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Taxonomy of *Aspergillus* section *Flavi* and their production of aflatoxins, ochratoxins and other mycotoxins

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Abstract: Aflatoxins and ochratoxins are among the most important mycotoxins of all and producers of both types of mycotoxins are present in *Aspergillus* section *Flavi*, albeit never in the same species. Some of the most efficient producers of aflatoxins and ochratoxins have not been described yet. Using a polyphasic approach combining phenotype, physiology, sequence and extrolite data, we describe here eight new species in section *Flavi*. Phylogenetically, section *Flavi* is split in eight clades and the section currently contains 33 species. Two species only produce aflatoxin B_1 and B_2 (*A. pseudotamarii* and *A. togoensis*), and 14 species are able to produce aflatoxin B_1, B_2, G_1 and G_2: three newly described species *A. aflatoxiformans, A. austwickii* and *A. cerealis* in addition to *A. arachidicola*, *A. minisclerotigenes*, *A. mottae, A. luteovirescens* (formerly *A. bombycis*), *A. nomius, A. novoparasiticus, A. parasiticus, A. pseudocaelatus, A. pseudonomius, A. sergii* and *A. transmontanensis*. It is generally accepted that *A. flavus* is unable to produce type G aflatoxins, but here we report on Korean strains that also produce aflatoxin G_1 and G_2. One strain of *A. bertholletius* can produce the immediate aflatoxin precursor 3-O-methylsterigmatocystin, and one strain of *Aspergillus sojae* and two strains of *Aspergillus alliaceus* produced versicolorins. Strains of the domesticated forms of *A. flavus* and *A. parasiticus*, *A. oryzae* and *A. sojae*, respectively, lost their ability to produce aflatoxins, and from the remaining phylogenetically closely related species (belonging to the *A. flavus*-, *A. tamarii*-, *A. bertholletius*- and *A. nomius*-clades), only *A. caelatus*, *A. subflavus* and *A. tamarii* are unable to produce aflatoxins. With exception of *A. togoensis* in the *A. coremiiformis*-clade, all species in the phylogenetically more distant clades (*A. alliaceus*-, *A. coremiiformis*-, *A. leporis*- and *A. avenaceus*-clade) are unable to produce aflatoxins. Three out of the four species in the *A. alliaceus*-clade can produce the mycotoxin ochratoxin A: *A. alliaceus s. str.* and two new species described here as *A. neoalliaceus* and *A. vandermerwei*. Eight species produced the mycotoxin tenuazonic acid: *A. bertholletius*, *A. caelatus, A. luteovirescens*, *A. nomius, A. pseudocaelatus*, *A. pseudonomius, A. pseudotamarii* and *A. tamarii* while the related mycotoxin cyclopiazonic acid was produced by 13 species: *A. aflatoxiformans, A. austwickii, A. bertholletius, A. cerealis, A. flavus, A. minisclerotigenes, A. mottae, A. oryzae, A. pipericola, A. pseudocaelatus*, *A. pseudotamarii, A. sergii* and *A. tamarii*. Furthermore, *A. hancockii* produced speradine A, a compound related to cyclopiazonic acid. Selected *A. aflatoxiformans, A. austwickii, A. cerealis, A. flavus, A. minisclerotigenes, A. pipericola* and *A. sergii* strains produced small sclerotia containing the mycotoxin aflatrem. Kojic acid has been found in all species in section *Flavi*, except *A. avenaceus* and *A. coremiiformis*. Only six species in the section did not produce any known mycotoxins: *A. aspearensis*, *A. coremiiformis, A. lanosus, A. leporis, A. sojae* and *A. subflavus*. An overview of other small molecule extrolites produced in *Aspergillus* section *Flavi* is given.

Key words: *Aspergillus*, Section *Flavi*, Aflatoxins, Cyclopiazonic acid, Tenuazonic acid.

Taxonomic novelties: *Aspergillus aflatoxiformans* Frisvad, Ezekiel, Samson & Houbraken, *Aspergillus aspearensis* Houbraken, Frisvad, Arzanlou & Samson, *Aspergillus austwickii* Frisvad, Ezekiel, Samson & Houbraken, *Aspergillus cerealis* Houbraken, Frisvad, Ezekiel & Samson, *Aspergillus neoalliaceus* A. Nováková, Hubka, Samson, Frisvad & Houbraken, *Aspergillus pipericola* Frisvad, Samson & Houbraken, *Aspergillus subflavus* Hubka, A. Nováková, Samson, Frisvad & Houbraken, *A. vandermerwei* Frisvad, Hubka, Samson & Houbraken.

https://doi.org/10.1016/j.simyco.2018.06.001.

INTRODUCTION

Aspergillus subgenus *Circumdati* section *Flavi* contains some of the most important species in the genus, which are of significance in biotechnology, foods and health (Varga *et al.* 2011). *Aspergillus flavus* is reported, after *A. fumigatus* (section *Fumigati*), as the second leading cause of invasive aspergillosis and it is the most common cause of superficial infection (Hedayati *et al.* 2007). *Aspergillus oryzae* and *A. sojae* appear to be the domesticated forms of the aflatoxigenic species *A. flavus* and *A. parasiticus*, respectively, and are used extensively in the food and biotechnology industries (Houbraken *et al.* 2014). A large number of species in *Aspergillus* section *Flavi* are common in crops, and some of them produce several mycotoxins, such as aflatoxins, 3-nitropropionic acid, tenuazonic acid and cyclopiazonic acid (Varga *et al.* 2011). Despite many publications in various research fields, the taxonomy of the aflatoxigenic species in *Aspergillus* section *Flavi* is still not fully elucidated, and several new species (some with aflatoxigenic potential) have been described since 2011, such as *A. novoparasiticus* (Gonçalves *et al.* 2012a,b), *A. mottae, A. transmontanensis*, *A. sergii* (Soares *et al.* 2012), *A. bertholletius* (Taniwaki *et al.* 2012), *A. hancockii* (Pitt *et al.* 2017) and *A. korhogoensis* (Carvajal-Campos *et al.* 2017). Additionally, there have also been some disagreements on the proper species names of strains formerly identified as *A. flavus* with large or small sclerotia (Probst *et al.* 2012, 2014).

Initially, *A. flavus* was reported to produce aflatoxin of the B and G type (Nesbitt *et al.* 1962, Codner *et al.* 1963). Later it was recognised that strains of *A. flavus* can only produce aflatoxin B_1 and B_2 (Varga *et al.* 2009, Amaike & Keller 2011) and that the

Peer review under responsibility of Westerdijk Fungal Biodiversity Institute.

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strains producing aflatoxin B and G were *A. parasiticus*, exemplified by strain NRRL 2999, which was initially identified as *A. flavus* (Christensen *et al.* 1973) and three years later re-identified as *A. parasiticus* (Buchanan & Ayres 1976). Although it was considered that *A. flavus* only produces B type aflatoxins, some reports indicate that *A. flavus* strains can also produce the G type aflatoxins (Camiletti *et al.* 2017, Barayani *et al.* 2015, Wicklow & Shotwell 1983, Okoth *et al.* 2018, Saldan *et al.* 2018). This contradictory data needs further investigation and it is important to determine whether *A. flavus sensu stricto* can produce aflatoxins of the G type or not. Most species in *Aspergillus* section *Flavi* produce both types of aflatoxins, while species outside section *Flavi* can only accumulate sterigmatocystin and aflatoxins of the B type (Geiser *et al.* 2007, Varga *et al.* 2009, Rank *et al.* 2011).

Raper & Fennell (1965) stated that *A. flavus* strains produced globose to subglobose sclerotia that are normally 400–700 µm in size, rarely exceeding 1 mm, but that some strains produced sclerotia that were uniformly and consistently smaller. They also mentioned strains that produced vertically elongate sclerotia, and such strains were later shown to be *A. nomius* or *A. pseudonomius* (Kurtzman *et al.* 1997, Varga *et al.* 2011, Massi *et al.* 2014). Also Hesseltine *et al.* (1970) reported *A. flavus* isolates with small sclerotia while most isolates had large sclerotia. They listed NRRL 3251 as one of the rare examples of a strain with small sclerotia that produced aflatoxin B_1 and B_2 only, and stated that this could represent a new species. Another strain similar to NRRL 3251 that also produce small-sized sclerotia is the genome sequenced strain ATCC MYA384 (= AF70) (Moore *et al.* 2015). These *A. flavus* strains with small sclerotia that produce B type aflatoxins (*A. flavus* S_B) are more common in USA than in Africa (Probst *et al.* 2014). Later Saito & Tsuruta (1993) found many strains with small sclerotia from agricultural soil in Thailand. They described their strains and NRRL 3251 as *A. flavus* var. *parvisclerotigenus*. In 2005, Frisvad *et al.* (2005) raised *A. flavus* var. *parvisclerotigenus* to species level and neotypified the species with a strain isolated from a peanut in Nigeria producing aflatoxin B_1, B_2, G_1 and G_2 (CBS 121.62 = IMI 093070 = NRRL A-11612). This neotypification is questionable as the original type only produced B type aflatoxins. Other strains producing small sclerotia, often referred to as *A. flavus* group S_{BG} (= "*A. flavus* strains producing small sized sclerotia and aflatoxin B and G") represent multiple species. One of the "*A. flavus* group S_{BG}" taxa was described as *A. minisclerotigenes* (from Argentina originally) (Pildain *et al.* 2008) and is also found in Central, East and Southern Africa and Australia (Probst *et al.* 2014), while *A. parvisclerotigenus sensu* Frisvad *et al.* (2005) has been found in West Africa: Benin, Burkina Faso, Nigeria, Senegal and Sierra Leone (Probst *et al.* 2014). Another important group of strains is identified as *A. flavus* S_B and these strains are regarded as the agent causing lethal levels of aflatoxins in Kenyan maize. It remains questionable whether these are truly *A. flavus* or that these strains represent a species that has not yet been named (Cotty and Cardwell, 1999, Cardwell and Cotty, 2002, Donner *et al.* 2009, Okoth *et al.* 2012, 2018, Probst *et al.* 2007, 2010, 2012, 2014). However, a later study shows *A. flavus sensu stricto* and *A. minisclerotigenes* are the predominant species in Kenyan maize (Okoth *et al.* 2018).

The genomes of *A. oryzae* RIB 40 (Machida *et al.* 2005, Galagan *et al.* 2005, Inglis *et al.* 2013, Umemura *et al.* 2013a,b), and other strains of *A. oryzae* (Zhao *et al.*2012, 2013, 2014), *A. flavus* NRRL 3357 (= ATCC 200026) (Payne *et al.* 2006, Fedorova *et al.* 2008, Nierman *et al.* 2015), ATCC MYA384 (= AF70) (Moore *et al.* 2015) and other strains (Faustinelli *et al.* 2016), *A. parasiticus* ATCC 56775 (= NRRL 5862 = SU-1) (Linz *et al.* 2014), *A. sojae* NBRC 4239 (Sato *et al.* 2011), *A. bombycis* NRRL 26010 (Moore *et al.* 2016), *A. nomius* NRRL 13137 (= NBRC 33223) (Horn *et al.* 2009c, Moore *et al.* 2015), *A. hancockii* FRR 3425 (Pitt *et al.* 2017) and *A. arachidicola* (Moore *et al.* 2018) have been published. Gene clusters for several secondary metabolites, and the regulation of these gene clusters in *A. flavus* are known, including those for aflatoxins, aflatrem, aflavarins, aflavinines, asparasones, cyclopiazonic acid, kojic acid, leporins and penicillin (Chang *et al.* 2009, Georgianna *et al.* 2010, Marui *et al.* 2010, Terebayashi *et al.* 2010, Chang & Ehrlich 2011, Marui *et al.* 2011, Amare & Keller 2014, Ehrlich & Mack, 2014, Tang *et al.* 2015, Cary *et al.* 2015a,b, 2017, Gilbert *et al.* 2016, Ammar *et al.* 2017, Chang *et al.* 2017, Ibara *et al.* 2018). Genome sequencing of more strains in section *Flavi* will help elucidating how the gene clusters for aflatoxins and ochratoxins evolved. Sexual reproduction appears to be important for the variation between isolates of *A. flavus*, so acquisition of new alleles and mitochondrial inheritance are factors that should be taken into consideration (Horn *et al.* 2016).

For food safety purposes, correct species identification is of high importance (Kim *et al.* 2014, Samson *et al.* 2006, Probst *et al.* 2007, 2010, 2012, 2014, Varga *et al.* 2011), as different species may have different mycotoxin profiles and physiology. For example, *A. flavus* strains used to prevent aflatoxin production in crops, themselves unable to produce aflatoxins, may produce other potentially toxic secondary metabolites (Ehrlich, 2014). Detection of these species in foods using sophisticated analytical techniques requires an accurate and reliable taxonomic system (Frisvad *et al.* 2007, Godet & Munaut, 2010, Luo *et al.* 2014a,b, Faustinelli *et al.* 2017, Kaya-Celiker *et al.* 2015). Occasionally, strains producing important mycotoxins are apparently misidentified. An example of a dubious link between fungal species and mycotoxins is the production of the *A. fumigatus* metabolites fumigaclavine A (Jahardhanan *et al.* 1984) and fumitremorgins (Ma *et al.* 2016) by an *A. tamarii* strain. There is evidence that aflatoxigenic species can hybridize (Olarte *et al.* 2012, 2015), so it should be examined whether some of the species producing aflatoxins may be hybrids. Furthermore, cells of *A. flavus* are multinucleate (Runa *et al.* 2015), and it is unknown whether such nuclei contain the same genetic material.

In this manuscript we present an update on the taxonomy of section *Flavi* and describe eight new species using a polyphasic approach combining physiology, morphology, sequence and extrolite data. A list of accepted species (and their synonyms) belonging to section *Flavi* is presented. The ability of the new species to produce aflatoxin and ochratoxin A is studied and an overview on the mycotoxin producing potential of all section *Flavi* species is presented.

MATERIALS AND METHODS

Isolation of microfungi

A part of the strains used in the study was recently isolated during various surveys in different countries (Czech Republic, Nigeria, Iran). Soil and drillosphere (soil in immediate proximity of