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SHORT COMMUNICATION

Molecular analysis of *Aspergillus* section *Flavi* isolated from Brazil nuts

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Abstract Brazil nuts are an important export market in its main producing countries, including Brazil, Bolivia, and Peru. Approximately 30,000 tons of Brazil nuts are harvested each year. However, substantial nut contamination by *Aspergillus* section *Flavi* occurs with subsequent production of aflatoxins. In our study, *Aspergillus* section *Flavi* were isolated from Brazil nuts (*Bertholletia excelsa*), and identified by morphological and molecular means. We obtained 241 isolates from nut samples, 41% positive for aflatoxin production. Eighty-one isolates were selected for molecular investigation. Pairwise genetic distances among isolates and phylogenetic relationships were assessed. The following *Aspergillus* species were identified: *A. flavus*, *A. caelatus*, *A. nomius*, *A. tamarii, A. bombycis*, and *A. arachidicola*. Additionally, molecular profiles indicated a high level of nucleotide variation within β-tubulin and calmodulin gene sequences associated with high genetic divergence from RAPD data. Among the 81 isolates analyzed by molecular means, three of them were phylogenetically distinct from all other isolates representing the six species of section *Flavi*. A putative novel species was identified based on molecular profiles.

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Introduction

The Brazil nut (*Bertholletia excelsa*) is a monotypic South American tree species of the Amazon Rain forests, native to Brazil, Peru, Bolivia, Guianas, Venezuela, and Columbia. It occurs as scattered trees in large forests along the banks of large river systems. Tree exploitation is a source of socio-economic and environmental impacts for the region. Activity generates employment and income for local people; at the same time, it inhibits the deforestation (see review Freitas-Silva and Venâncio 2011).

The Brazil nut is a tall tree, reaching heights of up to 50 m, one of the largest of the Amazon forest. The lower limits documented for the species are 30–45 m. The fruit is a spherical capsule of ligneous mesocarp, and is extremely hard. Seeds are found in average numbers of 18, with a ligneous and rough shell. The nut (i.e., seed) is recognized by its high nutritional value. The fruits are collected after falling, and therefore are in contact with the ground where nuts are exposed to fungal infection (Souza et al. 2004).

Among the main problems identified in Brazil nut production is contamination by fungi that produce aflatoxins, primarily by species of *Aspergillus* section *Flavi*. Aflatoxins are toxic secondary metabolites produced by some fungal species when humidity and temperature are favorable. The occurrence of aflatoxins in Brazil nuts is a critical constraint for its commercialization, which has been cited in several reports (Pohland 1993; FSA 2004; Marklinder et al. 2005; Arrus et al. 2005; CAC 2005; Pacheco and Scussel 2007; Olsen et al. 2008; Pacheco and Scussel 2009; Freitas-Silva and Venâncio 2011).

Producing countries (Bolivia, Peru and Brazil) have implemented restrictions to avoid aflatoxin contamination in an attempt to meet international sanitary standards and strengthen export markets. For example, "Safenut", a project coordinated by the European Union served to validate an aflatoxin level analysis method that is reliable and easy for industrialists to use for reducing and controlling nut aflatoxin contamination. The project completion in 2008 resulted in several recommendations; among them, the necessity for studies on the microbiota to better understand the infection process, and subsequent aflatoxin contamination (STDF 2008).

Aspergillus section *Flavi* taxonomy is complex and it is continually evolving. Usually, fungi of this section are subdivided into two classes based on their effects to food and human health (see Godet and Munaut 2010) i.e., aflatoxin producing and non-producing species. Aflatoxin producing species include *A. flavus*, *A. parasiticus* (Codner et al. 1963), *A. nomius* (Kurtzman et al. 1987), *A. pseudotamarii* (Ito et al. 2001), *A. bombycis (*Peterson et al. 2001), *A. parvisclerotigenus* (Saito and Tsurota 1993) *A. arachidicola*, and *A. minisclerotigenes* (Pildain et al. 2008). The first three species are the most damaging to foods such as rice, wheat, nuts, peanuts, and peppers (Geiser et al. 1998). The non-aflatoxin producing species are *A. sojae, A. oryzae, A. tamarii*, *A. caelatus*. *A. oryzae* and *A. sojae* are economically important species that are used in the preparation of fermented food products. At the molecular level, *A. oryzae* and *A. sojae* are closely related to *A. flavus* and *A. parasiticus*, respectively, but are maintained as separate species to avoid regulatory problems in the food industry (Geiser et al. 1998). Furthermore, recent studies have demonstrated that many previously described species in *Aspergillus* section *Flavi* are not supported by currently available data. Varga et al. (2011) used sequence data from three loci to clarify the taxonomy of this section. The phylogenetic analysis based on calmodulin and ITS sequence data encourage authors to included seven main clades with 20 or more taxa in the *Aspergillus* section *Flavi*. The main clades isolates form well-defined subclades on the trees, however, mostly are represented by a single isolate and further collections and studies are needed to clarify if they represent separated species.

Amongst the different types of aflatoxins (AF), the most important are aflatoxin B1, B2, G1, and G2. All these important aflatoxins have been detected in Brazil nuts (Olsen et al. 2008). *A. flavus* produces only AFB1 and AFB2, while the other aflatoxigenic species additionally produce AFG1 and AFG2. As reviewed by Freitas-Silva and Venancio (2011) there is a controversy concerning which species contribute greatest to aflatoxin production in Brazil nut. *Aspergillus flavus* has been described as dominant in Brazil nuts (Freire et al. 2000, Arrus et al. 2005), however this species are not able to produce AFG1 and AFG2. According to Olsen et al. (2008), *Aspergillus flavus* is not the main aflatoxin producer in contaminated Brazil nuts. Authors obtained evidences that *A. nomius* is an important producer, and recommended further studies to inspect for the presence of other B and G aflatoxin producers. The supposition that supports this controversy is the imprecise recognition of the fungal species (Freitas-Silva and Venancio 2011).

Since the identification of aflatoxin producer species may influence strategies for prevention and control of aflatoxins in Brazil nuts, the objectives of this study were to isolate and identify, by morphological and molecular data, fungal isolates from this important product extracted from the Amazonian region.

Materials and methods

Fungi isolation and identification by morphological analysis

A total of 20 samples from approximately 2 kg of Brazil nuts were collected in the Brazilian Amazon region. From each sample, approximately 100 g of shelled nuts (i.e., seeds) and 100 g of shells were disinfected separately by immersion in 0.4% sodium hypochlorite solution for 1 min. Fifty pieces of nuts or shells were plated separately onto Dichloran Glycerol (DG-18) agar containing chloramphenicol. Samples were incubated for 5 days at 25 °C. All *Aspergillus* section *Flavi* were isolated on Czapek yeast extract (CYA) agar, and incubated at 25 °C, 37 °C or 42 °C for 7 days. Identification was carried out according to Klich and Pitt (1988), and Pitt and Hocking (2009), based on macroscopic characteristics, such as diameter size, colony colour and texture, colour of colony reverse, presence of exudates and soluble pigment as well as microscopic characteristics, such as shape and size of vesicles and conidia, thickness of stipes and presence of phialides and metulas. For this evaluation, 40× and 100× microscopic magnifications were used.

Aflatoxin production

Isolates were evaluated for aflatoxin production following the protocol of Filtenborg et al. (1983). Briefly, the isolates were inoculated onto yeast extract agar containing 15% sucrose, and incubated at 25 °C for 7 days. A small piece of each colony was cut, and used for toxin extraction with chloroform:methanol (1:1). Plugs were placed on thin layer chromatography (TLC) plates (silica gel-G 500 mm thick), which were developed in toluene: ethyl acetate: 90% formic acid: chloroform (7:5:2:5), and visualized under UV

light at 356 and 254 nm. Aflatoxin B_1, B_2, G_1, and G_2 standards (Sigma Chemical, St Louis, USA) were used for qualitative comparison of retention time and fluorescence.

DNA extraction

Isolates were cultivated in yeast extract and lactose (YEL) solid medium for 7 days. From this culture, we performed a suspension of approximately 10^7 conidia in 2.5 mL of Tween 80. The suspension was inoculated into bottles containing 50 mL of YEL liquid, and incubated in a shaker (180 rpm) at 28 °C for 16 to 24 h. After incubation, mycelia were collected by vacuum filtration and washed in sterile water. Nucleic acids were extracted according to Azevedo et al. (2000), and treated with ribonuclease A.

RAPD analysis

DNA amplification was performed using the arbitrary primers OPX 3, OPX 7, and OPX 11 (Invitrogen ®) in a PTC-100 thermocycler (MJ Research, Inc.) according to Fungaro et al. (1996). Isolate genotyping was conducted based on analysis of polymorphic loci generated by RAPD markers. A similarity matrix using Jaccard's coefficient was constructed. Cluster analysis was performed using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) with the SAHN module (Sequential, Agglomerative, Hierarchic, Nonoverlapping Clustering Methods) in NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System) (Rohlf 2000). An Analysis of Molecular Variance (AMOVA) was conducted using the software Arlequin 3.0 (Excoffier et al. 2005) to examine divergence levels between groups evidenced by RAPD markers.

Partial amplification of the β-tubulin and calmodulin coding genes

Amplification of a β-tubulin gene region was performed using the following primer pair: Bt2a (5′ GGT AAC CAA ATC GGT GCT GCT TTC 3′) and Bt2b (5′ ACC CTC AGT GTA GTG ACC CTT GGC 3′), as described by Glass and Donaldson (1995). Similarly, a calmodulin gene region was amplified using the cmd5 (5′ CCG AGT ACA AGG AGG CCT TC 3′) and cmd6 (5′ CCG ATA GAG GTC ATA ACG TGG 3′) primers previously reported by Hong et al. (2006). Standard amplification reactions and cycling protocols were adopted, and amplicons submitted to direct sequencing in both directions (forward and reverse) in a MegaBaceTM 1,000 Molecular Dynamics system (Amersham, Pharmacia Biotech). The quality of the sequences was examined using the software package Phred/Phrap/Consed. The sequences obtained were aligned using *Aspergillus* section *Flavi* type species sequences deposited in the NCBI database (http://www.ncbi.nlm.nih.gov/) and MycoBank (http://www.mycobank.org). Editing and sequence alignment were performed using ClustalW (Thompson et al. 1994). The computer program package MEGA (Tamura et al. 2007) was used for building a phylogenetic tree based on the neighbor-joining method.

Results and discussion

Results from 241 isolates in *Aspergillus* section *Flavi* indicated 99 (41%) positive for aflatoxin production, with respective 72 (29.8%) and 27 (11.2%) B and G group aflatoxin production. Based on the differences found by means of macro- and microscopic characteristics, the 241 isolates were divided in 11 groups. Representatives of each morphological group (40 from nuts and 41 from shells) were selected to identify or confirm isolate taxonomy by means of molecular methods (Table 1). Figure 1 shows RAPD profiles for 41 isolates generated from nuts, and 40 from shells. One hundred and two RAPD loci were detected from nut isolates, which resulted in a dendrogram constructed with high confidence values, and isolates clustered into six groups. Among the groups, three (Groups II, IV, V) were aflatoxin non-producer isolates, two groups (Groups III, VI) were aflatoxin producers, and the major group (Group I) contained both types of isolates (aflatoxin non-producers and producers). Shell isolates generated 143 RAPD loci clustered into seven groups. Similarly, three groups (Groups II, IV, V) were comprised of aflatoxin non-producer isolates, three (Groups III, VI, VII) were aflatoxin producers, and the other group (Group I) had both types of isolates (aflatoxin non-producer and producer).

AMOVA performed with RAPD marker data obtained from nut samples indicated the main source of variation was between ($F_{ST} = 0.81$), rather than within groups ($F_{ST} = 0$ 19). The pairwise F_{ST} values ranged from 0.77 to 1.0. The largest difference was detected between Group V (ITAL 116 isolate) and Group VI (ITAL 89) (Table 2a). In addition, the main source of variation based on shell sample isolates was between groups ($F_{ST} = 0.83$). Similarly, the pairwise F_{ST} values ranged from 0.79 to 1.0. The largest divergence was detected between Group VI (ITAL 245 isolate) and Group VII (ITAL 189), followed by Group V (ITAL 259 and ITAL 262) and Group VI (ITAL 246) (Table 2b).

The high genetic distance values (not shown) and F_{ST} estimates suggest our isolate collection contains different species of *Aspergillus* section *Flavi*.

As the main source of genetic variation was between, rather than within RAPD groups, representatives from each group underwent nucleotide sequence analysis of β-tubulin and calmodulin coding genes. Compared to public databases using the BlastN algorithm, for most β-tubulin and calmodulin

Table 1 *Aspergillus* section *Flavi* isolates obtained from nuts and shells of Brazil nuts collected from different States of the Amazonian region

Code	RAPD group	Molecular identification	Aflatoxin	Substrate	State of origin[a]
ITAL 20	I	*A. flavus*	Np	Nut	Pará
ITAL 24	I	*A. flavus*	Np	Nut	Pará
ITAL 26	I	*A. flavus*	AFB_1/B_2	Shell	Pará
ITAL 31	I	*A. flavus*	AFB_1/B_2	Shell	Pará
ITAL 35	I	*A. flavus*	AFB_1/B_2	Shell	Pará
ITAL 40	I	*A. flavus*	AFB_1/B_2	Shell	Pará
ITAL 48	I	*A. flavus*	AFB_1/B_2	Nut	Pará
ITAL 49	I	*A. flavus*	AFB_1/B_2	Nut	Pará
ITAL 57	I	*A. flavus*	AFB_1	Nut	Amazonas (Humaitá)
ITAL 58	I	*A. flavus*	AFB_1	Nut	Amazonas (Humaitá)
ITAL 60	I	*A. flavus*	AFB_1	Nut	Amazonas (Humaitá)
ITAL 62	I	*A. flavus*	AFB_1	Nut	Amazonas (Humaitá)
ITAL 67	I	*A. flavus*	AFB_1/B_2	Nut	Amazonas (Coari)
ITAL 70	I	*A. flavus*	AFB_1	Nut	Amazonas (Coari)
ITAL 72	I	*A. flavus*	AFB_1/B_2	Nut	Amazonas (Coari)
ITAL 74	I	*A. flavus*	AFB_1	Nut	Amazonas (Coari)
ITAL 113	I	*A. flavus*	AFB_1	Nut	Amazonas
ITAL 205	I	*A. flavus*	AFB_1	Shell	Amazonas (Coari)
ITAL 254	I	*A. flavus*	AFB_1	Shell	Amazonas
ITAL 261	I	*A. flavus*	Np	Shell	Amazonas
ITAL 263	I	*A. flavus*	Np	Shell	Amazonas
ITAL 336	I	*A. flavus*	Np	Nut	Pará (Belém)
ITAL 371	I	*A. flavus*	AFB_1/B_2	Shell	Pará (Belém)
ITAL 375	I	*A. flavus*	Np	Shell	Pará (Belém)
ITAL 50	II	*A. caelatus*	Np	Nut	Pará
ITAL 79	II	*A. caelatus*	Np	Nut	Amazonas (Coari)
ITAL 87	II	*A. caelatus*	Np	Nut	Amazonas (Jarí)
ITAL 91	II	*A. caelatus*	Np	Nut	Amazonas (Jarí)
ITAL 95	II	*A. caelatus*	Np	Nut	Amazonas (Jarí)
ITAL 96	II	*A. caelatus*	Np	Nut	Amazonas (Jarí)
ITAL 121	II	*A. caelatus*	Np	Nut	Amazonas
ITAL 140	II	*A. caelatus*	Np	Nut	Amazonas
ITAL 201	II	*A. caelatus*	Np	Shell	Amazonas (Coari)
ITAL 212	II	*A. caelatus*	Np	Shell	Amazonas (Coari)
ITAL 216	II	*A. caelatus*	Np	Shell	Amazonas (Coari)
ITAL 225	II	*A. caelatus*	Np	Shell	Amazonas (Coari)
ITAL 243	II	*A. caelatus*	Np	Shell	Amazonas (Jarí)
ITAL 301	II	*A. caelatus*	Np	Shell	Pará
ITAL 330	II	*A. caelatus*	Np	Nut	Pará (Belém)
ITAL 94	III	*A. nomius*	AFB_1/G_1	Nut	Amazonas (Jarí)
ITAL 98	III	*A. nomius*	AFB_1/G_1	Nut	Amazonas (Jarí)
ITAL 114	III	*A. nomius*	AFB_1/G_1	Nut	Amazonas
ITAL 120	III	*A. nomius*	AFB_1/G_1	Nut	Amazonas
ITAL 122	III	*A. nomius*	AFB_1/G_1	Nut	Amazonas
ITAL 139	III	*A. nomius*	AFB_1/G_1	Nut	Amazonas
ITAL 144	III	*A. nomius*	AFB_1/G_1	Nut	Amazonas
ITAL 148	III	*A. nomius*	AFB_1/G_1	Nut	Amazonas
ITAL 192	III	*A. nomius*	AFB_1/G_1	Shell	Amazonas (Humaitá)

Table 1 continued

Code	RAPD group	Molecular identification	Aflatoxin	Substrate	State of origin[a]
ITAL 193	III	*A. nomius*	AFB_1/G_1	Shell	Amazonas (Humaitá)
ITAL 206	III	*A. nomius*	AFB_1/G_1	Shell	Amazonas (Coari)
ITAL 213	III	*A. nomius*	AFB_1/G_1	Shell	Amazonas (Coari)
ITAL 215	III	*A. nomius*	AFB_1/G_1	Shell	Amazonas (Coari)
ITAL 217	III	*A. nomius*	AFB_1/G_1	Shell	Amazonas (Coari)
ITAL 223	III	*A. nomius*	AFB_1/G_1	Shell	Amazonas (Coari)
ITAL 244	III	*A. nomius*	AFB_1/G_1	Shell	Amazonas (Jarí)
ITAL 255	III	*A. nomius*	AFB_1/G_1	Shell	Amazonas
ITAL 256	III	*A. nomius*	AFB_1/G_1	Shell	Amazonas
ITAL 333	III	*A. nomius*	AFB_1/G_1	Nut	Pará (Belém)
ITAL 76	IV	*A. tamarii*	Np	Nut	Amazonas (Coari)
ITAL 80	IV	*A. tamarii*	Np	Nut	Amazonas (Coari)
ITAL 81	IV	*A. tamarii*	Np	Nut	Amazonas (Coari)
ITAL 119	IV	*A. tamarii*	Np	Nut	Amazonas
ITAL 123	IV	*A. tamarii*	Np	Nut	Amazonas
ITAL 129	IV	*A. tamarii*	Np	Nut	Amazonas
ITAL 142	IV	*A. tamarii*	Np	Nut	Amazonas
ITAL 198	IV	*A. tamarii*	Np	Shell	Amazonas (Humaitá)
ITAL 207	IV	*A. tamarii*	Np	Shell	Amazonas (Coari)
ITAL 219	IV	*A. tamarii*	Np	Shell	Amazonas (Coari)
ITAL 226	IV	*A. tamarii*	Np	Shell	Amazonas (Coari)
ITAL 227	IV	*A. tamarii*	Np	Shell	Amazonas (Coari)
ITAL 248	IV	*A. tamarii*	Np	Shell	Amazonas
ITAL 249	IV	*A. tamarii*	Np	Shell	Amazonas
ITAL 250	IV	*A. tamarii*	Np	Shell	Amazonas
ITAL 251	IV	*A. tamarii*	Np	Shell	Amazonas
ITAL 252	IV	*A. tamarii*	Np	Shell	Amazonas
ITAL 116	V	*Aspergillus* sp	Np	Nut	Amazonas
ITAL 259	V	*Aspergillus* sp	Np	Shell	Amazonas
ITAL 262	V	*Aspergillus* sp	Np	Shell	Amazonas
ITAL 89	VI	*A. bombycis*	AFB_1/G_1	Nut	Amazonas (Jarí)
ITAL 246	VI	*A. bombycis*	AFB_1/G_1	Shell	Amazonas (Jarí)
ITAL 189	VII	*A. arachidicola*	AFB_1	Shell	Amazonas (Humaitá)

[a] Localities of the corresponding states are indicated in parentheses

Np Non-producer of aflatoxins

sequences we observed very low E-values and higher than 99% identity percentages with *Aspergillus* species described in the literature. However, for ITAL 116 (Group V collected from nuts), ITAL 262 and ITAL 259 (Group V collected from shells) isolates, the β-tubulin nucleotide sequences showed 89% maximum identity with those deposited in databases (*A. tamarii* and *A. caelatus*). Similarly, for the calmodulin gene, sequence analysis generated a maximum identity of 91% with *A. caelatus* sequences. The phylogenetic trees reconstructed from the comparisons of nucleotide sequences of our isolates and those of each type species of *Aspergillus* section *Flavi* are shown in Figs. 2 and 3.

Although RAPD data provided a random sample of the genetic variation dispersed throughout the genome, and gene sequence data offered information from genes recognized as suitable for distinguishing species in the *Aspergillus* section *Flavi*, the results here obtained from these two approaches were exactly in agreement. Isolates collected even from nuts or shells were clustered into approximately the same number of groups (6 or 7), independently if based on RAPD profiles or sequences data.

Among the 41 nut isolates, we identified the following species: *A. flavus*, *A. caelatus*, *A. nomius, A. tamarii*, and *A. bombycis*, corresponding to RAPD groups I, II, III, IV, and VI. Among the 40 shell isolates, we identified *A. flavus*, *A. caelatus*, *A. nomius*, *A. tamarii*, *A. bombycis* and *A. arachidicola*, corresponding to RAPD groups I, II, III, IV, VI, and VII (Table 1).

According to the literature, *A. nomius* and *A. flavus* were the only two aflatoxigenic species isolated from Brazil

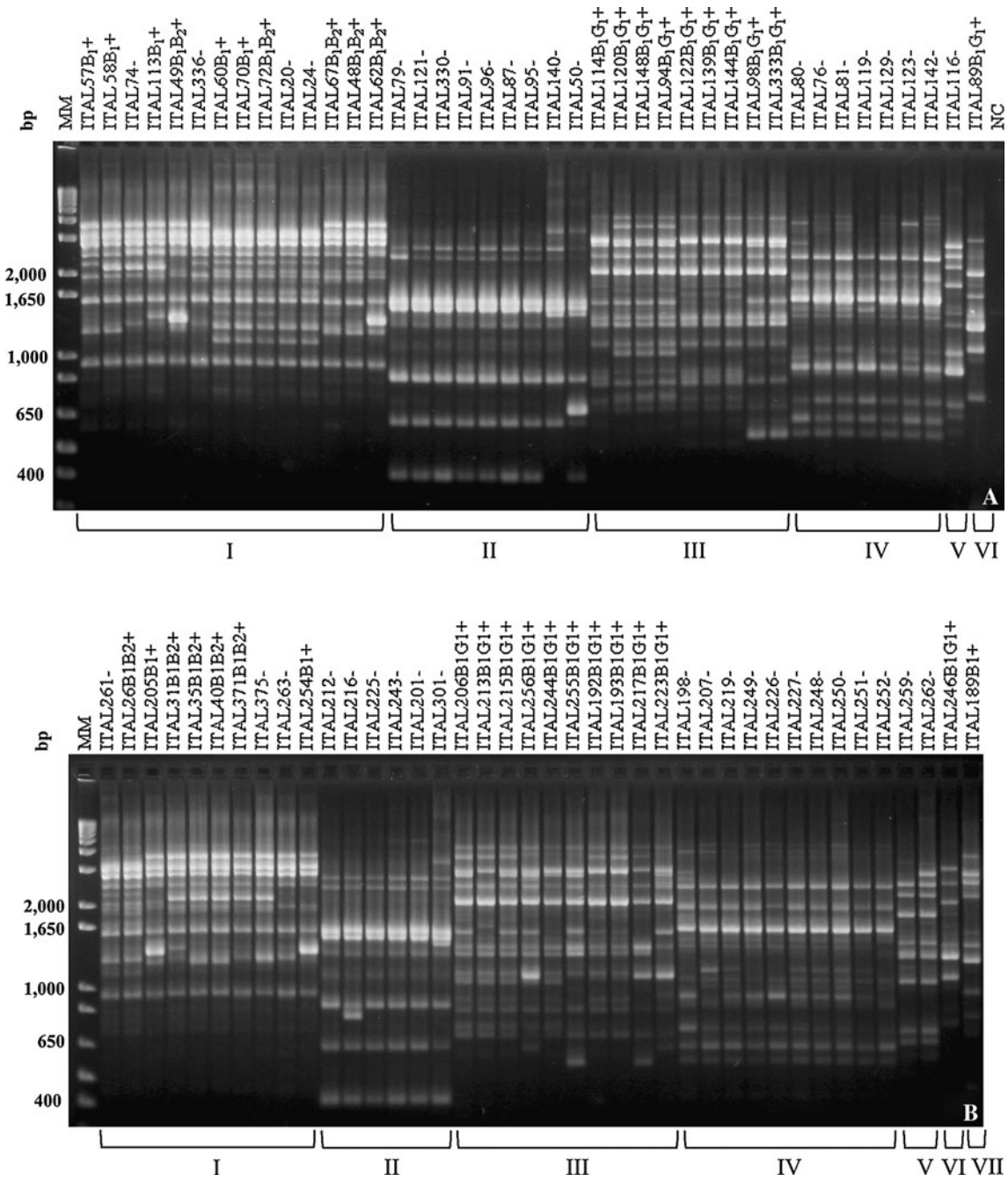

Fig. 1 RAPD profiles from *Aspergillus* section *Flavi* isolates collected from Brazil nuts. **a** and **b** Isolates obtained from nuts and shells, respectively. *MM* molecular weight marker 1 kb plus; *NC* negative control. Codes followed by + and − correspond to aflatoxin producers and non-producers

nuts. These species have been described as occurring in shell and shelled Brazil nuts. (De Mello and Scussel 2007, Olsen et al. 2008). In the present study, we identified in addition to *A. nomius* and *A. flavus*, two other aflatoxigenic species: *A. bombycis* and *A. arachidicola*. One of them, *A. bombycis* was found in both nuts and shells; however, *A. arachidicola* was found only in the shells. *A. arachidicola* was described in Agentinean peanuts and can produce both B and G-type aflatoxins (Pildan et al. 2008). *A. bombycis* was discovered by Peterson et al. (2001) during fungi isolation from insect frass collected in silkworm rearing houses in Japan, and they can also produce B and G-type aflatoxins. Among the four aflatoxin producing species identified in this study, *A. nomius* causes the greatest concern because of its frequency in Brazil nuts (23%), and all isolates produced aflatoxins B_1 and G_1.

Table 2 Pairwise F_{ST} values based on RAPD data among *Aspergillus* section *Flavi* isolate groups collected from Brazil nuts

Nut Isolates					
	I	II	III	IV	V
I					
II	0.78				
III	0.80	0.82			
IV	0.81	0.81	0.81		
V	0.80	0.85	0.75	0.85	
VI	0.80	0.84	0.77	0.87	1.00

Shell Isolates						
	I	II	III	IV	V	VI
I						
II	0.83					
III	0.81	0.82				
IV	0.86	0.85	0.81			
V	0.86	0.84	0.81	0.86		
VI	0.86	0.86	0.79	0.86	0.87	
VII	0.84	0.85	0.79	0.84	0.88	1.00

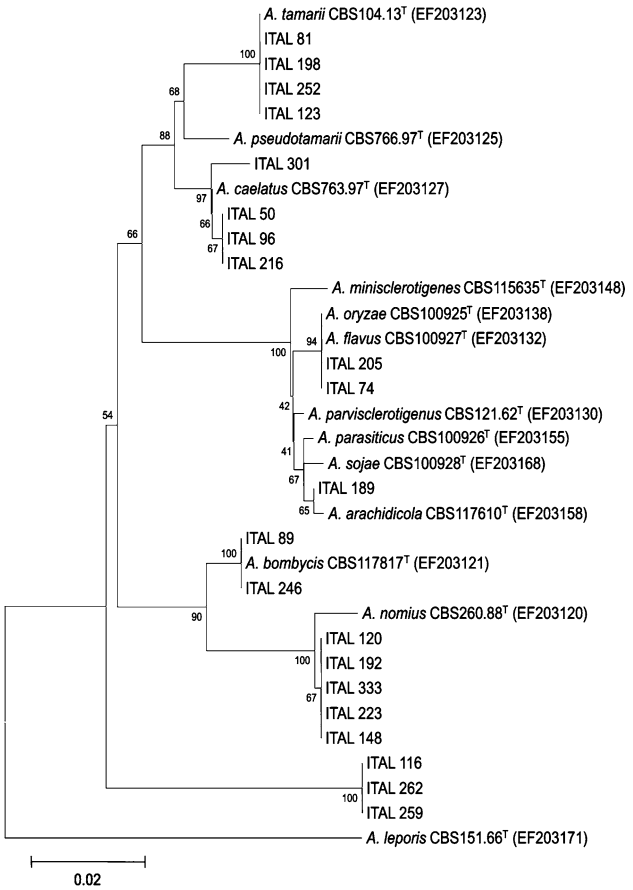

Fig. 2 Phylogenetic tree reconstructed from the β-tubulin gene (Bt2) sequences aligned with corresponding sequences of *Aspergillus* section *Flavi* type species deposited in public databases

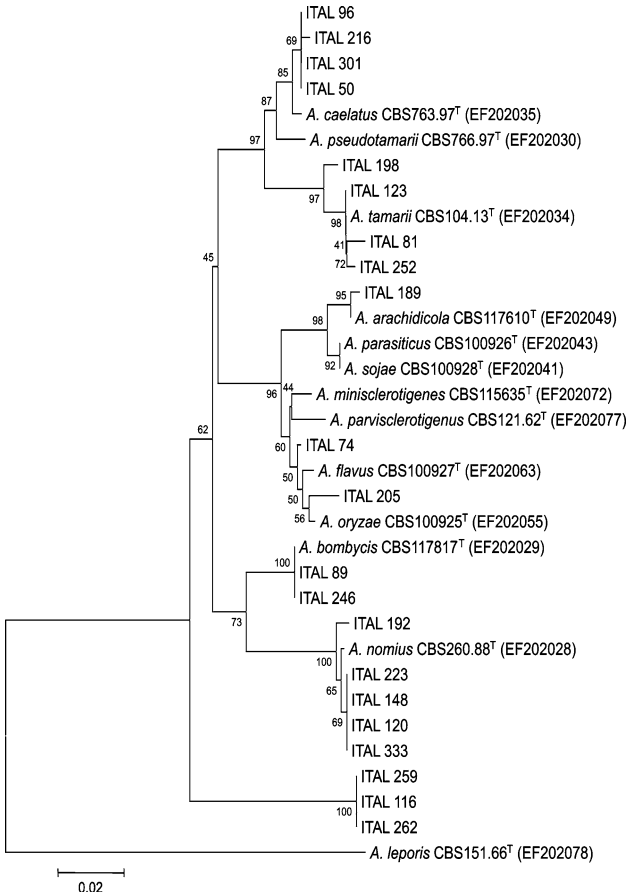

Fig. 3 Phylogenetic tree reconstructed from the calmodulin gene sequences aligned with corresponding sequences *Aspergillus* section *Flavi* type species deposited in public databases

The isolate denoted ITAL 116 collected from nuts (corresponding to Group V RAPD data), ITAL 262 and ITAL 259 collected from shells (corresponding to Group V RAPD data) formed a group phylogenetically distant from other *Aspergillus* species (Figs. 2, 3). Interestingly, a high number of nucleotide polymorphisms were detected on the β-tubulin gene Bt2 region of the three isolates relative to the type species presumed phylogenetically closest to the isolates i.e., *A. tamarii* and *A. caelatus*. A comparison with *A. tamarii* revealed 50 substitutions and three insertions, and 51 substitutions and two insertions were detected in *A. caelatus* (Fig. 4a). Sequence analysis of the calmodulin gene revealed 48 substitutions and one deletion compared to *A. tamarii*. Relative to *A. caelatus*, 38 substitutions were detected (Fig. 4b). Single nucleotide polymorphisms (SNP) were not found in β-tubulin and calmodulin genes (Fig. 4) of the ITAL 116, ITAL 262, and ITAL 259 isolates.

The high level of nucleotide variation within the β-tubulin and calmodulin sequences in the ITAL 116, ITAL 262, and ITAL 259 isolates compared with the type species sequences deposited in public databases, and the high genetic divergence revealed through RAPD data

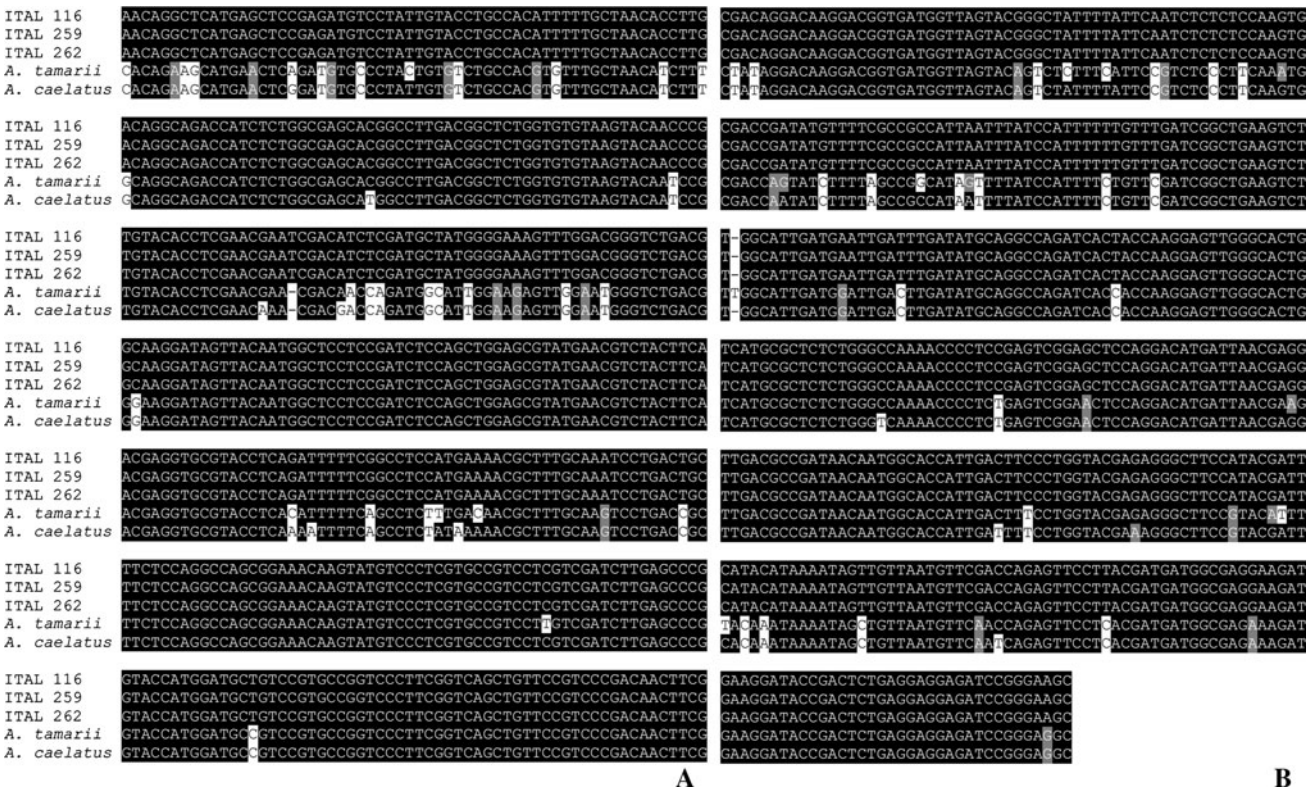

Fig. 4 Alignment of nucleotide sequences of the β-tubulin gene (Bt2) (**a**) and calmodulin gene (**b**) of the ITAL 116, ITAL 259, and ITAL 262 isolates with corresponding *Aspergillus tamarii* and *A. caelatus* sequences

suggests these three isolates from Brazil nuts represents a novel species not yet described in the literature.

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