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Secondary metabolite profiling, growth profiles and other tools for species recognition and important *Aspergillus* mycotoxins

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Abstract: Species in the genus *Aspergillus* have been classified primarily based on morphological features. Sequencing of house-hold genes has also been used in *Aspergillus* taxonomy and phylogeny, while extrolites and physiological features have been used less frequently. Three independent ways of classifying and identifying aspergilli appear to be applicable: Morphology combined with physiology and nutritional features, secondary metabolite profiling and DNA sequencing. These three ways of identifying *Aspergillus* species often point to the same species. This consensus approach can be used initially, but if consensus is achieved it is recommended to combine at least two of these independent ways of characterising aspergilli in a polyphasic taxonomy. The chemical combination of secondary metabolites and DNA sequence features has not been explored in taxonomy yet, however. Examples of these different taxonomic approaches will be given for *Aspergillus* section *Nigri*.

Key words: aflatoxins, carbohydrates, chemotaxonomy, extrolites, ochratoxins, phenotype.

INTRODUCTION

The genus *Aspergillus* and its teleomorphs contain a large number of species some of which have been exploited for biotechnologically interesting products for centuries (Bennett & Klich, 1992). In particular *Aspergillus niger* has been used for fermentation of Puer tea (Mo *et al.* 2005) and Awamori (Tamamura *et al.* 2001), citric acid production (Greval & Kalra, 1995; Magnuson & Lasure, 2004), extracellular enzyme production (Wösten *et al.* 2007), for biotransformations of chemicals (Schauer & Boris, 2004), and as a producer of antioxidants (Fang *et al.* 2007). All *A. niger* strains appear to be able to (over)produce citric acid (Moyer, 1953), suggesting that this ability is probably an essential feature of the species. It is therefore tempting to turn this phenomenon around and use such a chemical feature as a taxonomic diagnostic tool. Other species in the section *Nigri* such as *A. carbonarius* and *A. aculeatus* are able to produce citric acid (Greval & Kalra, 1995), so it is necessary to use a whole profile of such chemical features to circumscribe a species. Several types of tests and measurements can be used in *Aspergillus* taxonomy (Table 1), but some of these require special equipment and may not all be diagnostic. In some cases it is only the combination of some of those features that may work in classification and identification. Some features are specially suited for cladisitic studies, especially DNA sequence data. Both colour and physiological tests were used in early taxonomic research by Murakami (1976) and Murakami *et al.* (1979), including pigment production in Czapek agar, growth on nitrite as sole nitrogen source, acid production, extracellular enzyme production and reaction of broth with $FeCl_3$. However, these detailed studies were mostly ignored by the *Aspergillus* community. Raper & Fennell (1965) did not use any chemical, biochemical or physiological characters, but in later taxomomic studies of *Aspergillus* physiological tests (Klich & Pitt 1988) and secondary metabolites (for example Frisvad 1989; Frisvad *et al.* 1998a, 2004; Samson *et al.* 2004; Frisvad *et al.* 2007) have been introduced. In addition to their use in chemotaxonomy, many secondary metabolites have bioactive properties. Mycotoxins are of particular interest, because *Aspergillus* species produce some of the most important mycotoxins (Frisvad *et al.* 2007a). In this review we focus mainly on the use of secondary metabolites and nutritional tests in *Aspergillus* taxonomy and the reasons why they may work very efficiently in some cases, and less satisfactory in other cases. Aflatoxin production is used as an example case to the genetical background on why certain strains in a species do not produce mycotoxins and others do.

Extrolites in *Aspergillus*

The fungal exo-metabolome (Thrane *et al.* 2007), cell-wall metabolome and certain parts of the endo-metabolome are produced as a reaction to the biotic and abiotic environment, and consists of secondary metabolites, overproduced organic acids, accumulated carbohydrates (e.g. trehalose and polyols), extracellular enzymes, hydrophobins, adhesins, expansins, chaperones and other molecules. Those metabolites that are secreted or are accumulated in the cell wall are part of the exo-interactome. Exo-metabolites are secreted and consist mainly of secondary metabolites, overproduced organic acids, extracellular enzymes and other bioactive secreted proteins. The cell wall metabolome consists of structural components (melanin, glucan etc.), epitopes, and certain polyketides and alkaloids that probably protect fungal propagules in being eaten by insects, mites and other animals (Janzen 1977; Rohlfs *et al*. 2005). The endo-metabolome consists of primary metabolites in constant change and internal interaction (the interactome and fluxome). These

Table 1. Features used to characterise *Aspergillus* strains for taxonomic and phylogenetic purposes.

Type of feature	Specialized equipment needed?	Specialized equipment present in mycological labs?	Level of diagnostic power	In use
Micromorphology	Microscope	Yes	++	++
Macromorphology	(Camera, colourimeter)	Yes	++	++
Physiology	(Incubators etc.)	Yes	+	+
Nutritional tests	No	(Yes)	+	Rare
Secondary metabolites, volatiles	GC	Rarely	+	Rare
Secondary metabolites, non-volatile	TLC	Occasionally	++	Rare
	HPLC-DAD	Rarely	+++	Rare
	HPLC-MS	Rarely	+++	Rare
	diMS	Rarely	++	Rare
Extracellular enzymes	GE, CE	Rarely	+	Rare
DNA sequencing	PCR, sequencing	Occasionally	++	+

GC: gas chromatography; TLC: thin layer chromatography; HPLC: high performance liquid chromatography; DAD: diode array detection; MS: mass spectrometry; dims: direct inlet mass spectrometry; GE: gel electrophoresis; CE: capillary electrophoresis; PCR: polymerase chain reaction

primary metabolites are of no interest for taxonomy. However, the profile of accumulated carbohydrates, such as trehalose and mannitol, may change as a reaction to the environment in a more species-specific manner (Henriksen *et al*. 1988). The same may be the case for certain chaperones, i.e. those that participate in the reaction to changes in the environment or stress based on extreme environments. Only a fraction of all these molecules have been used in taxonomy (Frisvad *et al.* 2007b). In general those metabolites that are of ecological interest can be called extrolites, because they are outwards directed. The molecules used most in species recognition have been secondary metabolites, because the profiles of these are highly species specific (Frisvad *et al*. 1998a; Larsen *et al*. 2005). In some cases several isolates in a species do not produce the secondary metabolite expected and this is especially common concerning aflatoxin and ochratoxin production (see below). However the "chemoconsistency" is usually much more pronounced for other secondary metabolites. For example in the case of *Aspergillus* section *Nigri*, each species is characterised by a specific profile (see for a complete Table in Samson *et al.* 2007) which also shows relationships among the taxa. Based on such profiles a "chemophylogeny" can be seen in section *Nigri* (Table 2) or at least an agreement in taxonomic and phylogenetic grouping. Classification of the black aspergilli using morphological, physiological, and chemical features results in a grouping of the black aspergilli that is in very good agreement with a cladification of the same aspergilli using β-tubulin sequencing (Samson *et al*. 2004; Perrone *et al*. 2007). For example *A. carbonarius, A. sclerotioniger, A. ibericus* and *A. sclerotiicarbonarius* in the suggested series "*Carbonaria*" have relatively large rough-walled conidia, a relatively low growth rate at 37° C, moderate citric acid production and other characters in common and at the same time they belong to the same clade according to β-tubulin sequencing.

Some of the secondary metabolites are secreted as volatiles, especially terpenes and certain small alcohols. Other secondary metabolites stay in the conidia, sclerotia or other propagules or are secreted in to the growth medium. Volatile metabolites can be separated and detected by GC-MS, whereas most other secondary metabolites are extracted by organic solvents and separated and detected by HPLC-DAD-MS. Proteins of interest may be separated by 2D-gel electrophoreses or capillary electrophoresis and detected (and identified) by MS. A more indirect detection, followed by chemometric treatment of the data may also be used. For example, extracts of fungi may be analysed by direct inlet electrospray mass spectrometry (Smedsgaard *et al*. 2004).

Filamentous fungi can also be characterised by quantitative profiles of fatty acids (Blomquist *et al*. 1998), their pattern of utilisation of C- and N- sources, their temperature, water activity, pH, atmosphere, redox relationships (Frisvad *et al*. 1998b; Andersen & Frisvad 2002) etc.

Isolates of *Aspergillus* have mostly been characterised by their profiles of secondary metabolites, by their growth rate at certain temperatures and water activities, their growth on creatine-sucrose agar and the color of the conidia, in addition to morphology. As can be seen from the discussion above, many other potential means of characterising the phenome of aspergilli exist. Of all the phenotypic features it is strongly recommended to use secondary metabolites in species descriptions, in addition to morphological and DNA sequence features. However, water and temperature relationships should also be used, at least for culturable fungi such as the aspergilli. A minimum standard for the features that need to be characterised for a species description should be made as an international collaborative effort.

Chemotaxonomy and secondary metabolite profiling

As mentioned in the previous section, the molecules used most often in species recognition have been secondary metabolites, due to their high species specificity (Frisvad 1989; Larsen *et al*. 2005). In other words practically all species produce a unique combination of different types of small organic compounds such as polyketides, non-ribosomal peptides, terpenoids as well as many other compounds of mixed biosynthetic origin. Some of these compounds are even unique to a single species. The fact that secondary metabolites are indeed excellent phenotypic characters for species recognition is backed up by the recent studies on full genome sequencing of important aspergilli concluding that major genomic differences between species are often related to the number and similarity of

Table 2. Provisional serial classification of *Aspergillus* section *Nigri*.

Series *Nigri*:		
	Subseries *Nigri*:	
		Aspergillus niger
		Aspergillus lacticoffeatus
		Aspergillus brasiliensis
	Subseries *Tubingensis*:	
		Aspergillus tubingensis
		Aspergillus vadensis
		Aspergillus foetidus
		Aspergillus piperis
		Aspergillus costaricaensis
Series *Carbonaria*:		
	Aspergillus carbonarius	
	Aspergillus sclerotioniger	
	Aspergillus ibericus	
	Aspergillus sclerotiicarbonarius	
Series *Heteromorpha*:		
	Aspergillus heteromorphus	
	Aspergillus ellipticus	
Series *Homomorpha*:		
	Aspergillus homomorphus	
Series *Aculeata*:		
	Aspergillus aculeatus	
	Aspergillus aculeatinus	
	Aspergillus uvarum	
	Aspergillus japonicus	

polyketide and non-ribosomal peptide synthase genes (Galagan *et al*. 2005; Nierman *et al*. 2005; Pel *et al*. 2007).

Thus in various scenarios detection of a unique mixture or in some cases one or a few biomarkers can be used for species recognition. Given the chemical nature of such small organic molecules they can be detected by different spectroscopic tools such as IR, UV, FLD, MS and NMR each giving complementary structural information, which is why these techniques are often used in a combined setup in connection with either gas- or liquid chromatography (Nielsen *et al*. 2004).

More recently chemoinformatic tools have been developed and applied in order to deal with large amounts of spectroscopic data that can be generated from analysis of numerous fungal strains (Nielsen *et al*. 2004; Larsen *et al*. 2005) This includes analysis of raw extracts of secondary metabolites either by direct injection MS (diMS) or by NMR. "Fingerprints" obtained from both these types of analysis of the "global" chemistry of fungi can relatively easily by stored using the database facilities supplied with the standard commercial software, that is used for running of the analytical equipment. Especially diMS has proven excellent for identification as well as classification purposes of Penicillia grown on standard media and growth conditions (Smedsgaard & Frisvad 1996; Smedsgaard *et al*. 2004). A similar but very different approach for species recognition is the use of electronic nose technologies combined with neural network analysis as a kind of "black box" approach for detection of fungal growth associated to a certain feed or food stuff (Karlshøj *et al*. 2007).

In many cases it is of course of outmost importance to identify the production of individual secondary metabolite production from a given species. This is usually done by LC-DAD-FLD or LC-DAD-MS, even though TLC coupled to simple UV detection often can do the job. For example both ochratoxins and aflatoxins are excellent targets using FLD. Many types of polyketides and non-ribosomal peptides contain aromatic ring systems and other conjugated chromophore systems allowing detection using DAD, whereas non-ribosomal peptides and other alkaloids in general are readily protonated and thereby relatively easily detectable by electrospray MS analysis (Smedsgaard & Frisvad 1996; Smedsgaard *et al*. 2004; Larsen *et al*. 2005).

In conclusion spectroscopic based methods for detection of either fungal fingerprints or biomarkers are excellent tools for recognition of species and specific metabolites, such as mycotoxins, in various scenarios.

The use of growth and enzyme profiles for species recognition in the black aspergilli

Black aspergilli are found throughout the world except for the arctic regions. This means that these fungi encounter highly different biotopes with strong variations in the crude carbon sources they utilise for growth. This raises the question whether strains that were isolated from different biotopes have adapted to the carbon sources in their environment and are therefore different in their enzyme and growth profile with respect to a range of different carbon sources (nutritional tests). Also, one might expect that different black aspergilli occupy different ecological niches and therefore have different growth and enzyme profiles. Murakami *et al*. (1979) have studied this on some black aspergilli, but many new species have been described since. A comparison of *A. niger*, *A. vadensis*, *A. tubingensis*, *A. foetidus* and *A. japonicus* on 7 carbon sources revealed clearly different growth profiles for each species, and demonstrated that *A. niger* and *A. tubingensis* were most similar (de Vries *et al.* 2005). The growth profile of *A. vadensis* was remarkable in that growth on glycerol, D-galacturonate and acetate was poor compared to the other species. *A. foetidus* and *A. japonicus* grew poorly on xylitol, while *A. tubingensis* grew poorly on citrate. Recently, a more elaborate study was performed in which differences between *A. niger* isolates were compared to differences between the black *Aspergillus* species (Meijer, Houbraken, Samson & de Vries, unpubl. data). For this study 17 true *A. niger* isolates (verified by ITS and β-tubulin sequencing) from different locations throughout the world were compared to type strains of the different black *Aspergillus* species and grown on different monosaccharides. No differences in growth on specific carbon sources was observed between the *A. niger* isolates, while significant differences were observed compared to the different species, demonstrating that adaptation of strains to their environment with respect to carbon source utilisation does not occur in *A. niger*. Most remarkable was the finding that of all the black aspergilli, only *A. brasiliensis* was able to grow significantly on D-galactose, but growth differences between the species were also observed on D-fructose, D-xylose, L-arabinose and galacturonic acid (Meijer, Houbraken, Samson & de Vries, unpubl. data). The *A. niger* isolates and the different type strains were also grown in liquid medium with wheat bran or sugar beet pulp as a carbon source. Culture filtrate samples were taken after 1 and 2 d and analysed on SDS-PAGE. The SDS-PAGE profiles were found to be highly similar between the different *A. niger* isolates, while significant differences were observed between

the different species. This indicates that protein profiles could be used as a fast screen for species identification (Meijer, Houbraken, Samson & de Vries, unpubl. results).

As growth and protein profiles require only relatively low-tech infrastructure these characteristics could be extremely helpful in initial screens to determine the identity of an isolate. However, for conclusive identification, these tests should be followed by sequencing the ITS and the β-tubulin region and would be significantly strengthened by metabolite analysis as described in this paper. So far, using growth characteristics on defined media and specific carbon sources has received little attention in taxonomy where traditionally undefined media like malt extract agar, potato dextrose agar and mout extract agar are used for morphological analysis. The example of growth on minimal medium with D-galactose as sole carbon source for *A. brasiliensis* as the only species from the black aspergilli (Meijer, Houbraken, Samson & de Vries, unpubl. data), demonstrates that this is an unexplored area that might be a significant asset in multifactor species identification.

Use of Ochratoxin A in identification of aspergilli

There are more than 20 species cited as ochratoxin A-producing fungi in the genus *Aspergillus* (Abarca *et al*. 1997; Frisvad *et al*. 2004; Samson *et al*. 2004) However, few of them are known to be regularly the source of ochratoxin A (OTA) contamination of foods. OTA contamination of foods was until recently believed to be caused only by *Aspergillus ochraceus* and by *Penicillium verrucosum*, which affect mainly dried stored foods and cereals respectively, in different regions of the world. However, recent surveys have clearly shown that some *Aspergillus* species belonging to the section *Nigri* (e.g. *A niger* and *A. carbonarius*), are sources of OTA in food commodities such as wine, grapes and dried vine fruits. *Petromyces alliaceus* has been cited as a possible source for the OTA contamination, occasionally observed in figs (Bayman *et al*. 2002). Recently, new OTA-producing species have been described from coffee (e.g. *A. lacticoffeatus*, *A. sclerotioniger*, *A. westerdijkiae* and *A. steynii*) (Frisvad *et al*. 2004; Samson *et al*. 2004), and recent results indicated that *A. westerdijkiae*, *A. steynii*, *A. ochraceus*, *A. niger* and *A. carbonarius* are responsible for the formation of OTA in this product (Vega *et al*. 2006; Mata *et al*. 2007).

On the other hand, not all the strains belonging to an ochratoxigenic species are necessarily producers. Several methods have been developed to detect OTA producing fungi. Traditional mycological methods are time consuming and require taxonomical and chromatography expertise, however the agar plug method is quite simple (Filtenborg & Frisvad 1981; Filtenborg *et al*. 1983). Different molecular diagnostic methods for an early detection of ochratoxigenic fungi, using mainly PCR techniques, have been also proposed. One of the goals of these techniques is to differentiate between toxigenic and non-toxigenic strains belonging to species known to produce OTA. To date, one of the problems is that little is known about the genes involved in the OTA biosynthesis (O"Callaghan & Dobson 2006; O"Callaghan *et al*. 2006; Schmidt-Heydt & Geisen 2007). A full characterisation of the gene clusters responsible for ochratoxin A production in the different species will show whether **all** isolates in any of the species reported to produce OTA actually have the gene cluster required. The inability to produce OTA may be caused by silent genes or by mutations in functional or regulatory genes.

OTA production is included as a character for taxonomical purposes in classification (e.g. extrolite profiles for describing species) and also for identification (e.g. synoptic key to species). As is well known in taxonomy, one difficulty in devising identification schemes is that the results of characterisation tests may vary depending on different conditions such as the incubation temperature, the length of incubation period, the composition of the medium, and the criteria used to define a positive or negative mycotoxin or extrolite production. In general the presence of a secondary metabolite is a strong taxonomic character, while the absence of a secondary metabolite is simply no information. Ochratoxin A production is a very consistent property when monitored on YES agar for most species known to produce it, whereas other species, such as *A. niger*, have few strains producing it. Perhaps, for these reasons we can find some confusing or controversial data about the ability to produce OTA by some species in the literature (Frisvad *et al*. 2006). Very often a way to solve such a problem is to record the whole profile of secondary metabolites, because several other secondary metabolites than ochratoxin are consistently produced, in this example, by *Aspergillus niger*.

Aflatoxin biosynthesis and regulation

Aflatoxin is the best studied fungal polyketide-derived metabolite. Aflatoxins are produced by an array of different *Aspergillus* species, but have not yet been found outside *Aspergillus*. Aflatoxins have been found in three phylogenetically different groups of aspergilli: *A. flavus, A. parasiticus, A. parvisclerotigenus, A. nomius, A. bombycis*, and *A. pseudotamarii* in section *Flavi*, *A. ochraceoroseus* and *A. rambellii* in section *Ochraceorosei* and *Emericella astellata* and *E. venezuelensis* in section *Nidulantes* (Frisvad *et al*. 2005). However, sterigmatocystin is also produced by phylogenetically widely different fungi such as *Chaetomium* species (Udagawa *et al*. 1979; Sekita *et al*. 1981), *Monocillium nordinii* (Ayer *et al*. 1981) and *Humicola fuscoatra* (Joshi *et al*. 2002). The genes for production of sterigmatocystin in *E. nidulans* (*A. nidulans*) and aflatoxin in *A. flavus*, *A. parasiticus*, and *A. nomius* are clustered (Ehrlich *et al.* 2005b). At least some of the genes required for production of aflatoxins are present in species of *Aspergillus* not known to be able to make aflatoxins or its precursors, such as *A. terreus*, *A. niger*, and *A. fumigatus* (Galagan *et al.* 2005; Nierman *et al.* 2005; Pel *et al.* 2007). The ST gene cluster from *A. nidulans* contains most of the genes found in the *A. flavus*-type aflatoxin cluster, except that gene order and regulation of gene expression are different (Brown *et al.* 1996). In the aflatoxin biosynthesis gene cluster from *A. ochraceoroseus*, a species more related to *A. nidulans* than to *A. flavus*, the genes are similar to those in the biosynthesis cluster of *A. nidulans*, but are separated into at least two clusters (Cary & Ehrlich 2006). Dothistromin, produced by *D. septosporum*, is an oxidation product of the aflatoxin biosynthesis intermediate versicolorin A. The genes involved in dothistromin biosynthesis are organised into at least 3 different clusters (Bradshaw *et al.* 2006). These differences in cluster organisation could reflect the evolutionary processes involved in the formation of the AF biosynthesis cluster in section *Flavi* aspergilli (Ehrlich 2006).

The genes in the ST and AF cluster are presumably co-ordinately regulated by the Gal4-type ($Cys_6Zn_{2)}$ DNA-binding protein, AflR (Chang *et al.* 1995). Most of the AF biosynthetic genes in section *Flavi* aflatoxin-producing species have AflR-binding sites in their promoter regions and not in the promoter regions of genes neighbouring the cluster. In the ST cluster of *A. nidulans*, only a few genes have recognisable AflR-binding sites in their promoters.

This difference and the fact that globally acting transcription factors putatively affect gene expression could account for the differences in regulation of cluster gene transcription in response to environmental and nutritive signals of the different aflatoxin-producing species.

In addition to AflR, upstream regulatory proteins such as LaeA, a putative RNA methyltransferase, (Bok & Keller 2004; Bok *et al.* 2005; Bok *et al.* 2006; Keller *et al.* 2006) control secondary metabolism possibly by affecting chromatin organisation in subtelomeric regions, where most of these polyketide biosynthesis clusters are located (Bok *et al.* 2006). Location of the genes in the cluster is important to their abilities to be transcribed (Chiou *et al.* 2002). Protein factors that affect developmental processes such as formation of sclerotia and conidia also affect aflatoxin formation (Calvo *et al.* 1999; Calvo *et al.* 2004) (Lee & Adams 1994, 1996; Hicks *et al.* 1997).

Aflatoxin/ST/dothistromin biosynthesis begins with a hexanoylCoA starter unit synthesised by two non-primary metabolism FASs, encoded by genes in the cluster (Watanabe & Townsend 2002). These FASs form a complex with the PKS. This complex allows a unique domain in the PKS to receive hexanoylCoA prior to iterative addition of malonylCoA units. It was hypothesised that addition of malonylCoA continues until the polyketide chain fills the cavity of the PKS and is excised by a thioesterase that also acts as a Claisen-like-cyclase (Fujii *et al.* 2001). The starter unit ACP transacylase domain (SAT) is found near the N-terminus of the AF/ST/DT PKSs. SAT domains have now been implicated in the formation of many fungal polyketides (Crawford *et al.* 2006).

Although the functions of most of the oxidative enzymes encoded by AF/ST cluster genes are now well understood, there are still some enzymes whose role has not been established. The highly similar short chain alcohol dehydrogenases, NorB and NorA, may be necessary for the oxidative decarboxylation required to convert open chain AFB1 and AFG1 precursors to AFB1 and AFG1. Mutation of a gene, *nadA*, previously predicted to be part of a sugar cluster adjoining the AF cluster, prevents formation of AFG1, but not AFB1. NadA may be involved in ring opening of a putative epoxide intermediate formed in the conv. process. The genes *avfA* and *ordB* (*aflX*) also encode proteins predicted to have a catalytic motif for a flavin-dependent monooxygenase (Cary *et al.* 2006). Insertional inactivation of *ordB* led to a leaky mutant that accumulated versicolorin A at the expense of AF. Although *avfA* mutants accumulate averufin (Yu *et al.* 2000), the role of AvfA in the averufin oxidation to hydroxyversicolorone has not been established. Another enzyme, CypX, was proven to be required for the first step of the conv. process (Wen *et al.* 2005). AvfA may catalyze opening of an epoxide intermediate to an unstable aldehyde, which would be expected to immediately condense to hydroxversicolorone. A similar step can be imagined for the conv. of VerA to ST in which another predicted intermediate epoxide might require an enzyme to catalyze the opening of its ring to a form an unstable intermediate that would be subsequently by the enzymes Ver-1 and AflY to generate the expected precursor (Ehrlich *et al.* 2005a; Henry & Townsend 2005). The genes in the AF cluster, *hypB1* and *hypB2*, are predicted to encode hypothetical oxidases. Similar genes are found in other clusters, for example, in the *A. terreus* emodin biosynthesis cluster. Deletion of the gene for HypB2 gave leaky mutants that accumulate OMST and norsolorinic acid, while deletion of *hypB1* gave mutants with reduced ability to produce AF. From the chemical structures of HypB1 and HypB2 we predict they are dioxygenases that catalyze the oxidations, respectively, of the anthrone initially produced by PksA and the OMST epoxide intermediate resulting from oxidation of OMST by OrdA during the conv. of OMST to AFB1 and G1 (Udwary *et al.* 2002).

CONCLUSIONS

Several chemical features may be used for classifying, identifying and cladifying *Aspergillus* species, but only a fraction of these have been used to any great extent. However as shown here many of these features have shown to be promising in *Aspergillus* section *Nigri*. Nutritional tests, fatty acid profiling, extracellular enzyme production, volatile secondary metabolites have been used sparingly, while secondary metabolite profiling has been used quite extensively for taxonomic purposes in several *Aspergillus* sections. Together with morphology, physiology, nutritional tests and DNA sequence features, a stable polyphasic classification can be suggested for *Aspergillus* species. Any of those kinds of characterisation methods alone may give occasional unambiguous results, but together they are very effective in discovering species and identifying isolates of *Aspergillus*. A minimum standard for describing new species and for an unequivocal classification and identification of *Aspergillus* species should be developed.

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