and aspergillic acid. *Aspergillus* section *Flavi* incidence was calculated, as described by Lione et al. (2015), as the percentage of contaminated samples over the total samples. The experiment was repeated twice.

2.3 Molecular analysis

The isolates for the molecular studies were grown on Yeast Extract Sucrose Broth (20 g yeast extract, Merck; 150 g glucose, Sigma Aldrich, Germany; 1 L H_2O), incubated at 28°C in the dark. The mycelium was collected after 7 days, and DNA was extracted using Omega E.Z.N.A. Fungal DNA Mini Kit (VWR, USA), according to manufacturer's instructions. Partial amplification of the β-tubulin and calmodulin genes was obtained using the bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC - 3') and bt2b (5' - ACCCTCAGTGTAGTGACCCTTGGC - 3') primer pair and the cmd5 (5' - CCGAGTACAAGGARGCCTTC - 3') and cmd6 (5' - CCGATRGAGGTCATRACGTGG - 3') primer pair, respectively. PCR was carried out in a total volume of 25 µL containing: 2.5 µL of Buffer 10 X, 0.5 µL of MgCl2, 0.75 µL of dNTPs (10 mM), 1 µL of each primer (10 mM), 0.2 µL

of Taq DNA polymerase (Qiagen, Germany) and 40 ng of template DNA. Thermal cycling programs were performed according to Samson et al. (2014). The obtained amplicons were run on a 1% agarose gel with 1 µL of GelRed™ (VWR) at 100 V/cm for 45 minutes, and compared with positive controls. Gel Pilot Wide range Ladder (Qiagen) was used to compare the expected size of the bands. The amplified DNA fragments of both genes were purified using QIAquick© PCR purification Kit (Qiagen), and sequenced in both directions by Macrogen, Inc. (The Netherlands).

Forward and reverse sequences of each gene were used to create a consensus sequence, using a DNA Baser program (Heracle Biosoft S.R.L., Romania), and alignment was performed using CLUSTALW through Molecular Evolutionary Genetics Analysis (MEGA6) software, version 6.0. After cutting the trimmed regions and manual correction, a dataset was obtained of 484 bp for the β-tubulin genes and 529 bp for the calmodulin ones. The Neighbor-Joining algorithm was used for phylogenetic analysis, with MEGA version 6. The branch robustness was evaluated using bootstrap analysis with 1,000 replicates. All the reference sequences from the National Centre for Biotechnology Information (NCBI) are reported in Suppl. Tables 1 and 2.

Representative sequences of the FIE15, AFSP6, AFCAL9, AFLX4, AFCAL11 and AFSP8 strains, which included one for each species and haplotype, were deposited in GenBank with accession numbers KX400738, KX400739, KX400740, KX400741, KX400742 and KX400743 for the β-tubulin gene, and KX400732, KX400733, KX400734, KX400735, KX400736 and KX400737 for the calmodulin gene.

2.4 Macromorphological analysis

After molecular identification, a macro-morphological analysis of each isolate was performed. The isolates were 3-point inoculated with a spore suspension (10^5 spores/mL) on Yeast Extract Sucrose Agar (YES), Czapek Yeast Autolysate (CYA), and Malt Extract Autolysate (MEA) agar, and were observed for growth and sporulation after 7 days of incubation at 25°C in the dark (Samson et al., 2004).

2.5 Ehrlich test

Two methods were used to analyze the production of cyclopiazonic acid and the related alkaloids: the Ehrlich test, performed using the filter paper method (Frisvad and Samson, 2004), and a second method that was developed as follows. *Aspergillus* strains were grown on 30 mL of Czapek-Dox Broth (Fluka) at 25°C. After 5 days, 500 µL of filtered medium was added to 500 µL Ehrlich reagent (Sigma-Aldrich). If a reaction appeared within 5 minutes – from violet to pink - the culture was considered positive for cyclopiazonic acid or the related compounds. The color vanished after 10 min.

2.6 Pathogenicity assay

Aspergillus section *Flavi* isolates were tested for their pathogenicity through an *in vivo* assay. Thirty healthy chestnuts, divided into three replicates, were used to measure the moisture content (ISTISAN 1996/34). Healthy chestnuts were surface sterilized with 10% sodium hypochlorite and rinsed in sterile deionized water. Three wounds (1 cm long) were made per chestnut, and each chestnut was inoculated with a monoconidial spore suspension ($1x10^5$ spores/mL) prepared by growing in YES. Three nuts per strain were stored in a box at 28±1°C in the dark. The control chestnuts were prepared in a similar manner, but they were treated with sterile deionized water. After 7 days, a disease index (D.I.) was assigned on the basis of the observed symptoms: non-pathogenic (NP) = no symptoms; slightly virulent (SV) = 1-30% infected area; moderately virulent (MV) = 31-50% infected area; highly virulent (HV) = 51-100% infected area. Symptomatic chestnuts showed a whitish mycelium turning into yellow to green, due to conidia production. The mold was accompanied by dried, white, and chalky pulp. The experiment was performed twice, with three replicates for each strain.

2.7 In vitro *and* in vivo *aflatoxin production*

Aflatoxins were extracted from 10 mL of YES Broth, incubated for 7 days at 35 °C in the dark. Ten mL of ethyl acetate (Merck) was added twice, the mixture was shaken for 1 min, and the ethyl acetate extracts were then placed in flasks. The final extract was evaporated to dryness in a rotary evaporator. The residue was dissolved in 0.5 mL of methanol-water (50:50 v/v) for the HPLC-MS/MS analysis.

The inoculated chestnuts used for the pathogenicity assay were also used to *in vivo* analyze the aflatoxin production. Aflatoxins were extracted from 10 g of chestnuts with 20 mL of acetone (Merck), using a rotary-shaking stirrer for 30 min. After filtration through a folded filter paper, the samples were concentrated using a vacuum concentrator (Eppendorf, Germany). Dry samples were dissolved in 0.5 mL of methanol-water (50:50 v/v), vortexed for few seconds and then transferred to vials for HPLC-MS/MS analysis.

Analysis was performed using a Varian Model 212-LC micro pump (Palo Alto, CA, USA) with a Varian autosampler Model 410 Prostar, coupled with a Varian 310-MS triple quadrupole mass spectrometer, with an electrospray ion source operating in positive ionization mode (Prelle et al., 2014). Chromatographic separation was performed in isocratic mode on a Pursuit XRs Ultra C18 (100mm x 2.0 mm, 2.8 μm, Varian) column using H_2O, acidified with 0.05% HCOOH (Sigma Aldrich) and CH_3OH (Merck) (40:60 v/v), as the eluents; the flow rate was set at 0.2 mL/min for 10 minutes. The monitoring reaction mode (MRM) transitions used for the AF analyses were: 313>285 (CE 14 eV) and 313>241 (CE 34 eV) for AFB_1, 315>287 (CE 18 V) and 315>243 (CE 38 eV) for AFB_2, 329>243 (CE 18 V) and 329>311 (CE 18 eV) for AFG_1, and 331>245 (CE 24 V) and 331>313 (CE 23 eV) for AFG_2.

3. Results

3.1. Isolation of Aspergillus *section* Flavi

One hundred and ten isolates of *Aspergillus* section *Flavi* were collected from different chestnut sources, one per positive sample (Suppl. Table 3): 66 from fresh chestnuts (53% positive samples),

17 from dried chestnuts (19%), 5 from sorted dried chestnuts (8%), 17 from chestnut granulate (14%) and 12 from roasted chestnut granulate (10%). When considering the origin of the chestnut flour processing samples, it was found that the Italian ones showed the highest incidence (22% positive samples), compared to the 14% and 6% of the Albanian and Spanish ones, respectively. No *Aspergillus* section *Flavi* was isolated from the fresh produced chestnut flour. Additionally, 69 isolates of *Aspergillus* section *Flavi* were isolated in a commercial chestnut flour that had been stored for 6 months. Fifty-eight isolates were selected, on the basis of the isolation sources and morphotype, for further studies.

The production of aspergillic acid was assessed on AFPA (Table 2). Eighty-one percent of the isolates showed a bright orange or orange/yellow color on the reverse side of the colonies, after 24 and 48 hours of growth, respectively, as a result of a positive reaction between ferric citrate and aspergillic acid. Nineteen percent of the tested isolates (11) showed a cream color on the reverse side of the colonies, after 24 h of incubation, but some of them (6) turned dark-brown after 48 h of incubation.

3.2 Molecular analysis

Partial sequences of the β-tubulin and calmodulin genes were analyzed for the molecular analysis. BLAST analysis of the obtained sequences was only able to provide a low univocal identification of the isolates at the species level. A phylogenetical analysis was performed to classify the isolates, but resulted in an inconsistent species identification. The partial β-tubulin gene sequence was 484 bp for each strain, and was used to build a NJ tree (Fig. 1). The strains were divided into three main clades: Clade I (bootstrap 86), which included *A. flavus*, *A. oryzae* and *A. oryzae* var. *effusus* isolates, and which grouped most of the isolates; Clade II (bootstrap 92) with *A. parasiticus* and *A. toxicarius* isolates*;* and Clade III (bootstrap 99) with the *A. tamarii* isolates. The partial β-tubulin gene sequence did not allow the identification at species level.

A second NJ tree (Fig. 2) was built with partial calmodulin gene sequences (529 bp per isolate), and the three main clades were confirmed: Clade I, II and III, corresponding to the β-tubulin analysis.

The strains in clade I were further divided into two subclades, IA and IB (Suppl. Fig. 1). Subclade IA, which included most *A. flavus* isolates, showed two conserved polymorphisms, with a thymine at position 138 and a cytosine at position 149. The IB subclade was divided into two groups: group IBI, which included five *A. oryzae* var *effusus* isolates (AFCAL1, AFCAL5, AFCAL10, AFLX3 and AFLX4), and the IBII group, which included five *A. flavus* isolates (AFLX8, XN, AFSP6, AFLX7 and AFCAL2). The strains of group IBII showed an adenine and a thymine, respectively, at positions 138 and 149, shared with *A. oryzae*, and two conserved SNPs, one base deletion at position 124 and a thymine substitution at position 469.

The AFLX6, AFCAL11 and AFCAL8 strains in Clade II clustered together with *A. parasiticus* (bootstrap 64), while the AFSP8 strain clustered with *A. toxicarius* (bootstrap 67) in a subclade, IIA, with a conserved polymorphism (a thymine at position 246 instead of a cytosine).

A. tamarii strains formed a single group (Clade III, bootstrap 99), which showed 100% identity.

After strain identification at a species level, it was possible to relate the aspergillic acid reaction to the species (Table 2). *A. flavus*, *A. parasiticus* and *A. toxicarius* strains showed a bright orange or orange/yellow color. *A. oryzae* var *effusus* showed a cream color after 24 and 48 h of incubation, while *A. tamarii* showed a cream color at 24 h of incubation, and a dark-brown color at 48 h of incubation.

3.4 Macromorphological analysis

All the strains belonging to *Aspergillus* section *Flavi* confirmed the typical colony morphology and growth rates of that section. After 7 days of incubation at 25°C on YES, CYA and MEA, the mean diameters were measured, and the results are reported in Table 2. The colors of the colonies are shown in Fig. 3, where the typical yellow-green shades of conidia can be observed for *A. flavus*, darker green conidia for *A. parasiticus* and *A. toxicarius*, and green to reddish-brown conidia for *A.*

tamarii. *A. oryzae* var *effusus* isolates show a plain whitish surface with irregular margins on YES and MEA, poor sporulation on YES, and floccose with dominant green aerial mycelium on CYA.

A. flavus population, which is composed of 43 strains, includes a homogeneous group of 38 strains with a mean diameter of 4.13 cm on YES, 3.57 cm on CYA and 3.33 cm on MEA, and typical yellow-green to brown conidial heads (Table 2 and Fig. 3). The other five strains show different colony morphologies (Fig. 4) and growth rates (Table 2). All the strains displayed different morphologies, shades and sporulation on YES. The AFLX8 and XN strains were similar on CYA, and showed poor sporulation and olive to olive-brown conidia. The AFSP6 and AFLX7 strains showed velvety green conidia, with good sporulation on CYA. The AFCAL2 strain displayed smooth green to olive conidia on CYA. No difference was observed between the five strains on MEA.

3.5 Ehrlich test

All the strains of *Aspergillus* section *Flavi* tested negative for cyclopiazonic acid and the related alkaloids production with the filter paper method. However, with the second method, which was developed on a liquid medium, *Aspergillus* section *Flavi* strains were divided according to the reaction that had been observed, and 36% were positive (Table 1). All the *A. tamarii* strains showed a positive faint pink reaction within 5 min. *A. oryzae* var *effusus* strains produced a yellow reaction. *A. parasiticus* and *A. toxicarius* strains were negative. A higher variability was observed, within 5 minutes, among the *A. flavus* strains*:* a positive faint pink or violet reaction (21%), a positive dark violet reaction (14%), or a negative reaction (65%).

3.6 Pathogenicity

The moisture content of the fresh chestnut was about 45%. The pathogenicity test divided *Aspergillus* section *Flavi* strains into four categories, according to their virulence (Table 1): 21% of the strains were virulent (V), 28% moderately virulent (MV), 48% slightly virulent (SV), and 3%

were not pathogenic (NP). The same symptoms were observed in all the replicates. All the strains belonging to *A. parasiticus*, *A. toxicarius*, *A. tamarii*, *A. oryzae* var *effusus* species, and 41 out of 43 strains of *A. flavus* were virulent. The pathogenic strains of *A. flavus* and *A. tamarii* were slightly virulent, moderately virulent or virulent. *A. parasiticus* and *A. toxicarius* strains were slightly virulent. *A. oryzae* var *effusus* strains were slightly virulent, except for one strain, which was instead virulent.

3.7 Aflatoxins production

The production of aflatoxins was evaluated both *in vitro* and *in vivo* on the strains of all the species (Figure 5), except for *A. tamarii*, which had not been able to produce any aflatoxin. Five strains out of 43 (11.6%) *A. flavus* strains grown on YES-broth were only able to produce aflatoxin B1, while the other strains were not able to produce any aflatoxin. When inoculated on chestnuts, a higher number of isolates produced AFB_1 (39.5%), and three of these isolates (AFLX2, AFSP4, and AFSP7) were also able to produce AFB_2 (7.0%). *A. parasiticus* and *A. toxicarius* strains were able to produce the four aflatoxins, both *in vitro* and *in vivo*, with some exceptions. *A. oryzae* var *effusus* strains, on the contrary, did not produce any AFs.

4. Discussion

Most of the studies on chestnuts have focused on commercial products (Rodrigues et al., 2013; Wells and Payne, 1975), but the occurrence of *Aspergillus* species and their toxigenic potential in fresh chestnut and along the chestnut flour production chain have not been studied extensively. Strains belonging to the *Aspergillus* section *Flavi* are predominant in the *Aspergillus* genus on chestnuts (Abdel-Gawad and Zohri, 1993; Overy et al., 2003; Rodrigues *et. al*., 2013). *A. flavus* contamination in agricultural commodities could occur from the field to the processing, including storage (Berthier and Valla, 1998). In this work, the presence of different *Aspergillus* section *Flavi* species has been reported in the field and along the chestnut flour processing chain.

In this study, the incidence of *Aspergillus* section *Flavi* in the field was 53%, a higher percentage than that (16.8%) reported by Wells and Payne (1975). The environmental conditions found in field, with high humidity in October 2015 (ARPA, 2015), probably increased the susceptibility of chestnuts to fungal invasion during the pre-harvest, as already reported by the US Council for Agricultural Science and Technology (CAST, 2003). During nut processing, two steps, that are sorting and roasting, could be able to reduce the average number of *Aspergillus* section *Flavi* (FAO/WHO, 2012). The currently adopted chestnut production practices are not sufficient to eliminate the growth of aflatoxigenic species, as we were able to isolate aflatoxigenic strains from all the chestnut processing steps. A high occurrence of aflatoxins in chestnut flour could be related to post-harvest contamination or contamination during the production process of chestnuts, as reported by Bertuzzi et al. (2015).

In this study, a biological, molecular and chemical characterization was conducted on 58 *Aspergillus* section *Flavi* strains, isolated from fresh chestnuts in the field (16), during the chestnut flour production (33), and from a commercial flour (9). In order to avoid a species misidentification, the characterization of *Aspergillus* section *Flavi* requires a polyphasic approach, which should include morphological analysis, molecular typing, and aflatoxin production (Rodrigues et al., 2009, 2011; Samson et al., 2014).

In the molecular analysis, partial sequences of β-tubulin and calmodulin genes were explored. The beta tubulin dataset showed a simpler NJ tree, without any clear separation between *A. flavus* and *A. oryzae* var *effusus*, or between *A. parasiticus* and *A. toxicarius* (Rodrigues et al., 2011). Only *A. tamarii* was unambiguously identified. The phylogenetic analysis performed with the calmodulin gene was more effective in grouping the strains on the species basis, due to the presence of phylogenetically informative polymorphic sites, as demonstrated by Gallo and colleagues (2012) on a population of *A. flavus* from maize. Focusing on the strains of *A. flavus*, the present data have shown the existence of intraspecific variability, as already reported by Gallo et al. (2012), Pildain et al. (2004) and Vaamonde et al. (2003) on different substrates.

The production of aflatoxins was analyzed both *in vitro* and *in vivo*. The *in vitro* frequency of the aflatoxigenic strains was 19%, with AFB_1 production for *A. flavus* strains and both AFB_S, and AFGs production for *A. parasiticus* and *A. toxicarius*. The *in vivo* percentage of aflatoxigenic strains was higher (40%). As already reported by Probst and Cotty (2012), there is no correlation between *in vitro* and *in vivo* AF production, with a potential overestimation of false atoxigenic strains *in vitro*. The percentage of toxigenic strains of *A. flavus* was similar to the percentage observed by Mauro et al. (2013) and Nesci and Etcheverry (2002) in maize (about 50%), but lower than the incidence found by Astoreca et al. (2011), Barros et al. (2006) and Nakai et al. (2008) (about 90%) .

As far as the pathogenicity test is concerned, only two strains out of 58 were avirulent. Most strains showed high or medium virulence on chestnut. As already reported by Cotty (1989), for *Aspergillus flavus* in cotton, there was no correlation between the level of virulence and aflatoxin production. The results of both the *in vivo* aflatoxin production and the pathogenicity tests show the contamination potential of the whole chestnut production chain, from the field to the final products.

The developed Ehrlich test in a liquid medium permits the production of indoles, such as cyclopiazonic acid or relative alkaloids, to be detected from fungal strains, when the traditional filter paper method fails (Lund, 1995). As reported by Frisvad and Samson (2004) and Samson et al. (2007), this method could be useful to classify *Penicillium* subgenus *Penicillium* and *Aspergillus* section *Nigri*. In this work, this method has resulted to be a good diagnostic tool in discriminating *A. oryzae* var *effusus* from the closely related species *A. flavus*.

The present study has confirmed that a single method is not sufficient to discriminate the species inside the section *Flavi* of *Aspergillus*, as reported by Samson and Varga (2009). The species identity needs to be determined through a polyphasic approach, where a morphological examination and DNA sequences of at least two regions, which can be confirmed by aflatoxin analysis, are combined.

In conclusion, the present research is the first attempt that has been made to monitor and characterize aflatoxigenic fungi along the chestnut flour processing chain. Further studies should be

conducted to investigate the same samples, from the field to the shelf life focusing on temperature, humidity, atmosphere and time during drying and storage, which are critical points for the contamination of aflatoxigenic strains. In addition, a sampling from a large number of lots per origin could be considered.

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Tables

Table 1. Name of strain, source of isolation, geographical origin, molecular identification, Ehrlich test and virulence results of the *Aspergillus* section *Flavi* strains isolated in this study.

Strain	Source	Origin	Genus	Subgenus	Section	Species	Ehrlich test	Virulence
AFCAL1	DC	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. oryzae* var *effusus*	Y	SV
AFCAL2	DC	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-/+	SV
AFCAL3	DC	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	+	SV
AFCAL4	CG	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-/+	SV
AFCAL5	CG	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. oryzae* var *effusus*	Y	SV
AFCAL6	RCG	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	+	MV
AFCAL7	RCG	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	+	SV
AFCAL9	CG	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	++	MV
AFCAL10	DC	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. oryzae* var *effusus*	Y	HV
AFLX1	CG	AL	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	++	MV
AFLX2	CG	AL	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	MV
AFLX3	DC	AL	*Aspergillus*	*Circumdati*	*Flavi*	*A. oryzae* var *effusus*	Y	SV
AFLX4	DC	AL	*Aspergillus*	*Circumdati*	*Flavi*	*A. oryzae* var *effusus*	Y	SV
AFLX5	DC	AL	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	++	SV
AFLX7	CG	AL	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	+	MV
AFLX8	RCG	AL	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	+	MV
AFLX9	RCG	AL	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	++	HV
AFLX10	RCG	AL	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	HV
AFLX11	RCG	AL	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	HV
XN	RCG	AL	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	+	NP
AFSP1	CG	SP	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	MV
AFSP2	CG	SP	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	MV
AFSP3	CG	SP	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	HV
AFSP4	CG	SP	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	++	MV
AFSP5	CG	SP	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	MV
AFSP6	RCG	SP	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	+	SV
AFSP7	CG	SP	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	++	SV
AF2	CF	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	NP
AF3	CF	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	SV
AF4	CF	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	MV
AF11	CF	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	SV
AF17	CF	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	SV
AF20	CF	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	SV
AF24	CF	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	SV
AF12	CF	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	SV
AF18	CF	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	SV
AFCAL8	RCG	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. parasiticus*	-	SV
AFCAL11	RCG	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. parasiticus*	-	SV

Strain	Source	Origin	Genus	Subgenus	Section	Species	Ehrlich test	Virulence
AFLX6	SDC	AL	*Aspergillus*	*Circumdati*	*Flavi*	*A. parasiticus*	-	SV
AFSP8	CG	SP	*Aspergillus*	*Circumdati*	*Flavi*	*A.toxicarius*	-	SV
MCAL1	DC	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A.tamarii*	-/+	SV
MCAL2	DC	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A.tamarii*	-/+	SV
FIE1	F	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A.tamarii*	-/+	MV
FIE2	F	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	MV
FIE3	F	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	SV
FIE4	F	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A.tamarii*	-/+	MV
FIE5	F	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	SV
FIE6	F	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	HV
FIE7	F	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	HV
FIE8	F	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	MV
FIE9	F	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	SV
FIE10	F	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	HV
FIE11	F	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	HV
FIE12	F	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	SV
FIE13	F	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	HV
FIE14	F	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A. flavus*	-	HV
FIE15	F	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A.tamarii*	-/+	MV
FIE16	F	IT	*Aspergillus*	*Circumdati*	*Flavi*	*A.tamarii*	-/+	HV

Source: F: field; DC: dried chestnuts; SDR: sorted dried chestnuts; CG: chestnut granulate; RCG: roasted chestnut granulate; CF: chestnut flour. Origin: IT: Italy; AL: Albany; SP: Spain. Ehrlich test: (-/+) faint pink reaction; (+) faint violet reaction; (++) dark violet reaction; (-) negative reaction; Y (yellow reaction). Virulence: NP not pathogenic, SSV slightly virulent; MV moderately virulent; HV highly virulent.

Table 2 – Aspergillic acid production and colony diameter of *Aspergillus* section *Flavi* species isolated in this study.

Species	Aspergillic acid *		Colony diameter (cm) ± SD **		
	24h	48h	YES	CYA	MEA
A. flavus AF20***	Orange	Orange	4.13 ± 0.12	3.57 ± 0.21	3.33 ± 0.12
A. flavus AFLX8	Orange	Orange	3.93 ± 0.12	3.43 ± 0.06	3.27 ± 0.06
A. flavus AFCAL2	Orange	Orange	3.17 ± 0.29	2.50 ± 0.00	3.50 ± 0.00
A. flavus XN	Orange	Orange	4.37 ± 0.06	4.10 ± 0.10	3.93 ± 0.12
A. flavus AFSP6	Orange	Orange	3.17 ± 0.29	2.40 ± 0.17	3.50 ± 0.00
A. flavus AFLX7	Orange	Orange	4.17 ± 0.29	3.17 ± 0.29	3.67 ± 0.29
A. oryzae var. *effusus*	Cream	Cream	3.24 ± 0.06	3.45 ± 0.11	3.61 ± 0.12
A. parasiticus	Yellow	Yellow to Orange	4.33 ± 0.06	3.80 ± 0.10	3.87 ± 0.12
A. toxicarius	Orange	Orange	4.30 ± 0.10	3.70 ± 0.10	3.57 ± 0.12
A. tamarii	Cream	Cream to brown	4.13 ± 0.06	3.60 ± 0.20	3.67 ± 0.15

*Aspergillic acid production on AFPA medium incubated at 30°C for 2 days.

**Colony diameters (mean cm) of strains grown on YES, CYA and MEA incubated at 25°C for 7 days.

***The AF20 strain was considered representative for the 38 *A. flavus* strains with the same morphology.

Figures

Figure 1. Maximum Likelihood phylogeny inferred from the β-tubulin gene of *Aspergillus* section *Flavi* strains isolated in this study. Phylogeny was rooted by *A. niger*. The numbers at the major nodes indicate the percent ML bootstrap values from 1,000 bootstrapped datasets. Evolutionary analyses were conducted using MEGA6.

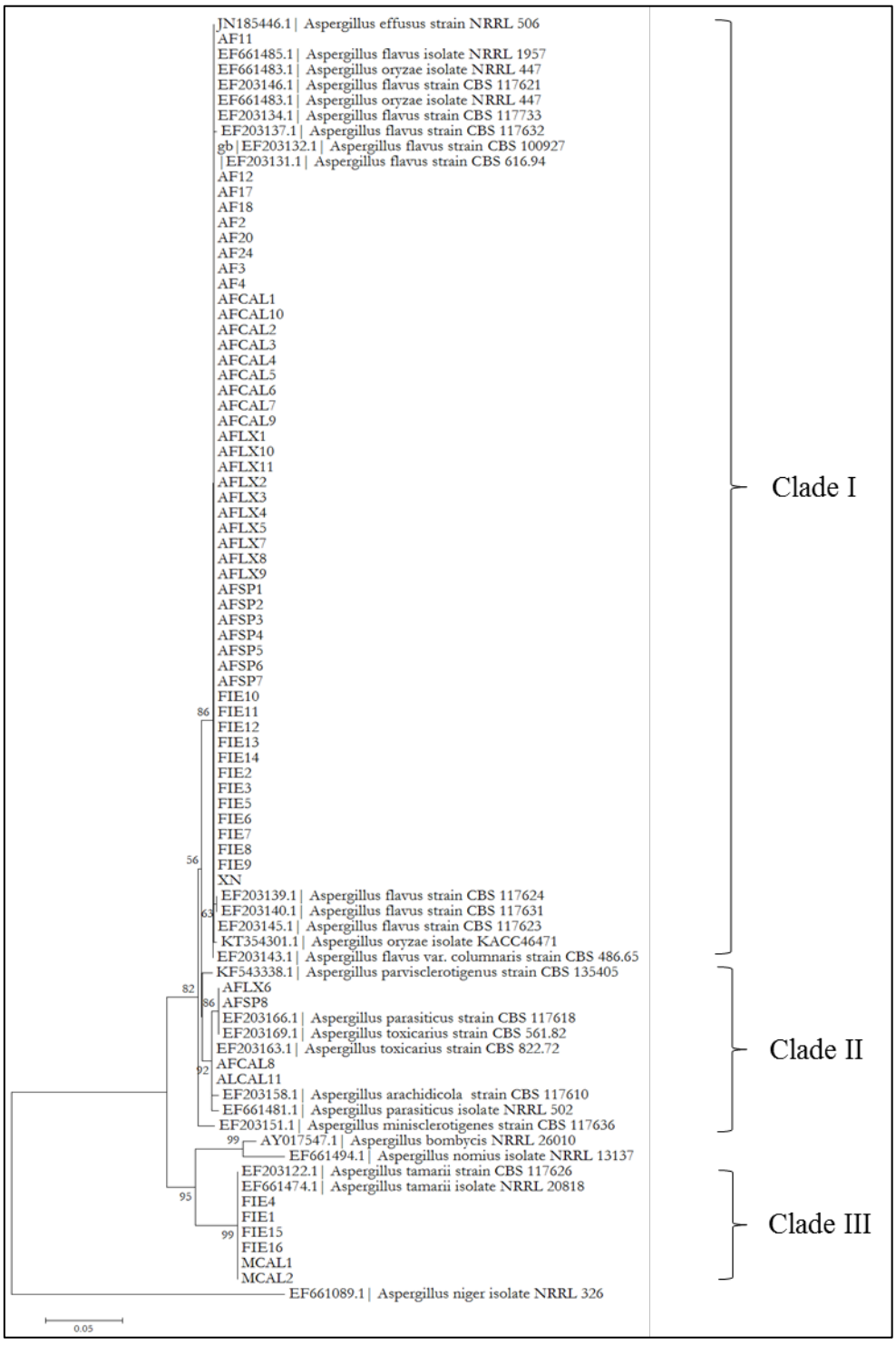


Figure 2. Maximum Likelihood phylogeny inferred from the calmodulin gene of *Aspergillus* section *Flavi* strains isolated in this study. Phylogeny was rooted by *A. niger*. The numbers at the major nodes indicate the percent ML bootstrap values from 1000 bootstrapped datasets. Evolutionary analyses were conducted using MEGA6.

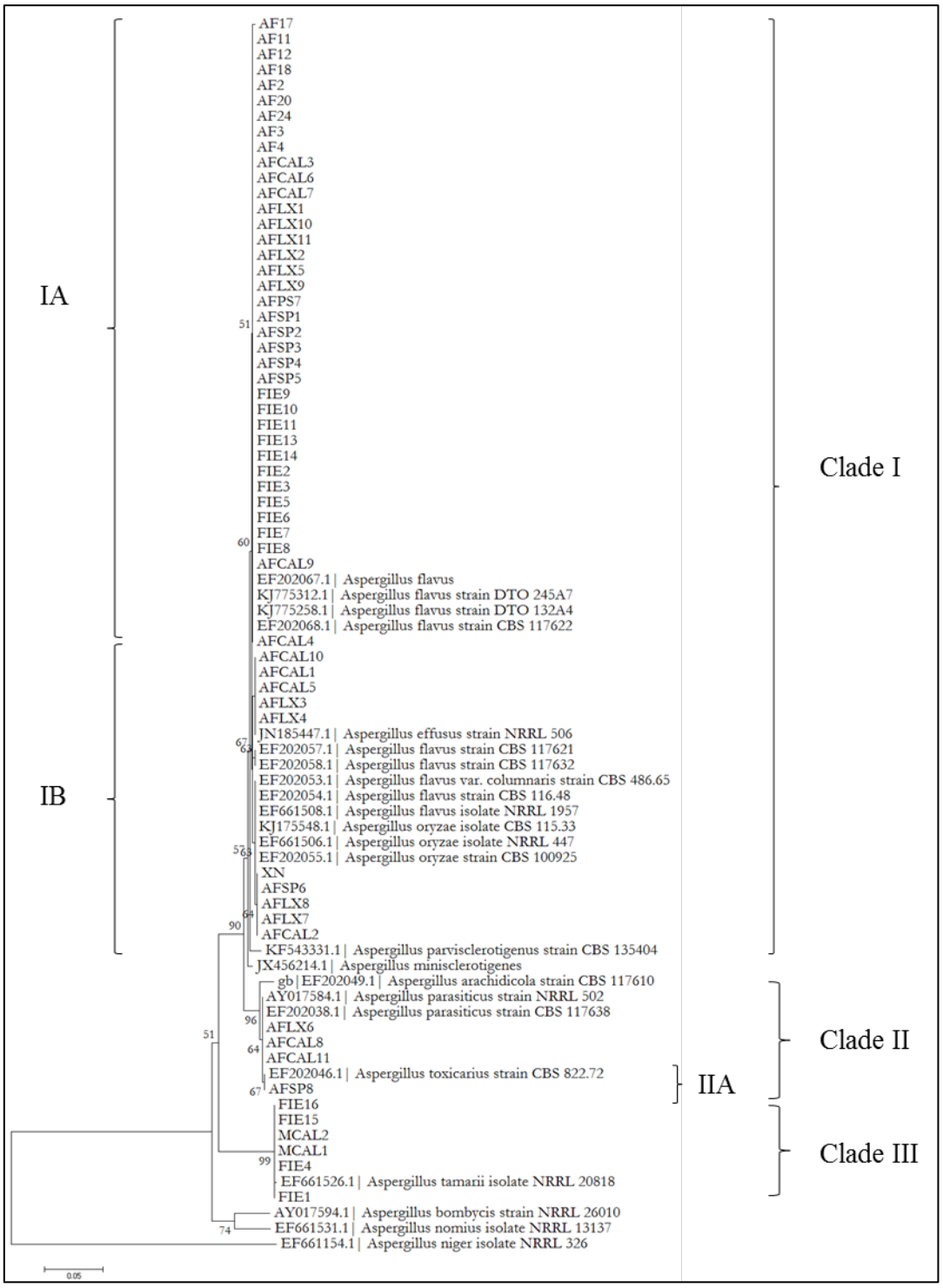


Figure 3 - *Aspergillus* section *Flav*i colonies after 7 days of incubation at 25°C on YES, CYA and MEA. **A.** *Aspergillus oryzae* var. *effusus*. **B.** *Aspergillus flavus*. **C.** *Aspergillus parasiticus.* **D.** *Aspergillus toxicarius*. **E.** *Aspergillus tamarii*.

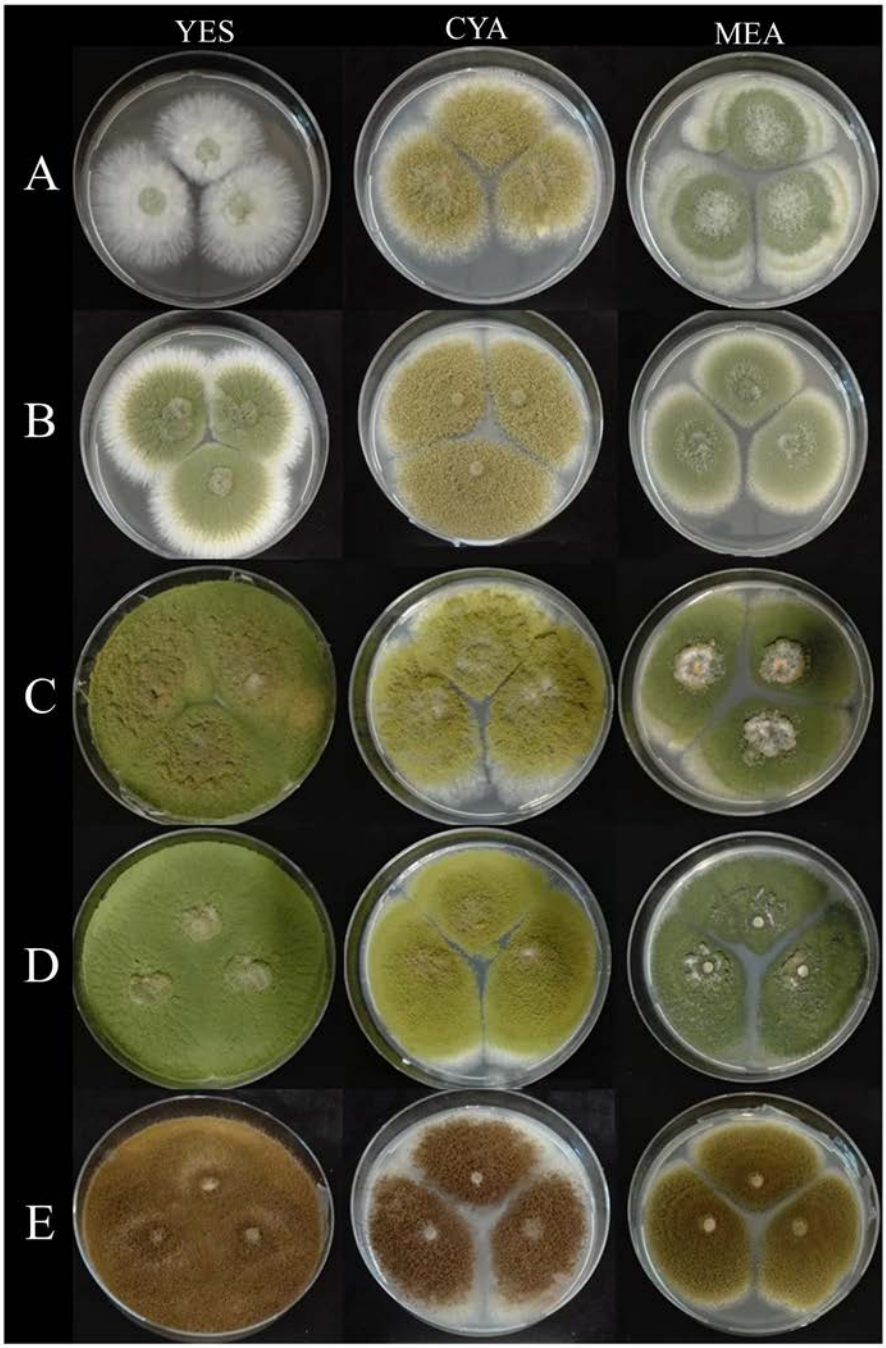


Figure 4. *Aspergillus flavus* AFLX8,AFCAL2, XN, AFSP6 and AFLX7 strains after 7 days of incubation at 25°C on YES, CYA and MEA.

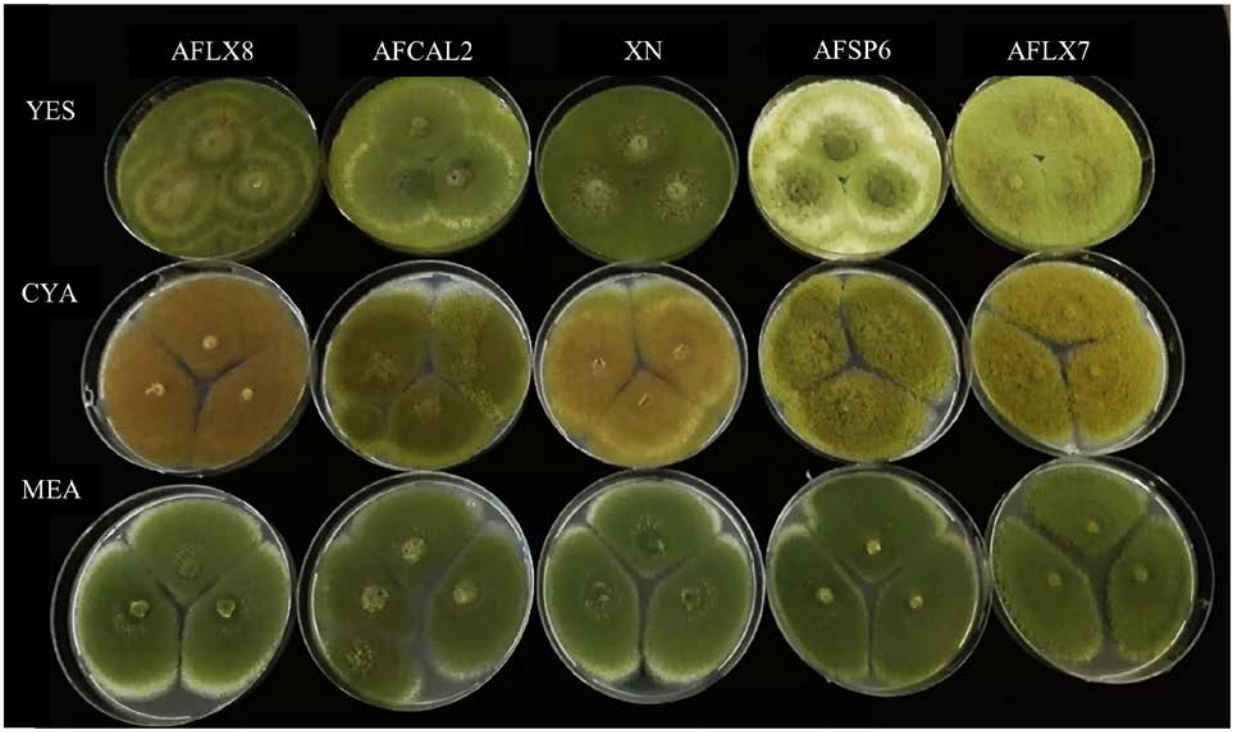
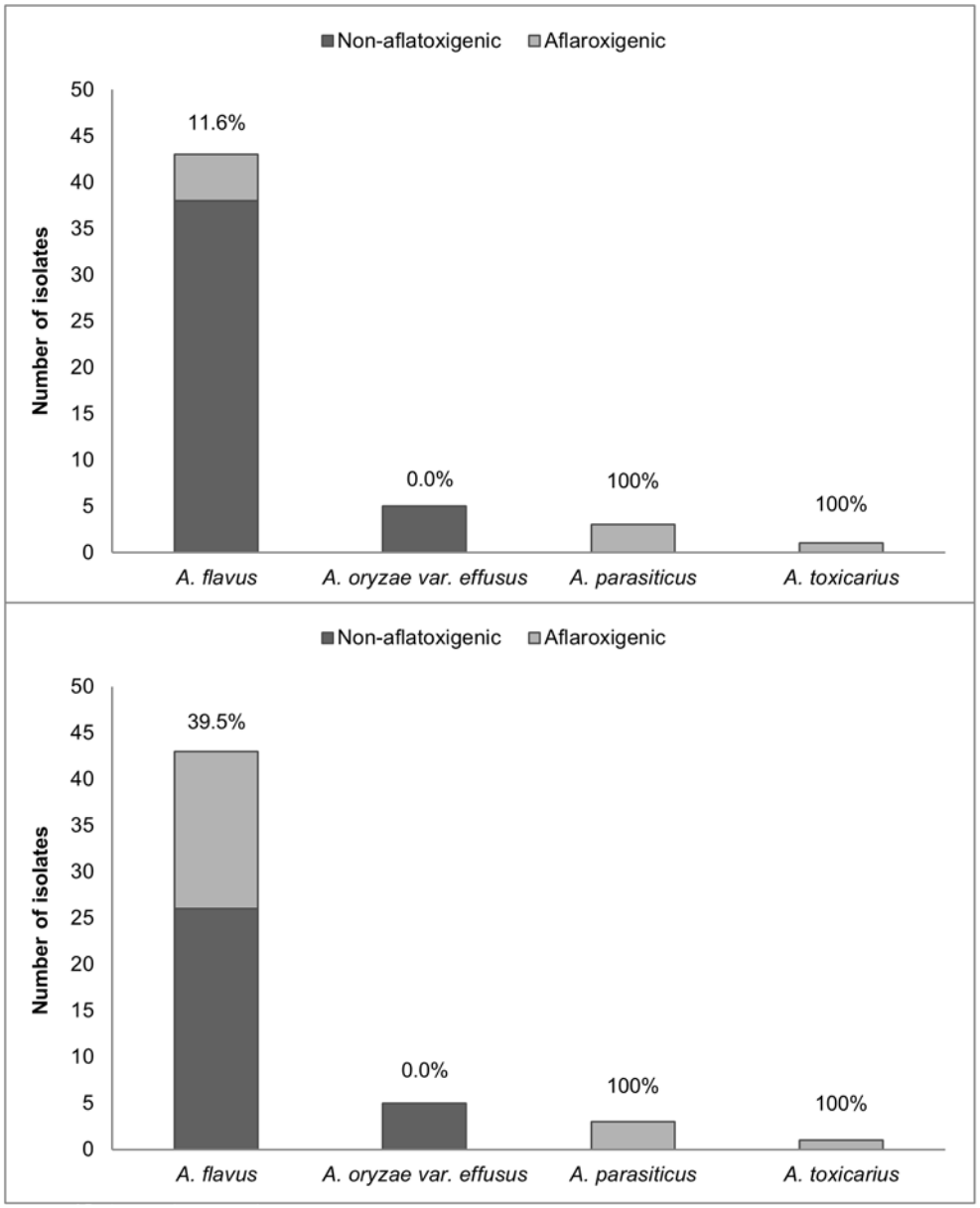
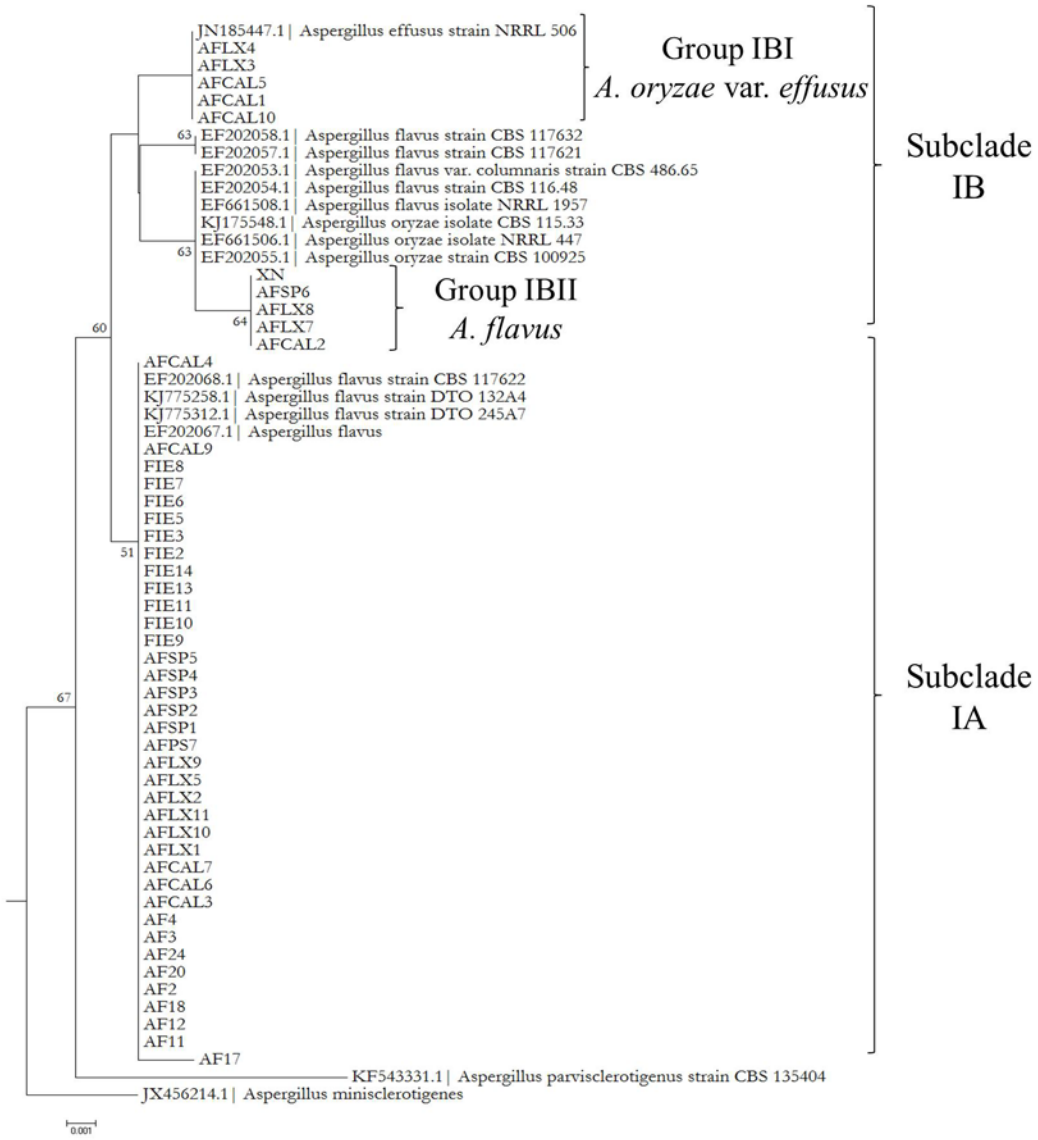


Figure 5. Number (and percentage) of aflatoxigenic strains of *Aspergillus* section *flavi in vitro* on YES Broth incubated in the dark at 35 °C for 7 days (A) and *in vivo* on fresh chestnuts incubated at 28±1°C in the dark for 7 days (B).



Supplementary figures

Supplementary Figure 1. Focus of the Maximum Likelihood phylogeny inferred from the calmodulin gene of *Aspergillus* section *Flavi* strains belonging to Clade I isolated from chestnut. Phylogeny was rooted by *A. minisclerotigenes*. The umbers at the major/main nodes indicate the ML bootstrap values percent from 1000 bootstrapped datasets. Evolutionary analyses were conducted using MEGA6.



Supplementary Tables

Supplementary Table 1. List of the accession numbers used as references, and the relative description of the name, designation, source of isolation and origin of the strains used for the phylogenetical analysis of the β-tubulin gene.

β-tubulin gene references				
Species name	**Strain designation**	**Isolation source**	**Origin**	**Accession number**
A. flavus	CBS 117631	*Arachis hypogaea* seed	Argentina	EF203140
	CBS 117624	*Arachis glabrata* leaf	Argentina	EF203139
	CBS 100927	Cellophane	South Pacific Island	EF203132
	CBS 117621	*Arachis hypogaea* seed	Argentina	EF203146
	CBS 117632	*Arachis hypogaea* seed	Argentina	EF203137
	NRRL 1957	Cellophane	South Pacific Island	EF661485
	CBS 616.94	Orbital tumor	Germany	EF203131
	CBS 117623	Seed	Argentina	EF203145
	CBS 486.65	Soil	California	EF203143
	CBS 117733	Hospital strain	The Netherlands	EF203134
A. oryzae	CBS 100925	Unknown	Japan	EF203138
	KACC46471	Soy sauce	Korea	KT354301
	NRRL 447	Insect	The USA	EF661483
A. oryzae var. *effusus*	NRRL 506	Unknown	Unknown	JN185446
A. parasiticus	CBS 117618	*Arachis glabrata* leaf	Argentina	EF203166
	NRRL 502	Sugarcane	Hawaii	EF661481
A. toxicarius	CBS 822.72	*Arachis hypogaea*	Uganda	EF203163
	CBS 561.82	Löss deposit	Nebraska	EF203169
A. tamarii	NRRL 20818	Activated charcoal	Unknown	EF661474
	CBS 822.72	*Arachis hypogaea* seed	Argentina	EF203122
A. arachidicola	CBS117610	*Arachis glabrata* leaf	Argentina	EF203158
A. bombycis	NRRL 26010	*Bombyx mori*, silkworm larva	Unknown	AY017547
A. minisclerotigenes	CBS 117636	*Arachis hypogaea* seed	Argentina	EF203151
A. nomius	NRRL 13137	Wheat	The USA	EF661494
A. parvisclerotigenus	CBS 135405	Sesame kernels	Nigeria	KF543338
A. niger	NRRL 326	Tannin-gallic acid fermentation	The USA	EF661089

Supplementary Table 2. List of accession numbers used as the references, and relative description of the name, strain designation, source of isolation and origin of the species used for the phylogenetical analysis of the calmodulin gene.

Calmodulin gene references				
Species name	**Strain designation**	**Isolation source**	**Origin**	**Accession number**
A. flavus	DTO 245A7	Indoor house dust	The United Kindom	KJ775312
	DTO 132A4	Indoor house dust	Thailandia	KJ775258
	CBS 486.65	Soil	California	EF202053
	CBS 116.48	Unknown	The Netherlands	EF202054
	CBS 117621	*Arachis hypogaea* seed	Argentina	EF202057
	CBS 117632	*Arachis hypogaea* seed	Argentina	EF202058
	-	Unknown	Unknown	EF202067
	CBS 117622	*Arachis hypogaea* seed	Argentina	EF202068
	NRRL 1957	Cellophane	South Pacific Island	EF661508
A. oryzae	CBS 100925	Unknown	Japan	EF202055
	NRRL 447	Insect	USA	EF661506
	CBS 115.33	Unknown	Poland	KJ175548
A. oryzae var *effusus*	NRRL 506	Unknown	Unknown	JN185447
A. parasiticus	CBS 117638	*Arachis hypogaea* seeds	Argentina	EF202038
A. parasiticus	NRRL 502	Sugarcane	Hawaii	AY017584
A. toxicarius	CBS 822.72	*Arachis hypogaea*	Uganda	EF202046
A. tamarii	NRRL 20818	Activated charcoal	Unknown	EF661526
A. arachidicola	CBS117610	*Arachis glabrata* leaf	Argentina	EF202049
A. bombycis	NRRL 26010	*Bombyx mori*, silkworm larv	Unknown	AY017594
A. minisclerotigenes	-	White pepper	Morocco	JX456214
A. nomius	NRRL 13137	Wheat	The USA	EF661531
A. parvisclerotigenus	CBS 135404	Sesame kernels	Nigeria	KF543331
A. niger	NRRL 326	Tannin-gallic acid fermentation	The USA	EF661154

Supplementary Table 3. Number of samples with *Aspergills* section *Flavi*/total samples and incidence (%).

Contaminated / total samples				
Sources of isolation	**Origin**			**Incidence (%)**
	Italy	**Albany**	**Spain**	
Dried chestnuts	14/30	3/30	-	**19%**
Sorted dried chestnuts	*	5/30	-	**8%**
Chestnut granulate	4/40	6/40	7/40	**14%**
Roasted chestnut granulate	6/40	5/40	1/40	**10%**
Chestnut flour	-	-	-	-
Incidence (%)	**22%**	**14%**	**6%**	-

*The processing phase of the sorted dried chestnuts was absent for the Italian lot.