Aspergillus systematics in the genomic era

Robert A. Samson and János Varga
Studies in Mycology

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Cover: Top from left to right. Conidiophores Aspergillus clavatus, A. fumigatus, A. longivesica.
Bottom from left to right. Scanning electron micrographs of Neosartorya denticulata, Petri dish with DG 18 agar and coffee beans from Thailand showing Aspergillus colonies, conidiophores of A. terreus.
Aspergillus systematics in the genomic era

edited by

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CONTENTS

D.M. Geiser, M.A. Klich, J.C. Frisvad, S.W. Peterson, J. Varga and R.A. Samson: The current status of species recognition and identification in *Aspergillus* ................................................................................................................................................................................................. 1


S.A. Balajee, J. Houbraken, P.E. Verweij, S-B. Hong, T. Yaguchi, J. Varga and R.A. Samson: *Aspergillus* species identification in the clinical setting....................................................................................................................................................................................................... 39

C.H.W. Klaassen and N. Osherov: *Aspergillus* Strain Typing in the Genomics Era......................................................................................................................................................................................................... 47


J.I. Pitt and R.A. Samson: Nomenclatural considerations in naming species of *Aspergillus* and its teleomorphs ....................................................................................................................................................................................................... 67

R.A. Samson, J. Varga, S.M. Witiak and D.M. Geiser: The species concept in *Aspergillus*: recommendations of an international panel.... 71

J. Varga, J.C. Frisvad and R.A. Samson: Polyphasic taxonomy of *Aspergillus* section *Candidi* based on molecular, morphological and physiological data................................................................................................................................................................................................... 75

J. Varga, M. Due, J.C. Frisvad and R.A. Samson: Taxonomic revision of *Aspergillus* section *Clavati* based on molecular, morphological and physiological data................................................................................................................................................................................................... 89

J. Houbraken, M. Due, J. Varga, M. Meijer, J.C. Frisvad and R.A. Samson: Polyphasic taxonomy of *Aspergillus* section *Usti* .................. 107

R.A. Samson, P. Noonim, M. Meijer, J. Houbraken, J.C. Frisvad and J. Varga: Diagnostic tools to identify black aspergilli .................. 129

R.A. Samson, S. Hong, S.W. Peterson, J.C. Frisvad and J. Varga: Polyphasic taxonomy of *Aspergillus* section *Fumigati* and its teleomorph *Neosartorya*................................................................................................................................................................................................... 147
Since Pier Antonio Micheli described *Aspergillus* in his *Nova Plantarum Genera* in 1729 the genus attracted an immense interest. Many species were found as spoilage agents, or responsible for human and animal diseases. On the other hand aspergilli were also found as beneficial microorganisms in the fermentation of Asian food and beverages. With the discovery of aflatoxins, the interest and research of the aspergilli increased even more. In the present days *Aspergillus* research has grown to such a level, that it could be stated that aspergilli might be the most studied fungi.

In all these applied fields, the basic systematics has always been very difficult and many mycologists have tried to propose a stable taxonomic classification based on their respective species concept. Although the taxonomy and subsequently the identification of most species has improved, the species concept of many species is still unclear. With the development of various molecular methods new approaches are proposed but it is clear that there is an urgent need to come to a standardisation and consensus how a species in *Aspergillus* is designated. *Aspergillus* is also in the forefront with respect to our genetic knowledge of fungi and with the complete genome sequences of nine *Aspergillus* species (*Emericella nidulans*, *A. oryzae*, *A. fumigatus*, *Neosartorya fischeri*, *A. terreus*, *A. clavatus*, *A. niger*, *A. flavus*, *A. parasiticus*) in advanced states of release, opportunities are upon us to generate data in characterising *Aspergillus* species.

The particular topic to discuss the species concept with a multidisciplinary audience was the aim of the international workshop entitled “*Aspergillus* systematics in the genomics era”. The idea was to bring investigators working on various aspects of *Aspergillus* and its species concept to come together to start to address the question - what is a species and how do we recognise it? The workshop was held at the CBS Fungal Biodiversity Centre, Utrecht, The Netherlands on 12–14 April and 39 mycologists participated. The programme consisted of 10 sessions dealing with various topics all related to the species concept. The sessions discussed the current species concept in *Aspergillus* and the use of secondary metabolite profiling and other tools for species recognition. The views of species recognition with regards to *Aspergillus* genomics and genetics were presented. Two sessions dealt with the species identification in the clinical setting and strains typing of human opportunistic aspergilli while the importance of the genus to agriculture and biotechnology was expressed. The constraints with the *Aspergillus* nomenclature in view of the Rules of the Botanical Code were debated while proposals to develop web-based initiatives and tools for analysis were provided. Finally a special session was organised to discuss the Guidelines for species description in *Aspergillus*. This issue of the Studies in Mycology is a summary of these presentations and discussions.

Additionally, the polyphasic methods applied recently on aspergilli resulted in four monographs included in this issue of *Studies in Mycology*, dealing with *Aspergillus* sections *Candidi*, *Clavati*, *Fumigati* and *Usti*. Diagnostic tools developed for the identification of the economically extremely important but taxonomically problematic black aspergilli (*Aspergillus* section *Nigri*) are also covered in a separate paper.

The organisation of this workshop would not have been possible without the financial aid of various international organisations and companies. We are grateful to the IUMS Mycology Division, Schering Plough, Novoymes, DSM, Merck Sharp & Dome, the Fungal Research Trust, ISHAM and Myconosticta for their financial support.

Robert A. Samson and János Varga  
December 1, 2007
The current status of species recognition and identification in *Aspergillus*

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Abstract: The species recognition and identification of aspergilli and their teleomorphs is discussed. A historical overview of the taxonomic concepts starting with the monograph of Raper & Fennell (1965) is given. A list of taxa described since 2000 is provided. Physiological characters, particularly growth rates and the production of extracellular enzymes, are often show differences that reflect phylogenetic species boundaries and greater emphasis should be placed on extracellular profiles and growth characteristics in species descriptions. Multilocus sequence-based phylogenetic analyses have emerged as the primary tool for inferring phylogenetic species boundaries and relationships within subgenera and sections. A four locus DNA sequence study covering all major lineages in *Aspergillus* using genealogical concordance theory resulted in a species recognition system that agrees in part with phenotypic studies and reveals the presence of many undescribed species not resolved by phenotype. The use of as much data from as many sources as possible in making taxonomic decisions is advocated. For species identification, DNA barcoding uses a short genetic marker in an organism’s DNA to quickly and easily identify it to a particular species. Partial cytochrome oxidase subunit I sequences, which are used for barcoding animal species, were found to have limited value for species identification among black aspergilli. The various possibilities are discussed and at present partial β-tubulin or calmodulin are the most promising loci for *Aspergillus* identification. For characterising *Aspergillus* species one application would be to produce a multilocus phylogeny, with the goal of having a firm understanding of the evolutionary relationships among species across the entire genus. DNA chip technologies are discussed as possibilities for an accurate multilocus barcoding tool for the genus *Aspergillus*.

Key words: multilocus phylogenetics, polyphasic taxonomy, species identification, species recognition

INTRODUCTION: Where things stand currently, and how we got here

The last complete monograph of *Aspergillus* was written in 1965 (Raper & Fennell 1965). They recognised 132 species and 18 varieties. This was a pragmatic treatise. Although they provided Latin descriptions for new species, they did not designate formal type specimens, instead using type strains. They described teleomorphs under the anamorph (*Aspergillus*) name. They divided the species into 18 informal groups based on the authors’ opinions of probable relationships. The groups established, which largely reflected groups defined in previous treatments (Thom & Church 1926; Thom & Raper 1945), have been amazingly stable through a great deal of morphological, physiological and molecular scrutiny over the subsequent 42 years. In an effort to bring the taxonomy of the genus in line with the International Code of Botanical Nomenclature, the species were typified (Samson & Gams 1985), the groups were revised and given formal taxonomic status as sections, and subgenera were added (Gams et al. 1985).

A number of changes have been made to the infrageneric taxa proposed by Gams et al. (1985). In his revision of the genus based on rDNA sequences, Peterson (2000) proposed eliminating three of the six subgenera established by Gams et al. (1985), retaining 12 of the 18 sections, modifying three of the sections and deleting the other three. Frisvad et al. (2005) proposed Section *Ochraceorosei* to accommodate the species *A. ochraceoroseus* and *A. rambellii*. The genus *Neopetromyces* was proposed by the same authors for the teleomorph of *A. muricatus* (Frisvad & Samson 2000).

Species named since the turn of the century are listed in Table 1. Raper and Fennell (1965) described 150 taxa in their monograph; the latest compilation of names in current use (Pitt et al. 2000) listed 182. Samson (2000) listed another 36 published between 1992 and 1999. More than 40 new species descriptions have been published since then (Table 1), bringing the total number to ~250. The number will continue to grow as we discover new species and refine species concepts.

*Aspergillus* is one of the most economically important genera of microfungi, so the rigor and stability of its taxonomy is of significant practical concern. We joke that everyone trying to break a patent is a “splitter”, and everyone trying to retain a patent is a “lumper”, but there is some truth in the joke. Some aspergilli produce metabolites toxic to animals that are highly regulated in many countries while other aspergilli cause allergies or mycoses. It is important that we carefully consider any taxonomic changes in order to keep the taxonomic system practical for economic and regulatory reasons. This has been accomplished through open discussion and consensus-building in meetings such as the Aspergillus Workshops, and in efforts such as the lists of accepted species and synonyms (Pitt et al. 2000).

*Aspergillus* taxonomists have a long tradition of an eclectic approach to our discipline. This is reflected in the methods used by many researchers to delineate new species (Table 1). In addition to morphological characters - the shapes and sizes of various structures, we have traditionally used physiological characters such as colony diam and production of coloured metabolites in our taxonomic systems. We now also use data on other metabolites as measured by chromatography and mass spectroscopy. As a rule, molecular data have supported relationships previously inferred based on morphological and physiological characters.
### Table 1. Characters used in delineating new species of *Aspergillus* since 2000.

<table>
<thead>
<tr>
<th>Section</th>
<th>Species</th>
<th>Morphology</th>
<th>Physiology</th>
<th>Molecular</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circumdati</td>
<td>A. persii</td>
<td>i</td>
<td>x</td>
<td></td>
<td>Zotti &amp; Corti (2002)</td>
</tr>
<tr>
<td></td>
<td>A. cretensis</td>
<td>i</td>
<td>x</td>
<td>BenA</td>
<td>Frisvad et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>A. rocculosus</td>
<td>i</td>
<td></td>
<td>BenA</td>
<td>Frisvad et al. (2004)</td>
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<td></td>
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<td>i</td>
<td>g</td>
<td>BenA</td>
<td>Frisvad et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>A. pseudoelegans</td>
<td>i</td>
<td>x</td>
<td>BenA</td>
<td>Frisvad et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>A. roseoglobulosus</td>
<td>i</td>
<td>x</td>
<td>BenA</td>
<td>Frisvad et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>A. steynii</td>
<td>i</td>
<td>g x</td>
<td>BenA</td>
<td>Frisvad et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>A. westerdijkiae</td>
<td>i</td>
<td>x</td>
<td>BenA</td>
<td>Frisvad et al. (2004)</td>
</tr>
<tr>
<td>Nigri</td>
<td>A. costaricaensis</td>
<td>i</td>
<td>g x</td>
<td>BenA</td>
<td>Samson et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>A. homomorphus</td>
<td>i</td>
<td>g x</td>
<td>BenA</td>
<td>Samson et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>A. lactocoffeatus</td>
<td>i</td>
<td>g x</td>
<td>BenA</td>
<td>Samson et al. (2004)</td>
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<tr>
<td></td>
<td>A. piperis</td>
<td>i</td>
<td>g x</td>
<td>BenA</td>
<td>Samson et al. (2004)</td>
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<tr>
<td></td>
<td>A. sclerotioringer</td>
<td>i</td>
<td>g x</td>
<td>BenA</td>
<td>Samson et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>A. vadensis</td>
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<td>g x</td>
<td>multi</td>
<td>Vries et al. (2005)</td>
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<td></td>
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<td>i</td>
<td>g x</td>
<td>multi</td>
<td>Serra et al. (2006)</td>
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<td></td>
<td>A. brasiliensis</td>
<td>i</td>
<td>g x</td>
<td>multi</td>
<td>Varga et al. (2007)</td>
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<td></td>
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<td>i</td>
<td>g x</td>
<td>multi</td>
<td>Perrone et al. (2007)</td>
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<td></td>
<td>A. aculeatinus</td>
<td>i</td>
<td>g x</td>
<td>multi</td>
<td>Noonim et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>A. scleroticolaborarius</td>
<td>i</td>
<td>g x</td>
<td>multi</td>
<td>Noonim et al. (2007)</td>
</tr>
<tr>
<td>Flavi</td>
<td>A. bombysis</td>
<td>i</td>
<td>g x</td>
<td>multi</td>
<td>Peterson et al. (2001)</td>
</tr>
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<td>i</td>
<td>g x</td>
<td>multi</td>
<td>Ito et al. (2001)</td>
</tr>
<tr>
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<td>A. arachidicola</td>
<td>i</td>
<td>g x</td>
<td>multi</td>
<td>Pildain et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>A. minisclerotogenes</td>
<td>i</td>
<td>g x</td>
<td>multi</td>
<td>Pildain et al. (2007)</td>
</tr>
<tr>
<td></td>
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<td>i</td>
<td>g x</td>
<td>BenA</td>
<td>Frisvad et al. (2005)</td>
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<td>Ochraceorosei</td>
<td>A. rambelli</td>
<td>i</td>
<td>g x</td>
<td>ITS, RAPD</td>
<td>Frisvad et al. (2005)</td>
</tr>
<tr>
<td>Fumigati</td>
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<td>i</td>
<td></td>
<td>multi</td>
<td>Balajee et al. (2005)</td>
</tr>
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<td>A. fumigatiiformis</td>
<td>i</td>
<td>x</td>
<td>multi</td>
<td>Hong et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>A. novofumigatus</td>
<td>i</td>
<td>x</td>
<td>multi</td>
<td>Hong et al. (2005)</td>
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<tr>
<td></td>
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<td>g x</td>
<td>multi</td>
<td>Hong et al. (2007)</td>
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<tr>
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<td>N. nishimurae</td>
<td>a i</td>
<td>g</td>
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<tr>
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<td>a l</td>
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<td>Takada et al. (2001)</td>
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<tr>
<td></td>
<td>N. takakii</td>
<td>a</td>
<td></td>
<td></td>
<td>Horie et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>N. indohii</td>
<td>a</td>
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<tr>
<td></td>
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<td></td>
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<td></td>
<td>N. coreana</td>
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<td></td>
<td>BenAcalm</td>
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<td></td>
<td>N. assulata</td>
<td>a i</td>
<td>g x</td>
<td>multi</td>
<td>Hong et al. (2007)</td>
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<tr>
<td></td>
<td>N. denticulata</td>
<td>a i</td>
<td>g x</td>
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<td>Hong et al. (2007)</td>
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<td></td>
<td>N. galapagensis</td>
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<td>g x</td>
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<td>Hong et al. (2007)</td>
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<td></td>
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<td>a i</td>
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<td></td>
<td>N. ferenczi</td>
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<td></td>
<td>N. papuensis</td>
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<td>a i</td>
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<td>Abiliz &amp; Horie (2001)</td>
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**Abbreviations:** a = ascospore characters; l = anamorph characters; g = Growth rate; x = Extrolites; BenA = β-tubulin; Calm = calmodulin; multi = three or more molecular probes.
There is no one method (morphological, physiological or molecular) that works flawlessly in recognising species. Perhaps this is why Aspergillus taxonomists have so readily embraced the eclectic approach. Morphological characters can vary. For instance, sclerotia which are “characteristic” of some species are not always present in all isolates of a species, and their production can vary among cultures of the same isolate. Currently we are beginning to question the past emphasis on ascospore wall characteristics in systematics. A case in point is the *Emeicella nidulans* variants that possess rough-walled ascospores normally characteristic of *Em. rugulosa* (Klich et al. 2001). Physiological characters may vary, or in the case of metabolites, be absent altogether in some isolates. Multilocus DNA sequence data are extremely useful for recognising species boundaries, but we do not have strict criteria as to where to draw the line between phylogenetic species and well-differentiated populations that are potentially capable of interbreeding, at the same time integrating them into a coherent species concept. Therefore, we advocate the use of morphological, physiological and molecular data in circumscribing Aspergillus species wherever possible.

Although the majority of fungal systematists now utilise molecular data to some extent, researchers give varying levels of weight to molecular versus other sources of information in making taxonomic decisions. Indeed, *Aspergillus* systematists are no exception, and a rather large diversity of opinions on this matter exists even among the authors of this paper! Rather than dwelling on these disagreements, we emphasise the considerable common ground among us, which is leading to a taxonomic system that reflects morphological, physiological, ecological and phylogenetic relationships. Dobzhansky (1951) noted that the discontinuous distribution of variation among organisms is a matter of observation consistent with species being real entities, and that discontinuity is as evident in the genus *Aspergillus* as it is anywhere. While there are inherent problems of logic with placing these discontinuous units into species using consistent rules (Hey 2001) we have to do the best job we can, and the approaches employed over recent decades appear to have served us well. We advocate the use of as much data from as many sources as possible in making taxonomic decisions (Samson *et al.* 2006).

The roles of molecular, morphological and physiological characters in species recognition

It is important to consider carefully the roles of different kinds of characters in drawing boundaries between species. Variable DNA sequence characters provide the best means for inferring relationships among organisms, simply because it is possible to sample very large numbers of variable characters, and for the most part, those characters vary because they are under little or very weak selection. Within and between sister species, the genealogies inferred from single genes do not reflect the underlying organisinal genealogies, because of the stochastic effects of the segregation of variation during speciation, and recombination. However, the point where different gene genealogies become concordant is a useful place to assign a species boundary, as it is likely to reflect a lack of historical recombination among species (Dykhuizen & Green 1991; Koufopanou *et al.* 1997; Geiser *et al.* 2000; Taylor *et al.* 2000). In practice, this has proven to be a powerful tool in the fungi and in the genus *Aspergillus*, as discussed below.

An inference of a species boundary based on multilocus data provides an objectively determined line of demarcation, but as a rule in fungi, it appears that reproductive isolation precedes morphological differentiation in speciation (Taylor *et al.* 2006). This can lead to some unsettling observations. On the one hand, it is comforting that there is usually a very strong correlation between biological species in fungi defined based on laboratory mating tests and those defined based on genealogical concordance principles (O’Donnell *et al.* 1998; O’Donnell 2000). In some cases, closely related phylogenetic species show some degree of cross-fertility (Dettman *et al.* 2006), allowing a window into the forces of hybridisation that affect fungal evolution as much as they do animals and plants, yet have gone mostly unobserved because fungal morphological species concepts tend to be too broad to allow hybridisation to be considered (O’Donnell *et al.* 2004). What is less satisfying about the genealogical concordance approach is that it tends to yield species that are morphologically and otherwise biologically cryptic (Koufopanou *et al.* 1997; Geiser *et al.* 1998; O’Donnell *et al.* 2004; Pringle *et al.* 2005; Balajee *et al.* 2005), in some cases leaving authors with little choice but to describe species using nucleotide characters as the primary descriptors (Fisher *et al.* 2002; O’Donnell *et al.* 2004). However, this may be a simple matter of not looking hard enough. Indeed, initial observations of the morphology and physiology of the cryptic human pathogenic species *Coccidioides immitis* and *C. posadasii* uncovered no morphological or physiological differences other than growth rates on media with high salt concentration (Fisher *et al.* 2002), but molecular evolutionary studies of a proline-rich antigen in these species showed different patterns of positive selection that need to be considered in vaccine design (Johansson *et al.* 2004). While these species may be cryptic to the human eye, perhaps they can be distinguished by the human immune system, and we are served well by recognising them as separate species.

Most *Aspergillus* species were defined based on morphology, with the additional consideration of molecular and extrrolite data used in recent years (Hong *et al.* 2005). Molecular phylogenetics has uncovered cryptic speciation in a number of taxa (Geiser *et al.* 1998; Pringle *et al.* 2005; Balajee *et al.* 2005), suggesting that morphological characters provide a very broad species concept that does not reflect the true extent of evolutionary divergence and reproductive isolation, as appears to be the rule in fungi (Geiser 2004). However, physiological characters, including growth rates and the production of extrrolites, often show differences that reflect phylogenetic species boundaries (Geiser *et al.* 2000). Considering this, greater emphasis should be placed on extrrolite profiles and growth characteristics in species descriptions.

Molecular characters provide the greatest number of variable characters for fungal taxonomy, they can be generated using a widely available technology, that technology comes with an extremely well-developed bioinformatic infrastructure that allows worldwide communication and comparison of results, and they produce results that generally correlate with reproductive barriers and physiological differences. This utility ensures that molecular characters will have a primary role in recognising fungal taxa. However, good taxonomy does not end with the recognition of a species and a Latin binomial. Species descriptions should include data from as many sources as possible, comprising morphology, physiology and molecular data, which can be used not only as tools for identifying an isolate, but understanding its biology.
Some insights on *Aspergillus* species recognition based on multilocus phylogenetics

In recent years, molecular tools such as RFLP’s, RAPD’s, AFLP, MLEE, ribosomal RNA sequences, and protein-coding gene sequences have been applied to taxonomic questions in the genus. Multilocus DNA sequence studies of some anamorphic species have shown that the patterns of polymorphisms in different genes are consistent with recombination in these asexual species (Geiser et al. 1998). Clonal lineages accumulate large numbers of deleterious mutations over time but perhaps aspergilli survive because they are not as clonal as we once thought. The discovery of MAT idiomorphs in populations of *A. fumigatus* showed that the patterns of polymorphisms in different genes are consistent with recombination in these asexual species (Geiser et al. 1998). The subsequent discovery of both MAT idiomorphs in the complete genome sequence of *A. fumigatus* (Paoletti et al. 2005; Dyer & Paoletti 2005; Nierman et al. 2006; Rydholm et al. 1998) strongly indicates that this species is heterothallic, consistent with population genetic patterns suggesting some level of recombination (Pringle et al. 2005). These indications putatively asexual *Aspergillus* species are actually recombining allowing the use of genealogical concordance methods for delimiting species (Taylor et al. 2000).

Single locus DNA sequence studies have been conducted in *Aspergillus* using different loci, and there are extensive databases available for nuclear ribosomal RNA genes (large subunit, internal transcribed spacers) and β-tubulin. Any of these gene regions alone may serve as an effective tool for identifying well-defined species, but a weakness of the single locus approach is that not all species can be identified from DNA polymorphisms therein.

In order to resolve the central question of species boundaries in *Aspergillus* and detection of those species using methodologies based on DNA sequences, a four locus DNA sequence study was undertaken covering all major lineages in *Aspergillus* and have included most of the known and accepted species. Interpretation of the results using genealogical concordance theory results in a species recognition system that agrees in part with phenotypic studies and reveals the presence of many undescribed species not resolved by phenotype.

A primary question of this study is whether the *Aspergillus* anamorph is a reliable marker for species that belong in the *Aspergillus* clade. The species of *Sclerotieista*, *S. ornata* and *S. thaxteri*, are vastly different from most *Aspergillus* species and form a distinct group distantly related to the main *Aspergillus* clade. *A. zonatus*, *A. clavatoiflavus* (section Flavi) and *Warcupiella spinosa* (section Ornati), like the *Sclerotieista* species are distant from the main body of species with *Aspergillus* anamorphs and should be placed in their own groups too. *Hemicarpenites paradoxus*, *A. malodoratus* and *A. crystallinus* are phylogenetically outside of all other *Aspergillus* species (Stevenson, Samson & Varga, unpubl. data).

In examining close relationships, it was found that *Aspergillus niveus* ex type is not the same species as *Fennelia nivea* ex type; *Chaetosartorya stromatoides* ex type is not the same species as *Aspergillus stromatoides* ex type (Peterson 1995) and *Fennelia flavipes* ex type is not the same species as *A. flavipes* ex type. This raises a nomenclatural challenge, because multilocus DNA analysis showed that the assignments of anamorph-teleomorph connections were incorrect. Additionally, among isolates identified as *A. flavipes* there are at least three new species.

In the *Eurotiun* lineage, a teleomorphic state was identified that belongs with *A. proliferans*. This species was placed in the *Eurotiun* clade even though it was anamorphic (Raper & Fennell, 1965). The isolates identified as telemorphs of *A. proliferans* had previously been described by Raper & Fennell (1965) as a colonial variant of *A. ruber* (NRRL 71) or a transitional strain somewhere between *A. ruber* and *A. mangini* in taxonomic terms (NRRL 114). Because these isolates are conspecific with *A. proliferans* they should have a distinct *Eurotiun* name and description. Separately, about 80 isolates of *A. restrictus* were sequenced at a single locus. Three of those isolates were identical with known *Eurotiun* species at that locus. Subsequent sequencing of the other three loci conclusively showed that the anamorphs of *E. intermedium*, *E. repens* and *E. amstelodami* can be found in nature apart from their teleomorph state.

*Aspergillus terreus* isolates contain a greater amount of intraspecific variation than in many other species, and additionally, among the many *A. terreus* isolates examined were several new species. In this species, protein coding genes seem to provide a good set of loci for strain typing. It remains to be seen whether the typing possible in this group with these loci will have relevance for medical treatments.

The internet provides a means for the rapid distribution of data, but not every site on the web contains data of equal quality. GenBank contains sequences of questionable quality and probable errors in the identity of source organisms. Proposals from the MLST community would create curated sites with a more narrow scope than GenBank. At such a site, researchers with a putative new species recognition system that agrees in part with phenotypic studies and reveals the presence of many undescribed species not resolved by phenotype.

From species recognition to species identification

DNA barcoding is a taxonomic method which uses a short genetic marker in an organism’s DNA to quickly and easily identify it as belonging to a particular species. A DNA sequence should meet several criteria to be used successfully for species identification. DNA sequences should be orthologous in the examined organisms, and variable enough to allow species identification, with low levels of intraspecific variation (Hebert et al. 2003). A DNA barcode should be easily accessible (universally amplified/sequenced by standardised primers from a wide set of organisms), relatively short (≤ ~500–600 bp), simple to sequence, easily alignable although this problem can be overcome by using Composition Vector Tree analysis (Chu et al. 2006), or other non-alignment based algorithms developed recently (Little & Stevenson 2007), with no recombination. An ~600 bp region of the 5′ end of the mitochondrial cytochrome oxidase subunit 1 (cox1, usually referred to as CO1 in barcoding studies) was proposed to be a good candidate for barcoding animal species including birds (Hebert et al. 2004b), fishes (Ward et al. 2005), and Lepidopteran insects (Hebert et al. 2004a; Hajibabaei et al. 2006). The cox1 region was also used successfully to develop DNA barcodes for red algae (Saunders 2005). However, recent studies have indicated that mtDNA based barcoding region on its own is not suitable for species identification in several cases. Factors such as interspecific hybridisation (Hurst & Jiggins 2005; Bachtrog et al. 2006), presence of mtDNA derived genes in the nuclear genome (Thalmann et al. 2004; Bensasson et al. 2001), and infection by maternally transmitted endosymbionts
In flowering plants, the nuclear ITS region and the plastid genes and non-coding spacers in the nuclear and plastid genomes have been suggested as DNA barcodes for plants, including coding of \( \text{cox1} \) (and other mitochondrial genes) in higher plants than in animals (Moritz & Cicero 2004; Dashamapatra & Mallet 2006). Attempts have been made to evaluate various regions of the nuclear ribosomal RNA gene cluster for barcoding animals (Sonnenberg et al. 2007; Schill & Steinbrück 2007; Vences et al. 2005a).

For some animal groups, other (usually nuclear) genomic regions have been proposed to be used for species identification. In nematodes, a small region of the 18S rRNA gene was proposed as a DNA barcode (Shady et al. 2006). For dinoflagellates and sponges the intergenic transcribed spacer (ITS) region was found to be the most promising for species identification (Park et al. 2007; Litaker et al. 2007), while for Cephalopoda \( \text{rRNA} \) sequences were suggested to be used as DNA barcodes (Strugnell & Lindgren 2007). Regarding plants, the \( \text{cox1} \) gene was found to be inappropriate for species identification because of a much slower rate of evolution than other mitochondrial genes (Kress et al. 2005; Chase et al. 2005). A variety of loci have been suggested as DNA barcodes for plants, including coding genes and non-coding spacers in the nuclear and plastid genomes. In flowering plants, the nuclear ITS region and the plastid \( \text{trnH-psbA} \) intergenic spacer are two of the leading candidates (Kress et al. 2005). More recently, the non-coding \( \text{trnH-psbA} \) spacer region coupled with the coding \( \text{rbcL} \) gene have been suggested as a two-locus global barcode for land plants (Kress & Erickson 2007). In protists, Scicluna et al. (2006) used successfully part of the ssu rRNA gene for species identification, while spliced leader RNA sequences have been used as barcodes in Trypanosomatidae (Maslov et al. 2007).

Regarding fungi, ITS and translation elongation factor 1-alpha (\( \text{tef1} \)) based DNA barcodes have been developed recently for identification of Trichoderma and Hypocrea species (Druzhina et al. 2005). The ITS region was also found to be useful for species identification in other fungal groups including Zygomycetes (Schwarz et al. 2006), dematiaceous fungi (Desnos-Öllivier et al. 2006) and Trichophyton species (Summerbell et al. 2007). For the identification of \( \text{Fusarium} \) species, Geiser et al. (2004) developed \( \text{FU.S.A.RIUM-ID v.1.0} \), a publicly available sequence database of partial \( \text{tef1} \) sequences. Recently, the applicability of the \( \text{cox1} \) region for species identification in \( \text{Penicillium} \) subgenus \( \text{Penicillium} \) has been examined by Seifert et al. (2007). They found that representatives from 38 to 58 species could be distinguished from each other using \( \text{cox1} \) sequences. Work is in progress in several laboratories to develop DNA barcodes for various fungal groups.

In this study, we wished to evaluate the usefulness of \( \text{cox1} \) for species identification in the \( \text{Aspergillus} \) genus. Our studies were focused on \( \text{Aspergillus section Nigri} \). Our previous work on mitochondrial genetics of aspergilli indicated that the \( \text{cox1} \) gene, or more generally any genes located on the mtDNA of aspergilli does not meet all criteria needed for a DNA barcode. Regarding intraspecific variability, several studies found high levels of intraspecific variability among black aspergilli (Varga et al. 1993, 1994; Hamari et al. 1997; Kevei et al. 1996), which is manifested not only in the presence or absence of intronic sequences, but also in exonic regions (Hamari et al. 2001; Juhász et al. 2007). Both inter- and intraspecific recombination has been detected in aspergilli even under non-selective conditions (Earl et al. 1981; Tóth et al. 1998; Kevei et al. 1997; Hamari et al. 2003; Juhász et al. 2004). Additionally, the \( \text{cox1} \) gene of \( \text{Aspergillus} \) species examined so far carries numerous introns which could make further work cumbersome (Hamari et al. 2003). Several studies also indicated that phylogenetic trees based on mitochondrial and nuclear sequence data are incongruent (Geiser et al. 1996; Wang et al. 2000). Although DNA barcodes are not meant to be used for phylogenetic analyses, the standard short barcode sequences (ca. 600 bp) were found to be unsuitable for inferring accurate phylogenetic relationships among fungi (Min & Hickey 2007). The authors proposed to extend the barcoding region from 600 to 1200 bp to be able to distinguish between these closely related species; however, longer sequences are impractical for use in high-throughput screening programs.

During this study, we concentrated on the \( \text{A. niger} \) species complex, which includes eight species: \( \text{A. niger} \), \( \text{A. tubingensis} \), \( \text{A. foetidus} \), \( \text{A. piperis} \), \( \text{A. brasiliensis} \), \( \text{A. vadensis} \), \( \text{A. costaricensis} \) and \( \text{A. lacticoffeatus} \) (Samson et al. 2004). According to our previous studies, all these species can be distinguished from each other using calmodulin sequence data, and all except one could be distinguished using \( \beta \)-tubulin sequence data \( \text{(A. lacticoffeatus)} \) had identical \( \beta \)-tubulin sequences to some \( \text{A. niger} \) isolates; Samson et al. 2004; Varga et al. 2007). The ITS data set delimited 4 groups: 1. \( \text{A. niger} \) and \( \text{A. lacticoffeatus} \) isolates; 2. \( \text{A. brasiliensis} \); 3. \( \text{A. costaricensis} \); 4. \( \text{A. tubingensis} \), \( \text{A. foetidus} \), \( \text{A. vadensis} \) and \( \text{A. piperis} \) (Varga et al. 2007). We also examined the applicability of the IGS (intergenic spacer region) for species identification; our data indicate that this region exhibits too high intraspecific variability to be useful for DNA barcoding. Other genomic regions examined by other research groups could also distinguish at least 2–5 species in the \( \text{A. niger} \) species complex, including pyruvate kinase, pectin lyase, polygalacturonase, arabinoxylarabinofuranohydrolase and several other genes (Gielkens et al. 1997; de Vries et al. 2005; Parenicova et al. 2001), translation initiation factor 2, pyruvate carboxylase, 70 kD heat shock protein, chaperonin complex component (TCP-1), ATPase (Witiak et al. 2007), and translation elongation factor 1-\( \alpha \), RNA polymerase II and actin gene sequences (S.W. Peterson, pers. comm.).

To evaluate the usefulness of the \( \text{cox1} \) gene for DNA barcoding, we amplified and sequenced part of the \( \text{cox1} \) gene from about 45 isolates of the \( \text{A. niger} \) species complex using the primer pairs developed by Seifert et al. (2007), and examined their properties for species delimitation. Sequence alignments and phylogenetic analysis were made by the MEGA 3 software package (Kumar et al. 2004). For parsimony analysis, the PAUP v. 4.0 software was used (Swoford 2003). Alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option with 100 random taxa additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1000 bootstrap replications (Hillis & Bull 1993). Thirty-seven variable sites were found in the 501 bp region, ten of which were parsimony informative (excluding \( \text{A. ellipticus} \),
which was used as outgroup, 8 of the 12 variable characters were parsimony informative). The topology of the Neighbour-joining tree shown in Fig. 1 is the same as one of the 76 maximum parsimony trees (length: 56 steps, CI = 0.9038, RI = 0.8936, RC = 0.8077). The intraspecific genetic distances calculated using Kimura’s 2-parameter model (Kimura 1980) were 0.003 both for A. niger and A. tubingensis, 0.007 for A. brasiliensis, and 0.002 for A. foetidus. The interspecific diversities were in the same range: 0.003 between A. niger and A. tubingensis or A. foetidus, 0.004 between A. tubingensis and A. foetidus, and 0.005–0.006 between A. brasiliensis and the other species examined. The genetic distance from A. ellipticus varied between 0.084–0.090 for the different species of the A. niger aggregate. Although most isolates of either A. tubingensis or A. niger were found to have identical cox1 sequences, several isolates of these species did not fit into these clades. We could not identify a single site which could be used without ambiguity for distinguishing A. niger from A. tubingensis using a character-based approach as suggested by DeSalle et al. (2005). Although at position 328 all A. tubingensis isolates contained an A nucleotide and most A. niger isolates contained C, some A. niger isolates also had A in this position (data not shown). This nonsynonymous substitution resulted in an amino acid change from leucine to isoleucine. Regarding other amino acid sequences, altogether 5 variable sites have been found, 3 of which

Fig. 1. Neighbour-joining tree based on cox1 sequences of the A. niger aggregate. Aspergillus ellipticus CBS 482.65 was used as outgroup. Bootstrap values above 50 % are shown on the branches. Partial cox1 sequences of the examined isolates have been deposited in the GenBank database under accession numbers EU021012–EU021046.
were parsimony informative. All these amino acid substitutions represented intraspecific variability within the species (data not shown).

Our data indicate that cox1 is not appropriate to be used as DNA barcode in black aspergilli since none of the eight species of the A. niger species complex could be identified unequivocally (Fig. 1). The cox1 gene sequences of the A. niger species complex do not obey either of the two most frequently used methods for species delimitation, reciprocal monophyly as proposed by Wiens 

& Penkroft (2002), or the “10× rule”, the observation of 10 times greater average pairwise genetic difference between the n1 individuals of the candidate species and the reference species than the average within-species pairwise differences found in the particular taxonomic group (Hebert et al. 2004). The phylogenetic tree constructed based on the cox1 sequences shows an overlap between intra- and interspecific variation possibly due to past mitochondrial DNA recombination events as suggested earlier (Tóth et al. 1998). Although a high degree of heterokaryon incompatibility was observed among isolates of the A. niger species complex (van Diepeningen et al. 1997), mtDNA transfers occur readily even between incompatible isolates (Tóth et al. 1998). Our data are in agreement with those of Min 

& Hickey (2007), who recently analyzed the cox1 sequences of A. niger and A. tubingensis isolates available from the GenBank database. The intra- and interspecific genetic diversity was found to be in the same range, and the amino acid sequences of the cox1 barcoding region of isolates of the two species were the same. In the barcoding region, 4 variant sites have been found, of which 2 represent intraspecific variability within A. niger, one substitution was present in an A. niger and an A. tubingensis isolate, and one substitution represented interspecific variability between A. niger and A. tubingensis. This very low level of interspecific variability compared to the similar level of intraspecific diversity in our view is unsatisfactory for a DNA barcode to be used successfully for species identification.

Recent attempts to use the cox1 gene for species identification in other fungal groups including Fusarium species and basidiomycetes have also met with limited success (K. Seifert, pers. comm.). Comparing the phylogenies based on cox1, ITS, β-tubulin and calmodulin sequences, either β-tubulin and calmodulin could serve as a suitable region for species identification among black aspergilli. Recently, participants of the All Fungi DNA Barcoding Planning Workshop (Smithsonian Conservation and Research Center, Front Royal, Virginia, 13–15 May, 2007) accepted the ITS region as the first choice for DNA barcoding of the Fungal Kingdom (www.allfungi.org). However, ITS does not always resolve very closely related phylogenetic species (Bruns 2001), whereas intron-rich protein coding genes generally do much better (Geiser 2004). In Fusarium, partial translation elongation factor 1-alpha sequences have emerged as the most useful single-locus identification tool (Geiser et al. 2004). In the case of aspergilli, our opinion is to use either β-tubulin or calmodulin sequences for accurate species identification because of their prevalence in public databases universality of application, and relative resolving power. However, studies in progress indicate that other regions of the genome might serve better both for species identification and for phylogenetic studies (V. Roberts, pers. comm.). Further studies are in progress to evaluate the usefulness of cox1 for DNA barcoding in another Aspergillus section, and work is in progress to examine the applicability of other loci for species identification.

Looking ahead: genomics, many-locus phylogenies, and multilocus sequence typing

With the complete genome sequences of eight Aspergillus species (Em. nidulans, A. oryzae, N. fischeri, A. teneus, A. clavatus, A. niger, A. flavus) in advanced states of release, opportunities are upon us to generate data from far more loci in characterising Aspergillus species. One application would be to produce a many-locus phylogeny, with the goal of having a firm understanding of the evolutionary relationships among species across the entire genus. Previous phylogenetic studies across the genus have utilised one or a few markers, and unsurprisingly, do not resolve backbone relationships among sections and subgenera (Berbee et al. 1995; Geiser et al. 1996; Ogawa & Sugiyama, 2000; Peterson, 2000). Rokas et al. (2003) showed that strongly inferred nodes could be inferred consistently in the genus Saccharomyces, when approximately 20 genes were used, or about 8 000 orthologous nucleotide sites. With the goal of producing a well-supported phylogeny for the genus, we are using the available complete genome sequences to design new sequence markers that can be applied across the genus. Primers were designed to amplify 500–1 300 bp regions of protein coding genes, using conserved stretches of amino acids as primer sites. Our goal is to generate data from up to 20 genes across the entire genus. Initial results based on ten genes and 36 taxa indicate that backbone node support remains elusive across the genus, but excellent bootstrap support is generally obtained for relationships among species within sections and subgenera (Wiltik & Geiser, unpubl.). Poor backbone support may be due to the overwhelming amount of sequence diversity in the genus, leading to saturation of third codon positions and long branch attraction artifacts. These problems may be averted by selecting genes exhibiting appropriate levels of resolving power (Townsend 2007) or perhaps with full taxon sampling breaking up long branches. Regardless of any inability to resolve early evolutionary events in the evolution of the genus, these data will provide the basis for multilocus sequence typing schemes that will allow for the precise identification of unknowns and the discovery of new taxa. With advances in the utility of DNA chip technologies accompanied by lower cost, we should be looking toward the application of an affordable and accurate multilocus barcoding chip for the genus Aspergillus in the foreseeable future.

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What can comparative genomics tell us about species concepts in the genus Aspergillus?


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Abstract: Understanding the nature of species* boundaries is a fundamental question in evolutionary biology. The availability of genomes from several species of the genus Aspergillus allows us for the first time to examine the demarcation of fungal species at the whole-genome level. Here, we examine four case studies, two of which involve intraspecific comparisons, whereas the other two deal with interspecific genomic comparisons between closely related species. These four comparisons reveal significant variation in the nature of species boundaries across Aspergillus. For example, comparisons between A. fumigatus and Neosartorya fischeri (the teleomorph of A. fischerianus) and between A. oryzae and A. flavus suggest that measures of sequence similarity and species-specific genes are significantly higher for A. fumigatus – N. fischeri pair. Importantly, the values obtained from the comparison between A. oryzae and A. flavus are remarkably similar to those obtained from an intra-specific comparison of A. fumigatus strains, giving support to the proposal that A. oryzae represents a distinct ecotype of A. flavus and not a distinct species. We argue that genomic data can aid Aspergillus taxonomy as serving as a source of novel and unprecedented amounts of comparative data, as a resource for the development of additional diagnostic tools, and finally as a knowledge database about the biological differences between strains and species.

Key words: comparative genomics, genetic diversity, genome sequences, identification of species boundaries

INTRODUCTION

The diversity of life on earth has two fundamental properties: the diversity is discontinuous and the discontinuity is hierarchically organised (Dobzhansky 1971). Understanding the nature and extend of this discontinuity, especially for microbial communities, is one of the great challenges in evolutionary biology. For example, how can we best identify the discontinuity breaks that demarcate microbial species boundaries? Are those species boundaries similar in kind across clades of the tree of life? Addressing these questions is important because the means by which microbial species are identified can have important consequences in understanding species* biomedically-important characteristics, such as their pathogenicity (Kasuga et al. 1999; Balaje et al. 2006) and their geographical distribution (Taylor et al. 2006).

A growing body of research is focused on investigations of microbial species boundaries and the design of appropriate methodologies for delimitating them (Taylor et al. 2000; Taylor et al. 2006; Ward et al. 2007). Briefly, three species concepts are prevalent in the fungal literature: the morphological species concept (MSC – species are recognised based on morphological characters); the biological species concept (BSC – recognition is based on the establishment of reproductive isolation), and the phylogenetic species concept (PSC – species are diagnosed on the basis of shared ancestry). The MSC has been the concept by which fungal species are classified, largely due to the ease of its application. In contrast, the lack of a recognisable sexual stage in many fungal species, such as Aspergillus, makes the application of the BSC across fungi impractical. The problems associated with the application of the MSC and BSC in fungi, coupled with the emergence of theoretical and experimental molecular systematics in the last two decades, have resulted in the increasing popularity of the PSC.

The nature of species boundaries has been experimentally investigated under the PSC for two Aspergillus species of relevance to human health, A. fumigatus and A. flavus. A. fumigatus is considered by many to be the world’s most harmful mould (Pringle et al. 2005), being both a primary and opportunistic pathogen as well as a major allergen (Denning 1998; Latgé 1999). Most A. fumigatus isolates belong to one phylogenetic subspecies with a global distribution (Pringle et al. 2005). A. fumigatus is characterised by surprisingly low levels of genetic diversity, a characteristic that sharply contrasts with the high levels observed in some of its closest sexual relatives, such as Neosartorya fischeri (Rydhom et al. 2006). A. flavus is one of the primary producers of aflatoxin, one of the most potent carcinogenic substances known (Geiser et al. 1998). Species boundaries in the A. flavus clade are more complex (Geiser et al. 1998). Examination of Australian A. flavus strains suggests the presence of at least two cryptic species, and provides convincing evidence that A. oryzae is a domesticated ecotype of A. flavus (Geiser et al. 1998).

The recent availability of several genomes from the genus Aspergillus (Galagan et al. 2005; Machida et al. 2005; Nieman et al. 2005; Baker 2006; Payne et al. 2006; Pel et al. 2007; Rokas & Galagan 2007) allows us for the first time to examine the demarcation of fungal species at the whole-genome level. Below, we discuss four case studies involving taxa from the genus Aspergillus. Two case studies focus on genome-scale intraspecific differences whereas the other two deal with genomic comparisons between closely related but taxonomically distinct species (interspecific).
MATERIALS AND METHODS

Genome sequences. The genome sequences discussed in this paper are available in public databases under the accession numbers: A. clavatus NRRL 1: AAKD00000000 (GenBank); N. fischeri NRRL 181: AAKE03000000 (GenBank); A. fumigatus A1163: ABDB01000000 (GenBank); A. flavus AP23: AAHF01000000 (GenBank); A. niger CBS 513.88: AM270980–AM27098 (GenBank); A. niger ATCC 1015: http://genome.jgi-psf.org/Aspn1/Aspn1.home.html (JGI); A. oryzae: AAIH01000000; A. oryzae: AP007150–AP007177 (DDJB).

Sequence identity at the genome level. The assemblies larger than 5 Kb were aligned using the MUMmer package (http://mummer.sourceforge.net/) (Delcher et al. 1999). Alignments longer than 100 Kb were used to determine average sequence identity to avoid highly repetitive and duplicated regions.

Sequence identity at the protein level. First orthologous groups in the Aspergillus genomes were identified by bidirectional best BLASTp clustering using a cut-off of 1e-05 and were analyzed using the Sybil software package (http://sybil.sourceforge.net/) and the Aspergillus Comparative database (http://www.tigr.org/sysbil/asp). Average sequence identity between reciprocal best matches, which were considered putative orthologs.

Sequence identity at the gene level. The PASA pipeline, initially developed to align EST data onto genomic sequences (Haas et al. 2003), was used to coding sequences (CDSs) from one strain to assemblies of another. The alignments were generated by the gmap program implemented in PASA. Average sequence identity was calculated only for CDSs that produced high quality alignments (more than 95 % identity and 90 % coverage).

Strain- and isolate-specific genes. The PASA pipeline was also applied to identify three classes of genes: (i) orthologous genes, which aligned well and passed validation criteria (more than 95 % identity and 90 % coverage); (ii) variable genes, which aligned poorly (less than 95 % identity and 90 % coverage); (iii) strain- or isolate-specific genes, which did not produce any alignments.

FOUR CASE STUDIES FROM THE GENUS ASPERGILLUS

To compare the extent of diversity within and between species we used three computational approaches based on comparisons between: (i) genomic alignments, (ii) coding sequences (CDS) and genomic alignments, and (iii) protein alignments (Tables 1, 2). Interestingly, whereas the highest similarity values (and presumably the most accurate ones) between pairs of A. fumigatus and A. niger strains, and between A. oryzae and A. flavus were obtained from the genomic alignment comparisons, the highest similarity value in the A. fumigatus – N. fischeri genomic comparison was produced by the protein alignments (Table 2). The most plausible explanation for the observed discrepancies is that many gene models are likely mis-annotated in Aspergillus genomes (annotation was done by six different sequencing centres, Rokas & Galagan 2007).

Intraspecific case study I: A. niger CBS 513.88 versus A. niger ATCC 1015

Aspergillus niger is a workhorse organism in the field of biotechnology and industrial mycology. This organism is a source of and product host for industrial enzymes as well as the basis for the most efficient filamentous fungal fermentation known, the production of citric acid. In addition to its role in the enzyme and chemical industries, A. niger is an important experimental model organism for the study of protein secretion, organic acid production and others areas of basic fungal biology. Genome sequence data have been generated for three different A. niger strains, ATCC 1015, ATCC 9029 and CBS 513.88 (Baker 2006). Strains ATCC 1015 and ATCC 9029 are wild type strains, while strain CBS 513.88 has been through limited mutagenesis. A detailed analysis of A. niger strain CBS 513.88 has recently been published (Pel et al. 2007).

The US Department of Energy Joint Genome Institute (JGI) made available a draft sequence of A. niger strain ATCC 1015 in April 2006. Following the release of the draft genome, the JGI-Stanford finishing team began a genome improvement project. Their work has resulted in a nearly complete sequence consisting of 24 scaffolds. At the time of this writing, there are not any internal gaps within these scaffolds that encompass 34.9Mb, 1Mb more than the published nucleotide content of the strain CBS 513.88 genome. While the size difference between the genome can be accounted for by the sequencing approaches and a larger number of intra-scaffold gaps in the strain CBS 513.88 sequence, there are significant differences between strains which are not due to uncaptured genomic sequence. The nucleotide sequence identity between the two strains is 99.3 % (Table 2).

The sequenced A. niger strains also differ in their morphology when grown on solid media. A. niger ATCC 1015 is characterised by dark black conidiophores with long strings of connected spores. In contrast, A. niger CBS 513.88 is brown in colour, has conidiophores bearing only a limited number of spores and has a sectored colony morphology. The phenotypic behaviour of the two strains is also different; A. niger ATCC 1015 was originally described in 1917 as a citric acid producing strain (Currie 1917) while CBS 513.88 is descended from NRRL 3122 which is described as an induced mutant with high glucoamylase production (Van Lanen & Smith).

The behaviour, morphology and sequence differences as well as others between strains are evidence that organisms classified as A. niger can differ considerably at both the levels of growth morphology and genome sequence. While for strains ATCC 1015 and CBS 513.88, it may not be readily obvious whether the differences between them are due to speciation, induced mutagenesis or both, the availability of genome sequences for these closely related strains raises the important issue of how differences in genomic and phenotypic characteristics can be used to define species within the black aspergilli.

An international team of researchers associated with the JGI strain ATCC 1015 project as well as the DSM researchers who led the strain CBS 513.88 genome project are currently analyzing and comparing the two genome sequences. One goal of this comparison will be the characterisation of phenotypic, genome organisation and sequence dissimilarities between the two strains. We anticipate cataloguing differences in morphology and extrolite profiles, single nucleotide polymorphisms, insertions, deletions and chromosomal rearrangements. While a definitive answer may not result from the study, the question of whether strains ATCC 1015 and CBS 513.88 are the same species or represent an early speciation event will help to guide the analysis.
Aspergillus fumigatus is the main causative agent of invasive aspergillosis, the most common fungal infection worldwide, and can also cause mycotoxicosis and severe allergic reactions in humans (Denning 1998). The most frequent source of A. fumigatus, a thermotolerant mesophile, is spent compost prepared for growing mushrooms. Aspergillosis, also known as “Mushroom Worker’s Lung Disease”, is a highly lethal invasive disease that affects people with compromised immune function and mushroom pickers working with mouldy compost, but is pathogenic to all humans in concentrated quantities. The therapeutic management options for invasive aspergillosis are limited due to high toxicity, low efficacy rates, and growing drug resistance and even with antifungal therapy the mortality rate is approximately 50 %. Despite medical and agricultural importance of this species, the biology of this human pathogen has only recently been actively investigated by more than a few groups.

Sequencing of the first isolate, strain Af293 (Nierman et al. 2005), has brought many unexpected discoveries including the possibility of a hidden sexual cycle and the presence of an impressive array of secondary metabolism clusters. The A. fumigatus clinical isolates have been shown to vary significantly in their pathogenicity (Paisley et al. 2005) and resistance to antifungals (Denning, unpubl. data). Therefore the availability of the second sequenced strain, A1163, which is a derivative of another clinical isolate CBS144-89/CEA10 (made available through Merck), will provide a unique opportunity to gain new insight into the nature of fungal pathogenicity. A1163 was obtained from CEA17, which is a uridine/uracil auxotroph pyrG mutant of CEA10 (d”Enfert 1996), via the ectopic insertion of the A. niger pyrG gene.

Preliminary comparative genomic analysis shows that the “core” A. fumigatus genome is very conserved as evidenced by the high identity between Af293 and A1163 orthologous sequences and the low number of isolate-specific genes (Tables 1, 2). In addition to this core genome, both isolates contain several small variable loci and large unique regions ranging in size from 10 to 400 Kb. The variable loci are orthologous and highly syntenic, but share very little sequence identity (37–90 % at the protein level). They contain several genes that encode encode NACHT, NB-ARC and Pfs domains predicted to function and non-self recognition and programmed cell death during hyphal fusion between genetically incompatible individuals (Fedorova et al. 2005).

### Table 1. Major characteristics and genetic diversity of the Aspergillus genomes.

<table>
<thead>
<tr>
<th>Species</th>
<th>A. fumigatus</th>
<th>A. niger</th>
<th>A. flavus</th>
<th>A. oryzae</th>
<th>A. fumigatus</th>
<th>N. fischeri</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain/Isolate</td>
<td>Af293</td>
<td>A1163</td>
<td>ATCC 1015</td>
<td>CBS 513.88</td>
<td>NRLL 3357</td>
<td>ATCC 42149</td>
</tr>
<tr>
<td>Genome Size (Kb)</td>
<td>28,81</td>
<td>29,133</td>
<td>36,63</td>
<td>33,976</td>
<td>36,51</td>
<td>37,111</td>
</tr>
<tr>
<td>Difference in size (Kb)</td>
<td>323</td>
<td>2,854</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No assemblies &gt; 5 Kb</td>
<td>19</td>
<td>20</td>
<td>49</td>
<td>19</td>
<td>20</td>
<td>26</td>
</tr>
<tr>
<td>No assemblies &gt; 100 Kb</td>
<td>18</td>
<td>11</td>
<td>23</td>
<td>18</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>Shared genome %</td>
<td>98.8 %</td>
<td>97.7 %</td>
<td>89 %</td>
<td>96.5 %</td>
<td>97.4 %</td>
<td>95.8 %</td>
</tr>
<tr>
<td>Unique genome %</td>
<td>1.20 %</td>
<td>2.30 %</td>
<td>11 %</td>
<td>3.5 %</td>
<td>2.6 %</td>
<td>4.2 %</td>
</tr>
</tbody>
</table>

Number of genes

| Total | 9630 | 9930 | 11200 | 14097 | 13515 | 12074 | 9630 | 10415 |
| Shared (blastp) | 9366 | 9366 | 10158 | 10158 | 10620 | 10620 | 8676 | 8676 |
| Aligned (gmap) | 9422 | 9610 | 10279 | 13242 | 13086 | 11507 | 8606 | 8649 |
| Divergent (80–95 %) | 60 | 102 | 396 | 386 | 151 | 236 | 365 | 340* |
| Unique (gmap) | 148 | 218 | 525 | 469 | 278 | 331 | 954 | 1766 |
| Unique (gmap) % | 1.5 % | 2.4 % | 4.7 % | 3.3 % | 2.1 % | 2.7 % | 9.9 %* | 16.7 %** |

*Alignments with identity from 80 % to 90 % were considered divergent
** Values were calculated for reciprocal best BLAST matches

### Table 2. Percent identity of the Aspergillus genomes at the genome, gene and protein level.

<table>
<thead>
<tr>
<th>Species</th>
<th>Within A. fumigatus</th>
<th>Within A. niger</th>
<th>A. flavus vs. A. oryzae</th>
<th>A. fumigatus Af293 vs. N. fischeri</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genome vs. genome</td>
<td>99.8 %</td>
<td>99.3 %</td>
<td>99.5 %</td>
<td>92.4 %</td>
</tr>
<tr>
<td>CDS vs. genome</td>
<td>99.6 %</td>
<td>99.1 %</td>
<td>99.1 %</td>
<td>94.3 %</td>
</tr>
<tr>
<td>Protein vs. protein</td>
<td>99.5 %</td>
<td>96.7 %</td>
<td>98 %</td>
<td>93.4 %</td>
</tr>
</tbody>
</table>

Intraspécific case study II: A. fumigatus Af293 versus A. fumigatus A1163

Aspergillus fumigatus is the main causative agent of invasive aspergillosis, the most common fungal infection worldwide, and can also cause mycotoxicosis and severe allergic reactions in humans (Denning 1998). The most frequent source of A. fumigatus, a thermotolerant mesophile, is spent compost prepared for growing mushrooms. Aspergillosis, also known as “Mushroom Worker’s Lung Disease”, is a highly lethal invasive disease that affects people with compromised immune function and mushroom pickers working with mouldy compost, but is pathogenic to all humans in concentrated quantities. The therapeutic management options for invasive aspergillosis are limited due to high toxicity, low efficacy rates, and growing drug resistance and even with antifungal therapy the mortality rate is approximately 50 %. Despite medical and agricultural importance of this species, the biology of this human pathogen has only recently been actively investigated by more than a few groups.

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Preliminary comparative genomic analysis shows that the "core" A. fumigatus genome is very conserved as evidenced by the high identity between Af293 and A1163 orthologous sequences and the low number of isolate-specific genes (Tables 1, 2). In addition to this core genome, both isolates contain several small variable loci and large unique regions ranging in size from 10 to 400 Kb. The variable loci are orthologous and highly syntenic, but share very little sequence identity (37–90 % at the protein level). They contain several genes that encode encode NACHT, NB-ARC and Pf domains predicted to function and non-self recognition and programmed cell death during hyphal fusion between genetically incompatible individuals (Fedorova et al. 2005).

While variable loci in Af293 and A1163 are randomly distributed along the A. fumigatus chromosomes, unique regions are located on all chromosomes and display a clear subtelomeric bias. In Af293, the five out of 9 largest genomic islands are located within 300 Kb
from chromosome ends. The unique regions contain mostly isolate-specific genes and numerous repeat elements. Af293-specific islands 1 and 2 house two recent segmental duplications, containing several rapidly evolving genes and apparent pseudogenes.

The Af293 and A1163 genomes have 208 and 320 unique genes, respectively, that aligned poorly or not at all to the other strain's assemblies (Table 1). About 80% of these genes are clustered together in isolate-specific genomic islands containing from 5 to 90 genes. Unexpectedly, more than 60% of Af293-specific genes can be assigned a biological function such as cellular metabolism, secondary metabolism, signalling and transcriptional regulation (Fig. 1A). The high percentage can be explained in part by the fact that the list of specific genes includes one arsenic resistance gene cluster and two putative secondary metabolite biosynthesis gene clusters. A similar pattern is observed for the A1163-specific genes (Fig. 1B).

**Interspecific case study III: A. oryzae versus A. flavus**

*Aspergillus* Section *Flavi* contains two economically important species. Accordingly, species within this section have received considerable attention, making it one of the best-studied groups of filamentous fungi. *A. flavus* is responsible for opening the modern field of mycotoxicology by its production of the potent carcinogen, aflatoxin. This toxin is responsible for millions of dollars in losses in the world and for significant health issues in developing countries, including human mortality. More recently, *A. flavus* has received notoriety as the second leading cause of aspergillosis in immunocompromised individuals. *A. oryzae*, on the other hand, has been widely used in Japanese fermentation industries to produce, *sake* (Japanese alcohol beverage), *miso* (soy bean paste) and *shoyu* (soy sauce) for more than a thousand years. *A. oryzae* secretes large quantities of diverse hydrolytic enzymes important for these fermentations. The extensive use of *A. oryzae* in food fermentation industries, and its long history as a safe food microbe, has lead to industrial applications of *A. oryzae* being listed as Generally Recognised as Safe (GRAS) by the Food and Drug Administration (FDA) in the United States of America (Tailor & Richardson 1979). *A. oryzae* is not considered to be either a plant pathogen or to produce aflatoxin (Murakami et al. 1967). The safety of this organism is also supported by the World Health Organisation (FAO/WHO 1987).

While morphological differences can be used to distinguish between the two species (Klich & Pitt 1985), these differences can be subtle (Thom & Raper 1945; Hesseltine et al. 1970; Wicklow 1983) and controversy remains as whether these two fungi represent separate species or different ecotypes of the same species. It has been suggested that *A. oryzae* is a domesticated ecotype of *A. flavus*. While this is an appealing hypothesis, and genetic and molecular evidence support this hypothesis (Kurtzman...
Comparative genomics and Aspergillus species concepts

et al. 1986; Klich & Mullaney 1987; Cruickshank & Pitt 1990; Geiser et al. 1998; Kumeda & Asao 2001; Montiel et al. 2003), there are little genomic data to support such a close relationship between the two species. The recently available whole genome sequences of A. flavus and A. oryzae present an opportunity to carefully examine two fungi with different ecologies but very similar morphology. We have initiated a comparative genomic analysis of these two species and our preliminary results are revealing some interesting similarities as well as differences. The sequence reads for A. flavus and A. oryzae have been deposited at National Center for Biotechnology Information (NCBI).

The results presented here represent an early peek into the similarities and differences between these two fungi basing on a comparison of their genomes. As may have been predicted from earlier morphological and genetic studies, these two fungi appear similar at the genomic level. The estimated A. flavus genome size of 36.8 Mb is similar to that for A. oryzae (36.7 Mb). Interestingly, the genomes of these two fungi are larger than those of either A. nidulans or A. fumigatus and contain several lineage-specific genes. These lineage-specific genes are extra copies of particular genes specifically existing in the A. oryzae and A. flavus genomes as compared to A. nidulans and A. fumigatus (Machida et al. 2005). In general, these extra homologs are located in non-syntenic blocks, which are less conserved among species, rather than in syntenic blocks (Machida et al. 2005). The high conservation of genes from these two species in the non-syntenic blocks indicates that the two species are genetically close relatives. A comprehensive comparison between the two genomes revealed that these A. flavus/A. oryzae specific genes were acquired before domestication of A. oryzae but not during breeding strains for fermentation. The non-syntenic blocks appear to have been acquired by horizontal gene transfer.

The genes on the non-syntenic blocks of A. oryzae generally have lower transcriptional levels than those on the syntenic blocks (Machida et al. 2005). This has been confirmed by the preliminary DNA microarray analysis (Fig. 2A). Further, the genes on the two blocks are regulated in a different manner as shown in Fig. 2B; most of the gene on syntenic and non-syntenic blocks were induced and repressed at heat shock, respectively. These results suggest that the genes on the two blocks may be regulated by the mechanisms specific to each block.

Comparative analysis between the A. oryzae and A. flavus genomes revealed extremely high similarity in their nucleotide sequences and the amino acid sequences. Sequence analysis comparing genes predicted to code for proteins with over 100 amino acids revealed 306 genes unique to A. flavus and 332 genes unique to A. oryzae. Most of the genes unique to the species have no predicted function. Some, however, are involved in secondary metabolism. A. flavus has 34 polyketide synthases, two more than A. oryzae. While they have the same number of predicted non-ribosomal peptide synthetases (24), each has two that are unique to the species. It remains unclear if these differences observed between the sequenced strains mirror that observed in population structure of these organisms. Natural variation is known to occur in populations of each fungus. For example, in each species there are strains that differ in the completeness of the aflatoxin biosynthetic cluster. As an example, the sequenced strain of A. oryzae contains the putative homolog of the entire gene cluster for aflatoxin biosynthesis, while some strains lack a part or the entire gene cluster. Overall, approximately half of the A. oryzae strains have nearly intact gene clusters. Strains of A. flavus are also known to differ in the completeness of their aflatoxin biosynthesis clusters (Chang et al. 2005).

There is also evidence of differences in gene expression between A. flavus and A. oryzae. While ESTs for all 25 of the aflatoxin pathway genes were found in A. flavus (Yu et al. 2004), no ESTs of these genes were detected in A. oryzae except for aflJ and norA (Akao et al. 2007). Mutations in some putative binding sites for the known transcription regulators may account for the lower expression levels of some genes (Tomina et al. 2006). A truncation mutation of the pathway specific regulator aflR is known to prevent expression of the aflatoxin biosynthesis homologs in A. sojae. However, complementation of the mutated aflR with an intact copy did not restore the ability of the fungus to produce aflatoxin (Matsushima et al. 2001; Takahashi et al. 2002).

It is possible that other mutations may have been introduced into the A. oryzae genome preventing the expression of most secondary metabolism genes. Recently, detailed sequence analysis of the aflR genes from A. oryzae and A. flavus revealed phylogenetic differences of sequence between the two species (Chang et al. 2006). These results indicate that consideration of particular phenotypes, which includes productivity of some metabolites, should be a useful means to distinguish species efficiently and accurately.

The availability of both oligo-based and Affymetrix GeneChip DNA microarrays provide a new genomic tool to examine genome
wide differences between A. flavus and A. oryzae. Such experiments are currently in progress. The observation that A. oryzae fails to produce many of the secondary metabolites that are present in A. flavus suggests that several genes will be differentially expressed between the two fungi. Affymetrix GeneChip DNA microarrays also provide a powerful tool to examine gene content and polymorphism among isolates of A. flavus and A. oryzae. For example, the high degree of DNA correspondence between A. flavus and A. oryzae allows the use of the A. flavus DNA microarrays for CGH (Comparative Genome Hybridisation) analysis across strains of both A. flavus and A. oryzae. Data obtained from a comparison between the two sequenced strains of A. flavus and A. oryzae show the power of this technique. The number of unique genes predicted for each strain by CGH was very similar to that predicted by sequence annotation. The difference between the two techniques was less that 10 % (+/- 3 %, a = 0.05). Currently three strains of each species are being compared for similarity.

In summary, genomic resources provide a new and powerful tool for distinguishing among closely related fungal species. An available genome sequence for both A. flavus and A. oryzae, and whole genome arrays for both fungi provide a means to carefully interrogate these two fungal species and learn more about their genetic relatedness, their evolution, and possibly the effect of domestication on changes in their genome.

Interspecific case study IV: A. fumigatus AF293 versus N. fischeri

Neosartorya fischeri (the teleomorph of A. fischerianus), a very close homothallic sexual relative to A. fumigatus, is found in soil, and its spores are found in many agricultural products. N. fischeri can cause keratitis and possibly pulmonary aspergillosis in transplant patients, but is an extremely rare invasive pathogen (Gerber et al. 1973; Chim et al. 1998). Its inadequacy as a pathogen is interesting in light of its close evolutionary relationship to A. fumigatus, and so comparison of the N. fischeri and A. fumigatus genomes should yield significant clues regarding A. fumigatus virulence and epidemiology. In addition, N. fischeri has a known sexual cycle, and elucidation of A. fumigatus sexual reproduction by comparative genomics would be of immense value to the Aspergillus research community. This would greatly advance A. fumigatus as an experimental system by facilitating genetic studies.

The J. Craig Venter Institute (JCVI) has sequenced the type culture of N. fischeri NRRL 181 by the whole genome random sequencing method (Nierman et al. 2005), using single spore subculture for genomic DNA extraction. Preliminary analysis of the N. fischeri genome, which was made available by the JCVI, has shown that it (32.6 Mb) is 10 % larger than the A. fumigatus AF293 genome (Table 1). There are currently 10 415 predicted protein-coding genes with a mean gene length of 1 466 bp. Comparisons to the genomes of A. fumigatus AF293 revealed 1 739 genes unique to N. fischeri, including several mycotoxin biosynthesis gene clusters. Other notable findings include a large number of transposable elements, which may have contributed to the genome size expansion observed in this species.

In contrast to the intraspecific comparison between A. fumigatus strains discussed above, the genomes of A. fumigatus and N. fischeri are much more divergent (Tables 1, 2). Only 35 % of the A. fumigatus-specific genes can be assigned any biological function (Fig. 1C). Comparison of the A. fumigatus – N. fischeri pair with the A. oryzae – A. flavus comparison, gives support to the proposal that A. oryzae represents a distinct ecotype of A. flavus and not a distinct species (Geiser et al. 1998). Firstly, the identity between orthogonal sequences is ~5–7 % lower for the A. fumigatus – N. fischeri pair than the A. oryzae – A. flavus pair (Table 2). Secondly, whereas more than 10 % of A. fumigatus and N. fischeri genes are species-specific, only < 3 % of genes are species-specific in the A. oryzae – A. flavus comparison, a value remarkably similar to the number of strain-specific genes exhibited by the intraspecific comparison in A. fumigatus.

CONCLUSIONS

These four comparisons highlight the potential usefulness of genomics for the accurate identification of species boundaries in the genus Aspergillus. Genomics can aid Aspergillus taxonomy by serving as a source of novel and unprecedented amounts of comparative data, as a resource for the development of additional diagnostic tools, and finally as a knowledge database about the biological differences between strains and species. It is unlikely that genomics, or any other discipline for that matter, will come up with a golden rule the application of which will solve all taxonomic problems in the clade. Rather, our hope is that genomics can provide an arsenal of data and molecular tools to aid taxonomists’ quests for a more accurate delineation of species boundaries in the genus Aspergillus.

ACKNOWLEDGEMENTS

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REFERENCES


Sexual and vegetative compatibility genes in the aspergilli

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Abstract: Gene flow within populations can occur by sexual and/or parasexual means. Analyses of experimental and in silico work are presented relevant to possible gene flow within the aspergilli. First, the discovery of mating-type (MAT) genes within certain species of Aspergillus is described. The implications for self-fertility, sexuality in supposedly asexual species and possible uses as phylogenetic markers are discussed. Second, the results of data mining for heterokaryon incompatibility (het) and programmed cell death (PCD) related genes in the genomes of two heterokaryon incompatible isolates of the asexual species Aspergillus niger are reported. Het-genes regulate the formation of anastomoses and heterokaryons, may protect resources and prevent the spread of infectious genetic elements. Depending on the het locus involved, hetero-allelism is not tolerated and fusion of genetically different individuals leads to growth inhibition or cell death. The high natural level of heterokaryon incompatibility in A. niger blocks parasexual analysis of the het-genes involved, but in silico experiments in the sequenced genomes allow us to identify putative het-genes. Homologous sequences to known het- and PCD-genes were compared between different sexual and asexual species including different Aspergillus species, Sordariales and the yeast Saccharomyces cerevisiae. Both het- and PCD-genes were well conserved in A. niger. However some point mutations and other small differences between the het-genes in the two A. niger isolates examined may hint to functions in heterokaryon incompatibility reactions.

Key words: apoptosis, ascomycete, Aspergillus fumigatus, Aspergillus nidulans, Aspergillus niger, heterokaryon incompatibility, MAT, mating type, Neurospora crassa, Podospora anserina, Saccharomyces cerevisiae, self/non-self recognition.

INTRODUCTION

Self and non-self recognition is a common requirement for all living organisms. Most taxa have developed specific systems to identify self and non-self. Fungi exhibit two types of compatibility systems based on self and non-self recognition, namely sexual compatibility and somatic or heterothallic (in)compatibility (Dyer et al. 1992, Leslie 1993). Such systems are of great significance as they govern the degree to which gene flow can occur between members of a species, with consequences for evolution and recognition of species (Taylor et al. 1999a). Maintaining gene flow within a species is advantageous as this may lead to increased genotypic variation allowing adaptation to changing environments (Milgroom 1996). However, unrestricted gene flow may lead to resource plundering by other genotypes (Debets & Griffiths 1998).

Many filamentous fungi are able to reproduce both sexually and asexually, depending on environmental conditions (Dyer & Paoletti 2005). In a nutritionally rich environment fungi generally produce mitotic spores (the anamorphic stage), but when conditions become unfavourable for vegetative growth, they may initiate sexual reproduction (teleomorphic stage). There are two main types of sexual breeding system evident in fungi: homothallism and heterothallism (Dyer et al. 1992). Homothallic strains are self-fertile, though may also be capable of outcrossing, whereas heterothallic strains are self-incompatible and require the presence of a compatible mating partner for the sexual cycle to occur. In addition there are numerous species which are apparently restricted to propagation by asexual means with no known sexual cycle (Taylor et al. 1999). Sexual compatibility in heterothallic fungi is governed by so called "mating-type" (MAT) genes, with two mating types MAT1-1 and MAT1-2 present in heterothallic filamentous ascomycetes (Turgeon & Yoder 2000). These differ according to DNA present at a single MAT locus, with highly divergent DNA sequence (termed an "idiomorph") present in isolates of opposite mating type. By convention MAT1-1 isolates contain an idiomorph including a MAT gene encoding a protein with an alpha-box domain. In contrast, MAT1-2 isolates contain an idiomorph including a MAT gene encoding a regulatory protein with a specific high mobility group (HMG) type DNA-binding domain (Turgeon & Yoder 2000). Mating-type genes regulate initial stages of the mating process such as pheromone signalling and plasmogamy leading to the production of ascogenous hyphae in ascomycetes, and may also have roles in later intranuclear recognition (Lengeler et al. 2000, Coppin et al. 2005, Debuchy & Turgeon 2006). Intriguingly, MAT genes have also been shown to be required for sexual reproduction in homothallic ascomycetes (Paoletti et al. 2007). Mating-type genes also have other functions in some species, for example affecting vegetative incompatibility in Neurospora crassa (Glass et al. 1988).

The lack of a known sexual cycle does not mean automatically the lack of recombination. Indeed, there is evidence of genotypic diversity and gene flow in some supposedly asexual fungal species (Geiser et al. 1998, Paoletti et al. 2005). One possible explanation for such observations is that during the vegetative state of its life a fungus is able to undergo hyphal fusion, karyogamy and mitotic recombination, in the so-called "parasexual cycle" (Pontecorvo 1956). Finding an appropriate partner for mitotic recombination is a crucial aspect of the parasexual cycle, similarly to the sexual mating process. A complex set of heterokaryon-incompatibility genes and associated network of cellular machinery are responsible for the...
acceptance or rejection of partners in parasexuality (Glass et al. 2000, Saupe 2000).

There are four steps in this parasexual cycle, which can be distinguished by the gene sets governing the steps (Fig. 1) (Leslie and Zeller 1996). The initial step, named pre-fusion, is controlled by genes involved in pheromone production and receptors, and in heterokaryon self-incompatibility (hsi genes). The fusion step, in which the interhyphal anastomoses formed and results in a heteroplasm, is governed by self/non-self recognition genes, like heterokaryon incompatibility (het) (Glass and Kulda 1992) or vegetative incompatibility (vic) genes (Leslie 1993). Effects of het-genes can be influenced by modifier (mod) genes. After fusion the biochemical pathways leading to non-self recognition and cell death may be influenced by genes including suppressor (sup) genes, which can modify the signal. In the final step programmed cell death (PCD) genes initiate processes leading to apoptosis.

The precise function(s) of heterokaryon incompatibility in fungi is not yet clear. There are three main theories to explain the existence of these vegetative self/non-self recognition systems in fungi. One theory, the so called allorecognition hypothesis, proposes that it is a bona fide reaction between genetically different individuals, which may limit the spread of harmful cytoplasmic or nuclear elements (Calen 1972) or prevent resource plundering (Debets and Griffiths 1998). In these examples the role of het-genes is to preserve genetic individuality. The second, alternative, theory suggests that het-genes simply arose accidentally during evolution. The existence of genes with dual function, like the mat-a/mat-A loci in N. crassa (Saupe 2000), supports the second theory. These genes sometimes behave as het-genes, whilst fulfilling other cellular functions at other times. Finally it has been suggested that vegetative incompatibility may promote the initiation of sexual reproduction in some species as a result of non-self recognition (Dyer et al. 1992).

There have been a limited number of studies attempting to test these hypotheses. For example, if het-genes are present to prevent formation of heterokaryons between genetically different individuals [i.e. to protect a local population from "invaders"] then an evolutionary trend towards generation of new alleles might be predicted, to avoid "pollution" of the isolates belonging to the same incompatibility groups (Saupe et al. 1994). However, population genetic modelling suggests that selection favouring more than a few alleles is expected to be extremely weak (Nauta and Hoekstra 1993). In contrast to positive selection in non-allelic systems, certain het loci are under maintaining/balancing selection. A well studied example is the N. crassa het-c with three known alleles. These alleles show trans-species polymorphism and balancing selection (Wu et al. 1998).

In this article, we describe experimental work assessing the occurrence of genes relating to mating and heterokaryon incompatibility processes in the aspergilli, and their possible roles in gene flow within species.

**Occurrence of mating-type genes in the aspergilli**

Although mating-type genes were first reported from filamentous fungi by Glass et al. in 1988, it has proved difficult to clone such genes by traditional molecular biology methods due to high sequence divergence between major ascomycete lineages. Indeed, the only regions conserved across divergent taxa are the alpha-domain in MAT1-1 family proteins, and the HMG-domain in MAT1-2 proteins, and even these show relatively poor sequence conservation (see examples in Debuchy & Turgeon 2006). This prevented initial attempts to identify MAT genes from Aspergillus species (e.g. Arie et al. 1997). However, two major developments in recent years have allowed mating-type genes to be identified from the aspergilli. The first was the use of a degenerate-PCR approach, using primers designed to anneal to conserved sequence present at the HMG MAT1-2 locus, to amplify MAT1-2 sequence from species of Aspergillus (Singh et al. 1999, Paoletti et al. 2005, 2007). The second was the public release of genome sequence data from certain species of Aspergillus, meaning that whole genomes could be screened by bioinformatic approaches (e.g. BLAST searches) to determine the presence of MAT genes (Archer & Dyer 2004, Galagan et al. 2005, Pel et al. 2007). The use of these two techniques has lead to a series of major discoveries concerning sexuality and gene flow in Aspergillus species, with implications for species identity, as will be described.

**Mating-type genes in sexual aspergilli**

The first MAT gene to be reported from the aspergilli was an HMG-domain family gene from the homothallic sexual species A. nidulans (teleomorph Emericella nidulans), which was identified by a degenerate PCR approach (Dyer 2002; Paoletti et al. 2007). Subsequent BLAST searching of the A. nidulans genome revealed the presence of an alpha-domain family gene within the same genome (Dyer et al. 2003). The genes were found to be on different chromosomes and were named MAT1 (alpha-domain on chromosome 6) and MAT2 (HMG-domain on chromosome 3) to recognise the fact that they occupied different genetic loci (Turgeon & Yoder 2000, Galagan et al. 2007). This compares to other homothallic ascomycetes in which alpha- and HMG-domain MAT genes, if both present, have most often been found linked at the same single MAT locus (DeBuchy & Turgeon 2006). It was suggested by Galagan et al. (2005) that the arrangement of MAT loci in A. nidulans may have arisen as a result of a chromosome break and translocation event(s) from an ancestral single Aspergillus MAT locus. The role of the MAT genes in A. nidulans was subsequently investigated by Paoletti et al. (2007) who used gene deletion/replacement, overexpression and RNA interference approaches to demonstrate that both MAT1 and MAT2 genes are required for completion of the sexual cycle in A. nidulans. ΔMAT1 and ΔMAT2 gene deletant mutants were unable to form ascospores,
although sterile cleistothecia were produced. This was a significant discovery, as it showed that MAT genes, normally associated with control of sexual compatibility in heterothallic species, are also required for sexual development in this model homothallic species. The only other MAT genes to be identified from a known sexual species of Aspergillus has been the identification of both alpha- and HMG-domain genes again together within the genome of the homothallic species *Neosartorya fischeri* (Rydholm et al. 2007). The genes were present at unlinked loci, and where therefore termed MAT1 and MAT2 respectively. However, the arrangement and synteny of MAT loci differed from that seen in *A. nidulans*, and it was suggested that homothallism in this species had arisen by a segmental chromosome duplication and translocation event (Rydholm et al. 2007). There have so far been no reports of MAT gene isolation from heterothallic *Aspergillus* species, reflecting the fact that the vast majority of sexual aspergilli are homothalllic, with only four heterothallic species so far identified (Dyer 2007).

**Mating-type genes in asexual aspergilli**

Ironically, all the other MAT genes reported from the aspergilli have come from asexual species, which supposedly lack a sexual cycle. The detection of MAT genes in asexual species might appear surprising, but is thought to reflect the fact that asexual species have evolved from sexual ancestors by loss of sexuality (Geiser et al. 1996), therefore "sex-related" genes may be retained in the genome though prone to mutation and loss by genetic drift. Both genomic and experimental work was first used to identify apparently functional (i.e. lacking any frameshift or stop codon mutation) MAT genes from the opportunistic pathogen *A. fumigatus* (Pöggeler 2002, Varga 2003, Dyer and Paoletti 2005, Paoletti et al. 2005). The species was later shown to contain a complement of other genes required for sexual reproduction (Galagan et al. 2005; Nieman et al. 2005). Intriguingly a survey of 290 worldwide clinical and environmental isolates of *A. fumigatus*, using a newly-developed multiplex mating-type PCR diagnostic, revealed that all isolates contained either a MAT1-1 alpha-domain gene or a MAT1-2 HMG-domain gene and that MAT1-1 and MAT1-2 genotypes were present in a near 1:1 ratio (Paoletti et al. 2005). This resembled the pattern that might be expected to be seen in a heterothallic sexual species. Furthermore, Paoletti et al. (2005) showed that mating-type, pheromone-precursor and pheromone-receptor genes were expressed, again consistent with heterothallism. In parallel work using either degenerate PCR or genomic screening, MAT genes have also been identified from other "asexual" species including *A. oryzae* (Galagan et al. 2005), *A. niger* (Pel et al. 2007), *A. clavatus*, *A. sojae*, *A. flavus* and *A. parasiticus* (Dyer 2007). Significantly isolates of both MAT1-1 and MAT1-2 genotype have been detected in near equal number for many of these species, the only exception being from the *A. niger* "black aspergilli" group which shows a strong bias towards isolates containing an alpha-domain MAT1-1 family gene (*Dyer 2007; Varga, Kocsbél, Pál, Debets, Eyres, Baker, Samson & Dyer unpubl. data). *A. niger* was also shown to contain a complement of genes required for sexual reproduction, although possible mutation was evident in at least one gene (Pel et al. 2007).

Taken as a whole, these results are highly significant because they suggest that certain "asexual" aspergilli might retain a latent ability for sexual reproduction. Indeed, there is evidence from population genetic studies of *A. flavus* (Geiser et al. 1998) and *A. fumigatus* (Dyer & Paoletti 2005, Paoletti et al. 2005, Pringle et al. 2005) of high genetic diversity and genetic recombination within populations of these species. It is possible that this is a result of meiotic exchanges in the near past. However, there is also the tantalising possibility that these, and perhaps other aspergilli categorised as "asexual", might posses an extant cryptic sexual state which has yet to be identified. This perhaps might be a result of a slow decline in sexual fertility in the majority of isolates due to selection for asexuality, but the retention of fertile isolates as a subset of wild populations (Dyer & Paoletti 2005).

**Mating-type genes and species identity**

Because MAT genes evolve at a relatively fast rate it has been suggested that they might be particularly suited to phylogenetic analysis to resolve species identity and inter-species taxonomic relationships (Turegno 1998). At present there is insufficient MAT sequence data available to allow meaningful phylogenies to be constructed in the aspergilli. A further obstacle is that it would be necessary to obtain homologous MAT sequence from all species under examination (e.g. MAT1-2 gene sequence from all species) and for some species the necessary MAT1-1 or MAT1-2 data may be lacking as the majority of isolates might be of the opposite mating type. However, there remains the prospect that accumulating MAT data may provide a means to resolve closely related aspergilli taxa, complementing the use of other genes and markers presently used in phylogenetic studies.

**Genetic control of heterokaryon incompatibility in ascomycete fungi**

For filamentous fungi the establishment of hyphal anastomoses, both within and between individuals of the same species, is considered to be of high importance. A limitation to intermycelial fusions is heterokaryon incompatibility which is widespread among fungi and prevents the coexistence of genetically dissimilar nuclei within a common cytoplasm. The adaptive significance of heterokaryon incompatibility is unclear but it may serve to limit the spread of detrimental cytoplasmic or nuclear elements (Caten 1972) or prevent resource plundering (Debets & Griffiths 1998). A het locus can be any locus, at which heteroalleles is not tolerated in a heterokaryon (*Saupe 2000*). When heterokaryon-incompatible strains fuse, the resulting heterokaryotic hyphae are rapidly compartmentalised and destroyed (often with surrounding cells) or seriously inhibited in their growth, depending on the involved het locus. Heterokaryotic cells are often destroyed within 30 min after hyphal fusion. The process of destruction of hyphal compartments shows similarity at the microscopic level in different fungi, and some steps have common features even with multicellular metazoan programmed cell death (PCD) (Glass & Kaneko 2003).

Since heterokaryon incompatible strains can be sexually compatible, the number of het-genes that segregate from a cross can be deduced from the progeny. Genetic analysis of heterokaryon incompatibility was performed in a few sexual fungi. The number of identified het-genes varied with species. There are at least eight and maximum 18 het loci in *A. nidulans* (Anwar et al. 1993), six in *Cryphonectria parasitica* (Cortesi & Milgroom 1998), at least 11 in *N. crassa* (Glass et al. 2000) and nine in *P. anserina* (*Saupe 2000*). The majority of the incompatibility reactions is regulated by allelic systems, where two (e.g. mat-a/mat-A in *N. crassa*, Saupe 2000) or more alleles (e.g. het-c Groveland, Oakridge and Panama alleles in *N. crassa*, Sarkar et al. 2002) of the same locus interact. In other cases, two distinct loci trigger non-allelic incompatibility (e.g.
het-cl/het-e in *P. anserina*, Saupe 2000). In non-allelic systems, incompatible alleles can be present in the same haploid nucleus in the progeny and thus vegetative incompatibility may occur also in homokaryotic cells, like in the het-r/het-v incompatibility in *P. anserina*. Such homokaryotic strains can be obtained for each non-allelic system, and are named self-incompatible (SI) strains (Bourges et al. 1998).

### Identification of heterokaryon (in)compatibility related genes in the aspergilli

Unlike ascomycete species which have a sexual and usually also an asexual reproduction cycle, for many asexual aspergilli the only way to achieve (mitotic) recombination is via the parasexual cycle. There is evidence from studies of field isolates of species including *A. niger*, *A. terreus*, *A. versicolor*, *A. glaucus* and *E. nidulans* that heterokaryon incompatibility is widespread in the aspergilli (Croft & Jinks 1977). Mitotic recombination has been used for genetic analysis of mutants in an isogenic background of *A. niger*. Mitotic recombination is via the parasexual cycle. As a result, mitotic recombination between genetically dissimilar isolates is also blocked, so genetic analysis cannot reveal the genetic basis of heterokaryon incompatibility in *A. niger*.

In this study we present the results of data mining in the genomes of two heterokaryon incompatible isolates of the asexual *A. niger* species. We compared incompatibility and cell death related proteins of the two *A. niger* isolates with each other and similar proteins of related sexual (*A. nidulans*) and asexual aspergilli (*A. fumigatus*, *A. oryzae*, *A. terreus*), two members of the Sordariales (P. anserina, *N. crassa*) and the yeast *Saccharomyces cerevisiae*. Our analyses identified the major putative het-genes in the genome of *A. niger* and related aspergilli. These findings can be used for further functional analysis of candidate het-genes.

### In silico comparison of yeasts and filamentous fungi

A list of known genes involved in either programmed cell death from *S. cerevisiae* or involved in heterokaryon incompatibility and/or programmed cell death in *N. crassa* or *P. anserina* was constructed based on the literature (Table 1). Protein forms of genes were blasted against genomic databases: *A. fumigatus* preliminary sequence data was obtained from The Institute for Genomic Research website. The *A. nidulans*, *N. crassa* (release 7) and *A. terreus* sequence data were from the Aspergillus nidulans, Neurospora crassa and Aspergillus terreus Sequencing Projects, Broad Institute of MIT and Harvard. The *A. oryzae* sequences were available on the server of National Institute of Technology and Evaluation (NITE). The *P. anserina* genome was published by the Institut de Génétique et Microbiologie (Université de Paris-Sud Espagne). The *A. niger* ATCC1015 sequence data were produced by the US Department of Energy Joint Genome Institute and *A. niger* CBS513.88 sequence data by the DSM Research BV.

### Table 1. Genes used in this study involved in heterokaryon incompatibility in *N. crassa* and *P. anserina* and in Programmed Cell Death (PCD) in *S. cerevisiae*. All genes are given with their presumed function and references. ID numbers for *N. crassa* and *S. cerevisiae* proteins refer to the numbers given in their respective sequencing projects, the ID numbers for the *P. anserina* proteins were taken from GenBank. The table is an expanded v. of the table in Glass and Kaneko (2003).

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<tr>
<th>Species</th>
<th>Class</th>
<th>Gene</th>
<th>Function</th>
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<td><em>N. crassa</em></td>
<td>Heterokaryon incompatibility genes</td>
<td><em>het-6</em>&lt;sup&gt;r&lt;/sup&gt; (NCU03533.2)</td>
<td>allelic het-gene, TOL/HET-6/HET-E domain</td>
<td>Saupe 2000</td>
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<td><em>het-c</em> (NCU3125.2)</td>
<td>allelic het-gene, signal peptide</td>
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<td>un-24 (NCU03539.2)</td>
<td>allelic het-gene, ribonucleotide reductase large subunit</td>
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<td>Suppressor genes</td>
<td>tol (NCU04453.2)</td>
<td>TOL/HET-6/HET-E domain</td>
<td>Shiu &amp; Glass 1999</td>
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<td></td>
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<td>vfb-1 (NCU03725.2)</td>
<td>regulation of conidiation and maybe of nrAPase</td>
<td>Xiang &amp; Glass 2002</td>
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<td></td>
<td>Incompatibility related genes</td>
<td><em>ham-2</em> (NCU03727.2)</td>
<td>hyphal fusion, putative transmembrane protein</td>
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<td>pin-c (NCU03494.2)</td>
<td>allelic gene with HET-domain, linked to <em>het-c</em></td>
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<td><em>mr-a</em> (NCU07887.2)</td>
<td>suppresses un-24 temperature sensitive mutation, ribonucleotide reductase small subunit</td>
<td>Kotierk &amp; Smith 2001</td>
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<td><em>P. anserina</em></td>
<td>Heterokaryon incompatibility genes</td>
<td><em>het-c2</em> (AA20542)</td>
<td>nonallelic het-gene interacts with <em>het-d</em> and <em>het-e</em>, glycolipid transfer protein</td>
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<td><em>het-d</em>&lt;sup&gt;y&lt;/sup&gt; (AAL37301)</td>
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<td><em>het-e</em>&lt;sup&gt;s&lt;/sup&gt; (AAL37297)</td>
<td>nonallelic het-gene against <em>het-c2</em>, GTP-binding, WD repeat, TOL</td>
<td>Espagne et al. 2002</td>
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### Table 1. Sexual and vegetative compatibility genes in the Aspergilli

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<td>idi-1</td>
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<td>idi-7</td>
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<td>rsm23</td>
<td>mitochondrial small ribosomal unit</td>
<td>Madeo et al. 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sart1</td>
<td>ER to Golgi transport</td>
<td>Ligr et al. 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>stm1</td>
<td>suppressor of pop2 and tom2</td>
<td>Ligr et al. 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tor1</td>
<td>regulation of cell death, phosphatidylinositol 3-kinase</td>
<td>Rohde et al. 2001</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td></td>
<td></td>
<td></td>
<td>Dementhon et al. 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fitzgibbon et al. 2005</td>
</tr>
</tbody>
</table>

### Table 2. Genome databases and their websites used in this research.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Website</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. fumigatus</td>
<td>AF293</td>
<td><a href="http://tigrblast.tigr.org/er-blast/index.cgi?project=afu1">http://tigrblast.tigr.org/er-blast/index.cgi?project=afu1</a></td>
<td>Nierman et al. 2005</td>
</tr>
<tr>
<td>A. niger</td>
<td>ATCC1015</td>
<td><a href="http://genome.jgi-psf.org/cgi-bin/runAlignment?id=Aspn1&amp;advanced=1">http://genome.jgi-psf.org/cgi-bin/runAlignment?id=Aspn1&amp;advanced=1</a></td>
<td>DOE-JGI</td>
</tr>
<tr>
<td>N. crassa</td>
<td>OR74A</td>
<td><a href="http://www.broad.mit.edu/annotation/genome/neurospora/Blast.html">http://www.broad.mit.edu/annotation/genome/neurospora/Blast.html</a></td>
<td>Galagan et al. 2003</td>
</tr>
<tr>
<td>P. anserina</td>
<td>S</td>
<td><a href="http://podospora.igmors.u-psud.fr/blast_ch.html">http://podospora.igmors.u-psud.fr/blast_ch.html</a></td>
<td>P. anserina genome project</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>S28C</td>
<td><a href="http://seq.yeastgenome.org/cgi-bin/nph-blast2sgd">http://seq.yeastgenome.org/cgi-bin/nph-blast2sgd</a></td>
<td>Saccharomyces Genome Database</td>
</tr>
</tbody>
</table>
The in silico experiments performed with the different sequenced and available genomes show that the majority of the PCD genes from \textit{S. cerevisiae} have homologs in the filamentous fungi, only \textit{hef10} and \textit{hel 13}, whose functions are unknown, are mostly missing (Table 3). In contrast the majority of vegetative incompatibility and cell death related genes from \textit{N. crassa} and \textit{P. anserina} can be found in the other filamentous fungi, but many of them, like \textit{het-6}, \textit{het-c}, \textit{tol}, \textit{vib-1}, \textit{pin-c}, \textit{het-c2}, \textit{het-s}, \textit{id}-1, \textit{id}-2, \textit{idi}-3, \textit{idi}-4 and \textit{mod-A}, are missing in the baker's yeast. Yeast and filamentous fungi diverged approximately 1.1 billion years ago (Cai et al. 2006) and yeast have a different, mainly unicellular, life style. Only the \textit{HET-D} and \textit{HET-E} proteins which have GTP binding capacity and the so-called WD (tryptophan-aspartate) repeats (Espagne et al. 2002) yielded many homologous sequences in \textit{S. cerevisiae}. Of course these genes may have pleiotropic functions and may have a different function in \textit{S. cerevisiae} than controlling anastomosis formation. The \textit{id}-7 gene of \textit{P. anserina} is classified as being an ortholog of the \textit{S. cerevisiae aut7p} gene coding for a protein whose binding to the membrane represents an early step in vesicle formation (Lang et al. 1998). Not surprisingly this protein is quite well preserved between both filamentous fungi and yeast as are the genes coding for ribonucleotide reductases and serine proteases.

Some genes like the un-24 gene, known from \textit{N. crassa} to be involved in heterokaryon incompatibility and coding for the ribonucleotide reductase large subunit, are well conserved in the filamentous fungi and have a highly similar homolog in \textit{S. cerevisiae} (Supplementary Table 1). Whereas the putative transmembrane protein \textit{HAM-2} is very well conserved within the filamentous fungi, it differs considerably from the \textit{S. cerevisiae} homolog.

These results show that most of the genes involved in programmed cell death are well conserved among both the filamentous fungi and the yeast \textit{S. cerevisiae}, but that many genes involved in heterokaryon incompatibility are not.

\textbf{In silico comparison of \textit{Sordariales} and \textit{Eurotiales}}

\textit{N. crassa} and \textit{P. anserina} belong to the Sordariales, whereas the asperrigill belong to the Eurotiales. Comparing the incompatibility/ apoptosis gene sets between these two groups of filamentous fungi (see Table 3 and Suppl. Table 1), the most remarkable difference is shown by \textit{P. anserina}, bearing many more \textit{id} (induced during incompatibility) gene homologs than other fungi and missing the suppressor gene \textit{stm1}. Among these fungi, \textit{P. anserina} bears the most different gene set and the largest set of genes with \textit{het}-domains: HET-6 has 35, HET-D 94, PIN-C 51 and TOL 48 homologs below the threshold of e-10. Some of these homologous sequences overlap between the different genes due to their conserved \textit{het}-domains. A possible explanation for this can be in its life cycle. \textit{P. anserina} is a saprophytic fungus, which feeds on partially digested materials in the dung of herbivorous animals. As a coprophilic fungus it grows in synchrony and under rather high density with competitors for the same ephemeral and limited substrate. Therefore, the risk of exploitation or genetic infection by others may be relatively high in comparison to most other fungi. An efficient way to limit exchange of genetic materials is heterokaryon incompatibility, which process is governed by the so called \textit{het}-genes. In \textit{P. anserina} the majority of the incompatibility reactions is due to non-allelic interactions (exception is het \textit{sisthet-S} reaction). \textit{Het-d} and \textit{het-e} trigger incompatibility with the \textit{het-c2}. Both \textit{het-d} and \textit{het-e} encode HET domain proteins, and due to the presence of this domain, these proteins seem to be responsible for nonself recognition in filamentous fungi, including \textit{P. anserina} (Kaneko et al. 2006). Whether the huge number of HET domain protein homologs thus reflects the relative importance of fusion-rejection systems in the life history of \textit{P. anserina} is unclear, though this has been suggested for coprophiles (Buss 1982). Of course, het-domain containing genes also may have other functions than just heterokaryon incompatibility reactions and there certainly seems to be a large family of het-domain genes.

Comparing the phylogenies of different proteins in the different species (see e.g. Fig. 2 with the HET-6 gene homologs from \textit{A. niger} and \textit{N. crassa}) one can see that the homologs of these genes show old polymorphisms. The sequences found in the \textit{A. niger} strains are often very similar. The most similar homologs in \textit{N. crassa}, however, can be quite different from the \textit{A. niger} sequences. Old duplications of the ancestor genes with possible new functions are also visible in the phylogenies.

Within the tested set of proteins, there are no exclusive proteins for members of the \textit{Sordariales}. Only the MOD-A protein has but one hit in the asperrigilli: the protein blast in \textit{A. oryzae} resulted in one hit with only low similarity (e-10) (Suppl. Table 1).

\textbf{In silico comparison of the different asperrigilli}

\textit{S. cerevisiae} PCD- and \textit{N. crassa} and \textit{P. anserina} HET-, modifier and suppressor protein sequences were used to search the genomes of \textit{A. fumigatus} \textit{Af 293}, \textit{A. nidulans} FGSC A4, \textit{A. oryzae} RIB40, and \textit{A. terreus} NIH2624 for homologs (Table 2). Bi-directional best hit analyses were performed with as criterion for homologs an accepted E-value of e-10.

Comparing the different \textit{Aspergillus} species for their putative heterokaryon incompatibility and cell death related proteins, there are no large differences between the species in the presence of certain proteins, in agreement with previous findings (Fedorova et al. 2005; Table 3). But, in the number of homologs of HET-6, TOL and TOL-related PIN-C proteins there is a surprisingly big difference between the asexual and (supposedly) sexual lines. Whereas there are at least 10 HET-6, 9 TOL and 11 PIN-C homologs in the asexual strains, in \textit{A. nidulans} and \textit{A. fumigatus} we found only 2 and 3 HET-6, 0 and 1 TOL, and 0 and 1 PIN-C homologs respectively (Suppl. Table 1). This finding could be explained by the capability of sex: \textit{A. nidulans} is able to reproduce sexually, and there are indirect proofs for the presence of a sexual life cycle in \textit{A. fumigatus} (Varga 2003, Paoletti et al. 2005). The other asperrigilli (\textit{A. niger}, \textit{A. oryzae}, \textit{A. terreus}) are known as asexual species. The question in this case is why do asexual species bear much more HET domain genes? As it was mentioned above these genes are the main components of non-allelic incompatibility, therefore there could be a disadvantage for fungi with a sexual cycle to have such genes.

Another reason could be the proposed function for heterokaryon incompatibility in limiting the spread of detrimental cyttoplasmic or nuclear elements (Caten 1972). In \textit{A. niger} and related black \textit{Aspergillus} species dsRNA mycoviruses occur in approximately 10 % of the natural isolates. These mycoviruses are effectively transferred to all asexual spores (van Diepeningen et al. 2006). Tests showed that heterokaryon incompatibility indeed efficiently blocks the transfer of mycoviruses in these black asperrigilli (van Diepeningen et al. 1997). In sexual \textit{A. nidulans} no dsRNA viruses were found in nature and also here artificially introduced viruses efficiently find their way to the asexual spores. However, when sexual spores are produced the mycoviruses are excluded from the offspring (Coenen et al. 1997). Therefore \textit{A. nidulans} has an extra option to get rid of parasitic elements through its sexual cycle and thus heterokaryon incompatibility may be less important between \textit{A. nidulans} strains.
Table 3. HET and incompatibility related proteins in filamentous fungi and yeast. ID numbers for *N. crassa* and *S. cerevisiae* proteins refer to the numbers given in their respective sequencing projects, the ID numbers for the *P. anserina* proteins were taken from GenBank.

<table>
<thead>
<tr>
<th>Species (Strains)</th>
<th>Species</th>
<th>Class</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. fumigatus (AI235)</td>
<td>A. nidulans (FGSC A4)</td>
<td>A. niger (CBS1388)</td>
</tr>
<tr>
<td>N. crassa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heterokaryon incompatibility genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HET-6 (NCU03533.2)</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td></td>
<td>HET-C (NCU03125.2)</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td></td>
<td>UN-24 (NCU03539.2)</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td></td>
<td>Suppressor genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TOL (NCU04463.2)</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td></td>
<td>VIB-1 (NCU03725.2)</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td></td>
<td>Incompatibility related genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HAM-2 (NCU03727.2)</td>
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<td>●</td>
</tr>
<tr>
<td></td>
<td>PIN-C (NCU03494.2)</td>
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<td>●</td>
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<tr>
<td></td>
<td>RNR-A (NCU07887.2)</td>
<td>●</td>
<td>●</td>
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<tr>
<td>P. anserina</td>
<td>Heterokaryon incompatibility genes</td>
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<td></td>
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<tr>
<td></td>
<td>HET-C2 (AAA20542)</td>
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<td>●</td>
</tr>
<tr>
<td></td>
<td>HET-D2Y (AAL37301)</td>
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<td>●</td>
</tr>
<tr>
<td></td>
<td>HET-E4S (AAL37297)</td>
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<td>●</td>
</tr>
<tr>
<td></td>
<td>HET-S (AAB88771)</td>
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<td>●</td>
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<tr>
<td></td>
<td>Incompatibility related genes</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>IDI-1 (AAC24119)</td>
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<td>●</td>
</tr>
<tr>
<td></td>
<td>IDI-2 (AAC24120)</td>
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</tr>
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<td></td>
<td>IDI-3 (AAC24121)</td>
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<td>●</td>
</tr>
<tr>
<td></td>
<td>IDI-4/ JLB-A (AAT40415)</td>
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<td>●</td>
</tr>
<tr>
<td></td>
<td>IDI-6/ PSP (AAC03564)</td>
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</tr>
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<td></td>
<td>IDI-7 (AAN41258)</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td></td>
<td>Modifier genes</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>MOD-A (AAC25496)</td>
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<td>●</td>
</tr>
<tr>
<td></td>
<td>MOD-D (AAC24768)</td>
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<td>●</td>
</tr>
<tr>
<td></td>
<td>MOD-E (AAB97626)</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>Programmed Cell Death genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATP4 (YPL078C)</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td></td>
<td>CDC48 (YDL126C)</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td></td>
<td>HEL10 (YNL208W)</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td></td>
<td>HEL13 (YOR309C)</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td></td>
<td>MCA1/YCA1 (YOR197W)</td>
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<td>●</td>
</tr>
<tr>
<td></td>
<td>NSR1 (YGR159C)</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td></td>
<td>PPA1 (YHR026W)</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td></td>
<td>RSM23 (YGL129C)</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td></td>
<td>SAR1 (YPL218W)</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td></td>
<td>STM1 (YLR150W)</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td></td>
<td>TOR1 (YJR066W)</td>
<td>●</td>
<td>●</td>
</tr>
</tbody>
</table>
In silico comparison of the two A. niger genomes

Using strains with different spore colors and different auxotrophic mutations or dominant resistances, one can test for the formation of heterokaryotic mycelium on media on which the single partners are unable to grow. Different mutant lines were isolated from DSM Research BV's Strain CBS 513.88, DOE Joint Genome Institute's culture collection strain ATCC1015 and our lab strain N400 (ATCC 9029; CBS 120.49), making it possible to test for heterokaryon incompatibility between these strains. Strains ATCC1015 and our laboratory strain N400 (ATCC 9029; CBS 120.49) proved heterokaryon compatible with one another. That they thus belong to the same vegetative compatibility group suggests that they share a common clonal ancestor. However, DSM Research BV's Strain CBS 513.88 proved incompatible with the two other strains. Thus the two genomes sequenced by DSM and DOE Joint Genome Institute respectively are from heterokaryon incompatible strains.

We searched the genomic databases of these two A. niger strains for incompatibility/apoptosis related genes (Table 2). For blastp searches we used the apoptosis-like PCD proteins of S. cerevisiae and the HET, modifier and suppressor protein sequences of N. crassa and P. anserina (Table 1). For validation of the identified A. niger sequences, a bi-directional best hit analysis was performed, using the polypeptide sequence of the identified A. niger ORFs as a query for a blastp search at the N. crassa, P. anserina, S. cerevisiae and GenBank database (http://ncbi.nih.gov/BLAST; Altschul et al. 1990). As criterion for homologs we used an accepted E-value of <e^-10.

Similarly to the other asexual aspergilli, we found a high number of HET domain proteins (PIN-C, HET-6, HET-D and TOL homologs). Nearly all het-genes were highly similar between both A. niger strains, differences were limited to a few substitutions but are potentially crucial for incompatibility reactions. However, the
two sequenced *A. niger* strains differ in their sets of heterokaryon incompatibility and apoptosis related genes. Although they largely possess the same gene set, strain CBS513.88 lacks a HET-S homologue and strain ATCC1015 lacks the IDI-4/JLB-A and HEL10 homologs (Table 3). In nearly all of their putative HET proteins the two *A. niger* isolates show little to no variation in sequence. The regions of these genes involved in self/non-self recognition may be under positive selection and single-amino-acid differences can be sufficient to trigger incompatibility (Saupe 2000). Thus, the

<table>
<thead>
<tr>
<th>Function</th>
<th>Protein</th>
<th>Type and size of difference</th>
<th>Identities</th>
<th>Gaps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterokaryon incompatibility genes (<em>N. crassa</em> &amp; <em>P. anserina</em>)</td>
<td>HET-6</td>
<td>only HET domain motifs are slightly conserved</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HET-C</td>
<td>indel: 622</td>
<td>791/793 (99 %)</td>
<td>1/793 (0 %)</td>
</tr>
<tr>
<td></td>
<td>UN-24</td>
<td>no difference</td>
<td>(100 %)</td>
<td>(0 %)</td>
</tr>
<tr>
<td></td>
<td>HET-C2</td>
<td>no difference</td>
<td>(100 %)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HET-D / HET-E</td>
<td>diverse proteins with WD40 repeats, but no remarkable similarity</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Suppressor genes (<em>N. crassa</em>)</td>
<td>TOL</td>
<td>very diverse proteins, with conserved HET domain motifs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VIIB-1</td>
<td>substitution: 195</td>
<td>585/586 (99 %)</td>
<td>(0 %)</td>
</tr>
<tr>
<td>Incompatibility related genes (<em>N. crassa</em> &amp; <em>P. anserina</em>)</td>
<td>HAM-2</td>
<td>substitution: 756</td>
<td>1066/1067 (99 %)</td>
<td>(0 %)</td>
</tr>
<tr>
<td></td>
<td>PIN-C</td>
<td>very diverse proteins, with conserved HET domain motifs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>RNR-A</td>
<td>no difference</td>
<td>(100 %)</td>
<td>(0 %)</td>
</tr>
<tr>
<td></td>
<td>IDI-6 / PSP</td>
<td>1st pair: indel: 534</td>
<td>531/535 (99 %)</td>
<td></td>
</tr>
<tr>
<td>(2 alleles)</td>
<td>substitutions: 398, 525</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2nd pair: substitutions: 398, 436–443, 450–459, 471–514</td>
<td>413/416 (99 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IDI-7</td>
<td>no difference</td>
<td>(100 %)</td>
<td></td>
</tr>
<tr>
<td>Modifier genes (<em>P. anserina</em>)</td>
<td>MOD-D (2 alleles)</td>
<td>Members of the two allele pairs are 100 % identical, between the pairs there are some differences: 1–167 variable part, 1–60 and 168–360 more conserved region.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MOD-E</td>
<td>substitution: 244</td>
<td>672/702 (96 %)</td>
<td>(0 %)</td>
</tr>
<tr>
<td>Programmed Cell Death genes (<em>S. cerevisiae</em>)</td>
<td>ATP4</td>
<td>no difference</td>
<td>100 %</td>
<td>0 %</td>
</tr>
<tr>
<td></td>
<td>CDC48</td>
<td>no difference</td>
<td>100 %</td>
<td>0 %</td>
</tr>
<tr>
<td></td>
<td>HEL13</td>
<td>present only in CBS 513.88</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MCA1 / YCA1</td>
<td>1st pair</td>
<td>438/438 (100 %)</td>
<td>(0 %)</td>
</tr>
<tr>
<td>(2 alleles)</td>
<td>substitutions: 57–75, 105–106, 236, 440–442</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2nd pair</td>
<td>341/441 (77 %)</td>
<td>1/333 (0 %)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NSR1</td>
<td>diverse proteins, with short conserved motifs</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PPA1</td>
<td>no difference</td>
<td>100 %</td>
<td>0 %</td>
</tr>
<tr>
<td></td>
<td>SAR1</td>
<td>no difference</td>
<td>100 %</td>
<td>0 %</td>
</tr>
<tr>
<td></td>
<td>STM1</td>
<td>indel region: 8–13</td>
<td>297/303 (98 %)</td>
<td>6/303 (1 %)</td>
</tr>
<tr>
<td></td>
<td>TOR1</td>
<td>substitution: 16</td>
<td>2389/2390 (99 %)</td>
<td>(0 %)</td>
</tr>
</tbody>
</table>
observed small differences may be an explanation for the observed heterokaryon incompatibility between the two A. niger strains. However, as both P. anserina and N. crassa seem to have selected different sets of heterokaryon incompatibility genes to block intermycelial transfer, aspergilli may use a completely different set of genes as well.

DISCUSSION

Little is known about the nature of gene flow in natural populations of aspergilli. However, there is clear evidence of recombination within populations (e.g. Geiser et al. 1994, Geiser et al. 1998, Paoletti et al. 2005). This may have arisen through sexual and/or parasexual means. The presence or absence of gene flow in populations has significant implications for speciation within the aspergilli, which may proceed at different rates depending on the presence of recombination or clonality (Taylor et al. 1999a).

In the particular case of the presumed asexual A. niger we used an in silico study to assess the genetic basis of heterokaryon incompatibility. Comparisons were made of genome sequences of two different A. niger strains that are heterokaryon incompatible, together with genome sequences of four closely related sexual and asexual species. We searched these databases with genes known to be involved in heterokaryon incompatibility or apoptosis in P. anserina, N. crassa and S. cerevisiae. Our aim was to find out whether the same genes may be involved in the incompatibility reactions between different A. niger isolates as the ones found to interact in N. crassa and P. anserina, fungi that have different sets of active het-genes. Few differences were found between the two sequenced A. niger genomes, but many of the known heterokaryon incompatibility genes were indeed present in the A. niger genomes. Some of the examined het-genes were even found to have many homologs.

Further practical research is needed to find a satisfying explanation for the high level of incompatibility in the natural populations of black aspergilli and to pinpoint functional het-genes in the species. As a result of our data mining, the sequences of the known incompatibility genes are available for functional analysis, to uncover the secrets of incompatibility between the natural isolates of black aspergilli.

ACKNOWLEDGEMENTS

We want to thank all institutions, companies and individuals involved in the genome projects of the species used in this study. A. fumigatus preliminary sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org. A. niger CBS513.88 sequence data were obtained from the DSM Research B.V., the A. niger ATCC1015 sequence data from the DOE Joint Genome Institute. The A. nidulans genome data are from the Aspergillus Sequencing Project, the Aspergillus terreus genome data from the Aspergillus terreus Sequencing Project, and the release 7 data of the N. crassa genome were all from the Broad Institute of Harvard and MIT (http://www.broad.mit.edu). A. oryzae data were obtained from the NITE Biological Resource Center. Data from the complete sequence of P. anserina were obtained from the P. anserina genome project and the S. cerevisiae genome data from the Saccharomyces Genome Database.

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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 (Grevat & 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 (Grevat & 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 cladiests 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.), epitopes, and certain polyketides and alkaloids that probably protect fungal propagules in being eaten by insects, mites and other animals (Janzen 1977; Rohloff et al. 2005). The endo-metabolome consists of primary metabolites in constant change and internal interaction (the interactome and fluxome). These

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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 “chemrophylogeny” 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. sclerotioring, A. ibericus and A. scleroticarbonarius 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
In many cases it is of course of utmost 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. tubingenensis*, *A. foetidus* and *A. japonicus* on 7 carbon sources revealed clearly different growth profiles for each species, and demonstrated that *A. niger* and *A. tubingenensis* 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 xylose, while *A. tubingenensis* 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 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-xylene, L-arabinose and galacturonate 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 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 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).

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

<table>
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<th>Series Nigri:</th>
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<tr>
<td>Subseries Nigri:</td>
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<td><em>Aspergillus niger</em></td>
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<td><em>Aspergillus laeticofaciatus</em></td>
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<td><em>Aspergillus brasilianensis</em></td>
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<td>Subseries Tubingenensis:</td>
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<td><em>Aspergillus tubingenensis</em></td>
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<td><em>Aspergillus vadensis</em></td>
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<td><em>Aspergillus costaricaensis</em></td>
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<td>Series Carbonania:</td>
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<td><em>Aspergillus carbonarius</em></td>
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<td><em>Aspergillus ibericus</em></td>
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<td><em>Aspergillus sclerotiorcarbonarius</em></td>
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<td>Series Heteromorpha:</td>
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<td>Series Homomorpha:</td>
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<td><em>Aspergillus homomorphus</em></td>
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<td>Series Aculeata:</td>
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<td><em>Aspergillus aculeatus</em></td>
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<td><em>Aspergillus aculeatius</em></td>
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<td><em>Aspergillus uvarum</em></td>
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<td><em>Aspergillus japonicus</em></td>
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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. brassiliensis 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. lactoaffaeatus*, *A. sclerotioriger*, *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. extrotile 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 extrotile 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. rambeili* in section *Ochraceorosei* and *Emericellae aspergilli* and *E. venezuelensis* in section *Nidulantes* (Frisvad et al. 2005). However, sterigmatocystin is also produced by phylogenetically widely different fungi such as *Chaeotium* species (Udagawa et al. 1979; Sekita et al. 1981), *Monocillium nordii* (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,Zn₂) DNA-binding protein, AfIR (Chang et al. 1995). Most of the AF biosynthetic genes in section *Flavi* aflatoxin-producing species have AfIR-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 AfIR-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 (Chiong 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 units continues until the polyketide chain fills the cavity of the PKS and is excised by a thiosterase that also acts as a Caisen-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 (afx) 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 hydroxversicolorone 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 AfY 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 norsoronic 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 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.

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Aspergillus species identification in the clinical setting

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Abstract: Multiple recent studies have demonstrated the limited utility of morphological methods used singly for species identification of clinically relevant aspergilli. It is being increasingly recognized that comparative sequence based methods used in conjunction with traditional phenotype based methods can offer better resolution of species within this genus. Recognising the growing role of molecular methods in species recognition, the recently convened international working group meeting entitled “Aspergillus Systematics in the Genomic Era” has proposed several recommendations that will be useful in such endeavors. Specific recommendations of this working group include the use of the ITS regions for inter species level identification and the β-tubulin locus for identification of individual species within the various Aspergillus sections.

Key words: Emericella, molecular phylogeny, pathogenic aspergilli, polyphasic taxonomy, section Aspergillus section Terrei, section Usti.

INTRODUCTION

aspergilli cause a wide spectrum of infections including cutaneous manifestations, otomycosis, and invasive infections such as pulmonary aspergillosis and endocarditis. Pulmonary aspergillosis may range from invasive pulmonary aspergillosis (IPA) in severely immunocompromised patients to chronic necrotising aspergillosis in mildly immunocompromised populations. The risk of IPA appears to be much higher in hematopoietic stem cell transplant patients and in patients with leukemia, where the attributable mortality rate was 38.5 % according to a recent study (Pagano et al. 2007). Aspergillus fumigatus remains the predominant agent of IPA, followed by either A. terreus or A. flavus depending on the medical center. Recently, IPA due to A. ustus and other rare aspergilli such as A. alliaceus (Balajee et al. 2007), A. lentulus (Balajee et al. 2005a) and A. udagawae (Balajee et al. 2006) have been reported.

The genus Aspergillus was originally divided into subgenera and groups (Raper & Fennell 1965) but the current classification scheme replaces the designation “group” with “section” (Gams et al. 1985) to conform to rules of the International Code of Botanical Nomenclature. Currently, the genus Aspergillus is classified into 7 subgenera that are in turn sub-divided into several sections comprised of related species (Gams et al. 1985). Clinical microbiology laboratories rely heavily on morphology-based identification methods for Aspergillus species wherein diagnostic criteria include the recognition of asexaul or sexual structures and their characteristics such as shape, size, color, ornamentation and/or mode of attachment. Unfortunately, numerous difficulties exist in such a phenotype-based scheme largely because these characteristics are unstable, and clinical aspergilli sometimes manifest atypically with slow sporulation and aberrant conidiophore formation. Additionally, members of the section Fumigati have overlapping morphological characteristics, with several genetically distinct species existing within a single morphospecies.

Clinically, identification of unknown Aspergillus clinical isolates to species may be important given that different species have variable susceptibilities to multiple antifungal drugs. Thus, knowledge of the species identity may influence the choice of appropriate antifungal therapy. For example, in vitro and in vivo studies have demonstrated that A. terreus isolates are largely resistant to the antifungal drug amphotericin B. A. ustus isolates appear to be refractory to azoles, and A. lentulus and Petromyces alliaceus have low in vitro susceptibilities to a wide range of antifungals including amphotericin B, azoles, and echinocandins (Balajee et al. 2005a, 2007). Comparative DNA sequence-based identification formats appear to be promising in terms of speed, ease, objectivity and economy for species identification. Multiple genes ranging from the universal ribosomal DNA regions ITS and the large ribosomal subunit D1–D2 to protein encoding genes such as the β-tubulin and calmodulin gene regions have been evaluated to delimit species within aspergilli. In spite of the shift of fungal identification formats into the molecular arena as evidenced by numerous publications, there is no consensus on the gene/genes that can be used for species identification in the genus Aspergillus. As a first step, a group of experts met at the “International Workshop on Aspergillus Systematics in the Genomic Era” [Utrecht, The Netherlands; April 2007] and presented research data on species identification strategies available to identify aspergilli. Throughout the meeting, research pertaining to the utility of the ITS region for inter species level classification of Aspergillus was presented. The session “Species identification in the clinical setting” was proposed specifically to deliberate on the utility of comparative sequence analyses of protein coding loci for intra section level species identification. This communication is a report of the research findings presented at this session. At the end of the report, we present the recommendations proposed by the Aspergillus working group for inter species level recognition of clinically relevant aspergilli and for identification of species within the sections Fumigati, Terrei, Usti and Emericella nidulans.
Pathogenic species in Aspergillus section Fumigati and species delimitation based on polyphasic taxonomy

The most common causative agent of aspergillosis is *A. fumigatus* with rare reports of invasive infections caused by species of *Neosartorya*. However, clinical isolates of *A. fumigatus* are not necessarily morphologically uniform, and mistaken identification of these taxa by morphological characteristics has occurred in the past. In order to develop diagnostic techniques, it is essential to clarify intra- and interspecies diversity in *A. fumigatus* and closely related species using robust techniques.

Recently, *A. lentulus* isolated from clinical specimens in the U.S.A. was described as a new species; members of this species were not able to survive at 48 °C, and this species has high in vitro MICs to several different classes of antifungals (Balajee et al. 2005a). Members of this species were distinct from the other species in this section, which includes the varieties of *A. fumigatus*. Two additional new species, *A. fumigati affinis* and *A. novofumigatus*, had also been proposed (Hong et al. 2005) by investigators who analyzed the species within the section *Fumigatus* using a polyphasic approach that included phenotypic characters such as macro- and micro-morphology, growth temperature regimes, and extrolite patterns, and genotypic characters including RAPD-PCR and multi-locus sequence typing (MLST) of partial β-tubulin, calmodulin and actin genes. From these results, 30 species were accepted within the section *Fumigatus* (Hong et al. 2006) and their taxonomic positions are shown in Fig. 1. Although *A. fumigatus* is the predominant agent of aspergillosis, several species in the section have also been reported from clinical samples: *A. fumisynnomatus* (Yaguchi et al. 2007), *A. lentulus* (Balajee et al. 2005), *A. viridinutans* species complex (Hong et al. 2005, 2006; Katz et al. 2005), *Neosartorya coreana* (Hong et al. 2006; Katz et al. 2005), *N. fennelliae* ((Kwon-Chung & Kim 1974), *N. fischeri* (Chim et al. 1998; Gori et al. 1998), *N. pseudofischeri* (often as the anamorph *A. thermomutans* status) (Balajee et al. 2005b; Coriglione et al. 1990; Padhye et al. 1994), *N. spinosa* (Summerbell et al. 1992; Gerber et al. 1973), *N. hiratsukae* (Guarro et al. 2002) and *N. udagawae* (Balajee et al. 2006). In the case of *N. coreana* and *N. fennelliae*, pathogenicity in humans has not been established yet.

Because of the re-evaluation of section *Fumigati* based on polyphasic methods, the molecular identification of the *A. fumigatus* isolates recovered as causative agents of mycosis in humans and animals at the Medical Mycology Research Center, Chiba University, Japan (MMRC) was investigated (Yaguchi et al. 2007). Several other species within the section *Fumigatus* were also included in the analyses. The phylogenetic relationships among *A. fumigatus* and related species, including *Neosartorya* species, were analyzed by sequencing partial regions of the β-tubulin, hydrophobin and calmodulin genes. The gene regions were sequenced directly from clinical specimens in Japan clustered together in clade I. The other strains from clinical specimens fell into clades II and IV, and none of the clinical isolates clustered within clades III and V.

Correlations among morphology, maximal growth temperatures, minimal inhibitory concentrations (MICs) of antifungal agents, and phylogeny of isolates within the section *Fumigati* were also analyzed (Fig. 2). Scanning electron microscopy examination of these isolates showed that the conidial ornamentations of isolates belonging to clades I and V were lobate-reticulate (Kozakiewicz 1989), while those of *A. viridinutans* were intermediate between lobate-reticulate and microtuberculate. All strains in clade II and the six variant isolates in clade IV (IFM 5058, 51744, 53867, 53868, 54302 and CBM FD-0143) had conidia with microtuberculate ornamentation. These six strains are very closely related to *N. udagawae* (Horie et al. 1995), a heterothallic species isolated from soil in Brazil. However, mating between these strains and *N. udagawae* did not occur. It is often difficult to perform successful mating experiments on clinical isolates and fungi that have been routinely sub-cultured. Therefore these strains need to be investigated further before they are identified as the anamorphic state of *N. udagawae*.

The maximal growth temperatures and MICs of antifungal agents were also examined. The maximal growth temperatures of clades I, II, III, IV and V were above 50 °C, 45 °C, 45 °C, 42 °C and 42 °C, respectively. These phenotypic data may be useful for classification of species within Aspergillus section *Fumigati*. The isolates of *A. lentulus* demonstrated lower in vitro susceptibilities to amphotericin B than other isolates in section *Fumigati* (Balajee et al. 2005a). In conclusion, these studies showed that species of Aspergillus section *Fumigati* were divisible into five distinct clades by molecular analyses. Further, results revealed good correlation between phylogenetic and phenotypic characteristics. Comparative sequence analyses of the *benA* and *cal* regions offered good resolution and can be used for species delimitation within the section *Fumigati*.

Molecular phylogeny in Aspergillus section Terrei

Although *A. terreus* is a less common cause of invasive pulmonary aspergillosis when compared to *A. fumigatus*, infections due to these aspergilli appear to be increasing in frequency in certain hospitals worldwide (Baddley et al. 2003; Lass-Florl et al. 2000). Infections due to these organisms are difficult to treat because of both *in vitro* and *in vivo* refractoriness of the organism to the antifungal drug amphotericin B. In addition, *A. terreus* often causes disseminated infection with increased lethality compared with other Aspergillus spp. (Iwen et al. 1998; Steinbach et al. 2004; Walsh et al. 2003). Aspergillus terreus may be nosocomial in origin with potential reservoirs including construction activity, soil of potted plants, and water distribution systems in hospital environments (Lass-Florl et al. 2000; Anaisse et al. 2002; Flynn et al. 1993). In spite of the emerging threat due to this opportunistic pathogen, little is known about the genetic diversity and population structure of *A. terreus*.

*A. terreus* grows on potato dextrose agar at 25 °C as beige to buff to cinnamon brown colonies with reverse of the colony.
**Aspergillus Species Identification in the Clinical Setting**

**Fig. 1.** Phylogenetic tree of *Aspergillus* section Fumigati species inferred from Neighbour-Joining analysis of partial β-tubulin gene sequence. The shaded species have been reported from clinical environment.
appearing yellow. Microscopically, conidial heads are biserial and columnar with smooth walled conidiophores; conidia are globose and smooth. Globose, sessile, hyaline accessory conidia are frequently produced on submerged hyphae and are also produced in vivo during infection. Based on these phenotypical characteristics, *A. terreus* has been described as the only member of section *Terrei* and includes two varieties – *A. terreus* var. *africanus* and *A. terreus* var. *aureus*. However, molecular studies using the D1–D2 regions of the 28S RNA and the ITS regions (intergenic spacer regions 1 and 2 including the 5.8S rRNA) have indicated that this section should be expanded to include a number of other species (Varga et al. 2005). Recently, a three locus phylogenetic approach using partial regions of the protein coding genes β-tubulin, calmodulin and enolase has been attempted to characterise the genetic variability of a large number of *A. terreus* isolates [Balajee et al. in prep.]. These results suggest that *A. terreus* var. *aureus* should be raised to species status and that several cryptic species probably exist within isolates identified as *A. terreus*. Interestingly, members of one cryptic species/clade included clinical isolates recovered predominantly as colonising agents in the immunocompetent population [Balajee et al. in prep.]. Although the results of this study demonstrated the usefulness of the three-locus sequence strategy for species recognition in section *Terrei*, comparative sequence analyses of the benA region alone appeared to be a good marker for species recognition in this section.

**Emericella** species causing invasive infections

Invasive infections caused by **Emericella** species are uncommon in humans. Infections due to *E. nidulans* (anamorph *Aspergillus nidulans*) appear to occur predominantly in patients with chronic granulomatous disease (CGD), a rare disorder of phagocytes in which absence of superoxide and hydrogen peroxide production in phagocytes predisposes patients to bacterial and fungal infections. Invasive *E. nidulans* infections in this patient group are associated with a higher mortality than those caused by *A. fumigatus* (Dotis et al. 2003). *E. nidulans* is rarely encountered in other patient populations at risk for aspergillosis, such as those with neutropenia from myeloablative chemotherapy or recipients of a hematopoietic stem cell transplant. The lung is the most common site of infection, followed by subcutaneous or liver abscess, supplicative adenitis, osteomyelitis, fungemia, cellulitis and meningitis (van't Hek et al. 1998; Winkelstein et al. 2000).

The identification of *E. nidulans* in clinical microbiology laboratories is commonly based on the characteristic microscopic morphology. The conidiophore typically shows metulae and phialides arranged on the upper part of the flask-shaped vesicle. *E. nidulans* produces dark green conidia (asexual spores) on brown-tined conidiophores, and characteristic cleistothecia contain ascii with 8 purple-red ascospores (Fig. 3). Hüll cells, thickened large cells associated with cleistothecia, are often abundant in *E.
Another species within the genus *Emericella* that causes infection in humans is *E. quadrilineata*, which was reported to cause onychomycosis in one patient and sinusitis in two patients with hematological malignancy (Drakos et al. 1993; Gugnani et al. 2004; Polacheck et al. 1992). The morphologic features that distinguish *E. nidulans* from *E. quadrilineata* can be detected only by electron microscopy: the ascospores of *E. nidulans* have two longitudinal crests, while *E. quadrilineata* has four short equatorial crests.

A recent cluster of infections caused by *Emericella* species involving four patients was found to be due to *E. quadrilineata* based on sequence-based analysis of ITS1 and ITS2 regions [Verweij et al. in prep.]. Morphologically, the isolates were identical to *E. nidulans*. The identification of the strains involved in the cluster was further confirmed to be *E. quadrilineata* by sequence analysis of partial regions of the β-tubulin and the calmodulin genes [Verweij et al. in prep.]. In addition, several strains from the collection of the Centraalbureau voor Schimmelcultures were included in the study as well as clinical *E. nidulans* and *E. quadrilineata* isolates from published cases or private culture collections (Dotis et al. 2004). Based on the sequence-based analysis, several isolates were reclassified. The availability of molecular techniques in addition to morphological identification was shown to describe a role of *E. quadrilineata* as opportunistic fungal pathogen. The use of these techniques will help to identify and discriminate more accurately within the current fungal species and give more insight into the pathogenesis of fungal infection.

### Pathogenic species in section *Usti*

*A. ustus* is a world-wide occurring species commonly found in food, soil and indoor environments (Samson et al. 2002). *Aspergillus ustus* is also isolated from clinical specimens; however, invasive infections caused by this species are uncommon. In the review by Panackal et al. (2006), 21 documented cases of human infection were reported in the literature. Since this review, two more studies, a case of fungal endophthalmitis and a cluster of eye infections, have been published (Saracil et al. 2007; Yildiran et al. 2006). The true incidence of infections caused by this species is probably higher than reported, as there are cases where the fungus has not been identified or other instances where the organism was identified but remains unpubl. (Chakrabarti et al. 1998). The mortality associated with invasive aspergillosis caused by *A. ustus* seems strikingly high – of the 22 reported cases only 6 patients survived. One main reason for this high mortality rate could be the decreased susceptibilities of *A. ustus* to various antifungal drugs. *In vitro* susceptibility testing showed that this species has decreased susceptibilities to the antifungal drugs amphotericin B, caspofungin, itraconazole, voriconazole and posaconazole but is susceptible to the allylamine terbinafine (Yildiran et al. 2006; Garcia-Martos et al. 2005; Gene et al. 2001; Pavie et al. 2005).

*A. ustus* (Bainier) Thom and Church (1926), described in 1881 by Bainier as *Sterigmatocystis usta*, was placed by Thom and Raper together with *A. granulosus*, in the *A. ustus* group. This group was revised by Raper and Fennell, who used a broad description of *A. ustus* and added *A. puniceus*, *A. panamensis*, *A. conjunctus*, and *A. deflectus*. The use of the name “groups”, a category without nomenclatural standing, was abandoned, and infrageneric taxa were formalised. Subgenera and sections were

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**Fig. 3.** Scanning electronmicroscopy of ascospores of *Emericella* species. A. *E. nidulans*. B. *E. echinulata*. C. *E. quadrilineata*. D. *E. rugulosa*. Scale bars = 5 µm.
created and A. ustus became the type species of section Usti, which was placed in the subgenus Nidulantes (Gams et al. 1985). Peterson (2000) compared this phenotype-based classification system with the phylogenetic relationships based on the D1 and D2 regions of the large subunit ribosomal RNA (lsu-DNA). These data show that the type strain of A. ustus (and therefore also the section Usti) was in the subgenus Nidulantes. Because the entire section Usti branched between Emericella species, Usti was deleted and placed in section Nidulantes. However, the invalidation of section Usti by Peterson is rejected here since multiple sections can be linked to one teleomorph, and since the species also form a distinct clade within the subgenus Nidulantes. Based on the study of Peterson (2000), A. ustus, A. puniceus and A. pseudodeflectus are true members of the section Usti, while A. deflectus is tentatively placed in section Nidulantes and A. panamensis and A. conjunctus are in section Sparsi.

Of all the members in the section Usti, A. ustus is most often reported to be a causal agent of invasive infection, whereas A. puniceus and A. pseudodeflectus have never been mentioned in relation with infections. Only one report in available about A. granulosus, where this organism was described as the causal agent of a disseminated infection in a cardiac transplant patient (Fakhri et al. 1995). A MLST study using the partial regions of β-tubulin, calmodulin and actin genes showed that A. ustus strains received from multiple centers (Panackal et al. 2006; Yildiran et al. 2006; Verweij et al. 1999) revealed the presence of a new species A. calidoustus (sp. nov.) from predominantly clinical samples [Varga et. al., unpubl. data]. Phenotypic differences between A. ustus and A. calidoustus are easy to recognise, since the latter grows rapidly at 37 °C, while the former does not. Nevertheless, other undescribed species, which are also able to grow at 37 °C, are present in section Usti and the occurrence of these species in clinical samples remains unknown. Thus, identification solely based on morphology appears difficult and unreliable. An additional problem with members of section Usti is that these species rarely cause invasive infections, which makes identification even more difficult. Combining all of above mentioned details, the use of morphology in combination with sequence data is recommended as an approach which will generate a less subjective and more reliable result.

Analyses of the D1–D2 sequences currently deposited in GenBank showed that two main clades are present in the section Usti. In one clade the type cultures of A. ustus (U29791; NRRL 275), A. puniceus (AY216673; CBS 495.65) and A. ustus var. laevis are present (U29788; NRRL 1852); the other clade includes the types of A. granulosus (AF454165; CBS 119.58) and A. pseudodeflectus (AF433123; NRRL 6135). However, the D1–D2 region does not have enough variation for species delimitation. The presence of these two main clades is also confirmed by a phylogenetic analysis of the ITS region of the ribosomal RNA (Varga et. al., unpubl. data). Although identification of medically important aspergilli based on ITS sequence data is more reliable than that based on D1–D2 data (Hinrikson et al. 2005), it is also known that the genetic variability within the ITS region is not sufficient and that some Aspergillus species share identical sequences. This is also the case within the section Usti, where A. pseudodeflectus and A. calidoustus could not be discriminated on ITS data alone [Varga et al. in prep.]. For correct species identification within this section, it is recommended to use the protein coding genes rather than ITS or D1–D2 data. Partial regions of β-tubulin, calmodulin and actin genes were tested and gave good resolution and are therefore excellent identification markers within the section Usti.

**RECOMMENDATIONS**

The preceding presentations clearly demonstrate that a multi locus sequence identification method where multiple genes (or portions thereof) are sequenced and the resultant data are analyzed by phylogenetic methods is a robust strategy for species recognition within the genus *Aspergillus*. However, this methodology involves significant cost and phylogenetic expertise that are limiting factors in most clinical microbiology laboratories. Additionally, consideration should also be given to the fact that most of these isolates may not be true causative agents of disease and therefore may not warrant species level identification in a diagnostic laboratory. Taken together, a universal single marker that would rapidly and accurately identify Aspergillus isolates to the species level would help support diagnostic microbiology laboratories in their routine identification efforts. Comparative sequence-based methods are finding a place in the clinical microbiology laboratory for fungal species identification, and there is a need for a consensus recommendation for such global markers that can be used with confidence for this purpose.

Recently, the international group of experts that gathered for the workshop entitled “Aspergillus Systematics in the Genomic Era” reviewed research data presented from research groups worldwide on recent genomic investigations, secondary metabolite analyses, multi locus phylogenetic analyses of the genus *Aspergillus*, and sequence based identification schemes for previously recognised human pathogens within the genus. Deriving from the entire proceedings of the workshop in general and from the session on clinically relevant aspergilli in particular, the following recommendations were proposed as a first step towards formulating a unified sequence-based identification scheme for the genus *Aspergillus*.

Although not discussed in this session, previous publications and research work presented elsewhere during the meeting revealed the utility of ITS region for identification of *Aspergillus* isolates to the section level. Thus, as a first recommendation, comparative sequence analyses of the ITS regions, specifically the ITS1 and ITS2 non-coding regions flanking the 5.8S rDNA, was suggested as an appropriate locus for identification of *Aspergillus* isolates to the level of genus/section. Use of the ITS sequence should be sufficient to place most isolates within the appropriate section, but will not provide sufficient sensitivity to discriminate among individual species within the section. Realising the limitations of the ITS regions to identify intrasection species, sequence comparison of the β-tubulin region for species identification within the section (discussed in detail throughout this manuscript) was proposed as the second recommendation.

The ITS region is a convenient universal marker for fungal species identification and most clinical microbiology laboratories still rely on morphology based identification; both these strategies will not identify species within the sections. Considering these two factors and to help support species reporting in clinical microbiology laboratories, the term “complex” was proposed as an alternate to “section”. Thus, whether clinical microbiology laboratories rely on morphological identification methods or an ITS based sequencing strategy, it is advised to report species within the sections *Fumigati*, *Flavi*, *Nidulantes*, *Usti* and *Terrei* as “species complex”, for instance, “A. fumigatus complex”. Results reported in this manner should be interpreted as indicating the placement of the isolate within the species complex, but not necessarily indicating a species within that section. The microbiologist and the clinician can then jointly decide
whether further DNA sequencing using a protein coding locus is required to identify the individual into a particular species within the section/complex. This information may be necessary when investigating an outbreak, when dealing with infections refractory to antifungal therapy, or when performing applied epidemiologic studies. In all cases, communication between the clinician and the microbiologist is critical to provide results that benefit patient care with the highest value and the least cost.

It must be remembered that these are tentative recommendations that are based on research data available at this time. It should also be reiterated that comparative sequence analyses should be used in tandem with morphological examination for an identification scheme to be successful. We believe that these recommendations will help stimulate further discussion, encourage validations of appropriate loci for comparative sequence strategies, and focus research studies on the clinical relevance of recovering these species from our patient populations.

The contributors to this session were: A. Balajee, Pathogenic species in Aspergillus section Terrei; T. Yaguchi, Pathogenic species in Aspergillus subgenus Fumigati; B. Hong, Speciation in Aspergillus subgenus Fumigati; P. Verweij, Emericella pathogens; J. Houbraken, Pathogenic species in section Usti.

DISCLAIMER

The findings and conclusions in this article are those of the author(s) and do not necessarily represent the views of the CDC.

REFERENCES


Aspergillus strain typing in the genomics era

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Abstract: Multiple reasons may justify a need for strain typing purposes, but the most common reason is to delineate the epidemiological relationships between isolates. The availability of whole genome sequences has greatly influenced our ability to develop highly targeted and efficient strain typing methods for these purposes. Some strain typing methods may serve dual goals: not only can they be used to discriminate between multiple isolates of a certain species, they can also aid in the recognition, identification, description and validation process of a fungal species.

Key words: AFLP, coding tandem repeats, high resolution typing, identification pathogenic aspergilli, MLST.

INTRODUCTION

Strain typing can fulfill many needs both in clinical settings and otherwise. Among the many potential applications for strain typing are outbreak analysis and environmental monitoring, patient monitoring and treatment follow-up, local and global epidemiology, database construction, strain identification (e.g. with production organisms) and many more. Apart from these applications at the subspecies level, molecular methods are also increasingly used at the genus level for the definition and recognition of fungal species.

Over the years, many different molecular methods have been developed for Aspergillus strain typing. Because of its clinical significance, these methods were primarily directed at A. fumigatus. The most promising techniques are either PCR based, such as analysis of microsatellite length polymorphisms (MLP)/short tandem repeats (STR) (Bart-Delabesse et al. 1998; de Valk et al. 2005) and amplified fragment length polymorphism (AFLP) analysis (Warris et al. 2003; de Valk et al. 2007b), or based on non-coding repetitive sequences (such as the Afut1 element) in combination with restriction fragment length polymorphisms (RFLP) (Girardin et al. 1993). Use of these and other methods has been reviewed by Varga (2006). Three recent additions to this diverse list are multilocus sequence typing (MLST) (Bain et al. 2007), coding tandem repeats (Balajee et al. 2003; Levadsky et al. 2007) and retrotransposon insertion-site context (RISC) typing (de Ruiter et al. 2007). Depending on the exact reason for strain typing and on the technical resources in a particular setting, the choice for either of these methods could be appropriate.

Classically, without the availability of genomic sequence information, the process of developing a new strain typing method often involved many laborious selection and optimisation experiments. At present, in the genomics era, the availability of whole genome sequences has had a great impact on our options to develop novel and state-of-the-art fingerprinting methods. We now can develop new fingerprinting methods using highly targeted approaches with much higher a priori chances of being successful than before. Naturally, as more genomic sequence information is becoming available, these chances will continue to increase.

Here, we will present a number of applications for several of these genotyping methods and discuss the impact of the availability of genomic sequence data on the applications of these methods.

High resolution exact strain typing using short tandem repeats

Microsatellites or STR’s are ubiquitously present in the genomes of many fungi including Aspergillus spp. Microsatellites, as tools for the identification of and discrimination between individual organisms, already have a relatively long history in human forensic applications where they currently comprise the global “gold standard” in the identification process of individuals. The use of STR’s offers a number of technical advantages over many other fingerprinting techniques including: ease of amplification, multiplex options, extremely high discriminatory power, an exact unambiguous (numerical) and highly portable and exchangeable typing result, ability to detect mixed samples, construction of databases, etc. Because of these advantages, there is a growing interest in the use of STR based methods for strain typing in the microbial field as well.

Bart-Delabesse et al. (1998) reported the first application of microsatellites for A. fumigatus. These markers were obtained by screening genomic DNA libraries of A. fumigatus for suitable, microsatellite containing sequences, a process that proved quite laborious in the pre-genomics era. A panel of 4 dinucleotide repeats was selected that performed well in comparative genotyping experiments (Lasker 2002). Recently, based on genomic sequence data that has become available, de Valk et al. (2005) reported a novel set of 9 tandem repeats for typing A. fumigatus isolates, the so-called STRAf assay (STR’s of A. fumigatus). In contrast to the previously developed typing scheme, this panel also contained tri- and tetranucleotide repeat markers and, in addition, all loci contained a single uninterrupted repeat element. By using multicolor multiplex approaches with these novel markers, large numbers of isolates can be analyzed in a short period of time. Because of the larger number of loci, the STRAf assay yielded a superior discriminatory power for typing A. fumigatus isolates. In Fig. 1, a graphical representation of...
the diversity with the *A. fumigatus* population is shown. The minimal spanning tree represents 99 presumably unrelated *A. fumigatus* isolates. Almost all isolates could be discriminated from each other. The ones that could not be discriminated by the STR \( Af \) assay also proved to be indistinguishable using other molecular methods such as AFLP analysis. Furthermore, the STR \( Af \) assay proved to be an extremely robust typing assay. It has been shown that deliberate and significant changes to the experimental protocol did not lead to wrong typing results with this assay (de Valk *et al.* 2007a).

The key element in the use of microsatellites is to translate the electrophoretic mobility of the obtained fragment (reflected as the size of the fragment in bp and obtained on a high resolution electrophoretic platform) to the corresponding number of repeats. Unfortunately, this mobility is dependent on many factors such as the presence/absence of denaturing compounds, the sieving matrix that is used, the exact base composition and sequence of the fragment, run temperature, presence of different fluorescent labels and even something that may appear only trivial such as the sizing marker (de Valk *et al.* 2007a; Tu *et al.* 1998; Vainer *et al.* 1997). In order to transfer a microsatellite based assay to a different electrophoresis platform, a careful calibration of the new platform has to be established. Similar to the situation in human forensics, a series of allelic ladder was constructed that contain reference fragments with established repeat numbers. By running these allelic ladders, every platform can be calibrated to yield exchangeable typing data with any given set of isolates (de Valk *et al.* submitted for publication). Thus, the STR\( Af \) assay has all the key ingredients to be successfully used for global standardisation of *A. fumigatus* typing.

**Simultaneous identification and strain typing**

In recent years, there has been a growing interest in the use of more accessible techniques such as MLST approaches for fungal identification purposes and strain typing. This approach that is exclusively based on sequencing data has the advantage of the development of accurate databases totally reliable for taxonomy. However, whereas MLST performs well at the genus and species level, in the case of Aspergillus (and in contrast to other species like Candida) the discriminatory power at the subspecies level turns out to be disappointing (Bain *et al.* 2007).

AFLP analysis is a highly discriminatory method at the intraspecies level. In AFLP analyses, fragments are amplified from random locations throughout an organism's genome in a highly reproducible manner (Vos *et al.* 1995). The discriminatory power of AFLP analysis equals that of the STR panels (de Valk *et al.* 2007b) and Afut1 RFLP analysis. However, like with any other fingerprinting method based on DNA banding patterns, its long-term stability and reproducibility may be quite challenging. Development of AFLP fingerprinting requires no prior sequence information. However, depending on the genome composition (GC-content and distribution, presence of multicopy elements), certain combinations

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*Fig. 1.* Minimal spanning tree of 99 presumably unrelated *A. fumigatus* isolates based on microsatellite data. The tree was generated using the multi-state categorical similarity coefficient. Each circle represents a unique genotype. The size of the circle corresponds to the number of isolates with the same genotype. Genotypes connected through a shaded background differ by a maximum of 2 out of 9 markers. The numbers correspond to the number of different markers between the genotypes. No less than 96 different genotypes were discriminated. Data are taken from de Valk *et al.* (2005).
Aspergillus Strain Typing in the Genomics Era

Fig. 2. Example of AFLP analysis showing the discriminatory power as well as the ability to discriminate between isolates belonging to different species. The figure shows 16 fingerprints from A. fumigatus cultured from 16 IA patients (1 isolate per patient). Based on the differential presence or absence of one or more bands, all isolates can be discriminated from each other. One isolate with a clearly different fingerprint turned out to be N. fisheri. The dendrogram was calculated by UPGMA clustering using the Pearson correlation coefficient. The scale bar indicates the percentage similarity.

of restriction enzymes and selective residues could prove to be more suitable than others. At present, based on available genomic sequence data, one can predict in silico which fragments will be obtained with any known genome (Bikandi et al. 2004).

Whereas AFLP has originally been presented as a tool for strain typing purposes, it is also very well suited to simultaneously resolve isolates belonging to different species from each other. This is relevant in the case where the identification of a microorganism may be uncertain such as the species from the morphologically very similar section Fumigati. If not properly identified, use of such typing data could easily lead to false conclusions. In a way, AFLP can be considered the perfect PCR alternative to DNA-DNA reassociation studies. Classical DNA-DNA reassociation studies rely on sequence similarities. If two species share a certain amount of sequence information, it is to be expected that they will also share a certain amount of similarity in banding patterns from an AFLP fingerprint. In fact, this has already been demonstrated for a variety of bacterial and fungal species. According to our own observations, AFLP fingerprints of isolates of the same species are usually > 60 % similar whereas fingerprints for isolates representing different species are usually < 40 % similar (Fig. 2). We also used AFLP analysis for confirmation of the identity of 67 isolates representing 26 species in Aspergillus section Fumigati. These isolates have previously been identified using a variety of other methods. Although the majority of isolates were correctly identified, this exercise clearly showed that: i some isolates were misidentified, ii that some recognised species are comprised of multiple clearly discernible subgroups and iii that several isolates that are currently recognised as different species should actually be grouped into a single species. Thus, AFLP can very well complement the use of MLST and other methods in the recognition and validation process of fungal species.

Strain typing based on coding tandem repeats

Coding tandem repeats are adjacent in-frame coding DNA sequences of 2 to 200 nucleotides in length that are directly repeated; these repeated units may be completely identical or partially degenerate (Li et al. 2004). The number of these coding-repeat copies often varies among different isolates leading to expansion or contraction of amino-acid blocks. Coding repeats have been observed in a number of prokaryotic and eukaryotic genomes where they play an important role in generating variability in cell-surface immunogenic antigens and adhesins, thereby evading the immune system or enhancing pathogenicity (Gravekamp et al. 1998; Jordan et al. 2003; Verstrepen et al. 2005). The inter-strain variability in the number of coding sequences can also serve as an extremely robust and rapid typing technique. Sequence analysis of a single, highly-variable gene, Protein A (spa) or clumping factor (cflb) has been successfully applied to strain differentiation amongst Staphylococcus aureus isolates which generally exhibit low variability and poorly discernible population genetic structure (Shopsin et al. 1999). Recently, an analysis of the genome of A. fumigatus identified as many as 292 genes with internal repeats. Fourteen of 30 selected genes showed size variation of their repeat–containing regions among 11 clinical A. fumigatus isolates. One of these, the cell wall protein Afu3g08990 is involved in conidial germination and adhesion (Levdansky et al. 2007).

Importantly, the repeat containing region of Afu3g08990 or CSP (cell-surface protein) was shown to vary significantly between A. fumigatus isolates from various origin (Levdansky et al. 2007; Balajee et al. 2007) (Fig. 3). By simply sequencing the Afu3g08990...
repeat region in the various isolates and performing a phylogenetic analysis using the maximum parsimony method, it was possible to successfully "sub-type" fifty-five epidemiologically linked \textit{A. fumigatus} isolates from six nosocomial outbreaks of invasive aspergillosis. The results were concordant with another discriminatory genotyping technique, the \textit{Afu}f RFLP typing method. However, while \textit{Afu}f typing is labor and time intensive, needs specialised equipment and is not high throughput, \textit{Afu}g08990 / CSP-typing requires only the ability to perform PCR and have access to an automated sequencer. Also, interpretation of the sequence information does not require sophisticated algorithms nor dedicated software and thus can be seamlessly integrated into any clinical microbiology laboratory.

It is worthy to note that in the \textit{A. fumigatus} genome there is a substantial enrichment of putative cell-surface and/or secreted proteins that contain internal repeats. While 2.9 \% of the ~9 900 genes in the \textit{A. fumigatus} genome contain coding repeats, at least 12.5 \% of all putative cell-wall encoding genes do so, a greater than 4-fold increase. This suggests that as found in a number of other fungal genomes, repeats in \textit{A. fumigatus} may play an important role in generating variability in cell-surface immunogenic antigens and adhesins, thereby evading natural predators in its natural environment and the immune system in its inadvertent host.

CONCLUSIONS

Many different reasons may exist to explain the wish for being able to discriminate between different isolates of a given species. In the light of increasing reports of misidentified isolates, there is also clearly a need for more accurate and accessible methods to identify a fungal isolate to the species level. Ideally, these two parameters are combined in one typing/identification method. Based on the availability of genomic sequence data, highly targeted approaches allow strain typing methods to be directed at (a) highly specific part(s) of a specific fungal genome. Several of these typing methods can be used for typing purposes and may simultaneously be used for identification confirmation of fungal isolates: e.g. any clinical \textit{A. fumigatus} isolate lacking the characteristic amplification barrier such as MLST and AFLP analysis are not only suitable for strain typing and identification purposes but they could additionally serve as parameter in the description and validation process of fungal species and in the delineation of the relationships between them.

REFERENCES


Valk HA de, Meis JF GM, Curfs IM, Muelheithaler K, Mouton JW, Klaassen CHW (2005). Use of a novel panel of nine short tandem repeats for exact and high-resolution typing of \textit{Aspergillus fumigatus} isolates, including the genome-sequenced reference strain Af293. This figure shows size variation of the TR region between the strains.


Biodiversity of *Aspergillus* species in some important agricultural products

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Abstract: The genus *Aspergillus* is one of the most important filamentous fungal genera. *Aspergillus* species are used in the fermentation industry, but they are also responsible of various plant and food secondary rot, with the consequence of possible accumulation of mycotoxins. The aflatoxin producing *A. flavus* and *A. parasiticus*, and ochratoxigenic *A. niger*, *A. ochraceus* and *A. carbonarius* species are frequently encountered in agricultural products. Studies on the biodiversity of toxigenic *Aspergillus* species is useful to clarify molecular, ecological and biochemical characteristics of the different species in relation to their different adaptation to environmental and geographical conditions, and to their potential toxigenicity. Here we analyzed the biodiversity of ochratoxin producing species occurring on two important crops: grapes and coffee, and the genetic diversity of *A. flavus* populations occurring in agricultural fields. Altogether nine different black *Aspergillus* species can be found on grapes which are often difficult to identify with classical methods. The polyphasic approach used in our studies led to the identification of three new species occurring on grapes: *A. brasiliensis*, *A. ibericus*, and *A. uvarum*. Similar studies on the *Aspergillus* species occurring on coffee beans have evidenced in the last five years that *A. carbonarius* is an important source of ochratoxin A in coffee. Four new species within the black aspergilli were also identified in coffee beans: *A. sclerotiorigen*, *A. lactofoeatus*, *A. sclerotiocarbonarius*, and *A. aculeatins*. The genetic diversity within *A. flavus* populations has been widely studied in relation to their potential aflatoxigenicity and morphological variants L- and S-strains. Within *A. flavus* and other *Aspergillus* species capable of aflatoxin production, considerable diversity is found. We summarise the main recent achievements in the diversity of the aflatoxin gene cluster in *A. flavus* populations, *A. parasiticus* and the non-toxigenic *A. oryzae*. Studies are needed in order to characterise the aflatoxin biosynthetic genes in the new related taxa *A. minisclerotigenes* and *A. arachidicola*.

**Key words:** aflatoxins, *Aspergillus* Sect. Nigri, Sect. Flavi, grapes, ochratoxin A, polyphasic identification coffee beans.

**INTRODUCTION**

Although they are not considered to be major cause of plant disease, *Aspergillus* species are responsible for several disorders in various plant and plant products. The most common species are *A. niger* and *A. flavus*, followed by *A. parasiticus*, *A. ochraceus*, *A. carbonarius*, and *A. alliaceus*. They can contaminate agricultural products at different stages including pre-harvest, harvest, processing and handling. Changes due to spoilage by *Aspergillus* species can be of sensorial, nutritional and qualitative nature like: pigmentation, discoloration, rotting, development of off-odors and off-flavors. However, the most notable consequence of their presence is mycotoxins contamination of foods and feeds. Because they are opportunistic pathogens, most of them are encountered as storage moulds on plant products (Kozakiewicz 1989). Various mycotoxins have been identified in foods and feeds contaminated by *Aspergillus* species, the most important are the aflatoxins and ochratoxin A (Varga et al. 2004). Aflatoxins B1, B2, G1, G2 are the most toxic and carcinogenic naturally occurring mycotoxins. Due to their extreme hepatocarcinogenicity, extensive research has been carried out on the natural occurrence, identification, characterisation, biosynthesis, and genetic regulation of aflatoxins (Payne & Brown 1998; Bennett & Klich 2003; Yu et al. 2004). Aflatoxins pose a risk to human health because of their extensive pre-harvest contamination of corn, cotton, soybean, peanuts and tree nuts, and because residues from contaminated feed may appear in milk. The most important aflatoxin producing species belong to *Aspergillus* section *Flavi*, including *A. flavus*, *A. parasiticus* and several other species (Bennett & Klich 2003). Extensive research has examined the role of the environment in fostering aflatoxin contamination episodes in corn and cottonseed (Coty 2006; Cleveland et al. 2003). However, there is still no firm understanding of why contamination occurs during certain years, but not in others. In this regard, the conflicting involvements of insect damage to the crop, drought, and natural microbiological competition in creating favorable conditions for aflatoxin contamination complicate research efforts.

Ochratoxin A (OTA) is a potent nephrotoxin which may contaminate various food and feed products (grains, legumes, coffee, dried fruits, beer and wine, and meat). It also exhibits carcinogenic, teratogenic and immunotoxic properties in rats and possibly in humans (IARC 1993). The genotoxicity of OTA remains controversial (EFSA 2006). OTA is receiving increasing attention worldwide because of its wide distribution in food and feed and human exposure that most likely comes from low level of OTA contamination of a wide range of different foods (Petzinger & Weidenbach 2002). The economically most important OTA producers belong to *Aspergillus* sections *Circumdati* and *Nigri* (Samson et al. 2004; Frisvad et al. 2004).

In this review we briefly analyze the biodiversity and the phylogenetic relationships within two of the most important sections: *Flavi* and *Nigri* occurring in some important agricultural products including grapes and derived products, coffee beans and other agricultural products. We find that, while *A. flavus* is involved...
### Table 1. Species concepts of black aspergilli according to different authors.

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*Results of various RLFP analysis by different authors: Kusters-van Someren et al. (1991); Megnegneu et al. (1993); Varga et al. (1993, 1994); Accensi et al. (1999); Parenicova et al. (1997, 2001)

### Table 2. Morphological and biochemical diversity of black aspergilli occurring on grapes.

<table>
<thead>
<tr>
<th>Species</th>
<th>Conidial size (μm)</th>
<th>Color and size of sclerotia (mm)</th>
<th>Source</th>
<th>OTA</th>
<th>Extrolites produced</th>
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<td><strong>Biseriates</strong></td>
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<td><strong>A. brasiliensis</strong></td>
<td>3.5–4.5</td>
<td>Found only in some strain, white, 1–1.5</td>
<td>Soil, grape</td>
<td>-</td>
<td>Naphtho-γ-pyrones (including aurasperone B), pyrophen, tensidol A &amp; B, dihydrocarolic acid, aflavinine</td>
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<td><strong>A. carbonarius</strong></td>
<td>7–9</td>
<td>Pink to brown, 1</td>
<td>Grape, cocoa, coffee, spicots, palm oil, soil, air</td>
<td>+</td>
<td>Pyranonigrin A, naphtho-γ-pyrones</td>
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<td>(Thom 1916)</td>
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<tr>
<td><strong>A. foetidus</strong></td>
<td>3.5–4.5</td>
<td>Found only in some strain, white, 1–1.5</td>
<td>Tomato, grape, bottled fruits</td>
<td>-</td>
<td>Antafumicins, asperazine, funalenone, naphtho-γ-pyrones, pyranonigrin A</td>
</tr>
<tr>
<td>(Thom &amp; Raper 1945)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A. ibericus</strong></td>
<td>5–7</td>
<td>-</td>
<td>Grape</td>
<td>-</td>
<td>Naphtho-γ-pyrones, pyranonigrin A</td>
</tr>
<tr>
<td>(Serra et al. 2006)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A. niger</strong></td>
<td>3.5–5</td>
<td>-</td>
<td>Grape, cocoa, coffee, cereals, soil, paper, date palm</td>
<td>+/-</td>
<td>Funalenone, kotanins, naphtho-γ-pyrones, pyranonigrin A, pyrophen, tensidol A and B</td>
</tr>
<tr>
<td>(Tieghem 1867)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A. tubingensis</strong></td>
<td>3–5</td>
<td>White to pink, 0.5–0.8</td>
<td>Grape, cocoa, coffee, soil, cereals</td>
<td>+/-</td>
<td>Asperazine, funalenone, naphtho-γ-pyrones, pyranonigrin A, tensidol A &amp; B</td>
</tr>
<tr>
<td>(Schober) Mosseray 1934</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Uniseriates</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A. aculeatus</strong></td>
<td>4–5</td>
<td>-</td>
<td>Grape, papaya, pistachio, rice, tomato</td>
<td>-</td>
<td>Secalonic acid D &amp; F</td>
</tr>
<tr>
<td>(Iizuka 1953)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A. japonicus</strong></td>
<td>4–5</td>
<td>white to cream, 0.5</td>
<td>Grape, green coffee berries, pineapple, sesame seed</td>
<td>-</td>
<td>Secalonic acid D &amp; F</td>
</tr>
<tr>
<td>(Saito 1906)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A. uvarum</strong></td>
<td>3–4</td>
<td>dark brown to black</td>
<td>Grape</td>
<td>-</td>
<td>Secalonic acid D, geodin, erdin, asterric acid</td>
</tr>
<tr>
<td>(Perrone et al. submitted)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
in the majority of the agricultural contamination episodes, at least in the United States, the specific role of the S-strain and L-strain *A. flavus* has not yet been established.

**Biodiversity of black aspergilli on grapes from Europe**

Black aspergilli, which comprises species belonging to *Aspergillus* section *Nigri*, are worldwide distributed and have a significant impact on modern society. Many species cause food spoilage, and several are used in the fermentation industry (Bennett & Klich 1992), or candidate in the biotechnology industries. *A. niger* has even been granted the GRAS (Generally Regarded As Safe) status in certain industrial production processes by the Food and Drug Administration of the US government. Although the main source of black aspergilli is soil, they are among the most common fungi causing food spoilage and biodeterioration of other material. Various reports evidenced that members of the *A. niger* species complex, together with *A. carbonarius* and *A. japonicus* are frequently responsible for post-harvest decay of fresh fruit (apples, pears, peaches, citrus, grapes, figs, strawberries, tomatoes, melons, etc.) and some vegetables (especially onions, garlic, and yams); furthermore it is also among the commonest fungi isolated from dried fruit, beans, oil seeds and nuts (peanuts, pecans, pistachios, hazelnuts, almonds, walnuts etc.) (JECFA 2001). Recently, the significance of these species has completely changed since some of them, in particular *A. carbonarius*, is considered as the main source of OTA in grape and wine (Cabanes et al. 2002; Da Rocha Rosa et al. 2002; Battilani & Pietri, 2002; Magnoli et al. 2003, Leong et al. 2007a). Over the past five years several surveys and reports were published dealing with the epidemiology, ecology and distribution of black aspergilli occurring in wine grape and dried grape vineyards. Most of the surveys were from Mediterranean and South American countries and Australia. These studies clarified that the biseriate species *A. niger* “aggregate” and *Aspergillus carbonarius*, and the uniseriate species *A. aculeatus* and *A. japonicus* are the prevalent species occurring on grapes (Da Rocha Rosa et al. 2002; Battilani et al. 2003; Serra et al. 2005; Leong et al. 2006; Ponsone et al. 2007). In general species of the *A. niger* aggregate appear to be the dominant black *Aspergillus* species in all the countries studied, although some vineyards and years showed higher incidence of *A. carbonarius* isolates (Cabanes et al. 2002; Tjamos et al. 2004). In particular, the occurrence and frequency of ochratoxigenic strains in *A. carbonarius* and *A. niger* “aggregate” on grape proved to be similar in the Mediterranean countries and in Australia. On the contrary, *A. niger* was reported as the main ochratoxigenic species occurring on grapes in South America, while *A. carbonarius* occurred in Argentina mainly on retailed dried vine fruits with a low capacity to produce OTA (Chulze et al. 2006).

Ochratoxin A production of black aspergilli occurring on grapes was widely studied in the last years with sometimes ambiguous reports on the toxigenicity and the percentage of toxigenic strains among the species. The OTA producing strains of *A. carbonarius* ranged between 70 and 100 % when grown in vitro and tested using HPLC, while the range of producing strains was around 2–20 % for *A. niger* and *A. tubingensis* (Battilani et al. 2006; Perrone et al. 2006a). Some reports claimed the production of OTA also by *A. japonicus* but it has not yet been confirmed (Dalcero et al. 2002; Battilani et al. 2003). Recently, Ponsone et al. (2007) studying the occurrence and toxigenicity of *Aspergillus* species in Argentinean vineyards found that *A. niger* aggregate was the most frequent species on grapes with 27 % of the isolates producing OTA. The authors also confirmed the production of OTA by *A. japonicus* and *A. aculeatus* strains, but this work lacks molecular identification of the strains.

Black aspergilli are one of the more difficult groups concerning classification and identification. The taxonomy of *Aspergillus* section *Nigri* has been studied by many taxonomists, leading to various species concepts (Table 1). The difficulties in species recognition within the *Aspergillus niger* “aggregate” and the fact that most of the studies carried out on black aspergilli occurring on grapes lack molecular characterisation of the strains perplexed the extent of their natural occurrence and species distribution on grapes and food. In this respect, in 2001–2002 a large survey of black aspergilli occurring on grape from 107 vineyards in different European countries was performed within the EU project Wine-Ochra Risk (QLK1-CT-2001-01761) in order to characterise the species diversity and the potential toxigenic strains in the Mediterranean basin. This survey led to the identification of four main populations separated molecularly using AFLP, RFLP and sequence analyses (Bau et al. 2006; Perrone et al. 2006a, 2006b). These populations included *A. carbonarius*, *A. tubingensis*, *A. niger*, and a group of *Aspergillus uniseriate* isolates morphologically indistinguishable from *A. japonicus* and *A. aculeatus* but clearly separated by molecular techniques (Fig. 1). The genetic variability of these four populations observed by AFLP polymorphisms ranged from 15 to 35 % in *A. carbonarius*, *A. tubingensis* and the *Aspergillus uniseriate* group and 45–85 % in the *A. niger* group. The higher genetic diversity encountered in *A. niger* reflect the complexity of this taxon/group and the difficulties of identification at species level. The main OTA producer was *A. carbonarius* (95–100 % of strains), while the production of OTA was limited to a smaller proportion of strains in *A. niger* and *A. tubingensis* (10–15 % of the strains). No OTA production was observed in strains belonging to *Aspergillus uniseriate* group.

This species diversity was also revealed by sequence analyses of partial calmodulin (660 bp) and β-tubulin (1360 bp) genes which confirmed a significant molecular divergence of *Aspergillus uniseriate* group from other *Aspergillus* species. The description of a new species named *A. uvarum* isolated only from grape has been recently submitted (Perrone et al. 2007). Furthermore, during these surveys *A. ibericus*, a new species closely related to *A. carbonarius* and unable to produce OTA, was also described (Serra et al. 2006). Recently, a further characterisation of five atypical *A. niger* strains (Fig. 1) collected from Portugal grapes evidenced their similarity with other black *Aspergillus* isolates collected worldwide, which did not fit into any species of *Aspergillus* section *Nigri*. This new species called *A. brasiliensis* has recently been described and characterised by a polyphasic taxonomic approach by Varga et al. (2007) using macro- and micromorphology, secondary metabolite profiles, partial sequences of the β-tubulin, calmodulin and ITS genes, and AFLP analysis.

The morphological and biochemical diversity of black aspergilli occurring on grapes is being summarised in Table 2. They differ both in their micromorphology and in extrolite profiles, but for some species like *A. niger*, *A. tubingensis*, *A. foetidus* and *A. brasiliensis* molecular data (chemical or DNA based) are needed for their correct identification. The most frequently occurring species, as underlined above, are the “biseriate” *A. niger*, *A. tubingensis* and *A. carbonarius*, together with the “uniseriate” *A. japonicus*, *A. aculeatus* and the new species *A. uvarum* currently found only on European grapes (Perrone et al. 2006b). The other three species *A. brasiliensis*, *A. ibericus* and *A. foetidus* are occasionally found on grapes; in particular *A. ibericus* and *A. brasiliensis* were found only in some
grape samples from the Iberian Peninsula. *A. foetidus* was only found on grapes in South American surveys, but its identity has not been confirmed by molecular data (Chulze et al. 2006; Ponsone et al. 2007). The molecular diversity of the species within section *Nigri* is shown in Figs 1 and 2. The AFLP dendrogram (Fig. 1) summarises the data obtained using four different primer combinations for strains isolated from grapes in Europe in comparison with the type-strains of section *Nigri*. The same grouping was obtained by phylogenetic analysis of partial calmodulin sequence data (Fig. 2) and part of the β-tubulin gene (data not shown). These data indicate the need for molecular characterisation of these populations for a better and comprehensive identification of the complex of species involved in the *Aspergillus* black rot disease of grapes. In this respect, the molecular diversity of black aspergilli using partial calmodulin gene sequence data was widely exploited in the last three years and led to the development of primer pairs and SSCP tools for the rapid identification of these fungi.
Fig. 2. Phylogenetic tree based on calmodulin sequence data of Aspergillus section Nigri. Numbers above branches are bootstrap values. Only values above 70% are indicated.

* Strains were labelled using accession number of ITEM, Culture Collection of Agri-Food Important Toxigenic Fungi, ISPA-CNR, Bari, Italy.
and robust identification of the main species within the section (Perrone et al. 2004; Susca et al. 2007a, 2007b). In particular the SSCP analysis was successfully used to detect sequence variations contained in an about 180 bp-region of the calmodulin gene in order to identify species of Aspergillus section Nigri. The method developed allows discrimination between 11 Aspergillus species belonging to section Nigri: A. aculeatus, A. japonicus, A. uvarum, A. ellipticus, A. heteromorphus, A. carbonarius, A. ibericus, A. brasiliensis, A. niger, A. foetidus, and A. tubingensis.

Furthermore, the distribution and species diversity of black aspergilli has recently been studied in 8 vineyards of Primitivo and Negroamaro varieties in Apulia (a region with high risk of OTA contamination in wine) during three grape growing seasons (2004–2006) within the Work supported by MIUR Project 12818 – SIVINA (D.M. n. 593/2000). Aspergillus niger “aggregate” was predominant from early veraison to ripening representing 80–85 % of contamination. A. carbonarius increased from veraison reaching 15–20 % at ripening stage, while the Aspergillus “uniseriate” were only found from early veraison to ripening decreasing from 15–20 % to 0–5 % of the population. About 600 strains of black aspergilli, representative of the sampling were isolated, identified and characterised for OTA production. Five percent of A. niger aggregate strains (360) resulted produced OTA, while all A. carbonarius strains (200) and none of the Aspergillus “uniseriate” strains (50) were positive to OTA production (Cozzi et al. 2007). Studies are in progress to characterise the A. niger “aggregate” strains to identify the percentage of A. niger, A. tubingensis and A. brasiliensis strains presence on this south Italian population from grapes. In order to establish a fully correct relationship between species and OTA production, the reported producing isolates and the chemical

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**Fig. 3.** A. Arabica coffee. Ripen cherries on tree. B. depulped cherries. C. dried parchment coffee beans in drying yard. D–F. direct plating of parchment coffee beans on MEA and DG18. G–I. direct plating of green coffee beans on MEA and DG18.
Biodiversity of black aspergilli on Thai coffee beans

Ochratoxin A contamination of coffee is a worldwide problem. The presence of OTA in green coffee bean has been reported by several authors in wide concentration ranging between 0.2 and 360 µg/kg (Levi et al. 1974; Taniwaki 2006). Extensive sampling of green coffee beans of both Arabica and Robusta types worldwide indicated that although OTA contamination is more frequent in some areas including mainly African countries, no producing country was found to be free of contamination (Taniwaki 2006). Although previously A. ochraceus was suggested to be sole source of OTA contamination on coffee (Stack et al. 1983), recent studies indicated that other species, including A. steynii, A. westerdijkiae, A. carbonarius, A. lacticoffeatus, A. sclerotiniger and A. niger are also able to produce OTA on coffee (Téren et al. 1997; Samson et al. 2004; Frisvad et al. 2004). Different types of black aspergilli were reported in coffee bean from different countries. A. niger and A. carbonarius occurred most frequently. Extensive studies have been carried out on the mycobiota of Brazilian coffee recently. From the study of arabica coffee beans by Taniwaki et al. (2003), the results showed that A. niger was the species found most commonly (63 % of potential OTA producers), but only 3 % of them produced OTA. A. ochraceus also occurred commonly (31 % of isolates), and 75 % of those studied were capable of OTA production, a much higher percentage than reported elsewhere. A. carbonarius was found (6 % of isolates) only in the hottest region sampled, and only from beans in the drying yard or in storage. However, 77 % of the A. carbonarius isolates were capable of producing OTA. Other studies reported similar species distribution on Brazilian coffee beans. Martins et al. (2003) used a conventional method to identify fungal flora in coffee bean. The predominant fungal genus was Aspergillus, including A. niger (83.3 %), A. ochraceus (53.3 %) and A. flavus (25 %). The incidence of other genera was substantially lower than that of aspergilli. Magnani et al. (2005) isolated and identified Aspergillus spp. that contaminate coffee beans by sequencing the ITS region of the isolates. The incidence of potentially ochratoxigenic species was 82 % with A. niger being found most frequently, followed by A. ochraceus and A. carbonarius. However, the mycobiota of coffee beans in other countries or different type of coffee beans can be significantly different, e.g. in Llic et al. (2007), Vietnamese Robusta coffee beans were studied, and A. niger was the only ochratoxigenic species recovered. However, in another study carried out by Leong et al. (2007b) A. carbonarius isolates have also been recovered from Vietnamese Robusta and Arabica coffee bean samples.

We examined the mycobiota of coffee beans came from Thailand to clarify which species could be responsible for OTA contamination in this region. Different types of coffee varieties are cultivated in Thailand. Coffea arabica is the one grown in the Northern mountain area with elevation of more than 2 500 feet above sea level and average temperature of 18–25 °C. Coffea canephora var. robusta is grown in the Southern region of Thailand characterised by a totally different geography and climate, with elevation of not more than 500 feet above sea level, much more rain fall and average temperature of 25–35 °C.

Molecular identifications have not been carried out in most studies dealing with the mycobiota of coffee beans, which could lead to mis-identification of some closely-related species. In this study we analyzed the black aspergilli isolated from coffee beans using a polyphasic approach including morphological examinations, analysis of extrolite profiles and sequence analysis.

For Arabica coffee bean samples from the North, two types of samples, parchment coffee bean and green coffee beans were examined. Overall results showed that approximately 75 % of the samples were contaminated by black aspergilli, and similar levels of contamination were observed for isolates belonging to Aspergillus section Circumdati. (Fig. 3) A. niger was the predominant species but there were sometimes more than two species colonising the same beans. The related species A. tubingensis and A. foetidus were also common. Discrimination between A. niger and related species could be easily archeived by partial β-tubulin gene sequencing (Fig. 4). All three species were clustered in separate clade. Compared to the molecular method using sequencing of the ITS regions and with RFLP analysis of rRNA by Magnani et al. (2005), β-tubulin gene sequencing is more applicable and proved to be more efficient for species identification. Surprisingly, A. carbonarius was not detected, possibly as a result of climate selection as A. carbonarius occurs more frequently in hot regions. So species belonging to both sections Circumdati and Nigri could be responsible for OTA contamination in this region.

Two types of Robusta coffee beans, dried coffee cherries and green coffee beans from the South were also studied. Black aspergilli were the predominant in the mycobiota, with 100 % contamination in coffee cherry samples and approximately 98 % contamination in green coffee bean samples (Fig. 5), much higher than those reported in Brazilian coffee beans (Taniwaki 2003). Both A. carbonarius and A. niger were common and predominant in both types of coffee bean. Aspergillus spp. belonging to section Circumdati (A. westerdijkiae) was detected only in one sample. These results confirm a previous study of Joosten et al. (2001), who found that most of the examined 14 green coffee samples came from Southern Thailand were contaminated by black aspergilli, half of them by A. carbonarius. Based on these data, we presume that black aspergilli, especially A. carbonarius may play an important role in OTA contamination of coffee beans in Southern Thailand.

As a result of the survey of ochratoxin-producing aspergilli in Thai coffee beans, we also identified 2 new black Aspergillus spp. One of them (A. aculeatus) is related to A. aculeatus and other uniseriate black aspergilli and could be recovered from both regions, while the other one (A. scleroticarbonarius) is related to A. carbonarius and A. ibericus, and was found only in the Southern region of Thailand. Formal description of these species is in progress.

The diversity of black aspergilli recovered from Thai coffee beans is summarised in Table 3. Comparing the occurrence of black aspergilli from different parts of Thailand, remarkable differences were observed. A. carbonarius and A. scleroticarbonarius were found only in Southern Thai while A. foetidus was found only in the Northern region. These differences could be due to differences in the geography, climate and methods used for coffee processing in the two regions. The so-called wet method is used for Arabica coffee processing while the dry method is used for Robusta coffee processing. Principally, the dry method has three basic steps:
Pe r r o n e et a l.


Table 3. Distribution and ochratoxin producing abilities of black aspergilli in Thai coffee beans.

<table>
<thead>
<tr>
<th>Arabica (Northern Thailand)</th>
<th>Robusta (Southern Thailand)</th>
<th>Ochratoxin A production</th>
<th>Ochratoxin B production</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. niger (44 %)</td>
<td>A. niger (28 %)</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>A. tubingensis (19 %)</td>
<td>A. tubingensis (17 %)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A. foetidus (28 %)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A. aculeatinus (9 %)</td>
<td>A. aculeatinus (15 %)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>A. carbonarius (35 %)</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>A. sclerotialcarinarius (5 %)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

In brackets = percent of isolates identified from each type of Thai coffee beans.
Fig. 5. Neighbour-joining tree based on phylogenetic analysis of the partial β-tubulin gene sequences of black aspergilli recovered from Thai coffee.
cleaning, drying and hulling. In Thailand, the whole Robusta cherry is directly dried with sun drying. Suarez-Quiroz et al. (2004) also reported that the dry method seemed to increase the presence of A. carbonarius on the coffee beans. The wet method involves one more processing step: a fermentation step followed by cleaning to separate the beans from the pulp. This may cause changes in the natural substrate leading to changes in the species composition of the fungi colonising the beans. Differences in contact surfaces during processing may also play an important role in fungal contamination.

Ochratoxin producing abilities of black aspergilli isolated from Thai coffee beans were examined by the agar plug method of Smedsgaard (1997). OTA production was analysed by high performance liquid chromatography. A total of 83 isolates representing 6 species, A. carbonarius, A. niger, A. tubingensis, A. foetidus, A. aculeatinus and A. sclerotiorcarbonarius, were analyzed. The results confirmed former studies, only A. carbonarius and A. niger could produce ochratoxins. In this study, 100 % of the A. carbonarius isolates tested could produce large amounts of OTA but none of them produced ochratoxin B (Table 3). This is in agreement with Joosten et al. (2001), who reported that all A. carbonarius strains isolated from Thai coffee produced a significant amount of OTA. Similarly, Pardo et al. (2004) found that all A. carbonarius isolates came from coffee beans from various countries produced OTA, and Leong et al. (2007b) also observed that almost all (110/113) of the examined A. carbonarius isolates came from Vietnamese coffee beans could produce OTA. However, Taniwaki et al. (2003) observed that only 77 % of the A. carbonarius isolates came from Brazilian coffee beans produced OTA. Differences in the ratio of A. carbonarius isolates able to produce OTA could be due to misidentification of the non-OTA producer A. ibericus as A. carbonarius in previous studies. In contrast with previous reports, where 2–3 % of A. niger isolates isolated from coffee beans could produce ochratoxins (Heenan et al., 1998, Taniwaki et al. 2003), 13 % of the A. niger strains came from Thai coffee could produce both OTA and ochratoxin B but in rather small amounts compared to A. carbonarius (Fig. 6). It is more likely that A. carbonarius is the source of OTA contamination in Thai coffee beans.

In conclusion, diversity of black aspergilli in coffee beans occurring in Thailand depends on a combination of various factors including coffee variety, geographic region, climate and processing method. Significantly, more Robusta than Arabica beans were infected by black aspergilli, in agreement with the findings of Leong et al. (2007b) and Pardo et al. (2004). A. niger and related species are more important as contaminants of Arabica coffee beans in Northern Thailand, while A. carbonarius is responsible for OTA contamination of Robusta coffee beans in Southern parts of Thailand.

**Genetic diversity in A. flavus and implications for agriculture**

Aspergillus flavus is the most common species associated with aflatoxin contamination of agricultural crops (Cotty et al. 1994, Cotty 1997) (Fig. 7). A. flavus populations are highly diverse and their stability in the soil and on the plant is not well understood. An atoxigenic relative of A. flavus, A. oryzae, is widely used in Asian fermentation processes. It is now increasingly clear that A. oryzae is not a separate species, but actually is only one many examples of atoxigenic variants of A. flavus (Geiser et al. 2000). As much as 40 % of the soil isolates of A. flavus are incapable of producing aflatoxins (Cotty et al. 1994). Addition of atoxigenic strains of A. flavus to the soil of susceptible crops to dilute out toxin-producing strains is being used to remediate aflatoxin contamination of cotton and peanuts (Cotty and Bayman 1993, Horn et al. 2000, Horn and Dorner 2002).

As with other haploid fungal species, genetic isolation in A. flavus may be maintained by a vegetative compatibility system (Leslie 1993). A typical soil population is usually composed of isolates from hundreds of different vegetative compatibility groups (VCGs) (Bayman and Cotty 1991). No genetic exchange was found among A. flavus atoxigenic VCG isolates and toxin-producing isolates collected from six geographically separated regions, suggesting that recombination among VCGs is rare (Ehrlich et al. 2007b).
A. flavus soil populations also contain isolates from two morphologically distinct sclerotial size variants, termed the L-strain for isolates with sclerotial size greater than 400 μm and the S-strain for isolates with sclerotial size less than 400 μm (Cotty 1997). On typical laboratory growth media S-strain isolates produce higher levels of aflatoxins, more abundant sclerotia, and generally fewer conidia. Atoxigenic S-strain isolates are very rarely found in natural environments. Another consistent difference between S- and L-strain isolates is the size of deletion of portions of the genes, norB and cypA in the aflatoxin cluster. The size of the deletion in the norB-cypA gene was 1.5 kb for S-strain isolates and 0.8 kb for L-strain isolates. The gene cypA encodes a P450 monooxygenase that is necessary for formation of G aflatoxins. The deletion, therefore, is the reason why A. flavus is incapable of producing G aflatoxins. (Ehrlich et al. 2004). Most interestingly, A. oryzae isolates have an S-strain type deletion even though they morphologically resemble L strain A. flavus and make abundant conidia. When this gap size is included in a phylogenetic dataset that includes polymorphisms in the omA gene region of the aflatoxin cluster, a clade was distinguished that contained members of both aflatoxin-producing S strain isolates and L strain isolates incapable of AF production. Another clade was distinguished that contained both A. oryzae and L-strain isolates incapable of AF production. From this data we reasoned that the L-strain is the ancestral species and that A. oryzae derived from an atoxigenic L-strain ancestor, whereas S-strain isolates derived from an aflatoxin-producing L-strain ancestor (Chang 2006).

The adaptation of A. flavus to the carbon-rich environment of certain agricultural communities is perhaps conducive to gene loss. Many of the isolates incapable of aflatoxin production have multiple mutations in their aflatoxin cluster genes. A careful study of deletion patterns in different L-strain A. flavus isolates from peanut fields found that, in these isolates, part or most of the aflatoxin biosynthesis gene cluster is missing (Chang et al. 2005). Isolates of A. oryzae also have large deletions of the aflatoxin gene cluster (Lee et al. 2006). In some of these isolates the remaining aflatoxin biosynthesis genes Neighbooured the telomere. Proximity to the telomere may make the cluster more unstable. In A. parasiticus when normal development is thwarted, by forced repeated mycelial transfer, the resulting isolate permanently loses some of its normal developmental functions (Kale et al. 2003). It does not form conidia properly or make aflatoxins. The defects in these isolates remain to be determined.

Production of aflatoxin and its precursor metabolites is associated with increased production of conidia (Willkinson et al. 2004), but so far, unlike the protective role of melanin, no evidence has been found that the conidia are protected by making the aflatoxin cluster metabolites. It is thought that the red pigmented dothistromin may be a virulence factor for D. septosporum responsible for its pathogenicity to pine (Bradshaw et al. 2002). Like dothistromin, most of the aflatoxin precursor metabolites are red or orange. Because of their color, the metabolites could have helped to foster dispersal. In addition, since section Flavi isolates are normally saprophytic, polyketide metabolites may increase fungal survival in soil. Such a benefit may be unnecessary in carbon-rich agricultural environments. In such environments, the ability to make aflatoxins could be a vestigial function. To support this conjecture, when section Flavi isolates are collected from non-agricultural soils, almost all of the isolates examined were capable of producing aflatoxins (Ehrlich et al. 2007a). Furthermore, in some soils, A. flavus was not the most prominent species. Understanding the role of aflatoxin production and in general secondary metabolite production may only be possible if attempts are made to duplicate in the laboratory the conditions of the natural environment in which these aspergilli evolved.

CONCLUSIONS

Complexes of pathogenic and opportunistic species of Aspergillus can colonise and induce disease symptoms in various plants and plant products, and produce toxic secondary metabolites (mycotoxins) in the infected tissue. In this chapter we evidenced how environmental conditions, geographical areas and crops can influence both fungal populations associated and production of mycotoxins. In this respect, the studies on economically important Aspergillus species by a polyphasic approach are innovative, strategic and helpful in assessing the biodiversity of the population/species and the potential risk of mycotoxin contamination of the agricultural products. In particular, the phylogenetic analysis of sequences of
β-tubulin and calmodulin genes, AFLP polymorphisms and extrolite profiles together with morphological analysis have led to reconsider in the last five years the taxonomy within the Aspergillus section Nigri (about seven new species has been described). Also the reports on the occurrence of black aspergilli in agricultural products and their potential toxigenicity must be reconsidered on the basis of the wide molecular biodiversity found within morphologically undistinguishable strains of this section. Furthermore, there is a need of molecular studies on South-American black Aspergillus populations occurring on grapes and other agricultural products in order to ascertain the species composition and potential toxigenicity. Finally, the presence of an Aspergillus uniseriate population typical of grapes in Europe, named A. uvarum, is an interesting finding that needs further investigation in grapevine areas outside Europe in order to evaluate the distribution of this new species at a global level.

Within A. flavus and other Aspergillus species capable of aflatoxin production, considerable diversity is found. Such diversity makes it more difficult to assign firm taxonomic identity to isolates from such populations. For example, should all A. flavus that are incapable of producing aflatoxins be considered to be A. oryzae? Such isolates are routinely found in agricultural fields, but only some are now classified as A. oryzae. We now know that loss of G-aflatoxin formation in A. flavus is a result of deletions in three genes encoding enzymes required for conv. of O-methylsterigmatocystin to aflatoxin G1 and G2, namely the cytochrome P450, cypA, and the reductases, nadA and norB. The aflatoxin clusters of A. parasiticus and the recently described related taxon, A. minisclerotigenes from Australia, West Africa, and Argentina that produces both B and G aflatoxins contain functional v.s of these genes (Pildain et al. 2007). Further studies are needed to clarify if the other newly described species, A. arachidicola, which is closely related to A. parasiticus, also carry these genes. The separation between A. parasiticus and A. flavus is estimated to have occurred more than 8 Mya. The conidia of A. minisclerotigenes resemble those of A. flavus while those of A. parasiticus are distinctly different in appearance. Further studies need to be done to sort out what selective factors, both environmental and genetic affect cluster gene stability in these related organisms. In this regard, we need to know if agricultural interactions play a role in causing gene instability? We expect that comparisons of different fungal genomes and developing a better understanding of regulatory relationships may help in answering some of these questions.

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Nomenclatural considerations in naming species of Aspergillus and its teleomorphs

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Abstract: The nomenclature of Aspergillus is important in many fields of research and therefore the strategies for stable and efficient naming are important. The conservation of species names as accepted by the Aspergillus community is described. Published lists of accepted names provide that people who use Aspergillus and Penicillium taxonomies need no longer fear the overturning of names currently used. Aspergillus is a good example of a genus where the naming of both anamorph and teleomorph has been applied and arguments are given for maintaining the system of dual nomenclature. A protocol for describing new taxa in Aspergillus and their teleomorphs is proposed, including the availability of living ex type cultures, deposit of type cultures in at least two recognised culture collections, deposits of sequence data in specialised data bases and registration of the new names in MycoBank.

Key words: accepted Aspergillus names, conservation of names, dual nomenclature, protocol for species description.

INTRODUCTION

Aspergillus is one of the most studied fungal genera and has a great impact in many applied areas. The nomenclature of the species has always been an important issue because applied researchers do not like to see changes in species names, in particular for those which are common. Like all other fungi the naming of Aspergillus species must follow the Rules of the International Botanical Code. In the past however taxonomists have neglected these rules and for example Raper & Fennell (1965) only used Aspergillus names for sexual taxa and refused to use the correct names for the teleomorph.

In recent years, strategies for maintaining a stable Aspergillus nomenclature have been in the forefront of Aspergillus taxonomy including proposals for the correct anamorph-teleomorph nomenclature, for subgeneric taxa and for a list of accepted names. In the following paper the conservation of species names and dual nomenclature are discussed. Furthermore a proposal for recommended procedure for the description of new species is set out.

Conservation of species names

For a long time, the International Code of Botanical Nomenclature (ICBN) has had provisions for the conservation of names. Conservation is a process that enables the use of a name – or more usually the continued use of a name – for a particular taxon that for one reason or another is not the name having priority, i.e. is not the earliest validly published name. Under the ICBN, it is mandatory to use the earliest valid name for any taxon. The purpose behind, and justification for, conservation is almost always to permit the continued use of a popular or important name, and thus to promote taxonomic stability. Examples of conservation include that of the yeast genus Candida Berkhout 1923, which includes the important pathogenic species C. albicans, against the older, validly published genus Syringospora Quinquaed 1868 and three others. Conservation avoided the need to transfer C. albicans to an essentially unknown genus, with consequent confusion among users of taxonomy. Many other examples exist of well known fungal genera that have been conserved, including Altemaria, Drechslera, Mucor and Rhizopus.

For a long time, the provisions for conservation in the ICBN extended only down to genus level. Until recently, it was not possible to conserve species names. When Pitt (1979) revised the taxonomy of Penicillium, the generic name was not in dispute, but a number of species names were. It became clear that some of the species names used by Thom (1930) and Raper and Thom (1949) were predated by earlier valid names. Examples are P. cyclopium Westling 1911 which is predated by P. aurantiogriseum Dierckx 1901, and P. nigricans Bainier 1930 (published by Thom 1930) by P. janczewskii K.M. Zelesy 1927. Nothing could be done about those situations, and the earlier valid names were taken up. However, the calamitous loss of P. chrysogenum Thom 1907, clearly predated by P. griseoroseum Dierckx 1901, was avoided by placing the two species in separate subgenera (Pitt 1979).

In due course the International Botanical Congress agreed to permit conservation of species names of "major economic importance" (the Berlin Code, Greuter et al. 1988). The problem of keeping the name P. chrysogenum was then able to be solved by conserving it (Kozakiewicz et al. 1992). The next meeting of the International Botanical Congress (the Tokyo Code, Greuter et al. 1994) made possible the conservation of any species name.

Conservation is a slow and often tedious process. It requires careful documentation of the problem, and establishment of the reasons for conservation. Any proposal must be approved by the Special Committee on Fungi and Lichens of the ICBN before ratification by a full Botanical Congress, a body that meets only every six years. Approval by the Special Committee is by no means automatic. A proposal for the conservation of the commonly used...
name Aspergillus nidulans – not a valid name for reasons that do not concern us here – was not approved in 1992.

The advent of molecular taxonomy enormously increased the importance of conservation of species names. Old herbarium specimens, unrecognisable by traditional techniques, can nevertheless often be recognised by DNA analysis. The case of species in Aspergillus is particularly serious. European herbaria contain many old Aspergillus specimens. These are long dead, so that cultural methods cannot be used to obtain a recognisable species, but they may still contain DNA of analysable quality. It can be safely stated that many of the names in use in Aspergillus are predated by names validly applied to specimens and recognisable by molecular techniques.

A solution to this type of problem by the validation of commonly used names was proposed by US botanists many years ago, but it was not acceptable to the wider botanical community. Dr Morten Lange, a distinguished Danish mycologist, took up this idea at the International Botanical Congress in Montreal, in 1959, but again without acceptance (D.L. Hawksworth, pers. comm.). In 1988, Dr David Hawksworth, then at the International Mycological Institute, Kew, Surrey, U.K., organised a meeting there to again put forward this proposal (Hawksworth 1988). This led to a Symposium at the Royal Botanical Gardens, Kew that he organised, covering all groups of organisms and several publications (Hawksworth 1991a, 1991b, 1992, 1993). In these works, he and others proposed that lists of “Names in Current Use” (NCU) be established. These would be developed by appropriate experts, circulated widely to obtain general agreement and then sanctioned by a Botanical Congress. Names in an NCU would in a sense be cast in stone – they should not be subject to nomenclatural challenge from earlier names, if earlier valid names were subsequently located. At the same time, taxonomy would not be affected – names in an NCU could be split or combined by taxonomic decisions, but could not be overturned by nomenclatural findings. This was a brilliant concept, as at a stroke it would provide taxonomic stability to species in genera such as Aspergillus, Penicillium and their teleomorphs.

At Dr Hawksworth’s request, we prepared an NCU list of all the names in the fungal family Trichocomaceae, including of course Aspergillus and Penicillium. That list was published (Pitt & Samson 1993) and presented to the Tokyo Botanical Congress in 1993, along with the overarching concept of Names in Current Use. Unfortunately, the concept failed to gain Congress approval, and the whole idea has gone into abeyance, though it has not been abandoned. However, as reported in the Preface to the Tokyo Code (Greuter et al. 1994), the Nomenclature Section of that Congress “was particularly impressed by the utility of the list of species names in Trichocomaceae” and so “urges taxonomists not to adopt names that would compete with or change the application of any names on that list.”

This concept appears to have met with general approval among the specialist taxonomists who work with Aspergillus, Penicillium and related genera in Trichocomaceae, and has provided the stability which has long been sought. People who use Aspergillus and Penicillium taxonomies need no longer fear the overturning of names currently used.

The conflict between dual nomenclature and “one name, one fungus”

The concept of “dual nomenclature”, which simply means the use of more than one name for a single taxon, was established in the International Code of Botanical Nomenclature (ICBN) in 1910, to accommodate the problem of naming fungi that exhibit pleomorphic life cycles (Cline 2005). Article 59 of the ICBN governs the naming of these fungi. The Article has implication for many common fungi that are holomorphic, i.e. that produce both a teleomorph and an anamorph. Dual nomenclature has permitted the use, for any taxon, of either the teleomorph or the anamorph name as appropriate.

Aspergillus is a good example of a genus where dual nomenclature has been applied. Five of the six subgenera in Aspergillus include one or more species that produce a teleomorph, and many more that do not. Teleomorph – anamorph relations in Aspergillus are complex, because Aspergillus is associated with eight teleomorph genera. Molecular evidence to date indicates that these are all phylogenetically related (Peterson 2000). However, the major teleomorph genera with Aspergillus anamorphs are quite distinct from each other, with large differences in both morphology and physiology. Eurotium species are xerophiles, and cause spoilage of essentially any low water activity (dry or concentrated) material. Ascospores are only produced on media with increased sugar concentrations. Neosartorya species are thermophiles, and are not xerophilic, inhabiting decaying vegetation. Ascospores are readily produced and have exceptional heat resistance, causing spoilage of pasteurised products. Emericella species are neither xerophiles nor thermophiles, and are soil inhabiting fungi seldom found elsewhere.

Neosartorya species are classified in section Fumigati, and related to Aspergillus fumigatus, a species of great importance in medical mycology. Species related to Emericella are classified in section Nidulantes, and this section includes a number of species which consistently produce teleomorphs. However, in both of these Aspergillus sections, a larger number of species never make ascospores, or any body that resembles an ascocarp.

One teleomorph genus, Petromyces, classified in section Flavi, includes only one or two species that have ever been shown to produce ascospores, and production has been observed only rarely. However, many species in section Flavi are clearly related both morphologically and molecularly to Petromyces (Peterson 2000). For example, Aspergillus flavus commonly produces black sclerotia analogous to, and undoubtedly related to, the ascomata of Petromyces, but none of the many thousands of isolates examined around the world has ever been recorded to produce ascospores.

So throughout Aspergillus there are some species that produce teleomorphs and these are clearly related to many species where teleomorphs are unknown. Dual nomenclature has provided a simple means for distinguishing those that make ascospores from those that do not, and that has been of great practical importance.

A similar situation exists with Penicillium. Some species classified in Eupenicillium include naturally occurring isolates that may (a) produce both the teleomorph and the anamorph; (b) produce sclerotal bodies (like A. flavus) that have never been known to differentiate into ascospores; and (c) make only the anamorph, with no hint of a teleomorph connection. A classic example is the species Eupenicillium cinnamomopurpureum. Isolates are encountered that belong to each of these three categories, and where only the anamorph is produced the fungus is correctly known as Penicillium phoeniceum, the earliest valid name for the anamorph (Pitt 1979). Other similar examples of the presence or absence of the teleomorph and/or sclerotial state are E. hiyayamae and E. pinetorum. It appears that evolution in both Aspergillus and Penicillium is towards production of the anamorph alone, but of course the speed of this evolution is unknown.

The system of dual nomenclature has worked well, and has been of particular importance in food mycology. Because the presence of
a teleomorph tells so much about physiology, spoilage capabilities and potential for mycotoxin production, food mycologists have used the teleomorph names for species known to produce telemorphs for more than 20 years. Species where telemorphs are not known, and that includes the great majority of Aspergillus species, as well as species in many other important genera including Penicillium, Fusarium, Paecilomyces and Alternaria, are called by their anamorph names. This has proved to be a sensible and practical approach to taxonomy, where the name used provides the maximum amount of basic knowledge about a species. If a species is reported as a Eurotium, people in the food industry know immediately they are dealing with a xerophilic organism, if as a Neosartorya or a Byssochlamys, the spoilage problem they confront will be due to heat resistant ascospores.

A proposal to abandon dual nomenclature – termed “one name one fungus” – has a great deal of appeal to the theoretical mycologist. Why should a fungus have more than one name when genetic studies will often determine that it is a single species based on DNA analysis? This topic has been debated at length elsewhere (Gams et al. 2003; Hawksworth 2004; Rossman & Samuels 2005; Gams 2005). It is pertinent here because Aspergillus happens to be one of the hardest genera to see a way forward. One group of scientists says “The teleomorph name has precedence in the ICBN, so all species should be named according to the teleomorph with which molecular science indicates they are associated”. That approach is simplistic. First, it is not clear to what teleomorph genus some anamorph species may be associated. This is not a serious problem in Aspergillus but becomes very complex with species of Trichoderma or Paecilomyces. Second, many industrial users of taxonomies are now well familiar with the fact that a teleomorph name on a fungus means ascospores: use of teleomorph names for species without ascospores can only cause loss of information. Third, that approach requires hundreds and perhaps thousands of name changes. It is most unlikely that practical users of taxonomies would ever accept those new names, and confusion would result.

An alternative is to apply the well known anamorph names, like Aspergillus and Penicillium, to both teleomorph and anamorph species. Again, this is a retrograde step, as applying anamorph names to Eurotium or Neosartorya species also results in loss of information. Ascosporic Aspergillus species are known to have special properties in many cases, and users rely on teleomorph names to alert them to those properties. Moreover, the number of name changes needed, and the resistance to those new names, would lead to confusion once more.

This very difficult nomenclatural problem will only be resolved when a practical compromise is reached. The most obvious and sensible one is to follow the lead given by the food mycologists. Teleomorph names should be used where these are known, and anamorph names for those species that have no teleomorph. Food and industrial mycologists – probably the most numerous among the group of mycologists – agreed upon with general consensus (Samson et al. 2007).

Proposal for describing new taxa in Aspergillus and their telemorphs

The taxonomy of Aspergillus has evolved from a simple morphological species concept in which morphological characters of the conidiophores and conidia together with colony colours and patterns were used, into a polyphasic approach with strong molecular and biochemical characters. This means that the traditional rules following the Botanical Code are insufficient. For the comparison with newly proposed species dried herbarium specimens of holotypes or iconotypes are not suitable anymore.

The following procedure is proposed:

- For species descriptions, a polyphasic approach is preferred including morphological, physiological, molecular and/or ecological data
- Type cultures of new Aspergillus and telemorph species should be deposited in at least two international recognised culture collections
- Type cultures should be available directly after the description has been published. If type cultures are not available for the scientific community within six months after the description the species will be considered invalid
- Latin descriptions can be short indicating differences with related taxa
- Good morphological and physiological descriptions are essential
- Media used for the description should be Malt and Czapek based and exact formulations indicated
- The new species name should be registered at MycoBank (see www.Mycobank.org)
- Sequence data should be deposited in recognised genetic databases

The following procedure was extensively discussed at the International workshop “Aspergillus systematics in the genomics era” (Utrecht, 12–14 April 2007) and agreed upon with general consensus (Samson et al. 2007).

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The species concept in Aspergillus: recommendations of an international panel

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INTRODUCTION

During an International Workshop held from 12–14 April 2007 in Utrecht, The Netherlands, participants discussed what an Aspergillus species is and how we delimit a species. In several sessions the species concept was presented by researchers in Aspergillus covering traditional and modern taxonomy, genetics, clinical, industrial and applied microbiology, biochemistry and genomics. Several points were discussed including the following:

- What and how many genes should be used to delimit an Aspergillus taxon?
- How does the phylogenetic species concept translate to practical and routine diagnoses?
- What is the impact of Aspergillus taxonomy in terms of epidemiology, case definitions and biological understanding of disease?
- What are the roles of Aspergillus databases for species identification?
- What is the value and impact of polyphasic approaches for species identification?
- What genes/methods can be used to design kits for rapid identification?
- How should new species be proposed?

The following issues are described below in more detail:

1. Which and how many genes are required to delimit a species?

During the discussion, several points were dealt with. It was suggested that genealogical concordance principles should be tested before we can answer the question of how many genes are needed to define a species. It was agreed that ITS sequences should be determined for later quick identification purposes, although sequence data are not required by the International Code of Botanical Nomenclature. For species delimitation, the polyphasic approach was suggested as the “gold standard” using a combination of multilocus sequence data, morphological, physiological characteristics and ecological data. However, no one character could be used as a “gold standard” to test the null hypothesis that this is not a new species, only applying the whole set of characters will enable us to define new species. For species descriptions it is recommended to examine several (2–3) gene sequences (e.g. ITS, calmodulin, β-tubulin, actin) and submit them to recognised sequence databases. If the description of the new species meets the current conditions of the Botanical Code, it is not possible to reject a species. However, such an action can be recommended in the review of manuscripts describing new Aspergillus species. Regarding extrolites, it was suggested that a set of 4–8 compounds to be used, rather than a single molecule – no recommendations for which molecule. Additional points: it would be good to have a database (DNA and other types of characters) for quick identification; characters that are used for identification versus characters that are used to delimit a species should be separated.

Summary: A polyphasic approach is the gold standard, but it is difficult to define a standard set of criteria that must be met. Morphological characteristics are sometimes variable and need to be combined with other characters. Some suggestions for other characters included DNA sequences, physiological and ecological data and extrolite analyses. Regarding sequence data, ITS has been suggested because it has been used widely, but ITS sequences frequently show little or no variation between otherwise easily recognised closely related species. Solid phylogenetic species recognition generally requires multiple, more variable loci such as β-tubulin, calmodulin, actin, and other intron-rich protein coding genes. Difficulties regarding sequence data include the fact that not everyone has access to PCR and sequencing facilities, it is still unknown if other genes are more informative, and sequence data are not required by the Botanical Code. Consequently the absence of sequence data is presently not ground for rejection of new species.

2. What do we do about dual nomenclature?

It was suggested to use the same species name for both the anamorph and teleomorph, although it is not invalid to use another name according to the Botanical Code. A single culture should have only one name, but again it is not invalid to use a second name. Several participants suggested using Aspergillus as the primary name and the teleomorph as a secondary name, but experts of the International Botanical Code of Nomenclature disagreed as the second name will be invalid. Another suggestion was to epitypify all
new species to prepare for a single name, although again, it is not in agreement with the Botanical Code. Regarding clinical researchers who depend on one of the names, it was suggested to use the name “group” (or complex) if identification is based on morphology, which cannot distinguish between closely related species. Most, although not all participants, agreed to give preference to the teleomorph name. Researchers working on Aspergillus genetics mentioned that in a specific field, a name has a certain meaning, and preferred the name Aspergillus for phylogeny and genetics. Another suggestion was to give the name as follows: teleomorph [anamorph genus] if both exist, but it was rejected by most participants because names should not become too long by combining anamorph and teleomorph names. In the final vote, it was accepted by most participants to use dual names where necessary, single name in normal use, depending on the state that is seen, and treat it as a recommendation. Regarding Latin description of new species, it was accepted to have a short Latin diagnosis, followed by a more detailed English description. The Botanical Code accepts a valid description or a diagnosis in Latin. The majority of the workshop saw the need for a separate fungal nomenclatural code such as the code which the bacteriologists use.

Summary: Dual nomenclature is not an ideal system, but there are several difficulties with changing this. The majority voted to keep two names, but there were dissenting opinions. According to the Botanical Code, two names are legal and changing the code is difficult. However, different research communities sometimes use different names for a single species, causing confusion. The different names signal different morphological and physiological characteristics that people are looking for. Using a terminology such as complex or group might be helpful, but not precise enough for some applications. Names should not become too long by combining anamorph and teleomorph names. Single names would simplify things now that DNA features are available and identification does not necessarily depend only on morphology. However, it is difficult to have users switch to different names, and there is no consensus on which (teleomorph or anamorph) name to use.

3. What are the standards for describing and storing type cultures?

It was proposed that ex type cultures of new species be deposited in 2–3 different culture collections, preferably located in different parts of the world (Asia, Europe, US) to deal with import/export issues. If there is no available type culture, the Aspergillus community has the option of declaring it invalid. It was also suggested that at least a single locus DNA sequence must be provided for publication. There should be a limit to the time (e.g. six months) between publishing and depositing in collections. These suggestions are not accommodated by the Botanical Code, but were accepted as recommendations for good practice. New taxa should always be compared with ex type cultures of related species.

Summary: There should be 2–3 different open sources of a type culture of a newly proposed species in order to be scientifically valid. These should be deposited in a timely manner. New descriptions that are not deposited will be considered invalid by the Aspergillus community.

4. What sorts of databases do we need?

It was agreed that different specialised databases are needed for key identification purposes, with good links between databases. However, each database requires funding and curation. The use of a Wikipedia approach for the databases was questioned because of a lack of quality control, although it is a strong possibility as a clearinghouse for general information regarding protocols and media. It was suggested to use the Aspergillus website as a clearing house by linking to other sites. It was accepted that links should show the focus of each individual database, and there is a need for links to other communities as well. The databases that are linked to the Aspergillus website should be of high quality. Some overlap between databases is not a real problem. Regarding a simple database for species identification, it was suggested to include basic sequences for identification, pictures and links to media protocols. Although such a database already exists, it is in a more complicated form. Finally, it was agreed that the Wikipedia approach, not suitable for databases, was a good idea for media and protocols.

Summary: Databases are critical for identification and biology. In general, focused databases that are linked to other related databases were encouraged. The links should give an idea about the content of the database. A simple database for identification was proposed which would include basic sequences, photos, links to media/growth protocols, or the possibility to make a phylogenetic tree to get around the nomenclature problem.

RECOMMENDATIONS

- A polyphasic approach is recommended for describing new Aspergillus species, including molecular, morphological, physiological and ecological data.
- Any new species should be compared to type strains of presumed relatives.
- Any proposed new species should show evidence for evolutionary divergence from other taxa, particularly unique DNA characters at multiple loci, in addition to any distinctive extrolites and morphological characters.
- Latin descriptions can be short diagnoses, indicating differences from related taxa.
- Detailed morphological and physiological descriptions should be provided.
- Media used for the description should be based on the use of media recommended by the International Commission of Penicillium and Aspergillus: Malt Extract Agar and Czapek’s Agar, with referenced formulas.
- Type cultures of new Aspergillus species should be deposited in at least two international recognized culture collections.
- If type cultures are not made available for the scientific community, the species will be considered invalid.
- New species names should be registered at MycoBank (www.MycoBank.org).
- For the description of new taxa, multiple, independent
loci are recommended for use, particularly loci for which large datasets already exist, such as ITS, β-tubulin, calmodulin, actin, RNA polymerase.

- Sequences must be deposited in recognized genetic databases.
- Use dual names where necessary, and a single name in normal use, depending on the state (teleomorph or anamorph) that is observed.
- Focused databases that are linked to other related databases are encouraged, with links giving an idea about the content of the database.

- A simple database for identification was proposed which would include basic sequences, photos, links to media/growth protocols, or the possibility to make a phylogenetic tree.

This protocol will be endorsed by the IUMS International Commission of Penicillium and Aspergillus.
Polyphasic taxonomy of *Aspergillus* section *Candidi* based on molecular, morphological and physiological data

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**Abstract:** *Aspergillus* section *Candidi* historically included a single white-spored species, *A. candidus*. Later studies clarified that other species may also belong to this section. In this study, we examined isolates of species tentatively assigned to section *Candidi* using a polyphasic approach. The characters examined include sequence analysis of partial β-tubulin, calmodulin and ITS sequences of the isolates, morphological and physiological tests, and examination of the extrolite profiles. Our data indicate that the revised section *Candidi* includes 4 species: *A. candidus*, *A. campestris*, *A. taichungensis* and *A. tritici*. This is strongly supported by all the morphological characteristics that are characteristic of section *Candidi*: slow growing colonies with globose conidial heads having white to yellowish conidia, conidiophores smooth, small conidiophores common, metulae present and covering the entire vesicle, some large *Aspergillus* heads with large metulae, presence of dimorphic heads in all species, conidia smooth or nearly so with a subglobose to ovoid shape, and the presence of sclerotia in three species (*A. candidus*, *A. taichungensis* and *A. tritici*). *Aspergillus* *Tritici* has been suggested to be the synonym of *A. candidus* previously, however, sequence data indicate that this is a valid species and includes isolates came from soil, wheat grain, flour and drums from India, Ghana, Sweden, The Netherlands and Hungary, making it a relatively widespread species. All species produce terphenyllins and candidusins and three species (*A. candidus*, *A. campestris* and *A. tritici*) produce chlorflavonins. Xanthoascsins have only been found in *A. candidus*. Each of the species in section *Candidi* produce several other species specific extrolites, and none of these have been found in any other *Aspergillus* species. *A. candidus* has often been listed as a human pathogenic species, but this is unlikely as this species cannot grow at 37 °C. The pathogenic species may be *A. tritici* or white mutants of *Aspergillus flavus*.

**Taxonomic novelty:** revalidation of *Aspergillus tritici* Mehrotra & Basu.

**Key words:** Ascomycetes, *Aspergillus* section *Candidi*, β-tubulin, calmodulin, Eurotiales, extrolites, ITS, polyphasic taxonomy.

**INTRODUCTION**

*Aspergillus* section *Candidi* (Gams et al. 1995; *A. candidus* species group according to Raper & Fennell 1965) was established by Thom & Raper (1945) to accommodate a single white-spored species, *A. candidus* Link. This species frequently contaminates stored food and feeding stuff (Kozakiewicz 1989; Park et al. 2005). *A. candidus* is moderately xerophilic, and able to grow on stored grains with 15 % moisture content (Lacey & Magan 1991), raising the moisture level of the infested grain to 18 percent or higher, and the temperature to up to 55 °C. This species is one of the most frequently encountered mould in cereal grains and flour (Rabie et al. 1997; Weidenbörner et al. 2000; Ismail et al. 2004; Hocking 2003). *A. candidus* causes loss of viability and germ discoloration in cereals (Papavizas & Christensen 1960; Battacharya & Raha 2002; Lugauskas et al. 2006). It also occurs in soil, usually on seeds or in the rhizosphere, and also in milk (Raper & Fennell 1965; Kozakiewicz 1989; Moreau 1976).

*A. candidus* enzymes has also been used in the fermentation industry for the production of galacto-oligosaccharides (Zheng et al. 2006), and D-mannitol (Smiley et al. 1976). While some *A. candidus* metabolites including terphenyllins has antioxidant and anti-inflammatory activities (Yen et al. 2001, 2003). *A. candidus* is also used in the meat industry for spontaneous sausage ripening (Gracia et al. 1988; Sunesen & Stahnke 2003).

*A. candidus* is claimed to be involved in a wide range of human infections including invasive aspergillosis (Rippon 1988; Ribeiro et al. 2005), ootmycosis (Yasin et al. 1978; Falser 1983), brain granuloma (Linares et al. 1971) and onychomycosis (Schonborn & Schmoranzer 1970; Zaror & Moreno 1980; Piracini et al. 2002). *A. candidus* has also caused various disorders in pigs (Moreau 1979) and was found to be the second most prevalent *Aspergillus* species in a hospital surveillance project in the U.S.A. (Curts et al. 2005). Concentration of *A. candidus* conidia can reach alarming levels in grain dust and was suggested to contribute to the development of the so-called organic dust toxic syndrome (Weber et al. 1993; Krysinska-Traczyk & Dutkiewicz 2000). *A. candidus* is able to induce both cellular and humoral response in animals (Krysinska-Traczyk & Dutkiewicz 2000). *A. candidus* metabolites including terphenyl compounds and terpenins exhibit immunomodulating capabilities and are highly cytotoxic (Shanahan et al. 1998; Krysinka & Dutkiewicz 2000). There is some evidence that *A. candidus* might be toxic to chickens and rats (Marasas & Smallay 1972) and has also been isolated from birds (Saiz 1970, Sharma et al. 1971). *A. candidus* has been reported to produce several secondary metabolites including candidusins (Kobayashi et al. 1982; Rahbaek et al. 2000), terpenins (Kamigauchi et al. 1998), chlorflavonin (Bird & Marshall 1969), dechlorochlorflavonin (Marchelli & Vining 1973), xanthoascsin (Takahashi et al. 1976b), kojac acid (Kinosita & Shikata 1969, Saruno et al. 1979, Cole & Cox 1983), 3-nitro-proapionic acid (Kinosita et al. 1968), and 6-sulfoaminopenicillanic acid (Yamashita et al. 1983). *A. candidus* is reported to produce citrinin but the first report of citrinin production by an *Aspergillus* confused *A. niveus* with *A. candidus* (Timmins & Roualt 1944; Raper & Fennell 1965). However, some later reports indicate that some isolates may produce citrinin (Kinosita & Shikata 1969; Cole & Cox 1981).
The description of \textit{A. candidus} is admittedly broad, encompassing considerable variability among the isolates (Raper & Fennell 1965, Kozakiewicz 1989). \textit{A. candidus} is characterised by white conidial heads, globose to subglobose vesicles, biseriate large and uniseriate small conidial heads, and smooth conidiophores and conidia (Raper & Fennell 1965, Kozakiewicz 1989). Several white-spored \textit{Aspergillus} species described in the past have been synonymised with \textit{A. candidus}, including \textit{A. albus}, \textit{A. okazakii}, or \textit{A. dubius} (Raper & Fennell 1965). Raper & Fennell (1965) also stated that “it is possible that our current concept of \textit{A. candidus} is too broad”. Recent studies indicated that other species including \textit{A. campestris} (Christensen 1982; Rahbaek et al. 2000; Peterson 2000; Varga et al. 2000) and \textit{A. taichungensis} (Yaguchi et al. 1995, Rahbaek et al. 2000) are also members of section \textit{Candidi}. Besides, two other white-spored species, \textit{A. tritici} (as \textit{A. triticus}, Mehrtra & Basu 1976) and \textit{A. implicatus} (Maggi & Persiani 1994) have also been suggested to belong to this section.

In this study, we examined available isolates of the species, proposed to belong to section \textit{Candidi}, to clarify the taxonomic status of this section. The methods used include sequence analysis of the ITS region (including internal transcribed spacer regions 1 and 2, and the 5.8 S rRNA gene of the rRNA gene cluster), and parts of the \(\beta\)-tubulin and calmodulin genes, macro- and micromorphological analysis, and analysis of extrolite profiles of the isolates.

### MATERIALS AND METHODS

#### Morphological examinations

The strains examined are listed in Table 1. The strains were grown for 7 d as 3-point inoculations on Czapek agar, Czapek yeast autolysate agar (CYA), malt extract agar (MEA), and oat meal agar (OA) at 25 °C (medium compositions in Samson et al. 2004).

#### Analysis for secondary metabolites

The cultures were analysed according to the HPLC-diode array detection method of Frisvad & Thrane (1987, 1993) as modified by Smidsgaard (1997). The isolates were analyzed on CYA and YES agar using three agar plugs (Smedsgaard 1997). The secondary metabolite production was confirmed by identical UV spectra with those of standards and by comparison to retention indices and retention times in pure compound standards (Rahbaek et al. 2000).

#### Isolation and analysis of nucleic acids

The cultures used for the molecular studies were grown on malt peptone (MP) broth using 10 % (v/v) of malt extract (Oxoid) and 0.1

### Table 1. The Aspergillus section Candidi isolates examined in this study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain No.</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Aspergillus campestris}</td>
<td>CBS 348.81(^t)</td>
<td>Soil, North Dakota, U.S.A.</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 119.28</td>
<td>IFO 5466; A. \textit{okazakii}</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 116945</td>
<td>Museum dust, Tiel, Netherlands</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 175.68</td>
<td>Mouse dung, Netherlands</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 114385</td>
<td>Air, Finland</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 120.38</td>
<td>No. 827/2; Unknown, J.C. Neill</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 225.80</td>
<td>Human nail, Netherlands</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 102.13</td>
<td>Japan, G. Kita</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 118.28</td>
<td>QM 9372; A. Blochwitz</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 566.65(^t)</td>
<td>ATCC 1002; IMI 091889; NRRL 303; unknown, J. Westerdijk</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 1-9F</td>
<td>TM 04.129 V11</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 13-C4</td>
<td>House, Utrecht, Netherlands</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 17-C2</td>
<td>House, Eindhoven, Netherlands</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 25-11</td>
<td>Indoor environment, Germany</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>IMI 091889</td>
<td>ATCC 1002, CBS 566.65</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 283.95</td>
<td>IFO 33019; JCM 10250; SRRC 310</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>IBT 19404(^t)</td>
<td>PF1167; Soil, Taiwan</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 567.65</td>
<td>ATCC 16871; IMI 230752; NRRL 312; unknown, Brazil</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 112449</td>
<td>Indoor environment, Germany</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 119225</td>
<td>SLV 541; wheat flour, Sweden</td>
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<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 117270</td>
<td>Djambee (drum), Ghana</td>
</tr>
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<td>\textit{Aspergillus candidus}</td>
<td>CBS 266.81(^t)</td>
<td>Wheat grain, India</td>
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<td>\textit{Aspergillus candidus}</td>
<td>CBS 11-H7</td>
<td>Feed ingredient, Netherlands</td>
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<td>\textit{Aspergillus candidus}</td>
<td>SZMC 0565</td>
<td>Viticultural Institute, Kecskemét, Hungary</td>
</tr>
<tr>
<td>\textit{Aspergillus candidus}</td>
<td>CBS 13686=IMI 78734=NRRL 2297; P.G. Stansly, B81</td>
<td></td>
</tr>
<tr>
<td>\textit{Aspergillus tritici}</td>
<td>CBS 263.95</td>
<td>ATCC 13686=IMI 78734=NRRL 2297; P.G. Stansly, B81</td>
</tr>
<tr>
<td>\textit{Aspergillus implicatus}</td>
<td>SZMC 0897</td>
<td>Agricultural Service, Bekes county, Hungary</td>
</tr>
<tr>
<td>\textit{Aspergillus implicatus}</td>
<td>CBS 848.95(^t)</td>
<td>Soil, Ivory Coast</td>
</tr>
</tbody>
</table>
% (w/v) bacto peptone (Difco), 2 mL of medium in 15 mL tubes. The cultures were incubated at 25 °C for 7 d. DNA was extracted from the cells using the Masterpure™ yeast DNA purification kit (Epicentre Biotechnol.) according to the instructions of the manufacturer. Fragments containing the ITS region were amplified using primers ITS1 and ITS4 as described previously (White et al. 1990). Amplification of part of the β-tubulin gene was performed using the primers Bt2a and Bt2b (Glass & Donaldson 1995). Amplifications of the partial calmodulin gene were set up as described previously (Hong et al. 2005). Sequence analysis was performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit for both strands, and the sequences were aligned with the MT Navigator software (Applied Biosystems). All the sequencing reactions were purified by gel filtration through Sephadex G-50 (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in double-distilled water and analyzed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The unique ITS, β-tubulin, and calmodulin sequences were deposited at the GenBank nucleotide sequence database under accession numbers EU076291–EU076311.

Data analysis

The sequence data was optimised using the software package Seqman from DNASTar Inc. Sequence alignments were performed by using CLUSTAL-X (Thompson et al. 1997) and improved manually. The neighbour-joining (NJ) method was used for the phylogenetic analysis. For NJ analysis, the data were first analysed using the Tamura–Nei parameter distance calculation model with gamma-distributed substitution rates (Tamura & Nei 1993), which were then used to construct the NJ tree with MEGA v. 3.1 (Kumar et al. 2004). To determine the support for each clade, a bootstrap analysis was performed with 1000 replications.

For parsimony analysis, the PAUP v. 4.0 software was used (Swofford 2002). Alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option with 100 random taxa additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1000 bootstrap replications (Hillis & Bull 1993). An A. flavus isolate was used as outgroup in these experiments.

RESULTS AND DISCUSSION

Phylogeny

We examined the genetic relatedness of section Candidi isolates using sequence analysis of the ITS region of the ribosomal RNA gene cluster, and parts of the calmodulin and β-tubulin genes. During analysis of part of the β-tubulin gene, 496 characters were analyzed, among which 68 were found to be parsimony informative. The Neighbour-joining tree based on partial β-tubulin genes sequences is shown in Fig. 1. The topology of the tree is the same as the single maximum parsimony tree constructed by

![Fig. 1. Neighbour-joining tree based on β-tubulin sequence data of Aspergillus section Candidi. Numbers above branches are bootstrap values. Only values above 70 % are indicated.](image-url)
The PAUP program (length: 240 steps, consistency index: 0.8833, retention index: 0.9263). The calmodulin data set included 532 characters, with 43 parsimony informative characters (Fig. 2). The topology of the Neighbour-joining tree was the same as that of one of the 78 maximum parsimony trees (tree length: 300, consistency index: 0.9396). The ITS data set included 492 characters with 5 parsimony informative characters. The Neighbour joining tree shown in Fig. 3 has the same topology as one of the more than 10⁵ maximum parsimony trees (tree length: 35, consistency index: 1.0000, retention index: 1.0000).

Phylogenetic analysis of both β-tubulin and calmodulin sequence data indicated that Aspergillus section Candidi includes 4 species, namely: A. candidus, A. campestris, A. taichungensis and A. tritici. Interestingly, the reference strain of A. candidus, CBS 283.95 was found to belong to the A. tritici species. Isolates CBS 597.65 and CBS 112449 were found to be related to the A. taichungensis type strain based on β-tubulin sequence data, and formed a distinct clade on the tree based on calmodulin sequences. Further studies are needed to clarify the taxonomic position of these isolates.

Comparison of our ITS sequence data to those available on the web site of the Japan Society for Culture Collections (http://www.nbrc.nite.go.jp/jscc/idb/search) indicated that several strains held as A. candidus represent other species. Three strains (NBRC 4389 = IFO 4389, NBRC 4037 = IFO 4037, and NBRC 4322 = IFO 4322) were found to be actually white-spored A. oryzae isolates, NBRC 5468 (= IFO 5468) and NBRC 33019 (= IFO 33019 = CBS 283.95 = SRRC 310) belong to A. tritici, while NBRC 32248 (= IFO 32248) has identical ITS sequence to A. campestris. However, further loci should also be analyzed to confirm their assignment. Other isolates including NBRC 8816, NBRC 4309, NBRC 4310 and NBRC 4311 are representatives of the A. candidus species based on their identical ITS sequences.

Aspergillus implicatus, another species previously assigned to this section (Maggi & Persiani 1994), was found to be more closely related to A. anthodesmis based on sequence data, which places this species close to Aspergillus section Sparsi (data not shown). Further studies are needed to clarify the taxonomic position of this white-spored species within the Aspergillus genus.

Chemotaxonomy

All strains of species in section Candidi produced terphenyllins and candidusins. Aspergillus candidus isolates produced candidusins A and B, terphenyllin, 3-hydroxyterphenyllin and some isolates...
also produced chlorflavonin and a chlorflavonin analogue. A. tritici isolates differed from A. candidus in not producing candidusin A and chlorflavonin. A. taichungensis produced candidusin C, terphenyllin, and 3-hydroxyterphenyllin, while the type strain of A. campestris also produced chlorflavonin. Xanthoascin was only found in some strains of A. candidus and not in any other species in Candidi. Each species produced a large number of as yet not structure elucidated extrolites. These extrolites, including terphenyllins, candidusins, chlorflavonins and xanthoascin, have only been found in section Candidi and not in any other aspergilli, except for A. ellipticus, that produces terphenyllin and candidusin (Samson et al. 2004, 2007).

Morphology

Aspergillus candidus is a wide-spread species throughout the world. According to Raper & Fennell (1965), “a typical strain of A. candidus differs little from members of the A. niger group except for the absence of both pigmentation and roughening in the conidia”. Another interesting feature observed in A. candidus is the production of diminutive conidial heads which are frequently uniseriate in contrast with the biseriate large heads. Colonies on CYA and MEA usually slow growing, colonies white to cream coloured, reverse usually uncoloured. Conidial heads usually biseriate, white to cream coloured, at first globose, with spore chains later adherent in loose divergent columns, diminutive heads commonly produced, conidiophores varying with the strain from less than 500 µm to up to 1000 µm long, thick walled, smooth, occasionally septate, vesicles globose to subglobose, ranging from 40 µm or more in diam in very large heads to less than 10 µm in small heads, typically fertile over the whole surface, phialides occasionally uniseriate in small heads but typically in two series, colourless, conidia globose or subglobose in most strains to elliptical in others, thin walled, 2.5–3.5 µm or occasionally 4 µm, smooth, colourless. Sclerotia, when produced, at first white, quickly becoming reddish purple to black, consisting of thick-walled parenchyma-like cells. A. candidus is unable to grow at 37 ºC.

Aspergillus taichungensis was described by Yaguchi et al. (1995) from soil, Taiwan. The species is characterised by restricted growth on CZA and MEA at 25 ºC, colonies white to pale yellow, velvety, reverse uncoloured. Conidial heads radiate, biseriate, conidiophores smooth, 300–450 µm long, often diminutive (90–250 µm long, biseriate), vesicles hemispherical to elongate, 5–20 µm in diam, fertile over the upper half to two-thirds, conidia hyaline, yellow in mass, globose to subglobose, microverrucose, 3–4 µm.

Fig. 3. Neighbour-joining tree based on ITS sequence data of Aspergillus section Candidi. Numbers above branches are bootstrap values. Only values above 70 % are indicated.
Dark brown sclerotia which appear on MEA after more than 25 d incubation. *A. taichungensis* is able to grow at 37 ºC on CYA.

*Aspergillus campestris* was described by Christensen (1982) from native prairie soil, North Dakota. The species is characterised by its restricted growth on CZA and MEA at 25 ºC, colonies velvety, sulphur yellow, reverse uncoloured. Conidial heads biseriate, radiate, conidiophores usually 400–800 µm but can be up to 1 300 µm long, often diminutive (10–75 µm, sometimes uniseriate), vesicles elongated, small (5–11 µm), conidia globose to subglobose, slightly roughened, 2.7–3.5 µm. At maturity conidia are embedded in a water drop giving the conidial heads a “slimy” appearance. The sclerotia are at first white, later becoming purple to black. *A. campestris* is unable to grow at 37 ºC on any media tested.

*Aspergillus tritici* was described as *A. triticus* by Mehrotra & Basu (1976) from wheat grains, India. Colonies are slow-growing on CZA and MEA, white to light cream coloured, reverse light brown. Conidial heads are biseriate, radiate, conidiophores thick-walled, septate, 130–700 µm long, often diminutive (10–75 µm, sometimes uniseriate), vesicles elongated, small (5–11 µm), conidia globose to subglobose, slightly roughened, 2.7–3.5 µm. At maturity conidia are embedded in a water drop giving the conidial heads a “slimy” appearance. The sclerotia are at first white, later becoming purple to black. *A. tritici* grows well at 37 ºC.

Based on a polyphasic investigation of *Aspergillus* section *Candidi*, the section includes four species: *A. candidus*, *A. campestris*, *A. taichungensis* and *A. tritici*. Phenotypic characteristics of these species are shown in Table 2. *A. campestris* was placed in section *Circumdati* because of its yellowish white conidia and it was not considered closely related to *A. candidus* by Christensen (1982). *A. taichungensis* was equivocally placed in either section *Versicolores*, *Terrei* or *Flavipedes* (Yaguchi et al. 1995). However, the phylogenetic and chemotaxonomic evidence presented here indicates that both species belong to section *Candidi*. This is strongly supported by all the morphological characteristics that are characteristic of the section *Candidi*: slow growing colonies with globose conidial heads having white to yellowish conidia, conidiophores smooth, small conidiophores common, metulae present and covering the entire vesicle, some large *Aspergillus* heads with large metulae, conidia smooth or nearly so with a subglobose to ovoid shape (albeit slightly ellipsoidal in *A. campestris*), and sclerotia present in *A. taichungensis*, *A. candidus* and *A. tritici*. Sclerotia have not been observed in *A. campestris*, but have been observed in *A. candidus* (light cream coloured turning purple to black in age). *Aspergillus tritici* has been suggested to be the synonym of *A. candidus* by Samson (1979). However, sequence data indicate that this is a valid species and includes isolates from soil, wheat grain, flour and drums from India, Ghana, Sweden, The Netherlands and Hungary, making it a relatively widespread species.

Table 2. Phenotypic characteristics of species in *Aspergillus* section *Candidi*.

<table>
<thead>
<tr>
<th></th>
<th><em>A. candidus</em></th>
<th><em>A. tritici</em></th>
<th><em>A. taichungensis</em></th>
<th><em>A. campestris</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphological characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colony colour</td>
<td>white</td>
<td>Light cream</td>
<td>Light cream</td>
<td>Sulphur yellow</td>
</tr>
<tr>
<td>Colony reverse</td>
<td>Un coloured to yellowish</td>
<td>Light brown</td>
<td>Un coloured</td>
<td>Un coloured</td>
</tr>
<tr>
<td>Conidial heads</td>
<td>Globose</td>
<td>Radiate</td>
<td>Radiate</td>
<td>Radiate</td>
</tr>
<tr>
<td>Conidiophores</td>
<td>Smooth, 500–1000 µm</td>
<td>Septate, 130–700 µm</td>
<td>Smooth, 300–400 µm</td>
<td>Smooth, 400–800 µm</td>
</tr>
<tr>
<td>Diminutive heads</td>
<td>Common</td>
<td>Common</td>
<td>Common</td>
<td>Common</td>
</tr>
<tr>
<td>Vesicles</td>
<td>Globose, 40 µm</td>
<td>Elongated, 5–11 µm</td>
<td>Hemispherical, 5–20 µm</td>
<td>Globose, 25–40 µm</td>
</tr>
<tr>
<td>Conidial ornamentation</td>
<td>Smooth</td>
<td>Slightly roughened</td>
<td>Microverrucose</td>
<td>Smooth</td>
</tr>
<tr>
<td>Conidial shape</td>
<td>(Sub)globose</td>
<td>(Sub)globose</td>
<td>(Sub)globose</td>
<td>Ellipsoidal</td>
</tr>
<tr>
<td>Size of conidia</td>
<td>2.5–3.5 µm</td>
<td>2.7–3.5 µm</td>
<td>3–5 µm</td>
<td>3–4 x 2.3–3 µm</td>
</tr>
<tr>
<td>Growth at 37 ºC</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sclerotia</td>
<td>Purple to black</td>
<td>Purple to black</td>
<td>Dark brown</td>
<td>-</td>
</tr>
<tr>
<td><strong>Extrolate production</strong></td>
<td></td>
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<tr>
<td>Candidus A</td>
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<tr>
<td>Candidus B</td>
<td>+</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>Candidus C</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Candidus analogue</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Terphenyllin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3-Hydroxyterphenyllin</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Chlorflavonin</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Chlorflavonin analogue</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
**Aspergillus campestris** Christensen, Mycologia 74: 212. 1982. Fig. 4.

*Type:* CBS 348.81, from soil from native prairie, North Dakota, U.S.A.

*Other no. of the type:* IBT 27921 = IBT 13382

**Description**
- Colony colour: sulphur yellow to pinard yellow
- Conidiation: abundant
- Reverse colour (CZA): uncoloured
- Colony texture: velvety
- Conidial head: diminutive, with few divergent spore chains
- Stipe: 400–800(–1300) × 7–12 µm
- Vesicle diam/shape: (18–)24–36(–46) µm, globose to subglobose
- Conidium size/shape/surface texture: 3–4 × 2.3–3 µm, ellipsoidal to egg-shaped, smooth
- Colony colour and diminutive conidial heads: restricted growth on all media, sulphur yellow
- Colony colour: sulphur yellow to pinard yellow
- Conidiation: abundant
- Reverse colour (CZA): uncoloured
- Colony texture: velvety
- Conidial head: diminutive, with few divergent spore chains
- Stipe: 400–800(–1300) × 7–12 µm
- Vesicle diam/shape: (18–)24–36(–46) µm, globose to subglobose
- Conidium size/shape/surface texture: 3–4 × 2.3–3 µm, ellipsoidal to egg-shaped, smooth

**Cultures examined:** CBS 119.28, CBS 116945, CBS 175.68, CBS 114385, CBS 120.38, CBS 225.80, CBS 102.13, CBS 118.28, CBS 566.65 1-F9, 13-C4, 17-C2, 25-I1, IMI 091889, CBS 283.95, NRRL 5214

**Diagnostic features:** phialides clustered on one side of the vesicle, echinulate conidia, slow growth rate and cream-yellow reverse on CYA; unable to grow at 37 °C

**Similar species:** *A. tritici*

**Distribution:** worldwide (Bangladesh, Pakistan, Kuwait, Sri Lanka, Japan, South Africa, Somalia, Chad, Libya, Egypt, Syria, Israel, Argentina, Bahama Islands, New Guinea, Solomon Islands, China, Central America, Chile, Russia, Nepal, U.S.A., Spain, Italy, Hungary, Austria, Czecho- Slovakia, Germany, France, Britain, Ireland, Netherlands, Denmark)

**Ecology and habitats:** stored products, especially cereals, soil, dried fruits, dung, dried fish, indoor air

**Extrolites:** terphenyllin, 3-hydroxyterphenyllin (Rahbaek et al. 2000), prenylterphenyllin, 4''-deoxyprenyterphenyllin, 4''-deoxyisoterphenyllin, 4''-deoxyterphenyllin (Wei et al. 2007), and other terphenyl-type compounds (Marchelli & Vining 1975 Kurobane et al. 1979; Kobayashi et al. 1985; Takahashi et al. 1976b), including candidusins (A & B) (Kobayashi et al. 1982) and other terpenins (Kamiguchi et al. 1998), chlorflavonin (Bird & Marshall 1969; Munden et al. 1970), dechlorochlorflavonin (Marchelli & Vining 1973), and xanthoascan (Takahashi et al. 1976a). The production of terphenyllin, 3-hydroxyterphenyllin, candidusin A, candidusin B, chlorflavonin and xanthoascan was confirmed by HPLC-DAD.

**Aspergillus candidus** Link, Mag. Ges. Naturf. Freunde Berlin 3: 16. 1809. Fig. 5.

= Aspergillus okazakii Okazaki (1907)

*Type:* CBS 566.65, from Westerdijk, 1909

*Other no. of the type:* ATCC 1002; IMI 091889; LSHB Ac27; NCTC 595; NRRL 303; QM 9915; WB 303

**Description**
- Colony diam: CZA25: 15–30 mm; CYA25: 13–20 mm, MEA25: 8–14 mm, YES25: 19–33 mm, OA25: 9–18 mm, CYA37: 0 mm, CREA25: poor growth and no acid production
- Colony colour: white
- Conidiation: limited
- Reverse colour (CZA): uncoloured to pale yellow
- Colony texture: submerged
- Conidial head: diminutive, with few divergent spore chains
- Stipe: 500–1000 × 5–10(–20) µm, walled, smooth, occasionally septate, colourless or slightly yellowed in age
- Vesicle diam/shape: 10–40 µm, globose to subglobose
- Conidium size/shape/surface texture: 2.5–3.5(–4) µm, globose to subglobose, smooth
- Colour and diminutive conidial heads: restricted growth on all media, sulphur yellow
- Colony colour and diminutive conidial heads: restricted growth on all media, sulphur yellow
- Colony colour: sulphur yellow to pinard yellow
- Conidiation: abundant
- Reverse colour (CZA): uncoloured
- Colony texture: velvety
- Conidial head: diminutive, with few divergent spore chains
- Stipe: 400–800(–1300) × 7–12 µm
- Vesicle diam/shape: (18–)24–36(–46) µm, globose to subglobose
- Conidium size/shape/surface texture: 3–4 × 2.3–3 µm, ellipsoidal to egg-shaped, smooth
- Colony colour and diminutive conidial heads: restricted growth on all media, sulphur yellow
- Colony colour: sulphur yellow to pinard yellow
- Conidiation: abundant
- Reverse colour (CZA): uncoloured
- Colony texture: velvety
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- Vesicle diam/shape: (18–)24–36(–46) µm, globose to subglobose
- Conidium size/shape/surface texture: 3–4 × 2.3–3 µm, ellipsoidal to egg-shaped, smooth
Fig. 6. Aspergillus taichungensis. A–B Colonies after 7 d at 25 °C. A. CYA. B. MEA. C. Conidial heads. D–H Conidiophores. I. Conidia. Scale bars = 10 µm.
**Aspergillus taichungensis** Yaguchi, Someya & Udagawa, Mycoscience 36: 421. 1995. Fig. 6.

**Type**: PF1167, from soil, Taiwan

**Other no. of the type**: IBT 19404

**Description**
Colony colour: yellowish white to primrose
Condidiation: moderate
Reverse colour (CZA): colourless (CZA), light yellow to pale luteous (MEA)
Colony texture: floccose (MEA)
Conidial head: loose radiate
Stipe: 300–440 × 5–9 µm
Vesicle diam/shape: 5–20 µm, hemispherical to elongate
Conidium size/shape/surface texture: 3–4 µm, globose to subglobose; sometimes ovoid, 3–5 × 3–4.5 µm, microverrucose

**Cultures examined**: IBT 19404, CBS 567.65, CBS 112449

**Diagnostic features**: slow growing colonies with globose conidial heads having white to yellowish conidia, presence of diminutive conidiophores and dark brown sclerotia

**Similar species**: *A. candidus, A. tritici*

**Ecology and habitats**: soil, air

**Distribution**: Taiwan, Brazil, Germany

**Extritolites**: candidusin C, terphenyllin, 3-hydroxyterphenyllin (Rahbaek et al. 2000, and confirmed in this study). A large number of additional extritolites, until now only found in this species, were also produced. These have not yet been structure elucidated, but had characteristic UV spectra

**Pathogenicity**: not reported

**Notes**: the type strain produces dark brown sclerotia 300–500 × 200–400 µm in size in 30 d (Yaguchi et al. 1995; Rahbaek et al. 2000); diminutive conidiophores present, 90–250 × 2–3 µm in size

**Aspergillus tritici** Mehrrota & Basu, Nova Hedwigia 27: 599, 1976. Fig. 7.

**Type**: CBS 266.81, from wheat grain, India

**Other no. of the type**: No. A x 194

**Morphological characteristics**
Colony colour: white to light cream coloured
Condidiation: moderate
Reverse colour (CZA): light yellow to light brown with age
Colony texture: radially furrowed
Conidial head: short radiate
Stipe: 130–700 × 4–8 µm (diminutive stipes 10–75 × 1.5–3.5 µm), septate
Vesicle diam, shape: 4.8–11 µm, small, only slightly enlarged at the end
Conidium size, shape, surface texture: 2.7–3.5 µm, globose to subglobose, slightly roughened

**Cultures examined**: CBS 119225, CBS 117270, CBS 266.81, CBS 112.34, 11-H7, SZMC 0565, CBS 283.95, SZMC 0897, IBT 23116, IBT 24170

**Diagnostic features**: colonies more yellowish than those of *A. candidus*; able to grow at 37 °C

**Similar species**: *A. candidus*

**Distribution**: India, Ghana, Sweden, Hungary, Slovenia, South Africa

**Ecology and habitats**: wheat, soil

**Extritolites**: candidusin B, candidusin analogue, terphenyllin, 3-hydroxyterphenyllin, chlorflavonin (Rahbaek et al. 2000, and confirmed in this study)

**Pathogenicity**: not reported, but since this species is able to grow at 37 °C, it may have caused some of the mycoses listed under *A. candidus*

**Notes**: some isolates produce sclerotia purple to black in colour; in some isolates conidia are embedded in a water drop with age (‘slimy’ appearance) and produces diminutive heads

**REFERENCES**


Taxonomic revision of *Aspergillus* section *Clavati* based on molecular, morphological and physiological data

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**Abstract:** Aspergillus section Clavati has been revised using morphology, secondary metabolites, physiological characters and DNA sequences. Phylogenetic analysis of β-tubulin, ITS and calmodulin sequence data indicated that Aspergillus section Clavati includes 6 species, *A. clavatus* (synonym: *A. apicilis*, *A. pallidus*), *A. giganteus*, *A. rhizopus*, *A. longivesica*, Neocarpenteles acanthosporus and *A. clavatonanicus*. Neocarpenteles acanthosporus is the only known teleomorph of this section. The sister genera to Neocarpenteles are Neosartorya and Dichotomomyces based on sequence data. Species in Neosartorya and Neocarpenteles have anamorphs with green conidia and share the production of tryptalevines, while Dichotomomyces was found to be able to produce gliotoxin, which is also produced by some Neosartorya species, and tryptalevines and tryptalevines produced by members of both section Clavati and Fumigati. All species in section Clavati are alkalitolerant and acidotolerant and they all have clavate conidial heads. Many species are coprophilic and produce the effective antibiotic patulin. Members of section Clavati also produce antifungal, tryptalevines, cytochalasins, sarcins, dehydrocarolic acid and kotanins (orlandin, desmethylkotanin and kotanin) in species specific combinations. Another species previously assigned to section Clavati, *A. ingratus* is considered a synonym of *Hemicarpenteles paradoxus*, which is phylogenetically very distantly related to Neocarpenteles and section Clavati.

**Key words:** Ascomycetes, Aspergillus section Clavati, β-tubulin, calmodulin, Dichotomomyces, Eurotiales, Hemicarpenteles, ITS, mycotoxin, Neocarpenteles, patulin, polyphasic taxonomy.

**INTRODUCTION**

Species in the section *Clavati* are alkalitolerant, often dung-borne species that produce several mycotoxins such as patulin (Varga et al. 2003), cytochalasins (Demain et al. 1976; Steyn et al. 1982), tryptalevines and tryptalevines (Clardy et al. 1975; Büchi et al. 1977), and other bioactive natural products, including the sarcins (Cole & Cox 1981; Lin et al. 1994). Weisner (1942) and Bergel et al. 1945 found that *A. clavatus* produces patulin, and Florey et al. (1944) reported on patulin production by *Aspergillus giganteus* in 1944. Clavatol (Bergel et al. 1944) and ascladiol (Suzuki et al. 1971) were also isolates from *A. clavatus* as antibiotics. Cytochalasin E and K are also mycotoxins known from *Aspergillus giganteus* (Demain et al. 1976). *A. clavatus* was also reported to produce kotanin and xanthocillin X dimethylether (Büchi et al. 1977). Among the mycotoxins produced, patulin is receiving world-wide attention due to its frequent occurrence in apple juices (Harrison 1989; Beretta et al. 2000). *Aspergillus clavatus*, *A. giganteus* and Neocarpenteles acanthosporus isolates also produce ribotoxins, which are promising tools for immunotherapeutic cancer (Martinez-Ruiz et al. 1999; Varga et al. 2003). The economically most important species of the section, *A. clavatus* is possibly a cosmopolitan fungus. It can be isolated mainly from soil and dung, but also occurs on stored products (mainly cereals) with high moisture content, e.g. inadequately stored rice, corn and millet (Flannigan & Pearce 1994). *A. clavatus* isolates appear to be particularly well adapted for growth during malting (Flannigan & Pearce 1994). *A. clavatus* was found to be responsible for an extrinsic allergic alveolitis known as malt worker’s lung, and in cases of mycotoxicoses of animals fed with by-products of malting (Flannigan & Pearce 1994; Lopez-Diaz & Flannigan 1997). The toxic syndromes observed in animals were suggested to result from the synergistic action of various mycotoxins produced by this species (Flannigan & Pearce 1994). Several species of section Clavati have phototrophic long conidiophores at temperatures around 20–23 °C (Fennell & Raper 1955; Trinci & Banbury 1967; Sarbhoj & Elphick 1968; Huang & Raper 1971; Yaguchi et al. 1993).

*Aspergillus* subgenus Fumigati section Clavati (Gams et al. 1985; Peterson 2000) was formerly the *Aspergillus* clavatus group was recognised by Thom & Church (1926) with two species, *A. clavatus* and *A. giganteus*. *A. clavatonanicus* was added by Batista et al. (1955). After Raper & Fennell (1965) published their monograph on aspergilli, several new species or varieties assigned to section Clavati were described. These were summarised by Samson (1979), who recognised *A. longivesica* (Huang & Raper 1971) as the fourth species within the section. None of these have known teleomorphs. Another species, *A. rhizopus* (Rai et al. 1975) was treated by Samson (1979) as a synonym of *A. giganteus*. *A. pallidus* Kamyschko has been treated as a white-spored synonym of *A. clavatus* by several authors (Peterson 2000; Varga et al. 2003). *A. acanthosporus* (Udagawa & Takada (1971), placed in subgenus Ornati (Samson 1979), was shown by Peterson (2000) to be more closely related to section Clavati than section Ornati. Also, their major ubiquinone systems point in this direction as section Clavati and *A. acanthosporus* have Q10, while *A. ornatus* has Q9 ubiquinones (Tamura et al. 1999). Although its teleomorph was originally placed into the *Hemicarpenteles* genus, recently Udagawa & Uchiyama (2002) proposed the new ascomycete genus *Neocarpenteles* to accommodate this species, and excluded *N. acanthosporus* from section Ornati. Similar conclusions were drawn...
<table>
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<tr>
<th>Species</th>
<th>Strain No.</th>
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by Varga et al. (2003) based on sequence analysis of the internal transcribed spacer regions and the 5.8 S rRNA gene (ITS region) of isolates belonging to Aspergillus section Clavati. Another species, A. apicalis Mehrtra & Basu (1976) (as A. apica), was placed in section Ornati by Samson (1979) because of morphological similarities to H. paradoxus (small clavate blue green aspergilla). Finally, A. ingratus has been described by Yaguchi et al. (1993), who stated that this sclerotium producing species belonged to section Clavati.

In this study, we examined the taxonomic assignment of these alkali tolerant species characterised by clavate aspergilla using molecular, morphological and chemotaxonomical methods. We also examined the relationships among teleomorphs of Aspergillus subgenus Fumigati, including Neocarpenteles and Neosartorya species to the Dichotomomyces genus using molecular approaches. Although the anamorphs of Dichotomomyces belong to the Polypaecilum, ascomata and ascospores of Dichotomomyces species have a similar morphology as those of Neosartorya and Neocarpenteles (Samson RA, unpubl. data).

MATERIALS AND METHODS

Source of microorganisms

The fungi examined included all species allocated to Aspergillus section Clavati, and some species assigned to section Ornati with clavate aspergilla (the Aspergillus ornatus group), which could possibly be related to A. clavatus. The strains examined are listed in Table 1.

Morphology and physiology

The strains (Table 1) were grown for 7 d as 3-point inoculations on Czapek agar (CZA), Czapek yeast autolysate agar (CYA), creatine sucrose agar (CREA) and malt extract agar (MEA) at 25 °C in artificial daylight (medium compositions in Samson et al. 2004).

Analysis for secondary metabolites

The cultures were analysed according to the HPLC-diode array detection method of Frisvad & Thrane (1987, 1993) as modified by Smedsgaard (1997). The isolates were analysed on CYA and YES agar using three agar plugs (Smedsgaard 1997). The secondary metabolite production was confirmed by identical UV spectra with those of standards and by TLC analysis using the agar plug method, the TLC plates were eluted in toluene: ethylacetate:formic acid (6:3:1) and chloroform:acetone:2-propanol (85:15:20) (Fittenborg et al. 1983; Samson et al. 2004). Standards of patulin, cytchalasin E, kotonin, and nortryptoquivalin known to be produced by these fungi, were also used to confirm the identity of the compounds.

Isolation and analysis of nucleic acids

The cultures used for the molecular studies were grown on malt peptone (MP) broth using 10 % (v/v) of malt extract (Brix 10) and 0.1 % (w/v) Bacto peptone (Difco), 2 mL of medium in 15 mL tubes. The cultures were incubated at 25 °C for 7 d. DNA was extracted from the cells using the Masterpur™ yeast DNA purification kit (Epicentre Biotechnol.) according to the instructions of the manufacturer. Fragments containing the ITS region were amplified using primers ITS1 and ITS4 as described previously (White et al. 1990). Amplification of part of the β-tubulin gene was performed using the primers Bi2a and Bi2b (Glass & Donaldson 1995). Amplifications of the partial calmodulin gene were set up as described previously (Hong et al. 2005). Sequence analysis was performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit for both strands, and the sequences were aligned with the MT Navigator software (Applied Biosystems). All the sequencing reactions were purified by gel filtration through Sephadex G-50 (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in double-distilled water and analyzed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The unique ITS, β-tubulin, actin and calmodulin sequences were deposited at the GenBank nucleotide sequence database under accession numbers EU078624–EU078678 and EU076312–EU076343.

Data analysis

The sequence data was optimised using the software package Seqman from DNASTar Inc. Sequence alignments were performed by using CLUSTAL-X (Thompson et al. 1997) and improved manually. The neighbour-joining (NJ) method was used for the phylogenetic analysis. For NJ analysis, the data were first analysed using the Tamura–Nei parameter distance calculation model with gamma-distributed substitution rates (Tamura & Nei 1993), which were then used to construct the NJ tree with MEGA v. 3.1 (Kumar et al. 2004). To determine the support for each clade, a bootstrap analysis was performed with 1000 replications.

For parsimony analysis, the PAUP v. 4.0 software was used (Swofford 2002). Alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option with 100 random taxa additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1000 bootstrap replications (Hillis & Bull 1993). A Neosartorya fischeri isolate was used as outgroup in these experiments.

RESULTS AND DISCUSSION

Phylogeny

We examined the genetic relatedness of section Clavati isolates and their presumed relatives using sequence analysis of the ITS region of the ribosomal RNA gene cluster, and parts of the calmodulin and β-tubulin genes. During analysis of part of the β-tubulin gene, 468 characters were analyzed. Among the 174 polymorphic sites, 102 were found to be phylogenetically informative. The Neighbour-joining tree based on partial β-tubulin genes sequences is shown in Fig. 1. The topology of the tree is the same as one of the more than 105 maximum parsimony trees constructed by the PAUP program (length: 233 steps, consistency index: 0.8798, retention index: 0.9728). The ITS data set included 448 characters with 8 parsimony informative characters. The Neighbour-joining tree shown in Fig. 2 has the same topology as one of the 4 maximum parsimony trees (tree length: 25, consistency index: 0.9600, retention index: 0.9896).

Phylogenetic analysis of β-tubulin sequence data indicated that Aspergillus section Clavati includes six species, namely: A. clavatus
(synonyms: A. pallidus, A. apicalis), A. giganteus, A. longipes, A. rhizopodus, A. clavatonanicus and N. acanthosporus. Some misidentifications have also been clarified: isolates previously identified as A. clavatus (CBS 105.45) and A. clavatonanicus (CBS 112.27) were found to belong to the A. giganteus species, while one isolate originally identified as A. clavatus (IMI 351309) was found to belong to the A. rhizopodus species. The ITS sequences of A. clavatonanicus and A. rhizopodus isolates, and A. giganteus and A. longipes isolates, respectively, were identical, indicating their close relationship.

A. ingratus (Yaguchi et al. 1993) was found to be the synonym of H. paradoxus based on sequence data, so it was excluded from section Clavati (data not shown). H. paradoxus isolates are only distantly related to section Clavati, with affinities to some Penicillium species (to be published elsewhere).

Chemotaxonomy

The extrolites produced by species of Aspergillus section Clavati are listed in Table 2. Based on the common production of patulin, tryptoquivalins, tryptoquivalons and kotanins, most of the species appear to be closely related. A. clavatus produces patulin (= clavatin = clavacin) (Weisner 1942; Waksman et al. 1942, 1943; Hooper et al. 1944) and has been reported to cause mycotoxicosis in calves as early as 1954 (Forgacs et al. 1954). This mycotoxin was detected on YES agar in all isolates of A. clavatus, A. giganteus and A. longipes. Previously the presence of the isoepoxydon dehydrogenase gene taking part in the biosynthesis of patulin has also been proved for A. clavatonanicus and A. pallidus isolates using primer pairs developed by Paterson et al. (2000) to identify potential patulin producing Penicillia (Varga et al. 2003). Other interesting metabolites produced by species of section Clavati are ribotoxins. Ribotoxins are a family of ribosome-inactivating proteins that have specific ribonucleolytic activity against a single phosphodiester bond in the conserved sarcin/ricin domain of 26 S rRNA (Martinez Ruiz et al. 1999). Ribotoxins have recently been found in a number of Aspergillus species including A. clavatus, A. giganteus and A. longipes. Previously the presence of the Isoepoxydon dehydrogenase gene taking part in the biosynthesis of patulin has also been proved for A. clavatonanicus and A. pallidus isolates using primer pairs developed by Paterson et al. (2000) to identify potential patulin producing Penicillia (Varga et al. 2003). Other interesting metabolites produced by species of section Clavati are ribotoxins. Ribotoxins are a family of ribosome-inactivating proteins that have specific ribonucleolytic activity against a single phosphodiester bond in the conserved sarcin/ricin domain of 26 S rRNA (Martinez Ruiz et al. 1999). Ribotoxins have recently been found in a number of Aspergillus species including A. clavatus, A. giganteus, A. viridinutans, A. fumigatus, A. restrictus, A. oryzae var. effusus, A. tamarii and A. ostianus. Anamorphs of Neosartorya fischeri, N. glabrata and N. spinosa also produced ribotoxins (Lin et al. 1994; Martinez-Ruiz et al. 1999). Using the PCR probe developed by Lin et al. (1994), Varga et al. (2003) examined the presence of ribotoxin genes in isolates of Aspergillus section Clavati; a DNA fragment of about 600 bp was amplified in some A. clavatus, A.
**Fig. 2.** Neighbour-joining tree based on ITS sequence data of *Aspergillus* section *Clavati*. Numbers above branches are bootstrap values. Only values above 70% are indicated.

Giganteus, *A. pallidus* and *N. acanthosporus* isolates, indicating that these isolates are able to synthesize ribotoxins (Varga et al. 2003). *Hemicarpenteles paradoxus*, however, including its synonym *A. ingratus* produces no secondary metabolites in common with these core species and appear to more distantly related to section *Clavati*. Thus this species appears to occupy a unique position in the *Aspergillus* genus with no obvious closely related species.

**Morphology**

All the isolates except the ex type culture of *A. clavatonanicus*, produced numerous conidiophores with blue green conidia, hyaline conidiophore stipes and clavate aspergilla. The isolates in three species were phototropic producing very long conidiophores: *A. giganteus*, *A. rhizopodus* and *A. longivesica*. Another common phenotypic similarity was the alkalophilic tendency already described for *A. rhizopodus* which was isolated from soil with pH 8.5–9 and other species in the group (Raper & Fennell 1965; Rai et al. 1975). Several species have been isolated from dung which is also an alkaline substrate. This is further confirmed by the strong growth of all isolates on creatine-sucrose agar. This medium has an initial pH of 8 and creatine is an alkaline amino acid. Morphological and physiological data confirmed that *Neocarpenteles acanthosporus* and *Aspergillus* section *Clavati* are closely related.

**Teleomorph relationships in *Aspergillus* subgenus *Fumigati***

*Aspergillus* subgenus *Fumigati* includes section *Clavati* with the *N. acanthosporus* teleomorph, and section *Fumigati* with *Neosartorya* telemorphs. We examined the relationships of these teleomorph taxa to another ascomycete genus, *Dichotomomyces*. *Dichotomomyces cejpii* was originally described by Saito (1949) as *D. albus*, later validated as *D. cejpii* by Scott (1970). This species belongs to the Trichocomaceae family (although Malloch & Cain (1971) placed it to Onygenaceae). This species is characterised by the production of aleurioconidia on short branched conidiophores, and ascospores embedded in cleithotheca (Scott 1970; Udagawa 1970). Isolates of *D. cejpii* are highly heat resistant and can be found world-wide in soil, heat treated products and marine environments (Pieckova et al. 1994; Jesenska et al. 1993; Mayer et al. 2007). *D. cejpii* isolates has been claimed to produce a range of secondary metabolites including gliotoxin (Seigle-Murandi et al. 1990), xanthocillin X (Kitahara & Endo 1981), and several metabolites with...
Fig. 3. Neighbour-joining tree based on β-tubulin sequence data of *Neosartorya*, *Neocarpenteles*, *Dichotomomyces* species and their asexual relatives. Numbers above branches are bootstrap values. Only values above 70% are indicated.

Table 2. Extralite production of species assigned to *Aspergillus* section *Clavati* and *D. cejpii*. These toxins were all verified or found for the first time in the species listed, the ribotoxins (including α-sarcin) and xanthocillin X in *D. cejpii* were not verified, however.

<table>
<thead>
<tr>
<th>Species</th>
<th>Extralites</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. clavatonanicus</em></td>
<td>antafumicins, glyanthrypine, kotanins, tryptoquivalines, tryptoquivalones</td>
</tr>
<tr>
<td><em>A. clavatus</em></td>
<td>patulin, cytochalasin E &amp; K, kotanins, antafumicin, (dehydrocarolic acid), tryptoquivalines, tryptoquivalines, ascladiol, ribotoxins</td>
</tr>
<tr>
<td><em>A. giganteus</em></td>
<td>patulin, antafumicin, ascladiol, tryptoquivalones; tryptoquivalines, glyanthrypine, pyripyropen, α-sarcin and other ribotoxins</td>
</tr>
<tr>
<td><em>A. longivesica</em></td>
<td>patulin, tryptoquivalones, tryptoquivalines, antafumicins, pyripyropen</td>
</tr>
<tr>
<td><em>A. rhizopodus</em></td>
<td>pseurotins, dehydrocarolic acid, tryptoquivalines, tryptoquivalines, kotanins, cytochalasins</td>
</tr>
<tr>
<td><em>N. acanthosporus</em></td>
<td>kotanins, tryptoquivalines, tryptoquivalones, ribotoxins</td>
</tr>
<tr>
<td><em>D. cejpii</em></td>
<td>gliotoxin, tryptoquivalones, rubratoxins, (xanthocillin X)</td>
</tr>
</tbody>
</table>
antibiotic and ciliostatic properties (Pieckova & Jesenska 1997a, 1997b; Pieckova & Roeijmans 1999).

We examined the genetic variability and relationships of Aspergillus section Clavati and Fumigati isolates, D. cejpii var. cejpii and D. cejpii var. spinosus (Mallow & Cain 1971; originally described as D. albus var. spinosus; Udagawa 1970). Both the ITS region and part of the β-tubulin gene were amplified and sequenced, and phylogenetic analyses were carried out as described above. The trees based on both ITS and β-tubulin data indicate that D. cejpii forms a sister group with Neosartorya and Neocarpenteles species (Figs 3–4). During analysis of part of the β-tubulin gene, 469 characters were analyzed. Among the 270 polymorphic sites, 214 were found to be phylogenetically informative. The Neighbour-joining tree based on partial β-tubulin genes sequences is shown in Fig. 4. The topology of the tree is the same as one of the more than 105 maximum parsimony trees (length: 124 steps, consistency index: 0.6233, retention index: 0.8614). The ITS data set consisted of 446 nucleotides, with 45 parsimony informative sites. The topology of the Neighbour joining tree depicted in Fig. 4 was the same as one of the more than 105 maximum parsimony trees (length: 124 steps, consistency index: 0.7419, retention index: 0.9229). Both trees indicate that the Dictyotomomyces genus should be transferred to Aspergillus subgenus Fumigati. Similar results were obtained during phylogenetic analysis of partial calmodulin gene sequences (data not shown). D. cejpii isolates have been found to produce gliotoxin in common with several species assigned to section Fumigati including some Neosartorya species (Larsen et al. 2007), tryptoquivalones also produced by several species assigned to sections Clavati and Fumigati (Hong et al. 2005), and rubratoxins, which are hepatotoxic mycotoxins produced by P. cratenformae (Frisvad 1989; Sigler et al. 1996; Richer et al. 1997) [misidentified as Penicillium purpureogenum (Natori et al. 1970) or P. rubrum (Moss et al. 1968)]. D. cejpii has also been claimed to produce xanthocillin X (Kitahara & Endo 1981), even though it could not be confirmed in our analyses. Xanthocillin and related compounds have also been found in H. paradoxus (Frisvad JC, unpubl. data) A. candidus (Rahbaek et al. 2000), Eupenicillium crustaceum (Turner & Aldridge 1983), E. egyptiacum (Versen 1979), P. italicum (Arai et al. 1989), P. flavigenum (Frisvad et al. 2004) and P. chrysogenum (Hagedorn et al. 1960; Achenbach et al. 1972; Pfeiffer et al. 1972; Frisvad et al. 2004; de la Campa et al. 2007). Since the anamorph of Dictyotomomyces was earlier found to belong to Polypaecilum, further morphological and molecular studies are needed to clarify the significance of the morphology of the anamorph in the taxonomic placement of these species, and to clarify the taxonomy of Polypaecilum species.

In conclusion, the polyphasic approach applied to clarify the taxonomy of Aspergillus section Clavati led to the assignment of six species, namely: A. clavatus (synonyms: A. pallidus, A. apicalis), A. giganteus, A. longivesica, A. rhizopodus, A. clavotonanicus and N. acanthosporus to this section. Hemicarpenteles paradoxus (synonym: A. ingratus) was found to be unrelated to section Clavati, but more closely related to Penicillium. Dictyotomomyces and Neosartorya were found to be sister clades to the genus Neocarpenteles. Further studies are needed to clarify the taxonomic status of Dictyotomomyces species with Polypaecilum anamorphs.

Fig. 4. Neighbour-joining tree based on ITS sequence data of Neosartorya, Neocarpenteles, Dictyotomomyces species and their asexual relatives. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

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Aspergillus clavatonanicus

**Batista, Maia & Alecrim, Anais Fac. Med. Univ. Recife 15: 197. 1955. Fig. 5.**

**Type:** CBS 474.65, from finger nail lesion, Recife, Brazil

**Other no. of the type:** ATCC 12413; DMUR 532; IMI 235352; WB 4741

**Description**

Colony diam (7 d): CYA25: 50–82 mm, MEA25: 45–78 mm, YESS25: 57–82 mm, OA25: 49–60 mm, CYA37: 8–17 mm, CREA: very good growth and acid production in the margin of the colony

Colony colour: greyish blue green

Reverse colour (CZA): dull tan

Conidiation: usually abundant

Colony texture: floccose

**Cultures examined:** CBS 474.65 = IBT 12370 = IBT 24678, CBS 112.27 = IBT 12369 = IBT 24677

**Diagnostic features:** conidial heads smaller than 1 mm

**Similar species:** A. clavatus

**Distribution:** Brazil

**Ecology and habitats:** human

**Extritolites:** antafumicins, glyanthrypine, kotanin, tryptoquivalins, tryptoquivalons

**Pathogenicity:** isolated from nail lesion (Batista et al. 1955)

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Aspergillus clavatus

**Desmazières, Ann. Sci. Nat., Bot. 2: 71, 1834. Fig. 6.**

*Aspergillus pallidus* Kamyschko (1963)

*Aspergillus apicalis* Mehrrota & Basu (1976)

**Type:** CBS 513.65, J. Westerdijk > 1909, C. Thom > NRRL

**Other no. of the type:** ATCC 1007; ATCC 9602; ATCC 9598; CECT 2674; DSM 816; IMI 015949; IMI 015949v; IMI 015949ii; LSHB Ac96; LSHB Ac95; NCTC 978; NCTC 3887; NRRL 1; NRRL 1656; QM 1276; QM 7404; WB 1

**Description**

Colony diam (7 d): CYA25: 28–45 mm; MEA25: 25–44 mm, YESS25: 29–45 mm, OA25: 31–47 mm, CYA37: 9–26 mm, CREA25: very good growth and moderate to very strong acid production (exceptions: CBS 514.65, NRRL 2, NRRL 8 and NRRL 2254 grow poorly on CREA and produce no or very little acid)

Colony colour: blue-green

Conidiation: abundant

Reverse colour (CZA): uncoloured to somewhat brownish with age in some isolates

Colony texture: velvety

Conidial head: clavate, commonly ranging from 300 to 400 µm by 150 to 200 µm when young, in age commonly splitting into two, three, or more divergent columns

Stipe: 150–3000 x 20–30 µm

Conidium size/shape/surface texture: 3–4.5 x 2.5–3 µm, elliptical, smooth

**Cultures examined:** CBS 104.45, CBS 105.45, CBS 106.45, CBS 114.48, CBS 513.65, CBS 514.65, CBS 470.91, CBS 116685, CBS 118451, DTO 6-F8, DTO 27-2C, SZMC 0918, SZMC JV4, SZMC JV1.1, IMI 351309, IMI 358435, CBS 117.45, CBS 119.48, CBS 118.49, CBS 122.53, CBS 117.56, CBS 101.64, CBS 515.65, CBS 526.65

**Diagnostic features:** conidial heads up to 4 mm in size

**Similar species:** A. clavatonanicus

**Distribution:** worldwide, mainly in tropical, subtropical and Mediterranean regions

**Ecology and habitats:** soil, cereals, malt, dung

**Extritolites:** Patulin, cytochalasin E, kotanins, antafumicin, (dehydrocarolic acid), tryptoquivalone, tryptoquivalinares, ascladiol (all found in this study), ribotoxins (Lin et al. 1995, Huang et al. 1997)

**Pathogenicity:** caused endocarditis (Opal et al. 1986), responsible for an extrinsic allergic alveolitis known as malt worker's lung (Grant et al. 1976; Lopez-Diaz & Flannigan 1997; Flannigan & Pearce 1994), and various toxic syndromes including neurological disorders (Shlosberg et al. 1991; McKenzie et al. 2004; Loretti et al. 2003; Gilmour et al. 1989; Kellerman et al. 1976) and other mycotoxicosis-related diseases (Byth & Lloyd 1971) observed in animals

**Notes:** some isolates carry dsRNA mycoviruses 35–40 nm in size (Varga et al. 2003)

Aspergillus giganteus

**Wehmer, Mem. Soc. Phys. Genève 33 (2): 85. 1901. Fig. 7.**

**Type:** CBS 526.65, dung of bat in cave, Yucatan, Mexico

**Other no. of the type:** ATCC 10059; DSM 1146; IFO 5818; IMI 227678; NRRL 10; QM 1970; WB 10; IBT 12368

**Description**

Colony diam: CYA25: (26–) 40–65 mm, MEA25: (29–) 43–65 mm, YESS25: 40–80 mm, OA25: 31–75 mm, CYA37: 10–29 mm, CREA: very good growth and poor or no acid production

Colony colour: first white, becoming pale blue-green near light celandine green to slate-olive

Conidiation: usually abundant

Reverse colour (CZA): dull tan

Colony texture: velvety

Conidial head: splitting into 2 or more columns with age, blue green

Stipe: two types: 2–3(–4) mm; or several cm in length

Vesicle diam/shape: two types: 100–250 x 30–50 µm on short conidiophores, 400–600 x 120–180 µm on long ones, clavate

Conidium size/shape/surface texture: 3.5–4.5 x 2.4–3 µm, elliptical, thick-walled, smooth

**Cultures examined:** CBS 117.45, CBS 119.48, CBS 118.49, CBS 122.53, CBS 117.56, CBS 101.64, CBS 515.65, CBS 526.65

**Diagnostic features:** produces clavate vesicles in contrast with the elongate ones of A. longivesica; do not produce rhizoidal foot
Fig. 5. Aspergillus clavatonanicus. A–B. Colonies after 7 d at 25 °C. A. CYA. B. MEA. C–J. Conidiophores. K. Conidia. Scale bars = 10 µm.
Fig. 6. Aspergillus clavatus. A. Colonies after 7 d at 25 °C on CYA. B–C. Macrophotograph of conidiophores. D–I. Conidiophores. J. Conidia. Scale bars = 10 µm, except D and E = 30 µm.
Fig. 7. Aspergillus giganteus. A. Colonies after 7 d at 25 °C on CYA. B–C. Macrophotograph of conidiophores. D–I. Conidiophores. J. Conidia. Scale bars = 10 µm, except D and E = 30 µm.
cells characteristic to *A. rhizopodus*; conidial heads can be up to 1–5 cm long

**Similar species:** *A. rhizopodus, A. longivesica*

**Distribution:** Nigeria, U.S.A., Egypt, Mexico, Panama, Germany, Suriname, Netherlands, Poland

**Ecology and habitats:** dung, soil, wood

**Extrtolites:** patulin, antafumicin, ascladiol, tryptoquivalone; tryptoquivalines, glyanthrypine, pyripyropen (found in this study), α-sarcin and other ribotoxins (Olson & Goerner 1965; Olson et al. 1966; Lin et al. 1995; Wirth et al. 1997; Martinez-Ruiz et al. 1999). Carotenoids are also produced (van Eijk et al. 1979)

**Pathogenicity:** not reported

**Note:** two types of conidial structures: (1) conidiophores commonly 2 to 3 mm, rarely exceeding 4 mm in height, bearing clavate heads 200 to 350 µm in length; (2) conidiophores one to several centimeters in length, bearing heads up to 1 mm in length; longer conidiophores are phototropic, and only elongate in the presence of light

**Aspergillus longivesica** Huang & Raper, *Mycologia* 63(1): 53. 1971. Fig. 8.

**Type:** CBS 530.71, from soil, rain forest, Nigeria

**Other no. of the type:** ATCC 22434; IMI 156966; QM 9698

**Description**
- Colony diam: CYA25: 31–51 mm; MEA25: 48–56 mm; YES25: 60–74 mm; OA25: 52–60 mm, CYA37: 0 mm; CREA25: weak growth and no acid production (CBS 187.77 grow very well on CREA, however)
- Colour: white to cream
- Conidiation: abundant, rarely less abundant
- Reverse colour (CYA): pale cinnamon buff
- Colony texture: thin floccose
- Conidial head: elongate, splitting into divergent columns with age, greyish blue green
- Stipe: two types: 80–420 × 7–11.2 µm, or 1.5–4.5 cm long, thick walled (5.6–7 µm)
- Vesicle diam/shape: two types: 2.2–3.2 mm × 130–200 µm, elongate, clavate, thick-walled, or 18–36 µm, globose to flask-shaped, thin-walled
- Conidium length/shape/surface texture: two types: 4.2–16.8 × 2.8–7 µm, globose to elliptical, or 3.5–5.2 × 2.5–3.5 µm, elliptical or pyriform

**Cultures examined:** CBS 530.71, CBS 187.77

**Diagnostic features:** produces variously shaped foot cells with finger-like projections

**Similar species:** *A. giganteus, A. longivesica*

**Distribution:** India, Yugoslavia

**Ecology and habitats:** soil

**Extrtolites:** pseurotins, dehydrocarolic acid, tryptoquivalines, tryptoquivalones, kotanins and cytochalasin (found in this study)

**Pathogenicity:** not reported

**Note:** large conidial heads formed only in the presence of light


= *Talaromyces cejpii* Milko (1964)
= *Dichotomomyces albus* Saito (1949)
= *Royella albida* Dwiveli (1960)

**Type:** CBS 157.66, from orchard soil, near Tiraspol, Moldova

**Description**
- Colony colour: white to cream coloured
- Conidiation: sparse
- Reverse colour (CZA): colourless
- Colony texture: floccose, granular
- Conidium size/shape/surface texture: 5–10 µm, subglobe to pyriform, smooth
- Homothallic
- Cleistothecia: variable in size, spherical, white to cream coloured

**Pathogenicity:** not reported

**Note:** longer conidiofhores are phototropic, and only elongate in the presence of light


**Type:** CBS 450.75, from usar soil, Lucknow, India

**Other no. of the type:** IMI 385057; WB5442

**Description**
- Colony diam (7 d): CYA25: 35–51 mm; MEA25: 48–56 mm; YES25: 60–74 mm; OA25: 52–60 mm, CYA37: 0 mm; CREA25: weak growth and no acid production
- Colony colour: blue green
- Conidiation: abundant
- Reverse colour (CYA): colourless
- Colony texture: slightly furrowed
- Conidial head: short columnar
- Stipe: two types: 208–800 × 11–32 µm, or 5–22 mm × 36 µm, thick walled, smooth
- Vesicle diam/shape: two types: 40–176 × 11–32 µm, or 288 × 79 µm, clavate
- Conidium size/shape/surface texture: 4–5.5 × 2.5–3 µm, ellipsoidal, smooth

**Cultures examined:** CBS 450.75, IMI 351309

**Diagnostic features:** produces longer and wider conidiophores, longer vesicles and larger conidia than *A. giganteus*; vesicles are elongate to fusoid-clavate for the long conidiophore and globose for the smaller ones, while those of *A. giganteus* are clavate

**Similar species:** *A. giganteus*

**Distribution:** Nigeria, Ivory Coast

**Ecology and habitats:** soil

**Extrtolites:** patulin, antafumicin, ascladiol, tryptoquivalone; tryptoquivalines, glyanthrypine, pyripyropen (found in this study)

**Pathogenicity:** not reported

**Note:** longer conidiofhores are phototropic, and only elongate in the presence of light

Fig. 9. *Aspergillus rhizopodus*. A. Colonies after 10 d at 25 °C on CYA. B. Macrophotograph of conidiophores. C–I. Conidiophores. J. Conidia. Scale bars = 10 µm, except D and E = 30 µm.
Fig. 10. Dichotomomyces cejpii. A–B. Ascomata on MEA after 10 d at 25 °C. C. Ascomata wall. D–E. Asci and ascospores. F–I conidiophores and conidia. Scale bars = 10 µm, except B and C = 30 µm.
Cultures examined: CBS 761.96, CBS 779.7, CBS 219.67, CBS 100192, CBS 474.77, CSB 780.70, CBS 397.68, CBS 345.68, CBS 159.67, CBS 157.66, CBS 212.50

Diagnostic features: conidiophore apices are dichotomously branched, and conidia are produced from these branches (Polypaecium anamorph); racquet hyphae are frequently produced; vegetative hyphae often bear rhizomorphs

Similar species: -

Distribution: Slovakia, Netherlands, Egypt, U.S.A., South Africa, Pakistan, Japan, Moldova, India

Ecology and habitats: soil, compost, pasteurised products

Extrrolites: gliotoxin (Seigle-Murandi et al. 1990, confirmed in this study), tryptoquivalenes (found in this study), ribotoxins (found in this study), xanthocillins X (Klahara & Endo 1981; could not be confirmed in this study), and several metabolites with antibiotic and cillostatic properties (Piekova & Roeijmans 1999; Piekova & Jesenska 1997a, 1997b)

Pathogenicity: not reported

Note: not illustrated here, for detailed description and illustration see Udagawa & Takada (1971); no growth at 37 °C

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REFERENCES


Polyphasic taxonomy of *Aspergillus* section *Usti*

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Abstract: Aspergillus ustus is a very common species in foods, soil and indoor environments. Based on chemical, molecular and morphological data, *A. insuets* is separated from *A. ustus* and revived. *A. insuets* differs from *A. ustus* in producing drimans and ophiobolin G and H and not producing ustic acid and austocystins. The molecular, physiological and morphological data also indicated that another species, *A. keveii* sp. nov. is closely related but distinct from *A. insuets*. *Aspergillus* section *Usti* sensustricto includes 8 species: *A. ustus*, *A. puniceus*, *A. granulosus*, *A. pseudodeflectus*, *A. calidoustus*, *A. insuets* and *A. keveii* together with *Emericella heterothallica*.

Taxonomic novelties: *Aspergillus insuets* revived, *Aspergillus keveii* sp. nov.

Key words: actin, *Aspergillus*, β-tubulin, calmodulin, extrolite profiles, ITS, phylogenetics, polyphasic taxonomy.

INTRODUCTION

*Aspergillus ustus* is a very common filamentous fungus found in foods, soil and indoor air environments (Samson et al. 2002). This species is considered as a rare human pathogen that can cause invasive infection in immunocompromised hosts. However, *A. ustus* has been noted increasingly as causes of invasive aspergillosis in tertiary care centres in the US (Malani & Kaufman 2007). Up to date, 22 invasive aspergillosis cases have been reported to be caused by *A. ustus* (Verweij et al. 1999; Pavie et al. 2005; Panackal et al. 2006; Yildiran et al. 2006). Several studies indicate that *A. ustus* isolates are resistant to amphotericin B, echinocandins and azole derivatives (Verweij et al. 1999; Pavie et al. 2005; Gene et al. 2001; Garcia-Martos et al. 2005). Other species related to *A. ustus* can also cause human or animal infections. *Aspergillus granulosus* was found to cause disseminated infection in a cardiac transplant patient (Fahk et al. 1995), while *A. deflectus* has been reported to cause disseminated mycosis in dogs (Robinson et al. 2000; Kahler et al. 1990; Jang et al. 1986).

*A. ustus* is a variable species. Raper & Fennell (1965) stated that "not a single strain can be cited as wholly representative of the species as described". Indeed, *A. ustus* isolates may vary in their colony colour from mud brown to slate grey, with colony reverse colours from uncoloured through yellow to dark brown (Raper & Fennell 1965; Kozakiewicz 1989). Molecular data also indicate that this species is highly variable; RAPD analysis carried out in various laboratories could be used to detect clustering of the isolates (Rath et al. 2002; Panackal et al. 2006), and sequence analysis of parts of the ribosomal RNA gene cluster also detected variability within this species (Henry et al. 2000; Peterson 2000; Hinrikson et al. 2005).

We examined a large set of *A. ustus* isolates and related species originating from environmental and clinical sources to clarify the taxonomic status of the species, and to clarify the taxonomy of *Aspergillus* section *Usti*. The methods used include sequence analysis of the ITS region (intergenic spacer region and the 5.8 S rRNA gene of the rRNA gene cluster), and parts of the β-tubulin, calmodulin and actin genes, analysis of extrolite profiles, and macro- and micromorphological analysis of the isolates.

MATERIALS AND METHODS

 Morphological examination. The strains examined are listed in Table 1. Both clinical and environmental strains were grown as 3-point inoculations on Czapek yeast agar (CYA), malt extract agar (MEA), creatine agar (CREA) and yeast extract sucrose agar (YES) at 25 °C, and on CYA at 37 °C for 7 d (medium compositions according to Samson et al. 2004). For micro morphological examination light microscopy (Olympus BH2 and Zeiss Axiolopk 2 Plus) was employed.

 Extrolite analysis. Extrolites were analysed by HPLC using alkylophenone retention indices and diode array UV-VIS detection as described by Frisvad & Thrane (1987), with minor modifications as described by Smedsgaard (1997). Standards of ochratoxin A and B, aflavine, asperazine, austamide, austdiol, kotanin and other extrolites from the collection at Biocentrum-DTU were used to compare with the extrolites from the species under study.

 Isolation and analysis of nucleic acids. The cultures used for the molecular studies were grown on malt peptone (MP) broth using 10 % (v/v) of malt extract (Brix 10) and 0.1 % (w/v) bacto peptone (Difco), 2 mL of medium in 15 mL tubes. The cultures were incubated at 25 °C for 7 d. DNA was extracted from the cells using the Masterpure™ yeast DNA purification kit (Epitcience Biotecnol.) according to the instructions of the manufacturer. Fragments containing the ITS region were amplified using primers ITS1 and ITS4 as described previously (White et al. 1990). Amplification of part of the β-tubulin gene was performed using the primers Bt2a and Bt2b (Glass 1995). Amplifications of the partial calmodulin and actin genes were set up as described previously (Hong et al. 2005). Sequence analysis was performed with the Big Dye Terminator

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Table 1. Isolates in *Aspergillus* section *Usti* and related species examined in this study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain No.</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 112452</td>
<td>Indoor air, Germany</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 113228</td>
<td>ATCC 38849; IBT 13091</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 114380</td>
<td>Wooden construction material, Finland</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 121601T</td>
<td>Bronchoalveolar lavage fluid, proven invasive aspergillosis, Nijmegen, The Netherlands†</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 121602</td>
<td>Bronchial secretion, proven invasive aspergillosis, Nijmegen, The Netherlands†</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 121589</td>
<td>Autopsy lung tissue sample, proven invasive aspergillosis, Nijmegen, The Netherlands†</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 121603</td>
<td>Elevator shaft in hospital, Nijmegen, The Netherlands</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 121604</td>
<td>Patient room, Nijmegen, The Netherlands</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 121605</td>
<td>Laboratory, Nijmegen, The Netherlands</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 121606</td>
<td>Sputum, Nijmegen, The Netherlands</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 121607</td>
<td>Feces, Nijmegen, The Netherlands</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 121608</td>
<td>Bronchoalveolar lavage, Nijmegen, The Netherlands</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>7843</td>
<td>Pasteur Institute, Paris, France</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>8623</td>
<td>Oslo, Norway</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>9331</td>
<td>Mouth wash, Nijmegen, The Netherlands</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>9371</td>
<td>Mouth wash, Nijmegen, The Netherlands</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>9420</td>
<td>Bronchial secretion, Nijmegen, The Netherlands</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>9692</td>
<td>Hospital ward, Nijmegen, The Netherlands</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>V02-46</td>
<td>Tongue swab, Nijmegen, The Netherlands</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>V07-21</td>
<td>Bronchial secretion, Nijmegen, The Netherlands</td>
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<tr>
<td><em>A. calidoustus</em></td>
<td>V17-43</td>
<td>Bronchial secretion, Nijmegen, The Netherlands</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>V22-60</td>
<td>Skin biopsy, Nijmegen, The Netherlands</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 121609</td>
<td>Post-cataract surgery endophthalmitis, Turkey</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>907</td>
<td>Post-cataract surgery endophthalmitis, Turkey</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>908</td>
<td>Post-cataract surgery endophthalmitis, Turkey</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>64</td>
<td>Post-cataract surgery endophthalmitis, Turkey</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>67</td>
<td>Post-cataract surgery endophthalmitis, Turkey</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 121610</td>
<td>Post-cataract surgery endophthalmitis, Turkey</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>351</td>
<td>Osteorickets</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>482</td>
<td>Post-cataract surgery endophthalmitis</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 121611</td>
<td>Patient 4, Washington, U.S.A.</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 121616</td>
<td>Environmental, Washington, U.S.A.</td>
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<tr>
<td><em>A. calidoustus</em></td>
<td>FH 165</td>
<td>Patient 5b, Washington, U.S.A.</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 121614</td>
<td>Patient 5a, Washington, U.S.A.</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 121615</td>
<td>Patient 6, Washington, U.S.A.</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 121613</td>
<td>Patient 2, Washington, U.S.A.</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>CBS 121612</td>
<td>Patient 1, Washington, U.S.A.</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>FH 91</td>
<td>Patient 1a, Washington, U.S.A.</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>NRRL 26162</td>
<td>Culture contaminant, Peoria, U.S.A.</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>NRRL 281</td>
<td>Thom 5634</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>NRRL 277</td>
<td>Thom 5698.754, Green rubber</td>
</tr>
<tr>
<td><em>A. granulosus</em></td>
<td>CBS 588.65T</td>
<td>Soil, Fayetteville, Arkansas, U.S.A.</td>
</tr>
<tr>
<td><em>A. granulosus</em></td>
<td>CBS 119.58</td>
<td>Soil, Fayetteville, Arkansas, U.S.A.</td>
</tr>
<tr>
<td><em>A. granulosus</em></td>
<td>CBS 1932</td>
<td>Soil, Fayetteville, Arkansas, U.S.A.</td>
</tr>
<tr>
<td><em>A. insuetus</em></td>
<td>CBS 107.25T</td>
<td>South Africa</td>
</tr>
<tr>
<td><em>A. insuetus</em></td>
<td>CBS 119.27</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>A. insuetus</em></td>
<td>CBS 102278</td>
<td>Subcutaneous infection left forearm and hand of 77-year-old woman</td>
</tr>
<tr>
<td><em>A. keveii</em></td>
<td>CBS 209.92</td>
<td>Soil, La Palma, Spain</td>
</tr>
<tr>
<td><em>A. keveii</em></td>
<td>CBS 561.65</td>
<td>Soil, Panama</td>
</tr>
<tr>
<td><em>A. keveii</em></td>
<td>IBT 10524 = CBS 113227 = NRRL 1254</td>
<td>Soil, Panama</td>
</tr>
</tbody>
</table>
Cycle Sequencing Ready Reaction Kit for both strands, and the sequences were aligned with the MT Navigator software (Applied Biosystems). All the sequencing reactions were purified by gel filtration through Sephadex G-50 (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in double-distilled water and analyzed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Data analysis. The sequence data was optimised using the software package Segman from DNASTar Inc. Sequence alignments were performed by using CLUSTAL-X (Thompson et al. 1997) and improved manually. The neighbour-joining (NJ) method was used for the phylogenetic analysis. For NJ analysis, the data were first analysed using the Tamura–Nei parameter distance calculation model with gamma-distributed substitution rates (Tamura & Nei 1993), which were then used to construct the NJ tree with MEGA v. 2.1 (Kumar et al. 1993), which were then used to construct the NJ tree with MEGA v. 3.1 (Kumar et al. 2004). To determine the support for each clade, a bootstrap analysis was performed with 1000 replications.

For parsimony analysis, the PAUP v. 4.0 software was used (Swofford 2000). Alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option with 100 random taxa additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1000 bootstrap replications (Hillis & Bull 1993). An Aspergillus versicolor isolate was used as outgroup in these experiments. Unique sequences of the ITS, actin, calmodulin and β-tubulin gene sequences have been deposited in the GenBank under accession numbers EU076344–EU76377.

RESULTS

Phylogenetic analyses

For the molecular analysis, four genomic regions, the ITS region, and parts of the actin, calmodulin and β-tubulin genes were amplified and sequenced. Phylogenetic analysis of the data was carried out using the neighbour-joining technique and parsimony analysis. The trees obtained by the different approaches were identical, neighbour-joining trees based on the different data sets are shown in Figs 1–4. During analysis of part of the β-tubulin gene, 487 characters were analyzed, 111 of which were found to be parsimony informative. The topology of the tree is the same as that of one of the more than 10^4 maximum parsimony trees constructed by the PAUP program (length: 216 steps, consistency index: 0.8148, retention index: 0.9679). The calmodulin data set included 474 characters, with 172 parsimony informative characters (1 MP tree, tree length: 360, consistency index: 0.8083, retention index: 0.9550). The actin data set included 406 characters, with 161 parsimony informative characters (3 MP trees, tree length: 292, consistency index: 0.8870, retention index: 0.9633). The ITS data set included 482 characters, 26 of which were parsimony informative (>10^4 MP trees, tree length: 71, consistency index: 0.9155, retention index: 0.9781).

Molecular data revealed that Aspergillus section Usti consists of eight species: A. ustus, A. puniceus, A. granulosus, A. pseudodeflectus, A. calidostus, A. insuetus and a new species including CBS 209.92 and some other isolates. We propose the name A. keveii sp. nov. for this set of isolates. The trees based on ITS, calmodulin and β-tubulin sequence data indicated that also E. heterothallica belongs to this section, although actin sequence data did not support this finding.

Table 1. (Continued).

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain No.</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. keveii</td>
<td>IBT 16751 = DMG 153</td>
<td>Galápagos Islands, Ecuador, D.P. Mahoney</td>
</tr>
<tr>
<td>A. pseudodeflectus</td>
<td>CBS 596.65</td>
<td>Sugar, U.S.A., Louisiana</td>
</tr>
<tr>
<td>A. pseudodeflectus</td>
<td>CBS 756.74</td>
<td>Desert soil, Egypt, Western Desert</td>
</tr>
<tr>
<td>A. puniceus</td>
<td>CBS 122.33</td>
<td>Unknown</td>
</tr>
<tr>
<td>A. puniceus</td>
<td>9377</td>
<td>Mouth wash, Nijmegen, Netherlands</td>
</tr>
<tr>
<td>A. puniceus</td>
<td>V41-02</td>
<td>Faeces, Nijmegen, Netherlands</td>
</tr>
<tr>
<td>A. puniceus</td>
<td>NRRL 29173</td>
<td>Indoor air, Saskatoon, Canada</td>
</tr>
<tr>
<td>A. puniceus</td>
<td>CBS 495.65</td>
<td>Soil, Zarcero Costa Rica</td>
</tr>
<tr>
<td>A. puniceus</td>
<td>CBS 128.62</td>
<td>Soil, Louisiana, U.S.A.</td>
</tr>
<tr>
<td>A. ustus</td>
<td>CBS 116057</td>
<td>Antique tapestries, Krakow, Poland</td>
</tr>
<tr>
<td>A. ustus</td>
<td>CBS 114901</td>
<td>Carpet, The Netherlands</td>
</tr>
<tr>
<td>A. ustus</td>
<td>CBS 261.67</td>
<td>Culture contaminant, U.S.A.</td>
</tr>
<tr>
<td>A. ustus</td>
<td>CBS 133.55</td>
<td>Textile buried in soil, Netherlands</td>
</tr>
<tr>
<td>A. ustus</td>
<td>CBS 239.90</td>
<td>Man, biopsy of brain tumor, Netherlands</td>
</tr>
<tr>
<td>A. ustus</td>
<td>CBS 113233</td>
<td>IBT 14495</td>
</tr>
<tr>
<td>A. ustus</td>
<td>CBS 113232</td>
<td>IBT 14932</td>
</tr>
<tr>
<td>A. ustus</td>
<td>NRRL 285</td>
<td>Soil, Iowa, U.S.A.</td>
</tr>
<tr>
<td>A. ustus</td>
<td>NRRL 280</td>
<td>Bat dung, Cuba</td>
</tr>
<tr>
<td>A. ustus</td>
<td>NRRL 1609</td>
<td>Bat dung, Cuba</td>
</tr>
<tr>
<td>A. ustus</td>
<td>NRRL 29172</td>
<td>Indoor air, Edmonton, Canada</td>
</tr>
<tr>
<td>E. heterothallica</td>
<td>CBS 489.65</td>
<td>soil, Costa Rica</td>
</tr>
<tr>
<td>E. heterothallica</td>
<td>CBS 486.65</td>
<td>soil, Costa Rica</td>
</tr>
</tbody>
</table>

†These samples were taken from the same patient (Verweij et al. 1999)
Fig. 1. Neighbour-joining tree based on β-tubulin sequence data of Aspergillus section Usti. Numbers above branches are bootstrap values. Only values above 70 % are indicated.
Fig. 2. Neighbour-joining tree based on calmodulin sequence data of Aspergillus section Usti. Numbers above branches are bootstrap values. Only values above 70% are indicated.
Fig. 3. Neighbour-joining tree based on actin sequence data of Aspergillus section Ustil. Numbers above branches are bootstrap values. Only values above 70 % are indicated.
Fig. 4. Neighbour-joining tree based on ITS sequence data of Aspergillus section Usti. Numbers above branches are bootstrap values. Only values above 70% are indicated.
Morphological and physiological studies

Phenotypic comparison of the different members of the section Usti showed that eight taxa could be distinguished. Various characters showed to be valuable for differentiation (see also Table 2). One of the main criteria is the growth rate on CYA at 37 °C. A. calidoustus, A. pseudodeflectus and A. granulosus had high growth rates at this temperature, while E. heterothallica only grew restrictedly. The other members of this section were unable to grow at 37 °C, which reduces the potential of these species to become opportunistic human pathogens. The growth rate and the mycelium colour on creatin agar (CREA) also proved to be a good tool to differentiate between the species examined. Some species, like A. ustus, A. puniceus, A. insuetus and A. keveii have a good growth on this medium. Since sporulation on this medium is often inhibited, this medium was also useful to determine the colour of the mycelium. The colours varied from bright yellow by A. puniceus and E. heterothallica to faint yellow in A. ustus to colourless in the other species. Another useful character was the use of the Ehrlich test to detect the presence of indol metabolites. This feature gave, with the exception of A. keveii, very clear-cut results. Besides these features, the colony diam on YES was also suitable to differentiate between A. insuetus and the other species.

Extrolite profiles

Aspergillus ustus has been claimed to produce a range of extrolites including austidiol (Vleggaar et al. 1974), Austin (Cheval et al. 1976), austocystins (Steyn & Vleggaar 1974; Krif et al. 1986), brevianamide A (Steyn 1973), sterigmatocystin (Rabie et al. 1977), austalides (de Jesus et al. 1987), austamide (Steyn 1971), dehydroaustin (Scott et al. 1986), pergillin (Cutler et al. 1980), dehydropergillin (Cutler et al. 1981), phenylahistin (Kanoh et al. 1997), ophiobolins G & H (Cutler et al. 1984), drimans (Hayes et al. 1996), diacetoxyisccipenol (Tuomi et al. 2000) and ustic acid (Raistrick & Strickings 1951).

The mycotoxins and other extrolites found to be produced by the examined species in this study are listed in Table 3. Species assigned to section Usti could clearly be divided in three chemical groups based on the extrolites produced by them. A. ustus, A. granulosus and A. puniceus produced ustic acids in common. A. ustus and A. puniceus also produced austocystins and versicolorins. In the second chemical group, A. pseudodeflectus produced drimans (Hayes et al. 1996) in common with the other species in this group, and also several unique unknown compounds. A. calidoustus isolates produced drimans and ophiobolins in common with A. insuetus and A. keveii, but also produced austins not identified in other species of section Usti. A. insuetus isolates also produced pergillin, while A. keveii together with some other isolates produced nidulol. In the third chemical group, E. heterothallica has been reported to produce emethallicins A–F (Kawahara et al. 1989, 1990a, 1990b), 5'-hydroxyaveranthin (Yabe et al. 1991), emeheretone (Kawahara et al. 1988), esteremones A & B (Hosoe et al. 1998), 5'-hydroxyaveranthan (Yabe et al. 1991), Mer-NF8054X (Mizuno et al. 1995). This latter compound is an 18,22-cyclosterol derivative, and was also identified in an A. ustus isolate (Mizuno et al. 1995). Apart from this chemical similarity Emericella heterothallica appear to be quite different from the anamorphic species in section Usti, in agreement with actin sequencing data. Austamide, deoxybrevianamide E and austidiol could not be detected in any of the strains examined here and the strain producing these mycotoxins should be reexamined.

Comparing the extrolites profiles of section Usti with other sections within subgenus Nidulantes, nidulol and versicolorins are also produced by members of sections Versicolores and Nidulantes (Cole & Schweikert 2003). Interestingly, versicolorins and 5'-hydroxyaveranthan are intermediates of the aflatoxin biosynthetic pathway and also produced by species assigned to Aspergillus section Flavi and Ochraceorosei (Yabe et al. 1991; Frisvad et al. 2005). However, while the versicolorins are precursors of sterigmatocystin in section Ochraceorosei, Versicolores and Nidulantes, they are precursors of austocystins in section Usti.

Section Usti contains the only Aspergillus species known to produce pergilliins, ophiobolins, austins, austocystins, ustic acids, drimans, Mer-NF8054X, austalides, deoxybrevianamides and austamide and thus this section is chemically unique. We have not examined the species for production of emethallicins, esteremones and emeheretones, as standards of these compounds were not available.

Table 2. Overview of morphological criteria to differentiate between the members of Aspergillus section Usti.

<table>
<thead>
<tr>
<th>Species</th>
<th>CYA37 (mm)</th>
<th>YES (mm)</th>
<th>Ehrlich reaction</th>
<th>Reaction on CREA</th>
<th>Conidial colour on MEA**</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. ustus</td>
<td>No growth</td>
<td>43–49</td>
<td>None</td>
<td>Good growth, faint yellow mycelium</td>
<td>Hair brown</td>
</tr>
<tr>
<td>A. puniceus</td>
<td>No growth</td>
<td>48–53</td>
<td>None</td>
<td>Moderate to good growth, yellow mycelium</td>
<td>Olive brown</td>
</tr>
<tr>
<td>A. calidoustus</td>
<td>20–35</td>
<td>36–41</td>
<td>Violet</td>
<td>Weak to moderate growth, hyaline mycelium</td>
<td>Brownish grey</td>
</tr>
<tr>
<td>A. insuetus</td>
<td>No growth</td>
<td>23–30</td>
<td>Violet</td>
<td>Good growth, hyaline mycelium</td>
<td>(Brownish) grey to light grey</td>
</tr>
<tr>
<td>A. keveii</td>
<td>No growth</td>
<td>40–46</td>
<td>Violet*</td>
<td>Good growth, hyaline mycelium</td>
<td>Brownish grey / pinkish brown</td>
</tr>
<tr>
<td>A. pseudodeflectus</td>
<td>15–20</td>
<td>20–30</td>
<td>None</td>
<td>Weak to moderate growth, hyaline mycelium</td>
<td>No sporulation</td>
</tr>
<tr>
<td>A. granulosus</td>
<td>30–35</td>
<td>35–40</td>
<td>Violet</td>
<td>Weak growth, hyaline mycelium</td>
<td>Buff to greyish brown</td>
</tr>
<tr>
<td>E. heterothallica</td>
<td>5–10</td>
<td>38–42</td>
<td>None</td>
<td>Weak growth, bright yellow mycelium</td>
<td>No sporulation</td>
</tr>
</tbody>
</table>

* All have violet reaction, except CBS 113227
** Colour according Melthuen handbook of colours

DISCUSSION

Raper and Fennell (1965) classified A. ustus in the Aspergillus ustus group together with four other species: A. panamensis, A. puniceus, A. conjunctus and A. deflectus. Later, Kozakiewicz (1989) revised the taxonomy of the group, and included A. ustus,
Table 3. Extrolites produced by species assigned to Aspergillus section Usti.

<table>
<thead>
<tr>
<th>Chemical group I</th>
<th>Extrolites produced</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. ustus</em></td>
<td>Ustic acids, austocystins (and versicolorins), austalides, a compound related to sterigmatocystin, nidulol</td>
</tr>
<tr>
<td><em>A. granulosus</em></td>
<td>Ustic acids, a compound resembling sterigmatocystin, nidulol, drimans</td>
</tr>
<tr>
<td><em>A. punicus</em></td>
<td>Ustic acids, austocystins (and versicolorins), phenylahistin, a compound related to sterigmatocystin, nidulol</td>
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</tbody>
</table>

<table>
<thead>
<tr>
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<tbody>
<tr>
<td><em>A. pseudodeflectus</em></td>
<td>Drimans, unknown compounds</td>
</tr>
<tr>
<td><em>A. calidoustus</em></td>
<td>Drimans, ophiobolins G and H, austins</td>
</tr>
<tr>
<td><em>A. insuetus</em></td>
<td>Drimans, ophiobolins G and H, pergillin-like</td>
</tr>
<tr>
<td><em>A. keveii</em></td>
<td>Drimans, ophiobolins G and H, nidulol</td>
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<tbody>
<tr>
<td><em>E. heterothallica</em></td>
<td>Emethallicins A, B, C, D, E &amp; F, emeheterone, emesterones A &amp; B, 5’-hydroxyaveranthin, Mer-NF8054X, sterigmatocystin, versicolorins</td>
</tr>
</tbody>
</table>

*A. pseudodeflectus, A. conjunctus, A. punicus, A. panamensis* and *A. granulosus* into the *A. ustus* species group, and established the *A. deflectus* group including *A. deflectus, A. pulvinus* and *A. silvaticus* based on morphological studies. Klich (1993) treated *A. granulosus* as member of section Versicolores, and found that *A. pseudodeflectus* is only weakly related to this section based on morphological treatment of section Versicolores. Peterson (2000) transferred most species of section Usti to section Nidulantes based on sequence analysis of part of the 28 S rRNA gene. On his cladogram, *A. ustus, A. pseudodeflectus, A. granulosus* and *A. punicus* form a well-supported branch closely related to *A. versicolor* and its allies, while *A. deflectus* is on another branch related to *A. elongatus* and *A. lucknowensis*. Peterson (2000) transferred *A. conjunctus, A. funiculosus, A. silvaticus, A. panamensis* and *A. anthodesmis* to section Sparsi. Recently Varga et al. (submitted) studied large numbers of isolates from clinical and other sources using molecular, morphological and physiological approaches. Phylogenetic analysis of partial β-tubulin, calmodulin, actin and ITS sequences indicated that none of the clinical isolates recognised previously as *A. ustus* belong to the *A. ustus* species. All but two of these isolates formed a well-defined clade related to *A. pseudodeflectus* based on sequence analysis of protein coding regions. Morphological and physiological examination of the isolates indicated that they are able to grow above 37 ºC, in contrast with *A. ustus* isolates, and give a positive Ehrlich reaction, in contrast with related species including *A. granulosus, A. ustus,* and *A. pseudodeflectus*. These isolates were described as *A. calidoustus*.

Aspergillus ustus* (Bainier) Thom & Church was redescribed by Thom & Church (1926) based on *Sterigmatocystis ustia* Bainier. In this manual, *A. insuetus* (Bainier) Thom & Church was also accepted based on *S. insuenta* Bainier (Thom & Chuch, 1926), but later *A. insuetus* was abandoned (Thom and Raper, 1945) and included in the broad description of *A. ustus* in Raper and Fennell (1965). Our studies clarified that *A. insuetus* is a valid species which can be distinguished from *A. ustus* and other species assigned to Aspergillus section Usti. *A. insuetus* could be separated from the other members of the section Usti by various phenotypic characters. The most important one is the slower growth rate on YES agar and clear differences in extrolite profiles (Table 2). This finding was supported by all the different data sets used to characterise section Usti. The molecular data showed that this species is more related to *A. calidoustus* and *A. pseudodeflectus* than *A. ustus*. Also different extrolite patterns were observed. There were many differences between *A. ustus* and *A. insuetus*, and, like the molecular data, this species was mostly related to *A. calidoustus* and *A. pseudodeflectus*. The main difference between the latter species was the production of a pergillin-like compound by *A. insuetus* (Table 3).

Our polyphasic taxonomic approach revealed that Aspergillus section Usti includes eight species: *A. ustus, A. punicus, A. granulosus, A. pseudodeflectus, A. calidoustus, A. insuetus* and *A. keveii* sp. nov. The phylogenetic trees based on ITS, calmodulin and β-tubulin sequence data indicated that *E. heterothallica* also belongs to this section. This species has similar morphology of the conidiophores and Hülle cells. In our study we were not able to observe ascospores by crossing the two mating strains but these are described by Raper and Fennell (1965: 502–503).
**Aspergillus calidoustus** 
Varga et al. Eukaryotic Cell submitted. Fig. 5.

**Type:** CBS 121604 from human, Netherlands

**Other no. of the type:** strain 677

**Description**

- **strains**
  - Colony diam, 7 d, in mm: CYA25 27–32; CYA37 20–35; MEA25 35–48; YES 36–41
  - Colony colour on CYA: blond/greyish yellow, brownish grey or greyish brown
  - Conidiation on CYA: abundant
  - Reverse colour (CYA): yellow with beige or olive brown centre
  - Colony texture: floccose
  - Conidial heads: loosely columnar
  - Stipe: 150–300 × 4–7 µm, smooth, brown
  - Vesicle diam/shape: 9–15 µm, pyriform to broadly spathulate
  - Conidium size/shape/surface texture: 2.7–3.5 x µm, globose, very rough ornamentation (0.5–0.8 µm high), inner and outer wall visible
  - Hülle cells: sparsely produced, irregularly elongated, in scattered groups
  - Ehrlich reaction: violet
  - Growth on creatine: weak to moderate growth with hyaline mycelium, no acid production

**Diagnostic features:**
- good growth at 37 °C, violet Ehrlich reaction, coarsely roughened to echinulate conidia

**Cultures examined:** CBS 121589, 121601–121616

**Similar species:**
- A. pseudodeflectus

**Distribution:**
- U.S.A., Turkey, Finland, Germany, Netherlands

**Ecology and habitats:**
- Indoor air, rubber, construction material, human

**Extrolites:**
- Drimans, ophiobolins G and H, austins

**Pathogenicity:**
- Pathogenic to humans (Verweij et al. 1999; Weiss & Thiemke 1983; Pavie et al. 2005; Panackal et al. 2006; Yildiran et al. 2006; Iwen et al. 1998)

**Aspergillus granulosus** 
Raper & Thom, Mycologia 36: 565. 1944. Fig. 6.

**Type:** CBS 588.65, from soil, Fayetteville, Arkansas, U.S.A.

**Other no. of the type:** ATCC 1033; IFO 4128; NRRL 279; NRRL 1726; Thom No. 4685.245

**Description**

- **strains**
  - Colony diam, 7 d, in mm: CYA 28–32; CYA37 no growth; MEA25 36–41; YES 23–30
  - Colony colour: almost black in center, shading through gray to white sterile floccose marginal areas
  - Conidiation on CYA: moderate to good
  - Reverse colour (CYA): yellow olive to blackish brown with age
  - Colony texture: floccose
  - Conidial head: radiate to hemispherical
  - Stipe: 300 × 4–8 µm, smooth, brown
  - Vesicle diam/shape: 11–16 µm, hemispherical to subglobose
  - Conidium size/shape/surface texture: 3.2–4 µm, globose, distinct roughened and inner and outer wall visible, fulgineous, the colour mostly aggregated into echinulations of the cell-wall, and even forming bars and tubercules at times
  - Hülle cells: variously coiled or curved, in scattered groups
  - Ehrlich reaction: violet
  - Growth on creatine: good growth with hyaline mycelium, no acid production

**Diagnostic features:**
- no growth at 37 °C, violet Ehrlich reaction, restricted growth on YES, coarsely roughened to echinulate conidia

**Cultures examined:** CBS 107.25, CBS 119.27, CBS 102278

**Similar species:**
- A. keveii

**Distribution:**
- South Africa, Spain

**Ecology and habitats:**
- Soil (?), human

**Extrolites:**
- Drimans, ophiobolins G and H, pergillin-like

**Pathogenicity:**
- caused subcutaneous infection (Gené et al. 2001)
Fig. 5. Aspergillus calidoustus. A–B. Colonies at 25 °C after 7 d. A. CYA. B. MEA. C–E, G–H Conidiophores. F. Hüle cells. I. Conidia. Scale bars = 10 µm, except F = 30 µm.
Fig. 6. Aspergillus granulosus. A–B. Colonies at 25 °C after 7 d. A. CYA. B. MEA. C–H Conidiophores. I. Conidia. Scale bars = 10 µm, except C = 30 µm.
Fig. 7. Aspergillus insuetus. A–B. Colonies at 25 °C after 7 d. A. CYA. B. MEA. C–H Conidiophores. I. Conidia. Scale bars = 10 µm, except C = 30 µm.
Aspergillus keveii sp. nov. Varga, Frisyad & Samson – MycoBank MB505570. Fig. 8.

Holotype of Aspergillus keveii, here designated as CBS 209.92° (dried culture) isolated from soil, Las Palmas, Spain.

Coloniae in 7 diebus et 25 °C in agaro MEA 36–41 mm, in CYA 30–39 mm, in YES 40–46 mm, in CREA 25–32 mm diam; auscult in 7 diebus et 37 °C in agaro CYA nullus. Sporulatio in CYA abundans; colonia brunneo-olivaceobrunnea; texture coloniae floccosa; colonia reversa flavide olivaceobrunnea vel atrobrunnea. Captula conidialia laxe columnaria; stipites 150–300 × 4–6 µm, pariete laevi, brunneo; vesiculae pyriformes, 9–13 µm in lat., biserratae; metulae 4.7–6.7 × 2.8–3.6 µm; phialides 5.7–7 × 2–3 µm; conidia globosa, 2.4–2.8 µm diam., ornamentum exasperato vel echinulato. Cellulae “hülle” irregulariter elongatae, (10–) 25–40(–65) µm in long., in cumulus dispersis.

Colonies on MEA36–41 mm, on CYA30–39 mm, on YES40–46 mm, on CREA25–32 mm in diam. after 7 d at 25 °C, no growth on CYA after 7 d at 37 °C. Conidial heads abundant on CYA, colony colour brownish grey to pinkish brown, colony texture floccose, reverse yellow olive brown to dark brown. Conidial heads loosely columnar; stipites 150–300 × 4–6 µm, smooth walled, brown in colour; vesicles 9–13 µm wide, pyriform, biseriate; metulae covering the upper half to three-fourths of the vesicle, measuring 4.7–6.7 × 2.8–3.6 µm µm; phialides 5.7–7 × 2–3 µm; conidia globosa 2.4–2.8 µm, coarsely roughened to echinulate. Hülle cells (10–)25–40(–65) µm in long., irregularly elongated, produced in scattered groups.

Etymology: named after Prof. Ferenc Kevei, eminent mycologist devoting his life to Aspergillus research.

Type: CBS 209.92

Ehrlich reaction: violet, with exception of CBS 113227

Growth on creatine: good growth with hyaline mycelium, no or weak acid production

Diagnostic features: no growth at 37 °C, good growth on CREA and YES, coarsely roughened to echinulate conidia; Hülle cells in scattered groups, violet Ehrlich reaction

Cultures examined: CBS 561.65, CBS 209.92 and CBS 113227

Similar species: A. insuetus

Distribution: Egypt, U.S.A., Turkey, Finland, Germany, Netherlands

Ecology and habitats: indoor air, rubber, construction material, human

Extrolites: Drimans, ophiobolins G and H, nidulol

Pathogenicity: not reported

Notes: CBS 113227 is deviating in having larger conidial heads and small (2.6 µm), finely roughened pinkish brown coloured conidia

Aspergillus pseudodeflectus Samson & Mouchacca, Antonie van Leeuwenhoek 41(3): 325. 1975. Fig. 9.

Type: CBS 756.74, from desert soil, Western Desert, Egypt

Other no. of the type: IMI 278381

Description

Colony diam, 7 d, in mm: CYA25 43–49; CYA37 15–20; MEA25 35–45; YES 20–30; CZAR25 25–26

Colonial shape/colour/texture: white mycelial felt intermixed with brown conidigenous structures

Conidiation: sparse

Reverse colour (CZA): yellow

Colony texture: velvety appearance, no sporulation

Conidial head: radiate, brown

Stipe: 35–200 × 2.5–3.5 µm, rough-walled with warty protuberances, brown

Vesicle diam/shape: 4–12 µm, globose to clavate

Conidium size/shape/surface texture: 3.5–5 µm, globose to ellipsoidal, brown, ornamented with small warts and colour bars

Hülle cells: absent

Ehrlich reaction: none

Growth on creatine: weak to moderate growth with hyaline mycelium, no acid production

Diagnostic features: Growth at 37 °C, curved brown conidiophores and the ornamented conidia, absence of Hülle cells

Cultures examined: CBS 756.74, CBS 596.65

Similar species: A. calidoustus

Distribution: Egypt, U.S.A.

Ecology and habitats: soil

Extrolites: Drimans (Hayes et al. 1996), unknown compounds

Pathogenicity: not reported

Aspergillus puniceus Kwon and Fennell, The genus Aspergillus: 547. 1965. Fig. 10.

≡ A. ustus var. laevis Blochwitz (1945)

Type: CBS 495.65, from soil, Zarcero, Costa Rica

Other no. of the type: ATCC 16800; IMI 126692; WB 5077

Description

Colony diam, 7 d, in mm: CYA 40–50; CYA37 no growth; MEA25 40–45; YES 48–53; CZAR25: 40–50 mm

Colony colour: pinkish orange near vinaceous pink, with wine red exudate droplets

Conidiation: moderate

Reverse colour (CYA): dark yellow brown or crème brown

Colony texture: floccose

Conidial head: radiate to short columnar, dull green becoming light drab with age

Stipe: 150–250(−300) × 5.5–6(−8) µm, airmly borne stipes up to 135 × 3–4 µm, straight, smooth

Vesicle diam/shape: 8–16 µm (subglobose), 15–18 × 13–15 µm (elliptical)

Conidium size/shape/surface texture: 2.5–3.3 µm, globose, roughened

Hülle cells: elongate, crescent shaped or irregularly twisted, often aggregated into yellowish masses

Ehrlich reaction: no reaction

Growth on creatine: moderate to good growth with bright yellow mycelium, no acid production (in some isolates weak acid production under colony)

Cultures examined: CBS 495.65, CBS 122.33, CBS 128.62, 9377, V41-02, NRRL 29173
Fig. 8. Aspergillus kerrei. A–B. Colonies at 25 °C after 7 d. A. CYA. B. MEA. C–H Conidiophores. I. Conidia. Scale bars = 10 µm.
Diagnostic features: No growth at 37 °C, good growth on creatine with brightly pigmented yellow mycelium. Hülle cells aggregated into yellowish masses

Similar species: A. ustus

Distribution: Costa Rica, U.S.A., Canada, Netherlands

Ecology and habitats: soil, indoor air, human

Extrolites: ustic acids, austocystins, nidulol, versicolorins, phenylahistin, sterigmatocystin-related compound (in CBS 128.62)

Pathogenicity: isolated from mouth wash and faeces

Aspergillus ustus (Bainier) Thom & Church, The aspergilli: 152. 1924. Fig. 11.

= Sterigmatocystis ustus Bainier (1881)
= Aspergillus humus Abbott (1926)

Type: CBS 261.67, culture contaminant, U.S.A.

Other no. of the type: ATCC 1041; ATCC 16818; IMI 211805; NRRL 275; QM 7477; WB 275; Thom 3556

Description
Colony diam, 7 d, in mm: CYA 36–43; CYA37 no growth; MEA 25–46; YES 42–50
Colony colour: greyish brown to dark brown
Conidiation on CYA: moderate
Reverse colour (CZA): yellow-olive edge with olive brown centre
Colony texture: floccose, plane, sulcate or umbonate
Conidial head: radiate to hemispherical
Stipe: 400 × 3–6 µm, aerially borne stipes up to 125 × 2–5 µm, smooth, brownish
Vesicle diam/shape: 7–15 µm, hemispherical to subglobose
Conidium size/shape/surface texture: 3.2–4.5 µm, globose, roughened, greenish to dark yellow brown
Hülle cells: irregularly ovoid or elongate, usually scattered
Ehrlich reaction: no reaction
Growth on creatine: good growth with faint yellow mycelium, no acid production

Cultures examined: CBS 116057, CBS 114901, CBS 261.67, CBS 133.65, CBS 239.90, CBS 113233, CBS 113232, NRRL 285, NRRL 280, NRRL 1609, NRRL 29172

Diagnostic features: No growth at 37 °C, good growth on creatine with faint yellow pigmented mycelium. Hülle cells typically scattered or form irregular masses and not associated with pigmented mycelium

Similar species: A. puniceus

Distribution: U.S.A., Poland, Netherlands, Canada

Ecology and habitats: soil, indoor air, bat dung

Extrolites: Ustic acids, austocystins, versicolorins, australides, a compound related to sterigmatocystin, nidulol

Pathogenicity: isolated from biopsy of man with brain tumour (CBS 239.90). However, this isolate does not grow at 37 °C on normal agar media and might therefore be a culture contamination.


Type: CBS 489.65, from soil, Costa Rica

Other no. of the type: ATCC 16824; IHEM 2064; IMI 139278; RV 34434; WB 5097; IBT 22604

Description
Colony diam, 7 d, in mm: CYA25 35–39; CYA37 5–8; MEA25 40–42; YES25 38–42
Colony colour: cream to yellow to orange
Conidiation: limited
Reverse colour (CYA): yellow to orange to pink becoming dark reddish brown
Colony texture: floccose
Conidial head: hemispherical to short columnar
Stipe: 185–410 × 5–11 µm, generally sinuous, brownish with age, smooth
Vesicle diam/shape: 13–20 µm
Conidium size/shape/surface texture: 2.5–4 µm, globose, echinulate, yellow green
Hülle cells: 600–700(–1000) µm, pyriform to oval to elongate to twisted, in globose to subglobose masses
Cleistothecia: produced in a heterothallic manner, 270–510 µm, cinnamon to dark purple, surrounded by Hülle cells
Ascospores: 4–4.5 × 3.5–4 µm, lenticular, orange brown in colour, with two pleated equatorial crests (1.5–2 µm), with convex smooth Ehrlich reaction: none
Growth on creatine: weak growth with yellow coloured mycelium, no acid production

Diagnostic features: heterothallic species, weak growth at 37 °C

Cultures examined: CBS 489.65, CBS 488.65 = IBT 22607

Similar species: -

Distribution: Costa Rica

Ecology and habitats: soil


Pathogenicity: not reported
ACKNOWLEDGEMENTS

We would like to thank the Danish Technical Research Council for support to Center for Microbial Biotechnology and Ellen Kirstine Lyhne for technical support. Dr Emory Simmons kindly provided a Latin diagnosis for the new species.

REFERENCES


A full list of references can be found in the original document.
Diagnostic tools to identify black aspergilli

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Abstract: The present taxonomy of the black aspergilli reveals that there are 19 accepted taxa. However, the identification of species of Aspergillus section Nigri is often problematic in spite of the existence of numerous methods proposed. An overview is provided of phenotypic and molecular methods to identify the accepted species of the black aspergilli. Colony morphology, conidial size and ornamentation of the ex type cultures is presented in a pictorial overview. The temperature range of all species is given and their growth characteristics on creatine agar and boscalid agar, a medium which was developed as a selective medium for the isolation of A. carbonarius are also shown. The extrolites produced by each species are listed while the response of the Ehrlich reaction is described. The literature on the various molecular methods to be used for species identification is reviewed and a critical evaluation of the usefulness of various techniques and genomic loci for species identification of black aspergilli is presented.

Key words: Boscalid medium, calmodin, colony morphology, CREA, Ehrlich reaction, extrolites, molecular tools, tubulin

INTRODUCTION

The black aspergilli (Aspergillus section Nigri) is an important group of species in food mycology, medical mycology and biotechnology. Many species cause food spoilage, but on the other hand are also used in the fermentation industry to produce hydrolytic enzymes, such as amylases or lipases, and organic acids, such as citric acid and gluconic acid (Varga et al. 2000). They are also candidates for genetic manipulation in the biotechnology industries since A. niger used under certain industrial conditions has been granted the GRAS (generally regarded as safe) status by the Food and Drug Administration of the US government. Although the main source of black aspergilli is soil, members of this section have been isolated from various other sources (Kozakiewicz 1989; Abarca et al. 2004; Samson et al. 2004). Besides their economical importance, black aspergilli are also important as ochratoxin producing organisms which contaminate several agricultural products including grape derived products, coffee and cocoa (Cabañes et al. 2002; Samson et al. 2004).

Black aspergilli are one of the more difficult groups concerning classification and identification and several taxonomic schemes have been proposed. New molecular approaches have shown that there is a high biodiversity, but that taxa are difficult to be recognised based solely on their phenotypic characters (Mosseray 1934a,b; Murakami 1976a,b; Murakami and Noro 1979; Murakami and Yoshida 1979a, b; Murakami et al. 1979; Al-Musallam, 1980). Murakami (1979d) only reluctantly recommended to use nitrite as sole nitrogen-source as a diagnostic medium in Aspergillus taxonomy. 20 % tannic acid agar seems to be less useful for diagnostic purposes as most black aspergilli can grow on it (van Diepeningen et al. 2004).

In this paper we have compiled the most relevant methods to be used in the diagnostics of the known and accepted species. Some additional methods have been listed by Frisvad et al. (2007).

MATERIAL AND METHODS

Morphological examinations

For this study we have used the (neo)type cultures of the accepted species (Table 1). In case where the (neo)type culture was deteriorated (e.g. A. niger, A. tubingensis and A. foetidus) we have used a recent isolate which identity was confirmed by phenotypic and molecular data.

Cultures were three-point inoculated on media in 9 cm plastic Petri dishes using a dense conidium suspension and incubated in the dark at 25 °C, except where otherwise noted. The fungi were also grown at 15, 30 and 37 °C on CYA. The cultures were examined after 7 d of growth and further examined after 14 d. Colony diameter were measured using a ruler.

Growth response of the ex type cultures of section Nigri at 15, 18, 21, 24, 27, 30, 33, 36 and 40 °C after 10 d incubation on MEA has also been recorded.

All species were examined using oil immersion with a Zeiss microscope with Nomarski contrast at up to 1000 × magnification. Digital micrographs of colonies were taken with a Nikon Coolpix 990 and 995 camera. Microscopic slides were prepared from MEA plates and 60 % lactic acid was used as a mounting medium.

Growth on specific media

Boscalid medium: In this study, we tested the growth abilities of all type strains of Aspergillus section Nigri on MEA-B (MEA according to Samson et al. (2004) with 10 mg/L boscalid (Sigma) added after autoclaving. Each strain was 3-point inoculated with a dense spore suspension onto MEA-B and inspected for growth and sporulation after 3 and 7 d of incubation at 25 °C.
Table 1. The type strains and isolates examined in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>CBS No.</th>
<th>Origin and information (abbreviation)</th>
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<tbody>
<tr>
<td>A. aculeatinus</td>
<td>CBS 121060^T</td>
<td>Thailand, Arabica green coffee bean</td>
</tr>
<tr>
<td>A. aculeatinus</td>
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<td>Thailand, Arabica green coffee bean</td>
</tr>
<tr>
<td>A. aculeatinus</td>
<td>CBS 121062</td>
<td>Thailand, Arabica green coffee bean</td>
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<td>A. aculeatus</td>
<td>CBS 172.66^T</td>
<td>Origin unknown</td>
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<tr>
<td>A. aculeatus</td>
<td>CBS 101.43</td>
<td>Origin unknown</td>
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<td>A. aculeatus</td>
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<td>CBS 101740^T</td>
<td>Brazil, Sao Paulo, Pedreira, soil</td>
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<td>A. brasiilensis</td>
<td>CBS 246.65</td>
<td>Australia, New South Wales, soil</td>
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<td>A. carbonarius</td>
<td>CBS 111.26^T</td>
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<td>A. costaricaensis</td>
<td>CBS 115574^T</td>
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<td>Costa Rica, soil</td>
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<td>A. ellipticus</td>
<td>CBS 707.79^T</td>
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<td>A. heteromorphus</td>
<td>CBS 117.55^T</td>
<td>Brazil, culture contaminant</td>
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<tr>
<td>A. homomorphus</td>
<td>CBS 101889^T</td>
<td>Israel, 2 km away from Dead Sea</td>
</tr>
<tr>
<td>A. ibicicus</td>
<td>CBS 121593^T</td>
<td>Portugal, grapes</td>
</tr>
<tr>
<td>A. japonicus</td>
<td>CBS 114.51^T</td>
<td>Origin unknown</td>
</tr>
<tr>
<td>A. japonicus</td>
<td>CBS 119560</td>
<td>Italy, grape</td>
</tr>
<tr>
<td>A. japonicus</td>
<td>CBS 522.78</td>
<td>Netherlands, air</td>
</tr>
<tr>
<td>A. lacticoffeatus</td>
<td>CBS 101883^T</td>
<td>Indonesia, South Sumatra, coffee bean</td>
</tr>
<tr>
<td>A. lacticoffeatus</td>
<td>CBS 101884</td>
<td>Venezuela, Rubio District, coffee bean</td>
</tr>
<tr>
<td>A. lacticoffeatus</td>
<td>CBS 101885</td>
<td>Venezuela, Rubio District, coffee bean</td>
</tr>
<tr>
<td>A. niger</td>
<td>CBS 554.65^T</td>
<td>U.S.A., Connecticut</td>
</tr>
<tr>
<td>A. niger</td>
<td>CBS 120.49</td>
<td>U.S.A.</td>
</tr>
<tr>
<td>A. niger</td>
<td>CBS 101698</td>
<td>Kenya, coffee bean</td>
</tr>
<tr>
<td>A. niger</td>
<td>CBS 121045</td>
<td>Thailand, Chiangmai Province, Arabica Coffee bean</td>
</tr>
<tr>
<td>A. piperis</td>
<td>CBS 112811^T</td>
<td>Denmark, black pepper</td>
</tr>
<tr>
<td>A. scleroticarbonarius</td>
<td>CBS 121057^T</td>
<td>Thailand, Robusta coffee bean</td>
</tr>
<tr>
<td>A. scleroticarbonarius</td>
<td>CBS 121056</td>
<td>Thailand, Robusta coffee bean</td>
</tr>
<tr>
<td>A. scleroticarbonarius</td>
<td>CBS 121058</td>
<td>Thailand, Robusta coffee bean</td>
</tr>
<tr>
<td>A. sclerotoniaiger</td>
<td>CBS 115572^T</td>
<td>India, Karnataka, coffee bean</td>
</tr>
<tr>
<td>A. tubingenensis</td>
<td>CBS 134.48^T</td>
<td>Origin unknown</td>
</tr>
<tr>
<td>A. tubingenensis</td>
<td>CBS 126.52</td>
<td>Origin unknown</td>
</tr>
<tr>
<td>A. tubingenensis</td>
<td>CBS 116.36</td>
<td>Origin unknown</td>
</tr>
<tr>
<td>A. tubingenensis</td>
<td>CBS 121047</td>
<td>Thailand, Chiangmai Province, Arabica Coffee bean</td>
</tr>
<tr>
<td>A. uvarum</td>
<td>CBS 121591^T</td>
<td>Italy, healthy Cisternino grape</td>
</tr>
<tr>
<td>A. uvarum</td>
<td>CBS 121590</td>
<td>Italy, healthy grapes</td>
</tr>
<tr>
<td>A. uvarum</td>
<td>CBS 121592</td>
<td>Italy, healthy Carpaneto grapes</td>
</tr>
<tr>
<td>A. vadensis</td>
<td>CBS 113365^T</td>
<td>Origin unknown</td>
</tr>
</tbody>
</table>
Creatine Sucrose Agar (CREA): The growth of the type strains have also been tested on CREA, which is a semi-selective medium useful for classification of various fungal cultures especially *Penicillium* species (Samson et al. 2004; Frisvad & Samson 2004). Each strain was 3-point inoculated with a dense spore suspension onto CREA and inspected for growth and sporulation after 3 and 7 d of incubation at 25°C.

Extrolites

Cultures were extracted according to the method of Smedsgaard (1987) using 500 µl ethylacetate/methanol/dichloromethane 3:2:1 (vol./vol./vol.) with 1% formic acid and ultrasonicated for 10 min. The organic solvent was transferred to another vial and evaporated at 1 mbar in a Rotavapor centrifuge evaporator. The extract was redissolved in 400 µl methanol and analysed by HPLC with diode array detection (DAD) or electrospray mass spectrometric detection (ES-MS) (Frisvad & Thrane 1987, 1993; Smedsgaard 1997; Nielsen & Smedsgaard 2003). The extrolites were identified by their UV spectra and MS characteristics. Authentic analytical standards were employed for retention time and retention index comparison with the extrolites detected.

Ehrlich test

The Ehrlich test was used by Frisvad & Samson (2004) to distinguish taxa of *Penicillium* subgenus *Penicillium* and is based on the detection of alkaloids reacting with Ehrlich reagent (Lund 1995) using a filter paper method. The Ehrlich reagent consists of 2 g of 4-dimethylamino-benzaldehyde in 96% ethanol (85 ml) added
to 15 ml 10 N HCl. An four mm agar plug is cut out from the centre of a colony grown on CYA (incubated for 5–9 d at 25°C) and a round piece (1 cm diam.) of the wetted filter paper (Whatman No. 1) is placed on the mycelial side of the plug. If a violet ring appears after 2–6 min, the culture contains cyclopiazonic acid or related alkaloids. If the reaction comes after 7–10 min, it is regarded as weak. After 10 min the violet ring will fade away. Some fungi produce alkaloids that will react with Ehrlich reagent to give pink to red or yellow rings.

Molecular analysis
Phylogenetic analysis of sequences of the ITS region of the rRNA gene cluster, and parts of the β-tubulin and calmodulin genes have been carried out as described previously (Varga et al. 2007; Noonim et al. 2008).

RESULTS

Colony morphology
Figures 1 and 2 show the growth characteristics of all ex type cultures of section Nigri on CYA and MEA after 7 d at 25 °C. The different species exhibit different growth characteristics. Several species have been found to be able to produce sclerotia, including A. carbonarius, A. ellipticus, A. aculeatus, A. costaricaensis, A. piperis, A. sclerotiniger, A. auleatinus and A. sclerotiocarbonarius (Table 2). Several species can easily be identified by their colony morphologies, including eg. A. sclerotiocarbonarius which produces yellow-orange sclerotia and bright yellow colony reverse on YES. However, other species including A. niger and its relatives or the uniseriate species cannot be distinguished by their growth pattern alone.

Conidia
Light microscopic photographs of the conidia of type strains and representative isolates of Aspergillus section Nigri are shown in Figs 3–4. Several species produce large conidia up to 7–9 µm including A. carbonarius, A. ibericus, A. homomorphus, A. sclerotiocarbonarius and A. sclerotiniger. Most other species produce conidia in the size range of (2.5–)3–4.5(–5) µm. The ornamentation of the conidia is also characteristic for some species, e.g. A. homomorphus and A. ibericus produce conidia with spiny appearance, while other species produce conidia which are smooth or nearly so, including A. vadensis and A. lactocephatus.

Growth at different temperatures
All the strains where incubated at 12 different temperatures: 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36 and 40 °C (Fig. 5). Most strains are not able to grow at 6 °C and 9 °C, with the exception of A. carbonarius, which was able to grow (within 96 h) at 9 °C. A. brasilienensis was not able to grow at 15 °C even after 240 h of incubation. Looking at the temperature curves the strains can be divided into 4 groups considering the maximum temperature where the strains were not able to grow. Group 1 consists of A. ellipticus (30 °C), group 2 consists of A. sclerotiocarbonarius and A. heteromorphus (33 °C), group 3 consists of A. sclerotiniger, A. uvarum, A. carbonarius, A. auleatinus, A. homomorphus, A. japonicus and A. aculeatus (36 °C), while group 4 consists of A. ibericus, A. foetiatus, A. tubingensis, A. piperis, A. costaricaensis, A. vadensis, A. niger, A. lactocephatus and A. brasiliensis (40 °C).

Growth on CREA
In this study, the growth abilities of all Aspergillus section Nigri type strains were tested on CREA medium (Fig. 6). Creatine Sucrose Agar (CREA) is the semi-selective media useful for classification of various fungal cultures especially Penicillium spp (Samson et al. 2004; Frisvad & Samson 2004). On CREA, characteristics of colonial growth, production of acid (turning of the medium from purple to yellow) and base production can be used as diagnostic features. CREA can be used as semi-selective medium for dividing all black aspergilli into groups. The most distinguishable species was A. sclerotiocarbonarius due to its inability to grow on CREA. Consequently, growth response on CREA can be used to distinguish A. sclerotiocarbonarius from closely related species also forming large conidia including A. carbonarius, A. sclerotiniger and A. ibericus.

For the other biseriate species, the group of species having moderate growth and good acid production resulting in large yellowish halo around the colonies included A. niger and closely related species, A. brasilienensis, A. foetiatus, A. tubingensis, A. vadensis and A. sclerotiniger. A. costaricaensis, A. piperis and A. lactocephatus also had moderate growth and good acid production. A second group which grows moderately well but produces less acid includes A. ellipticus, A. heteromorphus and A. homomorphus.

With respect to the uniseriate species, CREA is helpful for distinguishing between the 4 species. A. uvarum had poor growth and limited acid production while A. aculeatus and A. japonicus grew quite well and had medium acid production. In contrast, A. auleatinus had quite good growth and good acid production.

Growth on MEA-B
Pollastro et al. (2006) developed a semi-selective medium for A. carbonarius based on malt extract agar (MEA) amended with some antibiotics and fungicides. Among these, MEA-B (MEA with Boscalid) was found to be an efficient semi-selective medium to detect the presence of A. carbonarius while A. niger could not grow. In this study, we tested the growth abilities of all type strains of Aspergillus section Nigri on MEA-B with 10 mg/L boscalid. The results are shown in Table 3 and Fig. 7. After 3 d incubation, good growth could be detected only in A. carbonarius, A. sclerotiniger, A. homomorphus and A. sclerotiocarbonarius. No visual growth was detected in A. ellipticus, A. niger, A. brasilienensis, A. vadensis, A. piperis and A. costaricaensis.

After 7 d of incubation, many strains could recover and grow. However, only 3 strains were able to sporulate: A. carbonarius, A. sclerotiniger and A. sclerotiocarbonarius. So MEA-B is a helpful selective medium for differentiation of the ochratoxigenic A. carbonarius from many other species in section Nigri. Moreover, A. ibericus, a closely related non-OTA-producer species, could be differentiated from A. carbonarius as this species could not grow well on this medium.

Ehrlich reaction
Lund (1995) reported an easy useful reaction to identify some closely related Penicillia. The so-call Ehrlich reaction method detects some indole secondary metabolites produced by fungi by direct reaction
Table 2. Morphological characteristics of different species belonging to *Aspergillus* section *Nigri*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Conidial size (mm)</th>
<th>Vesicle size (µm)</th>
<th>Colour and size of sclerotia (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Uniseriate species</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. aculeatinus</em></td>
<td>2.5–4.5</td>
<td>45–80</td>
<td>Found only in some strains, white to cream, 0.4–0.6</td>
</tr>
<tr>
<td><em>A. aculeatus</em></td>
<td>3.5–5</td>
<td>60–80</td>
<td>Found only in some strains, cream, up to 0.5</td>
</tr>
<tr>
<td><em>A. japonicus</em></td>
<td>3.5–5</td>
<td>20–35</td>
<td>Found only in some strains, white to cream, up to 0.5</td>
</tr>
<tr>
<td><em>A. uvarum</em></td>
<td>3–4</td>
<td>20–30</td>
<td>Found only in some strains, dark brown to black, 0.5–0.8</td>
</tr>
<tr>
<td><strong>Biseriate species</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. brasiliensis</em></td>
<td>3.5–4.5</td>
<td>30–45</td>
<td>Found only in some strains, white, 1–1.5</td>
</tr>
<tr>
<td><em>A. carbonarius</em></td>
<td>7–9</td>
<td>40–80</td>
<td>Found only in some strains, Pink to yellow, 1.2–1.8</td>
</tr>
<tr>
<td><em>A. costaricaensis</em></td>
<td>3.1–4.5</td>
<td>40–90</td>
<td>Pink to grayish yellow, 1.2–1.8</td>
</tr>
<tr>
<td><em>A. ellipticus</em></td>
<td>3.3–5.5</td>
<td>75–100</td>
<td>Dull yellow to brown, 0.5–1.5*</td>
</tr>
<tr>
<td><em>A. foetidus</em></td>
<td>3.5–4.5</td>
<td>50–80</td>
<td>Found only in some strains, white, 1.2–1.8</td>
</tr>
<tr>
<td><em>A. heteromorphus</em></td>
<td>3.5–5</td>
<td>15–30</td>
<td>White, 0.3–0.6 (not observed by Al-Musallam 1980)</td>
</tr>
<tr>
<td><em>A. homomorphus</em></td>
<td>5–7</td>
<td>50–65</td>
<td>-</td>
</tr>
<tr>
<td><em>A. ibericus</em></td>
<td>5–7</td>
<td>50–60</td>
<td>-</td>
</tr>
<tr>
<td><em>A. lacticoffeatus</em></td>
<td>3.4–4.1</td>
<td>40–65</td>
<td>-</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>3.5–5</td>
<td>45–80</td>
<td>-</td>
</tr>
<tr>
<td><em>A. piperis</em></td>
<td>2.8–3.6</td>
<td>40–55</td>
<td>Yellow to pink-brown, 0.5–0.8</td>
</tr>
<tr>
<td><em>A. scleroticarbonarius</em></td>
<td>4.8–9.5</td>
<td>45–90</td>
<td>Yellow to orange to red-brown</td>
</tr>
<tr>
<td><em>A. sclerotioniger</em></td>
<td>4.5–6.4</td>
<td>30–50</td>
<td>Yellow to orange to red-brown</td>
</tr>
<tr>
<td><em>A. tubingensis</em></td>
<td>3–5</td>
<td>40–80</td>
<td>Found only in some strains, white to pink, 0.5–0.8</td>
</tr>
<tr>
<td><em>A. vadensis</em></td>
<td>3–4</td>
<td>25–35</td>
<td>-</td>
</tr>
<tr>
<td><em>A. uvarum</em></td>
<td>3.5–5</td>
<td>45–80</td>
<td>Found only in some strains, white to cream, 0.4–0.6</td>
</tr>
</tbody>
</table>

* the sclerotoid bodies are dull yellowish when young becoming brown in age, 500–800 µm in diam, and borne within terbunate masses up to 1.0–1.5 mm.

Table 3. Growth and sporulation on Malt Extract Agar with Boscolid (MEA-B) at 3 and 7 d of incubation at 25 °C.

<table>
<thead>
<tr>
<th>Name</th>
<th>CBS No.</th>
<th>3 days</th>
<th>7 days</th>
<th>Sporulation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. aculeatinus</em></td>
<td>121060</td>
<td>+</td>
<td>+++</td>
<td>No</td>
</tr>
<tr>
<td><em>A. aculeatus</em></td>
<td>172.66</td>
<td>+</td>
<td>+++</td>
<td>No</td>
</tr>
<tr>
<td><em>A. brasiliensis</em></td>
<td>101740</td>
<td>-</td>
<td>+</td>
<td>No</td>
</tr>
<tr>
<td><em>A. carbonarius</em></td>
<td>111.26</td>
<td>+++</td>
<td>++++</td>
<td>Heavy</td>
</tr>
<tr>
<td><em>A. costaricaensis</em></td>
<td>115574</td>
<td>-</td>
<td>++</td>
<td>No</td>
</tr>
<tr>
<td><em>A. ellipticus</em></td>
<td>707.79</td>
<td>-</td>
<td>+++</td>
<td>No</td>
</tr>
<tr>
<td><em>A. foetidus</em></td>
<td>564.65</td>
<td>+</td>
<td>+++</td>
<td>No</td>
</tr>
<tr>
<td><em>A. heteromorphus</em></td>
<td>117.55</td>
<td>+</td>
<td>++++</td>
<td>No</td>
</tr>
<tr>
<td><em>A. homomorphus</em></td>
<td>101889</td>
<td>++</td>
<td>+++</td>
<td>No</td>
</tr>
<tr>
<td><em>A. ibericus</em></td>
<td>121593</td>
<td>-</td>
<td>+</td>
<td>No</td>
</tr>
<tr>
<td><em>A. japonicus</em></td>
<td>114.51</td>
<td>+</td>
<td>+++</td>
<td>No</td>
</tr>
<tr>
<td><em>A. lacticoffeatus</em></td>
<td>101883</td>
<td>+</td>
<td>++</td>
<td>No</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>554.65</td>
<td>-</td>
<td>++</td>
<td>No</td>
</tr>
<tr>
<td><em>A. piperis</em></td>
<td>112811</td>
<td>-</td>
<td>++</td>
<td>No</td>
</tr>
<tr>
<td><em>A. sclerocarbonarius</em></td>
<td>121057</td>
<td>+++</td>
<td>++++</td>
<td>Heavy</td>
</tr>
<tr>
<td><em>A. sclerotioniger</em></td>
<td>115572</td>
<td>++</td>
<td>++++</td>
<td>Good</td>
</tr>
<tr>
<td><em>A. tubingensis</em></td>
<td>134.48</td>
<td>+</td>
<td>++</td>
<td>No</td>
</tr>
<tr>
<td><em>A. uvarum</em></td>
<td>121591</td>
<td>+</td>
<td>+++</td>
<td>No</td>
</tr>
<tr>
<td><em>A. vadensis</em></td>
<td>113365</td>
<td>-</td>
<td>+</td>
<td>No</td>
</tr>
</tbody>
</table>
Fig. 3. Light microscopic pictures of conidia of type strains and representative isolates of Aspergillus section Nigri. (A) *A. aculeatinus* (2.5–4.5 µm), (B) *A. aculeatus* (3.5–5 µm), (C) *A. brasiliensis* (3.5–4.5 µm), (D) *A. carbonarius* (7–9 µm), (E) *A. costaricaensis* (3.1–4.5 µm), (F) *A. ellipticus* (3.3–5.5 µm), (G) *A. foetidus* (3.5–4.5 µm), (H) *A. foetidus* wild type, (I) *A. japonicus* (3.5–5 µm), (J) *A. heteromorphus* (3.5–5 µm), (K) *A. homomorphus* (5–7 µm), (L) *A. ibericus* (5–7 µm). Scale bar in L for all = 10 µm.
Fig. 5. Growth rates of type cultures of Aspergillus section Nigri at 15, 18, 21, 24, 27, 30, 33, 36 and 40 °C after 10 d incubation. (A) A. ellipticus; (B) A. scleroticonarbonarius; (C) A. heteromorphus; (D) A. uvarum; (E) A. carbonarius; (F) A. aculeatus; (G) A. tubingensis; (H) A. ibéricus; (I) A. foetidus; (J) A. tubingensis; (K) A. piperis; (L) A. costaricaensis; (M) A. vanden; (N) A. niger; (O) A. lacticoffeatus; (S) A. brasiliensis.
Diagnostic tools to identify black Aspergilli
Fig. 6. Growth of type strains of Aspergillus section Nigri on creatine sucrose agar (CREA) plates. (A) A. aculeatinus; (B) A. aculeatus; (C) A. brasiliensis; (D) A. carbonarius; (E) A. costaricaensis; (F) A. ellipticus; (G) A. foetidus; (H) A. heteromorphus; (I) A. homomorphus; (J) A. ibericus; (K) A. japonicus; (L) A. lacticoffeatus; (M) A. niger; (N) A. piperis; (O) A. sclerotialcarbonarius; (P) A. sclerotioniger; (Q) A. tubingensis; (R) A. uvarum; (S) A. vadamensis.

Fig. 7. Growth and sporulation of type strains of Aspergillus section Nigri on malt extract agar with Boscoid (MEA-B) after 7 d incubation. (A) A. aculeatinus; (B) A. aculeatus; (C) A. brasiliensis; (D) A. carbonarius; (E) A. costaricaensis; (F) A. ellipticus; (G) A. foetidus; (H) A. heteromorphus; (I) A. homomorphus; (J) A. ibericus; (K) A. japonicus; (L) A. lacticoffeatus; (M) A. niger; (N) A. piperis; (O) A. sclerotialcarbonarius; (P) A. sclerotioniger; (Q) A. tubingensis; (R) A. uvarum; (S) A. vadamensis.
Fig. 8. Ehrlich colour reaction of some *Aspergillus* species in section *Nigri*. (A) blue in *A. costaricaensis*, (B) purple ring in *A. heteromorphus*, (C) purple ring in *A. homomorphus*, (D) yellow-green in *A. niger*, (E) purple-red at sclerotial area of *A. sclerotioniger* and (F) no reaction in *A. vadensis*.

Table 4. Ehrlich reaction results on CYA after 7 d incubation at 25 °C.

<table>
<thead>
<tr>
<th>Name</th>
<th>CBS No.</th>
<th>CYA</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. aculeatinus</em></td>
<td>CBS 121060T</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>A. aculeatus</em></td>
<td>CBS 172.66T</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>A. brasiliensis</em></td>
<td>CBS 101740T</td>
<td>++</td>
<td>Yellow reaction</td>
</tr>
<tr>
<td><em>A. carbonarius</em></td>
<td>CBS 111.26T</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>A. costaricaensis</em></td>
<td>CBS 115574T</td>
<td>+++++ Blue reaction</td>
<td></td>
</tr>
<tr>
<td><em>A. ellipticus</em></td>
<td>CBS 707.79T</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>A. foetidus</em></td>
<td>CBS 564.65T</td>
<td>++</td>
<td>Yellow reaction</td>
</tr>
<tr>
<td><em>A. heteromorphus</em></td>
<td>CBS 117.55T</td>
<td>+++++ Yellow reaction with purple ring</td>
<td></td>
</tr>
<tr>
<td><em>A. homomorphus</em></td>
<td>CBS 101898T</td>
<td>+++++ Yellow reaction with purple ring</td>
<td></td>
</tr>
<tr>
<td><em>A. ibericus</em></td>
<td>CBS 121593T</td>
<td>++</td>
<td>Yellow reaction</td>
</tr>
<tr>
<td><em>A. japonicus</em></td>
<td>CBS 114.51T</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>A. laticoffeatus</em></td>
<td>CBS 10183T</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>CBS 554.65T</td>
<td>++</td>
<td>Yellow reaction</td>
</tr>
<tr>
<td><em>A. piperis</em></td>
<td>CBS 11281T</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>A. sclerotiscarbonarius</em></td>
<td>CBS 121057T</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>A. sclerotioniger</em></td>
<td>CBS 115572T</td>
<td>+</td>
<td>* Violet reaction at sclerotia</td>
</tr>
<tr>
<td><em>A. tubingensis</em></td>
<td>CBS 134.48T</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>A. uvarum</em></td>
<td>CBS 121591T</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>A. vadensis</em></td>
<td>CBS 113365T</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

diagnostic tools to identify black *Aspergilli*

The production of the secondary metabolites is usually consistent in a species, however, ochratoxin A production in *A. niger* is only found in ca. 6% of the strains. Ochratoxin A producing species of section *Nigri* occurring on grapes, raisins and in wine include *A. carbonarius* which species produces this compound very consistently, and to a lesser extent *A. niger*. Four species recovered from coffee, *A. carbonarius*, *A. niger*, *A. lacticoffeatus* and *A. sclerotioniger*, all produce ochratoxin A (Table 5). None of the other species in section *Nigri* have been found to be ochratoxin producers. Very old culture collection strains may have lost the ability to produce some of the secondary metabolites otherwise characteristic of the species. The consistency in production of malformins in *A. niger* and *A. tubingensis* is not yet explored. Many of the secondary metabolites found are as yet of unknown structure, but are often diagnostic for one or more species in section *Nigri*.

Molecular tools to distinguish black *Aspergilli*

Several molecular tools have been used to distinguish black *Aspergillus* species. Among these, restriction fragment length polymorphisms (RFLPs) of both nuclear and mitochondrial DNAs (mtDNAs) have been used successfully to identify new species. Kusters-van Someren *et al.* (1990) used Western blotting and DNA hybridisation with a pectin lyase (*pelD*) gene to ascertain whether these methods could be used for rapid strain identification. The DNA hybridisation experiments showed that the *pelD* gene is conserved in all isolates belonging to the *A. niger* aggregate. Hybridisation was also observed in DNAs of all *A. foetidus* strains. The authors established three groups within the *A. niger* aggregate on the basis of presence or absence of three other bands which hybridised strongly to the *pelD* gene. As a continuation of this work, Kusters-
van Someren et al. (1991) carried out a more extensive study on nuclear DNA RFLPs of several black Aspergillus collection strains. Two groups of strains were distinguished according to their Smal-generated ribosomal DNA (rDNA) patterns. The two groups were also clearly distinguishable by their hybridisation patterns when peptin lyase genes (pelA, pelB) and the pyruvate kinase (pki) gene were used as probes in DNA hybridisation experiments. The two groups found were proposed to represent different species, namely A. niger and A. tubingensis. Examination of other species not belonging to the A. niger aggregate was also carried out. A. foetidus strains, classified into a different species by Al-Musallam (1980), showed the same nuclear DNA RFLPs as A. niger. A. helicothrix was found to represent only a morphological variant of A. ellipiticus, and A. aculeatus exhibited the same Smal-digested rDNA pattern as the A. japonicus strains examined. Jaap Visser’s group detected further differences in the nuclear genes encoding polygalacturonase II, arabinoxylan-arabinofuranohydrolase and xylanase enzymes of A. niger and A. tubingensis strains (Bussink et al. 1991; Graaff et al. 1994; Gielkens et al. 1997).

Smal digested repetitive DNA profiles hybridised with the ribosomal repeat unit of A. nidulans were found to have distinctive value among black aspergilli (Varga et al. 1994, 2000). A. ellipiticus, A. heteromorphus, A. japonicus and A. carbonarius exhibited species specific hybridisation patterns, with the exception of A. carbonarius strain IN7, which revealed a slightly different profile than the other A. carbonarius strains examined. Among the strains of the A. niger species complex, four profiles were observed, among which rDNA types I and II were shown by A. niger and A. brasilienis strains, respectively, while rDNA types II and II were characteristic of the A. tubingensis strains (Varga et al. 1994).

Parenicova et al. (2001) used RFLP analysis to distinguish A. japonicus and A. aculeatus isolates. The hybridisation probes were the A. niger pyruvate kinase (pkiA) and peptin lyase A (pelA) and Agaricus bisporus 28S rRNA genes, which revealed clear polymorphism between these two taxa. The A. niger pkiA and pelA probes placed six strains in an A. japonicus group and 12 isolates in an A. aculeatus group, which exhibited intraspecific variation when they were probed with the pelA gene. The application of these probes could also be used to distinguish other species in the section (Parenicova et al. 2000), including the recently described species A. vadensis (de Vries et al. 2005).

Wide-ranging mtDNA variation has also been observed both among collection strains and in natural populations of the A. niger species complex (Varga et al. 1993, 1994). Within the A. niger species complex, most isolates were classifiable as A. niger or A. tubingensis according to their HaeIII-BglII digested mtDNA patterns. The A. niger and A. tubingensis species could be grouped into 5 and 6 mtDNA types, respectively. Six of the 13 Brazilian isolates examined exhibited mtDNA and rDNA types different from those of all the other strains. Later these strains have been assigned to the A. brasilienis species (Varga et al. 2007). The sizes of the mtDNAs of the black Aspergillus strains examined were highly variable. The mtDNA of type 3 was the largest (35 kb) followed by those of types 2f and 2e (34 kb and 32.5 kb, respectively). The smallest mtDNA molecule (26 kb) was that of type 2c. All the other mtDNA types had sizes in the range 28–31 kb. For A. japonicus isolates, the strains could be classified into seven different mtDNA RFLP groups based on their HaeIII-digested mtDNA profiles. Hybridisation data suggest that six of these mtDNA types have certain common features in their organisation, while mtDNA type 7, which was exhibited by A. aculeatus strains, probably have quite different mtDNA structure (Hamari et al. 1997). The sizes of A. japonicus mtDNAs were in the range of 43–50 kb. Among the 16 collection strains and field isolates of Aspergillus carbonarius examined, the HaeIII-digested mtDNA profiles revealed only slight variations, except for one field isolate (IN7), which exhibited completely different mtDNA patterns (Kevei et al. 1996). The mtDNAs of these strains were found to be much larger (45 to 57

### Table 5. Extrolite production of species assigned to Aspergillus section Nigri.

<table>
<thead>
<tr>
<th>Species</th>
<th>Extrolites produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. aculeatus</td>
<td>neoxaline, secalonic acid D, secalonic acid F, aculeasins</td>
</tr>
<tr>
<td>A. aculeatus</td>
<td>secalonic acid D, secalonic acid F</td>
</tr>
<tr>
<td>A. brasilienis</td>
<td>naphtho-γ-pyrones (including aurasperone B), pyrophren, tensidol A &amp; B</td>
</tr>
<tr>
<td>A. carbonarius</td>
<td>ochratoxins (A, B, α, β), naphtho-γ-pyrones (including aurasperone B), pyroninigrin A</td>
</tr>
<tr>
<td>A. costaricaensis</td>
<td>aflavinines*, funalnone, naphtho-γ-pyrones (including aurasperone B)</td>
</tr>
<tr>
<td>A. ellipticus</td>
<td>austdol, candidusins, terpenylcin, xanthoacin</td>
</tr>
<tr>
<td>A. foetidus</td>
<td>antafumicins (only some strains), asperazine, funalnone, naphtho-γ-pyrones (including aurasperone B), pyroninigrin A</td>
</tr>
<tr>
<td>A. heteromorphus</td>
<td>lots of highly unique extrolites including indol-alkaloids, none of them structure elucidated</td>
</tr>
<tr>
<td>A. homomorphus</td>
<td>dehydrocarolic acid, secalonic acid D, secalonic acid F</td>
</tr>
<tr>
<td>A. hericites</td>
<td>naphtho-γ-pyrones (including aurasperone B), pyroninigrin A</td>
</tr>
<tr>
<td>A. japonicus</td>
<td>cycloclavine, festuclavine</td>
</tr>
<tr>
<td>A. lactoceoffeatus</td>
<td>kotanins, ochratoxin A, pyroninigrin A, tensidol A &amp; B</td>
</tr>
<tr>
<td>A. niger</td>
<td>funalnone, ochratoxin A (only some strains), malformins, naphtho-γ-pyrones (including aurasperone B), pyroninigrin A, tensidol A &amp; B, (nigragillin)</td>
</tr>
<tr>
<td>A. piperis</td>
<td>aflavinines, naphtho-γ-pyrones (including aurasperone B), pyroninigrin A</td>
</tr>
<tr>
<td>A. scleroticolorbarius</td>
<td>naphtho-γ-pyrones (including aurasperone B), pyroninigrin A, three unique indol-alkaloids at retention indices 1475, 1676 and 1838.</td>
</tr>
<tr>
<td>A. sclerotioranger</td>
<td>corymbeller lactones, funalnone, naphtho-γ-pyrones (including aurasperone B), ochratoxins (A, B, α, β), pyroninigrin A</td>
</tr>
<tr>
<td>A. tubingenesis</td>
<td>asperazine, funalnone, malformins, naphtho-γ-pyrones (including aurasperone B), pyroninigrin A, tensidol A &amp; B, (nigragillin)</td>
</tr>
<tr>
<td>A. uvarum</td>
<td>asteric acid, dihydrogrodinin, erdin, geodin, secalonic acid D and F</td>
</tr>
<tr>
<td>A. vadensis</td>
<td>nigragillin, asperazine, naphtho-γ-pyrones (including aurasperone B), a polar orlandin-like compound</td>
</tr>
</tbody>
</table>

Limited text is provided here due to the length of the original text. However, the key points include:

- A PCR-RFLP technique to distinguish among black aspergilli.
- Analysis of the ITS region using other restriction enzymes has also been used to distinguish species.
- DNA barcoding approaches using the mitochondrial cytochrome b gene.
- Development of species-specific primers based on AFLP fragments for the identification of A. carbonarius.
- Use of AFLP and RAPD techniques for species identification.
- Genetic variability among black aspergilli is studied using RAPD and AFLP.

For a comprehensive understanding, it is recommended to refer to the original source.
Fig. 9. Neighbour-joining tree based on (A) calmodulin, (B) β-tubulin and (C) ITS sequence data of type strains of Aspergillus section Nigri. Numbers above branches are bootstrap values. Only values above 70% are indicated.
### Table 6. Molecular tools applied for identification of species in *Aspergillus* section *Nigri*.

<table>
<thead>
<tr>
<th>Method used</th>
<th>Target region</th>
<th>Reference</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFLP</td>
<td>peIA, peIB, pki</td>
<td>Kusters-van Someren et al. 1991</td>
<td>A. niger and A. tubingensis could be distinguished</td>
</tr>
<tr>
<td>RFLP</td>
<td>rDNA (Smal)</td>
<td>Varga et al. 2000</td>
<td>A. niger, A. tubingensis, A. brasiliensis, A. ellipticus, A. heteromorphus, A. japonicus and A. carbonarius could be distinguished</td>
</tr>
<tr>
<td>RFLP</td>
<td>mtDNA (HaeIII/BglII)</td>
<td>Varga et al. 1993, 1994, Hamari et al. 1987</td>
<td>Could be used to distinguish A. niger and A. brasiliensis from A. tubingensis, and A. japonicus from A. aculeatus; several intraspecific mtDNA types identified</td>
</tr>
<tr>
<td>RFLP</td>
<td>peIA, pki, rDNA</td>
<td>Parenicova et al. 2001, de Vries et al. 2005</td>
<td>Could distinguish most species including A. aculeatus, A. japonicus and A. vandenboschii</td>
</tr>
<tr>
<td>PCR-RFLP</td>
<td>ITS (RsaI)</td>
<td>Martinez-Culebras &amp; Ramon 2007</td>
<td>Could distinguish between A. niger, A. tubingensis, A. carbonarius and A. aculeatus isolates</td>
</tr>
<tr>
<td>PCR-RFLP</td>
<td>ITS (RsaI, HhaI, NsiI)</td>
<td>Zanzotto et al. 2006</td>
<td>Could distinguish between potential OTA-producing and non-producing isolates of the A. niger aggregate (i.e. between A. niger and A. tubingensis)</td>
</tr>
<tr>
<td>AFLP</td>
<td>calmodulin</td>
<td>Perrone et al. 2006a, 2006b</td>
<td>All known species could be distinguished</td>
</tr>
<tr>
<td>PCR-SSCP</td>
<td>calmodulin</td>
<td>Susca et al. 2007b</td>
<td>11 species including A. brasiliensis, A. niger, A. tubingensis, A. foetidus, A. aculeatus, A. vandenboschii, A. ellipsoideus, A. foetidus, A. aculeatus, A. carbonarius and A. ibericus could be distinguished</td>
</tr>
<tr>
<td>OLISA</td>
<td>calmodulin</td>
<td>Bufflier et al. 2007</td>
<td>A. carbonarius, A. ibericus and A. japonicus/A. aculeatus could be distinguished</td>
</tr>
<tr>
<td>Sequence analysis</td>
<td>ITS</td>
<td>Varga et al. 2007</td>
<td>Several species have identical ITS sequences (e.g. A. niger and A. lacticoffeatus); A. tubingensis, A. foetidus, A. aculeatus, A. vandenboschii, A. ellipsoideus, A. japonicus, A. aculeatus, A. carbonarius, and A. ibericus could be distinguished</td>
</tr>
<tr>
<td>Sequence analysis</td>
<td>Mitochondrial cytochrome b</td>
<td>Yokoyama et al. 2001</td>
<td>A. japonicus/A. aculeatus, A. niger, A. tubingensis, A. carbonarius and A. ellipticus could be distinguished</td>
</tr>
<tr>
<td>Sequence analysis</td>
<td>β-tubulin</td>
<td>Samson et al. 2004, Varga et al. 2007</td>
<td>All except one species (A. lacticoffeatus) could be distinguished</td>
</tr>
<tr>
<td>Sequence analysis</td>
<td>Calmodulin</td>
<td>Varga et al. 2007</td>
<td>All species could be distinguished</td>
</tr>
<tr>
<td>Sequence analysis</td>
<td>Cytochrome oxidase I</td>
<td>Klich et al. 2007</td>
<td>A. niger and A. tubingensis could not be distinguished; not appropriate to be used for species identification in black aspergilli</td>
</tr>
<tr>
<td>Sequence analysis</td>
<td>IGS</td>
<td>Unpublished data</td>
<td>Too variable for species identification</td>
</tr>
<tr>
<td>PCR with species-specific primers</td>
<td>ITS</td>
<td>Gonzalez-Salgado et al. 2005</td>
<td>Species-specific detection of A. niger, A. tubingensis, A. heteromorphus, A. ellipticus and A. japonicus</td>
</tr>
<tr>
<td>PCR with species-specific primers</td>
<td>calmodulin</td>
<td>Susca et al. 2007a</td>
<td>Species-specific detection of A. carbonarius and A. niger</td>
</tr>
<tr>
<td>PCR with species-specific primers</td>
<td>RAPD fragment</td>
<td>Fungaro et al. 2004</td>
<td>Species-specific detection of A. carbonarius</td>
</tr>
<tr>
<td>PCR with species-specific primers</td>
<td>calmodulin</td>
<td>Perrone et al. 2004</td>
<td>Species-specific detection of A. carbonarius and A. japonicus</td>
</tr>
<tr>
<td>PCR with species-specific primers</td>
<td>ITS</td>
<td>Haugland and Vesper 2002</td>
<td>Species-specific detection of A. carbonarius and A. niger</td>
</tr>
<tr>
<td>PCR with species-specific primers</td>
<td>AFLP marker</td>
<td>Schmidt et al. 2004</td>
<td>Species-specific detection of A. carbonarius</td>
</tr>
<tr>
<td>PCR with species-specific primers</td>
<td>PKS</td>
<td>Lebrini et al. 2003</td>
<td>Species-specific detection of OTA producing A. carbonarius isolates</td>
</tr>
<tr>
<td>PCR with species-specific primers</td>
<td>PKS</td>
<td>Dobson &amp; O’Callaghan 2004</td>
<td>Species-specific detection of OTA producing A. carbonarius and A. niger isolates</td>
</tr>
<tr>
<td>Real time PCR</td>
<td>ITS</td>
<td>Haugland et al. 2004</td>
<td>Species-specific detection of A. carbonarius and A. niger</td>
</tr>
<tr>
<td>Real time PCR</td>
<td>calmodulin</td>
<td>Mule et al. 2006</td>
<td>Species-specific detection of A. carbonarius</td>
</tr>
<tr>
<td>Real time PCR</td>
<td>AT domain of the PKS gene</td>
<td>Atou et al. 2007</td>
<td>Species-specific detection of A. carbonarius</td>
</tr>
</tbody>
</table>
CONCLUSIONS AND RECOMMENDATION FOR THE IDENTIFICATION OF BLACK ASPERGILLI

Our studies and experience with the identification of the black aspergilli show that morphological structures can be helpful but that particularly the species related to A. niger are difficult to distinguish. CREA and Boscald agar are only good media when identifying some taxa. CREA is helpful when distinguishing the rare species A. sclerotiorum from closely related species also forming large conidia, A. carbonarius and A. ibericus. Boscald agar can be used as a selective medium for A. carbonarius, A. sclerotiorum, A. homomorphus and A. sclerotiorum carbonarius, because after three d incubation, good growth could be detected.

When using extrolite patterns it is noteworthy that asperazine can be used to distinguish A. tubingenensis, A. foetidus and A. vadensis from A. niger and A. brasiliensis, while pyronigrerin A is present in all species in the Aspergillus niger complex, except A. brasilienensis, A. costaricaensis and A. vadensis. Secalinic acid D is produced by the uniseriate species only, except A. japonicus. However the biseriate A. homomorphus also produces secalinic acid D. A. ellipticus is entirely unique and produces extrolites found in section Candidi (terphenyllin and candishiuins) and section Usti (austidiol). A. heteromorphus also has a unique combination of extrolites not found in any other Aspergillus species. Thus it seems that all section Nigri members can be identified based solely on extrolites.

In Aspergillus section Nigri, all species can be distinguished from each other using calmodulin sequence data, and all except one could be distinguished using β-tubulin sequence data. As discussed ITS can only be used for a rough classification of the uni- and biseriate species while only four groups of related taxa of A. niger can be identified.

REFERENCES

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Polyphasic taxonomy of *Aspergillus* section *Fumigati* and its teleomorph *Neosartorya*

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Abstract: The taxonomy of *Aspergillus* section *Fumigati* with its teleomorph genus *Neosartorya* is revised. The species concept is based on phenotypic (morphology and extrolite profiles) and molecular (β-tubulin and calmodulin gene sequences) characters in a polyphasic approach. Four new taxa are proposed: *N. australiensis* *N. ferenczi*, *N. papuaensis* and *N. warcupii*. All newly described and accepted species are illustrated. The section consists of 33 taxa: 10 strictly anamorphic *Aspergillus* species and 23 *Neosartorya* species. Four other *Neosartorya* species described previously were not available for this monograph, and consequently are relegated to the category of doubtful species.

**Taxonomic novelties:** *Neosartorya australiensis*, *N. ferenczi*, *N. papuaensis* and *N. warcupii*.

**Key words:** Aspergillus section *Fumigati*, extrolite profiles, *Neosartorya*, phylogenetics, polyphasic taxonomy.

**INTRODUCTION**

Aspergillus section *Fumigati* includes species characterised by uniseriate aspergilli, columnar conidial heads in shades of green and flask shaped vesicles (Raper & Fennell 1965). Teleomorphic species belonging to the "Aspergillus fischeri" series of the *A. fumigatus* group (Raper & Fennell 1965) were placed in the genus *Neosartorya* (family Trichocomaceae) by Malloch & Cain (1972). Section *Fumigati* includes more than 20 *Neosartorya* species and 10 anamorphic species (Pitt et al. 2000; Samson 2000; Horie et al. 2003; Hong et al. 2005, 2006, 2007).

*Aspergillus fumigatus* Fresenius is an ubiquitous filamentous fungus in the environment, and also an important human pathogen (Raper & Fennell 1965). Several *Neosartorya* species have been described as causal agents of human diseases including invasive aspergillosis, osteomyelitis, endocarditis and mycotic keratitis (Corriglione et al. 1990; Summerbell et al. 1992; Padhye et al. 1994; Lonial et al. 1997; Jarv et al. 2004; Balajee et al. 2005, 2006). All of the *Neosartorya* species produce heat-resistant ascospores that are frequently encountered in different food products (Gomez et al. 1994; Samson 1989; Tournas 1994). The several myxotoxins produced by these species may cause serious health hazard (Fujimoto et al. 1993; Frisvad & Samson 1990; Larsen et al. 2007).

Some species also have valuable properties for mankind; e.g. *N. fischeri* strains produce fiscalins which effectively inhibit the binding of substance P to the human neurokinin receptor (Wong et al. 1993), while *A. fumigatus* strains produce pyripyropenes, potent inhibitors of acyl-CoA:cholesterol acyltransferase (Tomoda et al. 1994), the immunosuppressant restrictocins (Müllbacher & Eichner 1984), ribotoxins (Lin et al. 1995) and fumagillin that has amebicidal activity (McCown et al. 1951). *Neosartorya* spinosa can be used for the complete enzymatic recovery of ferulic acid from corn residues (Shin et al. 2006).

Here we present an overview of the species belonging to *Aspergillus* section *Fumigati* based on analysis of macro- and micromorphology, extrolite profiles and β-tubulin, calmodulin, ITS and actin gene sequences of the isolates. We also describe four new homothallic *Neosartorya* species found in soil samples in Australia and Papua New Guinea using this polyphasic approach and list synonymies.

**MATERIALS AND METHODS**

Source of microorganisms

The fungi examined included type strains or representatives of all species available for examination in *Aspergillus* section *Fumigati*. Some atypical isolates collected in Australia and Papua New Guinea were also examined to clarify their taxonomic status (Table 1).

**Morphology and physiology**

The strains (Table 1) were grown for 7 d as 3-point inoculations on Czapek agar, Czapek yeast autolysate agar (CYA), oat meal agar (OA) and malt extract agar (MEA) plates at 25 °C, and on CYA at 37 °C. For *Neosartorya* species Hay infusion agar and SNA agar have also been used for inducing the anamorphs (medium compositions in Samson et al. 2004). In some species e.g. *N. tatenoi* the anamorph could only be produced when growing the cultures at 30 or 37 °C on MEA + 40 % sucrose.
Table 1. Aspergillus section Fumigati isolates used in this study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate No.*</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. brevipes</td>
<td>CBS 118.53T</td>
<td>Soil, Australia</td>
</tr>
<tr>
<td>A. duricaulis</td>
<td>CBS 481.65T</td>
<td>Soil, Buenos Aires, Argentina</td>
</tr>
<tr>
<td>A. fumigatiaffinis</td>
<td>IBT12703T</td>
<td>Soil, U.S.A.</td>
</tr>
<tr>
<td>A. fumigatus</td>
<td>CBS 133.61T = NRRL 163</td>
<td>Chicken lung, U.S.A.</td>
</tr>
<tr>
<td>A. fumisynnematus</td>
<td>IFM 42277T</td>
<td>Soil, Venezuela</td>
</tr>
<tr>
<td>A. lentulus</td>
<td>CBS 117887T = NRRL 35552 = KACC 41940</td>
<td>Man, U.S.A.</td>
</tr>
<tr>
<td>A. novofumigatus</td>
<td>IBT 16806T</td>
<td>Soil, Ecuador</td>
</tr>
<tr>
<td>A. unilateralis</td>
<td>CBS 126.56T</td>
<td>Rhizosphere, Australia</td>
</tr>
<tr>
<td>A. viridinutans</td>
<td>CBS 127.56T</td>
<td>Rabbit dung, Australia</td>
</tr>
<tr>
<td>A. turcosus</td>
<td>KACC 42090 = IBT 27920</td>
<td>Air conditioner, Inchon, Korea</td>
</tr>
<tr>
<td>N. assulata</td>
<td>KACC 41691T</td>
<td>Tomato soil, Buyeo, Korea</td>
</tr>
<tr>
<td>N. aurata</td>
<td>CBS 466.65T</td>
<td>Jungle soil, Brunei</td>
</tr>
<tr>
<td>N. aureola</td>
<td>CBS 105.55T</td>
<td>Soil, Tafo, Ghana</td>
</tr>
<tr>
<td>N. australensis sp. nov.</td>
<td>CBS 112.55T = NRRL 2392 = IBT 3021</td>
<td>Garden soil, Adelaide, Australia</td>
</tr>
<tr>
<td>N. coreana</td>
<td>KACC 41659T = NRRL 35590 = CBS 121594</td>
<td>Tomato soil, Buyeo, Korea</td>
</tr>
<tr>
<td>N. denticulata</td>
<td>CBS 598.74T = KACC 41183</td>
<td>Soil under Elaeis guineensis, Suriname</td>
</tr>
<tr>
<td>N. fennelliae</td>
<td>CBS 599.74T</td>
<td>Eye ball of Oryctolagus cuniculus, U.S.A.</td>
</tr>
<tr>
<td>N. ferenczii sp. nov.</td>
<td>CBS 121594T = IBT 27813 = NRRL 4179</td>
<td>Soil, Australia</td>
</tr>
<tr>
<td>N. fischeri</td>
<td>CBS 544.65T = NRRL 181</td>
<td>Canned apples</td>
</tr>
<tr>
<td>N. galapagensis</td>
<td>CBS 117522T = IBT 16756 = KACC 41935</td>
<td>Soil, Ecuador</td>
</tr>
<tr>
<td>N. glabra</td>
<td>CBS 111.55T</td>
<td>Rubber scrub from old tire, Iowa, U.S.A.</td>
</tr>
<tr>
<td>N. hiratsukae</td>
<td>CBS 294.93T</td>
<td>Aloe juice, Tokyo, Japan</td>
</tr>
<tr>
<td>N. laciniosa</td>
<td>KACC 41657T = NRRL 35589 = CBS 117721</td>
<td>Tomato soil, Buyeo, Korea</td>
</tr>
<tr>
<td>N. multiplicata</td>
<td>CBS 841.96T = IBT 27801</td>
<td>Bark of Podocarpus sp. (Podocarpaceae), bark, Myola, Owen Stanley Range, Northern Province, Papua New Guinea</td>
</tr>
<tr>
<td>N. multiplex</td>
<td>CBS 135.52T = NRRL 2154</td>
<td>Cardboard, York, U.K.</td>
</tr>
<tr>
<td>N. nishimurae</td>
<td>CBS 107078</td>
<td>Soil, Korea</td>
</tr>
<tr>
<td>N. patagonicus</td>
<td>CBS 100942</td>
<td>Fruit juice, Netherlands</td>
</tr>
<tr>
<td>N. spathulata</td>
<td>CBS 253.94</td>
<td>Canned oolong tea beverage, Japan (type strain of N. primulina)</td>
</tr>
<tr>
<td>N. spinosa</td>
<td>CBS 406.89T</td>
<td>Soil under Alocasia macrorhiza, Taiwan</td>
</tr>
<tr>
<td>N. stramenia</td>
<td>CBS 483.65T</td>
<td>Soil, Nicaragua</td>
</tr>
<tr>
<td>N. lutea</td>
<td>CBS 496.65T</td>
<td>Soil from maple-ash-elm forest, Wisconsin, U.S.A.</td>
</tr>
<tr>
<td>N. tamentii</td>
<td>CBS 407.93T</td>
<td>Soil of sugarcane, Timbauba, Brazil</td>
</tr>
<tr>
<td>N. undulata</td>
<td>CBS 101754</td>
<td>Fruit, Yunnan, China (type strain of N. delicata)</td>
</tr>
<tr>
<td>N. warcupii sp. nov.</td>
<td>CBS 114217T</td>
<td>Soil, Brazil</td>
</tr>
<tr>
<td>N. warcupii sp. nov.</td>
<td>CBS 114218</td>
<td>Soil, Brazil</td>
</tr>
</tbody>
</table>

* CBS = Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; IBT = Institute for Biotechnology, Lyngby, Technical University of Denmark; IFM = Institute for Food Microbiology (at present, the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University), Chiba, Japan; KACC = Korean Agricultural Culture Collection, Suwon, Korea; NRRL = Agricultural Research Service Culture Collection, Peoria, Illinois, U.S.A.; T = type strain.
Analysis for extrolites

Extrólites were analysed using the HPLC-diode array detection method of Frisvad & Thrane (1987, 1993) as modified by Smedsgaard (1997). Extrólites were analyzed from cultures grown on CYA, OA and YES agar using three agar plugs (Smedsgaard 1997).

Isolation and analysis of nucleic acids

Isolates used for the molecular studies were grown on 2 mL of malt peptone broth [10 % (v/v)] malt extract (Brix 10) and 0.1 % (w/v) bacto peptone (Difco), in 15 mL tubes. The cultures were incubated at 25 °C for 7 d. DNA was extracted from the cells using the Masterpure™ yeast DNA purification kit (Epicentre Biotechnol.) following the instructions of the manufacturer. Fragments containing the ITS region were amplified using primers ITS1 and ITS4 as described (White et al. 1990). Amplification of partial β-tubulin gene was performed using the primers Bt2a and Bt2b and methods of Glass & Donaldson (1995). Amplifications of the partial calmodulin and actin genes were as described (Hong et al. 2005, 2007). Sequencing reactions were performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit and carried out for both strands. All the sequencing reactions were purified by gel filtration through Sephadex G-50 (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in double-distilled water and analyzed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The complementary sequences were corrected with the MT Navigator index: software (Applied Biosystems). Unique ITS, β-tubulin, actin and calmodulin sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov) with accession numbers DQ534140, DQ534141 and EU20279–EU220287.

Data analysis

Sequence alignments were performed using CLUSTAL-X (Thompson et al. 1997) and improved manually. The neighbour-joining (NJ) method was used for the phylogenetic analysis. For NJ analysis, the data were first analysed using the Tamura–Nei distance calculation with gamma-distributed substitution rates (Tamura & Nei 1993), which were then used to construct the NJ tree with MEGA v. 3.1 (Kumar et al. 2004). A bootstrap analysis was performed with 1 000 replications to determine the support for each clade.

PAUP v. 4.0 b10 software was used for parsimony analysis (Swofford 2002). Alignment gaps were treated as a fifth character and all characters were unordered and of equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option with random addition order (100 reps) and tree bisection-reconnection (TBR) branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1 000 bootstrap replications (Hillis & Bull 1993). Sequences from an A. clavatus isolate were used as outgroups in these experiments.

RESULTS AND DISCUSSION

Phylogenetic analysis

We examined the phylogenetic relatedness of species belonging to Aspergillus section Fumigati using sequence analysis of partial β-tubulin, calmodulin and actin genes including sequences of all known species. ITS sequences were determined from the new species and the species most closely related to them in the β-tubulin tree. The partial β-tubulin gene alignment included 453 characters. Among the polymorphic sites, 102 were found to be phylogenetically informative. The Neighbour-joining tree based on partial β-tubulin genes sequences is shown in Fig. 1. The topology of the tree is the same as one of the 419 maximum parsimony trees constructed by the PAUP programME (length: 465 steps, consistency index: 0.6710, retention index: 0.6467). The calmodulin data set included 549 characters with 85 parsimony informative characters. The Neighbour-joining tree shown in Fig. 2 has the same topology as one of the 9 maximum parsimony trees (tree length: 323, consistency index: 0.7585, retention index: 0.6422).

The actin data set included 590 characters with 104 parsimony informative characters. The Neighbour joining tree shown in Fig. 3 has the same topology as one of the 312 maximum parsimony trees (tree length: 397, consistency index: 0.6675, retention index: 0.7130). The ITS data set included 501 characters with 26 parsimony informative characters. The Neighbour joining tree shown in Fig. 4 has the same topology as one of the 57 maximum parsimony trees (tree length: 77, consistency index: 0.7532, retention index: 0.7765).

The four Neosartorya isolates representing new species were found to be different from all known species of Aspergillus section Fumigati based on either their β-tubulin, calmodulin or actin gene sequences. However, one of them (NRRL 4179) had identical ITS sequences with N. denticulata (Fig. 4). This isolate was found to be closely related to a clade including N. fennelliae and N. denticulata on all other trees.

Possible synonymies of some species described previously have also been examined during this study. Based on multilocus sequence analyses Hong et al. (2007) discussed the synonymy of N. botucatensis, N. paulistensis and N. takaki with N. spinosa (Raper & Fennell) Kozak. (1972). N. spinosa and the synonyms have roughly circular arrangements of projections on the ascospore convex walls. N. spinosa produces echinulate ascospores with spines ranging from < 0.5 µm up to 5–7 µm long with verrucose and small triangular projections or sometimes with circularly arranged projections.

N. otanii Takada, Y. Horie & Abiliz (2001) was described on the basis of its rapid growth on Czapek and malt extract agars, lenticular ascospores with two widely separated equatorial crests, tuberculate or lobate-reticulate convex surface, and globose to broadly ellipsoidal conidia with a microtuberculate wall. The morphology of N. otanii resembles N. fennelliae, although Takada et al. (2001) reported small differences of the ascospore ornamentation, which was not confirmed in our SEM studies. The β-tubulin gene sequences of N. otanii (GenBank accession numbers AB201363 and AB201362) were identical with N. fennelliae (KACC 42228) (Fig. 5A). These N. fennelliae isolates produced ascospores after mating with the N. fennelliae type strains (data not shown). N. otanii is probably synonymous with N. fennelliae, but mating experiments with N. fennelliae and N. otanii are needed for its confirmation.
Fig. 1. Neighbour-joining tree based on β-tubulin sequence data of Aspergillus section Fumigati. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

Fig. 2. Neighbour-joining tree based on calmodulin sequence data of Aspergillus section Fumigati. Numbers above branches are bootstrap values. Only values above 70 % are indicated.
Fig. 3. Neighbour-joining tree based on actin sequence data of *Aspergillus* section *Fumigati*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

Fig. 4. Neighbour-joining tree based on ITS sequence data of selected species of *Aspergillus* section *Fumigati*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.
**Fig. 5.** Neighbour-joining tree based on β-tubulin sequences showing the relationship of *N. otanii* and *N. fennelliae*. **B.** Neighbour-joining trees based on β-tubulin, calmodulin and actin sequence data of *Neosartorya* spp. showing the relationship of *N. primulina*, *N. quadricincta*, *N. tatenoi* and *N. delicata*. 
These experiments could not be carried out because the ex type cultures of *N. otanii* were not available.

*Neosartorya primulina* Udagawa, Toyaz. & Tsub. (1993) was characterised by its restricted growth on Czapek agar, chalky-buff ascomata, and lenticular ascospores with a very irregular ornamentation composed of several narrow crests and verrucose hemispheres. The ascospore ornamentation and anamorph morphology resembles those of *N. quadricincta*. Furthermore, the ex type culture (CBS 253.94) of *N. primulina* showed nearly identical sequences with strains of *N. quadricincta* for β-tubulin, calmodulin and actin genes (Fig. 5B). Therefore, we consider *N. primulina* reduced to synonymy with *N. quadricincta*.

*Neosartorya delicata* H.Z. Kong (1997) was described based on its ellipsoid or nearly clavate vesicles, and ascospores with conspicuous spines, joining one spine to another by fairly prominent ridges and reticulate ornamentation, the ridges spreading to the equatorial crests. This species has identical ascospore morphology with *N. tatenoi* (Fig. 36), and both taxa were clustered into a clade in three gene trees (99.6 % in β-tubulin, 98.5 % in calmodulin and 97.3 % in actin gene sequences) (Fig. 5B). Therefore, we consider *N. delicata* as a synonym of *N. tatenoi*.

*Neosartorya nishimurae* (Takada et al. 2001), *N. indohii*, *N. tsurutae* (Horie et al. 2003), *N. takakii* (Horie et al. 2001) and *N. sublevispora* (Someya et al. 1999) ex-type cultures were not available for this monograph of *Aspergillus section Fumigati*, and because we could not study them, they are listed as doubtful species.

### Table 2. Extrolites produced by species assigned to *Aspergillus section Fumigati*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Extrolites produced</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus brevipes</em></td>
<td>roquefortine C, meleagrin-like</td>
</tr>
<tr>
<td><em>Aspergillus duricaulis</em></td>
<td>psurotin A, fumagillin, asperpentyn, duricaulic acid and asperptin, phthalides, chromanols, cyclopatic acid, 3-O-methylcyclopolic acid</td>
</tr>
<tr>
<td><em>Aspergillus fumigatiannisin</em></td>
<td>aurantane, cycloechinuline, fumigaclavines, helvolic acid, neosartorin, palitantin, pyripyropenes A, E, O &amp; S, tryptoquivaline, tryptoquivalone</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em></td>
<td>fumagillin, fumitiroxins, fumigaclavines A &amp; C, fumitremorgins, gliotoxin, tryspacidin, pseudotins, helvolic acid, pyripyropens, methyl-sulochrin, verruculogen, fumiquinazolines</td>
</tr>
<tr>
<td><em>Aspergillus fumisynnematus</em></td>
<td>neosartorin, pyripyropens, fumimycin</td>
</tr>
<tr>
<td><em>Aspergillus lentulus</em></td>
<td>cyclopazonic acid, pyripyropens A, E &amp; O, terrein, aurantane, neosartorin</td>
</tr>
<tr>
<td><em>Aspergillus novofumigatus</em></td>
<td>azsonalenin, cycloechinuline, fiscalin, helvolic acid, neosartorin, palitantin, terrein, terrein B</td>
</tr>
<tr>
<td><em>Aspergillus turcosus</em></td>
<td>kotanins and several unique but not yet elucidated secondary metabolites</td>
</tr>
<tr>
<td><em>Aspergillus unilateralis</em></td>
<td>mycophenolic acid, other unique secondary metabolites</td>
</tr>
<tr>
<td><em>Aspergillus viridinutans</em></td>
<td>viriditoxin, 13-O-methylviridin, phomaligin A, variotin, viriditoxin, wasabidienone B0, B1, viriditoxin, 4-acetyl-6,8-dihydroxy-5-methyl-2-benzopyran-1-1 A</td>
</tr>
<tr>
<td><em>Neosartorya assulata</em></td>
<td>indole alkaloids and apolar metabolites</td>
</tr>
<tr>
<td><em>Neosartorya aurata</em></td>
<td>helvolic acid, yellow unidentified compounds</td>
</tr>
<tr>
<td><em>Neosartorya aureola</em></td>
<td>fumagillin, tryptoquivaline, tryptoquivalone, psurotin A and viriditoxin (FRR 2269 also produces helvolic acid)</td>
</tr>
<tr>
<td><em>Neosartorya australensis</em></td>
<td>wortmannin-like, azsonalenin-like</td>
</tr>
<tr>
<td><em>Neosartorya coreana</em></td>
<td>azsonalenins</td>
</tr>
<tr>
<td><em>Neosartorya denticulata</em></td>
<td>gliotoxin, viriditoxin</td>
</tr>
<tr>
<td><em>Neosartorya fennelliae</em></td>
<td>asperfuran, azsonalenin, fumigaclavine, viritidicatumtoxin</td>
</tr>
<tr>
<td><em>Neosartorya ferczii</em></td>
<td>asperfuran, azsonalenin, fumigaclavine, viritidicatumtoxin, gliotoxin-like, fumigatins, azsonalenin-like</td>
</tr>
<tr>
<td><em>Neosartorya fischeri</em></td>
<td>terrein, fumitremorgins A &amp; C, tryptoquivaline A, lycacidin, TR-2, verruculogen, sarcin, azsonalenins, fischerin, neosartorin, fiscalin, helvolic acid</td>
</tr>
<tr>
<td><em>Neosartorya galapagensis</em></td>
<td>gretagnin</td>
</tr>
<tr>
<td><em>Neosartorya glabra</em></td>
<td>asperpentyn, avenaciolide, wortmannin-like compound</td>
</tr>
<tr>
<td><em>Neosartorya hiratsukae</em></td>
<td>avenaciolide</td>
</tr>
<tr>
<td><em>Neosartorya laciniosa</em></td>
<td>azsonalenins, tryptoquivaline, tryptoquivalone</td>
</tr>
<tr>
<td><em>Neosartorya multiplicata</em></td>
<td>helvolic acid</td>
</tr>
<tr>
<td><em>Neosartorya papuensis</em></td>
<td>wortmannin-like</td>
</tr>
<tr>
<td><em>Neosartorya pseudofischeri</em></td>
<td>asperfuran, cytochlasin-like compound, fiscalin-like compound, pyripyropens, gliotoxin</td>
</tr>
<tr>
<td><em>Neosartorya quadricincta</em></td>
<td>quinolactacin, azsonalenin</td>
</tr>
<tr>
<td><em>Neosartorya spinosa</em></td>
<td>azsonalenins, 2-pyrovoylaminobenzamide, psurotin</td>
</tr>
<tr>
<td><em>Neosartorya spathulata</em></td>
<td>xanthocilins, azsonalenins</td>
</tr>
<tr>
<td><em>Neosartorya stramenia</em></td>
<td>quinolactacin, avenaciolide</td>
</tr>
<tr>
<td><em>Neosartorya tatenoi</em></td>
<td>azsonalenins</td>
</tr>
<tr>
<td><em>Neosartorya udagawae</em></td>
<td>fumigatin, fumagillin, tryptoquivaline, tryptoquivalone</td>
</tr>
<tr>
<td><em>Neosartorya warcupii</em></td>
<td>wortmannin-like, azsonalenin-like, chromanols-like, tryptoquivaline-like and tryptoquivalone-like</td>
</tr>
</tbody>
</table>
Morphology and extrolite production

The atypical *N. glabra* isolate NRRL 4179 (Raper & Fennell 1965) produced asperfurans, aszonalenin, fumigacalvaine, viridicatumtoxins, and fumigatins, extrolites common in *N. fennelliae*, but none of the extrolites produced by *N. glabra*. However, in contrast with the heterothallic *N. fennelliae*, this isolate is homothallic. It is closely related to *N. denticulata* based on phylogenetic analysis of sequence data, although their ascospore ornamentations are strikingly different (Figs. 21, 23). Ascospore ornamentation of NRRL 4179 is similar to that of the heterothallic *N. fennelliae* (Fig. 22) with equatorial crests much narrower, while *N. denticulata* has denticulate ascospores without equatorial crests. Isolate NRRL 4179 exhibited 72% nuclear DNA relatedness to *N. fennelliae* and only 60% relatedness to *N. glabra* isolates (Peterson 1992). This isolate also yielded different mtDNA and Smal-digested repetitive DNA patterns from those of all the other *Neosartorya* strains examined (Rinyu et al. 2000). Hybridisation experiments were also carried out with *Neurospora crassa* mating type genes (the A idiomorph with about 6 kb flanking sequences, or the a idiomorph flanked by about 2 kb genomic DNA on either side) to the EcoRI digested DNA of several teleomorphic and asexual *Aspergillus* strains. Hybridisation to a 1.9 kb band was observed for both mating-type strains of *N. fennelliae* and isolate NRRL 4179 (Rinyu et al. 2000). Based on these observations, isolate NRRL 4179 seems to be closely related to *N. fennelliae* strains. These results are in agreement with those found using carbon source utilisation tests and isoenzyme analysis of these strains (Varga et al. 1997).

Strain NRRL 35723 was isolated from soil in Australia, and produced compounds structurally related to wortmannin, aszonalenin, chromanol, tryptoquatins and tryptoquivalons. This isolate was markedly different from all other known *Neosartorya* species in secreting a bluish pigment after 7 d incubation on MEA isolates (Peterson 1992). This isolate was isolated from garden soil in Adelaide, Australia, and produced compounds similar to wortmannin and aszonalenin and some unique metabolites, while CBS 841.96 was isolated from *Podocarpus* bark in Papua New Guinea, and produced a compound related to wortmannins and some unique compounds. The structures of which have not yet been elucidated (Table 2). The ascospore ornamentations of these isolates were microtuberculate, similarly to those of *N. glabra* and *N. galapagensis*. However, both isolates produced cream-coloured colonies on CYA in contrast with *N. glabra* which produces greyish green colonies. In phylogenetic analysis they were unrelated to any other *Neosartorya* species, justifying their treatment as new species. We propose four new homothallic and monotypic *Neosartorya* species; *N. ferenzii* (NRRL 4179), *N. warcupii* (NRRL 35723), *N. australensis* (CBS 112.55) and *N. papuensis* (CBS 841.96).

Identification

Traditionally the identification of members of section *Fumigati* were done using the colony patterns and the morphology of the conidiogenous structures, conidia, ascomata and ascospores. Ascospore ornamentation has been studied by Scanning electron microscopy, but our studies have shown that different species have similar ascospore shape and surface structure. Several species such *A. fumigatus*, *A. novofumigatus*, *fumigatiaffinis*, *A. fumisynnematus* and *A. lentulus* show strong morphological resemblance and in the light microscope these species can be difficult to be separated. The anamorphs of *Neosartorya udagawae* and *N. fennelliae* also show a similar morphology. Therefore we recommend that for a correct species identification, sequence analysis should be carried out. Our experience with sequencing the calmodin and β-tubulin gene revealed good species delimitation and recognition. All sequences of the ex type cultures of section *Fumigati* are available from specialised databases and also from GenBank.

List of accepted species belonging to *Aspergillus* section *Fumigati*

The list of known species of *Neosartorya* and anamorphic species from the section *Fumigati* (Horie et al. 2003; Hong et al. 2005, 2006, 2007) is still expanding. With the species proposed here, there are now 23 *Neosartorya* species (including four new taxa) and 10 *Aspergillus* species in this group, 33 species in total and they are illustrated below.

Strict anamorphic species:

*Aspergillus* brevipes Smith
*Aspergillus* duricaulis Raper & Fennell
*Aspergillus* fumigatiaffinis Hong, Frisvad & Samson
*Aspergillus* fumigatus Fresenius
  = *A. anomalus* Pidoplichko & Kirilenko
  = *A. fumigatus* var. *acolumnaris* Rai et al.
  = *A. fumigatus* var. *ellipticus* Raper & Fennell
  = *A. fumigatus* mut. *helvola* Rai et al.
  = *A. phialiseptus* Kwon-Chung
  = *A. neoellipticus* Kozakiewicz
  = *Aspergillus arvii* Aho, Horie, Nishimura & Miyaji
*Aspergillus* fumisynnematus Horie, Miyaji, Nishimura, Taguchi & Udagawa
*Aspergillus* lentulus Balajee & Marr
Aspergillus novofumigatus Hong, Frisvad & Samson
Aspergillus turcosus Hong, Frisvad & Samson
Aspergillus unilateralis Thrower
≡ A. brevipes var. unilateralis (Thrower) Kozakiewicz
Aspergillus viadinutans Ducker & Thrower
≡ A. fumigatus var. sclerotiorum Rai, Agarwal & Tewari

Teleomorph species:

Neosartorya assulata Hong, Frisvad & Samson [anamorph: A. assulatus Hong, Frisvad & Samson]
Neosartorya aurata (Warcup) Malloch & Cain [anamorph: A. igneus Kozakiewicz]
Neosartorya aureola (Fennell & Raper) Malloch & Cain [anamorph: A. aureoluteus Samson & Gams]
Neosartorya australensis Samson, Hong & Varga, sp. nov.
Neosartorya coreana Hong, Frisvad & Samson [anamorph: A. coreanos Hong, Frisvad & Samson]
Neosartorya denticulata Samson, Hong & Frisad [anamorph: A. denticulatus Samson, Hong & Frisad]
Neosartorya fennelliae Kwon-Chung & Kim [anamorph: A. fennelliae Kwon-Chung & Kim]
≡ Neosartorya otanii Takada, Horie & Abliz [anamorph: A. otanii Takada, Horie & Abliz]
Neosartorya ferenczii Varga & Samson, spec. nov.
Neosartorya fischeri (Wehmer) Malloch & Cain [anamorph: A. fischeranus Kozakiewicz]
Neosartorya galapagensis Frisvad, Hong & Samson [anamorph: A. galapagensis Frisvad, Hong & Samson]
Neosartorya glabra (Fennell & Raper) Kozakiewicz [anamorph: A. neoglaber Kozakiewicz]
Neosartorya hiratsukae Udagawa, Tsubouchi & Horie [anamorph: A. hiratsukae Udagawa, Tsubouchi & Horie]
Neosartorya labiniosa Hong, Frisvad & Samson [anamorph: A. laciniosus Hong, Frisvad & Samson]
Neosartorya multiplicata Yaguchi, Someya & Udagawa [anamorph: A. multiplicatus Yaguchi, Someya & Udagawa]
Neosartorya papuensis Samson, Hong & Varga, sp. nov.
Neosartorya pseudofischeri Peterson [anamorph: A. thermomutatus (Paden) Peterson]
Neosartorya quadricincta (Yuill) Malloch & Cain [anamorph: A. quadricincta Kozakiewicz]
≡ Neosartorya primulina Udagawa, Toyazaki & Tsubouchi [anamorph: A. primulina Udagawa, Toyazaki & Tsubouchi]
Neosartorya spathulata (Raper & Fennell) Kozakiewicz [anamorph: A. spinosus Kozakiewicz]
≡ Aspergillus fischeri var. spinosus Raper & Fennell 1965 (basionym)
≡ Sartorya fumigata var. verrucosa Udagawa & Kawasaki
≡ Neosartorya botucatensis Horie, Miyaji & Nishimura [anamorph: A. botucatensis Horie, Miyaji & Nishimura]
≡ Neosartorya paulistensis Horie, Miyaji & Nishimura [anamorph: A. paulistensis Horie, Miyaji & Nishimura]
Neosartorya stramenia (Novak & Raper) Malloch & Cain [anamorph: A. stramenia Samson & Gams]
Neosartorya tsurutae Horie, Miyaji, Yokoyama, Udagawa & Campos-Takagi [anamorph: A. tsurutae Horie, Miyaji, Yokoyama, Udagawa & Campos-Takagi]
≡ Neosartorya delicata Kong [anamorph: A. delicata Kong]
Neosartorya udagawae Horie, Miyaji & Nishimura [anamorph: A. udagawae Horie, Miyaji & Nishimura]
Neosartorya warcupi Peterson, Varga & Samson, sp. nov.

Doubtful species:

Neosartorya sublevispora Someya, Yaguchi & Udagawa [anamorph: A. sublevisporus Someya, Yaguchi & Udagawa]
Neosartorya indohii Horie [anamorph: A. indohii Horie]
Neosartorya tsurutae Horie [anamorph: A. tsurutae Horie]
Neosartorya nishimurae Takada, Horie & Abliz [anamorph: A. nishimurae Takada, Horie & Abliz]
Aspergillus brevipes Smith, Trans. Br. mycol. Soc. 35: 241. 1952. Fig. 6.

**Type:** CBS 467.91, from soil, New South Wales, Australia

**Other no. of the type:** ATCC 16899; CBS 118.53; IFO 5821; IMI 16034; IMI 51494; NRRL 2439; WB 4772 = IBT 22571; WB 4078 = IBT 22572

**Description**
Colony diam (7 d): CYA25: 12–15 mm; MEA25: 23–25 mm; OA25: 28–33 mm; CYA37: 16–19 mm; CREA: weak growth, no acid production
Colony colour: purple red
Conidiation: abundant
Reverse colour (CZA): dull yellow turning to reddish brown
Colony texture: velutinous
Conidial head: short columnar
Stipe: 15–50 (–100) µm, occasionally septate, heavy walled
Vesicle diam, shape: 10–18 µm, pear shaped
Conidium size, shape, surface texture: 2.8–3.5 µm, globose, spinulose

**Cultures examined:** CBS 467.91; WB 4772; WB 4078; CBS 118.523 = IBT 3051, all from the same original source

**Diagnostic features:** short heavy walled stipes, finely spinulose conidia, purple red colony colour, coloured vesicles and phialides and dark blue conidia; characterised by its vesicles borne at an angle to the stipe, as in *A. viridinutans* and *A. duricaulis*

**Similar species:** *A. brevipes*

**Distribution:** Argentina

**Ecology and habitats:** soil

**Extrrolites:** pseurotin A, fumagillin (found here), asperpentyn (Muhlenfeld & Achenbach 1988), duricaulic acid and asperdurin (Achenbach et al. 1985a), phthalides and chromanols (Achenbach et al. 1982a, 1985b), cyclopaldic acid and 3-O-methylcyclopolic acid (Brillinger et al. 1978; Achenbach et al. 1982b)

**Pathogenicity:** not reported

Aspergillus fumigatiaffinis Hong, Frisvad & Samson, Mycologia 97: 1326. 2005. Fig. 8.

**Type:** CBS 117186, from soil, Socorro County, Sevilleta National Wildlife Refuge, New Mexico, U.S.A..

**Other no. of the type:** KACC 41148; IBT 12703

**Description**
Colony colour: white, with center dull green
Conidiation: limited
Reverse colour (CZA): yellowish to greyish orange
Colony texture: flocose
Conidial head: short columnar
Stipe: 6–8 µm in diam.
Vesicle diam, shape: 18–24 µm, globose-subglobose
Conidium size, shape, surface texture: 2–3 µm, globose-subglobose, smooth

**Diagnostic features:** has comparatively small (sub)globose vesicles (16–24 µm); able to grow at 10 °C, and unable to grow at 50 °C

**Similar species:** *A. fumigatus, A. lentulus, A. novofumigatus, A. fumigatiaffinis*

**Distribution:** U.S.A., Spain

**Ecology and habitats:** kangaroo rat, soil, human

**Extrrolites:** auranthine, cycloechinuline, fumigaclavines, helvolic acid, neosartorin, palitantin, pyripyropenes A, E, O & S, tryptoquivaline, tryptoquivalone

**Pathogenicity:** pathogenic to humans (Alcazar-Fuoli et al. 2007)

**Note:** exhibits high MICs to amphotericin B and several triazoles (Alcazar-Fuoli et al. 2007)
**Aspergillus fumigatus** Fresenius, Beitr. Mykol. 81: 18. 1863. Fig. 9.

- Aspergillus fumigatus var. acolummatus Rai, Agarwal & Tekwani (1971)
- Aspergillus fumigatus var. coleculus Malchevska (1939)
- Aspergillus fumigatus var. ellipiticus Raper & Fennell (1965)
- Aspergillus fumigatus var. fumigatus Fresen. (1863)
- Aspergillus fumigatus var. helvoculus Yuill (1937)
- Aspergillus fumigatus var. luteolus Sartory, Sartory & Mey. (1943)
- Aspergillus fumigatus var. minimus Sartory (1919)
- Aspergillus phialocephalus Kwon-Chung (1975)
- Aspergillus bronchialis Blumentrit (1901)
- Aspergillus septatus Sartory & Sartory (1943)
- Aspergillus aniv Aho, Horie, Nishimura & Miyaji (1994)

**Type:** IMI 016152, from chicken lung, Connecticut, U.S.A.

**Other no. of the type:** Thom 118; QM 1981; WB 163; CBS 133.61; NRRL 163; ATCC 1022; LSHB Ac71; NCTC 982; KACC 41143

**Description**

Colony diam (7 d): CYA25: 21–67 mm; MEA25: 25–69 mm; YES25: 42–80 mm; OA25: 34–62 mm, CYA37: 57–61 mm, CREA: poor growth, no or very weak acid production

**Colony colour:** greyish turquoise or dark turquoise to dark green to dull green

**Reverse colour:** (CYA37): pale yellow to grey orange, greyish brown

**Colony texture:** velutinous, st. floccose

**Reverse texture:** floccose

**Conidial head:** short columnar

**Stipe:** 210 × 6–8.5(–10) µm

**Vesicle diam, shape:** 16–20(–25) µm, hemispherical

**Conidium size, shape, surface texture:** 2.8–3.2 × 2.4–2.8 µm, broadly ellipsoidal, verruculose

**Cultures examined:** IFM 42277

**Diagnostic features:** production of synnemata on MEA with age (1–4.3 mm in height, 30–40 µm in diam.)

**Similar species:** A. fumigatus, A. lentulus, A. novofumigatus, A. fumigatiaffinis

**Distribution:** Brazil, Venezuela, Spain

**Ecology and habitats:** soil, human

**Extraroles:** neosartorin, pyripyropens (found here), fumimycin (Kwon et al. 2007)

**Pathogenicity:** pathogenic to humans (Alcazar-Fuoli et al. 2007; Yaguchi et al. 2007)

**Note:** growth at 10 °C, no growth at 50 °C; some isolates carry dsRNA mycoviruses (Anderson et al. 1996)

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**Type:** IFM 42277, from soil, Sabaneta, Coro City, Falcon State, Venezuela

**Description**


**Colony colour:** greenish grey

**Conidiation:** limited

**Reverse colour:** (CZA): orange white to orange grey

**Colony texture:** floccose

**Conidial head:** short columnar

**Stipe:** 210 × 6–8.5(–10) µm

**Vesicle diam, shape:** 16–20(–25) µm, hemispherical

**Conidium size, shape, surface texture:** 2.8–3.2 × 2.4–2.8 µm, broadly ellipsoidal, verruculose

**Cultures examined:** IFM 42277

**Diagnostic features:** production of synnemata on MEA with age (1–4.3 mm in height, 30–40 µm in diam.)

**Similar species:** A. fumigatus, A. lentulus, A. novofumigatus, A. fumigatiaffinis

**Distribution:** Brazil, Venezuela, Spain

**Ecology and habitats:** soil, human

**Extraroles:** neosartorin, pyripyropens (found here), fumimycin (Kwon et al. 2007)

**Pathogenicity:** pathogenic to humans (Alcazar-Fuoli et al. 2007; Yaguchi et al. 2007)

**Note:** growth at 10 °C, no growth at 50 °C

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**Aspergillus lentulus** Balajee & Marr, Eukaryot. Cell 4: 631.2005. Fig. 11.

**Type:** FH5, from clinical specimens of patients hospitalised at the Fred Hutchinson Cancer Research Center, U.S.A.

**Other no. of the type:** KACC 41940, NRRL 35552; IBT 27201

**Description**

Colony diam: CYA25: (19–)25–56 mm, MEA25: (30)40–70 mm; YES25: 42–80 mm; OA25: 44–59 mm; CYA37: 54–70 mm, CREA: weak growth, no acid production

**Colour:** white with interspersed grey green conidia

**Conidiation:** usually poor, but abundant in some isolates

**Reverse colour (CYA):** pale yellow to grey orange, greyish brown

**Colony texture:** floccose

**Conidial head:** short columnar

**Stipe:** 20–500 × 4–7 µm, smooth, sometimes sinuous and constricted neck

**Vesicle diam, shape:** (6–)10–25 µm, globose to pyriform, usually subglobose
Fig. 10. Aspergillus fumisynnematus. A–B. Colonies 7 d 25 °C. A. CYA. B. MEA. C–H. Conidiophores. I. Conidia. Scale bars = 10 µm.
Conidia length, shape, surface texture: 2–3.2 µm, globose to broadly ellipsoidal, smooth to finely roughened.

**Cultures examined:** KACC 41391 = CBS 116886, KACC 41392, KACC 41393, KACC 41681, KACC 41682, KACC 41642, KACC 41394, KACC 41395, KACC 41939 = FH7 = IBT 27209, KACC 41941 = FH4 = IBT 27210, KACC 41942 = FH220 = IBT 27202, KACC 41940 = FH5 = IBT 27201 = NRRL 35552

**Diagnostic features:** slow and poor conidiation, floccose colony texture, short columnar conidial heads, thin stipe (<7 µm), globose vesicle; growth at 10 °C and no growth at 50 °C

**Similar species:** A. fumigatiaffinis, A. fumigatus, A. fumisynnematus, A. novofumigatus, A. viridinutans

**Distribution:** Korea, U.S.A., Japan, Australia, Netherlands, Spain etc. It is assumed that the species is distributed worldwide.

**Ecology and habitats:** soil, human, dolphin

**Extrrolites:** cyclopiazonic acid, pyripyropenes A, E & O, terrein, auranthine, neosartorin

**Pathogenicity:** pathogenic to humans (Balajee et al. 2005b; Alhambra et al. 2006; Alcazar-Fuoli et al. 2007; Yaguchi et al. 2007; Lau et al. 2007)

**Note:** exhibits high MICs to amphotericin B and several triazoles (Balajee et al. 2004, 2005b)

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**Fig. 12.**

**Type:** CBS 117520, from soil, Galapagos Islands, Ecuador

**Other no. of the type:** IBT 16806

**Description**

Colony diam (7 d): CYA25: 33–48 mm; MEA25: 48–60 mm; YES25: 44–55 mm; OA25: 54–67 mm; CYA37: 49–52 mm; CREA: weak growth, no acid production

Colony colour: deep green to grey green

Conidiation: in central areas

Reverse colour (CZA): greyish orange to yellowish orange

Colony texture: velutinous

Conidial head: short columnar

Stipe: 50–500 × 4–7 µm in diam

Vesicle diam, shape: (13–)15–30 µm subglobose to flask shaped

Conidium size, shape, surface texture: 2.5–3 µm, ellipsoidal, smooth

**Cultures examined:** CBS 117520 = IBT 16806, CBS 117519 = IBT 16755

**Diagnostic features:** has nearly flask-shaped and comparatively large vesicles (15–30 mm); growth at both 10 and 50 °C

**Similar species:** A. fumigatus, A. lentulus, A. fumisynnematus, A. fumigatiaffinis

**Distribution:** Galapagos Islands, Ecuador

**Ecology and habitats:** soil

**Extrrolites:** aszonalenin, cycloechinuline, fiscalins, helvolic acid, neosartorin, palitantin, terrein, territrem B

**Pathogenicity:** not reported

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**Fig. 13.**

**Type:** KACC 42091, from air conditioner, Seoul, South Korea

**Other no. of the type:** IBT 27921

**Description**


Colony colour: grey-turquoise to grey-green

Conidiation: abundant

Reverse colour (CZA): yellowish orange to greyish orange

Colony texture: velutinous

Conidial head: short columnar

Stipe: 80–100 × 4–7 µm

Vesicle diam, shape: 15–25 µm, flask shaped to globose

Conidium size, shape, surface texture: 2.5–3.5 µm, subglobose, smooth

**Cultures examined:** KACC 42091 = IBT 27921, KACC 42090 = IBT 27920, KACC 41955 = IBT 3016

**Diagnostic features:** Velutinous colony, grey-turquoise (green) colony colour and yellowish orange reverse on MEA and CYA, phialides cover distal two-thirds of the vesicle and growth at both 10 and 50 °C

**Similar species:** -

**Ecology and habitats:** air conditioner

**Distribution:** South Korea

**Extrrolites:** Kotanins and several unique compounds but not yet elucidated secondary metabolites

**Pathogenicity:** not reported

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≡ A. brevipes var. unilateralis (Thrower) Kozakiewicz

**Type:** CBS 126.56, from rhizosphere of Hibbertia fasciculata and Epacris impressa, Australia

**Other no. of the type:** ATCC 16902; IFO 8136; IMI 062876; NRRL 577, QM 8163; WB 4366; WB 4779; IBT 3210

**Description**

Colony diam: CZA25: 30 mm; MEA25: 60–70 mm in 14 d, CRWEA: poor growth, no acid production

Colony colour: slate olive

Conidiation: limited

Reverse colour (CZA): nearly black

Colony texture: thin, brittle, folded in central area

Conidial head: diminutive, with few divergent spore chains

Stipe: 5–30 × 1.2–2.2 µm

Vesicle diam, shape: 4–8.5 µm, irregularly globose

Conidium size, shape, surface texture: 2.5–3.5 µm, globose, coarsely echinulate

**Cultures examined:** CBS 126.56; CBS 283.66 = IBT 3211
**Diagnostic features**: phialides clustered on one side of the vesicle, echinulate conidia, slow growth rate and dark reverse on CYA

**Similar species**: -

**Distribution**: Australia

**Ecology and habitats**: soil

**Extralites**: mycophenolic acid, other unique secondary metabolites

**Pathogenicity**: not reported

**Aspergillus viridinutans** Ducker & Thrower, Austral. J. Bot. 2: 355. 1954. Fig. 15.

= *A. fumigatus var. sclerotiorum* J.N. Rai, S.C. Agarwal & J.P. Tewari

**Type**: CBS 127.56, from dung of rabbit, Frankston, Victoria, Australia

**Other no. of the type**: ATCC 16901; IMI 062875; IMI 062875ii; NRRL 4365; WB 4081; WB 4782; WB 4365

**Description**


Colony colour: Niagara green

Conidiation: limited on CZA, abundant on MEA

Reverse colour: colourless (CZA), yellowish green to light brownish olive (MEA)

Colony texture: centre raised, velutinous on MEA

Conidial head: columnar

Stipe: 20–35 × 3.3–4.4 µm

Vesicle diam, shape: 7.5–12 µm, flask shaped to subglobose

Conidium size, shape, surface texture: 2–2.8 µm, globose, delicately roughened

**Cultures examined**: CBS 127.56

**Diagnostic features**: “nodding” conidial heads, Niagara green colony colour

**Similar species**: none

**Ecology and habitats**: soil, dung, human

**Distribution**: Australia, Sri Lanka, Zambia, Russia (Varga et al. 2000b)

**Extralites**: viriditoxin, 13-O-methylviriditin, phomaligin A, variotin, viriditin, wasabidienone B0, B1, viriditin A (Omolo et al. 2000), 4-acetyl-6,8-dihydroxy-5-methyl-2-benzopyran-1-1 A (Aldridge et al. 1966)

**Pathogenicity**: pathogenic to humans (Katz et al. 2005, Yaguchi et al. 2007, Alcazar-Fuoli et al. 2007)

**Notes**: this is a highly variable species; further taxonomic studies needed to clarify the taxonomic position of the isolates assigned to it (Varga et al. 2000a, b); exhibits high MICs to some azoles (Alcazar-Fuoli et al. 2007)
**Neosartorya assulata** Hong, Frisvad & Samson [anamorph: *A. assulatus* Hong, Frisvad & Samson], Antonie van Leeuwenhoek (in press). Fig. 16.

**Type**: KACC 41691, from Tomato field soil, Buyeo, Korea

**Other no. of the type**: IBT 27911

**Morphological characteristics**
- Colony diam (7 d): CYA25: (19–)37–41 mm; MEA25: 47–58 mm; YES25: 28–31 mm; OA25: 36–40; CYA37: 32–68 mm
- Colony colour: white
- Conidiation: abundant
- Reverse colour (CYA): yellowish white to pale yellow
- Colony texture: radially sulcate
- Conidial head: short columnar
- Stipe: 3–7.5 µm wide
- Vesicle diam, shape: 10–18 µm, subclavate
- Conidium size, shape, surface texture: 2–3 µm, subglobose to ovoid, smooth
- Homothallic
- Cleistothecia: 120–250 µm, white to yellowish
- Ascospores: 5–6 µm, lenticular, with two well-separated equatorial crests and convex surface decorated with several large, round flaps

**Cultures examined**: KACC 41691 = IBT 27911, IBT 27910

**Diagnostic features**: well developed long and round flaps on convex surface of ascospore with two distinct equatorial crests; grow on MEA and CZA much slower than *N. pseudofischeri*

**Similar species**: *N. pseudofischeri*

**Distribution**: Korea

**Ecology and habitats**: soil

**Extrolites**: some indole alkaloids and some apolar metabolites

**Pathogenicity**: not reported

**Neosartorya aurata** (Warcup) Malloch & Cain [anamorph: *A. igneus* Kozakiewicz], Raper & Fennell 1965. Fig. 17.

**Type**: CBS 466.65, from jungle soil, Berakas, Muama, Brunei

**Other no. of the type**: ATCC 16896; IFO 8105; IMI 061451; IMI 061451ii; MUCL 13579; NRRL 2244; QM 1906; WB 2244; IBT 3027

**Morphological characteristics**
- Colony diam (7 d): CYA25: 64–80 mm; MEA25: 77–90 mm; YES25: 70–75 mm; OA25>: 55–59 mm; CYA37: 75–80 mm, CREA: poor growth, no acid production
- Colony colour (CZA): apricot to light cadmium yellow
- Conidiation: sparse
- Reverse colour (CZA): yellow ocher to ochraceus
- Colony texture: radially furrowed at center, slightly zonate
- Conidial head: loosely columnar
- Stipe: 50 × 2.5–4.5 µm
- Vesicle diam, shape: 6–9 µm, clavate to flask shaped
- Conidium size, shape, surface texture: 3–3.3 µm, globose to subglobose, delicately echinulate
- Homothallic
- Cleistothecia: 175–500 µm, pale lemon yellow, surrounded by loose wefts of dark golden yellow hyphae
- Ascospores: 6–7 × 4.4–5 µm, lenticular, with two prominent equatorial crests and with convex surfaces conspicuously echinulate

**Cultures examined**: CBS 105.55; WB 2391

**Diagnostic features**: yellow to golden pigmentation of hyphae surrounding the cleistothecia

**Similar species**: *N. udagawae, A. viridinutans*

**Distribution**: Suriname, Ghana, Liberia, Fiji

**Ecology and habitats**: soil, canned passionfruit

**Extrolites**: fumagillin, tryptoquivaline, tryptoquivalone, pseurotin A and viriditoxin (FRR 2269 also produces helvolic acid)

**Pathogenicity**: not reported
**Neosartorya australensis** Samson, Hong & Varga, sp. nov. (Fig. 19) – MycoBank MB492203.


Holotype of *Neosartorya australensis*, here designated as CBS 112.55T (dried culture), isolated from garden soil, Adelaide, Australia.

Homothallic, cleistothecia superficial, yellowish white to pale yellow, globose to subglobose, 150–380 µm in diam., surrounded by a loose covering of hyaline to yellowish white hyphae. Ascii 8-spored, globose to subglobose 12–14 µm, evanescent at maturity. Ascospores lens-shaped, 4.5–7.5 µm, with two equatorial crests, convex surfaces smooth to microtuberculate. Mycelium composed of hyaline, branched, septate, smooth-walled hyphae. Conidial heads short, columnar. Conidiophores arising from aerial hyphae often curling, uniseriate, stipes 12–30 µm; vesicles flask-shaped, 8–14 µm in diam.; phialides 7.5–9 × 2–3 µm, covering the upper half of vesicle. Conidia subglobose to ellipsoidal, smooth, 2.0–3.2 µm. Colonies on MEA growing rapidly, 40–45 mm in 7 d at 25 °C, white. Conidial heads produced few in number. Colonies on CYA, 30–35 mm in 7 d at 25 °C, creamy white, loosely overgrown by aerial hyphae in the centre. Conidial heads few in number. Reverse yellowish white to pale yellow.

**Etymology:** isolated from soil in Australia

**Extrolites:** wortmannin-like, aszonalenin-like

**Distinguishing features:** conidiophores often curled

**Other no. of the type:** IMI 061450; NRRL 2392; IBT 3021; WB 2392; Warcup SA14

**Diagnostic features:** smooth or microtuberculate 4.5–7.5 µm ascospores

**Similar species:** *N. glabra*

**Distribution:** Australia

**Ecology and habitats:** soil, strawberry

**Pathogenicity:** not reported

**Neosartorya coreana** Hong, Frisvad & Samson [anamorph: *A. coreanus* Hong, Frisvad & Samson], Int. J. Syst. Evol. Microbiol. 56: 477. 2006. Fig. 20.

**Type:** CBS 117059, from tomato field soil, Buyeo, Korea

**Other no. of the type:** KACC 41183 = NRRL 35590 = IBT 24945

**Morphological characteristics**

Colony diam (7 d): CYA25: 41–62 mm; MEA25: 57–66 mm; YES25: 50–74 mm; OA25: 54–58 mm; CYA37: 70–74 mm, CREA: poor growth, no acid production

Colony colour: white to yellowish white

Condiation: sparse

Reverse colour (CYA): pale to light orange

Colony texture: radially sulcate

Conidial head: columnar

Stipe: 3–4 µm wide

Vesicle diam, shape: 8–13(–15) µm, subclavate

Conidium size, shape, surface texture: 2.5–3.5 µm, subglobose to broadly elliptical, smooth

Homothallic

CLEISTOTHECIA: 200–300 µm, white to light yellow

Ascospores: 4–5 µm, with two well-separated but often bent equatorial crests up to 2 µm, convex surface reticulate

**Cultures examined:** CBS 117059

**Diagnostic features:** rugose to weak reticulate ascospores with two often bent crests, but without the equatorial rings of small projections

**Similar species:** *N. spinosa*, *N. laciniosa*

**Distribution:** South Korea, Australia

**Ecology and habitats:** soil

**Extrolites:** aszonalenins

**Pathogenicity:** not reported in humans (although isolated from the air sacs of an ostrich: Katz et al. 2005)

**Neosartorya denticulata** Samson, Hong & Frisvad [anamorph: *A. denticulatus* Samson, Hong & Frisvad], Antonie van Leeuwenhoek (in press). Fig. 21.

**Type:** CBS 652.73, from Soil under *Elaeis guineensis*, Suriname

**Other no. of the type:** KACC 41183

**Morphological characteristics**

Colony diam (7 d): CYA25: 22–24 mm; MEA25: 35–40 mm; CYA37: 35–38 mm; CREA: poor growth, no acid production

Colony colour: white

Condiation: only on the marginal area

Reverse colour (CYA): yellowish white to pale yellow

Colony texture: loosely overgrown by aerial hyphae in the centre, sulcate in marginal areas

Conidial head: short columnar

Stipe: 3.4–5.6 µm wide

Vesicle diam, shape: 7–12 µm, spatulate

Conidium size, shape, surface texture: 2.3–5 µm, subglobose to broadly elliptical, smooth

Homothallic

CLEISTOTHECIA: 140–230 µm, yellowish white to pale yellow

Ascospores: 4–5 µm, denticulate with a prominent equatorial furrow

**Cultures examined:** CBS 652.73

**Diagnostic features:** denticulate ascospore surface and lacking equatorial crests make this a distinctive species

**Similar species:** *N. fennelliae*, *N. ferenczii*
Polyphasic taxonomy of *Aspergillus* section *Fumigati* and *Neosartorya*

**Distribution**: Netherlands, Suriname  
**Ecology and habitats**: soil, sycamore  
**Extrrolites**: gliotoxin, viriditoxin  
**Pathogenicity**: not reported

**Neosartorya fennelliae** Kwon-Chung & Kim [anamorph: A. fennelliae Kwon-Chung & Kim], Mycologia 66: 628. 1974. Fig. 22.

**Type**: CBS 598.74 & CBS 599.74, from eye ball of Oryctolagus cuniculus, U.S.A.

**Other no. of the type**: ATCC 24325 & ATCC 24326, NRRL 5534 & NRRL 5535

**Morphological characteristics**


**Etyymology**: named after Prof. Lajos Ferenczy, eminent mycolologist.

**Extrrolites**: asperfuran, aszonalenin, fumigaclavine, viridicitumtoxin, gliotoxin-like, fumigatins and aszonalenin-like

**Type**: CBS 121594, from soil, Australia

**Other no. of the type**: IBT 27813, NRRL 4179; Warcup SA57

**Diagnostic features**: ascospore ornamentation similar to that of *N. fennelliae*, but with equatorial crests much narrower, and markedly different from those of *N. denticulata*

**Similar species**: *N. fennelliae, N. denticulata*

**Distribution**: Australia

**Ecology and habitats**: soil

**Extrrolites**: asperfuran, aszonalenin, fumigaclavine, viridicitumtoxin, gliotoxin-like, fumigatins, and aszonalenin-like

**Pathogenicity**: not reported


**Type**: CBS 544.65, from canned apples, Wehmer

**Other no. of the type**: ATCC 1020; DSM 3700; IMI 211391; NRRL 181; QM 1983; Thom 4651.2, WB 181; IBT 3018

**Morphological characteristics**


**Extrrolites**: gliotoxin, viriditoxin

**Type**: not reported


**Fig. 22.**

**Fig. 23.**

**Fig. 24.**
Colony texture: velutinous
Conidial head: columnar
Stipe: 300–500 × 4–7 µm
Vesicle diam, shape: 12–18 µm, flask shaped
Conidium size, shape, surface texture: 2–2.5 µm, globose to subglobose, microtuberculate
Homothallic
Cleistothecia: up to 400 µm, light cream, borne singly or in small clusters within a loose hyphal envelope
Ascospores: 7–8 × 3–4 µm, convex surfaces bearing anastomosing ridges (reticulate)

Cultures examined: CBS 544.65; WB 4075; CBS 317.89; CBS 584.90; CBS 118441; NRRL 181; NRRL 4075; NRRL 4161; NRRL 4585

Diagnostic features: reticulate ascospore ornamentation

Similar species: N. tatenoi

Distribution: worldwide
Pathogenicity: pathogenic to animals and humans (Coriglione et al. 1990; Lonial et al. 1997; Mellado et al. 2006; Chim et al. 1998; Gori et al. 1998)

Neosartorya galapagensis Frisvad, Hong & Samson [anamorph: A. galapagensis Frisvad, Hong & Samson], Antonie van Leeuwenhoek (in press).  Fig. 25.

Type: CBS 117522, from soil, Galapagos Islands, Ecuador
Other no. of the type: KACC 41935 = IBT 16756

Morphological characteristics
Colony diam (7 d): CYA25: 25–40 mm; MEA25: 26–35 mm; YES25: 39–44 mm; OA25: 34–41 mm; CYA37: 44–65 mm; CREA poor growth and no acid production
Colony colour: white
Conidiation: sparse
Reverse colour (CYA): golden yellow
Colony texture: strongly funiculose
Conidial head: columnar
Stipe: 2–4 µm wide
Vesicle diam, shape: 4–11 µm, (sub)clavate
Conidium size, shape, surface texture: 2.3–3 µm, globose to subglobose, smooth
Homothallic
Cleistothecia: 90–220 µm, yellowish white, surrounded by a loose covering of aerial hyphae
Ascospores: 5 µm, with two distinct equatorial crests 1–2 µm wide, convex surface of ascospores microtuberculate

Cultures examined: CBS 117522 = IBT 16756; CBS 117521 = IBT 16763

Diagnostic features: has smaller and whiter cleistothecia and relatively straight equatorial crests and smoother walled convex surfaces compared to N. laciniosa, N. coreana and N. spinosa; N. glabra grows somewhat slower than the other species and grows well at comparatively low temperatures; can be distinguished from N. papuensis and N. australensis using sequence data or extrolite profiles

Similar species: N. papuensis, N. australensis

Distribution: U.S.A., Morocco, Denmark, Australia, Netherlands, South Korea
Ecology and habitats: soil, foods, indoor
Extrtolites: asperpentyn, avenaciolide, wortmannin-like compound
Pathogenicity: not reported

**Type**: NHL 3008, from pasteurised aloe juice, Tokyo, Japan

**Other no. of the type**: CBS 294.93; NRRL 20819

**Morphological characteristics**
- Colony diam (7 d): CZA25: 14–15 mm; CYA25: 12–14 mm; MEA25: 26–39 mm; YES25: 42–45 mm; OA25: 42–45 mm; CYA37: 27–30 mm; CREA: rather poor growth and no acid production
- Colony colour: greyish green
- Conidiation: moderate
- Reverse colour (CZA): light brown
- Colony texture: velutinous
- Conidial head: short columnar
- Stipe: 120–380 × 5–7 µm
- Vesicle diam, shape: 15–24 µm, flask-shaped
- Conidium size, shape, surface texture: 2–2.5 µm, globose to subglobose, smooth or delicately roughened
- Homothallic
- Cleistothecia: 130–220 µm, light cream coloured
- Ascospores: 4.5–5 µm, lenticular, with two closely appressed equatorial crests, convex surfaces finely reticulate

**Cultures examined**: CBS 294.93; IFM 50770 = IBT 27913

**Diagnostic features**: restricted growth on CZA, small cleistothecia, finely reticulate ascospores

**Similar species**: N. fischeri, N. tatenoi

**Distribution**: Japan, Brazil, South Korea

**Ecology and habitats**: soil, fruit juice, indoor air, human

**Extrrolites**: aszonalenins, tryptoquivaline, tryptoquivalone

**Pathogenicity**: pathogenic to humans (Guarro et al. 2002; Mellado et al. 2006; Alcazar-Fuoli et al. 2007)

**Note**: no growth above 48 °C; some isolates carry dsRNA mycoviruses which are efficiently transmitted both through ascospores and conidia to the progeny (Varga et al. 1998)

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**Neosartorya laciniosa** Hong, Frisvad & Samson [anamorph: A. laciniosus Hong, Frisvad & Samson], Int. J. Syst. Evol. Microbiol. 56: 477. 2006. Fig. 28.

**Type**: CBS 117721, from tomato field soil, Buyeo, Korea

**Other no. of the type**: CBS 117721 = KACC 41657

**Morphological characteristics**
- Colony diam (7 d): CYA25: 38–58 mm; MEA25: 53–67 mm; YES25: 60–78 mm; OA25: 52–59 mm; CYA37: 41–80 mm; CREA: poor growth and no acid production
- Colony colour: white to pale yellow
- Conidiation: sparse
- Reverse colour (CYA): greyish yellow to olivaceous buff
- Colony texture: floccose
- Conidial head: loosely columnar
- Stipe: 20–160 × 2.5–4 µm
- Vesicle diam, shape: 4–8 µm, flask-shaped to irregular
- Conidium size, shape, surface texture: 2.5–4 µm, globose to subglobose, smooth
- Homothallic
- Cleistothecia: 100–300 µm, cream coloured
- Ascospores: 4–5 µm, with a shallow furrow but without distinct equatorial crests, ornamented on surfaces by several linear ridges presenting ribbed or somewhat reticulate pattern

**Cultures examined**: CBS 117721; IBT 6660; KACC 41648; CBS 117719 = KACC 41652; KACC 41644

**Diagnostic features**: cleistothecia surrounded by a loose covering of hyaline to yellowish white, 2–4 µm wide hyphae; microtuberculate ascospores with two bent crests and two distinct equatorial rings of small projections

**Similar species**: N. spinosa, N. coreana

**Distribution**: South Korea, U.S.A., Pakistan, Netherlands, Suriname, Dominican Republic, Kenya

**Ecology and habitats**: soil

**Extrrolites**: aszonalenins, tryptoquivaline, tryptoquivalone

**Pathogenicity**: not reported

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**Neosartorya multiplicata** Yaguchi, Someya & Udagawa [anamorph: A. multiplicatus Yaguchi, Someya & Udagawa], Mycoscience 35: 309. 1994. Fig. 29.

**Type**: PF 1154, from soil, Taiwan

**Other no. of the type**: CBS 646.95, IBT 17517

**Morphological characteristics**
- Colony diam (7 d): CYA25: 24–36 mm; MEA25: 35–50 mm; YES25: 38–42 mm; OA28–43 mm; CYA37: 41–80 mm; CREA: poor growth and no acid production
- Colony colour: white
- Conidiation: sparse
- Reverse colour (CYA): greyish orange to yellowish orange
- Colony texture: floccose
- Conidial head: loosely columnar
- Stipe: 20–160 × 2.5–4 µm
- Vesicle diam, shape: 4–8 µm, flask-shaped to irregular
- Conidium size, shape, surface texture: 2.5–4 µm, globose to subglobose, smooth
- Homothallic
- Cleistothecia: 100–300 µm, cream coloured
- Ascospores: 4–5 µm, with a shallow furrow but without distinct equatorial crests, ornamented on surfaces by several linear ridges presenting ribbed or somewhat reticulate pattern

**Cultures examined**: CBS 646.95

**Diagnostic features**: can be distinguished from other species of Neosartorya by its almost globose ascospores, which have ribbed ornamentation with several linear ridges, and by the reduced production of its conidial heads on common media

**Similar species**: none

**Distribution**: Taiwan

**Ecology and habitats**: soil

**Extrrolites**: helvolic acid

**Pathogenicity**: not reported
**Neosartorya papuensis** Samson, Hong & Varga, sp. nov. (Fig. 30) – MycoBank MB505571.


Holotype of *Neosartorya papuensis*, here designated as CBS 841.96T (dried culture), isolated from *Podocarpus* (Podocarpaceae), bark, Myola, Owen Stanley Range, Northern Province, Papua New Guinea.


**Etymology:** isolated in Papua New Guinea

**Extrolites:** wortmannin-like

**Distinguishing features:** smooth microtuberculate 5.5–7.5 µm, ascospores

**Other no. of the type:** IBT 27801

**Cultures examined:** CBS 841.96

**Similar species:** *N. galapagensis*, *N. glabra*, *N. australensis*

**Distribution:** Papua New Guinea

**Pathogenicity:** not reported

**Neosartorya pseudofischeri** Peterson [anamorph: *A. thermomutatus* (Paden) Peterson], Mycol. Res. 86: 547. 1992. Fig. 31.

**Type:** NRRL 20748, from human vertebrae, Atlanta, Georgia, U.S.A.

**Other no. of the type:** CBS 208.92

**Holotype:** 404.67, moldy cardboard, Victoria, British Columbia, Canada

**Morphological characteristics**
- Colony diam (7 d): CYA25: 60–70 mm; MEA25: 90 mm in 7 d
- Colony colour: white to pale creamish
- Conidiation: sparse
- Reverse colour (CZA): clear or faintly yellowish
- Colony texture: velutinous
- Conidial head: loosely columnar
- Stipe: 200–300 × 4–7 µm
- Vesicle diam, shape: 10–17 µm, subglobose
- Conidium size, shape, surface texture: 3–4 µm, globose to subglobose, smooth

Homothallic

Cleistothecia: 150–300 µm, white

Ascospores: 4.5–6 µm, subglobose, with two equatorial crests of 1 µm wide, convex surfaces with raised flaps resembling triangular projections

**Cultures examined:** CBS 208.92, CBS 404.67

**Diagnostic features:** distinctly ornamented ascospores

**Similar species:** -

**Distribution:** U.S.A., Canada, Netherlands, South Korea, Spain, Denmark, Estonia

**Ecology and habitats:** soil, indoor, human

**Extrolites:** asperfurane, cytochalasin-like compound, ficsalin-like compound, pyripyropens, gliotoxin

**Pathogenicity:** pathogenic to humans (Padhye et al. 1994; Matsumoto et al. 2002; Jarv et al. 2004; Baljee et al. 2005a; Alcazar-Fuoli et al. 2007; Lau et al. 2007) and animals (Barrs et al. 2007)


= *Neosartorya primulina* Udagawa, Toyaz. & Tsub. [anamorph: *A. primulinus* Udagawa, Toyaz. & Tsub.]

**Type:** CBS 135.52, from cardboard, York, U.K.

**Other no. of the type:** ATCC 16897; IMI 048583; IMI 048583ii; NRRL 2154; QM 6874; WB 2154

**Morphological characteristics**
- Colony diam (7 d): CYA25: 26–42 mm; MEA25: 52–59 mm; YES25: 36–59 mm; OA25: 47–55 mm; CYA37: 50–58 mm; CREA: poor growth and no acid production
- Colony colour (CZA): white to light tan
- Conidiation: sparse
- Reverse colour (CZA): colourless to flesh coloured
- Colony texture: floccose
- Conidial head: loosely columnar
- Stipe: 400–500 × 2–7 µm
- Vesicle diam, shape: 10–20 µm, flask shaped
- Conidium size, shape, surface texture: 2–3 µm, elliptical to globose, microtuberculate

Homothallic

Cleistothecia: up to 300 µm, buff to light tan
Ascospores: 4–5 µm, with two prominent equatorial crests, each duplicated by a somewhat less prominent band, reticulate

**Cultures examined**: CBS 135.52; WB 2221; WB 4175; CBS 100942

**Diagnostic features**: presence of 4 equatorial crests on ascospores, reticulate ascospore ornamentation

**Similar species**: -

**Distribution**: Suriname, South Korea, U.K., Netherlands, Australia

**Ecology and habitats**: Soil, pectin, cardboard, fruit juice, mango pulp

**Extrolites**: quinolactacin, aszonalenins

**Pathogenicity**: not reported

**Note**: some isolates carry dsRNA mycoviruses (Varga et al. 1998)

### Neosartorya spathulata

**Type**: CBS 408.89 & CBS 409.89, from cultivated soil under *Alocasia macrorrhiza*, Taiwan  
**Other no. of the type**: IMI 308593 & IMI 308593; NHL 2948, NHL 2949; NRRL 20549 & NRRL 20550

**Morphological characteristics**

- Colony diam (7 d): CZA25: 33–38 mm; MEA25: 80 mm; OA25: 40–46 mm
- Colony colour: greyish green
- Conidiation: abundant
- Reverse colour (CZA): uncoloured
- Colony texture: velutinous
- Conidial head: loosely columnar
- Stipe: 500–1500 × 11–18(–25) µm
- Vesicle diam, shape: 25–52 µm and 8–15 µm, flask-shaped
- Conidium size, shape, surface texture: 3–5.5 × 2–4.5 µm, ellipsoidal, smooth
- Heterothallic

**Cleistothecia**: 100–260 µm, pale yellow to light yellow

**Ascospores**: 3.5–4 µm, lenticular, with two equatorial crests, convex surfaces nearly smooth

**Cultures examined**: CBS 408.89 & CBS 409.89

**Diagnostic features**: yellowish cleistothecia, ascospores with large equatorial crests and smooth surface, two types of conidial heads (diminutive??)

**Similar species**: -

**Distribution**: Taiwan

**Ecology and habitats**: soil

**Extrolites**: aszonalenins, 2-pyrovoylaminobenzamide, pseurotin

**Pathogenicity**: pathogenic to humans (Summerbell et al. 1992; Mellado et al. 2006; Gerber et al. 1973)

### Neosartorya spinosa

**Type**: CBS 483.65, from soil, Nicaragua

**Other no. of the type**: ATCC 16898; IFO 8782; IMI 211390; NRRL 5034; WB 5034; IBT 3022

**Morphological characteristics**

- Colony diam (7 d): CYA25: 41–70 mm; MEA25: 55–75 mm; YES25: 55–80 mm; OA25: 56–64 mm; CYA37: 67–85 mm; CREA: poor growth and no acid production
- Colony colour (CZA): white to pale yellow to buff
- Conidiation: sparse
- Reverse colour (CZA): colourless to light pink
- Colony texture: velutinous
- Conidial head: columnar
- Stipe: 300–500 × 4–7 µm
- Vesicle diam, shape: 12–18 µm, flask shaped
- Conidium size, shape, surface texture: 2–2.5 µm, globose to subglobose, microtuberculate
- Homothallic

**Cleistothecia**: 200–300 µm, cartridge buff

**Ascospores**: 4.5 µm, with two widely separated equatorial crests, with convex surfaces bearing spinellike projections

**Cultures examined**: CBS 483.65

**Diagnostic features**: have echinulate ascospores with spines ranging from <0.5 µm up to 7 µm long, or with verruculose and small triangular, sometimes circularly arranged, projections

**Similar species**: *N. coreana*, *N. laciniosa*

**Distribution**: Nicaragua, Kenya, Denmark, Dominican Rebublic, U.S.A., Belgium, Sudaen, Japan, India, Pakistan, South Korea

**Ecology and habitats**: Soil, fruit juice, human

**Extrolites**: aszonalenins, 2-pyrovoylaminobenzamide, pseurotin

**Pathogenicity**: pathogenic to humans (Summerbell et al. 1992; Mellado et al. 2006; Gerber et al. 1973)

### Neosartorya stramenia

**Type**: CBS 498.65, soil from maple-ash-elm forest, Wisconsin, U.S.A.

**Other no. of the type**: ATCC 16895; IFO 9611; IMI 172293; WB 4652

**Morphological characteristics**

- Colony diam (7 d): CYA25: 10–40; MEA25: 40–59 mm; YES25: 58–62 mm; OA: 56–60 mm; CYA37: 45–49 mm; CREA: poor growth and no acid production

Type: CBM FA 0022, from soil, Brazil

Other no. of the type: CBS 407.93; IBT 21589

Morphological characteristics
Colony colour: pale yellow to yellowish white
Conidiation: abundant
Reverse colour (CZA): light orange to greyish orange
Colony texture: velutinous
Conidial head: columnar
Stipe: up to 530 × 4–6 µm
Vesicle diam, shape: 12–15 µm, hemispherical to flask shaped
Conidium size, shape, surface texture: 2.6–3.2 × 2.4–2.6 µm, subglobose to broadly ellipsoidal, smooth
Heterothallic
Cleistothecia: 310–620 × 280–530 µm, yellowish white to light yellow, surrounded by a loose covering of hyaline to pale yellowish brown hyphae
Ascospores: 5–5.5 × 4–5 µm, broadly lenticular, with two equatorial or often irregular crests, convex surfaces tuberculate

Cultures examined: CBS 407.93; NRRL 4584

Diagnostic features: heterothallic species, with characteristic tuberculate ascospore ornamentation

Similar species: N. aureola, A. viridinutans

Distribution: Brazil, U.S.A., Spain, Japan

Ecology and habitats: Soil, human

Extralites: fumigatin, fumagillin, tryptoquivaline, tryptoquivalone

Pathogenicity: pathogenic to humans (Balajee et al. 2006; Moragues et al. 2006)
Neosartorya warcupii

Peterson, Varga & Samson, sp. nov. (Fig. 38) – MycoBank MB505572.

Homothallia; cleistothecia superficialia, alba vel dilute lutea, globosa vel subglobosa, 200–350 µm diam, in hyphis hyalinis vel luteobrunnea obtectis. Asci octospori, globosi vel subglobosi, 4.5–7 µm diam, evanescentes. Ascosporae 5.5–7 µm diam, crista angustis, aequatorinis, pagina convexa sublaevigata. Mycelium ex hyphis hyalinis, rarissimi, septatis, laeviparietinis constans. Capitula conidialis curta, columnaria. Conidiophora ex hyphis aeris exorientia, uniseriata. Mycelium ex hyphis hyalinis, ramosis, septatis, subclavato-subglobosis 180–350 µm diam, subclavato to subglobose 12–18 µm in diam.; phialides 7.5–9 x 2–3 µm, dinitrium superficie vesicle obtengentes. Conidiospora subglobosa vel ellipsodea, laevia, 1.8–1.5 µm diam. Colonie in agaro MEA in 7–10 d; reliabilius and habitatibus. Distribution

Holotype of Neosartorya warcupii, here designated as NRRL 35723T (dried culture), isolated from soil, Finder’s Range, Australia.

Homothallia, cleistothecia superficialia, yellowish white to pale yellow, globose to subglobose, 180–350 µm in diam., surrounded by a loose covering of hyaline to yellowish white hyphae. Asci 8-spored, globose to subglobose 10–16 µm, evanescent at maturity. Ascospores lens shaped 4.5–7 µm, with two prominent equatorial crests, convex surface smooth to microtuberculate. Mycelium composed of hyaline, branched, septate, smooth-walled hyphae. Conidial heads short, columnar. Conidiophores arising from aerial hyphae, uniseriate, stipes 100–150 x 4–6 µm; vesicles subclavato to subglobose, 12–18 µm in diam; phialides 7.5–9 x 2–3 µm, covering the upper half of vesicle. Conidia globose to subglobose, smooth, 1.8–2.5 µm. Colonies on MEA growing rapidly, 35–40 mm in 7 d at 25 °C. Colonies on CYA, 18–22 mm in 7 d at 25 °C, creamish white, sectors frequently produced. Conidial heads few in number. Reverse bluish in colour.

Etymology: named after Prof. J. H. Warcup, eminent mycologist, who isolated this culture.

Extracellular: wortmannin-like, aszonalenin-like, chomanol-like, tryptophane-like and tryptophane-like

Distinguishing features: secretes a blue pigment to the medium in 7–10 d; relatively slow growth on CYA at 25 °C

Distribution: Australia

Ecology and habitats: soil

Pathogenicity: not reported

REFERENCES


PolypHycal Section Fumigati and Neosartorya


INDEX

aflatoxin biosynthesis 15, 34, 36–7, 65–6, 128
aflatoxins 8, 9, 11, 14–5, 17, 31–6, 53, 63–5
aflavine 140
aflavinina 140
alkaloids 153
antafumicin 94
antafumicins 8, 9, 11, 14–5, 17, 31–6, 53, 63–5
aspergillus aculeatinus 53, 59, 62, 130–1, 133
aspergillus aculeatus 31, 54–5, 58–9, 131, 133, 140–1, 143
aspergillus anomalus 41
aspergillus apicalis 89
aspergillus arvii 154, 160
aspergillus atroviolaceus 54
aspergillus brasiliensis 5, 6, 33–4, 53–5, 58, 130–1, 133, 139, 141, 143–4
aspergillus candidus 79, 87–8
aspergillus clavatonanicus 89, 92–3, 95–6
aspergillus clavatus 7, 21, 89, 91–2, 89, 95–6, 98, 104–6, 149
aspergillus clavatum 92–3, 95–6, 100
aspergillus clonatius 115
aspergillus coeruleus 160
aspergillus conjunctus 43–4, 115
aspergillus coreana 148, 176
aspergillus costaricaensis 5, 130–1, 133, 136, 140–1, 143–4
aspergillus deflectus 43–4, 115
aspergillus duricaulis 40–1, 148, 154, 156, 158, 201–2
aspergillus ellipticus 5, 6, 40–1, 54, 56, 58, 79, 133, 140–1, 143–4, 160
aspergillus flavus 16–7, 62, 64–5, 68, 75, 83
aspergillus fomitatus 40, 148, 154, 156, 159–60, 164
aspergillus fumigatus 4, 7, , 8, 11–3, 15–7, 19, 21–2, 24, 28–9, 34, 36, 42–4, 45–7, 50, 92, 126, 160–1, 164, 201–2
aspergillus fumisynnematus 40, 148, 154, 160, 162, 164
aspergillus giganteus 89, 92–3, 95–6, 100
aspergillus griseibrunneus 160
aspergillus hennebergii 54
aspergillus heteromorphus 58, 131, 133, 139–41, 143–4
aspergillus homomorphus 130–1, 133, 136, 144
aspergillus ibericus 32, 53, 55, 58–9, 62, 131, 133, 141, 143–4
aspergillus implicatus 76
aspergillus ingratus 89
aspergillus insuetus 107, 114–6, 120
aspergillus japonicus 33, 55, 58, 130–1, 133, 140–1, 143–4
aspergillus keveii 76
aspergillus lentulus 39, 40, 148, 154, 156, 160, 163–4
aspergillus longivesica 89, 92–3, 95–6, 100
aspergillus lunzinense 160
aspergillus neoellipticus 154, 160
aspergillus nidulans 7, 15, 19, 20–2, 24, 28–9, 35–6, 42–3, 45, 140
aspergillus niger 9, 12, 16, 19, 31, 34, 55, 58, 64–6, 144–5
aspergillus novofumigatus 40, 148, 154, 156, 160, 164–5
aspergillus ochraceus 34, 53, 59, 170
aspergillus oryzae 7, 8, 11–7, 21–2, 24, 29, 36, 53, 62–5, 78, 92
aspergillus pallidus 54, 89, 92–3, 95
aspergillus panamensis 43–4, 115
aspergillus parasiticus 21, 34, 53, 63–4
aspergillus phialiseptus 41, 154, 160
aspergillus piperis 5, 34, 53–4, 59, 130–1, 133, 136, 138, 141, 143–4
aspergillus pseudodeflectus 109, 114
aspergillus restrictus 92
aspergillus rhizopodus 89, 92–3, 95–6, 100
aspergillus sclerotiicarbonarius 32, 53, 59, 62, 130, 132, 133, 136, 144
aspergillus sclerotioniger 32, 34, 53–4, 59, 132, 133, 136, 139, 141, 143–4
aspergillus fumigatus var septatus 160
aspergillus taichungensis 75–80
aspergillus terreus 4, 7, 22, 24, 34, 39, 40, 42, 45–6
aspergillus thermomutatus 190
aspergillus tricci 7–5, 78–80, 83, 86
aspergillus tubingensis 8, 65, 132, 136, 138, 144–5
aspergillus turcosus 141, 164, 166
aspergillus unilateralis 148, 164
aspergillus ustus 39, 43, 45–6, 107, 114, 124, 126, 128
aspergillus uvarum 53, 55, 58–9, 64, 130, 132, 133, 136, 138, 141, 143
aspergillus vadensis 5, 33, 57, 130, 133–4, 140–1, 143–4
aspergillus viridinutans 40–1, 92, 148, 156, 160, 164, 168–70, 196
aspergillus warcupiensis 201
aspergillus westerdijkiae 34, 59
aspergillus asperpentyn 153
aspergillus aszonalenin 153, 178
aspergillus aszonalenins 153–4, 156, 174, 178, 182, 186, 193, 196, 201
asper gillus aurthine 153
aspergillus austalides 114–5, 124, 126
aspergillus austamides 107, 114, 128
aspergillus austidiol 140
aspergillus austins 114–6
aspergillus austocystins 107, 114–5, 124, 126, 128
aspergillus avenaciolide 153
aspergillus boscalid medium 129
aspergillus brevianamide 114
aspergillus brevipes 40, 148, 154, 156–7
aspergillus bronchialis 160
aspergillus candidusin 80
aspergillus candidusins 75, 78–9, 83, 86–7, 140, 144
aspergillus celluloses 160
aspergillus chlorflavonin 75, 79, 80, 83, 86–7
aspergillus chromatans 153–4, 156, 201
aspergillus clavacin 92
aspergillus clavatin 92
aspergillus crea 91, 96, 100, 107, 114, 120, 129, 132–3, 144, 156, 160, 164, 170, 174, 178, 186
aspergillus cycloclavine 140
aspergillus cycloechinuline 153, 164
aspergillus cyclopaldic 153
aspergillus cytochalasin 89, 91, 94, 96, 100, 104–5
aspergillus dechlorochlorflavonin 83
aspergillus dehydroaflavinide 140
aspergillus dehydroaustin 114
aspergillus dehydrocarotic acid 89, 94, 100
aspergillus dehydropergillin 114
aspergillus deoxyisoterpinren 83
deoxyprenylterphenyllin 83
deoxyterpenin 83
desmethylkotanin 89
diaacetoxyscirpenol 114
Dichotomomyces 89, 91, 93, 95
albus 100
cejpii 93–5, 100, 103, 105
dihydrogeodin 140
drims 114–6, 120
dual nomenclature 67–72
Ehrlich reagent 132–3
emeheterone 114–5, 124, 126
Emericella heterothallica 107, 114–5, 124, 126–7
quadrilineata 43
rugulosa 43
emesterones 114–5, 124, 126
emethallicins 114–5, 124, 126
erdin 140
festuclavine 140
fiscalins 153, 164
fischerin 153
fulviruber 160
fumagillin 153, 160
fumigaclavines 153, 160, 178
fumigatins 153–4, 178
fumimycin 153
fumiquinazolines 153, 160
fumitoxins 153, 160
funalenone 140
giothxin 89, 93, 95, 104–5, 178, 190
glyanthrypine 94, 96, 100
gregatins 153, 182
helvola 154
helvolic 153, 160
helvolus 160
Hemicarpenteles paradoxus 89, 91–2, 95
hydroxyaveranthin 114–5, 124
hydroxyterphenyllin 80
indole 153
isoperoxidon 104
kotanins 89, 91–2, 94, 96, 100, 104, 107, 164
lactones 140
malformins 140
meleagrin 153
methylcyclopolic 153
methyl–sulochrin 153
methylviriditin 153
minimus 160
mycophenolic acid 168
Neosartorya 8, 40, 45, 69, 89, 91, 94–5, 147, 149, 153–4, 157, 161, 165, 167, 185–7, 202–3
assulata 41, 148, 155, 170–1
aurata 41, 95, 148, 155, 172
areoa 41, 148, 155, 173, 196
australensis 148, 155, 174–5
deliciata 152–3, 155, 196
denticula 41, 148, 155, 155, 177–8
echinulata 43
fennelliae 40, 41, 95, 149, 152, 154–5, 178–9
ferenczi 147–8, 154–5, 178
fischeri 7, 11, 14, 16, 40, 45, 91–2, 148, 178, 181, 186, 196, 201
galapagensis 148, 155, 182–3
gabra 41, 92, 148, 153–4, 182, 184
hiratsukae 41, 148, 155, 186
lacinos 41, 148, 187
multiplicata 148, 155, 186
nishimurae 41, 148, 155
otanii 149, 152, 155
papuensis 148, 190, 196,
primulina 155, 190
pseudo fischeri 45, 95, 148, 190, 201–2
quadricincta 41, 148, 152–3, 155, 190, 192
spathulata 41, 148, 155, 193
spinoso 40, 92, 147–9, 182, 186, 193, 195
stramenia 41, 95, 148, 155, 193
takakii 155
tateno 41, 147, 152–3, 182, 186, 196, 198
tsuruae 155
udagawae 39–41, 148, 155, 170, 196, 199
warcupii 155, 201
Neocarpenteles 89, 91, 94–5, 106
acanthosporus 89, 92–3, 104
neosartorin 153, 156, 160, 164, 182
neoxaline 140
nidulol 120, 124
ochratoxins 34, 53, 55, 59, 62, 140, 143
ophiobolins 115–6, 120
orlandin 89
paitantin 153, 164
patulin 89, 91–2, 94, 96, 100, 104–5
pergillin 116
petromyces 68
phenylahistin 114
phomaligin 153
phthalides 153
prenylterphenyllin 83
pseurotin 153, 170, 193
pseurotins 94, 153, 160
pyrolypropen 94, 100, 153, 160
pyrolyaminobenzamide 193
quinolactacin 193
ribotoxins 89, 92, 94, 96, 104–5, 147, 202
roquefortine 153
rubratoxins 94
sarcins 89
Section Candidi 75, 77–9
Section Clavati 101
Section Flavi 4, 8, 17, 34, 44, 53, 64
Section Fumigati 40–2, 49, 147, 149–51, 153–4, 201–3
Section Nidulantes, 44, 114
Section Nigri 5, 9, 31–2, 34, 37, 53, 55, 57–9, 64–5, 129, 131–2, 134, 136, 139, 141–5
Section Usti 107, 110–5
stigmatocystin 8, 34–7, 114–6, 124, 126
Subgenus fumigati 45, 91, 93, 95
sulochrin 160
Talaromyces cejpii 100
terphenyllin 80, 140
terpenins 83
terrein 153, 164
territrem 153, 164
trypacidin 153, 160
tryptoquivalins 89, 92, 94, 96, 100, 104, 153–4, 156, 170, 182, 186, 201
tryptoquivalone 89, 92, 94, 95–6, 100, 104, 153–4, 156, 170, 186, 201

ustic acids 114, 116, 124, 126

variotin 153
versicolorins 35, 114–5, 124
viridicatumtoxin 153, 178
viriditin 153, 168
viirditoxin 153, 168, 170

wortmannin 153–4, 174, 190, 201

xanthoascin 75, 79, 83, 87, 140
SIM59 References for Citations


