Journal of Microbiological Methods 196 (2022) 106470

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Low-cost, specific PCR assays to identify the main aflatoxigenic species of *Aspergillus* section *Flavi*

Josué J. Silva [a,*], Maria H.P. Fungaro [b], Taynara Souza Soto [a], Marta H. Taniwaki [a], Beatriz T. Iamanaka [a]

[a] *Institute of Food Technology — ITAL, Campinas, SP, Brazil*
[b] *Londrina State University- UEL, Londrina, PR, Brazil*

ARTICLE INFO

Keywords:
Aspergillus novoparasiticus
Aspergillus section *Flavi*
Aflatoxigenic
Species-specific
RPB2

ABSTRACT

Aflatoxins are fungal metabolites that are present as contaminants in food globally. Most aflatoxigenic species belong to *Aspergillus* section *Flavi*, and the main ones are grouped in the *A. flavus* clade, where many cryptic species that are difficult to discriminate are found. In this study, we investigated inter- and intraspecific diversity of the *A. flavus* clade to develop low-cost, species-specific PCR assays for identifying aflatoxigenic species. A total of 269 sequences of the second largest subunit of RNA polymerase II (*RPB2*) locus were retrieved from GenBank, and primer pairs were designed using data mining to identify *A. flavus*, *A. parasiticus*, and *A. novoparasiticus*. Species-specific amplicons of approximately 620, 350, and 860 bp enabled identification of target species as *A. flavus*, *A. parasiticus*, and *A. novoparasiticus*, respectively.

1. Introduction

Aflatoxins (AFs) are leading natural food contaminants with several isoforms are known; however, the most frequently found and studied include B1 (AFB1), B2 (AFB2), G1 (AFG1), and G2 (AFG2) and biotransformed metabolites of AFB1 and AFB2, M1 (AFM1) and M2 (AFM2), respectively. AFs have hepatotoxic, immunosuppressive, mutagenic, teratogenic, and carcinogenic activity (Kumar et al., 2017; Coppock et al., 2018). The International Agency for Research on Cancer (IARC) has classified isoforms B1, B2, G1, and G2 as carcinogenic to humans, in group 1 (IARC, 1993).

AFs are present in many foods globally; however, they are of particular concern in developing countries, where production technologies are precarious and deficiencies exist in the surveillance of regulatory limits, associated with low government budgets (Wild, 2007; Elias, 2016; Jallow et al., 2021). Thus, one of the best alternatives to mitigate the problem of AF contaminations is to monitor potentially aflatoxigenic fungi, thereby treating the cause and not just the effect.

A. section *Flavi* (subgenus *Circumdati*) comprises 36 species, of which approximately 55% are AF producers, which makes this fungal group the main one responsible for the contamination of this class of mycotoxins in food. Among members of *A.* section *Flavi*, *A. flavus* and *A. parasiticus* are most frequently found in foods and are reported as the main producers of AFs (Caceres et al., 2020).

A. oryzae and *A. sojae* are nontoxigenic, domesticated variants of *A. flavus* and *A. parasiticus*, respectively (Geiser et al., 1998; Machida et al., 2005; Gibbons et al., 2012; Rokas et al., 2007; Kim et al., 2017; Hua et al., 2018). *A. oryzae* and *A. sojae* are widely used in the food industry, such as in the production of fermented foods and alcoholic beverages (especially in Asian countries) and production of various enzymes; these procedures have been given a generally recognized as safe status by the FDA (Chang et al., 2014; Kim et al., 2017).

The standard polyphasic taxonomy for the genus *Aspergillus* (Samson et al., 2014) does not allow for the discrimination of *A. flavus/oryzae* and *A. parasiticus/sojae*; however, *A. flavus, A. oryzae, A. parasiticus*, and *A. sojae* are still classified as separate taxa, only for economic and food safety, but not taxonomic, reasons.

A. flavus and *A. parasiticus* are present in the soil and contaminate a wide variety of agricultural products, such as peanuts and nuts (Bayman et al., 2002; Baquião et al., 2012; Baquião et al., 2013; Guezlane-Tebibel et al., 2013; Mimoune et al., 2018); herbs/teas and coffee (Batista et al., 2003; Tournas and Katsoudas, 2008; Dutta et al., 2008; Su et al., 2018; Silva et al., 2019a), grapes and derivatives (Freire et al., 2017; Ghuffar et al., 2020), milk and dairy products (Jodral et al., 1993; Ortiz-Martinez et al., 2017), cereals (Al-Wadai et al., 2013; Sebok et al., 2016; Mamo et al., 2018; Camiletti et al., 2018; Del Palacio and Pan, 2020), and fruits

* Corresponding author at: Institute of Food Technology (ITAL), P.O. Box 139, CEP 13078-170 Campinas, SP, Brazil.
E-mail address: josue.biomol@gmail.com (J.J. Silva).

https://doi.org/10.1016/j.mimet.2022.106470
Received 25 October 2021; Received in revised form 26 March 2022; Accepted 12 April 2022
Available online 18 April 2022
0167-7012/© 2022 Elsevier B.V. All rights reserved.

(Hussein et al., 2020; Saif et al., 2021). Contamination can occur at any stage of the production chain, pre- and postharvest (Akhund et al., 2017; Norlia et al., 2019).

In 2012, Gonçalves et al. (2012) described *A. novoparasiticus*, a species phylogenetically very close to *A. parasiticus*. Since then, this new species has been described in cassava, sugarcane, yerba-mate, maize, and rice (Adjovi et al., 2014; Viaro et al., 2017; Katsurayama et al., 2018; Iamanaka et al., 2019; Silva et al., 2019a; Ono et al., 2021).

Regarding the capacity to produce AFs, *A. parasiticus* and *A. novoparasiticus* can produce AFs of type B (B1 and B2) and G (G1 and G2). *A. flavus* is recognized as a type B AF-producing species (B1 and B2) (Varga et al., 2015), and until recently, one of the characteristic features of this species was the inability to produce type G AFs. However, recently, it has been recognized that some (rare) strains of *A. flavus* can produce G-type AFs (G1 and G2) (Frisvad et al., 2019), showing that knowledge about the toxigenic potential of *A.* section *Flavi* is constantly changing.

Recently, the occurrence of *A.* section *Flavi* in sugarcane has been revised by some authors, considering its new taxonomic status (Silva et al., 2019b; Abdallah et al., 2020). *A. parasiticus* and *A. flavus* were for a long time considered the most frequent aflatoxigenic species in this substrate. However, as demonstrated by Silva et al. (2019b), the strains previously identified as *A. parasiticus* in sugarcane actually belonged to *A. novoparasiticus*. Similarly, Abdallah et al. (2020) revisited a strain of *A. flavus* isolated from a sugarcane field in Egypt, which was then identified as *A. novoparasiticus* by them. In light of these new facts, *A. novoparasiticus* appears to be a ubiquitous species in the sugarcane crop. These examples illustrate the need to create tools to discriminate these species.

In this context, the importance of correct identification of *A. flavus* and *A. parasiticus* in food is evident, given their high frequency and aflatoxigenic and spoilage potential. This study aims to create fast and low-cost PCR assays that enable accurate identification of *A. flavus* and *A. parasiticus*, and unprecedentedly, a PCR assay for the identification and discrimination of *A. novoparasiticus.*

2. Materials and methods

2.1. Identification of reference strains for PCR assay validation

A total of 24 strains belonging to Microbiology Laboratory Collection of Instituto de Tecnologia de Alimentos (ITAL), previously identified morphologically as *A.* section *Flavi*, isolated from corn, sugarcane, peanuts, and cassava samples, were submitted for meticulous phylogenetic analysis using multilocus sequencing (calmodulin [*CaM*], beta-tubulin [*BenA*], and RNA polymerase II second largest subunit [*RPB2*]).

2.1.1. Genomic DNA extraction

The strains were inoculated on Czapek yeast autolysate agar (CYA) (Pitt, 1979) to obtain monosporic colonies. The purified strains were grown in yeast extract sucrose (YES) (Frisvad, 1981) liquid medium at 25 °C for 3 d until the formation of the mycelial skin, which was then manually macerated using liquid nitrogen. The macerated material was used to obtain genomic DNA through the gel DNA Purification kit (Mebep Bioscience, Nanshan District, Shenzhen, China) according to the protocol recommended by the manufacturer.

DNA was initially quantified using a spectrophotometric method, using a NanoDrop® (Thermo Scientific, Waltham, MA, USA), and then 0.8% agarose gel stained with ethidium bromide (0.5 μg/mL) was used to compare fluorescence intensities under UV light between the obtained DNA bands and a DNA sample of known concentration. After quantification, the samples were stored at −20 °C until use.

2.1.2. Multilocus analysis of reference strains

To identify the reference strains, amplification of *BenA, CaM*, and *RPB2* loci was performed, using primer pairs described by Glass and Donaldson (1995), Hong et al. (2005), and Houbraken et al. (2012), respectively. Conditions for amplification and sequencing were as described by Silva et al. (2020).

The sequences obtained were compared by local alignment using the BLAST tool (Altschul et al., 1990) against the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and MYCOBANK MLST databases (https://www.mycobank.org/page/Pairwise_alignment). Additionally, multiloci phylogenetic trees were inferred based on Maximum likelihood (ML) and Bayesian (BI) methods. The Kimura-2-parameter model (Kimura, 1980) with discrete Gamma distribution and invariant sites (G + I) was selected for ML and BI analysis. The software packages MrBayes v. 3.2.6 (Ronquist et al., 2012) and MEGA 7.0 (Kumar et al., 2016) were used to obtain phylogenetic inferences based on BI and ML analyses, respectively.

To estimate the level of support for the ML tree, bootstrap analysis was performed on 1000 replicates. For BI analysis, the Markov chain Monte Carlo (MCMC) algorithm was run for 5,000,000 generations. Sample frequency was set to 100, with 25% of trees removed as burn-in. Convergence diagnostics were monitored based on an average standard deviation of split frequencies below 0.01, potential scale reduction factor (PSRF) values close to 1.0, and effective sample size (ESS) values above 200. The trees were visualized using FigTree version 1.4.2, developed by Andrew Rambaut (http://tree.bio.ed.ac.uk/software/figtree).

2.2. Development of species-specific PCR assays

2.2.1. Searching, screening, and curating RPB2 sequences in GenBank

For designing species-specific primer pairs, a search for sequences of the *RPB2* locus in GenBank for all 13 species belonging to the *A. flavus* clade was performed. A total of 317 sequences were retrieved from GenBank (Aug 11, 2021) based on the search for the following keywords: *A. arachidicola*; *A. parasiticus* and synonyms *(A. sojae, A. toxicarius, A. chungii, A. terrícola* var. *americanus)*; *A. cerealis* and synonym *(A. korhogoensis)*; *A. aflatoxiformans* and synonym *(A. parvisclerotigenus)*; *A. flavus* and synonyms *(A. oryzae, A. fasciculatus, A. subolivaceus, A. thomii, A. kambarensis)*; *A. novoparasiticus*; *A. transmontanensis*; *A. sergii*; *A. pipericola*; *A. minisclerotigenes*; *A. subflavus*; and *A. mottae*, associated with "RPBII," "RNA polymerase II," or "RPB2."

An initial curation was performed based on multiple alignments using the ClustalW algorithm in BioEdit 7.2.5 (Hall, 1999), where very short sequences that prevented achieving higher quality alignment were removed. In the second phase of curation, a phylogenetic tree (ML) was constructed based on the alignment of *RPB2* deposits retrieved from GenBank with the sequences of type strains of *A.* section *Flavi*. Based on the genealogy presented, it was possible to detect identification/deposit errors, that is, sequences deposited in GenBank with the description of a species belonging to the *A. flavus* clade but clearly belonging to other clades or even other sections. These sequences identified as not belonging to the *A. flavus* clade were then removed from the final dataset.

The final dataset composed of 269 sequences was then submitted for haplotype analysis using DnaSP.v6 (Rozas et al., 2017). To elucidate the taxonomic position of the haplotypes, a representative of each haplotype was used in constructing the ML phylogenetic tree together with type strains of *A.* section *Flavi*. The ML tree was inferred based on the Tamura–Nei model (Tamura and Nei, 1993), and to determine the support for each clade, bootstrap analysis was performed with 1000 replicates using MEGA 7.0 (Kumar et al., 2016).

2.2.2. Design of species-specific primer pairs

With the haplotypes identified at the species level, species-specific primers were designed, based on intra- and interspecific variability available in GenBank for the *RPB2* locus of the *A. flavus* clade. The analysis involved sequence alignment, using ClustalW (Thompson et al., 1994), and a visual search for regions of complete similarity to all target

species haplotypes: *A. flavus*, *A. parasiticus*, and *A. novoparasiticus*, but not with other species belonging to the *A. flavus* clade. The designed primers were verified for their specificity/uniqueness against the GenBank database using the BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Melting temperature, formation of secondary structures, and other parameters were analyzed *in silico* using Oligoanalyzer 3.1 (https://www.idtdna.com/calc/analyzer). Primer sequences are shown in Table 1.

2.2.3. PCR assay validation and determination of specificity, sensitivity, and limit of detection

PCR conditions of the designed primer pairs were optimized and method validation was performed by testing the DNA of reference strains identified by multilocus analysis as *A. flavus*, *A. parasiticus*, and *A. novoparasiticus*. As a negative control, strains identified as *Aspergillus nomius*, *Aspergillus pseudonomius*, *Aspergillus tamarii*, *Aspergillus bertholethius*, *Aspergillus caelatus*, *Aspergillus pseudocaelatus*, and *A. arachidicola* were used. PCR amplification was performed according to the conditions shown in Table 1.

After assay validation, determination of detection limit and specificity and sensitivity values was performed. To investigate the detection limit of each set of primers, a serial dilution of the template DNA of the following reference strains was performed: ITAL 11a, ITAL 119a, and ITAL 240 (for *rpbflav*, *rpbpar*, and *rpbnov* PCR assays, respectively) at 100, 50, 10, 5, 4, 3, 2, 1, 0.5, 0.2, and 0.1 ng.

For determining specificity and sensitivity, 200 strains of *A.* section *Flavi* belonging to the Microbiology Laboratory Collection of ITAL, previously molecularly identified by our research group, were used. In this set of strains, the following species are included: *A. flavus* ($n = 50$ strains), *A. parasiticus* ($n = 50$ strains), *A. novoparasiticus* ($n = 47$ strains), *A. arachidicola* ($n = 15$ strains), *A. nomius* ($n = 5$ strains), *A. pseudonomius* ($n = 4$ strains), *A. pseudocaelatus* ($n = 7$ strains), *A. bertholletius* ($n = 5$ strains), and *A. tamarii* ($n = 17$ strains). Specificity and sensitivity were calculated according to Altman and Bland (1994) and Greenhalgh (1997), where specificity is given by the number of true negatives (TN)/the sum of TN and false positives (FP); sensitivity is given by the number of true positives (TP)/the sum of TP and false negatives (FN).

PCR reaction conditions for determining sensitivity, specificity, and limit of detection were similar to those used for validation (Table 1). Analyses of validation, detection limit and specificity/sensitivity, were conducted in triplicate.

3. Results and discussion

Based on the final dataset (269 *RPB2* sequences), 21 haplotypes were found. The ML tree shown in Fig. 1 allowed for the taxonomic positioning of these haplotypes in relation to the taxonomic status of *A.* section *Flavi*. Most sequences (194/269) were deposited in GenBank as *A. flavus*; our results confirmed this identification (Fig. 1). Complete haplotype analysis is available in Supplementary Material 1.

Based on the haplotypes, the design of species-specific primer pairs was performed for amplifying *A. flavus* (Fig. 2A), *A. parasiticus* (Fig. 2B), and *A. novoparasiticus* (Fig. 2C).

The primer pair rpbflav-F/rpbflav-R (Table 1) was successful in amplifying all strains of *A. flavus*, previously selected and meticulously identified by multilocus phylogenetic analysis (Fig. 3). The amplicon generated was approximately 620 bp (Fig. 4A), which corroborates the expected theoretical fragment (Fig. 2A). In this validation assay, none of the negative controls for *A. nomius* (ITAL 1000), *A. pseudonomius* (ITAL 638), *A. tamarii* (ITAL 117a), *A. bertholletius* (ITAL 270/06), *A. caelatus* (ITAL 8562), and *A. arachidicola* (ITAL 270v), *A. parasiticus* (ITAL 85v2), and *A. novoparasiticus* (ITAL 12y) were amplified, demonstrating that under these conditions primer pair rpbflav-F/rpbflav-R is suitable for the identification of *A. flavus*.

The *rpbflav* PCR assay had 99.3% specificity (149 TN / 149 TN + 1 FP) and 100% sensitivity (50 TP / 50 TP + 0 FN). The detection limit of this assay was 1 ng (Supplementary Fig. S1).

The rpbpar-F/rpbpar-R primer pair was also effective in identifying all *A. parasiticus* strains verified (Fig. 3). The amplicon generated was approximately 350 bp (Fig. 4B), which corroborates with the expected theoretical fragment (Fig. 2B). In this validation assay, none of the negative controls for *A. nomius* (ITAL 1000), *A. pseudonomius* (ITAL 638), *A. tamarii* (ITAL 117a), *A. bertholletius* (ITAL 270/06), *A. caelatus* (ITAL 8562), *A. arachidicola* (ITAL 270v), *A. flavus* (ITAL 191), and *A. novoparasiticus* (ITAL 12y) were amplified. Therefore, the PCR assay based on the rpbpar-F/rpbpar-R primer pair identified target species.

The *rpbpar* PCR assay showed 100% specificity (150 TN / 150 TN + 0 FP) and 100% sensitivity (50 TP / 50 TP + 0 FN). The detection limit of this assay was 0.5 ng (Supplementary Fig. S1).

The rpbnov-F/rpbnov-R primer pair was also effective in identifying all *A. novoparasiticus* strains verified (Fig. 3). The amplicon generated was approximately 860 bp (Fig. 4C), which corroborates with the expected theoretical fragment (Fig. 2C). In this validation assay, none of the negative controls for *A. nomius* (ITAL 1000), *A. pseudonomius* (ITAL 638), *A. tamarii* (ITAL 117a), *A. bertholletius* (ITAL 270/06), *A. caelatus* (ITAL 8562), *A. arachidicola* (ITAL 270v), *A. flavus* (ITAL 191), and *A. parasiticus* (ITAL 85v2) were amplified.

Table 1
Primers and conditions of the PCR assays developed in this study.

	Primers	Sequence (5′-3′)				
Primers (this study)	*rpbflav-F*	GCAAGTGCTAAGGCTGGC				
	rpbflav-R	CTCTACGATCCTCAGGG				
	rpbpar-F	ACAGAGAGATCTACCTCAAC				
	rpbpar-R	AATGATAGGTTCRCTGGGG				
	rpbnov-F	CTGACTGGTGGCTTGAAGTAT				
	rpbnov-R	TAGAGCGAACACGCTTGTTA				
			35 Cycles			
Thermal program	PCR assay	Initial denaturation	Denaturation	Annealing	Extension	Final extension
	rpbflav	95 °C (3 min)	94 °C (45 s)	55 °C (45 s)	72 °C (1 min 20 s)	72 °C (5 min)
	rpbpar	95 °C (3 min)	94 °C (45 s)	60 °C (45 s)	72 °C (1 min)	72 °C (5 min)
	rpbnov	95 °C (3 min)	94 °C (45 s)	65 °C (45 s)	72 °C (1 min 20 s)	72 °C (5 min)
PCR conditions -Final volume 25 μL	*Taq* DNA polymerase*	1 U				
	PCR buffer*	1×				
	Primer	0.4 μM -each primer				
	dNTP	0.2 mM				
	$MgCl_2$	1.5 mM				
	DNA template	10 ng				

* Thermo Fisher Scientific, USA.

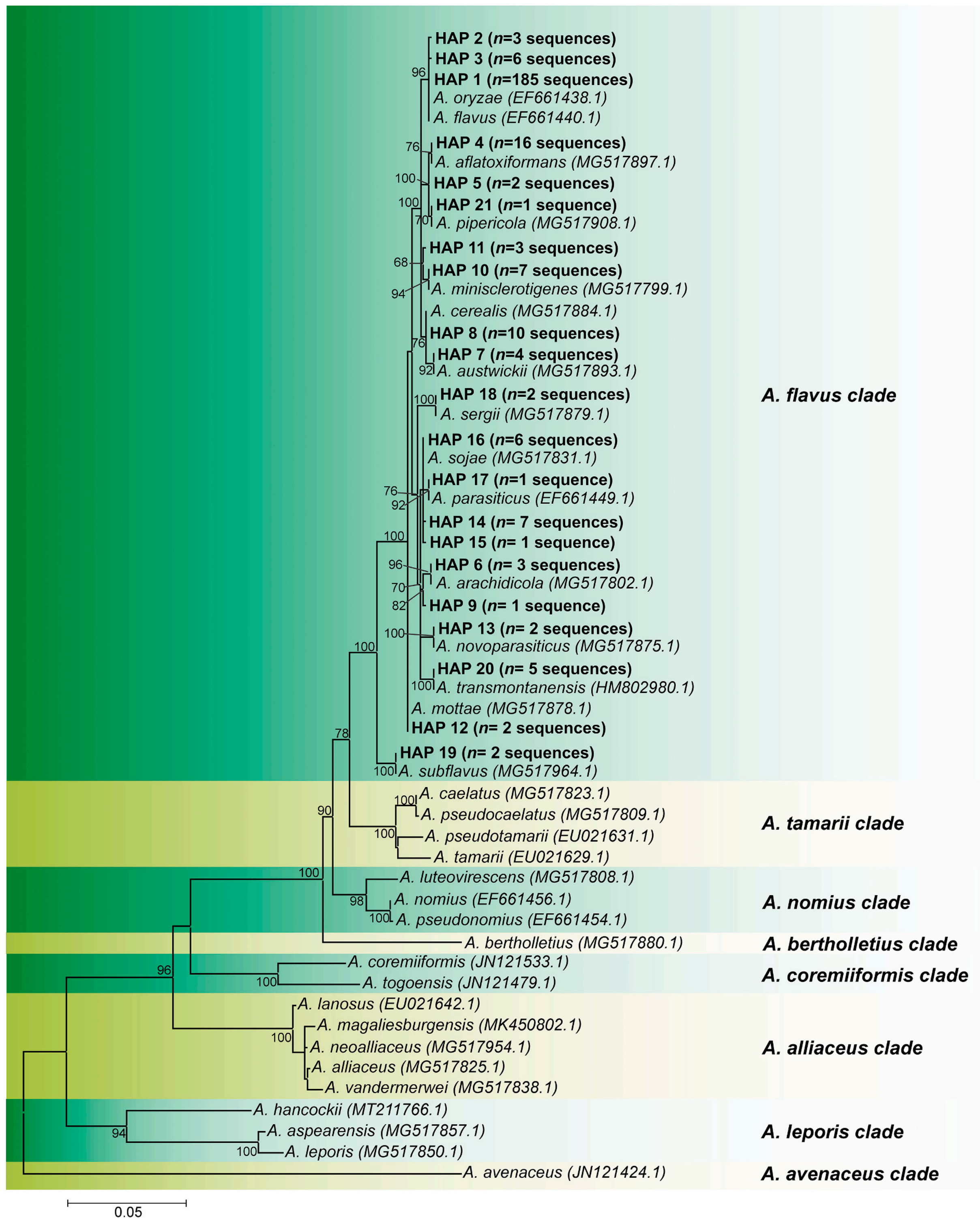

Fig. 1. Maximum likelihood tree of *Aspergillus* section *Flavi* species and *RPB2* haplotypes of *Aspergillus flavus* clade species retrieved from GenBank. Only bootstrap ≥60% values are shown. Haplotypes are in bold.

The *rpbnov* PCR assay showed the lowest detection limit of 0.1 ng (Supplementary Fig. S1). The specificity determined was 98.7% (151 TN / 151 TN+2 FP), whereas the sensitivity was 100% (47 TP / 47 TP + 0 FN).

The detection limits of the PCR assays developed in this study ranged from 1 to 0.1 ng of template DNA, a similar result was found by others who developed detection and identification methods using conventional PCR or derived techniques (Zha et al., 2010; Filleron et al., 2014). The PCR assays developed here aim for direct identification of pure cultures, and in these cases, DNA concentrations greater than 1 ng are easily obtained. However, depending on the user's needs, additional techniques, such as nested-PCR, can be used to improve the detection limit, *e.g.,* Grote et al. (2002) reported an increase of at least 1000× in the detection limit when using nested-PCR for detecting *Phytophthora nicotianae*.

Recently, Ortega et al. (2020) developed primers for detecting

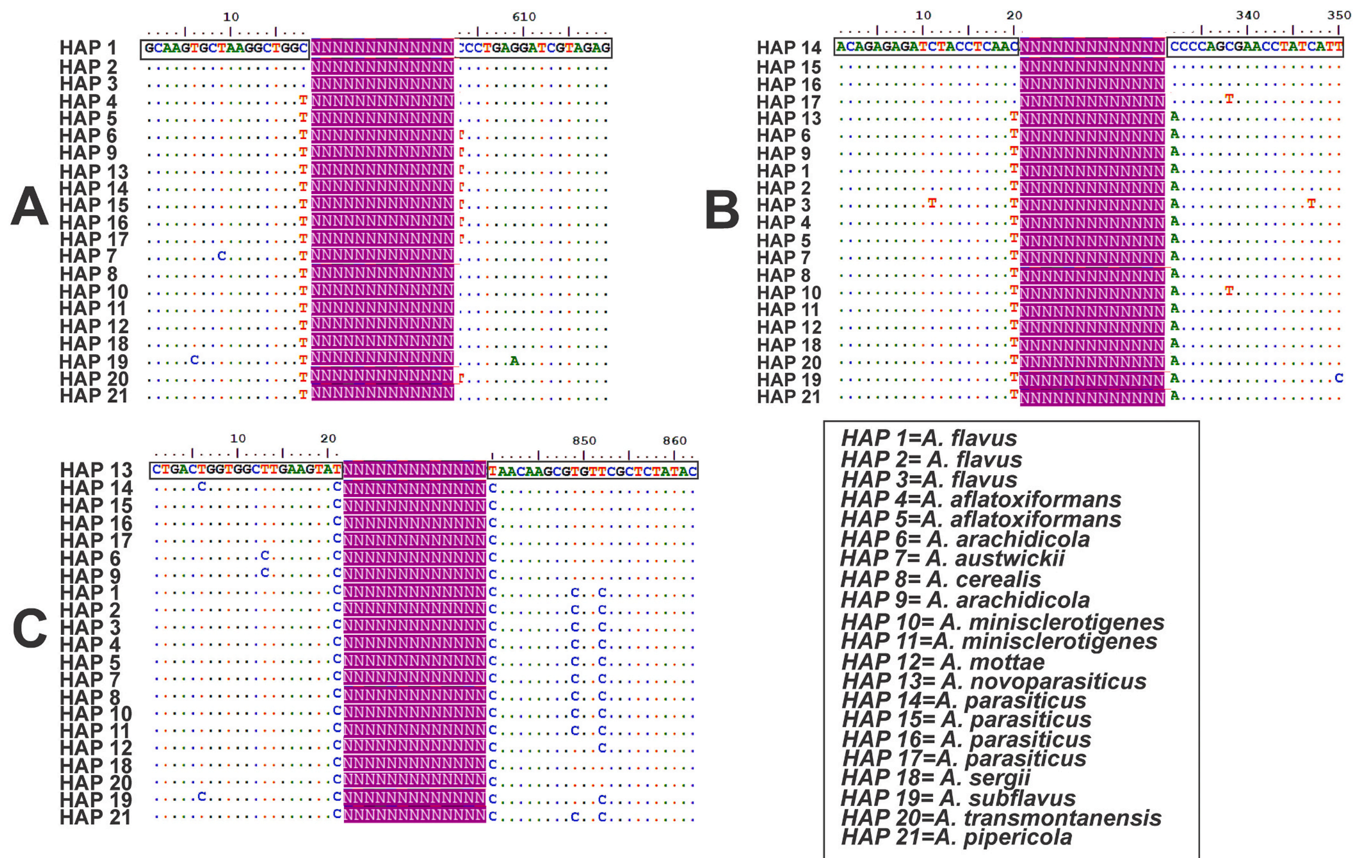

Fig. 2. Annealing sites of the species-specific primer pairs in the 21 *RPB2* haplotypes from *Aspergillus flavus* clade. Annealing of specific primers for A) *A. flavus*; B) *A. parasiticus*; and C) *A. novoparasiticus*.

aflatoxigenic strains of *A. flavus* and *A. parasiticus* found in hazelnuts and chestnuts. The authors targeted the *aflT* gene of the AF biosynthesis cluster for developing primers for the techniques of LAMP, PCR, and qPCR. The specificity and sensitivity of the PCR assay reported by the authors were 82% and 87.2%, respectively. In addition, there were also cases of amplification of species other than *A. flavus* and *A. parasiticus*, a fact that may be justifiable given the high similarity of the species in the *A. flavus* clade.

Al-Shuhaib et al. (2018) evaluated several primers for detecting *A. flavus* and concluded that, among the primers investigated, the *Fla* primer pair, described by González-Salgado et al. (2008), is optimal for diagnosing this species, presenting 100% specificity and 97% efficiency. However, the validation of the reference strains by Al-Shuhaib et al. (2018) was performed based on the ITS locus and another eight loci (*aflP, aflP-2, aflP-3, aflD, aflM, aflR*, *Gap*, and *PEP*). The ITS locus (ITS1-5.8S-ITS2), is part of the ribosomal RNA gene cluster, it is well known that this locus does not have discriminatory power for many species of *Aspergillus* (Samson et al., 2014); several species of *A.* section *Flavi* have identical ITS sequences (see Supplementary Fig. S2). The other sequenced loci are not commonly used for *Aspergillus* phylogeny (see Samson et al., 2014; Visagie et al., 2014; Chen et al., 2017; Frisvad et al., 2019), and the loci recommended by the International Commission of *Penicillium* and *Aspergillus* (ICPA) include *CaM*, *BenA*, and *RPB2* (Samson et al., 2014).

Furthermore, the *aflP, aflP-2, aflP-3, aflD, aflM, aflR, Gap*, and *PEP* loci do not allow comparison between species, because there are no sequence deposits in GenBank for many species of *A.* section *Flavi*, including species close to the authors' target (*A. flavus*). Moreover, the *Fla* primer pair itself, based on the ITS region, has 100% identity with other species of *A.* section *Flavi* (see BLASTn https://blast.ncbi.nlm.nih.gov/Blast.cgi).

A. flavus and *A. parasiticus* are included in the *A. flavus* clade, in addition to 11 other phylogenetically related species, including *A. arachidicola, A. transmontanensis, A. novoparasiticus, A. sergii, A. cerealis, A. subflavus, A. pipericola, A. minisclerotigenes, A. mottae, A. austwickii*, and *A. aflatoxiformans* (Frisvad et al., 2019). *A. flavus* and *A. parasiticus* are the most frequently found species of *A.* section *Flavi* isolated from food and the most toxigenically important (Caceres et al., 2020). The development of identification and detection methods for these species, isolated or in matrix, is a good alternative for controlling mycotoxin contamination.

Studies have reported the development of PCR assays (and derived techniques) for the identification and/or detection of *A. flavus* and/or *A. parasiticus* (Chen et al., 2002; Sugita et al., 2004; González-Salgado et al., 2008; Sardiñas et al., 2010; Luo et al., 2012; Wang et al., 2012; Ahmad et al., 2014; Bansal et al., 2019). However, many studies have been based on a restricted number of sequences for primer design and/or a small number of strains for assay development. In some cases, there was a deficiency in the validation of the analyzed strains as reference, which may compromise the accuracy of the test. In addition, many studies have invested in methodologies such as real-time PCR/qPCR (Godet and Munaut, 2010; Sardiñas et al., 2011; Mahmoud, 2015; Ortega et al., 2020; Abd El-Aziz et al., 2021) which although advantageous, are economically more burdensome than conventional PCR. This factor should be considered because the severest contamination by aflatoxigenic species occurs in developing countries.

We performed data mining using the largest public bank of sequences (GenBank) associated with the curation of these sequences. Thus, the primers designed in this study contain intraspecific diversity of the *A. flavus* clade available on GenBank. In addition, the strains used in the assay were validated based on multilocus analyses. The assays were designed and performed as conventional PCR, with an average final cost

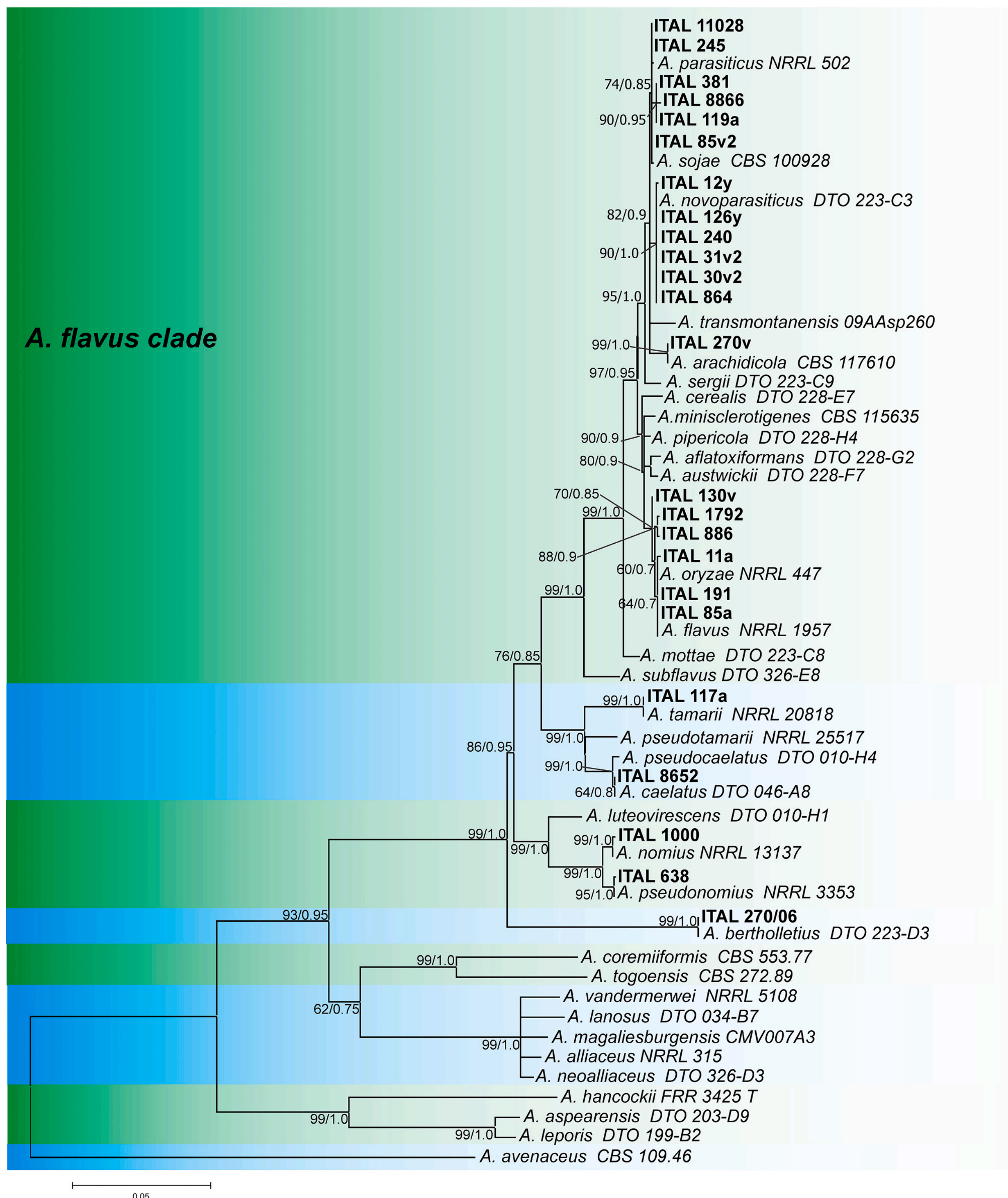

Fig. 3. Maximum likelihood tree based on multilocus data (*CaM* + *BenA* + *RPB2*) showing phylogenetic relationships between *Aspergillus* section *Flavi* species and Brazilian strains isolated from foodstuff (in bold), which were used for validating species-specific PCR assays. Bootstrap values (BS) and/or posterior probabilities values (pp) higher than 60% and 0.70, respectively, are shown.

of approximately US$ 0.7/reaction.

Other non-genotypic detection methods, such as the MALDI-TOF MS analysis, have been used for the identification and detection of *Aspergillus* species. Recently, Queró et al. (2020) used MALDI-TOF MS to identify isolates of *A.* section *Flavi* and *Penicillium roqueforti* and identified some species of *A.* section *Flavi*. However, the identification performance of *A. novoparasiticus* was 95%, as not all spectra obtained by the authors for this species were identified. In fact, the relationship between *A. parasiticus* group species is very close, and their separation either by phenotypic or genotypic methods is not easy.

As reported by Frisvad et al. (2019), even the *BenA* locus, which is a secondary barcode for *Aspergillus* (Samson et al., 2014), does not allow

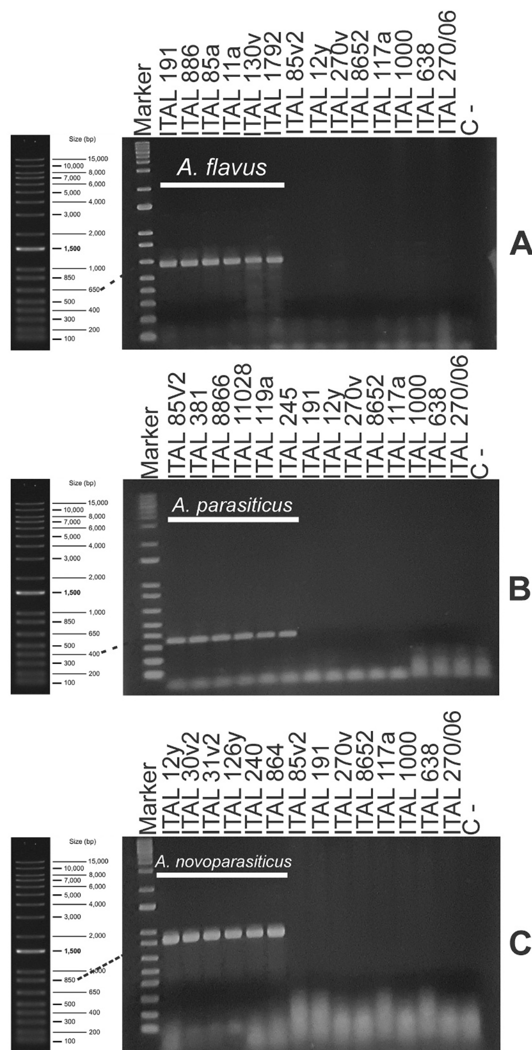

Fig. 4. Agarose gel electrophoresis 1% (*w/v*). A) primer pair rpbflav-F/rpbflav-R amplification; B) primer pair rpbpar-F/rpbpar-R amplification; C) primer pair rpbnov-F/rpbnov-R amplification. *Aspergillus flavus* (strains: ITAL 191, 886, 85a, 11a, 130v, and 1792); *A. parasiticus* (strains: ITAL 85v2, 381, 8866, 11,028, 119a, and 245); *A. novoparasiticus* (strains: ITAL 12y, 30v2, 31v2, 126y, 240, and 864*); A. nomius* (ITAL 1000); *A. pseudonomius* (ITAL 638); *A. tamarii* (ITAL 117a); *A. bertholletius* (ITAL 270/06); *A. caelatus* (ITAL 8562); and *A. arachidicola* (ITAL 270v). Marker (1 Kb Plus DNA Ladder, Thermo Fisher Scientific, Carlsbad, CA, USA).

discriminating between *A. parasiticus* and *A. novoparasiticus*, and in these cases, the use of the *CaM* or *RPB2* loci is recommended. The *RPB2* locus is recommended for evaluating the phylogeny of many fungal groups (Vetrovsky et al., 2016) and has been used in the creation of species-specific primers in *Fusarium* (Hong et al., 2010; Kang et al., 2011) and recently in *A.* section *Versicolores* (Kubosaki et al., 2020); however, this study is the first to report its use in developing species-specific primers for *A.* section *Flavi.*

In addition, many studies that have developed PCR assays for detecting and identifying *A. parasiticus* were performed before the description of *A. novoparasiticus* in 2012 (Shapira et al., 1996; Chen et al., 2002; Somashekar et al., 2004; Sardiñas et al., 2010; Khoury et al., 2011; Sardiñas et al., 2011). In other cases, later studies did not include *A. novoparasiticus* in their *in vitro* analyses (Hue et al., 2013; Ahmad et al., 2014; Nguyen et al., 2015; Atoui and Khoury, 2016; Singh et al., 2017; Ortega et al., 2020). Therefore, there is no way to be sure about the specificity of these PCR assays for these two species. To our knowledge, this is the first study to develop a species-specific PCR assay for identifying *A. novoparasiticus*.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2022.106470.

Funding

Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Process 2018/25552–0; J. J. Silva grant # 2018/25597–3).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank the technical and administrative staff of the Instituto de Tecnologia de Alimentos (ITAL) for the infrastructure provided to carry out the study. We acknowledge the funding agency Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

References

Abd El-Aziz, A., Shehata, S.M., Hisham, S.M., Alobathani, A.A., 2021. Molecular profile of aflatoxigenic and non-aflatoxigenic isolates of *Aspergillus flavus* isolated from stored maize. Saudi J. Biol. Sci. 28 (2), 1383–1391. https://doi.org/10.1016/j.sjbs.2020.11.073.

Abdallah, M.F., Audenaert, K., Saeger, S., Houbraken, J., 2020. Revisiting an *Aspergillus flavus* strain isolated from an Egyptian sugarcane field in 1930. Microorganisms 8, 1633. https://doi.org/10.3390/microorganisms8111633.

Adjovi, Y.S.C., Bailly, S., Gnonlonfin, B.J.G., Tadrist, S., Querin, A., Sanni, A., Oswald, I. P., Puel, O., Bailly, J.D., 2014. Analysis of the contrast between natural occurrence of toxigenic *aspergilli* of the *Flavi* section and aflatoxin B1 in cassava. Food Microbiol. 38, 151–159. https://doi.org/10.1016/j.fm.2013.08.005.

Ahmad, M.M., Ahmad, M., Ali, A., Hamid, R., Javed, S., Abdin, M.Z., 2014. Detection of *Aspergillus flavus* and *Aspergillus parasiticus* from aflatoxin-contaminated peanuts and their differentiation using PCR-RFLP. Ann. Microbiol. 64, 1597–1605. https://doi.org/10.1007/s13213-014-0803-5.

Akhund, S., Akram, A., Hanif, N.Q., Qureshi, R., Naz, F., Nayyar, B.G., 2017. Pre-harvest aflatoxins and *Aspergillus flavus* contamination in variable germplasms of red chillies from Kunri, Pakistan. Mycotoxin Res. 33, 147–155. https://doi.org/10.1007/s12550-017-0274-1.

Al-Shuhaib, M.B.S., Albakri, A.H., Alwan, S.H., Almandil, N.B., AbdulAzeez, S., Borgio, J. F., 2018. Optimal PCR primers for rapid and accurate detection of *Aspergillus flavus* isolates. Microb. Pathog. 116, 351–355. https://doi.org/10.1016/j.micpath.2018.01.049.

Altman, D.G., Bland, J.M., 1994. Diagnostic tests 1: sensitivity and specificity. BMJ. 308, 1552. https://doi.org/10.1136/bmj.308.6943.1552.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Al-Wadai, A.S., Al-Othman, M.R., Mahmoud, M.A., Aziz, Abd El-, 2013. Molecular characterizations of *Aspergillus flavus* and aflatoxin contamination of wheat grains from Saudi Arabia. Genet. Mol. Res. 12, 3335–3352. https://doi.org/10.4238/2013.September.3.10.

Atoui, A., Khoury, A.E., 2016. PCR-RFLP for *Aspergillus* species. In: Moretti, A., Susca, A. (Eds.), Mycotoxigenic Fungi, Methods in Molecular Biology, vol. 1542. Humana Press, New York, NY, pp. 313–320.

Bansal, S., Mangal, M., Tushir, S., Oberoi, H.S., Gupta, R.K., 2019. A rapid and reliable method for the specific detection of aflatoxigenic fungi in groundnut and rice samples. J. Food Process. Preserv. 43, 14127. https://doi.org/10.1111/jfpp.14127.

Baquião, A.C., Zorzete, P., Reis, T.A., Assunção, E., Vergueiro, S., Correa, B., 2012. Mycoflora and mycotoxins in field samples of Brazil nuts. Food Control 28, 224–229. https://doi.org/10.1016/j.foodcont.2012.05.004.

Baquião, A.C., Oliveira, M.M.M., Reis, T.A., Zorzete, P., Atayde, D.D., Correa, B., 2013. Polyphasic approach to the identification of *Aspergillus* section *Flavi* isolated from Brazil nuts. Food Chem. 139, 1127–1132. https://doi.org/10.1016/j.foodchem.2013.01.007.

Batista, L.R., Chalfoun, S.M., Prado, G., Schwan, R.F., Wheals, A.E., 2003. Toxigenic fungi associated with processed (green) coffee beans (*Coffea arabica L.*). Int. J. Food Microbiol. 85, 293–300. https://doi.org/10.1016/S0168-1605(02)00539-1.

Bayman, P., Baker, J.L., Mahoney, N.E., 2002. Aspergillus on tree nuts: incidence and associations. Mycophatologia 155, 161–169. https://doi.org/10.1023/a:1020419226146.

Caceres, I., Al Khoury, A., El Khoury, R., Lorber, S., Oswald, I.P., El Khoury, A., Atoui, A., Puel, O., Bailly, J.D., 2020. Aflatoxin biosynthesis and genetic regulation: a review. Toxins. 12, 150. https://doi.org/10.3390/toxins12030150.

Camiletti, B.X., Moral, J., Asensio, C.M., Torrico, A.K., Lucini, E.I., Pecci, M.P.G., Michailides, T.J., 2018. Characterization of Argentinian endemic *Aspergillus flavus* isolates and their potential use as biocontrol agents for mycotoxins in Maize. Phytopathology 108, 818–828. https://doi.org/10.1094/PHYTO-07-17-0255-R.

Chang, P.K., Horn, B.W., Abe, K., Gomi, K., 2014. *Aspergillus*: Introduction. In: Batt, C.A., Tortorello, M.L. (Eds.), Encyclopedia of Food Microbiology. Academic Press, Massachussetts, pp. 77–82.

Chen, R.S., Tsay, J.G., Huang, Y.F., Chiou, R.Y.Y., 2002. Polymerase chain reaction-mediated characterization of molds belonging to the *Aspergillus flavus* group and detection of *Aspergillus parasiticus* in peanut kernels by a multiplex polymerase chain reaction. J. Food Prot. 65, 840–844. https://doi.org/10.4315/0362-028X-65.5.840.

Chen, A.J., Hubka, V., Frisvad, J.C., Visagie, C.M., Houbraken, J., Meijer, M., Varga, J., Demirel, R., Jurjevic, Z., Kubátová, A., Sklenár, F., Zhou, Y.G., Samson, R.A., 2017. Polyphasic taxonomy of *Aspergillus* (formerly *Eurotium*), and its occurrence in indoor environments and food. Stud. Mycol. 88, 37–135. https://doi.org/10.1016/j.simyco.2017.07.001.

Coppock, R.W., Christian, R.G., Jacobsen, B.J., 2018. Chapter 69 - aflatoxins. In: Gupta, R.C. (Ed.), Veterinary Toxicology. Academic Press, Hopkinsville, KY, United States, pp. 983–994. https://doi.org/10.1016/B978-0-12-811410-0.00069-6.

Del Palacio, A., Pan, D., 2020. Occurrence and toxigenic potential of *Aspergillus* section *Flavi* on wheat nad sorghum silages in Uruguay. Mycology 11, 147–157. https://doi.org/10.1080/21501203.2020.1752321.

Dutta, B.K., Dutta, S., Nath, P.K., 2008. Mycotoxin production potential of mycoflora in tea. In: Jain, N.K., Rahman, F., Baker, P. (Eds.), Economic Crisis in Tea Industry. Studium press, USA, pp. 221–232. https://www.cabdirect.org/cabdirect/abstract/20083151442.

Elias, N.K.S., 2016. Aflatoxins: a silent thread in developing countries. Afr. J. Biotechnol. 15, 1864–1870. https://doi.org/10.5897/AJB2016.15305.

Filleron, A., Simon, M., Hantova, S., Jacquot, A., Cambonie, G., Marchandin, H., Jumas-Bilak, E., 2014. Tuf-PCR-temporal temperature gradient gel electrophoresis for molecular detection and identification of staphylococci: application to breast milk and neonate gut microbiota. J. Microbiol. Methods 98, 67–75. https://doi.org/10.1016/j.mimet.2013.12.022.

Freire, L., Passamani, F.R.F., Thomas, A.B., Nassur, R.C.M.R., Silva, L.M., Paschoal, F.N., Pereira, G.E., Prado, G., Batista, L.R., 2017. Influence of physical and chemical characteristics of wine grapes on the incidence of *Penicillium* and *Aspergillus* fungi in grapes and ochratoxin A in wines. Int. J. Food Microbiol. 241, 181–190. https://doi.org/10.1016/j.ijfoodmicro.2016.10.027.

Frisvad, J.C., 1981. Physiological criteria and mycotoxin production as aids in identification of commom asymmetric *Penicillia*. Appl. Environ. Microbiol. 41, 568–579. https://doi.org/10.1128/aem.41.3.568-579.1981.

Frisvad, J.C., Hubka, V., Ezekiel, C.N., Hong, S.B., Nováková, A., Al, C., Arzanlou, M., Larsen, T.O., Sklenár, F., Mahakarnchanakul, W., Samson, R.A., Houbraken, J., 2019. Taxonomy of *Aspergillus* section *Flavi* and their production of aflatoxins, ochratoxins and other mycotoxins. Stud. Mycol. 93, 1–63. https://doi.org/10.1016/j.simyco.2018.06.001.

Geiser, D.M., Pitt, J.I., Taylor, J.W., 1998. Cryptic speciation and recombination in the aflatoxin producing fungus *Aspergillus flavus*. PNAS USA. 95, 388–393. https://doi.org/10.1073/pnas.95.1.388.

Ghuffar, S., Irshad, G., Ahmed, M.Z., Zeshan, M.A., Ali, R., Haq, E., Anwaar, H.A., Abdullah, A., Ahmad, F., Haque, K., 2020. First report of *Aspergillus flavus* causing fruit rot of grapes (*Vitis vinifera*) in Pakistan. Plant Dis. 104, 3062. https://doi.org/10.1094/PDIS-04-20-0863-PDN.

Gibbons, J.G., Salichos, L., Slot, J.C., Rinker, D.C., McGary, K.L., King, J.G., Klich, M.A., Tabb, D.L., McDonald, W.H., Rokas, A., 2012. The evolutionary imprint of domestication on genome variation and function of the filamentous fungus *Aspergillus oryzae*. Curr. Biol. 22, 1403–1409. https://doi.org/10.1016/j.cub.2012.05.033.

Glass, N.L., Donaldson, G.C., 1995. Development of primer sets designed for use with PCR to amplify conserved genes from filamentous Ascomycetes. Appl. Environ. Microbiol. 61, 1323–1330.

Godet, M., Munaut, F., 2010. Molecular strategy for identification in *Aspergillus* section *Flavi*. FEMS Microbiol. Lett. 304 (2), 157–168. https://doi.org/10.1111/j.1574-6968.2009.01890.x.

Gonçalves, S.S., Stchigel, A.M., Cano, J.F., Martinez, P.C.G., Colombo, A.L., Guarro, J., 2012. *Aspergillus novoparasiticus:* a new clinical species of the section *Flavi*. Med. Mycol. 50, 152–160. https://doi.org/10.3109/13693786.2011.593564.

González-Salgado, A., González-Jaén, T., Vázquez, C., Patiño, B., 2008. Highly sensitive PCR-based detection method specific for *Aspergillus flavus* in wheat flour. Food Addit. Contam.Part A. 25, 758–764. https://doi.org/10.1080/02652030701765715.

Greenhalgh, T., 1997. How to read a paper: papers that reporting diagnostics or screening tests. BMJ. 315, 540–543. https://doi.org/10.1136/bmj.315.7107.540.

Grote, D., Olmos, A., Kofoet, A., Tuset, J.J., Bertolini, E., Cambra, M., 2002. Specific and sensitive detection of *Phytophthora nicotianeae* by simple and nested PCR. Eur. J. Plant Pathol. 108, 197–207. https://doi.org/10.1023/A:1015139410793.

Guezlane-Tebibel, N., Bouras, N., Mokrane, S., Benayad, T., Mathieu, F., 2013. Aflatoxigenic strains of *Aspergillus* section *Flavi* isolated from marketed peanuts (*Arachis hypogaea*) in Algiers (Algeria). Ann. Microbiol. 63, 295–305. https://doi.org/10.1007/s13213-012-0473-0.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for window 95/98/NT. Nucleic Acid Symp. Ser. 41, 95–98.

Hong, S.B., Go, S.J., Shin, H.D., Frisvad, J.C., Samson, R.A., 2005. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. Mycologia. 97, 1316–1329. https://doi.org/10.1080/15572536.2006.1183273877.

Hong, S.Y., Kang, M.R., Cho, E.J., Kim, H.K., Yun, S.H., 2010. Specific PCR detection of four quarantine *Fusarium* especies in Korea. J. Plant Pathol. 26, 409–416. https://doi.org/10.5423/PPJ.2010.26.4.409.

Houbraken, J., Spierenburg, H., Frisvad, J.C., 2012. *Rasamsonia*, a new genus comprising thermotolerant and thermophilic *Talaromyces* and *Geosmithia* species. Antonie Van Leeuwenhoek 101, 403–421. https://doi.org/10.1007/s10482-011-9647-1.

Hua, S.S.T., Parfitt, D.E., Sarreal, S.B.L., Lee, B.G., Wood, D.F., 2018. First report of an atypical new *Aspergillus parasiticus* isolates with nucleotide insertion in aflR gene resembling to *A. sojae*. Mycotoxin Res. 34, 151–157. https://doi.org/10.1007/s12550-018-0309-2.

Hue, N.T., Nhien, N.H., Thong, P.M., Thanh, C.N., An, P.V., 2013. Detection of toxic *Aspergillus* species in food by a multiplex PCR. In: Toi, V., Toan, N., Khoa, T.D., Phuong, T.L. (Eds.), 4th International Conference on Biomedical Engineering in Vietnam. IFMBE Proceedings, vol. 49. Springer, Berlin, Heidelberg, pp. 184–189. https://doi.org/10.1007/978-3-642-32183-2_48.

Hussein, M.A., El-Said, A.H.M., Yassein, A.S., 2020. Mycobiota associated with strawberry fruits, their mycotoxin potential and pectinase activity. Mycology 158–166. https://doi.org/10.1080/21501203.2020.1759719.

Iamanaka, B.T., Lopes, A.S., Martins, L.M., Frisvad, J.C., Medina, A., Magan, N., Sartori, D., Massi, F.P., Fungaro, M.H.P., Taniwaki, M.H., 2019. *Aspergillus* section *Flavi* diversity and the role of *A. novoparasiticus* in aflatoxin contamination in the sugarcane production chain. Int. J. Food Microbiol. 293, 17–23. https://doi.org/10.1016/j.ijfoodmicro.2018.12.024.

IARC, 1993. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. International Agency for Research on Cancer, Lyon, France. https://monographs.iarc.who.int/wp-content/uploads/2018/06/mono56.pdf.

Jallow, A., Xie, H., Tang, X., Qi, Z., Li, P., 2021. Worldwide aflatoxin contamination of agricultural products and foods: from occurrence to control. Compr. Rev. Food Sci. Food Saf. 20, 2332–2381. https://doi.org/10.1111/1541-4337.12734.

Jodral, M., Liñan, E., Acosta, I., Gallego, C., Rojas, F., Bentabol, A., 1993. Mycoflora and toxigenic *Aspergillus flavus* in Spanish milks. Int. J. Food Microbiol. 18, 171–174. https://doi.org/10.1016/0168-1605(93)90222-3.

Kang, M.R., Kim, J.H., Lee, S.H., Ryu, J.G., Lee, T., Yun, S.W., 2011. Detection of *Fusarium verticillioides* contaminated in corn using a new especies-specific primers. Res. Plant Dis. 17, 369–375. https://doi.org/10.5423/RPD.2011.17.3.369.

Katsurayama, A.M., Martins, L.M., Iamanaka, B.T., Fungaro, M.H.P., Silva, J.J., Frisvad, J.C., Pitt, J.I., Taniwaki, M.H., 2018. Occurrence of *Aspergillus* section *Flavi* and aflatoxins in Brazilian rice: from field to market. Int. J. Food Microbiol. 266, 213–221. https://doi.org/10.1016/j.ijfoodmicro.2017.12.008.

Khoury, A.E., Atoui, A., Rizk, T., Lteif, R., Kalassy, M., Lebrihi, A., 2011. Differentiation between *Aspergillus flavus* and *Aspergillus parasiticus* from pure culture and aflatoxin contaminated grapes using PCR-RFLP analysis of *aflR-aflJ* intergenic spacer. J. Food Sci. 76, M247–M253. https://doi.org/10.1111/j.1750-3841.2011.02153.x.

Kim, K.M., Lim, J., Lee, J.J., Lee, I., 2017. Characterization of *Aspergillus sojae* isolated from Meju, Korean traditional fermented Soybean Brick. J. Microbiol. Biotechnol. 27, 251–261. https://doi.org/10.4014/jmb.1610.10013.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120. https://doi.org/10.1007/BF01731581.

Kubosaki, A., Kobayashi, N., Watanabe, M., Yoshinari, T., Takatori, K., Kikuchi, Y., Hara-kudo, Y., Terajima, J., Konish, Y.S., 2020. A new protocol for the detection of sterigmatocystin-producing *Aspergillus* section *Versicolores* using a high discrimation polymerase. Biocontrol Sci. 25, 113–118. https://doi.org/10.4265/bio.25.113.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874. https://doi.org/10.1093/molbev/msw054.

Kumar, P., Mahato, D.K., Kamle, M., Mohanta, T.K., Kang, S.G., 2017. Aflatoxins: a global concern for food safety, human health and their management. Front. Microbiol. 7, 2170. https://doi.org/10.3389/fmicb.2016.02170.

Luo, J., Vogel, R.F., Niessen, L., 2012. Development and application of a loop-mediated isothermal amplification assay for rapid identification of aflatoxigenic molds and their detection in food samples. Int. J. Food Microbiol. 159, 214–224. https://doi.org/10.1016/j.ijfoodmicro.2012.08.018.

Machida, M., Asai, K., Sano, M., et al., 2005. Genome sequencing and analysis of *Aspergillus oryzae*. Nature. 438, 1157–1161. https://doi.org/10.1038/nature04300.

Mahmoud, M.A., 2015. Detection of *Aspergillus flavus* in stored peanuts using real-time PCR and the expression of aflatoxin genes in toxigenic and atoxigenic *A. flavus* isolates. Foodborne Pathog. Dis. 12, 289–296. https://doi.org/10.1089/fpd.2014.1854.

Mamo, F.T., Shang, B., Selvaraj, J.M., Wang, Y., Liu, Y., 2018. Isolation and characterization of *Aspergillus flavus* strains in China. J. Microbiol. 56, 119–127. https://doi.org/10.1007/s12275-018-7144-1.

Mimoune, N.A., Manzanares, N.A., Gracia, L.G., Campaña, A.M.G., Bouti, K., Sabaou, N., Riba, A., 2018. *Aspergillus* section *Flavi* and aflatoxins in dried figs and nuts in Algeria. Food Addit. Contam., Part B. 11, 119–125. https://doi.org/10.1080/19393210.2018.1438524.

Nguyen, H.N., Nguyen, T.M.T., Nguyen, T.H., 2015. Developing a PCR assay to detect *Aspergillus parasiticus*. Sci. Technol. Dev. J. 18, 43–51. https://doi.org/10.32508/stdj.v18i1.1033.

Norlia, M., Jinap, S., Nor-Khaizura, M.A.B., Radu, S., Samsudin, N.I.P., Azri, F.A., 2019. Aspergillus section Flavi and aflatoxins: Occurence, detection, and identification in

raw peanuts and peanut-based products along the supply chain. Front. Microbiol. 10, 2602. https://doi.org/10.3389/fmicb.2019.02602.

Ono, L.T., Silva, J.J., Doná, S., Martins, L.M., Iamanaka, B.T., Fungaro, M.H.P., Pitt, J.I., Taniwaki, M.H., 2021. *Aspergillus* section *Flavi* and aflatoxins in Brazillian cassava (M*anihot esculenta Crantz*) and products. Mycotoxin Res. 37, 221–228. https://doi.org/10.1007/s12550-021-00430-2.

Ortega, S.F., Siciliano, I., Prencipe, S., Gullino, M.L., Spadaro, D., 2020. Development of PCR, LAMP and qPCR assays for the detection of aflatoxigenic strains of *Aspergillus flavus* and *A. parasiticus* in Hazelnut. Toxins. 12, 757. https://doi.org/10.3390/toxins12120757.

Ortiz-Martinez, R., Valdivia-Flores, A., Quezada-Tristan, T., Rangel-Muñoz, E., Cruz-Vazquez, C., Luna-Lopez, M.C., Castañeda, C.A.M., 2017. *Aspergillus flavus* and aflatoxins in feeds and dairy products in Central Mexico. Toxicol. Lett. 280, S314–S315. https://doi.org/10.1016/j.toxlet.2017.08.048.

Pitt, J.I., 1979. The Genus *Penicillium* and its Teleomorphic States *Eupenicillium* and *Talaromyces*. Academic Press, London, UK, p. 634.

Queró, L., Courault, P., Cellière, B., Lorber, S., Jany, J.L., Puel, O., Girard, V., Vasseur, V., Nodet, P., Mounier, J., 2020. Application of MALDI-TOF MS to species complex differentiation and straind typing of food related fungi: case studies with *Aspergillus* section *Flavi* species and *Penicillium roqueforti* isolates. Food Microbiol. 86, 103311 https://doi.org/10.1016/j.fm.2019.103311.

Rokas, A., Payne, G., Fedorova, N.V., et al., 2007. What can comparative genomics tell us about species concepts in the genus *Aspergillus*? Stud. Mycol. 59, 11–17. https://doi.org/10.3114/sim.2007.59.02.

Ronquist, F., Teslenko, M., Mark, P., Ayres, D.L., Darling, A., Hohna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542. https://doi.org/10.1093/sysbio/sys029.

Rozas, J., Mata, A.F., DelBarrio, J.C.S., Rico, S.G., Librado, P., Onsins, S.E.R., Gracia, A. S., 2017. DnaSP 6: DNA sequence polymorphism analysis of large data sets. Mol. Biol. Evol. 34, 3299–3302. https://doi.org/10.1093/molbev/msx248.

Saif, F.A., Yaseen, S.A., Alameen, A.S., Mane, S.B., Undre, P.B., 2021. Identification and characterization of *Aspergillus* species of fruit rot fungi using Microscopy, FT-IR, Raman and UV-Vis spectroscopy. Spectrochim. Acta, Part A. 246, 119010 https://doi.org/10.1016/j.saa.2020.119010.

Samson, R., Visagie, C.M., Houbraken, J., Hong, S.B., Hubka, V., Klaassen, C.H., Perrone, G., Seifert, K.A., Susca, A., Tanney, J.B., Varga, J., Kocsubé, S., Szigeti, G., Yaguchi, T., Frisvad, J.C., 2014. Phylogeny, identification and nomenclature of the genus *Aspergillus*. Stud. Mycol. 78, 141–173. https://doi.org/10.1016/j.simyco.2014.07.004.

Sardiñas, N., Vázquez, C., Gil-Serna, J., González-Jaén, M.T., Patiño, B., 2010. Specific detection of *Aspergillus parasiticus* in wheat flour using a highly sensitive PCR assay. Food Addit. Contam., Part A. 27, 853–858. https://doi.org/10.1080/19440041003645779.

Sardiñas, N., Vázquez, C., Gil-Serna, J., González-Jaén, M.T., Patiño, B., 2011. Specific detection and quantification of Aspergillus flavus and Aspergillus parasiticus in wheat flour by SYBR® Green quantitative PCR. Int. J. Food Microbiol. 145, 121–125. https://doi.org/10.1016/j.ijfoodmicro.2010.11.041.

Sebok, F., Dobolyi, C., Zágoni, D., Risa, A., Krifaton, C., Hartman, M., Cserháti, M., Szoboszlay, S., Kriszt, B., 2016. Aflatoxigenic *Aspergillus flavus* and *Aspergillus parasiticus* strains in Hungarian maize fields. Acta Microbiol. Immunol. Hung. 63, 491–502. https://doi.org/10.1556/030.63.2016.012.

Shapira, R., Paster, N., Eyal, O., Menasherov, M., Mett, A., Salomon, R., 1996. Detection of aflatoxigenic molds in grains by PCR. Appl. Environ. Microbiol. 62, 3270–3273. https://doi.org/10.1128/aem.62.9.3270-3273.1996.

Silva, J.J., Puel, O., Lorber, S., Ferranti, L.S., Ortiz, L.F., Taniwaki, M.H., Iamanaka, B.T., Fungaro, M.H.P., 2019a. Occurrence and diversity of *Aspergillus* in commercial yerba mate elaborated for the Brazilian beverage 'chimarrão'. Food Res. Int. 121, 940–946. https://doi.org/10.1016/j.foodres.2019.01.023.

Silva, J.J., Iamanaka, B.T., Fungaro, M.H.P., Taniwaki, M.H., 2019b. Aflatoxins in sugarcane production chain: what could be the source? Curr. Opin. Food Sci. 29, 94–98. https://doi.org/10.1016/j.cofs.2019.08.010.

Silva, J.J., Iamanaka, B.T., Ferranti, L.S., Massi, F.P., Taniwaki, M.H., Puel, O., Lorber, S., Frisvad, J.C., Fungaro, M.H.P., 2020. Diversity within Aspergillus niger clade and description of a new species: Aspergillus vinaceus sp. J. Fungi. 6, 371. https://doi.org/10.3390/jof6040371.

Singh, R., Rai, T.S., Sharma, N.S., Arora, A.K., Kaur, P., 2017. Evaluation of a real time polymerase chain reaction assay for the detection of aflatoxin/sterigmatocystin producing strains of *Aspergillus spp*. Indian J. Anim. Res. 51, 676–678.

Somashekar, D., Rati, E.R., Chandrashekar, A., 2004. PCR-restriction fragment length analysis of *aflR* gene for differentiation and detection of *Aspergillus flavus* and *Aspergillus parasiticus* in maize. Int. J. Food Microbiol. 93, 101–107. https://doi.org/10.1016/j.ijfoodmicro.2003.10.011.

Su, C., Hu, Y., Gao, D., Luo, Y., Chen, A.J., Jiao, X., Gao, W., 2018. Occurrence of toxigenic fungi and mycotoxins on root herbs from chinese markets. J. Food Prot. 81, 754–761. https://doi.org/10.4315/0362-028X.JFP-17-405.

Sugita, C., Makimura, K., Uchida, K., Yamaguchi, H., Nagai, A., 2004. PCR identification system for the genus *Aspergillus* and three major pathogenic species: *Aspergillus fumigatus, Aspergillus flavus* and *Aspergillus niger*. Med. Mycol. 42, 433–437. https://doi.org/10.1080/13693780310001656786.

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10, 512–526. https://doi.org/10.1093/oxfordjournals.molbev.a040023.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment throught sequence weighting position-especific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680. https://doi.org/10.1093/nar/22.22.4673.

Tournas, V.H., Katsoudas, E.J., 2008. Microbiological quality of various medicinal herbal teas and coffee substitutes. Microbiol. Insights. 1, 47–55. https://doi.org/10.4137/2FMBI.S943.

Varga, J., Baranyi, N., Chandrasekaran, M., Vágvolgyi, C., Kocsubé, S., 2015. Mycotoxin producers in the *Aspergillus* genus: An update. Acta Biol. Szeged. 59, 151–167.

Vetrovsky, T., Kolarik, M., Zifcaková, L., Zelenka, T., Baldrian, P., 2016. The rpb2 gene represents a viable alternative molecular marker for the analysis of environmental fungal communities. Mol. Ecol. Resour. 16, 388–401. https://doi.org/10.1111/1755-0998.12456.

Viaro, H.P., Silva, J.J., Ferranti, L.S., Bordini, J.G., Massi, F.P., Fungaro, M.H.P., 2017. The first report of *A. novoparasiticus, A. arachidicola* and *A. pseudocaelatus* in Brazilian corn kernels. Int. J. Food Microbiol. 243, 46–51. https://doi.org/10.1016/j.ijfoodmicro.2016.12.002.

Visagie, C.M., Varga, J., Houbraken, J., Meijer, M., Kocsubé, S., Yilmaz, N., Fotedar, R., Seifert, K.A., Frisvad, J.C., Samson, R.A., 2014. Ochratoxin production and taxonomy of the yellow aspergilli (*Aspergillus* section *Circumdati*). Stud. Mycol. 78, 1–61. https://doi.org/10.1016/j.simyco.2014.07.001.

Wang, D.Y., Hadj-Henni, L., Thierry, S., Arné, P., Chermette, R., Botterel, F., Hadrich, I., Makni, F., Ayadi, A., Ranque, S., Huang, W.Y., Guillot, J., 2012. Simple and highly discriminatory VNTR-based multiplex PCR for tracing sources of *Aspergillus flavus* isolates. PLoS One 7, e44204. https://doi.org/10.1371/journal.pone.0044204.

Wild, C.F., 2007. Aflatoxin exposure in developing countries: the critical interface of agriculture and health. Food Nutr. Bull. Suppl. 28, 372–380. https://doi.org/10.1177/2F15648265070282S217.

Zha, D., Xing, X., Yang, F., 2010. A multiplex PCR assay for fraud identification of deer products. Food Control 21, 1402–1407. https://doi.org/10.1016/j.foodcont.2010.04.013.