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A polyphasic study of *Aspergillus* section *Flavi* isolated from corn in Guangxi, China- a hot spot of aflatoxin contamination

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ABSTRACT

Aspergillus section *Flavi* is widely known as a potential threat to contaminate agricultural products and food commodities. In this study, a polyphasic approach consisting of micro- and macro-morphological, chemical and molecular features, was applied to survey the *Aspergillus* section *Flavi* population in corn collected from Guangxi, China. Based on multigene phylogenies as well as morphological observations, *Aspergillus flavus* (192/195), *A. arachidicola* (1/195), *A. pseudonomius* (1/195) and *A. novoparasiticus* (1/195) were found to be the predominant section *Flavi* population. Among them, 31 representative isolates were selected for mycotoxin determination. The results showed that *Aspergillus flavus* chemotype I was most common, chemotype IV was also detected with low incidence and low CPA amounts, while chemotypes II and III were absent. Other tested species including *A. arachidicola*, *A. pseudonomius*, and *A. novoparasiticus* produced all types of aflatoxins, but none of them produced CPA. The polyphasic approach applied in this study permitted reliable understanding of the prevailing *Aspergillus* section *Flavi* population and their mycotoxin profiles. Knowledge of the prevailing section *Flavi* population will aid in developing a sustainable strategy to mitigate the effects of aflatoxin contamination. This study suggests that CPA contamination of food should be considered while conducting mycotoxigenic surveys of food commodities, and the same should be considered while planning a bio-control strategy to control aflatoxin contamination.

1. Introduction

Aflatoxins (AFs), derivatives of polyketide, are low molecular weight, secondary metabolites primarily produced by filamentous cosmopolitan species of genus *Aspergillus*: *Aspergillus flavus*, *A. parasiticus* and *A. nomius* (Malik et al., 2010; Paul, 1993) According to previous reports, 25% world agricultural commodities were contaminated by mycotoxins (Boutrif and Canet, 1998), more recently Lee and Ryu (2017) summarized that 45% of global corn and 33% of corn based products were contaminated by aflatoxins, but no specific surveys for cyclopiazonic contamination have been conducted so far. Among all the aflatoxins, aflatoxin B1 is the most potent toxin for being hepatocarcinogenic, teratogenic and mutagenic, posing the risk of hepatitis, edema, hemorrhage, neural tube defects, esophageal cancer and hepatic carcinoma in human and animals (Turner et al., 2005; Wu et al., 2014).

Species of *Aspergillus* section *Flavi* are of great importance in the food industry because they produce different kinds of mycotoxins which damage fresh and stored food commodities. *Aspergillus flavus* is economically and biologically of major concern, and it occupies a major position in section *Flavi*. *Aspergillus flavus* populations are very diverse and have been widely studied to establish a correlation between their phenotypic characters to aflatoxigenic profiles (Horn, 2007). Based on toxigenic profiles, five groups of chemotypes were proposed for *Aspergillus flavus*: (1) chemotype I for AFBs and Cyclopiazonic Acid (CPA) producers; (2) chemotype II for AFBs, AFGs and CPA producers; (3) chemotype III for AFBs producers; (4) chemotype IV for CPA producers; and (5) chemotype V for non-producers (Vaamonde et al., 2003). In another study, chemotype I can be further separated as (1) AFB1 > AFB2 and CPA producer and (2) AFB1 < AFB2 and CPA producer, and the seventh group was established for AFB and AFG producers (Giorni et al., 2007). It's well known that *A. flavus* populations and toxigenic profiles considerably vary with geographic region, strain, and the substrate. While other members of section *Flavi* are more consistent in their toxigenic profiles, *A. parasiticus* and *A. nomius* are usually strong aflatoxigenic and produce AFBs and AFGs but not CPA, and non aflatoxigenic strains are rare (Razzaghi-abyaneh et al., 2006; Vaamonde

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et al., 2003). Frisvad et al. (2019) updated the mycotoxin profile of section *Flavi* species, 15 more species including *A. aflatoxiformans*, *A. arachidicola*, *A. austwickii*, *A. cerealis*, *A. luteovirescens*, *A. minisclerotigenes*, *A. mottae*, *A. novoparasiticus*, *A. pipericola*, *A. pseudocaelatus*, *A. pseudonomius*, *A. pseudotamarii*, *A. sergii*, *A. togoensis*, and *A. transmontanensis* are able to produce aflatoxins.

Among agricultural products, corn is highly susceptible to aflatoxin contamination. Corn-based products occupy a major position in human and animal diet; hence, its consumers are at high risk of exposure to aflatoxin contamination. Aflatoxigenic fungi and aflatoxin contamination of corn have been investigated in several countries around the world such as China (Gao et al., 2011), Argentina (Razzazi-Fazeil et al., 2004), Turkey (Algül and Kara, 2014), Indonesia, Korea (Kim et al., 2013), Italy (Gallo et al., 2012), Pakistan (Sabahat et al., 2010), Kenya (Okoth et al., 2018), Brazil (Baquião et al., 2013) and in United States (Horn, 2007). These studies mainly identify *Aspergillus* species based on morphological characters and toxigenic profile. However, due to high interspecific similarities and intraspecific variabilities within *Aspergillus* (Ehrlich and Mack, 2014; Pildain et al., 2008), a polyphasic approach which combines morphological, chemical and molecular methods is necessary for the accurate identification.

Guangxi province located in the southwest is one of the largest corn zones of China. Due to the hot and humid weather here, corn is susceptible to aflatoxin contamination. Hence, a polyphasic study to investigate *Aspergillus* section *Flavi* population in Guangxi was needed, which may affect prevention and control strategies of fungi and mycotoxins in this region. The objectives of present study herein are; a) to investigate the biodiversity of *Aspergillus* section *Flavi* from corn growing areas of Guangxi province by a polyphasic approach which involves morphological, chemical and molecular methods, b) to investigate the toxigenic profiles of the prevailing *Aspergillus* section *Flavi* populations.

2. Materials and methods

2.1. Fungal isolates

Eighty-nine corn samples were collected from six cities of Guangxi province, China during 2016 and 2017. Isolations were made using dilution to extinction method (Collado et al., 2007), modified by Visagie et al. (2014). We used 48 well microplate instead of capped 1.5 mL tubes, 1 mL of Malt Extract Agar (MEA 20 g malt extract, 15 g agar with Chloramphenicol) was dispensed into each well using a multichannel pipette. Corn grains were milled to flour, and corn flour was suspended in carboxymethylcellulose (0.1%) solution and diluted stepwise up to 1:64. Ten µL aliquots were pipetted per well, and the dilution, yielding a maximum number of single colonies was selected for subsequent studies. Isolation plates were kept at 28 °C for 7–10 days and colonies that appeared to represent *Aspergillus* section *Flavi* species (Klich, 2002) were plated onto 9 cm MEA plates and further purified if necessary.

2.2. Morphological characterization

Spore suspensions of each isolate were made in 500 µl of 0.2% agar solution by transferring a loop full of spores from seven days old cultures. These suspensions were used for three-point fashioned inoculations onto 9 cm Petri dishes containing 20 mL of MEA and Czapek agar (CZ) (Czapek dox liquid medium: 33.4 g; bacteriological agar: 15 g; distilled water: 1 L.) and incubated at 25 °C for 7 days in the dark. Plates were investigated for macroscopic (colony color and morphology) and microscopic (head seriation, conidia morphology) characteristics under Nikon Eclipse 80i microscope equipped with NTS-Elements F 3.0, by following the identification key and guides for *Aspergillus* species (Klich, 2002). Colony diameters of all the isolates were measured on CZ media after 7 days of incubation at 25 °C, 37 °C and 42 °C, and group identification was confirmed according to available guidelines (Kurtzman et al., 1987; Peterson et al., 2001; Varga et al., 2011).

Sclerotia production by the tested isolates was determined by inoculating on CZ culture plates incubated at 30 °C for 15 days. Sclerotia were scraped from culture plates and washed on Whatman No.2 filter under a stream of water containing 0.1% tween 20. Ten sclerotia were selected, and their diameters were measured under Nikon Eclipse 80i microscope.

2.3. Molecular characterization

2.3.1. DNA extraction

DNA was extracted from cultures grown in 100 ml of Wickerham medium (40 g/L glucose, 5 g/L peptone, 3 g/L yeast extract, 3 g/L malt extract), for 2–4 days at 25 °C under shaking condition (150 rpm). Mycelia were harvested and washed with 0.5 M ethylenediamine tetraacetic acid (EDTA) and sterilized water. DNA was extracted by CTAB method briefly; frozen mycelia were ground fine by a glass rod in 1.5 mL tube added with 120 µL of CTAB and quartz sand. After grinding, 1 mL of CTAB was added, and tubes were incubated at 65 °C for one hour with occasional gentle inversion. Tubes were centrifuged at 14000 rpm for ten minutes, and 800 µL of supernatant were transferred to 2 mL centrifuge tube to which 800 µL of Chloroform:Phenol:Isoamyl alcohol (24:24:1) was added, inverted gently and centrifuged at 14000 rpm for 10 min. Supernatants (700 µL) were extracted with 700 µL of chloroform:isoamyl alcohol (24:1) and centrifuged at 14000 rpm for 10 min. This step was repeated two times, and 400 µL of clear supernatant was gently mixed with isopropanol and incubated at −20 °C for 1 h. After incubation, DNA pallets were precipitated by centrifugation and washed twice with 70% alcohol. DNA was suspended in 50 µL of DEPC water, quantified with Thermo Fisher Quawell Q3000 UV spectrophotometer. The ratio 260/280 = 1.6–1.85 showed that the DNA was of good quality.

2.3.2. PCR amplification and sequencing

PCR amplification of ITS, *BenA* and *CaM* were done for each isolate using primer set ITS1&ITS4 (White et al., 1990), Bt2a&Bt2B (Glass and Donaldson, 1995) and cmd5&cmd6 (Hong et al., 2006). PCR amplifications were performed in 25 µL of the reaction mixture, containing 50 mM $MgCl_2$, 10 mM of each dNTP, 5 U/uL Taq DNA Polymerase, 10 uM of each primer and 10 ng/µL DNA template. The PCR products were sequenced from BGI GuangZhou and obtained sequences were submitted in NCBI GenBank (*BenA* accession numbers MN213616-33, MN210919-30, MN240568 and CaM accession numbers MN240567, MN240569-98).

2.3.3. Phylogenetic analysis

For preliminary identification of isolates, obtained sequences were compared with already submitted sequences in GenBank (http://www.ncbi.nlm.nih.gov/) by using nucleotide Blast feature of NCBI. For precise identification, phylogenies of amplified ITS, *BenA* and *CaM* were established. Each sequence alignment was generated with MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/index.html) and manually adjusted. The most suitable substitution model was determined using FindModel (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html). Maximum likelihood analyses, including 500 bootstrap replicates were run using RAxML (Stamatakis et al., 2008). Bayesian analyses were performed with MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003). The sample frequency was set to 100, and the first 25% of trees were removed as burn-in. The resulting trees were read with FigTree v1.4.2 and annotated using Adobe Illustrator CS5. BI posterior probabilities (pp) values and bootstrap (bs) percentages of analysis are labeled at the nodes. Values < 0.95 pp. and < 70% bs are not shown. Branches with values > 0.98 pp. and 95% bs are thickened.

2.4. Determination of mycotoxin profiles

2.4.1. Fast screening of aflatoxin producers

2.4.1.1. Ammonia vapor test. Isolates were incubated on potato dextrose Agar (PDA) culture plates and incubated at 25 °C for three days. After the incubation period, dishes were placed upside down, a drop of 25% ammonia solution was put on plate lid, and reverse colony color was observed. Pinkish colony color change denotes aflatoxins production (Saito and Machida, 1999).

2.4.1.2. Carbon added medium fluorescence test. PDA was prepared with the addition of activated charcoal at a final concentration of 0.3 mg/mL and Cyclodextrin (CD) (αCD, βCD. mβCD, γCD) each at a concentration of 3 mg/mL (Suzuki and Iwahashi, 2016). Isolates were inoculated on modified PDA culture plates and incubated for 2–3 days at 25 °C. Cultures were observed for fluorescence signals from the reverse side of culture plates under long wavelength (365 nm) UV light. Colonies showing blue fluorescence signals were producers of aflatoxin B1 and B2 while colonies showing bluish-green fluorescence were producers of all four kinds of aflatoxins.

2.4.2. Fast screening of aspergillic acid

Isolates were inoculated on AFPA plates [bacteriological peptone 10 g; yeast extract 20 g; ferric ammonium citrate 0.5 g; bacteriological agar 15 g; dichloran 2 mg (0.2% in ethanol, 1.0 mL); distilled water 1 L] and incubated at 30 °C for 48 h. Aspergellic acid production was confirmed by observing orange color on the reverse side of the colony (Pitt et al., 1983).

2.4.3. HPLC determination of aflatoxins

All the tested isolates were incubated on 9 cm Yeast Extract Sucrose (YES) (yeast extract 20 g/L, sucrose 150 g/L, agar 15 g/L) petri plates and incubated at 25 °C in the dark for 7 days. Three agar plugs from each colony were taken with a cork borer and pooled in 4 mL capped vial, and then extracted with 1 mL of HPLC grade methanol for 60 min with occasional shaking. After 60 min, the extracts were filtered through a 0.22 µm syringe filter, and were stored at −20 °C until further analysis (Bragulat et al., 2001).

Aflatoxins of obtained extracts were analyzed using a Waters HPLC e2695 autosampler system equipped with a Waters fluorescence detector 2475 (365 nm excitation wavelength, 435 nm emission wavelength). Reverse phase Agilent C18 column (4.6 mm × 250 mm, 5 µm) was used for chromatographic separation. Aflatoxins were eluted with an isocratic mobile phase consisted of acetonitrile:methanol:water (2,2,6 v/v). The mobile phase flow rate was 1 mL/min, and the injection volume was 10 µL.

Aflatoxin standard solutions were purchased from Sigma and a mixture of a standard solution containing 2 µg/mL each of AFB1 and AFG1, and 0.5 µg/mL, each of AFB2 and AFG2, was used as a standard solution. Calibration curves (R^2 value 0.99) were constructed by standard solutions of different concentrations and samples were taken positive only when yielding a peak at a retention time similar to standard solution. Empower3 software was used to calculate the results of positive samples.

2.4.4. LC-MS/MS determination of cyclopiazonic acid (CPA)

Isolates were cultured on YES petri plates and incubated at 25 °C for 14 days in the dark. CPA was extracted with methanol and analyzed according to Novas et al. (2001).

Analysis of CPA was performed on Waters Acquity UPLC coupled with Xevo G2-XS QTof mass spectrophotometry. A ZORBAX Eclipse XDB C18 column (1.8 µm, 100 × 2.1 mm) was used for chromatographic separation. The mobile phase consisted of methanol (A) and water adjusted with 0.1% formic acid (B) at a flow rate of 0.4 mL/min. Mobile phase gradient elution program started with 90% A and 10% B then B decreased with a linear decrease to 30% in 5 min, at the same time B increased with a linear increase to 70% in 5 min. In the next 3 min, A was decreased to 1% and B was increased to 99% in 3 min and it was kept for 2 min. During the last 2 min A:B was kept at 90:10. Elution program was of 12 min, including re-equilibration. Nitrogen gas was applied as spray gas, and capillary voltage was 3 kv with source and desolvation temperatures set as 100 and 400 °C respectively. Cone gas flow was adjusted to 50 L/h while desolvation gas flow was set at 700 L/h.

CPA standard solutions were prepared by dissolving CPA solid standard in methanol. Precursor ion (*m/z*) of CPA was 337.15, and product ions (*m/z*) were 196.11 and 182.08.

3. Results

3.1. Identification of isolates

3.1.1. Phylogenetic identification

In total, 195 isolates were obtained from tested corn samples. In order to identify them, the nuclear ribosomal internal transcribed spacer (ITS), beta-tubulin (*BenA*), and calmodulin (*CaM*) genes sequences were used to construct phylogenetic trees. The optimal models were Kimura 2-parameter plus Gamma for *BenA* and Tamura-Nei plus Gamma for *CaM* respectively. The ITS tree could not distinguish each species in this section, thus was excluded here. Based on phylogenetic trees inferred from *BenA* and *CaM* genes, the obtained *Aspergillus* section *Flavi* population was identified as *Aspergillus flavus* (192/195), *A. arachidicola* (1/195), *A. pseudonomius*(1/195) and *A. novoparasiticus* (1/195) as shown in Figs. 1 and 2.

3.1.2. Morphological identification

Morphological characterization of the studied population is summarized in Table 1. Isolates of *Aspergillus flavus* are characterized by yellow-green colonies, smooth to finely rough conidia, predominantly biseriate conidiophores. *Aspergillus arachidicola* produces olive green colonies, rough conidia, routinely uniseriate conidiophores. *Aspergillus novoparasiticus* produces dark green colonies, rough conidia, and uniseriate conidiophores. *Aspergillus pseudonomius* produces yellow-green colonies, rough conidia, and predominantly uniseriate conidiophores. *Aspergillus flavus* isolates showed faster growth at 37 °C, the diameter of colonies of all the *A. flavus* isolates were > 50 mm on CZ at 37 °C except stain C20 which showed less growth (47 mm). Strain C20 also showed limited growth on CZ (6 mm) at 42 °C, which could lead its classification to species other than *A. flavus*, but we could not find any other distinguishing feature to support this argument. *Aspergillus pseudonomius* showed very limited growth (2 mm) on CZ at 42 °C and *A. arachidicola* isolates were on the second (9 mm). Colony diameters of all the *A. flavus* (except C20) and *A. novoparasiticus* isolates were > 10 mm on CZ at 42 °C.

Sclerotia production on CZ at 30 °C for 15 days placed *Aspergillus flavus* isolates into two groups: sclerotia producers and non-producers. Only eight isolates of *A. flavus* isolates produced sclerotia, among them, one isolate produced S-type sclerotia (< 400 nm), other seven isolates produced L-type sclerotia (> 400 nm). None of *A. arachidicola*, *A. novoparasiticus*, and *A. pseudonomius* isolates produced sclerotia (Table 1).

3.2. Toxigenic screening test

Mycotoxin profiles were screened on carbon added PDA at 25 °C (Fig. 3). Strains producing only aflatoxin B1 and B2 showed bluish fluorescence signals around margins of the colony (Fig. 3B), while strains producing both types of aflatoxins (B&G) showed bluish-green fluorescence signals (Fig. 3C). Ammonia vapor test showed pink pigmentation on reverse colony side of toxigenic and isolates (Fig. 4), but for few toxigenic isolates, it produced false results as a couple of isolates showed negative results, but they were toxigenic in carbon added PDA fluorescence test and in HPLC results (Table 2). Thus, carbon added

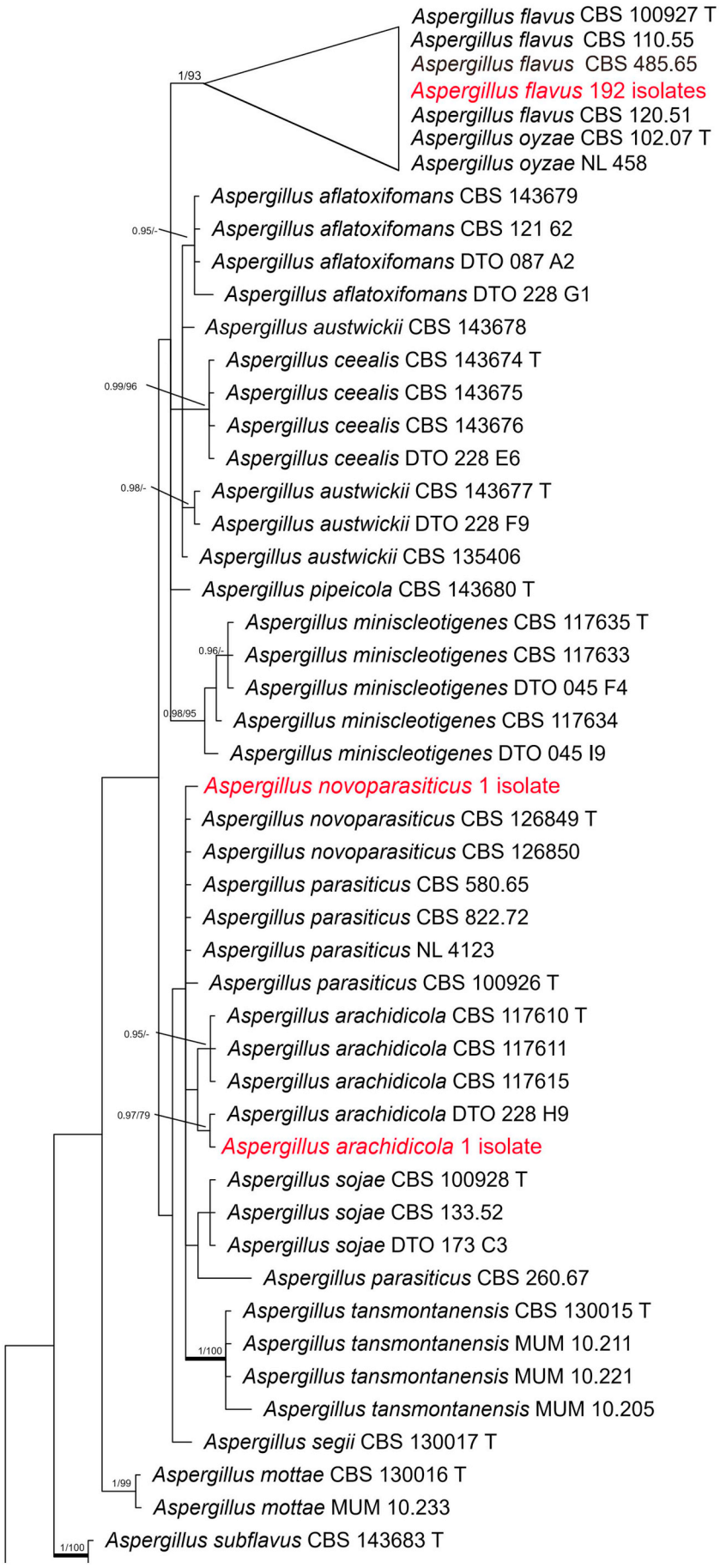

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Fig. 1. Phylogenetic tree inferred from the partial *BenA* sequences showing the relationships among members of *Aspergillus* section *Flavi*. ML bootstrap support values over 75%, and Bayesian posterior predictive values over 0.95 are shown above the branches. Strongly supported branches have bootstrap support values of 100, Bayesian posterior predictive values of 1.00 are shown in bold. The tree is rooted with *Aspergillus muricatus* NRRL 35674^{T}.

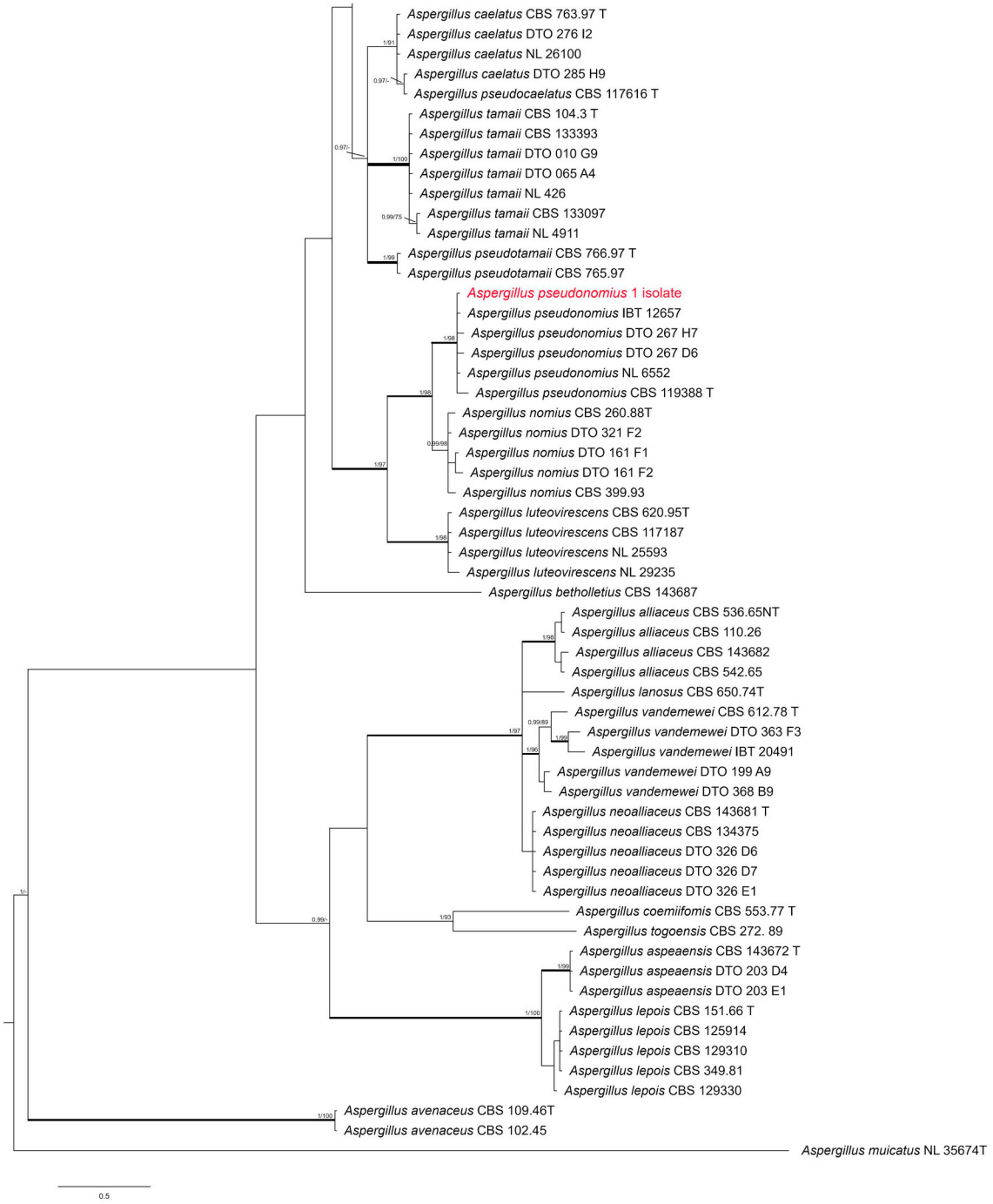

Fig. 1. (*continued*)

PDA fluorescence test was found to be more sensitive than the ammonia vapor test.

3.3. Mycotoxin analysis

Based on morphological characteristics and phylogeny, 31 representative isolates were submitted to HPLC and LC-MS/MS analysis. Fig. 5 shows the HPLC standard chromatogram of standard solution containing aflatoxins B1&G1(2 μg/mL) and aflatoxins B2&G2(0.5 μg/mL), and (B) showing the MS/MS standard spectrum of CPA with Precursor ion (*m/z*) of CPA 337.15 and product ions (*m/z*) are 196.11 and 182.08. The chromatogram of *Aspergillus pseudonomius* strain C39 and *Aspergillus flavus* strain C58 have been illustrated in Figs. 6 and 7, respectively.

Tested *Aspergillus* section *Flavi* isolates produced a diverse pattern of mycotoxigenic profiles (Table 2). *Aspergillus arachidicola*, *A. pseudonomius*, and *A. novoparasiticus* produced both types of aflatoxins, but none of them produced CPA. Mycotoxins (AFs and CPA) were produced by 26

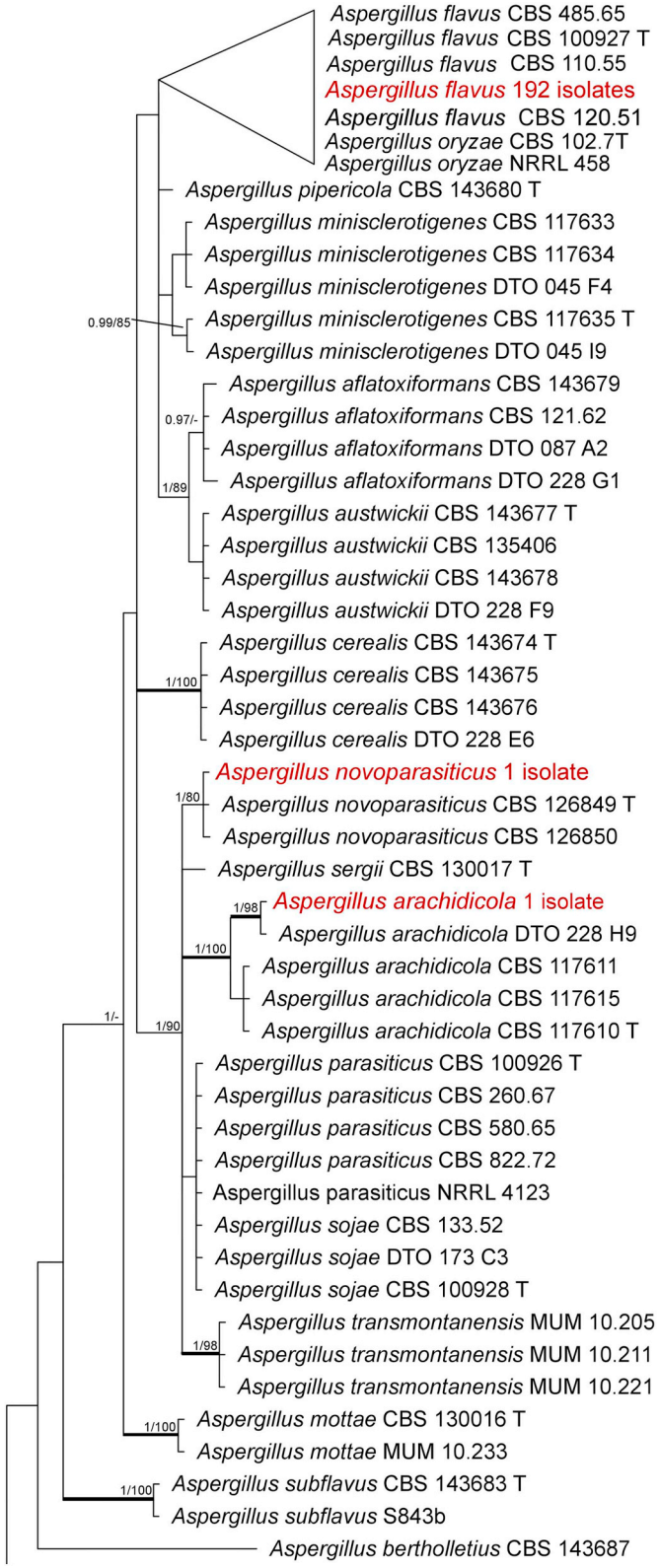

Fig. 2. Phylogenetic tree inferred from the partial *CaM* sequences showing the relationships among members of *Aspergillus* section *Flavi*. ML bootstrap support values over 75%, and Bayesian posterior predictive values over 0.95 are shown above the branches. Strongly supported branches have bootstrap support values of 100, Bayesian posterior predictive values of 1.00 are shown in bold. The tree is rooted with *Aspergillus muricatus* NRRL 35674T

isolates of *A. flavus* isolates. Two *A. flavus* isolates did not produce AFs and CPA and were identified as non-aflatoxigenic *A. flavus*. Five out of seven chemotypes of *A. flavus* proposed by Giorni et al. were found in our study (Table 2). Chemotype-I producing B-type aflatoxins and CPA was prominent, and most of the *A. flavus* isolates belonged to this chemotype. *Aspergillus flavus* isolates of chemotype II and III were not found in our study. Chemotype IV comprised three isolates producing only CPA. Only two isolates of chemotype V were found, and they were non-producers of aflatoxins and CPA. Interestingly, we found four isolates of chemotype VI which produced both types of aflatoxins and CPA, and they were strong producer of CPA, but their total AF (AFB and AFG) production was less than any other species of Section *Flavi*.

4. Discussion

Corn and corn-based products are susceptible to mycotoxin contaminations due to *Aspergillus* spp., infection of corn kernels during pre and post-harvest level. In this study, we have documented the incidence and toxigenicity of *Aspergillus* section *Flavi* from corn kernels in Guangxi province, China. Four species of section *Flavi* including *Aspergillus flavus*, *A. arachidicola*, *A. pseudonomius*, and *A. novoparasiticus* were found with diverse toxigenic profiles, *A. flavus* was the most commonly isolated among them.

Morphological identification of *Aspergillus* section *Flavi* is complicated due to diverse toxigenic profiles and interspecific similarities (Rodrigues et al., 2009). Characterization of section *Flavi* normally comprises colony color, conidial morphology, conidiophore seriation, toxigenic profile, and molecular markers. Kurtzman suggested a test to differentiate *A. nomius* from *A. flavus* by growing isolates at 42 °C on CZ. We found colony diameter at 42 °C (Kurtzman et al., 1987) as a useful tool to differentiate *A. flavus* from other species of section *Flavi* except for *A. novoparasiticus*, and it can be extended to differentiate *A. arachidicola* in the initial screening. According to Frisvad et al. (2019), the majority of species belonging to the *A. flavus*-clade were able to grow moderate or well at 42 °C (> 5 mm) on CYA, the only exceptions are *A. mottae*, *A. subflavus*, and *A. pipericola*. Our observations are in agreement with the growth range as they reported. Conidia color on MEA and/or CYA can be used to differentiate *A. flavus* and *A. novoparasiticus*, *A. flavus* produces yellow-brown conidia, while *A. novoparasiticus* produces dark green to brown conidia (Frisvad et al., 2019), these were confirmed in our study too, but yellow green colonies were observed in this study because 7-day old colonies were used for observation.

Preliminary screening of aflatoxigenic isolates is an essential diagnostic step in mycotoxigenic studies, and various techniques have previously been developed to accomplish this task. Fluorescence detection of aflatoxigenic isolates on coconut agar media was developed by Davis et al. (1987), and further modified to coconut liquid media by Degola et al. (2011). The ammonia vapor screening test was developed by Saito and Machida (1999). However, the inconsistencies of results have been reported by some authors (Rodrigues et al., 2009; Scherm et al., 2005; Yazdani et al., 2010). Same findings were documented in our study, false positive and false negative results of the ammonia vapor test were observed in the present study. Recently a fluorescence test was developed by Suzuki and Iwahashi (2016) by addition of carbon source and CDs (αCD, βCD. mβCD, γCD) in PDA. This test was found to be effective in the present study and showed a positive correlation with HPLC analysis.

Aspergillus flavus isolates are very diverse in their toxigenic profiles, and they have been classified into different chemotypes (Giorni et al., 2007). *Aspergillus flavus* isolates are able to produce aflatoxin type B and CPA simultaneously and are reported to produce these toxins in co-occurrence (Fernandez Pinto et al., 2001; Vaamonde et al., 2003). In the present study chemotype I (Aflatoxin B > B2 and CPA) was most common. The high frequency of chemotype I agrees with the finding of (Astoreca et al., 2011; Razzaghi-abyaneh et al., 2006; Rodrigues et al., 2009). We did not obtain any isolates of Chemotypes II and III; they

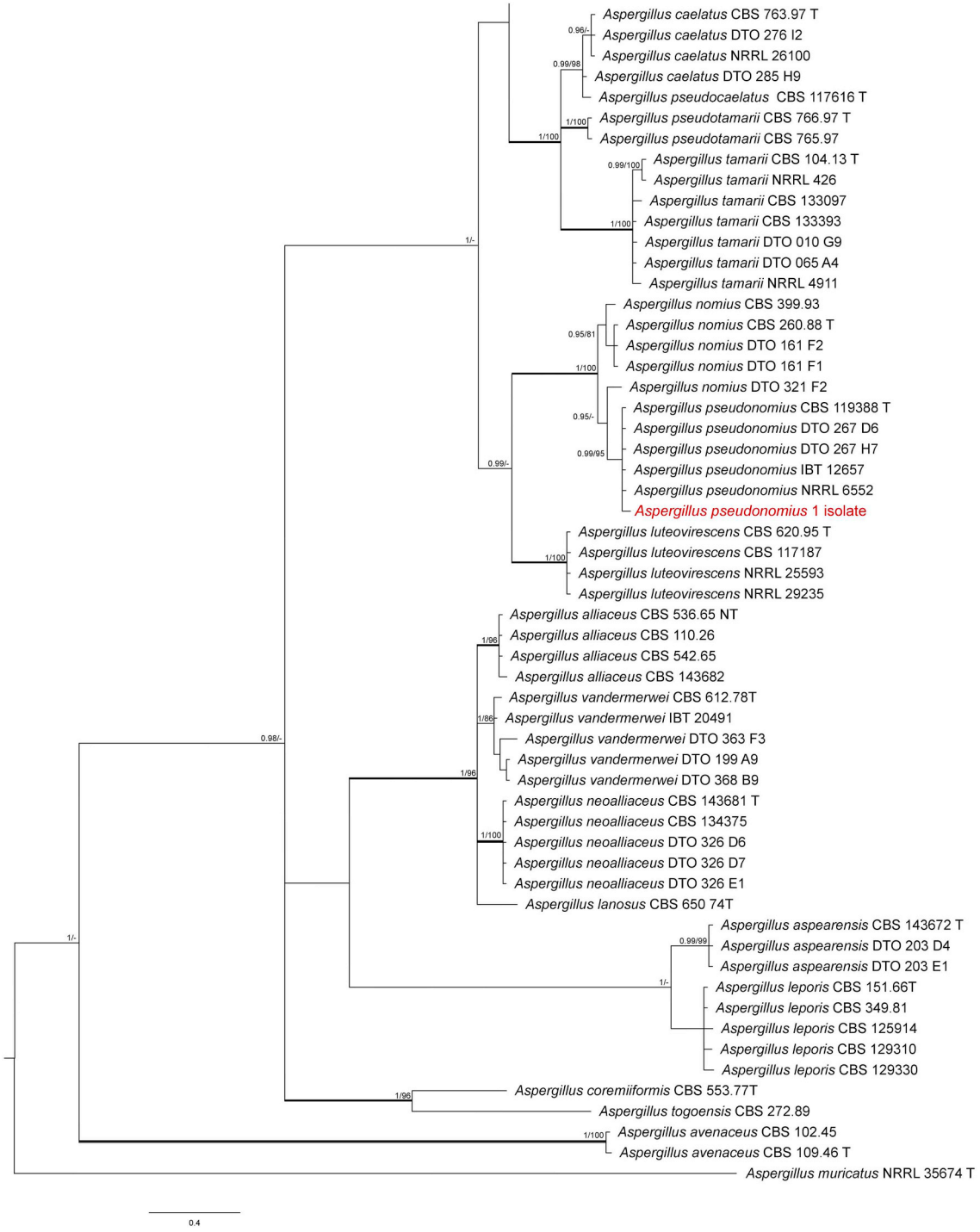

Fig. 2. (*continued*)

Table 1
Morphological characterization of tested *Aspergillus* section *Flavi* species.

Species	Colony diameter on CZ (mm)						
	Sclerotia size[a]	Seriation[b]	Conidia	Colony color	25 °C	37 °C	42 °C
Aspergillus flavus	If present, 325–860	B to b/u	Smooth to finely rough	Yellow green	30–51	40–63	6–25
Aspergillus arachidicola	–	U	Rough	Olive green	32	41	8.5
Aspergillus novoparasiticus	–	U	Rough	Dark green	42	44	11
Aspergillus pseudonomius	–	u/b	Rough	Yellow green	50	41	2

[a] Size: average size of 10 sclerotia in μm.
[b] U: uniseriate; B: biseriate; u/b: predominantly uniseriate; b/u: predominantly biseriate.

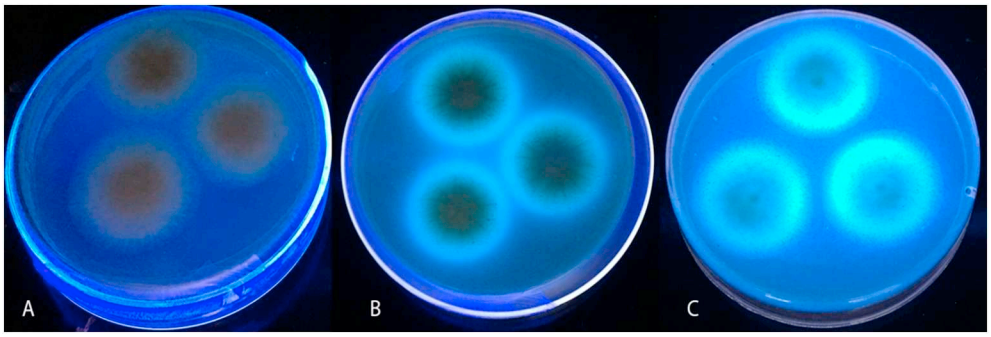

Fig. 3. Fluorescence signals around colony margins under UV light (λ365) after grown on carbon added media for 2–3 days. (A) Non-toxigenic isolates showing no fluorescence signals, (B) Toxigenic isolates showing blue fluorescence and producing only aflatoxin B1 and B2, (C) Toxigenic isolates showing greenish-blue fluorescence and producing all four kinds of aflatoxins. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

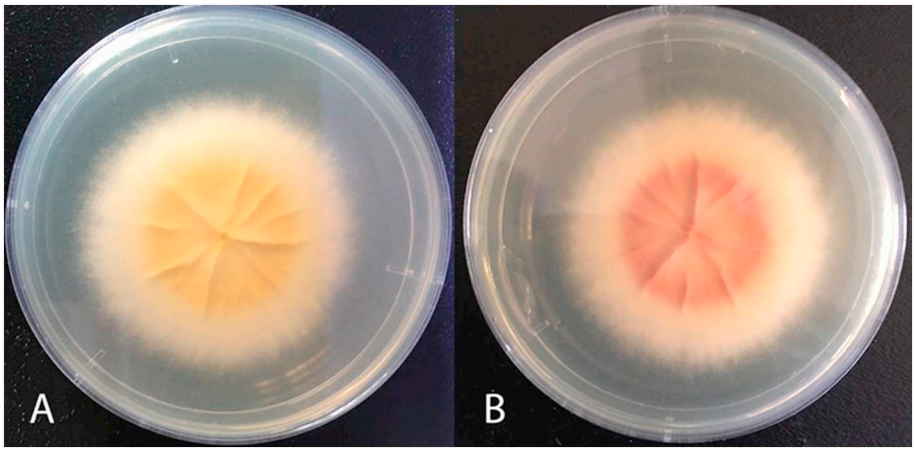

Fig. 4. Isolates grown on PDA media for 3 days showing color change after ammonia vapor test. (A) Colony color before the test and (B) Pink colony color after the test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Toxigenic profiles of tested *Aspergillus* section *Flavi* species.

Species	Chemotype	Isolates	AFB1 ng/g	AFB2 ng/g	AFG1 ng/g	AFG2 ng/g	Total AF ng/g	CPA ng/g	Aspergillic acid	Fast toxigenic screening test	
										Carbon added	Ammonia vapor
A. flavus	I	19	3864	94	NA	NA	3958	47,139	+	+	+ (−)
A. flavus	l	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
A. flavus	III	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
A. flavus	IV	3	NA	NA	NA	NA	NA	14,194	+	NA	NA
A. flavus	V	2	NA	NA	NA	NA	NA	NA	+	−	−
A. flavus	VI	4	2140	85	419	2	2646	71,562	+	+	+
A. arachidicola	VII	1	1845	156	507	24	2532	NA	+	+	+
A. pseudonomius		1	19,958	542	48,122	912	69,534	NA	−	+	+
A. novoparasiticus		1	11,995	6	12,775	187	24,963	NA	+	+	+

ND: not found in this study.
NA: not applicable.
+: Positive result; (−) false negative results (a couple of isolates showed negative results, but there were positive for toxins in carbon added media test and in HPLC results).
-: Negative results.

were not frequently distributed according to other studies. Chemotype IV (producers of CPA only) was also identified in the present study with low incidence and low CPA amounts. This finding may be attributed as a high amount of CPA is produced in co-occurrence with aflatoxins. Chemotype VI producing both types of aflatoxins (B and G type) and CPA is unusual in *Aspergillus flavus* (Blaney et al., 1989; Geiser et al., 2000; Vaamonde et al., 2003) four isolates of chemotype VI were identified in our work and incidence of chemotype VI is rare as reported by Frisvad et al. (2019). Our chemotype VI strains showed elevated levels of CPA production, which might suggest that chemotype VI have the propensity to produce higher amounts of CPA. Chemotype V comprises non-toxigenic members of section *Flavi* as they don't produce aflatoxins or CPA. The incidence of non-aflatoxigenic *Aspergillus flavus* isolates (chemotypeV) was relatively low (6.5%), and the same results have previously been reported (Astoreca et al., 2011; Baquião et al., 2013).

For other tested species, both types of aflatoxins (B&G) were produced by tested isolates of *A. arachidicola*, *A. novoparasiticus*, and *A. pseudonomius*, but no CPA production was observed. These results are consistent with previous results (Baquião et al., 2013; Razzaghi-abyaneh et al., 2006; Rodrigues et al., 2009; Pildain et al., 2008; Vaamonde et al., 2003). Frisvad et al. (2019) reported 13 CPA producers in *Aspergillus* section *Flavi*. CPA is immunotoxic and cytotoxic for human, and its immunosuppressive effects in the human cell have been studied *in vivo* (Hymery et al., 2014). CPA is speculated to be associated with “Kodo poisoning” toxicity characterized by unconsciousness,

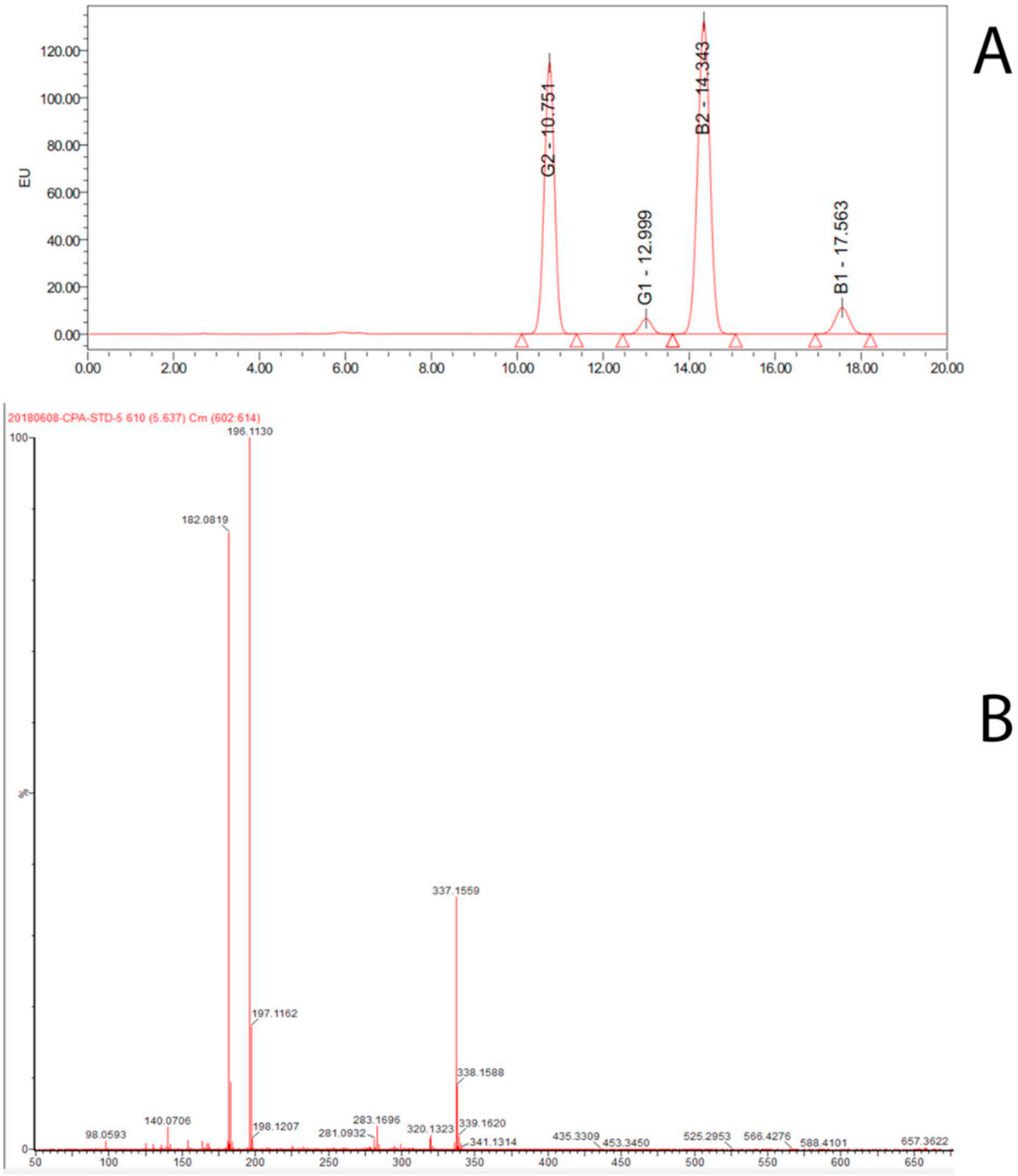

Fig. 5. (A) Standard chromatogram of Aflatoxins AFB1, AFB2, AFG1 and AFG2 and (B) MS/MS spectrum of CPA.

intoxication, depression, vomiting and nausea after consumption CPA contaminated Kodo millet in north India (Antony et al., 2003). *Aspergillus flavus* was reported as main CPA producer in different substrates in Argentina (Vaamonde et al., 2003). In the present study, the ratio of CPA producers among *A. flavus* was high (92%), and it has previously been reported as 89% (Blaney et al., 1989), 93% (Horn et al., 1996), and 97% (Resnik et al., 1996). Considering the high occurrence of CPA in *A. flavus* and other section *Flavi* species, we should pay more attention to CPA contamination for food safety purposes.

Sclerotia production by *Aspergillus flavus* is not a routine phenomenon, and it is suggested to be related to environmental factors (Giorni et al., 2007). In the present study, we found eight isolates of *A. flavus* produced sclerotia, and only one out of them produced S-type sclerotia (< 400microm). Among sclerotial producer, one isolate was non-aflatoxigenic, so we could not differentiate toxigenic and non-aflatoxigenic isolates on the basis of sclerotia production. Similar results have been documented by(Astoreca et al., 2011; Giorni et al., 2007; Rodrigues et al., 2009, 2011). Some studies have documented a positive correlation between elevated aflatoxin level and sclerotia production (Cotty, 1989; Novas et al., 2001), whereas others had contradictory results, and they found no co-relation of sclerotia size and elevated toxin level (Giorni et al., 2007; Razzaghi-abyaneh et al., 2006; Rodrigues et al., 2009), similar results are documented in the present study. However, average aflatoxin production was high in sclerotial producers, the same

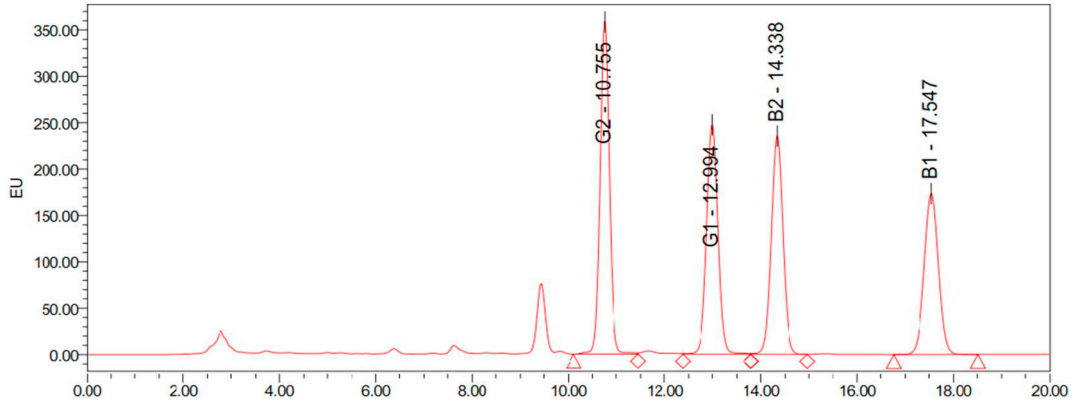

Fig. 6. Chromatogram of *Aspergillus pseudonomius* strain C39.

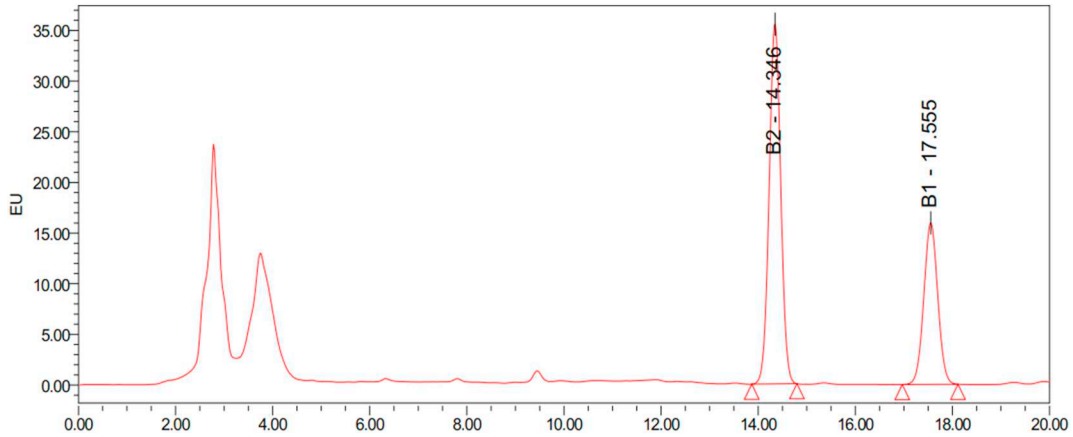

Fig. 7. Chromatogram of *Aspergillus flavus* strain C58.

as reported by (Baquião et al., 2013).

For food safety purposes, correct species identification is of high importance. *Aspergillus* section *Flavi* population is highly variable in terms of morphological characteristics, toxigenic profiles, and molecular features. Occasionally, strains producing important mycotoxins are apparently misidentified, a polyphasic approach combining physiology, morphology, sequence, and extrolites data is necessary for full understanding of section *Flavi* population (Frisvad et al., 2019). And this method has been widely applied to study the taxonomy and biodiversity of *Aspergillus* in the indoor environment and food (Chen et al., 2017), peanut (Norlia et al., 2018). Partial calmodulin gene sequencing, the recommended method for identification of Aspergilli (Samson et al., 2014) works well in the identification of *Aspergillus* section *Flavi* species. Based on our observation, morphological characteristics such as conidia color and growth diameter on certain temperature (42 °C for this section) are useful for differentiating phylogenetically closely related species. The interspecific and intraspecific mycotoxin profiles are diverse for section *Flavi* species, to have full knowledge of mycotoxic population, a combination of morphological, chemical, and molecular methods is recommended.

5. Conclusions

The polyphasic approach applied in this study permitted a reliable understanding of the prevailing *Aspergillus* section *Flavi* population in Southern China. Knowledge of the prevailing mycotoxic section *Flavi* population will aid in developing a sustainable strategy to mitigate the effects of aflatoxin contamination and in developing preventive measures. The present study highlighted the cooccurrence of CPA and aflatoxins and high CPA amounts produced by isolated mycobiota. This study suggests that CPA contamination of food should be considered while conducting mycotoxigenic surveys of food commodities, and the same should be considered while planning a bio-control strategy to control aflatoxin contamination.

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Author contributions

U. R., H. W. and B. L. conceived study design; U. R. performed the experiments and wrote the manuscript. J.F.W., X.Y. Ou, B.S.Q. and X.H·Y assisted with the experiment; H.C. and A.J.C. constructed phylogenetic trees; A.J.C. and B.L. supervised the research, edited and approved the final manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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