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A small growth study of *Aspergillus* section *Flavi*, and potentially aflatoxigenic fungi and aflatoxin occurrence in Brazil nuts from local markets in Manaus, Brazil

Ellinor Andersson

Department of Microbiology
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Ellinor Andersson

Supervisor: Su-lin Leong, Swedish University of Agricultural Sciences, Department of Microbiology

Assistant Supervisor: Marta Taniwaki, Instituto de Technologia de Alimentos (ITAL), Campinas, Brazil

Examiner: Volkmar Passoth, Swedish University of Agricultural Sciences, Department of Microbiology

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Abstract

This study aimed to gain more knowledge about morphological features of eight different species of *Aspergillus* section *Flavi,* which may aid in identification of these species. The presence of potentially toxigenic *Aspergillus* section *Flavi,* and occurrence of aflatoxins, in ready-to-eat Brazil nuts (*Bertholletia excelsa*) were also investigated.

Seventeen different strains, representing eight species from section *Flavi,* were grown at different water activities and different temperatures. After 7 days, as well as at 14 and 28 days for the lower water activities, colonies were visually inspected, and diameter and macroscopic characteristics were noted. Ten samples of Brazil nuts were collected from different market stalls in the city of Manaus, Brazil. *Aspergillus* section *Flavi* were isolated and identified by morphology and toxin profile. Toxin production was evaluated by the agar plug technique and quantification of aflatoxins in the Brazil nuts was performed by high-performance liquid chromatography.

It was found that macromorphology can be used to differentiate between some of the species if the different methods were combined, but for more certain identification, other available methods are better. One hundred and thirty five strains of suspected *Aspergillus* section *Flavi* were isolated and aflatoxin production was observed in 100 of them (74%). The most common species was *Aspergillus nomius*, followed by *A. flavus* and *A. tamarii*. Total aflatoxin content detected in the nuts did not exceed the European legislative limit of 10 µg/kg for ready-to-eat Brazil nuts, which would be considered safe to be consumed.

Key words: Aspergillus section *Flavi*, Aflatoxin, Brazil nut, mould growth, Brazil, morphological identification

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1 Introduction

The Brazil nut (*Bertholletia excelsa)* comes from a tall tree in the family *Lecythidaceae* and is native to the Amazon rain forest in South America. The nut is mainly found in Brazil and Bolivia but also in Peru, Guiana, Venezuela, Suriname and Colombia (FAO, 1993) and the trees are scattered along the Amazonian river systems in dense groups. The Brazil nut tree is one of the tallest in Amazonas and can reach a height of almost 60 m. It bears hard, large, spherical fruit pods weighing up to 3 kg, with a capsule that contains 10–25 edible seeds. The seeds are 3.5–5 cm long and have the distinct triangular shape characteristic of Brazil nuts. Upon ripening in the rainy season (December to March), the fruit falls to the ground with the capsule still closed, and once on the ground it can be collected and cut open. The principal use of the Brazil nut is raw consumption, as well as an ingredient in food products due to its high nutritional value (Shanley, 2002). It is rich in fat (approximately 66 %) and protein (approximately 14 %) and has higher energy content than peanuts (NFA, 2012). Brazil nut protein contains all the essential amino acids and is especially rich in methionine. Brazil nuts are also high in vitamins and minerals. These qualities make the nut very important in the socio-economic setting, providing income and work for the local Amazon communities (Newing & Harrop, 2000). Approximately 20,000 tons of Brazil nuts are produced annually (Collison *et al.,* 2002) with the majority being exported to Europe. The complex ecological chain of the pollination by specific bees has made plantations unsuccessful, and the nuts are mainly supplied from the wild (Newing & Harrop, 2000). Being produced organically and with a low technical production chain, the Brazil nut is considered an environmentally friendly product.

One of the main problems identified in the production of Brazil nut is contamination by moulds, which make the nut visually unattractive, inedible and increase the risk of toxic metabolites such as aflatoxins and ochratoxins. This problem has been a strong obstacle to marketing the product, mainly in foreign markets, given the strict regulations of European countries and the United States regarding levels of toxins in food. A common contaminant is *Aspergillus,* a mould that grows well in a warm and humid environment. *Aspergillus* is known to spoil foods such as peanuts, corn, figs, spices, rice, cocoa and coffee beans as well as Brazil nuts.

Some *Aspergillus* species, mainly in the *Flavi*-group, produce secondary metabolites called aflatoxins. Aflatoxins can occur in the above-mentioned foods as a result of fungal contamination before and after harvest (EFSA, 2012). The International Agency of Research on Cancer (IARC, n.d.) has stated that aflatoxins are among the most potent natural carcinogens known affecting animals and humans. Chronic low-level exposure may increase the risk of developing hepatic cancer, especially in individuals with chronic Hepatitis B viral infection (Tillet, 2010). Aflatoxins also have acute toxic effects. Although not as common, acute high-level exposures occur sporadically and have been known since the 60's when 100 000 domesticated birds in England died after consuming peanuts contaminated with *Aspergillus* (FAO, 1991). On a few rare occasions, aflatoxins have also caused the death of humans (Pitt & Hocking, 2009). In these cases, a very high dose was consumed and the lethal dose is approximately 10 mg.

There are four different naturally occurring aflatoxins: B_1, B_2, G_1 and G_2 where B_1 is considered to be the most potent carcinogen. The letters refer to the colour they fluoresce under UV-light; blue and green, respectively.

A recent overview of *Aspergillus* section *Flavi* by Varga *et al.* (2011) concluded that the section includes 22 species. Despite the ability of aflatoxin production by nine of these species, *Aspergillus flavus* has been considered the most important for food products in general (Pitt & Hocking, 2009) and *A. nomius* an important producer in Brazil nuts (Olsen *et al.,* 2008). Taxonomic concepts within section *Flavi* have evolved in recent years, with some species producing aflatoxins and others not. Thus, development of reliable and economical methods to differentiate between the various species is an important research field. Molecular techniques and extrolite studies are the most precise for differentiating species, but for a smaller laboratory without the possibility of these techniques, morphological methods are more practical.

The occurrence of high levels of aflatoxins in Brazil nuts has been known for several years. In 1998, the European Union (Commission Regulation, 1998) set regulations regarding maximum residue levels for aflatoxin in food. To guarantee a safe consumption, the limits for Brazil nuts were set to 4 µg/kg for total aflatoxins and 2 µg/kg for aflatoxin B_1. However, the industry faced a hard time meeting the new quality requirements and formal complaints were made to the World Trade Organization (WTO) from producing countries. The strict regulation led to a decline in sales, mainly to the European market, of almost 90 % between the years 2000–2004 (STDF, 2010). Recently new total aflatoxin limits have been set by Codex alimentarius (CAC, 2010) with 10 µg/kg for shelled, ready-to-eat nuts and 20 µg/kg for in-shell nuts. The European Commission (2010) also relaxed their levels to meet the new Codex alimentarius limits, and in 2011, Brazil adapted similar regulations in order to meet food safety standards. Still, many nuts sold in

local markets have not gone through any sorting or processing, and high levels of toxins might occur.

There were two main objectives of this study. The first one focused on growing 17 different strains of *Aspergillus* section *Flavi,* from previously collected samples of Brazil nuts (and 1 peanut) on agar media of different water activities and at different temperatures, in order to gather more knowledge about morphological features which may aid in identification of these species. The second objective was to isolate and identify toxigenic fungi in samples of Brazil nuts, both from the kernel and the shell, that have not gone through any processing, and to quantify aflatoxins in these samples.

2 Methods

2.1 Growth study of 17 isolates available at Instituto de Technologia de Alimentos (ITAL), Campinas, Brazil.

2.1.1 Media preparation and isolates

Two media were prepared, Czapek yeast extract (CYA) and Aspergillus Flavus and Parasiticus (AFPA) agar. All media used in the experiment were prepared according to Pitt & Hocking (2009) and autoclaved at 121 °C for 15 minutes. Before incubation, all Petri-dishes were stacked in piles of approximately 16 plates and wrapped with common household cling film. ITAL codes and scientific names of isolates can be found in Table 1.

Table 1. Isolates used in growth and temperature study. All, except isolate 103/06 F, which was found on peanut, had been isolated from Brazil nuts. A. Flavi *is a collective name for unknown species from the* Flavi *group.*

ITAL code	Identified species
243 F	*A. caelatus*
201 F	*A. caelatus*
252 F	*A. tamarii*
249 F	*A. tamarii*
4207 F	*A. pseudotamarii*
5328 F	*A. pseudotamarii*
6942 F	*A. Flavi (group)*
7052 F	*A. Flavi (group)*
7581 F	*A. Flavi (group)*
532 F	*A. nomius*
699 F	*A. nomius*
189 F	*A. arachidicola*
1445 F	*A. arachidicola*
89 F	*A. bombycis*
1271 F	*A. flavus*
1250F	*A. flavus*
103/06 F	*A. pseudocaelatus*

2.1.2 Inoculation

A total of 17 strains of *Aspergillus* section *Flavi,* 16 previously obtained from Brazil nuts and 1 from peanut and preserved on silica gel, were inoculated on CYA by pouring the silica balls straight onto the media; plates were incubated at 25 °C for 5 days. Due to contamination, the strains were sub-cultured a second time to obtain pure cultures: all *Aspergillus* section *Flavi* were isolated on CYA again by carefully collecting spores from each plate with a needle and inoculating at three points on the new plates. The plates were incubated at 25 °C for 4 days. The pure isolates were three-point inoculated in one replicate on CYA and incubated at 25 °C, 37 °C and 42 °C for 7 days. In order to screen for potentially toxigenic species, three-point inoculation was also made on Aspergillus Flavus and Parasiticus (AFPA) agar and incubated at 25 °C. Potentially aflatoxigenic species will show an orange reverse on this media due to production of ferric chelates of aspergillic acids (Samson *et al,* 2010). All isolates were also inoculated in duplicates on CYA media with different water activities (see 2.1.3) and incubated at 25 °C for 7 days.

2.1.3 Water activity and inspection

The water activity of CYA was modified by adding different amounts of glycerol to the media. The water activities of the plates were measured in triplicate in a Decagon Aqualab Series 3TE instrument at 25 °C, and came to a_w 0.79, 0.87, 0.91, 0.94 and 0.97 (Appendix 6.1). After 7 days, three out of the total six colonies, were randomly chosen for macroscopic studies. Macroscopic characteristics such as colony diameter, colour, texture and AFPA reverse colour were noted. At the lower water activities (a_w 0.87 and 0.79, respectively), colonies were also inspected after 14 and 28 days.

2.2 Isolation and identification of potentially toxigenic fungi from Brazil nuts collected from market stalls.

2.2.1 Sampling

Samples of Brazil nuts were collected from 10 different market stalls in the city of Manaus, Brazil. The nuts arrive at market early in the morning and are not subject to any sorting or processing. In each market stall, approximately 2–4 kilos of nuts were purchased. All nuts were ready to eat, three of the samples with the husks still on, and seven without, depending on availability.

2.2.2 Water activity

Before further experiments, the water activity of the nuts was determined by analyzing the samples in triplicate in a Decagon Aqualab Series 3TE instrument at 25 °C (Appendix 6.2).

2.2.3 Fungal isolation and identification of potential aflatoxigenic species

From each sample, approximately 100 g of nuts or shells were sub-sampled. The nuts and shells were surface disinfected separately with freshly prepared hypochlorite solution (equivalent to 0.4 % chlorine) for 2 minutes and 50 pieces were aseptically direct plated onto Dichloran 18% Glycerol (DG18) agar, five particles per plate (Pitt & Hocking, 2009). The plates were stacked and wrapped with household cling film and incubated at 25 °C. After 5–7 days the plates were inspected for fungal growth, and total infection percentage of the Brazil nuts was calculated. Infection percentage with *Aspergillus* section *Flavi*, section *Nigri* and with *Penicillium* spp. was also noted. All possible *Aspergillus* spp. were isolated onto Czapek Yeast Extract (CYA) agar to obtain pure cultures (Pitt & Hocking, 2009). After successful isolation, the fungi were grown on AFPA media for 7 days at 25ºC and reverse colour was noted. Fungi identified as potential producers of aflatoxins were inoculated onto Yeast Extract Sucrose (YES) agar for 7 days at 25ºC and the capability of isolates to produce aflatoxins was evaluated by the agar plug technique (Samson et al., 2010). Small pieces of the fungus and agar were cut out with a scalpel and extracted with chloroform: methanol (1:1). The plugs were placed on thin layer chromatography (TLC) plates, developed in a toluene: ethyl acetate:90% formic acid: chloroform (7:5:2:5) mobile phase, and visualized under UV light at 365 nm. A positive control with a mixture of aflatoxins B_1, B_2, G_1 and G_2(Sigma, St. Louis, USA) was used for comparison. With aid from supervisors Beatriz T. Iamanaka and Marta H. Taniwaki, the colonies were identified based on both macroscopic and microscopic characteristics on CYA, AFPA reverse colour and aflatoxin profile.

2.2.4 Aflatoxin analysis

Brazil nut kernels (25 g) from each sample were finely ground using a hand-held mixer. Extraction was performed by adding 2.5 g NaCl and 100 ml of methanol:water solution (8:2, v/v) and blending for 3 min at high speed (10,000 rpm) using an Ultra-Turrax homogenizer (Polytron, Switzerland). The homogenized solution was filtered twice, first through Nalgon 3551 filter paper and then Vicam microfiber filters. An aliquot of 10 ml filtrate was diluted in 60 ml of previously prepared phosphate buffered saline (PBS), after which it was passed through an Aflatest WB immunoafffinity column (Vicam, USA) at a flow rate of 2–3 ml/min, followed by washing with 30 ml distilled water. The columns were conditioned with PBS before application of the sample. The aflatoxins were eluted by first adding 500 µl HPLC-grade methanol and after 1 minute adding another 750 µl. The eluate was diluted with 1750 µl Milli Q water. A positive control was prepared by spiking a clean sample of nuts with no known growth of *Aspergillus* section *Flavi* with 100 µl of a mixture of aflatoxins B_1, B_2, G_1 and G_2(Sigma, St. Louis, USA). The control was treated in the same way as the samples.

For the quantification of aflatoxins in the samples, a chromatographic method was used. A Shimadzu LC-10VP HPLC system (Shimadzu, Japan) was used with a fluorescence detector set at 362 nm excitation and 455 nm emission for aflatoxins G_1 and G_2, and 425 nm emission for aflatoxins B_1 and B_2. A Shimadzu CLC G-ODS (4×10 mm) guard column and Shimadzu Shimpack (4.6×250 mm) column were employed. The mobile phase consisted of water: acetonitrile: methanol (6:2:3) containing 119 mg/lKBr and 4 M, 350 µl/l nitric acid. The flow rate was 1 ml/min. A post-column derivatization of aflatoxins B_1 and G_1 was performed with bromine using a KobraCell (R-Biopharm Rhône Ltd, Scotland). The injection volume was 100 µL.

3 Results and discussion

3.1 Growth study

In this part of the study, 17 isolates from *Aspergillus* section *Flavi* were grown on CYA at different temperatures, on CYA with different water activity and on AFPA. The isolates represented eight different species: *A. bombycis, A. arachidicola, A. caelatus, A. flavus, A. nomius, A. pseudocaelatus, A. pseudotamarii and A. tamarii,* as well as three unidentified species from section *Flavi.*

3.1.1 Colony appearance

In general, the colonies were in shades of yellowish to brownish green (Figure 1) with the exception of some more cream-coloured colonies due to low sporulation. Some differences between the species could be seen, but were not sufficient to consistently differentiate among the species. The reverse colour on AFPA varied between bright orange to weaker orange and maroon. In some of the isolates, contamination had occurred, making distinguishing between them even harder. The importance of working with pure, uncontaminated colonies was highlighted.

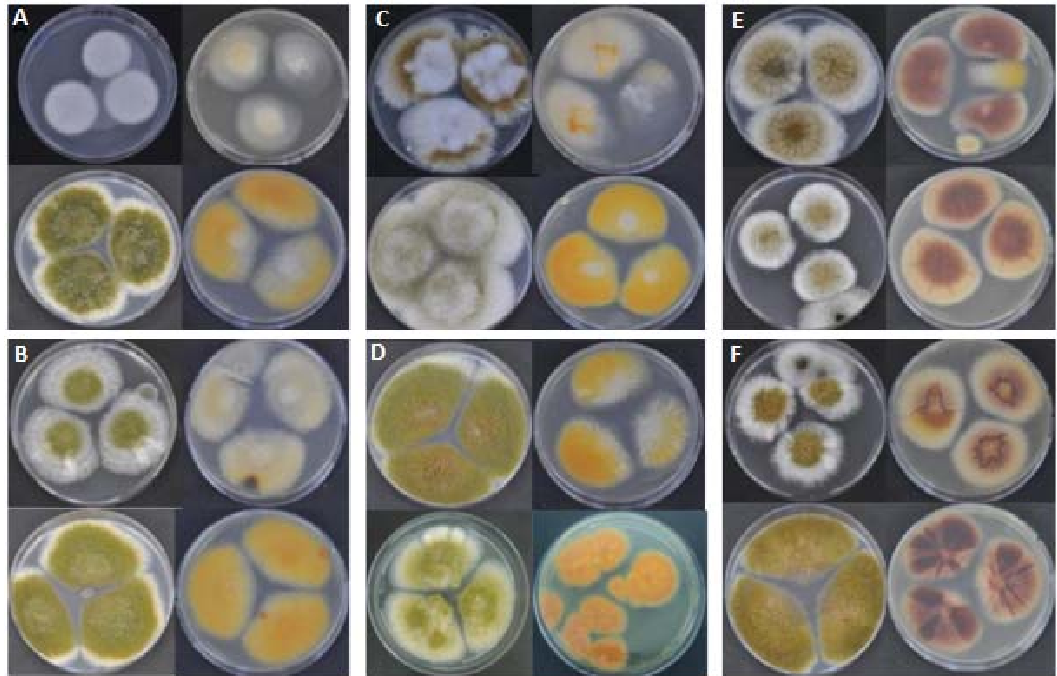

Figure 1. Twelve of the 17 isolates of *Aspergillus* section *Flavi*. The left column shows the colonies on CYA and the right column, reverse on AFPA, both after 7 days at 25 °C. There are two isolates from each species. A: *A. arachidicola*, B: *A. caelatus*, C: *A. nomius*, D: *A. flavus*, E: *A. pseudotamarii* and F: *A. tamarii. A. bombycis* and *A. pseudocaelatus,* as well as the three unknown isolates, were omitted from the picture, as only one isolate of each species were available.

3.1.2 Colony size at different temperatures and different water activities

Colony diameters for growth on CYA are listed in Table 2. All isolates grew well on CYA at 25 ° C and 37 °C, most reaching 40–60 mm in 7 days. A few of the isolates, 6942 F, 4207 F, 189 F and the three unknown species attained a diameter of only slightly more than 30 mm. At 42 °C, nine isolates grew, including both isolates of *A. tamarii, A. nomius, A. flavus* and the three unknown isolates. The ability to grow at 42 °C has previously proven a useful way to distinguish *A. bombycis* from *A. nomius* (Peterson *et al.*, 2001). As both produce aflatoxins type B and G, methods to differentiate between these two similar-looking species are desirable. *A. flavus* can be distinguished from the other two by its inability to produce G toxins. For the rest of the isolates, extent of growth at various temperatures did not yield useful characteristics for separation of the species, namely, the small variations in growth among the species at 25 °C and 37 °C was not enough to tell them apart. In addition, differences in growth were often observed for the two isolates belonging to the same species, i.e. intra-specific variation. The next step to obtain more robust results on potentially differential characteristics could be growth studies with several strains, possibly in combination with a wider range of temperatures.

Table 2. Growth of 17 isolates of Aspergillus *section* Flavi *on CYA at different temperatures and AFPA reverse colour after 7 days; colony diameter given in mm.*

Species	CYA 25°C	CYA 37°C	CYA 42°C	AFPA
A. caelatus	62.3	24.7	ng[1]	(+)[2]
A. caelatus	47.0	24.0	ng	(+)
A. tamarii	65.3	57.7	6.0	Maroon
A. tamarii	41.3	45.7	8.0	Maroon
A. pseudotamarii	34.3	31.3	ng	Maroon
A. pseudotamarii	55.7	30.0	ng	Maroon
A. Flavi (group)	36.0	46.3	4.7	(+)
A. Flavi (group)	48.7	59.0	12.0	(+)
A. Flavi (group)	56.0	44.7	9.3	(+)
A. nomius	45.0	44.3	8.0	(+) weak
A. nomius	60+	51.0	9.0	(+) weak
A. arachidicola	33.7	56.7	ng	(+) weak
A. arachidicola	55.7	26.0	ng	(+)
A. bombycis	54.0	14.0	ng	(+)
A. flavus	64.0	50.0	11.5	(+)
A. flavus	60.7	58.7	23.7	(++)
A. pseudocaelatus	57.7	60.7	ng	(+)

[1]ng, no growth

[2](+) indicates a bright orange colour and (++) a darker orange colour

Growth at different water activities is shown in Figure 2. All isolates grew well at a_w 0.97 and 0.94, moderately well at a_w 0.91 and slowly at a_w 0.87. No growth occurred at a_w 0.79 and this water activity was excluded from the graph. There are some differences among the species but not enough to differentiate among them by only using the impact of water activity on growth. Measuring growth at regular intervals to calculate the linear growth rate could yield useful data for differentiation, and possibly growth at particular a_w in combination with different temperatures.

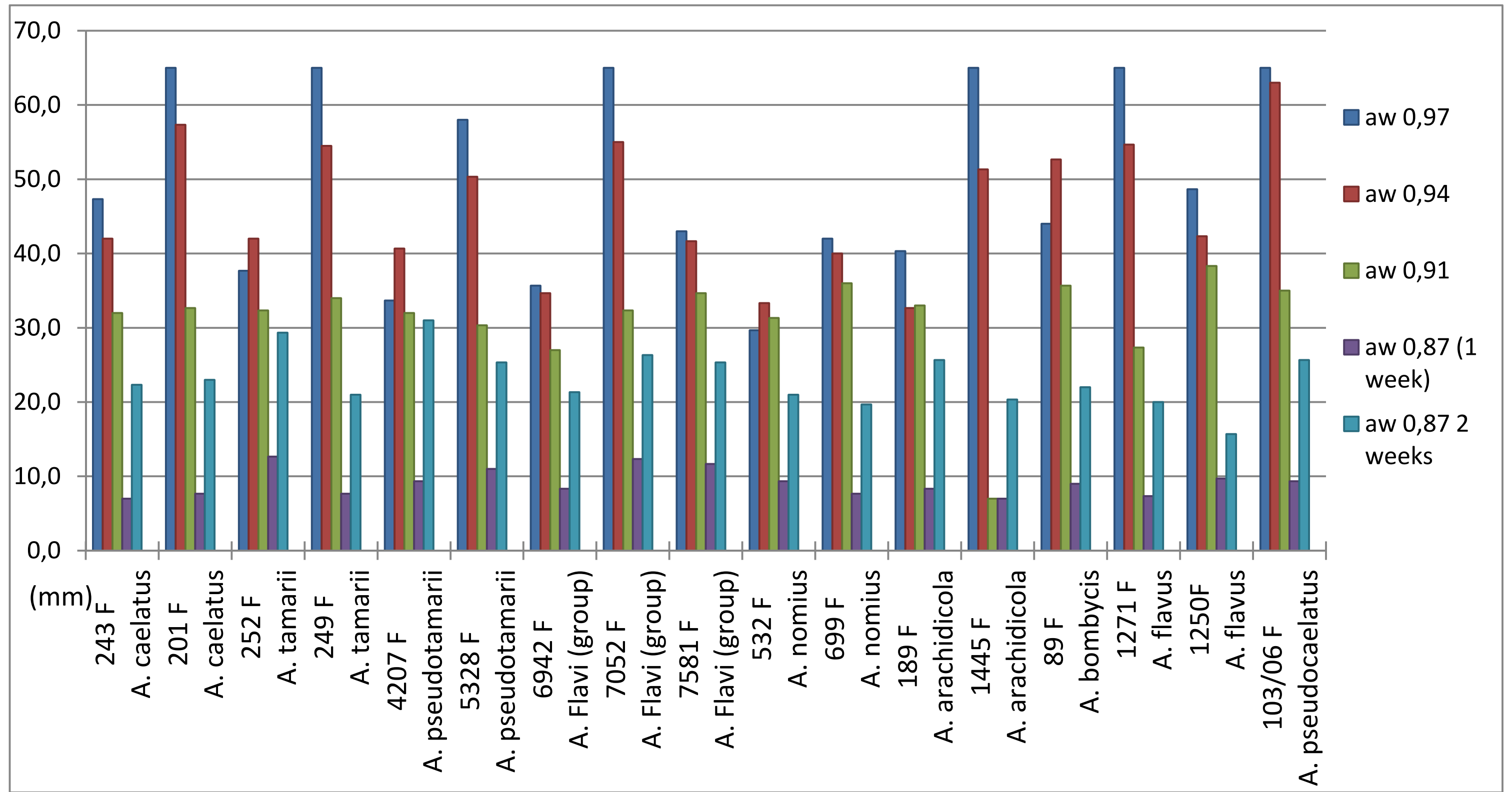

Figure 2. Colony diameters (mm) of 17 isolates of *Aspergillus* section *Flavi* at four different water activities after 1week. The 0.87 water activity is also shown after 2 weeks.

3.1.3 Summary: Growth

Individual characteristics, colony size, colony colour, growth at various water activities, and temperature tolerance, were not sufficient to distinguish the species within section *Flavi*. Macromorphology could be used to differentiate some of the species from others, but it was not enough to separate very similar looking species. Identification based on looking at the fungi takes practice, and requires some background knowledge of how the fungi should look. Temperature tolerance can be a good way of separating some species, but only at certain temperatures. Water activity seems to be the least reliable option, as the eight different species in this study gave similar results. In order to partly identify the species in section *Flavi*, one has to combine these three common identification features with each other. Further studies should also include micromorphology and probably also molecular methods. However, since many of the species are relatively new, appropriate keys are not yet available. It is important that a sufficient amount of replicates are used and preferably grown on the same batch of media to avoid differences within the same species that might complicate the identification. Other methods used for full identification include the production of aflatoxins and other extrolites (secondary metabolites), and molecular techniques.

3.2 Isolation and identification of toxigenic fungi from collected Brazil nuts.

The second part of this study aimed at isolating and identifying potentially toxigenic fungi in samples of Brazil nuts, and to evaluate the presence of aflatoxins in these samples.

3.2.1 Infection

In total, 10 samples of Brazil nut kernels were examined for total fungal and individual percentage endogenous infection by *Aspergillus* section *Flavi, Aspergillus* section *Nigri* and *Penicillium* spp. From three of these samples, the shell was also examined. Percentage infection is shown in Table 3 (raw data in Appendix 6.3). The average percentage fungal infection of the combined samples (nuts and shells) was 61 %, with a variation of 4–100 % infection between the samples. The highest infection rates occurred in the three shell samples (8, 9 and 10), and also in sample 8 kernel. The high presence of fungi in these samples correlated with the water activity, where sample 8 with the highest infection rate also had the highest water activity (Table 3). The samples with the highest overall infection rates also had the highest infection with *Aspergillus* section *Flavi*, with sample 8 again being the highest. The water activity of the shells was not tested. The lowest infection rate occurred in sample 6. The most common contaminants were *Aspergillus* section *Flavi*, with an average contamination of 21 %, closely followed by *Penicillium*

species with an average contamination of 20 %, both with a high variation between the samples. *Aspergillus* section *Nigri* had an infection percentage of 4.5 %, and 9 % of kernels were infected by other fungi not important from a mycotoxin perspective. In general, the samples with a high infection percentage of one fungal group were low in the others. For example, sample 10, which had no *Aspergillus* section *Flavi* infection, had the highest *Penicillium* infection. There is a competition between *Aspergillus* and *Penicillium* species with *Aspergillus* being more competitive at lower water activities than *Penicillium* (Pitt & Hocking, 2009). Sample 10 had a slightly lower a_w, which should give an advantage for *Aspergillus*. On the other hand, Penicillia tend to sporulate faster, which could explain the increased *Penicillium* growth on this sample.

Table 3. Water activity and infection percentage of Aspergillus *section* Flavi*, section* Nigri *and* Penicillium *species on Brazil nuts.*

Sample	Average water activity	Total infection (%)	A. section *Flavi* (%)	A. section *Nigri* (%)	*Penicillium* (%)	Others
1	0.55	12	0	2	2	4
2	0.49	54	2	2	6	20
3	0.54	38	0	4	6	14
4	0.57	62	4	0	10	24
5	0.61	18	2	0	2	7
6	0.67	4	0	2	0	1
7	0.65	22	0	14	2	3
8	0.96	100	100	28	18	2
9	0.88	90	26	2	44	9
10	0.78	92	0	0	80	19
8 Shell	NA	100	100	2	0	1
9 Shell	NA	100	2	0	24	7
10 Shell	NA	100	12	2	60	7
Average		61	19	4,5	20	9
Variation		4–100	0–102	0–28	0–80	

3.2.2 *Aspergillus* section *Flavi* from Brazil nuts

From the 10 samples of Brazil nuts and the 3 shell samples, 135-suspected *Aspergillus* section *Flavi* was isolated (Table 4). They were isolated from kernel sample 2, 4, 5, 8, 9 and 10 and from all shell samples. Samples 1, 3, 6 and 7 did not contain any *Aspergillus* section *Flavi* (Table 3).

Aflatoxin production was observed in 100 (74 %) out of the total 135 isolates (Table 4). Ninety-seven isolates produced aflatoxins B_1, B_2, G_1 and G_2, and three isolates produced only B_1, B_2 (Appendix 6.4).

Based on macromorphological differences, AFPA reverse colour (Appendix 6.4), and the ability to produce aflatoxins, the isolates were divided into different groups. In one group, the colony colour was white to cream with no sporulation. The reverse had a yellow colour on CYA and they were AFPA positive with an orange reverse. Aflatoxins B_1, B_2, G_1 and G_2 were produced. Isolates in the second group were also white with no sporulation, yellow reverse on CYA, and produced aflatoxins B_1, B_2, G_1 and G_2. However, isolates in this group were AFPA negative. AFPA results might not be fully reliable, as the medium was originally developed for *A. flavus* and *A. parasiticus.* For other species, such as *A. nomius,* results may vary. The third group of isolates had a green colour with abundant sporulation and produced aflatoxins B_1, B_2, G_1 and G_2. They were AFPA positive. In the first inoculation on CYA, two different batches of Czapek solution were used in the media, giving a consistent difference in colony appearance between the two batches. A second inoculation, using a freshly made Czapek solution showed that these isolates did indeed sporulate. Based on the new morphological results, production of aflatoxins B_1, B_2, G_1, G_2 and microscopic studies following appropriate keys, these first three groups were presumptively identified as *A. nomius* (Table 4). *A. nomius*, which produces all four aflatoxins, has previously been found to be of importance in Brazil nuts because it is frequently isolated from this substrate (Goncalves *et al.*, 2011).

The next two groups were combined and listed as possible *A. flavus*. One group had isolates with only little sporulation, were all aflatoxin negative and AFPA positive, whereas the other group had more sporulation, were AFPA positive and produced B_1 and B_2 toxins. Not all isolates of *A. flavus* are toxigenic and when they are, they solely produce the B-type aflatoxins (Johnsson *et al.,* 2008). These isolates grew on CYA at 37 and 42 °C, and combining this information with colony appearance and microscopic studies, they were presumptively identified as *A. flavus* (Table 4).

The last group, consisting of possible *A. tamarii* (Table 4), had brown colonies on CYA with abundant sporulation, a maroon reverse on AFPA and was aflatoxin negative. Representatives from each group were selected and inoculated onto CYA at 37 and 42 C°. The representatives all grew at these temperatures. One isolate was never identified due to problems with repeated contamination and the time limitations of this study; this isolate was non-toxigenic.

Table 4. Identified species, toxin production and positive AFPA results.

Identified species	Number of isolates	Toxin production	Positive AFPA reverse
A. nomius	97	100%	86%
A. flavus	34	9%	100%
A. tamarii	3	0%	0%
A. Flavi	1	0%	0%
Total	135	74%	87%

3.2.3 Aflatoxin in Brazil nuts

Aflatoxins were not detected in kernel samples 1, 2, 3, 4, 5, 6, 9 and 10. In samples 7 and 8, the total aflatoxin contents were 0.054 µg/kg and 0.089 µg/kg respectively (Table 5), both well below the European legislative limit of 10 µg/kg. It is of note that *Aspergillus* section *Flavi* was not isolated in sample 7, and yet aflatoxins were detected, indicating production of the toxins at an earlier stage in the kernels, after which the fungi then died. The Brazil nuts in sample 7 had low water activity, 0.65, which could be the result of sufficient drying, killing off the fungi. Correlation between fungal presence and aflatoxin contamination is not always observed (Iamanaka *et al.*, 2006). Sample 8 had 100 % infection with *Aspergillus* section *Flavi* and a higher water activity (Table 3). In sample 9, 22% of the nuts were infected with *Aspergillus* section *Flavi*, but no aflatoxins were found.

Table 5. Aflatoxin content in Brazil nuts.

Sample	µg/kg
1–6	0
7	0.054
8	0.089
9–10	0

3.2.4 Summary: Fungal infection and aflatoxin production in Brazil nuts

Samples 7 and 8 were the only two samples in which aflatoxins were detected. The total aflatoxin contents were 0.054 µg/kg and 0.089 µg/kg respectively. *A. nomius* was the most commonly isolated species of section *Flavi,* followed by *A. flavus,* and a few *A. tamarii.* In general, the water activity of the Brazil nut kernels was low, with only two of the samples having a_w over 0.87. As the growth study results showed (section 3.1.2), these species do not grow well at lower water activities (<0.87 a_w).

3.3 Conclusions

Colony appearance at different temperature is not a good way of distinguishing between *Aspergillus* section *Flavi* species used in this experiment. Most colonies were yellowish-green with *A. tamarii* and *A. pseudotamarii* standing out with a more brown-green colour. Colour, texture and size seemed to depend on the amount of sporulation, which could vary with different batches of media. No conclusion could be drawn regarding growth at different water activities, as a larger-scale growth study is needed, comparing the growth over time combined with different temperatures and with more replicates. The information gained from temperature and growth studies has to be combined with microscopic characters,

extrolite production such as toxin- and exudate production, and possibly molecular identification in order to fully identify the fungi. However, after working with the fungi over a longer period, one might become more skilled in using the above information, as it takes a lot of practice to see small differences between colonies by eye.

All species in this experiment grew well at the temperatures 25 and 30°C, and at the water activities 0.91, 0.94 and 0.97, meaning that the fungus is very capable of growing in the warm and humid environment of the Amazonas rainforest. As not only one species contributes to aflatoxin content, it may not be necessary to always identify the *Aspergillus* strains down to species level in order to assess the risk of aflatoxin contamination, even though some species are non-toxigenic. For producers, merchandisers and re-sellers of Brazil nuts, focus should be on drying and storing the nuts in such a way that the risk of *Aspergillus* section *Flavi* contamination in general is kept to a minimum.

Aflatoxin was detected in 2 out of 10 samples. In both samples, the toxin-level was well below the new European legislative limit of 10 µg/kg. Water activity played a role for the amount of aflatoxigenic fungi found on the Brazil nuts, and nuts purchased with the shell still on had the highest water activity. Buying and consuming pre-shelled nuts with adequate drying probably reduces the risk of aflatoxigenic fungi in general, but does not completely reduce the risk of aflatoxins. As one sample result showed, toxins may be present in the nuts even though the water activity is low and no fungi are found. However, all the nuts from the 10 different market stalls in this study would be considered safe to consume.

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5 References

CAC (2010) Codex Alimentarius Commission. *Report of the Fourth Session of the Codex Committee on Contaminants in Foods*. CL 2010/13-CF. Izmir, Turkey. Joint FAO/WHO Food Standards Program, Rome, Italy.

Collinson, C., Burnett, D., Agreda, V. (2000). Peru. Natural Resources and Ethical Trade Program, Natural Resources Institute, University of Greenwich.

Commission Regulation (1998) No 1525-98. *Amending Commission Regulation (EEC) No 752/93 laying down provisions for the implementation of Council Regulation (EEC) No 3911/92 on the export of cultural goods*. Official Journal of the European Communities, L 201/47

Commission Regulation (2010) No 165/2010.*Amending Regulation (EC) No 1881/2006 Setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins*. Official Journal of the European Union**,** L 50/8

EFSA (2012) European Food Safety Authority - Aflatoxins in food. Available at: http://www.efsa.europa.eu/en/topics/topic/aflatoxins.htm?wtrl=01. Accessed 2012-08-22

FAO (1991) Food and Agriculture Organization. *Mycotoxins and food supply***.** Food, Nutrition and Agriculture. Available at: http. Accessed 2012-08-22

FAO (1993) Food and Agriculture Organization. *Selected species and strategies to enhance income generation from Amazonian forests*. Working Paper. Available at: http://www.fao.org/docrep/V0784E/V0784E00.htm. Accessed 2012-08-22

Goncalves J.S., Ferracin, L.M., Carneiro Vieira, M.L., Iamanaka, B.T., Taniwaki, M.H., Pelegrinelli Fungaro, M.H. (2012) *Molecular analysis of Aspergillus section Flavi isolated from Brazil nuts.* World Journal of Microbiology and Biotechnology 28:1817-1825

Iamanaka, B.T., Castle de Menezes, H., Vicente, E., Leite, R.S.F., Taniwaki, M.H. (2007) *Aflatoxigenic fungi and aflatoxins occurrence in sultanas and dried figs commercialized in Brazil.* Food Control 18: 454–457

IARC (n.d.). International Agency for Research on Cancer (IARC). Monograph 100F – Aflatoxins. Available at: http://monographs.iarc.fr/ENG/Monographs/vol100F/mono100F-23.pdf. Accessed 2012-08-22

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 Journal, 1: 127-137

Newing, H., Harrop, S. (2000). *European Health Regulations and Brazil nuts: Implications for Biodiversity Conservation and Sustainable Rural Livelihoods in the Amazon*. Journal of International Wildlife Law and Policy 3: 109-124.

Olsen, M., Johnsson, P., Möller, T., Paladino, R., Lindblad, M. (2008). *Aspergillus nomius*, an important aflatoxin producer in Brazil nuts? World Mycotoxin Journal 1: 123–126.

Pitt, J.I., & Hocking, A.D. (2009). *Fungi and food spoilage*. Springer, New York, USA.Third edition.

Samson, R.A., Houbraken, J., Frisvad, J.C., Thrane, U., Andersen, B. (2010). Food and Indoor fungi.CBS Laboratory Manual series 2. Utrecht : CBS-KNAW Fungal Biodiversity Centre.

Shanley, P., Pierce, A.R., Laird, S.A., Guillen, A. (2002). *Tapping the Green Market: Certification and Management of Non-Timber Forest Products*. Earthscan Publications Ltd, United Kingdom. First edition.

NFA (2012) Swedish National Food Agency. Livsmedelsverkets livsmedelsdatabas version 2012-01-26 Available at: http://www7.slv.se/Naringssok/Naringsamnen.aspx. Accessed: 2012-08-22

STDF (2008) Standards and Trade Development Facility, Project 114 - SafeNut. Available at: http://www.stdf-safenutproject.com. Accessed 2012-08-22

Tillet, T. (2010). *Carcinogenic Crops: Analyzing the Effect of Aflatoxin on Global Liver Cancer Rates.* Environmental Health Perspectives, 118:A 258.

6 Appendices

6.1 Water activity of CYA media

Replicates	Water activity				
1	0.787	0.867	0.909	0.940	0.970
2	0.788	0.869	0.914	0.940	0.968
3	0.785	0.865	0.912	0.942	0.973
Averagea_w	0.79	0.87	0.91	0.94	0.97

6.2 Water activity of Brazil nut kernels

Sample	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
	0.542	0.513	0.529	0.606	0.607	0.665	0.672	0.955	0.885	0.778
	0.556	0.487	0.551	0.567	0.613	0.672	0.672	0.962	0.875	0.783
	0.548	0.481	0.55	0.539	0.605	0.677	0.618	0.963	0.882	0.783
Average	0.55	0.49	0.54	0.57	0.61	0.67	0.65	0.96	0.88	0.78

6.3 Infection with total fungi, *Aspergillus* section *Flavi,* section *Nigri* and *Penicillium* species on Brazil nuts

Sample	Total infection	Infection (%)	A. section *Flavi*	A. section *Flavi* %	A. section *Nigri*	*Nigri* %	*Penicillium*	*Penicillium* %	Others
1	6	12	0	0	1	2	1	2	4
2	27	54	1	2	1	2	3	6	22
3	19	38	0	0	2	4	3	6	14
4	31	62	2	4	0	0	5	10	24
5	9	18	2	4	0	0	1	2	7
6	2	4	0	0	1	2	0	0	1
7	11	22	0	0	7	14	1	2	3
8	50	100	52	104	14	28	9	18	2
9	45	90	22	44	1	2	22	44	9
10	46	92	0	0	0	0	40	80	19
8 Shell	50	100	50	100	1	2	0	0	1
9 Shell	50	100	1	2	0	0	12	24	7
10 Shell	50	100	6	12	1	2	30	60	7
Average		61		21		4.5		20	9
Variation		4-100		0-102		0-28		0-80	

6.4 Aflatoxin profile and AFPA reverse of presumptive species of *Aspergillus* section *Flavi* isolated from Brazil nuts

ITAL number	Presumptive identification	Toxin production	AFPA reverse
8021	*A. flavus*	(-)	(+)
8188	*A. nomius*	B1, B2, G1, G2	(-)
8189	*A. nomius*	B1, B2, G1, G2	(+)
8176 A	*A. flavus*	(-)	(+)
8176 B	*A. tamarii*	(-)	Maroon
8027	*A. flavus*	(-)	(+) centre
8028	*A. nomius*	B1, B2, G1, G2	(+)
8029	*A. nomius*	B1, B2, G1, G2	(+)
8030	*A. nomius*	B1, B2, G1, G2	NA
8031	*A. nomius*	B1, B2, G1, G2	(+)
8032	*A. flavus*	(-)	(++)
8033	*A. nomius*	B1, B2, G1, G2	(+)
8034	*A. nomius*	B1, B2, G1, G2	(+)
8035 A	*A. nomius*	B1, B2, G1, G2	(+)
8035 B	*A. flavus*	(-)	(+) centre
8036	*A. nomius*	B1, B2, G1, G2	(+)
8037	*A. nomius*	B1, B2, G1, G2	(+)
8038	*A. nomius*	B1, B2, G1, G2	(+)
8039	*A. nomius*	B1, B2, G1, G2	(-)
8040	*A. flavus*	(-)	(++)
8041	*A. nomius*	B1, B2, G1, G2	(+)
8042	*A. nomius*	B1, B2, G1, G2	(-)
8043	*A. nomius*	B1, B2, G1, G2	(++)
8044	*A. flavus*	(-)	(+) centre
8045	*A. nomius*	B1, B2, G1, G2	(-)
8046	*A. nomius*	B1, B2, G1, G2	(+)
8047	*A. nomius*	B1, B2, G1, G2	(-)
8048	*A. nomius*	B1, B2, G1, G2	(+) centre
8049	*A. flavus*	(-)	(+) centre
8050	*A. nomius*	B1, B2, G1, G2	(+) weak
8051	*A. nomius*	B1, B2, G1, G2	(+)
8052	*A. nomius*	B1, B2, G1, G2	(+)
8053	*A. nomius*	B1, B2, G1, G2	(+) weak
8054	*A. nomius*	B1, B2, G1, G2	(+) weak
8055	*A. nomius*	B1, B2, G1, G2	(+)
8056	*A. nomius*	B1, B2, G1, G2	(+) weak
8057	*A. nomius*	B1, B2, G1, G2	(+)
8058	*A. flavus*	(-)	(++)

8059	*A. nomius*	B1, B2, G1, G2	(++)
8060	*A. flavus*	(-)	(++)
8061	*A. nomius*	B1, B2, G1, G2	(+)
8062	*A.flavus*	(-)	(+)
8063	*A. flavus*	(-)	(++)
8064 A	*A. nomius*	B1, B2, G1, G2	(+)
8064 B	*A. nomius*	B1, B2, G1, G2	(+) centre
8065	*A. flavus*	(-)	(++)
8066	*A. nomius*	B1, B2, G1, G2	(+)
8067	*A. nomius*	B1, B2, G1, G2	(+)
8068	*A. nomius*	B1, B2, G1, G2	(+)
8069	*A. nomius*	B1, B2, G1, G2	(+)
8070	*A. flavus*	(-)	(++)
8071	*A. flavus*	(-)	(++)
8072	*A. nomius*	B1, B2, G1, G2	(+)
8073	*A. nomius*	B1, B2, G1, G2	(+) centre
8075	*A. nomius*	B1, B2, G1, G2	(+)
8076	*A. nomius*	B1, B2, G1, G2	(+) weak
8132	*A. nomius*	B1, B2, G1, G2	(+) weak
8077	*A. nomius*	B1, B2, G1, G2	(-)
8078	*A. nomius*	B1, B2, G1, G2	(+)
8079	*A. flavus*	(-)	(++)
8080	*A. nomius*	B1, G1	(+) centre
8081	*A. nomius*	B1, B2, G1, G2	(+)
8082	*A. nomius*	B1, B2, G1, G2	(+)
8083	*A. nomius*	B1, B2, G1, G2	(+)
8084	*A. nomius*	B1, B2, G1, G2	(+)
8085	*A. flavus*	B1, B2	(++)
8086	*A. nomius*	B1, B2, G1, G2	(++)
8087	*A. flavus*	(-)	(++)
8088	*A. flavus*	(-)	(+) centre
8090	*A. flavus*	(-)	(++)
8091	*A. nomius*	B1, B2, G1, G2	(+)
8092	*A. nomius*	B1, B2, G1, G2	(+)
8093	*A. nomius*	B1, B2, G1, G2	(+) weak
8094	*A. flavus*	(-)	(++)
8095	*A. nomius*	B1, B2, G1, G2	(+) weak
8096	*A. nomius*	B1, B2, G1, G2	(-)
8097	*A. nomius*	B1, B2, G1, G2	(+)
8098 A	*A. nomius*	B1, B2, G1, G2	(++)
8098 B	*A. nomius*	B1, B2, G1, G2	(+)
8099	*A. nomius*	B1, B2, G1, G2	(+) weak
8100	*A. nomius*	B1, B2, G1, G2	(+)
8101	*NA*	(-)	NA

8102	*A. flavus*	(-)	(+) centre
8103	*A. nomius*	B1, B2, G1, G2	(+) weak
8104	*A. nomius*	B1, B2, G1, G2	(+)
8105	*A. nomius*	B1, B2, G1, G2	(++)
8106	*A. nomius*	B1, B2, G1, G2	(+)
8107	*A. nomius*	B1, B2, G1, G2	(+)
8108	*A. nomius*	B1, B2, G1, G2	(+)
8109 A	*A. nomius*	B1, B2, G1, G2	(+) weak
8110	*A. flavus*	(-)	(++)
8111	*A. nomius*	B1, B2, G1, G2	(-)
8112	*A. flavus*	(-)	(++)
8113	*A. nomius*	B1, B2, G1, G2	(++)
8114	*A. flavus*	(-)	(++)
8115	*A. nomius*	B1, B2, G1, G2	(-)
8116	*A. flavus*	(-)	(++)
8117	*A. flavus*	(-)	(+) centre
8118	*A. nomius*	B1, B2, G1, G2	(+)
8119	*A. nomius*	B1, B2, G1, G2	(+)
8120	*A. nomius*	B1, B2, G1, G2	(+)
8122	*A. nomius*	B1, B2, G1, G2	(-)
8123	*A. nomius*	B1, B2, G1, G2	(-)
8124	*A. nomius*	B1, B2, G1, G2	(-)
8125	*A. nomius*	B1, B2, G1, G2	(-)
8126	*A. nomius*	B1, B2, G1, G2	(+)
8135	*A. nomius*	B1, B2, G1, G2	(+)
8136	*A. nomius*	B1, B2, G1, G2	(+)
8137	*A. nomius*	B1, B2, G1, G2	(++)
8138	*A. nomius*	B1, B2, G1, G2	(+)
8139	*A. nomius*	B1, B2, G1, G2	(+)
8140	*A. nomius*	B1, B2, G1, G2	(++)
8141	*A. nomius*	B1, B2, G1, G2	(+)
8142	*A. nomius*	B1, B2, G1, G2	(+)
8143	*A. nomius*	B1, B2, G1, G2	(+)
8144	*A. nomius*	B1, B2, G1, G2	(++)
8145	*A. nomius*	B1, B2, G1, G2	(+)
8146	*A. nomius*	B1, B2, G1, G2	(+)
8147	*A. nomius*	B1, B2, G1, G2	(+)
8151	*A. nomius*	B1, B2, G1, G2	(+)
8154	*A. nomius*	B1, B2, G1, G2	(+)
8155	*A. nomius*	B1, B2, G1, G2	(+)
8156	*A. nomius*	B1, B2, G1, G2	(+)
8157	*A. flavus*	(-)	(++)
8158	*A. nomius*	B1, B2, G1, G2	(+) weak
8159	*A.flavus*	(-)	(++)

8160	*A. nomius*	B1, B2, G1, G2	(+)
8161	*A. nomius*	B1, B2, G1, G2	(+)
8150	*A. tamarii*	(-)	Maroon
8165	*A. flavus*	B1, B2	(++)
8166	*A. flavus*	(-)	(++)
8167	*A. flavus*	(-)	(+)
8168	*A. flavus*	B1, B2	(++)
8169	*A. tamarii*	(-)	Maroon
8170	*A. flavus*	(-)	(++)