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Brazil nuts are subject to infection with B and G aflatoxin-producing fungus, *Aspergillus pseudonomius*

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ABSTRACT

The exploitation of the Brazil nut is one of the most important activities of the extractive communities of the Amazon rainforest. However, its commercialization can be affected by the presence of aflatoxins produced by fungi, namely *Aspergillus* section *Flavi*. In the present study, we investigated a collection of *Aspergillus nomius* strains isolated from Brazil nuts using different approaches, including morphological characters, RAPD and AFLP profiles, partial β-tubulin and calmodulin nucleotide sequences, aflatoxin patterns, as well as tolerance to low water activity in cultured media. Results showed that most of the isolates do belong to *A. nomius* species, but a few were re-identified as *Aspergillus pseudonomius*, a very recently described species. The results of the analyses of molecular variance, as well as the high pairwise F_{ST} values between *A. nomius* and *A. pseudonomius* suggested the isolation between these two species and the inexistence of gene flow. Fixed interspecific nucleotide polymorphisms at β-tubulin and calmodulin loci are presented. All *A. pseudonomius* strains analyzed produced aflatoxins AFB1, AFB2, AFG1 and AFG2. This study contains the first-ever report on the occurrence in Brazil nuts of *A. pseudonomius*. The G-type aflatoxins and the mycotoxin tenuazonic acid are reported here for the first time in *A. pseudonomius*.

1. Introduction

The Brazil nut tree (*Bertholletia excelsa* Humb. & Bonpl.: Lecythidaceae) is considered one of the most economically important species of the Amazon rainforest. The genus is monotypic, endemic to upland areas and distributed along almost the entire region of the Amazon. The Brazil nut has been traditionally exploited by local extractive communities; however, more recently, *B. excelsa* has been used as an alternative for cultivation in areas degraded by agriculture or livestock, reconciling reforestation and income generation. The fruit is a spherical capsule of ligneous mesocarp, which is extremely hard. Inside the fruit, the seeds (or nuts) have a ligneous, rough shell and are found in average numbers of 18.

Brazil nuts are much appreciated for human consumption due to their high nutritional value and health benefits. The nut contains substantial amounts of phenolic antioxidants that can effectively control oxidative stress in the body (John and Shahidi, 2010). However, some reports state that Brazil nut commercialization could be affected by the presence of aflatoxins (AF), a group of secondary metabolites produced by *Aspergillus* section *Flavi* that are extremely toxic and carcinogenic. The fruit of *B. excelsa* remains on the tree for 15 months. It then falls from the top of the tree that can grow to a height of up to 45 m, one of the tallest trees of the Amazon rainforest. This means that the fruit is not harvested from the plant but collected off the ground where it can remain for periods of a few days to several months, with ample opportunity for interaction with the environment. Some studies indicate that the soil is the main source of fungal contamination of Brazil nuts (Arrus et al., 2005; Baquião et al., 2013).

The taxonomy of *Aspergillus* section *Flavi* is very complex and continually changing; β-tubulin and calmodulin gene sequences have been increasingly considered to be essential for appropriate

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species identification. As reported in a recent review by Varga et al. (2011), *Aspergillus* section *Flavi* contains 21 species, which can be grouped into seven clades: *Aspergillus flavus* (8 species); *Aspergillus tamari* (4 species); *Aspergillus nomius* (3 species); *Petromyces alliaceus* (2 species); *Aspergillus togoensis* (2 species); *Aspergillus leporis* (1 species); and *Aspergillus avenaceus* (1 species). Our group has subsequently described a new species of *Aspergillus* section *Flavi* that represents a new phylogenetic clade, *Aspergillus bertholletius* (Taniwaki et al., 2012).

The *A. nomius* clade includes *A. nomius*, *Aspergillus bombycis* and *Aspergillus pseudonomius*. According to several authors, *A. nomius* is of particular importance because it is the main culprit responsible for the presence of aflatoxins in Brazil nuts (Baquião et al., 2012; Calderari et al., 2013; Olsen et al., 2008; Reis et al., 2012). *A. bombycis* also infects Brazil nuts, but at very low frequency (Calderari et al., 2013). In terms of their aflatoxin-producing ability, *A. nomius* and *A. bombycis* can produce both types of aflatoxin, AFB and AFG, but do not produce cyclopiazonic acid.

A. pseudonomius was recently described by Varga et al. (2011), reporting that the species is related to *A. nomius* and produces aflatoxin B1 (but not G-type aflatoxins). The *A. pseudonomius* culture ex-type CBS 119388T = NRRL 3353 was isolated from bees by D. Shimanuki (cited by Peterson et al., 2001), and treated as *A. nomius* until Varga's study was published. Based on in silico analysis of ITS, β-tubulin and calmodulin nucleotide sequences, this new species includes several other isolates from insects and soil from the North American states of Louisiana, Texas, Wyoming and Wisconsin (Peterson et al., 2001). However, only the NRRL 3353 isolate was available for mycotoxin analysis.

Recently, there has been an increase in the number of molecular studies characterizing toxigenic *Aspergillus*, but little data is available from South American countries. In the present study, we investigated the genetic variation of 40 isolates collected from Brazil nuts, all previously identified as *A. nomius*. A number of different approaches were used, including RAPD and AFLP profiles, partial β-tubulin and calmodulin nucleotide sequences, aflatoxin patterns, as well as tolerance to low water activity.

2. Materials and methods

2.1. Fungal isolates

A total of 40 isolates previously identified as *A. nomius* (Calderari et al., 2013), collected from Brazil nuts at different stages in the production chain, were randomly selected for investigation. Sequence data from several other *A. nomius* isolates (40) available in the GenBank database were used for comparison and constructing phylogenetic trees.

2.2. RAPD and AFLP procedures

RAPD and AFLP protocols were performed as proposed by Fungaro et al. (1996) and Ferri et al. (2012). Amplified fragments were scored 1 for the presence and 0 for the absence at each polymorphic locus across all 40 isolates. Genetic similarities among the isolates were calculated using Jaccard's coefficient. A dendrogram was then constructed on the basis of UPGMA in the SAHN clustering program of the NTSYS-pc software (v 2.1; Setauket, NY) (Rohlf, 2000). Bootstrap analysis with 1000 resamples was completed with WINBOOT software (IRRI, Manila, Philippines) to determine the confidence limits of the dendrogram. In addition, each isolate was probabilistically assigned to a genetically distinct cluster based on Bayesian models using STRUCTURE (v 2.3.4; Department of Human Genetics, University of Chicago) (Pritchard et al., 2000), assuming 'no admixture model', ten runs with burn-in period of 20,000 and 50,000 repetitions.

2.3. Partial amplification and sequencing of the β-tubulin and calmodulin coding genes

Fungal DNA extraction and amplification of the β-tubulin (*benA*) and calmodulin (*cmd*) genes were performed as described by Gonçalves et al. (2012). Briefly, the primer-pairs Bt2a–Bt2b (Glass and Donaldson, 1995) and cmd5–cmd6 (Hong et al., 2006) were used to amplify the *benA* and *cmd* gene regions respectively, adopting standard amplification reactions and cycling protocols. Fragments generated by PCR were purified with Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The amplicons were submitted to direct sequencing in both directions using a BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA) under the following conditions: denaturation at 95 °C for 60 s, followed by 30 cycles of denaturation at 95 °C for 20 s, annealing at 50 °C for 15 s, extension at 60 °C for 1.5 min, and a final extension at 60 °C for 1.5 min. A volume of HiDiformamide (10 μL) was added to the sequencing products, which were processed in an ABI 3500XL Genetic Analyzer (Applied Biosystems, USA).

Sequences were assembled using the Phred/Phrap/Consed package (University of Washington, Seattle) applying a quality threshold value ≥80. If the Phred score of a base exceeded this threshold, the base was considered of high quality. The sequences were aligned with those from *Aspergillus* section *Flavi* available on the NCBI site. Phylogenies are inferred separately from each gene using MEGA 5.1 (Tamura et al., 2011). The Tamura 3-parameter (T92) model was used for building phylogenetic trees from the distance data on the basis of Neighbor-Joining (NJ) method and nonparametric bootstrap analysis (1000 replicates) as a method for assessing node confidences.

To estimate the molecular variance within and between clusters, a hierarchical analysis of molecular variance (AMOVA) was performed using ARLEQUIN v 3.5 (Excoffier and Lischer, 2010), also used to calculate the fixation index (F_{ST}).

A partial β-tubulin gene sequence is deposited in GenBank as accession numbers KJ789977, KJ789978, KJ789979, KJ789980, KJ789981, KJ789982, KJ789983, KJ789984, KJ789985 and KJ789986, and partial calmodulin gene sequences are deposited in GenBank as accession numbers KJ816323, KJ816324, KJ816325, KJ816326, KJ816327, KJ816328, KJ816329, KJ816330, KJ816331 and KJ816332.

2.4. Macromorphological and micromorphological observations

Isolates were inoculated at three points on plates containing Czapek Yeast Autolysate agar (CYA), Malt Extract Agar (MEA), Yeast Extract Sucrose Agar (YESA) and *A. flavus* and *parasiticus* Agar (AFPA) (Pitt et al., 1983). After incubation in culture media (25 °C, 7 days) macromorphological observations were made. For micromorphological observation using scanning electron microscopy (SEM), samples were prepared as follows: plugs (0.5 cm × 0.5 cm) containing the strain ITAL 286 (grown on solid MEA) were cut and immersed in 2% glutaraldehyde solution at 4 °C, for 24 h. Then specimens were washed in 0.1 M phosphate buffer (three times, 15 min each) and post-fixed with 1% osmium tetroxide aqueous solution at room temperature for 2 h. Each plug was washed again in 0.1 M phosphate buffer (three times, 15 min each) and dehydrated through a graded ethanol series (70, 80, 90, and 100%, at least 10 min each). Afterwards, samples were transferred to a critical point dryer (Bal-Tec, CSDC 030), coated with gold in a sputter coater (Bal-Tec, SDC 050), viewed and photographed (FEI Quanta 200®).

2.5. Aflatoxin and other extrolite analyses

Isolates were evaluated for aflatoxin production following the protocol of Filtenborg et al. (1983). Briefly, isolates were inoculated onto yeast extract agar containing 20% sucrose (YESA), and incubated at 25 °C for 7 days. A small piece of each colony was cut out, and used for toxin extraction with chloroform:methanol (1:1). Plugs were placed

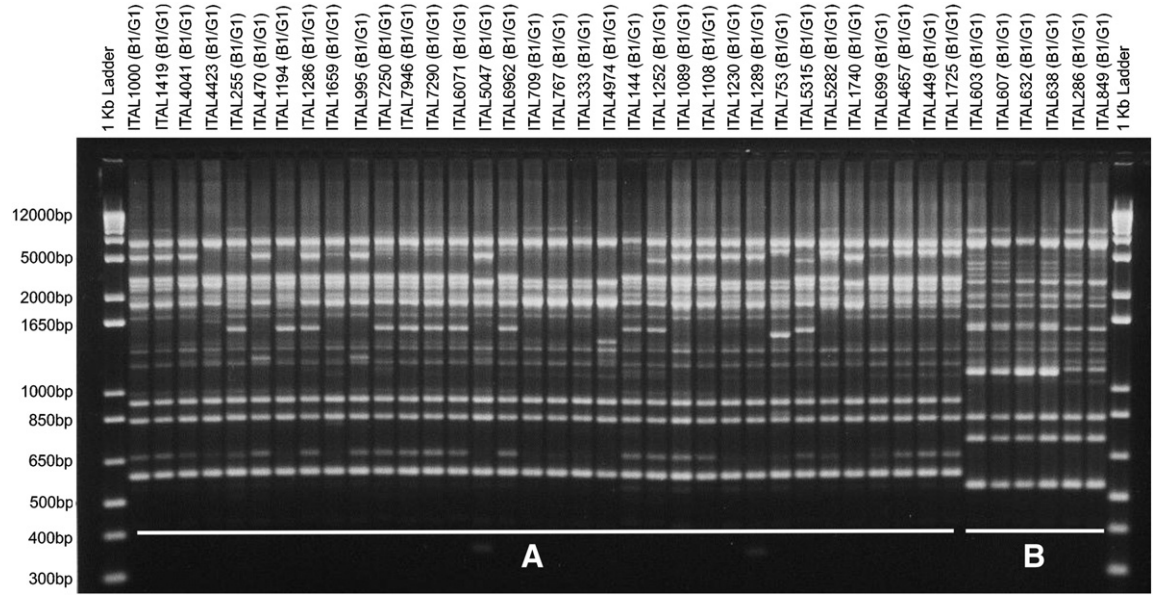

Fig. 1. RAPD profile of *Aspergillus nomius* (A-group) and *A. pseudonomius* (B-group) strains using the 10-mer OPAM-07 *primer*.

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 B1, B2, G1 and G2 standards (Sigma Chemical, St Louis, USA) were used for qualitative comparison of retention time and fluorescence. Production of the B1, B2, G1 and G2 aflatoxins by *A. pseudonomius* strains was confirmed and quantified by HPLC. A Shimadzu LC-10VP HPLC system (Shimadzu, Japan) was used with a fluorescence detector set to 362 nm excitation and 455 nm emission for aflatoxins G1 and G2 and 425 nm emission for aflatoxins B1 and B2. A Shimadzu CLC G-ODS (4 × 10 mm) guard column and Shimadzu Shimpack (4.6 × 250 mm) column were employed. The mobile phase used was water:acetonitrile:methanol (6:2:3, v/v/v) containing KBr (119 mg/L) and nitric acid (4 M, 350 μL/L). The flow rate was 1 mL/min. A standard mix of aflatoxins B1, B2, G1 and G2 was used to construct a five point calibration curve of peak areas versus concentration (μg/mL). The injection volume was 100 μL. Post-column derivatization of aflatoxins B1 and G1 was performed with bromine using a Kobra Cell (R-Biopharm Rhône Ltd, Scotland). Other extrolites were analyzed as described by Frisvad and Thrane (1987), with minor modifications according to Smedsgaard (1997), or by the improved methods described by Klitgaard et al. (2014), Nielsen and Smedsgaard (2003), and Nielsen et al. (2011).

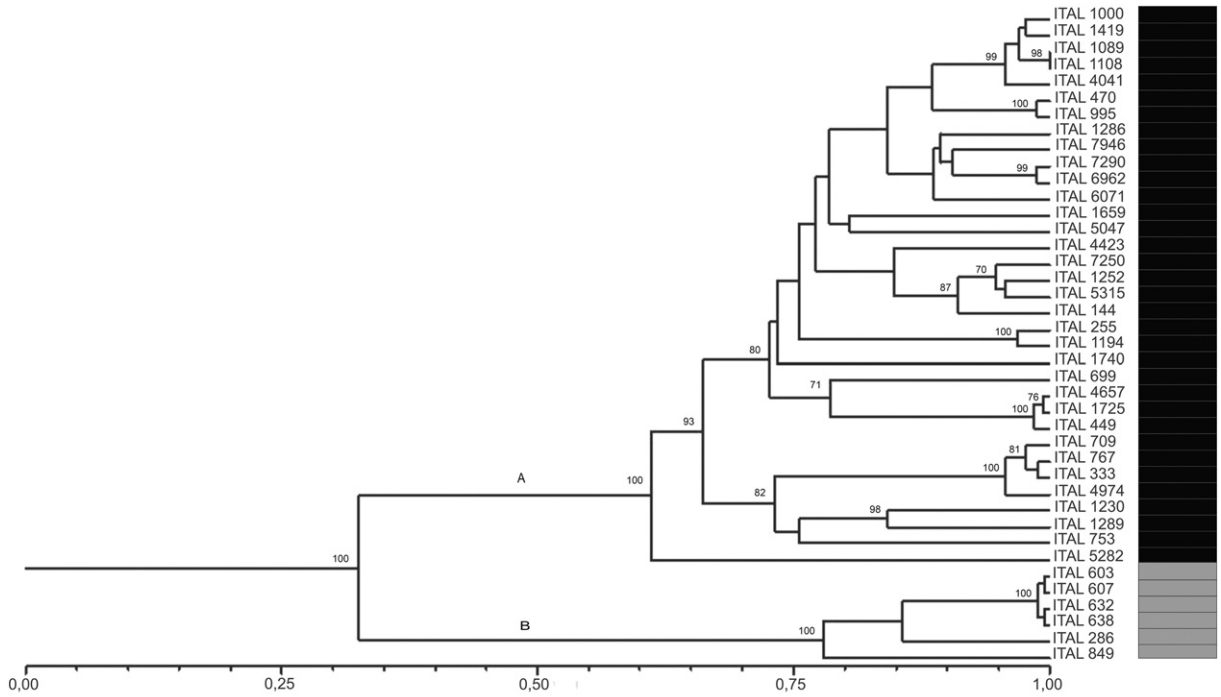

Fig. 2. UPGMA-based dendrogram generated from the distance matrix of the Jaccard coefficients using RAPD and AFLP data from 40 isolates collected from Brazil nuts, previously identified as *A. nomius*. Nodes supported by bootstrap values > 70% are indicated by numeric values. The column on the right corresponds to the hierarchical organization of genetic relatedness of the same group of isolates obtained using the STRUCTURE computational program (K = 2).

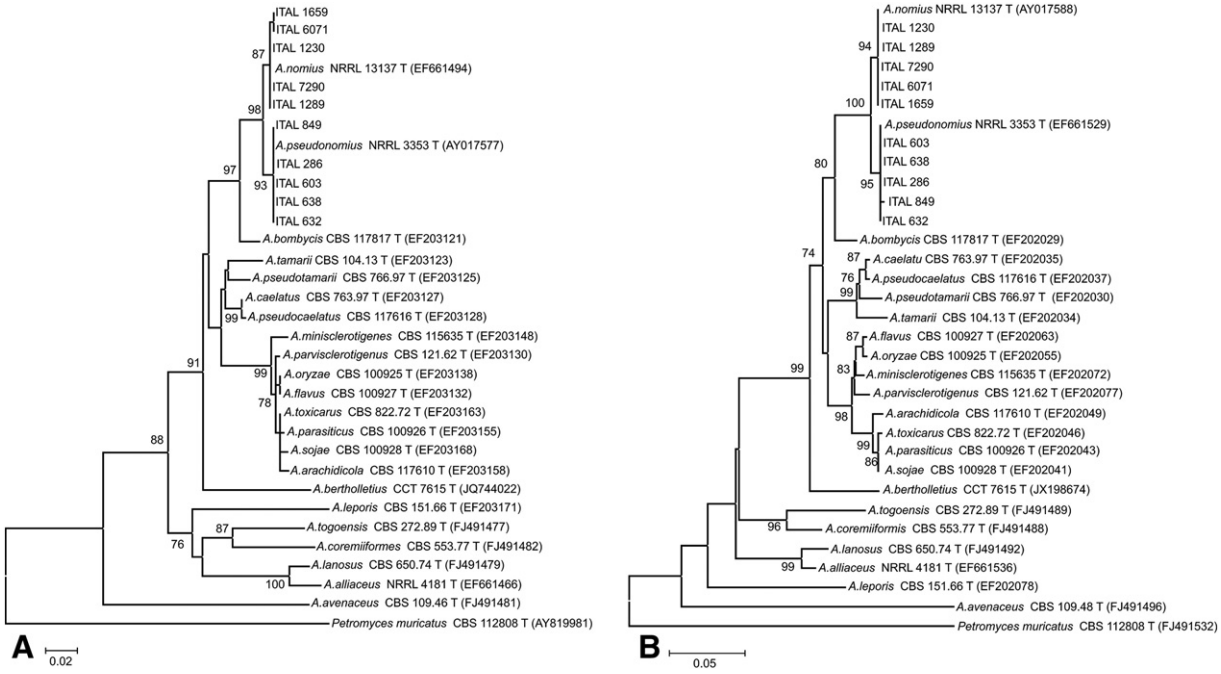

Fig. 3. Neighbor-Joining trees of *Aspergillus* section *Flavi* type strains and *Aspergillus* isolates from Brazil nuts based on β-tubulin (A) and calmodulin (B) sequence data. Nodes supported by bootstrap values >70% are indicated by numeric values.

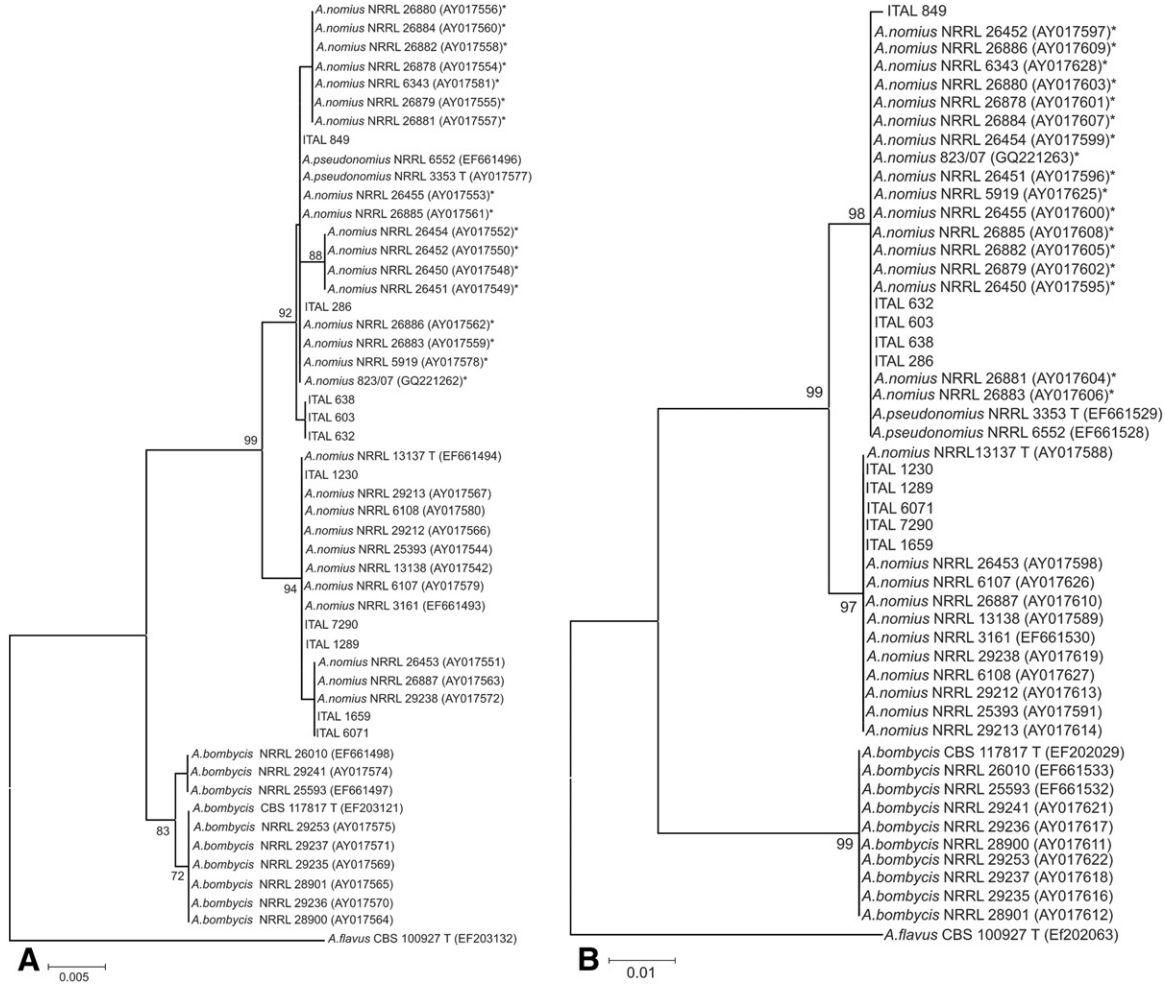

Fig. 4. Neighbor-Joining of 50 taxa belonging to the *Aspergillus nomius* clade based on β-tubulin (A) and calmodulin (B) tree sequence data. *Aspergillus flavus* (CBS 100927) was chosen as an outgroup. Nodes supported by bootstrap values >70% are indicated by numeric values.

2.6. Partial norB and cypA gene sequence amplification

A portion of the intergenic region and the 5′ coding sequences of *norB* (an aryl alcohol dehydrogenase coding gene) and *cypA* (a cytochrome P450 monooxygenase coding gene) was amplified by PCR using a slightly modified version of the protocol described by Chang et al. (2005). Briefly, amplifications were performed in a Veriti® Thermal Cycler (Applied Biosystems) using a 25 μL reaction volume containing 10 ng of DNA template, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U of Taq DNA polymerase (Invitrogen, Life Technologies), 0.2 μM of primers 5′-GTG CCC AGC ATC TTG GTC CA-3′, and 5′-AGG ACT TGA TGA TTC CTC GTC-3′, at an annealing temperature of 57 °C. Amplicons were resolved by electrophoresis on 1.0% agarose gels stained with ethidium bromide.

2.7. CYA medium preparation adjusted to different levels of water activity, incubation, and colony diameter measurements

CYA media were adjusted to various levels of water activity (a_w) by substituting a part of water by glycerol (w/w). An AquaLab a_w meter (Decagon Devices) was used to measure the water activity of the CYA medium in triplicate. Petri plates were inoculated at three equidistant points and incubated (25 °C, for 7 days). Colony diameters were measured from cultures grown under 12 levels of a_w (0.99, 0.98, 0.97, 0.95, 0.93, 0.91, 0.87, 0.86, 0.85, 0.84, 0.83 and 0.81) in a completely randomized factorial design (12 × 10, 120 treatments), replicated 3 times. After performing a preliminary ANOVA, the effect of treatments was partitioned into the main factors: levels of water activity (A), strain (S); and the interaction of (A × S) factors. The S factor and the interaction (A × S) were subsequently split into two groups, according to the strain species, and contrasting the two species. After verifying the existence of significant effects of all sources of variation, including the interaction effect, polynomial regression procedure and coefficient of determination (R^2) estimations were performed.

3. Results and discussion

The genetic variation of an *A. nomius* collection isolated from Brazil nuts was investigated using RAPD and AFLP profiles and partial β-tubulin (*benA*) and calmodulin (*cmd*) nucleotide sequences. We chose RAPD and AFLP because they are multilocus approaches that provide an overview of the genome since the DNA fragments generated are randomly distributed. Moreover, they have already been described as good discriminators of *Aspergillus* section *Flavi* strains (Barros et al., 2007; Gonçalves et al., 2012). We also used partial *benA* and *cmd* gene sequence analysis since this procedure was successfully used in a very recent overview on the taxonomy of *Aspergillus* section *Flavi* (Varga et al., 2011).

Nine RAPD primers and three AFLP primer–enzyme combinations produced 226 polymorphic fragments from 40 DNA samples. Fig. 1 shows one of the RAPD profiles obtained. Thirty-nine haplotypes were detected, including one that was found to be shared by two isolates (ITAL 1089 and ITAL 1108) that were collected in the same geographic region. The polymorphic markers (226) allowed to build a Jaccard similarity matrix, used to generate an UPGMA-based dendrogram. The 40 isolates were grouped into two major clusters (A and B) supported by high bootstrap values. Model-based clustering obtained from the STRUCTURE analysis (at K = 2) revealed a similar pattern of grouping (Fig. 2).

The AMOVA performed with RAPD and AFLP markers confirmed the occurrence of a high level of genetic variability in the fungal isolate collection, and showed that most of the variance (~73%) was due to differences between clusters (A and B) rather than within clusters (~27%). The amount of between-cluster divergence relative to overall diversity (F_{ST}) was very high (0.73), which is unusual for a group of isolates belonging to the same fungal species.

As the main source of genetic variation was between clusters, five representatives from each cluster were subjected to *benA* and *cmd* partial nucleotide sequence analyses. When the sequences obtained were compared to those of the *Aspergillus* section *Flavi* type strains (22) available in GenBank (http://www.ncbi.nlm.nih.gov), the isolates belonging to the A-cluster determined by AFLP/RAPD analyses grouped into one cluster that includes the *A. nomius* ex-type strain (NRRL 13137 = CBS 260.88), and the B-cluster isolates were included in another cluster that includes the *A. pseudonomius* ex type strain (NRRL 3353 = CBS 119388) (Fig. 3).

We also performed a second comparison using all the sequences available in GenBank from the clade *A. nomius* (*A. nomius*, *A. pseudonomius* and *A. bombycis*). The alignment of our *benA* sequences (10) with those deposited (40) revealed 24 polymorphic sites in a total of 459 sites (5.2%). The alignment of our *cmd* sequences with those deposited revealed 35

Table 1
Haplotypes of the *Aspergillus nomius* clade defined on the basis of β-tubulin (*benA*) and calmodulin (*cmd*) genes. Sequences from strains denoted as ITAL were obtained in the present study; all others were obtained from GenBank. Roman numerals designate identical sequences.

Species	Strain	*benA*-based haplotype	*cmd*-Based haplotype
A. nomius	NRRL 13137 (T)	I (EF661494)	I (AY017588)
	ITAL 1230	I	I
	ITAL 1289	I	I
	ITAL 7290	I	I
	NRRL 6108	I	I
	NRRL 29212	I	I
	NRRL 25393	I	I
	NRRL 13138	I	I
	NRRL 6107	I	I
	NRRL 29213	I	I
	NRRL 3161	I	I
	NRRL 26887	II (AY017563)	I
	ITAL 1659	II	I
	ITAL 6071	II	I
	NRRL 26453	II	I
	NRRL 29238	II	I
A. pseudonomius	NRRL 3353 (T)	III (AY017577)	II (EF661529)
	NRRL 6552[a]	III	II
	ITAL 286	III	II
	ITAL 849	III	III
	NRRL 26455[a]	III	II
	NRRL 26886[a]	III	II
	NRRL 26885[a]	III	II
	NRRL 26883[a]	III	II
	NRRL 5919[a]	III	II
	823/07[a]	III	II
	NRRL 26881[a]	IV (AY017557)	II
	NRRL 26879[a]	IV	II
	NRRL 6343[a]	IV	II
	NRRL 26878[a]	IV	II
	NRRL 26882[a]	IV	II
	NRRL 26880[a]	IV	II
	NRRL 26884[a]	IV	II
	NRRL 26454[a]	V (AY017552)	II
	NRRL 26452[a]	V	II
	NRRL 26450[a]	V	II
	NRRL 26451[a]	V	II
	ITAL 638	VI (KJ789979)	II
	ITAL 603	VI	II
	ITAL 632	VI	II
A. bombycis	CBS 117817 (T)	VII (EF203121)	IV (EF202029)
	NRRL 29253	VII	IV
	NRRL 29237	VII	IV
	NRRL 29235	VII	IV
	NRRL 28901	VII	IV
	NRRL 29236	VII	IV
	NRRL 28900	VII	IV
	NRRL 25593	VIII (EF661497)	IV
	NRRL 26010	VIII	IV
	NRRL 29241	VIII	IV

T: type strain;
[a] A strain whose sequences were annotated as *A. nomius*, but identified as *A. pseudonomius* in this study.

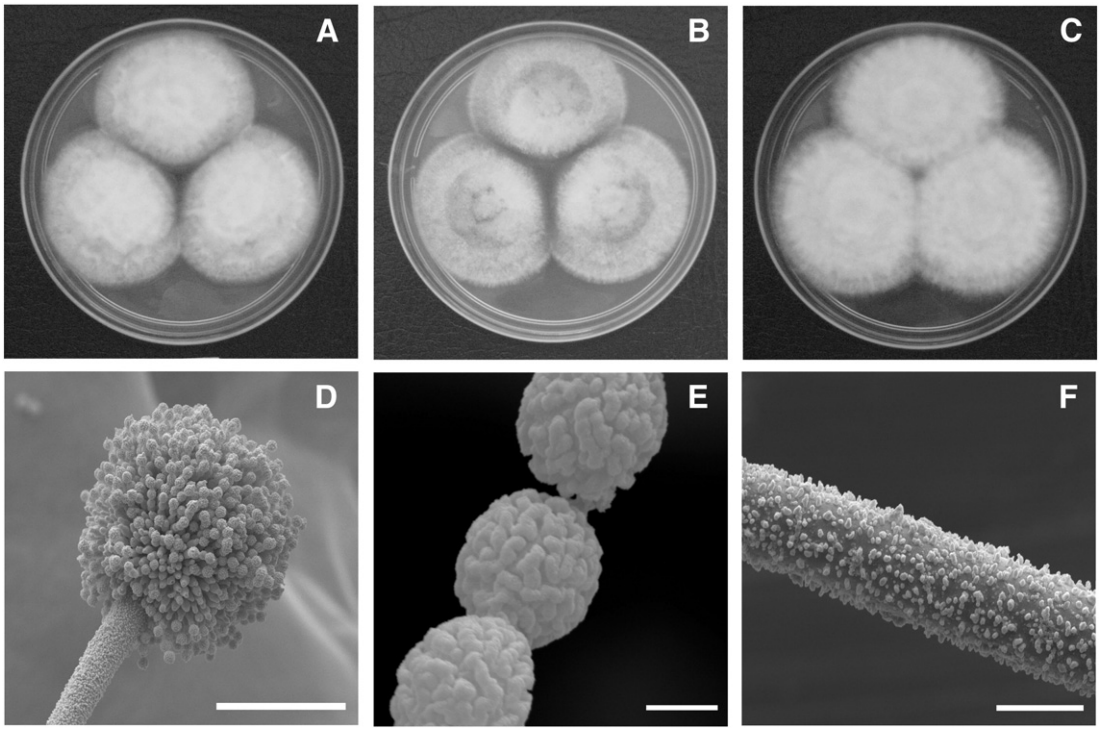

Fig. 5. *Aspergillus pseudonomius* strain ITAL 286: Colonies grown on CYA (A), MEA (B), and YESA (C) at 25 °C for 7 days. Scanning electron micrographs of conidiophore (D), conidia (E) and stipe (F). Bars: 1 = 50 μm; 2 = 2 μm; and 3 = 10 μm.

polymorphic sites in a total of 528 sites (6.6%). The Neighbor-Joining clustering analyses for both genes were congruent and revealed that the sequences of *A. nomius* strains deposited in GenBank extend over two distinct groups: one including the *A. nomius* ex type strain (NRRL 13137) and the other the *A. pseudonomius* ex type strain (NRRL 3353) (Fig. 4).

The relationships among all the sequences analyzed revealed that 19 strains previously accepted as *A. nomius* (NRRL 3353; NRRL 6552, NRRL 26455; NRRL 26886; NRRL 26885; NRRL 26883; NRRL 5919; 823/07; NRRL 26454; NRRL 26452; NRRL 26450; NRRL 26451; NRRL 26881; NRRL 26879; NRRL 6343; NRRL 26878; NRRL 26882; NRRL 26880; NRRL 26884) could be recognized as *A. pseudonomius*. While NRRL 25393, isolated from tea field soil in Okinawa Prefecture, Japan, was *A. nomius*, an isolate from silkworm excrement in Kagawa Prefecture, Japan, originally identified as *A. nomius*, (Ito et al., 1998) was in fact *A. pseudonomius*. Both species were reported to produce more aflatoxin of the G type than the B type, in addition to kojic acid, and both species were reported to produce sclerotia, but not cyclopiazonic acid, in line with the results obtained in our study.

The AMOVA performed on the same data set but based on the above-mentioned classification showed the structure of the genetic variation of the group strains analyzed. The fixation index (F_{ST}), which reveals the amount of between-group genetic differentiation, was very high for both partial nucleotide sequence analyses (F_{ST} = 0.92 and F_{ST} = 0.99, respectively for *benA* and *cmd*). All pairwise F_{ST} values were also high (0.87 to 1.00), indicative of complete isolation due to the absence of gene flow between these phylogenetic species, in line with the conclusions proposed by Varga et al. (2011) to the effect that the *A. nomius* clade includes at least three species. Interestingly, the *cmd* sequence data allowed better discrimination between clusters as only 0.2% of the variance was apportioned within groups, compared to 8.1% when the analysis was based on partial *benA* gene sequences. In regard to the genetic differentiation between *A. nomius* and *A. pseudonomius* groups, the pairwise F_{ST} values were also high for both genes (F_{ST} = 0.87 and F_{ST} = 0.99, *benA* and *cmd*, respectively), suggesting an absence of gene flow (i.e. full genetic isolation) between these two phylogenetic species. The sequence variations of all taxa belonging to the *A. nomius* clade (50) allowed us to define eight distinct haplotypes based on the *benA* locus, and four based on the *cmd* locus. Among the *A. nomius* sequences analyzed (16), two distinct haplotypes were found based on the *benA* locus, and one based on the *cmd* locus. For *A. pseudonomius*, four distinct haplotypes at the *benA* locus and two at the *cmd* locus were found among the 24 sequences analyzed (Table 1). Fixed nucleotide differences between *A. nomius* and *A. pseudonomius* at both loci are shown in Fig. A.1.

A. pseudonomius was first described by Varga et al. (2011), reporting that the species is related to *A. nomius* and produces aflatoxin B1 (but not G-type aflatoxins), chrysogine and kojic acid. The macro- and micromorphological characteristics of the strains isolated in the present study were in line with those described by these pioneering authors, except that under scanning electron microscopy the stipes were found to be rough-walled (Fig. 5). The colony sizes grown on YESA, MEA and CYA (incubated 7 days at 25 °C) reached a diameter of approximately 6.0 cm; their surfaces were floccose with dominant aerial mycelium, and sporulation was remarkably poor in comparison to that of *A. nomius* strains. Conidial heads were similar to *A. nomius*, but predominantly uniseriate. Conidia were globose to subglobose and superficially rough.

Table 2
Aflatoxin production by five strains of *Aspergillus pseudonomius* grown on YESA for 7 days at 25 °C.

Strain	Production of aflatoxins (μg/g)				
	B1	B2	G1	G2	Total
ITAL 286	117.53	5.33	210.00	6.58	339.43
ITAL 603	0.94	0.02	1.39	0.02	2.37
ITAL 632	2.74	0.18	3.74	0.16	6.82
ITAL 638	39.00	1.14	51.4	0.79	92.33
ITAL 849	1.34	0.016	1.61	0.01	2.97

Table 3
Production of mycotoxins and other extrolites by strains of *Aspergillus nomius* and *A. pseudonomius*.

Species	Strain	Mycotoxins and other extrolites
A. nomius	NRRL 13137	Aflatoxin B1, B2, G1, G2, anominine, aspernomine, kojic acid, a miyakamide, 3-O-methylsterigmatocystin, parasiticol, paspaline, paspalinin, a pseurotin, tenuazonic acid, versicolorins, (NOM[a], NOL[a], NON[a])
	NRRL 13138	Aflatoxin B1, B2, G1, G2, anominine, aspernomine, kojic acid, a miyakamide, 3-O-methylsterigmatocystin, paspaline, paspalinine, tenuazonic acid, versicolorins, (NOM[a], NOL[a])
	NRRL 3161	Aflatoxin B1, B1, G1, G2, anominine, aspernomine, kojic acid, 3-O-methylsterigmatocystin, a miyakamide, parasiticol, a pseurotin, a versicolorin, (NOM[a], NOL[a])
A. pseudonomius	NRRL 3353	Aflatoxin B1, chrysogine, kojic acid, two miyakamides, (VOP[a])
	ITAL 849	Aflatoxin B1, B2, G1, G2, aspergillic acid, kojic acid, 3-O-methylsterigmatocystin, tenuazonic acid
	NRRL 5919	Aflatoxin B1, B2, G1, G2, aspergillic acid, chrysogine, kojic acid, a miyakamide
	823/07	Aflatoxin B1, B2, G1, G2, kojic acid, tenuazonic acid
	ITAL 638	Aflatoxin B1, B2, G1, G2, kojic acid, tenuazonic acid, a versicolorin, (HUTTI[a])
	ITAL 603	Aflatoxin B1, B2, G1, G2, kojic acid, a miyakamide, parasiticol, tenuazonic acid, a versicolorin, (HUTTI[a])
	ITAL 632	Aflatoxin B1, B2, G1, G2, kojic acid, (HUTTI[a])

[a] These metabolites had characteristic UV spectra, but the structures of the compounds have not yet been elucidated.

In contrast to the initial description of the species, all five *A. pseudonomius* strains analyzed in the present study produced different amounts of AFB1, AFB2, AFG1 and AFG2 (Table 2). It is important to note that only one *A. pseudonomius* strain was available for mycotoxin analysis by Varga and collaborators (culture ex-type CBS 119388 = NRRL 3353). Because of this inconsistency, we reexamined the metabolite profile of some strains of *A. nomius* and *A. pseudonomius* using HPLC-DAD. In general most strains of each species produced AFB1, AFB2, AFG1 and AFG2, often including their precursors, such as versicolorins, parasiticol, and 3-O-methylsterigmatocystin. *A. nomius* and *A. pseudonomius* both produced kojic acid, miyakamides (Rank et al., 2012; Shiomi et al., 2002), and tenuazonic acid (Varga et al., 2011). The mycotoxin tenuazonic acid is reported for the first time in *A. pseudonomius*. Anominine (Gloer et al., 1989) and aspernomine (Staub et al., 1992) were produced by all *A. nomius* isolates, but were not found in all the *A. pseudonomius* isolates that we analyzed. Anominine was first called nominine, but the name has been changed to anominine (Bradshaw et al., 2008). Pseurotin A was produced by two isolates of *A. nomius* (NRRL 13137 and NRRL 3161), but not by any *A. pseudonomius*. Chrysogine was produced by some isolates of haplotype III (*A. pseudonomius*), while isolates of haplotype VI of the same species produce "HUTTI", an extrolite that has not yet been fully characterized (Table 3).

As mentioned in the Materials and methods section, we used PCR to investigate the length of a region denoted *norB–cypA*. According to the literature, when using the primer pair norB–cypA-F/norB–cypA-R (Chang et al., 2005), an amplicon of approximately 1.8 kb is obtained for the *A. parasiticus* and *A. nomius* strains, both producers of B and G aflatoxins (Ehrlich et al., 2004). This region of the aflatoxin biosynthesis pathway gene cluster includes a portion of the intergenic region and the 5′ coding sequences of *norB* (an aryl alcohol dehydrogenase) and *cypA* (a cytochrome P450 monooxygenase). There is already sufficient evidence that the *cypA* gene is required for the formation of G-type aflatoxins in *A. parasiticus*. As shown in Fig. A.2, we obtained a PCR product of 1.8 kb from each *A. pseudonomius* isolate, which was identical to those of *A. nomius*. The two *A. flavus* strains used as non-producers of aflatoxin G showed different gel profiles, one strain generating a 0.9 kb amplicon and the other a 0.3 kb amplicon. These results can be fully explained by the fact that *A. flavus* and *Aspergillus oryzae* lack the ability to produce G aflatoxins as a consequence of a 0.8 to 1.5 kb gap near the 5′ end of the aflatoxin gene cluster (Ehrlich et al., 2004). Finally, we

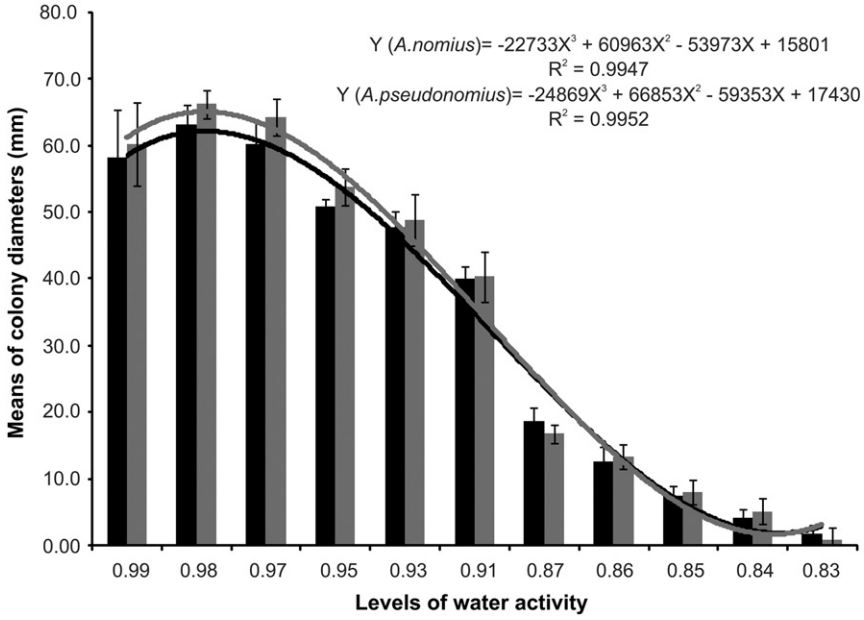

Fig. 6. Polynomial regression curve showing the relationship between the variables, colony diameter means (after 7 days) (y) and levels of water activity in CYA medium (x). *Aspergillus nomius* and *A. pseudonomius* data are shaded in black and dark gray, respectively.

demonstrated that *A. pseudonomius* strains have similar PCR profiles to those obtained from other aflatoxin G producer species.

Water activity (a_w) is one of major factors controlling fungal growth. *A. pseudonomius* and *A. nomius* are capable of producing aflatoxin and their potential growth is of serious concern. Referring to a personal communication from the late C. W. Hesseltine (NRRL, USA), Varga et al. (2011) mention that they considered the NRRL 3353 strain (now renamed *A. pseudonomius*) different from other *A. nomius* strains in respect to its tolerance to low water activity. According to these authors, this attribute should be further investigated in *A. pseudonomius* (Varga et al., 2011). In order to meet this requirement, the colony diameters of five isolates each of *A. nomius* and *A. pseudonomius* were recorded after 7 days of incubation under 12 levels of a_w (0.99 to 0.81). Data from both species investigated have shown that fungal growth can occur over a wide range of a_w levels, and colony sizes were larger under 0.98 a_w. For the majority of strains the minimal required for growth was 0.83 a_w (Fig. 6). Although significant differences in the mean sizes of the colonies at different a_w levels were detected between the species, it is worth mentioning that intraspecific variability was also found among the strains of both species. Consequently, this trait is not suitable for easily distinguishing between isolates of *A. nomius* and *A. pseudonomius*.

4. Conclusions

Based on several approaches, our study led us to draw the following conclusions: Brazil nuts may harbor *A. pseudonomius*, a sibling species of *A. nomius*. To date, the best way of distinguishing these sibling species is to analyze β-tubulin or calmodulin gene sequence. We also concluded that *A. pseudonomius* strains might be able to produce both B and G-type aflatoxins.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2014.06.006.

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