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

J.C. Frisvad1, T.O. Larsen1, R. de Vries2, M. Meijer2,3, J. Houbreken1, F.J. Cabañas4, K. Ehrlich5 and R.A. Samson3

1Center for Microbial Biotechnology, BioCentrum-DTU, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark; 2Microbiology, Utrecht University, Utrecht, The Netherlands; 3CBS Fungal Biodiversity Centre, Utrecht, The Netherlands; 4Veterinary Mycology Group, Departament de Sanitat i d’Anatomia Animals, Universitat Autònoma de Barcelona, Bellaterra, Spain; 5Southern Regional Research Center/ARS/USDA, New Orleans, LA 70124, U.S.A.

*Correspondence: Jens C. Frisvad, jcf@biocentrum.dtu.dk

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 Puér tea (Mo et al. 2005) and Awamori (Tamamura et al. 2001), citric acid production (Grewal & 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 (Grewal & 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 cladistic 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 FeCl3. 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 taxonomic 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 genetic background on why certain strains in a species do not produce mycotoxins and others do.

Extrolites in Aspergillus

The fungal exo-metabolome (Thran 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.), epilopes, and certain polyketides and alkaloids that probably protect fungal propagules in being eaten by insects, mites and other animals (Janzen 1977; Rohlfis et al. 2005). The endo-metabolome consists of primary metabolites in constant change and internal interaction (the interactome and fluxome). These
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 2002). 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. sclerotiorum, A. ibericus and A. sclerotiorum A 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 phenotype 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 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                    | GC                            | Rarely                                          | +                         | Rare  |
| Secondary metabolites, volatiles     | HPLC-DAD                      | Occasionally                                    | ++                        | Rare  |
| Secondary metabolites, non-volatile  | TLC                           | Rarely                                          | +++                       | Rare  |
|                                     | HPLC-MS                       | Rarely                                          | ++                        | Rare  |
|                                     | dMS                           | Rarely                                          | +                         | Rare  |
| Extracellular enzymes                | GE, CE                        | Rarely                                          | Occasionally              | ++    |
| 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
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 Aspergillus 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-galacturonic acid 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. brassiliensis was able to grow significantly on D-galactose, but growth differences between the species were also observed on D-fructose, D-xylene, 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

---

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

| Series Nigri:                          |
|---------------------------------------|
| Subseries Nigri:                      |
| Aspergillus niger                    |
| Aspergillus lactocefactus            |
| Aspergillus brasiliensis             |
| Subseries Tubingensis:               |
| Aspergillus tubingensis              |
| Aspergillus vaderi                  |
| Aspergillus foetidus                 |
| Aspergillus piperis                  |
| Aspergillus costaricaensis           |

| Series Carbonaria:                   |
|-------------------------------------|
| Aspergillus carbonarius             |
| Aspergillus sclerotum niger         |
| Aspergillus ibericus                |
| Aspergillus scleroticonarbonarius   |

| Series Heteromorpha:                |
|-------------------------------------|
| Aspergillus heteromorphus           |
| Aspergillus elliotticus             |

| Series Homomorpha:                  |
|-------------------------------------|
| Aspergillus homomorphus             |

| Series Aculeata:                    |
|-------------------------------------|
| Aspergillus aculeatus               |
| Aspergillus aculeatn                 |
| Aspergillus uvarum                  |
| Aspergillus japonicus                |

polypeptide 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 be 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).
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 malt 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. ramblilli 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 & Ehrich 2006). Dohlstomin, produced by D. septosporum, is an oxidation product of the aflatoxin biosynthesis intermediate versicolorin A. The genes involved in dohlstomin biosynthesis are organised into at least 3 different clusters (Bradhaw 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 (Ehrich 2008).

The genes in the ST and AF cluster are presumably coordinately regulated by the Gal4-type (Cys2-Zn2+) 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 AfIR, 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 (Choiu 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 Diothistromase that also acts as a Claissen-like-cyclase (Fuji & 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 (alFx) 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 found 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 hydroxyversicolorone. 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 AfF 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 catalize 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 clarifying 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.

REFERENCES

Abarca ML, Accensi F, Bragulat MR, Castella G, Cabafes FJ (2003). Aspergillus carbonarius as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. Journal of Food Protection 66: 504–506.

Abarca ML, Bragulat MR, Castella G, Accensi F, Cabafes FJ (1997). New ochratoxigenic species in the genus Aspergillus. Journal of Food Protection 60: 1590–1592.

Abarca ML, Accensi F, Cano J, Cabafes FJ (2004). Taxonomy and significance of black aspergilli. Antonie van Leeuwenhoek 86: 33–49.

Andersen B, Frisvad JC (2002). Physiological studies of Alternaria and Penicillium species from similar substrata. Systematic and Applied Microbiology 25: 162–172.

Ayer WA, Pena-Rodriguez L, Vedera JC (1981). Identification of sterigmatocystin as a metabolite of Monillicium nordinii. Canadian Journal of Microbiology 27: 846–847.

Bayman P, Baker JL, Doster MA, Michailides TJ, Mahoney NE (2002). Ochratoxin production by the species Aspergillus ochraceus group and Aspergillus aflaceus. Applied and Environmental Microbiology 68: 2326–2329.

Bennett JW, Kilch MA (eds.) (1992). Aspergillus: Biology and Industrial applications. Butterworth–Heinemann, Boston.

Blomquist G, Anderson B, Anderson K, Brondz I (1992). Analysis of fatty acids. A new method for characterization of molds. Journal of Microbiological Methods 16: 59–68.

Bok JW, Balajea SA, Marr KA, Andes D, NielsenKF, Frisvad JC, Keller NP (2005). LaeA, a regulator of morphogenetic fungal virulence factors. Eukaryotic Cell 4: 1574–1582.

Bok JW, Keller NP (2004). LaeA, a regulator of secondary metabolism in Aspergillus spp. Eukaryotic Cell 3: 527–535.

Bok JW, Noordermeer D, Kale SP, Andes D, Nielsen KF, Frisvad JC, Keller NP (2005). Secondary metabolic gene cluster silencing in Aspergillus nidulans. Molecular Microbiology 61: 1636–1645.

Bradshaw RE, J. Hen, Morgan BS, Schwelm A, Teddy OR, Young CA, Zhang S (2006). A polyketide synthase gene required for biosynthesis of the aflatoxin-like toxin, dothistromin. Mycopathologia 161: 283–294.

Brown DW, Yu JH, Kelkar HS, Fernandes M, Niesbitt TC, Keller NP, Adams TH, Leonard TJ (1996). Twenty-five co-regulated transcripts define a sterigmatocystin gene cluster in Aspergillus nidulans. Proceedings of the National Academy of Sciences U.S.A. 93: 1418–1422.

Cabafes F, Accensi F, Bragulat MR, Abarca ML, Castellà G, Minguèz S, Pons A (2002). What is the source of ochratoxin A in wine? International Journal of Food Microbiology 79: 213–215.

Calvo AM, Bok JW, Brooks W, Keller NP (2004). veA is required for toxin and sclerotial production in Aspergillus parasiticus. Applied and Environmental Microbiology 70: 4733–4739.
Calvo AM, Hinze LL, Gardner HW, Keller NP (1999). Sporogenic effect of polyunsaturated fatty acids on development of Aspergillus sp. Applied and Environmental Microbiology 65: 3686–3677.

Cary JW, Ehrlich KC (2006). Aflatoxin biosynthesis cluster gene, aflX, encodes an oxidoreductase involved in conv. of versicolorin A to demethylsterigmatocystin. Applied and Environmental Microbiology 72: 1096–1101.

Chang PK, Ehrlich KC, Yu J, Bhatnagar D, Cleveland TE (1995). Increased expression of Aspergillus parasiticus asfR, encoding a sequence-specific DNA-binding protein, relieves nitrate inhibition of aflatoxin biosynthesis. Applied and Environmental Microbiology 61: 2372–2377.

Chen CM, Miller WS, Davis DL, Trail F, Linz JE (2002). Chromosomal location plays a role in regulation of aflatoxin gene expression in Aspergillus parasiticus. Applied and Environmental Microbiology 68: 306–315.

Crawford JM, Dancy BC, Hill EA, Udowy DW, Townsend CA (2008). Identification of a starter unit acyl-carrier protein transacylase domain in an iterative type I polyketide synthase. Proceedings of the National Academy of Sciences U.S.A. 105: 16728–16733.

Ehrlich KC (2006). Evolution of the aflatoxin gene cluster. Mycotoxin Research 22: 9–15.

Ehrlich KC, Montalbano B, Boue SM, Bhatnagar D (2005a). An aflatoxin biosynthesis cluster gene encodes a novel oxido-reductase required for conv. of versicolorin A to 3-O-methylsterigmatocystin. Applied and Environmental Microbiology 71: 8963–8965.

Ehrlich KC, Yu J, Cotty PJ (2005b). Aflatoxin biosynthesis gene clusters and flanking regions. Journal of Applied and Environmental Microbiology 71: 518–527.

Fang HL, Lai JJ, Lin WL, Lin WC (2007). A fermented substance from Aspergillus phoenicis reduces liver fibrosis induced by carbon tetrachloride in rats. Bioscience Biotechnology Biochemistry 71: 1541–1545.

Filtenborg O, Frisvad JC (1986). A simple screening method for toxicogenic fungi in pure culture. Lebensmittel Wissenschaft und Technologie 19: 128–130.

Filtenborg O, Frisvad JC, Svendsen JA (1983). Simple screening method for moulds producing intracellular mycotoxins in pure cultures. Applied and Environmental Microbiology 45: 581–585.

Frisvad JC (1989). The use of high-performance liquid chromatography and diode array detection in fungal chemotaxonomy based on profiles of secondary metabolites. Botanical Journal of the Linnean Society 99: 81–95.

Frisvad JC, Bridge PD, Arora DK, Frisvad JC, Bridge PD, Arora DK (eds.) (1998a). Aspergillus. CRC Press, Boca Raton, pp. 135–159.

Frisvad JC, Bridge PD, Arora DK (eds.) (1998b). Aspergillus. CRC Press, Boca Raton, pp. 19–35.

Frisvad JC, Bridge PD, Arora DK (eds.) (1998c). Aspergillus. CRC Press, Boca Raton, pp. 306–315.

Frisvad JC, Bridge PD, Arora DK (eds.) (1998d). Aspergillus. CRC Press, Boca Raton, pp. 466–470.

Frisvad JC, Bridge PD, Arora DK (eds.) (1998e). Aspergillus. CRC Press, Boca Raton, pp. 471–479.

Frisvad JC, Bridge PD, Arora DK (eds.) (1998f). Aspergillus. CRC Press, Boca Raton, pp. 518–527.

Frisvad JC, Bridge PD, Arora DK (eds.) (1998g). Aspergillus. CRC Press, Boca Raton, pp. 581–585.

Frisvad JC, Bridge PD, Arora DK (eds.) (1998h). Aspergillus. CRC Press, Boca Raton, pp. 672–693.

Frisvad JC, Bridge PD, Arora DK (eds.) (1998i). Aspergillus. CRC Press, Boca Raton, pp. 952–955.

Gomi K, Griffith-Jones S, Gwilliam R, Haas B, Harris D, Horiuchi H, Huang J, Humphray S, Jimenez J, Keller N, Khoure H, Kitiyako K, Kobayashi T, Konzaa K, Kulkarni K, Tanikato Y, Kato M, Kuroiwa M, Tekaia F, Turner G, Urita K, Vazquez de Aldana CR, Weidman J, White O, Woodward J, Yu J-H, Fraser C, Galagan JE, Asai K, Machida M, Hall N, Barrett B, Denning D (2005). Genomic sequence of the pathogenic and allergenic filamentous fungus Aspergillus fumigatus. Nature 438: 1151–1156.

Greish R, Moyer Z, Karoliewicz A, Farber P (2004). Development of a real-time PCR system for detection of F. nivoricum and for monitoring ochratoxin A production in foods based on the ochratoxin polyketide synthase gene. Systematic and Applied Microbiology 27: 501–507.

Grevel HS, Klaras KL (1995). Fungal production of citric acid. Biotechnology Advances 13: 209–234.

Hendeen CN, Shaw KJ, Pitt JI (1998). Ochratoxin A production by Aspergillus carbonarius and A. niger isolates and detection using coconut cream agar. Journal of Food Mycology 1: 67–72.

Henry KM, Townsend CA (2005). Synthesis and fate of a-carboxybenzophenones in the biosynthesis of aflatoxin. Journal of the American Chemical Society 127: 3300–3309.

Hicks JK, Yu JH, Keller NP, Adams TH (1997). Aspergillus sporulation and mycotoxin production both require inactivation of the Flg A alpha protein-dependent signaling pathway. EMBO Journal 16: 4916–4923.

Joshik BK, Groer JB, Wickoff DT (2002). Bioactive natural products from a sclerotium-colonizing isolate of Humicola fuscoatra. Journal of Natural Products 65: 1734–1737.

Karlshøj K, Nielsen PV, Larsen TO (2007). Fungal volatiles: Biomarkers of good and bad food quality. In: Dijksterhuis J, Samson RA (eds.) Food Mycology. A multifaceted approach to fungus and food. CRC Press, Boca Raton, pp. 279–302.

Keller N, Bok J, Chung D, Perrin RM, Keats Shwab E (2005). A novel regulatory region of Aspergillus toxins. Medical Mycology 43: S83–S85.

Klich M, Pitt JI (1986). A laboratory manual for study of Aspergillus species and their teleomorphs. CSIRO Division of Food Processing, North Ryde, NYS, Australia.

Larsen TO, Smidsgaard J, Nielsen FF, Hansen ME, Frisvad JC (2005). Phenotypic taxonomy and metabolite profiling in microbial drug discovery. Natural Product Reports 22: 672–693.

Lee BN, Adams TH (1994). Overexpression of fbaB, an early regulator of Aspergillus aflatoxin biosynthesis, leads to activation of hfr and premature initiation of chromosome replication. Molecular Microbiology 14: 323–334.

Lee BN, Adams TH (1996). Flg and fba function interdependently to initiate conidiospore development in Aspergillus nidulans through flg beta activation. EMBO Journal 15: 299–309.

Magnuson JK, Lasure LL (2004). Organic acid production by filamentous fungi. In: Tkacz JS, Lange L (eds.). Advances in fungal biotechnology for industry, agriculture and medicine. Kluwer Academic, New York, pp. 307–340.

Mata MM, Tanaka WI, Ishamaka BT, Sartori D, Oliveira ALM, Furlaneto MC, Fangaro MHP (2007). Aflatoxin production by Altersonia alternata in the ochratoxin polyketide synthase gene. Applied and Environmental Microbiology 73: 518–527.

Mayer AJ (1983). Effect of alcohols on the mycological production of citric acid and for monitoring ochratoxin A production. Lebsemschwissenschaft und Technologie 13: 9–15.

Meyerson M, Nierden WC, Denning DW, Caddick M, Hynes M, Paoletti M, Fischer R, Klich M, Pitt JI (1986). Advances in fungal biotechnology for industry, agriculture and medicine. Kluwer Academic, New York, pp. 19–35.

Miyoshi K, Muroyama K, Tekaia F, Turner G, Urita K, Vazquez de Aldana CR, Weidman J, White O, Woodward J, Yu J-H, Fraser C, Galagan JE, Asai K, Machida M, Hall N, Barrett B, Denning D (2005). Genomic sequence of the pathogenic and allergenic filamentous fungus Aspergillus fumigatus. Nature 438: 1151–1156.
O’Callaghan J, Dobson ADW (2006). Molecular characterization of ochratoxin A biosynthesis and producing fungi. Advances in Applied Microbiology 58: 227–243.

O’Callaghan J, Stapleton PC, Dobson ADW (2006). Ochratoxin A biosynthetic genes in Aspergillus ochraceus are differentially regulated by pH and nutritional stimuli. Fungal Genetics and Biology 43: 213–221.

Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, Turner G, Rohlfs M, Obmann B, Petersen R (2005). Competition with filamentous fungi and its implication for a gregarious lifestyle in insects living on ephemeral resources. Systematic and Evolutionary Microbiology 57: 195–203.

Samson RA, Noonim P, Meijer M, Houbraken J, Frisvad JC, Varga J, Kocsubé S, Tóth B, Szakács G, Varga G, Yu J, Woloshuk CP, Bhatnagar D, Cleveland TE (2000). Cloning and characterization of avfA and omrB genes involved in aflatoxin biosynthesis in three Aspergillus species. Gene 248: 157–167.

Smedsgaard J, Hansen ME, Frisvad JC (2004). Classification of tervicillate Penicillia by electrospary mass spectrometric profiling. Studies in Mycology 49: 235–251.

Tamura T, Wada K, Takara K, Ishikawa N, Iwabuchi K, Nakasone Y, Chinen I (2001). Analysis of volatile compounds in Awamori using solid state phase extraction. Journal of the Japanese Society for Food Science and Technology – Nippon Shokuhin Kagaku Kogaku Kaishi 48: 202–209.

Tanikwahi MH (2006). An update on ochratoxigenic fungi and ochratoxin A in coffee. Advances in Experimental Medicine and Biology 571: 189–202.

Thrane U, Andersen B, Frisvad JC, Smedsgaard J (2007) The exo-metabolome in filamentous fungi. In: Nielsen J, Hewitt J (eds.). Metabolomics. A powerful tool in systems biology, Springer, Berlin. Topics in Current Genetics 18: 235-252.

Udagawa S, Muroi T, Kurata H, Sekita S, Yoshikura K, Natori S (1979). The production of chaetoglobosins, sterigmatocystin, O-methylsterigmatocystin and chaetocin by Chaetomium spp. and related fungi. Canadian Journal of Microbiology 25: 170–177.

Udway DW, Casillas LK, Townsend CA (2002). Synthesis of 11-hydroxyl C-methylsterigmatocystin and the role of a cytochrome P-450 in the final step of aflatoxin biosynthesis. Journal of the American Chemical Society 124: 5294–5302.

Varga G, Varga J, Susca A, Frisvad JC, Staag G, Kocsubé S, Toth B, Kozakiewicz Z, Samson RA (2007). Aspergillus uvarum sp. nov., an uniseriate black Aspergillus species isolated from grapes in Europe. International Journal of Systematic and Evolutionary Microbiology (in press).

Rohfis M, Obmann B, Petersen R (2005). Competition with filamentous fungi and its implication for a gregarious lifestyle in insects living on ephemeral resources. Ecological Entomology 30: 556–563.

Samson RA, Houbenwan JAMP, Kuipers AFA, Frank JM, Frisvad JC (2004). New ochratoxin A or sclerotium producing species of Aspergillus section Nigri. Studies in Mycology 50: 45–61.

Samson RA, Hong S, Frisvad JC (2006). Old and new concepts of species differentiation in Aspergillus. Medical Mycology 44: S133–S148.

Samson RA, Noonim P, Meijer M, Houbenwan J, Frisvad JC, Varga J (2007). Diagnostic tools to identify black aspergilli. Studies in Mycology 59: 129–145.

Schauer F, Borriss R (2004). Biocatalysis and biotransformation. In: Tkacz JS, Samson RA, Noonim P, Meijer M, Houbraken J, Frisvad JC, Varga J, Kocsubé S, Tóth B, Szakács G, Varga G, Yu J, Woloshuk CP, Bhatnagar D, Cleveland TE (2000). Cloning and characterization of avfA and omrB genes involved in aflatoxin biosynthesis in three Aspergillus species. Gene 248: 157–167.

Watanabe CM, Townsend CA (2002). Initial characterization of a type I fatty acid synthase and polyketide synthase multienzyme complex NorG in the biosynthesis of aflatoxin B1. Chemistry and Biology 9: 981–998.

Wen Y, Hatabayashi H, Atari H, Kitamoto HK, Yabe K (2005). Function of the cypX and moxY genes in aflatoxin biosynthesis in Aspergillus parasiticus. Applied and Environmental Microbiology 71: 3192–3198.

Wosten HAB, Scholtmeijer K, de Vries RP (2007). Hyperproduction of enzymes by fungi. In: Dijkstra R, Samson RA (eds.). Food Mycology. A multifaceted approach to fungi and food. CRC Press, Boca Raton, pp. 183–196.

Yu J, Woloshuk CP, Bhatnagar D, Cleveland TE (2000). Cloning and characterization of avfA and omrB genes involved in aflatoxin biosynthesis in three Aspergillus species. Gene 248: 157–167.