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Application of loop-mediated isothermal amplification assays for direct identification of pure cultures of *Aspergillus flavus*, *A. nomius*, and *A. caelatus* and for their rapid detection in shelled Brazil nuts

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ABSTRACT

Brazil nuts have a high nutritional content and are a very important trade commodity for some Latin American countries. Aflatoxins are carcinogenic fungal secondary metabolites. In Brazil nuts they are produced predominantly by *Aspergillus* (*A.*) *nomius* and *A. flavus*. In the present study we applied and evaluated two sets of primers previously published for the specific detection of the two species using loop-mediated isothermal amplification (LAMP) technology. Moreover, a primer set specific for *A. caelatus* as a frequently occurring non-aflatoxigenic member of *Aspergillus* section *Flavi* in Brazil nuts was newly developed. LAMP assays were combined with a simplified DNA release method and used for rapid identification of pure cultures and rapid detection of *A. nomius* and *A. flavus* from samples of shelled Brazil nuts. An analysis of pure cultures of 68 isolates representing the major *Aspergillus* species occurring on Brazil nuts showed that the three LAMP assays had individual accuracies of 61.5%, 84.4%, and 93.3% for *A. flavus*, *A. nomius*, and *A. caelatus*, respectively when morphological identification was used as a reference. The detection limits for conidia added directly to the individual LAMP reactions were found to be 10^5 conidia per reaction with the primer set ID9 for *A. nomius* and 10^4 conidia per reaction with the primer set ID58 for *A. flavus*. Sensitivity was increased to 10^1 and 10^2 conidia per reaction for *A. nomius* and *A. flavus*, respectively, when sample preparation included a spore disruption step. The results of LAMP assays obtained during the analysis of 32 Brazil nut samples from different regions of Brazil and from different steps in the production process of the commodity were compared with results obtained from mycological analysis and aflatoxin analysis of corresponding samples. Compared with mycological analysis of the samples, the Negative Predictive Values of LAMP assays were 42.1% and 12.5% while the Positive Predictive Values were 61.5% and 66.7% for *A. nomius* and *A. flavus*, respectively. When LAMP results were compared with the presence of aflatoxins in corresponding samples, the Negative Predictive Values were 22.2% and 44.4% and the Positive Predictive Values were 52.2% and 78.3% for aflatoxins produced by *A. nomius* and *A. flavus*, respectively. The LAMP assays described in this study have been demonstrated to be a specific, sensitive and easy to use tool for the survey of Brazil nuts for contaminations with potential aflatoxin-producing *A. nomius* and *A. flavus* in low tech environments where resources may be limited.

1. Introduction

The Brazil nut tree (*Bertholletia excela* Humb. and Bonpl.) is an Amazonian tree that prefers non-flooded rainforest habitats in the Guianas, Venezuela, Colombia, Ecuador, Peru, Bolivia and Brazil (Pacheco and Scussel, 2007). It represents a very important trade commodity for the Northern Brazilian States and the abovementioned countries. Due to their nutritional properties, Brazil nuts are well known for their high content of oil, protein, and selenium (Andrade et al., 1999; Chang et al., 1995; Ryan et al., 2006). Nearly 20,000 tons of Brazil nuts are harvested each year. The bulk of production is exported, with less than 3% used for domestic consumption (Collinson et al., 2000). The Brazil nut tree is large, reaching 50 m (160 ft) in height and 1 to 2 m (3.3 to 6.6 ft) in diameter, making it one of the largest trees in the Amazon Rainforests. Only a few examples exist in which the tree has been grown in botanical gardens or plantations. Therefore, the productivity of Brazil nut production chain is low and it is currently not economically viable for agro-industrial companies (Mori, 1992). Similar to cashew nuts or nutmeg, Brazil nuts are collected manually in indigenous regions by peasants after the ripe seed pods have fallen to the ground during the rainy season which is December to March. Depending on collection frequency, the pods and nuts are in contact with the ground where they may become exposed to fungal infection.

Some factors have decreased the volume of Brazil nut exports, such as maximum levels of total aflatoxins (4.0 μg · kg^{-1}) imposed by the

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European Union (EU) in 1998 (European Union, 1998), the destruction of native trees (Kitamura and Müller, 1984; Mori and Prance, 1990), and the market competition with other nut products (Gunneroed, 1994). Brazil nuts have been found to be frequently contaminated by aflatoxins in relatively high concentrations (Pacheco and Scussel, 2007). In 2010, the EU adopted the maximum levels of aflatoxin B_1 and total aflatoxins in Brazil nuts for further processing and ready to eat of 8.0 and 15.0 μg/kg and 5.0 and 10.0 μg/kg, respectively, based on Codex Alimentarius (European Union, 2010). Aflatoxins are naturally occurring toxic secondary metabolites produced by several fungal species within the genus *Aspergillus* (*A.*). They are highly carcinogenic and cause organic changes, and, in high concentrations, they are lethal to animals and humans (International Agency for Research on Cancer, 1997). Foods and feeds, especially in warm climates, are susceptible to invasion by aflatoxigenic *Aspergillus* species and to the subsequent production of aflatoxins during pre-harvesting, processing, transportation and storage (Ellis et al., 1991). Brazil nut production occurs in environments with temperatures of 30–35 °C and with high relative humidity (80–95%). The environmental conditions in the Amazon region influence the level of water activity and moisture in Brazil nuts, and favor aflatoxin production (Johnsson et al., 2008). Previous studies reported the presence of *A. flavus* in in-shell and shelled Brazil nuts (Lopez Castrillon and Purchio, 1988; Freire et al., 2000; Bayman et al., 2002; Arrus et al., 2005a). However, Olsen et al. (2008) examined the relation between aflatoxin B_1 and G_1 in 199 lots of in-shell Brazil nuts imported to Europe and reported a near 1/1 ratio between aflatoxins B_1 and G_1 in most samples. The authors concluded that *A. flavus* cannot be the only species responsible for aflatoxin production in Brazil nuts since it produces solely aflatoxins of the B series. Moreover, no *A. parasiticus* was found during the study, which was in agreement with earlier studies (Arrus et al., 2005a,b). Therefore, Olsen et al. (2008) suggested that the regularly occurring *A. nomius* may be another important producer of aflatoxins in Brazil nuts. As a consequence, this fungus as well as other potential producers of B and G aflatoxins should be further examined since the findings may influence strategies for prevention and control of aflatoxins in Brazil nuts. Recently, Calderari et al. (2013) also found *A. nomius* and *A. flavus* as the main aflatoxin producers at different stages of the Brazil nut production chain. In addition, the authors indicated that *A. caelatus*, which showed the same conidium color on CYA, is a common non-aflatoxin producing species with very high frequency of occurrence in Brazil nut.

For the detection of molds, methods such as the traditional mycological methods (Pitt and Hocking, 2009), enzyme-linked immunosorbent assay (Notermans et al., 1986), PCR and RT-PCR (Shapira et al., 1996; Haugland et al., 2002) can be applied. However, the traditional mycological method being used to assess mold presence in commodities is time-consuming, labor-intensive, requires facilities and mycological expertise, and above all, does not allow the specification of mycotoxigenic strains. In addition, PCR and RT-PCR have been described as more sensitive and specific methods for detection of molds (Shapira et al., 1996; Haugland et al., 2002). Nevertheless, these methods are costly and require trained personnel for their implementation. As an alternative technology, loop-mediated isothermal amplification (LAMP) of DNA was described as a specific, rapid, cost-effective, and easy-to-use method by Notomi et al. (2000). The method takes advantage of the high processivity of the *Bst* DNA polymerase from *Bacillus stearothermophilus*. Since *Bst* DNA polymerase has a very high activity, vast amounts of high molecular weight DNA are produced within a short time. The exceptionally high specificity of LAMP is due to the fact that a set of four primers with six binding sites must hybridize correctly to their target sequence before DNA biosynthesis occurs. Detection of amplified product has been accomplished by AGE (agarose gel electrophoresis) or by in-tube detection methods such as direct staining of double stranded DNA using intercalating dyes, e.g. SYBR green 1, SYTO-9 (Njiru et al., 2008) or by precipitation of the LAMP product with a fluorescently labeled cationic polymer (Mori et al., 2006). Indirect in-tube detection of LAMP product can be done using pyrophosphate as a specific by-product of enzymatic DNA synthesis resulting in precipitation of a magnesium–pyrophosphate complex which can be detected in a dedicated turbidimeter (Mori et al., 2001). In the current study, indirect calcein fluorescence was used to detect DNA synthesis by removing the manganese quencher from the molecule by complexing with pyrophosphate during production of DNA (Tomita et al., 2008). Only few applications of LAMP for the detection of fungal organisms have been described so far. Most recently, Niessen et al. (2013) reviewed the application of LAMP-based methods in detecting and identifying food-borne bacterial pathogens and toxicants as well as mycotoxin producing food-borne fungi.

Built upon our previous report (Luo et al., 2012) which verified the usefulness of LAMP assays for the detection of three aflatoxin-producing *Aspergillus* species, the present study was aimed at developing analytical protocols involving the use of LAMP-based assays in combination with a simple and rapid procedure for the extraction of amplifiable DNA from fungal spores of aflatoxin-producing *A. nomius* and *A. flavus*, respectively, in pure cultures and in naturally contaminated Brazil nuts. Moreover, to aid analysis of the biodiversity of *Aspergillus* section *Flavi* in Brazil nuts, a primer set for LAMP-based detection of *A. caelatus* was developed.

2. Materials and methods

2.1. Fungal strains, media and Brazil nut samples

2.1.1. Fungal strains

A total of 69 fungal isolates listed in Table 1 were obtained from Brazil nuts as described by Gonçalves et al. (2012) and Calderari et al. (2013). Working cultures were maintained on 2% malt extract agar (MEA, see below) at ambient temperature. For long term storage mycelia were grown in 50 ml 2% malt extract broth (w/v) with a spoonful of sterilized porous clay granules (3–5 mm diameter) at 25 °C. After 5 d granules were removed from the medium and transferred into 80% sterile glycerol in 2 ml screw cap cryo-vials (Sarstedt, Nümbrecht, Germany) and frozen at −80 °C.

2.1.2. Media

All media were sterilized by autoclaving at 121 °C, 1.2 bar for 15 to 20 min. Malt Extract agar (MEA) comprised 2% (w/v) malt extract (AppliChem, Darmstadt, Germany) with 0.2% (w/v) soy peptone (Oxoid, Basingstoke, Hampshire, England) and 15 g/l agar (European agar, DB, Le Pont de Claix, France), with pH 5.6 adjusted. Malt extract broth was prepared accordingly without the agar.

Dichloran Glycerol (DG18) agar comprised peptone 5 g/l, KH_2PO_4 1 g/l, $MgSO_4 \times 7H_2O$ 0.5 g/l, dichloran 0.002 g/l, chloramphenicol 0,1 g/l, glucose 10 g/l, glycerol (100%) 220 g/l and 20 g/l agar, pH 5.6. Glycerol was added after the agar had dissolved and glucose was autoclaved separately in 100 ml H_2O and mixed with the other components after autoclaving.

Czapek yeast extract agar (CYA) comprised Czapek concentrate 10 ml/l, $K_2HPO_4 \times 3H_2O$ 1.2 g/l, yeast extract 5 g/l, saccharose 3% (w/v), agar 20 g/l, pH 6.0. Saccharose was autoclaved separately in 100 ml H_2O and mixed with the other components after autoclaving.

2.1.3. Brazil nut samples

A total of 32 of the 288 Brazil nut samples analyzed by Calderari et al. (2013) were available for the current study. Samples had been stored at −20 °C for 16–55 months prior to analysis. Nuts had been collected from the Amazonian rainforest, at local markets, supermarkets and during processing in different Brazilian States (see Table 2). There were 5 samples obtained from the state of Amazonas; 19 from Pará; and 8 from São Paulo.

Table 1
Fungal isolates used during the current study and results of LAMP analysis.

Morphological identification	Strain (see Soares Gonçalves et al., 2012)	Clone	LAMP identification		
			Anom ID9	Afla ID58	Aca ID56
A. arachidicola	ITAL 189	TMW 4.2312	+	−	+
A. caelatus	ITAL 91	TMW 4.2353	−	−	+
A. caelatus	ITAL 97	TMW 4.2348	−	+	+
A. caelatus	ITAL 201	TMW 4.2303	−	+	+
A. caelatus	ITAL 212	TMW 4.2359	−	+	+
A. caelatus	ITAL 243	TMW 4.2287	−	−	+
A. caelatus	ITAL 467	TMW 4.2360	−	−	+
A. caelatus	ITAL 504	TMW 4.2286	−	+	+
A. caelatus	ITAL 562	TMW 4.2295	−	−	+
A. caelatus	ITAL 566	TMW 4.2349	−	+	+
A. caelatus	ITAL 584	TMW 4.2281	−	+	+
A. caelatus	ITAL 695	TMW 4.2308	−	−	+
A. caelatus	ITAL 787	TMW 4.2310	−	−	+
A. caelatus	ITAL 1574	TMW 4.2327	−	+	+
A. caelatus	ITAL 1576	TMW 4.2362	−	+	+
A. flavus	ITAL 58	TMW 4.2342	−	+	−
A. flavus	ITAL 59	TMW 4.2301	−	+	−
A. flavus	ITAL 71	TMW 4.2333	−	+	−
A. flavus	ITAL 73	TMW 4.2356	−	+	−
A. flavus	ITAL 92	TMW 4.2289	−	+	−
A. flavus	ITAL 99	TMW 4.2363	−	+	−
A. flavus	ITAL 112	TMW 4.2374	−	+	−
A. flavus	ITAL 136	TMW 4.2354	−	+	−
A. flavus	ITAL 168	TMW 4.2355	−	+	−
A. flavus	ITAL 179	TMW 4.2313	−	+	−
A. flavus	ITAL 295	TMW 4.2332	−	+	−
A. flavus	ITAL 758	TMW 4.2346	−	+	−
A. flavus	ITAL 1257	TMW 4.2364	−	+	−
A. flavus	ITAL 1306	TMW 4.2302	−	+	−
A. flavus	ITAL 1836	TMW 4.2361	−	+	−
A. flavus	ITAL 3919	TMW 4.2337	−	+	−
A. nomius	ITAL 255	TMW 4.2330	+	−	−
A. nomius	ITAL 256	TMW 4.2331	+	−	−
A. nomius	ITAL 438	TMW 4.2305	+	−	−
A. nomius	ITAL 486	TMW 4.2314	+	−	−
A. nomius	ITAL 532	TMW 4.2285	+	−	−
A. nomius	ITAL 587	TMW 4.2350	+	−	−
A. nomius	ITAL 608	TMW 4.2343	+	−	−
A. nomius	ITAL 618	TMW 4.2300	+	−	−
A. nomius	ITAL 638	TMW 4.2329	+	−	−
A. nomius	ITAL 648	TMW 4.2328	+	−	−
A. nomius	ITAL 650	TMW 4.2357	−	−	−
A. nomius	ITAL 657	TMW 4.2315	+	−	−
A. nomius	ITAL 696	TMW 4.2336	+	−	−
A. nomius	ITAL 699	TMW 4.2334	+	−	−
A. nomius	ITAL 709	TMW 4.2341	+	−	−
A. nomius	ITAL 723	TMW 4.2307	+	−	−
A. nomius	ITAL 730	TMW 4.2347	−	+	−
A. nomius	ITAL 741	TMW 4.2335	+	−	−
A. nomius	ITAL 743	TMW 4.2344	+	−	−
A. nomius	ITAL 764	TMW 4.2297	−	−	−
A. nomius	ITAL 849	TMW 4.2292	+	−	−
A. nomius	ITAL 1228	TMW 4.2298	+	−	−
A. nomius	ITAL 1230	TMW 4.2299	+	−	−
A. nomius	ITAL 1325	TMW 4.2283	−	−	−
A. nomius	ITAL 3052	TMW 4.2304	+	−	−
A. nomius	ITAL 3087	TMW 4.2311	+	−	−
A. nomius	ITAL 3333	TMW 4.2345	+	−	−
A. nomius	ITAL 4466	TMW 4.2294	+	−	−
A. nomius	ITAL 6071	TMW 4.2365	+	−	−
A. nomius	ITAL 6226	TMW 4.2284	−	+	−
A. nomius	ITAL 6245	TMW 4.2358	−	−	−
A. nomius	ITAL 6251	TMW 4.2309	+	−	−
A. pseudotamarii	ITAL 791	TMW 4.2339	−	−	−
A. pseudotamarii	ITAL 792	TMW 4.2338	−	−	−
A. tamarii	ITAL 119	TMW 4.2296	−	−	−
A. tamarii	ITAL 129	TMW 4.2352	−	−	−
A. tamarii	ITAL 226	TMW 4.2306	−	−	−

2.2. Mycological analysis and aflatoxin production

Isolation of fungal pure cultures from surface disinfected seeds and shells of Amazonian Brazil nuts followed by the identification by morphological and molecular analyses of the isolates given in Table 1 were carried out as described in Gonçalves et al. (2012) and Calderari et al. (2013). Mycological analysis of Brazil nut samples as given in Table 2 was done according to the following protocol. Fifty shelled

Table 2
LAMP conditions and sequences of LAMP primers for *A. caelatus*.

Target sequence cource	LAMP condition	Primer designation	5′-3′ Oligonucleotide sequence
acl1	64 °C	FIP-Aca ID56	CCA ACT CTG ACC TGG AAA CCG CGT GGA TAC CAG CCT CCC T
A. caelatus	3%(v/v)formamide	BIP-Aca ID56	CAG CGT GAC CGA ACT GGA CCC ATC ACC AAG CCC ATT GTT G
		F3-Aca ID56	TTG GGC ATG TCC TCA AAG G
		B3-Aca ID56	CAT CGA GGC CGT CAA GAA C
		loopF-Aca ID56	TGT TGC TAA GAA CAA CGC CAT G
		loopB-Aca ID56	TGG CGC AGG TAC CAA TAG C

nuts (seeds) were surface-disinfected by immersion into 0.4% sodium hypochlorite for 1 min and plated immediately onto Dichloran Glycerol (DG18) agar containing chloramphenicol. Samples were incubated for 5 d at 25 °C. Fungal colonies resembling *Aspergillus* spp. section *Flavi* were transferred to Czapek yeast extract agar (CYA) for pure cultivation. Pure cultures were transferred to CYA and incubated at 25 °C, 37 °C, and 42 °C, respectively for 7 d to assess macroscopic and microscopic characters for species identification. Frequency of isolation was recorded for *A. flavus* and *A. nomius* in each sample.

Aflatoxin results as given in Table 2 were obtained from shelled Brazil nuts as described by Calderari et al. (2013) using HPLC with a fluorescence detector.

2.3. Preparation of spore suspensions

Fungal colonies were grown on MEA plates at ambient temperature until abundant sporulation occurred. Spores were harvested by two repetitive cycles of adding sterile 5 mm glass beads and 3 ml of sterile tap water per plate before shaking for 1 min and collecting the solution in a sterile 15 ml Falcon tube (Sarstedt, Nümbrecht, Germany). Spores were spun at 6000 ×*g* for 5 min at ambient temperature and the clear supernatant was discarded. The pellet was washed twice by re-suspending in 2 ml sterile deionized water and spinning at 6000 ×*g* for 5 min at ambient temperature before the supernatant was discarded. Washed spores were re-suspended in 2 ml sterile deionized water and conidial concentrations were assessed by counting in a Thoma type counting chamber (depth 0.1 mm).

2.4. DNA preparation

Highly purified fungal genomic DNA of *A. nomius*, *A. flavus*, and *A. caelatus* was extracted from the mycelia of reference strains *A. nomius* CBS 260.86, *A. flavus* CBS 113.32, and *A. caelatus* IBT 29700 and used as positive controls in the respective assays throughout the study. The mycelia were finely ground using the method described by Luo et al. (2012). Ground mycelia were subjected to DNA-extraction according to the method described by Niessen and Vogel (2010).

Spore suspensions used for direct LAMP analysis of plate grown pure cultures were obtained using a sterile toothpick to take spores from cultures by touching the culture surface. Spores were immersed into 100 μl of ultrapure water. Two alternative methods for the preparation of target DNA used in LAMP analyses of either pure cultures or spore containing debris in washings of Brazil nut samples were applied: (i) 10 μl of spore or debris suspensions was added directly into the LAMP master mix; (ii) 100 μl of the spore or debris suspension was disrupted by vigorous shaking together with 0.3 g zirconia/silica beads (diameter 0.1 mm: diameter 0.5 mm = 1:1; Carl Roth, Karlsruhe, Germany) for 10 min in a vortex at maximum speed. Samples were boiled for 10 min. 10 μl of the supernatant was used as LAMP template after centrifugation at 16,000 ×*g* for 5 min at ambient temperature.

Prior to extraction of fungal DNA from Brazil nut samples, 10 nuts were washed three times with sterile deionized water containing 0.1% Tween-20 by shaking for 10 min. The washing solutions were pooled and the solid debris was spun down at 7000 ×*g* for 5 min. The supernatant was discarded and the pellet was washed three times by re-suspending in 2 ml water and spinning under the same conditions. For DNA extraction, bead-beating treatment was used as described previously. The supernatant was used as LAMP template after 10-fold dilution in ultra-pure water.

2.5. Sequencing of the target gene acl1 of A. caelatus

To design the primer set for LAMP assay of *A. caelatus*, the target gene acl1 of *A. caelatus* was sequencing after amplification by PCR. The genomic DNA of *A. caelatus* IBT 29700 was used as the template in the PCR. For PCR amplification, the PCR *Taq* core kit (MP Biomedicals) with primers ACL1-615f (5′-GGY ATG ATG GAC AAC ATY GT-3′) and ACL1-1386r (5′GCU ATU ARC ATA GRA CCA TC-3′) was used. The DNA amplification was performed in a PCR-Cycler (MWG Biotech, AG, Ebersberg, Germany) with an initial cycle of denaturation (5 min at 95 °C) followed by 35 cycles of denaturation (1 min at 95 °C), annealing (1 min at 52 °C), and extension (1 min at 72 °C) followed by a final incubation step (10 min at 72 °C). After detection on a 1% agarose gel the product was purified and sequenced by GATC Biotech AG (Konstanz, Germany).

2.6. LAMP assays

Loop-mediated isothermal amplification (LAMP) assay of spore samples and Brazil nut samples was carried out according to the protocol described by Niessen and Vogel (2010) using the *A. flavus* and *A. nomius* specific primer sets and master mixes as well as reaction conditions described in Luo et al. (2012). The reaction conditions for the two LAMP reactions were those described by Luo et al. (2012) with the following modifications: *Bst* polymerase 2.0 was used instead of *Bst* polymerase and master mixes had formamide contents of 4.5% and 5% for the *A. nomius* and *A. flavus* assays, respectively. For the reaction conditions of the LAMP assay for *A. caelatus*, formamide content was 3% and incubation temperature of 64 °C (Table 2). Water was added instead of DNA in negative controls. DNA of *A. nomius* CBS 260.86, *A. flavus* CBS 113.32, and *A. caelatus* IBT 29700 was used as positive controls throughout the study, respectively. In order to inspect samples for the presence of a bright green fluorescence, LAMP reaction tubes were placed before a background of black photographic cardboard and illuminated with a 365 nm UV lamp (MinUVIS, 191 Desaga, Heidelberg, Germany). Results were documented using a handheld digital camera (IXUS 95, Canon).

3. Results

3.1. Primer design and specificity testing of the A. caelatus specific LAMP assay

After sequencing the 746 bp PCR product, a primer set for the LAMP-based detection of *A. caelatus* was designed using the PrimerExplorer V.4 software tool available on the Eiken Genome site (http://primerexplorer.jp/e/) provided by Eiken Chemical Co., Ltd. (Tokyo, Japan). The sequences of the designed primers Aca ID56 and loop primers designed manually are given in Table 2. Specificity of the primer sets for the detection of *A. caelatus* was analyzed using genomic DNA isolated from pure cultures of 173 fungal strains as applied in a previous report by Luo et al. (2012). Among the strains, all non-target DNA tested

negative with the Aca ID56 primer set with exception of *A. archidicola* and *A. bombycis*.

3.2. Sensitivity of the LAMP assays for spores from pure cultures

Fig. 1 shows that LAMP reactions run with primer sets Anom ID9 and Afla ID58 had different levels of detection for spores of *A. flavus* and *A. nomius*. When untreated spore suspensions were added directly to the LAMP assays, the detection limits were 10^4 spores for *A. flavus* and 10^5 spores for *A. nomius*, respectively. When spores were disrupted and boiled prior to addition as template in either LAMP assay, detection limits could be considerably decreased to 100 spores per reaction and 10 spores per reaction for *A. flavus* and *A. nomius*, respectively (Fig. 1). The results suggest that although enough DNA seems to leak out of spores during the LAMP reaction, highly sensitive detection can only be achieved by spore disruption prior to LAMP analysis.

3.3. LAMP identification of fungal isolates from Brazil nut seeds and shells

Table 1 shows the results of three LAMP assays with primer sets Afla ID58, Anom ID9 and Aca ID56, respectively, for identification of *Aspergillus* section *Flavi* species isolated from Brazil nut samples. Comparing the results obtained previously by Gonçalves et al. (2012) and Calderari et al. (2013) for the identification of *Aspergillus* section *Flavi* isolates from Brazil nut samples, LAMP assays identified *A. flavus*, *A. nomius* and *A. caelatus* with an accuracy of 85.3%, 92.6%, and 100%, respectively. Using primer set Afla ID58, all of the strains assigned to *A. flavus* were confirmed by a positive LAMP reaction. With the exception of 8 *A. caelatus* and 2 *A. nomius* strains, all strains assigned to other species were tested negative with the *A. flavus* specific primer set. Results revealed a cross reaction of the *A. flavus* specific primer set with *A. caelatus* which was previously undetected by Luo et al. (2012). The LAMP assays run with primer set Aca ID56 showed positive reactions with all isolates assigned to *A. caelatus* as well as with one strain of *A. arachidicola*. Reactions with all other tested species were negative. Analysis of the tester Brazil nut strains using the *A. nomius* specific primer set Anom ID9 revealed a false positive result only with a singular isolate assigned to *A. arachidicola*. However, the LAMP assay failed to identify four of the isolates assigned to *A. nomius* by morphological analysis.

3.4. LAMP-based detection of A. nomius and A. flavus on naturally infected Brazil nuts

The Brazil nut samples available to the study had been analyzed for the presence of *A. flavus* and *A. nomius* as well as for the presence of total aflatoxins in previous studies (Gonçalves et al., 2012; Calderari et al., 2013). The results of the microbiological analysis given in Table 2 revealed that *A. flavus* was present in 24 of the 32 samples with frequencies (number of nuts in a sample that contained the respective fungal species/total number of nuts analyzed per sample) varying between 2% and 62%. *A. nomius* was present in 13 of the samples with frequencies ranging from 2% to 20%. This species always co-occurred with *A. flavus*. Eight of the samples were virtually free from infection by either species. Analysis of surface washings obtained by shaking 10 Brazil nut seeds with water containing 0.1% Tween-20 and using the debris fraction as LAMP template after disruption and boiling showed that 30 of the samples (94%) were contaminated with either of the two fungi. Nineteen of 32 samples (59%) showed a positive result in the Anom ID9 LAMP assay for *A. nomius* whereas 23 of the samples (72%) were positive in the Afla ID58 LAMP assay for *A. flavus*. Twelve of the samples (37%) were positive in both assays and two samples (6%) showed no reaction in either LAMP assay. Eleven of the samples showed the presence of *A. nomius* according the Anom ID9 LAMP assay but were negative for the fungus according to mycological analysis. In turn, the LAMP assay was negative in five other samples, from which *A. nomius* had been detected by mycological analysis. In one of the LAMP negative samples (no. 179) the fungus even had a frequency of 16% according to mycological analysis. The *A. flavus* specific Afla ID58 LAMP assay detected contamination in 7 samples which had no contamination with this fungus according to plating results and in turn were not detected in another 7 samples even though they tested positive according to mycological analysis. Positive and Negative Predictive Values of the LAMP assays for the respective target species were calculated from the comparison of results assuming that the results of mycological analysis as reference method showed the correct contamination state in samples. The Positive Predictive Value was defined as the number of samples positive in LAMP and mycological analysis for the respective species (= true positive) divided by the number of samples positive in the respective LAMP assay. The Negative Predictive Value was defined in analogy. The Afla ID58 LAMP assay had Positive and Negative Predictive Values of 69.6% and 11.1%, respectively. The Anom ID9 LAMP assay had Positive and Negative Predictive Values of 42.1% and 61.5%, respectively.

The results obtained by HPLC analysis of the 32 Brazil nut samples revealed that 23 (72%) contained measurable concentrations of total aflatoxins (B_1, B_2, G_1, G_2) whereas 9 (28%) samples had no detectable aflatoxin contamination. Comparison of results of mycotoxin analysis, mycological analysis, and both LAMP assays showed that there was only one out of 32 samples (no. 222) in which results agreed completely among the analyses with no mycotoxins, fungal contamination and LAMP signal detected. Predictive Values (see above for definition) of the LAMP assays were analyzed in regard to the presence of aflatoxin contamination of samples. The Afla ID58 LAMP assay had Positive and

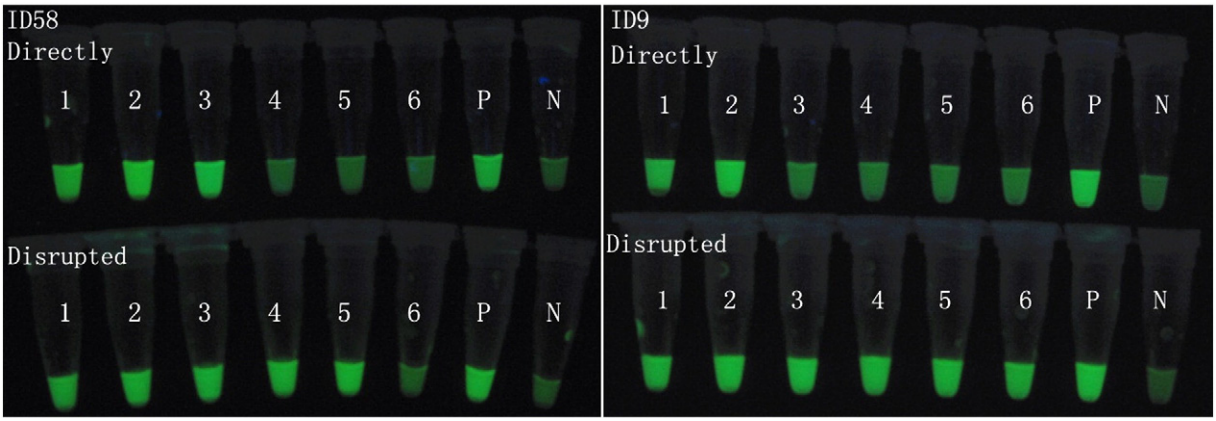

Fig. 1. LAMP reactions with primers sets Afla ID58 (left panel) and Anom ID9 (right panel) using a serial dilution of spores added without previous treatment (upper row) and after previous treatment of spores in a cell disrupter. 1–6: spores of the respective species with concentrations from 1 = 106 to 6 = 10 spores per reaction; P: positive control (pure DNA of *A. flavus* and *A. nomius*, resp.); N: negative control (water added instead of template DNA or sample).

Negative Predictive Values for total aflatoxin contamination of samples being above the LOD of 78% and 44%, respectively. Values for the Anom ID9 LAMP assay were found to be 63% and 15% for Positive and Negative Predictive Values, respectively. Combining the results of both LAMP assays showed the Positive Predictive Value for total aflatoxin contamination in the samples increased to 77%. The Negative Predictive Value could not be improved substantially by combining assay results.

4. Discussion

As with fungi in general, *Aspergillus* taxonomy is complex and ever evolving. Geiser et al. (2007) reviewed the current status of species recognition and identification in *Aspergillus* and suggested that there is no one method (morphological, physiological or molecular) that works flawlessly in recognizing species. Moreover, there may be disadvantages of classical identification methods in the fact that they are highly time consuming and require considerable mycological knowledge to be accurately performed. Molecular biological techniques are time-consuming and are complicated due to cumbersome preparation of high quality DNA and will only result in a reliable identification when there is sufficient data available with which to compare the analyzed sequence. Studies on dissemination, ecology or biodiversity of microorganisms generally result in the isolation of vast amounts of culture specimens and involve further processing in order to generate the information needed.

Pure cultures and samples analyzed during the current study came from such a survey and may therefore be useful as an example for the application of LAMP-based rapid identification and detection methods to support extensive microbiological studies. Recently, Calderari et al. (2013) and Gonçalves et al. (2012) described the results from the analysis of 173 and 115 samples of seeds and shells of Brazil nuts, respectively, which resulted in a total of 2447 fungal isolates, showing the biodiversity of *Aspergillus* section *Flavi* in Brazil nuts from the Amazonian rain forest to the consumer's household. With high frequency of occurrence in Brazil nut, *A. caelatus* is a very common species. A LAMP assay for identification of the species may be helpful to the analysis of the biodiversity of *Aspergillus* section *Flavi* in Brazil nuts. The assay showed cross reactions with *A. archidicola* and *A. bombycis*, either of which species is infrequent in Brazil nuts so that this will not pose any problems with the specificity of the assay. The LAMP assays used during the current study have been applied previously to identify *A. nomius* and *A. flavus* using DNA isolated from pure fungal cultures (Luo et al., 2012). In the same study, authors demonstrated the use of the method for identification of pure cultures of the target fungi without previous DNA purification. However, as a prerequisite the cultures had to be grown on a synthetic agar medium (SNA) in the dark and mycelia had to be harvested before sporulation occurred.

In order to account for the use of fungal spore material from cultures grown on typical laboratory media as a template for identification, a protocol for rapid sample preparation from spores of *Aspergillus* without previous DNA purification was developed during the current study. The results show that direct analysis of pure cultures is possible by adding spores from MEA grown cultures directly to the master mix before the LAMP reaction is started. Although it was demonstrated that the sensitivity of the assay is considerably higher if spores were disrupted and boiled before addition to the master mix, sensitivity of detecting non-treated spores should be still sufficiently high to identify pure cultures since spore numbers are no limitation in *Aspergillus* pure cultures. Moreover, the fact that minimizing time needed for sample preparation was found to be a big advantage of using unprocessed spores rather than processed spores for identification by LAMP. However, with only a little more input in time and handling the sensitivity of spore detection could be increased by several orders of magnitude after processing

Table 3
Detection of the presence of *A. nomius* and *A. flavus* in naturally infected Brazil nuts using microbiological plating and LAMP assays together with detection of aflatoxins.

Sample ID	Origin and state	Result plating (no. of infected nuts in lots of 50)		Result LAMP assay		Aflatoxins present in sample
		A. nomius	*A. flavus*	*A. nomius* Anom ID9	*A. flavus* Afla ID58	
2	Rainforest, Parà	0	12	+	−	−
8		5	21	+	−	+
10	Supermarket, Campinas	1	1	+	−	+
12		0	1	+	−	+
15	Market, Parà	0	0	+	+	−
17		0	0	+	+	+
21	Supermarket, Sao Paulo	0	0	−	+	+
26	Market, Amazon	8	1	+	−	−
32	Market, Parà	1	24	−	−	−
37	Processing, Parà	0	10	+	+	−
41		0	0	+	+	−
51		2	12	−	+	+
53		0	31	−	+	+
80	Market, Amazon	5	1	+	+	+
169	Processing, Parà	0	1	+	+	+
170		0	0	−	+	+
172		5	1	+	−	+
177		0	1	−	+	+
178		3	18	−	+	+
179		8	17	−	+	+
180		1	1	−	+	+
181		0	1	+	+	+
182		0	0	−	+	+
195		0	2	+	−	+
196	Supermarket, Sao Paulo	0	1	+	+	+
197		0	7	−	+	+
198		0	2	+	+	+
199		3	2	+	+	+
202		0	0	−	+	+
222	Market, Amazon	0	0	−	−	−
233		10	18	+	+	−
234		5	11	+	+	−

of samples involving treatment of spores with a mixture of two different sizes of zirconia beads and subsequent boiling. Zhou et al. (2000) identified bead-beating as the most effective way for disruption of spores and subsequent release of fungal DNA. Williams et al. (2001) demonstrated by microscopic examination that airborne fungal spores remained visibly intact even after a 10-min heating step at 95 °C and that disrupting the spores was found to be essential for achieving maximum sensitivity of a PCR-based detection assay for fungal spores. In the current study it was shown that spore disruption had a higher effect on the sensitivity of the *A. nomius* specific LAMP assay than the treatment of *A. flavus* spores. Conidia of *A. nomius* have a coarser surface ornamentation than conidia of *A. flavus* (Feibelman et al., 1998). This difference in surface ornamentation may contribute to enhanced friction between *A. nomius* spores and beads which in turn may lead to increased efficiency of spore disruption in that particular species.

The paper published by Luo et al. (2012) demonstrated both LAMP assays to have high specificity and sensitivity using pure fungal DNA and SNA grown mycelia as the template. During the current study we screened 68 fungal isolates from Brazil nuts for identification by LAMP assays. The rate of accuracy was found to be 78%. Ten of the isolates tested were misidentified by LAMP as *A. flavus* and one as *A. nomius*. The fact that neither of the two LAMP assays had shown any cross reactions previously with pure DNA of the respective other species might indicate that the original identification of the strains was not correct. Moreover, six *A. caelatus* strains which were negative in the *A. flavus* and *A. nomius* LAMP assays showed a positive signal in an *A. caelatus* specific LAMP assay while the other 8 strains gave positive results in both the *A. flavus* and *A. caelatus* specific LAMP assays. Due to the repeated re-isolation of the analyzed cultures, we could rule out the possibility that the tested cultures had been contaminated. Sequencing of the calmodulin gene amplified from genomic DNA of all *A. caelatus* strains given in Table 3 revealed that all isolates represented the species with the exception of strain ITAL 91F which was identified as *A. tamarii*. The results of a BLAST analysis of calmodulin gene sequences deposited in GenBank showed that the *cmd* gene of *A. caelatus* shares a very high degree of homology with that of *A. flavus*. Peterson et al. (2000) stated that *A. caelatus* strains could mistakenly be identified as *A. tamarii* as aflatoxin-producing strains of *A. tamarii* were found to be taxonomically more closely related to *A. caelatus* than to *A. tamarii*. Moreover, Wang et al. (2001) described a strain defined as *A. flavus* which was closely related to *A. tamarii* based on the mitochondrial cytochrome *b* gene, similarly brownish colonies, and similarities of the spore surface profile by SEM. Recently, Varga et al. (2011) described *A. pseudocaelatus* producing aflatoxins B and G as a new species closely related to the non-aflatoxin producing *A. caelatus*. From the facts described, we deduced that the strains of *A. caelatus* which tested positive in both LAMP assays for *A. flavus* and *A. caelatus* may have been misidentified in morphological analysis.

The current paper is the first report analyzing the capability of LAMP for identification and detection of *A. nomius*, *A. flavus*, and *A. caelatus* in naturally infected Brazil nut samples. Disruption and boiling of surface washings were applied during the current study as a pre-treatment for the detection of *A. flavus* and *A. nomius* in samples of naturally infected Brazil nuts. In this study, 32 Brazil nut samples obtained from different regions of Brazil and from different steps in the production process were analyzed microbiologically for fungal contamination, chemically for aflatoxins and by a molecular biological technique for the presence of target fungi using LAMP assays in parallel studies. Comparing the results of the LAMP assays and of the microbiological analysis, the Positive Predictive Value was about 65% for both species. This means that 65% of samples with a contamination by either *A. flavus* or *A. nomius* will be detected by LAMP assays under the assumption that the microbiological analysis showed the real contamination state of Brazil nuts. This high deviation between results of LAMP and microbiological analysis in some of the samples can be explained by the fact that LAMP analysis was performed based on surface contamination but for the microbiological analysis, slices of whole nuts were plated with no differentiation between surface and internal infections. This may explain the fact that the number of samples with an infection by one of the two species was higher after microbiological analysis. Even though Pacheco et al. (2010) found that *A. flavus* occurred with high frequencies and cfu values on the surface of Brazil nuts, also the number of whole nuts used for LAMP analysis may have been too small to discover low levels of contamination which were better detected by plating of samples. However, our experiments showed that washing solutions for DNA release should not be collected from more than 10 nuts because of the high oil content of Brazil nuts (Ryan et al., 2006) which decreased the sensitivity of LAMP assays in our study (data not shown). During the study, LAMP assays of five of the Brazil nut samples showed that they were infected by one or both *Aspergillus* spp. while no aflatoxin was detected in the samples. The high Positive Predictive Value of LAMP assays based on the analysis of aflatoxin indicated that positive samples in LAMP assays had high possibility to be contaminated with aflatoxin, whereas the negative samples may not be free of aflatoxin. It has been established that, depending on the source of isolation, only a portion of *A. flavus* strains (25% (Rodrigues et al., 2006) to 69% (Vaamonde et al., 2003)) are capable of producing aflatoxins. Although 100% of *A. nomius* strains produce aflatoxins, their toxin production is influenced by environmental factors such as temperature, humidity and substrate composition (Northolt et al., 1977). Hence the presence of non-producing strains or adverse physiological conditions during growth could explain positive LAMP results with no aflatoxins found in corresponding samples.

The experiments described in the current study show an interesting potential of LAMP assays to detect and identify potentially aflatoxin producing *A. nomius* and *A. flavus*, as well as non-aflatoxin producing *A. caelatus* in pure cultures and in infected Brazil nuts. LAMP assays could be useful tools to rapidly analyze the presence or absence of typical aflatoxin producers in collection areas in order to study the natural distribution of those fungi and possible sources of contamination in situ. Moreover, assays could be applied to analyze and evaluate the processes involved in the production of Brazil nuts in order to detect those steps which might favor fungal contamination and aflatoxin production. Such information may help to improve the quality and safety of the product. Furthermore, assays can be useful for institutional and governmental programs that have been set up to survey and improve Brazil nut quality thus increasing the competitiveness of a sustainable product with high economic impact on indigenous populations within the Amazonian Basin. As was recently reviewed by Niessen et al. (2013), further developments in assay set up and in the use of novel technologies such as nanotechnology will soon integrate LAMP into handheld systems facilitating its application in rapid and easy to handle on site testing systems which would be ideally suited for testing Brazil nuts and other commodities directly at the point of production.

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References

Andrade, E.H.A., Maia, J.G.S., Streich, R., Marx, F., 1999. Seed composition of Amazonian Lecythidaceae species: part 3 in the series "Studies of Edible Amazonian Plants". J. Food Compos. Anal. 12, 37–51.

Arrus, K., Blank, G., Abramson, D., Clear, R., Holley, R.A., 2005a. Aflatoxin production by *Aspergillus flavus* in Brazil nuts. J. Stored Prod. Res. 41, 513–527.

Arrus, K., Blank, G., Clear, R., Holley, R.A., Abramson, D., 2005b. Microbiological and aflatoxin evaluation of Brazil nut pods and the effects of unit processing operations. J. Food Prot. 68, 1060–1065.

Bayman, P., Baker, J.L., Mahoney, N.E., 2002. *Aspergillus* on tree nuts: incidence and associations. Mycopathologia 155, 161–169.

Calderari, T.O., Iamanaka, B.T., Frisvad, J.C., Pitt, J.I., Sartori, D., Pereira, J.L., Pelegrinelli Fungaro, M.H., Taniwaki, M.H., 2013. The biodiversity of *Aspergillus* section *Flavi* in Brazil nuts: from rainforest to consumer. Int. J. Food Microbiol. 160, 267–272.
Chang, J.C., Gutenmann, W.J., Reid, C.M., Lisk, D.J., 1995. Selenium level of Brazil nuts from two geographic locations in Brazil. Chemosphere 30, 801–802.
Collinson, C., Burnett, D., Agreda, V., 2000. Economic Viability of Brazil Nut Trading in Peru. Natural Resources Institute, University of Greenwich, Kent, UK.
Ellis, W.O., Smith, J.P., Simpson, B.K., 1991. Aflatoxins in food-occurrence, biosynthesis, effects on organisms, detection and methods of control. Crit. Rev. Food Sci. Nutr. 30, 403–439.
European Union, 1998. Commission Regulation 1525/98 of 16 July 1998, amending Regulation (EC) N. 194/97 of January 1997 setting maximum levels for certain contaminants in foodstuff. Off. J. Eur. Communities 93–101.
European Union, 2010. Commission Regulation 165/10 of 26 February 2010, amending Regulation (EC) N. 1881/06 of December 2006 setting maximum levels for certain contaminants as regards aflatoxins. Off. J. Eur. Communities 8–12.
Feibelman, T.P., Cotty, P.J., Doster, M.A., Michailides, T.J., 1998. A morphologically distinct strain of *Aspergillus nomius*. Mycologia 90, 618–623.
Freire, F.D.C.O., Kozakiewicz, Z., Paterson, R.R.M., 2000. Mycoflora and mycotoxins in Brazilian black pepper, white pepper and Brazil nuts. Mycopathologia 149, 13–19.
Geiser, D.M., Klich, M.A., Frisvad, J.C., Peterson, S.W., Varga, J., Samson, R.A., 2007. The current status of species recognition and identification in *Aspergillus*. Stud. Mycol. 59, 1–10.
Gonçalves, J.S., Ferracin, L.M., Carneiro Viera, M.L., Iamanaka, B.T., Taniwaki, M.H., Pelegrinelli Fungaro, M.H., 2012. Molecular analysis of *Aspergillus* section *Flavi* isolated from Brazil nuts. World J. Microbiol. Biotechnol. 28, 1817–1825.
Gunneroed, P.K., 1994. Tropical nuts: strong demand in the United Kingdom. International Trade Forum. International Trade Forum 2/1994, pp. 24–27.
Haugland, R.A., Brinkman, N., Vesper, S.J., 2002. Evaluation of rapid DNA extraction methods for the quantitative detection of fungi using real-time PCR analysis. J. Microbiol. Methods 50, 319–323.
International Agency for Research on Cancer—IARC, 1997. Evaluation of Carcinogenic Risks to Humans: Some Naturally Occurring Substances: Aromatic Amines and Mycotoxins. IARC Monographs, Lyon 245–395.
Johnsson, P., Lindblad, M., Thim, A.M., Jonsson, N., Vargas, E.A., Medeiros, N.L., Brabet, C., Quaresma de Araújo, M., Olsen, M., 2008. Growth of aflatoxigenic moulds and aflatoxin formation in Brazil nuts. World Mycotoxin J. 1, 127–137.
Kitamura, P.C., Müller, C.H., 1984. Castanhais nativas de Marabá-PA: Fatores de depredação e bases para a sua preservação. EMBRAPA-CPATU (Centro de Pesquisa Agropecuária do Trópico Umido). Documentos 30, 1–32.
Lopez Castrillon, A.L., Purchio, A., 1988. Fungos contaminantes e produtores de aflatoxinas em castanhas do Pará (*Bertholletia excelsa* Humb. & Bonpl. 1808). Acta Amazon. 18, 173–183.
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.
Mori, S.A., 1992. The Brazil nut industry—past, present, and future. In: Plotkin, M., Famolare, L. (Eds.), Sustainable Harvest and Marketing of Rain Forest Products. Island Press, Washington D.C., USA, pp. 241–251.
Mori, S.A., Prance, G.T., 1990. Taxonomy, ecology, and economic botany of the Brazil nut (*Bertholletia excelsa* Humb. and Bonpl.:Lecythidaceae). Adv. Econ. Bot. 8, 130–150.
Mori, Y., Nagamine, K., Tomita, N., Notomi, T., 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Biophys. Res. Commun. 289, 150–154.
Mori, Y., Hirano, T., Notomi, T., 2006. Sequence specific visual detection of LAMP reactions by addition of cationic polymers. BMC Biotechnol. 6, 3. http://dx.doi.org/10.1186/1472-6750-6-3.
Niessen, L., Vogel, R.F., 2010. Detection of *Fusarium graminearum* DNA using a loop-mediated isothermal amplification (LAMP) assay. Int. J. Food Microbiol. 140, 183–191.
Niessen, L., Luo, J., Denschlag, C., Vogel, R.F., 2013. The application of loop-mediated isothermal amplification (LAMP) in food testing for bacterial pathogens and fungal contaminants. Food Microbiol. 36, 191–206.
Njiru, Z.K., Mikosza, A.S.J., Matovu, E., Enyaru, J.C.K., Ouma, J.O., Kibona, S.N., Thompson, R.C.A., Ndung'u, J.M., 2008. African trypanosomiasis: sensitive and rapid detection of the sub-genus *Trypanozoon* by loop-mediated isothermal amplification (LAMP) of parasite DNA. Int. J. Parasitol. 38, 589–599.
Northolt, M.D., van Egmond, H.P., Paulsch, W.E., 1977. Differences between *Aspergillus flavus* strains in growth and aflatoxin B1 production in relation to water activity and temperature. J. Food Prot. 40, 778–781.
Notermans, S., Heuvelman, C.J., van Egmond, H.P., Paulsch, W.E., Besling, J.R., 1986. Detection of mold in food by enzyme-linked immunosorbent assay. J. Food Prot. 49, 786–791.
Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, e63. http://dx.doi.org/10.1093/nar/28.12.e63.
Olsen, M., Johnsson, P., Moeller, T., Paladino, R., Lindblad, M., 2008. *Aspergillus nomius*, an important aflatoxin producer in Brazil nuts? World Mycotoxin J. 1, 123–126.
Pacheco, A.M., Scussel, V.M., 2007. Selenium and aflatoxin levels in raw Brazil nuts from the Amazon basin. J. Agric. Food Chem. 55, 11087–11092.
Pacheco, A.M., Lucas, A., Parente, R., Pacheco, N., 2010. Association between aflatoxin and aflatoxigenic fungi in Brazil nut (*Bertholletia excelsa* H.B.K.). Cienc. Tecnol. Aliment. 30, 330–334.
Peterson, S.W., Horn, B.W., Ito, Y., Goto, T., 2000. Genetic variation and aflatoxin production in *Aspergillus tamarii* and *A. caelatus*. Classification of *Penicillium* and *Aspergillus*: integration of modern taxonomic methods. 447–458.
Pitt, J.I., Hocking, A.D., 2009. Methods for isolation, enumeration, and identification. In: Pitt, J.I., Hocking, A.D. (Eds.), Fungi and Food Spoilage. Springer, New York, USA, pp. 19–40.
Rodrigues, P., Venâncio, A., Kozakiewicz, Z., Lima, N., 2006. A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* Section *Flavi* isolated from Portuguese almonds. Int. J. Food Microbiol. 129, 187–193.
Ryan, E., Galvin, K., O'Connor, T.P., Maguire, A.R., O'Brien, N.M., 2006. Fatty acid profile, tocopherol, squalene and phytosterol content of Brazil, pecan, pine, pistachio and cashew nuts. Int. J. Food Sci. Nutr. 57, 219–228.
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.
Tomita, N., Mori, Y., Kanda, H., Notomi, T., 2008. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat. Protoc. 3, 877–882.
Vaamonde, G., Patriarca, A., Fernández Pinto, V., Comerio, R., Degrossi, C., 2003. Variability of aflatoxin and cyclopiazonic acid production by *Aspergillus* section *Flavi* from different substrates in Argentina. Int. J. Food Microbiol. 88, 79–84.
Varga, J., Frisvad, J.C., Samson, R.A., 2011. Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. Stud. Mycol. 69 (1), 57–80.
Wang, L., Yokoyama, K., Takahasi, H., Kase, N., Hanya, Y., Yashiro, K., Miyaji, M., Nishimura, K., 2001. Identification of species in *Aspergillus* section *Flavi* based on sequencing of the mitochondrial cytochrome *b* gene. Int. J. Food Microbiol. 71 (1), 75–86.
Williams, R.H., Ward, E., McCartney, H.A., 2001. Methods for integrated air sampling and DNA analysis for detection of airborne fungal spores. Appl. Environ. Microbiol. 67, 2453–2459.
Zhou, G., Whong, W.Z., Ong, T., Chen, B., 2000. Development of a fungus-specific PCR assay for detecting low-level fungi in an indoor environment. Mol. Cell. Probes 14, 339–348.