Characterization of Aspergillus section Flavi : molecular markers as tools to unmask cryptic species

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▶ **To cite this version:**

Amaranta Carvajal-Campos. Characterization of Aspergillus section Flavi : molecular markers as tools to unmask cryptic species. Toxicology. Université Paul Sabatier - Toulouse III, 2018. English. NNT : 2018TOU30036 . tel-02073580

HAL Id: tel-02073580

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Université Fédérale
Toulouse Midi-Pyrénées

THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :

Université Toulouse 3 Paul Sabatier (UT3) Paul Sabatier

Présentée et soutenue par :

Amaranta CARVAJAL CAMPOS

Titre:

Characterization of *Aspergillus* section *Flavi*: molecular markers as tools to unmask cryptic species

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Discipline ou spécialité :
ED SEVAB : Pathologie, Toxicologie, Génétique et Nutrition

Unité de Recherche :
UMR 1331 INRA-ToxAlim

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Hoje de manhã saí muito cedo,
Por ter acordado ainda mais cedo
E não ter nada que quisesse fazer...

Não sabia que caminho tomar
Mas o vento soprava forte, varria para um lado,
E segui o caminho para onde o vento me soprava nas costas.

Assim tem sido sempre a minha vida, e
Assim quero que possa ser sempre --
Vou onde o vento me leva e não me
Sinto pensar.

Alberto Caeiro (Fernando Pessoa)

ACKNOWLEDGMENTS

Becoming a PhD candidate means to start a journey, an intellectually and personal one. Intelectually it forces the person to learn, to become flexible, while increasing his/her skills to survive in the laboral world. It is not a smooth trip, it comes with ups and downs, yet it is a path that teaches you to become a better professional. For me, besides of the aforementioned, it also opened the door into the world of toxic mold and their impact in Human Health. At a personal level it has also been quite an adventure, sometimes more a roller coaster. This thesis gave me the opportunity to experience a new culture and to learn a new language, which is not necessary a piece of cake. Despite the difficulties during this period, I have been supported by a bunch of amazing people, that have helped me intellectually and personally. Find in these acknowledgements my most sincere gratitude to all of you, and if I forgot someone, I apologize to you in advance.

This Doctoral Thesis was sponsored by an scholarship from the Ecuadorian Government entity SENESCYT (from it initials in Spanish, Secretaria de Educación Superior, Ciencia, Tecnología e Innovación).

Firstly, I would like to express my sincere gratitude to my advisor Dr. Isabelle Oswald, Director of the Biosynthesis and Toxicity of Mycotoxins Team, who gave me the opportunity to perform my thesis at Toxalim INRA and welcome me in the team. Isabelle, I am really grateful to you for the opporunity and also for all your advice and support during this process. I have learnt a lot from you.

I would also thank Mr. Bernard Salles, former Director of UMR 1331 Toxalim INRA, Toulouse for letting me perform my research in the facilities.

I would also like to express my special appreciation and gratitude to my advisor Dr. Olivier Puel. I would like to thank you for encouraging my research and for your support during these years, I known that has not always being an easy task, and I appreciate all your efforts. I am grateful for your sense of humor, your taste in music, your open door whenever I needed, for letting me got lost and for guiding me to find the way out, for the practical and intelectual help and advice. I must also thank Dr. Sophie Lorber for always being there, I sincerely appreciate all your advice, your help in the lab, your smile, your support, intellectual and personal, and your guide in this proccess. It has been a pleasure learning from you. I would also thank Dr. Jean-Denis Bailly and Ms. Sylviane Bailly, I really appreciate a lot your advice during these years, I have learnt a lot from both of you, you have helped me to understand fungi world and how complicated they can be, in addition I have felt your support at different crucial moments, thanks for that and your cheerful spirit.

I would also thank the members of my thesis committee, Dr. Hervé Gryta, Dr. Vessela Anatovasova-Penichon, Dr. Jean-Denis Bailly and MSc Sylviane Bailly for all your advice and suggestions regardless the work performed during these years.

I would also thanks the members of the jury, my “rapporteurs”, Pr. Schorr-Galindo and Pr. Dupont, and my “examinateurs”, Dr. Gryta and Dr. Brabet, for accepting to read, judge, comment and provide advice for this manuscript and for being part of the jury.

I thanks as well Soraya Tadrist for all her help in the lab, we have sweat together, and without you finishing my experiments would have been even harder. I am grateful that we learnt to get along, and for the nice moments and laughs that we shared. As well I want to express my gratitude to Joëlle, I have learnt a lot from you, your enthusiasm, your professionalism and your character have been an example during these years. I cannot forget all the people that are part of the team, and wich plays a keyrole in my journey even though we do not necessarily work together. Thank you Phillipe for having your door always open and your smile all the time, I felt that I could always count on you, and that is priceless. In addition I want to say thanks to Sylvie, Anne-Marie and Arlette, you are a really good example of proffesionalism, enthusiastic and approachable people, I enjoyed the moments we shared. Selma, thanks for your help and advice in the lab and with French, you gave me a good push at the begining. I am also deeply grateful for the opportunity to known, shared and learnt from the different people that have passed by the team, Immourana, Pascal, Joanna, Delphine, Gisella, Leticia, Viviane, Delphine, Abdula, Su, Ophelie, Crisitine, Claire, Yannick, Tarek, Christian.

I am also really grateful to Dr. Catherine Brabet and Lethicia Manizan for our colaboration, which has lead to an important part of the work presented in this manuscript. Our collaboration is a good example of the importance of sharing between scientific groups.

I would also thank Sarah, Jeannette, Vincent, Vu, Pierre, Aurore and Lolita…. Each one of you are an important part in my journey, the first three for the administrative and computational stuff, and the good spirit and the laughs, Vu for your company in the latest hours and for being so nice and kind (eventhough I am the most desorganized person of the team), and the last three for keeping me alive and in good health by feeding me with amazing food. Thanks for doing your jobs professionally, with enthusiasm and with a smile, you made my doctoral experience easier and more enjoyable.

During this period of time, and in the lab, I have the opportunity to met several women to whom I really grateful… I have shared with all of you unforgetable moments….

Isaura, I have not enough words to express my gratitude to you. At the begining you guided me in this strange and complex country, providing me with the bases of the language, and the clues to not get lost in Toulouse. You welcome me not only in your office, you had not option, but also as a friend. I appreciate enourmously the way you share your knowledge, you are an incredible lab teacher. Thanks for always been in a good mood, even if you are feeling blue. We have shared several moments, mostly good ones, and English does not let me express to you how glad I am to met you and to have your friendship, thanks a lot for coping with my moody character and for being there!! You are an amazing friend. Te quiero chiqui!!!

Christelle, habibie, you were also forced to share your office with me, so you could not scape, as you were obligated to get along with me. Thanks for being there, for your support, your frienship, for the fun, for your crazyness, for letting me destroy your French, for sharing Toulouse, Poland, Leban, Ghent and the long days hanging out with me. I am really glad to met you and to say that you are my friend for lifetime. Also I am grateful with all your familly for making me feel at home.

Bianca, my darling, thanks for everything, you frienship means a lot to me, thank you for the laughs, for your support in the good and bad times, for all the experiences shared. Joya, thanks a lot for your friendship, your good mood, your smile, your kindness. Alix, thanks for the French lessons, for the rides to the lab, for the laughs, the cakes, the carefree spirit, the remarks, for the carnival, Halloween and the game nights. Laura C., thank you for you help, your patience, your kindness, your jokes, your draws, your French, your crazyness, the moments shared and most important for your friendship, you mean a lot to me. Rhoda, thank you for your advice, your sense of humor, your fiendship, your skills with languages, your cupcakes. Manon, thanks for the laughs, the crazyness, the French lessons, your opinions, your friendship, your sense of humor. Chloé, thanks for the French, laughs, and support. Laura S., thank you for putting me in perspective, for your jokes, for your crazyness and lovely spirit, for the music, the gifs, the tiny dances.

Outside the lab, I also want to thank several people. Thanks Ana L., Sebas A. and Micaela for being there and listened to me, and for gave me good advice, intellectually and personally. Francisca Hervas for your help. Pierre T. thanks for your wife (just a joke), your sense of humor and jokes, your French lessons, and for welcome me in your home. Ana P. thanks for all, you are always there, you are my soulmate. Sofy thanks for always being there, does not matter the km between us, you are always there to make me feel safe. Thanks to the clan of correziens and others for the great and funny experiences, for the French free lessons, for showing me another part of France, basically for letting me hang out with you and appreciate even more your country, especially to Melina, Hugo and Julien. To Pablo and Marian for the good memories and delicious food. To Sebastian D. for the friendship.

Last but not the least, I am deeply grateful for the continuous support, insight and patience of my family. Aita, thank you for always being there, I really appreciate how you handle my roller coaster of emotions. Thank you for being by best friend, my dad, a guide, for pushing me to follow my dreams, for recall me that life is a journey and we have to enjoy and learn from it… and to keep going, does not matter if the road looks impossible, it always will bring you to a new place. Thank you ñaño and mamu for being there, for recall me all the time that we can be apart geographically, but we have always each other. I love you, and I cannot be more grateful for having you as my family.

CONTENT

PUBLICATIONS

ARTICLES

- **Carvajal-Campos, A.,** Manizan, A.L., Tadrist, S., Akaki, D.K., Koffi-Nevry, R., Moore, G.G., Fapohunda, S.O., Bailly, S., Montet, D., Oswal, I.P., Lorber, S., Brabet, C., Puel, O. (2017). ***Aspergillus korhogoensis*, a Novel Aflatoxin Producing Species from the Côte d'Ivoire**. *Toxins*, *9*(11), 353.

- Manizan, A.L., **Carvajal-Campos, A.,** Akaki, D.K., Koffi-Nevry, R., Montet, D., Oswald, I.P., Lorber S., Puel, O., Brabet, C. **Biodiversity of *Aspergillus* isolates potentially aflatoxigenic recovered from peanuts in Côte d'Ivore.** (Manuscript in preparation)

- Makhlouf J., **Carvajal-Campos A.**, Querin A., Tadrist S., Puel O., Lorber S., Oswald I.P., Hamze M., Bailly J.D., Bailly S. **Biodiversity of *Aspergillus* section *Flavi* in spices marketed in Lebanon.** (Manuscript submitted)

REVIEW

- **Carvajal-Campos, A**., Oswald, I.P., Lorber, S., Puel, O. **Biodiversity of *Aspergillus* section *Flavi*: following the traces of crypsis.** (Manuscript in preparation)

COMMUNICATIONS

ORAL

- **Carvajal-Campos A.,** Manizan A.L., Tadrist S., Akaki, D.K., Koffi-Nevry, R., Moore, G.G., Fapohunda, S.O., Bailly, S., Montet, D., Oswal, I.P., Lorber, S., Brabet, C., Puel, O. **Diving into Guinean Gulf Aspergillus section Flavi diversity: description of a new aflatoxin producing species**. 1 st International MycoKey conference, Ghent, Belgium (14-17, September, 2017).

- **Carvajal-Campos A.,** Manizan A.L., Tadrist S., Akaki, D.K., Koffi-Nevry, R., Bailly, S., Montet, D., Oswal, I.P., Lorber, S., Brabet, C., Puel, O. **Description of a new aflatoxigenic species of Aspergillus section Flavi from Guinean Gulf.** 39th mycotoxins workshop, Bydgoszcz, Poland (19-21, June, 2017).

- Caceres I., **Carvajal-Campos A.,** Oswald I.P., Puel O., Bailly J.D. **Des remèdes naturels pour barrer la route aux toxines des moisissures.** Tête à tête avec des jeunes chercheurs, Musée de Toulouse, Toulouse, France (3, April, 2016).

POSTER

- Orlando, B., Bailly, S., El Mahgubi A., **Carvajal-Campos, A**., Puel, O., Oswald, I.P., Bailly, J.D. **Occurrence and identification of *Aspergillus* of the *Flavi* section and Aflatoxins emergence in French maize.** 10th conference of The World Mycotoxin Forum, Amsterdam, The Netherlands (12-14, March, 2018)

FIGURE LIST

01 INTRODUCTION

02 EXPERIMENTAL WORK

2.1 CHAPITRE 1: MOLECULAR *FLAVI* TOOL

2.2 CHAPITRE 2: Biodiversity of *Aspergillus* isolates potentially aflatoxigenic recovered from peanuts in Côte d'Ivore

2.3 CHAPITRE 3: *Aspergillus korhogoensis*, a novel aflatoxin producing species from the Côte d'Ivoire

2.4 CHAPITRE 4: Identification of *Aspergillus* section *Flavi* in French maize

03 GENERAL DISCUSSION AND PERSPECTIVES

TABLE LIST

ABREVIATIONS LIST

Amds	Acetamidase gen
AF(s)	Aflatoxin(s)
AFB	Aflatoxin B
AFG	Aflatoxin G
AflP	O-methyltransferase A gene
aflF-aflU	Aflp-AlfU region
BenA	β-tubulin gen
BI	Bayesian Inference
BIC	Bayesian Inference Criterion
bp	Base pairs
BP	Bootstrap percentages
CAST	Council for Agricultural Science and Technology
CC	Climate change
CmdA	Calmodulin gen
CPA	Cyclopiazonic acid
CYA	Czapek Yeast Autolysate Agar
DNA	Deoxyribonucleic acid
EU	European Union
FB	Fumonisin B
FAO	Food and Drug Administration
IARC	International Agency for Research on Cancer
ITS	Nuclear Ribosomal Internal Transcribed Spacer
MAT	Mating type loci
MEA	Malt Extract Agar
Mcm7	Minichromosome maintenance protein
ML	Maximum Likelihood
NRPS	Non-ribosomal Peptide Synthase
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PKS	Polyketide Synthase
PP	Posterior probability
Ppga	Pheromone precursor ppgA
PreA	Pheromone receptors preB
PreB	Pheromone receptors preA
OMST	O-methylsterigmatocystin

OTA	Ochratoxin A
Rpb1	RNA polymerase II, largest subunit
SBG	Strains of Aspergillus flavus clade with small sclerotia that synthetize AFBG
ST	Sterigmatocystin
TeA	Tenuazonic acid
VERA	Versicolorin A
VERB	Versicolorin B
WHO	World Health Organization
YES	Yeast Extract Agar
ZEA	Zearalenone

01

INTRODUCTION

1.1 BACKGROUND

Quality of food supplies has always being an issue in human societies. Having access to proper food supplies is necessary to avoid potential risk to human and animal health. Some fungi, especially from Ascomycota, are capable to synthesize a plethora of products as part of their metabolism, some of them toxic to humans and vertebrates, named mycotoxins. The ubiquitous presence of fungi in staples cannot be avoided, thus, their presence become a potential health risk for humans and livestock.

Mycotoxin contamination of staples is an important risk to public health because these compounds produce detrimental effects on vertebrates and humans. Since their discover, several studies have been performed to identify the principal mycotoxins depending on the geographical areas, the minimal doses of their toxicity, the fungi responsible of their production, and to develop strategies to control them in order to avoid their effects on human health, as well on animal health and to reduce economic losses. Once the amount of mycotoxins exceeds the levels permitted by the regulations, it is hardly recommended to eliminate staples from the food chain. However, in some regions worldwide, especially those including developing countries, monitoring and policies against mycotoxin presence in food and feed for human and animal consumption unfortunately are not well regulated. As consequence, the risk of entrance of mycotoxins in the food chain is high (Bhatnagar et al. 2002).

Aspergillus section *Flavi* is one of the most economically important groups of molds; their detrimental effects are an important public health issue, furthermore the stability of its taxonomy is of practical concern (Geiser et al. 2007; Pildain et al. 2008). The section encloses species able to produce several mycotoxins, among them, aflatoxins are a major concern because their deleterious effects in vertebrates (IARC 2003). Due to their physiological requirements, these species grow principally in tropical and subtropical regions worldwide. In these areas, they are a problem because harvest and storage conditions are not always the most appropriated ones to avoid mold development and mycotoxins production, besides, environmental conditions generally contributes to their production. Resulting in two main issues, the first is the risk on human and animal health, and the second, staples that are contaminated cannot be exported, which affects negatively some countries' economies because they are based on exportation. In fact, staples contamination of mycotoxins lead to great economic losses. In temperate regions, the importance of section *Flavi* is linked to the importation of contaminated raw material, as well as the possibility of the colonization of harmful species due to climate change, which could result in new niches for these species (Perrone et al. 2014).

The present introduction is divided in three parts: the first introductive part encloses a general overview of molds and the principal mycotoxins; the second part includes an overview of *Aspergillus* section *Flavi*, and the third part that summarizes the principal secondary metabolites yield by them.

1.2 FUNGI OVERVIEW

Fungi is a diverse eukaryotic kingdom containing an estimated of 3.5 to 5.1 million organisms, from unicellular to macroscopic multicellular, that inhabit a wide range of ecological niches worldwide (O'Brien et al. 2005). These organisms play a key role in nutrient cycle as decomposers, and include saprophages, symbionts and pathogens. As heterotrophs fungi fed from others organism by extracellular digestion, yielding enzymes that able them to digest and absorb nutrients. Fungi development requires certain elements that are used in their primary and secondary metabolisms, principally sources of carbon and nitrogen, and in a lesser extent potassium, phosphorus and magnesium, among others trace elements. Additionally, environmental factors, such as pH, light, temperature and water availability, are crucial for their development (Dix 2013; Dighton 2016).

Due to fungi diversity of life history strategies, several species are widely studied and applied in biotechnological industries to produce enzymes, medicines, biocontrol agents, natural fertilizers, natural pigments, cosmetics, alcoholic drinks, and food (Galagan et al. 2005; Schoch et al. 2009; Dupont et al 2016; Blackwell 2011; Jayasiri et al. 2015; Bill and Gloer 2016). Taking into account their diversity, the number of infectious species is low, yet those species have detrimental effects in organisms' health, including plants, animals and humans, and can be a worldwide threat for food security. Similarly, during the last decades, novel diseases produced by fungi have been discovered, and in some cases host population infected have decreased in alarming numbers, almost disappearing (Fischer et al. 2013). Summarizing, several fungi are economically important organisms, making their study mandatory (Mitchell 2010).

Fungi reproductive cycles include sexual and asexual reproduction, both mechanisms are mediated by spores (conidia, ascospores etc.), which have reproductive and dispersal functions. Some species present only sexual or asexual cycles while others a combination of both reproductive mechanisms. A holomorph fungus present both types of reproduction, an anamoph fungus presents the asexual type and a teleomorph fungus the sexual type. A fungus can have strains in anamorph state and others in teleomorph state; phenotypically they might be different and hence, be classified under different names. Moreover, some fungi show different anamorphic states, like some species of *Neurospora*, *Fusarium* and *Botrytis*, which show strong differences between their micro- and macroconidia (Webster and Weber 2007; Dix 2013).

Fungi are divided in ascomycetes, basidiomycetes, zygomycetes, and chytrids (Figure 1); the first two, Basidiomycota and Ascomycota, contain most species, including the most important to humankind. Ascomycota contains approximately 33.000 described species, including most lichens known and about 90% of pathogenic fungi (ca 400 species). Ascomycota fungi are characterized by their reproductive structures, ascus, nevertheless most species produce also asexual spores (Pitt and

Hocking, 2009), in fact they are most commonly found in their conidial state, and for some species, the sexual reproduction seems to be lost; this phenomenon seems to have occurred several times in the evolution of the group. The conidial states of *Aspergillus* and *Penicillium* are generally arranged in phialides, and their arrangements are generally used as diagnostic state. In *Aspergillus*, the conidiophore tip is swollen, forming the vesicle, and phialides start directly on its surface (uniseriate) or present a palisade of sterile cells, metulae, followed by phialides (biseriate) (Raper and Fennell 1965). *Penicillium* lacks vesicles, and the conidiophore tip has directly a monoverticillate arrangement or series of metulae followed by philiades, the levels of ramification could be from one to several series of metulae (Raper and Thon 1968). The cell walls in Ascomycetes are composed mainly by chitin and glucans and in general the septum is incomplete, forming a central pore that result in coenocytic mycelia (Webster and Weber 2007). Ascomycota probably arose around 500 to 900 million years ago; it is subdivided in three main groups, Archiascomycetes, Hemiascomycetes (yeasts), and the large Euascomycetes (molds) (Mitchell 2010).

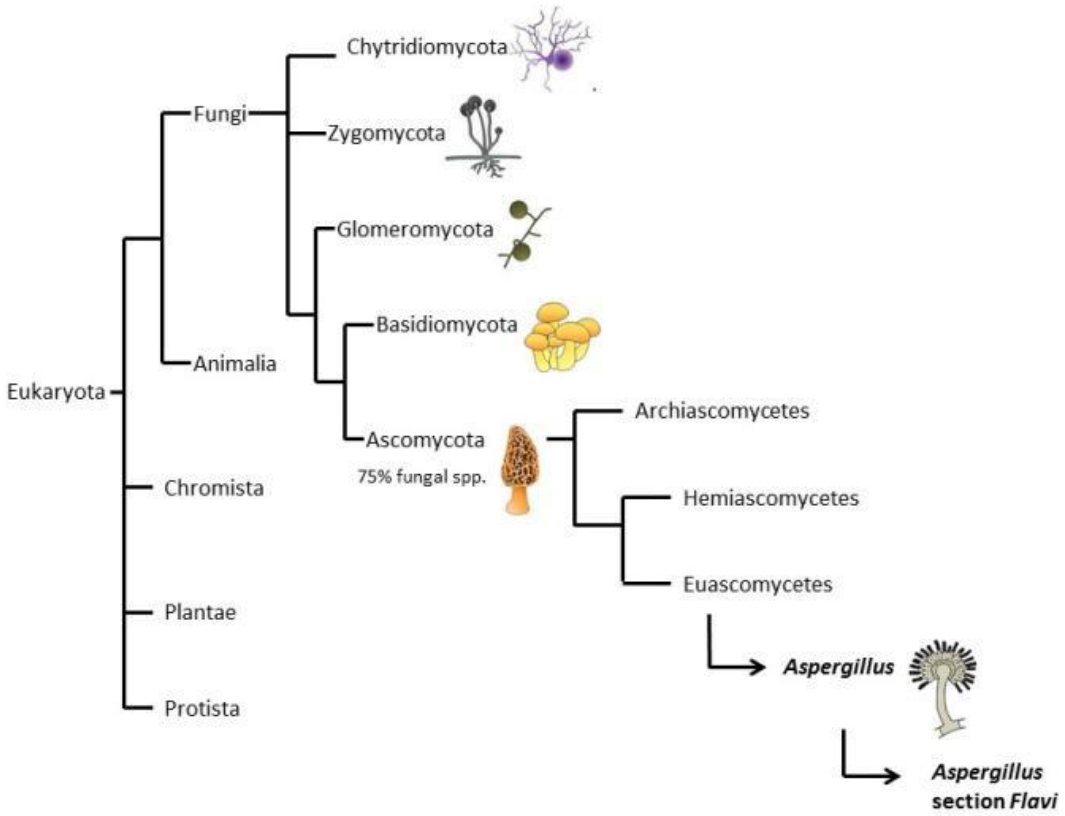

Figure 1: Fungi classification. The figure shows the relationships of fungi with other groups of Eukaryotes. In addition, it shows the main groups with Fungi, and the division of Ascomycota, and the placement of *Aspergillus*. The figure is adapted from Pitt and Hocking (2009).

Climate change presents a challenge for fungal relationships, between different fungi, between fungi and other organisms, and between fungi and their ecosystems. Resulting in shifts in community composition, creating new ecological niches, therefore, producing changes in the symbiotic associations between fungi and other organisms, changes in the distribution patterns of species, and the increment of detrimental effects caused by harmful fungi (Jayasiri et al. 2015). Climatic model predictions suggest that climatic conditions will vary over the next two decades, atmospheric concentrations of CO_2 are expected to double or triple (from 350 to 700 or 900-1000 ppm) and the regional cycles are going to change, some areas will become drier, and global temperatures will increase by approximately 2-5 °C (Medina et al. 2014). These environmental changes would result in homeostatic stress in crops. Hence, environmental changes would modify the agricultural cycles, affecting mycobiota composition in soils and crops, and the mycotoxinogen species, therefore the mycotoxins yield (Medina et al. 2014).

1.3 FILAMENTOUS FUNGI AND THEIR SECONDARY METABOLITES

Filamentous fungi are considered as the main producers of mycotoxins. It is a paraphyletic group, enclosing the Ascomycota phylum and some species from Mucorophyta (zygomycetes). Nevertheless, Ascomycota species are the most diverse and the most important at economical level, as they are linked to staple spoilage during harvesting or storage processes. In fact, the genera *Aspergillus*, *Fusarium* and *Penicillium* are considered as the main source of mycotoxins (Pitt and Hocking 2009).

Aspergillus and *Penicillium* genera are important to humankind not only because of their detrimental effects, but also because of their use in biotechnology; enzymes and other compounds synthesized as part of their primary and secondary metabolisms are used, as well as a direct inoculation of fungi on foodstuff. *Aspergillus* and *Penicillium* have been used in food production for several centuries in fermentation processes to produce beverages, sauces and in the cheese industry. Likewise, proteases, amylases, lipases and pectinases are important in the manufacture of dairy, bakery, distillery and brewery products, juices and leather, and in the starch industry. Furthermore, they have been used to synthesize antibiotics, such as penicillins and cephalosporins that comprise around the 50% of antibiotics production worldwide (Kavanagh 2017); or griseofulvin used as anti-tumoral and in dermatology (Banani et al. 2016).

1.4 WHAT IS A SECONDARY METABOLITE? DEFINITION, FUNCTION AND MYCOTOXINS

In order to cope with their environment, fungi have developed the ability to produce several extracellular chemicals, called secondary metabolites, which are not essential in the primary metabolism of fungi (*i.e.* growth, reproduction, respiration), and not required for their survival when growth in laboratory conditions. These compounds are low-weight molecules (< 1000 Daltons) produced by their secondary metabolism, which encloses the molecular pathways that are not essential for the survival of the organism (Bennet 1987; Bennet and Klich 2003). These molecules are diverse in their chemical nature, including polyketides, non-ribosomal peptides, terpene, indole terpenes and hybrids (Figure 2). These organisms are capable to produce a large number of these compounds, and their secondary metabolic profile will vary depending on the genetic information (presence of secondary metabolic gene clusters), environmental conditions (mainly nutrients and water availability), and community composition (Brakhage 2013; Bills and Gloer 2016).

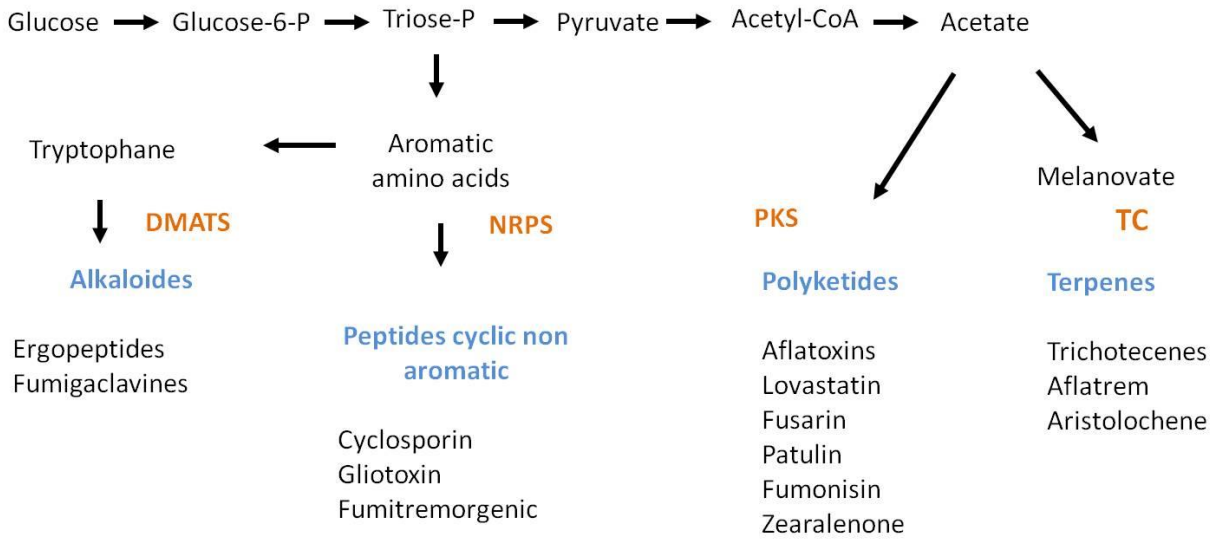

Figure 2: Biosynthetic pathways of secondary metabolites. In blue the groups of secondary metabolites. In black the main mycotoxins produced by these pathways. In orange the enzymes associated with each pathway; NRPS: non-ribosomal peptide synthetase, PKS: polyketide synthetase, TC: terpene cyclase, DMAT: dimethyl allyl transferase.

Polyketides are the most diverse group of secondary metabolites, including polyphenols, polyenes and macrolides. Due to their diversity, they exert different biological activities, some of them exploited in industrial processes. Fungal polyketides are synthesized by type I polyketide

synthases (PKSs) that are multidomain proteins linked to eukaryotic fatty acid synthases. Polyketides result from the metabolization of acetate, and are formed by the consecutive polymerization of ketide groups (CH_2-CO). The main difference between fungal polyketides and fatty acids is that polyketides are formed by PKSs able to use other carboxylic acids, rather than acetyl-coenzyme A as substrate. In addition, the oxidation state is variable and β-carbon is not necessary fully reduced in polyketides synthesis, for which the ketoacyl CoA synthase (KS), acyltransferase (AT) and acyl-carrier (ACP) domains are essential. Fungal PKSs are considered 'iterative PKSs' because they present just one module for the addition of methylmalonyl CoA, so the processes of addition require repeated biosynthetic reactions. Fungal PKSs are divided in three groups: non-reduced, partially reduced and highly reduced synthases. In addition, five of the six mycotoxins regulated to date by the EU, aflatoxins, ochratoxin A, patulin, fumonisins and zearalenone belongs to this group (Keller et al. 2005; Cano et al. 2016).

Terpenes are yielded by fungi, bacteria and plants. Plant terpenes are the best known, and are essential for plant growth, development, and interactions with their environment. Terpenes play a main role in interaction with pollinators and predators (*i.e.* herbivores), in the protection against photo-oxidative stress, in thermoregulation, among others (Tholl 2006). Aristolochenes, carotenoids, gibberellins and trichothecenes are some important terpenes characterized in fungi. Terpenes are formed by the combination of dimethylallyl pyrophosphate (DAMPP) and isopentenyl diphosphate (IPP), this reaction is catalyzed by isoprenyl diphosphate synthases, which belong to the family of phenyl transferases. Based on their chemical structures terpenes are classified as: (i) monoterpenes or geranyl diphosphate that are rarely yield by fungi, (ii) triterpenes that are mainly produce by plants, (iii) sesterterpenes, tetraterpenes or carotenoids linked to the defense against UV radiation, and (iv) sesquiterpenes that enclose the tricothecenes family known as an important group of mycotoxins (Cano et al. 2016).

Non-ribosomal peptides (NRPs) are compounds not involved in the primary metabolism. As their name suggest, their synthesis does not include proofreading mechanisms, making their structure highly variable, in fact, at the moment several hundreds of substrates of NRPs have been identified in comparison to the 20 amino acid involved in protein synthesis (Finking and Marahiel 2004). The synthesis of NRPs is catalyzed by non-ribosomal peptide synthetases (NRPSs), which have functions similar to those of enzymes catalyzing ribosomal peptides. When compared, fungal NRPs are reported to achieve bigger sizes than bacteria NRPs, and could be explained because their synthesis in fungi is generally catalyzed by one NRPS. These enzymes have a dual function, working as temperate and as biosynthetic machinery; actually, they are organized in different modules that integrate amino acids into the polypeptide chain. The synthesis of NRPs requires the presence of at least three domains: (i) the A-domain that determines the amino acid to be included and activates

the amino acid or the hydroxyl acid; (ii) the T or PCP domain, a thiolation or peptidyl-carrier protein, which transports the activated units between active sites of the domains; and (iii) the C-domain, a condensation domain, where the formation of the peptide bond (C-N) occurs between the polypeptide chain and the new amino acid. Other domains could also play a role in the synthesis, by adding special features, like non-proteinogenic amino acids, fatty acids, carboxylic acids, among others (Pang et al. 2016).

Indole alkaloids are mainly synthesized from tryptophan and DAMPP, but sometimes they include other amino acids as precursors. The steps of biosynthesis are yet to be elucidated, for some known compounds three processes were described that include steps of tryptophane prenylation catalyzed by DMATS, followed by the methylation of dimethylallyl tryptophan, and finally, a series of oxidation steps, which can be catalyzed by NRPSs. Some other enzymes can be involved in the biosynthetic pathways, like oxidases, methylases and prenyl transferases (Keller et al. 2005).

The development of genomic, transcriptomic, and proteomic is unmasking processes linked to compound synthesis in fungi. In fact, the genome characterization of several species has enabled to elucidate the biosynthetic pathways of several mycotoxins and the processes occurring in fungal cells, including an increase of knowledge of the biology of harmful fungi. For instance, pathogenic ascomycetes present more genes for polyketides, peptides, terpenes and other secondary metabolites than those non-pathogens such as *Neurospora crassa* (Desjardins 2006). Likewise, genome studies have shown that secondary metabolic yield depends on global transcriptional factors, encoded by unrelated genes with a specific biosynthetic pathway (*e.g. VeA* and *LaeA*), and on specific enzymes for each biosynthetic pathways that differ from primary metabolism enzymes. In Ascomycetes, biosynthetic pathways of secondary metabolites are often clustered together, which makes them different from other eukaryotes. The purpose of secondary metabolites is still not completely understood, however it is believed that they confer selective advantages to fungi under natural conditions, especially under stress conditions (*e.g.* environmental stress, nutrient availability, interspecies competition, predator defense) (Magan and Adred 2007; Fox and Howlett 2008, Schwab and Keller 2008; Brakhage 2013).

As aforementioned, several secondary metabolites are beneficial to humankind and are used in pharmacology, food industry, cosmetics, energy and construction (Bhatnagar et al. 2002). Some others, known as mycotoxins, are toxic and could exert deleterious effects on vertebrates, including humans (Peraica et al. 1999). Mycotoxins are amply studied due to their detrimental effects on vertebrates' health and their impact in agriculture and economy. Nowadays, over 1000 secondary metabolites are described, ca 400 are considered mycotoxins, 30 are considered as important mycotoxins for their effects, and from them just 7 are legally regulated by the European Union. Best-

known mycotoxins include aflatoxins, ergot alkaloids, fumonisins, ochratoxin, patulin, trichothecenes and zearalenone (Bennet and Klich 2003; Cano et al. 2016) (Table 1).

Table 1: Principal mycotoxins and producing species, frequent sources and effects (AFSSA 2009; CAST 2003, Bbosa et al. 2013). In red the mycotoxin and their principal producer.

MYCOTOXIN	TYPE	MAIN PRODUCERS	CONTAMINATED PRODUCTS	EFFECTS	CHEMICAL NATURE
Aflatoxins	B_1, B_2, G_1, G_2	*Aspergillus flavus*, *A. parasiticus*, *A. nomius*, Several spp. in *A.* section *Flavi*	Cereals: maize, wheat, rice, sorghum; spices, sunflower, nuts, almonds, pistachio, coconut, cotton, dried fruit	Hepatoxic, Carcinogenic, Immunotoxic, Teratogenic, Acute toxicity	Polyketide
Trichotecenes	T-2 Toxin and HT-2	*F. tricinctum*, *F. langsethiae*, *F. sporotrichioides*, *F. poae*, *F. equiseti*	Cereals: wheat, maize, rice, soy, beans and barley	Genotoxic, Immunotoxic, Reprotoxic, Neurotoxic	Terpene
	Deoxynivalenol	*Fusarium graminearum*, *F. culmorum*, *F. sporotrichoides*, *F. langsethiae*, *F. tricinctum*, *F. poae*, *F. solani*, *F. equiseti*	Cereals: wheat, maize, rice and sorghum	Immunotoxic, Digestive problems, Haematopoietic	Terpene
Fumonisis	B_1, B_2, B_3	*F. verticillioides*, *F. proliferatum*	Cereals: maize, rice, sorghum	Carcinogenic, Neurotoxic	Polyketide
Ochratoxin A		*Penicillium verrucosum*, *Penicillium nordicum* *A. ochraceus*, *A. carbonarius*	Cereals, cacao, coffee, wine, grape juice and spices	Nephrotoxic, Immunotoxic, Teratogenic	Polyketide
Zearalenone	F-2 Toxin	*F. graminearum*, *F. culmorum*, *F. crookwellense*	Cereals: maize, soy, sorghum, wheat, rice and oat	Reprotoxic, Immunotoxic	Polyketide
Patulin		*P. expansum*, *Byssochlamys nivea*	Apples, pears and derivates juices	Neurotoxic, Genotoxic, Cytotoxic	Polyketide
Ergot alkaloids		*Claviceps purpurea*, *C. paspali*, *C. africana*, *C. fusiformis*	Rye, wheat and triticale	Neurotoxic, Digestive problems, Vasoconstriction	Alkaloids

1.4.1 Mycotoxins

Mycotoxins are classified based on fungus that produce them, chemical structure and/or mode of action. Their degradation is a challenge because most are heat-stable and form toxic compounds while degradation processes are applied. Generally this compounds are hydrophobic (except for the fumonisins), allowing them to accumulate in lipophylic tissues in plants and animals (Hussein and Brasel 2001).

Mycotoxicosis symptoms depend on several variables that interacts synergistically, including the mycotoxin chemical nature, the exposure time (duration and dose), the organism that intakes the mycotoxin (species, sex, age, health, diet), and the mixed effects of mycotoxin with other xenobiotics. The effects exerted on vertebrates could be chronic (low doses, long periods of time) or acute toxic (high doses, short periods of time), mutagenic, teratogenic, carcinogenic, nephrotoxic, hepatotoxic, immunotoxic and estrogenic. The main target organs depend on the mycotoxin and the organisms that ingest it, and include the liver, kidney, lungs and the nervous, digestive, endocrine and immune systems (Bhatnagar et al. 2002). In general, more than one mycotoxin is found in staples, and a mix of them is thus usually ingested. The interaction between mycotoxins can produce different effects in the organism: antagonists, additive or synergic, which are linked to the mycotoxin nature, the decontamination pathway, of the host species, the time of exposure, and the doses and ratio of mycotoxins (Peraica et al. 1999; Alassane-Kpembi et al. 2017).

Mycotoxins have being around humans for as long as agriculture was developed, or even before, when recollection started as mechanisms for food storage (Richard 2007). Some episodes of mycotoxicosis can be traced in the literature, myths, and arts. For instance, they could be tracked in the Bible, as part of the Seven Plagues of Egypt or in the Dead Sea Scrolls (Richard 2007). Withal, the decline of Etruscan civilization (5^{th} century B.C.) could be related to fusariotoxins (toxin T2 and ZAE) (Yiannikouris and Jouany 2002). Howbeit, the episodes of hallucinations of “Saint Antony’s fire” or ergotism (11^{th} century), produced by alkaloids of *Claviceps purpurea* on rye, might be the best-known example of mycotoxicosis in ancient times (Figure 3). During the Middle Ages outbreaks were common, some registered epidemic episodes occurred during 8^{th} and 15^{th} century A.D.; also some more recently episodes are also suggested to be caused by ergotism, like witchcraft in Salem, USA, and Finnmar, Norway. Symptoms of ergotism include delirium, prostration, acute pain, abscess and gangrene of the limbs, and sometimes death (Peraica et al. 1999; Richard 2007; AFSSA 2009). Likewise, “Shoshin-kakke” or “yellow rice disease” is another well-known example of mycotoxicosis outbreak, this disease that causes acute cardiac beri-beri, was reported in Japan, affecting especially the colder regions. This illness is caused by the exposure to citreoviridin, a *Penicillium citreonigrum* mycotoxin. The fungus contaminated rice during the storage processes due to poor conditions and

practices. Once these conditions were controlled, the disease disappeared from the region, and has not been reported lately (Udagawa and Tatsuno 2004). Although fungal contamination occurred and relations with some diseases were perceived, the awareness of mycotoxins and their toxic effects arose for the first time in London (England) in 1962. Poultry presented a strange disease, "turkey X syndrome", which killed at least 10,000 birds. Interestingly, while tracing the origin of the illness, it was discovered that peanuts used to feed poultry were contaminated by secondary metabolites, aflatoxins, named after *Aspergillus flavus* (Bennet and Klich 2003); years later, cyclopiazonic acid was also proved to interfere in this outbreak (Richard 2008). Other compounds were also recognized as mycotoxins and their study became under scope. Mycotoxins are found in a wide variety of staples, use as animal and human food, principally cereals (maize, wheat, rye, rice, etc.), oligenosus seeds (peanuts, cotton, nuts, pistachios, etc.), and spices (Bath et al. 2010).

Figure 3: Art as evidence of mycotoxins contamination. Above: Paint exemplifying an ergotism outbreak. Down left: "Saint Anthony's hallucinations" by Mathias Grünewald (effects of hallucinations associated to ergotism). Down right: rye ear contaminated by *Claviceps purpurea*.

1.4.2 How mycotoxins enter in the chain food and their distribution

Occurrence of mycotoxin contamination can be divided in pre-harvest (crops and recollect) and post-harvest steps (mainly storage). The fungi that contaminate staples will vary depending on the geographical region, but also of the agriculture methods, crop cycles, harvesting and storage conditions. Humans' exposure to mycotoxins can occur by direct intake of staples of vegetal origin contaminated by mycotoxins, or by the intake of contaminated animal products. There is also the risk of dermal, respiratory and maternal exposure routes (Bath et al. 2010; IARC 2015).

In general, mycotoxigenic fungi are divided in two main groups, one enclosing species more prone to colonize staples and yield mycotoxins in crops (principally *Fusarium* species), and another group more prone to colonize and yield mycotoxins during storage (principally *Aspergillus* and *Penicillium*), yet, some fungi are able to colonize during both steps, like *A. flavus* (AFSS 2009; Antonissen et al. 2014). For example, *Fusarium* generally colonized grains before harvest, where moisture is high, whereas maize and peanuts are generally colonized in post-harvest, where temperatures and drought are more suitable for *Aspergillus* section *Flavi* colonization (Bryden 2012). As aforementioned, fungi reproduce by spores that are dispersed principally by the wind and insect vectors. Once the spores reach a suitable nutrient source, suitable environmental and atmospheric conditions, such water availability, humidity and drought conditions, pH, and temperature, they will germinate (Figure 4). Besides, these environmental conditions can trigger stress and predisposition of cultivars, helping fungi developpment. The optimal for these variables depend on the fungus, for example, *Aspergillus glaucus* requires approximately 10% less of water availability than *A. flavus*. Substrate is also important; some fungi are generalist, while others have more constrained niches. Generalist fungi might prefer a substrate that suits better their nutrimental and physiological requirements, like *A. flavus* that will prefer maize (though it colonized several commodities), whereas some *Fusarium* species prefer cereals with small grains (Dierkman and Green 1992; AFSSA 2009; Pinnoti et al. 2016).

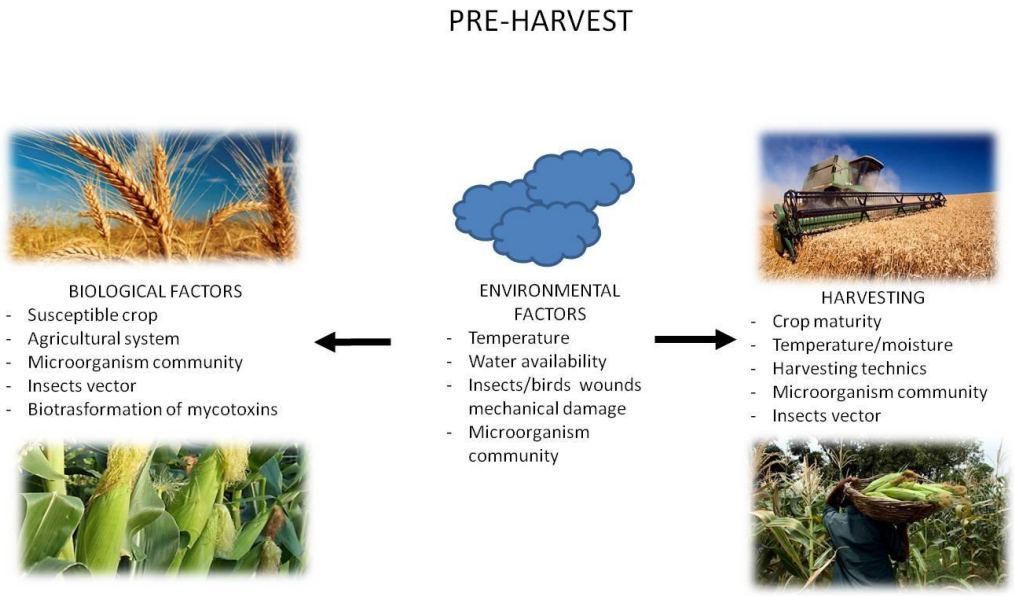

Figure 4: Pre-harvest contamination and main factors affecting mold contamination. Modified from Paterson and Lima 2010.

Insects have a key role in fungi contamination; they not only disperse spores, but also damage raw products, allowing an easier colonization by fungi. In fact, insects wound maize kernels, and transport spores of *Aspergillus* and *Fusarium*, resulting in disturbance of the natural barrier (leaves protecting kernels) (Aiko and Mehta 2015) (Figure 5).

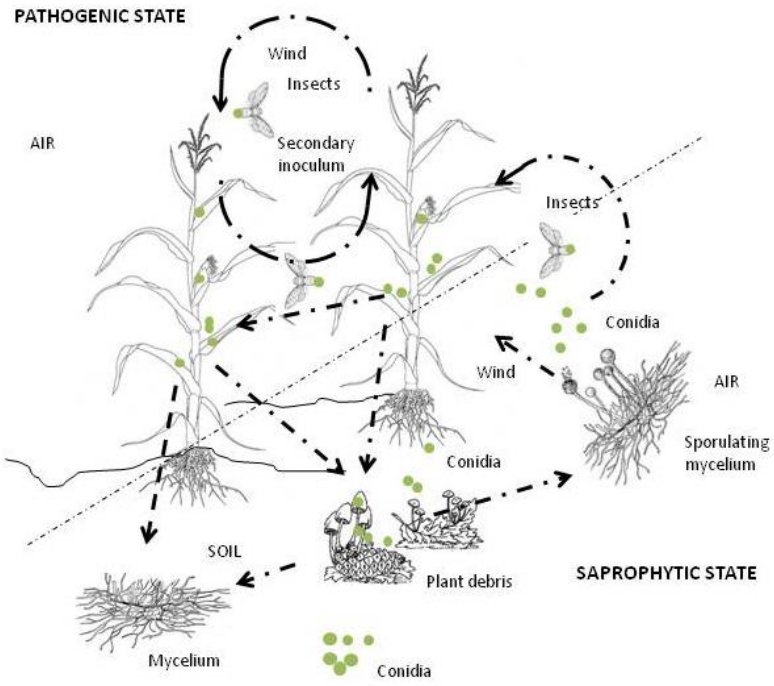

Figure 5: Cycle of fungi contamination (Modified from Abbas et al. 2009).

Agricultural practices are part of the factors that interact in pre-harvest contamination, some of the main practices include the variety of plants used (more or less sensitive), the type of crop rotation and soil tillage. Some techniques used to diminish fungi contamination includes growing resistant crop varieties, management of crop rotation, the type of soil tillage, chemical and biological control of plant diseases, and insect control (Rychlik et al. 2014).

Post-harvest contamination includes steps from crop maturation to feed and food consumption. Abiotic conditions, such as water availability, temperature, oxygen availability, are more easily controlled than the pre-harvest steps, facilitating the control of mold growth. Nevertheless, methods to storage following all the requirements are expensive, and easier to obtain in products that have higher markets, making this process not always achievable for small production or in some countries (Dierkman and Green 1992; Magan et al. 2003; Paterson and Lima 2010). Grain storage is a good example of how mycotoxins can contaminate feed and food supplies. In general, a community of microorganisms, most of them innocuous, colonizes grains. Mycotoxigenic fungi can be good competitors, and under proper temperatures and water availability, they can develop and produce mycotoxins. Insects play a similar role in the storage processes as well (Magan et al. 2003; Paterson and Lima 2010) (Figure 6).

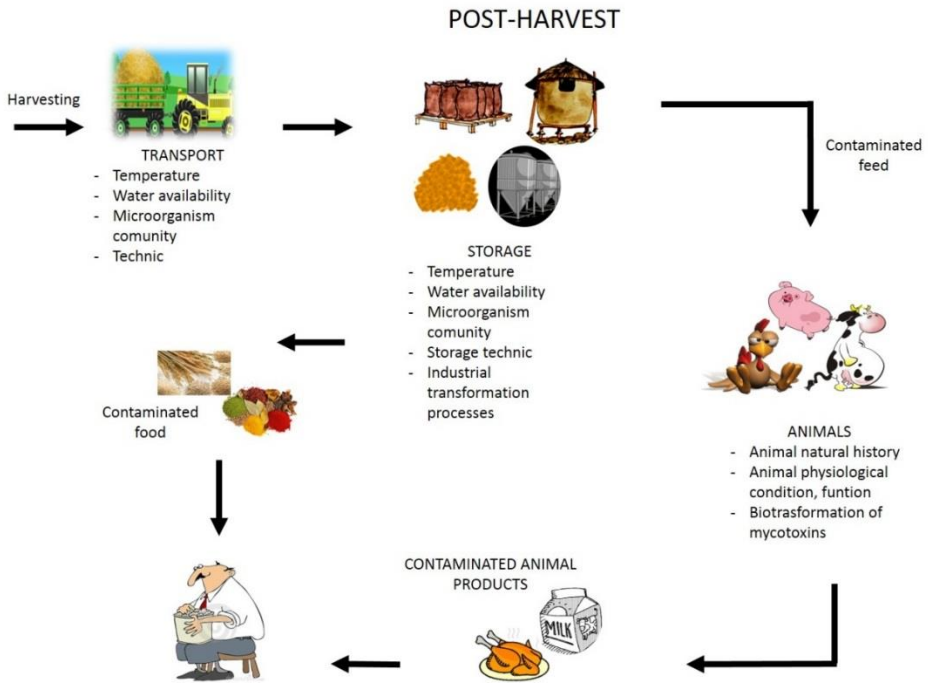

Figure 6: Post-harvest contamination and main factors affecting mold contamination. Modified from Paterson and Lima 2010.

Once the mycotoxins are present in feed and food commodities, they can contaminate the whole food chain. As said above, humans can ingest mycotoxins in two ways, by direct consumption

of contaminated vegetal commodities or by ingesting contaminated animal products, and in general are contaminated with more than one mycotoxin. A study case of nephropathy in Bulgarian pigs and chickens was caused by cocktail of mycotoxins, including ochratoxin A, penicillic acid, FB1 and an uncharacterized metabolite (Bryden 2012).

In animals, ruminants have a gastric system with a rich microbiota that facilitates the degradation of mycotoxins, whereas monogastric species, like pork and poultry, are especially sensible to mycotoxicosis because intestinal microbiota is less diverse. Poultry is less prone to biotransform toxins to less toxic compounds before the intestine absorbs them. For ruminants it has been documented that rumen function is nevertheless affected negatively by the presence of mycotoxins, as well some biotrasformation can produce toxic products, which can be excreted thus making it available, like the case of AF_{M1} (Hussein and Brasel 2001; AFSSA 2009). The major problem for livestock and poultry associated with ingestion of mycotoxins is the poor animal performance, which can be difficult to diagnose and quantify because of the diversity of life histories, physiological status, biotransformation pathways, detoxification mechanisms and the intra- and inter variability of species that ingested them. Similarly, the type and level of mycotoxin in feed, the time of the exposure, and the interaction between mycotoxins are also a problem (Bryden 2012; Alassane-Kpembi et al. 2015).

In addition, there are the 'masked' or conjugated mycotoxins that occur in vegetal food supplies, and are often linked with livestock and poultry feed intake, resulting in a decrease in their performance. These types of mycotoxins are the result of biotransformation processes occurring in plants (Bryden 2012; Pierron et al. 2016). Some examples are zearalenone-4-glucoside, a conjugate of zearalenone, and deoxynivalenol-3-glucoside a conjugate of deoxynivalenol. There is some evidence that OTA and fumonisins can also be conjugated in plants (Bryden 2012). The discovery of these mycotoxins has put them under scope.

1.4.3 Impact of mycotoxins

The presence of mycotoxins in staples is a major concern, not only for public health, but also for their economic impact. Food commodities losses due to mycotoxin contamination represent above 25% of spoiled food (FAO 2003). For instance, only in the United States the Food and Drug Administration (FDA) has estimated that the losses exceed $900 million per year (CAST report 2003). Due to its impacts, food security associated to mycotoxin contamination is a major issue worldwide; public health commissions all over the world try to ensure safe and healthy feed and food for animals and humans (Stoev 2013). In developed countries, food security is carried out better than in developing countries, in which food quality monitoring and the infrastructure to avoid mycotoxin

contamination are more difficult to settle. Nevertheless, regulation of mycotoxins reduced the intake in those countries that have proper regulation and monitoring, and increase exportation standards around the world. On the other hand, it could result in a higher risk of consumption of mycotoxins by human and animal populations of developing countries, as the best quality staples are exported, whereas the poor quality ones remain for domestic consumption (Wild and Gong 2009; Stoev 2013).

As aforementioned, the global distribution of mycotoxins is not homogenous, the conditions in each region will favor the development of certain fungi over others, thus favor some mycotoxins over others. In addition, climate change is shifting distribution and prevalence of some fungi, and thereby, mycotoxin distribution. Streit et al. (2013) determined the presence of the main mycotoxins (aflatoxins, zearalenone, deoxynivalenol, fumonisins and ochratoxins) around the world for a period of eight years. Their results showed that most of the samples (72%) were mycotoxin positive, and 38% showed a multicontamination (more than one mycotoxin). In addition, they determined that the percentages of each mycotoxin were more or less stable during the years, with the exception of aflatoxins, their level increased between 2005 and 2009 in tropical regions. Another study, that also screened mycotoxin presence in long term, showed the risk of mycotoxin contamination depending of the geographical distribution worldwide (Figure 7).

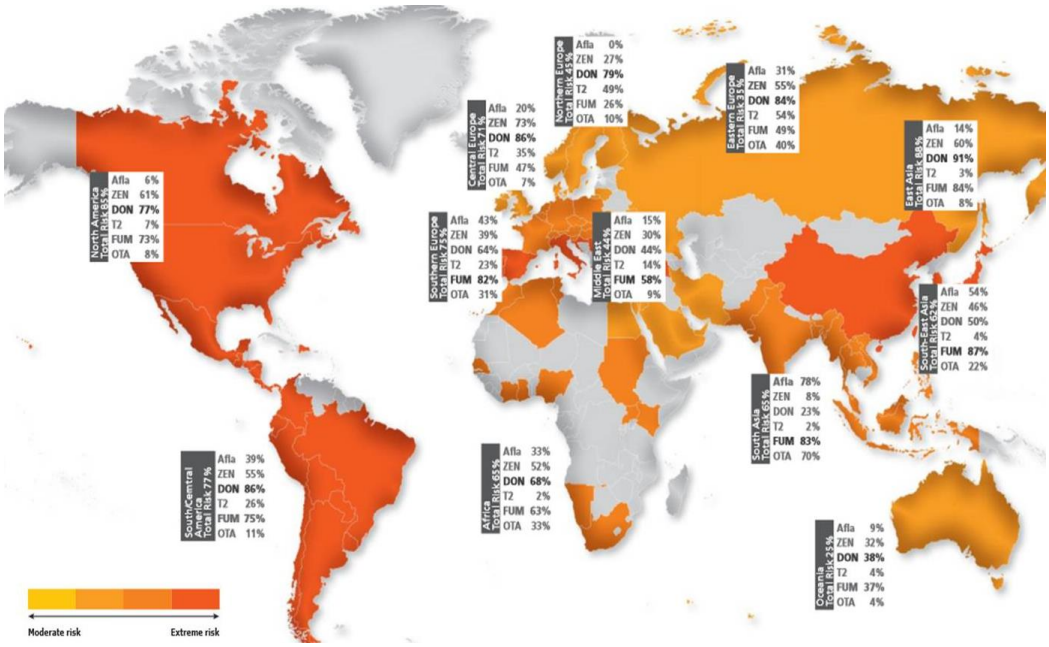

Figure 7: Global distribution of the main mycotoxins. Survey performed by Biomin 2017 based on more than 3715 samples and 14244 analyses in 54 countries. Afla: aflatoxins, ZEN: zearalenone, DON: deoxynivalenol, T-2: T-2 toxin, FUM: fumonisins and OTA: ochratoxin A. Moderate risk: 0-25% of samples above risk threshold; High risk: 26-50% of samples above risk threshold; Severe risk: 51-75% of samples above risk threshold; Extreme risk: 76-100% of samples above risk threshold.

1.5 BIODIVERSITY OF *ASPERGILLUS* SECTION *FLAVI*: FOLLOWING THE TRACES OF CRYPSIS

Aspergillus is a group of filamentous ascomycetes that encloses some of the most widespread fungi, containing approximately 350 recognized species. It encloses species of high economic importance for their compound production. Some species are used in biotechnology (enzymes, organic acids, bioactive metabolites), other species are harmful and considered as foodborne contaminants (food spoilage and mycotoxin contamination) or as causal agents of human mycoses (pulmonary, otomycosis, keratitis) (Kocsubé et al. 2016), and others use as model species to understand eukaryotic cell biology and molecular processes (*i.e. A. nidulans*) (Whiteway and Bachewich 2017). This genus is endowed with a diagnostic morphological trait reminding the holy water sprinkler, the 'aspergillum', which consists on a conidiophore that ends in a spherical vesicle bearing phialides and metulae that generate chains of conidia (Dyer and O'Gorman 2012) (Figure 8). The classification of *Aspergillus* has undergone several modifications over the past years using different approaches aiming to group the growing number of species according to its phylogenetic relationships (Scheidegger and Payne 2003). Aspergilli classification was traditionally based on morphological traits, and has nowadays extended to include the secondary metabolic profile and molecular approaches. This review will focus on the *Flavi* section of the *Circumdati* subgenus. This section bears a particular interest since it includes human pathogens, important mycotoxin producers, especially of aflatoxins, as well as safe enzyme producers commonly used in the food industry.

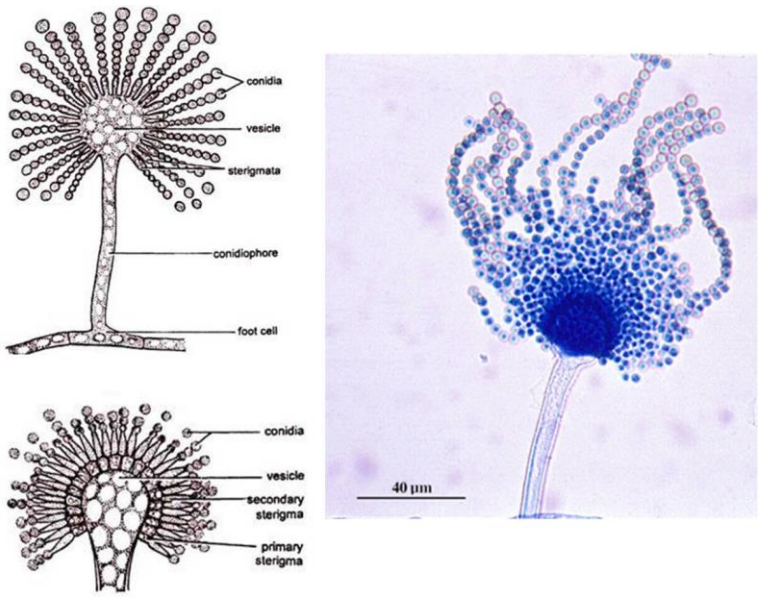

Figure 8. Aspergillum structure. Figures on left represent the mature conidiophore with primary sterigmata (above) and secondary sterigmata (below); right figure microscopic photo of *Aspergillus flavus.*

1.5.1 A compendium of section *Flavi*

Aspergillus section *Flavi* is mainly composed of saprophytic molds occurring in diverse ecological niches and playing a keystone role in the first steps of the nutrient cycle (Cotty et al. 1994; Rodrigues et al. 2012). Among their general phenotypical characteristics are the conidial heads yellow-green to brown shades, uniseriate or biseriate and the production of black sclerotia (Varga et al. 2011; Houbraken et al. 2014). The characteristic secondary metabolites in the group include aflatoxins (AF), paspaline, kojic acid, aspergillic acid, and cyclopiazonic acid (CPA) (Frisvad and Samson 2000). These molds grow better under environmental conditions of humidity (around 0.85 to 0.99 a_w) (Medina et al. 2015; Yogendrarajah et al. 2016) and temperatures ranging from 28 to 42 °C and several grow faster at 37 °C (Varga et al. 2011). The environmental humidity and temperature preferences make the species within the section *Flavi* suitable to grow in the tropics and subtropics over the world, yet some of them are able to grow in temperate regions, like *A. flavus*. Furthermore, climate change affects principally these two environmental variables, creating new niches in areas that in the past were not suitable for *Aspergillus* section *Flavi* species development, which could favor their colonization into temperate regions (Perrone et al. 2014).

Extrolites produced by these fungi make the section interesting for studying purposes. Some species, such as *A. flavus* and *A. parasiticus* have an impact on human and animal health as well as on international economy, as they are able to produce aflatoxins, especially aflatoxin B_1 (AF_{B1}), considered as mycotoxins of high health risk due to their carcinogenic, mutagenic and teratogenic potential (IARC 2012). In addition, species belonging to this section are also able of producing a wide range of other mycotoxins such as CPA, aflatrems, versicolorins, sterigmatocystin, ochratoxin A (OTA), etc. Albeit, other species are not toxinogenic and are used in biotechnology for producing enzymes and organic compounds commonly used in several industrial processes (Houbraken et al. 2014). For example, *A. oryzae* and *A. sojae* synthesize kojic acid, a secondary metabolite used in the production of soy sauce, a market with estimated shares of billions of dollars worldwide (Chang et al. 2007a).

Although there are some morphological characters and secondary metabolites that allow identification at species level, when cryptic species are present they become insufficient for taxonomical differentiation. In addition, morphological analyses to discriminate among isolates can be tricky, because mold phenotype is affected by environmental and nutritional conditions, creating overlapping phenotypical traits (Chang et al. 2007a). Inclusion of molecular analyses to the methods aforementioned is crucial for species identification and to clearly define relations within the *Flavi* section (Samson et al. 2014). However, finding differences at the molecular level can be challenging since species of this group shares several conserved traits. For instance, *Aspergillus flavus* and *A.*

parasiticus share approximately 97-99% nucleotide identity of their genomes (Chang et al. 2007a). ITS gene is usually used as a barcode gene to differentiate fungal species, but in this section, it is highly conserved, making it almost uninformative (Varga et al. 2011; Houbraken et al. 2014). Even though, there are challenges to characterize these organisms at species level, it is important to keep a practical taxonomic system because it is the base to the development of regulations to favor food safety and control (Geiser et al. 2007; Godet and Munaut 2010).

Over the last two decades, *Aspergillus* section *Flavi* has suffered several modifications in their composition; currently 26 species have been described based on a polyphasic approach, which includes phylogenetic, morphological and secondary metabolites analyses. The section is constituted by *Aspergillus flavus*, *A. oryzae*, *A. parvisclerotigenus*, *A. minisclerotigenes*, *A. parasiticus*, *A. sojae*, *A. arachidicola, A. novoparasiticus*, *A. sergii*, *A. transmontanesis*, *A. mottae*, *A. nomius*, *A. pseudonomius*, *A. bombycis*, *A. tamarii*, *A. pseudotamarii*, *A. caelatus*, *A. pseudocaelatus*, *A. bertholletius*, *A. coremiformiis*, *A. togoensis, A. leporis*, *A. hancockii, A. alliaceaus*, *A. lanosus,* and *A. avenaceus.* From these, the genome (strain = GenBank assembly accession numbers) of *A. flavus* (NRRL 3357 = EQ963472, AF70 = ASM95283v1)*, A. oryzae* (RIB40 = GCA_000965245.1)*, A. parasiticus* (SU-1= GCA_000956085.1, 68-5 = GCA_001576805.1), *A. nomius* (NRRL 13137 = GCA_001204775.1)*, A. bombycis* (NRRL 26010 = GCA_001792695.1)*, A. hancockii* (FRR 3425 = GCA_001696595.1), and *A. arachidicola* (CBS 117610 = GCA_002749805.1) have been sequenced.

1.5.2 Reproduction in *Aspergillus* section *Flavi*

Most *Aspergillus* fungi are only known in an asexual state (64%) (Dyer and O'Gormann 2011), nevertheless, there is evidence that cryptic reproduction occurs in some species. In ascomycetes, sexual identity and later stages of sexual development are partially regulated by the *MAT* locus, conformed by two idiomorphs *MAT1-1* and by the *Mat1-2* genes, encoding a protein with a α-box motif and a protein of the high mobility group (HMG), respectively (Ramirez-Prado et al. 2008; Dyer and O'Gorman 2011). In heterothallic species, only one of the idiomorphs is present, whereas in homothallic both idiomorphs are present and they occur in the same loci or in different chromosomes (Dyer and Kück 2017). The section *Flavi* is mainly composed by heterothallic species, and asexual reproduction seems to occur more frequently. Sexual reproduction is reported only for six species and from them only one species is homothallic, *A. alliaceus* (Horn et al. 2011; Dyer and Gorman 2012). It has been hypothesized that homothallic type can be the ancestral state in this section because *A. alliaceus* is a basal species (Ramirez-Prado et al. 2008). The presence of both idiomorphs in most analyzed species, however, suggests that heterothallic type could be the ancestral trait in *Aspergillus* (Ramirez-Prado et al. 2008). Sexual forms in this section are clustered in

the genus *Petromyces*, erected to include the teleomorph of *A. alliaceus*. Later on, sexual states of *A. flavus, A. parasiticus, A. nomius* and *A. oryzae* were incorporated, based on morphological evidence, like cleistothecia structure, and to maintain the monophyly of the group (Moore et al. 2009; Horn et al. 2009). Another important trait in the section is the production of sclerotia, which occur in several species. In asexual species, it has been hypothesized that sclerotia aid species to cope with adverse environmental conditions and predators, which is supported by the type of secondary metabolites produced (McAlpin and Wicklow 2005; Cary et al. 2015b), while in sexual species they also play a role in the formation of cleistothecia (Horn et al. 2009).

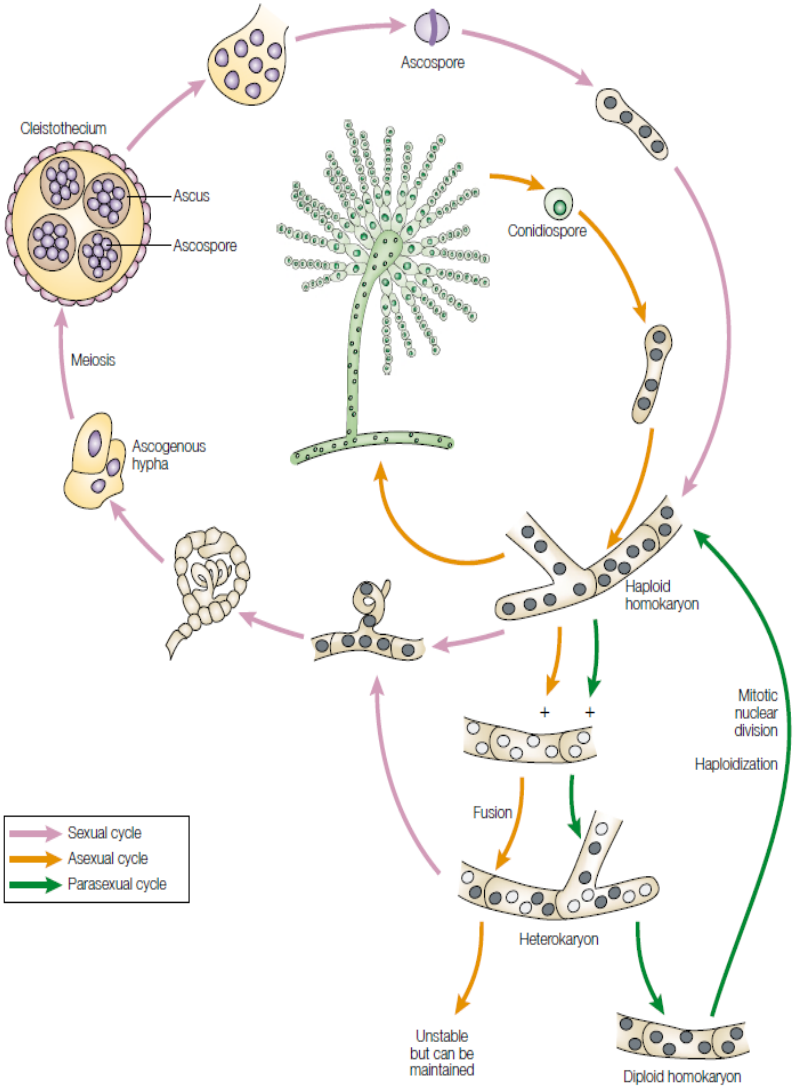

Figure 9. Reproductive cycle in *Aspergillus* (Image representing *A. nidulans*) (reprinted from Casselton and Zolan 2002)

Another way *Aspergillus* species recombinate is by the formation of a stable heterokaryon, resulting from hyphal anastomosis between strains sharing the same alleles of *het* loci (Pál et al. 2007). These heterokaryons are used to test species diversity, which might limit heterokaryosis and asexual gene flow in communities (Barros et al. 2007). Species from the section *Flavi* present different level of diversity of vegetative compatibility groups (VCGs), for instance *A. flavus* presents higher diversity than *A. parasiticus*, the first one tends to present several VCGs in one community, whereas the latest presents few. Making the study of VCGs appropriated to estimate diversity at community level, niche use, life cycles, and to control aflatoxigenic strains (Barros et al. 2007; Ramirez-Prado 2008; Grubisha and Cotty 2015).

1.6 DIVERSITY IN THE SECTION

A. flavus, A. parasiticus and *A. nomius* were considered as the only producers of aflatoxins. *A. flavus* produces type B aflatoxins (AF_B), whereas *A. parasiticus* and *A. nomius* produce B and G aflatoxins (Perrone et al. 2014). The discovery of other species, including species phylogenetically close to *A. flavus* able to produce AF_B and AF_G and non-aflotoxigenic species, accentuated the complexity of the section *Flavi*. Varga et al. (2011), in an attempt to organize the section, suggested the division in seven clades using a polyphasic approach: *Aspergillus flavus* clade (7 species), *A. tamari* clade (4 species), *A. nomius* clade (3 species), *A. alliaceus* clade (2 species), *A. togoensis* clade (2 species), *A. leporis* (2 species) and *A. avenaceus* (Figure 10). The addition of new species in the section increased the number of clades to ten. The principal modification is the division of *Aspergillus flavus* clade in two groups, *A. flavus* and *A. parasiticus* clades. *Aspergillus flavus* clade contains *A. flavus*, *A. oryzae*, *A. parvisclerotigenus*, and *A. minisclerotigenes*, whereas *A. parasiticus* clade contains *A. parasiticus*, *A. sojae*, *A. arachidicola, A. novoparasiticus*, *A. sergii* and *A. transmontanensis* (Soares et al. 2012). The other important modification is the inclusion of two clades, *A. mottae* and *A. bertholletius*, both composed by a single species (Taniwaki et al. 2012; Soares et al. 2012).

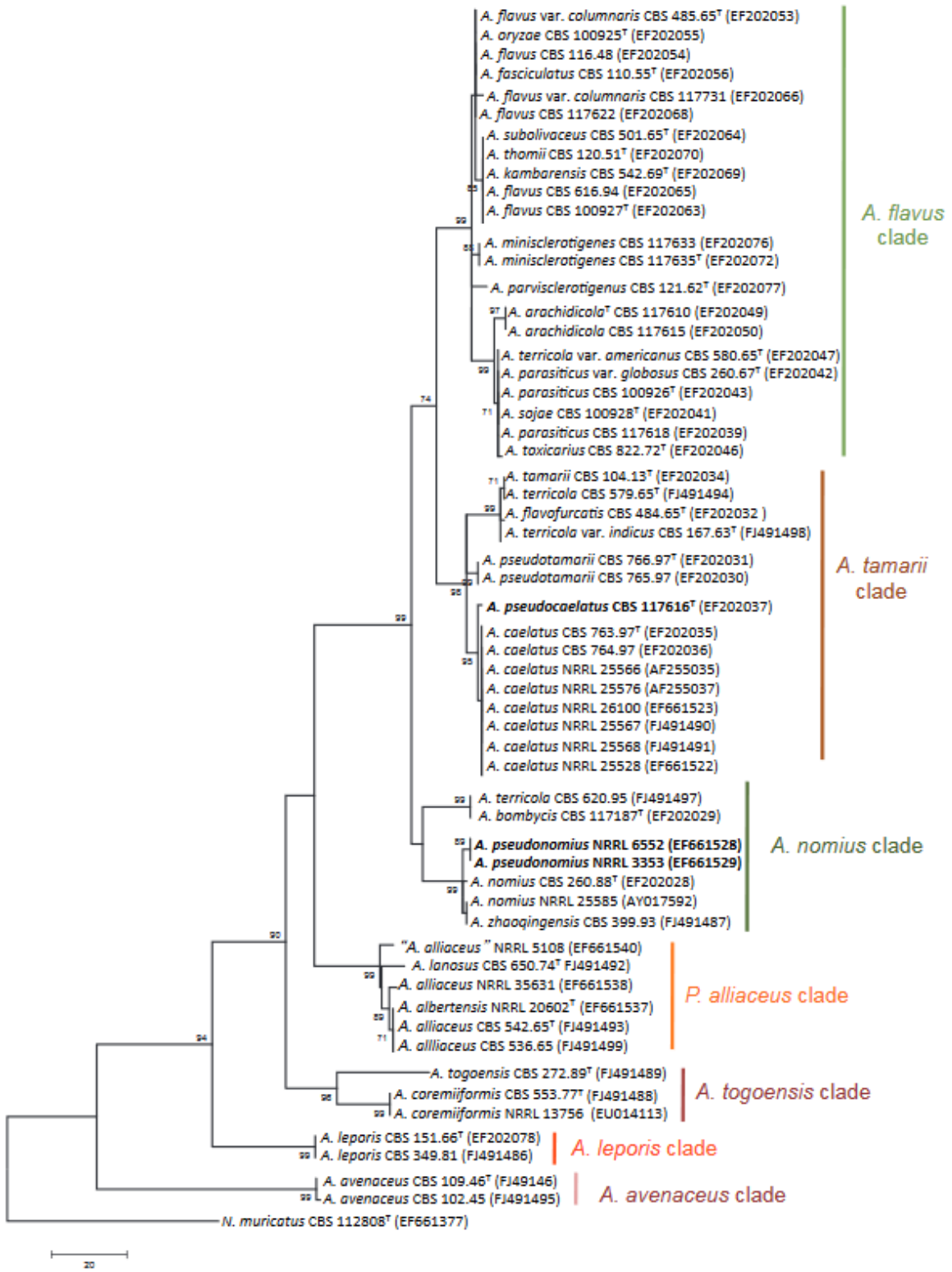

Figure 10. Maximum parsimony phylogenetic tree based on *β-tubulin* gene. Bootstrap values are indicated above 70% (reprinted from Varga et al. 2011).

1.6.1 Basal species

Basal species are less studied because they are rare and do not produce secondary metabolites considered as main mycotoxins. *Aspergillus avenaceus, A. leporis* and *A. alliaceus* are characterized by a Q-10 ubiquinone system, while derivate species often have Q-10 (H2) ubiquinone system (Yamatova et al. 1990; Kuraishi et al. 1990; Rigo et al. 2002; Varga et al. 2003). Conidia of *A. leporis* and *A. alliaceaus* are mostly globose, like the majority of species in the section, though they are smaller, whereas the conidia of *A. togoensis* clade are irregular and larger (Varga et al. 2011).

- *Aspergillus avenaceus* clade

Aspergillus avenaceus clade is formed by a single species, which is the basal taxon of the section (Figure 11). *Aspergillus avenaceus* home range in restrained to the USA, and it has been isolated from soil, seeds, peanuts and cornmeal. Phenotypic traits are colonies in olive shades, long conidiophores and black and long sclerotia, small and relative globose conidia, biseriate and radiate heads, and a restricted growth up to 37 °C (Christensen 1981). It is a heterothallic fungus that forms multiple nonostiolate ascocarps within the matrix of sclerotia (Horn et al. 2011). Though it is unable to produce aflatoxins, it is able to produce ochratoxin A, and avenaciolide, an extrolite with antibiotic properties (Bayman et al. 2002 ; Varga et al. 2011).

Figure 11. Colonies of *Aspergillus avenaceus* CBS109.46. Cultures on CYA and MEA, 7 days at 25 °C (modified Varga et al. 2011)

- *Aspergillus togoensis* clade

Aspergillus togoensis clade is a basal group formed by two species, *A. coremiiformis* and *A. togoensis* (Figure 12). Both species have been isolated from forest environments in Africa and are rare (Wicklow et al. 1989; Christensen 1990). Evidence that these species are sister taxa includes phenotypic traits, gene sequences, presence of synnemata (Varga et al. 2003), and it is the only basal clade presenting a Q-10 (H2) ubiquinone system in the section (Yamatova et al. 1990). These fungi have radiate and biseriate conidial heads, yellowish to brown colonies, and sclerotia (Wicklow et al.

1989; Christensen 1990). *A. togoensis* and *A. coremiformiis* were included in the section *Flavi* for their phenotypic characteristics, which overlap those of *A. tamarii,* aside from the presence of septate phialides in the first two species (Rigo et al. 2002). Molecular data confirmed that they belong to *A.* section *Flavi,* but they are not closely related to *A. tamarii* (Varga et al. 2011; Taniwaki et al. 2012). Available isolates of both species are scant, making difficult to elucidate the relations between them, and to fully characterize their secondary metabolic profiles. *Aspergilllus togoensis* distributes in Central Africa, and grows on seeds in tropical forests (Wicklow and McAlpin 1990). It produces AF_B and is the only species documented to storage sterigmatocystin in the section *Flavi* (Rank et al. 2011; Varga et al. 2015). *Aspergilllus coremiiformis* has been isolated in Côte d'Ivoire from soil, their colonies are olive to brown shades, and conidiophores are in coremiform arrangements (Christensen 1981; Kozakiewicz 1989).

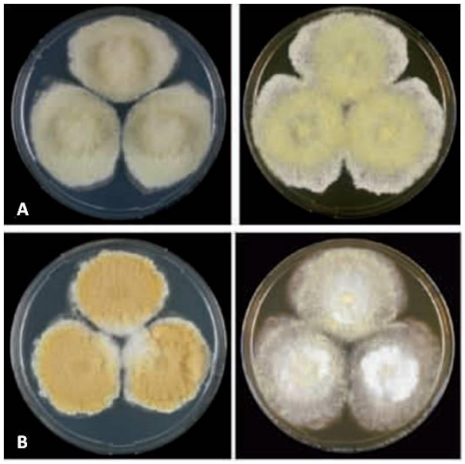

Figure 12. Colonies of *Aspergillus togoensis* clade. A= *A. togoensis* CBS272.89; B= *A. coremiiformis* CBS553.77. Cultures on CYA and MEA, 7 days at 25 °C (modified Varga et al. 2011).

➢ *Aspergillus alliaceus* clade

Aspergillus alliaceus clade encloses two species, *A. alliaceus* and *A. lanosus* (Figure 13). The classification of these species was complicated, at the beginning they were placed in the *A. ochraceus* group based on their metabolite production and phenotypical traits, and later on in the *A. wentii* group (Kozakiewicz 1989). Finally, the clade was moved to *A.* section *Flavi* based on a more complete profile of its secondary metabolites and molecular markers (Varga et al. 2000a). In addition, Varga et al. (2011) synonymized *A. albertensis* with *A. alliaceus* based on their secondary metabolic profiles, phenotypic and molecular traits. Literature described *A. alliaceus* and *A. lanosus* as different species, although, overlapping traits are observed while analyzing them, and generally both species do not show important differences (Varga et al. 2000a), with the exception of the amino acidic sequence of

Mat1.1 (Soares et al. 2012), which has an histidine and a tyrosine at the position 43, respectively. More analyses are required to understand the relations between these species.

Aspergillus alliaceus was described as a pathogen of onion bulbs, it has a cosmopolitan distribution, and it is isolated from grassland soils, groundnuts, nuts, figs, and air (Christensen 1981; Wagacha et al. 2013). *A. alliaceus* is found in its homothallic state (formerly *Petromyces alliaceus*), loci *MAT1-1* and *MAT1-2* are linked in the same chromosome, which differs from other homothallic genera were both genes are not placed together, like some species of *Emericella* (Horn et al. 2011). *A. alliaceus* teleomorphic state has smooth ascospores with several equatorial lines and a fine ridge, they are found as ascocarps encapsulated in stromata, their germination is slow (Horn et al. 2009). Colonies are in ochre shades, conidia heads yellow to cinnamon, smooth and ovate, biseriate, stipes smooth, abundant sclerotia, and growth intensified at 37 °C (Chistensen 1981; Horn et al. 2009). It is non-aflatoxigenic, but it produces several others mycotoxins, like ochratoxins A and B, the first one being linked to figs contamination in California (McAlpin and Wicklow 2005). Furthermore, *A. alliaceus* is associated to certain cases of othorrea, invasive aspergillosis and pulmonary infection; it also produces asperlicins, an antagonist of cholecystokinin, affecting the pancreatic hormonal regulation, gastric secretion, gallbladder contraction and gut motility. Howbeit, some enzymes are used in industry to perform steroid and alkaloid transformations, as peptin degrading enzymes and for their insecticidal properties (in Varga et al. 2000b), also it produces kojic acid and kotanins (Frisvad and Varga 2000). Its sister taxon, *Aspergillus lanosus*, is an asexual rare species occurring in India in teak forest soil. *A. lanosus* is characterize by a colony surface with spicular and trailing hyphae in shades yellow becoming in ochre shades with time, conidia smooth, ovate, globose to subglobose, teratological heads are rare, and lacks sclerotia (Christensen 1982; Varga et al. 2000a).

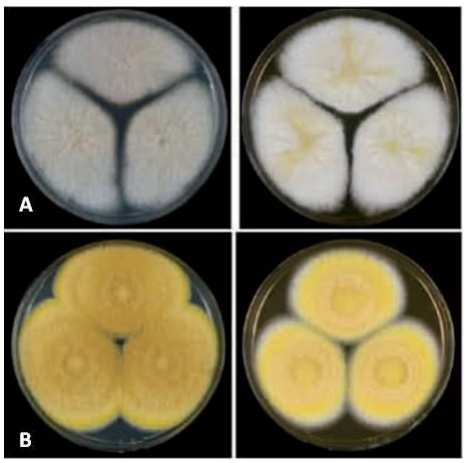

Figure 13. Colonies of *Aspergillus alliaceuss* clade. A= *A. alliaceus* 110.26; B= *A. lanosus* 654.74. Cultures on CYA and MEA, 7 days at 25 °C (modified Varga et al. 2011).

➢ *Aspergillus leporis* clade

Aspergillus leporis clade encloses two non-aflatoxigenic species, *A. leporis*, and the recently described species *A. hancockii* (Figure 14).

Aspergillus leporis occurs in soils associated with shrub communities in the desert areas and woodlands in the USA, where its frequency could be important, and in rabbit feces (Wicklow 1985). Its conidia are globose and small, conidial heads in olive shades, reverse uncolored or pale yellow and white-tipped cinnamon long sclerotia, and slow growth at 37 °C (Christensen 1981). Sclerotia production has been reported on rabbit dung, whereas on Czapek Yeast Autolysate Agar (CYA) or on Malt Extract Agar (MEA) is absent (Wicklow 1985). The sclerotia of *A. leporis* produce the N-alkoxypyridone antiinsectan metabolite, leporin A, suggested to be useful in control of Lepidoptera pests (Varga et al. 2011). The second species, *A. hancockii* is a rare species reported near Kumbia, Australia. Based on phenotypic traits and phylogenetic analyses it is clustered as a sister taxon of *A. leporis.* Its colonies are floccose, with a low sporulation rate, conidial heads radiate, greyish green to olive, sclerotia produced mainly in the center of the colonies, conidia small, spherical to sub-spheroidal, greyish green to olive. New metabolites were reported as part of the screening of its secondary metabolic profile, hancockiamides A-F, dehydroterrestric acid and 7-hydroxytrichothecolon. Additionally, *A. hancockii* yields onychoins A and B, speradine F, kojic acid, fumitremorgin A and eupenifeldin. Its genome was sequenced and deposited in GeneBank, under the BioProject accession PRJNA328536 (Pitt et al. 2017).

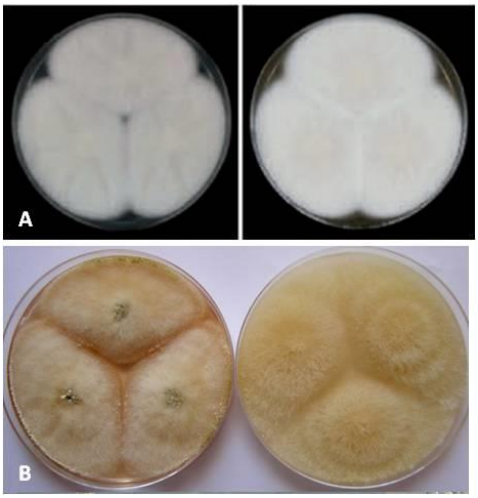

Figure 14. Colonies of *Aspergillus leporis* clade. A= *A. leporis* CBS151.66; B= *A. hancockii* Cultures on CYA and MEA, 7 days at 25 °C (modified from Varga et al. 2011 and Pitt et al. 2017).

➢ *Aspergillus bertholletius* clade

Aspergillus bertholletius is a mold associated with *Bertholletia excelsa* nuts (Figure 15). It is the sister taxon of a group that includes *A. nomius* clade, *A. tamarii* clade, *A. mottae*, *A. parasiticus* clade and *A. flavus* clade (Taniwaki et al. 2012). It occurs in Brazilian Amazonia, it is rare and it has been isolated from soil nearby the trees, nuts and shell nuts of *B. excelsa*. Its optimal temperature varies from others species in the section, growing slowly at 37 °C. Phylogenetic analyses suggested that is a monophyletic group, composed by a unique species (Taniwaki et al. 2012). Its conidia are profuse and brown on CYA and greenish on MEA, there is a lack of exudate, and reverse coloration is pale. The isolates of *A. bertholletius* do not produce aflatoxins, but some isolates can produce intermediary products (*i.e*. O-methylsterigmatocystin), suggesting the presence of an in complete aflatoxin gene cluster. Other secondary metabolites include CPA and its precursors, tenuazonic acid, kojic acid, ustilaginoidin C and indole alkaloids (Taniwaki et al. 2012).

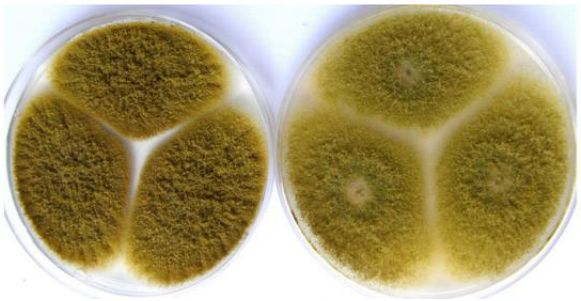

Figure 15. Colonies of *Aspergillus bertholletius*. Cultures on CYA and MEA, 7 days at 25 °C (modified from Taniwaki et al. 2012).

1.6.2 Clades of species of major economical interest

➢ *Aspergillus flavus* clade

Aspergillus flavus was considered as a unique species, nevertheless, several studies support that *A. flavus sensu lato* is a species complex, which includes cryptic species, making their differentiation complicated as they overlap several phenotypic and molecular traits. Species nested in the clade present colonies green to yellowish shades, conidial heads mostly biseriate and splitting radiate (Varga et al. 2011), and a Q-10 (H2) ubiquinone system (Varga et al. 2003). Species nested in the clade are heterothallic, and both idiomorphs of mating type gene *Mat1.1 and Mat1.2* have been amplified (Soares et al. 2012). *Aspergillus flavus sensu lato* has been divided in two morphotypes based on the amount of conidia and sclerotia size: (1) “L” morphotype: high production of conidia,

large sclerotia (> 400 µm), and variable production of aflatoxin B and cyclopiazonic acid; and (2) "S" morphotype: low production of conidia, conspicuously production of small sclerotia (< 400 µm) and high production of aflatoxins and generally produce cyclopiazonic acid. The morphotype "S" is less frequently found, and is subdivided into two groups distributed differently in the world, and with a different synthesis of aflatoxins: the "S_B", strains that are only able to produce B aflatoxins and found in USA; and the "S_{BG}" strains that are able to produce B and G aflatoxins (Cotty 1989; Geiser et al. 2000; Chang et al. 2009b). Furthermore, based on molecular data *A. flavus sensu lato* was also split in two reproductively isolated groups, I and II, which were not necessary monophyletic (Geiser et al. 1998). Ehrlich et al. (2004) suggested, based on molecular analyses, that strains belonging to the morphotype "L" were a monophyletic group, and strains belonging to "S_{BG}" should be place into a different taxon. Later on, isolates classified as "S_{BG}" and belonging to the group II have been reclassified as *A. minisclerotigenes* and *A. parvisclerotigenus* (Frisvad et al. 2005; Pildain et al. 2008). Further analyses determined that the lack of production of G aflatoxins was linked to deletions in the *norB-cypA* (*AflF-AflU*) region of the aflatoxin gene cluster on *A. flavus sensu stricto*. Two types of deletion were found, type I deletion (1.5 kb) mostly overlaps the type II deletion (1 kb), both deletions have arisen from independent evolutionary events. The type II deletion comprises two gaps, one absent in type I deletion, this gap of 32 bp in the *norB* (*aflF*) gene that encodes for amino acid residues 300-310 of the *NorB* aryl alcohol dehydrogenase (Chang and Erlich 2010). Further, Chang et al. (2006) showed that *A. flavus* L morphotype generally presents a 0.8 kb deletion, whereas *A. flavus* S_B morphotype present of 1.5 kb. The inability of producing G- aflatoxin is suggested to have occurred several times in the group (Ehrlich and Yu 2010).

Aspergillus flavus is the most well known species in section *Flavi* (Figure 16). This fungus is thermotolerant, growing at temperatures between 12 – 48 °C, yet its optimal temperature range is 28 – 37 °C (Yu et al. 2005). Its sexual state has been characterized in laboratory conditions and named as *Petromyces flavus,* and cryptic sexual reproduction is accepted to occur in nature (Horn et al. 2009). Among fungi in the section, it has the wider distribution worldwide, being more common between latitudes 35 N to 35 S, and less frequent in temperate regions (Mehl et al. 2012). It is normally associated with cultivars and different storage commodities that are principally colonized by airborne conidia. Maize is its optimal substratum, followed by cottonseed, peanuts, nuts, spices, oil seed crops, among others (Horn 2003). *A. flavus* is considered as a minor phytopathogen associated with rot in certain crops like maize, peanuts and cotton seeds (Klich 2007). This species is the main producer of AFB_1 worldwide, which is considered as the most potent natural compound with carcinogenic, teratogenic and mutagenic characteristics (IARC 1993), despite the majority of isolates identified are not AFB_1 producers (60–70 %) (Varga et al. 2011). The genomic analysis showed that *A. flavus* and *A. oryzae* genomes present more genes than *A. fumigatus* and *A. nidulans*

(Machida et al. 2008). The acquisition of the genes can occur via two mechanisms, horizontal gene transfer (HGT) and gene duplication and divergence (GDD). These processes were suggested based on the mosaic distribution of anomalous genes on the chromosomes. *Aspergillus flavus* is also the second source for aspergillosis (Gonçalves et al. 2013). As Amaike and Keller (2011) reported that this illness is caused by the spores, principally via inhalation and less frequently by wounds. Aspergillosis affects mostly immunosuppressed patients, and has a high level of mortality (Gonçalves et al. 2013). It is also associated to invasive cutaneous aspergillosis in low-birth-weight infants in neonatal intensive care unit (McAlpin et al. 2005). *A. flavus* is the primary causative agent of keratitis that results in corneal damage and sight loss in patients from tropical regions, especially from India (Srinivasan 2004). Animals can also suffer from this illness, especially rabbits, poultry and bees (Amaike and Keller 2011). As aforementioned, *Aspergillus flavus* is characterized by its phenotypic and genotypic plasticity. Morphological traits include greenish to yellowish colonies, conidial heads usually biseriate and radiate, vesicles globose to elongate, conidia globose to ellipsoidal, smooth to finely roughened walls, sclerotia if present usually "L" and in some population of the USA "S_B" morphotypes (Klich 2007). The production of mycotoxins is extremely variable, from highly toxigenic to nontoxigenic strains (Horn 2003).

The most important extrolites associated with *A. flavus* are AFB_1, cyclopiazonic acid, kojic acid, aspergillic acid, aflatrems, aflavinin and versicolorins. Nowadays, somewhat 56 putative gene clusters associated to secondary metabolites synthesis are presumed based on polyketide synthases (PKSs), nonribosomal peptide synthetases (NRPSs), hybrid PKS-NRPSs, and prenyltransferases (PTRs) genes (Georgianna et al. 2010; Arroyo-Manzanares et al. 2015; Cary et al. 2015b). The study of gene clusters and biosynthetic pathways in this fungus is ample; to date, secondary metabolites belonging to gene clusters linked to aflatoxins, cyclopiazonic acid, aflatrem, asparasone, leporins, bicumarins, piperazines, ditryptophenaline have been identified experimentally (Arroyo-Manzanares et al. 2015; Cary et al. 2015a; Cary et al. 2015b). These studies also evidenced the correlation of extrolite production and fungus physiology. Cary et al. (2015b) showed the conspicuous expresion of genes belonging to the cluster 39 in sclerotia, linked to the production of aflavarin that has anti-insectan activity. The authors suggested that aflavarin could play a key role in *A. flavus* ecology and survival, a part of its effects on insects.

Several strategies to avoid the presence of aflatoxins in crops and storage have been developed, and most of them targets *A. flavus* as the main producer. The use of non-aflotoxigenic strains of *A. flavus* (*e.g*. AF36) as biocontrol in crops is already use for over a decade. This biocontrol benefits from parasexual reproduction, by controlling VCGs ratios in natural populations. In this type of reproduction, as aforementioned, stable hyphal fusions take place between VCGs with compatible *het* loci (Amaike and Keller 2011). Each VCG could comprise a variable number of aflatoxigenic and

non-aflatoxigenic strains. Non-aflatoxigenic strains are inoculated as part of a mix that includes the fungus and all the nutritional requirements for its development. As a result, changes in the genotype composition of *A. flavus* communities occur, reducing ratios of aflatoxigenic strains. It is suggested that is a safe type of biocontrol because there is not recombination between AF36 and strains belonging to other VCGs (Grubisha and Cotty 2015).

Aspergillus oryzae is recognized as a domesticated *A. flavus* (Geiser et al. 2000), that is non-aflatoxigenic and is considered as safe (GRAS status, FAO 2016) (Figure 16). This species is reported in Japan and China, and not in nature. *A. oryzae* is widely use in industrial processes, some linked to koji, as starters in the first steps of fermentation to digest ingredients such as steamed rice, soybean, and wheat, being its conidia the target structure (Machida et al. 2008; Ogawa et al. 2010). Similarly, it is also used as source for enzymes, like glucoamylase, alpha-amylases and proteases, for the production of starch, baking, and brewing worldwide (Machida et al. 2008). The lack of aflatoxin production is not a unique feature of *A. oryzae* considering that approximately 60% of *A. flavus* strains are non-producers (Cotty and Cardwell 1999), making their species status more practical than evolutionary, as it is easier to differentiate these isolates from the potential aflatoxigenic isolates of *A. flavus*. Amongst the evidence of its domestication is that both species shares 99.5% genome-wide nucleotide similarity (Chang and Erlich 2010). *A. oryzae* appears to come from a group of non-aflatoxigenic *A. flavus* group I, since they share phenotypical traits, like olive green and floccose colonies, large conidia, large sclerotia if present, and molecular analyses clustered them (Geiser et al. 1998, 2000; Chang and Erlich 2010). Genome data have shown that both species share common characters, like 8 chromosomes, and a common entire genome size around 37.6 Mb (Machida et al. 2008; Cleveland et al. 2009). Further, deletions of type I and II in the *aflF-aflU (norB-cypA*) region, and a complete deletion of the region were also observed in *A. oryzae* (Chang et al. 2015).

The most important genetic differences between *A. flavus* and *A. oryzae* are linked with the aflatoxin biosynthesis gene cluster, such as the deletion of *AflT* gene, a frameshift mutation in gene *norA* (*AflE*) and a nucleotide substitution in *VerA* (*AflN*) in *A. oryzae*, which leads to the non production of this mycotoxin (Lee et al. 2006; Tominga et al. 2006). Regarding the aflatoxin cluster gap, *A. oryzae* could be splitted in groups: (i) group 1 has the *pksA* (*AflC*), *fas1* (*AflB*), *aflR, ver1* (*AflM*) and *vbs* (*AflK*) orthologs; (ii) group 2 has the *ver1* (*AflM*) and *vbs* (*AflK*) orthologs, in addition strains have a unique structure adjacent to the 'breakdown and restoration' region, located upstream of the *ver1* (*AflM*) gene, suggesting a monophyletic group; and (iii) group 3 has the *vbs* (*AflK*) orthologs (Chang and Erlich 2010). Some other genomic differences are associated to other processes, like the presence of two or three copies of α-amylase (versus one copy in *A. flavus*), which facilitates starch degradation, as well as genotypic differences on the glutaminase and sesquiterpene loci (Gibbons et al. 2012). In addition, the absence of cyclopiazonic acid in *A. oryzae* is linked to gaps in the

chromosome 3 subtelomeric region, which interferes with the biosynthesis pathway (Geiser et al. 2000; Chang and Erlich 2010).

Gibbons et al. (2012) suggested that differences between *A. oryzae* and *A. flavus* primary and secondary metabolisms could partially be explained by the domestication of the first. The principal secondary metabolite produced by *A. oryzae* is kojic acid, which is used in industrial processes. In addition, Rank et al. (2012) identified some secondary metabolites that are not produced by *A. flavus*, an aflatrem precursor (13-dehydroxypaxilline), and two analogues of parasiticolide A (dide- and 14-deacetyl parasiticolide A).

Regardless the presence of mating type genes *Mat-1* in *A. oryzae*, Wada et al. (2012) isolated *Mat1-1* and *Mat1-2* idiomorphs in a study performed with 164 tane-koji strains. Abundance ratios of both idiomorphs were close to 1:1, suggesting that sexual reproduction could occur under certain circumstances, even though the scarce production of sclerotia.

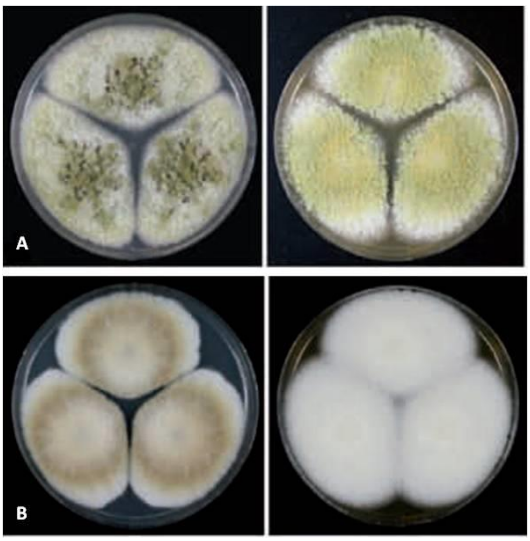

Figure 16. Colonies of *Aspergillus flavus* clade. A= *A. flavus* CBS100927; B= *A. oryzae* CBS100929; Cultures on CYA and MEA, 7 days at 25 °C (modified from Varga et al. 2011).

➢ Species S_{BG}

Morphotype strains "S_{BG}" had been reclassified as *Aspergillus minisclerotigenes* and *A. parvisclerotigenus*, one of the most conspicuous traits is the small size of their sclerotia, as both names suggest (Figure 17). *A. minisclerotigenes* is reported in different world regions (Africa, South and North America and Australia), whereas, *A. parvisclerotigenus* has a restricted distribution, it has been found in the Guinean Gulf, in Africa (Perrone et al. 2014; Adjovi et al. 2014). At the beginning both species where considered as *A. flavus*, nevertheless data accumulated suggested that the differences were enough to rise both at species level, like the production of B- and G- aflatoxins, and

the molecular evidence inferred from gene markers *benA, cmdA* and mating type genes *MAT1* (Pildain et al. 2008; Soares et al. 2012).

Moreover, phylogenetic inference studies suggest that "S_{BG}" morphotype is a paraphyletic group, which places *A. minisclerotigenes* as the sister taxon of a group formed by *A. flavus* and *A. oryzae*, whereas *A. parvisclerotigenus* is placed as the sister taxon of a group that includes the aforementioned species (Soares et al. 2012). Both species seems to share similar metabolic profiles, AF_{BG}, CPA, kojic acid, aspergillic acid, aflatrem, and aflavarins. However, *A. minisclerotigenes* produces aflavinins, which are not reported for *A. parvisclerotigenus*, and the latest produces aspirochorin not produced by the former (Varga et al. 2011).

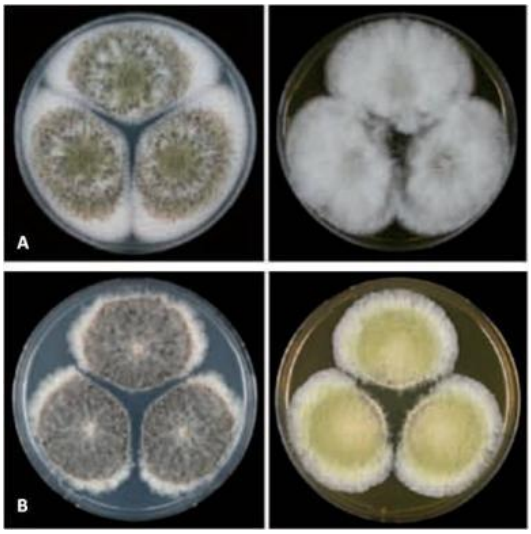

Figure 17. Colonies of *Aspergillus flavus* clade S_{BG}. A= *A. minisclerotigenus* CBS117635; B= *A. parvisclerotigenus* CBS121.62*;* Cultures on CYA and MEA, 7 days at 25 °C (modified from Varga et al. 2011).

➢ *Aspergillus parasiticus* clade

Aspergillus parasiticus clade was proposed by Soares et al. (2012), before the species and species related to *A. parasiticus* were shown to be nested in *A. flavus* clade. The number of species related to *A. parasiticus* has increased during the last decade grace to the use of the polyphasic approach. These species have colonies in green shades, darker than those of *A. flavus*, conidial heads mostly uniseriate, a Q-10(H2) ubiquinone system (Varga et al. 2003), species are heterothallic, and present both idiomorphs for mating type gene *Mat1* loci (Soares et al. 2012). They have smaller home ranges, and have more constrained niches regarding their substrate preferences while compared to *A. flavus.*

Aspergillus parasiticus is considered as the second producer of aflatoxins, it is able to produce B- and G- aflatoxins, and only 3 to 6% of isolates are considered non-aflatoxigenic (Chang et

al. 2007a) (Figure 18). It is mostly associated with ground crops and with almonds, and rarely in other commodities (Amaike and Keller 2011). Like *A. flavus,* it occurs frequently in tropical and subtropical areas, however it can also be found in temperate regions (Varga et al. 2011; Baranyi et al. 2015). This species is heterothallic, and its populations are genetically diverse, which suggests that cryptic sexual reproduction occurs in nature (Horn 2007). Horn et al. (2009), after crossing different vegetative strains in laboratory conditions, identified the sexual form that was named *Petromyces parasiticus*. Morphological characters include conidial heads mostly uniseriate, rarely biseriate, and loosely radiate. Conidia globose to subglobose, conspicuously roughened. Sclerotia and stromata external appearance globose to ellipsoidal, becoming darker with time. Stroma contains fertile, infertile, or a combination of both types of ascospores. Ascospores oblate, finely tuberculate, presence of a thin equatorial ridge, hyaline to pale brown, frequently an oil droplet present, globose to subglobose or irregularly shaped, nonostiolate, with white to light brown interior (Horn et al. 2009). *Aspergillus parasiticus* produces aflatoxins, kojic acid and aspergillic acid but not cyclopiazonic acid (Varga et al. 2015).

Over the last decade *Aspergillus parasiticus* composition has changed, for instance, *A. toxicarius* has been synonymized based on phenotypical and molecular data (Rigo et al. 2002; Varga et al. 2011). On the other hand, some populations are questioned to belong to the same species. For instance, Garber and Cotty (2014) suggested that the populations of *A. parasiticus* associated to sugarcane from Japan and Rio Grande Valley (USA) presented considerable differences at molecular, phenotypical and VCGs level from *A. parasiticus*. These populations associated with sugarcane could be a new species that has coevolved with its host, and could play an important role in the community dynamics of sugar cane crops (Kumeda et al. 2003; Garber and Cotty 2014), however further analysis are required to understand their phylogenetic relationships.

Aspergillus sojae is considered the domesticated species of *A. parasiticus*, and similarly to *A. oryzae*, this decision is based on practical purposes because it produces kojic acid, which is used in fermentation processes (Rigo et al. 2002). This species occurs in China, India and Japan, but it has not been reported in agricultural soils (Chang et al. 2007b; Varga et al. 2011). Morphologically, its isolates traits overlaps those of *A. parasiticus*; however differences in colonies coloration and texture, and conidia diameter are used as diagnostic traits. For instance, *A. sojae* colonies tend to be brownish olive versus dark olive green in *A. flavus* (Chang et al. 2007) (Figure 18). Genetically, the haplotypes of this species present differences from *A. parasiticus*. The main mutations are related to the aflatoxin biosynthetic pathway, especially differences observed on *AflR* (an insert of six-base repeat CTCATG in the amino-terminal coding region and a transition on nucleotide 1153 C for T) that creates a premature stop codon, resulting in a suppression of gene expression, and a disruption of interaction between *AflR* and the *AflJ* co-activator. In addition, the polyketide synthase gene presents

abnormalities (Chan et al. 2007). These differences between *A. parasiticus* and *A. sojae* explain why the latest does not produce aflatoxins and can be safely used.

Description of *Aspergillus arachidicola* was based on an isolate from *Arachis glabrata* leaves in Argentina (Figure 18). Morphologically, the colony surface is floccose with plenty conidial heads olive to olive brown, uniseriate to biseriate, conidia globose to subglobose, echinulate, greenish, absence of sclerotia, vesicles globose to subglobose. *A. arachidicola* extrolites profile includes B- and G- aflatoxins, kojic acid and aspergillic acid, chrysogine, parasiticolide, ditryptophenaline and "NO_2" metabolite (Pildain et al. 2008; Varga et al. 2011; Varga et al. 2015). The genome of the type strains is being sequenced (Moore et al. in preparation 2018).

Aspergillus novoparasiticus (Gonçalves et al. 2012) is morphologically similar to *A. parasiticus* and is an aflatoxin producer. It was described in South America in hospital environments, yet latter it was isolated from crops (Viaro et al. 2017), and its home range shifted, including a population in the Guinea Gulf (Adjovi et al. 2014). In Africa, it was reported to grow on cassava (Adjovi et al. 2014). Morphologically this species is characterized by greenish-yellow to olive colonies, with profuse amount of conidiophores, conidia globose to subglobose, lobate-reticulate, green conidial heads columnar olive yellow, generally uniseriate, rare biseriate, absence of sclerotia, presence of exudates, vesicles spatulate to pyriform (Figure 18). Extrolites associated to this species are B- and G- aflatoxins and kojic acid.

Soares et al. (2012) described three new aflatoxin producer species from Portugal, whose phenotypical features overlap those of *A. parasiticus*, including the production of B- and G- aflatoxins, *A. sergii*, *A. transmontanensis* and *A. mottae*. All species are rare species. *Aspergillus sergii* grows in almond shells (*Prunus dulcis*), it is able to produce aspergillic acid and cyclopiazonic acid in addition to aflatoxins (Varga et al. 2015). Morphologically it differs from *A. parasiticus* since it has rough conidia and mostly uniseriate conidial heads, presence of dark large sclerotia, and its conidia coloration tends to be lighter (Soares et al. 2012) (Figure 18). *A. transmontanesis* grows in almond shells (*Prunus dulcis*). Morphologically this species resembles to *A. parasiticus*, but it presents biseriate conidial heads and larger, darker and profuse sclerotia (Soares et al. 2012). Apart of aflatoxins, it produces aspergillic acid (Varga et al. 2015) (Figure 18).

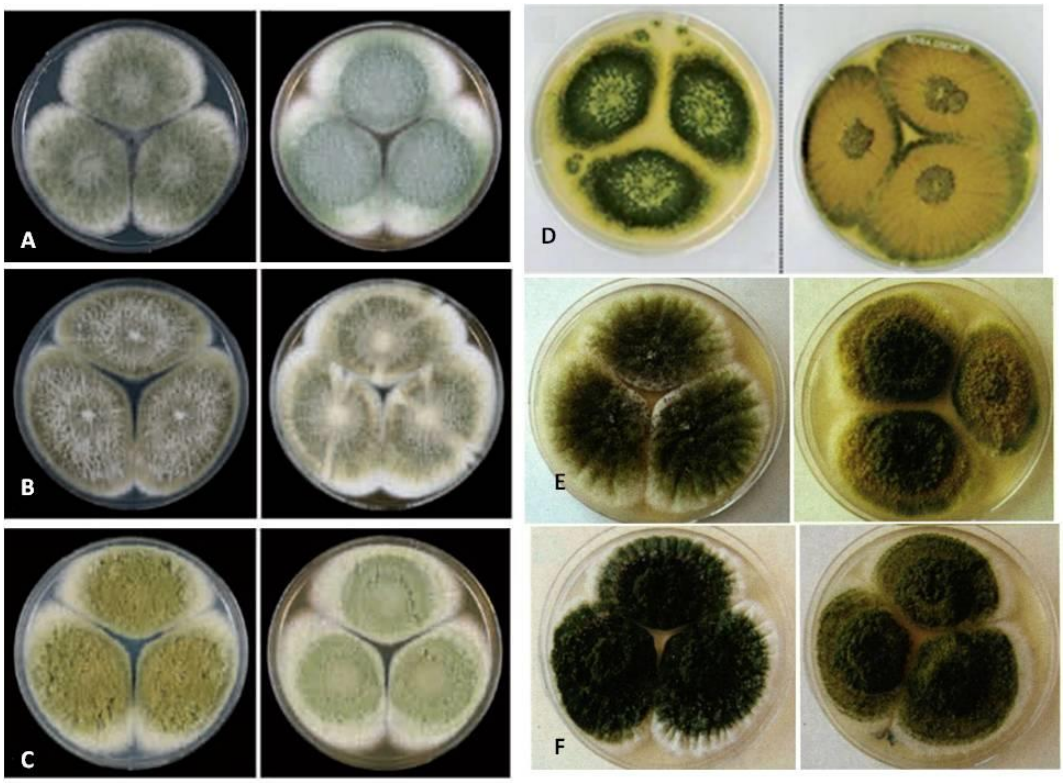

Figure 18. Colonies of *Aspergillus parasiticuss* clade. A= *A. parasiticus* CBS100926; B= *A.sojae* CBS100928*;* C= *A. arachidicola* CBS117610; D= *A. novoparasiticus* CBS126849*;* E= *A. sergii* MUM10.219; F= *A. transmontanensis* MUM10.214*;* Cultures on CYA and MEA, 7 days at 25 °C (modified from Pildain et al. 2008; Varga et al. 2011; Soares et al. 2012).

A. mottae is the sister taxon of the group formed by *A. flavus* and *A. parasiticus*. It was isolated from maize kernels in Portugal and its occurrence is rare. In addition to aflatoxins, it produces aspergillic acid and cyclopiazonic acid. Morphologically, it is characterized by flat colonies, with scarce yellow-green conidia heads, small and profuse dark sclerotia, generally biseriate heads, rarely uniseriate, and vesicles globose to subglobose (Soares et al. 2012) (Figure 19).

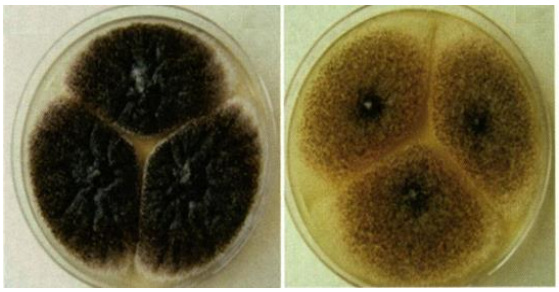

Figure 19. Colonies of *Aspergillus mottae* MUM10.231. Cultures on CYA and MEA, 7 days at 25 °C (modified from Varga et al. 2011).

➢ *Aspergillus tamarii* clade

Aspergillus tamarii clade is composed by cryptic species with ubiquinone system Q-10 (H_2), and overlapping phenotypic traits, for instance their colonies are initially in green or yellowish shades becoming brown to brown-bronze over time (Ito et al. 2001; Varga et al. 2003). Data from the genes *benA* and *cmdA* allowed the differentiation of four species in this group: *A. tamari* and *A. caelatus* that do not produce aflatoxins, and *A. pseudotamarii* and *A. pseudocaelatus* that produce B and G AFs (Varga et al. 2011). Metulae and philiades of *A. caelatus* and *A. pseudotamarii* are smaller than those of *A. tamarii*. In addition, temperature requirements are different among species, *A. tamarii* grows at 37 and 42° C whereas *A. pseudotamarii* and *A. caelatus* grow only at 37 ° C. Relationships among the species within the clade have been confirmed by molecular markers and secondary metabolic profiles (Ito et al. 2001; Soares et al. 2012).

Aspergillus tamarii isolates are considered as a low risk species and several of its extrolites are used in fermentation processes in the food industry (Varga et al. 2011). Some health problems were related to it, for example the human keratitis in Southern India (Kredics et al. 2007) and several potential allergens that could be spread by spores in the air (Vermani et al. 2010). *A. tamarii* has been found in tropical and subtropical areas in Africa, Asia, North and South America on nuts, coconuts, coffee beans, soil, sugarcane, spices, cereals, *Xylotrechus* insects and soils (Rigo et al. 2002; Kumeda et al. 2003; Pitt and Hocking 2009). Phenotypic traits are colonies in brown to bronze shades, colorless to pinkish reverse, radiate and biseriate conidial heads, globose to subglobose conidia, roughened with tubercles, and rare sclerotia (Ito et al. 2001; Rigo et al. 2002) (Figure 20). Among its secondary metabolites, kojic acid, speradine A, cyclopiazonic acid, fumigaclavines, amylases, proteases and xylanolytic enzymes are produced.

Aspergillus pesudotamarii occurs in South America and Japan (Varga et al. 2011). It is morphologically similar to some isolates of *A. tamarii*, showing an orange-brown or brownish bronze coloration on mature colonies. However, coloration differs at the first days of colony development; in *A. pseudotamarii* it is green or yellowish green, whereas in *A. tamari* it is mainly brown. Colonies are generally velvety and present abundant sporulated conidia, reverse pale yellowish brown. Conidial heads globose to radiate, generally splitting into several columns, biseriate. Sclerotia small and pyriform (Goto et al. 1996; Ito et al. 2001) (Figure 20). *Aspergillus pseudotamarii* produces AFB_1, cyclopiazonic acid and kojic acid, yet its association with commodities contamination is unknown (Varga et al. 2015).

Aspergillus caelatus is reported in the USA and Japan, it has been isolated from agricultural field soils, peanut damaged by insects and tea fields (McAlpin et al. 2005). Phenotypical traits include colonies in shades olive that become brownish-olive over time of maturation, reverse pale yellowish.

Conidiophores, vesicles and stipes are smaller than *A. tamarii* (Goto et al. 1996; Ito et al. 2001) (Figure 20). As well, *A. caelatus* can form synnemata and sclerotia sessile and stipitate, which resemble those of *A. togoensis*, but smaller (McAlpin 2004). It produces kojic acid and aspirochlorin, but not cyclopiazonic acid (Rigo et al. 2002; Varga et al. 2011). McAlpin and Wicklow (2005) suggested that VCGs in tea plantations in Japan are less diverse that VCGs in the USA, and this could be related to the agricultural systems; in the USA, fields uses a rotation crop system that causes more disruption in microbial communities and creates different niches, allowing the colonization by other genotypes.

Aspergillus pseudocaelatus was described by Varga et al. (2011) based on a sample found in Argentina on a leaf of *Arachis burkartii*. Its distribution is restricted to South America, in some areas of Argentina (Corrientes) and Brazil (Varga et al. 2011; Taniwaki et al. 2017; Viaro et al. 2017). Colonies have a velvet surface and abundant conidial heads. Conidial heads olive to brown, uniseriate or biseriate. Conidia are greenish, globose to subglobose and echinulate; it does not produce sclerotia, and its vesicles are globose to subglobose (Figure 19). It produces AFBG, cyclopiazonic acid and kojic acid.

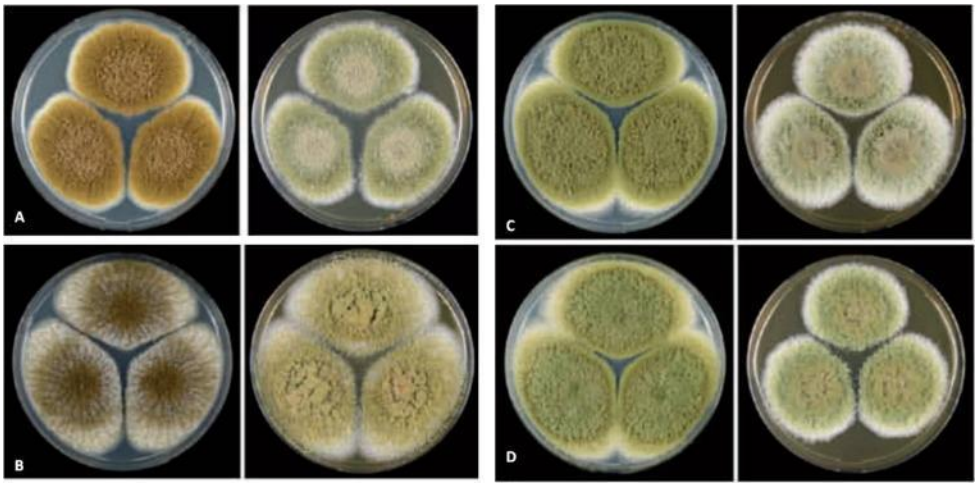

Figure 20. Colonies of *Aspergillus tamarii* clade. A= *A. tamari* CBS104.13; B= *A. pseudotamarii* CBS766.97; C= *A. caelatus* CBS763.27; D= *A. pseudocaelatus* CBS117616; Cultures on CYA and MEA, 7 days at 25 °C (modified from Varga et al. 2011).

➢ *Aspergillus nomius* clade

The lack of production of cyclopiazonic acid is a diagnostic trait within the clade *nomius* and can be considered a synapomorphy of the clade. Moore et al. (2016) showed that the three genes responsible of CPA production are present in *A. bombycis*, but a deletion of a nucleotide at position

954 in the pks-nrps gene suppresses CPA production. Moore et al. (2016) also showed in *A. bombycis* that the gene cluster of aflatoxins is located in a different chromosome than the CPA gene, whereas in *A. flavus and A. parasiticus* both clusters are juxtaposed. Lack of information on the other two species of the clade does not allow confirming that this arrangement is fixed in the clade, but studies of CPA gene cluster could elucidate the evolution of the pathway, in addition to shed some light on the evolution of the *Flavi* section. Species in this clade have colonies in green shades, are aflatoxigenic, and sclerotia if present are small and oval (Barros et al. 2007).

Aspergillus nomius is an important aflatoxigenic species (Figure 21), just below *A. flavus* and *A. parasiticus*. This species has a restricted distribution area, nevertheless, over the last years new reports suggest that it is widespread, occurring mainly in tropical and subtropical areas, and less frequently in temperate regions (Baranyi et al. 2015). In some regions (Brazil), the ability of *A. nomius* to produce aflatoxins is higher than that of the other two producers (Baquião et al. 2014). At the beginning, it was only associated with insects, but it also grows on other substrates, including Brazilian nuts, sugarcane, nuts, crops and seeds (Horn et al. 2011, Baquião et al. 2014). Ehrlich et al. (2007) suggested that this species is a complex species; based on DNA regions, it could be divided into three groups, likewise, sclerotia size of some strains are unusually big (1.00 to 4.00 mm), several small (> 400 µm) and coloration varies from tan to black. *A. nomius* is able to produce B and G aflatoxins, kojic acid, aspergillic acid, tenuazonic acid, miyakamides, anominine, aspernomine, pseurotin, parasiticol, paspaline, paspalinin, pseurotin A, tenuazonic acid, versicolorins, 3-O-methylsterigmatocystin (Massi et al. 2014). This species is heterothallic and its sexual state was identified in laboratory as *Petromyces nomius*, yet the majority of crosses resulted in infertile crosses (76%) (Horn et al. 2010).

Aspergillus pseudonomius (Varga et al. 2011) (Figure 21) is the sibling species of *A. nomius*, though their traits overlap, some are use as diagnostic, like floccose colonies, profuse aerial mycelium and low sporulation, lack of sclerotia, uniseriate conidia heads, globose to subglobose vesicles and stipes rough-walled when observed under scanning electron microscope (Massi et al. 2014). *A. pseudonomius* has a restricted distribution in South America and it has been found on Brazilian nuts and peanuts (Baquião et al. 2014). Its produces B and G AFs, chrysogine, kojic acid and miyakamides, aspergillic acid, 3-O-methylsterigmatocystin, tenuazonic acid, a versicolorin and parasiticol (Massi et al. 2014).

Aspergillus bombycis was described in 2001, the isolates were obtained from silkworm excreta in Japan and the home range settled in Asia (Peterson et al. 2001) (Figure 21). This species was previously misidentified as *A. nomius* since they have common traits (Peterson et al. 2001; Ehrlich et al. 2007; Moore et al. 2016), some of the diagnostic traits are related to temperature, *A. bombycis* grows slowly at 37 °C, and growth is inhibited at 42 °C. Colony texture is loose and deep,

green or yellowish, rarely brownish; roughened, globose to subglobose conidia; conidial heads arranged into loose columns; globose vesicles. Although this species is able to produce B and G AFs, it is not a pathogen for humans or animals. It also produces aspergillic acid and kojic acid (Varga et al. 2015; Moore et al. 2016).

Proper species identification is important at regional level, for instance, *A. nomius* and *A. pseudonomius* are large aflatoxinogenic contaminants of commodities in Brazil. Baquião et al. (2013) suggested that *A. nomius* contamination was more important than that of A. *flavus* and *A. parasiticus* in Brazil nuts, *A. nomius* producing higher amounts of aflatoxins and being more suitable for storage conditions. Later, Massi et al. (2014) suggested that part of the strains found in Brazilian studies corresponded to *A. pseudonomius*. Both examples underline the importance of a proper recognition at species level.

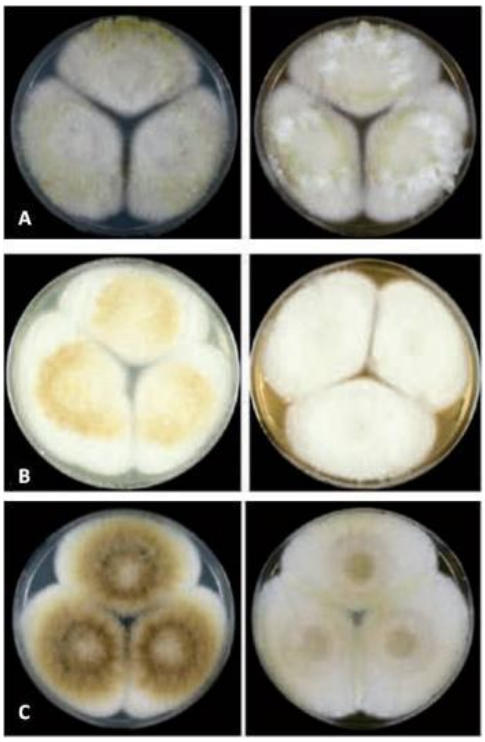

Figure 21. Colonies of *Aspergillus nomius* clade. A= *A. nomius* CBS260.88; B= *A. pseudonomius;* C= *A. bombycis* CBS117187*;* Cultures on CYA and MEA, 7 days at 25 °C (modified from Varga et al. 2011).

1.7 SECONDARY METABOLITES IN SECTION *FLAVI*

Aspergillus section *Flavi* includes a plethora of secondary metabolites and only a small portion has been characterized that includes some beneficial compounds used in biotechnological processes and some mycotoxins. Among the most important mycotoxins in the group are aflatoxins (AFs) and their biosynthetic intermediates such as sterigmatocystin (ST). In addition, among the most

important secondary metabolites are other toxic compounds, such cyclopiazonic acid (CPA), ochratoxin A (OTA) and emergent mycotoxins such as tenuazonic acid (TeA). Here, only a dozen are shown, and their selection is based on the importance in the group (AFs, CPA, ST, aflavarin), or because of its importance as mycotoxins (OTA, TeA).

➢ Aflatoxin biosynthetic pathway

The aflatoxin biosynthetic pathway has been extensively studied because it contains the genes for the biosynthesis of at least ten mycotoxins, including AFs as final products, and sterigmatocystin (Georgianna et al. 2010). Among the species presenting this cluster are species from *Aspergillus* sections *Flavi, Nidulantes* and *Ochraceorosei.*

In *A. flavus*, the gene cluster in charge of AF synthesis is the cluster 54. In several species, it is located near the telomere of the chromosome 3 (Georgianna and Payne 2009). In *A. flavus,* the cluster contains at least 25 genes and it spans a region of approximately 70 kb. The cluster is flanked on the distal end by four putative sugar-use genes and on the proximal end by the cyclopiazonic acid cluster (Amare and Keller 2014). The main cascade regulator genes are *aflR* and *aflS* (Yu 2012).

Studies on several genomes of aflatoxin producer species (*A. flavus, A. oryzae, A. parasiticus, A. fumigatus and A. terreus*) have shown that the aflatoxin gene cluster is arranged into seven modules: *aflA* (*fas2*)/*aflB* (*fas1*), *aflR*/*aflS* (*aflJ*), *aflX* (*ordB*)/*aflY* (*hypA*), *aflF* (*norB*)/*aflE* (*norA*), *aflT*/*aflQ* (*ordA*), *aflC* (*pksA*)/*afW* (*moxY*) and *aflG* (*avnA*)/*aflL* (*verB*) (Carbone et al. 2007a) (Figure 22). Cluster evolution studies suggest that the cluster was transferred vertically and that the retention or loss of the modules occurred differently among species, resulting in divergent lineages. In *A. fumigatus* and *A. terreus*, the clusters have five genes, *aflC, aflS, aflR, aflX* and *aflY,* it has been suggested that they derive from an ancestor with a more complete cluster and that several genes have been lost, as well as that the ancestral cluster may had other functions (Chang and Ehrlich 2010). In *A. fumigatus* and *A. terreus*, these genes are implicated in the synthesis of trypacidin and geodin (Nielsen et al. 2013; Mattern et al. 2015; Trockmorton et al. 2016). In species that have the cluster of aflatoxin, some genes are conserved through evolution, some are duplicated and some genes are transcribed bidirectionally from a single promoter (*aflA-aflB* and *aflR-aflS*) (Chang and Ehrlich 2010).

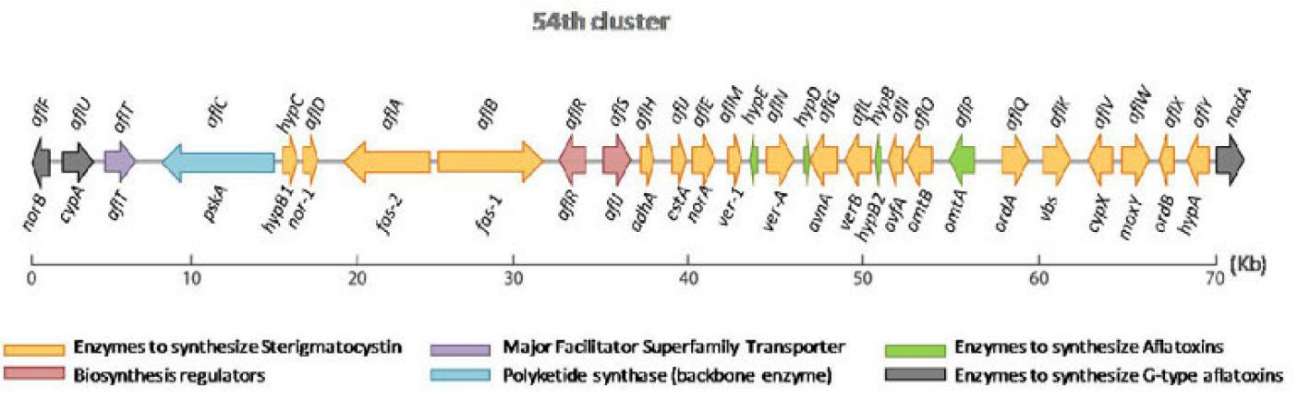

Figure 22. Aflatoxin gene cluster arrangement. Arrows represent the gene position and transcription orientation. Genes are named with the old nomenclature (below arrows) and with the actual nomenclature (above arrows) (modified from Amaike and Keller 2011).

In the next paragraphs, the biosynthetic pathway of AFs in *A. flavus* will be summarized. Some genes have homologs in other species that produce these compounds.The first steps of the synthesis is the transformation of acetyl-CoA and malonyl-CoA into norsoloric acid (NOR). This step requires four genes (enzyme encoded in parenthesis), *aflA* and *aflB* (fatty acid synthases), *aflC* (polyketide synthase) and *hypC* (anthrone oxidase) (Ehrlich 2009; Yu et al. 2004b; Roze et al. 2013). AflA and AflB are in charge of the transformation of the acetyl-CoA and malonyl-CoA in their polyketide structure; AflC catalyzes the formation of the polyketide skeleton and the formation of norsoloric acid anthrone (NAA), which is the substrate for the formation of NOR, and this transformation is catalyzed by HypC (Yu et al. 2004b; Roze et al. 2013) (Figure 23). Then, NOR is transformed into averatin (AVN), and the reaction is catalyzed by a norsolorinic acid ketoreductase encoded by the gene *aflD* (or *nor-1*). In this reaction, the enzyme converts the 1'-keto group of NOR into the 1'-hydroxyl group of AVN (Zhou and Linz 1999) (Figure 23).

The next steps include the transformation of AVN in 5'hydroxyaverantin (HAVN); this hydrolysis reaction is catalyzed by a cytochrome P-450 monooxygenase (cytP450) encoded by *aflG* (*avnA*) (Yu et al. 2000a). Then, HAVN is transformed into 5'-oxoaverantin (OAVN) by the action of an alcohol dehydrogenase encoded by *aflH* (*adhA*). OAVN is the substrate for averufin (AVN); this reaction is catalyzed by a cyclase encoded by *aflK* (*vbs*) (Sakuno et al. 2005). The next reactions include the conversion of AVN into versiconal hemiacetal acetate (VHA). Three genes are involved in this two-step process. The first step is the transformation of AVN into hydroxyversicolorone (HVN) (Yu et al. 2000b), which is carried out by a cytP450 encoded by the gene *aflI* (*avfA*) that is supposed to catalyze the ring formation. HVN is then used as a substrate for VHA. Two enzymes are involved in this step, a cytP450 (cyclization) and a monooxygenase (transformation by a Baeyer-Villiger reaction), encoded by *aflV (cypX)* and *aflW* (*moxY*), respectively (Wen et al. 2005; Yu et al. 2004a). Then,

versiconal hemiacetal acetate is transformed into versiconal (VAL) by an esterase encoded by *aflJ* (*estA*); this is a reaction in both directions. Another gene that interacts in this reaction is the aforementioned *aflK,* which encodes a cyclase, and transforms versiconal (VAL) in versicolorin B (VERB) (Yu et al. 2004b; Ren et al. 2017). The transformation of VERB to versicolorin A (VERA) is carried out by a cytP450 encoded by *aflL* (*verB*). Formation of VERA and VERB are important steps in the production of B and G aflatoxins (Yabe et al 1991) (Figure 23).

The conversion of VERA into demethylsterigmatocystin (DMST) is encoded by 4 genes: i) *aflM* (*ver-1*) encodes a ketoreductase (Hong et al. 2013); ii) *aflN* (*verA*) encodes a cytP450 whose role is unknown, but it is suggested that it can be linked to the formation of an intermediate and the formation of sterigmatocystin (Yu et al. 2004b; Cary et al. 2006); iii) *aflY* (*hypA*) seems to play a role in two hypothetical intermediate structures between VERA and DMST, catalyzed by a Baeyer-Villiger reaction (Ehrlich et al. 2005; Cary et al. 2006); iv) and *aflX* (*ordB*) encodes an oxido-reductase that catalyzes an oxidative decarboxylation and a ring-closure using a Baeyer-Villiger intermediate that results from AflY-catalyzed oxidation (Cary et al. 2006; Ehrlich 2009). Demethylsterigmatocystin is the substrate for *O*-methylsterigmatocystin (OMST) and two genes are involved in this process, *aflO* (*omtB*) and *aflP* (*omtA*). Both codes for *O*-methyltransferases, and the reaction catalyzed by AflP (OmtA) is reversible (Caceres 2016) (Figure 23).

The transformation of demethylsterigmatocystin into *O*-methylsterigmatocystin involves 4 genes: *aflQ* (*ordA*), *hypB* (*hypB2*), *aflE* and *hypE,* the functions of the first two were elucidated. *AflQ* encodes a cytP450 monooxygenase that is important for oxidation of the A-ring of OMST and leads to the AFB1 precursor, 11-hydroxyOMST (HOMST). *HypB* encodes an oxidase, linked with the conversion of HOMST into a 370 Da 7-ring lactone (Ehrlich 2009) (Figure 23).

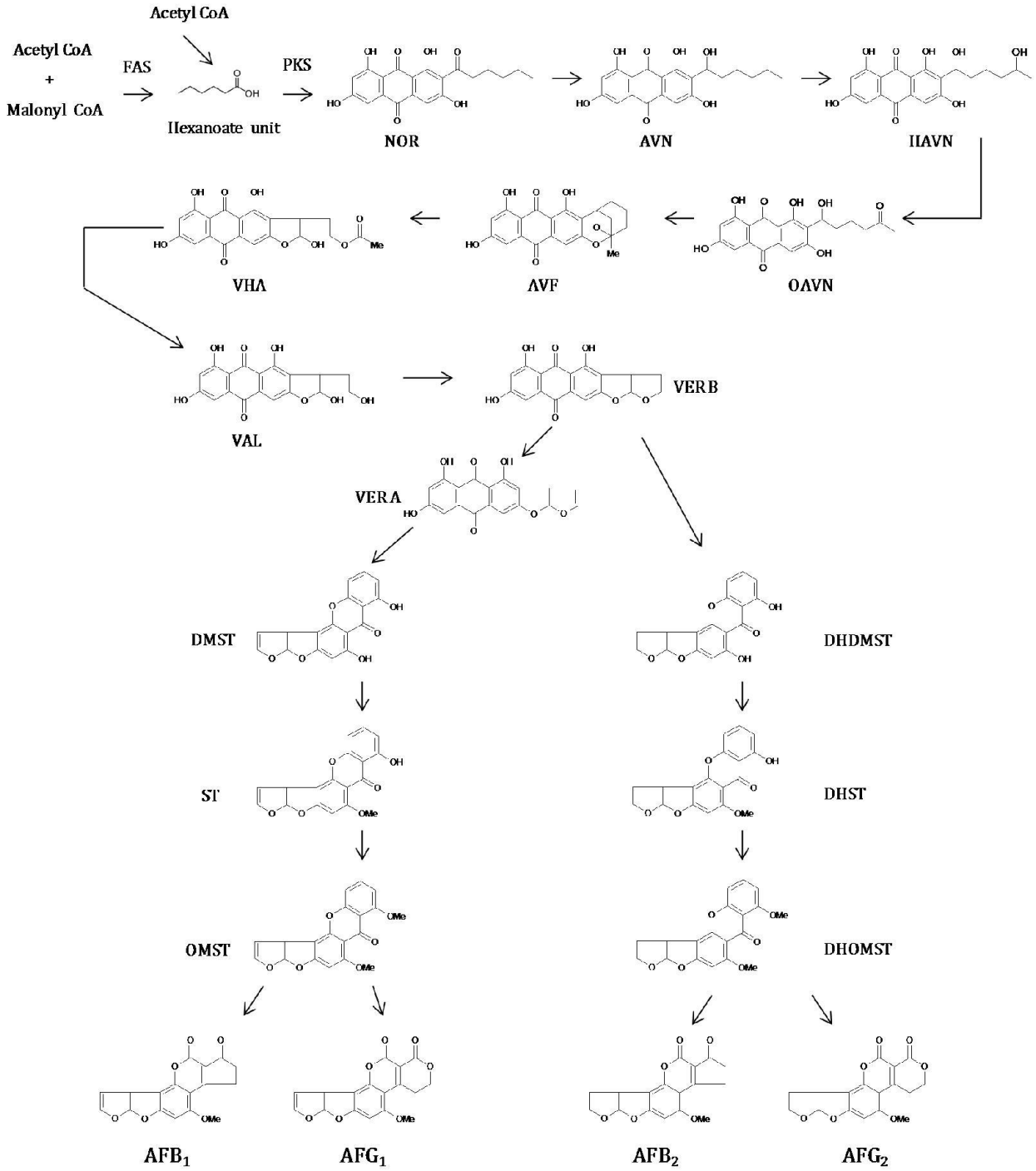

Figure 23. Biosynthetic pathway of aflatoxin in *Aspergillus flavus* (modified from Yu et al. 2004).

➢ Aflatoxins

As aforementioned, aflatoxins were isolated and characterized for the first time after being identified as the cause of turkey X syndrome, an acute aflatoxicosis outbreak that killed over 100,000 poultry in England in the 60s'. Poultry were intoxicated by eating Brazilian peanut (*Arachis hypogaea*)

cake contaminated with *Aspergillus flavus* (Blount 1961). Further studies based on the symptomatology suggested that CPA was also involved (Chang et al. 2009a). AFs are mainly produced by species of the *Flavi* section, however some species of *Aspergillus* section *Ochraceorosei (i.e. A. ochraceoroseus and A. rambellii*) and *A. section Nidulantes* (*i.e. A. astellatus*) are also producers (Varga et al. 2009). The role of AFs is not fully understood, however, they do not seem to be essential for the development of the fungus. Assumptions of AF functionality include the removal of excess carbon in carbon-rich substrates, chemical signals between species, compounds linked to some development processes, protection against soil microbial and insect competitors, and being a vestigial trait that has survived through gene clusters and horizontal gene transfer mechanisms. Conversely, they do not appear to have phytotoxic functions (Ehrlich et al. 2004).

Aflatoxins are derivatives of difuranocoumarin formed by two furans and one coumarin ring synthesized by a polyketide pathway. This family of secondary metabolites includes around 20 compounds (*e.g.* AFB1, AFB2, AFG1, AFG2, AFM1, P1, Q1, B2a, G2a, D1, B3) (Ashiq et al. 2014). This group of mycotoxins is known to be the most dangerous of the mycotoxins due to their carcinogenic, teratogenic and mutagenic effects on vertebrates. The most studied for their potential adverse effects are AFB1, AFB2, AFG1, and AFG2 (Figure 24), which are produced by fungi while the other compounds are the result of biotransformation processes (Groopman et al. 2008). In fact, AFB1 is the most potent naturally occurring chemical liver carcinogen known. Mixtures of naturally occurring aflatoxins have been classified as a Group 1 human carcinogens by the International Agency Research on Cancer (IARC) and have demonstrated carcinogenicity in many animal species, including rodents, nonhuman primates, and fish (IARC 1993; Groopman et al. 2008). B and G aflatoxins have been named based on their fluorescent characteristics under longwave ultraviolet light (λ=365 nm), AFBs have a blue fluorescence, while AFGs have a green fluorescence, in addition to having an extra oxygen atom in the A-ring (Ehrlich t al. 2008, Abbas et al. 2009). The absence of AFG in some species, like *A. flavus,* is due to deletion in the *aflF* (*norB*) – *aflU* (*cypA*) region, which is linked to the AFG promoter, and the expression of *afly* (*nadA*) gene (Ehrlich et al. 2008; Chang and Ehrlich 2010). *AflF* encodes an aryl alcohol dehydrogenase, *AflU* encodes a cyt P450 monooxygenase and *nadA* encodes a reductase (Ehrlich et al. 2004; 2008). The inability to produce AFG is suggested to have occurred several times in the group (Ehrlich and Yu 2010). In the *Flavi* section, the AFB and AFG producing species have the same orientation for this cluster and have similar genomic distances (*A. bombycis* = 68.1 kb, *A. parasiticus* = 68.3 kb and *A. nomius* = 68.4 kb) (Moore et al. 2016). AFB2 and AFG2 are the dihydro-derivatives of the parent compounds AFB1 and AFG1 (Pitt and Tomaska 2001).

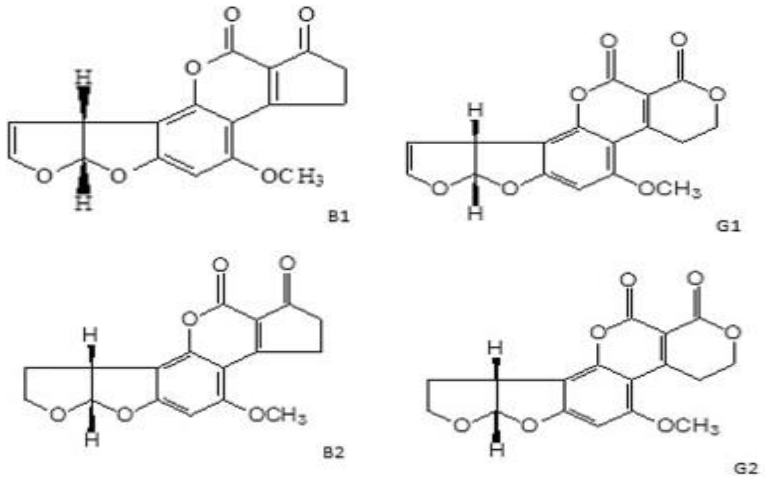

Figure 24. Aflatoxin structure

Once AFs have contaminated staples, toxins are hard to remove because of their chemical conformation; their denaturation temperature is above 200° C (IARC 2002), they are liposoluble, therefore soluble in polar solvents like methanol and dimethyl sulfoxide, and slightly soluble in water (10–20 mg/L) (Jalili 2015).

Effects of aflatoxins depend on the doses and time exposure and on the characteristics of the organism that ingest them (species, gender, age, tolerance and health conditions). For instance, sheep, rats and dogs are more sensitive than monkeys, chicken, mice and humans (Bbosa *et al.,* 2013). Acute exposure can cause jaundice, vomiting, hemorrhages, abdominal pain, acute liver failure, problems with absorption of nutrients, and can be lethal (IARC 2015). Outbreaks in India (during the 70s) and in Kenya (2004) caused the death of 100 and 125 people, respectively (Lewis et al. 2005; Azziz-Baumagarner et al. 2005). Chronic exposure is associated to high risk of hepatocarcinoma, immunosuppression, teratogenic and mutagenic effects, reduction in nutrient absorption , child stunting, effects in the endocrinal system and liver failure (Stack and Carlson 2003; Turner et al. 2005; Bbosa et al. 2013). Teratogenic effects occur during pregnancy, AFs are transferred into the placenta, causing congenital malformations of the fetus, and can also lead to a high risk of childhood cancer (Wangikar et al. 2007). In world regions where incidence of hepatitis B is high and where AFs contaminate food and feed, hepatocarcinoma is more common, suggesting a synergistic association between the two (Liu and Wu 2010).

The biotransformation of AFs occurs mainly in the liver. However, the most studies pathways concern those related to AFB1, as this aflatoxin is the most frequent and dangerous (Figure 25). In general, biotransformation is carried out by different cytP450s (Wild and Gong 2009). AFB1 enters the human body when contaminated products are ingested and then biotransformed. Once AFB1 reaches the intestine, it is absorbed by the intestinal cells and from there it reaches the blood and

reaches the liver. In the liver, AFB1 is metabolized by cytP450s (in particular CYP3A4 and CYP1A2) to an unstable intermediate compound, AFB1-exo-8,9-epoxide (Wu et al. 2013). The molecule AFB1-exo-8,9-epoxide is likely to form covalent bonds with different cellular components such as proteins to form AFB1-albumin and other protein adducts. AFB1-exo-8,9-epoxide also binds with DNA guanines to the N^7position of the *p53* gene, codon 249, which results in a transversion GC→TA, forming AFB1-guanine adducts that lead to more than 60% of AFB1- linked hepatocarcinoma (Hsu et al. 1991; Bennet and Klich 2003; Groopman et al. 2008; Xia et al. 2010; Bbosa et al. 2013). Another compound formed by the biotransformation of AFB1 is AFM1, which is sometimes bioaccumulated in lipophylic tissues of vertebrates and can be excreted by different fluids such as urine, bile, feces and the most important milk from which it takes its name. This AF is also heat resistant, so it cannot be eliminated by pasteurization or other food processing (Vidal et al. 2013). It has been suggested that the cytP450 involved and the adducts formed differ according to the species that has digested the AFs (Bbosa et al. 2013).

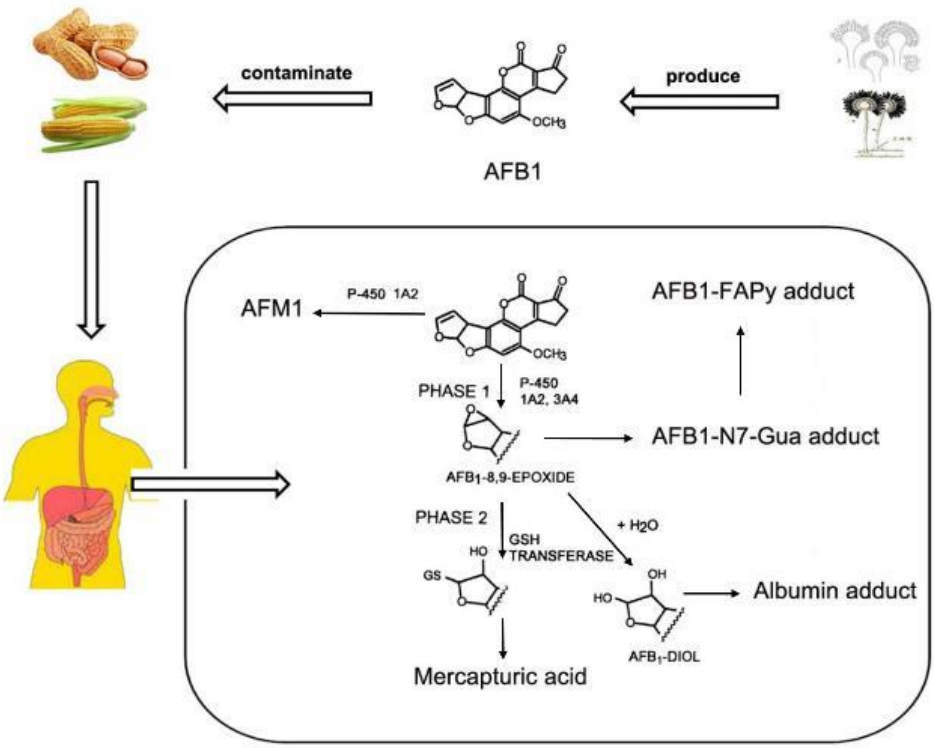

Figure 25. Biotransformation pathways of AFB1. AFB1, when ingested by humans, is metabolized by cytochrome P450 enzymes to its reactive form, AFB1-8,9-epoxide (AFB1-epoxide). Then, AFB1-epoxide forms covalent bonds with DNA strands, forming AFB1-DNA adducts (AFB1-N7-Gua adduct and AFB1-FAPy adduct) (reprinted from Xia et al. 2013).

➢ Sterigmatocystin (ST)

Sterigmatocystin is regarded as an emerging mycotoxin and is synthesized by several *Aspergillus* (*e.g. A. flavus, A. parasiticus, A. togoensis, A. nidulans, A. versicolor, A. ochraceoroseus*), *Penicillium, Bipolaris* and *Chaetomium* fungi. This mycotoxin is one of the last precursors of aflatoxin and is synthesized via the AF pathway. In some non-AF producing species such as *A. nidulans*, ST is the last product and the cluster has fewer genes (Gruber-Dorninger et al. 2017; Bertuzzi et al. 2017). This mycotoxin is prevalent in the environment (Jakšić et al. 2012). It is suggested that the ST cluster was transferred horizontally and evolved independently in different groups of fungi (Rank et al. 2011). In *A. nidulans*, the ST cluster has 20 genes that are homologous to AF genes, but some of the open reading frames (ORFs) in *A. nidulans* cluster (e.g. *ST* and *STD*) have not been found in the AF cluster (Ehrlich et al. 2005). Generally, the homologous genes in the AF and ST clusters have similar lengths and rarely differ in number of introns (Yu et al. 2004a).

Sterigmatocystin has been recognized as potentially carcinogenic, mutagenic and teratogenic in animals and was classed as group 2B carcinogen (possibly carcinogen for humans) (IARC 2013; Bertuzzi et al. 2017). As well as AFs, this compound is a polyketide and its toxic effect is linked to its furofuran ring structure that forms DNA adducts after metabolic activation to an epoxide (Gruber-Dorninger et al. 2016) (Figure 26). The role of this metabolite is unknown, yet it may have synergistic effects with other toxic secondary metabolites. ST has active effects against fungivorous insects, probably provides chemical protection for conidia and sclerotia. ST biosynthesis is related to conidiation, so it can play a role in survival fitness (Rank et al. 2011).

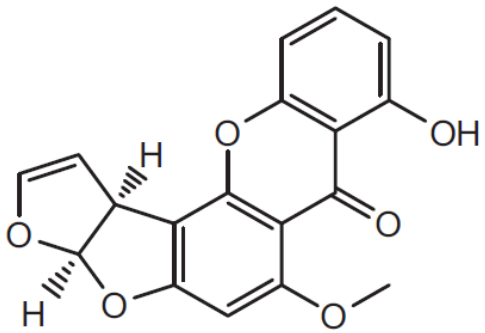

Figure 26. Sterigmatocystin structure

It is suggested that *Flavi* section species are weak producers of sterigmatocystin because most of it is biotransformed into AFs. The main producers of ST are the non-aflatoxigenic species, in particular *A. nidulans* and *A. versicolor* as they do not produce AFs (Varga et al. 2003; Bertuzzi et al. 2017).

The biosynthetic pathway includes the genes previously mentioned of the AF pathway. The *fluG* and *flbA* genes regulate asexual development and ST production. *fluG* acts upstream of *flbA* and appears to be involved in the production of extracellular low-molecular-weight diffusible factors that activate conidiation and ST production (Calvo et al. 2002).

➢ Versicolorin A and B (VerA and VerB)

A and B versicolorins are part of the AF and ST biosynthetic pathways (Figure 23 and 27). These compounds can be present in mold particles and conidia and can be taken by the respiratory system. Cytotoxic and genotoxic effects of verA and verB were tested in pulmonary A549 cell line and have shown positive effects, IC_{50} of 109 ± 3.5 µM and 172 ± 4 µM, respectively. However, their effects were 10 – to 20 times and 30- to 50 times less toxic than AFs and ST, respectively (Jakšić et al. 2012). It would be interesting to check the concentrations of these compounds in natural environments because they are metabolized to AFs and ST, so they effects can be less strong.

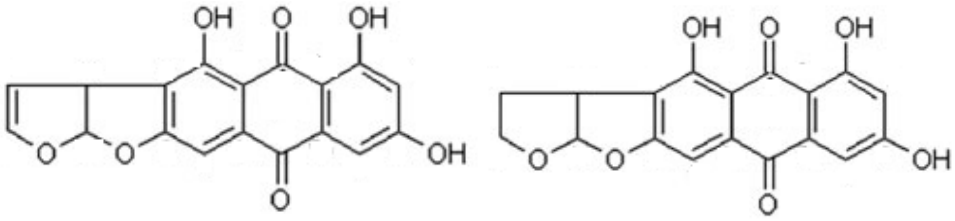

Figure 27. Versicolorin A and versicolorin B structures. Left = VerA and right = VerB.

➢ Cyclopiazonic Acid (CPA)

Cyclopiazonic acid is an indole-tetramic acid compound, synthesized by PKS-NRPS enzymes (Chang et al. 2009b). CPA is produced by species of *Penicillium, Aspergillus* section *Flavi, A.* section *Versicolores* and *A.* section *Fumigati*. Amongst the principal producer are *P. camembertii, P. chrysogenum, P. verrucosum, A. flavus, A. minisclerotigenes, A. oryzae, A. parvisclerotigenus, A. pseudocaelatus, A. pseudotamarii, A. tamarii* and *A. bertholletius* (Varga et al. 2015). CPA was first identified in *Penicillium cyclopium*, but it is not known how the cluster was inherited in both genera, anyway horizontal gene transfer mechanisms are not discarded (Moore et al. 2016). Its main effect is the inhibition of the calcium-dependent ATPase in the sarcoplasmic reticulum, leading to increased muscle contractions. Its effects vary on different vertebrates. In rodents, it could cause liver, kidney,

pancreatic, spleen and heart damage. In poultry, it could cause ulcerative proventriculitis, mucosal necrosis and lead to a significant mortality rate, and in mammals, dogs are more sensitive than pigs, and the two species are more sensitive than other animals. In addition, it is suggested that it could be involved in the 'kodo poisoning', which occurred in India when contaminated kodo millet was ingested (Vaamonde et al. 2006, Chang et al. 2009a). Moreover, in some species such as poultry and pigs, CPA has additive effects with AFs and OTA, respectively (Chang et al. 2009a) (Figure 28).

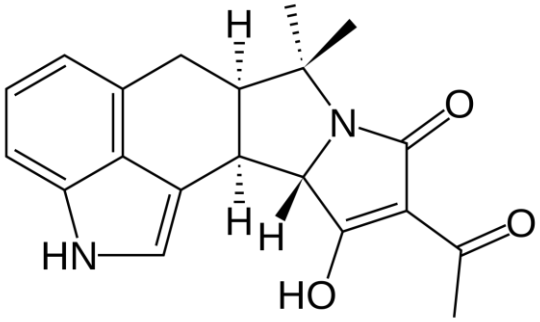

Figure 28. Cyclopiazonic acid structure

The function of CPA is not known, but it might play a role in niche adaptation, providing an advantage in fungus fitness under specific environmental conditions (Georgianna et al. 2010), or it can act as sequestering agent of Fe^{3+} because it is an excellent chelator. CPA might have partially fulfilled the function of ion-chelation before siderophore molecules were incorporated in the fungus genome. In *A. flavus*, *sidC and sidT* (*msf2*), which encode a siderophore and a siderochrome-iron transporter, are settled in the same subtelomeric region than the CPA gene cluster, yet they are located at the terminus of the chromosome 3, suggesting that they were incorporated later (Chang et al. 2009a).

In *Aspergillus flavus* and *A. oryzae,* CPA is synthesized by the cluster 55, which is located in the subtelomeric region of chromosome 3, close to the AF cluster (Chang et al. 2005; Tominaga et al. 2006). On the other hand, in *A. bombycis,* this cluster is settled in a genomic region different from that of the AF cluster and it has some deletions, such as a deletion in the 11.7-kb *pks-nrps* gene at position 954, which results in a frameshift stop codon at position 1096, stopping the translation of 3541 amino acids and thus the lack of production of CPA (Moore et al. 2016).

The CPA biosynthetic cluster contains three essential genes, identified in the genome of *A. flavus* and *A. oryzae* (Figure 29). CPA precursors include a tryptophan residue, two units of acetic acid and an isoprenoid moiety (dimethylallyl diphosphate—DMAPP) in a three-enzyme biochemical

pathway. Two stable intermediates are produced in the synthesis of CPA: cyclo-acetoacetyl-L-tryptophan (cAATrp) and β-cyclopiazonic acid (β-CPA), by the action of CpaS, CpaD and CpaO, which form an hybrid two-module polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS). CpaS (or CpaA), catalyzes the formation of the tetramic acid cyclo-acetoacetyl-L-tryptophan (cAATrp). It is prenylated by the prenyltransferase CpaD (or dmaT), producing a β-CPA, which is the ultimate tricyclic precursor of α-CPA. The conversion of β- to α-CPA is catalyzed by a cyclo-oxidase CpaO (or maoA) in a redox reaction forming two rings (ring C and D) (Uka et al. 2017). Gaps in the CPA cluster have been identified, and depending on the size of the gap differences, the synthesis of this mycotoxin can be altered. For instance, the non-toxigenic strains of *A. flavus,* NRRL 21882 and NRRL 35739, lack the CPA cluster.

Figure 29. Cyclopiazonic acid cluster. Arrows represent the gene position and transcription orientation (modified from Tokuoka et al. 2015)

➢ Kojic acid (KA)

Kojic acid is a beneficial secondary metabolite produced by the species *Acetobacter*, *Penicillium* and *Aspergillus*, including several species in the *Flavi* section, but the main producers are *A. oryzae, A. sojae* and *A. tamarii* (the three species are used in biotechnology processes) (Burdock et al. 2001; Terabayashi et al. 2010) (Figure 30). This metabolite is used in the production of several food processes including miso (soybean paste), shoyu (soy sauce) and fermented beverages such as sake, amazake, shouchu and mirin (mostly Asian products) during fermentation processes. KA is used for its antioxidant properties in food processing for example in beef, fried bacon and fruits to prevent the formation of warmed-over flavor, nitrosopyrrolidine production and fruit oxidation, respectively. It has also been used as starting material for the synthesis of the food enhancers: maltol and ethyl maltol. In the cosmetic industry, it is used as a skin-lightening agent, as KA is a copper-sequestrating agent; it inhibits the activation of tyrosinase and thus inhibits melanine production; additionally, KA has UV protective properties. Finally, KA has been used as an antibiotic and pesticide (Burdock et al. 2001; Bentley 2006; Terabayashi et al. 2010; Sanchez et al. 2012). Toxicological studies suggested

that it is a safe compound for vertebrates because it has no acute toxic effects, is not mutagenetic, and although chronic exposure tests have shown a tendency to affect pituitary function, these effects were easily reversible and not linked to a genotoxic pathway (Burdock et al. 2001; Bentley 2006).

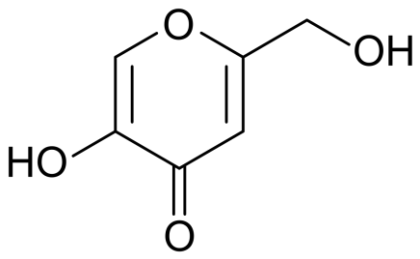

Figure 30. Kojic acid structure

Although KA has been known for over a century, its biosynthesis is not fully understood. During the last decade, some of the genes in *A. oryzae* have been identified. The cluster is located on the chromosome 5, in the genomic region between AO090113000132 and AO090113000145 that includes approximately 14 genes. Three genes have been identified and tested to play a role in KA synthesis, one gene (*kojR* or AO090113000137) encoding a transcription factor, one gene (*kojA* or AO090113000136) encoding an enzyme and one gene (*kojA* or AO090113000136) encoding a transporter (*kojT* or AO090113000138). *kojR* encodes a fungal-specific $Zn(II)_2Cy_6$ transcription factor located between *kojA* (upstream 743 bp) and *kojT* (downstream 383 bp). The experiments have shown that KojR regulates the transcription of *kojA* and *kojT* (Terabayashi et al. 2010; Yamada et al. 2014) (Figure 31). It is postulated that *kojR* is expressed at low levels, causing an accumulation of kojA and kojT transcripts, which leads to the synthesis of KA. When KA synthesis reaches a threshold, it induces a higher production of *kojA* and *kojT*, which increases the synthesis of KA (Maraui et al. 2010)

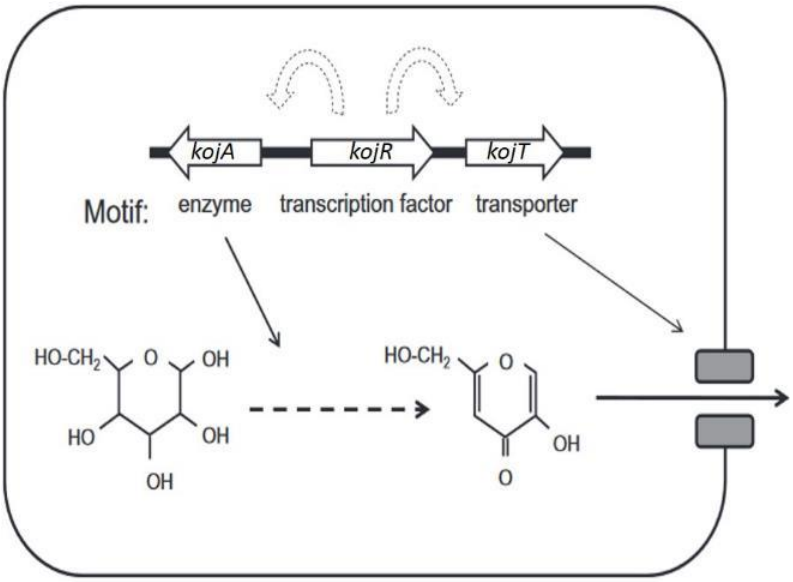

Figure 31. Schematic diagram showing the genes in the kojic acid cluster. KojR (transcription factor) activates one gene (*KojA*) encoding an enzyme and one gene (*KojT*) encoding a transporter (reprinted from Terabayashi et al. 2010).

➢ Aflatrem

This mycotoxin belongs to the indole terpene family and is classified as a potent tremorgenic compound that causes neurological disorders. The mechanism by which aflatrem exerts its effects in mammals is unknown, however it appears that this could be related to interaction with receptors and interference with the release of neurotransmitters in the central and peripheral nervous systems (Zhang et al. 2004).

Studies in *A. flavus* and *A. oryzae* have elucidated the biosynthesis of aflatrem, two clusters are in charge: cluster 32 (chromosome 7) and cluster 15 (chromosome 5). Cluster 32 loci (*ATM1*) is located telomere proximal and contains three genes, *atmG*, *atmC*, and *atmM*, while cluster 15 loci (ATM2) is located telomere distal and contains five genes, *atmD*, *atmQ*, *atmB*, *atmA*, and *atmP* (Nicholson et al. 2009). The backbone enzyme for cluster 15 is a dimethylallyl tryptophan synthase, whereas there is no backbone for cluster 32 (Dolezal et al. 2013). One of the regulators of this mycotoxin is *VeA* and mutants for this gene no longer produce the mycotoxin, besides, exposure to light increases the synthesis of this mycotoxin (Duran et al. 2007) (Figures 32-33).

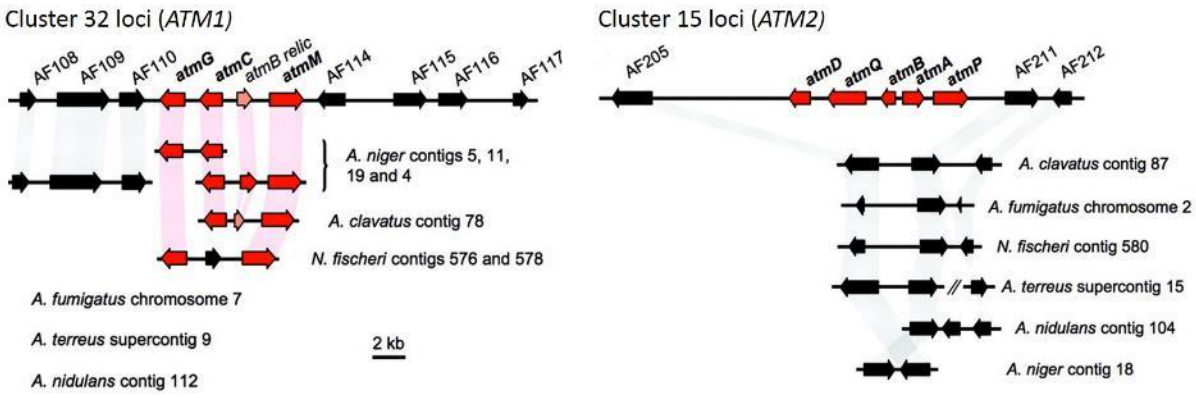

Figure 32. Aflatrem clusters in *Aspergillus flavus* and *A. oryzae.* Physical maps of *ATM1* and *ATM2* loci and the syntenic regions of other *Aspergillus* species. Arrows represent the gene position and transcription orientation (modified from Nicholson et al. 2009).

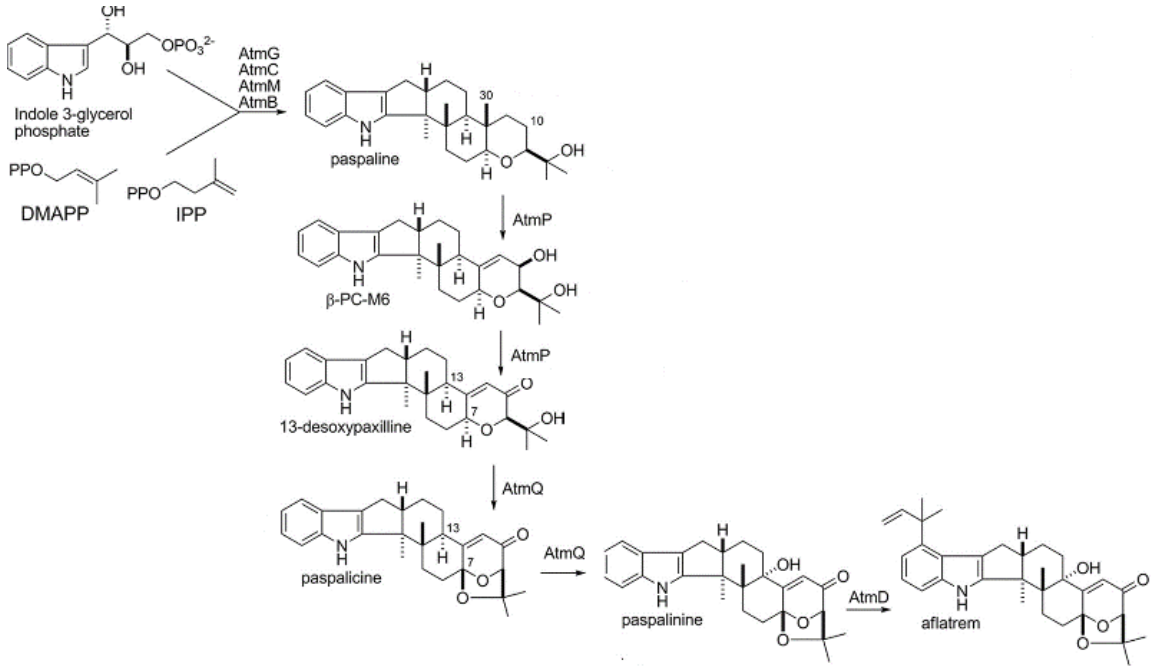

Figure 33. Biosynthetic pathway of aflatrem in *Aspergillus flavus* (modified from Nicholson et al. 2009).

➢ Aflavinine

This secondary metabolite is part of the indole-diterpenes, they have a cyclic diterpene backbone in their structure derived from geranylgeranyl diphosphate and an indole group that is derived from indole-3-glycerol phosphate (Figure 34). These compounds are associated with

antiinsectan activities and can confer an ecological advantage to fungi that produce them. In fact, aflavinines occur in the sclerotia of some species of *A.* section *Flavi* and they belong to the non-paxilline indole-diterpenes (Saikia et al. 2008). It is suggested that the production of aflavinines and their derivates is linked to sclerotia production (Gloer et al. 1980). Among the antiinsectan activity are their effects ion the fungivorous beetle *Carpophilus hemipterus* and the Lepidoptera *Helicoverpa zea* (Parker and Scott 2005). To date, the cluster responsible from its synthesis has not been elucidated.

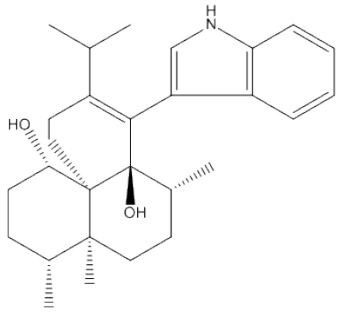

Figure 34. Aflavinine structure.

➢ Aflavarins

Aflavarins are bicoumarins and their synthesis is hypothesized to occur by the dimerization of monomeric coumarins (Figure 35). Aflavarins are produced in sclerotia and can have antiinsectan and actibacterial properties, but they are not cytotoxic; hence, they play an important ecological role in producing species and contribute to their survival (TePaske et al. 1992, Cary et al. 2015b). Besides, the cluster in charge of aflavarin biosynthesis is necessary for normal production of sclerotia (Cary et al. 2015b). This polyketide secondary metabolite is found in some *Aspergillus* species, including several species of *A.* section *Flavi.*

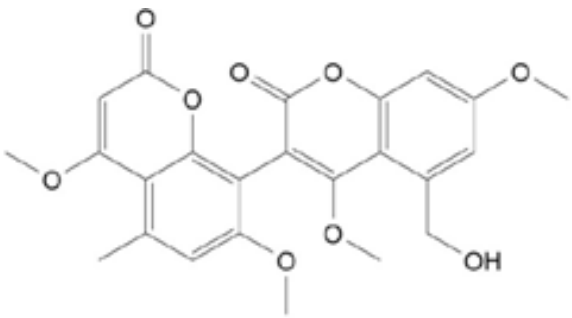

Figure 35. Aflavarin structure

The cluster responsible for the production of these metabolites has recently been characterized by Cary et al. (2015) in *Aspergillus flavus* and corresponds to the cluster 39. The cluster is conserved in some species (*e.g. A. oryzae, A. nidulans, A. niger*, *A. terreus, A. fumigatus, A. fischerianus* and *A. clavatus*), and homologs for the genes are found. There are at least four isomers of aflavarin and its diversity is linked to the the C-C bridge (biaryl axis) (Figure 36).

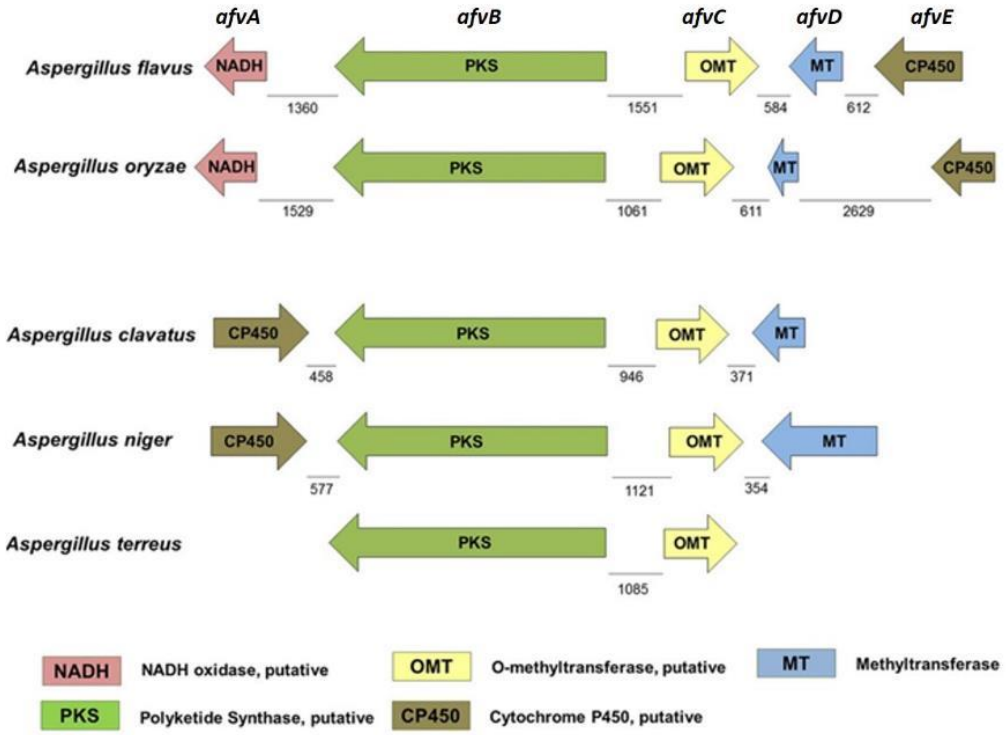

Figure 36. Comparison of cluster 39 among *Aspergillus* species. The scheme shows the cluster 39 in *A. flavus* NRRL3357 (above the arrows the names of genes encoding each enzyme), and compares it with others species of the genus, showing the level of conserved genes (reprinted from Cary et al. 2015).

➢ Tenuazonic Acid (TeA)

Tenuazonic acid is a tetramic acid derivative mainly synthesized by species of *Alternaria* followed by *Phoma sorghina* and *Magnaporthe oryzae* and rarely by *Aspergillus nomius, A. caelatus* and *A. bertholletius* of *Flavi* section (Varga et al. 2011; Taniwaki et al. 2012; Gruber-Dorninger et al. 2016). This mycotoxin is non-mutagenic and its effects might be associated with the interference of protein biosynthesis. The effects of this mycotoxin include tremors, diarrhea, vomiting, and hemorrhages; for some species like rodents it can be lethal. Toxicity experiments have shown that in mice, rats, beagle dogs, chickens and monkeys, it has a certain degree of toxicity. A survey performed

by EFSA (2011) has shown that TeA was present in 15% of the samples (n = 300) of European feed and agricultural commodities at concentrations of about 500 and 4310 µg/kg, and in feed and feed raw materials in 65% of the samples (n = 83) at median and maximum concentrations of 68 and 1980 µg/kg, respectively. The product that presented the higher levels of TeA (up to 1200 µg/kg) was Sorghum-based infant food. EFSA (2011) concluded that TeA is unlikely to be of human health concern, but more recent studies have nonetheless suggested that because of the high doses in Sorghum-based infant food, TeA can pose health risks, so further experiments are needed. In addition, more experiments are needed to confirm that TeA is not a risk for poultry (chickens) (Gruber-Dorninger et al. 2016) (Figure 37).

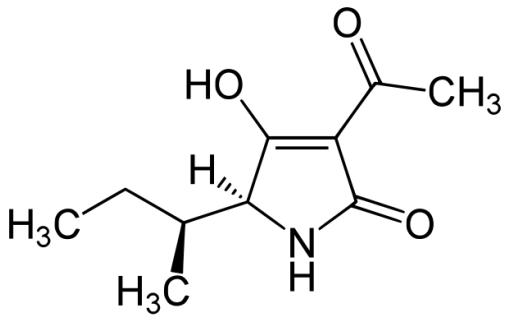

Figure 37. Tenuazonic acid structure

➢ Ochratoxin A (OTA)

Ochratoxins are mycotoxins produced by *Aspergillus* and *Penicillium* species. It was first described in *A. ochraceus*, and later found in *P. verrucosum, P. nordicum* and in the *Nigri* and *Flavi* sections of subgenus *Circumdati* and in the *Flavi* section it is produced by *A. alliaceus* clade (Varga et al. 2015). Ochratoxins are 3,4-dihydromethylisocoumarin derivatives linked with an amide bond to the amino group of L-phenylalanine (Dirheimer and Creppy 1991, Fungaro and Sartori 2009) (Figure 38). The staples that are contaminated by this mycotoxin include cereals, spices, coffee, cocoa and grape derived products. Like other mycotoxins, OTA is very stable and very little degradation occurs during food processing such as cooking, washing, and fermenting, therefore, it is found in staples (Fungaro and Sartori 2009).

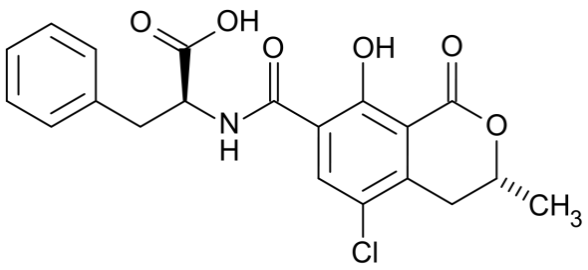

Figure 38. Ochratoxin A structure

Ochratoxin A is known for nephrotoxic, immunosuppressive, teratogenic and carcinogenic effects. Toxicity of OTA is caused by the lactone moiety, which has a structure similar to that of tRNA phenylalanine synthetases, therefore it competes with it and binds to the substrate, thus interrupting protein synthesis (Dirheimer and Creppy 1991). OTA appears to act as a competitive inhibitor of ATPase, succinate dehydrogenase and cytochrome C oxidase in rat liver mitochondria. In addition, OTA produces cellular damage through the formation of hydroxyl radical and lipid peroxidation. Amongst the vertebrates sensitive to the toxin are poultry, rats and mice. Experiments *in vitro* on human and dog kidney cells at concentrations of 100 nmol/L resulted in apoptosis. Besides, OTA is associated with several nephropathies in humans and mammals (Hussein Brasel 2001; Varga et al. 2015). OTA was classified by IARC (2012) in 2b group. Although the mechanisms by which OTA produces its carcinogenic and teratogenic effects is not totally clear, the formation of DNA adducts and single strand breaks have been reported (Lühe et al. 2003).

AIM OF THE STUDY

Aspergillus section *Flavi* is a group of molds that are at risk to health and the economy and widespread worldwide, formed by species able to produce a plethora of secondary metabolites, including several mycotoxins and beneficial compounds. The principal mycotoxins, which have already been highlighted, are AFs, CPA, ST, aflatrems, OMST, versicolorins and OTA. To date, this group comprises 26 described species, including a number of cryptic species. The majority of species are generally isolated from tropical and subtropical regions worldwide; hence, these regions are more affected by AFs and other mycotoxins, causing human health impacts and economically issues. The presence of *A.* section *Flavi* is not only an issue for countries in these geographical regions, but also a problem in temperate regions due to the importation of potentially contaminated raw materials. In addition, climate change is leading to environmental shifts that might alter the home range and frequency of *Aspergillus* section *Flavi* worldwide.

As aforementioned, the ability of species to produce secondary metabolites is in part species-specific, hence the need for adequate characterization from a food safety perspective. The characterization of species in the section is traditionally morphological, yet currently secondary metabolite screening and the inclusion of molecular markers are also performed, which facilitates identification. Molecular marker techniques used to characterize *Flavi* section include RFLPs, AFLPs, RAPDs and phylogenetic inference. Regardless of these methods usefulness, to date there is no agreement on which are the best molecular markers and combinations to distinguish between the species in the *Flavi* section.

The general aim of this dissertation was to identify the molecular markers that allow appropriate characterization at species level in *Aspergillus* section *Flavi*.

The first aim of the study was to develop a molecular tool based on phylogenetic inference to identify species from *Flavi* section. To achieve this goal, a pool of 11 genes has been selected from the literature. At the same time, a collection of fungi, including most of the section's species, was created. Genes were amplified when possible, and their potential applicability as molecular markers using phylogenetic inference was tested using Maximum Likelihood and Bayesian Inference (Chapter 2).

The second aim was to test the effectiveness of the molecular tool with unidentified strains of *Aspergillus* from the section. We collaborated with Dr. Catherine Brabet for a screening of the *Flavi* section in the peanut production chain in the Côte d'Ivoire. This collaboration allowed us to test the molecular tool and identify the best combination of the different genes (Chapter 2.2 and 2.3). We also collaborated in the project ARVALIS for screening maize samples in France, this work also allowed to test the molecular tool (Chapter 2.4).

02

EXPERIMENTAL WORK

2.1 CHAPITRE 1

Molecular *Flavi* Tool

As mentioned before, species identification in *Aspergillus* can be challenging, and section *Flavi* is one of the best examples of their complexity. Species have high inter and intra variability of traits, making only one of identification methods (morphological, molecular characterization and secondary metabolites profile characterization) futile (Taylor et al. 2000; Geiser et al. 2007). Conversely, the development of molecular tools over the last two decades has allowed the development of new techniques that are useful for species identification in *Aspergillus*, nevertheless a combination of methods is recommended to achieve robust results (Geiser et al. 2007; Varga et al. 2011).

2.1.2 Polyphasic approach, ways of determining section *Flavi* species

➢ Species concept

There are several concepts of species, depending on the field the definition of a species is slightly different (Table 1). However, several authors suggested that all these concepts had a main core, and that the species concept should be based on it, and so the unified species concept was born (De Queiroz 1998; 2005; 2007). Under this concept, a species is a lineage that has evolved separately from other lineages, so it is not mandatory to be verified under the limits of other species concepts, it does not need to be recognized for its phenotype, diagnose as monophyletic, nor reproductively isolated, etc. (de Queiroz 2007).

Table 1. Species concepts (Reprinted from de Queiroz 2007)

CLASS OF SPECIES DEFINITION	PROPERTY UPON WHICH IT IS BASED
BIOLOGICAL	Interbreeding (natural reproduction resulting in viable and fertile offspring)
Isolation	Intrinsic reproductive isolation (absence of interbreeding between organisms of different species based on intrinsic properties, as opposed to geographic barriers)
Recognition	Shared specific mate recognition or fertilization system (mechanisms by which organisms of the same species, or their gametes, recognize one another for mating and fertilization)
Ecological	Same niche or adaptive zone (all components of the environment with which the organisms interact)
PHYLOGENETIC	Heterogeneous
Monophyletic	Monophyly (consisting of an ancestor and all of its descendants; commonly inferred from possession of shared derived character states)
Genealogical	Exclusive coalescence of alleles (all alleles of a given gene are descended from a common ancestral allele not shared with

	those of other species)
Diagnosable	Form a diagnosable group (qualitative difference)
EVOLUTIONARY	Form a diagnosable group (qualitative difference)/ Separation of lineages (intrinsic or extrinsic)
PHENETIC	Form a phenetic cluster (quantitative difference)
GENOTYPIC CLUSTER	Form a genotypic cluster (inferred from deficits of genetic intermediates, *e.g.* heterozygotes)

Species classification in *Aspergillus,* and especially in section *Flavi* include differences in the approach of the terms, nevertheless, it is possible to use the unified species concept to include all the information obtained, hence, better acknowledging the relationships in the genus. There are two main currents in species classification of section *Flavi*, one more conservative proposed by Cotty and collaborators, and other more flexible, supported by Samson, Ehrlich, Frisvad, Pitt, Varga and collaborators. The first one suggests that *A. flavus* clade and *A. parasiticus* clade are, each of them, one species, and both species are plastic. Cotty and collaborators in some studies have accepted the possibility of species complexes; however, they do not openly incorporate the recently described species, especially for *A. minisclerotigenes* and *A. parvisclerotigenus.* These species are mainly supported by the *ITS* gene, VCGs, and phenetic traits. From all of them, *A. flavus* is the more plastic species, presenting strains with large and small sclerotia, and producers of B- and G- AFs. The more lax current suggests that *A. flavus, A. parasiticus* and *A. nomius* are species complexes that enclose cryptic species that are more feasible to be recognized by the combination of phenetic and physiological traits, secondary metabolic compounds, and molecular markers. The idea of species complex in section *Flavi* is increasingly been accepted as it is well supported by secondary metabolic profiles and molecular data. With the acquisition of more information of gene expression and omics, these cryptic species are better supported.

➢ Morphology and physiology

Conventional classification of the *Aspergillus* section *Flavi* includes several phenotypic traits, which, when used together, allow most species to be grouped. This approach presents some difficulties especially when cryptic species are present. In addition, phenotypic traits are variable and depend on environmental conditions, such as temperature, nutrient availability, moisture, competitors, microorganism communities, host, etc. The morphological traits include macro and microscopic traits. For example, colony color and texture, arrangement of conidial heads (globose to

radiate) and coloration in shades of yellow-green to brown; globose to subglobose or flask shapes vesicles; conidia form, coloration and texture; uni- and biseriate sterigmata; dark sclerotia (Raper and Fennell 1965; Varga et al. 2011). Other traits include ubiquinone systems, growth at different temperatures, media color shifts due to metabolite production (AFPA and CREA media) (Varga et al. 2003; Rigo et al. 2002; Gonçalves et al. 2012).

➢ Secondary metabolites

The secondary metabolic profile of a species comprises all the secondary metabolites that a species can produce, including toxins, antibiotics, among others. Although, several species can produce similar secondary metabolites, the secondary metabolic profile is species specific, working like a fingerprint, and is helpful for species characterization in ascomycetes like *Alternaria, Aspergillus, Fusarium* and *Penicillium* (Frisvad et al. 2008). However, some species present a highly similar metabolic profile, especially those species that are closely related. Furthermore, the expression of the genes encoding for secondary metabolites in fungi depends on abiotic and biotic factors, triggering different genes. Some of the secondary metabolites frequent synthesized by *Aspergillus* section *Flavi* are kojic acid, aspergillic acid, aflatoxin B (Samson et al. 2014). The disadvantages of this approach includes the intra species variability (*e.g*. *AffF-AflU* region in *A. flavus*), the development of the fungus (during different steps in their life different secondary metabolites can be expressed), and the response to stimuli including environmental, like pH, temperature, carbon and nitrogen sources, and stimuli derived from other organisms (Frisvad et al. 2008; Brakhage 2013).

➢ Molecular markers

The advantage of molecular markers over phenotypic markers (including those linked to secondary metabolites) is that they are more stable. In addition, the development of biotechnology and the development of biostatistics approaches and software facilitate the analyses, making them quicker, less expensive and more robust (Mitchell 2010). The use of molecular markers has helped to resolve taxonomic questions that have not been resolved by morphological and physiological approaches (Perrone et al. 2004).

Molecular markers are defined as any region of the genome that could be identified, and must be designed with the purpose of the study in mind, targeting the genomic regions that will be more informative to accomplish it. Molecular markers include DNA, RNA, and amino acid sequences of proteins. In fact, the design must consider the potential variability between gene sequences, a

conserved marker will be preferred to determine higher taxonomic groups than the marker used at population level (Taylor 2000; Aguileta et al. 2008; Mitchell 2010). In strains identification studies, the use of markers that include noncoding regions, hence that lack selective pressure, are preferred. Some uses of molecular markers include fingerprinting of allozymes of fungi with medical purposes, electrophoretic karyotypes, DNA hybridization probes, PCR-based genotypes, endonuclease restriction fragment length polymorphisms (Table 1) (Mitchell 2010).

Several molecular markers and techniques have been tested in an attempt to properly classify the species in *A.* section *Flavi*, including RAPDs (random amplified polymorphic DNA), AFLPs (amplified fragment length polymorphism), RFLPs (restriction fragment polymorphism), sequence analyses of cytochrome *b* gene, *ITS* region, and analyses of the aflatoxin gene cluster. However, the use of a single molecular marker has not been sufficient to solve the identification issues (Geiser et al. 2007; Godet and Manaut 2010).

Table 2. Screening methods to determine fungal species (Reprinted from Mitchell, 2010)

	APPLICATION			
METHOD	**Strain identification, molecular epidemiology, population genetics**	**Species identification**	**Phylogenetics, systematics**	**Examples *Aspergillus* section *Flavi****
Electrophoretic karyotype	**X**			
RFLP	**X**	**X**		**Somashekar et al. 2004**
Southern hybridation	**X**	**X**		**Kumeda and Asao 1996**
RAPD, AFLP, PCR fingerprint	**X**	**X**		**Massi et al. 2014; Viaro et al. 2017**
Microsatellites	**X**	**X**		**Tran-Dinh et al. 2009**
Microarrays	**X**	**X**		**Guo et al. 2011**
SNP, MLST, DNA sequencing	**X**	**X**	**X**	**Pildain et al. 2008; Varga et al. 2011**

*= There are only shown a couple of examples per method

➢ Phylogenetic inference

Nowadays, the use of molecular markers for phylogenetic inference is not only applied to understand inter species relationships, it is also used to identify the relationships between genes, to decipher evolutionary history at several levels (kingdoms, families, genera, species, populations, cellular lineages, genes), and to compare and understand pathogens dynamics, metagenomic regions, regulatory elements, and non-coding RNAs (Taylor et al. 2000; Ziheng and Rannala 2012).

The use of molecular markers in fungi is controversial. Depending on the target group, some markers could be more informative than others. However, markers can include genes that have biases or are not good enough for unmasking relationships in certain groups (Aguileta et al. 2008; Schimitt et al. 2009). For instance, in *Aspergillus* the most preferred molecular markers are *ITS, β-tubulin* and *calmodulin* genes, yet the first two have been reported to have problems in relationships inference, particularly *ITS* (internal transcribed spacer located between RNA subunit genes) (Geiser 2007). Withal, the robustness of the analysis will increase when multiple independent loci lead to a congruent answer (Taylor et al. 2000; Begerow et al. 2004; Townsend 2007), and the inclusion of molecular markers have to take into account their plausibility for molecular inference (Aguileta et al. 2008).

The use of phylogenetic inference to identify species in the section *Flavi* was included as part of the polyphasic approach. Generally, it includes one or more genes, which are mostly analyzed independently or together. The inclusion of this technique has allowed addressing the complexity of this group, and has reinforced the idea that species, such as *A. parasiticus sensu lato* and *A. flavus sensu lato* corresponded to species complexes (Peterson 2008; Pildain et al. 2008; Varga et al. 2001; Soares et al. 2012; Taniwaki et al. 2012).

2.1.3 Objective

To screen the species relationships in this group based on different markers, and subsequently, identify a combination of several molecular markers that allows species delimitation and classification in the section *Flavi* by phylogenetic inference.

2.1.4 Material and methods

Creating the database of Aspergillus section *Flavi*, "THE *FLAVI* TOOL"

GENES

For the present study, we chose twelve genes based on their function and their use in phylogenetic studies on fungi, in addition, for some strains the region *aflF-aflU* was analyzed (Figure 1).

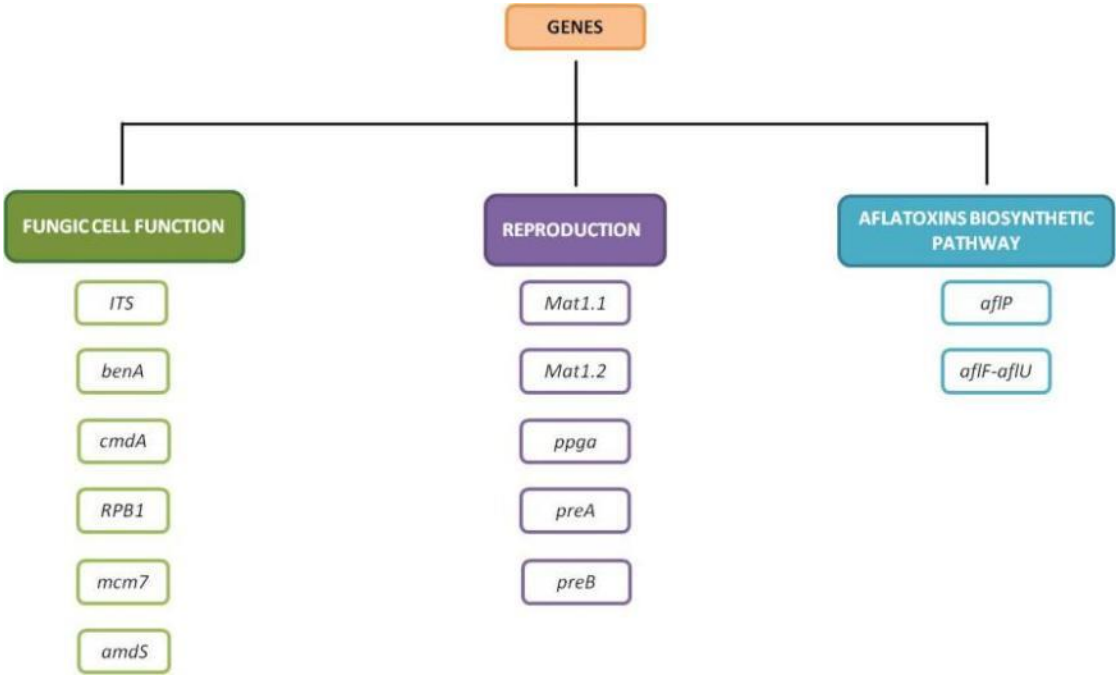

Figure 1. Genes used during the study, and their main classification.

Fungal cell function genes

➢ Nuclear Ribosomal Internal Transcribed Spacer (*ITS*):

This ribosomal region is universally used as bar coding gene for fungi. ITS is formed by a rRNA cistron constituted of 18S, 5.8S, and 28S rRNA genes, that suffer post-transcriptional processes resulting in the cleavage of the cistron by removing two internal transcribed spacers (White et al. 1990; Schoch et al. 2012). These loci are useful to infer relationships among fungi species, as they have different segments with different resolution at different scales; ITS1 has a fast evolution rate, 5.8S is highly conserved and ITS2 displays a moderately rapid evolution rate (Nilsson et al. 2008). Studies suggested that it is informative to delimit at genus level, and in some groups at species level (Nilsson et al. 2008; Scorzetti et al. 2002). ITS is suggested to be useful to obtain an idea of genera and species at community level (Buchan et al. 2002). However, the use of this gene as barcode for Ascomycota is sometimes insufficient; it can present pleomorphisms and alignment difficulties (Scorzetti et al. 2002; Nilsson et al. 2008; Seifert 2009). Furthermore, it has already been shown that this marker is insufficient to discriminate at species level in some groups of fungi. In some sections of *Aspergillus* and *Penicillium* it is not suitable because of difficulties in the alignment and/or the regions are highly conserved, indeed, the use of this markers is advise to use while other markers are also present (not necessarily in a multilocus dataset) (Varga et al. 2003; Geiser et al. 2007; Seifert 2009).

In the specific case of *A.* section *Flavi* it is informative for certain taxa, yet its ability to be useful at species level is questionable (Rigo et al. 2002).

➢ β-tubulin (*benA*)

Tubulins are crucial proteins for cells, they are an important component of the cytoskeleton, and essential for several processes such as chromosome segregation, cell division, cell shape, intracellular transport, and flagellar or ciliar movement (Einax and Voigt 2013). The family of tubulin proteins contains seven groups, α-tubulin and β-tubulin being the most frequent in Eukaryotes. β-tubulin genes are useful for phylogenetic studies in Eukaryotes because they are easily amplified, they have some highly conserved regions, especially at N-terminal ends, which share approximately 65–70% of their sequence (Glass and Donaldson 1995; Baldauf et al. 2000; Einax and Voigt 2003). On the other hand, intron sequences are more variable, as they can vary in number and position, which contributes to the variability of this gene, and enables the resolution of relationships at species level in certain clades (Einax and Voigt 2003; Peterson 2008; Samson et al. 2014). The use of *benA* for phylogenetic inference is currently widespread in different fungi taxa, and is generally accepted as a good phylogenetic marker (Begerow et al. 2004; Soares et al. 2012). Conversely, some studies suggest that the presence of orthologs of this gene can be an issue in some taxa (Begerow et al. 2004; Ziheng and Rannala 2012). The inclusion of this gene can improve the robustness of the inference analysis (Schoch et al. 2012).

➢ Calmodulin (*cmdA*)

Calmodulin is an important intracellular Ca^{2+} receptor among eukaryotes. This small acidic protein complex has several functions, such as regulation of cell growth and cycle. It activates phosphodiesterases, Ca^{2+}-ATPase, protein kinases, and adenylate cyclase (Yasui et al. 1995). Similarly to *β-tubulin*, *calmodulin* has been chosen as marker because it is easily amplified and it has conserved and variable regions (Geiser et al 2000; Samson et al. 2014). The region works for most *Aspergillus* groups as a reliable marker (Samson et al. 2014). The primers of calmodulin used in this study contain approximately 580 bp, and includes introns 2, 3 and 4, and exons 2, 3, 4 and partial exon 5 (Hong et al. 2006).

➢ Minichromosome maintenance protein (*mcm7*):

The minichromosome maintenance protein encodes for essential proteins in the first steps of eukaryotic replication (Raja et al. 2011). This gene is present as a single copy in the genome, giving it

some advantages over other markers, such as *β-tubulin* or small ribosomal subunits 18S and 28S (Schmitt et al. 2009; Raja et al. 2011). Schmitt et al. (2009) suggested that *mcm7* has characteristics that make it a good gene marker for phylogenetic inference among fungi, at large and fine scale. They tested this molecular marker across a wide range of unrelated taxa, resulting in good and robust topologies, with good resolution power and branch support. In fungi, phylogentic studies using this marker have showed that it is a suitable marker (Raja et al. 2011; Morgenstern et al. 2012; Schoch et al. 2012; Tretter et al. 2013). Raja et al. (2012) suggested that, although *mcm7* did not always provide reliable relationships, it should be considered as useful marker for fungi, and Ascomycota.

➢ RNA polymerase II, largest subunit (*rpb1*)

RNA polymerase II is in chart of transcribing all mRNAs and several noncoding RNAs. *Rpb1* has a C-terminal domain (CTD), consisting of about 25 repeats of the heptamer sequence Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7; of which five amino acids can be phosphorylated, and prolines can be in *cis* or *trans* configurations (Suh et al. 2016). RNA polymerase genes are used as markers to understand evolutionary relationships between several eukaryotic taxa, including plants, fungi and protists (Cheney et al. 2004; Nickerson and Grouin 2004; James et al. 2006; Morgenstern et al. 2012). Among its advantages as a marker are that *rpb1* is present as a single copy in the genome (Stockinger et al. 2014), Rpb1 protein features include nine amino acid regions highly conserved among eukaryotes, bacteria, and archaea, named regions A–I. In addition, it has different evolutionary rates, allowing some studies at finer scale. For instance, the use of *rpb1* has facilitated the comprehension of the evolution of arthropods, rhodophytes and protists (Nickerson and Grouin 2004). In fungi, its use can facilitate the understanding of evolutionary relationships at different levels, like in *Glomeromycota* (Stockinger et al. 2014) and *Inocybe* (Matheny et al. 2002).

➢ Acetamidase (*amdS*)

Certain *Aspergillus* can use acetamide as source of nitrogen thanks to the presence in their genomes of the enzymes belonging to the acetemidase family. The search of these genes in *Aspergillus* section *Flavi* arose because of their use in industrial processes (Katsuya et al. 1991). *A. oryzae* was one of the first species of the genus where this gene was characterized (Katsuya et al. 1991). Later on, Geiser et al. (1998) used this gene to infer relationships among *A. flavus* strains. This gene presents exons and introns, hence, it has conserved and variable regions that can be useful for phylogenetic inference (Geiser et al. 1998; Gonçalves et al. 2012).

Reproduction genes

➢ MAT genes

As aforementioned, sexual reproduction in fungi can be heterothallic, requiring fungi with vegetative compatibility groups strains with opposite *MAT* loci, or homothallic, self-reproduction where the fungus present the two *MAT* loci (Paoletti et al. 2007). It is suggested that besides of the sexual reproduction, *MAT* loci are involved in several other cellular processes (Dyer and O'Gorman 2012). *MAT* loci are formed by two gene regions, called *MAT1-1* and *MAT1-2* in euascomycetes. *MAT1-1* encodes an α-domain transcription factor, and *MAT1-2* encodes for a transcription factor with a high-mobility group (HMG) domain (Dyer and Kück 2017) (Figure 2). Some studies have tested that *MAT* loci and their ratios in the section *Flavi* and have shown that the vast majority of fungi are heterothallic (Geiser et al. 1998; Carbonne et al. 2007; Ramirez-Prado et al. 2008; Horn et al. 2011), nevertheless it is not known if all species present both idiomorphs. These genes are not suitable for phylogenetic inference per se, yet their analysis is interesting in order to increase the knowledge of the species in the section.

➢ Pheromone precursor *ppgA* and pheromone receptors *preA* and *preB*

These genes have important roles in mating recognition. *PpgA* encodes for an α-pheromone precursor that binds to PreB, whereas *preA* and *preB* encode a-pheromone and α-pheromone receptors target by MAT, currently the gene that codes for the α-pheromone has not been identified (Dyer et al. 2003; Dyer and O'Gorman 2012) (Figure 2). Although some studies have compared these genes among different fungi taxa (Pöggeler 2002; Dyer et al. 2003; Hoff et al. 2008), their use as phylogenetic markers has not been performed.

Pöggeler (2002) identified in *A. fumigatus* a type of gene that seemed to play a role as pro-α-factor-like- pheromone precursor, *ppgA*, and suggested that it was involved in cell recognition and mating in filamentous ascomycetes. Moreover, she identified that the polypeptide encoded by *ppgA* had two identical repeats of a non-peptide hydrophilic pheromone sequence, and these regions were flanked by maturation signals that can lead to cleavage, like α-factor precursors found in *Saccharomyces cerevisiae*.

Pöggeler (2002) also identified an ORF in *A. fumigatus* that had significant similarity with pheromone receptors of other filamentous ascomycetes, *preA,* whose protein sequence blasted with some Ascomycota and Basidiomycota pheromone receptors. In the same study, she identified another region similar to an α-factor receptor, *preB*. Although sequences are not identical, their

structures resemble and belong to the 7-transmembrane-type receptor family. Later on, Hoff et al. (2008) identified similar genes in *Penicilium chrysogenum.*

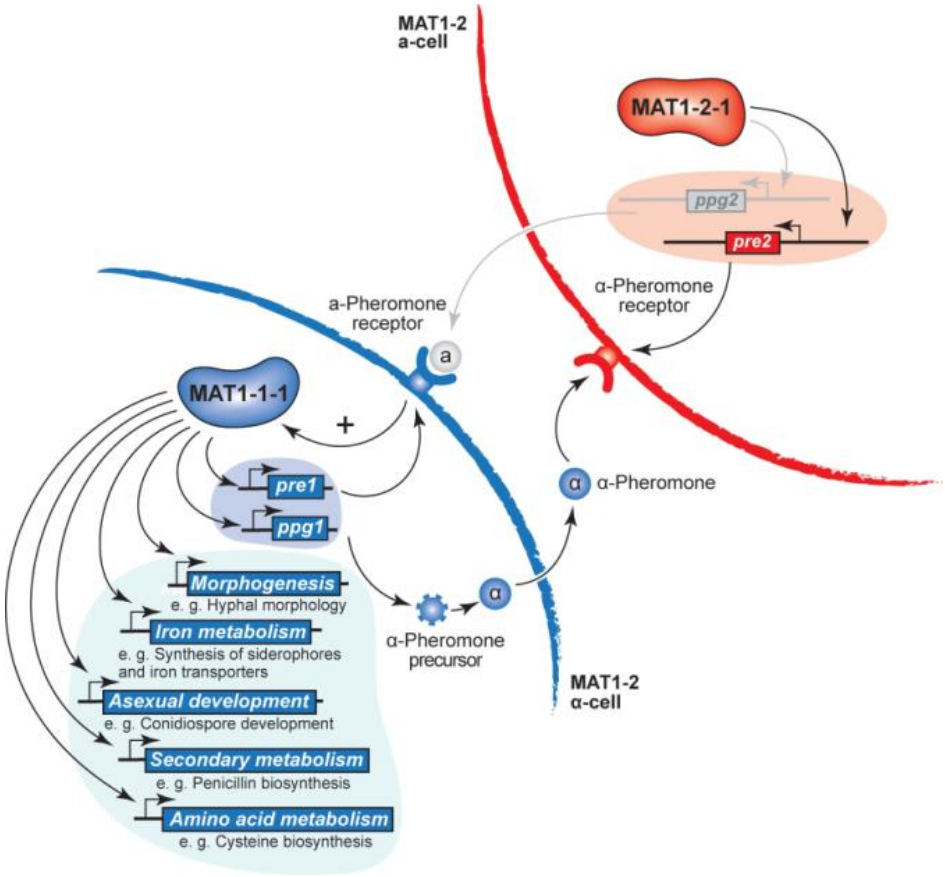

Figure 2. Scheme of reproduction genes. *MAT1*loci, target genes of *MAT1-1*, and encoded transcription factors inferred from functional genomics experiments in *Penicillium chrysogenum*. The scheme shows that MAT1-1 regulates several functions (reprinted from Dyer and Kück 2017).

Genes linked to aflatoxin pathway

➢ *aflP*

AflP encodes an O-methyltransferase, an enzyme that is expressed only in suitable conditions for aflatoxin production and catalyzes the transformation of sterimatocystin into O-methylsterigmatocystin (Yu et al. 1995; Scherm et al. 2005). It is suggested that this enzyme can also be involved in conidiation (Lee et al. 2002). The presence of this gene has been investigated in some aflatoxin producing species (Yabe et al. 1989; Yu et al. 1993; 1995). In fact, a comparison between *A.*

parasiticus and *A. flavus* suggested that *aflP* is similar in both species, sharing the same number of exons and introns, and at the same positions, as well as similar nucleotides and amino acidic sequences (Yu et al. 1995). Despite, some studies linking the function of the gene and its presence in section *Flavi* species have been performed, its use as a phylogenetic marker is not common (Geiser et al. 1998; Gonçalves et al. 2012). This gene can be interesting for phylogenetic inference in *A.* section *Flavi* because it is linked with aflatoxin production.

➢ *aflF-aflU* region

Like *aflP* gene, the comparison of *AflF-aflU* amongst species of *A.* section *Flavi* can be interesting as a marker to understand evolution of aflatoxins, especially AFGs. This section can be amplified and compared between species that produce these aflatoxins. In addition, for *A. flavus* it is an interesting genomic region to address question at population level. In several species of *Aspergillus* section *Flavi, aflF* and *aflU* are close together, *aflF* encodes for an aryl alcohol dehydrogenase and a*flU* encodes for a cytochrome P450 monooxygenase. Ehrlich et al. (2008) suggested that aflF may catalyze the biosynthesis step after the rearrangement and decarboxylation of the NadA-reduced 386 Da intermediate. As mentioned before, *A. flavus* is incapable to produce G-aflatoxins because it presents a deletion of 1-1.5 kb, this gaps occurs nearby 0.4 to 0.6 kb from the translational stop codon of *aflF* (Ehrlich et al. 2004) (Figure 3).

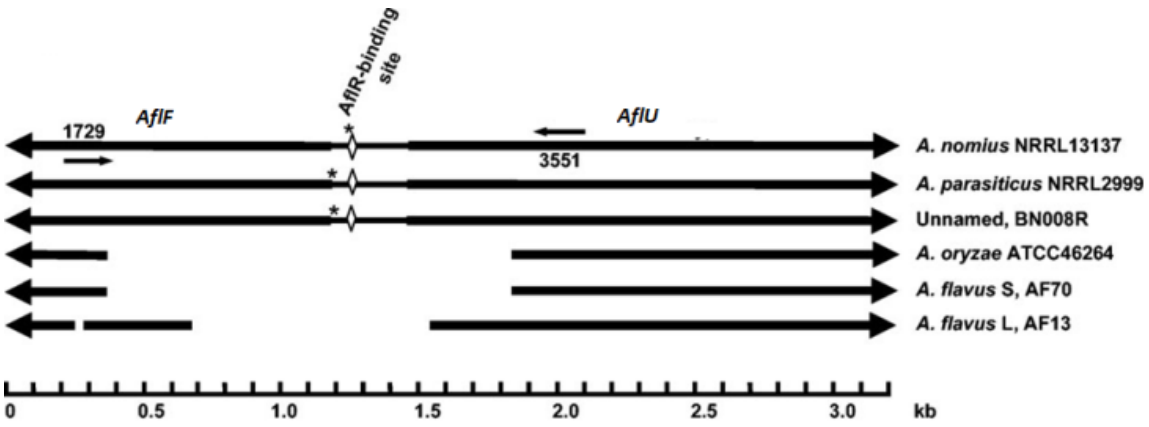

Figure 3. Scheme of the *aflF-aflU* genomic region in different strains of *Aspergillus* section *Flavi*. Arrows shows the coding region and the transcription direction of *aflF* and *aflU.* Gaps are present in all samples. Asterisks show small gaps in G aflatoxin producers: *A. nomius* (13 bp)*, A. parasiticus* (11 bp) and strain BN008R (4 bp). *Aspergillus flavus* and *oryzae* have different gaps that inhibits the synthesis of aflatoxins G. *A. flavus* AF70 and *A. oryzae* ATCC46264: Gap = 1516 bp; Strain AF13: gaps = 32 and 854 bp. Small arrows = position of oligonucleotide primers AP1729 and AP3551 (reprinted from Ehrlich et al. 2004).

ISOLATES

➢ Fungal Strains and Culture Conditions

A collection of *Aspergillus* section *Flavi* strains that includes at least one isolate of almost all species of the section are kept at Toxalim, INRA under controlled conditions (on Malt Extract Agar slope cultures at 4 °C). The strains used in this study and the isolates available in GenBank database that were included for constructing phylogenetic trees are listed in the Annexes and tables of chapters 2.2, 2.3 and 2.4.

In addition, some isolates were included in the analyses to test the application of the tool and its robustness. Seventy-one strains from Côte d'Ivoire were obtained from a collaboration with CIRAD, and fifteen isolates from a study linked to the project ARVALIS (see Chapter 2.2 and Chapter 2.4).

➢ DNA Extraction, Amplification and Sequencing

The DNA extraction protocol, and the amplification and sequencing conditions and protocols are described on chapters 2.2 and 2.3 (Figure 4).

PHYLOGENETIC APPROACH: Alignment, Model Selection and Phylogenetic Inference

➢ Alignment and Model selection

We performed several analyses, each one contained different genes and gene mixes, to test the best combination with most robust results (Figure 4).

We used BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) to assemble, align and trim the databases for each gene, and to trim concatenated datasets. The alignments were performed using ClustalW algorithm in this program, and checked with the naked eye to avoid errors. Concatenated dataset were performed using Mesquite v3.2 (Maddison and Maddison 2017).

To perform phylogenetic analyses, the determination of the best-fit model of nucleotide or amino acid substitution for each gene or each partition is required. Models of evolutionary substitution are based on the likelihood that a nucleotide or an amino acid changed into another one, resulting in a set of probabilistic assumptions that are accepted over others. Choosing a substitution model is problematic because assuming the wrong model would provide an evolutionary scenario that does not fit the real relationships among set of sequences. Thus, leading to a topology that does not correspond to the true evolutionary processes and that shows the wrong relationships among sequences (Posada 2009).

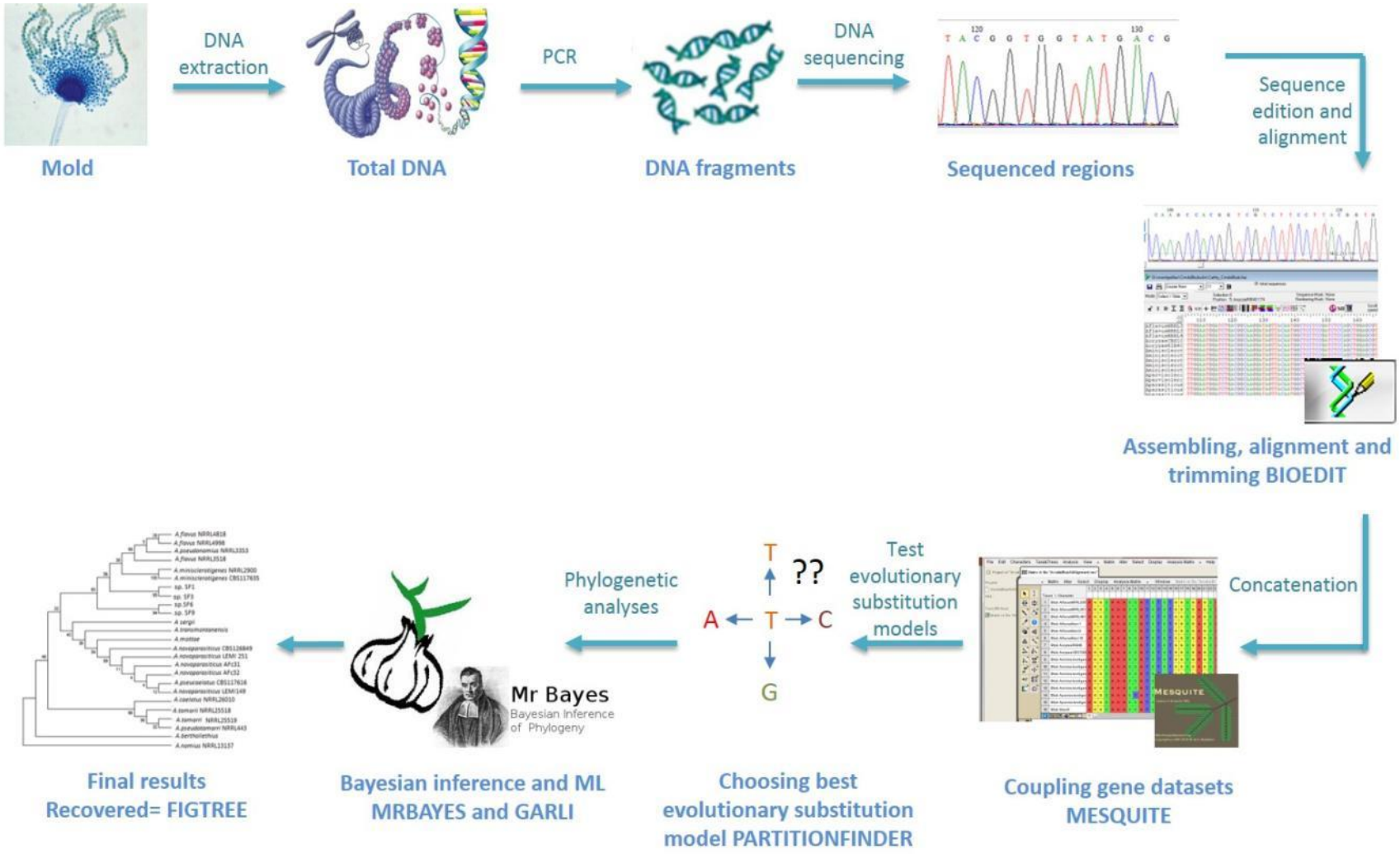

Figure 4. Phylogenetic inference process. The scheme shows all the steps and the software used.

Another issue in phylogenetic inference is to choose between two analyzing approaches, create a consensus from different single topologies or performing concatenated analyses. Consensus analyses have the advantage that, when performed properly, they produce branching pattern with high resolution, thus the topology provides a safe estimate of the phylogeny. It is easy to perform evolutionary model analyses for each gene, but with this method polytomies can increase, because there are not as many variation sites than in a concatenated matrix (Gadagkar et al. 200; Kubatko and Degnan 2007). Additionally, genes do not evolve at the same rates, or necessary present the same scenarios, and one gene can lead to more incongruences between topologies (Ziheng and Rannala 2012). On the other hand, the use of concatenated dataset can increase the number of variable sites, adding important information to clarify relationships. However, one of the problems that rise while using concatenated datasets is that different genes can be assumed to follow the same type of evolutionary model for nucleotide variation, leading to misinterpretation of the data, and a poor inference of the relationships (Gadagkar et al. 2005). Lanfear et al. (2016) partially solved this issue by providing software that allows testing different partitions in a dataset, and including complex algorithms and mathematical processes to improve the analyses. Nowadays, is also recommended to test for coalescence, especially when various genes are used, and some test has been developed to verify if data follows the parameters proposed by software (Degnan and Rosenberg 2009; Liu et al. 2009).

In the present study, depending on the purpose of the analysis and the amount of genes used, one gene datasets and multilocus dataset were analyzed. To choose the best substitution model for nucleotide evolution in single gene datasets we used jModelTest v2.1.6 (Darriba and Posada 2012), and for concatenated datasets we used PartitionFinder v2.00 (Lanfear et al. 2016). jModelTest analyses were run using three different criterion: Akaike Information Criterion corrected (AICc) (Sugiura 1978), decision-theoretic performance-based approach (DT) (Minim et al. 2003) and Bayesian Information Criterion (BIC) (Kass and Wasserman 1995). Conversely, the best-fit nucleotide substitution model for concatenated dataset and partitioning scheme were calculated under BIC. To search for the best scheme we used the "greedy" algorithm with branch lengths of alternative partitions "linked". The use of Bayesian Information Criterion increased over the last decade in the phylogenetic field (Lanfear et al. 2016). This criterion gives an approximate solution to the natural log of the Bayes factor, facilitating the analyses of large samples and nested competing hypotheses, and usually chooses simpler models than AIC (Posada 2009). In general, each gene has been analyzed alone and with a varying number of isolates (depending on the goal of each analysis). In some other cases, different genes were concatenated to obtain results that were more robust.

➢ Maximum Likelihood and Bayesian Inference

Maximum Likelihood

Maximum likelihood (ML) inference is one of the most widely used statistical methods for phylogenetic inference. This method is based on a priori conditions (evolutionary models), that are used as baseline for further probability analysis. ML searches for the best evolutionary model that has the highest likelihood with the given data, and also finds the best tree. Calculations are based on the probability that the pattern of variation of a site occurs given a particular substitution process, a particular tree and the overall observed base frequencies. The likelihoods for all the sites are multiplied to provide an overall likelihood. A good tree should have as many sites with high likelihoods, so the product of likelihoods is high (Brinkman and Leipe 2001; Ziheng and Rannala 2012).

Bayesian Inference (BI)

This statistical method has become more popular over the last two decades. The main difference between Bayesian inference (BI) and ML is that for the first the parameters in the evolutionary model are considered random variables, whereas for the latest they are considered fixed constants. In BI the parameters are assigned a prior distribution, which is combined with the likelihood for the given data to calculate posterior distribution. The strength of the method lies in the Markov Chain Monte-Carlo (MCMC) algorithms, which enables independent branch lengths on unrooted trees (Ziheng and Rannala 2012).

Advantages and Disadvantages of Both Methods

The two inference methods are based on evolutionary models, hence, it is important to choose and adequate substitution model. Likewise, both require complex calculations that are computationally demanding. Nevertheless, both methods have advantages over the maximum parsimony methods, making them widely used nowadays for systematics and phylogenetic analyses (Ziheng and Rannala 2012).

These methods are based on maximum likelihood, however, the way in which statistic are inferred varies from one method to the other. For example, Bayesian statistics answers biological questions directly and their results are easy to interpret. Posterior probability of a tree is interpreted as the probability that the tree is correct for the data under a provided model, yet sometimes it is inflated. In the case of likelihood analysis, concepts, such as the confidence interval, requires a basic

knowledge of statistics for a proper interpretation. The confidence interval for the tree widely used bootstrap method, which could be difficult to interpret (Ziheng and Rannala 2012)

Analyses

Once best nucleotide substitution model was chosen, we performed ML and BI statistical methods to test the accuracy of our results, and to identify the best gene combination. ML analyses were performed in MEGA v6.0.6 (Tamura et al. 2013) or in GARLI v2.01 (Zwickl 2006). Analyses in MEGA 6.0.6 were performed using just one gene and when the model existed in the parameters. GARLI analyses were performed following the manual. Four independent analyses were carried out, two rounds were carried out following random starting tree, and two more following stepwise starting tree. We compared the trees/lnL scores, checked if they were similar, and then we chose to run with the best tree/lnL score. Boostrap was performed using 200 replicates, and Mesquite was used to visualize the results.

For the Bayesian analyses, four independent runs were carried out for 10^7 generations, each with four MCMC chains, and sampling every 500 generations. More generations were added if the p-value was higher than 0.01, because significance was not achieved after finishing the analysis. We confirmed, for each analysis that the average standard deviation of split frequencies between chains approaches to values of ≤ 0.01, and the potential scale factor reduction factor (PSRF) to 1. For all the analyses, 25% were arbitrarily discarded as “burn-in” from the total number of trees per run. The remaining trees were used to calculate posterior probabilities (PP) for each bipartition in a 50% majority-rule consensus tree using Tracer v1.6 (Rambaut et al. 2014). Phylogenetic trees were visualized and edited with FigTree v1.4.2 (Rambaut 2014).

2.2 CHAPITRE 2

Biodiversity of *Aspergillus* isolates potentially aflatoxigenic recovered from peanuts in Côte d'Ivoire

2.2.1 BACKGROUND

The present study resulted from a collaboration between CIRAD and Toxalim. The goal was to characterize at species level some strains from *Flavi* section isolated along the peanut paste production chain at Korhogo region (Côte d'Ivoire). Some strains were already characterized using the PCR-DDGE, yet some strains were not recognized using this technique. Additionally, the confirmation of the results of PCR-DDGE was necessary. This collaboration enabled us to test the "Phylogenetic Molecular Tool" developed. The phylogenetic inference analyses were performed using *ITS*, *benA* and *cmdA* genes.

Risk of mycotoxin contamination is a global issue; however, some regions are more exposed to them, like sub-Saharan Africa, Latin America, and Asia. In fact, it is estimated that in these regions ca 500 million people are at high mortality and morbidity risk (IARC 2015).

In Africa, the presence of fungi belonging to section *Flavi* is frequent. The screening reports of mycotoxins are commonly positive for aflatoxins. Reasons for their presence in staples include suitable environmental variables, staples inadequate storage and transport conditions, and the unawareness of mycotoxin risk (Shephard 2003, Ezekiel et al. 2013; Wagacha and Muthomi 2008). In some African regions, aflatoxin contamination is a main public health problem, affecting people of all ages, including in utero infants (Lewis et al. 2005, Shephard 2008, Streit 2013). Actually, over the last decades, several aflatoxicosis outbreaks were reported, and were caused by the consumption of highly contaminated staples (Lewis et al. 2005; Azziz-Baumagarner et al. 2005). Moreover, aflatoxin contamination impacts African countries economy, since exportation of raw materials has to suit international mycotoxin policies (PACA 2013).

Peanut is an important staple in African countries, is nutritionally rich, and an important economic source. Unfortunately, it is a host for several species from section *Flavi*, including aflatoxinegic ones (Matumba et al. 2014, IARC 2015). The frequent presence of G1 and G2 aflatoxins (AFG1 and AFG2) in peanut products (Kamika et al. 2014, Matumba et al. 2014; 2015) suggests that *Aspergillus flavus* is not the only species contaminating this kind of commodities, making peanuts an excellent product to test the "Phylogenetic Molecular Tool".

The manuscript is been prepared in order to be submitted in a journal.

Biodiversity of *Aspergillus* isolates potentially aflatoxigenic recovered from peanuts in Côte d'Ivoire

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ABSTRACT

Mycotoxin contamination of staples is an issue in Sub-Saharan regions, especially aflatoxins and fumonisins, resulting in severe health and economy risks. Aflatoxins are important mycotoxins because they have detrimental effects in vertebrates, principally aflatoxin B1, which is consider as the most potent liver carcinogenic natural compound (IARC 1993). Maize and peanuts are important staples in Sub-Saharan region, and are among the staples more affected by AFs contamination. In the region, the main producers of aflatoxins are species belonging to *Aspergillus* section *Flavi*, the principal producer is *A. flavus* followed by other species from *A. flavus* clade and *A. parasiticus.* In the present study the biodiversity of *Aspergillus* section *Flavi* was assessed using a polyphasic approach along the peanuts chain process in Côte d'Ivoire. Experiments included morphological analyses, aflatoxins production, DGGE-PCR and phylogenetic inference. The results showed that in Korhogo region, in Côte d'Ivoire, three species of *Aspergillus flavus* clade grow on peanuts, the most frequent *A. flavus*, followed by *A. parvisclerotigenus* and the novel species *A. korhogoensis.* The lasts two species produced B- and G- aflatoxins, and in higher rates than *A. flavus.* The results also showed that the DGGE-PCR and multilocus phylogenetic analyses are elegant strategies for recognizing species of section *Flavi*, especially for *A. flavus* clade.

KEYWORDS

Aspegillus section *Flavi*, aflatoxins, peanuts, PCR-DGGE, phylogenetic analyses, polyphasic approach.

1 INTRODUCTION

Mycotoxins are low-weight molecules (< 1000 Daltons), yield by the secondary metabolism of filamentous fungi, which are detrimental to vertebrates and humans. Mycotoxins are common contaminants of food and feed staples that contaminate approximately 25% of crops worldwide (CAST 2003), and generate important health and economic risks (Wu et al. 2014). Only in the sub-Saharan Africa, Latin America, and Asia, an estimated number of 500 million people are exposed to mycotoxins at high levels, putting them at high mortality and morbidity risk (IARC 2015). Nowadays, 400 putative fungal toxins have been described, seven of which are characterized as major mycotoxins and regulated by the European Union (EU) based on their effects and prevalence: aflatoxins, fumonisins, ochratoxin A, trichothecenes (specifically deoxynivalenol), zearalenone, ergot alkaloids and patulin (Bennet and Klich 2003; Cano et al. 2016).

Among mycotoxins, aflatoxins have received lot of attention for their detrimental effects. Aflatoxin B1 (AFB_1) is the most potent liver carcinogenic natural compound (IARC 1993). Chronic exposure to aflatoxins is linked with teratogenic, mutagenic and immune suppression effects, child stunting, hepatotoxic effects with a high risk of hepatocarcinoma (Turner et al. 2005; IARC 2015). Acute aflatoxicosis can cause hemorrhages, acute liver damage, issues in the absorption and/or metabolism of nutrients, and death (Bbosa et al. 2013). Several episodes of acute toxicosis due to the consumption of high-contaminated foods have been reported in the last decades. The most important occurred in Kenya on 2004, causing the death of 125 people (Lewis et al. 2005; Azziz-Baumagarner et al. 2005). Aflatoxins are mainly produced by fungi of *Aspergillus* section *Flavi* in a variety of matrices, especially maize, peanuts, cotton seeds, oleaginous seeds, cereals and spices and are commonly found in tropical and subtropical regions worldwide, where the environmental conditions favor their production (Klich 2007). Aflatoxin B1 is the most recurrent aflatoxin and *Aspergillus flavus* is recognized as the main source of this toxin in warm and wet regions of the world.

In Africa, aflatoxins are frequently found in mycotoxin screenings, favored by a combination of suitable environmental variables, inadequate storage and transport conditions of staples and the unawareness of mycotoxin risk (Shephard 2004, Ezekiel et al. 2013; Wagacha and Muthomi 2008). Regardless the fact that in certain Sub-Saharan African regions the level of aflatoxins is controlled, in some other regions it is a main public health issue, affecting people of all ages, including *in utero* infants (Lewis et al. 2005, Shephard 2008, Streit 2013). Chronic exposure in these areas can start as early as in uterus, and continue through adulthood (Turner et al. 2013). Infant population present a high risk of aflatoxin exposure, in some Western African regions 99% of children are positive to aflatoxins in blood (Gong et al. 2002), likewise, breastfed infants are also at risk of B1, B2 M1 and M2 aflatoxin intake via their mothers' milk (Shepard 2004). Additionally, aflatoxin contamination has an economic impact in African countries since international regulations were applied, especially EU

legislation, export rejection has increased. For instance, during 2007-2012, the EU issued 346 notifications (PACA 2013).

Aflatoxin intake within West Africa involves principally maize and groundnuts (IARC 2015). The latter is an important staple in some of these countries; it plays a role in nutrition as a cheap source of protein and nutrients, as well as income (Matumba et al. 2014). Fungi of *Aspergillus* section *Flavi* can contaminate peanuts during pre- and post-harvesting steps, and the main factors facilitating their development are plant stress, insect damage, environmental conditions, specially temperatures and humidity, inadequate storage, transporting and marketing conditions (Pitt et al. 2013). *Aspergillus flavus* is not able to produce G-aflatoxins due to a deletion of 0.9-2.2 kb in the *norB* (*aflF*)-*cypA* (*aflU*) region, which modifies the promoter and coding regions (Ehrlich et al. 2004; Probst et al. 2012). Therefore, the frequent presence of G1 and G2 aflatoxins in peanut products (Kamika et al. 2014, Matumba et al. 2014; Matumba et al. 2015; Manizan et al. in press) suggests that *A. flavus* is not the only species contaminating this kind of commodities. Therefore, while G-aflatoxins are detected on staples, it is necessary to target other species as producers. Historically, peanuts' aflatoxin contamination was associated with *A. parasiticus* (Pitt et al. 2013), however, in certain areas of the world aflatoxin contamination of peanuts produced by species such as *A. minisclerotigenes*, *A. pseudocaelatus, A. pseudotamarii* and *A. flavus* were also reported (Pildain et al. 2008; Martins et al. 2017; Oyedele et al. 2017).

The aim of the present work is to address the diversity of the section *Flavi* in peanuts in the Côte d'Ivoire. In pursuance of species identification, a polyphasic approach, enclosing macro- and microscopic analyses, characterization of aflatoxin production and two different molecular approaches were carried out. We developed a Polymerase Chain Reaction-Denaturin Gradient Gel Electophoresis (PCR-DGGE) analysis using a fragment of *β-tubulin* gene, in addition phylogenetic inference analyses were performed using *ITS*, *β-tubulin* (*benA*) and calmodulin (*cmdA*) genes.

2 MATERIALS AND METHODS

2.1 Fungal strains

The biological material used in this study consists of 256 strains of *Aspergillus* isolated from peanut samples (pods, seeds, paste) collected along the peanut chain in Côte d'Ivoire. Peanuts samples were collected precisely in the Korhogo area in northern Côte d'Ivoire, in the villages of Gbandokaha (9°32'N 5°33'W) and Pokaha (9°24'N 5°30'W) and in the markets of Korhogo city (9°29'N 6°49'W). Strains were isolated after inoculation of samples on *Aspergillus flavus/Aspergillus parasiticus* Agar (AFPA) (Merck KGaA, Darmstadt, Germany) incubated at 30°C for 48 h (Pitt et al. 1983). Then, isolated strains were stored at 4°C on inclined Potato Dextrose Agar (PDA) (Biokar

Diagnostics, Allonne, France), and at -80°C in physiological saline solution (NaCl 9 g/L) containing 20% glycerol in cryotubes (MAST, Biovalley, Nanterre, France).

2.2 Morphological characterization

For experiments, calibrated semi-solid spore suspensions (10^6 conidia/mL) were prepared from the 256 strains conserved on inclined PDA medium. For macro- and microscopic analyses, 3 x 5 µL of calibrated spore suspension were inoculated at three equidistant points on Czapek Yeast Agar (CYA) and on Malt Extract Agar (MEA) (Difco Bacto, France); cultures were incubated at 25°C for 7 days. Macroscopic analyses were carried on CYA and MEA (Pildain et al. 2008), whereas microscopic analyses were observed from colonies on PDA after 5 days of incubation at 25°C, using a microscope (Leitz Laborux S) at X400 and X1000.

2.3 Aflatoxinogenesis tests of Aspergillus isolated

2.3.1 Cultures and aflatoxin extraction

After macroscopic and microscopic characterization, the 256 strains were divided into 179 groups, from which one strain was tested for aflatoxin production potential. Test of potential of aflatoxin production were performed on PDA following the method described by Dachoupakan et al. (2009).

Cultures stored at -80°C were used to inoculate the 179 strains on PDA at 25°C for seven days. Then, spore suspensions calibrated at 10^6 conidia/mL were calculated using a THOMA cell. For experiments, 5 µL of calibrated spore suspension (10^6 conidia / mL) were centrally inoculated on PDA and incubated at 25°C for 7 days. Inoculations were carried out in duplicate.

For aflatoxin extraction, 4 plugs of 6 mm diameter were taken (2 plugs at the centre and 2 at the margins of the colony), and weighed in 4 mL amber vials. Then, 2.5 mL of methanol: formic acid (25: 1, v/v) solution was added. Next, samples were agitated for 20 min in an ultrasound bath (Brasonic, 3510E-MT, Danbury, USA). Extracts were obtained by filtration of the samples with a syringe (10cc) through a 0.45 µm PTFE syringe filter (Teflon PTFE, Interchim, France). Next, samples were evaporated under a flux of nitrogen at 45°C. Finally, the dry extracts were suspended in 1 mL of mobile phase (qH_2O: methanol, 55:45, v/v, 350 µL of 4 M nitric acid, 119 mg potassium bromide) and passed in an ultrasound bath for 10 min before HPLC analyses.

2.3.2 Determination of AFs by HPLC/ FLD

The aflatoxins recovered were quantified by reversed-phase high performance liquid chromatography (RP-HPLC) with fluorometric detection (Shimadzu RF 20A, Kyoto, Japan) according to the method of R-Biopharm, Aflaprep IFU (P07.V18) (2013). An aliquot of 100 µL of the extract was

injected into a C18 grafted column (250×4.6 mm, 5 µm, Uptisphere 120 Å OBD silica, Interchim, Montluçon, France) heated to 40°C. The flow rate of the mobile phase was 0.8 mL min^{-1}. Aflatoxins were detected by fluorescence (λexc 362 nm, λem 425 nm) after post-column derivatization in a 100 µÅ electrochemical cell (Kobra Mobile™ R. Biopharm Rhone Ltd., Glasgow, United Kingdom). The results were expressed in µg of aflatoxins g^{-1} of culture medium.

2.4 Molecular analyses

2.4.1 Extraction and purification of DNA using Cethy Trimethyl Ammonium Bromure (CTAB) method

The DNA extraction method was adapted from the protocols of Prabha et al. (2012) and Borges et al. (2009). It was performed in three steps. 1) cell lysis: 5-day-old mycelium and conidia were suspended in 500 µL of CTAB buffer (1 M Tris pH 8, 5 M NaCl, 0.5 M EDTA, 20 g CTAB, 2.5 µL β-mercaptoethanol) in an Eppendorf tube containing 0.3 g of glass beads (425 to 600 µm, Merck, KGaA). The suspension was vigorously homogenized for 2 min in a bead-beater instrument (Vortex Genie 2 SI-A256, USA Scientific, Orlando, FL, USA), then incubated at 65°C for 15 min in a water bath. The suspension was homogenized again for 1 min and incubated at 65°C for 15 min in a water bath. 2) Inactivation of cellular nucleases: after incubation, 500 µL of a chloroform: isoamyl alcohol (24: 1, v/v) solution were added to the suspension and centrifuged for 5 min at 17000xg (centrifuge Heraeus Pico 21, ThermoFisher Scientific, Illkirch, France). 3) Purification of the DNA: 64 µL of 3 M sodium acetate (Merck, KGaA) and 233 µL of isopropanol (CARLO ERBA Reagents, Val de Reuil, France) were added to supernatant and centrifuged at 21,000xg for 5 min. The pellet was washed by adding 500 µL of 70% glacial ethanol. After centrifugation at 21,000xg for 5 min, the supernatant was removed and the pellet dried under a hood at room temperature for at least 4 h. The dry pellet was resuspended in 50 µL of sterile water and quantified using a Nanodrop (Biospec Nano, Shimadzu, Kyoto, Japan).

2.4.2 PCR-DGGE analyses

2.4.2.1 Protocol Polymerase Chain Reaction (PCR) amplification

A region of the *benA* gene was amplified for the 179 isolates using the primer pair Bt2a and Bt2b-GC (Table 1). Using of the GC clamp prevents complete separation of the DNA strand during polyacrylamide gel migration (Huang et al. 2016). The amplification of the DNA was done according to the following steps: 1) Pre-denaturation at 94° C for 4 min. 2) Denaturation at 94°C for 40 s. 3) Annealing at 58°C for 1 min. 4) Extension at 72°C for 1 min. Steps 2 to 4 were carried out for 35 cycles. 5) Final extension at 72°C for 5 min. 6) Final temperature 4°C. Negative controls and contamination checks were performed for all amplifications. PCR amplicons were analyzed on 2% agarose gels by horizontal electrophoresis in TAE 1X (Tris-acetate EDTA pH 8.3; EUROMEDEX,

Souffelweyersheim, France), molecular weight markers of 100 pb (DNA ladder, Promega, Madison, USA) were used to estimate the size of target fragments.

Table 1: Primer sequences

PRIMERS	SEQUENCES	USING
Bt2a *Bt2b*-GC	5'-GGTAACCAAATCGGTGCTGCTTTC-3' 5'-GCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGG GACCCTCAGTGTAGTGACCCTTGG C-3'	PCR-DGGE analyses
ITS1 *ITS4* *Bt2a* *Bt2b* *cmd5* *cmd6*	5'-TCCGTAGGTGAACCTGCGG-3' 5'-TCCTCCGCTTATTGATATGC-3' 5'-GGTAACCAAATCGGTGCTGCTTTC-3' 5'-ACCCTCAGTGTAGTGACCCTTGGC-3' 5'-CCGAGTACAAGGAGGCCTTC-3' 5'-CCGATAGAGGTCATAACGTGG-3'	Phylogenetic analyses

2.4.2.2 Denaturing Gradient Gel Electrophoresis (DGGE) Analyses

PCR products of DGGE were analyzed using a Bio-Rad DcodeTM universal mutation detection system (Bio-Rad Laboratories, USA). Samples containing 40 µL of PCR amplicons were loaded into 8% (w/v) polyacrylamide gels (acrylamide: bisacrylamide, 37.5:1, v/v, Biosolve Chimie, Dieuze, France) in 1X TAE buffer (40 mM Tris-HCl pH 7.4, 20 mM sodium acetate, 1.0 mM Na_2-EDTA). All electrophoresis experiments were performed at 60°C using a denaturing gradient ranging from 30 to 60% (100% corresponded to 7 mol L^{-1} urea and 40% [v/v] formamide, Promega, Charbonnières-les-Bains, France). The electrophoreses were performed at 20 V for 10 min and then at 80 V for 16 h (El Sheikha and Montet 2011). After electrophoresis, gels were stained for 45 min with a solution of Gelred® (Biotium, Fremont, CA, USA) at 0.1 µg mL^{-1} and visualized on a UV transilluminator with the Gel Smart 7.3 system (Clara Vision, Les Ulis, France). Two series of analyses were carried out by DGGE. First, the 179 strains were compared against on the reference strains on *A. flavus*, *A. parasiticus* and *A. nomius*, and then against on the reference strains of *A. parvisclerotigenus*, *A. minisclerotigenes* and *A. arachidicola*. The reference strains used are listed in Annex 1.

2.4.2.3 Image Analyses

Images were processed using ImageQuantTL® Version 2003 software (Amersham Biosciences, Piscataway, NJ, USA). This software automatically covers the DNA bands constituting the DGGE profiles and generates the migration fronts. Each band corresponds to an individual sequence (Kowalchuk et al. 1997; Nakatsu et al. 2000) representing a genus or a species of mold. The DNA of reference strains were used as a marker (*Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. arachidicola*, *A. minisclerotigenes* and *A. parvisclerotigenus*). These control DNAs account for the good migration in

the DGGE gel, and their migration position constitutes the reference position for each species in the analyses.

2.4.3 Phylogenetic analyses

2.4.3.1 Strain selection and PCR

Strains classified as group 2, 3, and 4 were selected for phylogenetic inference analyses using other molecular markers. Some of the strains classified as group 1 were also selected. In total, 71 strains were selected for *ITS* phylogenetic analyses (group 1 = 20, group 2 = 45, group 3 = 4, and group 4 = 1); and a subsample of 40 strains for *benA* and *cmdA* analyses (group 1 = 10, group 2 = 25, group 3 = 4, and group 4 = 1).

All genes were amplified as follows: 1) Pre-denaturation at 94°C for 5 min. 2) Denaturation at 94°C for 1 min. 3) Annealing at 55°C for 1 min. 4) Extension at 72°C for 1 min. Steps 2 to 4 were carried out for 40 cycles. 5) Final extension at 72°C for 10 min. 6) Final temperature 4°C. PCRs were performed in the C1000 Touch™ thermocycler (BioRad, Marnes-la-Coquette, France). Primers used in the study are shown in Table 1. Negative controls and contamination checks were performed for all amplifications. PCR amplicons were analyzed on 1% agarose gels by horizontal electrophoresis, molecular weight markers were used to estimate the size of target fragments. Purification of PCR amplicons were carried out with GeneElute™ PCR Clean-Up Kit (Merck KGaA). Double stranded sequencing was performed in both directions by Plateau de Génomique GeT-Purpan (Toulouse, France). New sequences were deposited in GenBank under the accession numbers indicated in the Table 2.

Table 2: Isolates and accession numbers deposited in GenBank. In normal sequences deposited in GenBank. In bold sequences recovered from a previous study. ^{T} = type strain, — = sequences not determined.

Table continues in following pages

ISOLATE NUMBER	ACCESSION NUMBER			DGGE-GROUP
	ITS	*benA*	*cmdA*	
		A. flavus		
MACI1	KY689211	KY628762	KY661255	Group 1
MACI3	KY689212	KY628763	KY661256	Group 1
MACI16	—	KY628764	KY661257	Group 1
MACI18	KY689213	—	—	Group 1
MACI21	KY689214	—	—	Group 1
MACI22	KY689215	—	—	Group 1
MACI26	KY689216	—	—	Group 1
MACI30	KY689217	—	—	Group 1
MACI36	KY689218	—	—	Group 1
MACI69	KY689219	—	—	Group 1

ISOLATE NUMBER	ACCESSION NUMBER			DGGE-GROUP
	ITS	*benA*	*cmdA*	
MACI70	—	KY628765	KY661258	Group 1
MACI72	KY689220	—	—	Group 1
MACI77	—	KY628766	KY661259	Group 1
MACI79	KY689221	—	—	Group 1
MACI83	KY689222	—	—	Group 1
MACI84	KY689223	KY628767	KY661260	Group 1
MACI97	KY689224	KY628768	KY661261	Group 1
MACI99	KY689225	—	—	Group 1
MACI121	KY689226	—	—	Group 1
MACI126	KY689227	—	—	Group 1
MACI133	—	KY628769	KY661262	Group 1
MACI145	—	KY628770	KY661263	Group 1
MACI156	KY689228	—	—	Group 1
MACI165	KY689229	—	—	Group 1
MACI204	—	KY628771	KY661264	Group 1
MACI250	KY689230	—	—	Group 1
A. parvisclerotigenus				
MACI5	**KY689161**	**KY628772**	**KY661269**	Group 2
MACI6	KY689162	—	—	Group 2
MACI8	**KY689163**	**KY628794**	**KY661270**	Group 4
MACI12	KY689164	KY628795	KY661271	Group 2
MACI14	KY689165	KY628773	KY661272	Group 2
MACI15	KY689166	KY628774	KY661273	Group 2
MACI20	KY689167	KY628775	KY661274	Group 2
MACI62	KY689168	—	—	Group 2
MACI63	KY689169	—	—	Group 2
MACI65	KY689170	KY628776	KY661275	Group 2
MACI118	KY689171	—	—	Group 2
MACI122	KY689172	—	—	Group 2
MACI139	KY689173	KY628777	KY661276	Group 2
MACI140	KY689174	—	—	Group 2
MACI142	KY689175	KY628778	KY661277	Group 2
MACI143	KY689176	—	—	Group 2
MACI177	KY689177	KY628796	KY661278	Group 2
MACI179	KY689178	—	—	Group 2
MACI180	KY689179	KY628779	KY661279	Group 2
MACI184	KY689180	KY628797	KY661280	Group 2
MACI185	KY689181	KY628780	KY661281	Group 2
MACI188	KY689182	—	—	Group 2
MACI191	KY689183	KY628781	KY661282	Group 2
MACI192	KY689184	—	—	Group 2
MACI198	KY689185	—	—	Group 2
MACI200	KY689186	KY628782	KY661283	Group 2
MACI201	KY689187	KY628783	KY661284	Group 2
MACI202	KY689188	—	—	Group 2
MACI203	KY689189	KY628784	KY661285	Group 2
MACI206	KY689190	—	—	Group 2

ISOLATE NUMBER	ACCESSION NUMBER			DGGE-GROUP
	ITS	*benA*	*cmdA*	
MACI208	KY689191	—	—	Group 2
MACI210	KY689192	KY628785	KY661286	Group 2
MACI213	KY689193	KY628798	KY661287	Group 2
MACI214	KY689194	—	—	Group 2
MACI217	KY689195	KY628799	KY661288	Group 2
MACI218	KY689196	KY628800	KY661289	Group 2
MACI220	KY689197	KY628786	KY661290	Group 2
MACI221	**KY689198**	**KY628787**	**KY661291**	Group 2
MACI222	KY689199	KY628801	KY661292	Group 2
MACI223	KY689200	—	—	Group 2
MACI224	KY689201	KY628788	KY661292	Group 2
MACI226	KY689202	—	—	Group 2
MACI238	KY689203	—	—	Group 2
MACI255	KY689204	—	—	Group 2
MACI258	**KY689205**	**KY628789**	**KY661293**	Group 2
MACI262	MG745384	MG757370	MG757370	Group 2
***Aspergillus* spp.**				
MACI46	**KY689207**	**KY628790**	**KY661265**	Group 3
MACI219	**KY689208**	**KY628791**	**KY661266**	Group 3
MACI254^{T}	**KY689209**	**KY628792**	**KY661267**	Group 3
MACI264	**KY689210**	**KY628793**	**KY661268**	Group 3

2.4.3.2 Alignment, model selection and phylogenetic analyses

Sequences obtained were combined with published available sequences for species of *Aspergillus* section *Flavi* (Annex 1). BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) was used to assemble, align and trim the databases for genes *ITS*, *benA*, and *cmdA*, using ClustalW algorithm. The best-fit nucleotide substitution model for *ITS* was chosen using jModelTest v2.0 (Darriba and Posada 2012). Evolution model analyses were run using three different criterion: Akaike Information Criterion corrected (AICc), decision-theoretic performance-based approach (DT) and Bayesian Information criterion (BIC); however, for the analyses, BIC criterion was chosen (*ITS* = TPM2uf + G). *BenA* and *cmdA* datasets were concatenated using Mesquite v3.2 (Maddison and Maddison 2017), and resulted in a matrix of 845 bp. The best-fit nucleotide substitution model for the concatenated matrix and its partitioning scheme were calculated using PartitionFinder v2.0 (Lanfear et al. 2016) under BIC. To search for the best scheme the "greedy" algorithm with branch lengths of alternative partitions "linked" was used, and resulted in one partition: *benA* + *cmdA* (K80 + G).

Both, maximum likelihood (ML) and Bayesian inference (BI) statistical methods were carried out, using the best-fit substitution models, to obtain tree topologies for *ITS* and *benA* + *cmdA*. *ITS* ML analyses were performed in MEGA 6.0.6 (Tamura et al. 2013) with a modification of the best

substitution model (HYK), whereas multilocus *benA* + *cmdA* ML analyses was performed in GARLI 2.01 (Zwickl 2006). Two hundred bootstrap replicates were run for bootstrap support statistics. For the Bayesian analyses, four independent runs were carried out for 107 generations, each with four MCMC chains, and sampling every 1,000 generations. For each analysis it was confirmed that the average standard deviation of split frequencies between chains approaches to values of ≤ 0.01, and the potential scale factor reduction factor (PSRF) to 1. For all the analyses, from the total number of trees per run (*ITS* = 10.001; *benA + cmdA* = 30.004), 25% were arbitrarily discarded as "burn-in". The remaining trees were used to calculate posterior probabilities (PP) for each bipartition in a 50% majority-rule consensus tree. *Aspergillus niger* was used as outgroup for *ITS* and *benA + cmdA* inference analyses. Phylogenetic trees were visualized and edited with FigTree v1.4.2 (Rambaut 2014).

3 RESULTS

3.1 Macroscopic and microscopic strains characterization

Macroscopic analysis of 256 strains isolated from peanuts and grown on MEA and CYA media allowed a preliminary classification into 179 groups. Several macroscopic traits were taken into account for the classification. On MEA, discriminating traits included colony coloration: white and brown shades, orangish brown (less frequent), dark brown, green shades, green and white, yellowish green or less frequent yellow-orange; floccose or flat colonies. Presence of sclerotia and exudates was rarely observed (43% of strains) on this medium. Colony diameter ranged from 30 to70 mm. For colony reverse, any strain penetrated the agar, and reverse coloration was in white, yellow or orange shades (Figure 1). On CYA, discriminating traits included colony coloration, brown, white or green, floccose or flat colonies, presence or absence of sclerotia (74% and 26%, respectively) and presence of exudates, abundant or reduced sporulation. Colony diameter ranged from 50 to 70 mm. Colony reverse was characterized by regular or irregular concentric penetration of agar, in beige, orange or brown shades (Figure 1).

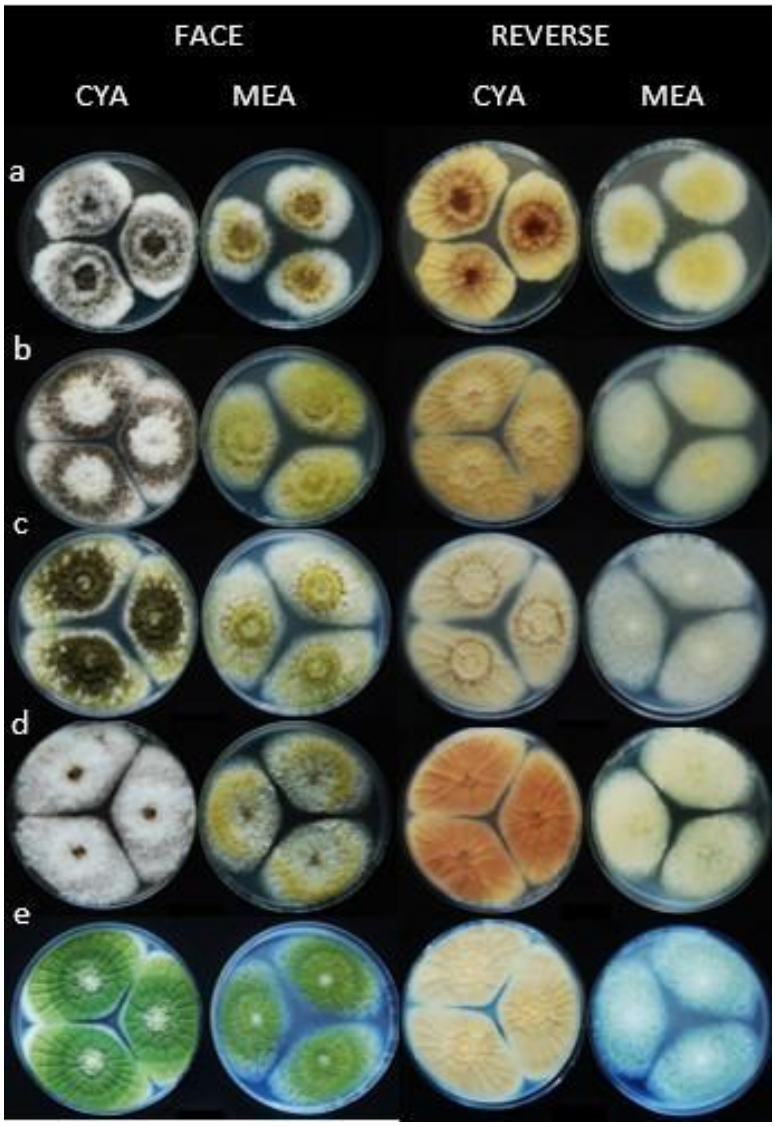

Figure 1. Macroscopic comparison of strains on CYA and MEA media. a: MACI5; b: MACI12; c: MACI84; d: MACI219; e: MACI250. Cultures were grown on CYA and MEA at 25°C for 7 days.

Microscopic analyses were performed on 51 strains. Strains observed, had conidial heads characteristic of *Aspergillus* with a radial or apical head. Conidial heads were mainly biseriate, rarely uniseriate. Conidia were usually round and the hyphae septate. Microscopic analysis confirmed that the isolated strains belonged to *Aspergillus* genus (Figure 2).

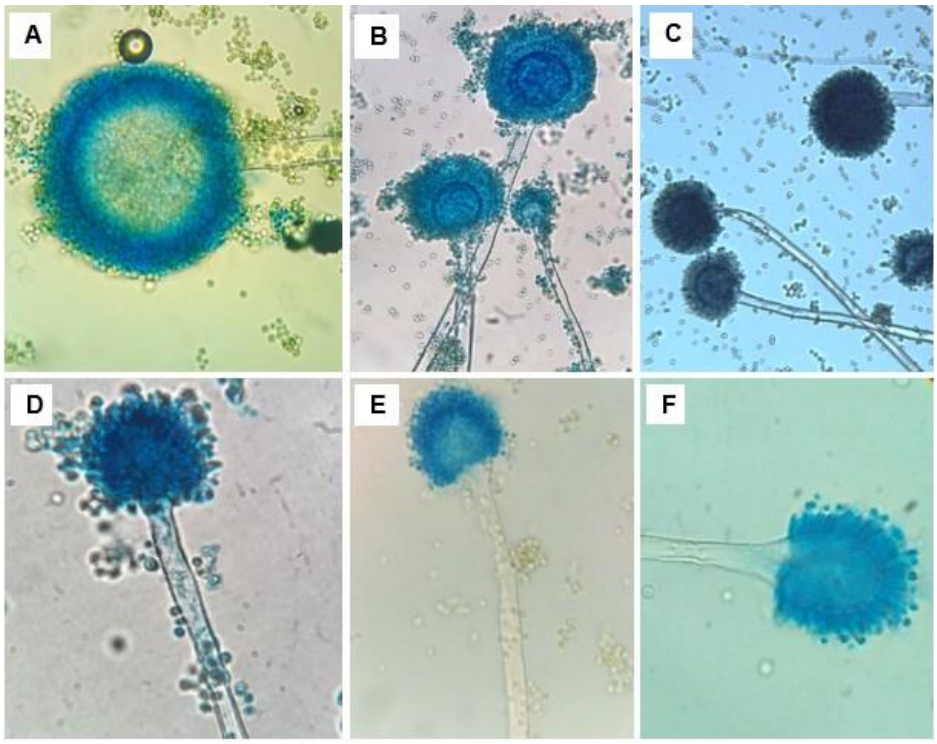

Figure 2. Microscopic comparison of conidial heads. A: MACI3; B: MACI12; C: MACI179; D: MACI46; E: MACI214; F: MACI254. Analysis performed at 400x

3.2 Toxigenic potency of isolated *Aspergillus* strains

The 179 strains, representing all the groups, were split according to their aflatoxin yield; 150 strains (83.8%) were aflatoxigenic, while 29 strains (16.2%) were non-aflatoxigenic. Aflatoxigenic samples (150 strains) were once again divided based on their aflatoxin yield, 8 strains produced AFB1, 92 produced AFB1 and AFB2, and 50 produced the four aflatoxins (AFB1, AFB2, AFG1 and AFG2). Strains that yielded the four AFs presented the highest levels of aflatoxin production. AFB1 and AFG1 yield were the highest among strains, reaching maximum levels of 108.37 µg/g and 103.89 µg/g, respectively. AFB2 and AFG2 were yield at lower levels, ranging from 0.02 to 2.02 µg/g and 0.07 to 3.44 µg/g, respectively.

3.3 PCR-DGGE analyses

The group of 179 strains obtained from the macroscopic analysis were subjected to DGGE analyses. Fungal genomic DNA of each sample was amplified by PCR. All bands had a molecular weight around 550 bp, which corresponds to the fragment of *β-tubulin* gene; hence, amplicons were used for DGGE analysis.

DGGE migration allowed the differentiation amplicons with the same size according to the composition of their DNA. GC-rich DNAs are less rapidly denatured than GC-poor ones and therefore migrate much further (El Sheikha and Montet 2011). DNA migration of reference strains indicates

that DGGE was perfectly performed. Each vertical lane represents one strain. The migration distance of the strains was compared with each other and with the reference strains.

The results for the first DGGE analysis compared the 179 strains against the reference strains of *A. flavus*, *A. parasiticus* and *A. nomius.* Based on this analysis, the strains were divided into 4 groups. Group 1 comprised 129 strains, and was characterized as *A. flavus* (Figure 3). The remaining 50 strains were divided into 3 groups according to their migration distance: group 2 (45 strains), group 3 (4 strains) and group 4 (1 strain). The 50 strains were reanalysed by PCR DGGE in a finer analysis that included the reference strains of *A. arachidicola, A. parvisclerotigenus* and *A. minisclerotigenes* (Figure 4). This second analysis permitted to identify group 2 as *A. parvisclerotigenus*, conversely groups 3 and 4 were not characterized at species level. *A. parasiticus*, *A. nomius*, *A. arachidicola* and *A. minisclerotigenes* were not detected in this study.

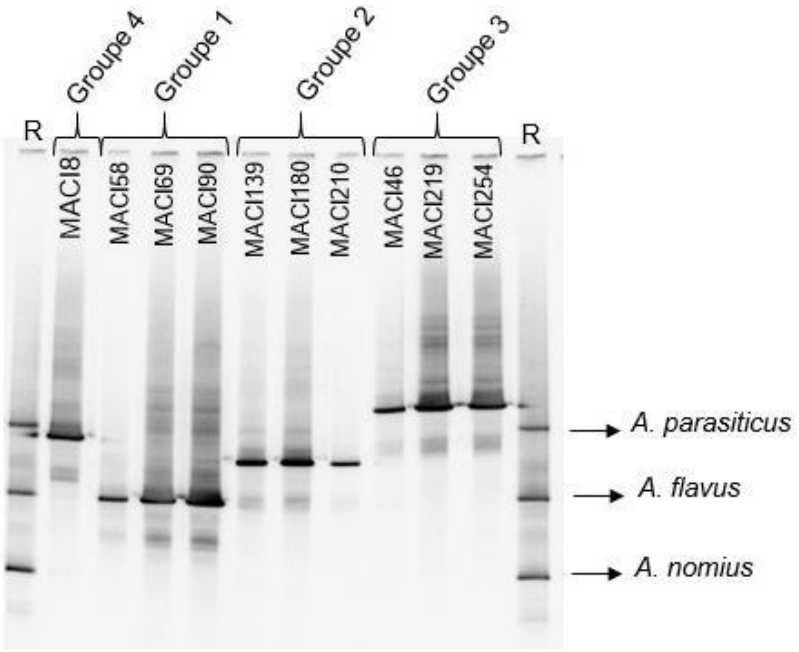

Figure 3. PCR-DGGE β-tubulin profile of *Aspergillus* strains. Reference strains (R): *A. flavus, A. parasiticus* and *A. nomius*.

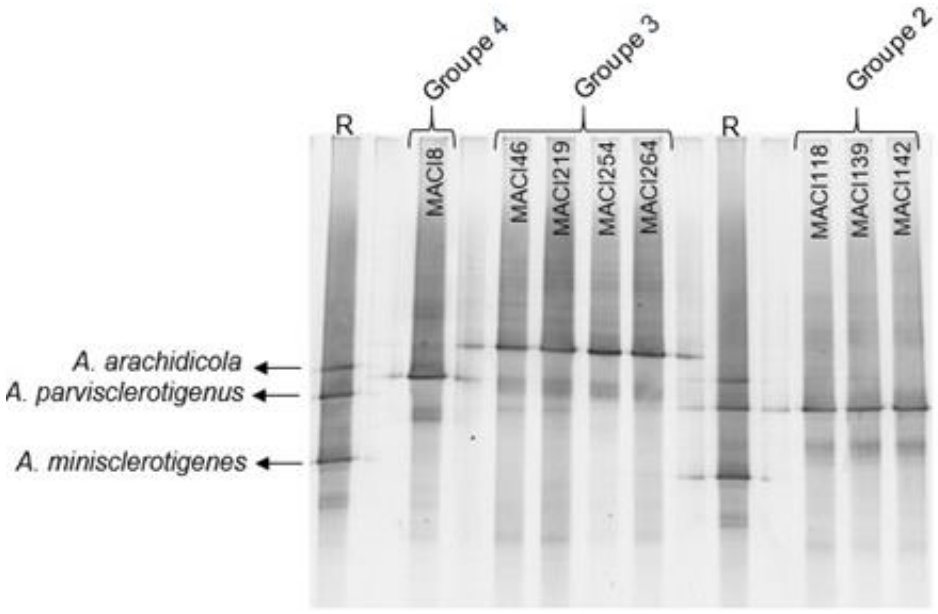

Figure 4. PCR-DGGE β-tubulin profile of *Aspergillus* strains. Reference strains (R): *A. arachidicola, A. parvisclerotigenus* and *A. minisclerotigenes.*

3.4 Phylogenetic analyses

3.4.1 *ITS* analyses

ITS 1-2 region was sequenced for 71 isolates from Côte d'Ivoire (Group 1 = 20, Group 2 = 45, Group 3 = 4, and Group 4 =1). All sequences were blasted against NBCI database, and characterized as *Aspergillus flavus*/*Aspergillus oryzae*. In addition, a subsample of 49 isolates (Group 1 = 18, Group 2 = 22, Group 3 = 4, and Group 4 =1), was analyzed using a phylogenetic approach. The alignment of these sequences contained 507 bp. The results from Bayesian Inference (BI) and Maximum Likelihood (ML) suggested that samples from Côte d'Ivoire cluster together with strains of *A. flavus*, its domesticated species *A. oryzae*, *A. minisclerotigenes* and *A. parvisclerotigenus*. However, the relations within the cluster were not clear because all samples presented polytomies, hence the result suggested that strains belonged to *A. flavus* clade (PP= 98) (Figure 5).

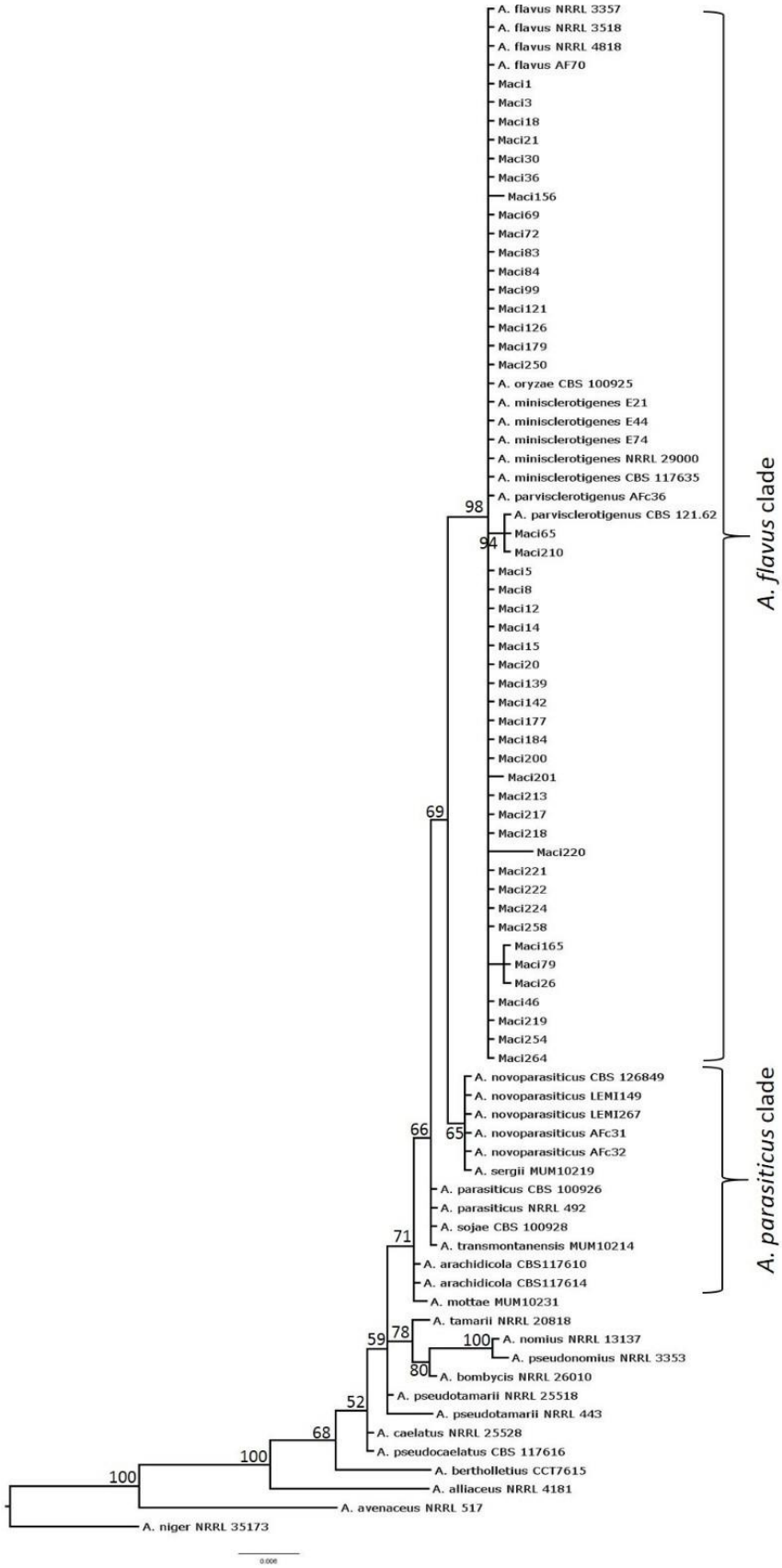

Figure 5. Phylogenetic tree of *Aspergillus* section *Flavi* based on *ITS* data. Bayesian tree calculated from 82 strains of *Aspergillus* section *Flavi,* which includes the reference strain of economically most important species. Species isolates numbers are indicated in each terminal, isolates coded as MACI corresponds to the strains used in this study. *A. niger* NRRL 35173 was used as outgroup.

3.4.2 Multilocus analysis

In order to better place strains of the different groups, a phylogenetic analysis comprising genes *benA* and *cmdA* was also performed. The results obtained from BI and ML analyses were consistent for the basal groups, however, for more derived taxa (*A. parasiticus* clade and *A. flavus* clade) important differences were observed. The topology from the Bayesian analysis was selected as the best hypothesis for inferring the phylogenetic relationships since it was more congruent with other analyses previously performed in the section (Pildain et al. 2008; Rodrigues et al. 2009; Varga et al. 2011; Soares et al. 2012). However, samples isolated from Côte d'Ivoire were clustered following the same pattern under both statistical analyses, forming three clusters. Samples named group 1 clustered together with *A. flavus* and *A. oryzae* strains, samples named group 3 clustered together, and strains named groups 2 and 4 clustered together with *A. parvisclerotigenus* strains.

The topology of BI for the aforementioned derived taxa suggested three clusters. A first one comprising strains belonging to *A. parasiticus* clade, including the strains of *A. transmontanensis*, *A. sergii*, *A. arachidicola*, *A. novoparasiticus*, *A. sojae* and *A. parasiticus* (PP=1). A second cluster comprising strains belonging to group 4, which did not cluster with any described species (PP=1); and a third cluster formed by *A. flavus* clade (PP=1). The latter enclosed three clusters: one formed by *A. parvisclerotigenus* and samples from groups 2 and 3 (PP=1), allowing the identification of these strains as *A. parvisclerotigenus*. A second group formed by *A. minisclerotigenes* strains (PP=0.98), and a third group formed by *A. flavus*, its domesticated species *A. oryzae*, and samples from group 1 (PP=1), hence the group 1 was confirmed as *A. flavus* (Figure 6).

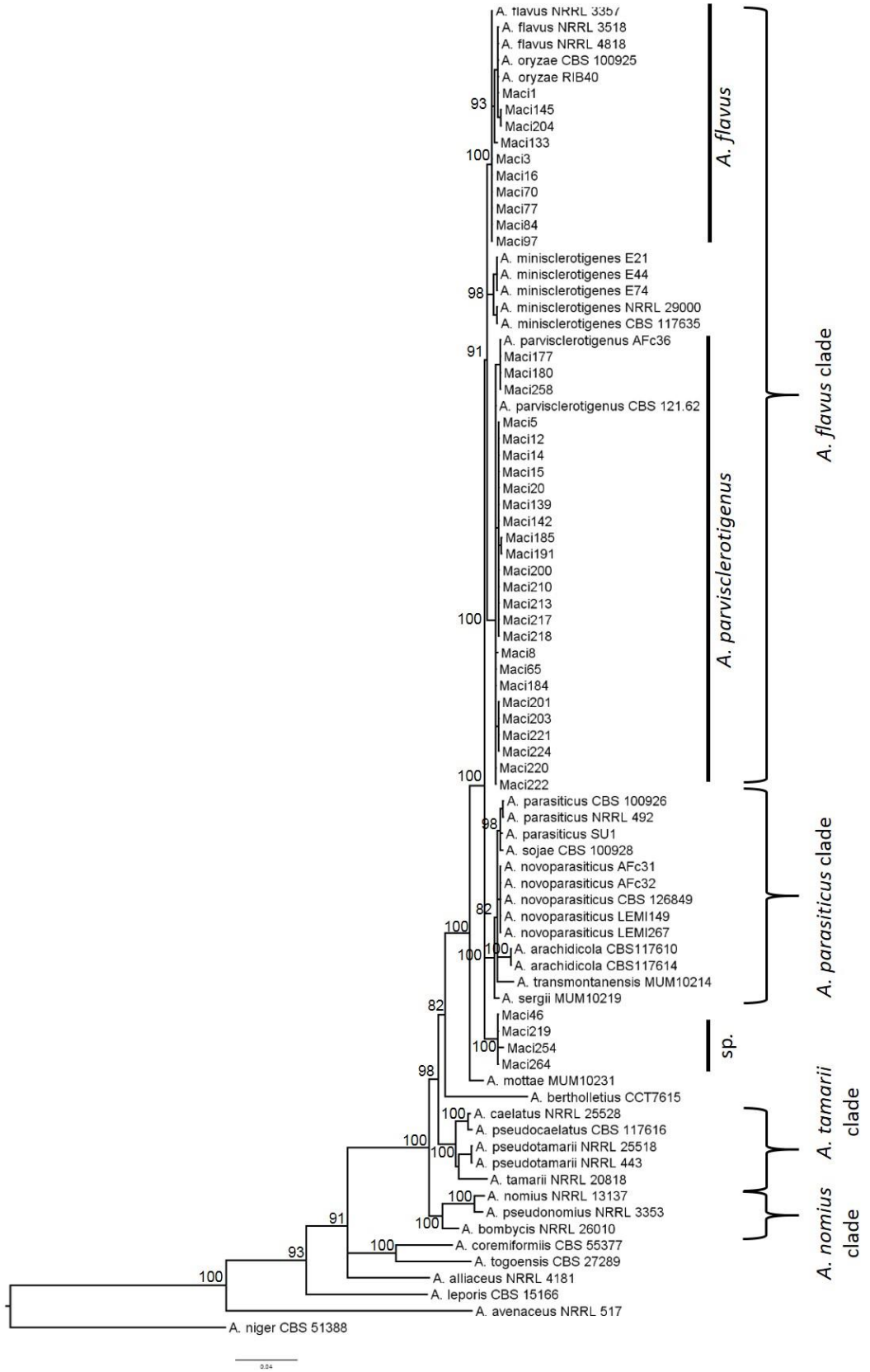

Figure 6. Phylogenetic tree of *Aspergillus* section *Flavi* based on *benA* + *cmdA* combined data. Bayesian tree calculated from 81 strains of *Aspergillus* section *Flavi,* which includes the reference strains of economically most important species. Posterior probability values are shown. The name of each strain is indicated in each terminal; isolates coded as Maci corresponds to the strains used in this study. *A. niger* CBS51388 was used as outgroup.

The combination of morphological analyses, aflatoxigenicity test, PCR-DGGE analyses, and phylogenetic analyses allowed the characterization of 256 isolates collected along the peanut paste production chain to species level. Group 1 was characterized as *A. flavus,* and strains were divided in three groups, AFB1 producers, AFB1 and AFB 2 producers, and non– aflatoxigenic. Groups 2 and 4 were identified as *A. parvisclerotigenus,* and all strains were able to produce the 4 aflatoxins. Group 3 was identified as a potential new species that produced the 4 aflatoxins, and was recently described as a new species, closely related to *Aspergillus parvisclerotigenus*: *A. korhogoensis* (Carvajal-Campos et al. 2017).

4. DISCUSSION

Peanuts are important sources of nutrients and economic income in Sub-Saharan regions, especially in rural areas (Ndung'u et al. 2013; Wagacha and Muthomi 2008; Wagacha et al. 2013; Matumba et al. 2014). In fact, peanuts are ranked sixth among oil producing crops and eighth among nutritional crops because they are nutrient-rich, providing carbohydrates, lipids, proteins, vitamins, minerals, fiber and some organic acids (Mupunga et al. 2017). Peanuts are considered to be one of the main commodities that are frequently contaminated by aflatoxins. In Asian countries, such as Thailand, Philippines and Indonesia, the prevalence of *A. flavus* in peanut samples is very high (Pitt et al. 1993; Pitt et al. 1998) while *A. parasiticus* is the most frequent aflatoxigenic species recovered from United States (Horn 2007; Moore et al. 2017). Several studies have been performed to address the microbiota that grows on peanuts in Africa (Ndung'u et al. 2013; Wagacha and Muthomi 2008; Wagacha et al. 2013; Kamika et al. 2014; Waliyar et al. 2015). In these reports, the most frequent species is *Aspergillus flavus* (aflatoxic and non-aflatoxic strains) and *A. flavus* S_{BG} is less frequent. In East Africa, studies on this staple suggested that species contaminating peanuts included *Penicillium, A. flavus* L *sensu stricto, A. flavus* S_{BG}*, A. parasiticus, A. niger, A. tamarii, A. alliaceus* and *A. caelatus* (information from Kenya and Malawi) (Ndung'u et al. 2013; Wagacha et al. 2013). In West Africa, in particular Nigeria, peanuts are mainly contaminated by *A. flavus* although contamination by G aflatoxins is frequent (Oyedele et al. 2017). One of the problems with the characterization of *Flavi* species in Africa is the definition of *A. flavus*, generally the definition of the *A. flavus sensu lato* species is used, which encloses *A. flavus* L and *A. flavus S* morphotypes. It has already been suggested that *A. flavus S* morphotypes are divided in *S* strains that are AFB producers and the S_{BG} morphotype, which includes at least three cryptic species, *A. minisclerotigenes* (Pildain et al. 2008)*, A. parvisclerotigenus* (Frisvad et al., 2005) and the novel species *A. korhogoensis* isolated in this study and recently described (Carvajal-Campos et al. 2017). Thus, *A. flavus sensu stricto* includes L and S morphotypes incapable of producing G-aflatoxins (Ehrlich 2004). As a result, in the past the biodiversity of the *Flavi* section has been underestimated.

The goal of the present study was to identify the biodiversity of *Aspergillus* section *Flavi* along the peanut value chain in Côte d'Ivoire. The species were identified using a polyphasic approach consisting of morphological, biochemical and molecular traits. Although morphological characterization is basic for identification in this section, overlapping traits resulted in a poor discrimination.

In order to quickly gather strains in definite groups, PCR-DGGE analysis was performed. This allowed the discrimination among DNA strains based on differences in the nucleotide sequence of a target gene. For these analyses is important to choose molecular markers that contain conserved and variable DNA regions (Sheffield et al. 1989; Yoshikawa et al. 2010). In this study, analyses of the *β-tubulin* gene by PCR-DGGE allowed the discrimination of isolates into four groups. Group 1 was characterized as *A. flavus*, groups 2 and 4 were characterized as *A. parvisclerotigenus* and group 3 was characterized as *Aspergillus korhogoensis*. Laforgue et al. (2009) performed PCR-DGGE using *β-tubulin* gene as molecular marker and showed that several species of *Aspergillus*, *A. niger*, *A. aculeatus*, *A. carbonarius*, *A. fumigatus* and *A. japonicus* could be discriminated with this method. Our study confirmed that PCR-DGGE using *β-tubulin* as molecular marker is useful for *Aspergillus* species characterization not only in sections of the genus, but also at a finer scale. Although no isolates belonging to *A. parasiticus*, *A. arachidicola* and *A. minisclerotigenes* were recovered from peanuts, the results obtained from PCR-DGGE analysis of their respective reference strains showed that the methodology is sufficiently accurate to discriminate these species. The results obtained with PCR-DGGE were validated by a phylogenetic approach based on *benA* and *cmdA* genes, which are also often used for the identification of *Aspergillus* species (Pildain et al. 2008; Soares et al. 2012; Varga et al. 2011). The two genes are considered informative, and when used together, the results are more robust. Moreover, it is a good method to rapidly identify species from the section *Flavi*. By contrast, our results confirmed that *ITS* sequence was highly conserved in *Aspergillus*, making it a poor marker for species identification in this genus (Geiser et al. 2007; Schoch et al. 2012) and not suitable for discrimination of *A. flavus* clade species.

Aflatoxin production was also measured in this study. Among the 179 strains, 83.8 % were aflatoxin producers and 16.2 % were non-aflatoxigenic *A. flavus*. Among the aflatoxin producers, 66.6 % were *A. flavus sensu stricto* and produced B-aflatoxins but at smaller rates (28%) than the 33 % B- and G-aflatoxin producers (*A. parvisclerotigenus* and *A. korhogoensis*), which reached levels of 108.37 µg/g for AFB1 and 103.89 µg/g for AFG1. Our results showed that G-aflatoxin peanut contamination in Côte d'Ivoire was mainly caused by *A. parvisclerotigenus,* followed by *A. korhogoensis*.

Before their description, the presence of these species in Benin has been evoked as *A. flavus* S_{BG} (Cotty and Cardwell, 1999) and has been reported in several parts of Sub-Saharan Africa. A study

of soil biota reported that *A. flavus* S_{BG} morphotype was significantly more frequent in the northern areas of Benin (Cardwell and Cotty, 2002). This observation has been strengthened by subsequent studies. S_{BG} morphotype or *A. parvisclerotigenus* strains was isolated from maize samples collected in Mokwa (9°12 N, Nigeria) (Atehnkeng et al. 2008; Perrone et al. 2014), Abuja (9°03N, Nigeria) (Atehnkeng et al. 2008), Akwanga (8°55N, Nigeria) (Atehnkeng et al. 2008), from sesame harvested in Plateau State (8°38N-9°10N, Nigeria) (Ezekiel et al. 2014) and from cassava in Sinissou (9°51N, Benin) (Adjovi et al. 2013). In our study, the high frequency of *A. flavus* S_{BG} (*A. parvisclerotigenus* and *A. korhogoensis*) can be explained by the sampling area, which is limited to the northern region of the Côte d'Ivoire (9° and 10°N latitude). These latitudes correspond to the agro-ecological zone of Northern Guinea savannah. In Senegal, S_{BG} strains were present in maize and sesame samples from two successive agro-ecological zones: sub-humid Guinea savannah and semi-arid Sudan savannah (Diedhiou et al., 2010). The highest frequency of S_{BG} isolates was also observed in poultry feed samples from Northern Guinea savannah and Sudanese savannah zones of Nigeria (Ezekiel et al. 2014). The impact of the presence of *A. flavus* S_{BG} (*A. parvisclerotigenus* and *A. korhogoensis*) on the aflatoxin contamination of staples is not well evaluated. While the studies above-quoted have shown that 40 % to 60 % *A. flavus* L strains are not aflatoxigenic, all *A. flavus* S_{BG} strains produced the four aflatoxins at higher rates. In their founder study, Cardwell and Cotty suggested that crop contamination with G-aflatoxins in Northern Benin could be caused by the S_{BG} strains. By contrast, in the study of Diedhiou et al. (2010), although S_{BG} strains were sometimes present at high frequency, the aflatoxin content of maize and sesame samples has not been greatly influenced by the S_{BG} isolates.

Conversely, better knowledge of the species that contaminate peanuts can contribute to the biocontrol already performed. Some attempts to perform biological control have been done in maize using cultivars resistant to pre-harvest contamination, but they have been limited by the lack of resistance genes. Although some resistant varieties have been developed to increase the germplasm in crops, resistance to aflatoxins in peanuts has not yet been achieved (Torres et al. 2014). Besides, the use of the Aflasafe™ biocontrol containing non-aflatoxigenic *A. flavus* strains has been used in Nigeria, Australia and Argentina. The first essay, in USA, reduced aflatoxin yield by approximately 85 % on peanut stock and to a maximum of 98 % on shelled stock. Additionally, a novel strategy to target resistance-related genes in peanuts has been developed. The idea was to identify resistance-related genes involved in defense response against *A. parasiticus* infection and subsequent aflatoxin contamination by developing expressed sequence tags (ESTs) from contaminated peanut seeds; the data obtained were used to create a microarray to identify candidate genes that confer resistance to *A. flavus* infection (Torres et al. 2014).

Aspergillus flavus is the main mold responsible of food and feed aflatoxin contamination worldwide (Perrone et al. 2014), and its toxinogenicity varies according to the geographical origin and the substrate (Rodrigues et al. 2009). Strains of *A. flavus* clade have a wide distribution in Africa and contaminate important staples, like maize and peanuts. Strains that do not belong to *A. flavus sensu stricto* are important in the region and have been associated with the outbreak in Kenya in 2004. It is important to recognize these species, even though they are less frequent than *A. flavus sensu stricto,* they are a risk to human and animal health due to their B- and G-aflatoxin yield is higher (Wagacha and Muthomi 2008; Wagacha et al. 2013), hence when population booms occur, the likelihood of outbreaks increases (Probst et al. 2010). The two molecular analyses performed in this study showed excellent results to discriminate among species of section *Flavi,* providing fine results to discriminate amongst species of *A. flavus sensu lato*. Analyses are feasible and could be easily performed to unmask biodiversity and therefore, to perform better risk assessment.

PCR-DGGE *β-tubulin* and phylogenetic analyses based on *benA* and *cmdA* are robust methods to characterize species from *Aspergillus* section *Flavi.* Results from the two studies were congruent suggesting that the methods can be useful tools; especially in areas were B- and G-aflatoxins are widespread.

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Annex 1: *Aspergillus* isolates used in the study

Species	Strain	Sampling Data		Reference	GeneBank accession number		
		Substrate	Country		*ITS*	*benA*	*cmdA*
A. alliaceus	NRRL 4181 =CBS 54265^{T}	Soil	Australia	D. I. Fennell, University of Wisconsin, Madison, Wisconsin (in NRRL database)	EF661556	AY160978	EF661536
A. arachidicola	CBS 117610T =IBT 25020	Arachis glabatra leaf	Argentina	Pildain et al. 2008	HM560045	EF203158	EF202049
A. arachidicola	CBS 117614 = IBT 27183	Arachis glabatra leaf	Argentina	Pildain et al. 2008	KY937923	KY924665	KY924677
A. avenaceus	NRRL 517^{T}	Seed peas	England	Smith 1943	EF661556	EF661501	EF661503
A. bertholletius	CCT 7615^{T}	Soil nearby Betholletia excelsa trees	Brazil	Taniwaki et al. 2012	KY937924	KY924666	KY924678
A. bombycis	NRRL 26010^{T} =CBS 117187	Silk worm excrement	Japan	Goto, National Food Research Institute, Japan (in NRRL database)	AF104444	AY017547	AY017594
A. caelatus	NRRL 25528^{T}=ATCC 201128 =CBS 763.97=JCM 10151	Peanut field soil	Georgia, USA	Bruce W. Horn, National Peanut Lab., Dawson, GA (in NRRL database)	AF004930	EF661470	EF661522
A. coremiiformis	CBS 553.77^{T}=ATCC 38576	Soil	Ivory Coast	Centralbureau voor Schimmelcultures, Baarn, The Netherlands (in NRRL database)	FJ491474	FJ49482	FJ491488
A. flavus	NRRL 3518	Wheat flour	Illinois, USA	D. Graves NRRL isolate (in NRRL database)	EF661552	EF661487	EF661510
A. flavus	NRRL 4818=CBS 16870	Food, butter	USA	D. I. Fennell, University of Wisconsin, Madison, Wisconsin (in NRRL database)	EF661563	EF661489	EF661510
A. flavus *	NRRL 3357 = CBS 128202	Peanuts cotyledons	USA	Nierman et al. 2015	MF966967	M38265	EED55330
A. flavus *	AF70	Seed of upland cotton, *Gossypium hirsutum*	Arizona, USA		ASM95283v1 :751:1381:22 30:-1	—	—
A. leporis	CBS 151.66^{T}= NRRL 3216	Dung of *Lepus townsendii*	USA	States and Chistensen 1966	AF104443	EF203171	EF202078
A. minisclerotigenes	CBS 117635^{T}	*Arachis hypogaea* seed	Argentina	Pildain et al. 2008	KY937925	KY924667	KY924679
A. minisclerotigenes	E21	Cumin	Morocco	El Mahgubi et al. 2013	KY937926	JX456195	JX456196

A. minisclerotigenes	E44	White pepper	Morocco	El Mahgubi et al. 2013	KY937927	JX456210	JX456214
A. minisclerotigenes	E74	Paprika	Morocco	El Mahgubi et al. 2013	KY937928	JX456211	JX456212
A. minisclerotigenes	NRRL 29000	Peanut field soil	Australia	David Gaiser , Pennsilvania State University (in Rodrigues et al 2011)	KY937929	KY924668	KY924680
A. mottae	MUM 10.231^{T}=CBS 130016	Maize seed	Portugal	Rodrigues et al. 2011	JF412768	HM803086	HM803015
A. nomius	NRRL 13137=CBS 260.88	Wheat	Illinois, USA	A.F. Schindler, Food and Drug Administration, Wasington D.C. (in NRRL database)	AF027860	AY017541	EF661531
A. novoparasiticus	AFc31	Cassava	Benin	Adjovi et al. 2014	KC9640099	KY924669	KY924681
A. novoparasiticus	AFc32	Cassava	Benin	Adjovi et al. 2014	KC964100	KY924670	KY924682
A. novoparasiticus	LEMI 149	Hospital air	São Paulo, Brazil	Gonçalves et al. 2012	KY937931	KY924671	KY924683
A. novoparasiticus	LEMI 267	Sputum, leukemic patient	São Paulo, Brazil	Gonçalves et al. 2012	KY937932	KY924672	KY924684
A. novoparasiticus	CBS 126849^{T}=LEMI 250	Sputum, leukemic patient	São Paulo, Brazil	Gonçalves et al. 2012	KY937930	KY924673	KY924685
A. oryzae	CBS 100925^{T}=IMI 16266	Unkown source	Osaka, Japan	Varga et al. 2011	MF668185	EF203138	EF202055
A. oryzae *	RIB40	Cereal (broad bean)	Kyoto, Japan	Machida et al. 2005	AP007173	BAE64122	XP_001820302
A. parasiticus	CBS 100926	*Pseudococcus calceolariae*, sugar cane mealy bug	Hawaii, USA	Spaere 1912 (in Varga et al. 2011*)*	KY937933	EF203155	EF202043
A. parasiticus	NRRL 492	Unkown source	China	Shin, China (in NRRL database)	KY937934	KY924674	KY924686
A. parasiticus *	SU-1	Unkown source	Unkown	Yu et al. 2015	—	ASM95608v1:498:14522:16215:1	KJK65439
A. parvisclerotigenus	CBS 121.62^{T}	*Arachis hypogaea*	Nigeria	Frisvad and Samson 2005	MF668183	EF203130	EF202077
A. parvisclerotigenus	AFc36	Cassava	Benin	Adjovi et al. 2014	KC964102	KC954604	KC954606
A. pseudocaelatus	CBS 117616	Arachis burkartii leaf	Argentina	Varga et al. 2011	KY937935	EF203128	EF202037

A. pseudonomius	NRRL 3353^{T}=CBS 119388	Diseased alkali bees,	USA	Varga et al. 2011	AF338643	EF661495	EF661529
A. pseudotamarii	NRRL 443	Soil	Brazil	Da fonseca, Brazil (in NRRL database)	AF004931	EF661476	EF661520
A. pseudotamarii	NRRL 22518	Tea field soil	Miyazaki, Japan	Tetsu Goto (in NRRL database)	KY937937	KY924675	KY924687
A. sergii	MUM 10.219^{T}=CBS 130017	Almond shell,	Portugal	Rodrigues et al. 2011	KY937936	HM803082	HQ340097
A. sojae	CBS 100928	Soy sauce,	Japan	Sakag and Yamada ex Murak 1971	NR_111545	KJ175494	KJ17555
A. tamarii	NRRL 20818=CBS 104.13=QM 9374	Activated carbon	Unkown	CBS database	AF004929	EF661474	EF661526
A. togoensis	CBS 272.89^{T} = NRRL 13550	Seed, near La Maboké	Central Africa	Samson and Seifert 1985 (in CBS database)	AJ874113	FJ491477	FJ491489
A. transmontanensis	MUM 10.214^{T}=CBS 130015	Almond shell,	Portugal	Rodrigues et al. 2011	JF412771	HM803101	HM803020
*A. niger**	NRRL 35173	Cofffe	Unkown	Peterson 2007 (unpublished)	AM270982	—	—
*A. niger**	CBS 513.88	Unkown source	Unkown	CBS database	—	GU296692	NT_166539.1 :c1747409-1745942

CBS, Centraalbureau voor Schimmelcultures, Utrech, The Netherlands; NRRL: National Center for Agricultural Utilization Research, U.S. Department of Agriculture, Peoria, IL, USA; LEMI: Laboratório Especial de Micologia, São Paulo, Brazil; MUM: Micoteca da Universidade de Minho, Braga, Portugal; CCT: Coleção de Cultura Tropical, Campinas, Brazil. SF: Southern Regional Research Center, U.S. Department of Agriculture, New Orleans, USA.

^{T} = type strain; * = genome sequenced strains

2.3 CHAPITRE 3

Aspergillus korhogoensis, a novel aflatoxin producing species from the Côte d'Ivoire

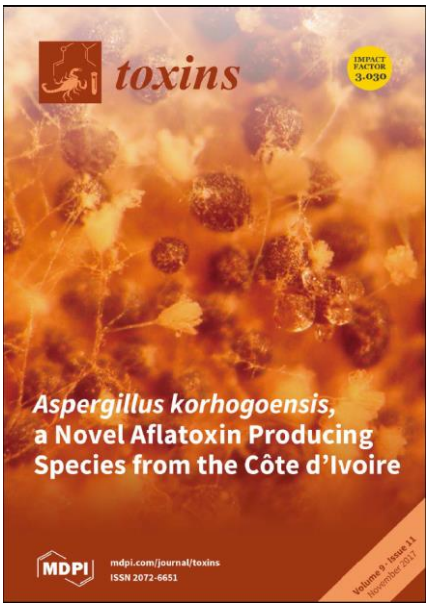
2.3.1 BACKGROUND

The present study is also a result from the collaboration between CIRAD and Toxalim. The goal of the present work was to characterize the four strains named Group 3 in the previous study, and that were identified as a possible new species. These strains were interesting for further analyses for three main reasons:

1) They grouped like a new clade while using *benA* and *cmdA,* suggesting a new species.
2) Topologies for the genes *ITS, benA and cmdA* placed the strains in differently, making them interting to test the "phylogenetic molecular tool".
3) The four strains produce B- and G- aflatoxins, making them hazardous, even though they occur at low frequency.

In order to characterize the four strains, phylogenetic analyses using ten genes (*ITS, benA, cmdA, mcmc7, ppgA, amdS, rbp1, preA, preB, AflP*) were performed (single genes analyses and a combination of them). The publication of the new species was based on the ML (annex 1) and BI results for the phylogenetic analyses using nine genes, and the results of BI were shown. By this approach the description of a new species, belonging to *Aspergillus* section *Flavi*, was possible. Aditionally, morphological analyses, and secondary profile characterization were performed. The mating type MAT loci were analyzed for several species in the section, allowing a better understanding of these genes in the section *Flavi*.

This work was published in Toxins, 2017, 9: 353, and was the cover of that issue. In addition, a summary of the study has been lectured in the 39th mycotoxins workshop (Poland, 2017), and in the 1st International MycoKey conference (Belgium, 2017).



toxins

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Article

Aspergillus korhogoensis, a Novel Aflatoxin Producing Species from the Côte d'Ivoire

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Academic Editor: Ana Calvo
Received: 26 September 2017; Accepted: 26 October 2017; Published: 31 October 2017

Abstract: Several strains of a new aflatoxigenic species of *Aspergillus*, *A. korhogoensis*, were isolated in the course of a screening study involving species from section *Flavi* found contaminating peanuts (*Arachis hypogaea*) and peanut paste in the Côte d'Ivoire. Based on examination of four isolates, this new species is described using a polyphasic approach. A concatenated alignment comprised of nine genes (*ITS*, *benA*, *cmdA*, *mcm7*, *amdS*, *rpb1*, *preB*, *ppgA*, and *preA*) was subjected to phylogenetic analysis, and resulted in all four strains being inferred as a distinct clade. Characterization of mating type for each strain revealed *A. korhogoensis* as a heterothallic species, since three isolates exhibited a singular *MAT1-1* locus and one isolate exhibited a singular *MAT1-2* locus. Morphological and physiological characterizations were also performed based on their growth on various types of media. Their respective extrolite profiles were characterized using LC/HRMS, and showed that this new species is capable of producing B- and G-aflatoxins, aspergillic acid, cyclopiazonic acid, aflavarins, and asparasones, as well as other metabolites. Altogether, our results confirm the monophyly of *A. korhogoensis*, and strengthen its position in the *A. flavus* clade, as the sister taxon of *A. parvisclerotigenus*.

Keywords: *Aspergillus* section *Flavi*; aflatoxins; cyclopiazonic acid; polyphasic approach; versicolorins

1. Introduction

The presence of mycotoxins in agricultural commodities poses serious economic and health risks [1–3]. Among the mycotoxins, aflatoxins are by far the most studied since their ingestion can cause deleterious health effects in humans and animals including hepatic cancer and, in some instances,

death [4]. Aflatoxin B_1 is the potent compound of this chemical family as it displays mutagenic, teratogenic and hepatocarcinogenic effects in humans and animals [5]. To date, it is considered as the most carcinogenic, teratogenic and genotoxic substance of natural origin [6,7].

Species that have so far been reported to produce aflatoxins are all classified in *Aspergilllus* subgenus *Circumdati* and section *Flavi*, with the exception of two other species originally sampled in the Côte d'Ivoire, *A. ochraceoroseus* and *A. rambelli* [8,9]. Species from *Aspergillus* section *Flavi* represent a well-known group of saprophytic filamentous fungi, several of which have the ability to produce beneficial secondary metabolites or enzymes used in food fermentation and biotechnology, such as kojic acid and α-amylase [10]. Conversely, some of these species have the potential to produce one or more harmful mycotoxins, such as aflatoxins, cyclopiazonic acid, versicolorins, and aflatrems [11,12]. Due to extensive research into their aflatoxin production, *A. flavus*, *A. parasiticus*, and *A. nomius* are considered major species in section *Flavi*. Among them, *A. flavus* is the most important because of its worldwide distribution, and it represents the largest source of aflatoxin B_1 contamination of several staple crops, including maize, tree nuts, peanuts, cottonseed, grains, cassava, and spices [13–16].

Although some species relationships in section *Flavi* are still unclear, the section can be separated into one of seven main clades based on a polyphasic approach: *A. flavus* clade, *A. parasiticus* clade, *A. tamarii* clade, *A. nomius* clade, *A. alliaceus* clade, *A. togoensis* clade, or *A. avenaceus* clade, and *A. mottae* and *A. bertholletius* [17–19]. Each clade may contain cryptic species that are difficult to identify, based solely on morphological characters or extrolite profiles, but can be delineated using a polyphasic approach that also includes molecular analyses [12,17,18]. It has been suggested that two cryptic species, *A. minisclerotigenes* and *A. parvisclerotigenus*, belong to the *A. flavus* clade [9,20]. However, in addition to B-aflatoxins these species produce G-aflatoxins, which *A. flavus* is incapable of producing [17,21] due to a deletion of genomic sequence between the aflatoxin pathway genes, *norB* (*aflF*) and *cypA* (*aflU*), thereby altering the promoter and coding regions [22].

In this paper, we describe *A. korhogoensis* sp. nov. as a novel cryptic species within the *A. flavus* clade, based on a polyphasic analysis of four strains isolated from peanuts collected in the region of Korhogo, Côte d'Ivoire.

2. Results

2.1. Molecular Analyses

2.1.1. Multilocus Phylogenetic Analysis

The phylogenetic tree inferred from nine concatenated genes (*ITS*, *benA*, *cmdA*, *mcm7*, *amdS*, *rpb1*, *preB*, *ppgA*, and *preA*), obtained from Bayesian and ML analyses, yielded largely similar topologies, particularly congruent for *Aspergillus flavus* clade. Here, we chose the Bayesian topology as hypothesis of phylogenetic relationships because the results were of greater robustness (Figure 1). Our results support previous phylogenetic inferences involving species from section *Flavi*. *Aspergillus bertholletius* was used as the outgroup taxon. The *A. nomius* clade, which included *A. bombycis* and *A. nomius*, was monophyletic and appeared as a basal group (Posterior Probability, PP = 1). The topology then split in two robust groups, one formed by the *A. tamarii* clade, which included *A. caelatus*, *A. pseudocaelatus* and *A. pseudotamarii*, and a second monophyletic group, which included the *A. parasiticus* and *A. flavus* clades, as well as *A. mottae*. This latter was placed as the ancestral taxon of the group including *A. parasiticus* and *A. flavus* clades. *Aspergillus parasiticus* clade was consistent with Soares et al. results [18] and included *A. parasiticus*, *A. sojae*, *A. arachidicola*, *A. novoparasiticus*, *A. sergii* and *A. transmontanensis*. *A. sergii* and *A. transmontanensis* are basal taxa, respectively. The *A. flavus* clade included *A. flavus*, *A. oryzae*, *A. minisclerotigenes*, *A. parvisclerotigenus* and *A. korhogoensis*, the herein described new species.

The *A. flavus* clade is comprised of two main groups: one that includes *A. flavus*, its domesticated species *A. oryzae* and *A. minisclerotigenes* (PP = 1); and the other group encompasses *A. parvisclerotigenus* and *A. korhogoensis* sp. nov. (PP = 1). The four isolates (MACI46, MACI219, MACI254 and

MACI264) putatively identified as *A. korhogoensis* were tightly clustered, suggesting they were a distinct species from *A. parvisclerotigenus*. Strains from the latter species included isolates from different populations, which were clustered together, suggesting a monophyletic group with no major differences among populations.

Aspergillus novoparasiticus was represented by two well-supported groups, which segregated based on geography since one group corresponds to the strains isolated from Brazil [23], while the other group corresponds to strains isolated from Benin [14]. Besides their geographical distributions, both groups were isolated from different environments, such as hospital environments (Brazil samples) or foodstuffs (Benin samples).

2.1.2. Mating Type Analysis

Results from our mating type diagnostic PCR revealed that isolates MACI46, MACI254 and MACI264 contained a single *Mat1-1* gene, and that isolate MACI219 contained a single *Mat1-2* gene. These findings demonstrate that *A. korhogoensis* sp. nov. is likely a heterothallic (self-infertile) fungus. Whether these mating-type genes are functional is unknown. Future mating tests will be required to determine this.

The MAT1-1 amino acid sequence of *A. bertholletius* was used as the reference sequence to compare with *Mat1-1* genes from other examined taxa. Basal taxa (*A. bertholletius*, *A. nomius*, *A. caelatus*, *A. pseudocaelatus*, *A. tamarii* and *A. pseudotamarii*) presented alanine, asparagine, histidine and threonine at position 36, 46, 61 and 65, respectively, which changed in derived species into serine, lysine, asparagine and asparagine (Figure 2). The *A. tamarii* clade presented four apomorphies that are specific to the clade, and one apomorphy that was fixed in the derived species. The *A. parasiticus* and *A. flavus* clades exhibited a highly conserved MAT1-1 amino acid sequence, except for one amino acid substitution in *A. parasiticus* at position 101, and two substitutions in both *A. flavus* and *A. oryzae* at positions 49 and 75. Although haplotypes of *A. minisclerotigenes*, *A. parvisclerotigenus* and *A. korhogoensis* sp. nov. shared identity for their respective MAT1-1 amino acid sequences (Figure 2), there were single nucleotide polymorphism (SNP) differences that did not result in an amino acid replacement. *Aspergillus minisclerotigenes* exhibited two apomorphies, whereas *A. parvislerotigenus* and *A. korhogoensis* sp. nov. each exhibited only one (except MACI264, which exhibited the conserved ancestral state).

In the case of *Mat1-2* gene, amino acid sequences were identical for *A. nomius*, *A. pseudonomius*, *A. sergii*, *A. transmontanensis*, *A. arachidicola*, *A. parasiticus*, *A. parvisclerotigenus* and *A. minisclerotigenes*. There was one substitution in the amino acid sequence for *A. korhogoensis* sp. nov. (S168P), and one for *A. flavus* (E181K) (Figure 3). Basal taxa, *A. avenaceus* and *A. alliaceus*, exhibited several differences in their amino acid sequences.

2.2. Secondary Metabolism Characterization

An analysis of secondary metabolites produced by the four *A. korhogoensis* strains was performed and the results are summarized in Table 1. Metabolites were identified according to the Metabolomics Standard Initiative level definitions [24]. Metabolites were identified at level 1 when they displayed the same retention time, and UV and MS/MS spectra as the authentic standard. They were identified at level 2 when the metabolites shared the same UV spectrum and/or the same MS/MS fragmentation pattern in accordance with the literature.

Toxins **2017**, *9*, 353

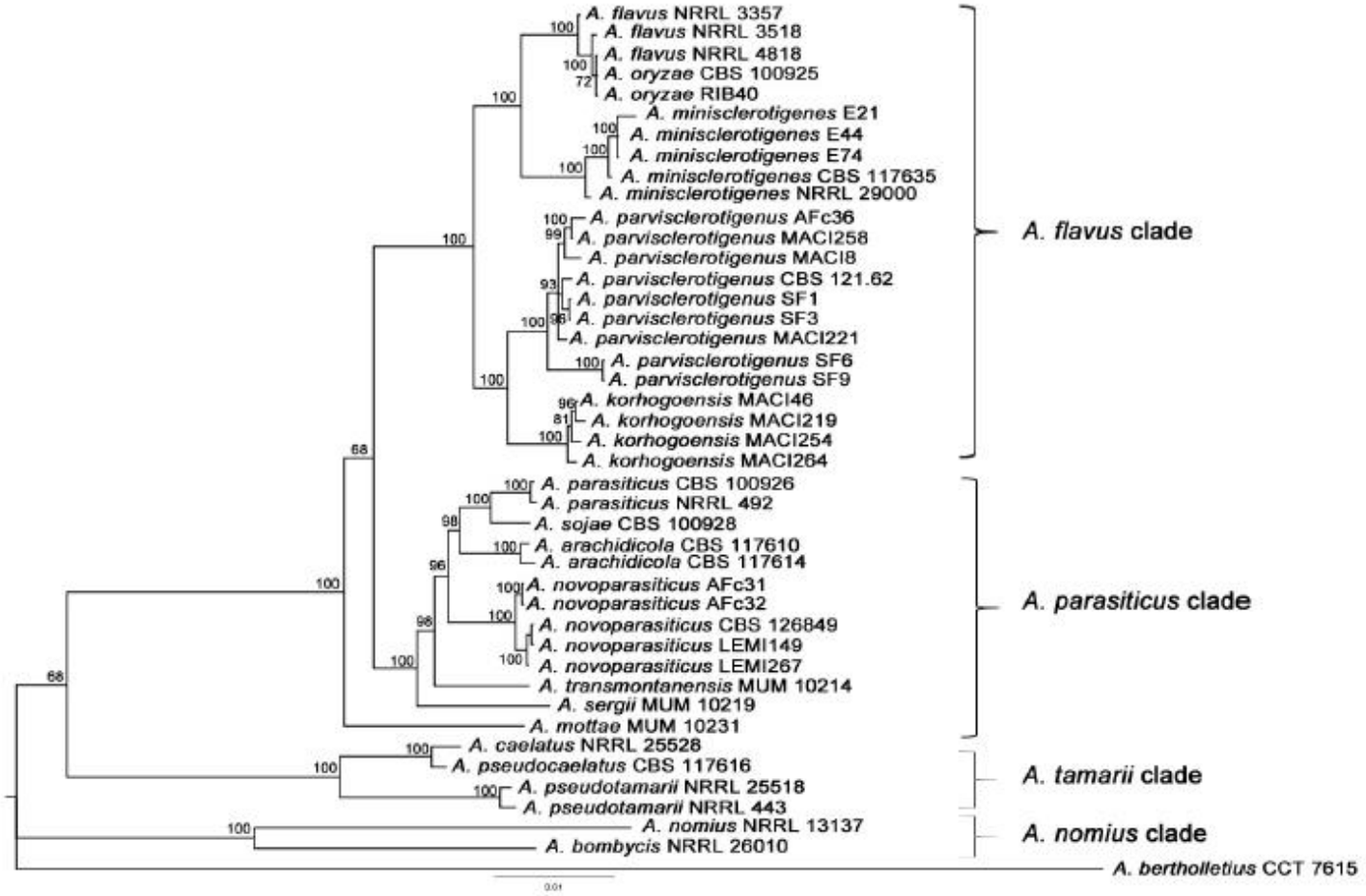

Figure 1. Phylogenetic tree of *Aspergillus* section *Flavi* based on concatenated sequences from nine genomic loci (*ITS*, *benA*, *cmdA*, *mcm7*, *amdS*, *rpb1*, *preB*, *ppgA*, and *preA*). Bayesian tree was calculated from 41 strains, and includes the Type strain for most species. Strong bootstrap values are shown at branch nodes. Species isolate numbers are indicated at branch tips. *A. bertholletius* CCT 7615 was used as the outgroup taxon.

Toxins **2017**, *9*, 353

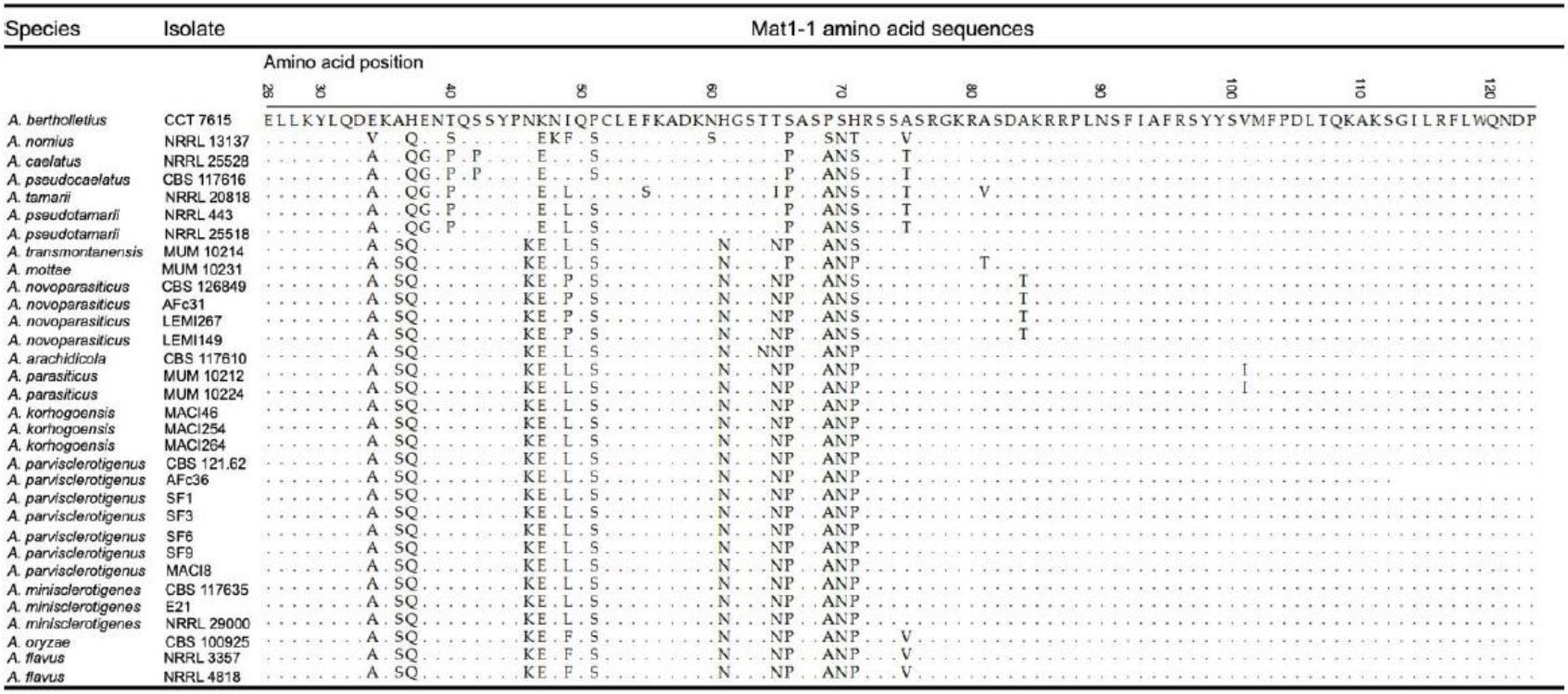

Figure 2. Amino acid sequence alignment for the *Mat1-1* locus in examined strains representing several *Aspergillus* species. The amino acid positions were determined based on the complete amino acid sequence of *A. flavus* NRRL 3357 strain (accession number = EED46656). Accession numbers recovered from GenBank: *A. parasiticus* MUM 10.212 = HM80303, *A. parasiticus* MUM 10.224 = HM803058, and *A. tamarii* NRRL 20818 = HM803044. Other accession numbers are given in Table S2.

Toxins **2017**, *9*, 353

Species	Isolate	Mat1-2 amino acid sequences
		Amino acid position
		140 150 160 170 180 190
A. avenaceus	NRRL 517	HHPRIKEAYPEFTNNEISIILGKQWKSETEEIKVQFRGMADELKKKHAEDHPDYHYTP
A. alliaceus	NRRL 4181	.....D.....M.....A.S..A.....S..E...R.....
A. bombycis	NRRL 26010	.....D.....A.S..V.M...N..ED.....
A. pseudonomius	NRRL 3353	.....D.....A.S..V.M...N..E.....
A. transmontanensis	MUM 10223	.....D.....A.S..V.M...N..E.....
A. sergii	MUM 10219	...D.....A.S..V.M...N..E.....
A. arachidicola	CBS 117614	.....D.....A.S..V.M...N..E.....
A. parasiticus	CBS 100926	.....D.....A.S..V.M...N..E.....
A. korhogoensis	MACI219	.....D.....A.P..V.M...N..E.....
A. parvisclerotigenus	MACI5	.....D.....A.S..V.M...N..E.....
A. minisclerotigenes	E44	.....D.....A.S..V.M...N..E.....
A. minisclerotigenes	E74	.....D.....A.S..V.M...N..E.....
A. flavus	NRRL 3518	.....D.....A.S..V.M...N..EK.....

Figure 3. Amino acid sequence alignment for the *Mat1-2* locus in examined strains representing several *Aspergillus* species. The amino acid positions were determined based on the complete amino acid sequence of *A. bombycis* NRRL 26010 strain (accession number = OGM45987). Accession numbers recovered from GenBank: *A. avenaceus* NRRL 517 = HM802955, *A. alliaceus* NRRL 4181 = HM802964, and *A. transmontanensis* MUM 10223 = HM802958. Other accession numbers are given in Table S2 except for *Aspergillus parvisclerotigenus* MACI5 (MF966968). *Aspergillus parvisclerotigenus* MACI5 species identification was based on genomic sequences from ITS (KY689161), *benA* (KY628772) and *cmdA* (KY661269). The *A. sergii* sequence available from GenBank is shorter.

Table 1. Principal secondary metabolites produced by *Aspergillus korhogoensis*.

Metabolite	Elemental Composition	*m/z*	Ion	Retention Time (min)	MS/MS	Error (ppm)	ID Level *	References
AFLATOXIN BIOSYNTHESIS PATHWAY								
Aflatoxin B_1	$C_{17}H_{12}O_6$	313.07	$[M+H]^+$	17.37	285 (100), 298, 284, 270, 257, 243, 229	−0.398	1, 2	[25]
Aflatoxin B_2	$C_{17}H_{14}O_6$	315.07	$[M+H]^+$	14.95	297, 287 (100), 259, 269, 273	−5.920	1, 2	[25]
Aflatoxin G_1	$C_{17}H_{12}O_7$	329.08	$[M+H]^+$	15.25	311 (100), 301, 300, 283, 243	−0.119	1, 2	[25]
Aflatoxin G_2	$C_{17}H_{14}O_7$	331.08	$[M+H]^+$	12.84	313 (100), 303, 285, 275, 257, 245	−0.511	1, 2	[25]
O-methyl-sterigmatocystin	$C_{19}H_{14}O_6$	339.08	$[M+H]^+$	24.21	324 (100), 311, 306, 295	2.817	1	
Sterigmatocystin	$C_{18}H_{12}O_6$	325.07	$[M+H]^+$	33.59	310 (100), 297, 282	0.570	1, 2	[26]
Versicolorin A	$C_{18}H_{10}O_7$	337.03	$[M-H]^-$	35.95	309 (100), 319, 308, 293, 265, 253	−2.094	1, 2	[27]
Versicolorin B	$C_{18}H_{12}O_7$	339.05	$[M-H]^-$	34.40	311 (100) 310, 309, 295, 297, 283	−0.578	1, 2	[27]
Norsolorinic acid	$C_{20}H_{18}O_7$	369.10	$[M-H]^-$	42.07	351 (100), 341, 325, 308, 297, 270	1.528	1	
CYCLOPIAZONIC ACID BIOSYNTHETIC PATHWAY								
α-cyclopiazonic acid	$C_{20}H_{20}N_2O_3$	337.15	$[M+H]^+$	36.77	182 (100), 196, 154, 140	0.561	1, 2	[28]
β-cyclopiazonic acid	$C_{20}H_{22}N_2O_3$	339.17	$[M+H]^+$	37.58	198 (100), 324, 283, 183, 144, 130	−1.289	2	[28]
2′-oxo-cyclopiazonic acid	$C_{20}H_{20}N_2O_4$	353.15	$[M+H]^+$	36.20	335 (100), 311, 293, 252, 224, 212	−1.174	2	[28]
3′-hydroxy-speradine A	$C_{21}H_{22}N_2O_5$	383.16	$[M+H]^+$	21.19	355 (100), 365, 182, 184, 226, 254, 323, 347, 337	−1.144	2	[28]
Speradine C	$C_{20}H_{22}N_2O_5$	371.16	$[M+H]^+$	18.19	353 (100), 287, 269, 259, 226, 184	2.780	2	[28]
Speradine D	$C_{20}H_{22}N_2O_6$	387.16	$[M+H]^+$	20.80	369 (100), 269, 226, 184	2.679	2	[28]
Speradine F	$C_{21}H_{22}N_2O_7$	415.15	$[M+H]^+$	18.99	397 (100), 379, 369, 355, 353, 337, 311, 297, 281, 269, 253, 226, 184	−0.644	2	[28]
Cyclopiamide J	$C_{22}H_{24}N_2O_7$	429.17	$[M+H]^+$	23.96	287 (100), 411, 497, 379, 369, 337, 269, 259, 226, 184	−0.693	2	[28]
KOJIC ACID BIOSYNTHETIC PATHWAY								
Kojic acid	$C_6H_6O_4$	143.03	$[M+H]^+$	1.87	143 (100) 125, 113, 97	1.432	1, 2	[29]

Table 1. *Cont.*

Metabolite	Elemental Composition	*m/z*	Ion	Retention Time (min)	MS/MS	Error (ppm)	ID Level *	References
AFLATREM BIOSYNTHETIC PATHWAY								
α-aflatrem	$C_{32}H_{39}NO_4$	502.29	$[M+H]^+$	41.45	444 (100), 484, 426, 412, 376, 198	1.144		
Paspalinine	$C_{27}H_{31}NO_4$	434.23	$[M+H]^+$	39.22	376 (100), 416, 419, 362, 358, 344, 130	0.726	2	[30]
Paspaline	$C_{28}H_{39}NO_2$	422.31	$[M+H]^+$	43.96	130 (100), 404, 407	−0.583	2	[30]
Hydroxyaflatrem	$C_{32}H_{39}NO_5$	518.29	$[M+H]^+$	38.22	460 (100), 500, 482, 442, 446, 428	−0.347		
Paxilline	$C_{27}H_{33}NO_4$	436.25	$[M+H]^+$	38.64	418 (100), 421, 400, 378, 360, 346, 130	−2.762	1, 2	[30]
13′-desoxypaxilline	$C_{27}H_{33}NO_3$	420.25	$[M+H]^+$	40.31	402 (100), 405, 362, 130	−0.320	2	[30]
ASPARASONE BIOSYNTHESIS PATHWAY								
Asparasone A	$C_{18}H_{14}O_8$	357.06	$[M-H]^-$	22.13	339 (100) 299	1.315	2	[31]
1,3,4,6,8 pentahydroxy-2-(1′-hydroxy-3′-oxobuty)anthraquinone	$C_{18}H_{14}O_9$	373.04	$[M-H]^-$	9.36	355 (100) 315	0.629	2	[31]
1,3,6,8 tetrahydroxy-2-(1′-hydroxyethyl) anthraquinone	$C_{16}H_{12}O_7$	315.05	$[M-H]^-$	27.98	297 (100)	0.775	2	[31]
1,3,6,8 tetrahydroxy-2-(3′oxobut 1′-en-1′-yl) anthraquinone	$C_{18}H_{12}O_7$	339.05	$[M-H]^-$	29.77	297 (100) 321, 296, 295, 311, 306	1.428	2	[31]
LEPORINS BIOSYNTHESIS PATHWAY								
Leporin B	$C_{22}H_{25}NO_3$	352.19	$[M+H]^+$	40.78	216 (100), 230, 244, 258, 270, 282, 296, 306	−1.505	2	[25]
Leporin B precursor	$C_{22}H_{25}NO_2$	336.20	$[M+H]^+$	37.97	200 (100), 214, 228, 242, 254, 266, 280	0.102	2	[25]
AFLAVARIN BIOSYNTHESIS PATHWAY								
Aflavarin	$C_{24}H_{22}O_9$	455.13	$[M+H]^+$	18.22	413 (100), 425, 437, 395, 379, 364, 348, 303	−3.732	1, 2	[32]
7′-demethyl-siderin	$C_{11}H_{10}O_4$	207.07	$[M+H]^+$	13.58	163 (100), 177, 175, 148, 147, 135, 133, 131, 115, 107	0.312	2	[32]
Aflavarin precursor 6	$C_{22}H_{18}O_8$	411.11	$[M+H]^+$	20.69	369 (100), 381, 379, 352, 343, 337, 279, 207, 177, 147	−0.569	2	[32]
Aflavarin precursor 5	$C_{23}H_{20}O_8$	425.12	$[M+H]^+$	26.75	383 (100), 393, 369, 363, 357, 349	0.484	2	[32]
Aflavarin precursor 4	$C_{24}H_{22}O_8$	439.14	$[M+H]^+$	30.52	397 (100), 383, 371, 367, 365, 351, 341, 321	−0.624	2	[32]
AFLAVININE BIOSYNTHESIS PATHWAY								
20′-hydroxyaflavinine	$C_{28}H_{39}O_2N$	404.29	$[M-H_2O+H]^+$	37.53	386 (100), 287, 269, 243, 144, 130	0.071	1	
Unknown aflavanine	$C_{28}H_{39}O_2N$	404.29	$[M-H_2O+H]^+$	38.14	386 (100), 287, 269, 224	0.170		

* ID Level 1: Metabolites that displayed the same retention time, UV and MS/MS spectra than the authentic standard. Level 2: Metabolites that displayed the same UV spectrum and/or the same MS/MS fragmentation pattern in accordance with the literature.

All strains produced aflatoxins B_1, B_2, G_1, and G_2, as well as several aflatoxin biosynthetic intermediates including 3-*O*-methylsterigmatocystin, sterigmatocystin and versicolorins A and B. Cyclopiazonic acid (CPA) and different other members of the CPA family were detected in the whole fungal extract of each strain. This new species also produced kojic acid, aflatrem, and its precursors or related compounds (paspaline, paspalinine, paxilline, and 13-desoxypaxilline). Aflatrem and paspaline were also present in the sclerotium extracts, as well as another related compound that appears to be an uncharacterized hydroxylated form of aflatrem ($[M + H]^+/z$ = 518.28992; deviation = −0.347 ppm) previously evoked by Nicholson et al. [33]. Leporin B and its precursor were previously detected in culture extracts [25].

The HPLC-DAD analysis of sclerotium extracts revealed the presence of members of at least three families. First, five compounds showed a typical anthraquinone UV spectrum (nm): 223 (100%), 269sh, 293, 319sh, 455. On the bases of UV and MS/MS fragmentation patterns, this compound was identified as asparasone A [31,34]. Three other asparasone-derived anthraquinones were identified in sclerotium extracts (Table 1). Six compounds displayed an aflavarin UV spectrum (nm): 221 (100%), 238sh, 291, 310, 322sh [35]. The LC-HRMS allowed the identification of aflavarin and four aflavarin-associated compounds previously reported by Cary et al. [32]. The last chemical family identified in *A. korhogoensis* sclerotia were aflavinines [36,37]. Indeed, three compounds with a typical aflavinine UV spectrum (nm): 224 (100%), 283, and 291, were present in the sclerotium extracts of each tested strain. The comparison with the 20-hydroxyaflavinine standard confirmed the presence of this metabolite.

2.3. Taxonomy

Aspergillus korhogoensis A. Carvajal-Campos, A.L. Manizan, S. Tadrist, D.K. Akaki, R. Koffi-Nevry, G.G. Moore, S.O. Fapohunda, S. Bailly, D. Montet, I.P. Oswald, S. Lorber, C. Brabet and O. Puel sp. nov. (Figures 4–7).

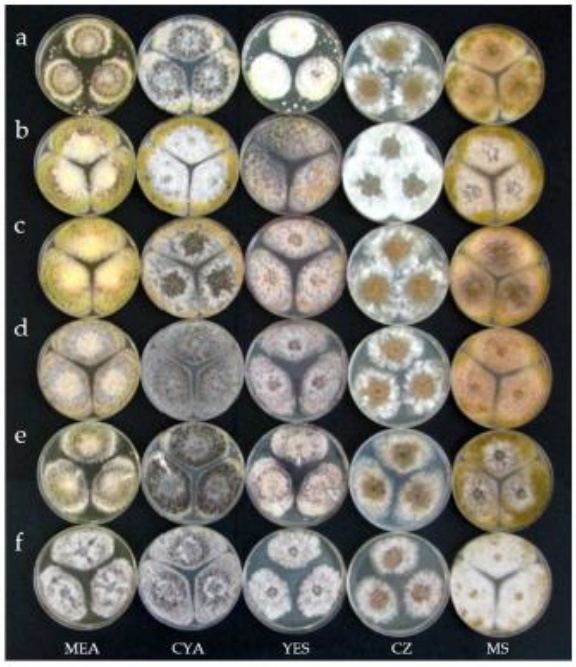

Figure 4. Comparison between cultures of *Aspergillus korhogoensis* sp. nov. and other species from the *A. flavus* clade: (**a**) *A. korhogoensis* MACI46; (**b**) *A. korhogoensis* MACI219; (**c**) *A. korhogoensis* MACI254; (**d**) *A. korhogoensis* MACI264; (**e**) *A. parvisclerotigenus* CBS 121.62; and (**f**) *A. minisclerotigenes* J117c. Cultures were grown on MEA, CYA, YES, CZ, and MS at 25 °C for Seven days.

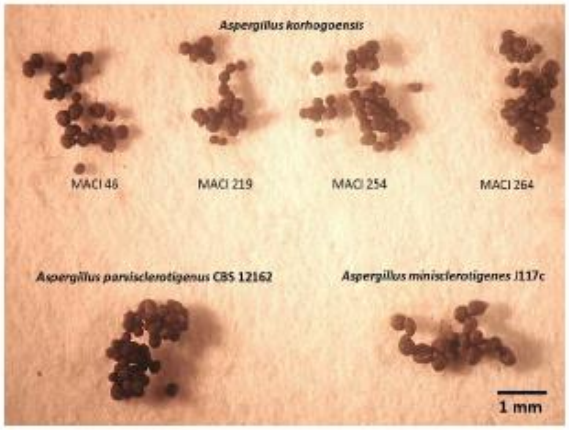

Figure 5. Comparison between sclerotia of *A. korhogoensis* sp. nov. and other species from *A. flavus* clade. Sclerotia recovered from cultures grown on MEA at 25 °C for seven days.

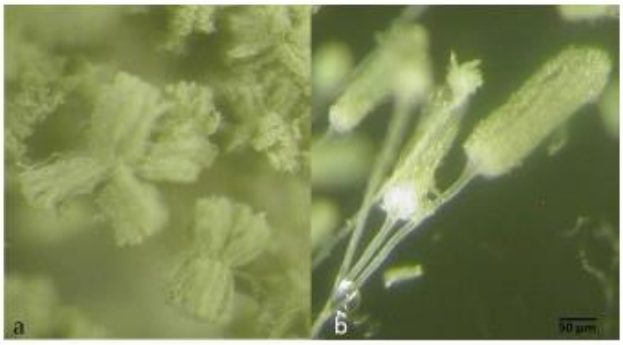

Figure 6. Conidial heads of *A. korhogoensis* MACI254 (100×): (**a**) radiate splitting conidial heads; and (**b**) columnar conidial heads (100×).

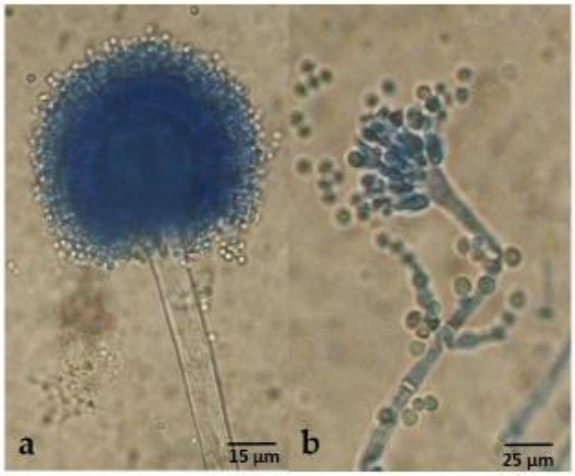

Figure 7. Conidiophores of *A. korhogoensis* MACI254 (400×): (**a**) typical conidiophore, radiate and biseriate, mostly observed in basal mycelium; and (**b**) atypical conidiophore uniseriate, found in aerial mycelium.

Etymology: The specific epithet "*korhogoensis*" is a noun in the genitive case and refers to the Korhogo region located in the Côte d'Ivoire, from where the new species was isolated.

Diagnosis: Colonies on MEA deeply floccose with a dominant white aerial mycelium. Sporulation dull yellowish green. Abundant sclerotia (especially on MEA and CYA), mostly at the colony surface, small size (<400 µm), dark brown at mature state; conspicuous amber exudate produced by sclerotia. Reverse orange to brownish orange, more conspicuous on MS, and on MEA and CYA presence of concentric rings on orange shades. Conidial heads typically radiate, fertile upper 75% of their surface and splitting, less frequent narrow and long columnar to short columnar, rarely micro-heads. Conidiophores of radiate heads are hyaline, long, large and slightly roughened, whereas conidiophores of columnar heads and micro-heads are short, narrow and smooth. Conidial heads biseriate for radiate heads, and uniseriate for the others. Vesicles oblong to spatulate, 25–47 µm in diam; metulae 6.7–11.2 µm X 4–5.5 µm; phialides 7–10 µm X 3–5.7 µm; conidia yellowish green to green, oblong and smooth or slightly rough, 3–5 µm diam.

Colony diameters: After seven days at 25 °C, colonies reached 37–60 mm on MEA, 59–67 mm on MS, 36–57 mm on YES, and 57–80 mm on CYA. Colonies kept seven days at 37 °C on MEA reached 38–57 mm, whereas colonies kept at 42 °C reached 7.5–12 mm.

Physiological studies: All strains analyzed on AFPA showed a bright orange reverse, a sign of aspergillic acid production. The colonies did not sporulate and presented reduced aerial mycelia. On CREA, the strains showed a positive production of organic acids, except for MACI219.

Extrolite production: Aflatoxins B_1, B_2, G_1, G_2, 3-*O*-methylsterigmatocystin, versicolorins A and B, aspergillic acid, α- and β-CPA, 2 oxo-CPA, aflatrem, paspaline, paspalinine, aflavarins, asparasones, aflavinines, leporin B.

Aspergillus korhogoensis sp. nov. exhibited phenotypic characters that place it within the *A. flavus* clade, such as conidial heads typically radiate that split into several columns in green shades. Phenotypically, the new species resembled *A. parvisclerotigenus*. Both species shared several common traits, making difficult to distinguish between them; however, some subtle differences were observed. The new species grew faster on MEA and CYA at 25 °C than *A. parvisclerotigenus*, and the reverse coloration on MEA and MS was orange for *A. korhogoensis* and cream for *A. parvisclerotigenus*. The size of sclerotia was also comparatively smaller in *A. korhogoensis*. On MEA at 42 °C, *A. parvisclerotigenus* grew faster (15.5–20 mm) than *A. korhogoensis*, for which growth was reduced (7.5–12 mm) or inexistent in strain MACI219. On AFPA, *A. parvisclerotigenus* colonies were mildly to highly floccose, produced profuse sclerotia and conidia in yellowish shades, whereas *A. korhogoensis* sp. nov. colonies exhibited sparse aerial mycelium and sclerotia, and conidia were almost non-existent.

Holotype: Isolated from Gbandokaha. Deposited in the NRRL collection.

Isolates examined: MACI254 (NRRL 66710), Côte d'Ivoire, Gbandokaha (9°32′ N, 5°33′ W), from peanut pods, 15 November 2014, A.L. Manizan MACI254. MACI46 (NRRL 66708), Côte d'Ivoire, Korhogo (9°29′ N, 6°49′ W), from peanut seeds, 19 November 2014, A.L. Manizan MACI46. MACI219 (NRRL 66709), Côte d'Ivoire, Pokaha (9°24′ N, 5°30′ W), from peanut pods, 15 November 2014, A.L. Manizan MACI219. MACI264 (NRRL 66711), Côte d'Ivoire, Gbandokaha (9°32′ N, 5°33′ W), from peanut pods, 17 November 2014, A.L. Manizan MACI264.

Habitat: Found on peanuts.

Distribution: Korhogo region, North Côte d'Ivoire.

3. Discussion

The number of species in section *Flavi*, in direct correlation with the number of species capable of producing aflatoxins, has increased over the last decade [17–19,23]. Although unable to produce aflatoxins, a 26th species, *A. hancockii* sp. nov., was very recently identified and grouped with section *Flavi* species [38]. The use of a polyphasic approach to characterize a species, based on the unified species concept [39,40], has enabled mycologists to acknowledge cryptic diversity in *Aspergillus* section *Flavi*. By using this approach, morphological, physiological, and molecular characters are integrated to understand species relationships [12,17–20,23,41]. It is accepted that the use of a single approach will mask the exact relationships among species, not only in Aspergilli, but also in other fungi [12].

In the present study, we included an ensemble of six genomic regions already tested to be informative for section *Flavi* (*ITS*, *benA*, *cmdA*, *mcm7*, *rpb1* and *amdS*). Three other genes (*ppgA*, *preA*, *preB*), reportedly involved in sexual development, were added and the set resulted in a concatenated sequence of 4624 bp. These three genes are required in the specific mating recognition. *PreA* and *preB* are MAT target genes that encode a-pheromone and α-pheromone receptors, respectively [42]. *PpgA* encodes the α-pheromone precursor that binds to PreB [43]. To our knowledge, this is the first study to make a phylogenetic inference that includes genes *PreA*, *PreB* and *PpgA*. Some authors have suggested that the accuracy of the phylogenetic approach could be increased by adding molecular data with different evolutive rates, diminishing possible artifacts caused by polymorphic haplotypes [44,45] and providing more robust information to elucidate potential complexity within a clade. The use of nine concatenated genes resulted in a robust phylogenetic tree topology, which includes the most important species of the section in terms of economic and public health impact, as well as species described in the last decade (not basal taxa). The results of the present study were congruent with several studies performed involving species from section *Flavi* [17–20,23]. The tree obtained from nine concatenated genes showed a clear partition of *A. novoparasiticus* strains in two, one subgroup containing South American isolates and another containing African isolates. More *A. novoparasiticus* strains isolated from both continents would be needed in order to confirm this observation. Additionally, the ensemble allowed to determine a cryptic species, *A. korhogoensis* sp. nov.

Most species in section *Flavi* are considered heterothallic, containing either the *MAT1-1* or *MAT1-2* idiomorph [15,18,46], and from the present study so is *A. korhogoensis*. Thus far, *A. alliaceus* is the only homothallic species in section *Flavi* [46]. Reportedly, strains of *A. nomius* may contain both idiomorphs, but only one is functional [11]. Diversity from sexual reproduction in these fungi is expected to arise from out-crossing of heterothallic species, between complementary strains that are able to produce sclerotia [47,48]. Laboratory crosses between sexually compatible strains showed that sexual reproduction was possible in *A. parasiticus*, *A. flavus*, and *A. nomius* [11,49,50]. Inter-specific hybridization was shown to be a possibility via laboratory crosses that resulted in viable ascospores, including recombinant offspring, being produced [51]. Moreover, sexual reproduction is more likely to occur within populations having a 1:1 ratio of both idiomorphs [11,42,48], although asexual reproduction is still a large component to the life cycle of micro-fungi such as the Aspergilli [52]. Presence of both idiomorphs in *A. parvisclerotigenus*, and *A. korhogoensis* suggests that cryptic sexuality might occur in natural populations, yet laboratory mating experiments involving these species is necessary to yield conclusions. As well, more population-scale field sampling of strains from these species are necessary to determine if they have a history of recombination as observed in *A. flavus* and *A. parasiticus* populations [53,54]. Indeed, the ratio 1:1 is not discernable due to the few strains isolated and curated in different collections.

The present study increases the number of species in the *A. flavus* clade, which is comprised of many heterothallic species that share common morphological characters, such as biseriate heads, greenish to brownish colony coloration, ability to produce sclerotia, among others [17]. Likewise, species in this clade are capable of producing aflatoxins, aspergillic acid, CPA, kojic acid, versicolorins, aflatrem, etc. [20,55]. Within the clade, the main difference between *A. flavus* and the remaining species (*A. minisclerotigenes*, *A. parvisclerotigenus* and *A. korhogoensis* sp. nov.), is that *A. flavus* has lost the ability to produce G-aflatoxins [22]. Another important difference is that *A. flavus* is comprised of two morphotypes: small sclerotium producers and those that are able to produce larger sclerotia than the other three species. *Aspergillus flavus* is a ubiquitous fungus, being readily sampled across the globe, but *A. minisclerotigenes*, *A. parvisclerotigenus*, and *A. korhogoensis* sp. nov. have smaller geographic distributions. For example, *A. minisclerotigenes* has been isolated from Africa, South and North America, Europe, and Australia, whereas *A. parvisclerotigenus* has been isolated from Guinea Gulf [16], and *A. korhogoensis* has only been found in the Côte d'Ivoire.

Herein, we proposed that *Aspergillus korhogoensis* sp. nov. is the sister taxon of *A. parvisclerotigenus*, based on secondary metabolite analyses, morphology and molecular evidence. Both species share

a similar secondary metabolic profile according to Frisvad et al. [9]. However, there are some differences in their secondary metabolite production. Unlike *A. parvisclerotigenus*, the production of A-30461 (aspirochlorin) was not observed in any *A. korhogoensis* extracts. On the other hand, *A. korhogoensis* produced aflavinines, asparasones and leporin B. A pattern close to each other could be appreciated while comparing morphological characters, though subtle differences were observed between strains of both species. On AFPA, *A. parvisclerotigenus* had a trend to produce yellowish spores and highly floccose colonies, whereas *A. korhogoensis* sp. nov. tended to have flatter colonies and reduced sporulation. At the molecular level, the concatenation of nine different loci strongly suggested that they were two different species. The inclusion of strains from different populations of *A. parvisclerotigenus* (Benin, Nigeria and Côte d'Ivoire) reduced the possibility of any artifact linked with polymorphisms.

Sub-Saharan West Africa, and especially the Guinea Gulf, displays an interesting diversity of species from section *Flavi*, including several cryptic species, although *A. flavus* continues to be the most frequent species sampled [56]. Despite the prevalence of *A. flavus*, it is noteworthy that other S-strain species are present at lower rates, are usually G-aflatoxin producers, and their production of aflatoxins is usually higher than that of *A. flavus* sensu stricto [57,58]. Some strains, previously characterized as *A. flavus* S_{BG}, are nowadays being classified as *A. minisclerotigenes*, *A. parvisclerotigenus*, and in this study as *A. korhogoensis*. In different countries of the Guinea Gulf, strains exhibiting the S_{BG} chemotype have been associated with drier agroecological zones bordering the Sahara desert [14,59,60]. Cadwell and Cotty [59] suggested that production of G-aflatoxins in Northern Benin could be mainly due to S_{BG} strains. Likewise, in this area, the environmental conditions could allow the presence of species that could be more sensitive to climate changes, resulting in a shift of the frequency of these strains [59]. Inter-specific sex is possible for these fungi, which has been shown via laboratory crosses [51]. All that is required for these strains to override heterokaryon incompatibility, or even species boundaries, is the need to circumvent an unfavorable environmental situation [61]. It may eventually be determined that many of the recently characterized novel species, with such similar morphological, genetic, metabolic and physiological characteristics, are hybrids resulting from cryptic inter-specific sex that comprise a species complex. However, much more research is required before this can be proven or refuted. Moore and co-workers [62–64] are sequencing the genomes of aflatoxigenic fungi in an effort to determine the relatedness of these fungi and to elucidate the evolution of aflatoxin production. The comparison of the genomes of species close to *A. flavus* such as *A. minisclerotigenes*, *A. parvisclerotigenus* and *A. korhogoensis* will also help to understand the genetic determinants of the *A. flavus* success.

4. Materials and Methods

4.1. Chemicals

Solvents (phenol, chloroform, ethanol, ethyl acetate, methanol, and acetonitrile) of analytical grade used in the extraction and high-performance liquid chromatography (HPLC) were obtained from ThermoFisher Scientific (Illkirch, France). Ultrapure water used for HPLC with Diode Array Detector (DAD), LC/MS analyses, and for molecular biology experiments was purified from a MilliQ purification system (Millipore, Billerica, MA, USA). Unless otherwise specified, chemicals were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

4.2. Fungal Isolates and Culture Conditions

Seven atypical S_{BG} *A. flavus* strains were isolated in 2014 from peanuts in the Northern Korhogo region of Côte d'Ivoire. Four other atypical S_{BG} strains isolated from food, decaying leaves, and logs of wood collected in Southwest Nigeria [65] were added. To identify these eleven S_{BG} strains to species level, we compared them against a dataset comprised of strains obtained from different international collections and stored under controlled conditions at Research Center in Food Toxicology TOXALIM, Toulouse. We included at least one strain belonging to most species within the section *Flavi* (Table 3).

The isolates were cultured on Malt Extract Agar (MEA) (Biokar Diagnostics, Allone, France) at 25 °C for seven days, and stored as spore suspensions on 20% glycerol for further analyses. The *A. korhogoensis* Type strain, along with three other strains, were deposited at Agricultural Research Service Culture Collection (NRRL) (Peoria, IL, USA).

Table 2. *Aspergillus* isolates used in this study.

Strain	Sampling Data		Reference
	Substrate	Country	
A. arachidicola			
CBS 117610^T = IBT 25020	*Arachis glabatra* leaf	Argentina	[20]
CBS 117614 = IBT 27183	*Arachis glabatra* leaf	Argentina	[20]
A. bertholletius			
CCT 7615^T	Soil near *Bertholletia excelsa* trees	Brazil	[19]
A. bombycis			
NRRL 26010^T = CBS 117187	Frass, silkworm rearing house	Japan	[66]
A. caelatus			
NRRL 25528^T = ATCC 201128 = CBS 763.97 = JCM 10151	Peanut field soil	Georgia, USA	Horn B.W., National Peanut Lab, Dawson, GA (in NRRL database)
A. flavus			
NRRL 3518	Wheat flour	Illinois, USA	Graves NRRL isolate (in NRRL database)
NRRL 4818 = CBS 16870	Food, butter	USA	Fennell D.I., University of Wisconsin, Madison, Wisconsin (in NRRL database)
NRRL 3357 = CBS 128202	Peanut cotyledons	USA	[67]
A. minisclerotigenes			
CBS 117635^T	*Arachis hypogaea* seed	Argentina	[20]
NRRL 29000	Peanut soil	Australia	Geiser D., Pennsylvania State University (in [21])
E21	Cumin	Morocco	[15]
E44	White pepper	Morocco	[15]
E74	Paprika	Morocco	[15]
A. mottae			
MUM 10.231^T = CBS 130016	Maize seed	Portugal	[18]
A. nomius			
NRRL 13137^T = CBS 260.88	Wheat	Illinois, USA	Schindler A.F., FDA, Washington D.C. (in NRRL database)
A. novoparasiticus			
CBS 126849^T = LEMI 250	Sputum, leukemic patient	São Paulo, Brazil	[23]
LEMI 149	Hospital air	São Paulo, Brazil	[23]
LEMI 267	Sputum, leukemic patient	São Paulo, Brazil	[23]
AFc31 = NRRL 62794	Cassava	Benin	[14]
AFc32 = NRRL 62795	Cassava	Benin	[14]
A. oryzae			
CBS 100925^T = IMI 16266 = NRRL 447	Unknown source	Japan	[17]
RIB40	Cereal (broad bean)	Kyoto, Japan	[68]
A. parasiticus			
CBS 100926^T	*Pseudococcus calceolariae*, sugar cane mealy bug	Hawaii, USA	[17]
NRRL 492	Unknown source	China	[23]

Table 3. *Aspergillus* isolates used in this study.

Strain	Sampling Data		Reference
	Substrate	Country	
A. parvisclerotigenus			
CBS 121.62^T	*Arachis hypogea*	Nigeria	[9]
AFc36 = NRRL 62796	Cassava	Benin	[14]
MACI8	Peanuts	Côte d'Ivoire	This study
MACI221	Peanuts	Côte d'Ivoire	This study
MACI258	Peanuts	Côte d'Ivoire	This study
SF1	Rain forest soil	Nigeria	[65]
SF3	Rain forest soil	Nigeria	[65]
SF6	Rain forest soil	Nigeria	[65]
SF9	Food item	Nigeria	[65]
A. pseudocaelatus			
CBS 117616^T	*Arachis burkartii* leaf	Argentina	[17]
A. pseudotamarii			
NRRL 443	Soil	Brazil	[69]
NRRL 25518	Tea field soil	Miyazaki, Japan	[70]
A. sergii			
MUM 10.219^T = CBS 130017	Almond shell	Portugal	[18]
A. sojae			
CBS 100928^T	Soy sauce	Japan	[17]
A. transmontanensis			
MUM 10.214^T = CBS 130015	Almond shell	Portugal	[18]
***A. korhogoensis* sp. nov.**			
MACI254^T	Peanuts	Côte d'Ivoire	This study
MACI46	Peanuts	Côte d'Ivoire	This study
MACI219	Peanuts	Côte d'Ivoire	This study
MACI264	Peanuts	Côte d'Ivoire	This study

CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; NRRL: National Center for Agricultural Utilization Research, U.S. Department of Agriculture, Peoria, IL, USA; LEMI: Laboratório Especial de Micologia, São Paulo, Brazil; MUM: Micoteca da Universidade de Minho, Braga, Portugal; CCT: Coleção de Cultura Tropical, Campinas, Brazil; SF: Southern Regional Research Center, U.S. Department of Agriculture, New Orleans, USA.

4.3. DNA Extraction and Amplification

A loopful of spores from each of the examined *Aspergillus* isolates was inoculated on Yeast Extract Sucrose (YES) liquid medium, and kept in agitation in an orbital incubator at 170 rpm at 27 °C for five days. DNA extraction was performed according to Girardin et al. [71] by grinding a portion of mycelium in a 5 mL mortar on ice, followed by the addition of 5.5 mL lysis buffer 2 (5 mL Tris-HCL 1 M, 3.65 g NaCl, 12.5 mL EDTA 0.5 M pH 8, 2.5 g SDS, H_2O qsp 250 mL). The content was transferred to a 15 mL tube, and 12.5 µL of proteinase K were added, before being incubated from 30 min to 1 h at 37 °C, and then incubated for 10 min at 65 °C. Afterwards, one volume of phenol/chloroform (7:3, *v*/*v*) was added and samples were then centrifuged at 3000× *g* for 1 h. The supernatant was recovered into a new tube, where 6 µL RNAse were added, and it was subsequently incubated for 2–3 h at 37 °C. Next, one volume of chloroform was added and centrifuged at 3000× *g* for 10 min. The supernatant was recovered into a new tube and one volume of isopropanol was added. At this point, samples were softly shaken for 2 h in a horizontal shaker and kept overnight at 4 °C. The following day, samples were centrifuged at 10,000× *g* for 30 min. The supernatant was eliminated and the pellet carefully washed with 300 µL of 70% ethanol, and centrifuged at 10,000× *g* for 15 min, followed by a gentle aspiration of the supernatant. Finally, the pellet was resuspended with 30 µL of pure water. DNA of samples was quantified using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

4.4. Amplification and Sequencing of Genomic Loci

Genes were amplified as follows: (1) pre-denaturation at 94 °C for 5 min; (2) denaturation at 94 °C for 45 s; (3) annealing at 55–57.3 °C for 1 min (55 °C = *ITS*, *benA*, *cmdA*, *mcm7*, *preB* and *preA*; 56 °C = *ppgA*; 57.3 °C = *amdS* and *rpb1*); (4) extension at 72 °C for 1 min (Steps 2 to 4 were carried out for 40 cycles); (5) final extension at 72 °C for 10 min; and (6) final temperature hold at 4 °C. Primers used in the study are shown in Table S1. Polymerase Chain Reaction (PCR) amplifications were performed in a C1000 Touch™ thermal cycler (BioRad, Marnes-la-Coquette, France). PCR amplicons were purified with GeneElute™ PCR Clean-Up Kit (Sigma-Aldrich, Saint Quentin Fallavier, France). Sanger sequences were obtained by using the Applied Biosystems Big Dye Terminator v3.1 chemistry (ThermoFisher Scientific, Illkirch, France), they were then purified with the Applied Biosystems Big Dye XTerminator protocol (Thermofisher Scientific, Illkirch, France) and finally processed on the ABI 3130xl Genetic Analyzer (Thermofisher Scientific, Illkirch, France), available on the GeT-Purpan technological facility (Genome and Transcriptome, GenoToul, Toulouse, France). New sequences were deposited in GenBank and accession numbers are reported in Table S2. Sequence data of some isolates, obtained from previously accessioned data in the GenBank database, were included for constructing phylogenetic trees (Table S2).

4.5. Alignment, Model Selection, and Molecular Analyses

Data were assembled, aligned and trimmed in BioEdit/ClustalW (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Gene regions with multiple gaps were aligned to minimize indels and optimize nucleotide identities among different strains. Sequences from multiple genomic regions were concatenated using Mesquite v3.2 [72], but the mating type loci were analyzed independently. For concatenated data, the best-fit nucleotide substitution models and partitioning scheme were chosen using PartitionFinder v2.0.0 [73] under BIC. To search for the best-fit scheme, a greedy algorithm with linked branch lengths of alternative partitions was used. Partitions obtained consisted of four subsets that corresponded to a specific model (noted in parentheses): Subset 1 included *ppgA*, *cmdA*, *benA*, *rpb1* and *mcm7* = 2129 bp (K80+G); Subset 2 included *ITS* = 778 bp (TRNEF+I); Subset 3 included *preB* and *preA* = 1223 bp (HKY+G); and Subset 4 included *amdS* = 491 bp (K80+G).

Bayesian inference statistical methods were used to obtain tree topologies for concatenated data, using the best-fit substitution models listed above. For Bayesian analyses, MrBayes v3.2 [74] was used, and four independent runs were carried out for 10^7 generations, each with four chains, Markov Chain Monte Carlo, and sampling every 10^3 generations. We confirmed for each analysis that the average standard deviation of split frequencies between chains approached values of ≤ 0.01, and the potential scale factor reduction factor (PSRF) to 1. For all the analyses, and from the total number of trees per run, 25% were arbitrarily discarded as "burn-in". The remaining trees were used to calculate posterior probabilities (PP) for each bipartition in a 50% majority-rule consensus tree using Tracer v1.6 [75]. Phylogenetic trees were visualized and edited with FigTree v1.4.2 [76].

4.6. Morphological and Physiological Studies

Morphological and growth analyses were carried on MEA, High Salt MEA (MS) (MEA complemented with NaCl 60 g/L), Czapek agar (CZ) (Oxoid, Dardly, France), Czapek Yeast Autolysate agar (CYA), YES agar. Physiological analyses were carried out on creatine agar (CREA) [77], and *Aspergillus flavus/parasiticus* agar (AFPA) [78]. Strains MACI46 (NRRL 66708), MACI219 (NRRL 66709), MACI254 (NRRL 66710), MACI264 (NRRL 66711), *A. parvisclerotigenus* CBS 121.62 and *A. minisclerotigenes* J117e were used for the analyses on MS, CYA, CZ, YES, and CREA. In addition, four strains belonging to *A. parvisclerotigenus* were included for the analyses on MEA and AFPA: MACI8, MACI258, MACI221, and AFc36.

Cultures on MEA, MS, CZ, CYA, YES, CREA, and AFPA were seeded with three calibrated inoculates of 500 spores, and incubated in the dark at 25 °C for seven and ten days. Macroscopic

characters were observed with a stereomicroscope SZX9—X12-120 (Olympus, Rungis, France). Microscopic characters were observed on MEA at 7 and 10 days using a microscope CX41—X400 and X1000 (Olympus, Rungis, France). In addition, growth analyses were calculated from MEA cultures, which were centrally inoculated with 10^3 spores, and incubated at 25 °C, 37 °C and 42 °C for seven days [17,79,80].

4.7. LC/MS Secondary Metabolic Characterization

4.7.1. Secondary Metabolic Characterization of Whole Fungal Culture

Pre-cultures of strains MACI46 (NRRL 66708), MACI219 (NRRL 66709), MACI254 (NRRL 66710) and MACI264 (NRRL 66711) incubated in the dark at 27 °C for seven days. For metabolite profile characterization, isolates were cultured in four different media: MEA, CYA, YES agar, and Potato Dextrose Agar medium (PDA) (Sigma-Aldrich, Saint Quentin Fallavier, France). For each medium, three biological replicates were inoculated centrally with 10 µL of calibrated spore suspensions (10^5 spores/mL) prepared from seven-day cultures on 7.5 cm Petri dishes. The samples were incubated in the dark at 27 °C for seven days.

To perform extrolite extractions, culture media were macerated and placed in 50 mL sterilized tubes, and thereafter 35 mL of ethyl acetate was added to each sample. Samples were agitated 48 h in an orbital incubator at 170 rpm at room temperature. Ethyl acetate was filtered through a Whatman 1PS phase separator (GE Healthcare Life Sciences, Vélizy-Villacoublay, France) and evaporated at 60 °C until dry. Samples were then dissolved in 400 µL of methanol. To eliminate possible impurities, each sample was filtered through a 0.45 µm disk filter (ThermoFisher Scientific, Illkirch, France) [81].

4.7.2. Secondary Metabolic Characterization of Sclerotia

The isolates were cultured on MEA whereby a loopful of spores was taken from seven-day cultures and streaked onto 9 cm Petri dishes. The MEA samples were incubated in the dark at 27 °C for eight days. To recover sclerotia from culture media, 10 mL of 0.01% Triton-X solution were added to each Petri dish. Sclerotia were gently scraped and transferred into 15 mL tubes. To remove mycelium and conidium debris, 10 mL of 0.01% Triton-X were added to each tube and homogenized in a vortex. Once the sclerotia precipitated, the supernatant was discarded. This step was carried out 4 to 5 times to eliminate possible residual debris [82].

Sclerotia were transferred into a 5 mL mortar. Then 5 mL of ethyl acetate were gently added while grinding the sclerotia. This step was carried out three times. Next, 5 mL of chloroform were gently added and the same procedure followed. This step was repeated three times. Samples were evaporated at 60 °C until dry, and resuspended in 0.5 mL solution methanol/acetonitrile/H_2O qsp (30:30:40, *v*/*v*/*v*) [31,82]. To remove possible impurities, samples were filtered through 0.45 µm disk filters (ThermoFisher Scientific, Illkirch, France).

4.7.3. Secondary Metabolic Analysis

Extrolite analyses were carried out on a HPLC apparatus coupled to an LTQ Orbitrap XL high-resolution mass spectrometer (HRMS) (ThermoFisher Scientific, Illkirch, France). Extracted samples contained 10 µL of each replicate diluted with 170 µL methanol. For the analyses, 10 µL of each sample were injected into a reverse-phase 5 µm Luna C18 column (150 mm × 2.0 mm) (Phenomenex, Torrance, CA, USA) operated at a flow rate of 0.2 mL/min. A gradient program of 0.1% formic acid in water (phase A) and 100% acetonitrile (phase B) was executed as follows: 0 min 20% B, 30 min 50% B, from 35 to 45 min 90% B, from 50 to 60 min 20% B. HRMS acquisitions were obtained by electrospray ionization (ESI) in the positive and negative modes under the subsequent parameters: (1) positive mode: spray voltage + 4.5 kV, capillary temperature 350 °C, sheath gas (N_2) flow rate 40 au (arbitrary units), auxiliary gas (N_2) flow rate 6 au; and (2) negative mode: spray voltage—3.7 kV, capillary temperature 350 °C, sheath gas (N_2) flow rate 30 au, auxiliary gas (N_2) flow rate 10 au.

Full MS spectra were accomplished at a resolution of 60,000 with a mass-to-charge ratio (m/z) range 50–800. The MS/MS spectra were generated by collision-induced dissociation (CID) according the following parameters: collision energy = 35 eV, resolution = 7500, isolation width = 1.5 Da, activation Q = 0.250, and activation time = 30 ms.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6651/9/11/353/s1, Table S1: Primers used to amplify multiple genomic regions within *Aspergillus* species, Table S2: Isolates examined and accession numbers deposited in GenBank.

Acknowledgments: The authors would like to sincerely thank J.C. Frisvad for providing us the strain of *Aspergillus bertholletius* CCT 7615. Aflavarin standard was a generous gift from J. Diana Di Mavungu. The isolation of the four *A. korhogoensis* spp. nov. and three *A. parvisclerotigenus* strains from peanuts in Korhogo region, Côte d'Ivoire is resulting from the EU project 3CIvoire (EuropeAid/129596/L/ACT/CI DCI-NSAPVD/2010/64), and was also supported by the Service of co-operation and cultural action of the French Embassy in Côte d'Ivoire. The views expressed are not necessarily those of the EU. This work was funded by Aflafree (ANR-11-ALID-0003). Doctoral studies of Amaranta Carvajal-Campos were funded by the Secretaría de Educación Superior, Ciencia, Tecnología e Inovación (SENESCYT), Ecuador. We would like to thank H. Gryta for helpful advice on phylogeny.

Author Contributions: Isabelle P. Oswald, Olivier Puel, Sophie Lorber and Catherine Brabet conceived, supervised, and designed the experiments. Amaranta Carvajal-Campos performed the experiments, analyzed the data and contributed to experiment design. Souria Tadrist performed experiments. Ama Lethicia Manizan, David Koffi Akaki and Rose Koffi-Nevry performed the fieldwork in Côte d'Ivoire. Ama Lethicia Manizan isolated the new species strains from the samples of Côte d'Ivoire. Geromy G. Moore and Stephen O. Fapohunda isolated the strains of *A. parvisclerotigenus* from Nigeria. Sylviane Bailly performed morphological analyses. Olivier Puel performed the HPLC-DAD and LC-MS analyzes. Didier Montet coordinated the partnership between Côte d'Ivoire and France. Amaranta Carvajal-Campos, Geromy G. Moore, Sophie Lorber and Olivier Puel wrote the paper.

Conflicts of Interest: The authors declare that they have no competing interest. The funding sponsors had no role in the design of the project; collection, analyses or data interpretation; in writing the manuscript; or in the decision to publish the results.

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Toxins 2017, *9*, 353; doi: 10.3390/toxins9110353

Supplementary Materials: *Aspergillus korhogoensis*, a Novel Aflatoxin Producing Species from the Côte d'Ivoire

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Table S1. Primers used to amplify multiple genomic regions within *Aspergillus* species.

Gene	Length bp*	Primers	Sequence	Reference
ITS	1152	Its1	5′ – GGAAGTAAAGTCGTAACAAGG-3′	[1]
		D2r	5′ – TTGGTCCGTGTTTCAAGACG-3′	[2]
benA	541	Btub2a	5′ – GGTAACCAAATCGGTGCTGCTTTC-3′	[3]
		Btub2b	5′ – ACCCTCAGTGTAGTGACCCTTGGC-3′	[3]
cmdA	543	cmd5	5′ – CCGAGTACAAGGAGGCCTTC-3′	[4]
		cmd6	5′ – CCGATAGAGGTCATAACGTGG-3′	[4]
rpb1	860	FORWARD	5′ – GARTGYCCDGGDCAYTTYGG-3′	This study
		REVERSE	5′ – CCNGCDATNTCRTTRTCCATRTA-3′	This study
mcm7	544	mcmF	5′ – CAATGCCTACACTTGTGATCGC-3′	This study
		mcmR	5′ – CTCCAATGAGCAAAAGAAGCAAG-3′	This study
amdS	566	amdS1	5′ – CCATCGGTATAGGAACTGA-3′	[5]
		amdS2	5′ – AGGGTGCCACGGTATGTC-3′	[5]
Mat1-1	241	M1F	5′ – ATTGCCCATTTGGCCTTGAA-3′	[6]
		MAT1R3*	5′ –ACMGARTARTTGGTMGAAATATCGGCTTC-3′	This study
Mat1-2	645	MAT2F2*	5′ – GAYGCTYTGCGTCACCTYGAG-3′	This study
		M2R	5′ – GCTTCTTTTCGGATGGCTTGCG-3′	[6]
preA	1336	PreAF2	5′ – TGCTSACCATCMCTCCSTTGATCTT-3′	This study
		PreAR3	5′ - GCTNGTNCCTGCCCATGCATTWG-3′	This study
preB	777	PreBF1	5′ – ATCCAGATCTGCATCAACT-3′	This study
		PreBR1	5′ – AGCGGGAGAGAGATAGTGACCAG-3′	This study
ppgA	258	ppgAF	5′ – GCAGCCACCAGTGTACAGGC-3′	This study
		ppgAR	5′ – CCATAGCATCCGCAAGGGCATC-3′	This study

* Amplicon size corresponds to *A. flavus* NRRL3357 sequences except for *Mat1-2*. For *Mat1-2*, amplicon size corresponds to *A. bombycis* sequence.

Toxins 2017, *9*, 353; doi: 10.3390/toxins9110353

Table S2: Isolates examined and accession numbers deposited in GenBank. In bold sequences recovered from GenBank; normal sequences obtain in this study. ᵀ = type strain, * = strains of which the whole genome is sequenced; ** = also found under accession number JX456214; / = gene not present.

Table continues in following pages

SPECIES AND ISOLATE NUMBER	ACCESSION NUMBER										
	ITS	*benA*	*cmdA*	*mcm7*	*amdS*	*rpb1*	*preB*	*ppgA*	*preA*	*Mat1-1*	*Mat1-2*
A. arachidicola											
CBS 117610ᵀ	MF668184	EF203158	EF202049	MF427568	GU203491	MF448475	MF427533	MF427635	MF427600	MF966969	/
CBS 117614	KY937923	KY924665	KY924677	MF427569	MF427670	MF448476	MF427534	MF427636	MF427601	/	MF448470
A. bertholletius											
CCT 7615ᵀ	KY937924	KY924666	KY924678	MF427570	MF427671	MF448477	MF427535	MF427637	MF427602	MF448449	/
A. bombycis											
NRRL 26010ᵀ	AF104444	AY017547	AY017594	JQ690064	MF427672	MF448478	OGM45283	OGM40854	OGM42599	/	OGM45987
A. caelatus											
NRRL 25528ᵀ	AF004930	EF661470	EF661522	JQ690063	MF427673	MF448479	MF427536	MF427638	MF427603	MF448450	/
A. flavus											
NRRL 3518	EF661552	EF661487	EF661510	MF427572	MF427675	MF448481	MF427537	MF427640	MF427605	/	MF448471
NRRL 4818	EF661563	EF661489	EF661510	MF427571	MF427674	MF448480	MF427538	MF427639	MF427604	MF448451	/
NRRL 3357*	MF966967	M38265	EED55330	EED52746	EED48415	EED56055	EED51811	EED56518	EED51899	EED46656	/
A. minisclerotigenes											
CBS 117635ᵀ	KY937925	KY924667	KY924679	MF427573	MF427677	MF448483	MF427539	MF427642	MF427607	MF448453	/
E21	KY937926	JX456195	JX456196	MF427574	MF427678	MF448484	MF427540	MF427643	MF427608	JX456194	/
E44	KY937927	JX456210	JX456214	MF427575**	MF427679	MF448485	MF427541	MF427644	MF427609	/	JX456216
E74	KY937928	JX456211	JX456212	MF427576	MF427680	MF448486	MF427542	MF427645	MF427610	/	JX456213
NRRL 29000	KY937929	KY924668	KY924680	MF427577	MF427676	MF448482	MF427543	MF427641	MF427606	MF448452	/
A. mottae											
MUM 10.231ᵀ	JF412768	HM803086	HM803015	HM803059	MF427681	MF448487	MF427544	MF427646	MF427611	HM803042	/

Toxins 2017, *9*, 353; doi: 10.3390/toxins9110353

SPECIES AND ISOLATE NUMBER	ACCESSION NUMBER										
	ITS	*benA*	*cmdA*	*mcm7*	*amdS*	*rpb1*	*preB*	*ppgA*	*preA*	*Mat1-1*	*Mat1-2*
A. novoparasiticus											
CBS 126849^T	KY937930	KY924673	KY924685	MF427583	GU203478	MF448490	MF427549	MF427649	MF427614	MF448456	/
AFc31	KC964099	KY924669	KY924681	MF427579	KC921994	MF448488	MF427545	MF427647	MF427612	MF448454	/
AFc32	KC964100	KY924670	KY924682	MF427580	KC921995	MF448489	MF427546	MF427648	MF427613	MF448455	/
LEMI 149	KY937931	KY924671	KY924683	MF427581	MF427682	MF448491	MF427547	MF427650	MF427615	MF448457	/
LEMI 267	KY937932	KY924672	KY924684	MF427582	GU203480	MF448492	MF427548	MF427651	MF427616	MF448458	/
A. oryzae											
CBS 100925^T	MF668185	EF203138	EF202055	MF427584	MF427683	MF448493	MF427550	MF427652	MF427617	MF448459	/
RIB40*	AP007173	BAE64122	XP_001820302	BAE65095	D10492	XP_001819667	BAE62296	AP007155	XP_001823505	BAE63328	/
A. parasiticus											
CBS 100926^T	KY937933	EF203155	EF202043	MF427585	GU203493	MF448494	MF427551	MF427653	MF427618	/	MF448472
NRRL 492	KY937934	KY924674	KY924686	MF427586	GU203494	MF448495	MF427552	MF427654	MF427619		MF495344
A. parvisclerotigenus											
CBS 121.62^T	MF668183	EF203130	EF202077	MF427587	MF427684	MF448496	MF427553	MF427655	MF427620	MF448460	/
AFc36	KC964102	KC954604	KC954606	MF427588	MF427685	MF448497	MF427554	MF427656	MF427621	MF448461	/
MACI8	KY689163	KY628794	KY661270	MF427589	MF427686	MF448498	MF427555	MF427657	MF427622	MF448462	/
MACI221	KY689198	KY628787	KY661291	MF427590	MF427687	MF448499	MF427556	MF427658	MF427623	MF495342	/
MACI258	KY689205	KY628789	KY661293	MF427591	MF427688	MF448500	MF427557	MF427659	MF427624	MF495343	/
SF1	MF668179	MF521634	MF521638	MF521646	MF521642	MF521662	MF521658	MF521650	MF521654	MF537434	/
SF3	MF668180	MF521635	MF521639	MF521647	MF521643	MF521663	MF521659	MF521651	MF521655	MF537435	/
SF6	MF668181	MF521636	MF521640	MF521648	MF521644	MF521664	MF521660	MF521652	MF521656	MF537436	/
SF9	MF668182	MF521637	MF521641	MF521649	MF521645	MF521665	MF521661	MF521653	MF521657	MF537437	/
A. pseudocaelatus											
CBS 117616^T	KY937935	EF203128	EF202037	MF427592	MF427689	MF448501	MF427558	MF427660	MF427625	MF448463	/
A. pseudotamarii											
NRRL 443	AF004931	EF661476	EF661520	MF427593	MF427690	MF448502	MF427559	MF427661	MF427626	MF448464	/

Toxins 2017, *9*, 353; doi: 10.3390/toxins9110353

SPECIES AND ISOLATE NUMBER	ACCESSION NUMBER										
	ITS	*benA*	*cmdA*	*mcm7*	*amdS*	*rpb1*	*preB*	*ppgA*	*preA*	*Mat1-1*	*Mat1-2*
NRRL 25518	KY937937	KY924675	KY924687	MF427594	MF427691	MF448503	MF427560	MF427662	MF427627	MF448465	/
A. sergii											
MUM 10.219^T	KY937936	**HM803082**	**HQ340097**	**HM803071**	MF427692	MF448504	MF427561	MF427663	MF427628	/	**HM802967**
A. sojae											
CBS 100928^T	MF668186	**KJ175494**	**KJ175550**	MF427595	**GU203490**	MF448505	MF427562	MF427664	MF427629	MF537438	/
A. transmontanensis											
MUM 10.214^T	**JF412771**	**HM803101**	**HM803020**	**HM803065**	MF427693	MF448506	MF427563	MF427665	MF427630	**HM803050**	/
A. korhogoensis spp. nov.											
MACI46	KY689207	KY628790	KY661265	MF427596	MF427694	MF448507	MF427564	MF427666	MF427631	MF448466	/
MACI219	KY689208	KY628791	KY661266	MF427597	MF427695	MF448508	MF427565	MF427667	MF427632	/	MF448474
MACI254^T	KY689209	KY628792	KY661267	MF427598	MF427696	MF448509	MF427566	MF427668	MF427633	MF448467	/
MACI264	KY689210	KY628793	KY661268	MF427599	MF427697	MF448510	MF427567	MF427669	MF427634	MF448468	/

CBS, Centraalbureau voor Schimmelcultures, Utrech, The Netherlands; NRRL: National Center for Agricultural Utilization Research, U.S. Department of Agriculture, Peoria, IL, USA; LEMI: Laboratório Especial de Micologia, São Paulo, Brazil; MUM: Micoteca da Universidade de Minho, Braga, Portugal; CCT: Coleção de Cultura Tropical, Campinas, Brazil. SF: Southern Regional Research Center, U.S. Department of Agriculture, New Orleans, USA.

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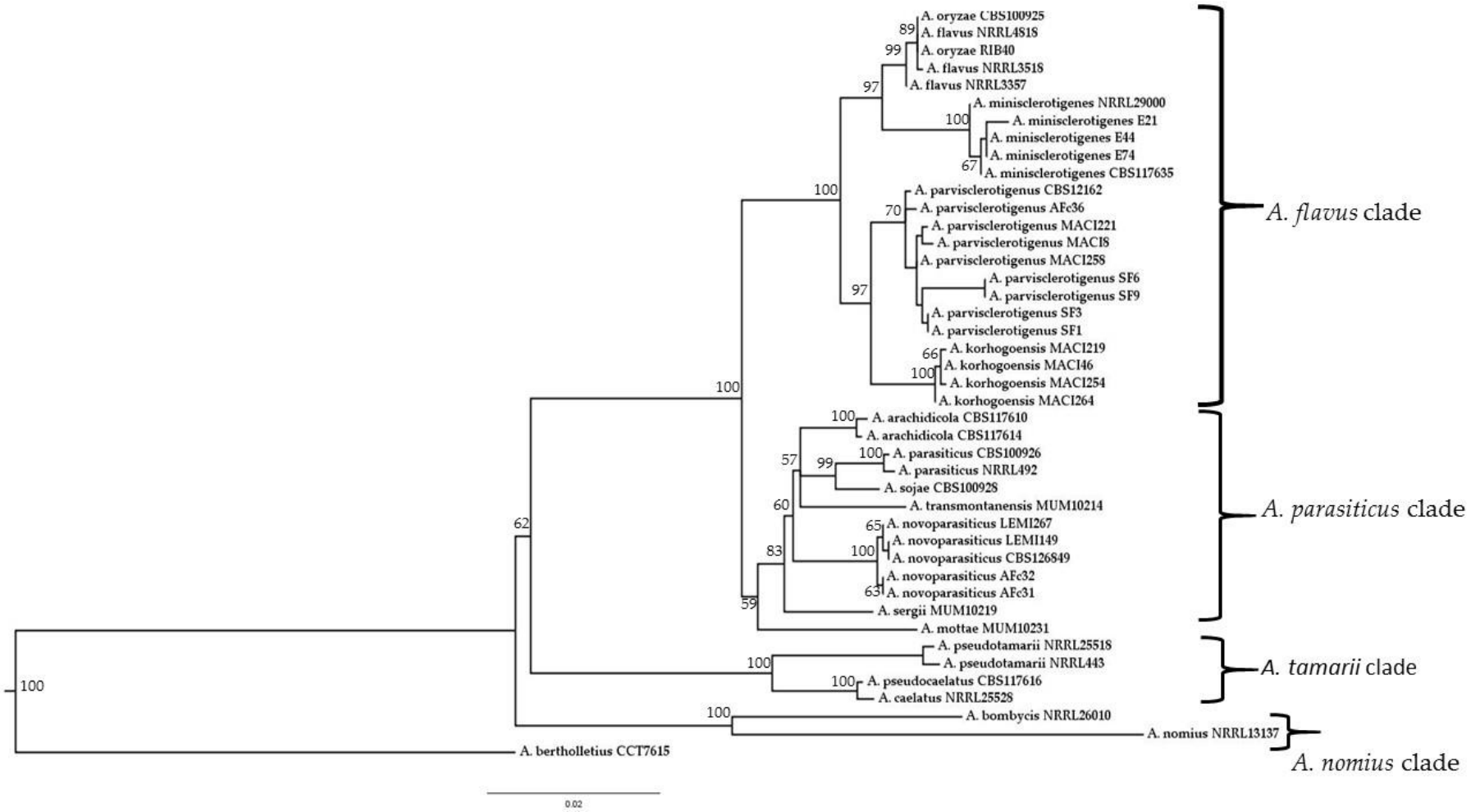

Annex 1**.** Maximum likelihood phylogenetic tree of *Aspergillus* section *Flavi* (based on concatenated sequences from nine genomic loci: *ITS, benA, cmdA, mcm7, amdS, rpb1, preB, ppgA*, and *preA*). Maximum likelihood tree was calculated from 41 strains, and includes the Type strain for most species. Strong bootstrap values are shown at branch nodes. Species isolate numbers are indicated at branch tips. *A. bertholletius* CCT 7615 was used as the outgroup taxon.

2.4 CHAPITRE 4

Identification of *Aspergillus* section *Flavi* in French maize

4.1 BACKGROUND

The present study is part of a preliminary survey on French maize in 2015. The interest of the survey was to evaluate the risk of aflatoxin production on maize kernels and to identify the species that synthesize these compounds. The study consisted in testing the mycobiota in 19 maize samples contaminated with AFB and 24 none contaminated. The mycobiota was characterized based on a polyphasic approach. Isolation, morphological test and analyses of aflatoxin potential production (HPLC MS/MS) were performed by our colleagues from the Ecole Nationale Vétérinaire de Toulouse (France) (Sylviane and Jean-Denis Bailly). Here, phylogenetic analyses of selected strains to confirm the species identification are shown.

Climatic conditions in Southern France were atypical that year, with warmer than normal temperatures and a drier summer. Maize was analyzed because under these climatic conditions kernels were prone to the development of *Aspergillus* section *Flavi* species.

The results showed that all samples had *Fusarium* in their growing mycobiota. Regardless *Aspergillus* section *Flavi* diversity, mycological analyses resulted in the isolation of 67 strains from the section, recovered from samples contaminated and no contaminated with AF. The most frequent species was *A. flavus*, followed by *A. parasiticus*, and *A. tamarii*. To our knowledge, this is the first time that *A. tamari* is reported on maize in France. These results showed that *Aspergillus* section *Flavi* may be part of the soil mycobiota in France, and that two species, *A. flavus* and *A. parasiticus* represent a potential risk, the latter being able to produce B and G aflatoxins.

4.2 IDENTIFICATION OF *ASPERGILLUS* SECTION *FLAVI* IN FRENCH MAIZE

➢ Climate change

Climate change threatens food availability worldwide, alters primary agricultural systems, and therefore, affecting livestock and plant production (Van der Fels-Klerx et al. 2016). Climate change would have negative effects worldwide, though in some regions it could favor the development of some crops. Abiotic factors that have the greatest influence on crop changes are temperature, precipitation patterns and CO_2 availability, which change due to climate change (Paterson and Lima 2010; Medina et al. 2017). Overall, projections suggest that annual precipitations will increase in high latitudes and tropical regions, whereas in mid-latitudes conditions will become extreme, arid regions will become drier and wet regions wetter. Worldwide, extreme events will become more frequent, more intense and longer (Qin et al. 2013). According to some projections, atmospheric CO_2 concentrations will double or triple in the next 25 to 50 years (increasing to 800 - 1200 ppb), temperatures will rise in 2-5° C range, and extreme rain/drought conditions are expected in parts of Europe, Asia, and Central and South America. In these regions, several crops, such as wheat, maize and soya, are produced (Medina et al. 2017).

Temperature, precipitation patterns and CO_2 are important for agriculture, because they are key factors in plant development. Actually, changes in these factors are altering plant distribution patterns in the world and the associated pathosystems, because they influence host–pathogen dynamics, including mycotoxin distribution patterns (Paterson and Lima 2011; Battilani et al. 2016, Van der Fels-Klerx et al. 2016). In addition, some pathogens can be favored due to plant stress conditions, becoming more frequent, and the development of some pathogens can also be favored by climatic changes, increasing the number of their populations (Paterson and Lima 2010; Ehrlich 2014; Van der Fels-Klerx et al. 2016). In general, there is a tendency of species to migrate towards the poles, some pests are suggested to migrate at a rate of 3-5 km/year (Medina et al. 2017). Abiotic and biotic pressures on plants can create new niches or/and create new conditions for the production of certain mycotoxins (Van der Fels-Klerx et al. 2016; Medina et al. 2017).

➢ Europe, climate change effects on crops and mycotoxins

Climate change in Europe may have different effects depending on the region; the positive or negative effects will depend mainly on increased temperature, the precipitation pattern, and physiological response of crops enriched with CO_2. As consequence, crops, grazing livestock and plant pests may change. The biogeographic agricultural scenario for Europe suggests that crop production and arable areas will expand northwards, making northern Europe more suitable for agricultural

production, while southern regions may experience a reduction of production and a decrease of arable areas. Maize, sunflowers, and soybean are crops expected to follow these patterns (Miraglia et al. 2009, Medina et al. 2017).

In northern Europe (Norway, Sweden, Finland and Baltic States) temperatures are expected to increase around 3–4.5 °C, resulting in mild temperatures, increase of rainfall, and higher risk of floods. Agriculture could benefit from these changes by increasing crop production (European Commission, 2007; Miraglia et al. 2009). In southern and south-Eastern Europe (Portugal, Spain, Southern France, Italy, Slovenia, Greece, Malta, Cyprus, Bulgaria, and Southern Romania), the projections suggest an increase in average annual temperature around 4–5 °C, a reduction of precipitations, especially in summer, a decrease in water availability, and an increase of CO_2. These phenomena will lead to a decrease in agricultural production in the range of 10–30% in many regions, drought, heat waves, soil and ecosystem degradation, and ultimately desertification. In central Europe (Poland, Czech Republic, Slovakia, Hungary, Northern Romania, Southern and Eastern Germany, and Eastern Austria) annual temperatures will rise by 3–4.5° C, rainfall will increase in winter and decrease in summer, increasing the risk of flooding. Impacts on agriculture include soil erosion, drought and higher temperatures in summer. In addition, in southern and central Europe, the changes of abiotic factors will affect the development of pathogens and insects, causing earlier flowering and ripening of cereals (Schröter et al. 2005; European Commission 2007; Miraglia et al. 2009; Medina et al. 2017).

➢ Aflatoxins in Europe

In Europe, the main mycotoxins are produced by *Fusarium* spp., being *F. graminearum* one of the main contaminating fungi. However, since 2003 *A. flavus* has been recognized as an emerging problem (Piva et al. 2006). The increasing risk of AF contamination in Europe is due to the effects of climate change. Battilani et al. (2016), based on a study in maize, suggested that *Aspergillus flavus* can increase its home range in Europe in temperature rise scenarios of 2-5 °C, and that the most threatened areas are southern and central Europe.

Although, species of *Aspergillus* section *Flavi* are not common in Europe, there are reports of some species, which present a risk because they are aflatoxins producers. The species most frequently reported is *A. flavus*, and staples contaminated with AFs included maize, nuts, barley and milk (Perrone et al. 2014; Battilani et al. 2016; Giorni et al. 2016; Prencipe et al. 2018). Another species that have been reported in Europe in isolated cases was *A. parasiticus,* which were identified in chestnuts (Prencipe et al. 2018), other rare species, reported only in Europe, are *A. sergii, A.*

transmontanensis, and *A. mottae,* found in Portugal in restricted niches (Soares et al. 2012). The non-aflatoxigenic species *A. tamarii* has also been reported (Prencipe et al. 2018).

Outbreaks of AFs in Europe have shown that it is important to perform analyses targeting AF contamination in the region. In fact, a major survey conducted by the European Food Safety Authority [EFSA] (2007) revealed that aflatoxins were an emerging problem, as contaminated corn, almonds, pistachios were identified, and *A. flavus* was the main producer. Contamination of milk with AFM1 has also been reported in Europe. In 2006 in Sweden, cattle feed with contaminated rice produced milk with AFM1 concentration that exceeded the EU legal limit of 0.05 µg/kg, therefore thousands of tons of milk were discarded; unfortunately, this was not an isolated event (Perrone et al. 2014).

➢ Maize and aflatoxins

Maize is an important crop in Europe and is used for different purposes, like grains for food, feed and processing, and green maize for silage or biogas production. It is one of the region main cereals and is grown in at least 27 countries (Figure 1) (Battilani et al. 2012). France is the leading producer of grain maize and the third largest corn silage producer in Europe. Grain maize is the second largest crop after wheat, covering about 6% of the agricultural area. Maize is mainly grown in two regions with different climatic conditions: the northeast (Grand-Est) and the south-west (Nouvelle Aquitaine, Occitanie) (Caubel et al. 2018).

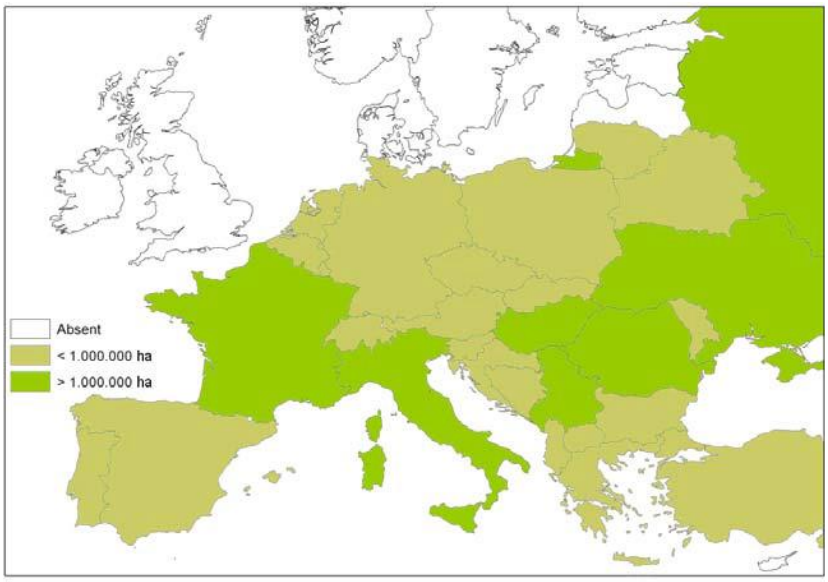

Figure 1. European distribution of maize crops (reprinted from Battilani et al. 2012).

The main fungi that attack maize in the region are *Fusarium* species; therefore, their mycotoxins are a more frequent. Dispersion of *Fusarium* benefits from intense rainfall during the anthesis period, to disperse in maize ears, and from the prolonged period of warm humid conditions to infect the kernels. However, in recent years, the occurrence of species belonging to *Flavi* section, in particular *A. flavus*, has become more frequent (Battilani et al. 2012). Contamination by *A. flavus* is enhanced by plant stress, insect damage, especially the European corn borer *Ostrinia nubilalis,* and warmer and drier conditions (Van der Fels-Klerx et al. 2016). Although *Aspergillus* section *Flavi* is not frequent in the area, a survey in northern Italy on 2003 showed that out of 110 maize samples, 75% was positive for AFBs with an average of 4.4 and a maximum of 154.5 µg/kg (Piva et al. 2006). Under the changing climatic conditions, species belonging to *Flavi* section could become prevalent (Bunyavanich et al., 2003), especially in Romania and Italy (Battilani et al. 2012).

Several studies have tested the possible effects of climate change on maize production, particularly those associated with plant performance under patterns of seasonal drought-stress conditions. As a result, maize sensitive traits, including phenology (maturity), anthesis-silking synchrony, kernels per ear and ears per plant will be altered (Harrison et al. 2014). The same conditions of drought and high temperatures during kernel development are also the suitable conditions for fungi colonization and aflatoxin production (Bruns 2003). *A. flavus* colonization of kernels starts after silking (female flowering) and continues during the season, and it is only observed when kernels are mature (Giorni et al. 2016).

4.2 MATERIALS AND METHODS

➢ FUNGAL STRAINS

Twenty-two strains belonging to *Aspergillus* section *Flavi,* which did not displayed the *A. flavus* characteristic morphological traits, were selected for performing phylogenetic analyses. All samples were previously identified morphologically and their ability to synthesize aflatoxins and cyclopiazonic acid was analyzed by HPLC (Table 1). Of these samples, three strains were of particular interest because they showed interesting morphological traits: G641b, G649b and G644a (Figure 1). The first two were characterized as *A. parasiticus* with a profuse sclerotia production, and the latter as *A. flavus* with special sclerotia (Figure 2).

Table 1. Strains selected for phylogenetic analyses.

STRAINS	TOXINOGENIC POTENTIAL			MORPHOLOGICAL IDENTIFICATION
	AFB	AFG	CPA	
G632b	+	+	–	*A. parasiticus*
G632c	–	–	–	*A. parasiticus*
G638b	+	+	–	*A. parasiticus*
G639	+	+	–	*A. parasiticus*
G640a	+	+	–	*A. parasiticus*
G641b	–	–	–	Section *Flavi*
G644a	–	–	–	*A. flavus*
G644b	+	+	–	*A. parasiticus*
G648b	+	+	–	*A. parasiticus*
G649a	+	+	–	*A. parasiticus*
G649b	+	+	–	Section *Flavi*
G650d	+	+	–	*A. parasiticus*
G651a	+	+	–	*A. parasiticus*
G651c	–	–	+	*A. tamarii*
G651e	–	–	–	*A. parasiticus*
G652b	+	+	–	*A. parasiticus*
G622Rc	+	+	–	*A. parasiticus*
G622Rd	+	+	–	*A. parasiticus*
G628Ra	+	+	–	*A. parasiticus*
G629Ra	-	-	–	*A. parasiticus*
G643Rb	+	+	–	*A. parasiticus*
G647Rc	+	+	–	*A. parasiticus*

Figure 2. Non identified strains by morphological exam. Cultures grown on MEA at 25° C for 7 days (Photos: S. Bailly).

Figure 3. Sclerotia of weird strains. Sclerotia isolated from 7 days-old cultures of at 25° C (Photos: S. Bailly).

➢ MOLECULAR ANALYSES

Molecular analyses were performed on the 22 strains following the processes previously explained: i) cultures and DNA extraction, ii) amplicon amplification by PCR of *ITS* 4-5*, benA* and *cmdA* genes, iii) annealing, trimming and alignment of sequences, and iv) phylogenetic analyses by ML and BI (see chapters 2.1-2.3).

4.3 RESULTS

For the *ITS* gene, the best-fit nucleotide substitution model was TIM2+I+G (jModelTest), whereas for the concatenated data *benA + cmdA* a partition was obtained, and the best substitution model was K80+G (PartitionFinder).

The results from *ITS* 4-5 showed several polytomies, the *A. flavus* clade was nested in the *A. parasiticus* clade. Resolution of *A. nomius* and *A. tamarii* clades was low. For the maize strains, two (G529Ra and G644a) were nested in "*A. flavus* clade", 19 strains (G638b, G622Rc, G622Rd, G628Ra,

G632b, G632c, G639, G640a, G641b, G643Rb, G644, G647Rc, G648b, G649a, G649b, G650d, G651a, G651b, G651e) in *A. parasiticus* clade, and one strain (G651c) was nested as basal taxon of a group including *A. tamarii* and *A. nomius* strains (Figure 4).

On the other hand, the results from the concatenated genes *benA*+*cmdA* showed a better representation of the clades, the *A. flavus* clade species clustered together (with the exception of *A. korhogoensis* that formed a new branch, as in previous analyses). Members of the *A. parasiticus* clade were clustered together; *A. mottae* was settled as basal taxon of the former clusters. *A. nomius* and *A. tamarii* clusters contained the species expected. For the samples recovered from French maize, we observed that G529Ra and G644a clustered with *A. flavus* and *A. oryzae* (PP=1). The sequences belonging to the 19 samples G638b, G622Rc, G622Rd, G628Ra, G632b, G632c, G639, G640a, G641b, G643Rb, G644, G647Rc, G648b, G649a, G649b, G650d, G651a, G651b, G651e) were grouped with *A. parasiticus* and *A. sojae* (PP=89). Finally, G651c clustered with *A. tamarii* (PP=1), both sequences forming the sister taxon of *A. pseudotamarii* (Figure 5).

Hence, based on phylogenetic inference, three species of *A.* section *Flavi* were identified, *A. flavus, A. parasiticus* and *A. tamarii.* The atypical strains G644a and G649b were confirmed as *A. parasiticus* and G641b was confirmed as *A. flavus.*

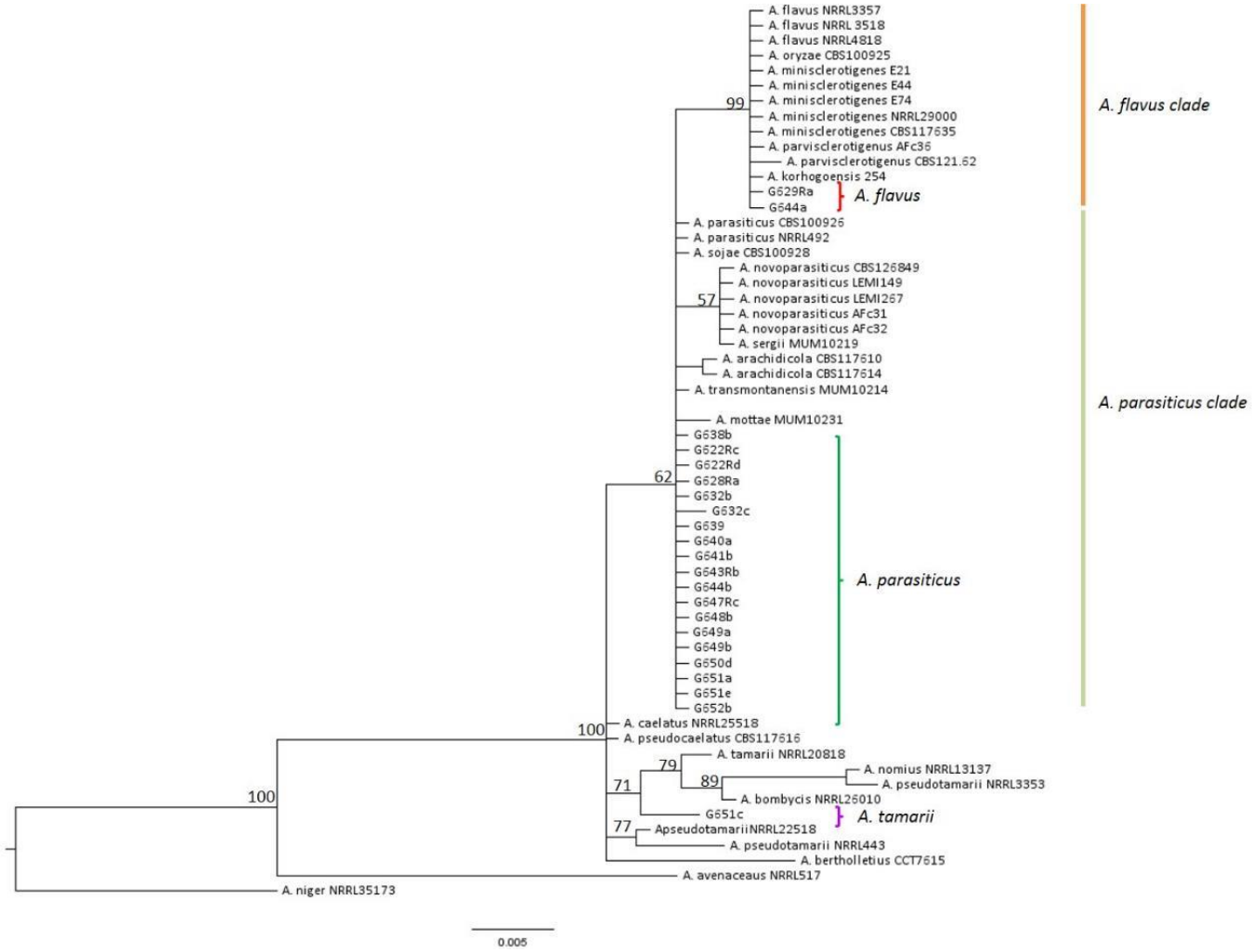

Figure 4. *ITS* 4-5 BI tree. The tree included 58 isolates: 22 sequences from French maize samples and 36 recovered from GenBank (including the reference strain for most species in the section). PP values are shown. Strains for this study are indicated by brackets, clades are indicated with bars (in red shades: *A. flavus*; in green shades: *A. parasiticus* and in purple shades: *A. tamarii*).

Figure 5. *BenA-cmdA* BI tree. The tree included 67 isolates: 22 sequences from French maize samples, and 36 recovered from GenBank (including the reference strain for most species in the section). PP values are shown. Strains for this study are indicated by brackets, clades are indicated with bars (in red shades: *A. flavus*; in green shades: *A. parasiticus* and in purple shades: *A. tamarii*).

4.4 DISCUSSION

The results confirmed the presence of *A.* section *Flavi* species in French maize. In this study, three species were isolated, *A. flavus, A. parasiticus* and the non-aflatoxigenic species *A. tamarii. Aspergillus flavus* represented the 69% of the 67 strains isolated from section *Flavi.* From the 67 strains, 22 produced important quantities of AF, and in 8 non aflatoxigenic strains traces of CPA were observed. The weird isolate G641b with pigmented sclerotia was confirmed as *A. flavus* (PP=1, *benA+cmdA*). The presence of *A. parasiticus*, especially in post-harvest samples, suggests that this species is important in soil in France; and as expected, the majority of the strains were aflatoxigenic. Eventhough, samples G644a and G649b were atypical, molecular result showed that they are clustered with strains of *A. parasiticus* (PP=0.89, *benA+cmdA*).

Aspergillus tamarii has a wide distribution in tropical and subtropical areas. It is frequently isolated in nuts, such as betel nuts (Misra and Misra 1981), peanuts (Martins et al. 2017), pecans, and in a wide variety of sources like spices, peppercorns, cocoa and yams (Pitt and Hocking 1985; 2009). Although, its isolation in cereals is less frequently reported, most reports involve maize. In fact, several publications have reported *A. tamarii* in harvested maize in different African countries (Perrone et al. 2014; Probst et al. 2014). The occurrence of *A. tamarii* in Europe has been rarely reported and the majority of these reports are recent. In a Portuguese study, 8.2% of *Aspergillus* section *Flavi* isolates from almonds harvested in Morocco and Faro regions were identified as *A. tamarii* (Rodrigues et al. 2012). Another report concerns the presence of *A. tamarii* in “vinho verde” (Lago et al. 2014). In Italy, this species was reported in chestnuts mycoflora (Prencipe et al. 2018). To our knowledge, this is the first time that the presence of *A. tamarii* in European maize is reported.

Studies of climate change scenarios for maize in France suggest that maize is likely to remain an important crop. Nevertheless, climatic change challenges the region by the increasing temperatures and the risk of water stress. In fact, Occitanie and Ile-de-France regions should be sensitive to water stress during grain development; especially Occitanie, where rainfall is expected to decrease. It is important to understand the effects of abiotic factors in maize and the risks for its production. Abiotic factors change phenology, vegetative and reproductive growth, grain quality (sugar or protein content) and the performance of cultural practices, which can lead to the development of maize grain under drier and warmer conditions (Olesen et al. 2011; Caudal et al. 2018). These plants changes can make maize more vulnerable to contamination of *A. Flavi* section species. Actually, climate change scenarios in France suggest than maize will be prone to the colonization of *A. Flavi* section species, resulting in reduce safe maize production and economical losses. Therefore, the major challenge in France is to reduce drought stress, which is an important factor to reduce *A. flavus* colonization and AF production (Battilani et al. 2012)

Biocontrol is used to inhibit the growth of fungi and AF production. For instance, the use of maize cultivars more resistant to water stress is a strategy to limit the impact of water deficit on maize production and quality (Caudal et al. 2018). Similarly, the use of non-aflatoxigenic strains of *A. flavus* can also reduce the increasing risk of aflatoxigenic strains in fields. The isolation of “safe” strains during survey studies contributes to the search for French strains of *A. flavus* that could be used as biocontrol. The use of strains from the same region increases the reliability of the biocontrol (Battilani et al. 2012; Bandyopadhyay et al. 2016).

03

GENERAL DISCUSSION PERSPECTIVES AND CONCLUSION

3.1 GENERAL DISCUSSION

➢ A proper identification of species: why is it crucial?

A proper characterization of species from *Aspergillus* section *Flavi,* which are potentially contaminants of staples, is necessary to ensure consumers safety (RASFF 2011; Prencipe et al. 2018). A proper identification of species prevents food and feed contamination, as life histories and ecological features of species are better addressed, improving risk assessment and reducing the potential risk of these species on human and animal health. This is particularly crucial for potential AF producer species, and for other important mycotoxin producers, though characterizing species and the strains occurring in different worldwide areas is also important (Vaamonde et al. 2003). Some species appear to have reduced home ranges and niches that are closely related to their host, such as *A. sergii, A. mottae, A. transmontanensis,* which have only been isolated in Portugal and are linked to specific substrates (Soares et al. 2012), or like *A. parvisclerotigenus* and *A. korhogoensis* that have been found in the Guinean Gulf (Adjovi et al. 2014; Chapter 2.2). A proper identification of strains may show that the home ranges are wider or even identify more species.

Species characterization of molds, especially *Flavi* section, is not straightforward because the section present intra- and inter- variability, which results in phenotypic and physiologic overlapping traits. Additionally, some studies of species characterization include only one approach, leading to poor classification at species level. Several species have been misidentified, some of which were subsequently recognized as novel species, such as *A. minisclerotigenes* and *A. parvisclerotigenus* considered to be part of *A. flavus;* or *A. pseudonomius* considered to be *A. nomius,* which is the main aflatoxigenic species in Brazilian nuts (Pildalin et al. 2008; Varga et al. 2011; Massi et al. 2014), and the possible new species from *A. parasiticus* that grows in sugar cane crops (Kumeda et al. 2003; Garber et al. 2014). Likewise, there are studies where G-aflatoxins were identified, yet the species were not characterized, *e.g.* Matumba et al. (2014) found high levels of G-aflatoxins in Malawi peanuts, but they did not identify the producer.

Species description in *Flavi* section is still under debate, *A. flavus*, *A. parasiticus* and *A. nomius* species are defined differently depending on the research team. Some authors (Cotty and collaborators) consider that phenotypic, molecular and physiology traits and in the production of secondary metabolites of these species are highly variable. Some others (Frisvad, Samson, Ehrlich and collaborators) consider these species as species complexes and that cryptic species embedded in them can be identify by a polyphasic approach. The latest statement is in agreement with the unified species concept and the phylogenetic concept. The recognition of these cryptic species is becoming each day increasingly accepted by researchers in the field because these new cryptic species can be considered as lineages that have evolved separately from other lineages, which has been proven by

phenetic and physiological data, secondary metabolic compounds, phylogenetic and molecular evidence, as well as some insights of ecology and life histories. I agree with the statement that *A. flavus, A. parasiticus* and *A. nomius* are species complexes and that most species described over the last decade are cryptic species.

The plethora of mycotoxins synthesized by fungi belonging to *Aspergillus* section *Flavi*, includes AFs, VER A and B, STC, OMST, CPA, OTA, aflatrems, TeA, amongst others (Varga et al. 2011; 2015). It is important to take into account that mycotoxins yield depend on inter- and intra- species variability. To recognize species, and the specific isolates in specific geographical areas, is thus important to have an overview of the secondary metabolites yield in those areas and to assess the potential health risks (FAO 2003; IARC 2015). Generally, a mixture of secondary metabolites is yield by a strain, resulting in staples contaminated by several mycotoxins. As aforementioned, a mixture of mycotoxins leads to antagonist, additive or synergic effects, depending on the mycotoxin structure, as well as the amount of each compound, the host (species, sex, age, health, diet), and the intake pathway (Paterson and Lima 2010; IARC 2015).

A good example of the importance and applicability of proper recognition of strains is the use of “non-toxigenic” strains to reduce the impact of “toxigenic-strains”. This method is based on the importance of microbiota diversity and the competition between strains, in order to reduce the prevalence and effects of undesirable strains. In fact, this method seems to have excellent results, and its use is widespread in the USA, Africa and Eastern Europe (IARC 2015; Bandyopadhyay et al. 2016). Rodrigues et al. (2013) already underlined the importance of a diverse microbiota, which could explain the low rates of detrimental species and isolates of *Aspergillus* identified in their chestnut samples. Similarly, Prencipe et al. (2018) explained their results by the diversity of species and non-aflatoxigenic strains that reduce the presence of aflatoxigenic strains.

Aspergillus from *Flavi* section also synthesizes beneficial compounds, some of which are linked to billion dollar markets. *A. sojae, A. oryzae* and *A. tamarii* are used for the production of kojic acid and several enzymes, like α-amylase, glucoamylase, and proteases, for the production of starch, baking, and brewing worldwide (Rigo et al. 2002; Machida et al. 2008). In this section, non-aflatoxigenic species in particular are interesting to study and to identify new beneficial compounds.

On the other hand, climate change is challenging fungal relationships because fluctuations in temperature and rainfall are creating new environmental conditions that lead to shifts in community composition and the formation of new ecological niches. These changes will vary depending on the worldwide areas, and will create new pressures and scenarios; in general, these environmental changes will affect the agricultural cycles. Therefore, climate change affects microbiota composition in soils and crops, modifying associations (symbiotic or not) between fungi and other organisms, and creates changes in the species distribution patterns. Modeling projections suggest a worldwide

increase of detrimental effects caused by harmful fungi (Wu et al. 2011; Medina et al. 2014; Jayasiri et al. 2015).

In Europe, climate change could increase the risk of AF contamination. Battilani et al. (2016) suggested that the risk of *Aspergillus flavus* presence increases in scenarios with a temperature rise of 2 to 5 °C, and its home range could expand. In general, areas that are most at risk are the south and central parts of Europe, with migrations northwards. Moreover, these scenarios place AF production above the threshold set by the European Regulations in food and feed (Commission Regulation 2001/466/EC). Battilani et al. (2016) study focused on *A. flavus* and maize, its principal substrate. Likewise, other species from the section, that have not been studied, can follow the same patterns (Prencipe et al. 2018). For instance, some AF producers, like *A. parasiticus, A. sergii, A. transmontanensis,* and *A. mottae,* contaminate other staples, in particular nuts and oily grains. Although, these species are less frequent in staples, they produce B- and G- aflatoxins, as well of other mycotoxins, and may cause health risks. Regardless the preliminary results of ARVALIS project, this is the first report of *A. parasiticus* and *A. tamarii* on maize in France, and the frequency of *Flavi* section is expected to increase under the climate change scenarios.

In tropical and subtropical areas, species belonging to *Flavi* section are more frequent, growing principally on maize, peanuts, nuts, spices, which are ingested on daily basis by the inhabitants. In these areas, an important part of smallholder farmers consume the products as home-grown foods. Besides, commercialization of the products in small markets is usual; conversely, the methods to control mycotoxins contamination in food and feed are scarce. Altogether, *Flavi* species place the population at high risk of mycotoxin consumption (Paterson and Lima 2011; IARC 2015; Bandyopadhyay et al. 2016). Depending on the region, climate change would not be favorable either, as these areas would become more arid, and rainfall would fluctuate. For instance, in Africa and Oceania the projections suggest a decrease in suitable areas for agriculture, whereas in Asia and Latin America they suggest more savanna and fewer tropical forests (Figure 1) (Paterson and Lima 2011). Once again, the lack of knowledge of the diversity of *Aspergillus* section *Flavi,* and the poor information of the life history and ecology of most species could lead to more health risk in these areas. Under climatic changing conditions there is a possibility that in Africa, *A. minisclerotigenes, A. parvisclerotigenus* and *A. korhogoensis* could expand their home ranges; the scenarios of climate change suggest drier and warmer conditions that apparently favor their frequency. A recent study on *Aspergillus flavus* clade showed that strains from Benin grouped together, forming a group evolutionary different from the other strains of the clade (Moore et al. 2017). These strains correspond to *A. parvisclerotigenus* strains and support the results showed in this study. To summarized, *Aspergillus flavus* is the most frequent species in Africa, however, some areas have high

presence of *A. flavus* S_{BG} morphotype, in the agro-ecological zone of Northern Guinea savannah, *A. parvisclerotigenus*, and in Kenya, *A. minisclerotigenes.*

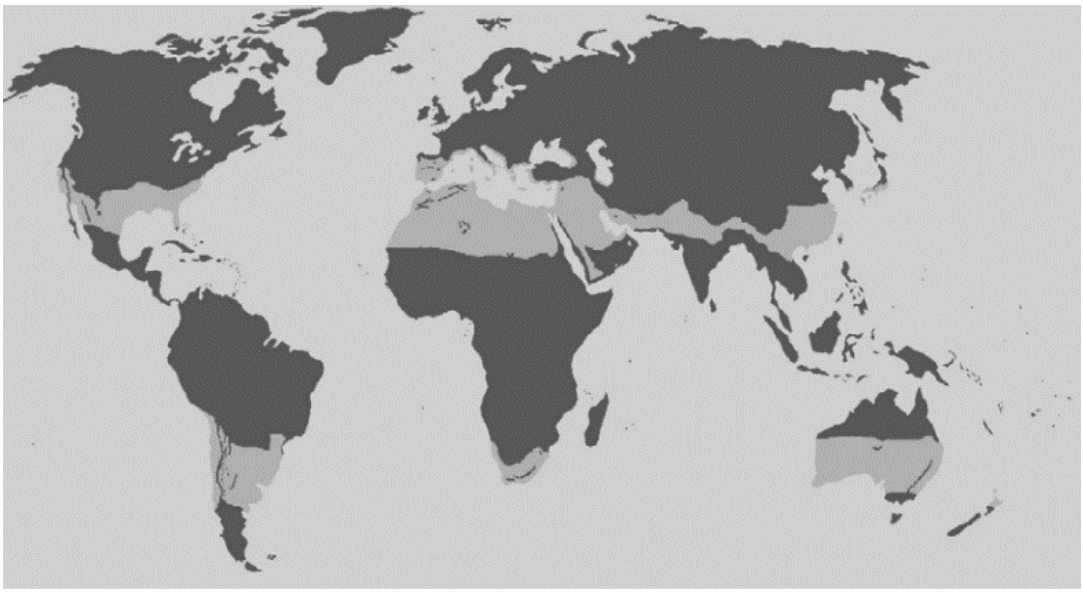

Figure 1: Predicted areas to become tropical. In light color, the predicted areas to become tropical, therefore more suitable to mycotoxin contamination (reprinted from Paterson and Lima 2011).

➢ Phylogenetic inference vs. other molecular techniques

The use of molecular markers revolutionized science. They opened a new understanding of the world, and provided new tools to comprehend life. They enabled the recognition of the biodiversity and to clarify relatedness between different groups, from kingdom to species. Fungi were thus recognized as the closest group of Animalia thanks to molecular markers, which also helped to recognize among cryptic species. In a practical way, molecular markers have allowed the identification of pathogens and their biological machinery. In addition, they can also be used for screening species in different environments.

Mitochondrial RFLPs in fungal systematics gave fruitful results, resolving some relatedness in *Aspergillus*, and has been suggested to be relevant for *Flavi* section screening. Several restriction enzymes were used, but the procedure sometimes failed to discriminate strains, which can be linked to section variability (Quirk and Kupinsky 2002). To date, this technique is less applied because it is time consuming and unfriendly, requires important amounts of DNA, and has screening limitations (Grover and Sharma 2014). RAPDs have also been widely used as screening tool in the section. Tran-Dinh et al. (1999) reported a population study that used a battery of RAPDs combining several primers to distinguish isolates of *A. flavus* and *A. parasiticus.* They have shown that *A. flavus* is more polymorphic, which contributes to the hypothesis that its plasticity confers advantages in its life

history and pathogenicity (Hedayatii et al. 2007). Gonçalves et al. (2012b) used RAPDs as screening technique for section *Flavi* in Brazilian nuts, and identified a high variation in the fungal populations. They complemented their studies with other techniques, including phylogenetic inference. Godet and Munaut (2010) performed another example of RAPDs, showing interesting results. They developed a molecular analytical tool that included several RAPDs and a small digestion; this technique used several markers and real-time PCR to discriminate among species. Using this technique, they could distinguish important species from the section, albeit, they could not differentiate *A. parvisclerotigenus*. Again, the use of more than one molecular marker showed better results than the use of a single marker. However, there are some drawbacks, RAPDs often target unknown dominant markers, the results are not always reproducible because of low annealing temperatures, and differences due to Taq polymerases are found. To solve these problems there are variations of the technique that can improve it (Grover and Sharma 2014). Another technique useful to screen in this section is the PCR-DGGE based on *β-tubulin* (Chapter 2.2), since, it was able to distinguish between *A. flavus* clade and *A. parasiticus* clade species, and highlighted the four strains belonging to *A. korhogoensis.*

Although these techniques showed good results, phylogenetic inference is a finer tool for species screening. Its robustness makes it the third axis of the polyphasic approach, which is mandatory for species description in *Aspergillus* section *Flavi.* Phylogenetic inference uses identified gene markers, and compares the sequences based on complex mathematical algorithms that test the plausibility of evolutionary scenarios based on their nucleotides. Since it includes all these data, it is an excellent tool when used properly. An example of the ability to discriminate among strains in *Flavi* section, is the comparison with PCR-DGGE, although this technique is innovative, its accurateness was lower than that of phylogenetic inference. The latter resolved the relationships for group 4 (Maci 8 sample), which was characterized as *A. parvisclerotigenus*, and showed robust evidence of group 3 as a novel species.

As suggested in the last paragraph, phylogenetic inference is convenient to unmask relationships amongst fungal species and can be used as screening tool for taxa, like *Aspergillus* (Taylor et al. 2002; Peterson 2008; Mitchell 2010; Raja et al. 2011). Some of the characteristics that make it a robust molecular tool are highlighted. First, phylogenetic inference allows the use of more than one molecular marker to solve questions regardless relationships (Peterson 2008; Soares et al. 2012). Second, this technique is friendly and quick. Third, the algorithms used by the software are complex and include several evolutionary scenarios that enable to test complex assumptions to test relatedness between isolate sequences. Fourth, the data generated for each marker can be reused, and by anyone, once deposited in GenBank; whereas other molecular techniques requires new control cultures for each new analysis to compare results. Fifth, free software for analysis are

available, as well as tutorials and manuals explaining how to use them. Besides, on line courses become frequent and inexpensive.

Conversely, the main issues with phylogenetic inference are to properly perform the steps. Molecular markers should be selected according to the aim of the study, markers has to be informative, and can be one or several. The same gene has to be amplified from different organisms, and amplicons have to be confirmed as the correct sequences in order to compare the same gene, and avoid the amplification of ortholog sequences or genes with multiple copies in the genome. A proper alignment of sequences must be performed, to date there are several software that performs this step, usually using CrustalW algorithm, but alignments must always be checked by the naked eye. This is a crucial step, because if there are some errors in the alignment, a completely different inference can be obtained from the data. Depending on the aim of the study, introns can be used; and gaps must be treated carefully. The best evolutionary nucleotide model must always be tested and software that test different evolutionary scenarios should be chosen (*e.g.* jModelTest, PartitioningFinder). If a concatenated dataset was chosen, it is important to run tests of evolutionary nucleotide model for each partition and coalescence test (Beast and Mesquite, among other programs include complex test to try to find the likelihood of all possible scenarios). Once these steps are performed, the next step, which is time consuming, is to perform the analyses. This step must include all the priors of the alignment, and it is suggested to run likelihood and Bayesian analyses to infer properly the relationships between the sequences of the sample. There are different software to perform these analyses, such as BEAST, GARLI, MrBayes, as well as some platforms developed to save computing time that are online, like CIPRES. Finally, once the analyses have been performed, the interpretation of topologies has to be performed thinking in the question to be solved and including the knowledge of the group that is being studied.

➢ Single locus vs. multilocus

Phylogenetic studies to discriminate among species in *Flavi* section are based in a few genes already considered as informative markers (*ITS, β-tubulin*, *cmdA, mcm7, rpb2, tsr1,* etc.). Some authors analyzed single genes and subsequently compared their topologies to classify species (Varga et al. 2011; Gonçalves et al. 2012b; Taniwaki et al. 2012; Tam et al. 2014; Pitt et al. 2017; Prencipe et al. 2018); while others used a mixture of single and multilocus analyses to classify them (Probst et al. 2012; Gonçalves et al. 2012; Soares et al. 2012). However, the use of multilocus datasets has been suggested as a good tool to perform robust phylogenetic analyses (Taylor et al. 2000; Samson and Varga 2009; Houbraken et al. 2014). The advantage of using concatenated matrices over the use of single genes analyses lies in the addition of informative sites to the final analysis, whereas in single

genes analyses the branches should be compared at the end of the analysis in order to obtain a consensus between the genes. Before, the main issue with a concatenated approach was that the analysis of all genes was done as a single gene, which can be reflected in the loss of coalescence, but nowadays this problem can be solved by analyzing the evolutionary models of each gene and performing coalescence tests (Gadagknar et al. 2005; Degnan and Rosenberg 2009; Lanfear et al. 2016). It is important to recall that evolution rates are directly associated to selection pressures, and their effects differ between genomic region, genes loci, and the sections of a gene (exons, introns, and nucleotide position in the codon) (Huelsenbeck et al. 1996; Degnan and Rosenberg 2009). Herein, I agree with this statement, based on the slightly differences observed by each gene, that the incorporation of more genes to the analyses can help to discriminate in a proper way the species in this section.

The use of a single gene can be useful to have a picture of the relatedness of species in the section. As part of the work conducted in this study, several single gene analyses were performed using variable number of haplotypes to test among genes. As a result, the use of at least three genes is hardly recommended. The results observed during the analyses are also comparable to those reported in the literature (previously mentioned).

I agree with the suggestion of several authors (Geiser et al. 2007; Seifert 2009; Schoch et al. 2012) that explain that the use of *ITS* is useless to predict the relatedness within section *Flavi*. The use of this gene permits to split the section *Flavi* into the main clades: *A. nomius*, *A. tamarii*, and *A. flavus sensu* Varga et al. (2011). Albeit, *ITS* gene is too conserved to clarify relationships within these clades, making it not recommended to screen these fungi because it does not add valuable information and its amplification and sequencing increase the cost and time of the analyses.

The genes *benA* and *cmdA* are widely used to characterization of *Aspergillus* species, and in the *Flavi* section (Taniwaki et al. 2012; Gonçalves et al. 2012b; Soares et al. 2012; Prencipe et al. 2018). They are generally used in single gene analyses, and topologies are then compared. However, the resulting topologies often differ slightly in the placement of species in the clades (Hong et al. 2006). The same tendency was observed in the analyses performed in this study. The results for *benA* and *cmdA* had the same tendency, but slightly differences were identified in certain species relationships. A main difference was observed when *A. korhogoensis* strains were analyzed. The four strains were always nested in the *A. flavus* clade (following Varga et al. 2011 classification), but there were differences between both topologies. Topology of *benA* showed several polytomies in the clade *A. flavus* (following Varga et al. 2011 classification); in fact, *A. novoparasiticus, A. korhogoensis* and some strains of *A. parvisclerotigenus* are not resolved with this gene, whereas, *cmdA* had a tendency to group the species as expected (following the classification of Soares 2012).

Mcm7 and *rpb1* genes have also been tested for phylogenetic inference in *Aspergillus,* although their use is less frequent than the previous two genes. Single gene analyses for *mcm7* and *rpb1* tested in this study showed that both genes discriminate the *A. parasiticus* clade from the *A. flavus* clade, but they are not informative enough to solve relationships within each clade. Similarly, *A. mottae* was placed differently in both analyses; it was nested in *A. flavus* clade with *mcm7*, and in *A. parasiticus* clade with *rpb1*.

Mcm7 topology clustered together species belonging to *A. flavus* clade, and the strains nested inside were grouped more or less as expected, yet it was not useful to divide the sister species *A. parvisclerotigenus* and *A. korhogoensis.* Conversely, the results of *A. parasiticus* clade were less robust, the topology showed polytomies for almost all species. The topology for *rpb1* showed clear differences between *A. flavus* clade and *A. parasiticus* clade, but it was not informative enough to solve the relationships within each clade, in particular for *A. parasiticus* clade. *Rpb1* divided *A. flavus* clade in three main groups (*A. flavus, A. minisclerotigenes* and *A. parvisclerotigenus/A. korhogoensis*), but strains of *A. minisclerotigenes* did not formed a group with a node, they were placed as polytomies in the cluster. *A. tamarii* clade and *A. nomius* clade were placed as expected with the genes *mcm7* and *rpb1*. *AmdS* followed a similar trend; *A. bertholletius* was settled as a basal group, followed by the clades *A. nomius* and *A. tamarii.* The clades *A. parasiticus* and *A. flavus* were evidenced using *amdS*. *A. flavus* clade was divided in three polytomic clusters, *A. flavus/A. oryzae*, *A. minisclerotigenes* and *A. parvisclerotigenus/A. korhogoensis*. The clade *A. parasiticus* was clustered as expected, except for *A. sergii,* which was settled as the ancestral taxon of *A. parasiticus/A. flavus* clades and *A. mottae* was nested as the sister taxon of *A. novoparasiticus. AmdS* was quite informative, but it is not so easy to amplify because there are several copies in the genome, hence, this gene is not the best option for phylogenetic inference in *Aspergillus*.

To our knowledge, the use of genes related to reproduction is not common in phylogenetic analyses of Aspergilli. Here, we tested *ppgA*, *preA* and *preB.* The results for the three genes showed interesting topologies. *PreA* and *PreB* had similar topologies, which were congruent with the expected results as they split the *A. flavus* clade from the *A. parasiticus* clade. The relatedness within each of these clades was congruent with the expected topologies; in general, all species were clustered together. They differed slightly in the position of *A. korhogoensis,* which was settled as basal taxon of *A. flavus* clade with *PreA,* and as sister taxon of *A. parvisclerotigenus* with *PreB*. The placement of *A. transmontanesis* changed in the *A. parasiticus* clade. *A. mottae* was embedded in *A. parasiticus* clade with both genes. The results with *ppgA* were different; the topology clustered species of the *A. flavus* clade together, but the relationships within the clade were poorly resolved, showing several polytomies. Conversely, the relationships in *A. parasiticus* clade were better explained and species were clustered together as expected.

In general, the use of a single gene solves poorly the relationships within *A. flavus* and *A. parasiticus* clades, particularly in the last one. *A. mottae* was nested differently with the different genes. All genes placed *A. bertholletius* as basal group and all genes placed *A. tamarii* and *A. nomius* clades as expected.

On the other hand, *AflP* showed poor results. First, amplification of the genes was not straightforward. Secondly, the species were generally poorly clustered, with the exception of *A. minisclerotigenes, A. parvisclerotigenus/A. korhogoensis* and *A. tamari* clade; whose haplotypes were grouped as expected. Polytomies were identified in all the clades, and *A. novoparasiticus* was split in two basal clades. This gene was not informative at any level and was quite complicated to amplify, so it is not recommended for use in phylogeny.

The use of concatenated genes helped to clarify the relationships within the group. As explained above, genes have different evolutionary rates, which were expressed as differences in the topologies. The use of concatenated genes incorporates the different evolutionary histories, providing more robust results (Huelsenbeck et al. 1996; Seifert 2009; Taylor et al. 2000), so the use of concatenated matrix is therefore recommended, and the advantages and disadvantages of three genes matrices will be explained in the next section.

➢ Selecting the best genes for the "phylogenetic molecular tool"

The use of *benA* and *cmdA* are highly recommended to unmask the relationships in this section. Both markers are highly recommended for use in a concatenated matrix, because the relationships in the group are better explained than when single gene matrices are used, thus, both genes are necessary to cluster isolates and species properly. Moreover, the copious amount of published data on both genes is an advantage, because haplotypes of several strains can be integrated into the matrices, adding more information to the analyses. Nevertheless, I observed that they were not sufficient to place properly *A. korhogoensis.* Further, partition analyses had sometimes integrated both genes into the same partition and under the same evolutionary model, whereas, in some other cases both genes were included in different partitions, indicating the need of evolutionary model analyses each time than an analysis is performed.

In this study, two good examples of the applicability of *benA* and *cmdA* as molecular markers for *Flavi* section are shown. The first shows how the use of both genes facilitates the identification of strains in this section (chapters 2.2 and 2.4). In the study of strains isolated along the peanut production chain, both genes allowed the classification of strains as *A. flavus, A. parvisclerotigenus* and four strains as a new clade, *A. korhogoensis.* Albeit, the two genes used independently or together were not sufficient to nest the new species in *Aspergillus flavus* clade. These results were

improved by including at least one gene. In the ARVALIS study, we observed that the strains were placed consistently with morphological results when both molecular markers were used.

Despite this, the addition of more genes resulted always in an improvement of the analyses; with results more consistent with the information already known from the literature and previous analyses (species classification in the section).

Three gene matrices (*benA* and *cmdA* + one more gene) were tested to identify the advantages and disadvantages of each combination. The tested genes included *mcm7* (Figure 2)*, rpb1* (Figure3)*, ppgA, preA* (Figure 4) and *preB* (Figures 5)*.* The inclusion of either of them settled *A. mottae* as the basal taxon of *A. flavus + A. parasiticus* clades*.* The clades *A. nomius* and *A. tamarii* with all the combinations were settled as basal clusters of the previous taxa (*A. mottae* (*A. parasiticus* clade + *A. flavus* clade)). However, the matrices including *preB and ppgA* clustered *A. nomius* and *A. tamarii* as a sister group of the aforementioned taxa (*A. mottae* (*A. parasiticus* clade + *A. flavus* clade)), whereas *mcm7*, *rpb1* and *preA* settled *A. nomius* as the most ancestral clade, followed by the *A. tamari* clade and the more derived clades. Perhaps the lack of some taxa in both clades did not allow a more precise resolution for *preB* and *ppgA.*

In the case of *A. parasiticus* clade, all combinations clustered species together, and settled *A. sergii* as the basal taxon in the cluster, although *A. transmontanensis* and *A. arachidicola* were placed differently depending on the gene combination. For *A. flavus* clade, all genes related *A. flavus* strains (*A. flavus* + *A. oryzae*) with *A. minisclerotigenes*, and overall all showed acceptable results. *A. parvisclerotigenus,* in most cases, was settled in a cluster as sister taxon of the group composed by *A. flavus, A. oryzae* and *A. minisclerotigenes* (with or without *A. korhogoensis*). Despite, the main difference among the topologies was the settlement of *A. korhogoensis*. The topology of *benA, cmdA* and *mcm7* genes did not nested *A. korhogoensis* in *A. flavus* clade, placing *A. korhogoensis* as a cluster related with *A. flavus* and *A. parasiticus clades*. Conversely, *A. korhogoensis* was nested with *A. parvisclerotigenus,* as it sister taxon, with *preB*, and as basal taxon of *A. flavus* clade when either *rpb1*, *preA*, *ppgA* genes were added. It is important to recall that *A. korhogoensis* is a cryptic species that was nested with *A. parvisclerotigenus* after analyses based on a polyphasic approach that included analyses of a matrix concatenating all genes, morphological analyses and secondary metabolic profile, this result is robust and confirms *A. korhogoensis* position as sister taxa of *A. parvisclerotigenus.*

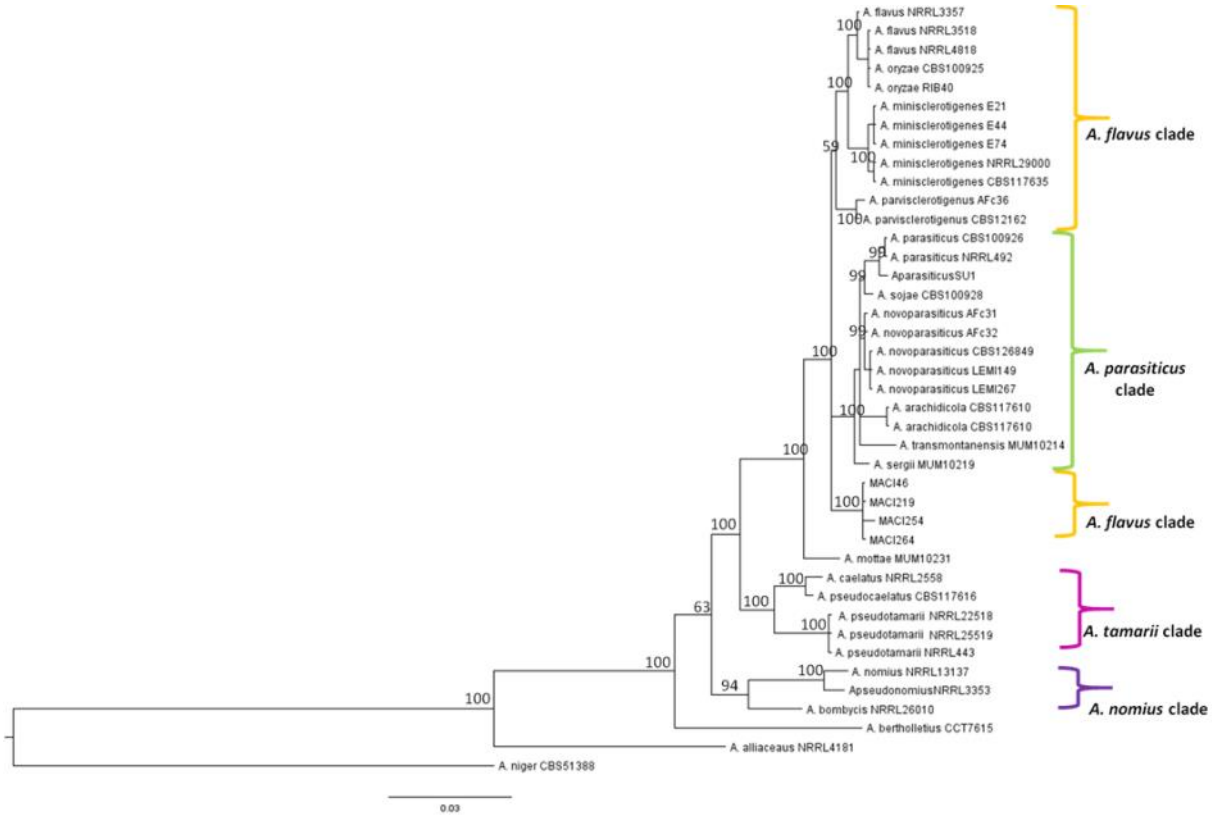

Figure 2: Bayesian topology for concatenated tree using *benA, cmdA* and *mcm7*. PP values are shown. In red brackets *A. flavus* clade; and in blue brackets *A. parasiticus* clade.

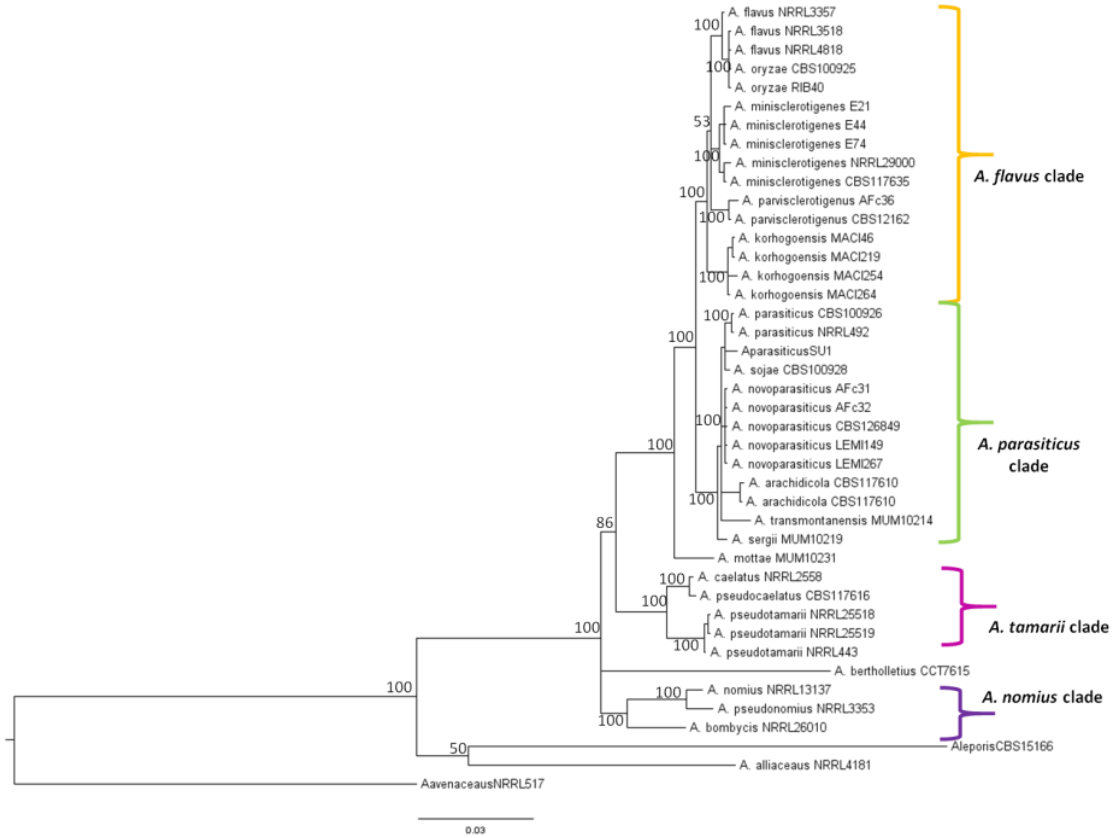

Figure 3: Bayesian topology for concatenated tree using *benA, cmdA* and *rpb1*. PP values are shown. In red brackets *A. flavus* clade, and in blue brackets *A. parasiticus* clade.

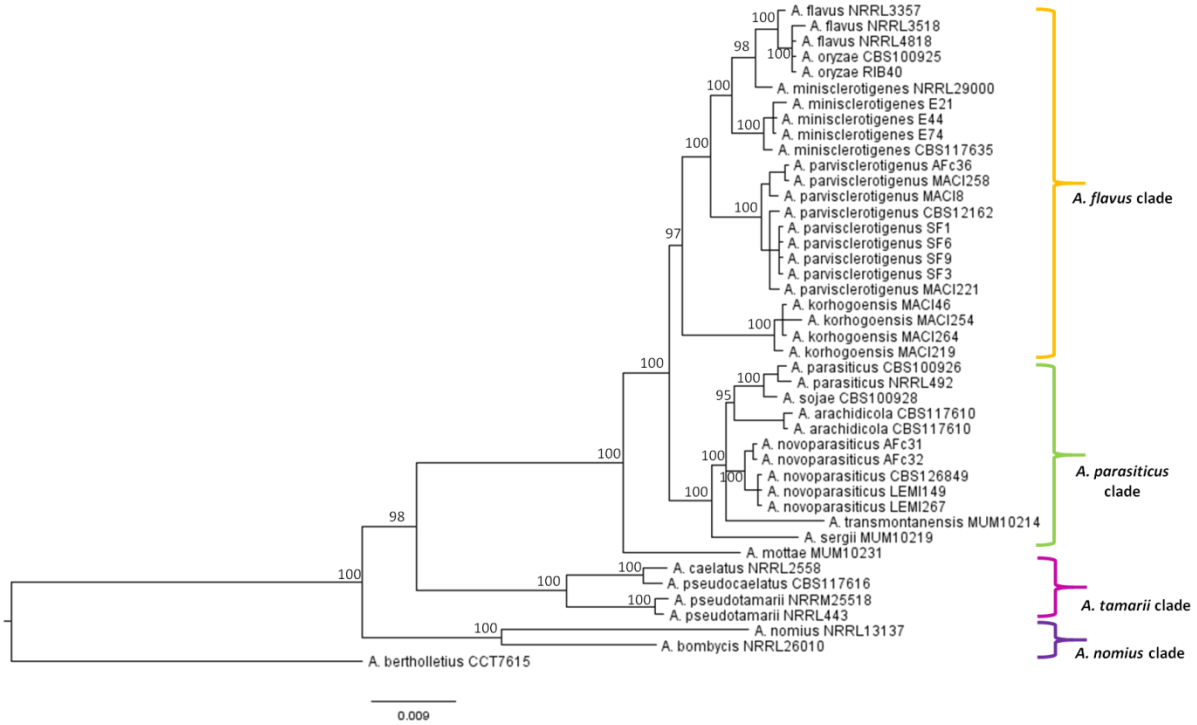

Figure 4: Bayesian topology for concatenated tree using *benA, cmdA* and *preA*. PP values are shown. In red brackets *A. flavus* clade; and in blue brackets *A. parasiticus* clade.

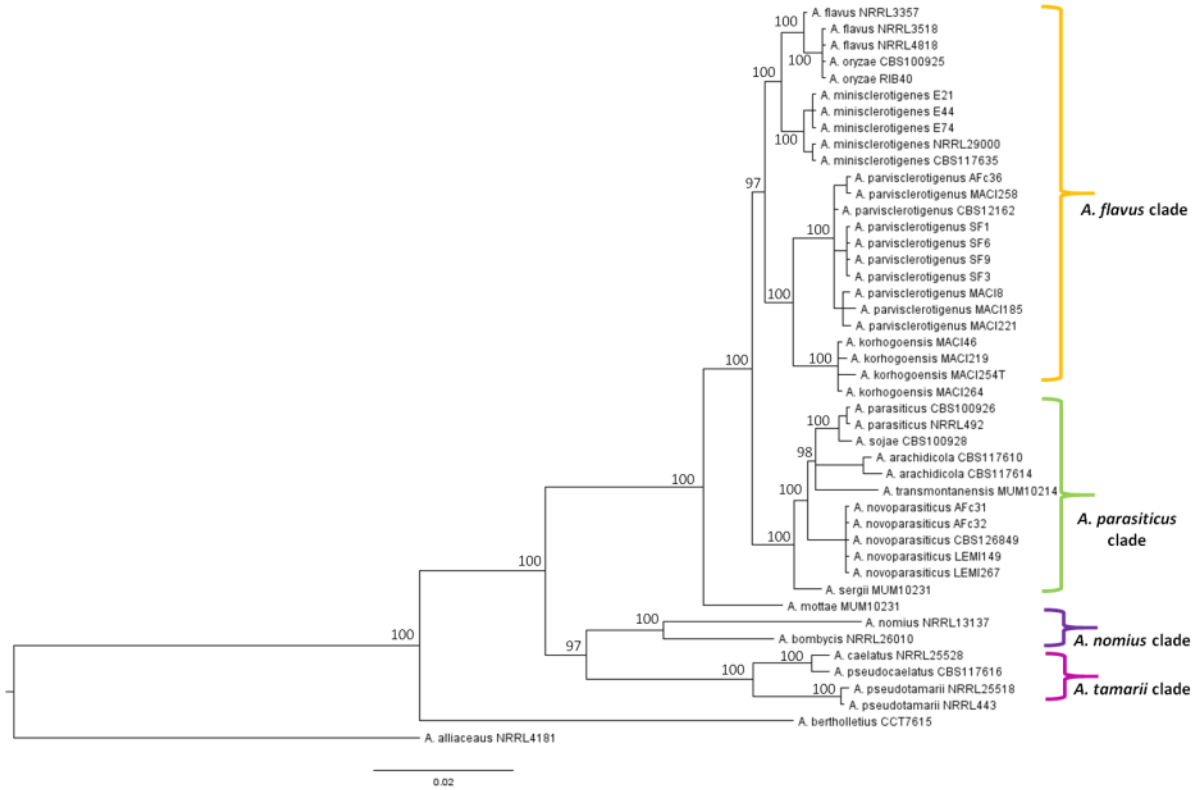

Figure 5: Bayesian topology for concatenated tree using *benA, cmdA* and *preB*. PP values are shown. In red brackets *A. flavus* clade; and in blue brackets *A. parasiticus* clade.

An overview of the results confirms that the use of at least one of these genes is strongly recommended for performing a robust topology of the section. As described before, these genes are presented as a single copy in the genome. Further, these genes could help to solve discrepancies obtained by the use of only *benA* and *cmdA,* as explained above and through chapter 2.3.

Sexual genes *Preb* and *PreA* showed interesting topologies, grouping most clades as expected, which make them suitable for phylogenetic analyses in section *Flavi*. They were more easily amplified for derived taxa. In the case of *preA*, it was not easily amplified, and it is necessary to develop new primers in order to be use in other sections of *Aspergillus.* For *preB* even though it was easier to amplify, it is also necessary to develop more universal primers for its use in other sections of *Aspergillus.* On the contrary, for the discrimination among "economically important species" from section *Flavi,* both genes add information to the multilocus analyses. The use of *mcm7* did not increase the reliability of the analysis for *A. korhogoensis,* but it is informative for the other clusters of the section *Flavi.* Furthermore, it has the advantage that is more frequently amplified, making more feasible to obtain data of several species, and also it is a gene that was quite easily amplified, it has already used with good results in studies on *A. section versicolores* (Jurgevic et al. 2012). *Mcm7* has been suggested to be a good marker in other section of *Aspergillus,* in Ascomycota, and fungi (Schmitt et al. 2009). Based on that, it can also be recommended as a third marker for other sections of *Aspergillus*, and when *A. korhogoensis* is not included in the matrix, it is robust for this section. *Rpb1* and *ppgA*, show as well good results, making them also good candidates for analyses. *Rpb1* is a longer region and includes exons and introns, adding more informative sites, there are also evidence that it is informative in studies comprising several genes, like in *A.* section *versicolores* (Jurgevic et al. 2012), or in combination with the gene tsr1, which allowed the description of *A. tanneri* species (Sugui et al. 2012).

To summarize, the genes tested are informative for the section *Flavi,* and they might be informative for other economically important sections of *Aspergillus,* such as section *Nigri, Fumigat,* and *Nidulans* and in *Penicillium.* Before testing these genes in other sections, the development of primers that targets these regions might be necessary, especially for sexual genes. The inclusion of one or two more genes (*mcm7*, *rpb1* or a sexual gene), add robustness to the analyses in section *Flavi,* and are therefore necessary to be included. *ITS* gene is not useful for characterization at species level, and its use should be avoided. In addition, phylogenetic inference is a robust approach to screen species of section *Flavi,* it is true that slightly differences can be appreciated while different genes are used, but the species clustered together and most combination allowed settling correctly the species within their clusters.

3.2 CONCLUSION

Molecular markers have highlighted the diversity of fungi, including species that cannot be grown under laboratory conditions, which confirms the use of molecular inference as an appropriate tool. This is not only restricted to fungi. The inclusion of molecular data has allowed unmasking the biodiversity of several taxa, including archeas, bacteria, vertebrates, plants and algae. In relation with the molecular approach, the results obtained during this study have evidenced that the use of phylogeny inference based on a concatenated matrix is a fine tool to discriminate among species of *Aspergillus* section *Flavi*. This technique was used to confirm the characterization performed by other approaches, and proved to be a feasible and robust technique. Several species described after 2012 that were not analyzed together, were included in the analyses and their position within the section was strengthened by adding information from more genes. Furthermore, a novel species belonging to the section was described, *A. korhogoensis*. Based on the literature of the selected markers, it may be interesting to apply the same combination of markers to discriminate between other sections of *Aspergillus* and *Penicillium*.

A good combination of molecular markers is required; based on the results, I recommend the use of a multilocus matrix that includes at least *benA* and *cmdA*, which have proven several times to be two excellent markers. To increase the power of the analysis, at least one additional marker must be added, such as *mcm7*, *rpb1*, *preB, preA* and *ppgA* are recommended. Conversely, the use of *ITS* in *Aspergillus* can lead to an underestimation of the diversity because it is a too conserved gene. The study of mating type MAT1 loci in the section is useful for increasing knowledge of reproduction. Further analyses could help to understand better the many functions of these genes in the biological machinery of fungi, and in the future can be used in biotechnological processes to control development of strains.

I agree with the statement that *A. flavus, A. parasiticus* and *A. nomius* are species complexes, and that the majority of species described over the last decade are cryptic (except *A. bertholletius* and *A. hancockii*). Further, the recognition of the new species is increasingly accepted by researchers as they were described using several traits, which show the reliability of these independent evolutive lineages. More studies should be done in the section *Flavi* because there is a high probability that the number of species will increase, as well as the ecological knowledge and information regardless the species life histories.

Even though the use of phylogenetic inference is a good method to identify species at fine scale, and that I hardly recommend its application, it is important to keep in mind that it is not the only available tool. It has to be used cautiously, and bearing in mind that a morphological approach

and a secondary metabolic characterization are also important. There are several critical moments that can lead to misidentification.

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AUTEUR : Amaranta CARVAJAL

TITRE : Caractérisation d'*Aspergillus* section *Flavi* : les marqueurs moléculaires comme outils pour démasquer les espèces cryptiques

DIRECTEURS DE THESE : Dr. Olivier PUEL et Dr. Isabelle P. OSWALD

RESUME :

Certains champignons, notamment des Ascomycètes, peuvent synthétiser des métabolites secondaires toxiques pour les hommes et les vertébrés, appelés mycotoxines. Étant donné que la présence de ces champignons dans les aliments de base constitue un risque potentiel pour la santé humaine et animale, les aliments de base sont éliminés lorsqu'ils sont contaminés.

La section *Flavi* est un des groupes de champignons les plus importants du point de vue économique et sanitaire car il comprend des espèces productrices de mycotoxines. Parmi les mycotoxines produites par ce groupe se trouvent les aflatoxines (AF), considérées comme une préoccupation majeure en raison de leurs effets délétères chez les vertébrés. Les espèces de la section *Flavi* se développent principalement dans les régions tropicales et subtropicales car elles bénéficient de conditions environnementales optimales. De plus, les conditions de récolte et de stockage sont souvent inappropriées, favorisant ainsi leur développement. Dans les régions tempérées, ces espèces se rencontrent moins fréquemment. Cependant, le réchauffement climatique pourrait favoriser leur colonisation.

L'identification des espèces d'*Aspergillus* de la section *Flavi* est un défi, en raison de l'inter- et intra-variabilité des caractères. Par conséquent, l'utilisation d'une seule méthode d'identification (caractérisation morphologique, moléculaire ou du profil des métabolites secondaires) est insuffisante. Inversement, le développement d'outils moléculaires a facilité la tâche. Le but de notre étude était de déterminer les relations entre les espèces d'*Aspergillus* de la section *Flavi* à partir de différents marqueurs moléculaires (*ITS, benA, cmdA, amdS, préA, perB, ppgA, aflP,* gènes *Mat1*), puis d'identifier ceux qui permettent une classification des espèces par inférence phylogénétique.

L'utilisation de l'inférence phylogénétique dans cette étude a montré qu'il s'agit d'une approche robuste pour identifier les espèces d'*Aspergillus* de la section *Flavi*, notamment en confirmant certaines hypothèses déjà proposées pour les espèces de la section *Flavi*. En effet, l'ajout de marqueurs moléculaires a permis de confirmer le placement phylogénétique des espèces dans la section *Flavi*. De plus, une nouvelle espèce cryptique a pu être décrite : *Aspergillus korhogoensis* (appartenant au clade *A. flavus*). Notre étude a également pu mettre en évidence que les marqueurs moléculaires sélectionnés (*benA*, *cmdA, mcm7*, rpb1, *preB, preA* et *ppgA*) sont de bons candidats pour l'étude d'autres sections d'*Aspergillus*. L'utilisation de l'inférence phylogénétique est une méthode élégante permettant d'identifier de façon précise les espèces. Sur la base de nos résultats, il est recommandé d'utiliser des matrices concaténées pour effectuer une inférence phylogénétique dans cette section, et la meilleure combinaison inclut les gènes *benA, cmdA*, et l'inclusion d'un autre gène : *mcm7, rpb1, preB, preA* ou *ppgA*. A l'inverse, l'utilisation du gène ITS chez Aspergillus peut conduire à une sous-estimation de la diversité car le gène est très fortement conservé. L'étude des gènes du loci *Mat1* dans la section est utile pour accroître les connaissances sur la reproduction sexuée chez les ascomycètes. De plus, plusieurs fonctions de la machinerie biologique fongique sont liées aux gènes du loci *Mat1*.

La caractérisation du profil métabolique secondaire chez les souches d'*Aspergillus* de la section *Flavi* doit être utilisée, non seulement comme outil d'identification, mais également pour discriminer les souches toxinogènes et atoxinogènes. La section *Flavi* renferme des espèces capables de produire à la fois de mycotoxines et de composés bénéfiques. Parmi les mycotoxines qui devraient faire l'objet d'une attention particulière figurent les AF, l'acide cyclopiazonique, les versicolorines a et b, la stérigmatocystine. Une étude plus approfondie du métabolisme secondaire sera également utile pour la recherche de nouveaux composés bénéfiques.

MOT-CLES : *Aspergillus* section *Flavi*, aflatoxines, acide cyclopiazonique, mycotoxines, analyse phylogénétique, approche polyphasique

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