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Aspergillus systematics in the genomic era

Robert A. Samson and János Varga

CBS Fungal Biodiversity Centre,
Utrecht, The Netherlands

An institute of the Royal Netherlands Academy of Arts and Sciences

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Cover: Top from left to right. Conidiophores *Aspergillus clavatus, A. fumigatus, A. longivesica*.
Bottom from left to right. Scanning electron micrograhs of *Neosartorya denticulata*, Petri dish with DG 18 agar and coffee beans from Thailand showing *Aspergillus* colonies, conidophores of *A. terreus.*

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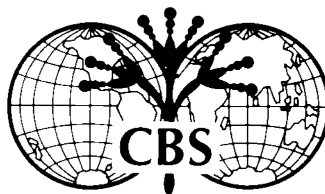

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PREFACE

Since Pier Antonio Micheli described *Aspergillus* is 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 micro organisms 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, Novozymes, DSM, Merck Sharp & Dome, the Fungal Research Trust, ISHAM and Myconosticta for their financial support.

Robert A. Samson and János Varga December 1, 2007

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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 extrolites, often show differences that reflect phylogenetic species boundaries and greater emphasis should be placed on extrolite 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 1 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: multolocus 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" they established, which largely reflected groups defined in previous treatises (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.

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Table 1. Characters used in delineating new species of *Aspergillus* since 2000.

Section	Species	Morphology	Physiology	Molecular	Reference
Circumdati	*A. persii*	i	x		Zotti & Corti (2002)
	A. cretensis	i	x	*BenA*	Frisvad *et al.* (2004)
	A. flocculosus	i		*BenA*	Frisvad *et al.* (2004)
	A. neobridgeri	i	g	*BenA*	Frisvad *et al.* (2004)
	A. pseudoelegans	i	x	*BenA*	Frisvad *et al.* (2004)
	A. roseoglobosus	i	x	*BenA*	Frisvad *et al.* (2004)
	A. steynii	i	g x	*BenA*	Frisvad *et al.* (2004)
	A. westerdijkiae	i	x	*BenA*	Frisvad *et al.* (2004)
Nigri	*A. costaricaensis*	i	g x	*BenA*	Samson *et al.* (2004)
	A. homomorphus	i	g x	*BenA*	Samson *et al.* (2004)
	A. lactocoffeatus	i	g x	*BenA*	Samson *et al.* (2004)
	A. piperis	i	g x	*BenA*	Samson *et al.* (2004)
	A. sclerotioniger	i	g x	*BenA*	Samson *et al.* (2004)
	A. vadensis	i	g x	multi	Vries *et al.* (2005)
	A. ibericus	i	g x	multi	Serra *et al.* (2006)
	A. brasiliensis	i	g x	multi	Varga *et al.* (2007)
	A. uvarum	i	g x	multi	Perrone *et al.* (2007)
	A. aculeatinus	i	g x	multi	Noonim *et al.* (2007)
	A. sclerotiicarbonarius	i	g x	multi	Noonim *et al.* (2007)
Flavi	*A. bombysis*	i	g x	multi	Peterson *et al.* (2001)
	A. pseudotamarii	i	g x	multi	Ito *et al.* (2001)
	A. arachidicola	i	g x	multi	Pildain *et al.* (2007)
	A. minisclerotigenes	i	g x	multi	Pildain *et al.* (2007)
	A. parvisclerotigenus	i	gx	*BenA*	Frisvad *et al.* (2005)
Ochraceorosei	*A. rambellii*	i	g x	ITS, RAPD	Frisvad *et al.* (2005)
Fumigati	*A. lentulus*	i		multi	Balajee *et al.* (2005)
	A. fumigatiaffinis	i	x	multi	Hong,*et al.* (2005)
	A. novofumigatus	i	x	multi	Hong *et al.* (2005)
	A. turcosus	i	g x	multi	Hong *et al.* (2007)
Neosartorya	*N. nishimurae*	a i	g		Takada *et al.* (2001)
	N. otanii	a I	g		Takada *et al.* (2001)
	N. takakii	a			Horie *et al.* (2001)
	N. indohii	a			Horie *et al.* (2003)
	N. tsurutae	A			Horie *et al.* (2003)
	N. coreana	a		*BenA*/calm	Hong *et al.* (2006)
	N. laciniosa	a		*BenA*/calm	Hong *et al.* (2006)
	N. assulata	a i	g x	multi	Hong *et al.* (2007)
	N. denticulata	a i	g x	multi	Hong *et al.* (2007)
	N. galapagensis	a i	g x	multi	Hong *et al.* (2007)
	N. australensis	a i	g x	multi	Samson *et al.* (2007)
	N. ferenczii	a i	g x	multi	Samson *et al.* (2007)
	N. papuensis	a i	g x	multi	Samson *et al.* (2007)
	N. warcupii	a i	g x	multi	Samson *et al.* (2007)
Emericella	*Em. qinqixianii*	a i			Horie *et al.* (2000)
	Em. venezuelensis	a	x	*BenA*	Frisvad & Samson (2004)
Eurotium	*Eu. taklimakanense*	a i	g		Abliz & Horie (2001)

Abbreviations: a = ascospore characters; I = 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 *Emericella 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 organismal 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 (Johannesson *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 extrolite 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 extrolites, often show differences that reflect phylogenetic species boundaries (Geiser *et al.* 2000). Considering this, greater emphasis should be placed on extrolite 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 the complete genome sequence of *A. fumigatus* and the subsequent discovery of both MAT idiomorphs in populations of *A. fumigatus* (Paoletti *et al.* 2005; Dyer & Paoletti 2005; Nierman *et al.* 2006; Rydholm *et al.* 2007) strongly indicate that this species is heterothallic, consistent with population genetic patterns suggesting some level of recombination (Pringle *et al.* 2005). These indications that putatively asexual *Aspergillus* species are actually recombining allow 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 *Sclerocleista*, *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. clavatoflavus* (section *Flavi*) and *Warcupiella spinosa* (section *Ornati*), like the *Sclerocleista* species are distant from the main body of species with *Aspergillus* anamorphs and should be placed in their own groups too. *Hemicarpenteles 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 *Fennellia nivea* ex type; *Chaetosartorya stromatoides* ex type is not the same species as *Aspergillus stromatoides* ex type (Peterson 1995) and *Fennellia 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 *Eurotium* lineage, a teleomorphic state was identified that belongs with *A. proliferans*. This species was placed in the *Eurotium* clade even though it was anamorphic (Raper & Fennell, 1965). The isolates identified as teleomorphs 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 *Eurotium* name and description. Separately, about 80 isolates of *A. restrictus* were sequenced at a single locus. Three of those isolates were identical with known *Eurotium* 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 teleomorphic 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 sequence type submit their DNA sequence along with corroborating information, such as the tracings from the DNA sequencer, to the curator of the site. The curator verifies the quality of the data and either asks for more information from the submitting scientist or posts the new sequence type to the web site. In this way, data that has gone through a third party quality check quickly appears at a central site accessible from all over the globe.

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

such as *Wolbachia* are known to cause flow of mitochondrial genes between biological species, so species groupings created using mtDNA can differ from the true species groupings (Hurst & Jiggins 2005; Withworth *et al.* 2007). Infra-individual heterogeneity of mitochondrial sequences can also cause problems (Sword *et al.* 2007). Besides, data presented by Wiemers & Fiedler (2007) indicate that the "barcoding gap" (i.e. the difference between inter- and intraspecific variability) is an artifact of insufficient sampling, and suggested that other characters should be examined to identify new species. Hickerson *et al.* (2006) claimed that single-locus mtDNA based barcodes can only consistently discover new species if their populations have been isolated for more than 4 million generations. Other authors also suggested that mtDNA-based barcodes should be supplemented with nuclear barcodes (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 (Bhadury *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 rRNA sequences were suggested to be used as DNA barcodes (Strugnell & Lindgren 2007). Regarding plants, the *cox1* region was found to be inappropriate for species identification because of a much slower rate of evolution of *cox1* (and other mitochondrial genes) in higher plants than in animals (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 *trnH-psbA* intergenic spacer are two of the leading candidates (Kress *et al*. 2005). More recently, the non-coding *trnH-psbA* spacer region coupled with the coding *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 (*tef1*) based DNA barcodes have been developed recently for identification of *Trichoderma* and *Hypocrea* species (Druzhinina *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-Ollivier *et al.* 2006) and *Trichophyton* species (Summerbell *et al.* 2007). For the identification of *Fusarium* species, Geiser *et al.* (2004) developed FU.S.A.RIUM-ID v.1.0, a publicly available sequence database of partial *tef1* sequences. Recently, the applicability of the *cox1* region for species identification in *Penicillium* subgenus *Penicillium* has been examined by Seifert *et al.* (2007). They found that representatives from 38 of 58 species could be distinguished from each other using *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 *cox1* for species identification in the *Aspergillus* genus. Our studies were focused on *Aspergillus* section *Nigri*. Our previous work on mitochondrial genetics of aspergilli indicated that the *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 *cox1* gene of *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 *A. niger* species complex, which includes eight species: *A. niger, A. tubingensis, A. foetidus, A. piperis, A. brasiliensis, A. vadensis, A. costaricensis* and *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 β-tubulin sequence data (*A. lacticoffeatus* had identical β-tubulin sequences to some *A. niger* isolates; Samson *et al.* 2004; Varga *et al.* 2007). The ITS data set delimited 4 groups: 1. *A. niger* and *A. lacticoffeatus* isolates; 2. *A. brasiliensis*; 3. *A. costaricaensis*; 4. *A. tubingensis, A. foetidus, A. vadensis* and *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 *A. niger* species complex, including pyruvate kinase, pectin lyase, polygalacturonase, arabinoxylan-arabinofuranohydrolase and several other genes (Gielkens *et al.* 1997; de Vries *et al.* 2005; Parenicova *et al.* 2001), translation intitiation factor 2, pyruvate carboxylase, 70 kD heat shock protein, chaperonin complex component (TCP-1), ATPase (Witiak *et al.* 2007), and translation elongation factor 1-α, RNA polymerase II and actin gene sequences (S.W. Peterson, pers. comm.).

To evaluate the usefulness of the *cox1* gene for DNA barcoding, we amplified and sequenced part of the *cox1* gene from about 45 isolates of the *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 (Swofford 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 *A. ellipticus*,

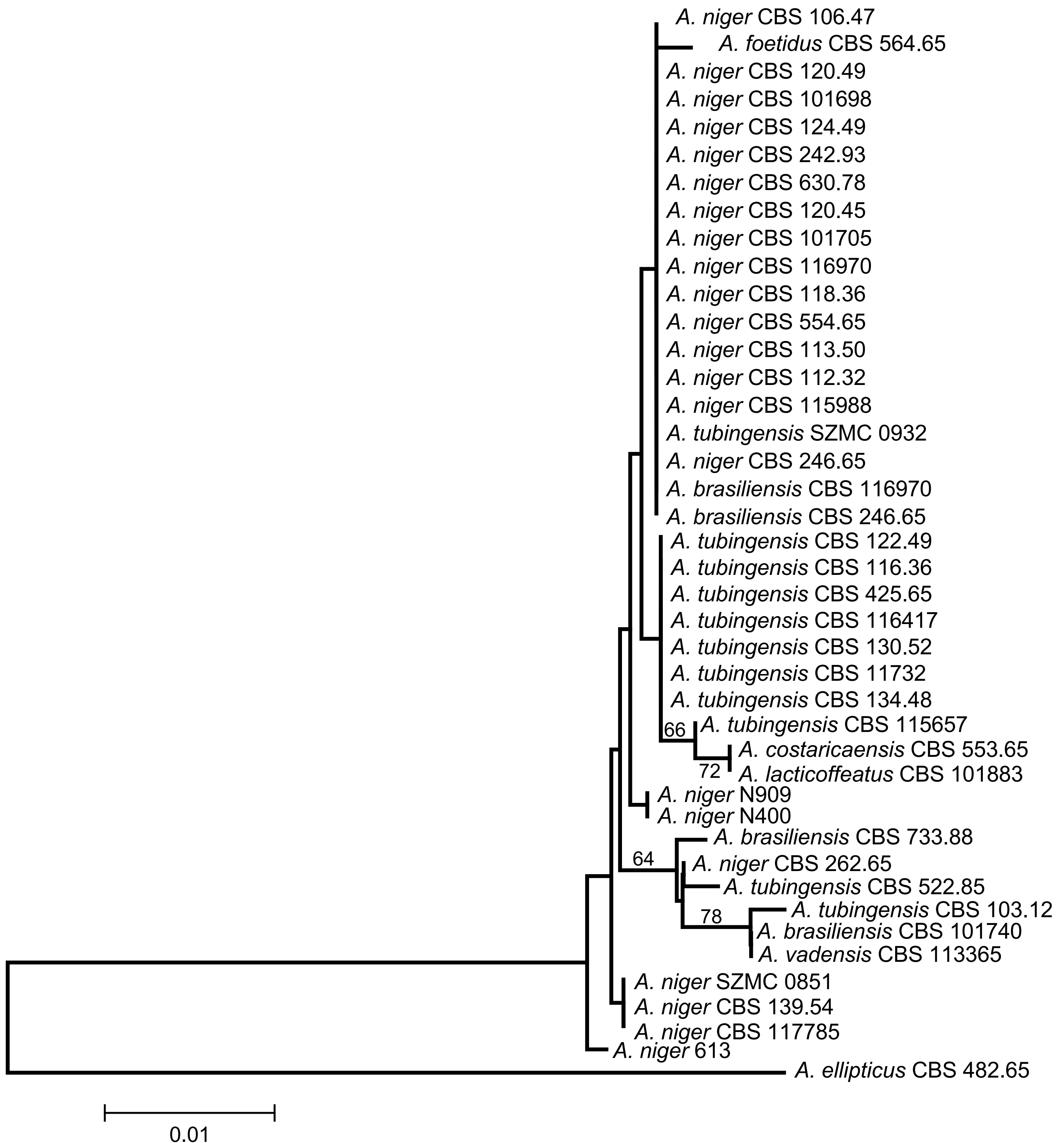

Fig. 1. Neighbour-joining tree based on cox1 sequences of the *A. niger aggregate. Aspergillus ellipticus CBS 482.65 was used as outgroup.* Bootstrap values above 50 % are shown on the branches. Partial *cox1* sequences of the examined isolates have been deposited in the GenBank database under accession numbers EU021012–EU021046.

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

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 *n*1 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, A. fumigatus, N. fischeri*, *A. terreus, 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 utlised 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 (Witiak & 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 the *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 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.

Key words: comparative genomics, genetic diversity, genome sequences, identifcation 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; Balajee *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* (Rydholm *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; Nierman *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).

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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. fumigatus* Af293: AAHF01000000 (GenBank); *A. niger* CBS 513.88: AM270980–AM27998 (GenBank); *A. niger* ATCC 1015: http://genome.jgi-psf.org/Aspni1/Aspni1.home.html (JGI); *A. flavus*: 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/sybil/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 then 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 then 95 % identity and 90 % coverage); (ii) variable genes, which aligned poorly (less then 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.

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

Species	***A. fumigatus***		***A. niger***		***A. flavus***	***A. oryzae***	***A. fumigatus***	***N. fischeri***
Strain/Isolate	Af293	A1163	ATCC 1015	CBS 513.88	NRLL 3357	ATCC 42149	Af293	NRRL 181
Genome								
Size (Kb)	28,81	29,133	36,83	33,976	36,51	37,111	28,81	33,289
Difference in size (Kb)	323		2,854		601		4,479	
No assemblies > 5 Kb	19	20	49	19	20	26	19	74
No assemblies > 100 Kb	18	11	23	18	16	19	18	14
Shared genome %	98.8 %	97.7 %	89 %	96.5 %	97.4 %	95.8 %	n/a	n/a
Unique genome %	1.20 %	2.30 %	11 %	3.5 %	2.6 %	4.2 %	n/a	n/a
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.

	Within *A. fumigatus*	**Within *A. niger***		
Species	**Af293 vs. A1163**	**ATCC 1015 vs. CBS 513.88**	***A. flavus* vs. *A. oryzae***	***A. fumigatus* Af293 vs. *N. fischeri***
Genome vs. genome	99.8 %	99.3 %	99.5 %	92.4 %
CDS vs.genome	99.6 %	99.1 %	99.1 %	94.3 %
Protein vs. protein	99.5 %	96.7 %	98 %	93.4 %

Intraspecific case study II: *A. fumigatus* Af293 versus *A. fumigatus* A1163

Aspergillus fumigatus is the main causative agent of invasive aspergilliosis, 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).

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

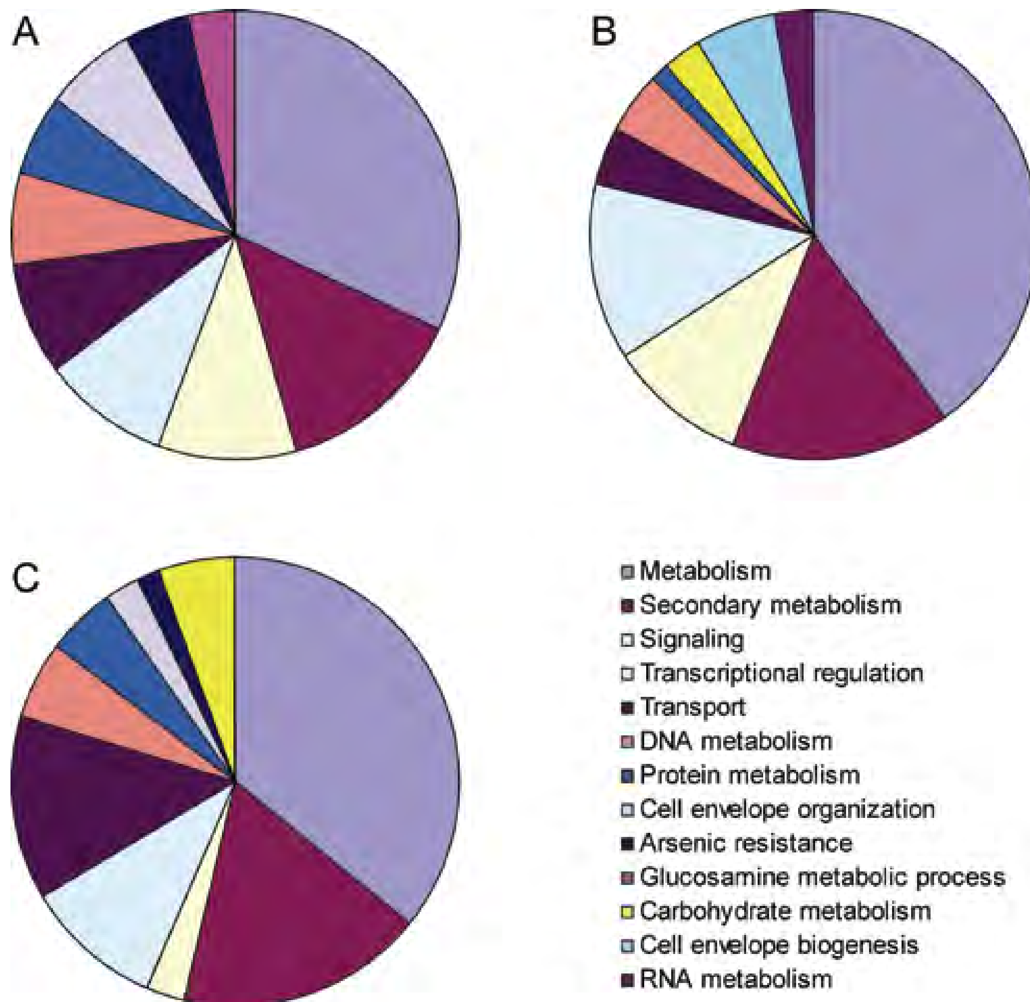

Fig. 1. Functional characterisation of strain- and species-specific *A. fumigatus* genes. (A) Functional characterisation of 88 *A. fumigatus* strain Af293-specific genes absent in strain A1163; (B) Functional characterisation of 70 *A. fumigatus* strain A1163-specific genes absent in strain Af293; (C) Functional characterisation of 322 *A. fumigatus* strain Af293-specific genes absent in *N. fischeri*. Only the ten largest functional categories are shown and genes of unknown function are excluded.

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 on animal 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

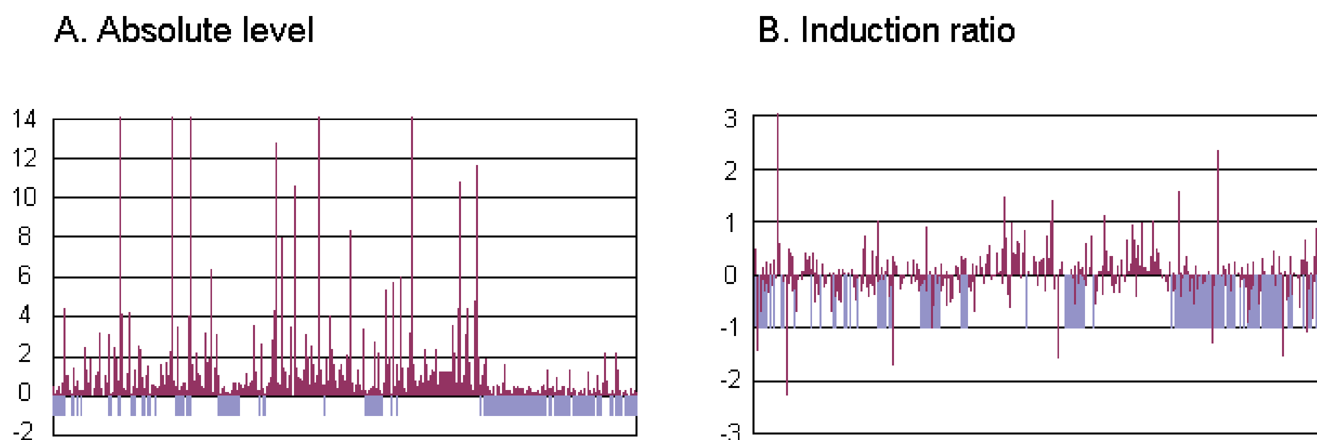

Fig. 2. Gene expression analysis and of chromosome 6 of *A. oryzae*. Expression levels (A) and induction ratios (B) of all the genes in chromosome 6 are shown. The *A. oryzae* mycelia used in the oligonucleotide microarray analysis were incubated at 42 °C, using as a reference a mycelium incubated at 30 °C. Light blue bars indicate genomic regions (blocks) of A. oryzae that are absent in *A. fumigatus* and *A. nidulans*. The scale of the vertical axis is logarithmic.

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 based 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 synthases (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*. 2006).

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 (Tominaga *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 orthologous 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*.

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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 state), but when conditions become unfavourable for vegetative growth, they may initiate sexual reproduction (teleomorphic state). 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 internuclear 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

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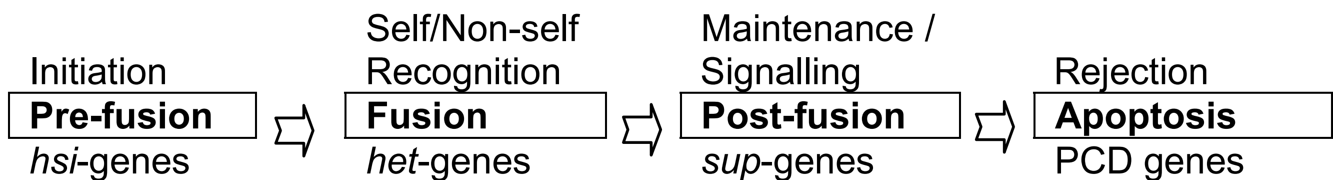

Fig. 1. Gene families involved in steps of vegetative incompatibility.

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 heteroplasmon, is governed by self/non-self recognition genes, like heterokaryon incompatibility (*het*) (Glass and Kuldau 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 heterokaron 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 (Caten 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, Paoletti *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*. Δ*MAT1* and Δ*MAT2* 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 homothallic, 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; Nierman *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, Kocsubé, 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 sub-set 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 (Turgeon 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 heteroallelism 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-c/*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 incompatibily 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* and the construction of a genetic map (Debets *et al.* 1993). But, natural isolates of black aspergilli are highly incompatible with each other, efficiently blocking virus transfer as well as the formation of heterokaryons (van Diepeningen *et al.* 1997). 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 heterokayon 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 XI / CNRS). *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 2).

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).

Species	Class	Gene	Function	References
N. crassa				
	Heterokaryon incompatibility genes			
		*het-6^OR^* (NCU03533.2)	allelic *het*-gene, TOL/HET-6/HET-E domain	Saupe 2000
		het-c (NCU3125.2)	allelic *het*-gene, signal peptide	Sarkar *et al.* 2002
				Saupe *et al.* 2000
		un-24 (NCU03539.2)	allelic *het*-gene, ribonucleotide reductase large subunit	Saupe 2000
				Smith *et al.* 2000
	Suppressor genes			
		tol (NCU04453.2)	TOL/HET-6/HET-E domain	Shiu & Glass 1999
		vib-1 (NCU03725.2)	regulation of conidiation and maybe of nrAPase	Xiang & Glass 2002
	Incompatibility related genes			
		ham-2 (NCU03727.2)	hyphal fusion, putative transmembrane protein	Xiang *et al.* 2002
		pin-c (NCU03494.2)	allelic gene with HET-domain, linked to *het-c*	Kaneko *et al.* 2006
		rnr-a (NCU07887.2)	suppresses *un-24* temperature sensitive mutation, ribonucleotide reductase small subunit	Kotierk & Smith 2001
P. anserina				
	Heterokaryon incompatibility genes			
		het-c2 (AAA20542)	nonallelic *het*-gene interacts with *het-d* and *het-e*, glycolipid transfer protein	Saupe *et al.* 1994
		het-d2y (AAL37301)	nonallelic *het*-gene against *het-c2*, GTP-binding, WD repeat, TOL/HET-6/HET-E domain	Espagne *et al.* 2002
		het-e4s (AAL37297)	nonallelic *het*-gene against *het-c2*, GTP-binding, WD repeat, TOL/HET-6/HET-E domain	Espagne *et al.* 2002

Table 1. (Continued).

Species	Class	Gene	Function	References
		het-S (AAB88771)	allelic *het*-gene, prion analog	Coustou *et al.* 1997
	Incompatibility related genes			
		idi-1 (AAC24119)	induced by *het-c/e* and *r/v* incompatibility, signal peptide	Dementhon *et al.* 2003
				Bourges *et al.* 1998
		idi-2 (AAC24120)	induced by *het-r/v* incompatibility, signal peptide	Bourges *et al.* 1998
		idi-3 (AAC24121)	induced by *het-c/e* and *r/v* incompatibility, signal peptide	Bourges *et al.* 1998
		idi-4 (*jlb-a*)	bZIP motif, putative trans-activation domain	Dementhon *et al.* 2004, 2005
		(AAT40415)		
		idi-6 (*pspA*)	induced by *het-c/e* and *r/v* incompatibility, subtilisin-like serine protease	Paoletti *et al.* 2001
		(AAC03564)		Reichard *et al.* 2000
		idi-7 (AAN41258)	ortholog of the *S. cerevisiae aut7p*	Pinan-Lucarre *et al.* 2003
	Modifier genes			
		mod-A (AAC25496)	modifier of *het-c/e*, *c/d* and *r/v* incompatibility, SH3-binding motif	Barreau *et al.* 1998
				Bourges *et al.* 1998
		mod-D (AAC24766)	modifier of *het-c/e* incompatibility, G protein α subunit	Loubradou *et al.* 1999
		mod-E (AAB97626)	modifier of *het-r/v* incompatibility, HSP90	Loubradou *et al.* 1997
S. cerevisiae				
	Programmed Cell Death genes			
		atp4 (YPL078C)	F_0F_1-ATPase	Matsuyama *et al.* 1998
		cdc48 (YDL126C)	cell division cycle, AAA ATPase, fusion of ER-derived vesicles	Madeo *et al.* 1997
		hel10 (YNL208W)	unknown	Ligr *et al.* 2001
		hel13 (YOR309C)	unknown	Ligr *et al.* 2001
		mca1/*yca1* (YOR197W)	metacaspase	Madeo *et al.* 2002
		nsr1 (YGR159C)	rRNA processing	Ligr *et al.* 2001
		ppa1 (YHR026W)	vacuolar H^+-ATPase	Ligr *et al.* 2001
		rsm23 (YGL129C)	mitochondrial small ribosomal unit	Madeo *et al.* 2002
		sar1 (YPL218W)	ER to Golgi transport	Ligr *et al.* 2001
		stm1 (YLR150W)	suppressor of *pop2* and *tom2*	Ligr *et al.* 2001
		tor1 (YJR066W)	regulation of cell death, phosphatidylinositol 3-kinase	Rohde *et al.* 2001
				Dementhon *et al.* 2003
				Fitzgibbon *et al.* 2005

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

Species	Strain	Website	References
A. fumigatus	Af293	http://tigrblast.tigr.org/er-blast/index.cgi?project=afu1	Nierman *et al.* 2005
A. nidulans	FGSC A4	http://www.broad.mit.edu/annotation/genome/aspergillus_nidulans/Blast.html	Galagan *et al.* 2005
A. niger	CBS513.88	http://www.dsm.com/en_US/html/dfs/genomics_aniger.htm	Pel *et al.* 2007
A. niger	ATCC1015	http://genome.jgi-psf.org/cgi-bin/runAlignment?db=Aspni1&advanced=1	DOE -JGI
A. oryzae	RIB40	http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao	Machida *et al.* 2005
A. terreus	NIH2624	http://www.broad.mit.edu/annotation/genome/aspergillus_terreus/Blast.html	Broad Institute of Harvard and MIT
N. crassa	OR74A	http://www.broad.mit.edu/annotation/genome/neurospora/Blast.html	Galagan *et al.* 2003
P. anserina	S	http://podospora.igmors.u-psud.fr/blast_ol.html	*P. anserina* genome project
S. cerevisiae	S288C	http://seq.yeastgenome.org/cgi-bin/nph-blast2sgd	*Saccharomyces* Genome Database

The *in silico* experiments performed with the different sequenced and available genomes show that the majority of the PCD genes from *S. cerevisiae* have homologs in the filamentous fungi, only *hel10* and *hel 13*, whose functions are unknown, are mostly missing (Table 3). In contrast the majority of vegetative incompatibility and cell death related genes from *N. crassa* and *P. anserina* can be found in the other filamentous fungi, but many of them, like *het-6*, *het-c*, *tol*, *vib-1*, *pin-c*, *het-c2*, *het-s*, *idi-1*, *idi-2*, *idi-3*, *idi-4* and *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 HET-D and 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 *S. cerevisiae*. Of course these genes may have pleiotropic functions and may have a different function in *S. cerevisiae* than controlling anastomoses formation. The *idi-7* gene of *P. anserina* is classified as being an ortholog of the *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 *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 *S. cerevisiae* (Supplementary Table 1). Whereas the putative transmembrane protein HAM-2 is very well conserved within the filamentous fungi, it differs considerably from the *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 *S. cerevisiae*, but that many genes involved in heterokaryon incompatibility are not.

In silico comparison of Sordariales and Eurotiales

N. crassa and *P. anserina* belong to the Sordariales, whereas the aspergilli 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 *P. anserina*, bearing many more *idi* (induced during incompatibility) gene homologs than other fungi and missing the suppressor gene *stm1*. Among these fungi, *P. anserina* bears the most different gene set and the largest set of genes with *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 *het*-domains. A possible explanation for this can be in its life cycle. *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 *het*-genes. In *P. anserina* the majority of the incompatibility reactions is due to non-allelic interactions (exception is het *het-s*/*het-S* reaction). *Het-d* and *het-e* trigger incompatibility with the *het-c2*. Both *het-d* and *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 *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 *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 *A. niger* and *N. crassa*) one can see that the homologs of these genes show old polymorphisms. The sequences found in the *A. niger* strains are often very similar. The most similar homologs in *N. crassa* however, can be quite different from the *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 *Sordariales*. Only the MOD-A protein has but one hit in the aspergilli: the protein blast in *A. oryzae* resulted in one hit with only low similarity (e^{-11}) (Suppl. Table 1).

In silico comparison of the different aspergilli

S. cerevisiae PCD- and *N. crassa* and *P. anserina* HET-, modifier and suppressor protein sequences were used to search the genomes of *A. fumigatus* Af 293, *A. nidulans* FGSC A4, *A. oryzae* RIB40, and *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 *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 *A. nidulans* and *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: *A. nidulans* is able to reproduce sexually, and there are indirect proofs for the presence of a sexual life cycle in *A. fumigatus* (Varga 2003, Paoletti *et al.* 2005). The other aspergilli (*A. niger*, *A. oryzae*, *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 cytoplasmic or nuclear elements (Caten 1972). In *A. niger* and related black *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 aspergilli (van Diepeningen *et al.* 1997). In sexual *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 *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 *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.

Species	Class	Protein	Species (Strains)								
			A. fumigatus (Af 293)	*A. nidulans* (FGSC A4)	*A. niger* (CBS513.88)	*A. niger* (ATCC*1015*)	*A. oryzae* (RIB40)	*A. terreus* (NIH 2624)	*N. crassa* (OR74A)	*P. anserina* (S)	*S. cerevisiae* (S288C)
N. crassa											
	Heterokaryon incompatibility genes										
		HET-6 (NCU03533.2)	●	●	●	●	●	●	●	●	
		HET-C (NCU03125.2)	●	●	●	●	●	●	●	●	
		UN-24 (NCU03539.2)	●	●	●	●	●	●	●	●	●
	Suppressor genes										
		TOL (NCU04453.2)	●		●	●	●	●	●	●	
		VIB-1 (NCU03725.2)	●	●	●	●	●	●	●	●	
	Incompatibility related genes										
		HAM-2 (NCU03727.2)	●	●	●	●	●	●	●	●	●
		PIN-C (NCU03494.2)	●		●	●	●	●	●	●	
		RNR-A (NCU07887.2)	●	●	●	●	●	●	●	●	●
P. anserina											
	Heterokaryon incompatibility genes										
		HET-C2 (AAA20542)	●	●	●	●	●	●	●	●	
		HET-D2Y (AAL37301)	●	●	●	●	●	●	●	●	●
		HET-E4S (AAL37297)	●	●	●	●	●	●	●	●	●
		HET-S (AAB88771)		●		●		●			
	Incompatibility related genes										
		IDI-1 (AAC24119)								●	
		IDI-2 (AAC24120)								●	
		IDI-3 (AAC24121)								●	
		IDI-4/ JLB-A (AAT40415)			●			●	●	●	
		IDI-6/ PSP (AAC03564)	●	●	●	●	●	●	●	●	●
		IDI-7 (AAN41258)	●	●	●	●	●	●	●	●	●
	Modifier genes										
		MOD-A (AAC25496)					●		●	●	
		MOD-D (AAC24766)	●	●	●	●	●	●	●	●	●
		MOD-E (AAB97626)	●	●	●	●	●	●	●	●	●
S.cerevisiae											
	Programmed Cell Death genes										
		ATP4 (YPL078C)	●	●	●	●	●	●	●	●	●
		CDC48 (YDL126C)	●	●	●	●	●	●	●	●	●
		HEL10 (YNL208W)	●		●						●
		HEL13 (YOR309C)			●						●
		MCA1/YCA1 (YOR197W)	●	●	●	●	●	●	●	●	●
		NSR1 (YGR159C)	●	●	●	●	●		●	●	●
		PPA1 (YHR026W)	●	●	●	●	●	●	●	●	●
		RSM23 (YGL129C)							●	●	●
		SAR1 (YPL218W)	●	●	●	●	●	●	●	●	●
		STM1 (YLR150W)	●	●	●	●	●	●	●		●
		TOR1 (YJR066W)	●	●	●	●	●	●	●	●	●

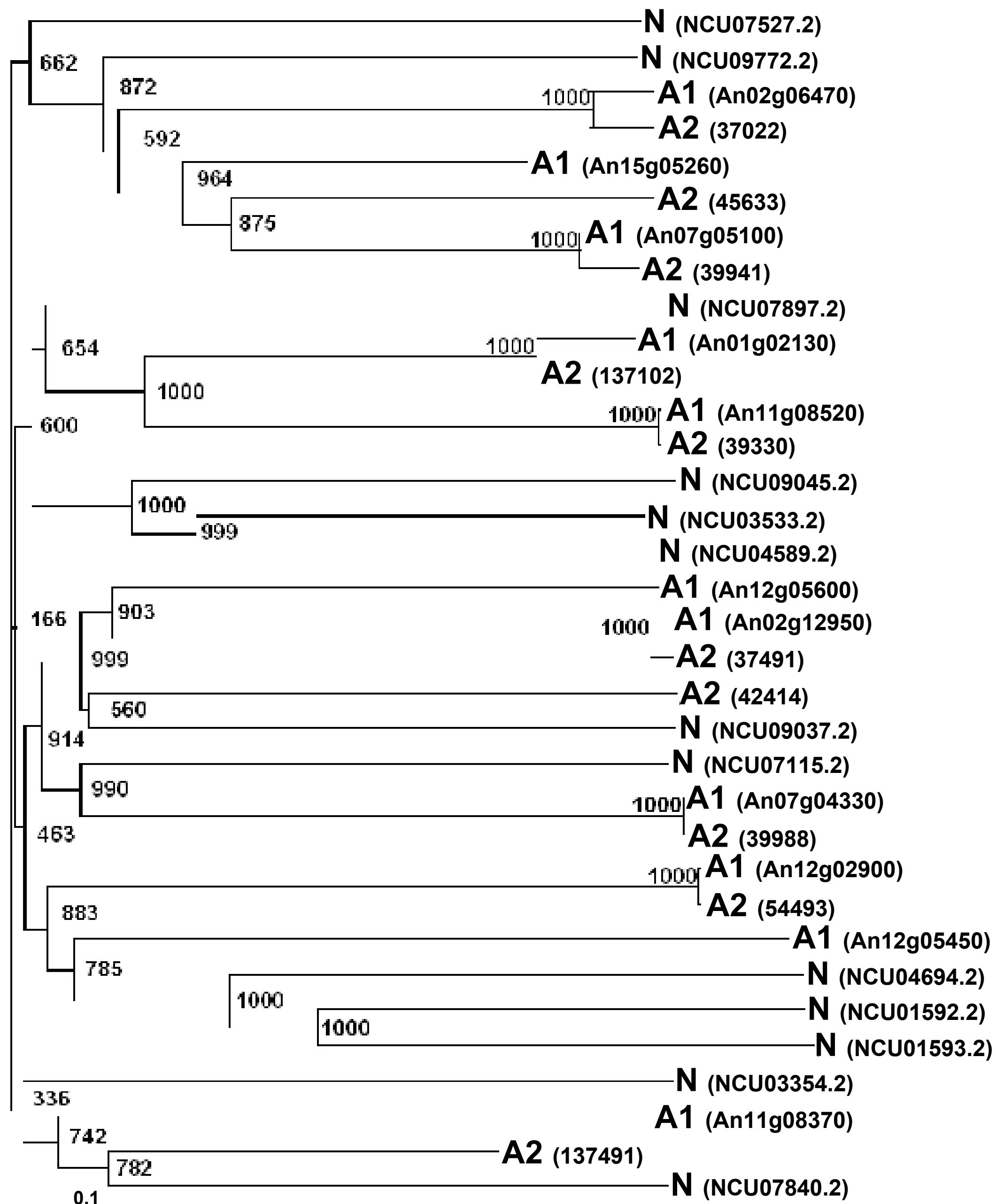

Fig. 2. Neighbour-joining tree of HET-6 proteins from *N. crassa* and two *A. nigers*. Strains on the tree: A1: *A. niger* CBS513.88; A2: *A. niger* ATCC1015; N: *N. crassa* OR74A. Accession numbers of the different homologs are given from the respective databases. Sequence alignment and bootstrapping was performed with ClustalX (Thompson *et al.* 1997). Trees were visualised by Treeview (Page, 1996).

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

Table 4. A comparison between the different heterokaryon incompatibility and programmed cell death related genes in the two sequenced *A. niger* genomes (CBS513.88 and ATCC1015). If two proteins differ in size, the longer one is the basis for counting percentage of identities, similarities and gaps. Gaps are counted only in the homologous region.

Function	Protein	Type and size of difference		Identities	Gaps
Heterokaryon incompatibility genes (*N. crassa* & *P. anserina*)					
	HET-6	only HET domain motifs are slightly conserved		–	–
	HET-C	indel: 622		791/793 (99 %)	1/793
		substitution: 196			(0 %)
	UN-24	no difference		(100 %)	(0 %)
	HET-C2	no difference		(100 %)	
	HET-D / HET-E	diverse proteins with WD40 repeats, but no remarkable similarity		–	
Suppressor genes (*N. crassa*)					
	TOL	very diverse proteins, with conserved HET domain motifs		–	–
	VIB-1	substitution: 195		585/586 (99 %)	(0 %)
Incompatibility related genes (*N. crassa* & *P. anserina*)					
	HAM-2	substitution: 756		1066/1067 (99 %)	(0 %)
	PIN-C	very diverse proteins, with conserved HET domain motifs		–	–
	RNR-A	no difference		(100 %)	(0 %)
	IDI-6 / PSP	1st pair: indel: 534		531/535 (99 %)	
	(2 alleles)	substitutions: 398, 525			
		2nd pair: substitutions: 398, 436–443, 450–459, 471–514		413/416 (99 %)	
	IDI-7	no difference		(100 %)	
Modifier genes (*P. anserina*)					
	MOD-D (2 alleles)	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.			
	MOD-E	substitution: 244		672/702 (96 %)	(0 %)
Programmed Cell Death genes (*S. cerevisiae*)					
	ATP4	no difference		100 %	0 %
	CDC48	no difference		100 %	0 %
	HEL13	present only in CBS 513.88		–	–
	MCA1 / YCA1		1st pair	438/438 (100 %)	(0 %)
	(2 alleles)				
		2nd allele: indel region: 1–56, 75–104, 237, 443–447			
		subtitutions: 57–75, 105–106. 236, 440–442	2nd pair	341/441 (77 %)	1/333 (0 %)
	NSR1	diverse proteins, with short conserved motifs		–	–
	PPA1	no difference		100 %	0 %
	SAR1	no difference		100 %	0 %
	STM1	indel region: 8–13		297/303 (98 %)	6/303
					(1 %)
	TOR1	substitution: 16		2389/2390 (99 %)	(0 %)

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 two strains are heterokaryon incompatible and the differences –sometimes only simple substitutions, sometimes small stretches of amino acids - in the known indel regions of some of the putative *het*-genes may explain the observed incompatibility reaction between the two strains (Table 4). For the *idi-6* (*psp*) genes two alleles are present, both very similar with very few substitutions. However, for the MCA1/YCA1 (a meta-caspase) one pair is completely identical, whereas the second pair shows more differences and is only 77 % identical.

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

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.

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

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

INTRODUCTION

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

Extrolites in *Aspergillus*

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

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

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

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

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

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

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

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

Chemotaxonomy and secondary metabolite profiling

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

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

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

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

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

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

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

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

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

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

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

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

Use of Ochratoxin A in identification of aspergilli

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

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

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

Aflatoxin biosynthesis and regulation

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

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

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

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

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

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

CONCLUSIONS

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

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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 recognised 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 section 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 asexual 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 section 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*.

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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. fumigatiaffinis* and *A. novofumigatus*, had also been proposed (Hong *et al.* 2005) by investigators who analyzed the species within the section *Fumigati* 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 *Fumigati* (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. fumisynnematus* (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 Fumigati 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 the PCR products by using primer pairs Bt2a and Bt2b, rodA1 and rodA2, and cmd5 and cmd6, respectively. PCR products were sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) and phylogenetic analyses were performed by the maximum parsimony (MP) and Neighbour-joining (NJ) methods. Comparative analyses of tree topologies from MP and NJ analyses showed no differences, and the three trees based on the three loci were similar.

Results of this study showed that the species within the section *Fumigati* could be divided into five clades: clade I, typical strains of *A. fumigatus* including *A. fumigatus* var. *ellipticus* (Raper & Fennell 1965) and *A. arvii* (Aho *et al.* 1994); clade II, species including *A. lentulus* and *A. fumisynnematus* (Horie *et al.* 1993); clade III, species including *A. fumigatiaffinis* and *A. novofumigatus*; clade IV, atypical strains of *A. fumigatus* including *A. viridinutans* Katz *et al.* 1998; Varga *et al.* 2000); and clade V, species including *A. brevipes, A. duricaulis* and *A. unilateralis*. Most of the examined strains 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; Anaissie *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

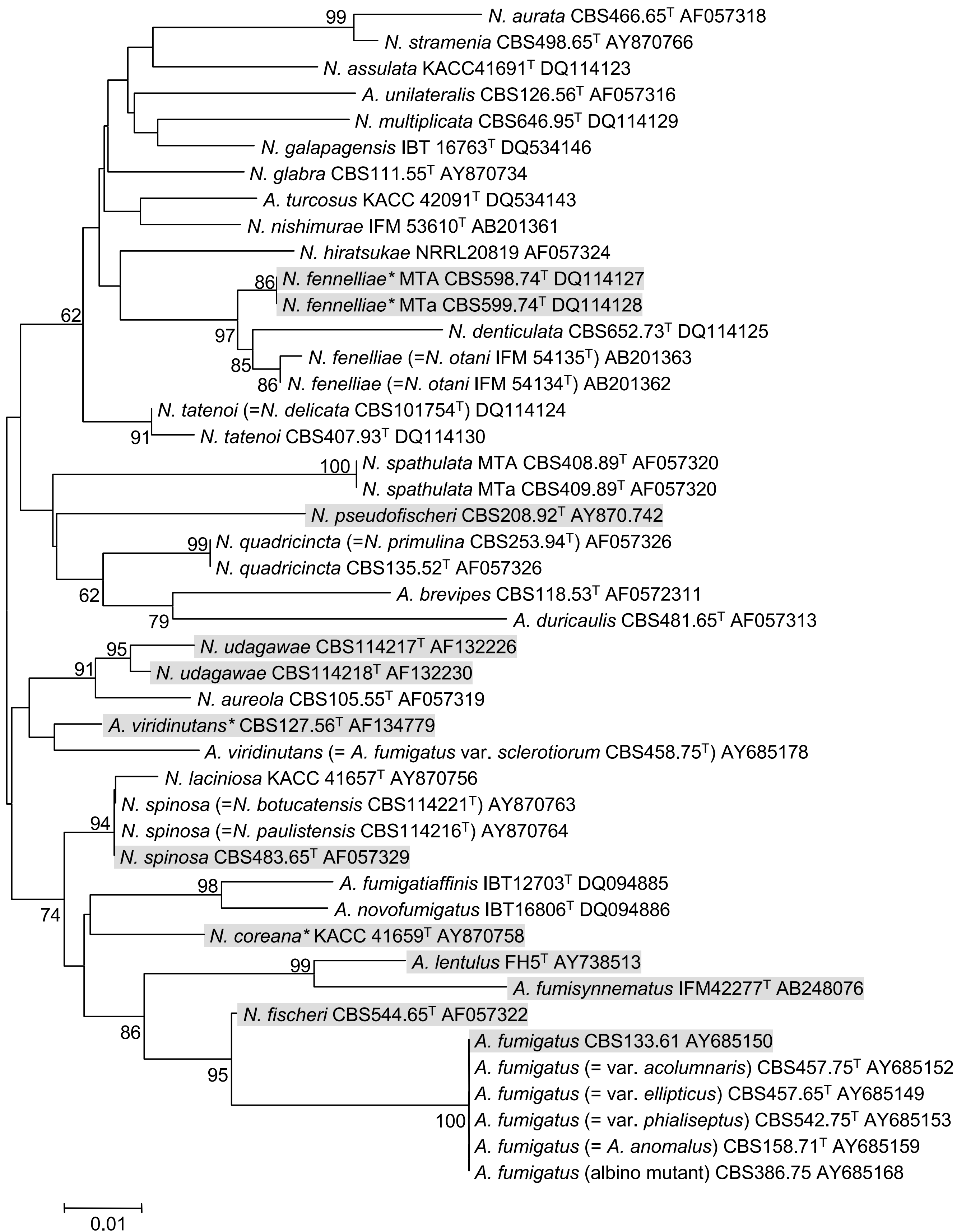

Fig. 1. Phylogenetic tree of *Aspergillus* section *Fumigati* species inferred from Neighbour-Joining analysis of partial β-tubulin gene sequence. The shaded species have been reported from clinical environment.

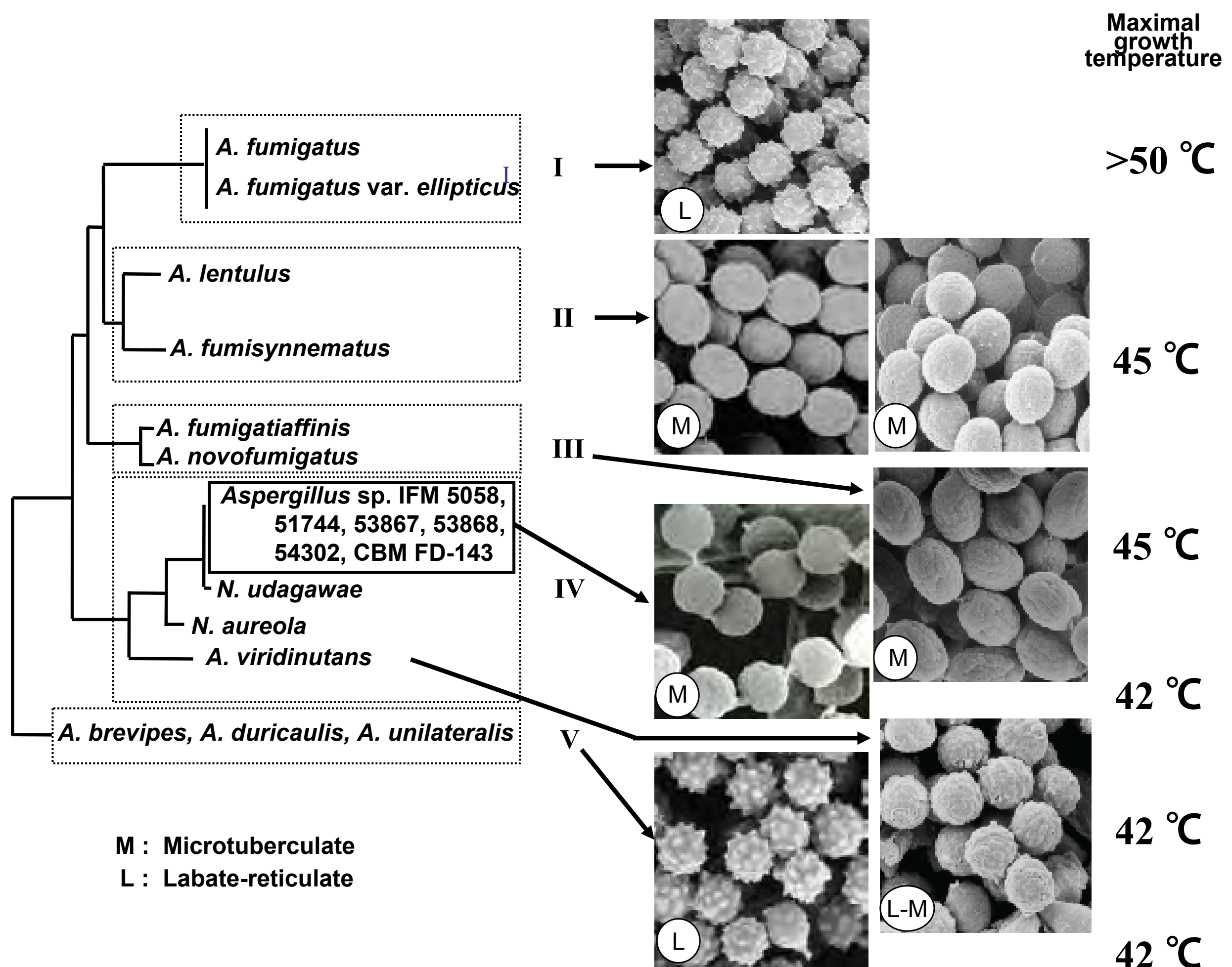

Fig. 2. Correlation among phylogeny, detailed morphology and maximal growth temperatures on *Aspergillus* section *Fumigati.* The letters L and M refer to the conidial ornamentation as observed by scanning electron microscopy.

appearing yellow. Microscopically, conidial heads are biseriate 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, suppurative 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-tinged conidiophores, and characteristic cleistothecia contain asci with 8 purple-red ascospores (Fig. 3). Hülle cells, thickened large cells associated with cleistothecia, are often abundant in *E.*

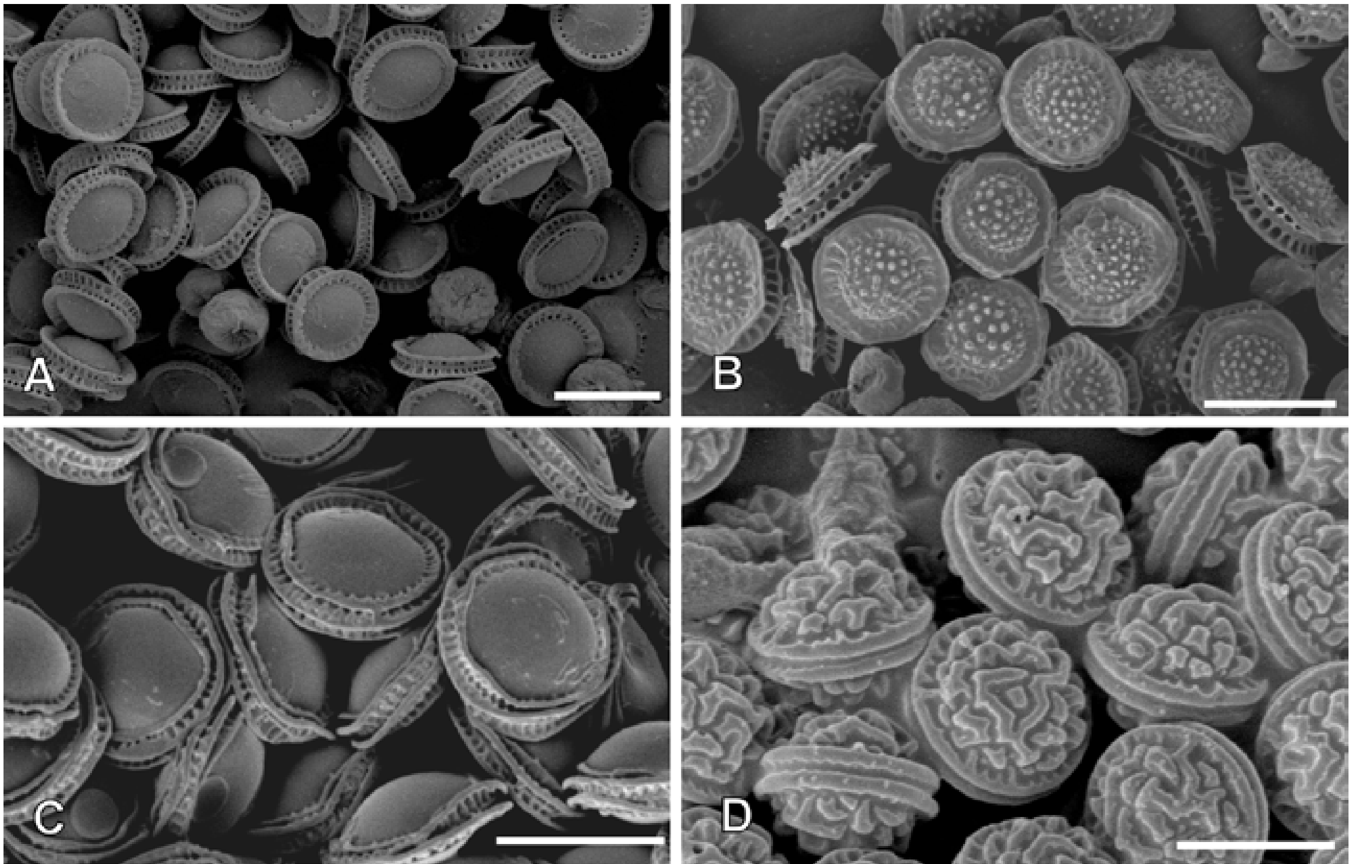

Fig. 3. Scanning electronmicroscopy of ascospores of *Emericella* species. A. *E. nidulans*. B. *E. echinulata.* C. *E. quadrilineata.* D. *E. rugulosa*. Scale bars = 5 µm.

nidulans. 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 (Saracli *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

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 (Fakih *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 there of) 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 subgenus/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.

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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 fur 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.* 2007; Levdansky *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 *à 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 libaries 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 STR*Af* 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 STR*Af* assay yielded a superior discriminatory power for typing *A. fumigatus* isolates. In Fig. 1, a graphical representation of

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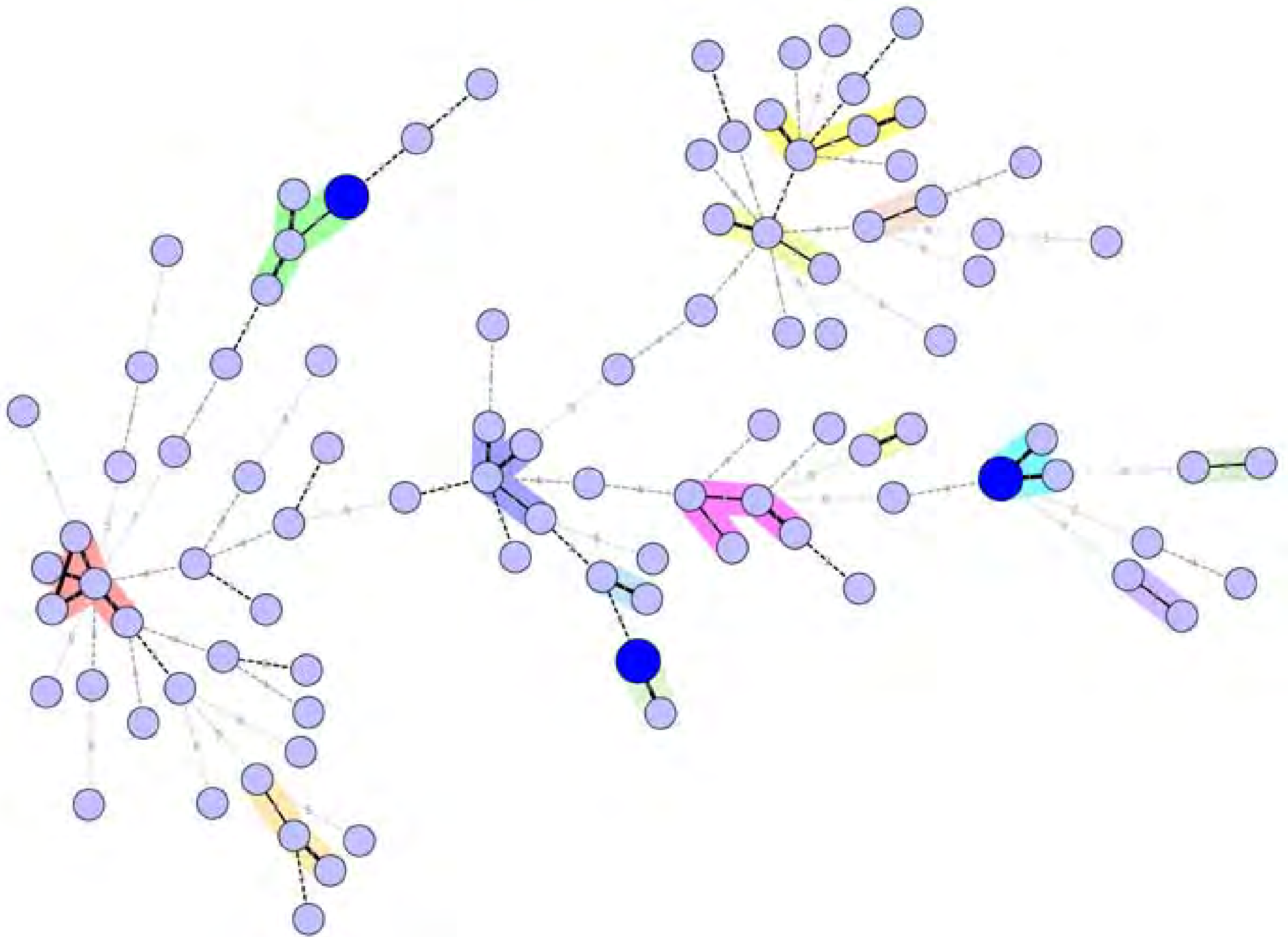

Fig. 1. Minimal spanning tree of 99 presumably unrelated *A. fumigatus* isolates based on microsatellite data. The tree was generated using the multi-state categorical similarity coefficient. Each circle represents a unique genotype. The size of the circle corresponds to the number of isolates with the 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).

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 organisms" 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

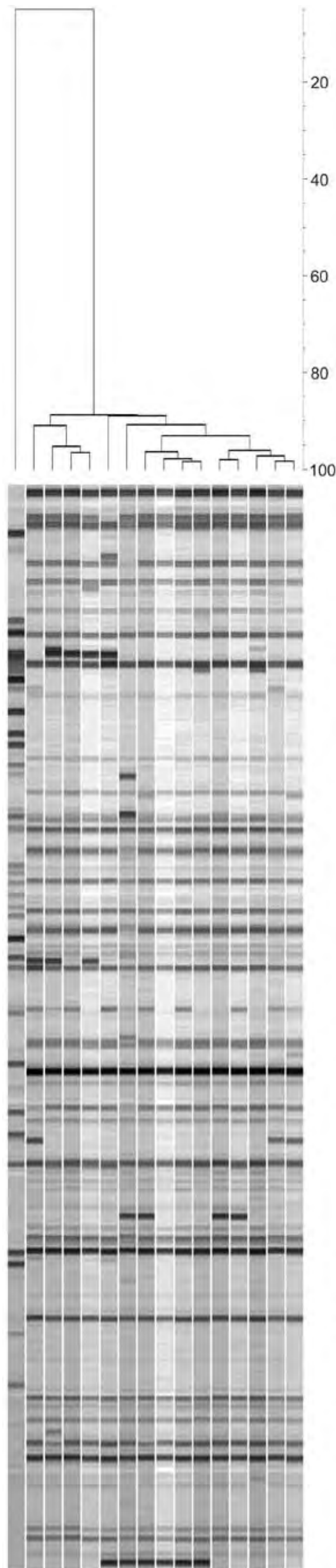

Fig. 2. Example of AFLP analysis showing the discriminatory power as well as the ability to discriminate between isolates belonging to different species. The figure shows 16 fingerprints from *A. fumigatus* cultured from 16 IA patients (1 isolate per patient). Based on the differential presence or absence 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*

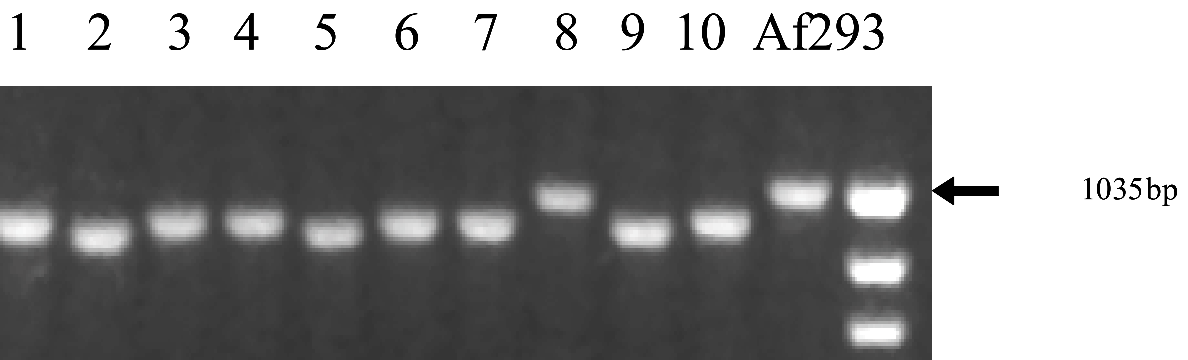

Fig. 3. The TR region in *A. fumigatus* gene *Afu3g08990* containing a leader sequence and GPI anchor shows size variability. The TR region of *Afu3g08990* was amplified by PCR in 11 clinical *A. fumigatus* isolates, including the genome-sequenced reference strain Af293. This figure shows size variation of the TR region between the strains.

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 *A. fumigatus* isolates from six nosocomial outbreaks of invasive aspergillosis. The results were concordant with another discriminatory genotyping technique, the *Afut*1 RFLP typing method. However, while *Afut1* typing is labor and time intensive, needs specialised equipment and is not high throughput, *Afu3g08990* / 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 *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 *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 *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 inadvertant 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 simultaneous be used for identification confirmation of fungal isolates: e.g. any clinical *A. fumigatus* isolate lacking the characteristic amplification products using a highly species specific typing method such as the STR*Af* assay or any isolate lacking the typical banding patterns obtained with the *Afut1* RFLP method is most likely not a true *A. fumigatus*. The true identity of such an isolate yet remains to be established using other methods. In contrast, more universally applicable typing methods that are not hampered by the species 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.

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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 ochratoxinogenic *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. sclerotioniger*, *A. lacticoffeatus*, *A. sclerotiicarbonarius*, and *A. aculeatinus*. 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 B_1, B_2, G_1, G_2 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 (Cotty 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

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Table 1. Species concepts of black aspergilli according to different authors.

Raper and Fennell (1965)	Al-Musallam (1980)	Kozakiewicz (1989)	RLFP* analysis	Samson *et al.* (2004)
A. japonicus	*A. japonicus* var. *japonicus*	*A. japonicus*	*A. japonicus*	*A. japonicus*
A. aculeatus	*A. japonicus* var. *aculeatus*	*A. atroviolaceus*	*A. aculeatus*	*A. aculeatus*
A. carbonarius	*A. carbonarius*	*A. carbonarius*	*A. carbonarius*	*A. carbonarius*
		A. fonsecaeus		
A. heteromorphus	*A. heteromorphus*	*A. heteromorphus*	*A. heteromorphus*	*A. heteromorphus*
A. ellipticus	*A. ellipticus*	*A. ellipticus*	*A. ellipticus*	*A. ellipticus*
	A. helicothrix	*A. helicothrix*		*A. sclerotioniger*
				A. homomorphus
***A. niger* aggregate:**				
A. niger	*A. niger* var. *niger*	*A. niger* var. *niger*	*A. niger*	*A. niger*
A. tubingensis	*A. niger* var. *niger f. hennebergii*	*A. niger* var. *tubingensis*	*A. tubingensis*	*A. tubingensis*
A. phoenicis	*A. niger* var. *phoenicis*	*A .niger* var. *phoenicis*	*A. foetidus*	*A. foetidus*
A. polverulentus	*A. niger* var. *phoenicis f. pulverulentus*	*A.niger* var. *polverulentus*	*A. brasiliensis*	*A. brasiliensis* (Varga *et al.* 2007)
A. awamori	*A. niger* var. *awamori*	*A. niger* var. *awamori*		*A. costaricaensis*
A. ficuum	*A. niger* var. *nanus*			*A. lacticoffeatus*
A. foetidus	*A. niger* var. *usamii*	*A. niger* var. *ficuum*		*A. piperis*
A. foetidus var. *pallidus*	*A. niger* var. *intermedius*	*A. citrus* var. *citrus*		*A. vadensis*
A. foetidus var. *acidus*	*A. foetidus*	*A. acidus*		*A. ibericus* (Serra *et al.* 2006)
		A. citrus var. *pallidus*		*A. uvarum* (Perrone *et al.* 2007)

*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.

Species	Conidial size (µm)	Color and size of sclerotia (mm)	Source	OTA	Extrolites produced
Biseriates					
A. brasiliensis (Varga *et al.* 2007)	3.5–4.5	Found only in some strain, white, 1–1.5	Soil, grape	-	Naphtho-γ-pyrones (including aurasperone B), pyrophen, tensidol A & B, dihydrocarolic acid, aflavinine
A. carbonarius (Bainier) (Thom 1916)	7–9	Pink to brown, 1	Grape, cocoa, coffee,spices, palm oil, soil, air	+	Pyranonigrin A, naphtho-γ-pyrones
A. foetidus (Thom & Raper 1945)	3.5–4.5	Found only in some strain, white, 1–1.5	Tomato, grape, bottled fruits	-	Antafumicins, asperazine, funalenone, naphtho-γ-pyrones, pyranonigrin A
A. ibericus (Serra *et al.* 2006)	5–7	-	Grape	-	Naptho-γ-pyrones, pyranonigrin A
A. niger (Tieghem 1867)	3.5–5	-	Grape, cocoa, coffee, cereals, soil, paper, date palm	+/-	Funalenone, kotanins, naphtho-γ-pyrones, pyranonigrin A, pyrophen, tensidol A and B
A. tubingensis ((Schober) Mosseray 1934)	3–5	White to pink, 0.5–0.8	Grape, cocoa, coffee, soil, cereals	+/-	Asperazine, funalenone, naphtho-γ-pyrones, pyranonigrin A, tensidol A & B
Uniseriates					
A. aculeatus (Iizuka 1953)	4–5	-	Grape, papaya, pistachio, rice, tomato	-	Secalonic acid D& F
A. japonicus (Saito 1906)	4–5	white to cream, 0.5	Grape, green coffee berries, pineapple, sesame seed	-	Secalonic acid D& F
A. uvarum (Perrone *et al.* submitted)	3–4	dark brown to black	Grape	-	Secalonic acid D, geodin, erdin, asterric acid

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/aculeatus* 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 45–55 % 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

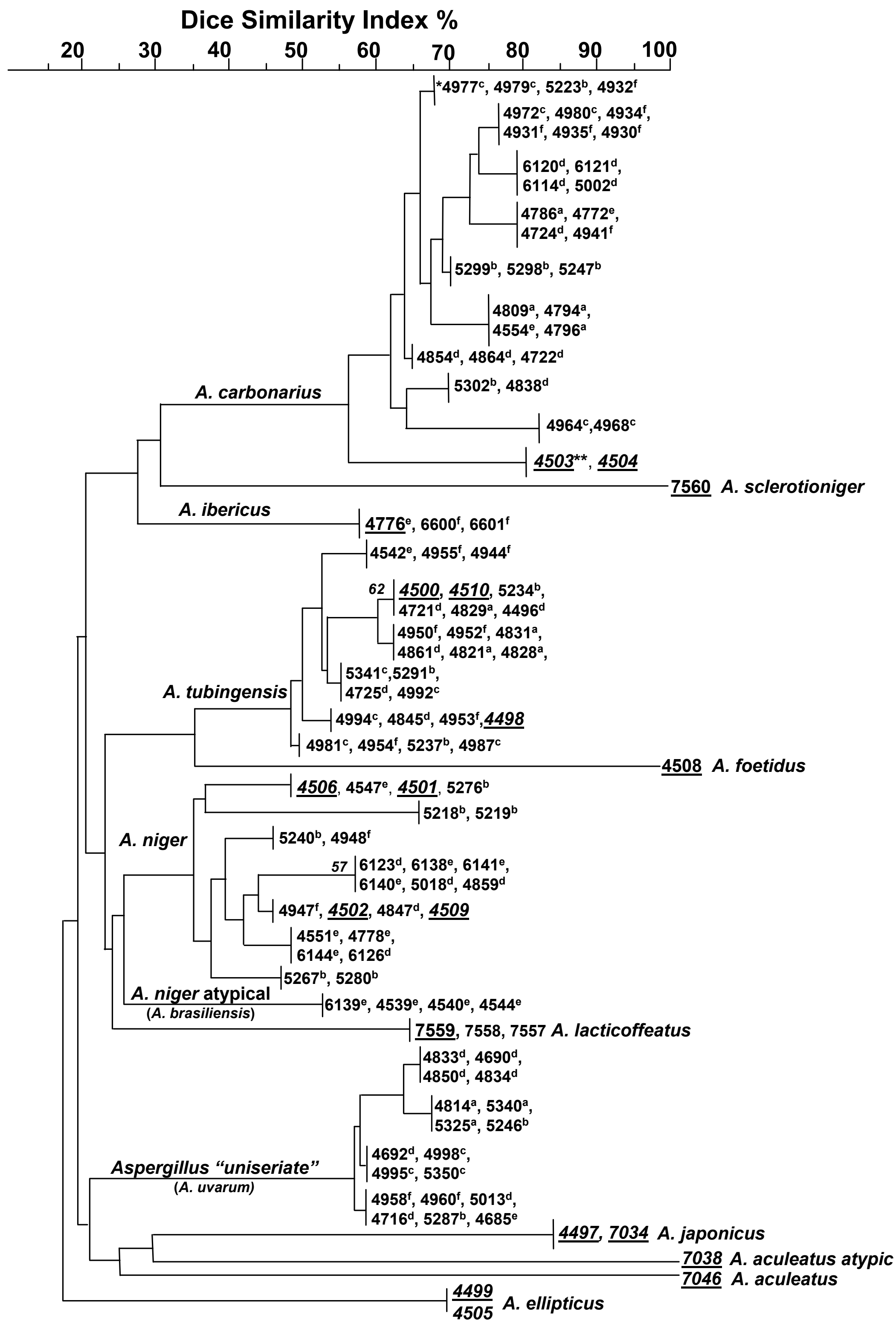

Fig. 1. AFLP dendrogram evidencing molecular biodiversity of representative black aspergilli isolated from grape in Europe.

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

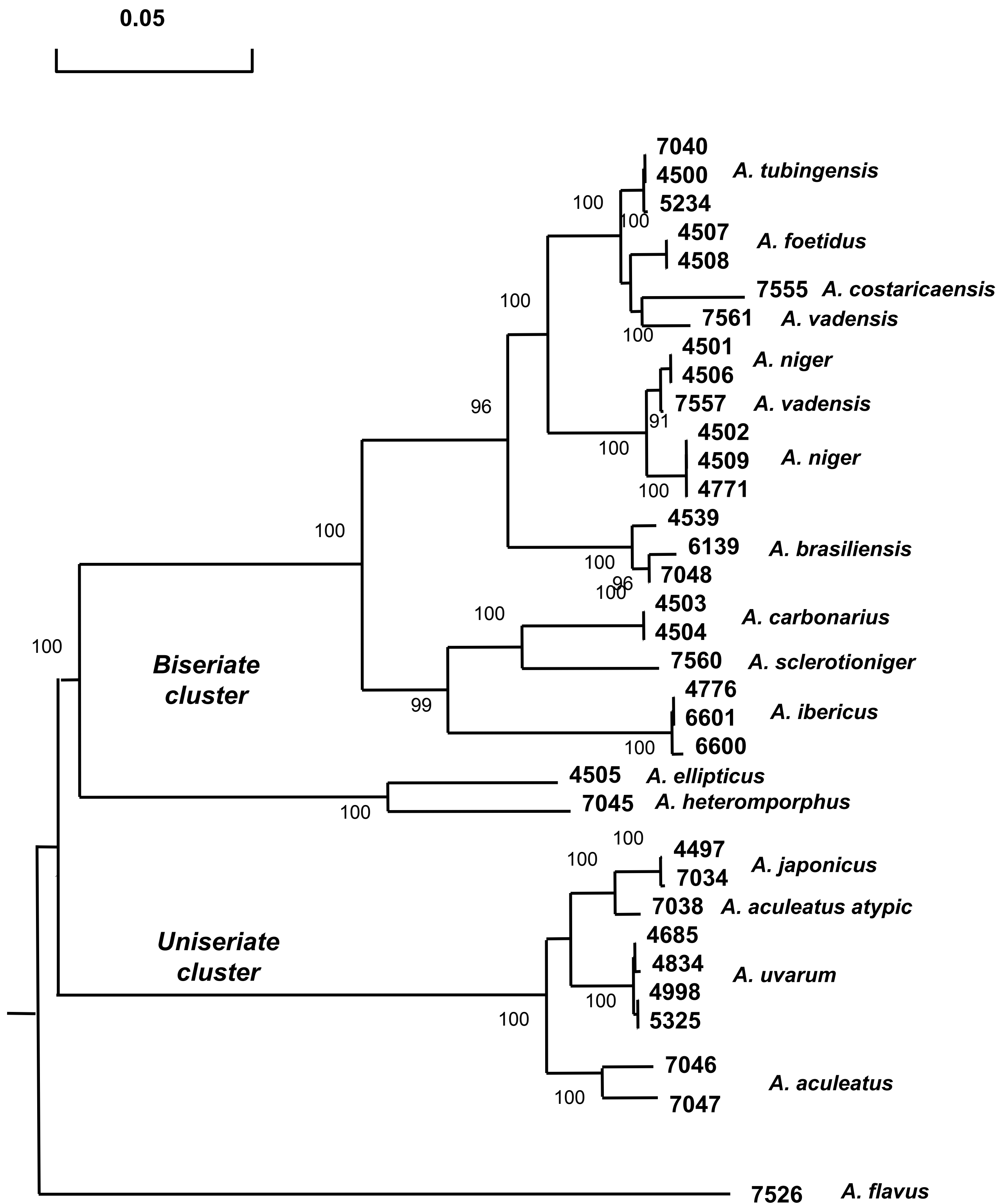

Fig. 2. Phylogenetic tree based on calmodulin sequence data of *Aspergillus* section *Nigri*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.
* Strains were labelled using accession number of ITEM, Culture Collection of Agri-Food Important Toxigenic Fungi, ISPA-CNR, 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

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.

evidence need to be further confirmed as recommended by Frisvad *et al*. (2006).

In conclusion, a different species distribution of black aspergilli may occur in Europe in relation with metereological conditions (Battilani *et al.* 2006) and geographical areas: *A. tubingensis* and *A. niger* proved to be the dominant species in all countries, while *A. carbonarius* appears to be prevalent in southern Mediterranean areas (south of France, Southern Italy, Portugal and Greece). The distribution of *A. ibericus* is limited to Spain and Portugal, while *A. uvarum* occurs more frequently in Italy, France, Greece and Israel.

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. sclerotioniger* 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* occured 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 Ilic *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* species. One of them (*A. aculeatinus)* is related to *A. aculeatus* and other uniseriate black aspergilli and could be recovered from both regions, while the other one (*A. sclerotiicarbonarius)* 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. sclerotiicarbonarius* were found only in Southern Thailand 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:

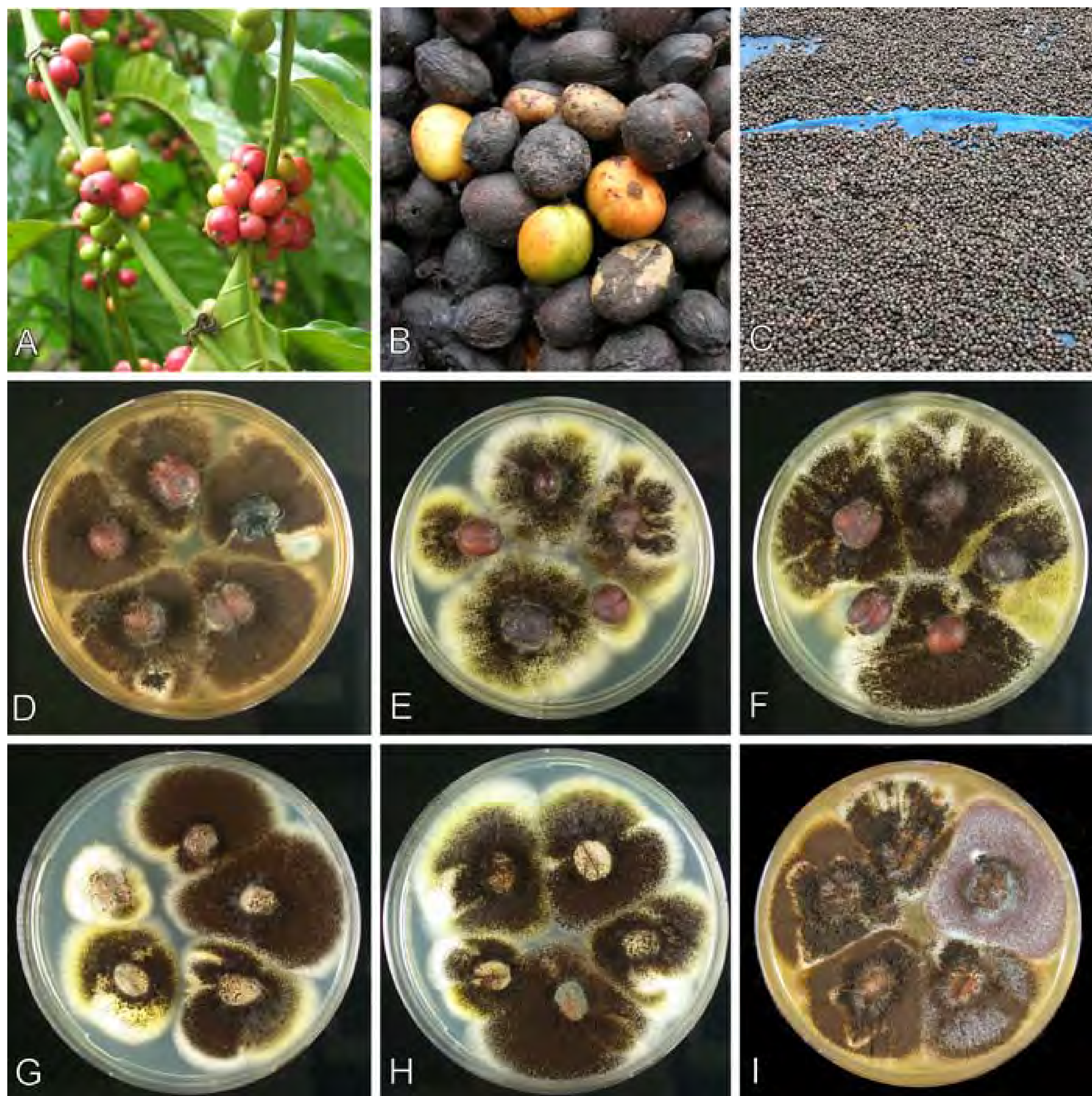

Fig. 4. Robusta coffee. A. Ripe cherries on tree. B. dried cherries. C. dried coffee beans in drying yard. D–F. direct plating of coffee cherries on MEA and DG18. G–I. direct plating of green coffee beans on MEA and DG18.

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

Arabica (Northern Thailand)	Robusta (Southern Thailand)	Ochratoxin A production	Ochratoxin B production
A. niger (44 %)	*A. niger* (28 %)	++	++
A. tubingensis (19 %)	*A. tubingensis* (17 %)	-	-
A. foetidus (28 %)	-	-	-
A. aculeatinus (9 %)	*A. aculeatinus* (15 %)	-	-
-	*A. carbonarius* (35 %)	+++++	-
-	*A. sclerotiicarbonarius* (5 %)	-	-

In brackets = percent of isolates identified from each type of Thai coffee beans.

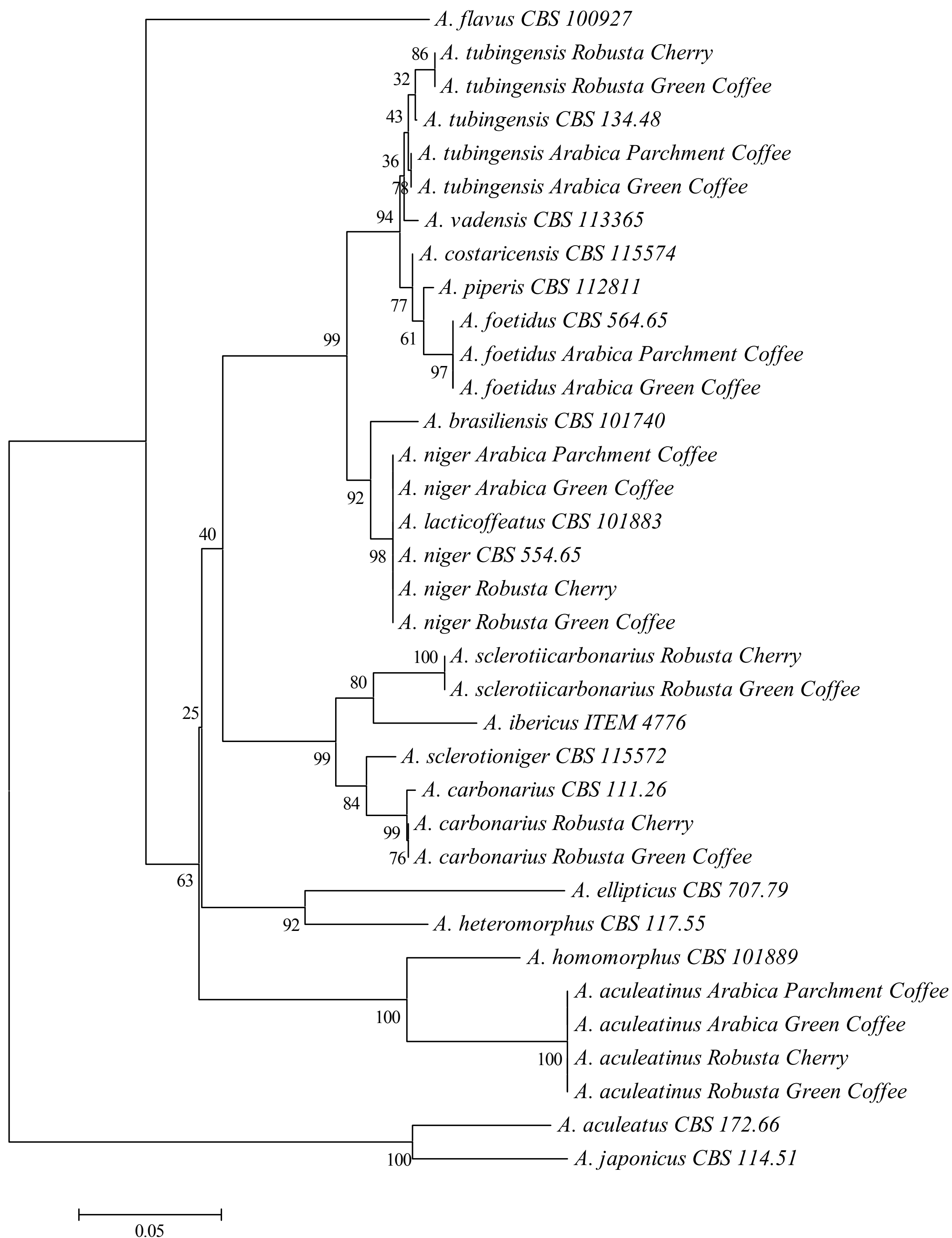

Fig. 5. Neighbour-joining tree based on phylogenic analysis of the partial β-tubulin gene sequences of black aspergilli recovered from Thai coffee.

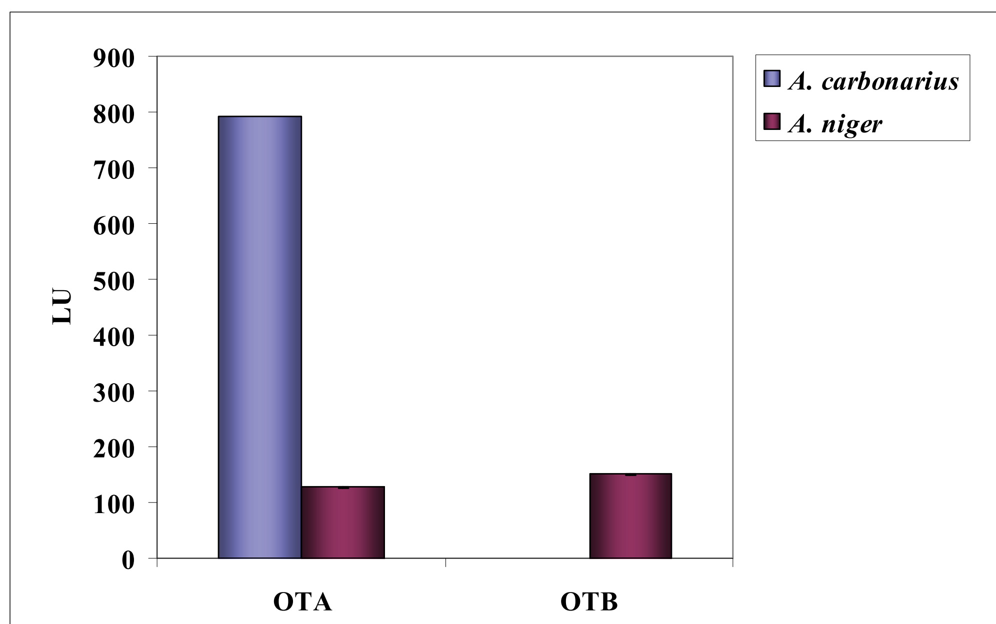

Fig. 6. Comparison of relative average abilities (expressed as Luminescence Unit from Fluorescence detector: LU) to produce ochratoxins of *A. carbonarius* and *A. niger*.

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. niger* 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. sclerotiicarbonarius*, 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).

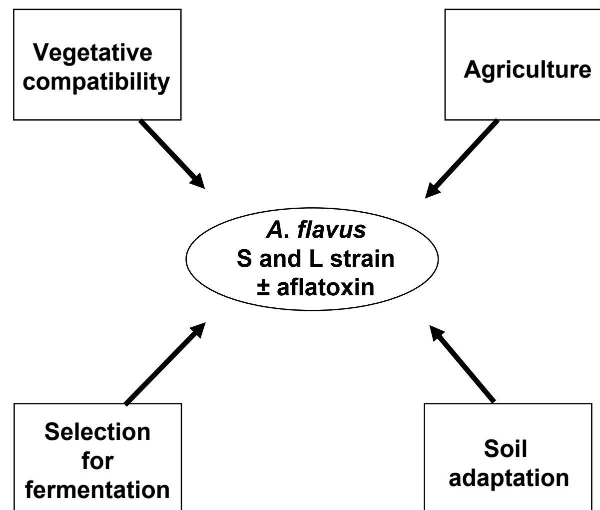

Fig. 7. Causes of *A. flavus* diversity.

A. flavus soil populations also contain isolates from two morphologically distinct sclerotial size variants, termed the L-strain for isolates with average sclerotial size greater than 400 µm and the S-strain for isolates with sclerotial size less that 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 omtA 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 Neighboured 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 (Wilkinson *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 neded 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* Quinquaud 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 *Alternaria, 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. Zalessky 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

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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 telomorph 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 sclerotial 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 cinnamopurpureum*. 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. hirayamae* 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 teleomorphs for more than 20 years. Species where teleomorphs 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 users of dual nomenclature – have been applying this principle for the past 20 years, with a notable improvement in understanding and communication with users Pitt & Hocking, 1997, Samson *et al.* 2004). This system is totally consistent with that of the ICBN system, that also provides precedence to teleomorphs when they exist.

There is one further important point. If this particular approach to “one name, one fungus” was put into practice, dual nomenclature could be laid to rest, for it is only occasionally that *any particular species* has more than one name in common use for it. As noted above, *E. cinnamopurpureum*, *E. hirayamae* and *E. pinetorum* are examples of that. However, the loss of information from using a single name for these species would be a small price to pay.

Proposal for describing new taxa in *Aspergillus* and their teleomorphs

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 teleomorph 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

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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*.

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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 diminutive 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. Xanthoascins 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 accomodate 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 ºC. 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 discolouration 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.* 1969), 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.* 1986; 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), otomycosis (Yasin *et al*. 1978; Falser 1983), brain granuloma (Linares *et al.* 1971) and onychomycosis (Schonborn & Schmoranzer 1970; Zaror & Moreno 1980; Piraccini *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. (Curtis *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 terprenins exhibit immunomodulating capabilities and are highly cytotoxic (Shanan *et al.* 1998; Krysinka & Dutkiewicz 2000). There is some evidence that *A. candidus* might be toxic to chickens and rats (Marasas & Smalley 1972) and has also been isolated from birds (Saez 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), terprenins (Kamigauchi *et al.* 1998), chlorflavonin (Bird & Marshall 1969), dechlorochlorflavonin (Marchelli & Vining 1973), xanthoascin (Takahashi *et al.* 1976b), kojic acid (Kinosita & Shikata 1969, Saruno *et al*. 1979, Cole & Cox 1981), 3-nitro-propionic 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* (Timonin & Rouatt 1944; Raper & Fennell 1965). However, some later reports indicate that some isolates may produce citrinin (Kinosita & Shikata 1969; Cole & Cox 1981).

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

Species	Strain No.	Origin
Aspergillus campestris	CBS 348.81[T]	Soil, North Dakota, U.S.A.
Aspergillus candidus	CBS 119.28	IFO 5468; *A. okazakii*
Aspergillus candidus	CBS 116945	Museum dust, Tiel, Netherlands
Aspergillus candidus	CBS 175.68	Mouse dung, Netherlands
Aspergillus candidus	CBS 114385	Air, Finland
Aspergillus candidus	CBS 120.38	No. 827/2; Unknown, J.C. Neill
Aspergillus candidus	CBS 225.80	Human nail, Netherlands
Aspergillus candidus	CBS 102.13	Japan, G. Kita
Aspergillus candidus	CBS 118.28	QM 9372; A. Blochwitz
Aspergillus candidus	CBS 566.65[T]	ATCC 1002; IMI 091889; NRRL 303; unknown, J. Westerdijk
Aspergillus candidus	1-F9	TM 04.129 V11
Aspergillus candidus	13-C4	House, Utrecht, Netherlands
Aspergillus candidus	17-C2	House, Eindhoven, Nertherlands
Aspergillus candidus	25-I1	Indoor environment, Germany
Aspergillus candidus	IMI 091889	ATCC 1002, CBS 566.65
Aspergillus candidus	CBS 283.95	IFO 33019; JCM 10250; SRRC 310
Aspergillus taichungensis	IBT 19404[T]	PF1167; Soil, Taiwan
Aspergillus taichungensis	CBS 567.65	ATCC 16871; IMI 230752; NRRL 312; unknown, Brazil
Aspergillus taichungensis	CBS 112449	Indoor environment, Germany
Aspergillus tritici	CBS 119225	SLV 541; wheat flour, Sweden
Aspergillus tritici	CBS 117270	Djambee (drum), Ghana
Aspergillus tritici	CBS 266.81[T]	Wheat grain, India
Aspergillus tritici	11-H7	Feed ingredient, Netherlands
Aspergillus tritici	SZMC 0565	Viticultural Instittute, Kecskemet, Hungary
Aspergillus tritici	CBS 283.95	ATCC 13686=IMI 78734=NRRL 2297; P.G. Stansly, B81
Aspergillus tritici	SZMC 0897	Agricultural Service, Bekes county, Hungary
Aspergillus implicatus	CBS 484.95[T]	Soil, Ivory Coast

The description of *A. candidus* is admittedly broad, encompassing considerable variability among the isolates (Raper & Fennell 1965, Kozakiewicz 1989). *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 *Aspergillus* species described in the past have been synonymised with *A. candidus*, including *A. albus, A. okazakii*, or *A. dubius* (Raper & Fennell 1965). Raper & Fennell (1965) also stated that "it is possible that our current concept of *A. candidus* is too broad". Recent studies indicated that other species including *A. campestris* (Christensen 1982; Rahbaek *et al.* 2000; Peterson 2000; Varga *et al.* 2000) and *A. taichungensis* (Yaguchi *et al.* 1995, Rahbaek *et al.* 2000) are also members of section *Candidi*. Besides, two other white-spored species, *A. tritici* (as *A. triticus*, Mehrotra & Basu 1976) and *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 *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 β-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 Smedsgaard (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

% (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

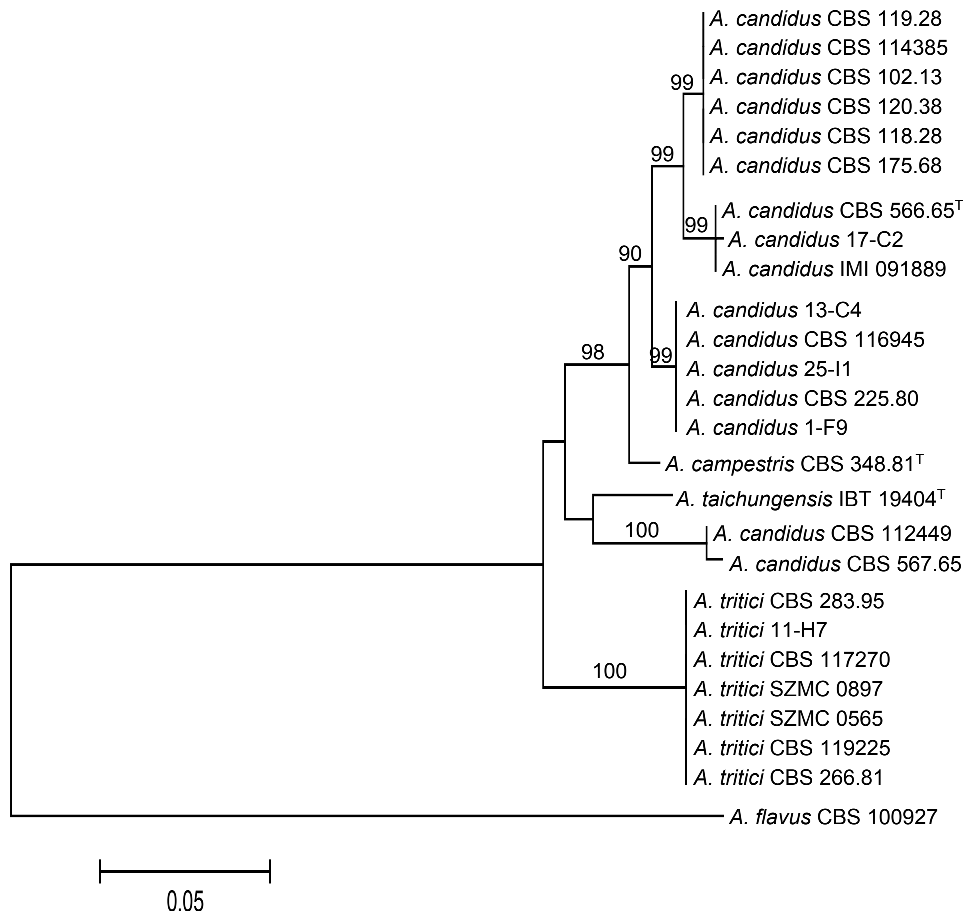

Fig. 1. Neighbour-joining tree based on β-tubulin sequence data of *Aspergillus* section *Candidi*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

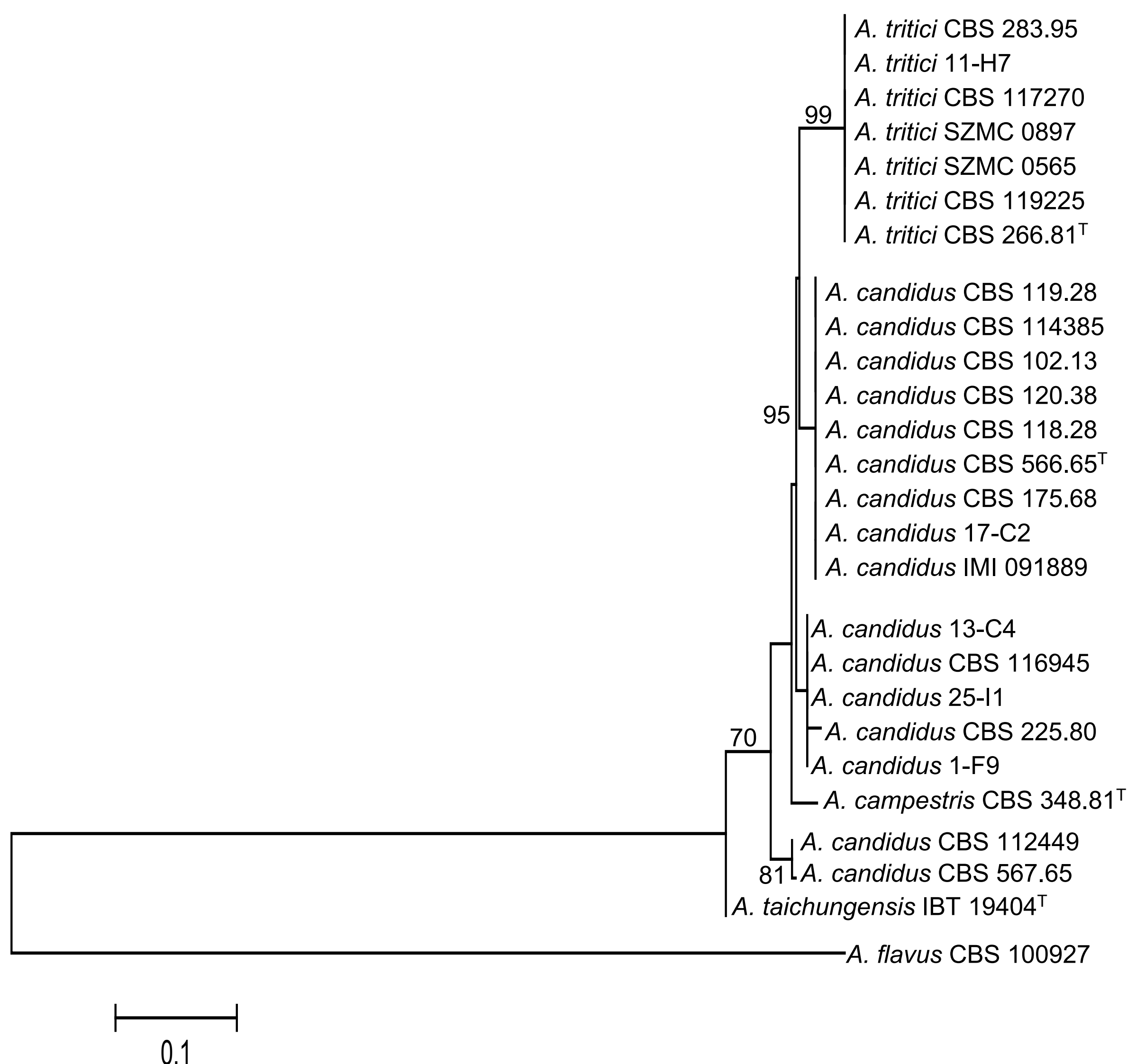

Fig. 2. Neighbour-joining tree based on calmodulin sequence data of *Aspergillus* section *Candidi*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

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: 0.9633, retention 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 10^5 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

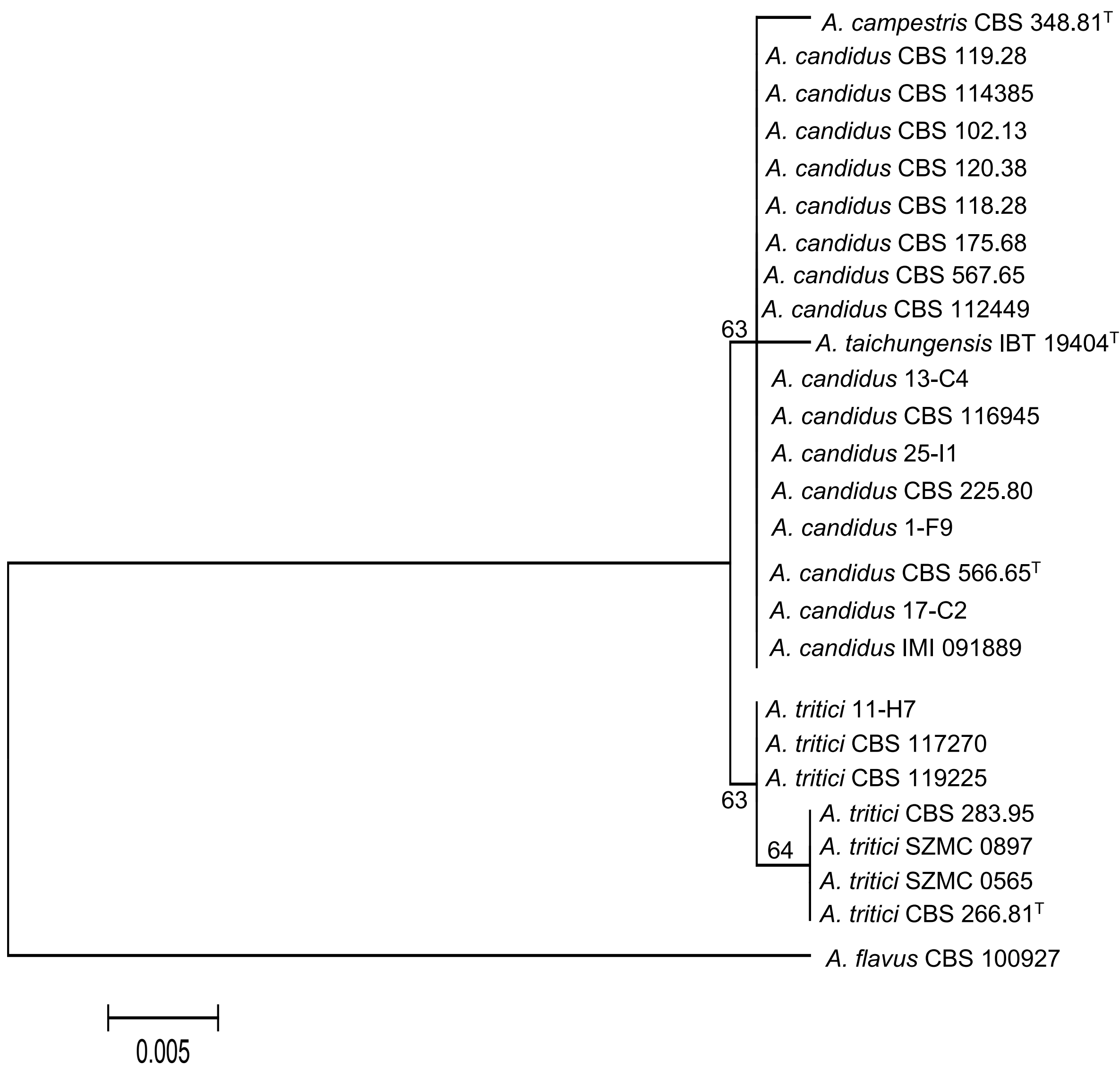

Fig. 3. Neighbour-joining tree based on ITS sequence data of *Aspergillus* section *Candidi*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

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.

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

	A. candidus	*A. tritici*	*A. taichungensis*	*A. campestris*
Morphological characteristics				
Colony colour	white	Light cream	Light cream	Sulphur yellow
Colony reverse	Uncoloured to yellowish	Light brown	Uncoloured	Uncoloured
Conidial heads	Globose	Radiate	Radiate	Radiate
Conidiophores	Smooth, 500–1000 µm	Septate, 130–700 µm	Smooth, 300–400 µm	Smooth, 400–800 µm
Diminutive heads	Common	Common	Common	Common
Vesicles	Globose, 40 µm	Elongated, 5–11 µm	Hemispherical, 5–20 µm	Globose, 25–40 µm
Conidial ornamentation	Smooth	Slightly roughened	Microverrucose	Smooth
Conidial shape	(Sub)globose	(Sub)globose	(Sub)globose	Ellipsoidal
Size of conidia	2.5–3.5 µm	2.7–3.5 µm	3–5 µm	3–4 × 2.3–3 µm
Growth at 37ºC	-	+	+	-
Sclerotia	Purple to black	Purple to black	Dark brown	-
Extrolite production				
Candidusin A	+	-	-	-
Candidusin B	+	+	-	-
Candidusin C	-	-	+	+
Candidusin analogue	-	+	-	-
terphenyllin	+	+	+	+
3-hydroxyterphenyllin	+	+	+	-
chlorflavonin	+	+	-	+
chlorflavonin analogue	+	-	-	-

Dark brown sclerotia which appear on MEA after more that 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, smooth, often diminutive (up to 100 µm long, biseriate), vesicles globose to slightly elongate, 25–40 µm in diam, fertile over the entire surface, conidia thin-walled, hyaline, pale yellow in mass, slightly ellipsoidal, 3–4 × 2.3–3 µm. Sclerotia not observed. *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.

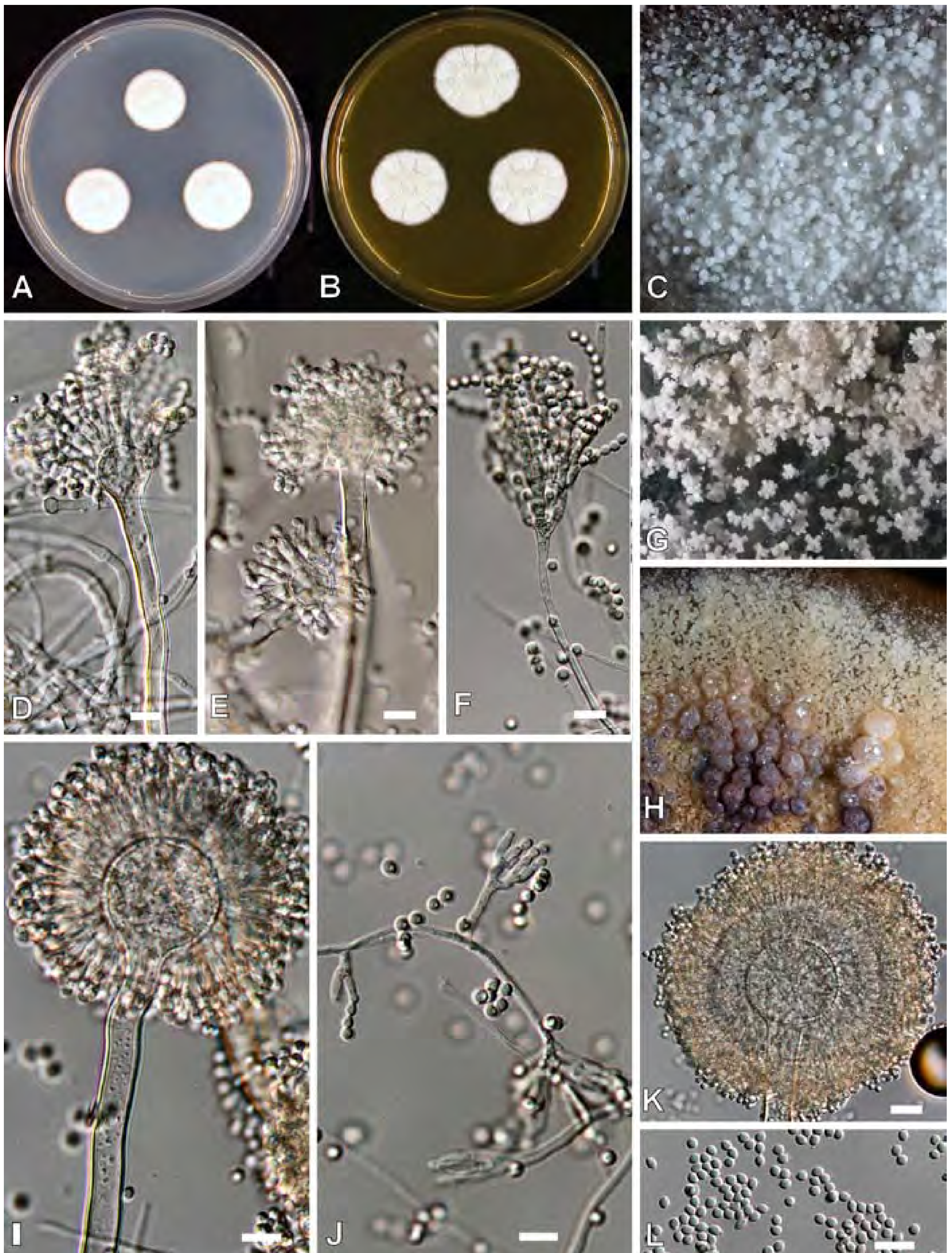

Fig. 4. *Aspergillus candidus*. A–B Colonies after 7 d at 25 °C A. CYA. B. MEA. C, G. Conidial heads. D–F, H–K. Conidiophores. H. Sclerotia. L. Conidia. Scale bars = 10 µm.

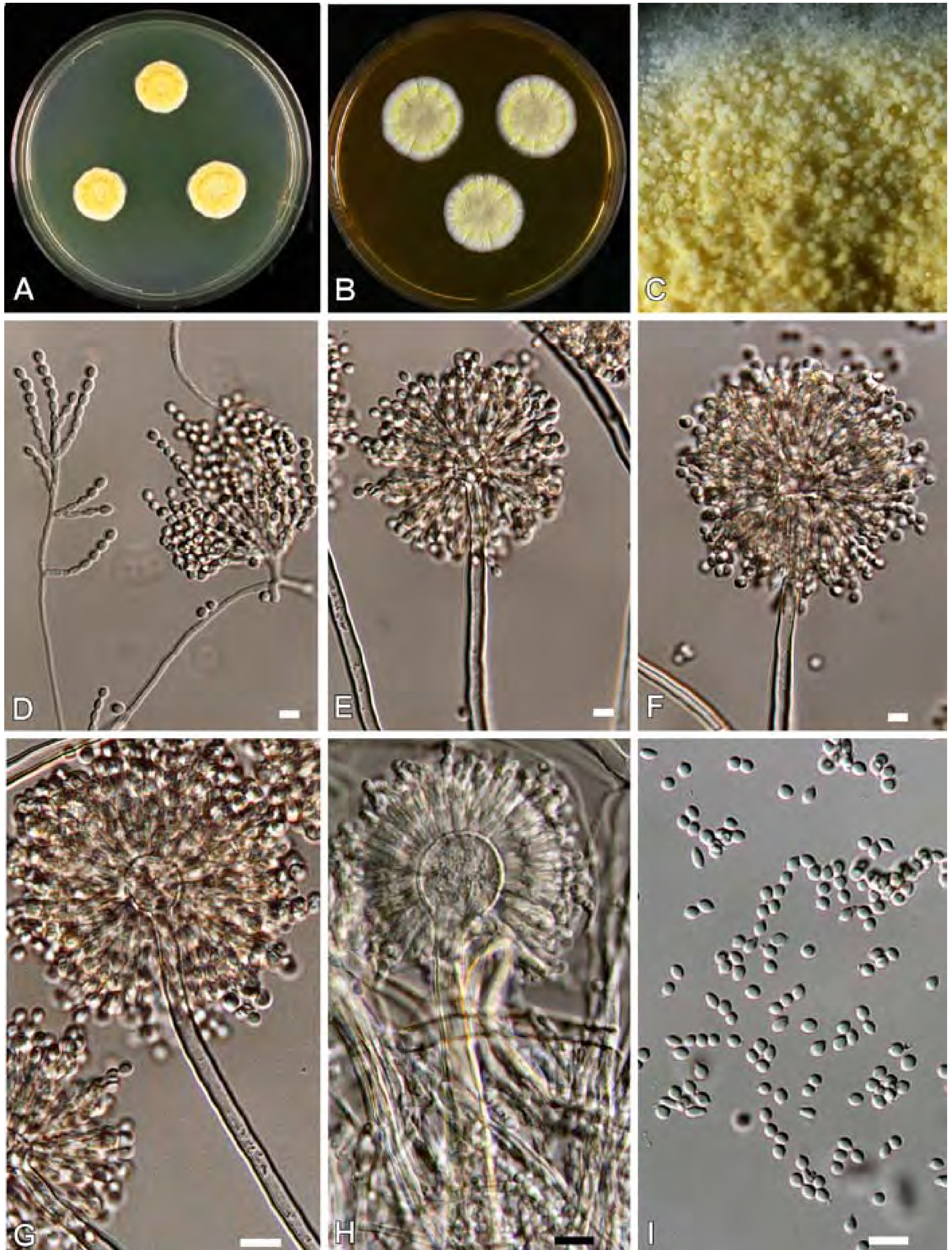

Fig. 5 *Aspergillus campestris.* A–B Colonies after 7 d at 25 °C A. CYA. B. MEA. C. Conidial heads. D–H. Conidiophores. I. Conidia. Scale bars = 10 µm.

Aspergillus 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 diam: CZA25: 10–12 mm; CYA25: 10–15 mm, MEA25: 7–10 mm, YES25: 18–24 mm, OA25: 9–12 mm, CYA37: 0 mm, CREA25: poor growth, no acid production
Colony colour: sulphur yellow to pinard yellow
Conidiation: abundant
Reverse colour (CZA): uncoloured
Colony texture: velvety
Conidial head: radiate, splitting in age
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: KACC 42091, KACC 42090 = IBT 27920, KACC 41955 = IBT 3016, UAMH 1324 (from mouse, Canada, as *A. sulphureus*), IBT 17867

Diagnostic features: restricted growth on all media, sulphur yellow colony colour and diminutive conidial heads

Similar species: -

Ecology and habitats: soil

Distribution: U.S.A., Canada

Extrolites: candidusin C, terphenyllins, chlorflavonin (Rahbaek *et al.* 2000), confirmed in this study

Pathogenicity: not reported

Note: Diminutive conidial heads commonly produced (100 × 10–12 µm)

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 1995; 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

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, Czechoslovakia, 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""-deoxyprenylterphenyllin, 4""-deoxyisoterprenin, 4""-deoxyterprenin (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 terprenins (Kamigauchi *et al.* 1998), chlorflavonin (Bird & Marshall 1969; Munden *et al.* 1970), dechlorochlorflavonin (Marchelli & Vining 1973), and xanthoascin (Takahashi *et al.* 1976a). The production of terphenyllin, 3-hydroxyterphenyllin, candidusin A, candidusin B, chlorflavonin and xanthoascin was confirmed by HPLC-DAD.

Extrolites not produced by *A. candidus*: kojic acid (Kinosita & Shikata 1969; Cole & Cox 1981), and 3-nitro-propionic acid (Kinosita *et al.* 1968) were reported from the same strain of *A. candidus* of which ATCC 44054 is representative. A re-examination of that strain showed that it was a white-spored mutant of *Aspergillus flavus*, a known producer of these two metabolites. The asterriquinone analogs, neoasterriquinone and isoasterriquinone (Alvi *et al.* 1999) have not been found in any strains of *A. candidus* by us. These asterriquinone analogues are probably produced by *A. niveus*, but this has to be confirmed by examination of isolates of the latter species. Citrinin production was observed in some studies (Timonin & Rouatt 1944; Kinosita & Shikata 1969), but the producing fungus was later identified as *A. niveus* (NRRL 1955, Raper and Fennell, 1965). The production of 6-sulfoaminopenicillanic acid by *A. candidus* (Yamashita *et al.* 1983) has not been confirmed

Pathogenicity: Pathogenicity of *A. candidus* is rather improbable, as this species cannot grow at 37 °C., however pathogenicity has often been reported: *A. candidus* has been claimed to be involved in a wide range of human infections including invasive aspergillosis (Rippon 1988; Ribeiro *et al.* 2005), pulmonary aspergillosis (Iwasaki *et al.* 1991), aspergilloma (Avanzini *et al.* 1991), otomycosis (Yasin *et al*. 1978; Falser 1983), brain granuloma (Linares *et al.* 1971) and onychomycosis (Kaben 1962; Fragner & Kubackova 1974; Cornere & Eastman 1975; Piraccini *et al.* 2002; Schonborn & Schmoranzer 1970; Zaror & Moreno 1980); also caused various disorders in pigs (Moreau 1979). In these cases it is more likely caused by white spored mutants of *A. flavus* or by *A. tritici*

Note: young heads varying in the same culture from globose masses 200 to 300 µm in diam to small heads less than 100 µm in diam; some isolates produce purple to black sclerotia

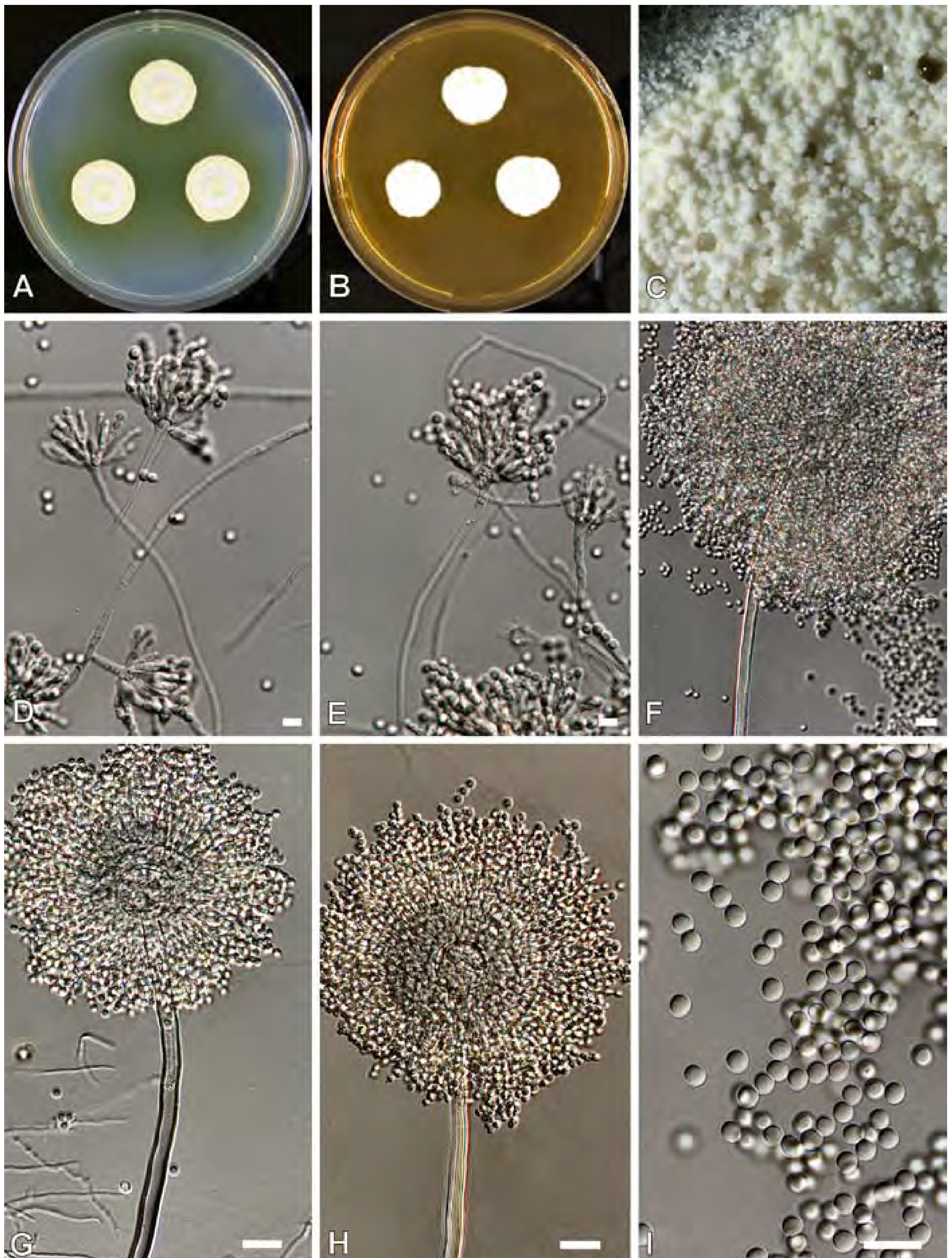

Fig. 6. *Aspergillus taichungensis*. A–B Colonies after 7 d at 25 °C A. CYA. B. MEA. C. Conidial heads. D–H Conidiophores. I. Conidia. Scale bars = 10 µm.

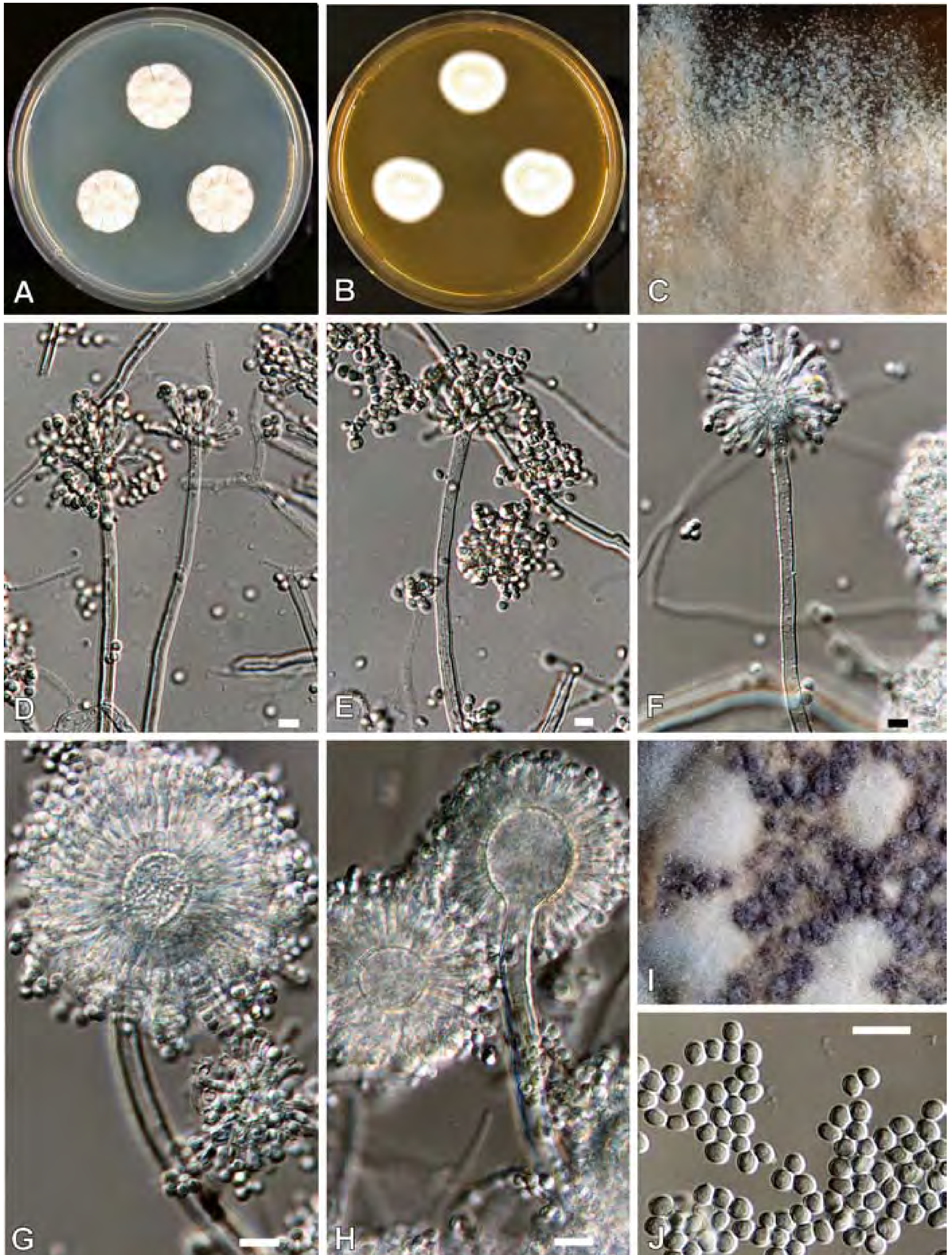

Fig. 7. *Aspergillus tritici*. A–B Colonies after 7 d at 25 °C A. CYA. B. MEA. C. Conidial heads. D–F, G–H.Conidiophores. I. Sclerota. J. Conidia. Scale bars = 10 µm.

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 diam: CZA25: 12–15 mm; CYA25: 17–20 mm, MEA25: 9–13 mm in 7 d, YES25: 25–28 mm, OA25: 12–16 mm, CYA37: 7–10 mm, CREA25: poor growth, no acid production
Colony colour: yellowish white to primrose
Conidiation: 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

Extrolites: candidusin C, terphenyllin, 3-hydroxyterphenyllin (Rahbaek *et al.* 2000, and confirmed in this study). A large number of additional extrolites, 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 Mehrotra & 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 diam (7 d): CZA25: 18–23 mm; CYA25: 16–29 mm, MEA25: 11–17 mm, YES25: 18–41 mm, OA25: 13–25 mm, CYA37: 7–21 mm, CREA25: poor growth, no acid production
Colony colour: white to light cream coloured
Conidiation: 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

Extrolites: 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

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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* (synonyms: *A. apicalis*, *A. pallidus*), *A. giganteus, A. rhizopodus*, *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 tryptoquivalins, while *Dichotomomyces* was found to be able to produce gliotoxin, which is also produced by some *Neosartorya* species, and tryptoquivalines and tryptoquivalones 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 antafumicin, tryptoquivalines, 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 *Aspergillus* 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), tryptoquivalines and tryptoquivalones (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*. 1943 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 clavatus* (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 immunotherapy of 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; Sarbhoy & Elphick 1968; Huang & Raper 1971; Yaguchi *et al.* 1993).

Aspergillus subgenus *Fumigati* section *Clavati* (Gams *et al.* 1985; Peterson 2000), 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. rhizopodus* (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 to section *Ornati*. Also, their major ubiquinone systems point in this direction as section *Clavati* and *A. acanthosporus* have Q10, while *H. 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

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Table 1. The *Aspergillus* section *Clavati* isolates examined in this study.

Species	Strain No.	Origin
A. clavatus	CBS 104.45	ATCC 9600; Czech Republic, Pribram
	CBS 105.45	Church, No. Ac 87
	CBS 106.45	*Humulus lupulus* (Cannabaceae), G. Smith
	CBS 114.48	Culture contaminant, Netherlands
	CBS 513.65[T]	ATCC 1007; IMI 015949; NRRL 1; Thom 107
	CBS 514.65	ATCC 10058; IMI 321306; NRRL 4; Thom 4754.3
	CBS 470.91	Toxic feed pellets, Hungary
	CBS 116685	Milled rice, Netherlands
	CBS 118451	Medicine, Germany
	DTO 6-F8	Air, ciabatta factory, Netherlands
	DTO 27-C2	Bakery, Netherlands
	SZMC 0918	Soil, Hungary
	SZMC JV4	Stored wheat, Hungary
	SZMC JV1.1	Human mucosa, Hungary
	IMI 358435	Feed pellet, Hungary
A. giganteus	CBS 117.45	IMI 024256; P. Biourge
	CBS 119.48	H. Burgeff, No. 382, Germany
	CBS 118.49	Wood of ship (*Virola surinamensis*), Suriname
	CBS 122.53	Tail borad, Nigeria
	CBS 117.56	Wood in swimming pool, Netherlands
	CBS 101.64	Unknown, Poland
	CBS 515.65[T]	ATCC 16439; IMI 235601; NRRL 7974; mouse dung, U.S.A.
	CBS 526.65	ATCC 10059; IMI 227678; NRRL 10; Thom 5581.13A
	CBS 112.27	A. Blochwitz
A. rhizopodus	CBS 450.75[T]	Usar soil, India, Lucknow
	IMI 351309	Soil, Yugoslavia
A. pallidus	CBS 344.67[T]	ATCC 18327; IMI 129967; soil, Moldova
	SZMC JV6	Culture contaminant, Hungary
A. clavatonanicus	CBS 474.65[T]	ATCC 12413; IMI 235352; WB 4741; finger nail lesion, Brazil
A. longivesica	CBS 530.71[T]	ATCC 22434; IMI 156966; soil, Nigeria
	CBS 187.77	Soil, Ivory Coast, Tai
A. apicalis	CBS 236.81[T]	Wheat bran, India
N. acanthosporus	CBS 558.71[T]	Solomon Islands, Bougainville Island
	CBS 445.75	Solomon Islands, Bougainville Island, Buin, Malapita
	CBS 446.75	Solomon Islands, Bougainville Island, Buin, Batubatuai
	CBS 447.75	Solomon Islands, Bougainville Island, Kieta
D. cepjii var. *cejpii*	CBS 761.96	spent mushroom compost, Netherlands
D. cepjii var. *cejpii*	CBS 779.70	Soil, Cincinatti, U.S.A.
D. cepjii var. *cejpii*	CBS 100192	Soil, Bratislava, Slovakia
D. cepjii var. *cejpii*	CBS 474.77	Soil, Egypt
D. cepjii var. *cejpii*	CBS 780.70	Pasturised milk, Cincinatti, U.S.A.
D. cepjii var. *cejpii*	CBS 397.68	Soil, South Africa
D. cepjii var. *cejpii*	CBS 345.68	rhizosphere of *Hordeum vulgare*, Pakistan
D. cepjii var. *cejpii*	CBS 159.67	Soil, Kominato, Japan
D. cepjii var. *cejpii*	CBS 157.66[T]	Orchard soil, Moldova, near Tiraspol
D. cepjii var. *spinosus*	CBS 219.67[T]	Soil, Kyoto, Japan

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* Mehrotra & 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 alkalitolerant 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 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 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) (Filtenborg *et al*. 1983; Samson *et al*. 2004). Standards of patulin, cytochalasin E, kotanin, 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 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, 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 10^5 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*

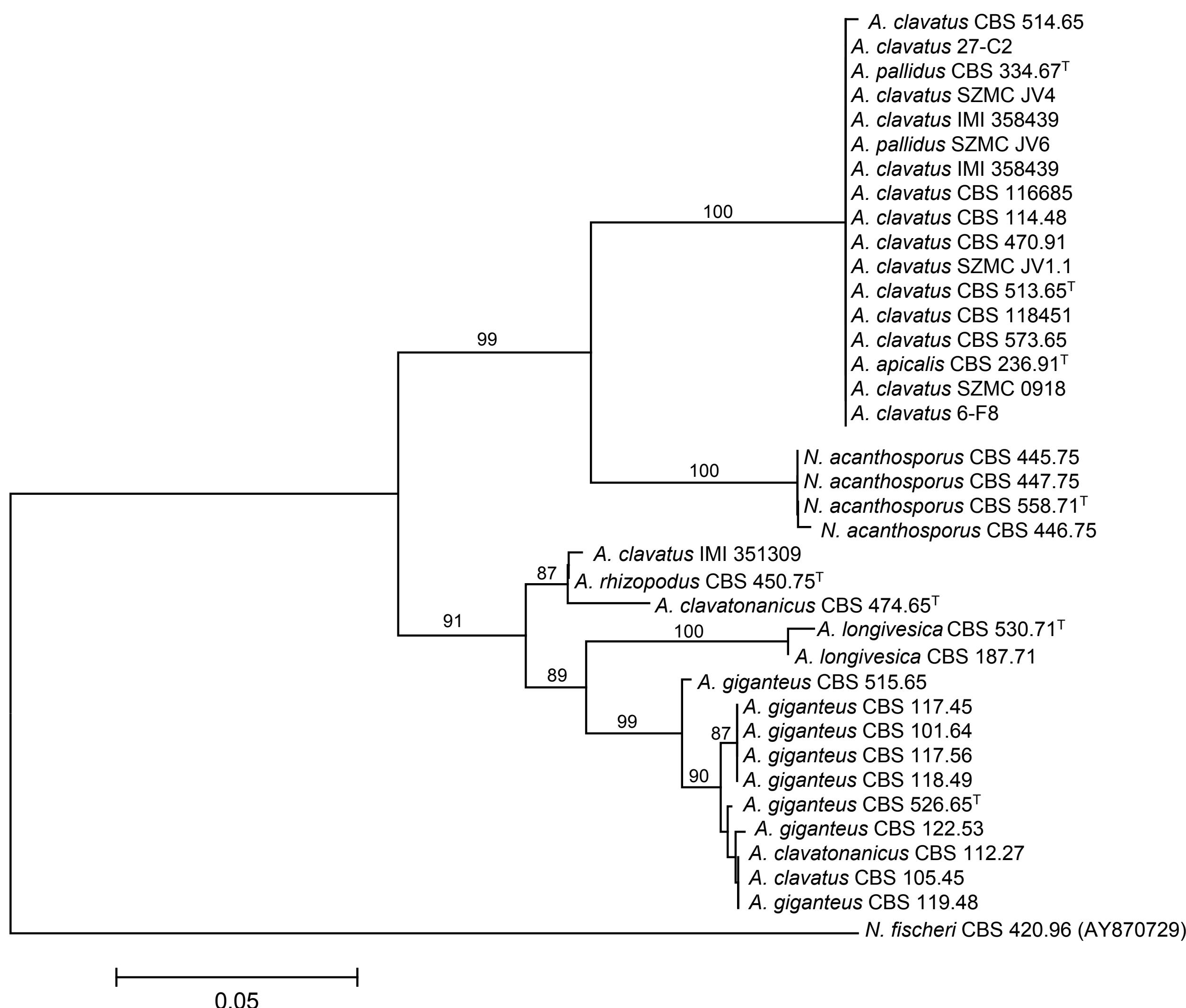

Fig. 1. Neighbour-joining tree based on β-tubulin sequence data of *Aspergillus* section *Clavati*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

(synonyms: *A. pallidus, A. apicalis*), *A. giganteus, A. longivesica, 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. longivesica* 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. longivesica*. 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 phospodiester 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. glabra* 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.*

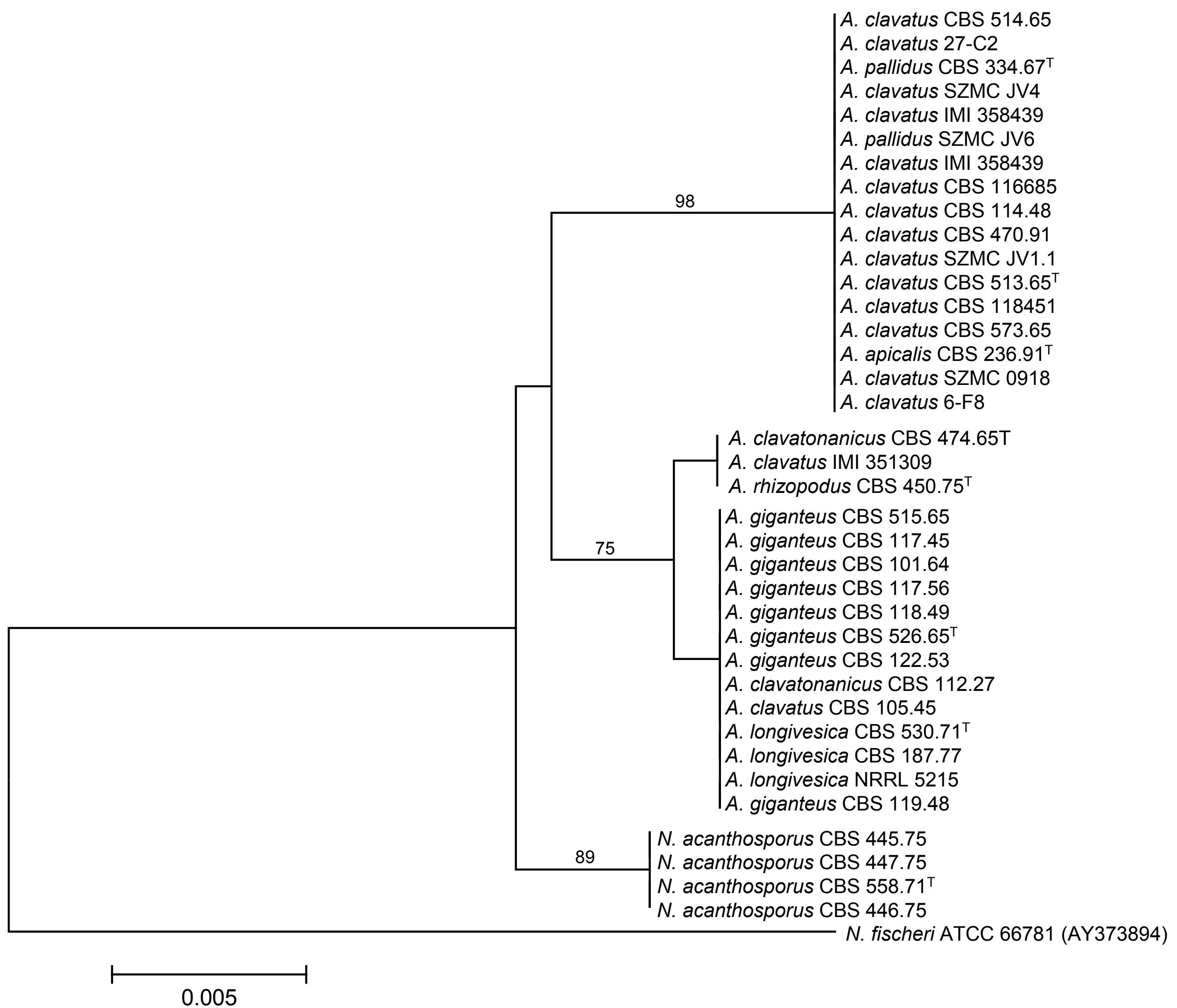

Fig. 2. Neighbour-joining tree based on ITS sequence data of *Aspergillus* section *Clavati*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

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* teleomorphs. We examined the relationships of these teleomorphs 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 cleithothecia (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

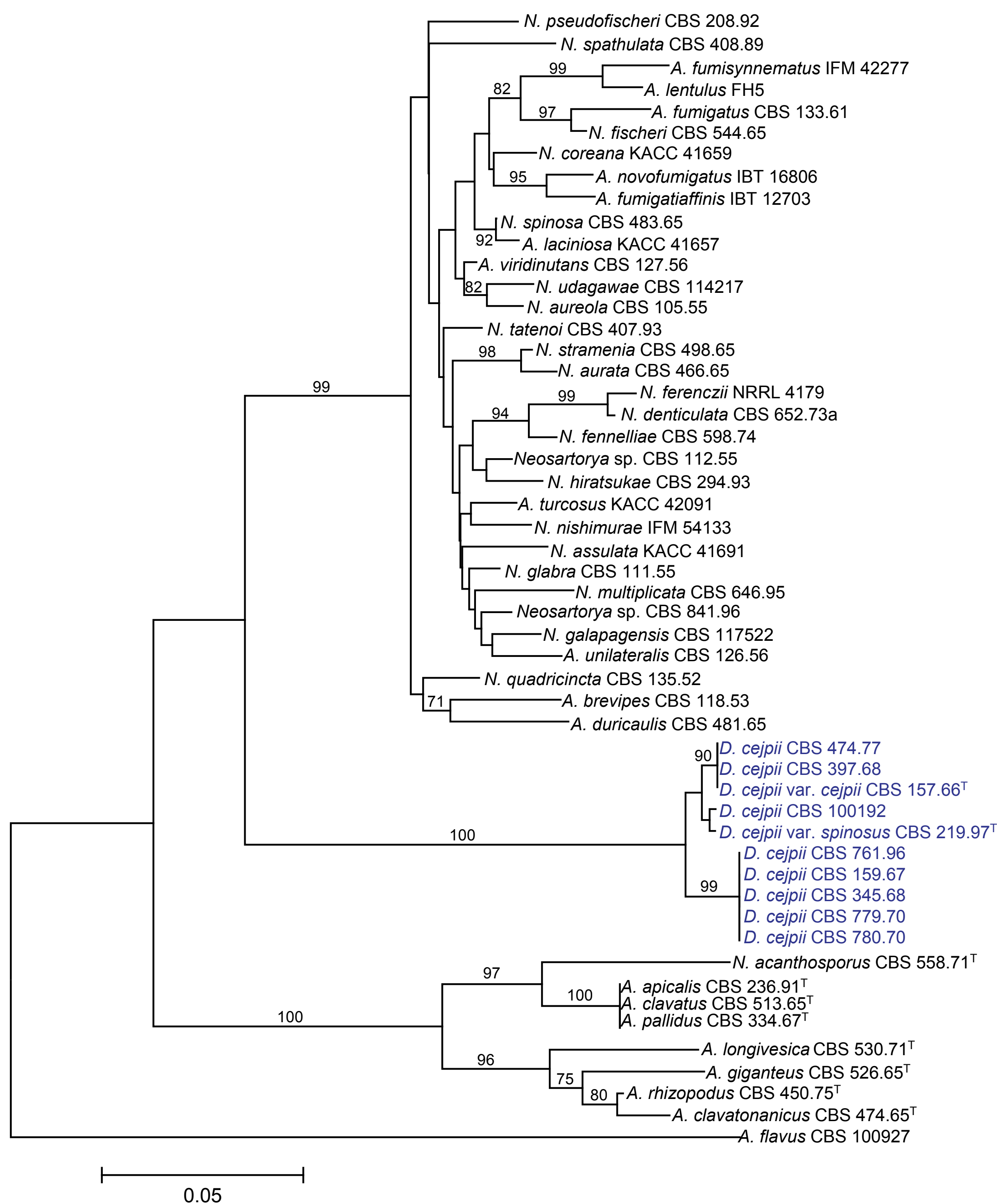

Fig. 3. Neighbour-joining tree based on β-tubulin sequence data of *Neosartorya*, *Neocarpenteles*, *Dichotomomyces* species and their asexual relatives. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

Table 2. Extrolite 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.

Species	Extrolites
A. clavatonanicus	antafumicins, glyanthrypine, kotanins, tryptoquivalines, tryptoquivalones
A. clavatus	patulin, cytochalasin E & K, kotanins, antafumicin, (dehydrocarolic acid), tryptoquivalones, tryptoquivalines, ascladiol, ribotoxins
A. giganteus	patulin, antafumicin, ascladiol, tryptoquivalones; tryptoquivalines, glyanthrypine, pyripyropen, α-sarcin and other ribotoxins
A. longivesica	patulin, tryptoquivalones, tryptoquivalines, antafumicins, pyripyropen
A. rhizopodus	pseurotins, dehydrocarolic acid, tryptoquivalines, tryptoquivalones, kotanins, cytochalasins
N. acanthosporus	kotanins, tryptoquivalines, tryptoquivalones, ribotoxins
D. cejpii	gliotoxin, tryptoquivalones, rubratoxins, (xanthocillin X)

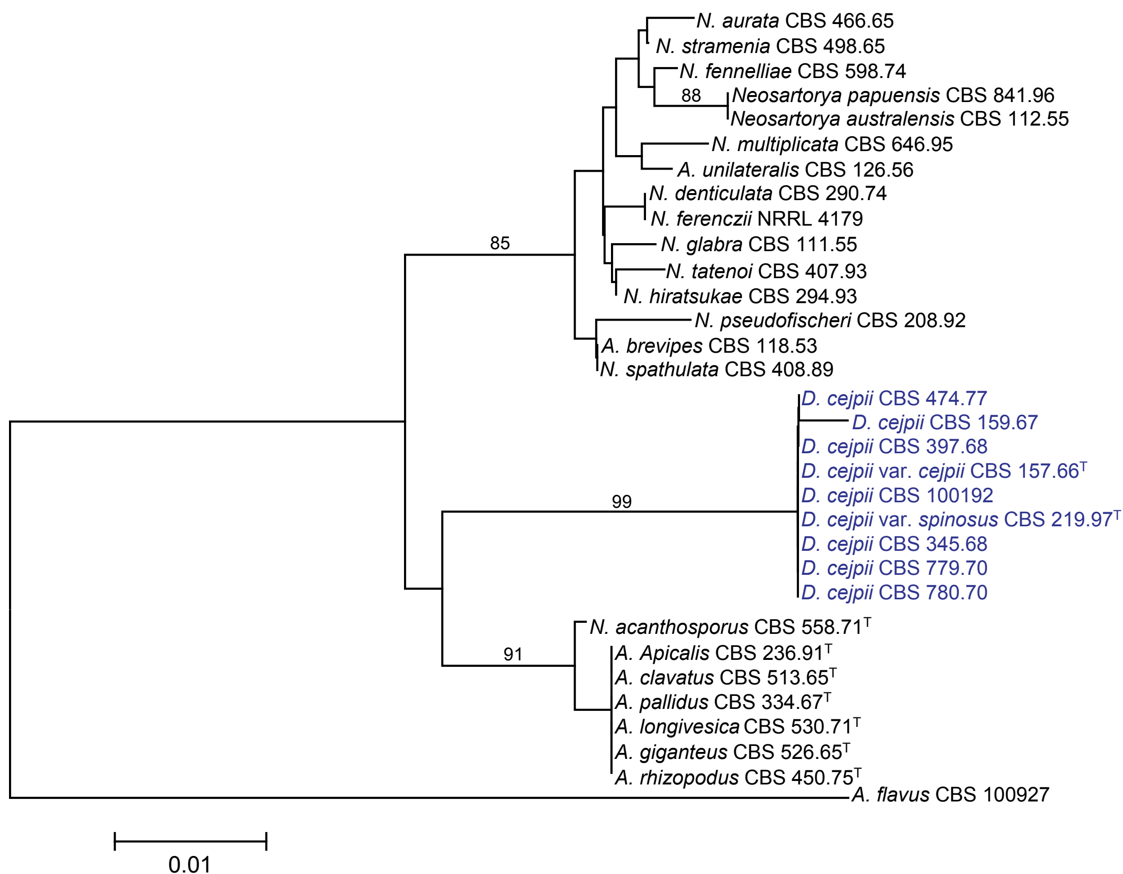

Fig. 4. Neighbour-joining tree based on ITS sequence data of *Neosartorya*, *Neocarpenteles*, *Dichotomomyces* species and their asexual relatives. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

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* (Malloch & 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. 3. The topology of the tree is the same as one of the 22 maximum parsimony trees constructed by the PAUP program (length: 738 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 *Dichotomomyces* 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. crateriforme* (Frisvad 1989; Sigler *et al.* 1996; Richer *et al*. 1997) [misidentified as *Penicillium purpurogenum* (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* (Vesonder 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 *Dichotomomyces* 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. clavatonanicus* and *N. acanthosporum* to this section. *Hemicarpenteles paradoxus* (synonym: *A. ingratus*) was found to be unrelated to section *Clavati*, but more closely related to *Penicillium*. *Dichotomomyces* and *Neosartorya* were found to be sister clades to the genus *Neocarpenteles*. Further studies are needed to clarify the taxonomic status of *Dichotomomyces* species with *Polypaecilum* anamorphs.

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, YES25: 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
Conidiation: abundant
Reverse colour (CZA): uncoloured to light brownish
Colony texture: floccose
Conidial head: clavate, up to 145–360 × 120–180 µm
Stipe: 40–470 × 6–16 µm, rough walled
Vesicle diam/shape: 22–125 × 5–22 µm, clavate
Conidium size/shape/surface texture: 5–8.5 × 5–6.5 µm, ellipsoid or cylindrical, smooth

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

Extrolites: antafumicins, glyanthrypine, kotanin, tryptoquivalins, tryptoquivalons

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

Aspergillus clavatus Desmazières, Ann. Sci. Nat., Bot. 2: 71, 1834. Fig. 6.
= *Aspergillus pallidus* Kamyschko (1963)
= *Aspergillus apicalis* Mehrotra & 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 015949iv; IMI 015949iii; LSHB Ac86; 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, YES25: 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: 1500–3000 × 20–30 µm
Vesicle diam/shape: 200–250 × 40–60 µm, clavate
Conidium size/shape/surface texture: 3–4.5 × 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-C2, 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

Extrolites: Patulin, cytochalasin E, kotanins, antafumicin, (dehydrocarolic acid), tryptoquivalone, tryptoquivalines, 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, YES25: 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 × 30–50 µm on short conidiophores, 400–600 × 120–180 µm on long ones, clavate
Conidium size/shape/surface texture: 3.5–4.5 × 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

Diagnostic features: produces clavate vesicles in contrast with the elongate ones of *A. longivesica*; do not produce rhizoidal foot

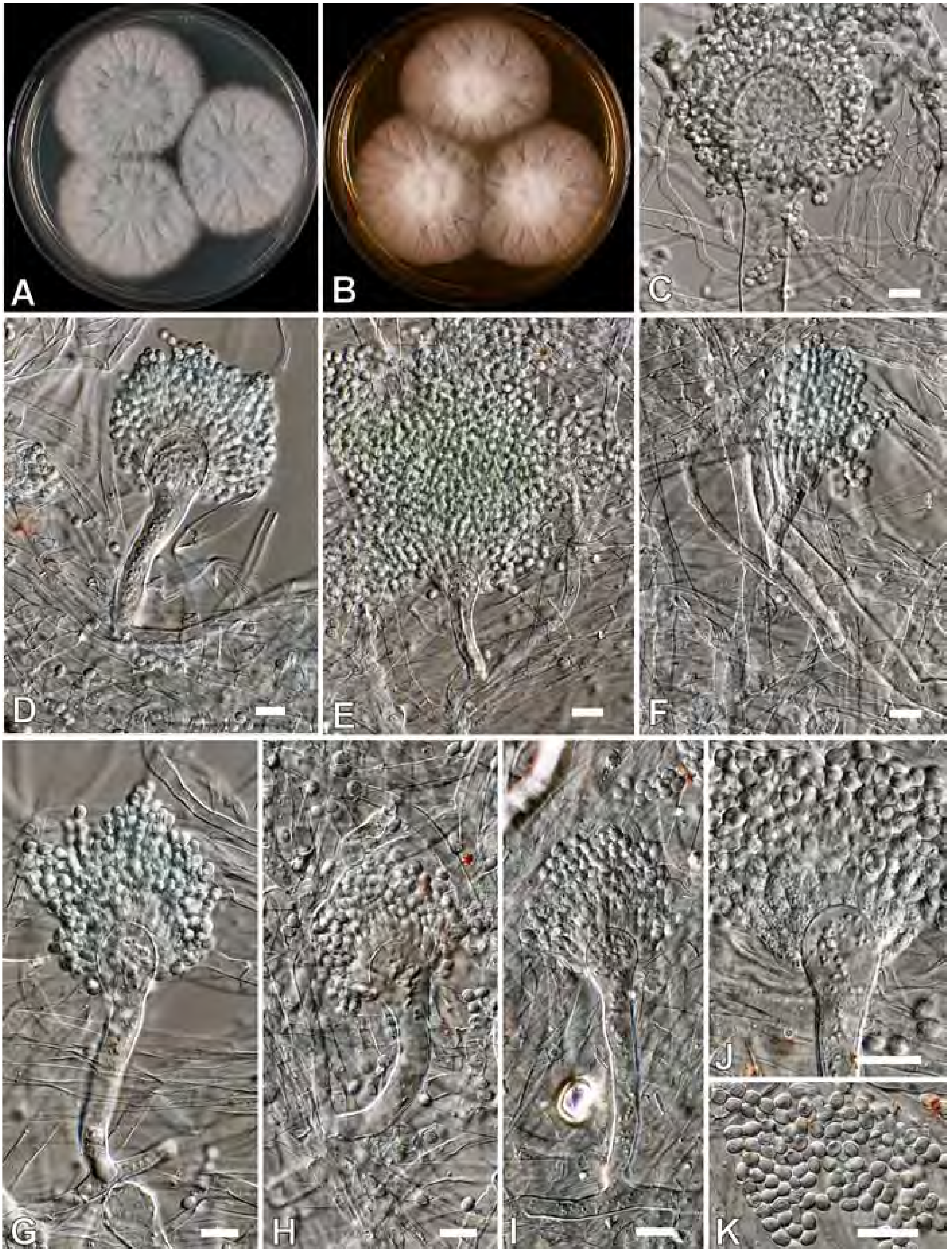

Fig. 5. *Aspergillus clavatonanicus*. A–B. Colonies after 7 d at 25 °C. A. CYA. B. MEA. C–J. Conidiophores. K. Conidia. Scale bars = 10 µm.

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.

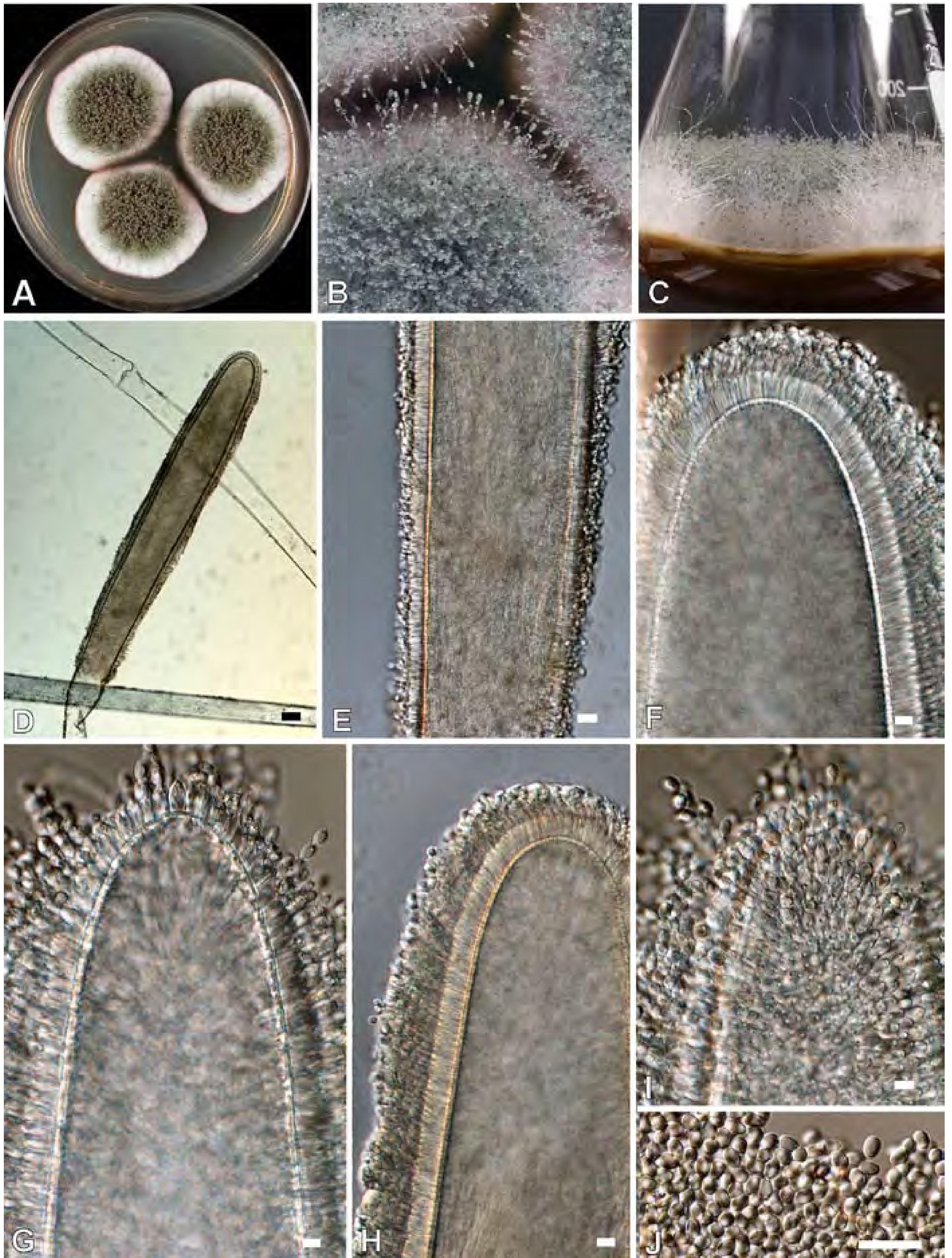

Fig. 7. *Aspergillus giganteus*. A. Colonies after 7 d at 25 °C on CYA. B–C. Macrophotograph of conidiophores. D–I. Conidiophores. J. Conidia. Scale bars = 10 µm, except D and E = 30 µm.

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

Extrolites: patulin, antafumicin, ascladiol, tryptoquivalone; tryptoquivalines, glyanthrypine, pyripyropen (found in this study), α-sarcin and other ribotoxins (Olson & Goerner 1965; Olson *et al.* 1965; Lin *et al.* 1995; Wirth *et al.* 1997; Martinez-Ruiz *et al.* 1999). Carotens 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
Conidia 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 longer and wider conidiophores, longer vesicles and larger conidia than *A. giganteus*; vesicles are elongate to fusoid-clavate for the long conidiophre and globose for the samml ones, while those of *A. giganteus* are clavate

Similar species: *A. giganteus*

Distribution: Nigeria, Ivory Coast

Ecology and habitats: soil

Extrolites: patulin, tryptoquivalone, tryptoquivalines, antafumicins, pyripyropens (found in this study)

Pathogenicity: not reported

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

Aspergillus rhizopodus Rai, Wadhwani & Agarwal, Trans. Br. Mycol. Soc. 64: 515. 1975. Fig. 9

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

Other no. of the type: IMI 385057; WB5442

Description
Colony diam (7 d): CZA30: 40 mm; CYA25: 38–42 mm; MEA25: 50–55 mm; YES25: 68–72 mm; OA25: 43–47 mm; CYA37: 17–19 mm; CREA25: rather good growth and no acid production
Colony colour: blue green
Conidiation: abundant
Reverse colour (CZA): 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 variously shaped foot cells with finger-like projections

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

Distribution: India, Yugoslavia

Ecology and habitats: soil

Extrolites: 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

Dichotomomyces cejpii (Milko) D.B. Scott, Trans. Brit. Mycol. Soc. 47: 428, 1970. Fig. 10.

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

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

Description
Colony diam (7 d): CYA25: 25–47 mm; MEA25: 35–58 mm; YES25: 47–50 mm; OA25: 38–48; CYA37: 24–32 mm; CREA: poor growth and noa cid production
Colony colour: white to cream coloured
Conidiation: sparse
Reverse colour (CZA):
Colony texture: floccose, granular
Conidium size/shape/surface texture: 5–10 µm, subglobose to pyriform, smooth
Homothallic
Cleistothecia: variable in size, spherical, white to cream coloured

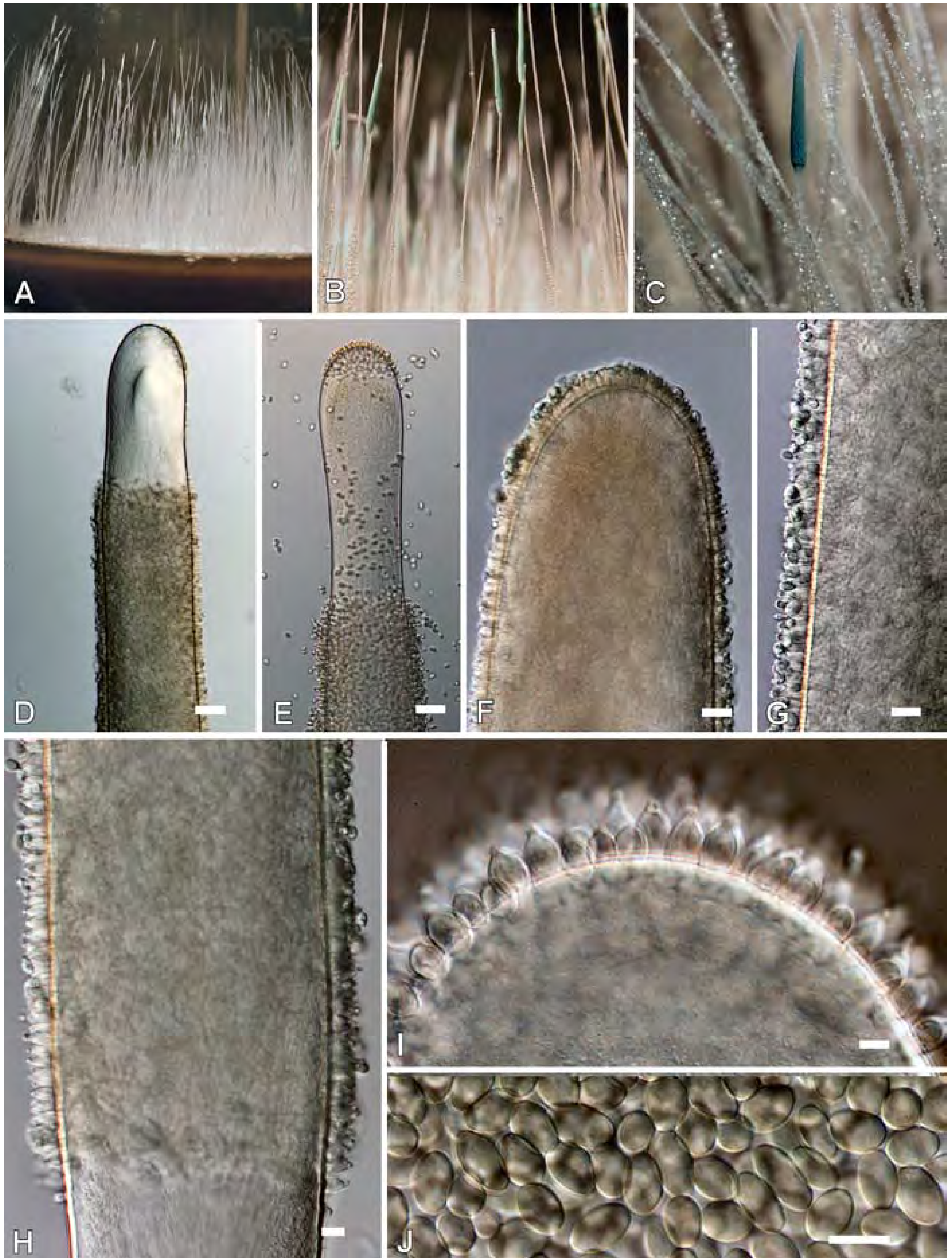

Fig. 8. *Aspergillus longivesica*. A. Colonies after 10 d at 25 °C on CYA. B–C. Macrophotograph of conidiophores. D–I. Conidiophores. J. Conidia. Scale bars = 10 µm, except D and E = 30 µm.

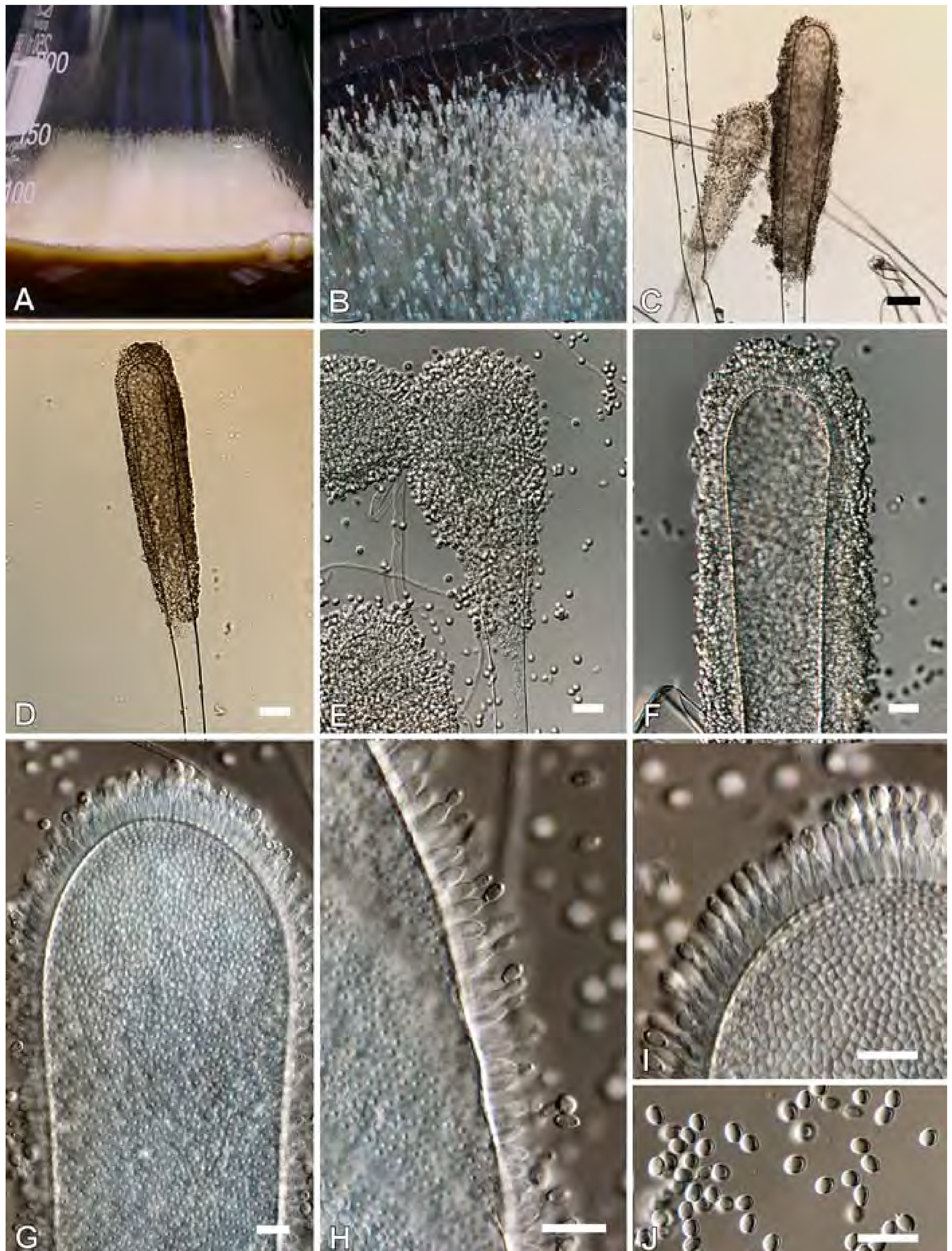

Fig. 9. *Aspergillus rhizopodus.* A. Colonies after 10 d at 25 °C on CYA. B. Macrophotograph of conidiophores. C–I. Conidiophores. J. Conidia. Scale bars = 10 µm, except D and E = 30 µm.

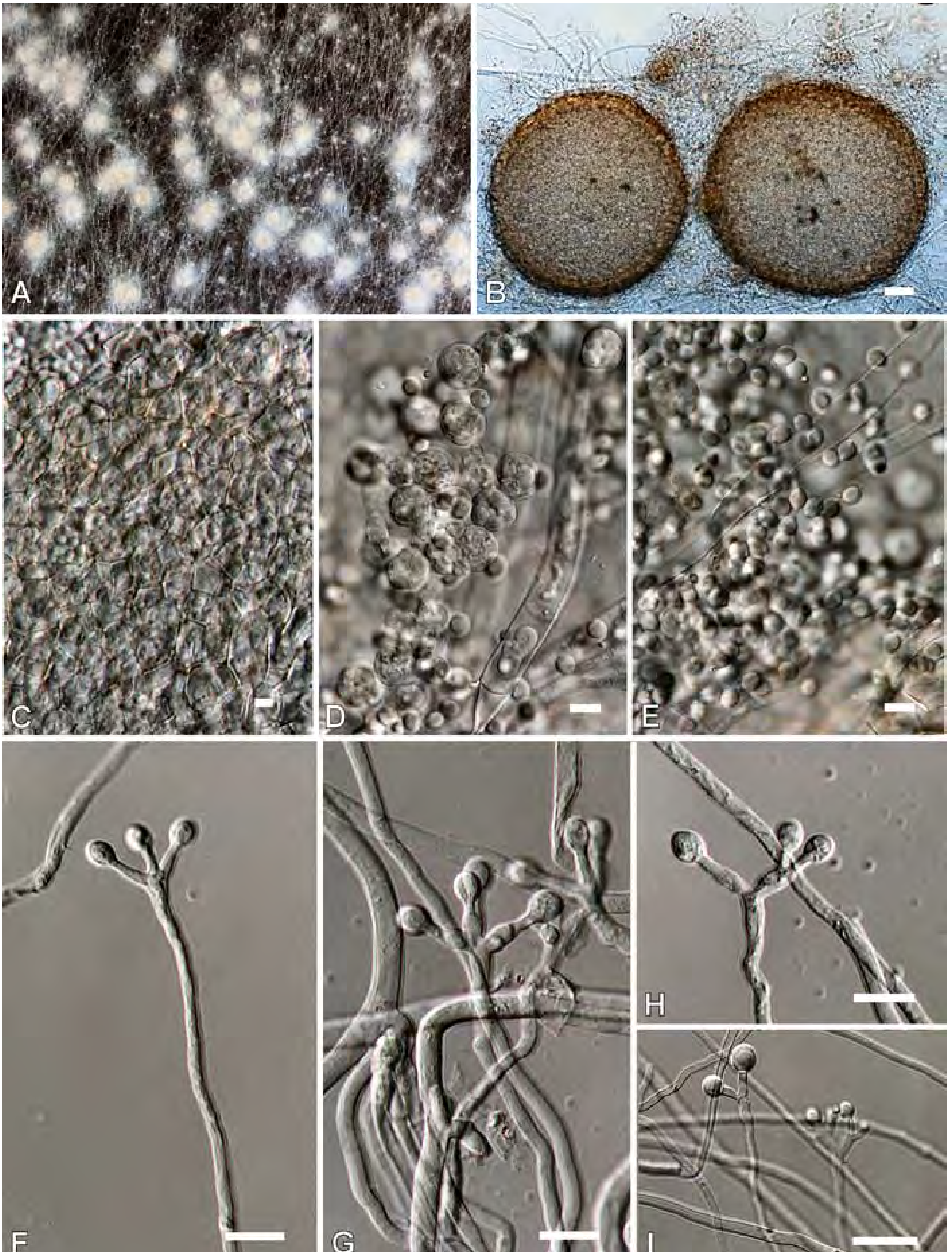

Fig. 10. *Dichotomomyces cejpii*. A–B. Ascomata on MEA after 10 d at 25 °C. C. Ascomata wall. D–E. Asci and ascospores. F–I conidiophores and conidia. Scale bars = 10 µm, except B and C = 30 µm.

Ascospores: 3–3.5 × 4–4.5 µm, lenticular, with two closely appressed very thin equatorial crests and convex walls smooth

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 (*Polypaecilum* 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

Extrolites: gliotoxin (Seigle-Murandi *et al.* 1990, confirmed in this study), tryptoquivalons (found in this study), rubratoxins (found in this study), xanthocillin X (Kitahara & Endo 1981; could not be confirmed in this study), and several metabolites with antibiotic and ciliostatic properties (Pieckova & Roeijmans 1999; Pieckova & Jesenska 1997a, 1997b)

Pathogenicity: not reported

Note: this species is reported as a heat resistant fungus causing food spoilage (Pieckova *et al.* 1994; Jesenska *et al.* 1993; Mayer *et al.* 2007)

Neocarpenteles acanthosporus (Udagawa & Takada) Udagawa & Uchiyama [anamorph: *A. acanthosporus* Udagawa & Takada], Mycoscience 43(1): 4. 2002.
= *Hemicarpenteles acanthosporus* Udagawa & Takada (1971)

Type: CBS 558.71, from soil, Bougainville Island (Solomon Islands), Papua New Guinea

Other no. of the type: ATCC 22931; IMI 164621; NHL 2462

Description
Colony diam (7 d): CYA25: 37–47 mm; MEA25: 72–85: mm; YES25: 62–82; OA25: 40–49 mm; CYA37: 0 mm; CREA: poor growth and no acid production
Colour: white to brownish orange
Conidiation: sparse
Reverse colour (CYA): greyish-orange
Colony texture: floccose
Conidial head: radiate to loosely columnar
Stipe: (50–)100–400 × 5–12 µm, smooth, septate
Vesicle diam /shape: 10–26 µm, flask shaped
Conidia length/ shape/ surface texture: 4.5–7 µm, globose to subglobose, spinulose
Homothallic
Cleistothecia: 350–1000 × 250–850 µm, sclerotioid, subglobose to ovoid, fawn, covered with dense aerial hyphae
Ascospores: 4–4.5 × 3.5–4 µm, lenticular, with two thin equatorial crests and convex walls ornamented with raised flaps

Cultures examined: CBS 558.71, CBS 445.75, CBS 446.75, CBS 447.75

Diagnostic features: small dull green readiate conidial heads, short conidiophores with small flask-shaped vesicle, production of ascospores, and large globose conidia distinguish this species from other members of section *Clavati*

Distribution: Papua New Guinea (Bougainville Island), Japan

Ecology and habitats: soil

Extrolites: kotanins, tryptoquivalines, tryptoquivalones (found in this study), ribotoxins (Varga *et al.* 2003). (+)-isoepoxydon has also been reported (Kontani *et al*. 1990)

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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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. insuetus* is separated from *A. ustus* and revived. *A. insuetus* 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. insuetus*. *Aspergillus* section *Usti sensu stricto* includes 8 species: *A. ustus, A. puniceus*, *A. granulosus, A. pseudodeflectus, A. calidoustus*, *A. insuetus* and *A. keveii* together with *Emericella heterothallica*.

Taxonomic novelties: *Aspergillus insuetus* 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 (Fakih *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 Axioskop 2 Plus) was employed.

Extrolite analysis. Extrolites were analysed by HPLC using alkylphenone 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, aflavinine, 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 (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 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.

Species	Strain No.	Source
A. calidoustus	CBS 112452	Indoor air, Germany
A. calidoustus	CBS 113228	ATCC 38849; IBT 13091
A. calidoustus	CBS 114380	Wooden construction material, Finland
A. calidoustus	CBS 121601[T]	Bronchoalveolar lavage fluid, proven invasive aspergillosis, Nijmegen, The Netherlands[†]
A. calidoustus	CBS 121602	Bronchial secretion, proven invasive aspergillosis, Nijmegen, The Netherlands[†]
A. calidoustus	CBS 121589	Autopsy lung tissue sample, proven invasive aspergillosis, Nijmegen, The Netherlands[†]
A. calidoustus	CBS 121603	Elevator shaft in hospital, Nijmegen, The Netherlands
A. calidoustus	CBS 121604	Patient room, Nijmegen, The Netherlands
A. calidoustus	CBS 121605	Laboratory, Nijmegen, The Netherlands
A. calidoustus	CBS 121606	Sputum, Nijmegen, The Netherlands
A. calidoustus	CBS 121607	Feces, Nijmegen, The Netherlands
A. calidoustus	CBS 121608	Bronchoalveolar lavage, Nijmegen, The Netherlands
A. calidoustus	7843	Pasteur Institute, Paris, France
A. calidoustus	8623	Oslo, Norway
A. calidoustus	9331	Mouth wash, Nijmegen, The Netherlands
A. calidoustus	9371	Mouth wash, Nijmegen, The Netherlands
A. calidoustus	9420	Bronchial secretion, Nijmegen, The Netherlands
A. calidoustus	9692	Hospital ward, Nijmegen, The Netherlands
A. calidoustus	V02-46	Tongue swab, Nijmegen, The Netherlands
A. calidoustus	V07-21	Bronchial secretion, Nijmegen, The Netherlands
A. calidoustus	V17-43	Bronchial secretion, Nijmegen, The Netherlands
A. calidoustus	V22-60	Skin biopsy, Nijmegen, The Netherlands
A. calidoustus	CBS 121609	Post-cataract surgery endophthalmitis, Turkey
A. calidoustus	907	Post-cataract surgery endophthalmitis, Turkey
A. calidoustus	908	Post-cataract surgery endophthalmitis, Turkey
A. calidoustus	64	Post-cataract surgery endophthalmitis, Turkey
A. calidoustus	67	Post-cataract surgery endophthalmitis, Turkey
A. calidoustus	CBS 121610	Post-cataract surgery endophthalmitis, Turkey
A. calidoustus	351	Osteorickets
A. calidoustus	482	Post-cataract surgery endophthalmitis
A. calidoustus	CBS 121611	Patient 4, Washington, U.S.A.
A. calidoustus	CBS 121616	Environmental, Washington, U.S.A.
A. calidoustus	FH 165	Patient 5b, Washington, U.S.A.
A. calidoustus	CBS 121614	Patient 5a, Washington, U.S.A.
A. calidoustus	CBS 121615	Patient 6, Washington, U.S.A.
A. calidoustus	CBS 121613	Patient 2, Washington, U.S.A.
A. calidoustus	CBS 121612	Patient 1, Washington, U.S.A.
A. calidoustus	FH 91	Patient 1a, Washington, U.S.A.
A. calidoustus	NRRL 26162	Culture contaminant, Peoria, U.S.A.
A. calidoustus	NRRL 281	Thom 5634
A. calidoustus	NRRL 277	Thom 5698.754, Green rubber
A. granulosus	CBS 588.65[T]	Soil, Fayetteville, Arkansas, U.S.A.
A. granulosus	CBS 119.58	Soil, Texas, U.S.A.
A. granulosus	IBT 23478 = WB 1932 = IMI 017278iii = CBS 588.65	Soil, Fayetteville, Arkansas, U.S.A.
A. insuetus	CBS 107.25[T]	South Africa
A. insuetus	CBS 119.27	Unknown
A. insuetus	CBS 102278	Subcutaneous infection left forearm and hand of 77-year-old woman
A. keveii	CBS 209.92	Soil, La Palma, Spain
A. keveii	CBS 561.65	Soil, Panama
A. keveii	IBT 10524 = CBS 113227 = NRRL 1254	Soil, Panama

Table 1. (Continued).

Species	Strain No.	Source
A. keveii	IBT 16751 = DMG 153	Galápagos Islands, Ecuador, D.P. Mahoney
A. pseudodeflectus	CBS 596.65	Sugar, U.S.A., Louisiana
A. pseudodeflectus	CBS 756.74 [T]	Desert soil, Egypt, Western Desert
A. puniceus	CBS 122.33	Unknown
A. puniceus	9377	Mouth wash, Nijmegen, Netherlands
A. puniceus	V41-02	Faeces, Nijmegen, Netherlands
A. puniceus	NRRL 29173	Indoor air, Saskatoon, Canada
A. puniceus	CBS 495.65 [T]	Soil, Zarcero Costa Rica
A. puniceus	CBS 128.62	Soil, Louisiana, U.S.A.
A. ustus	CBS 116057	Antique tapestries, Krakow, Poland
A. ustus	CBS 114901	Carpet, The Netherlands
A. ustus	CBS 261.67 [T]	Culture contaminant, U.S.A.
A. ustus	CBS 133.55	Textile buried in soil, Netherlands
A. ustus	CBS 239.90	Man, biopsy of brain tumor, Netherlands
A. ustus	CBS 113233	IBT 14495
A. ustus	CBS 113232	IBT 14932
A. ustus	NRRL 285	Soil, Iowa, U.S.A.
A. ustus	NRRL 280	Bat dung, Cuba
A. ustus	NRRL 1609	Bat dung, Cuba
A. ustus	NRRL 29172	Indoor air, Edmonton, Canada
E. heterothallica	CBS 489.65[T]	soil, Costa Rica
E. heterothallica	CBS 488.65	soil, Costa Rica

[†]These samples were taken from the same patient (Verweij *et al.* 1999)

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 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 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. calidoustus*, *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.

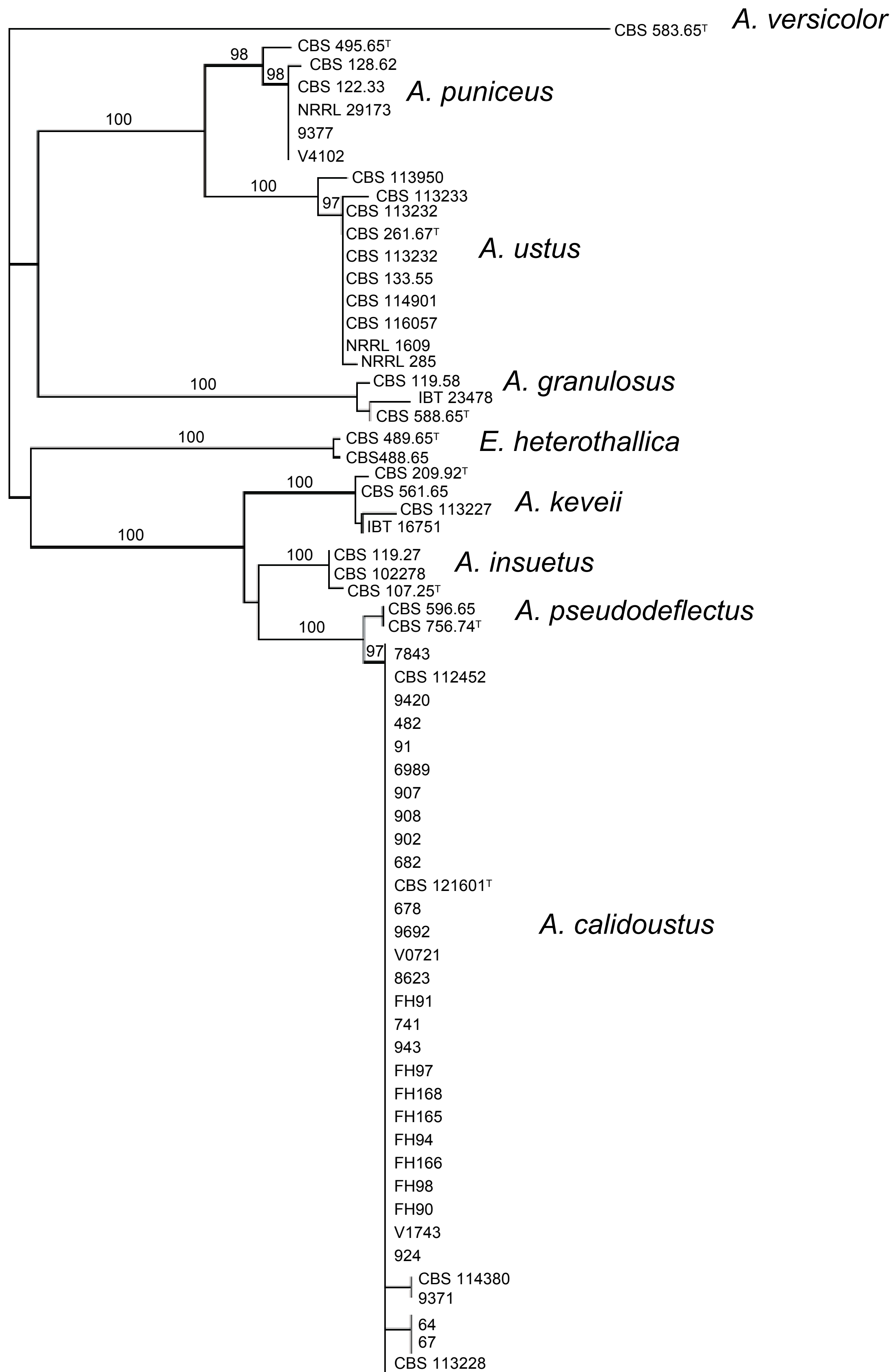

Fig. 1. Neighbour-joining tree based on β-tubulin sequence data of *Aspergillus* section *Usti*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

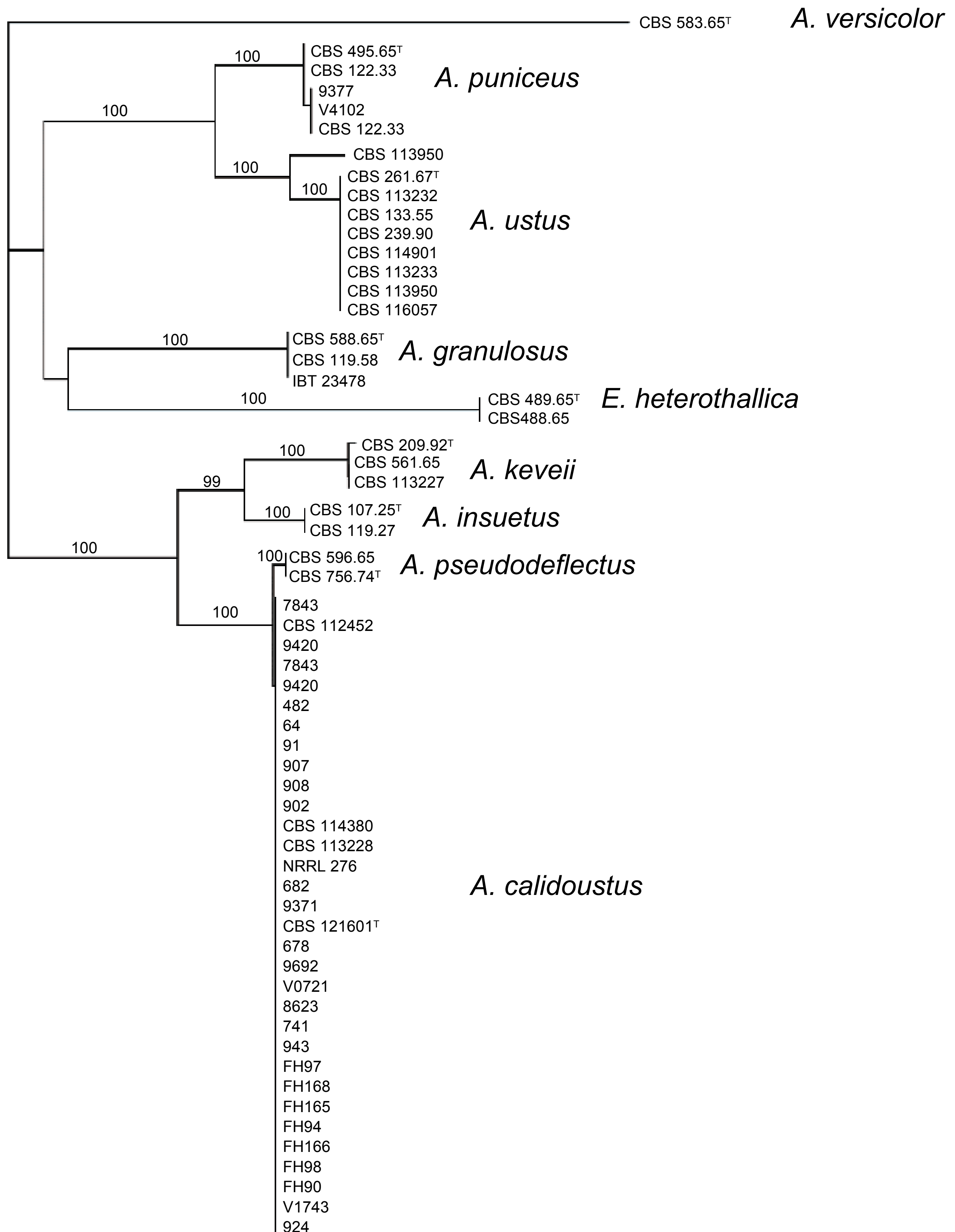

Fig. 2. Neighbour-joining tree based on calmodulin sequence data of *Aspergillus* section *Usti*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

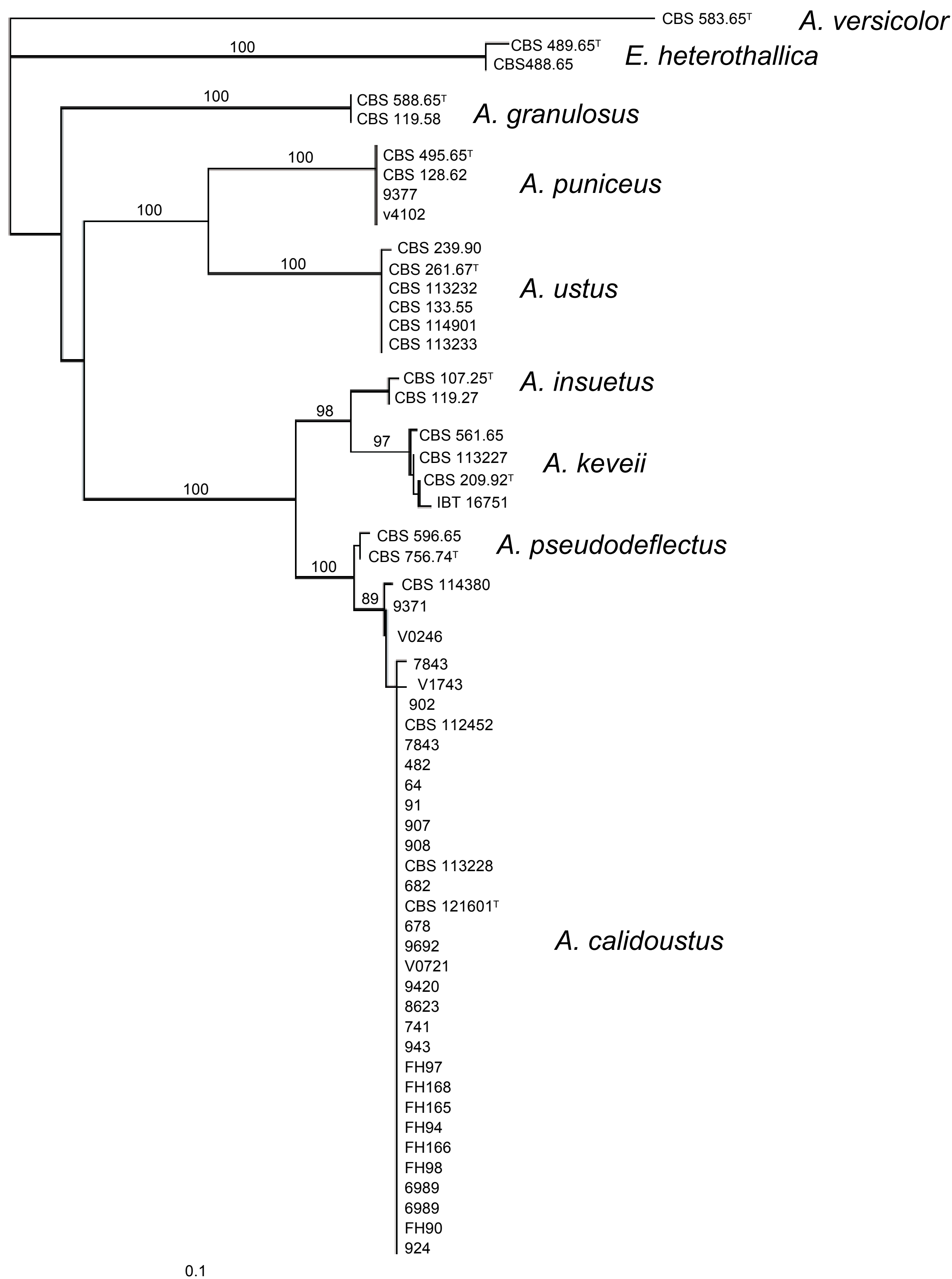

Fig. 3. Neighbour-joining tree based on actin sequence data of *Aspergillus* section *Usti*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

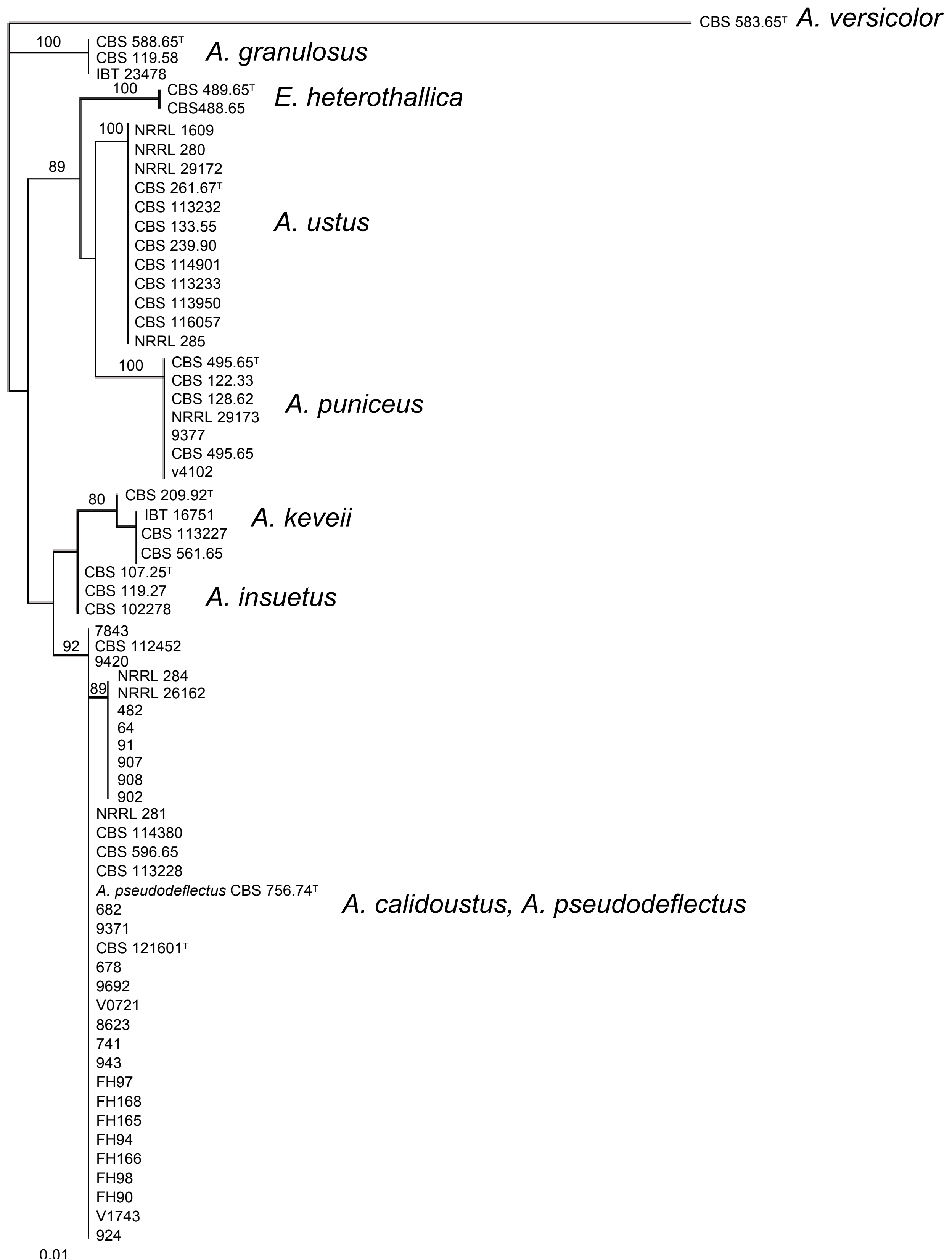

Fig. 4. Neighbour-joining tree based on ITS sequence data of *Aspergillus* section *Usti*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

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

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

* All have violet reaction, except CBS 113227

** Colour according Methuen handbook of colours

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 austdiol (Vleggaar *et al.* 1974), Austin (Chexal *et al.* 1976), austocystins (Steyn & Vleggaar 1974; Kfir *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), diacetoxyscirpenol (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), emeheterone (Kawahara *et al.* 1988), emesterones A & B (Hosoe *et al.* 1998), 5''-hydroxyaveranthin (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 austdiol 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''-hydroxyaveranthin 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 pergillins, 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, emesterones and emeheterones, as standards of these compounds were not available.

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*.

Species	Extrolites produced
Chemical group I	
A. ustus	Ustic acids, austocystins (and versicolorins), austalides, a compound related to sterigmatocystin, nidulol
A. granulosus	Ustic acids, a compound resembling sterigmatocystin, nidulol, drimans
A. puniceus	Ustic acids, austocystins (and versicolorins), phenylahistin, a compound related to sterigmatocystin, nidulol
Chemical group II	
A. pseudodeflectus	Drimans, unknown compounds
A. calidoustus	Drimans, ophiobolins G and H, austins
A. insuetus	Drimans, ophiobolins G and H, pergillin-like
A. keveii	Drimans, ophiobolins G and H, nidulol
Chemical group III	
E. heterothallica	Emethallicins A, B, C, D, E & F, emeheterone, emesterones A & B, 5''-hydroxyaveranthin, Mer-NF8054X, sterigmatocystin, versicolorins

A. pseudodeflectus, A. conjunctus, A. puniceus, 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. puniceus* 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 usta* Bainier. In this manual, *A. insuetus* (Bainier) Thom & Church was also accepted based on *S. insueta* 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. puniceus*, *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 strain
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 16837, NRRL 1932, WB 1932, CBS 452.93

Description
Colony diam, 7 d, in mm: CYA25 30–48; CYA37 30–51; MEA25 25–37; YES25 35–45; CZA25 17–25
Colony colour: buff to dull brown
Conidiation: moderate
Reverse colour (CYA): dull yellow to red brown
Colony texture: floccose, plane or irregularly furrowed
Conidial head: hemispherical to radiate
Stipe: 100–600 × 5.5–8 µm, thin-walled, smooth, straight, tan to light brown
Vesicle diam/shape: 15–25 × 12–18 µm, ovoid to elliptical
Conidium size/shape/surface texture: (3.3–)4–4.5(–5.5) µm, globose, delicately echinulate
Hülle cells: irregularly globose, ovoid to elongate, 12–30 µm, in colourless clusters at colony margins
Ehrlich reaction: violet
Growth on creatine: poor growth with inconspicuous mycelium, no acid production

Cultures examined: CBS 119.58, CBS 588.65, IBT 23478

Diagnostic features: small colourless clusters of irregularly globose Hülle cells, giving the colony a characteristic granular appearance, good growth at 37 °C and violet Ehrlich reaction

Similar species: -

Distribution: U.S.A.

Ecology and habitats: soil

Extrolites: Ustic acids, a compound resembling sterigmatocystin, nidulol, drimans

Pathogenicity: pathogenic to humans (Fakih *et al.* 1995)

Aspergillus insuetus (Bainier) Thom & Church, Manual of the aspergilli: 153. 1929. Fig. 7.
= *Sterigmatocystis insueta* Bainier (1908)

Type: CBS 107.25, from South Africa, Sartory

Other no. of the type: ATCC 1033; IFO 4128; NRRL 279; NRRL 1726; Thom No. 4658.245

Description
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, fuligeneous, 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

Cultures examined: CBS 107.25, CBS 119.27, CBS 102278

Similar species: *A. keveii*

Distribution: South Africa, Spain

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

Ecology and habitats: soil (?), human

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

Pathogenicity: caused subcutaneous infection (Gené *et al.* 2001)

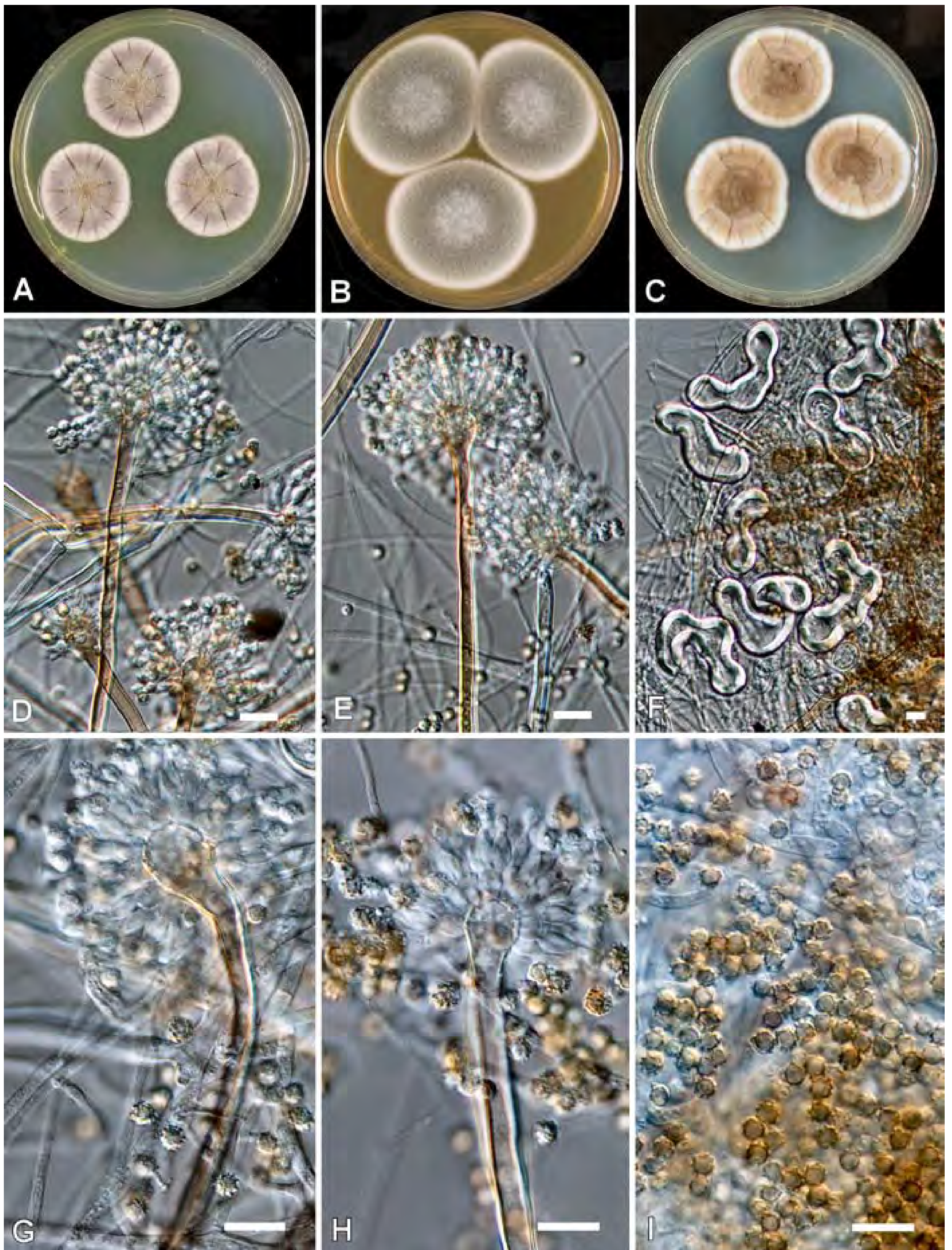

Fig. 5. *Aspergillus calidoustus*. A–B. Colonies at 25 °C after 7 d. A. CYA. B. MEA. C–E, G–H Conidiophores. F. Hülle cells. I. Conidia. Scale bars = 10 µm, except F = 30 µm

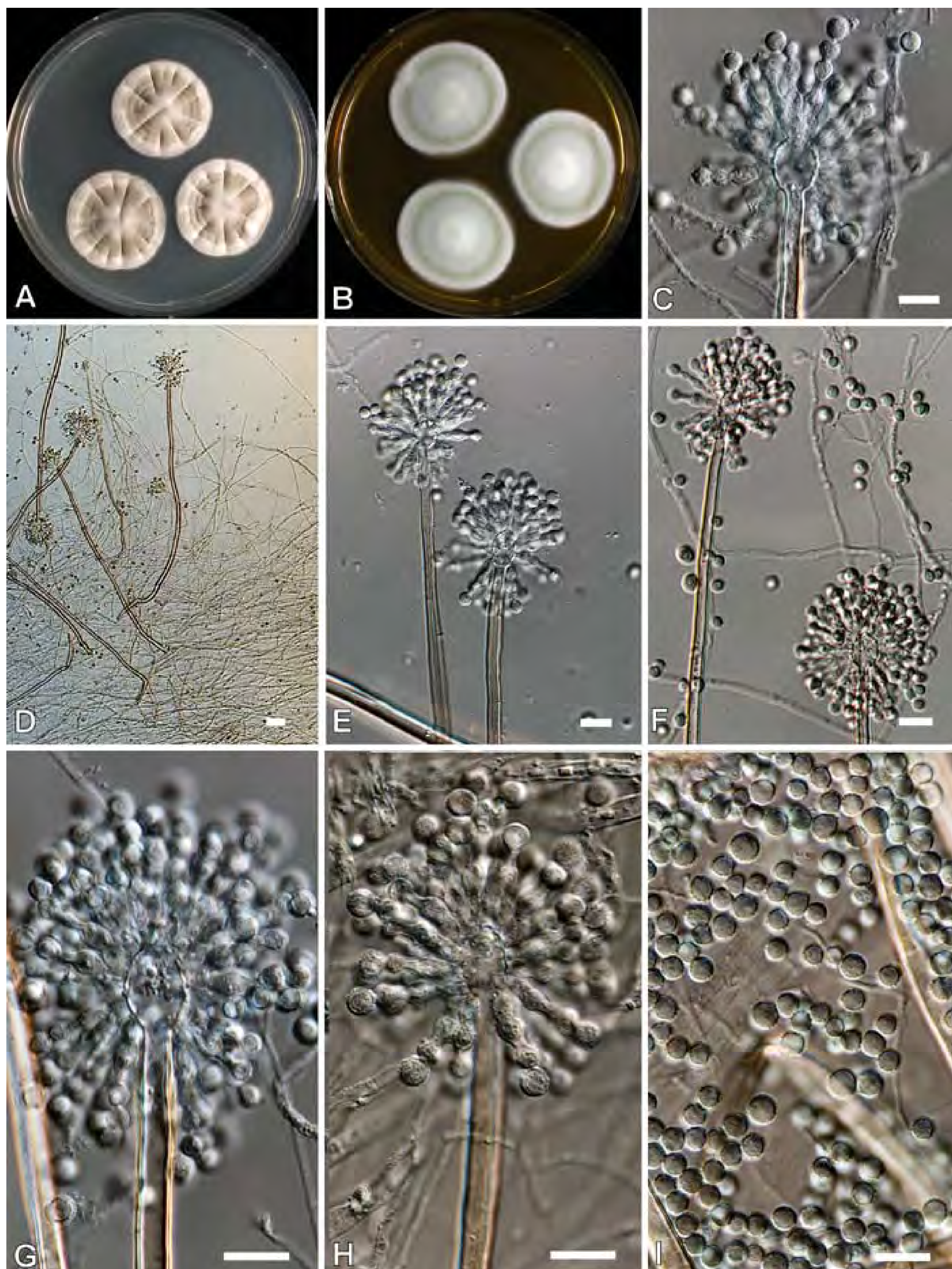

Fig. 6. *Aspergillus granulosus*. A–B. Colonies at 25 °C after 7 d. A. CYA. B. MEA. C–H Conidiophores. I. Conidia. Scale bars = 10 µm, except C = 30 µm.

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, Frisvad & Samson – MycoBank MB505570. Fig. 8.

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

Coloniae in 7 dieibus 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; auctus in 7 dieibus et 37 °C in agaro CYA nullus. Sporulatio in CYA abundans; colonia brunneogrisea vel subroseobrunnea; textura coloniae floccosa; colonia reversa flavide olivaceobrunnea vel atrobrunnea. Capitula conidialia laxe columnaria; stipites 150–300 × 4–6 µm, pariete laevi, brunneo; vesciculae pyriformes, 9–13 µm in lat., biseriatae; 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., ornamento exasperato vel echinulato. Cellulae "hülle" irregulariter elongatae, (10–) 25–40(–65) µm in long., in cumulis dispersis.

Colonies on MEA 36–41 mm, on CYA 30–39 mm, on YES 40–46 mm, on CREA 25–32 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; stipes 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 globose 2.4–2.8 µm, coarsely roughened to echinulate. Hülle cells (10–)25–40(–65) µm, 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: 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; CZA25 25-26
Colony colour: white mycelial felt intermixed with brown conidiogenous 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; CZA25: 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, aerially 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

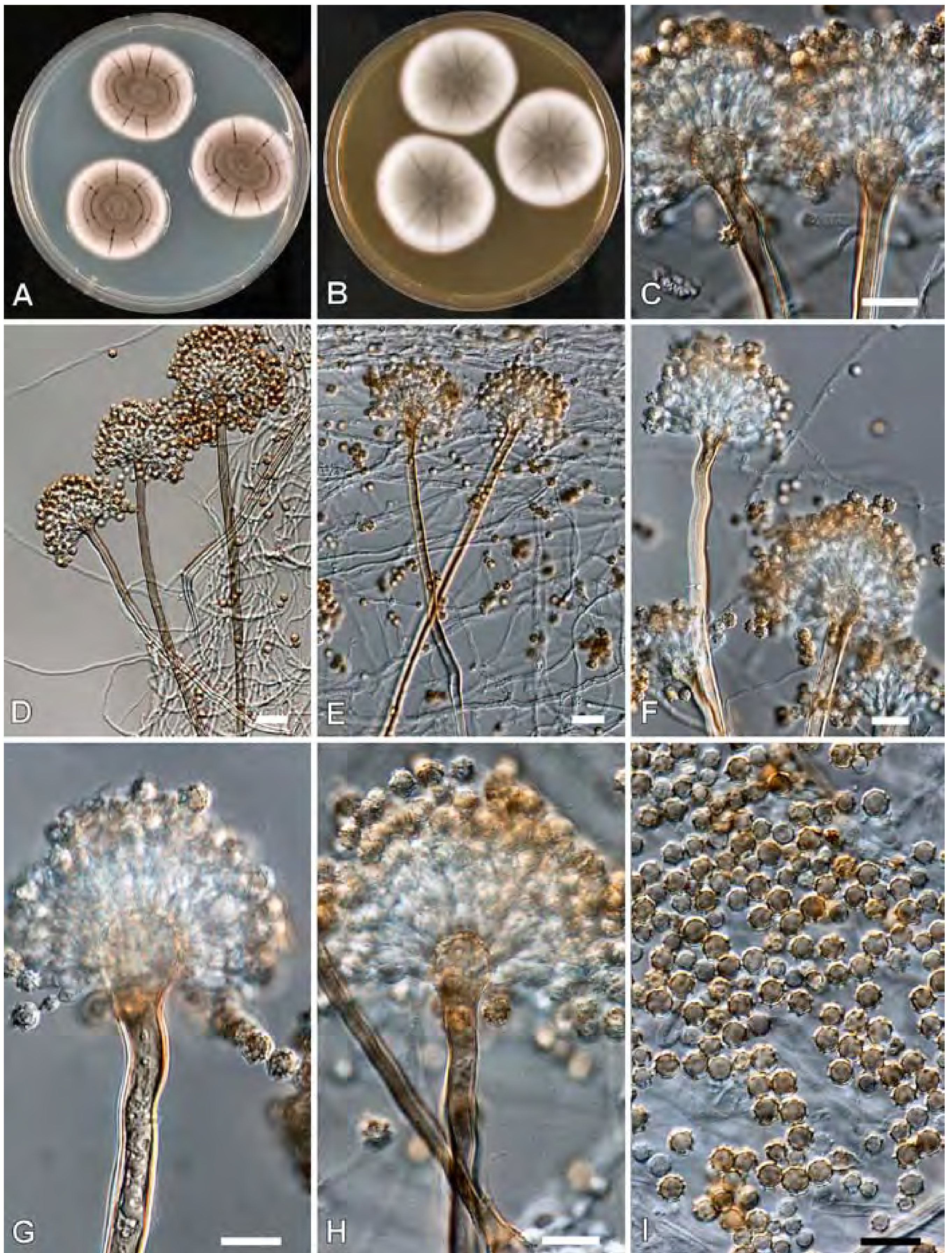

Fig. 8. *Aspergillus kerveii*. A–B. Colonies at 25 °C after 7 d. A. CYA. B. MEA. C–H Conidiophores. I. Conidia. Scale bars = 10 µm.

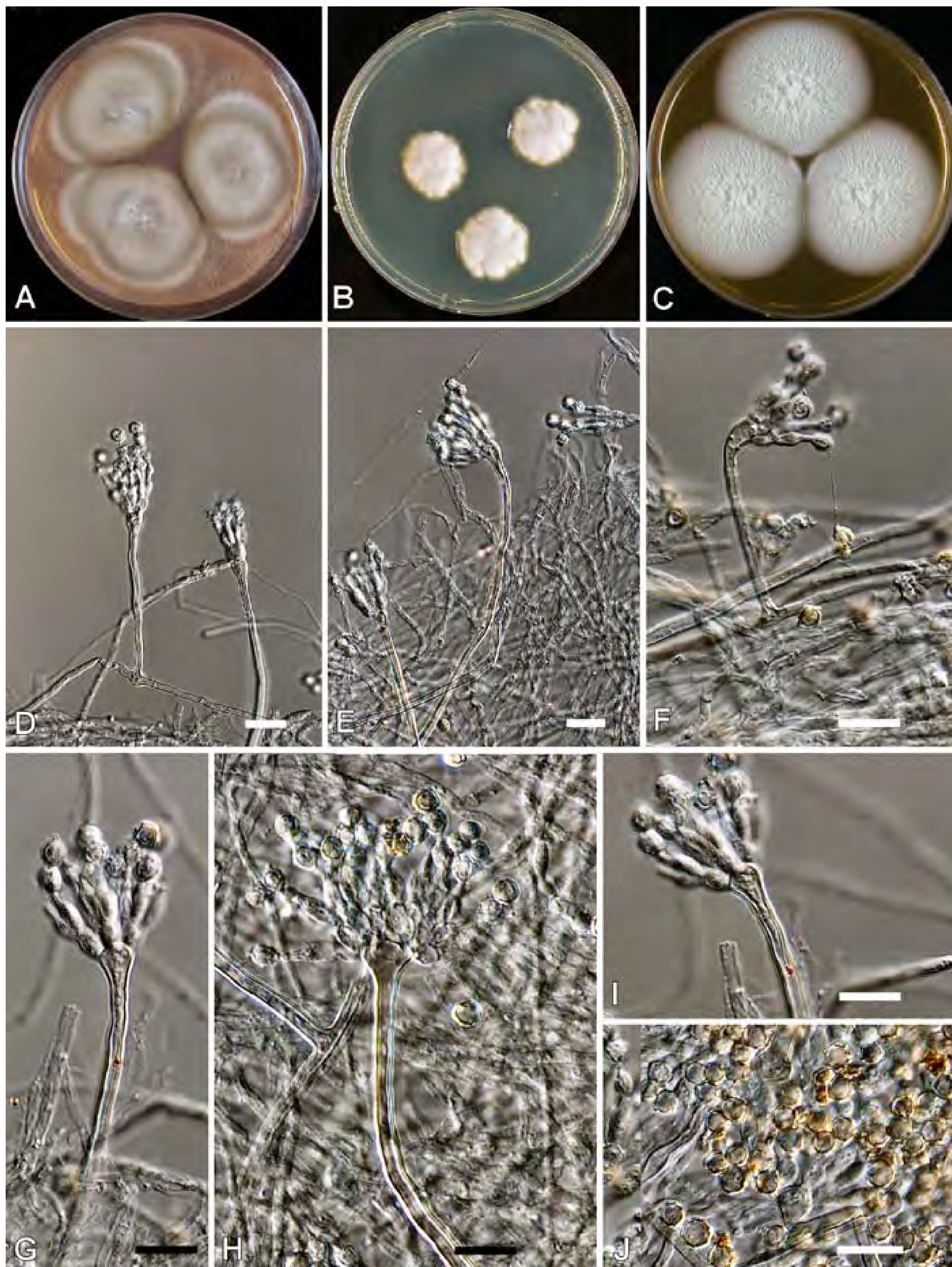

Fig. 9. *Aspergillus pseudodeflectus*. A–C. Colonies at 25 °C after 7 d. A. MEA + 40 % sucrose. B. CYA + 20 % sucrose. C. MEA. D–I. Conidiophores. H. Conidia. Scale bars = 10 µm.

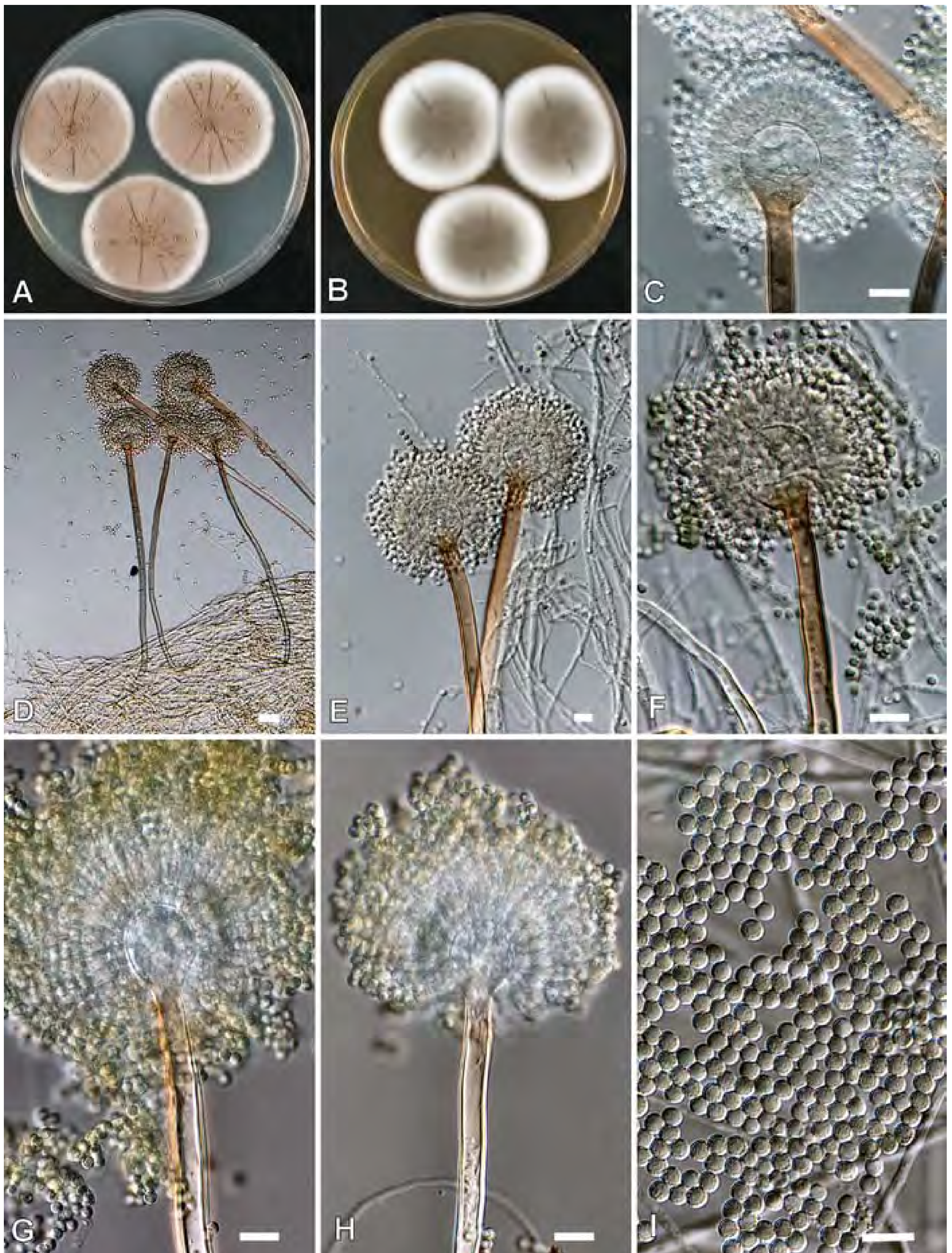

Fig. 10. *Aspergillus puniceus*. A–B. Colonies at 25 °C after 7 d. A. CYA. B. MEA. C–H Conidiophores. I. Sclerotia. J. Conidia. Scale bars = 10 µm, except D = 30 µm.

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 usta* 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; MEA25 39–46; 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 133.55, 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, austalides, 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.

Emericella heterothallica (Kwon-Chung, Fennell & Raper) Malloch & Cain [anamorph: *A. compatibilis* Samson & Gams], Can. J. Bot. 50: 62. 1972. Fig. 12.

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

Extrolites: Found in this study: Sterigmatocystin, versicolorins, Mer-NF8054X. Literature data: emethallicins A–F (Kawahara *et al.* 1989, 1990a), 5"-hydroxyaveranthin (Yabe *et al*. 1991), emeheterone (Kawahara *et al.* 1988), emesterones A & B (Hosoe *et al.* 1998), 5"-hydroxyaveranthin (Yabe *et al.* 1991), Mer-NF8054X (Mizuno *et al.* 1995).

Pathogenicity: not reported

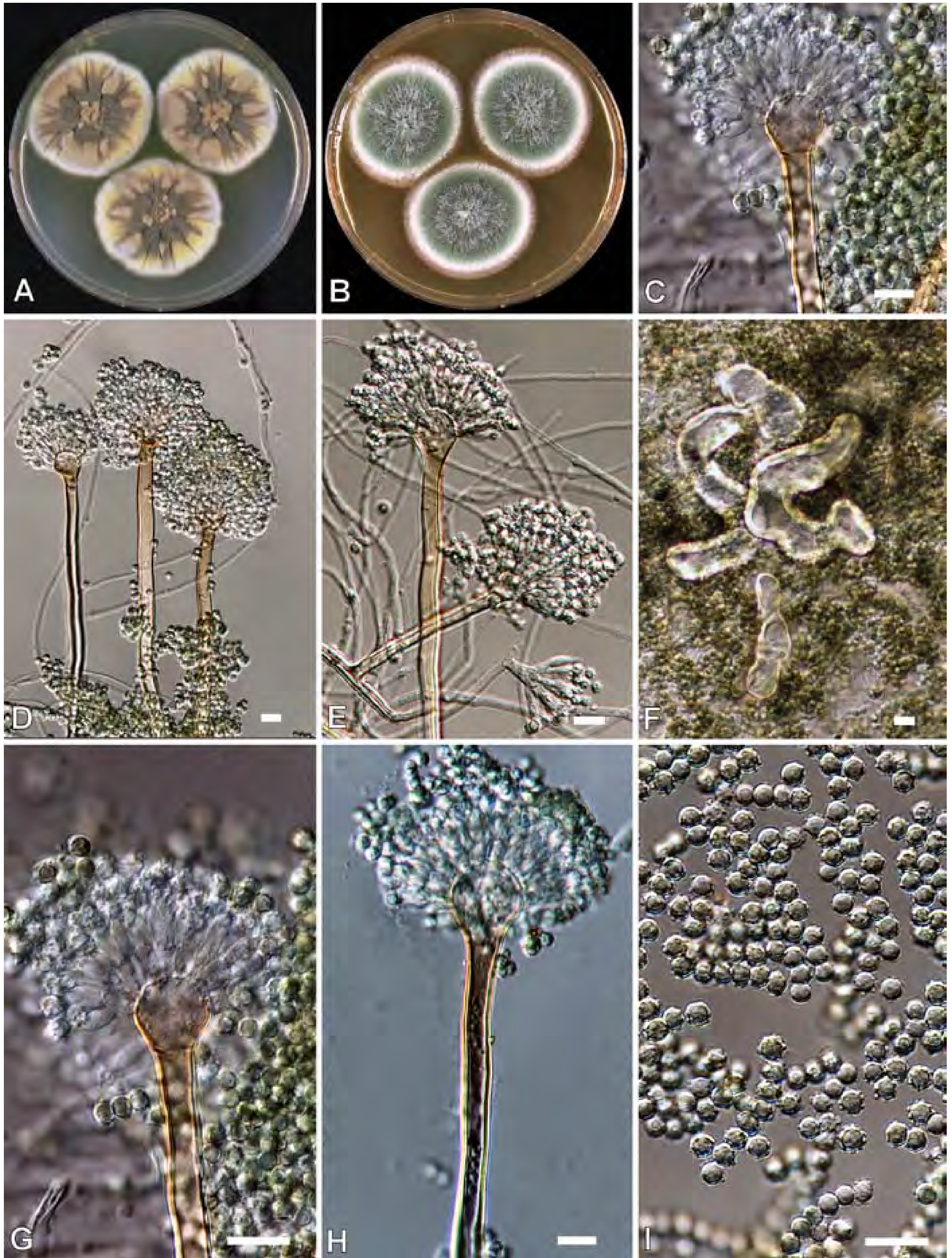

Fig 11. *Aspergillus ustus*. A–B. Colonies at 25 °C after 7 d. A. CYA. B. MEA. C–E. G–H Conidiophores. F. Hülle cells. I. Conidia. Scale bars = 10 µm, except F = 30µm.

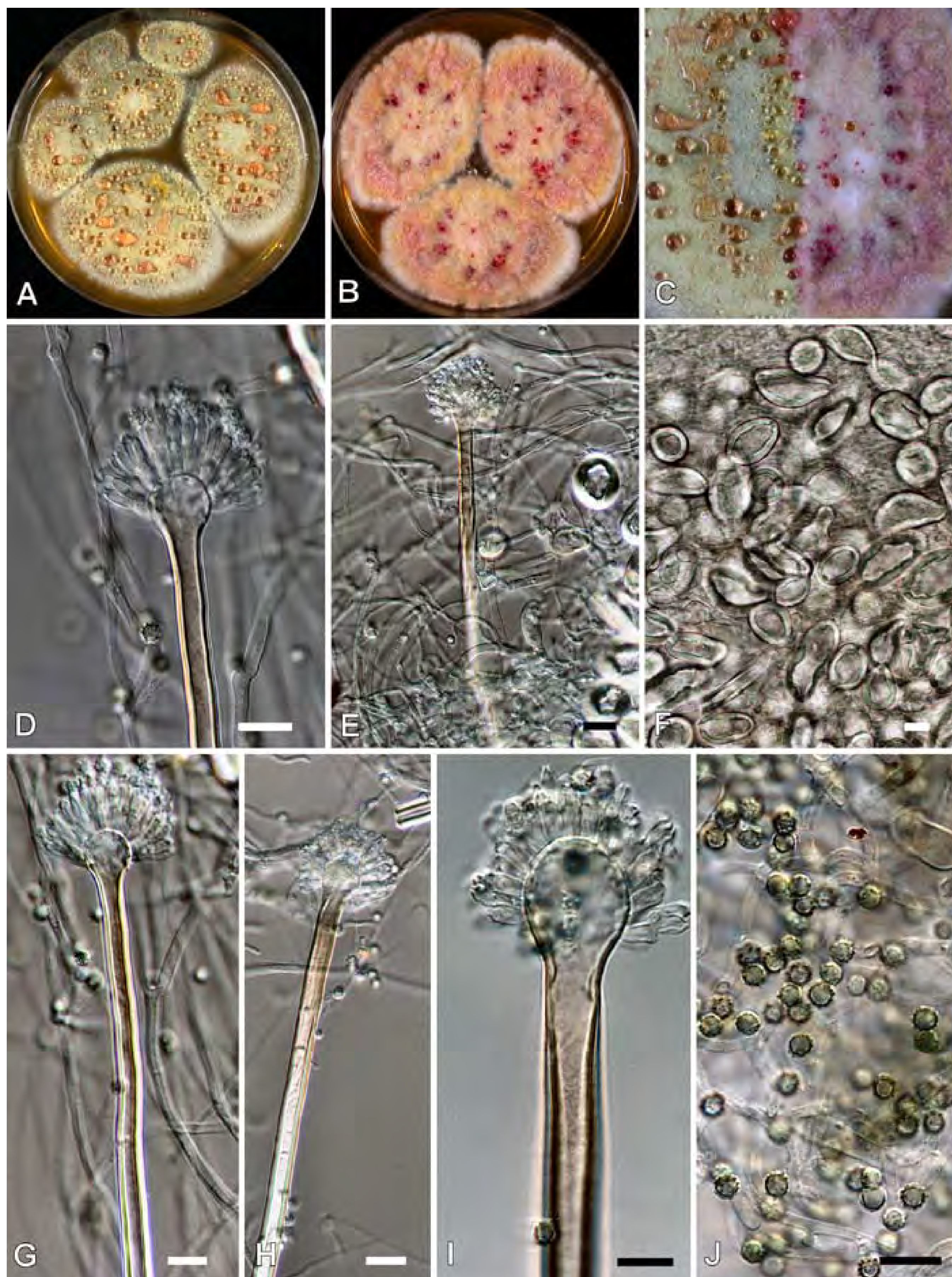

Fig. 12. *Emericella heterothallica*. A–C. Colonies at 25 °C after 7 d. A. CYA. B. MEA. C. Crossing of mating strains. D–E, F–H. Conidiophores. I. Conidia. Scale bars = 10 µm.

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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 1979a–d; 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 diam 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 Normarski 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.

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Table 1. The type strains and isolates examined in this study.

Name	CBS No.	Origin and information (abreviation)
A. aculeatinus	CBS 121060^{T}	Thailand, Arabica green coffee bean
A. aculeatinus	CBS 121061	Thailand, Arabica green coffee bean
A. aculeatinus	CBS 121062	Thailand, Arabica green coffee bean
A. aculeatus	CBS 172.66^{T}	Origin unknown
A. aculeatus	CBS 101.43	Origin unknown
A. aculeatus	CBS 610.78	Tropical soil
A. brasiliensis	CBS 101740^{T}	Brazil, Sao Paulo, Pedreira, soil
A. brasiliensis	CBS 246.65	Australia, New South Wales, soil
A. brasiliensis	CBS 116970	Netherlands, production plant
A. carbonarius	CBS 111.26^{T}	Origin unknown, paper
A. carbonarius	CBS 113.46	U.S.A.
A. carbonarius	CBS 110.49	Indonesia, Java, air
A. costaricaensis	CBS 115574^{T}	Costa Rica, Taboga Island, Gauguin garden, soil
A. costaricaensis	CBS 553.65	Costa Rica, soil
A. ellipticus	CBS 707.79^{T}	Costa Rica, soil
A. ellipticus	CBS 482.65	Costa Rica, soil
A. foetidus^{T}	CBS 564.65^{T}	Japan, unknown substratum
A. foetidus	CBS 106.47	Switzerland, Basel
A. foetidus	CBS 124.49	Central America, unknown substratum
A. foetidus	CBS 121050	Thailand, Chiangmai Province, Arabica Coffee bean
A. heteromorphus	CBS 117.55^{T}	Brazil, culture contaminant
A. homomorphus	CBS 101889^{T}	Israel, 2 km away from Dead Sea
A. ibericus	CBS 121593^{T}	Portugal, grapes
A. japonicus	CBS 114.51^{T}	Origin unknown
A. japonicus	CBS 119560	Italy, grape
A. japonicus	CBS 522.78	Netherlands, air
A. lacticoffeatus	CBS 101883^{T}	Indonesia, South Sumatra, coffee bean
A. lacticoffeatus	CBS 101884	Venezuela, Rubio District, coffee bean
A. lacticoffeatus	CBS 101885	Venezuela, Rubio District, coffee bean
A. niger	CBS 554.65^{T}	U.S.A., Connecticut
A. niger	CBS 120.49	U.S.A.
A. niger	CBS 101698	Kenya, coffee bean
A. niger	CBS 121045	Thailand, Chiangmai Province, Arabica Coffee bean
A. piperis	CBS 112811^{T}	Denmark, black pepper
A. sclerotiicarbonarius	CBS 121057^{T}	Thailand, Robusta coffee bean
A. sclerotiicarbonarius	CBS 121056	Thailand, Robusta coffee bean
A. sclerotiicarbonarius	CBS 121058	Thailand, Robusta coffee bean
A. sclerotioniger	CBS 115572^{T}	India, Karnataka, coffee bean
A. tubingensis	CBS 134.48^{T}	Origin unknown
A. tubingensis	CBS 126.52	Origin unknown
A. tubingensis	CBS 116.36	Origin unknown
A. tubingensis	CBS 121047	Thailand, Chiangmai Province, Arabica Coffee bean
A. uvarum	CBS 121591^{T}	Italy, healthy Cisternino grape
A. uvarum	CBS 121590	Italy, healthy grapes
A. uvarum	CBS 121592	Italy, healthy Carpaneto grapes
A. vadensis	CBS 113365^{T}	Origin unknown

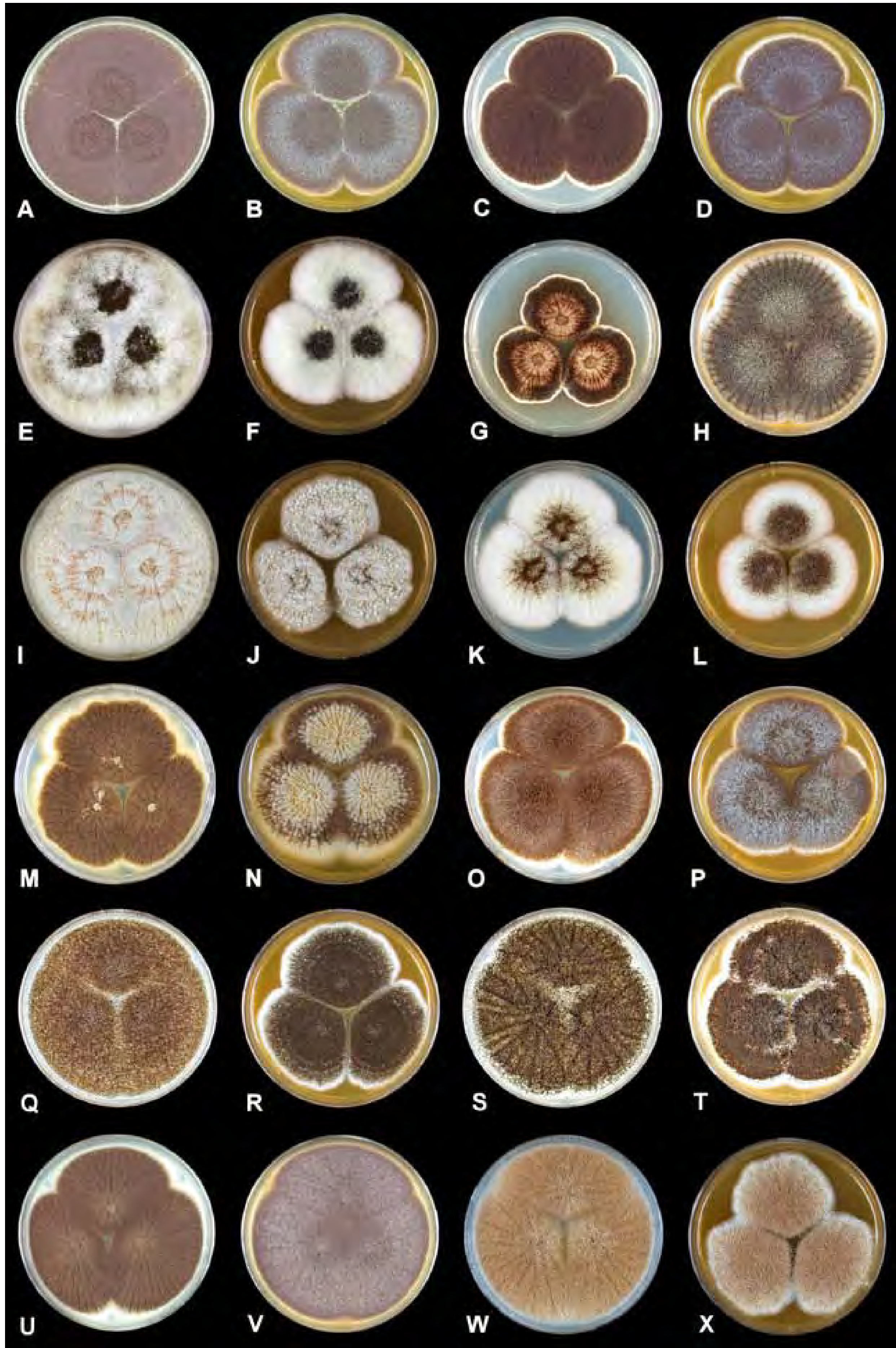


Fig. 1. Colony morphologies of type strains of species assigned to *Aspergillus* section *Nigri* grown on CYA and MEA plates at 25 °C for 7 d. (A–B) *A. aculeatinus*, (C–D) *A. aculeatus*, (E–F) *A. brasiliensis*, (G–H) *A. carbonarius,* (I–J) *A. costaricaensis*, (K–L) *A. ellipticus*, (M–N) *A. foetidus*, (O–P) *A. japonicus*, (Q–R) *A. heteromorphus*, (S–T) *A. homomorphus*, (U–V) *A. ibericus,* (W–X) *A. lacticoffeatus.*

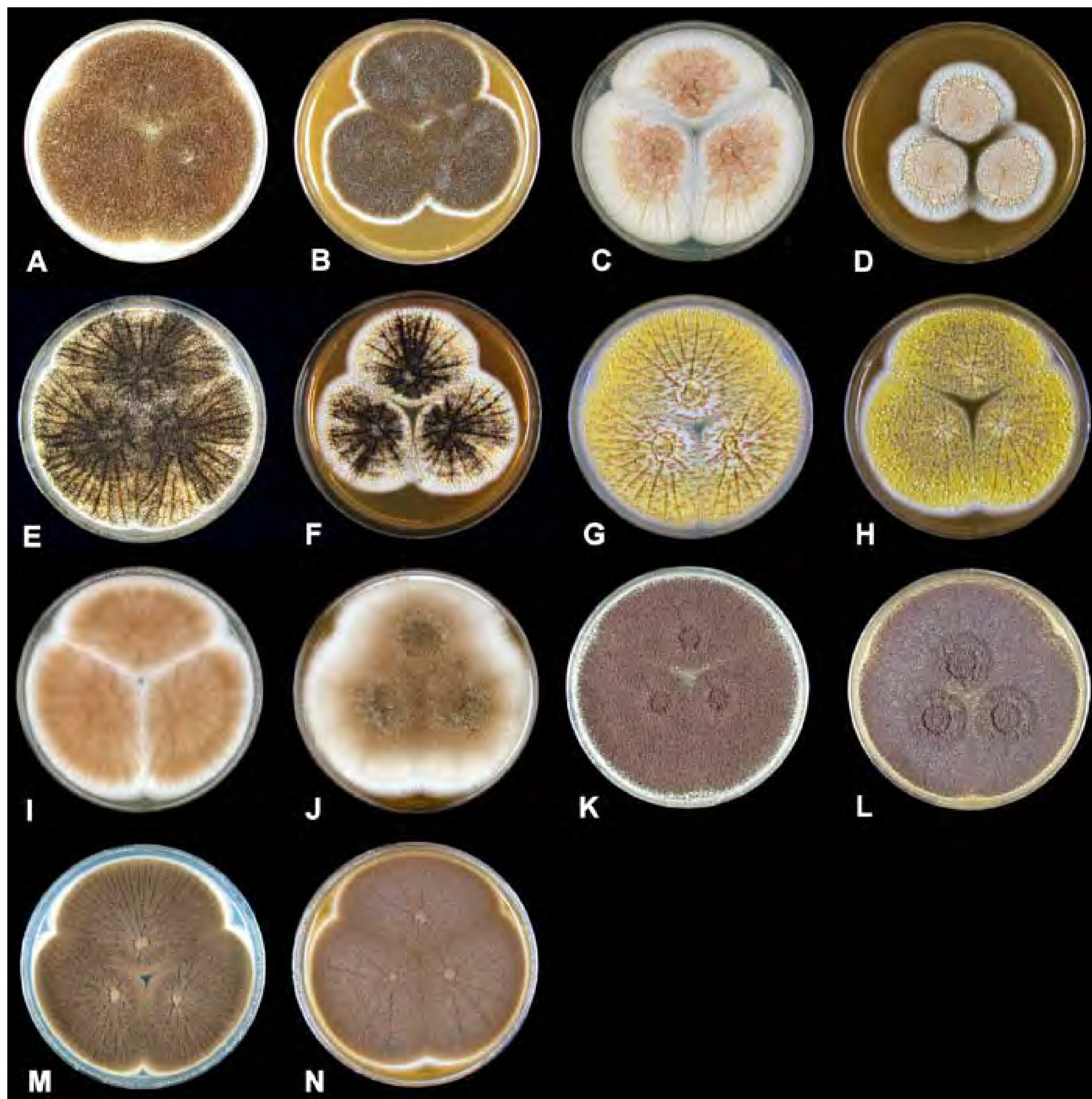


Fig. 2. Colony morphologies of type strains of species assigned to *Aspergillus* section *Nigri* grown on CYA and MEA plates at 25 °C for 7 d. (A–B) *A. niger*, (C–D) *A. piperis*, (E–F) *A. sclerotiicarbonarius*, (G–H) *A. sclerotioniger*, (I–J) *A. tubingensis*, (K–L) *A. uvarum,* (M–N) *A. vadensis*.

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 25ºC) 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. sclerotioniger, A. aculeatinus* and *A. sclerotiicarbonarius* (Table 2). Several species can easily be identified by their colony morphologies, including eg. *A. sclerotiicarbonarius* 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. sclerotiicarbonarius* and *A. sclerotioniger*. 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. lacticoffeatus*.

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*^{T}, which was able to grow (within 96 h) at 9 °C. *A. brasiliensis*^{T} 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. sclerotiicarbonarius*^{T}and *A. heteromorphus* (33 °C), group 3 consists of *A. sclerotioniger*, *A. uvarum*, *A. carbonarius*, *A. aculeatinus*, *A. homomorphus*, *A. japonicus* and *A. aculeatus* (36 °C), while group 4 consists of *A. ibericus, A. foetidus*, *A. tubingensis*, *A. piperis*, *A. costaricaensis*, *A. vadensis*, *A. niger*, *A. lacticoffeatus* 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. sclerotiicarbonarius* due to its inability to grow on CREA. Consequently, growth response on CREA can be used to distinguish *A. sclerotiicarbonarius* from closely related species also forming large conidia including *A. carbonarius, A. sclerotioniger* 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. brasiliensis*, *A. foetidus*, *A. tubingensis*, *A. vadensis* and *A. sclerotioniger*. *A. costaricaensis*, *A. piperis* and *A. lacticoffeatus* 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. aculeatinus* 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. sclerotioniger*, *A. homomorphus* and *A. sclerotiicarbonarius*. No visual growth was detected in *A. ellipticus*, *A. niger*, *A. brasiliensis*, *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. sclerotioniger* and *A. sclerotiicarbonarius*. 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.*

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

* the sclerotioid 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.

		Relative growth[a]		
Name	**CBS No.**	**3 days**	**7 days**	**Sporulation**
A. aculeatinus[T]	121060	+	+++	No
A. aculeatus[T]	172.66	+	+++	No
A. brasiliensis[T]	101740	-	+	No
A. carbonarius[T]	111.26	+++	+++++	Heavy
A. costaricaensis[T]	115574	-	++	No
A. ellipticus[T]	707.79	-	+++	No
A. foetidus[T]	564.65	+	+++	No
A. heteromorphus[T]	117.55	+	++++	No
A. homomorphus[T]	101889	++	+++	No
A. ibericus[T]	121593	-	+	No
A. japonicus[T]	114.51	+	+++	No
A. lacticoffeatus[T]	101883	+	++	No
A. niger[T]	554.65	-	++	No
A. piperis[T]	112811	-	++	No
A. sclerocarbonarius[T]	121057	+++	++++	Heavy
A. sclerotioniger[T]	115572	++	++++	Good
A. tubingensis[T]	134.48	+	++	No
A. uvarum[T]	121591	+	+++	No
A. vadensis[T]	113365	-	+	No

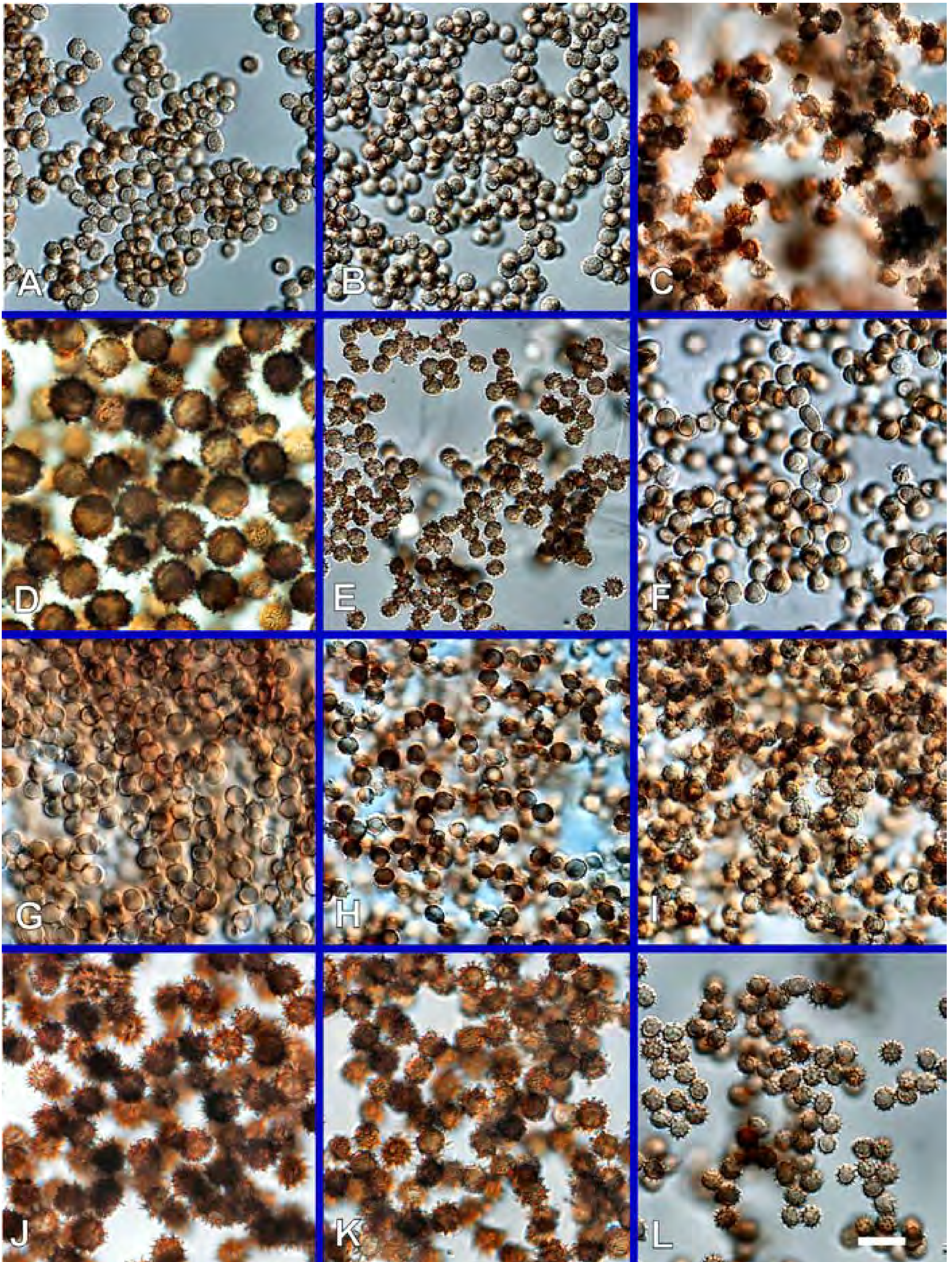


Fig. 3. Light microscopic pictures of conidia of type strains and representative isolates of *Aspergillus* section *Nigri*. (A) *A. aculeatinus*^{T} (2.5–4.5 µm), (B) *A. aculeatus*^{T} (3.5–5 µm), (C) *A. brasiliensis*^{T} (3.5–4.5 µm), (D) *A. carbonarius*^{T} (7–9 µm), (E) *A. costaricaensis*^{T} (3.1–4.5 µm), (F) *A. ellipticus*^{T} (3.3–5.5 µm), (G) *A. foetidus*^{T} (3.5–4.5 µm), (H) *A. foetidus* wild type, (I) *A. japonicus*^{T} (3.5–5 µm), (J) *A. heteromorphus*^{T} (3.5–5 µm), (K) *A. homomorphus*^{T} (5–7 µm), (L) *A. ibericus*^{T} (5–7 µm). Scale bar in L for all = 10 µm.

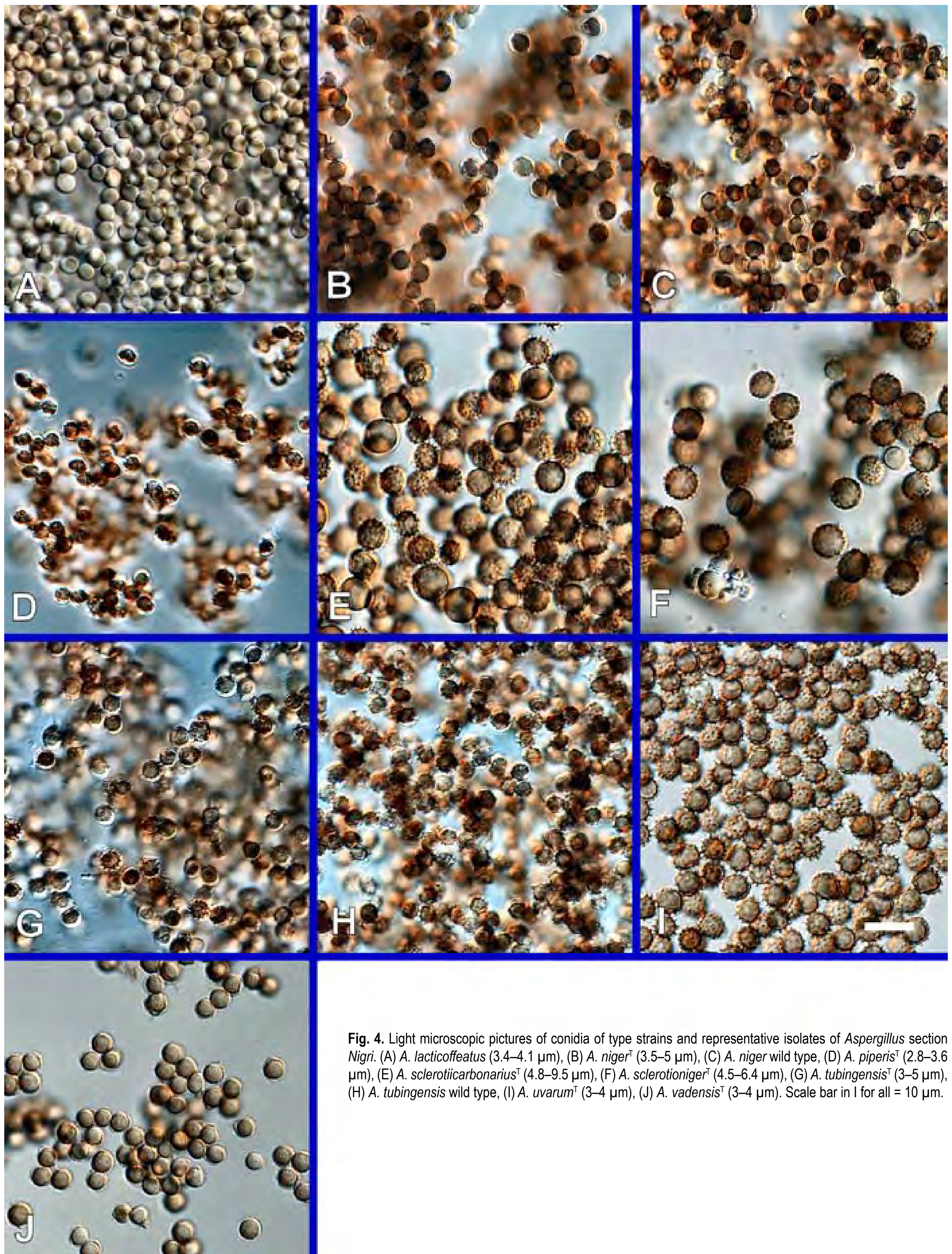
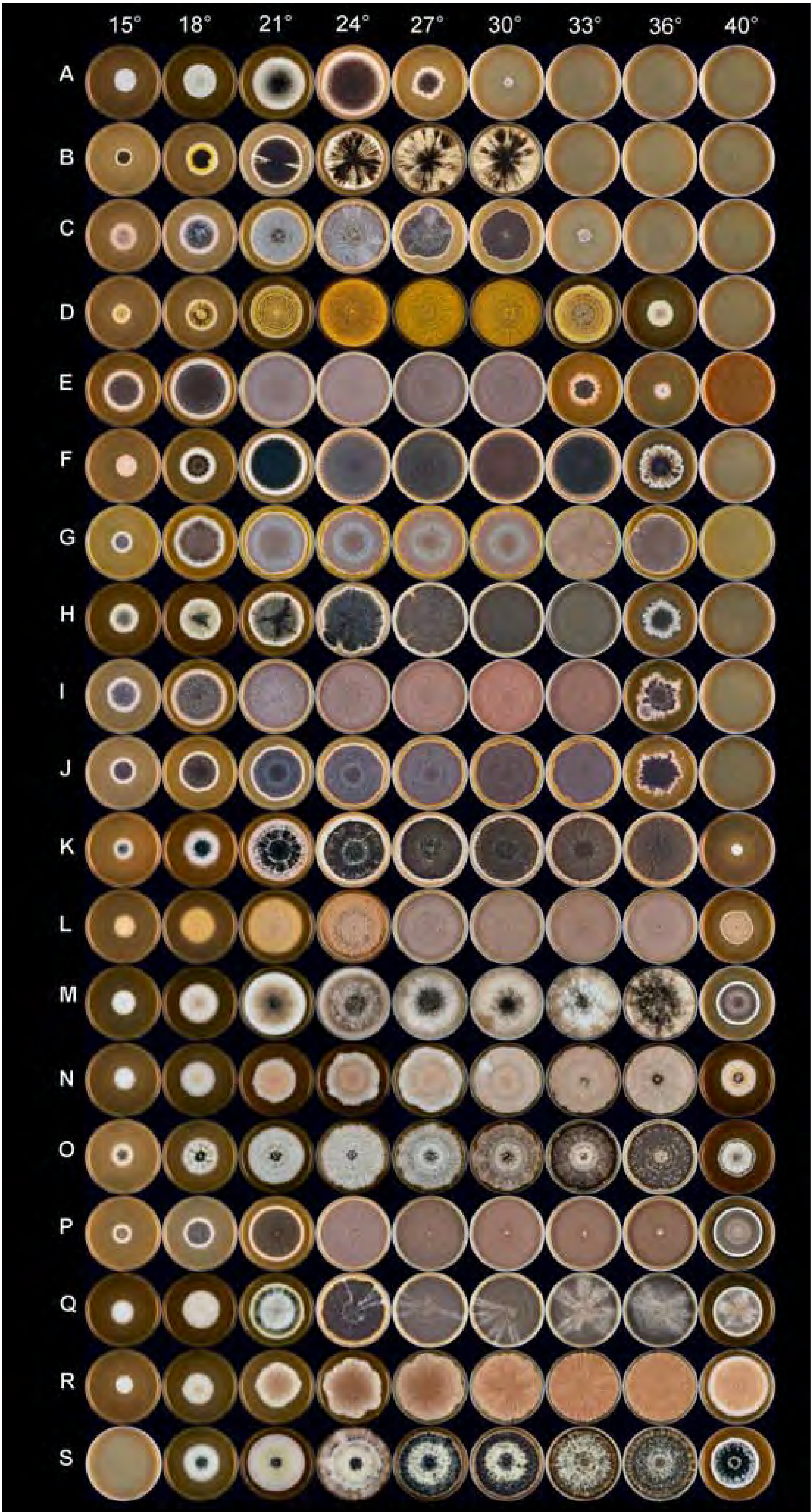


Fig. 4. Light microscopic pictures of conidia of type strains and representative isolates of *Aspergillus* section *Nigri*. (A) *A. lacticoffeatus* (3.4–4.1 µm), (B) *A. niger*[T] (3.5–5 µm), (C) *A. niger* wild type, (D) *A. piperis*[T] (2.8–3.6 µm), (E) *A. sclerotiicarbonarius*[T] (4.8–9.5 µm), (F) *A. sclerotioniger*[T] (4.5–6.4 µm), (G) *A. tubingensis*[T] (3–5 µm), (H) *A. tubingensis* wild type, (I) *A. uvarum*[T] (3–4 µm), (J) *A. vadensis*[T] (3–4 µm). Scale bar in I for all = 10 µm.

Fig. 5. (Page 137). 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*[T]; (B) *A. sclerotiicarbonarius*[T]; (C) *A. heteromorphus*[T]; (D) *A. sclerotioniger*[T]; (E) *A. uvarum*[T]; (F) *A. carbonarius*[T]; (G) A. *aculeatinus*[T]; (H) *A. homomorphus*; (I) *A. japonicus*[T]; (J) *A. aculeatus*[T]; (K) *A. ibericus*[T]; (L) *A. foetidus*[T]; (M) *A. tubingensis*[T]; (N) *A. piperis*[T]; (O) *A. costaricaensis*[T]; (P) *A. vadensis*[T]; (Q) *A. niger*[T]; (R) *A. lacticoffeatus*[T]; (S) *A. brasiliensis*[T].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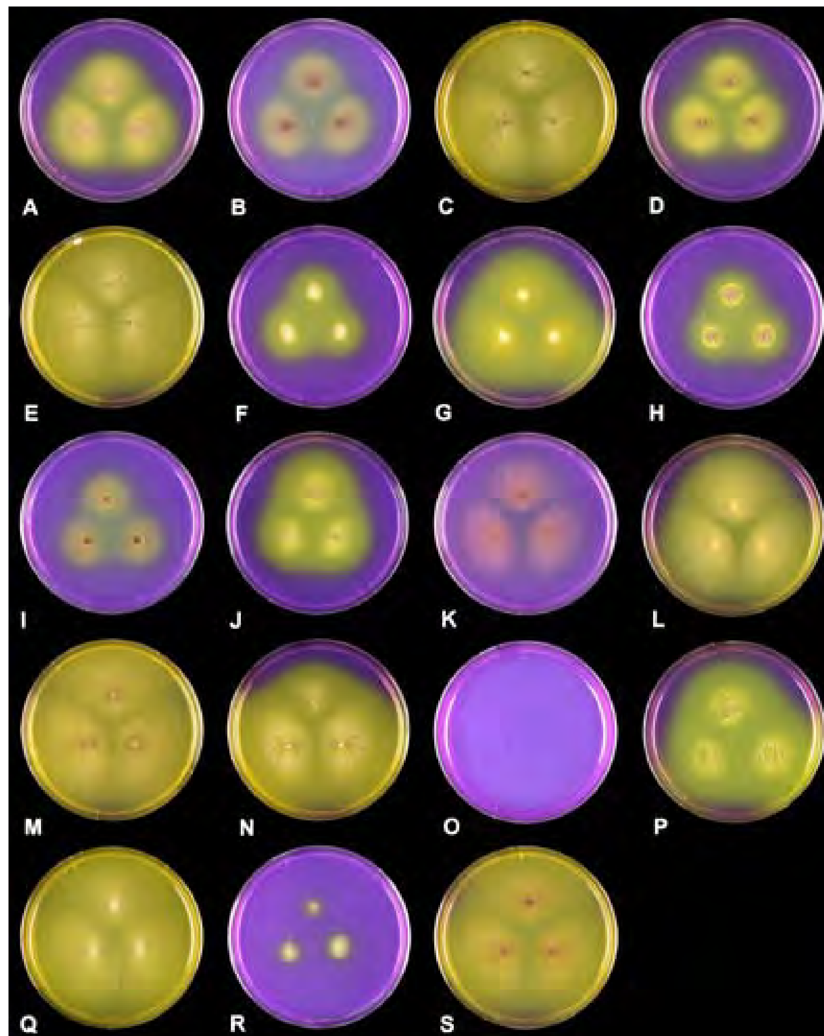

Fig. 6. Growth of type strains of *Aspergillus* section *Nigri* on creatine sucrose agar (CREA) plates. (A) *A. aculeatinus*[T]; (B) *A. aculeatus*[T]; (C) *A. brasiliensis*[T]; (D) *A. carbonarius*[T]; (E) *A. costaricaensis*[T]; (F) *A. ellipticus*[T]; (G) *A. foetidus*[T]; (H) *A. heteromorphus*[T]; (I) *A. homomorphus*[T]; (J) *A. ibericus*[T]; (K) *A. japonicus*[T]; (L) *A. lacticoffeatus*[T]; (M) *A. niger*[T]; (N) *A. piperis*[T]; (O) *A. sclerotiicarbonarius*[T]; (P) *A. sclerotioniger*[T]; (Q) *A. tubingensis*[T]; (R) *A. uvarum*[T]; (S) *A. vadensis*[T].

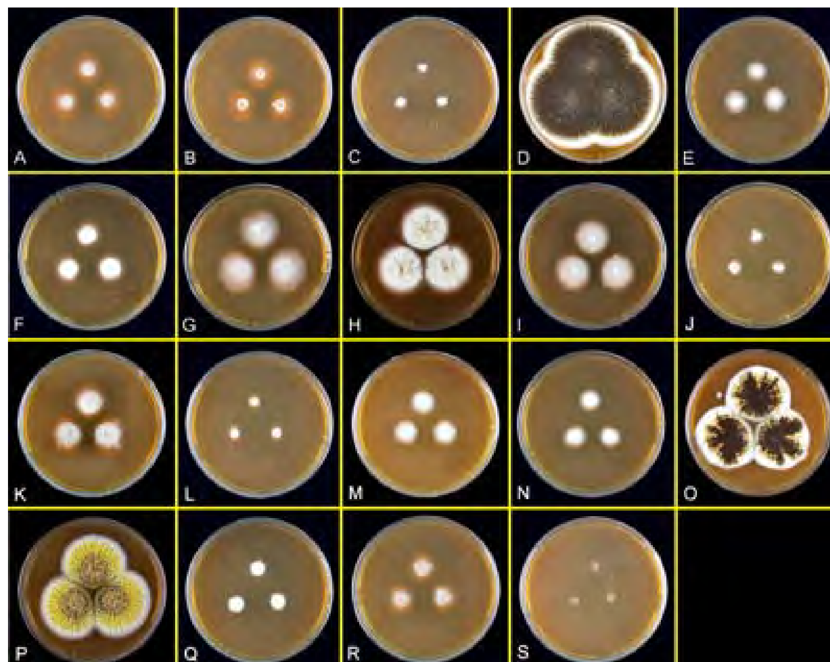

Fig. 7. Growth and sporulation of type strains of *Aspergillus* section *Nigri* on malt extract agar with Boscolid (MEA-B) after 7 d incubation. (A) *A. aculeatinus*; (B) *A. aculeatus*; (C) *A. brasiliensis*; (D) *A. carbonarius*; (E) *A. costaricaensis*; (F) *A. ellipticus*; (G) *A. foetidus*; (H) *A. heteromorphus*; (I) *A. homomorphus*; (J) *A. ibericus*; (K) *A. japonicus*; (L) *A. lacticoffeatus*; (M) *A. niger*; (N) *A. piperis*; (O) *A. sclerotiicarbonarius*; (P) *A. sclerotioniger*; (Q) *A. tubingensis*; (R) *A. uvarum*; (S) *A. vadensis*.

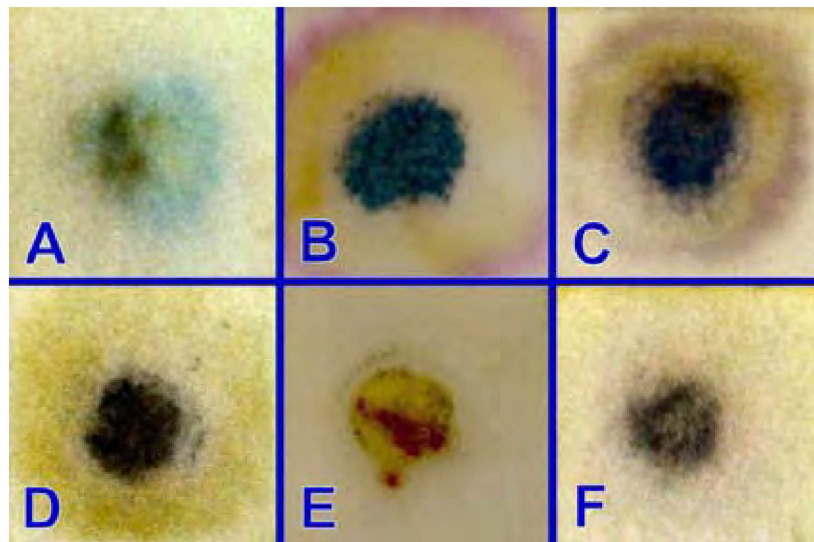

Fig. 8. Ehrlich colour reaction of some *Aspergillus* species in section *Nigri*. (A) blue in *A. costaricaensis*, (B) purple ring in *A. heteromorphus*, (C) purple ring in *A. homomorphus*, (D) yellow-green in *A. niger*, (E) purple-red at sclerotial area of *A. sclerotioniger* and (F) no reaction in *A. vadensis*.

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

Name	CBS No.	CYA	Notes
A. aculeatinus	CBS 121060ᵀ	-	
A. aculeatus	CBS 172.66ᵀ	-	
A. brasiliensis	CBS 101740ᵀ	++	Yellow reaction
A. carbonarius	CBS 111.26ᵀ	-	
A. costaricaensis	CBS 115574ᵀ	+++++	Blue reaction
A. ellipticus	CBS 707.79ᵀ	-	
A. foetidus	CBS 564.65ᵀ	++	Yellow reaction
A. heteromorphus	CBS 117.55ᵀ	+++++	Yellow reaction with purple ring
A. homomorphus	CBS 101889ᵀ	+++++	Yellow reaction with purple ring
A. ibericus	CBS 121593ᵀ	++	Yellow reaction
A. japonicus	CBS 114.51ᵀ	-	
A. lacticoffeatus	CBS 101883ᵀ	-	
A. niger	CBS 554.65ᵀ	++	Yellow reaction
A. piperis	CBS 112811ᵀ	-	
A. sclerotiicarbonarius	CBS 121057ᵀ	-	
A. sclerotioniger	CBS 115572ᵀ	+	* Violet reaction at sclerotia
A. tubingensis	CBS 134.48ᵀ	-	
A. uvarum	CBS 121591ᵀ	-	
A. vadensis	CBS 113365ᵀ	-	

of the Ehrlich reagent wetted on filter paper with mycelial side of an agar plug. Samson & Frisvad (2004) also suggested that this method is useful for classification of *Penicillium* subgenus *Penicillium*. In this paper, we tried this method to classify some *Aspergillus* spp. in section *Nigri*. All type strains were examined and the results were shown in Table 4 and Fig. 8.

In positive results, violet ring or blue colour compounds appeared. Based on their response, species of *Aspergillus* section *Nigri* can be classified into groups. *A. heteromorphus* and *A. homomorphus* had the same positive results with yellow reaction with purple ring occurring within 5 min. *A. costaricaensis* also gave a positive result with the test but it reacted and formed a strikingly blue colour. *A. brasiliensis*, *A. foetidus* and *A. niger* gave positive results in the form of a yellow reaction. *A. tubingensis*, and the other related species gave negative result so this method is useful to discriminate them from the others. Furthermore, *A. sclerotioniger* could also give purple colour positive result at the sclerotial area.

Extrolites found in the 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-

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

Species	Extrolites produced
A. aculeatinus	neoxaline, secalonic acid D, secalonic acid F, aculeasins
A. aculeatus	secalonic acid D, secalonic acid F
A. brasiliensis	naphtho-γ-pyrones (including aurasperone B), pyrophen, tensidol A & B
A. carbonarius	ochratoxins (A, B, α, β), naphtho-γ-pyrones (including aurasperone B), pyranonigrin A
A. costaricaensis	aflavinines[a], funalenone, naphtho-γ-pyrones (including aurasperone B)
A. ellipticus	austdiol, candidusins, terpenyllin, cf. xanthoascin
A. foetidus	antafumicins (only some strains), asperazine, funalenone, naphtha-γ-pyrones (including aurasperone B), pyranonigrin A, (nigragillin)
A. heteromorphus	lots of highly unique extrolites including indol-alkaloids, none of them structure elucidated
A. homomorphus	dehydrocarolic acid, secalonic acid D, secalonic acid F
A. ibericus	naphtho-γ-pyrones (including aurasperone B), pyranonigrin A
A. japonicus	cycloclavine, festuclavine
A. lacticoffeatus	kotanins, ochratoxin A, pyranonigrin A, tensidol A & B
A. niger	funalenone, ochratoxin A (only some strains), malformins, naphtho-γ-pyrones (including aurasperone B), pyranonigrin A, tensidol A & B, (nigragillin)
A. piperis	aflavinins, naphtho-γ-pyrones (including aurasperone B), pyranonigrin A
A. sclerotiicarbonarius	naphtho-γ-pyrones (including aurasperone B), pyranonigrin A, three unique indol-alkaloids at retention indices 1475, 1676 and 1838.
A. sclerotioniger	corymbiferan lactones, funalenone, naphtho-γ-pyrones (including aurasperone B), ochratoxins (A, B, α, β), pyranonigrin A
A. tubingensis	asperazine, funalenone, malformins, naphtho-γ-pyrones (including aurasperone B), pyranonigrin A, tensidol A & B, (nigragillin)
A. uvarum	asterric acid, dihydrogeodin, erdin, geodin, secalonic acid D and F
A. vadensis	nigragillin, asperazine, naphtho-γ-pyrones (including aurasperone B), a polar orlandin-like compound

[a] aflavinins are: 14-epi-14-hydroxy-10,23, dihydro-24,25-dehydroaflavinine, 10.23-dihydro-24,25-dehydroaflavinine and 10,23-dihydro-24,25-dehydro-21-oxo-aflavinine

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 *Sma*I-generated ribosomal DNA (rDNA) patterns. The two groups were also clearly distinguishable by their hybridisation patterns when pectin 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. ellipticus*, and *A. aculeatus* exhibited the same *Sma*I-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).

*Sma*I 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. ellipticus*, *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 III were shown by *A. niger* and *A. brasiliensis* 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 pectin 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 *pel*A 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 *Hae*III-*Bgl*II 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. brasiliensis* 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 *Hae*III-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 *Hae*III-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

kb) than those found earlier in the *A. niger* aggregate. The physical maps of the mtDNAs of *A. carbonarius* strain IN7 (which later was found to belong to the *A. ibericus* species; Varga J., unpubl. data) and the other *A. carbonarius* strains are quite different from each other, however, the order of the genes on these molecules seems to be conserved (Hamari *et al.* 1999).

Among other approaches, Megnegneau *et al.* (1993) applied the random amplified polymorphic DNA (RAPD) technique for examining variability among black aspergilli. By applying six random primers, they could differentiate *A. carbonarius*, *A. japonicus*, *A. aculeatus*, *A. heteromorphus* and *A. ellipticus* from each other, and could divide the *A. niger* species complex into two groups corresponding to the *A. niger* and *A. tubingensis* species. The RAPD technique could also be used successfully for the examination of genetic variability within *A. carbonarius* and *A. japonicus* species. *A. carbonarius* strain IN7 could readily be distinguished from the other *A. carbonarius* strains examined (Kevei *et al.* 1996). The strains representing the *A. aculeatus* species could also be distinguished from the other *A. japonicus* strains by using 4 random primers (Hamari *et al.* 1997), and *A. brasiliensis* could also be distinguished from the other strains of the *A. niger* species complex by RAPD analysis (Varga *et al.* 2000). AFLP analysis could successfully be used to distinguish among black *Aspergillus* species by Perrone *et al.* (2006a, 2006b), Serra *et al.* (2006) and Varga *et al.* (2007). Analysis of electrophoretic karyotypes among black aspergilli revealed the presence of high levels of intraspecific variability of the banding patterns observed (Megnegneau *et al.* 1993; Swart *et al.* 1994). However, the estimated total genome sizes did not differ significantly, ranging from 35.9 Mb in an *A. niger* strain to 43.8 Mb in an *A. ellipticus* strain. The average genome size of strains belonging to the *A. niger* species complex was 38.3 Mb. In general, electrophoretic karyotyping seems to be of little taxonomic value in such a variable group as black aspergilli.

Among the PCR based approaches, Accensi *et al.* (1999) used a PCR-RFLP technique to distinguish *A. niger* and *A. tubingensis* isolates. The authors used the restriction enzyme *Rsa*I to digest the amplified ITS region of the isolates, and observed that isolates of the *A. niger* species complex exhibit two different RFLP patterns, N and T corresponding to *A. niger* and *A. tubingensis* isolates, respectively. The ITS region of *A. niger* contains the recognition site of *Rsa*I (5"-GT/AC-3") at position 75, while that of *A. tubingensis* does not. However, *in silico* examination of the ITS region of black aspergilli indicated that pattern T is also shared by *A. foetidus, A. vadensis, A. piperis* and *A. costaricaensis* isolates, while all other species exhibit pattern N (data not shown). However, this method has been used to distinguish *A. niger* from *A. tubingensis* (Medina *et al.* 2005, Accensi *et al.* 2001, Martinez-Culebras & Ramon 2007, Bau *et al.* 2006). PCR-RFLP analysis of the ITS region using other restriction enzymes has also been used for species identification recently (Martinez-Culebras & Ramon 2007). The authors used *Hha*I, *Nla*III and *Rsa*I to distinguish between *A. niger, A. tubingensis, A. carbonarius* and *A. aculeatus* isolates came from grapes. Some "*A. tubingensis*-like" isolates exhibited characteristic RFLP profiles when *Nla*III was used to digest the amplified fragment, which was found to be caused by a single point mutation in the ITS region. Gonzales-Salgado *et al.* (2005) developed species-specific primer pairs designed based on sequences of the ITS region for the identification of *A. niger, A. tubingensis, A. heteromorphus, A. ellipticus* and *A. japonicus*. Zanzotto *et al.* (2006) used PCR-RFLP analysis of the ITS, IGS and β-tubulin genes to distinguish between OTA-producing and non-producing isolates of the *A. niger* aggregate. Schmidt *et al.* (2004) developed species specific PCR primers based on AFLP fragments for the identification of *A. carbonarius* on coffee beans, while Atoui *et al.* (2007) and Mule *et al.* (2006) developed real time PCR approaches to identify *A. carbonarius* on grapes. The latter two groups used species-specific primer pairs designed from the acyltransferase (AT) domain of the polyketide synthase sequence and the calmodulin gene, respectively. Susca *et al.* (2007a) also developed species-specific primers based on partial calmodulin gene sequences to identify *A. carbonarius* and *A. niger* by PCR. Recently, Susca *et al.* (2007b) developed a PCR-single-stranded conformational polymorphism (SSCP) screening method based on the detection of sequence variation in part of the calmodulin gene. Using this approach, 11 species including *A. brasiliensis, A. niger, A. tubingensis, A. foetidus, A. aculeatus, A. uvarum, A. japonicus, A. ellipticus, A. heteromorphus, A. carbonarius* and *A. ibericus* could be distinguished based on their different PCR-SSCP profiles. A low-complexity oligonucleotide microarray (OLISA) has also been developed based on oligonucleotide probes obtained from sequences of the calmodulin gene for the detection of black aspergilli (*A. carbonarius, A. ibericus* and *A. aculeatus/A. japonicus*) from grapes (Bufflier *et al.* 2007).

Nowadays, sequence-based identification methods are widely used for species identification. 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 (*A. lacticoffeatus* had identical β-tubulin sequences to some *A. niger* isolates; Samson *et al.* 2004, Varga *et al.* 2007; Fig. 9). The ITS data set can be used to distinguish 4 groups within the *A. niger* species complex: 1. *A. niger* and *A. lacticoffeatus* isolates; 2. *A. brasiliensis*; 3. *A. costaricaensis*; 4. *A. tubingensis, A. foetidus, A. vadensis* and *A. piperis* (Varga *et al.* 2007). Among the other black aspergilli, *A. carbonarius* and *A. sclerotioniger* exhibit identical ITS sequences, while most uniseriate species also have identical ITS sequences (including *A. japonicus, A. aculeatus* and *A. uvarum*). Yokoyama *et al.* (2001) used sequences of the mitochondrial cytochrome b gene to infer phylogenetic relationships among black aspergilli. *A. japonicus/A. aculeatus, A. niger, A. tubingensis, A. carbonarius* and *A. ellipticus* could be distinguished from each other based on phylogenetic analysis of amino acid data. However, *A. tubingensis* and *A. niger* isolates could not be clearly distinguished when nucleotide sequences were subjected to phylogenetic analysis.

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 *A. niger* species complex, including pyruvate kinase, pectin lyase, polygalacturonase, arabinoxylan-arabinofuranohydrolase and several other genes (Gielkens *et al.* 1997, de Vries *et al.* 2005, Parenicova *et al.* 2001), translation intitiation factor 2, pyruvate carboxylase, 70 kD heat shock protein, chaperonin complex component (TCP-1), ATPase (Witiak *et al.* 2007), and translation elongation factor 1-α, RNA polymerase 2 and actin gene sequences (S.W. Peterson, personal communication). According to recent data, *cox1* is not appropriate to be used for species identification in black aspergilli (Geiser *et al.* 2007). 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. The different molecular techniques applied for species delimitation in *Aspergillus* section *Nigri* are summarised in Table 6.

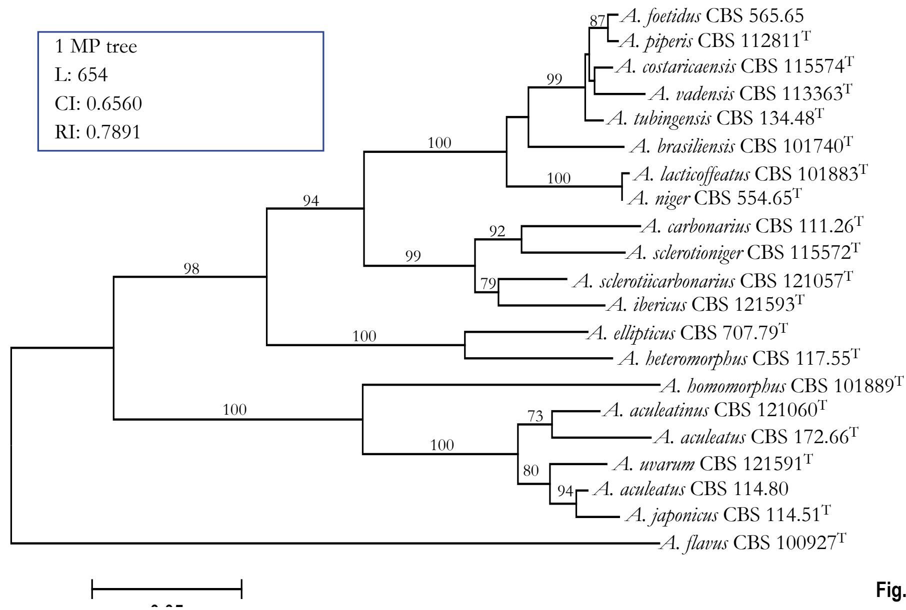

Fig. 9A.

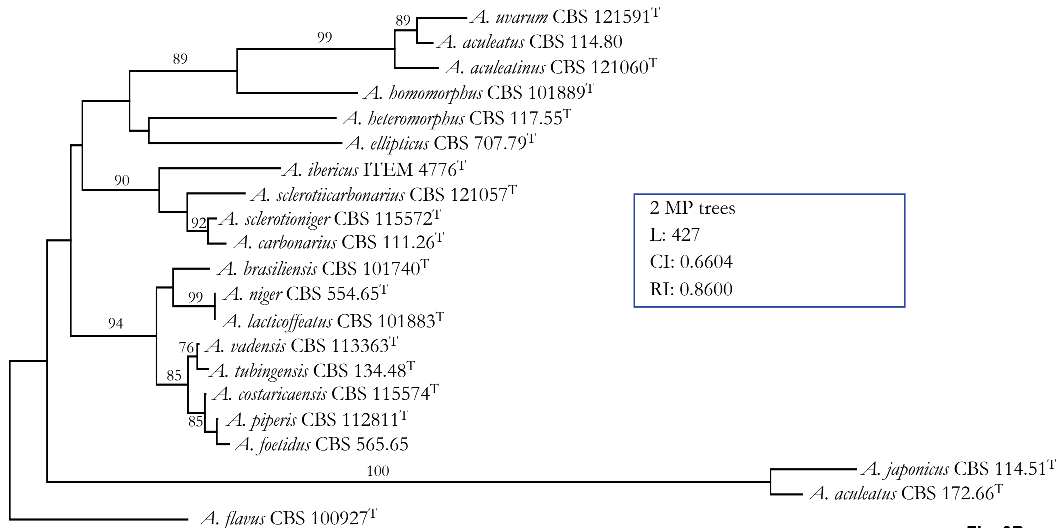

Fig. 9B.

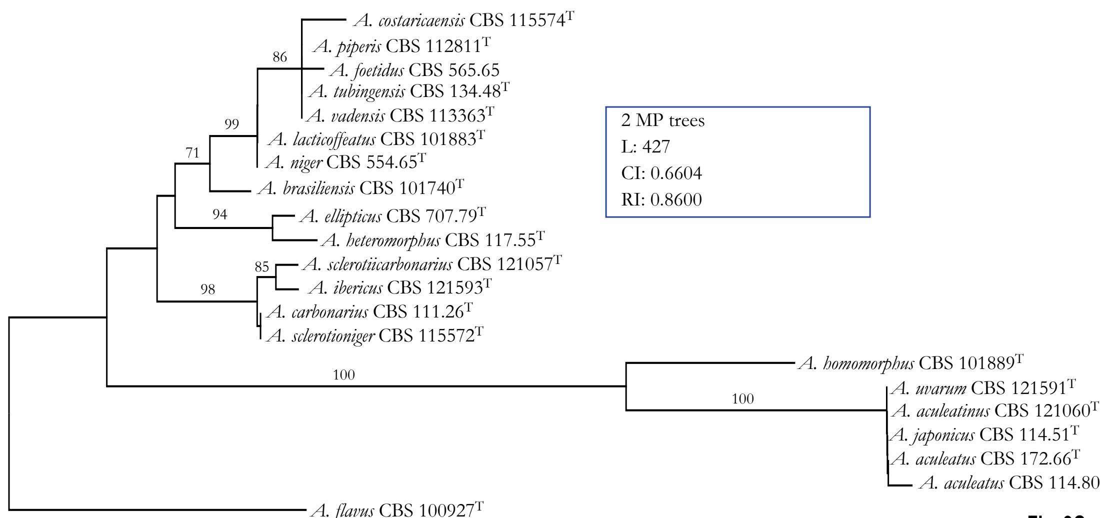

Fig. 9C.

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*.

Method used	Target region	Reference	Comments
RFLP	*pelA*, *pelB, pki*	Kusters-van Someren *et al.* 1991	*A. niger* and *A. tubingensis* could be distinguished
RFLP	rDNA (*Sma*I)	Kusters-van Someren *et al.* 1991, Varga *et al.* 2000	*A. niger, A. tubingensis, A. brasiliensis, A. ellipticus*, *A. heteromorphus*, *A. japonicus* and *A. carbonarius* could be distinguished
RFLP	mtDNA (*Hae*III/*Bgl*II)	Varga *et al.* 1993, 1994, Hamari *et al.* 1997	Could be used to distinguish *A. niger* and *A. brasiliensis* from *A. tubingensis*, and *A. japonicus* from *A. aculeatus*; several intraspecific mtDNA types identified
RFLP	*pelA, pki,* rDNA	Parenicova *et al.* 2001, de Vries *et al.* 2005	Could distinguish most species including *A. aculeatus, A. japonicus and A. vadensis*
PCR-RFLP	ITS(*Rsa*I)	Accensi *et al.*1999, 2001, Medina *et al.* 2005, Bau *et al.* 2005	N and T types distinguished among species of the *A. niger* aggregate; pattern T is shared by A. tubingensis, *A. foetidus, A. vadensis, A. piperis* and *A. costaricaensis* isolates, while all other species exhibit pattern N
PCR-RFLP	ITS (*Rsa*I, *Hha*I, *Nla*III)	Martinez-Culebras & Ramon 2007	Could distinguish between *A. niger, A. tubingensis, A. carbonarius* and *A. aculeatus* isolates
PCR-RFLP	ITS (*Rsa*I), IGS (*Hin*fI), β-tubulin (*Rsa*I)	Zanzotto *et al.* 2006	Could distinguish between potential OTA-producing and non-producing isolates of the *A. niger* aggregate (ie. between *A. niger* and *A. tubingensis*)
AFLP		Perrone *et al.* 2006a, 2006b	All known species could be distinguished
PCR-SSCP	calmodulin	Susca *et al.* 2007b	11 species including *A. brasiliensis, A. niger, A. tubingensis, A. foetidus, A. aculeatus, A. uvarum, A. japonicus, A. ellipticus, A. heteromorphus, A. carbonarius* and *A. ibericus* could be distinguished
OLISA	calmodulin	Bufflier *et al.* 2007	*A. carbonarius, A. ibericus* and *A. japonicus/A. aculeatus* could be distinguished.
Sequence analysis	ITS	Varga *et al.* 2007	Several species have identical ITS sequences (eg. *A. niger* and *A. lacticoffeatus; A. tubingensis, A. foetidus, A. vadensis* and *A. piperis; A. carbonarius and A. sclerotioniger; A. japonicus, A. aculeatus* and *A. uvarum)*
Sequence analysis	Mitochondrial cytochrome b	Yokoyama *et al.* 2001	*A. japonicus/A. aculeatus, A. niger, A. tubingensis, A. carbonarius* and *A. ellipticus* could be distinguished
Sequence analysis	β-tubulin	Samson *et al.* 2004, Varga *et al.* 2007	all except one species (*A. lacticoffeatus)* could be distinguished
Sequence analysis	Calmodulin	Varga *et al.* 2007	All species could be distinguished
Sequence analysis	Cytochrome oxidase I	Klich *et al.* 2007	*A. niger* and *A. tubingensis* could not be distinguished; not appropriate to be used for species identification in black aspergilli
Sequence analysis	IGS	Unpublished data	Too variable for species identification
PCR with species-specific primers	ITS	Gonzales-Salgado *et al.* 2005	Species-specific detection of *A. niger, A. tubingensis, A. heteromorphus, A. ellipticus* and *A. japonicus*
PCR with species-specific primers	calmodulin	Susca *et al.* 2007a	Species-specific detection of *A. carbonarius* and *A. niger*
PCR with species-specific primers	RAPD fragment	Fungaro *et al.* 2004	Species-specific detection of *A. carbonarius*
PCR with species-specific primers	calmodulin	Perrone *et al.* 2004	Species-specific detection of *A. carbonarius* and *A. japonicus*
PCR with species-specific primers	ITS	Haugland and Vesper 2002	Species-specific detection of *A. carbonarius* and *A. niger*
PCR with species-specific primers	AFLP marker	Schmidt *et al.* 2004	Species-specific detection of *A. carbonarius*
PCR with species-specific primers	PKS	Lebrihi *et al.* 2003	Species-specific detection of OTA producing *A. carbonarius* isolates
PCR with species-specific primers	PKS	Dobson & O"Callagfhan 2004	Species-specific detection of OTA producing *A. carbonarius* and *A. niger* isolates
Real time PCR	ITS	Haugland *et al.* 2004	Species-specific detection of *A. carbonarius* and *A. niger*
Real time PCR	calmodulin	Mule *et al.* 2006	Species-specific detection of *A. carbonarius*
Real time PCR	AT domain of the PKS gene	Atoui *et al.* 2007	Species-specific detection of *A. carbonarius*

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 Boscalid agars are only good media when identifying some taxa. CREA is helpful when distinguishing the rare species *A. sclerotiicarbonarius* from closely related species also forming large conidia, *A. carbonarius* and *A. ibericus*. Boscalid agar can be used as a selective medium for *A. carbonarius*, *A. sclerotioniger*, *A. homomorphus* and *A. sclerotiicarbonarius*, 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. tubingensis*, *A. foetidus* and *A. vadensis* from *A. niger* and *A. brasiliensis*, while pyranonigrin A is present in all species in the *Aspergillus niger* complex, except *A. brasiliensis, A. costaricaensis* and *A. vadensis*. Secalonic acid D is produced by the uniseriate species only, except *A. japonicus*. However the biseriate *A. homomorphus* also produces secalonic acid D. *A. ellipticus* is entirely unique and produces extrolites found in section *Candidi* (terphenyllin and candidusins) and section *Usti* (austdiol). *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.

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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. australensis N. ferenczii, 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 australensis*, *N. ferenczii*, *N. papuaensis*, *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 (Coriglione *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 mycotoxins 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 (McCowen *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.

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Table 1. *Aspergillus* section *Fumigati* isolates used in this study.

Species	Isolate No.*	Source
A. brevipes	CBS 118.53 T	Soil, Australia
A. duricaulis	CBS 481.65T	Soil, Buenos Aires, Argentina
A. fumigatiaffinis	IBT12703T	Soil, U.S.A.
A. fumigatus	CBS 133.61T = NRRL 163	Chicken lung, U.S.A.
A. fumisynnematus	IFM 42277T	Soil, Venezuela
A. lentulus	CBS 117887T = NRRL 35552 = KACC 41940	Man, U.S.A.
A. novofumigatus	IBT 16806T	Soil, Ecuador
A. unilateralis	CBS 126.56T	Rhizosphere, Australia
A. viridinutans	CBS 127.56T	Rabbit dung, Australia
A. turcosus	KACC 42090 = IBT 27920	Air conditioner, Inchen,Korea
	KACC 42091T = IBT 27921	Air conditioner, Seoul, Korea
	KACC 41955 = CBS 117265= IBT 3016	Car air conditioner, Seoul, Korea
N. assulata	KACC 41691T	Tomato soil, Buyeo, Korea
N. aurata	CBS 466.65T	Jungle soil, Brunei
N. aureola	CBS 105.55T	Soil, Tafo, Ghana
N. australensis sp. nov	CBS 112.55T = NRRL 2392 = IBT 3021	Garden soil, Adelaide, Australia
N. coreana	KACC 41659T = NRRL 35590 = CBS 121594	Tomato soil, Buyeo, Korea
N. denticulata	CBS 652.73T = KACC 41183	Soil under *Elaeis guineensis*, Suriname
	CBS 290.74 = KACC 41175	*Acer pseudoplatanus*, Netherlands
N. fennelliae	CBS 598.74T	Eye ball of *Oryctolagus cuniculus*, U.S.A.
	CBS 599.74	Eye ball of *Oryctolagus cuniculus*, U.S.A.
N. ferenczii **sp. nov.**	CBS 121594T = IBT 27813 = NRRL 4179	Soil, Australia
N. fischeri	CBS 544.65T = NRRL 181	Canned apples
N. galapagensis	CBS 117522T = IBT 16756 = KACC 41935	Soil, Ecuador
	CBS 117521 = IBT 16763 = KACC 41936	Soil, Ecuador
N. glabra	CBS 111.55T	Rubber scrab from old tire, Iowa, U.S.A.
N. hiratsukae	CBS 294.93T	Aloe juice, Tokyo, Japan
N. laciniosa	KACC 41657T = NRRL 35589 = CBS 117721	Tomato soil, Buyeo, Korea
N. multiplicata	CBS 646.95T = IBT 17517	Soil, Mouli, Taiwan
N. nishimurae	IFM 54133 = IBT 29024	Forest soil, Kenya
N. nishimurae	CBS 116047	Cardboard, Netherlands
N. papuensis **sp. nov.**	CBS 841.96T = IBT 27801	Bark of *Podocarpus* sp. (Podocarpaceae), bark, Myola, Owen Stanley Range, Northern Province, Papua New Guinea
N. pseudofischeri	NRRL 20748T = CBS 208.92	Human vertebrate, U.S.A.
N. quadricincta	CBS 135.52T = NRRL 2154	Cardboard, York, U.K.
	CBS 107078	Soil, Korea
	CBS 100942	Fruit juice, Netherlands
	CBS 253.94	Canned oolong tea beverage, Japan (type strain of *N. primulina*)
N. spathulata	CBS 408.89T	Soil under *Alocasia macrorrhiza*, Taiwan
N. spinosa	CBS 483.65T	Soil, Nicaragua
N. stramenia	CBS 498.65T	Soil from maple-ash-elm forest, Wisconsin, U.S.A.
N. tatenoi	CBS 407.93T	Soil of sugarcane, Timbauba, Brazil
	CBS 101754	Fruit, Yunnan, China (type strain of *N. delicata*)
N. udagawae	CBS 114217T	Soil, Brazil
	CBS 114218	Soil, Brazil
N. warcupii **sp. nov.**	NRRL 35723 T	Arid soil, Finder"s Range, Australia

* 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

Extrolites were analysed using the HPLC-diode array detection method of Frisvad & Thrane (1987, 1993) as modified by Smedsgaard (1997). Extrolites 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 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 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 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 390 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 verruculose and small triangular projections or sometimes with circularly arranged projections.

N. otanii Takada, Y. Horie & Abliz (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.

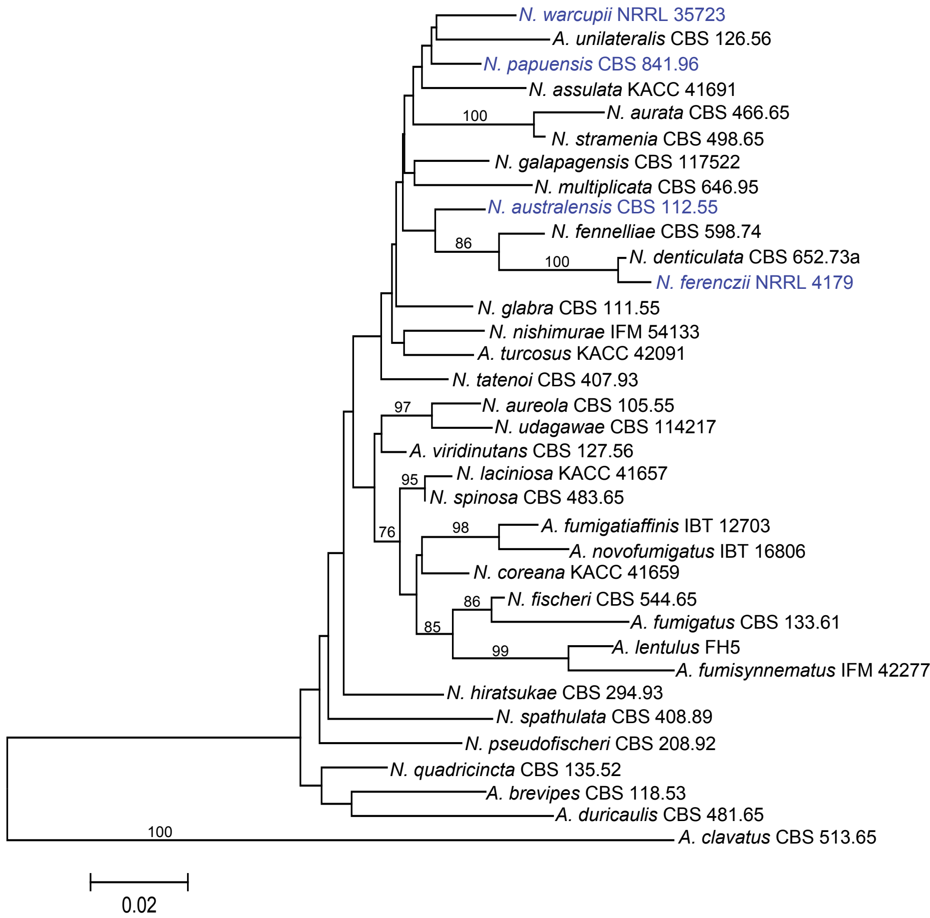

Fig. 1. Neighbour-joining tree based on β-tubulin sequence data of *Aspergillus* section *Fumigati*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

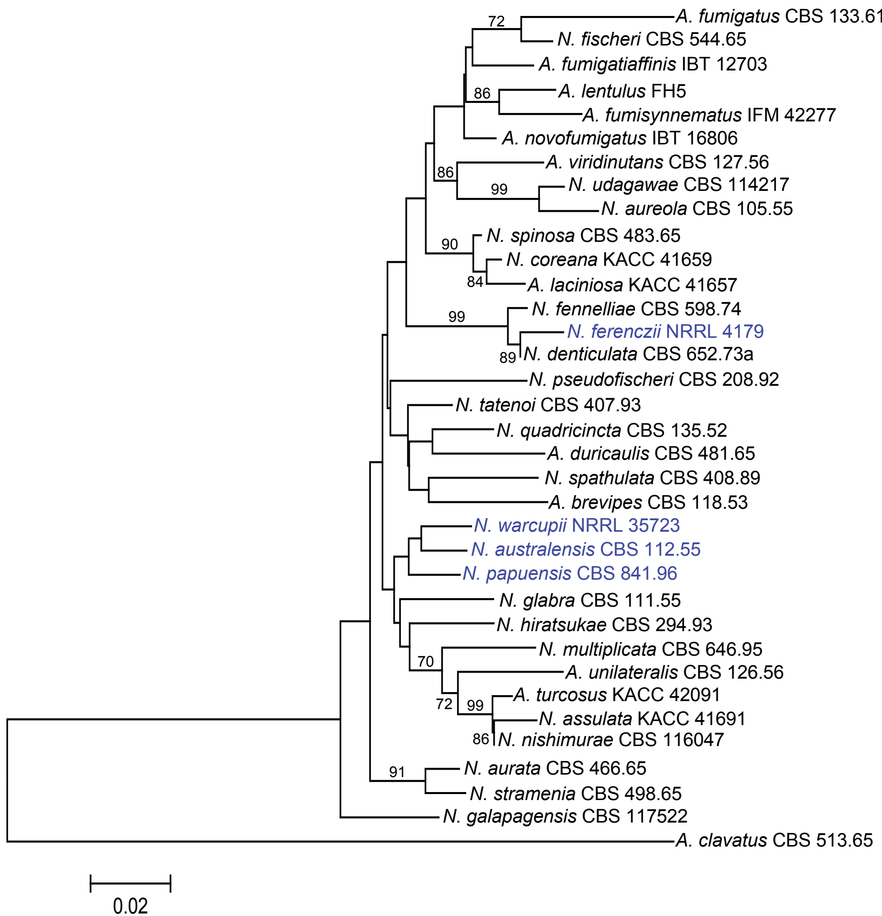

Fig. 2. Neighbour-joining tree based on calmodulin sequence data of *Aspergillus* section *Fumigati*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

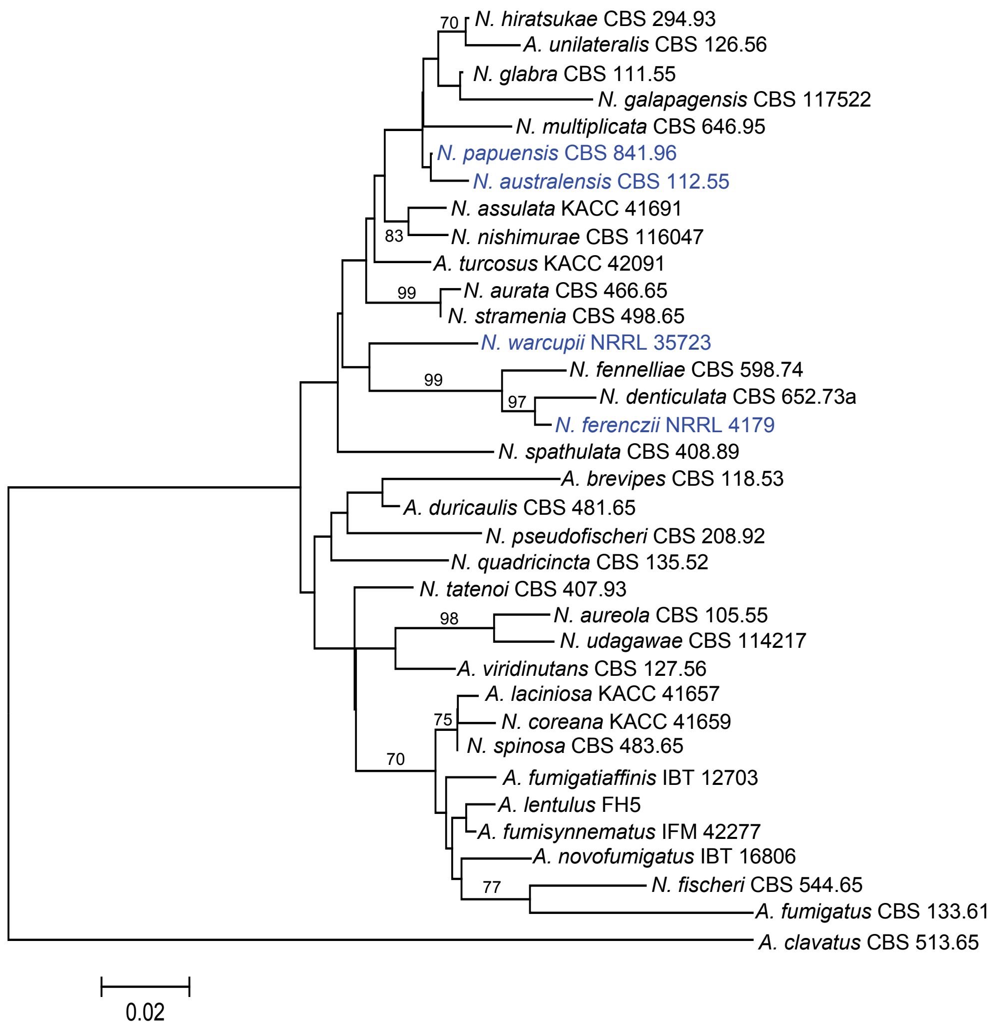

Fig. 3. Neighbour-joining tree based on actin sequence data of *Aspergillus* section *Fumigati*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

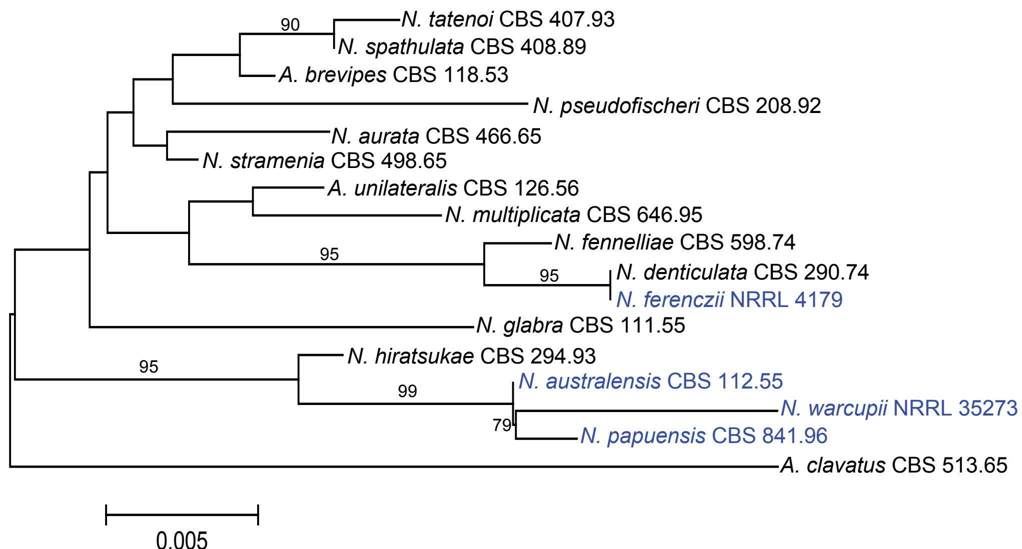

Fig. 4. Neighbour-joining tree based on ITS sequence data of selected species of *Aspergillus* section *Fumigati*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

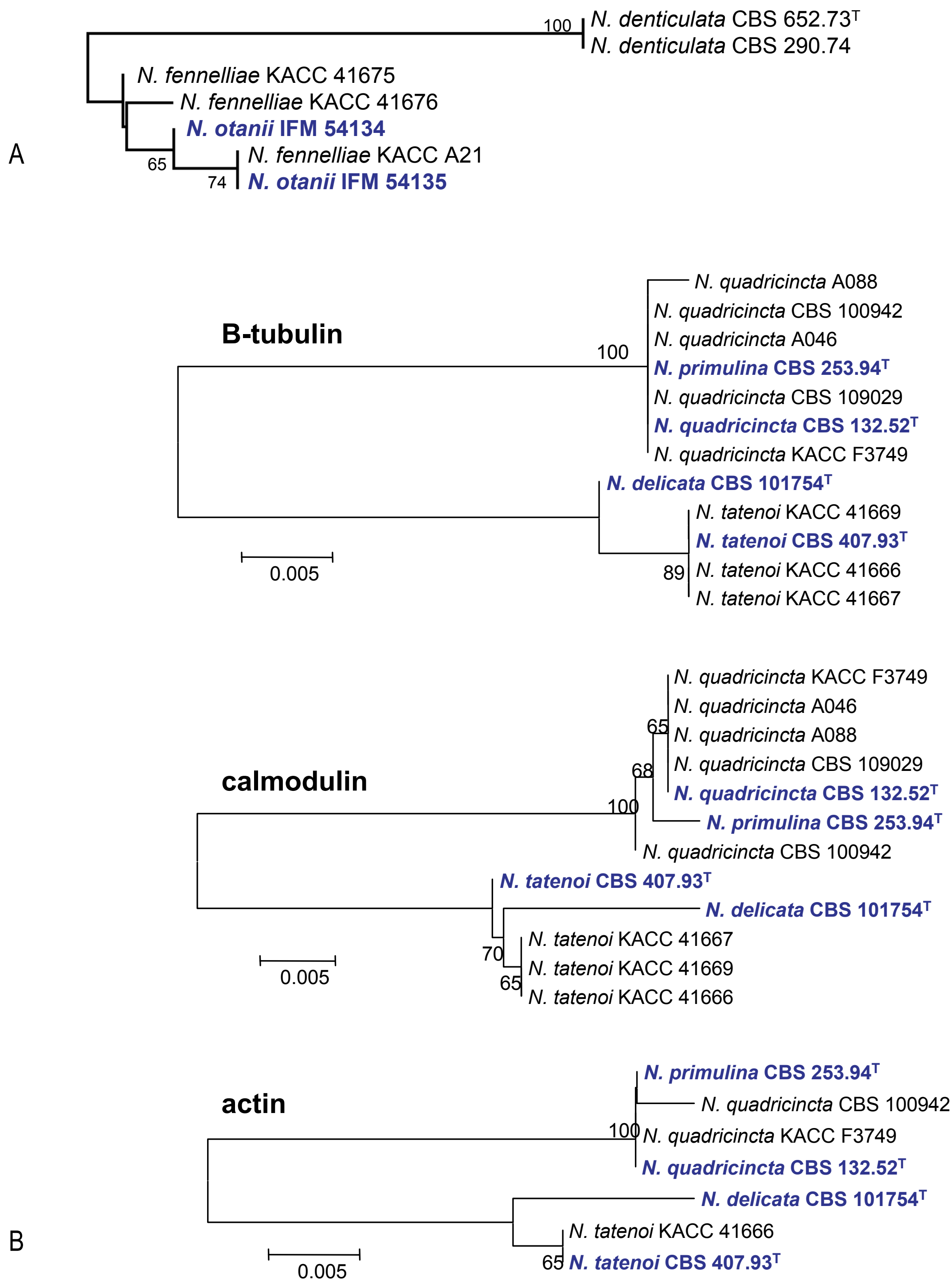

Fig. 5 A. Neighbour-joining tree based on β–tubulin sequences showing the relationship of *N. otanii* and *N. fennelliae*. **B**. Neighbour-joining trees based on β-tubulin, calmodulin and actin sequence data of *Neosartorya* spp. showing the relationship of *N. primulina, N. quadricincta, N. tatenoi* 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). *N. primulina* is 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*.

Species	Extrolites produced
Aspergillus brevipes	roquefortine C, meleagrin-like
Aspergillus duricaulis	pseurotin A, fumagillin, asperpentyn, duricaulic acid and asperdurin, phthalides, chromanols, cyclopaldic acid, 3-O-methylcyclopolic acid
Aspergillus fumigatiaffinis	auranthine, cycloechinuline, fumigaclavines, helvolic acid, neosartorin, palitantin, pyripyropenes A, E, O & S, tryptoquivaline, tryptoquivalone
Aspergillus fumigatus	fumagillin, fumitoxins, fumigaclavines A &C, fumitremorgins, gliotoxin, trypacidin, pseurotins, helvolic acid, pyripyropens, methyl-sulochrin, verruculogen, fumiquinazolines
Aspergillus fumisynnematus	neosartorin, pyripyropens, fumimycin
Aspergillus lentulus	cyclopiazonic acid, pyripyropenes A, E & O, terrein, auranthine, neosartorin
Aspergillus novofumigatus	aszonalenin, cycloechinuline, fiscalins, helvolic acid, neosartorin, palitantin, terrein, territrem B
Aspergillus turcosus	kotanins and several unique but not yet elucidated secondary metabolites
Aspergillus unilateralis	mycophenolic acid, other unique secondary metabolites
Aspergillus viridinutans	viriditoxin, 13-O-methylviriditin, phomaligin A, variotin, viriditin, wasabidienone B0, B1, viriditin, 4-acetyl-6,8-dihydroxy-5-methyl-2-benzopyran-1-1 A
Neosartorya assulata	indole alkaloids and apolar metabolites
Neosartorya aurata	helvolic acid, yellow unidentified compounds
Neosartorya aureola	fumagillin, tryptoquivaline, tryptoquivalone, pseurotin A and viriditoxin (FRR 2269 also produces helvolic acid)
Neosartorya australensis	wortmannin-like, aszonalenin-like
Neosartorya coreana	aszonalenins
Neosartorya denticulata	gliotoxin, viriditoxin
Neosartorya fennelliae	asperfuran, aszonalenin, fumigaclavine, viridicatumtoxin
Neosartorya ferenczii	asperfuran, aszonalenin, fumigaclavine, viridicatumtoxin, gliotoxin-like, fumigatins, aszonalenin-like
Neosartorya fischeri	terrein, fumitremorgins A & C, tryptoquivaline A, trypacidin, TR-2, verruculogen, sarcin, aszonalenins, fischerin, neosartorin, fiscalins, helvolic acid
Neosartorya galapagensis	gregatins
Neosartorya glabra	asperpentyn, avenaciolide, wortmannin-like compound
Neosartorya hiratsukae	avenaciolide
Neosartorya laciniosa	aszonalenins, tryptoquivaline, tryptoquivalone
Neosartorya multiplicata	helvolic acid
Neosartorya papuensis	wortmannin-like
Neosartorya pseudofischeri	asperfuran, cytochalasin-like compound, fiscalin-like compound, pyripyropens, gliotoxin
Neosartorya quadricincta	quinolactacin, aszonalenins
Neosartorya spinosa	aszonalenins, 2-pyrovoylaminobenzamide, pseurotin
Neosartorya spathulata	xanthocillins, aszonalenins
Neosartorya stramenia	quinolactacin, avenaciolide
Neosartorya tatenoi	aszonalenins
Neosartorya udagawae	fumigatin, fumagillin, tryptoquivaline, tryptoquivalone
Neosartorya warcupii	wortmannin-like, aszonalenin-like, chromanols-like, tryptoquivaline-like and tryptoquivalone-like

Morphology and extrolite production

The atypical *N. glabra* isolate NRRL 4179 (Raper & Fennell 1965) produced asperfuran, aszonalenin, fumigaclavine, viridicatumtoxin, 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 *Sma*I-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 *Eco*RI 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, chromanols, tryptoquivalins and tryptoquivalons. This isolate was markedly different from all other known *Neosartorya* species in secreting a bluish pigment after 7 d incubation on MEA and CYA plates. The microtuberculate ascospore ornamentation of this isolate is similar to those of *N. laciniosa, N. glabra* and *N. galapagensis* (Hong *et al.* 2007). However, it grew more slowly on CYA than these species, and phylogenetic data also indicate that this isolate represents a new species.

CBS 112.55 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. ferenczii* (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 ascsopores. 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 lightmicroscope 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 gen 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 viridinutans 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. coreanus* Hong, Frisvad & Samson]
Neosartorya denticulata Samson, Hong & Frisvad [anamorph: *A. denticulatus* Samson, Hong & Frisvad]
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 laciniosa Hong, Frisvad & Samson [anamorph: *A. laciniosus* Hong, Frisvad & Samson]
Neosartorya multiplicata Yaguchi, Someya & Udagawa [anamorph: *A. muliplicatus* 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. quadricingens* Kozakiewicz]
  = *Neosartorya primulina* Udagawa, Toyazaki & Tsubouchi [anamorph: *A. primulinus* Udagawa, Toyazaki & Tsubouchi]
Neosartorya spinosa (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 takakii* Horie, Abliz & Fukushima [anamorph: *A. takakii* Horie, Abliz & Fukushima]
Neosartorya spathulata Takada & Udagawa [anamorph: *A. spathulatus* Takada & Udagawa]
Neosartorya stramenia (Novak & Raper) Malloch & Cain [anamorph: *A. paleaceus* Samson & Gams]
Neosartorya tatenoi Horie, Miyaji, Yokoyama, Udagawa & Campos-Takagi [anamorph: *A. tatenoi* Horie, Miyaji, Yokoyama, Udagawa & Campos-Takagi]
  = *Neosartorya delicata* Kong [anamorph: *A. delicatus* Kong]
Neosartorya udagawae Horie, Miyaji & Nishimura [anamorph: *A. udagawae* Horie, Miyaji & Nishimura]
Neosartorya warcupii 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: 30–34 mm; YES25: 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. duricaulis*

Distribution: Australia

Ecology and habitats: soil

Extrolites: Roquefortine C, cf. meleagrin, red metabolite (not structure elucidated)

Pathogenicity: not reported

Note: previous reports on viriditoxin production of *A. brevipes* (Weisleder & Lillehoj 1971; Cole & Cox 1981) were based on studies of a mixed culture of *A. brevipes* and *A. viridinutans* (Peterson SW, pers. comm.)

Aspergillus duricaulis Raper & Fennell, The genus *Aspergillus*, 249. 1965. Fig. 7.

Type: CBS 481.65, from soil, Buenos Aires, Argentina

Other no. of the type: ATCC 16900; IMI 172282; JCM 01735; IBT 23177; NRRL 4021; VKM F-3572; WB 4021

Description
Colony diam (7 d): CYA25: 21–25 mm; MEA25: 20–22 m; YES25: 40–44 mm; OA25: 40–44 mm, CYA37: 21–25 mm, CREA: poor growth, no acid production
Colony colour: lily green to slate olive
Conidiation: heavy in central areas
Reverse colour (CZA): colourless to pinkish drab
Colony texture: velutinous
Conidial head: loosely columnar
Stipe: 5–50 × 3.5–5.5 µm, smooth thick walled
Vesicle diam, shape: 7–14 µm, flask shaped
Conidium size, shape, surface texture: (2.8–)3–3.3(–3.3) µm, globose, echinulate

Cultures examined: IMI 172282 = IBT 23177; CBS 481.65

Diagnostic features: echinulate conidia and weakly coloured reverse on CYA distinguish it from other anamorphic species

Similar species: *A. brevipes*

Distribution: Argentina

Ecology and habitats: soil

Extrolites: 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 diam (7 d): CYA25: 46–49 mm; MEA25: 53–60 mm; YES25: 67–74; CYA37: 65–70; CREA: weak griowth, good acid production
Colony colour: white, with center dull green
Conidiation: limited
Reverse colour (CZA): yellowish to greyish orange
Colony texture: floccose
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

Extrolites: 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)

Fig. 6. *Aspergillus brevipes*. A–B. Colonies 7 d 25 °C. A. CYA. B. MEA. C–I. Conidiophores. J. Conidia. Scale bars = 10 µm.

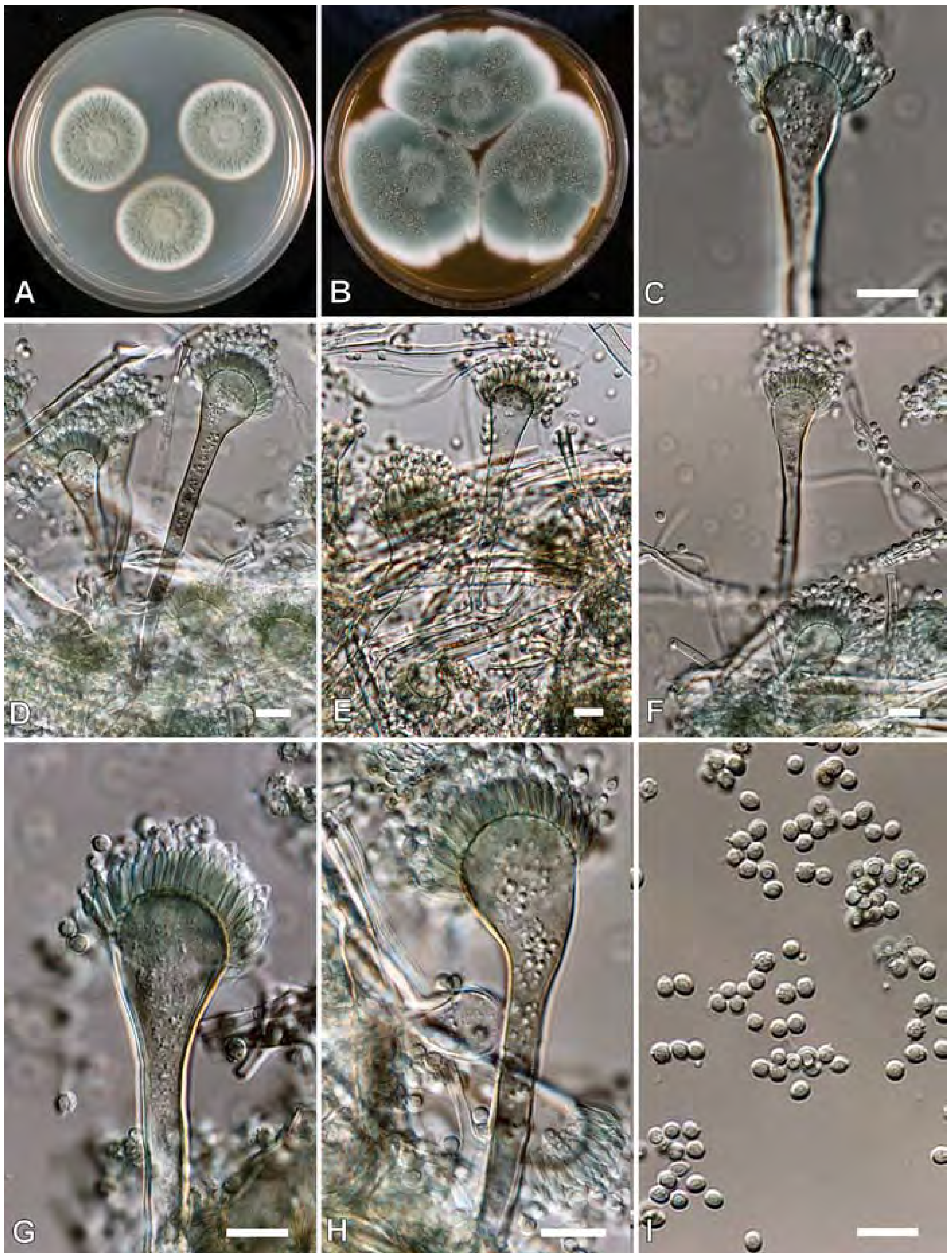

Fig. 7. *Aspergillus duricaulis*. A–B. Colonies 7 d 25 °C. A. CYA. B. MEA. C–H. Conidiophores. I. Conidia. Scale bars = 10 µm.

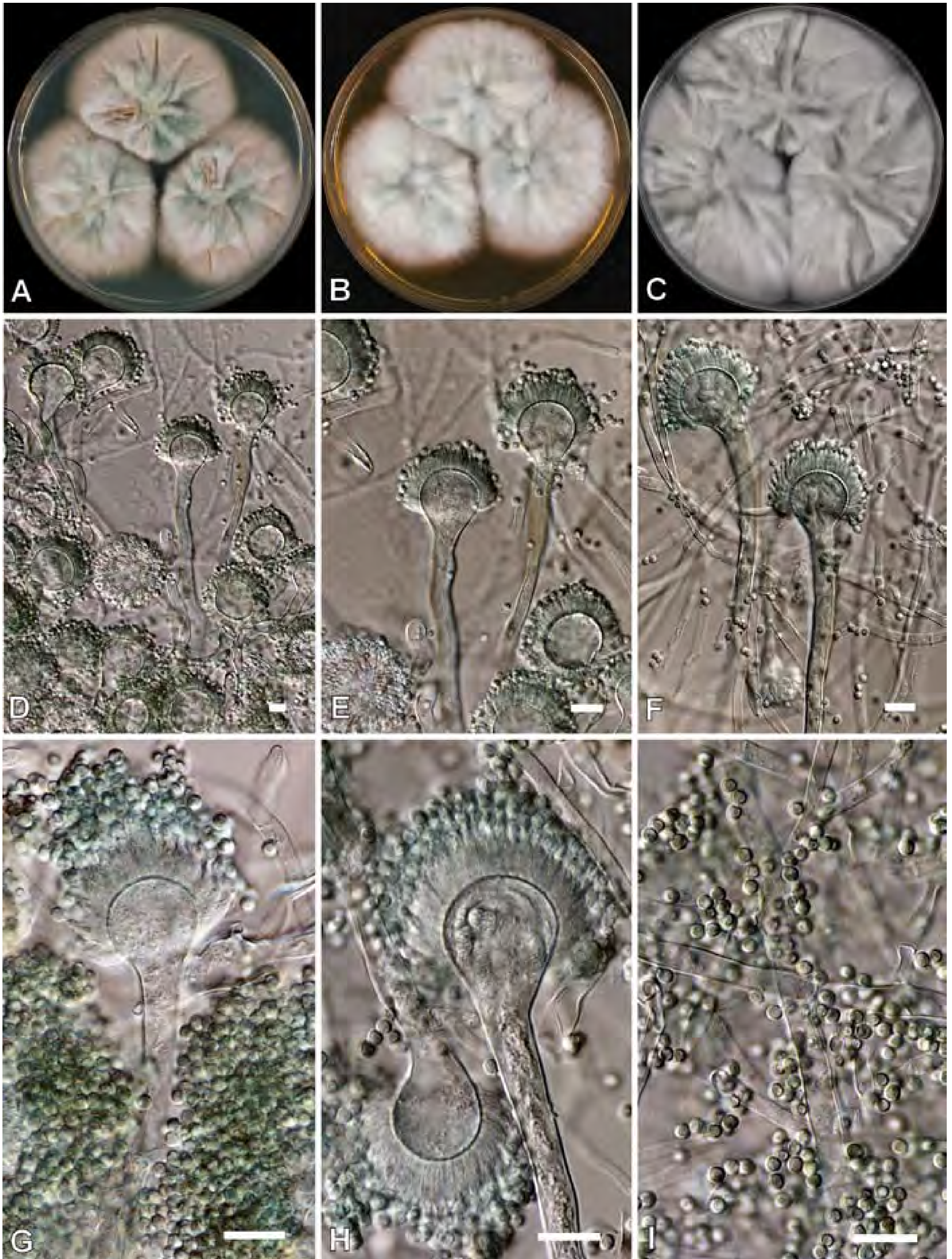

Fig. 8. *Aspergillus fumigatiaffinis*. A–C. Colonies 7 d 25 °C. A. CYA. B. MEA 25 °C. C. MEA 37 °C. D–H. Conidiophores. I. Conidia. Scale bars = 10 µm.

Aspergillus fumigatus Fresenius, Beitr. Mykol. 81: 18. 1863. Fig. 9.

= *Aspergillus fumigatus* var. *acolumnaris* Rai, Agarwal & Tewari (1971)
= *Aspergillus fumigatus* var. *albus* Rai, Tewari & Agarwal (1974)
= *Aspergillus fumigatus* var. *cellulosae* Sartory, Sartory & Mey. (1935)
= *Aspergillus fumigatus* var. *coeruleus* Malchevsk. (1939)
= *Aspergillus fumigatus* var. *ellipticus* Raper & Fennell (1965)
= *Aspergillus fumigatus* var. *fulviruber* Rai, Tewari & Agarwal (1974)
= *Aspergillus fumigatus* var. *fumigatus* Fresen. (1863)
= *Aspergillus fumigatus griseibrunneus* var. Rai & Singh (1974)
= *Aspergillus fumigatus* var. *helvolus* Yuill (1937)
= *Aspergillus fumigatus* var. *lunzinense* Svilv. (1941)
= *Aspergillus fumigatus* var. *minimus* Sartory (1919)
= *Aspergillus neoellipticus* Kozak. (1989)
= *Aspergillus phialoseptus* Kwon-Chung (1975)
= *Aspergillus bronchialis* Blumentritt (1901)
= *Aspergillus septatus* Sartory & Sartory (1943)
= *Aspergillus arvii* 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: 48–74 mm; OA25: 34–62 mm, CYA37: 60–75 mm, CREA: poor growth, no or very weak acid production
Colour: greyish turquoise or dark turquoise to dark green to dull green
Conidiation: abundant, rarely less abundant
Reverse colour (CYA): creamy, yellow to orange
Colony texture: velutinous, st. floccose (define the abreviation st.)
Conidial head: columnar
Stipe: 50–350 × 3.5–10 µm
Vesicle diam, shape: 10–26 µm, pyriform to subclavate, sometimes subglobose, but rarely globose
Conidia length, shape, surface texture: 2–3.5(–6) µm, globose to ellipsoidal, smooth to finely rough

Cultures examined: ATCC 32722, AF71, AF 293, AF294, CBS 112389, CBS 487.65, CBS 133.61, CBS 545.65, CBS 457.75, CBS 542.75, CBS 113.26, CBS 110.46, CBS 120.53, CBS 132,54, CBS 123.59, CBS 158.71, CBS 180.76, CBS 143.89, CBS 148.89, CBS 488.90, CBS 287.95, CBS 100076, CBS 109032, CBS 386.75, CBS 286.95, CEA10, IMI 376380, NRRL 1979

Diagnostic features: Rapid growing velutinous colonies, abundant and fast conidiation, thick stipe (*ca.* 6–10 um), large pyriform to semi-clavate vesicle is representative morphological features of the species. However, the characteristics are various according to strains, and some stains have exceptional characteristics. The species grows at 50 °C, no growth at 10 °C.

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

Distribution: Worldwide distribution, cosmopolitan fungus (Pringle *et al.* 2005)

Ecology and habitats: soil, human

Extrolites: fumagillin, fumitoxins, fumigaclavines A & C, fumitremorgins, fumiquinazolines, gliotoxin, helvolic acid, pseurotins, pyripyropens, methyl-sulochrin, trypacidin, verruculogen

Pathogenicity: pathogenic to humans (Raper & Fennell 1965; Marr *et al.* 2002)

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

Aspergillus fumisynnematus Horie, Miyaji, Nishimura, Taguchi et Udagawa, Trans. Mycol. Soc. Japan: 34: 3–7. 1993. Fig. 10.

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

Description
Colony diam (7 d): CYA25: 44–48 mm; MEA25: 56–60 mm; YES25: 35–39 mm; OA25: 42–46; CYA37: 57–61 mm, CREA: poor growth and no acid production
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 (1-4-2.3 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

Extrolites: 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

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

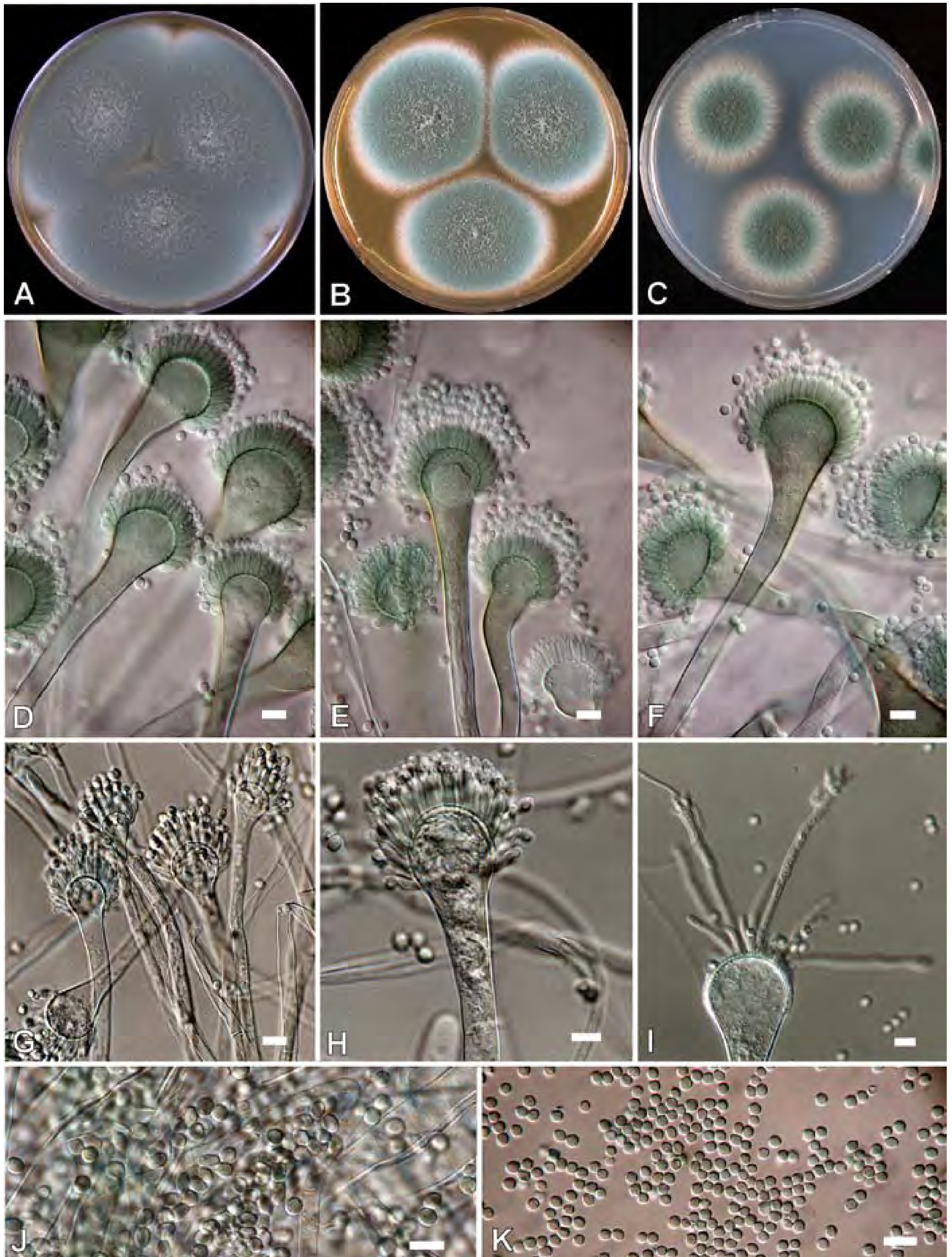

Fig. 9. *Aspergillus fumigatus*. A–C. Colonies 7 d 25 °C. A. CYA. B. MEA. C. CYA 37 °C. after 3 d. D–I. Conidiophores. D–F. A. fumigatus. G–H. *A. fumigatus* var. *ellipticus*. I. Atypical conidiophore of CBS 133.61. J. Conidia of *A. fumigatus* var. *ellipticus*. K. Conidia of *A. fumigatus*. Scale bars = 10 µm.

Fig. 10. *Aspergillus fumisynnematus*. A–B. Colonies 7 d 25 °C. A. CYA. B. MEA. C–H. Conidiophores. I. Conidia. Scale bars = 10 µm.

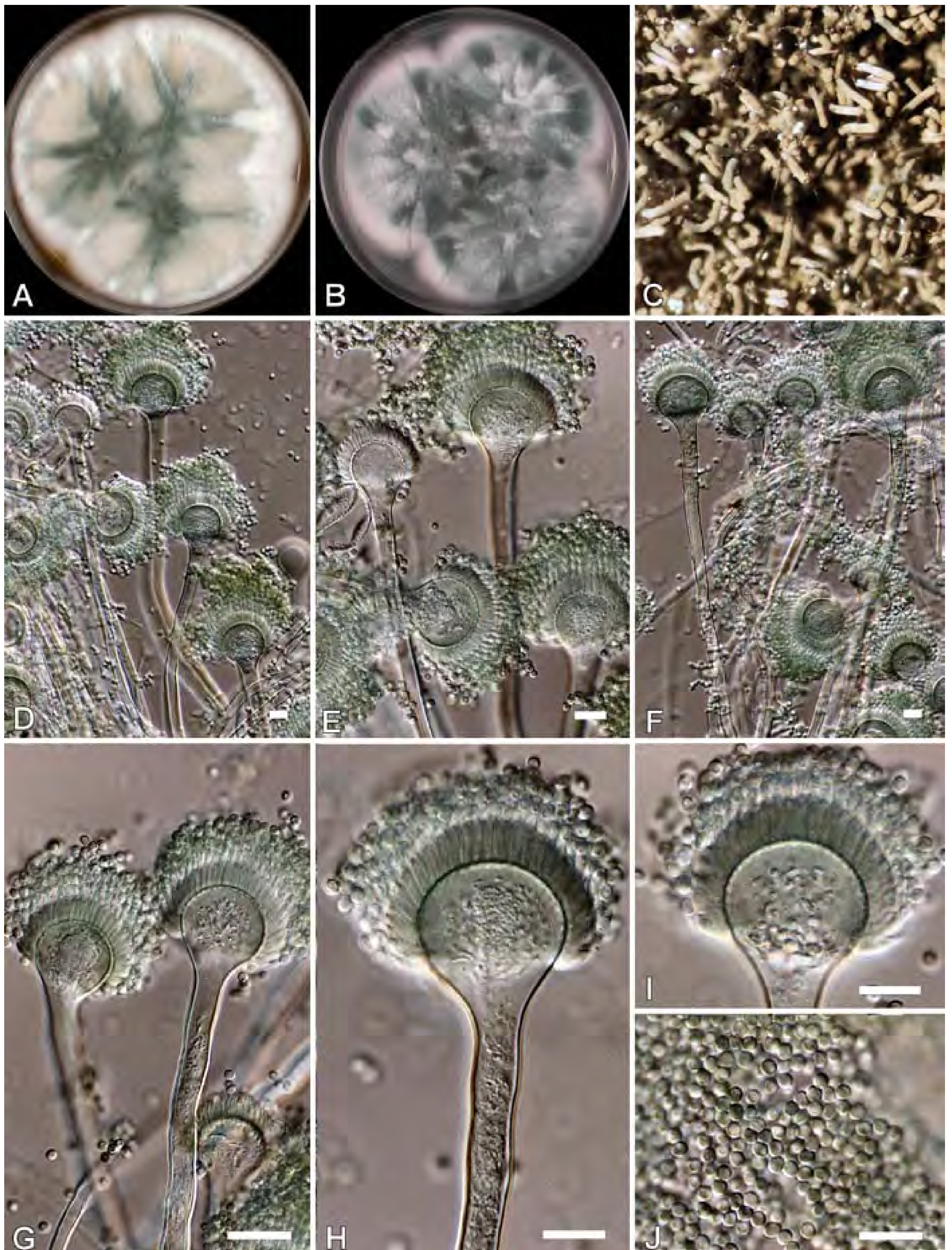

Fig. 11. *Aspergillus lentulus*. A–B. Colonies 7 d 25 °C. A. CYA. B. MEA. C. Macroscopic vieuw of the columnar conidial heads. D–I. Conidiophores. J. Conidia. Scale bars = 10 µm.

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 (<7um), 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

Extrolites: 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)

Aspergillus novofumigatus Hong, Frisvad & Samson, Mycologia 97: 1326. 2005. 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 10 °C, no growth at 50 °C

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

Distribution: Galapagos Islands, Ecuador

Ecology and habitats: soil

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

Pathogenicity: not reported

Aspergillus turcosus Hong, Frisvad & Samson, Antonie van Leeuwenhoek (in press). Fig. 13.

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

Other no. of the type: IBT 27921

Description
Colony diam: CYA25: 32–41 mm; MEA25: 42–53 mm; YES25: 48–52 mm; OA25: 46–52 mm; CYA37: 48–56; CREA poor growth, no acid production
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

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

Pathogenicity: not reported

Aspergillus unilateralis Thrower, Austral. J. Bot. 2: 355. 1954. Fig. 14.

≡ *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

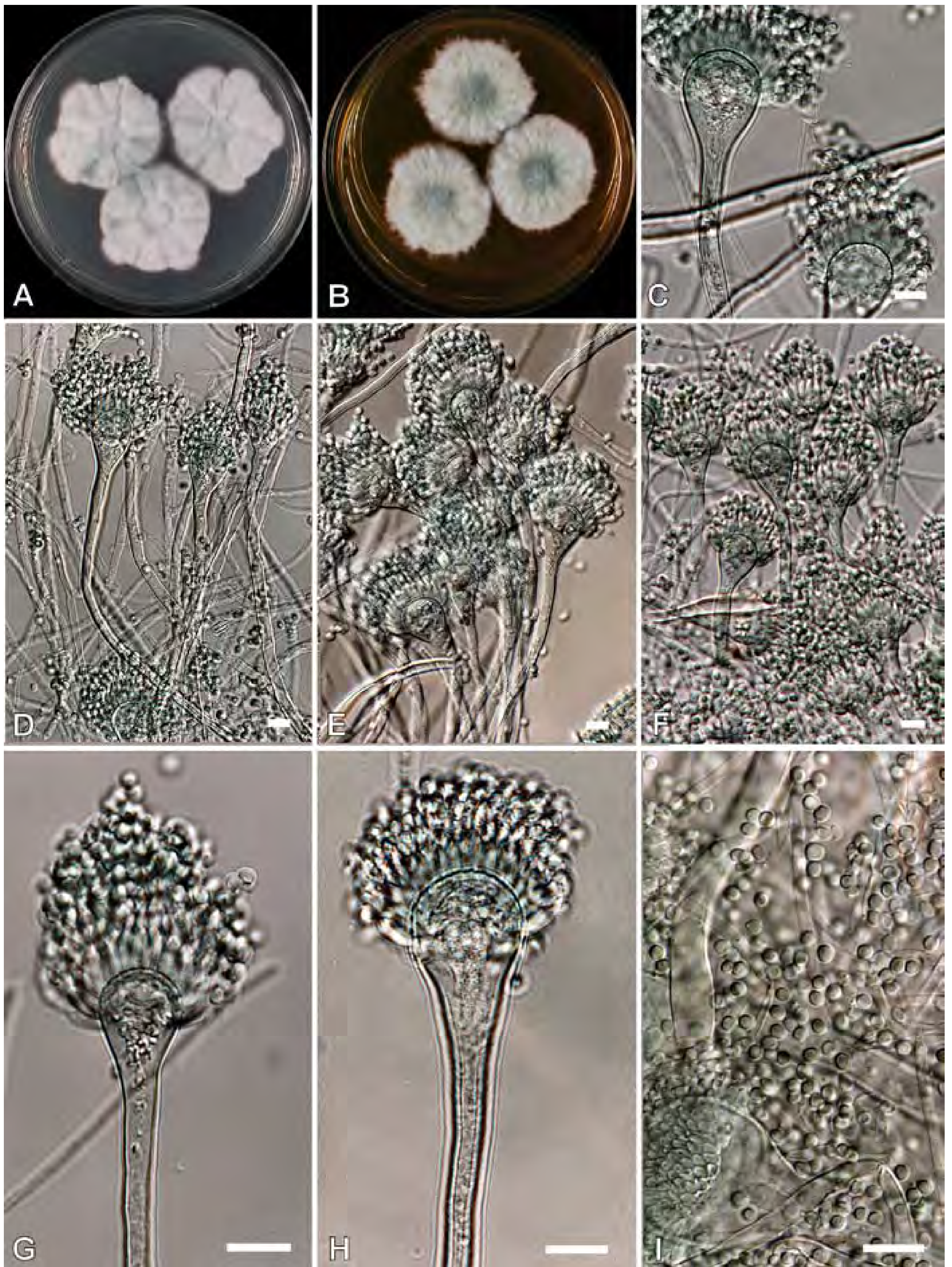

Fig. 12. *Aspergillus novofumigatus*. A–B. Colonies 7 d 25 °C. A. CYA. B. MEA. C–H. Conidiophores. I. Conidia. Scale bars = 10 µm.

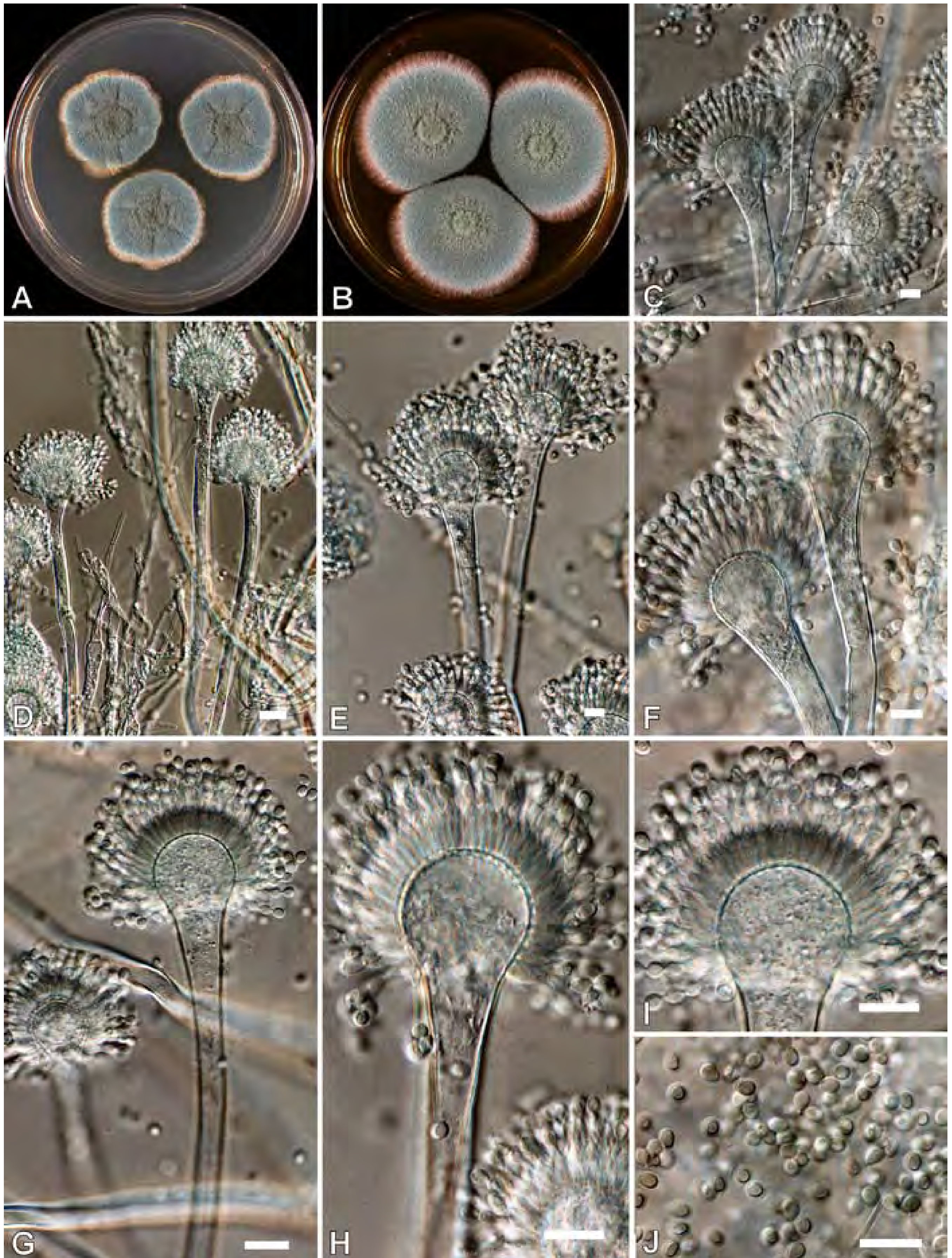

Fig. 13. *Aspergillus turcosus*. A–B. Colonies 7 d 25 °C. A. CYA. B. MEA. C–I. Conidiophores. J. Conidia. Scale bars = 10 µm.

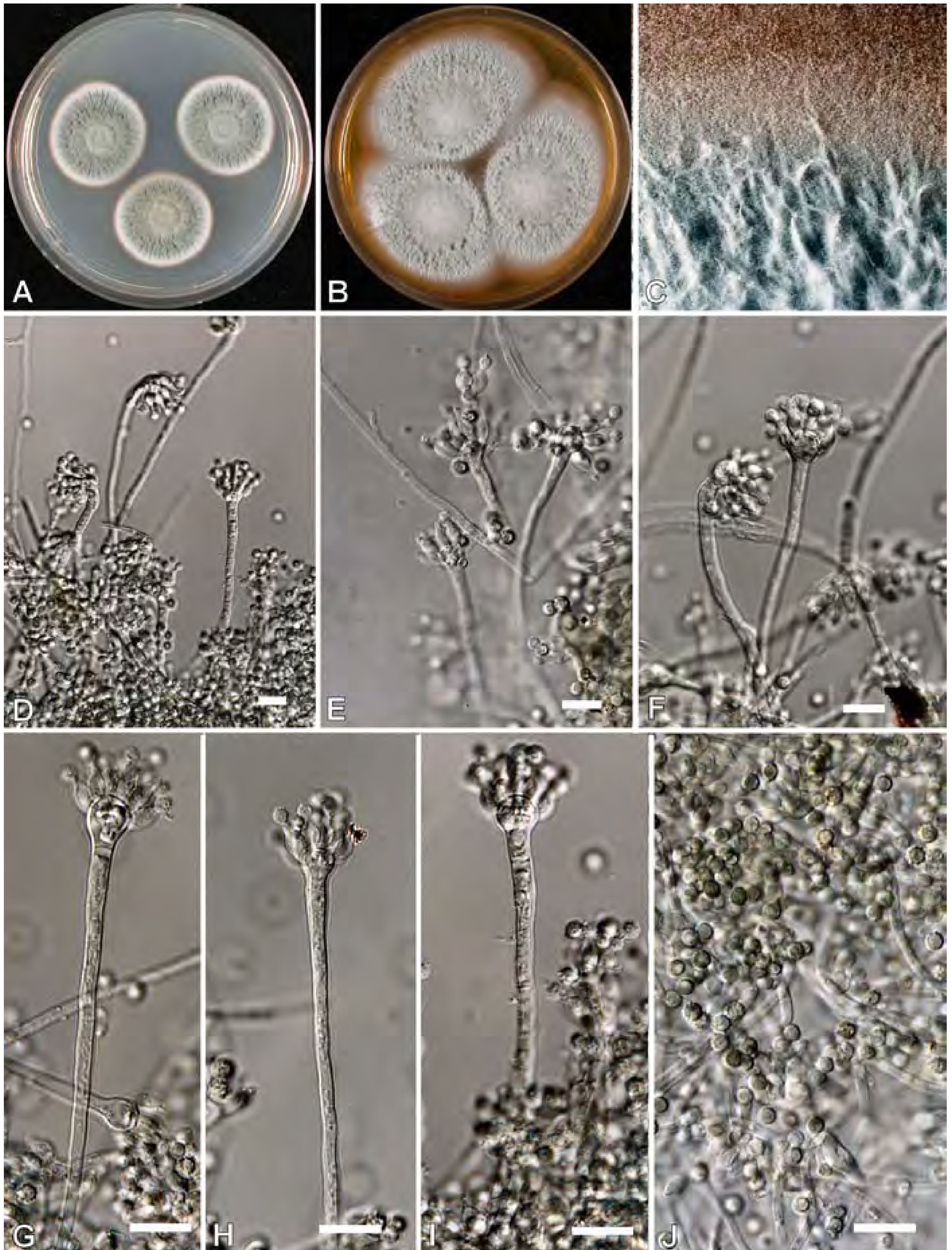

Fig. 14. *Aspergillus unilateralis*. A–B. Colonies 7 d 25 °C. A. CYA. B. MEA. C. Macroscopic view of the conidial heads. D–I. Conidiophores. J. Conidia. Scale bars = 10 µm.

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

Extrolites: 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 diam (7 d): CYA25: 20–40 mm; MEA25: 11–15 mm; YES25: 24–28 mm; OA25: 29–31 mm; CYA 37: 25–28 mm; CREA: poor griowth, no acid production
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)

Extrolites: 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)

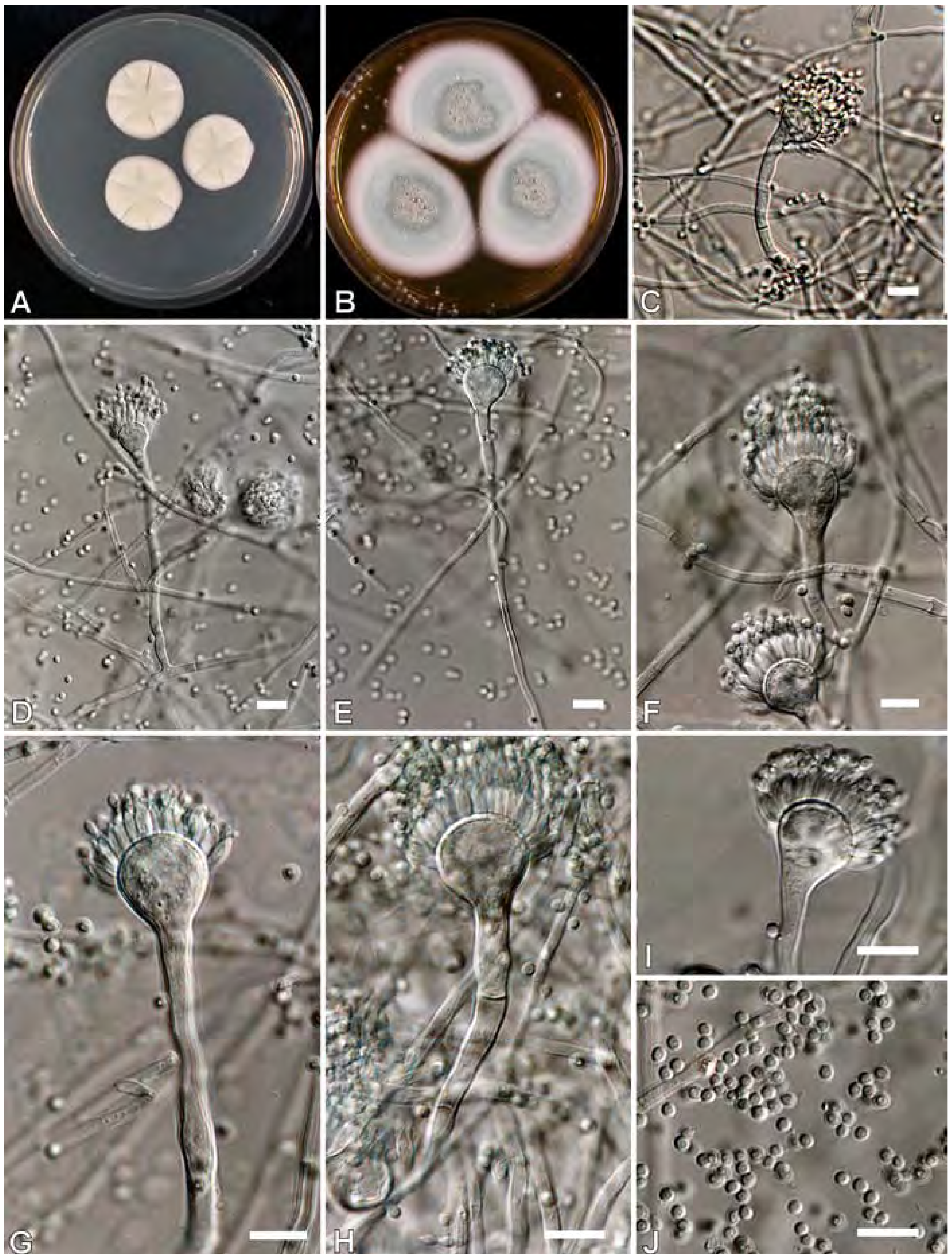

Fig. 15. *Aspergillus viridinutans*. A–B. Colonies 7 d 25 °C. A. CYA. B. MEA. C–I. Conidiophores. J. Conidia. Scale bars = 10 µm.

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 16894; IFO 8783; IMI 075886; IMI 075886ii; NRRL 4378; QM 7860; WB 4378; IBT 3028

Morphological characteristics
Colony diam (7 d): CYA25: 13–15 mm; MEA25: 30–42 mm; YES25: 17–29 mm; OA25: 31–35 mm; CYA37: 13–16 mm, CREA: weak growth and no acid production
Colony colour (MEA): orange to ochraceus orange
Conidiation: sparse
Reverse colour (CZA): orange to dull brown
Colony texture: velutinous
Conidial head: loosely columnar
Stipe: 60–120 × 2–4 µm
Vesicle diam, shape: 10–16 µm, flask shaped
Conidium size, shape, surface texture: 2.5–3 µm, globose, punctate
Homothallic
Cleistothecia: 50–150 µm, orange, surrounded by a loose tangle of encrusted orange hyphae
Ascospores: 6–6.5 × 4.5–5 µm, lenticular, with two narrow equatorial crests and convex walls finely reticulate

Cultures examined: CBS 466.65; WB 4379; IFO 9817

Diagnostic features: bright orange colour of the colony on MEA, restricted growth on CZA

Similar species: *N. stramenia*

Distribution: Brunei

Ecology and habitats: soil

Extrolites: helvolic acid, yellow unindentified compounds

Pathogenicity: not reported

Neosartorya aureola (Fennell & Raper) Malloch & Cain [anamorph: *A. aureoluteus* Samson & Gams], Mycologia 47: 71–75. 1955. Fig. 18.

Type: CBS 105.55, from soil, Tafo, Ghana

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 prodution
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

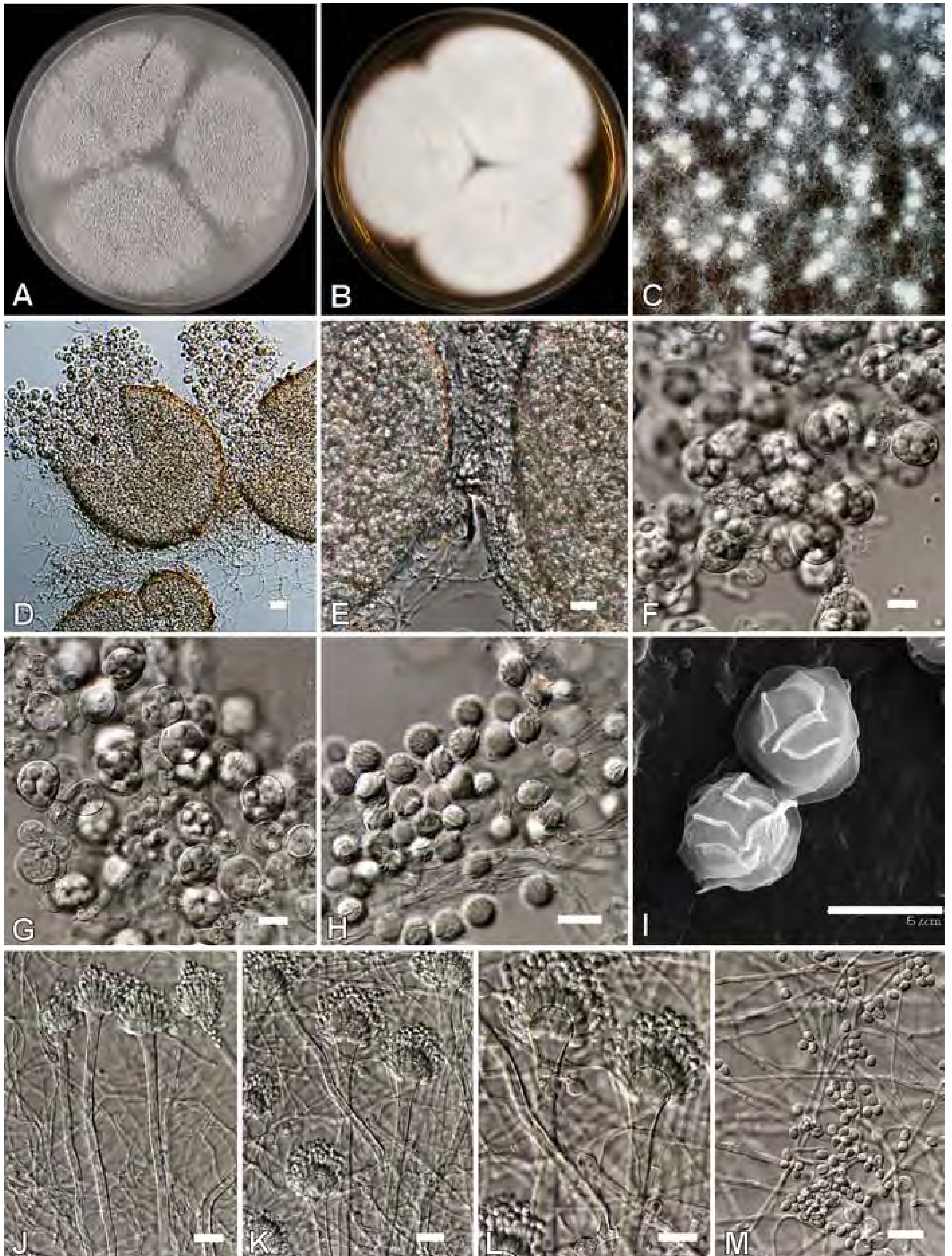

Fig. 16. *Neosartorya assulata*. A–B. Colonies 14 d 25 °C. A. OA. B. MEA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 5 µm.

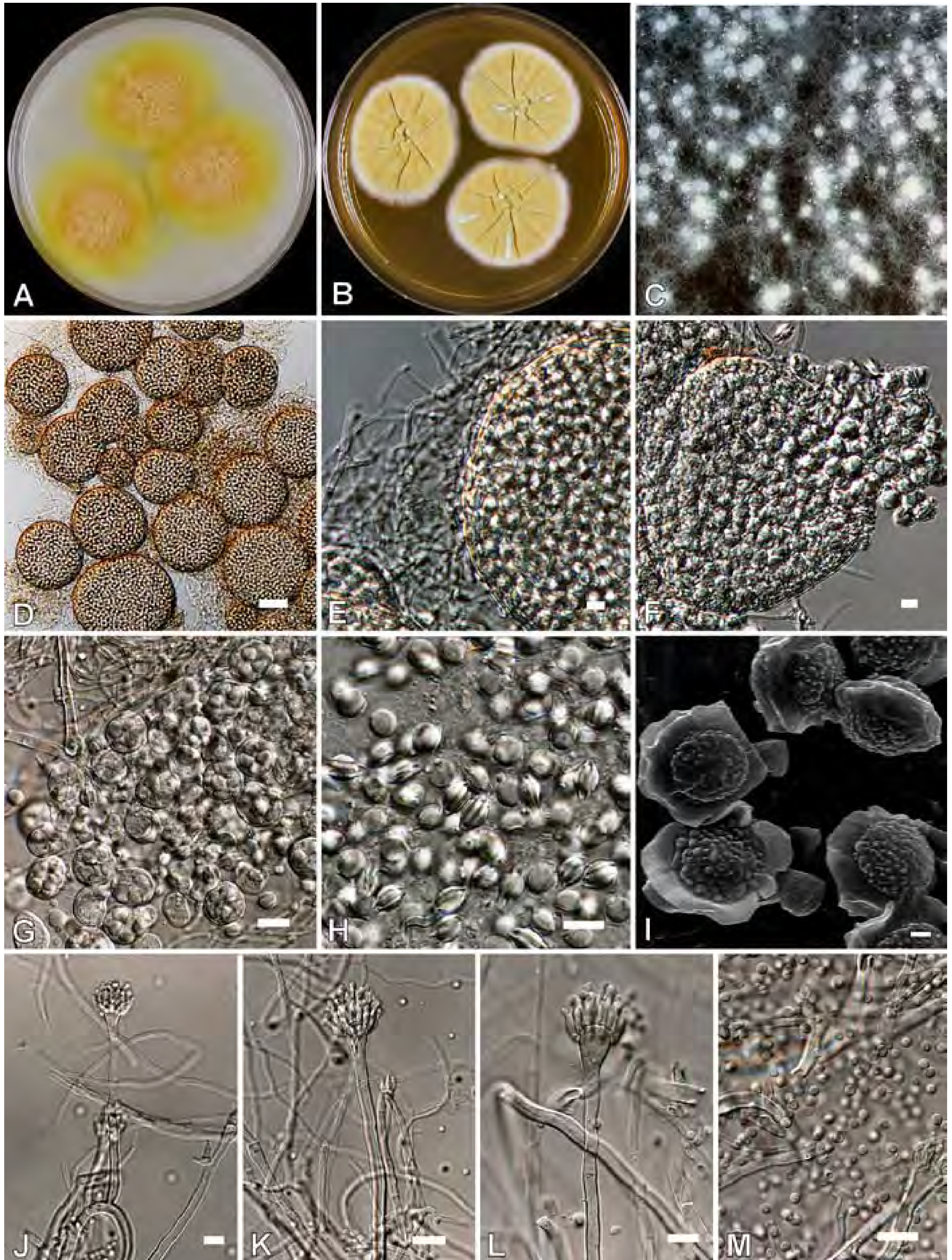

Fig. 17. *Neosartorya aurata*. A–B. Colonies 14 d 25 °C. A. OA. B. MEA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 1 µm.

Fig. 18. *Neosartorya aureola*. A–B. Colonies 14 d 25 °C. A. MEA. B. OA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 1 µm.

Neosartorya australensis Samson, Hong & Varga, **sp. nov.** (Fig. 19) – MycoBank MB492203.

Homothallica; cleistothecia superficialia, luteoalba vel dilute lutea, globosa vel subglobosa, 150–380 µm diam, in hyphis hyalinis vel luteoalbis laxe obtextis. Asci octospori, globosi vel subglobosi, 12–14 µm diam, evanescentes. Ascosporae 4.5–7.5 µm diam, cristis angustis, aequatoriis binis, pagina convexa sublaevigata. Mycelium ex hyphis hyalinis, ramosis, septatis, laeviparietinis constans. Capitula conidialia curta, columnaria. Conidiophora ex hyphis aeriis exorientia, uniseriata, stipitibus 8–14 µm; vesiculae ampulliformes, 12–30 µm diam; phialides 7.5–9 × 2–3 µm, dimidium supernum vesiculae obtegentes. Conidia subglobosa vel ellipsoidea, laevia, 3.5–5 µm diam. Coloniae in agaro MEA in 7 dieibus et 25 °C celeriter crescentes, 40–45 mm diam, albae, capitulis conidialibus paucis. Coloniae in agaro CYA in 7 dieibus et 25 °C 30–35 mm diam, cremeoalbae, centro ab hyphis aerialibus laxe obtecto; capitula conidialia pauca; colonia reversa luteoalba vel luteobrunnea.

Holotype of *Neosartorya australensis,* here designated as CBS 112.55[T] (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. Asci 8-spored, globose to subglobose 12–14 µm, evanescent at maturity. Ascospores lensshaped, 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 × 2–3µm,, 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 center. 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

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 41659 = 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
Conidiation: 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, strawberry

Extrolites: aszonalenins

Pathogenicity: not reported in humans (although isolated from the air sacks 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
Conidiation: 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 µm wide
Vesicle diam, shape: 7–12 µm, spathulate
Conidium size, shape, surface texture: 2–3 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*

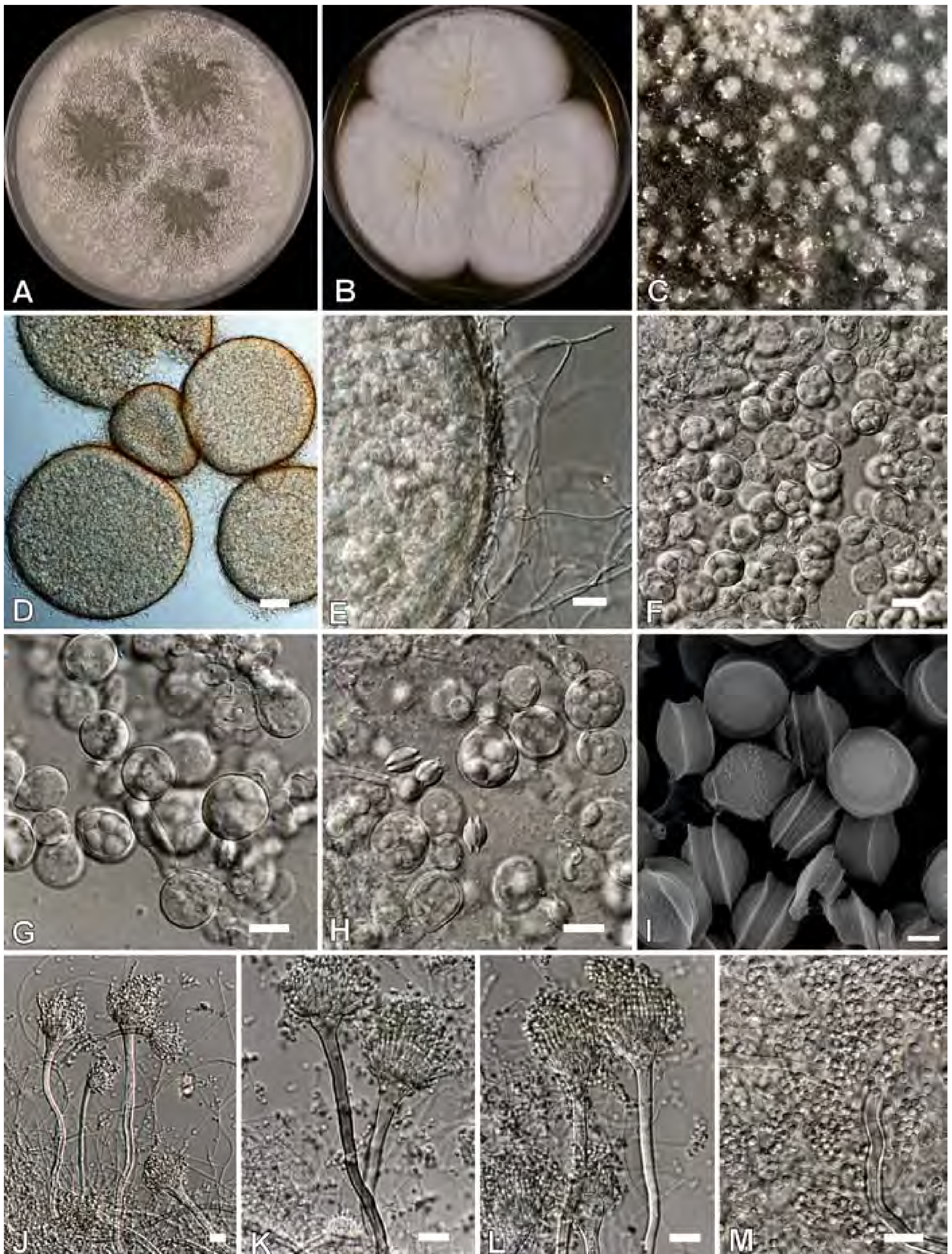

Fig. 19. *Neosartorya australiensis*. A–B. Colonies 14 d 25 °C. A. OA. B. MEA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 1 µm.

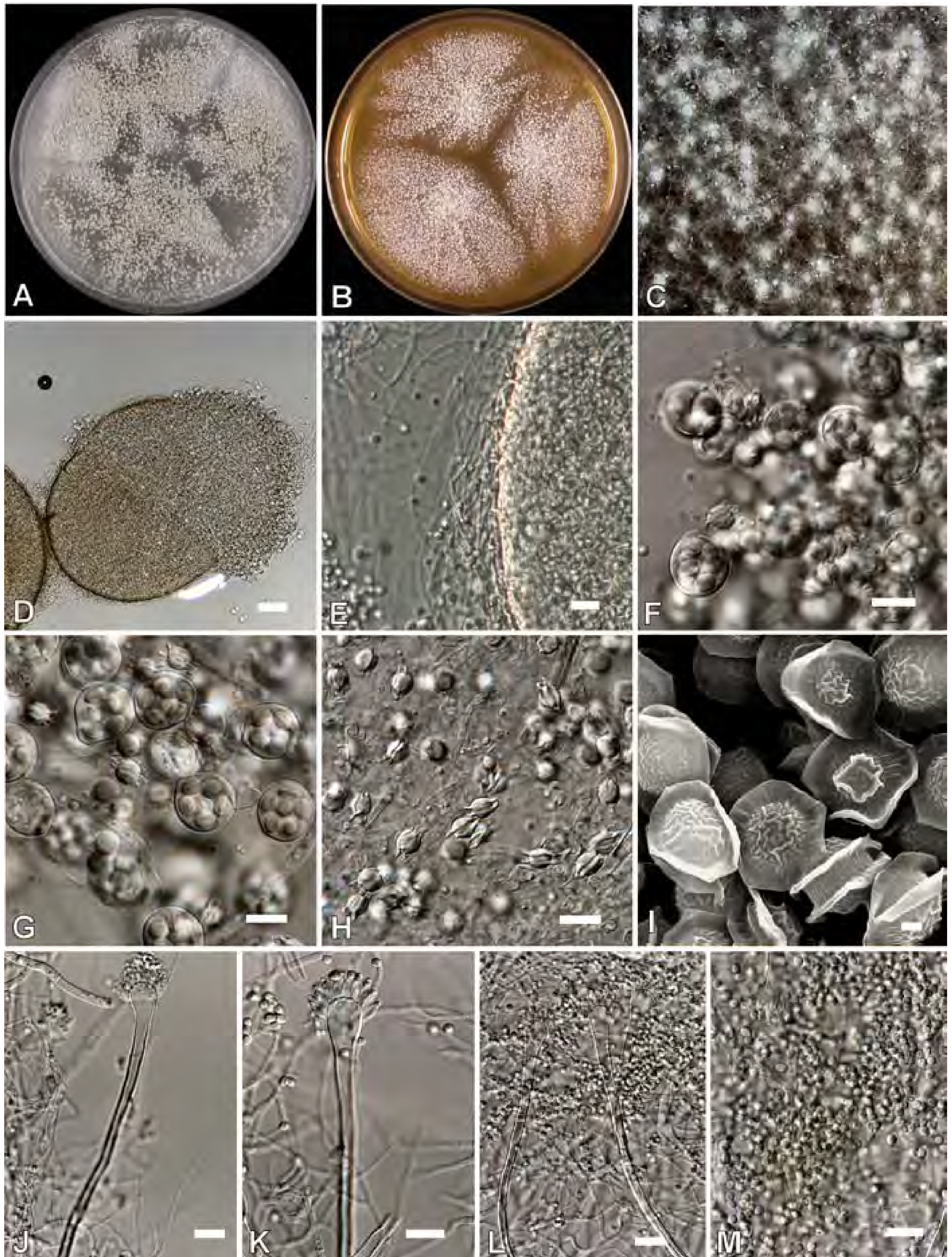

Fig. 20. *Neosartorya coreana*. A–B. Colonies 14 d 25 °C. A. OA. B. MEA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 1 µm.

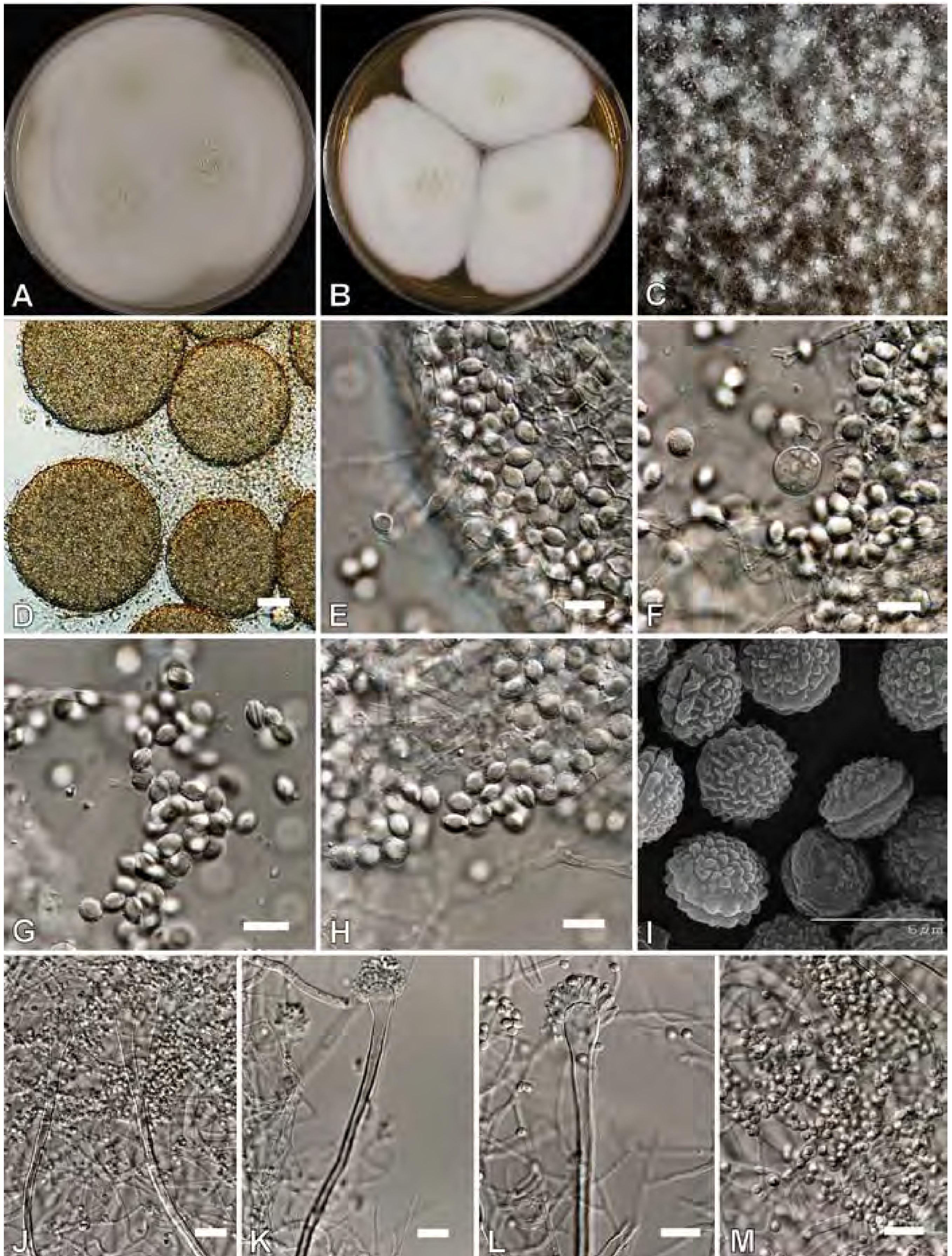

Fig. 21. *Neosartorya denticulata*. A–B. Colonies 14 d 25 °C. A. OA. B. MEA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 5 µm.

Distribution: Netherlands, Suriname

Ecology and habitats: soil, sycamore

Extrolites: 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
Colony diam (7 d): CYA25: 25–30 mm; MEA25: 44–48 mm; YES25: 30–34 mm; OA25: 34–38 mm; CYA37: 50–58 mm; CREA: poor growth and no acid production
Colony colour: grey
Conidiation: abundant
Reverse colour (CZA): white
Colony texture: velutinous
Conidial head: short columnar
Stipe: 150–250 × 4–6 µm
Vesicle diam, shape: 10–17 µm, flask-shaped
Conidium size, shape, surface texture: 2.2–2.5(–2.8) µm, globose to subglobose to ellipsoid, smooth or finely roughened
Heterothallic
Cleistothecia: 150–450 µm, white
Ascospores: 5.5–7.7 × 3.2–5 µm, with two equatorial crests, convex surfaces delicately roughened

Cultures examined: CBS 598.74, CBS 599.74

Diagnostic features: heterothallic

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

Distribution: U.S.A., Japan, South Korea

Ecology and habitats: soil, mirne sludge, rabbit

Extrolites asperfuran, aszonalenin, fumigaclavine, viridicatumtoxin

Pathogenicity: not reported in humans

Note: no growth at 47 °C

Neosartorya ferenczii Varga & Samson, **sp. nov.** (Fig. 23) – MycoBank MB504847.

Homothallica; cleistothecia superficialia, luteoalba vel dilute lutea, globosa vel subglobosa, 180–350 µm diam, in hyphis hyalinis vel luteoalbis laxe obtextis. Asci octospori, globosi vel subglobosi, 12–16 µm diam, evanescentes. Ascosporae 3.5–5.5 µm diam, cristis angustis, aequatoriis binis, pagina convexa sublaevigata. Mycelium ex hyphis hyalinis, ramosis, septatis, laeviparietinis constans. Capitula conidialia curta, columnaria. Conidiophora ex hyphis aeriis exorientia, uniseriata, stipitibus 100–150 × 4–5 µm; vesiculae ampulliformes, 10–14 µm diam; phialides 7.5–9 x 2–3 µm, dimidium supernum vesiculae obtegentes. Conidia globosa vel subglobosa, laevia, 2–2,5 µm diam. Coloniae in agaro MEA in 7 dieibus et 25 °C celeriter crescentes, 35–40 mm diam, albae, capitulis conidialibus paucis. Coloniae in agaro CYA in 7 dieibus et 25 °C 20–30 mm diam, cremeoalbae, centro ab hyphis aerialibus laxe obtecto; capitulis conidialibus paucis; colonia reversa luteoalba vel pallide lutea.

Holotype of *Neosartorya ferenczii,* here designated as CBS 121594[T] (dried culture), isolated from soil in Australia.

Homothallic, cleistothecia superficial, 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 12–16 µm, evanescent at maturity. Ascospores lens shaped, 3.5 × 5.5 µm, with two narrow equatorial crests, convex surface nearly smooth, microtuberculate. Mycelium composed of hyaline, branched, septate, smooth-walled hyphae. Conidial heads short, columnar. Conidiophores arising from aerial hyphae, uniseriate, stipes 100–150 × 4–5 µm; vesicles subclavate, 8–14 µm in diam; phialides 7.5–9 x 2–3 µm, covering the upper half of vesicle. Conidia globose to subglobose, smooth, 2–2,5 µm. Colonies on MEA growing rapidly, 35–40 mm in 7 d at 25 °C, white. Conidial heads produced few in number. Colonies on CYA, 20–30 mm in 7 d at 25 °C, creamish white, loosely overgrown by aerial hyphae in center. Conidial heads few in number. Reverse yellowish white to pale yellow (12A23) (Kornerup & Wanscher 1978).

Etymology: named after Prof. Lajos Ferenczy, eminent mycologist.

Extrolites: asperfuran, aszonalenin, fumigaclavine, viridicatumtoxin, 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

Extrolites: asperfuran, aszonalenin, fumigaclavine, viridicatumtoxin, gliotoxin-like, fumigatins, and aszonalenin-like

Pathogenicity: not reported

Neosartorya fischeri (Wehmer) Malloch & Cain [anamorph: *A. fischeranus* Kozakiewicz], Can. J. Bot. 50: 2621. 1973. Fig. 24.

= *Aspergillus fischeri* Wehmer, Centr. Bakteriol. Parasitenk. Abt. II 18: 390. 1907.
= *Sartorya fumigata* Vuill., Compt. rendu Acad. Sci. Paris 184: 136. 1927.

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
Colony diam (7 d): CYA25: 45–68 mm; MEA25: 66–80 mm; YES25: 70–80 mm; OA25: 58–80 mm; CYA37: 65–84 mm; CREA: poor growth and no acid production
Colony colour (CZA): white to pale yellow to buff
Conidiation: sparse
Reverse colour (CZA): colourless to flesh coloured

Fig. 22. *Neosartorya fennelliae*. A–B. Colonies 14 d 25 °C. A. MEA. B–C. Crossing of mating types on MEA. D–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 1 µm.

Fig. 23. *Neosartorya ferencii*. A–B. Colonies 14 d 25 °C. A. OA. B. MEA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 1 µm.

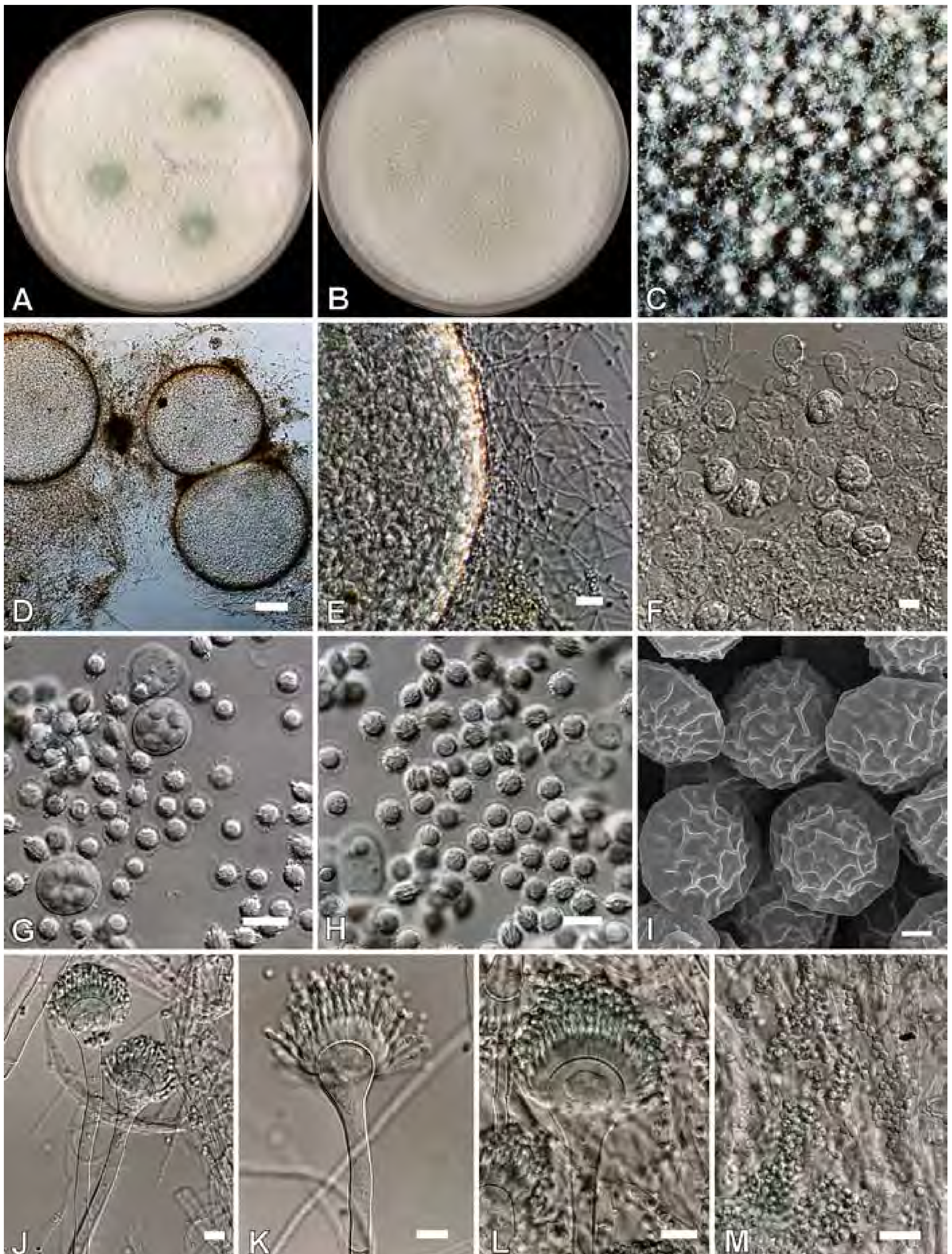

Fig. 24. *Neosartorya fischeri*. A–B. Colonies 14 d 25 °C. A. OA. B. MEA. C. Macroscopic view of the columnar conidial heads. D–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 1 µm.

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

Ecology and habitats: Soil, (milled) rice, cotton, potatoes, groundnuts, leather, paper products, canned products, human

Extrolites: terrein, fumitremorgins A & C, tryptoquivaline A, trypacidin, TR-2, verruculogen, sarcin, aszonalenins, fischerin, neosartorin, fiscalins, helvolic acid

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: colonies funiculose, the *Aspergillus* anamorph arises from bundles of aerial hyphae, ascospores with two wide conspicuous equatorial crests and with microtuberculate convex surface

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

Distribution: Galapagos Islands (Ecuador)

Ecology and habitats: soil

Extrolites: gregatins

Pathogenicity: not reported

Neosartorya glabra (Fennell & Raper) Kozakiewicz [anamorph: *A. neoglaber* Kozakiewicz], Mycol. Pap. 161: 56. 1989. Fig. 26.

Type: CBS 111.55, from rubber scrab of an old tire, Iowa, U.S.A.

Other no. of the type: ATCC 16909; IFO 8789; IMI 061447; IMI 061447ii; NRRL 2163; QM 1903; WB 2163

Morphological characteristics
Colony diam (7 d): CYA25: 24–43 mm; MEA25: 49–66 mm; YES25: 45–54 mm; OA25: 55–76 mm; CYA37: 30–80 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: 10–18 µm, flask shaped
Conidium size, shape, surface texture: 2.5–3.5 µm, globose to subglobose, microtuberculate
Homothallic
Cleistothecia: 100–500 µm, yellowish white
Ascospores: 6.5–7.5 × 4.5–5 µm, lenticular, with two equatorial crests of 1–1.5 µm, convex surfaces finely roughened

Cultures examined: CBS 111.55; IMI 144207; IMI 102073; CBS 165.63

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

Extrolites: asperpentyn, avenaciolide, wortmannin-like compound

Pathogenicity: not reported

Fig. 25. *Neosartorya galapagensis*. A–B. Colonies 14 d 25 °C. A. CYA. B. MEA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 5 µm.

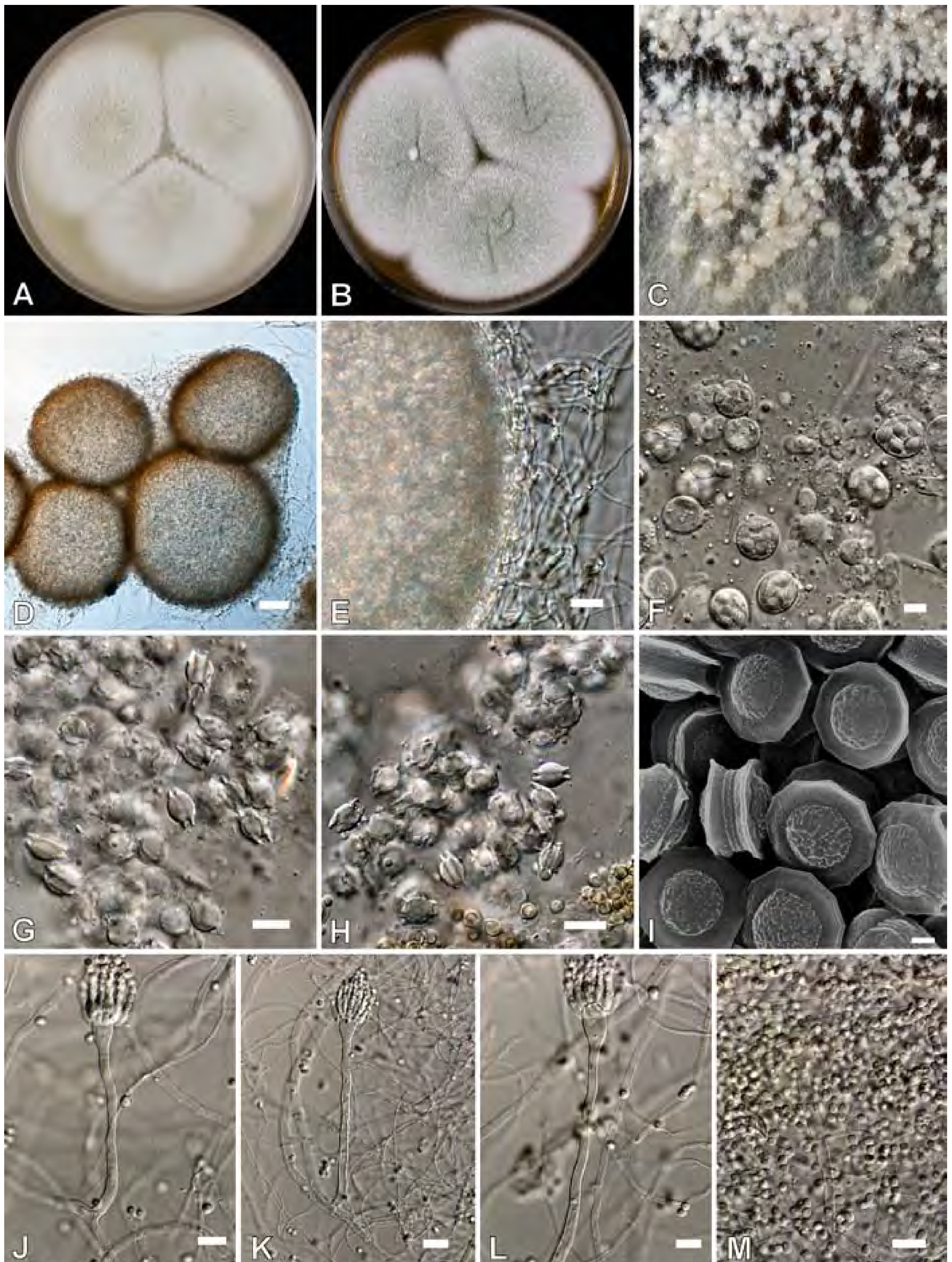

Fig. 26. *Neosartorya glabra*. A–B. Colonies 14 d 25 °C. A. OA. B. MEA, C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 1 µm.

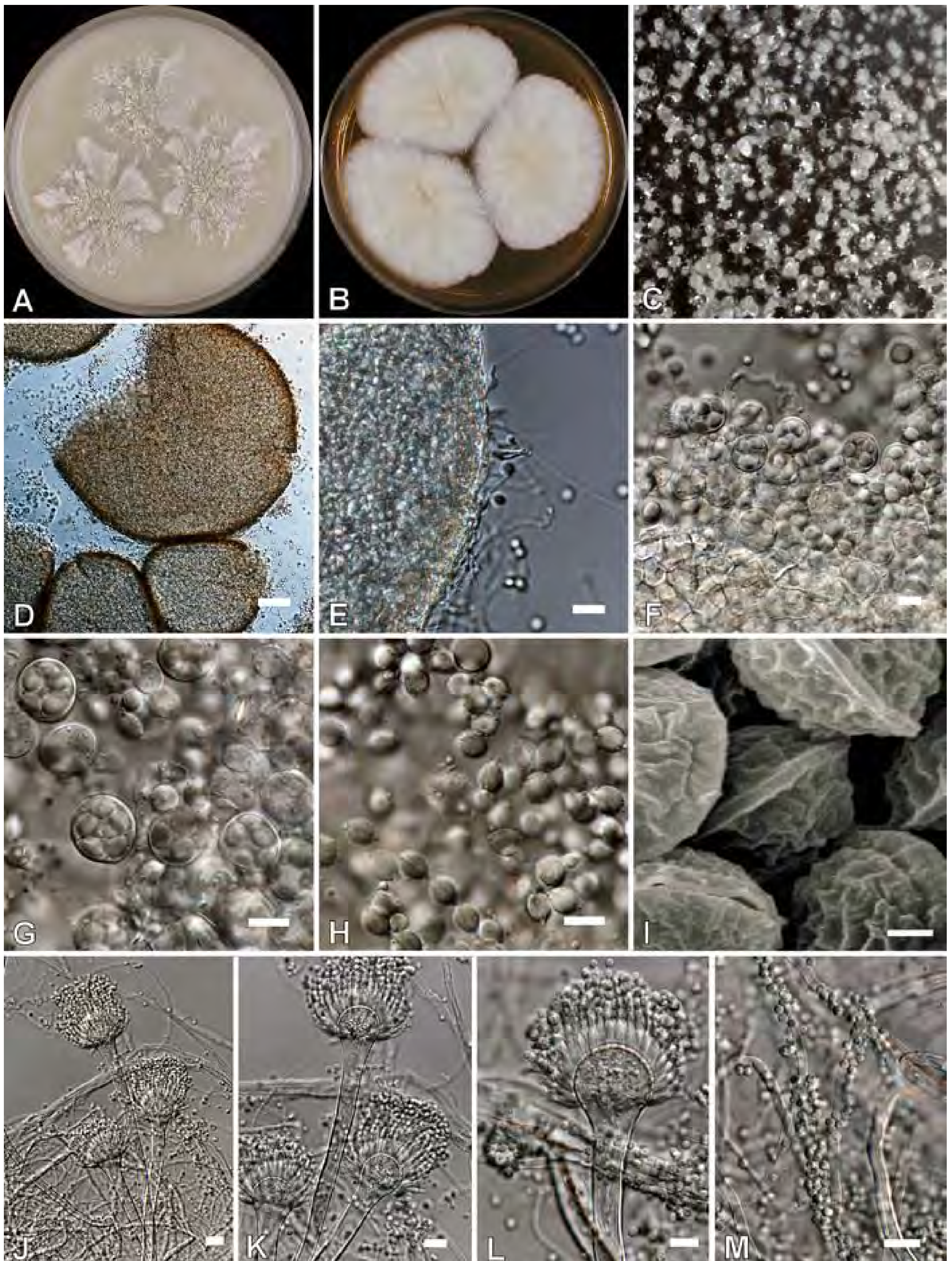

Fig. 27. *Neosartorya hiratsukae*. A–B. Colonies 14 d 25 °C. A. OA. B. MEA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 1 µm.

Neosartorya hiratsukae Udagawa, Tsubouchi & Horie [anamorph: *A. hiratsukae* Udagawa, Tsubouchi & Horie], Trans. Mycol. Soc. Japan 32: 23. 1991. Fig. 27.

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

Extrolites: avenaciolide

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)

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: NRRL 35589 = 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: 70–80 mm; CREA: poor growth and no acid production
Colony colour: white to pale yellow
Conidiation: sparse
Reverse colour (CYA): greyish orange to yellowish orange
Colony texture: sulcate, granular
Conidial head: columnar
Stipe: 3–4 µm wide
Vesicle diam, shape: 10–14 µm, subclavate
Conidium size, shape, surface texture: 2.5–3.5 µm, globose to subglobose, smooth
Homothallic
Cleistothecia: 300–400 µm, white to light yellow
Ascospores: 4–5 µm, broadly lenticular, with two distinct straight equatorial crests which are up to 2 µm

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

Extrolites: aszonalenins, tryptoquivaline, tryptoquivalone

Pathogenicity: not reported

Neosartorya multiplicata Yaguchi, Someya & Udagawa [anamorph: *A. muliplicatus* 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 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 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

Extrolites: helvolic acid

Pathogenicity: not reported

Fig. 28. *Neosartorya laciniosa*. A–B. Colonies 14 d 25 °C. A. MAA. B. CYA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 1 µm.

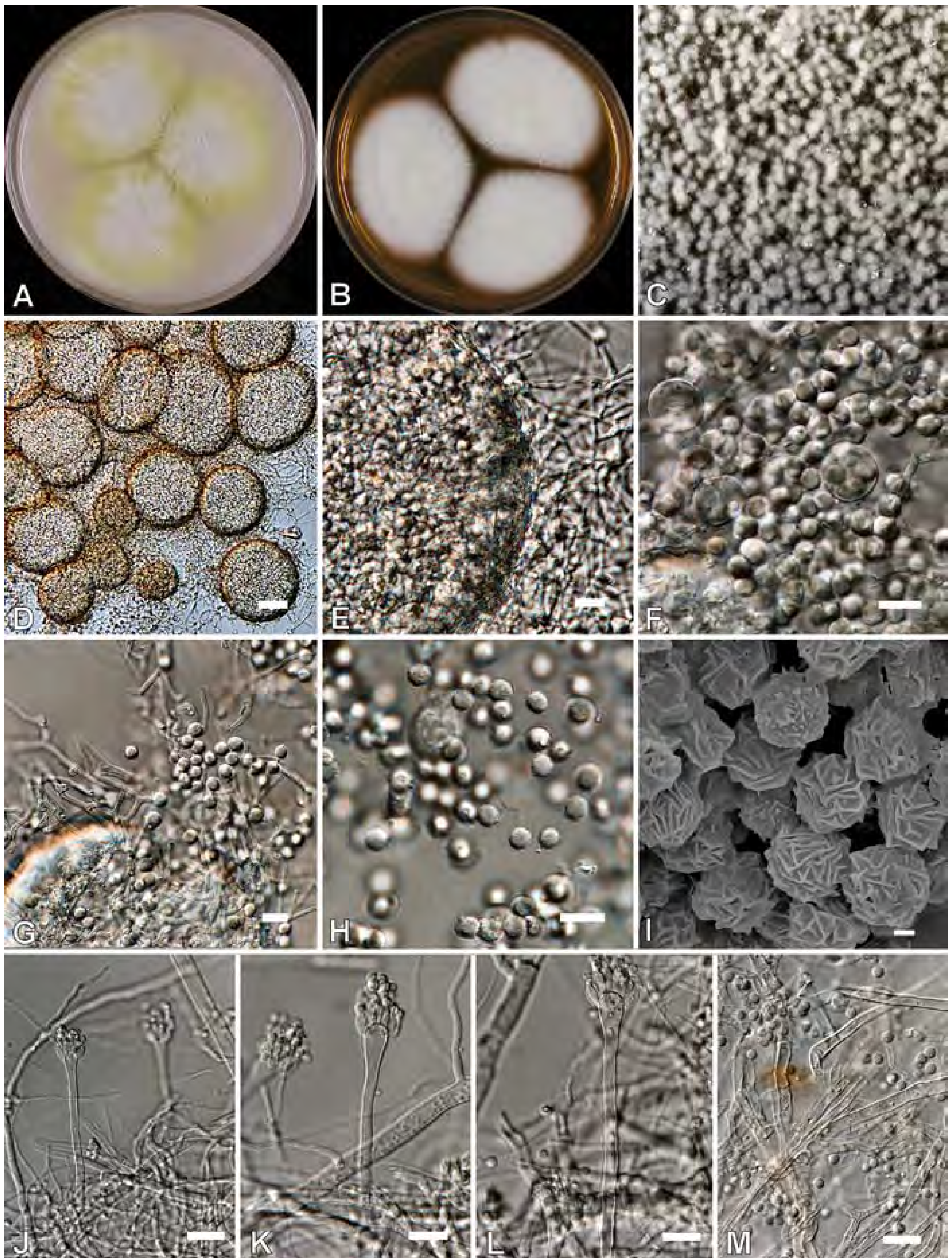

Fig. 29. *Neosartorya multiplicata*. A–B. Colonies 14 d 25 °C. A. OA. B. MEA. C. Macroscopic view of the columnar conidial heads D–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 1 µm.

Fig. 30. *Neosartorya papuensis*. A–B. Colonies 14 d 25 °C. A. OA. B. MEA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 1 µm.

Neosartorya papuensis Samson, Hong & Varga, **sp. nov.** (Fig. 30) – MycoBank MB505571.

Homothallica; cleistothecia superficialia, luteoalba vel dilute lutea, globosa vel subglobosa, 200–350 µm diam, in hyphis hyalinis vel luteoalbis laxe obtextis. Asci octospori, globosi vel subglobosi, 14–20 µm diam, evanescentes. Ascosporae 5.5–7.5 µm diam, cristis angustis, aequatoriis binis, pagina convexa sublaevigata. Mycelium ex hyphis hyalinis, ramosis, septatis, laeviparietinis constans. Capitula conidialia curta, columnaria. Conidiophora ex hyphis aeriis exorientia, uniseriata, stipitibus 80–120 x 4–5 µm; vesiculae ampulliformes, 10–14 µm diam; phialides 7.5–9 x 2–3 µm, dimidium supernum vesiculae obtegentes. Conidia globosa vel subglobosa, laevia, 2–3 µm diam. Coloniae in agaro MEA in 7 dieibus et 25 °C celeriter crescentes, 35–40 mm diam, albae, capitulis conidialibus paucis. Coloniae in agaro CYA in 7 dieibus et 25 °C 20–30 mm diam, cremeoalbae, centro ab hyphis aerialibus laxe obtecto; capitula conidialia pauca; colonia reversa luteoalba vel pallide lutea.

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

Homothallic, cleistothecia superficial, yellowish white to pale yellow, globose to subglobose, 200–350 µm in diam., surrounded by a loose covering of hyaline to yellowish white hyphae. Asci 8-spored, globose to subglobose 14–20 µm, evanescent at maturity. Ascospores 5.5–7.5 µm, with two equatorial crests, convex surface smooth microtuberculate. Mycelium composed of hyaline, branched, septate, smooth-walled hyphae. Conidial heads short, columnar. Conidiophores arising from aerial hyphae, uniseriate, stipes 100–150 × 4–5 µm; vesicles flask-shaped, 10–14 µm in diam.; phialides 7.5–9 x 2–3 µm, covering the upper half of vesicle. Conidia globose to subglobose, smooth, 2–3 µm. Colonies on MEA growing rapidly, 35–40 mm in 7 d at 25 °C, white. Conidial heads few in number. Colonies on CYA, 30–35 mm in 7 d at 25 °C, producing sectors, creamy white, loosely overgrown by aerial hyphae in center. Conidial heads few in number. Reverse yellowish white to pale yellow (12A23) (Kornerup and Wanscher 1978).

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 raiased 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: asperfuran, cytochalasin-like compound, fiscalin-like compound, pyripyropens, gliotoxin

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

Neosartorya quadricincta (J.L. Yuill) Malloch & Cain [anamorph: *A. quadricingens* Kozakiewicz], Can. J. Bot. 50: 2621. 1973. Fig. 32.

= *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

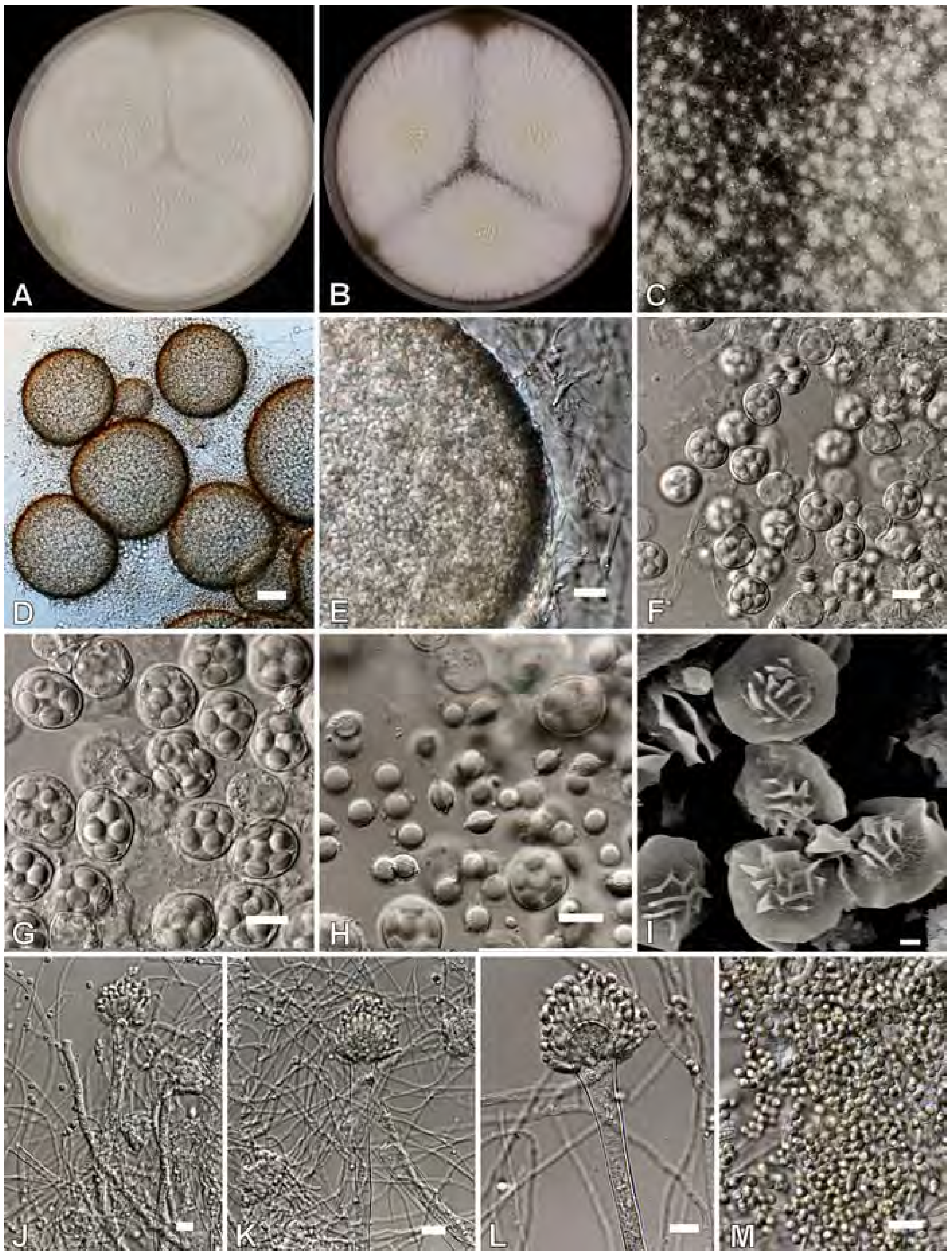

Fig. 31. *Neosartorya pseudofischeri*. A–B. Colonies 14 d 25 °C. A. OA. B. MEA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 1 µm.

Fig. 32. *Neosartorya quadricincta*. A–B. Colonies 14 d 25 °C. A. OA. B. MEA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm , I = 1 µm.

Ascospores: 4–5 µm, with two prominent equatorial crests, each duplicated by a some-what 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 Takada & Udagawa [anamorph: *A. spathulatus* Takada & Udagawa], Mycotaxon 24: 395. 1985. Fig. 33.

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 and 60–250 × 4–10 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: xanthocillins, aszonalenins

Pathogenicity: not reported

Neosartorya spinosa (Raper & Fennell) Kozakiewicz [anamorph: *A. spinosus* Kozakiewicz], Mycol. Pap. 161: 58. 1989. Fig. 34.

≡ *Aspergillus fischeri* var. *spinosus* Raper & Fennell 1965 (basionym)
= *Sartorya fumigata* var. *verrucosa* Udagawa & Kawasaki
= *Neosartorya botucatensis* Y. Horie, Miyaji & Nishim. [anamorph: *A. botucatensis* Y. Horie, Miyaji & Nishim.]
= *Neosartorya paulistensis* Y. Horie, Miyaji & Nishim. [anamorph: *A. paulistensis* Y. Horie, Miyaji & Nishim.]
? = *Neosartorya takakii* Horie, Abliz & K. Fukush. [anamorph: *A. takakii* Horie, Abliz & K. Fukush.]

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 spinelike 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, Sudasn, 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 (R.O. Novak & Raper) Malloch & Cain [anamorph: *A. paleaceus* Samson & Gams], Can. J. Bot. 50: 2622. 1972. Fig. 35.

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

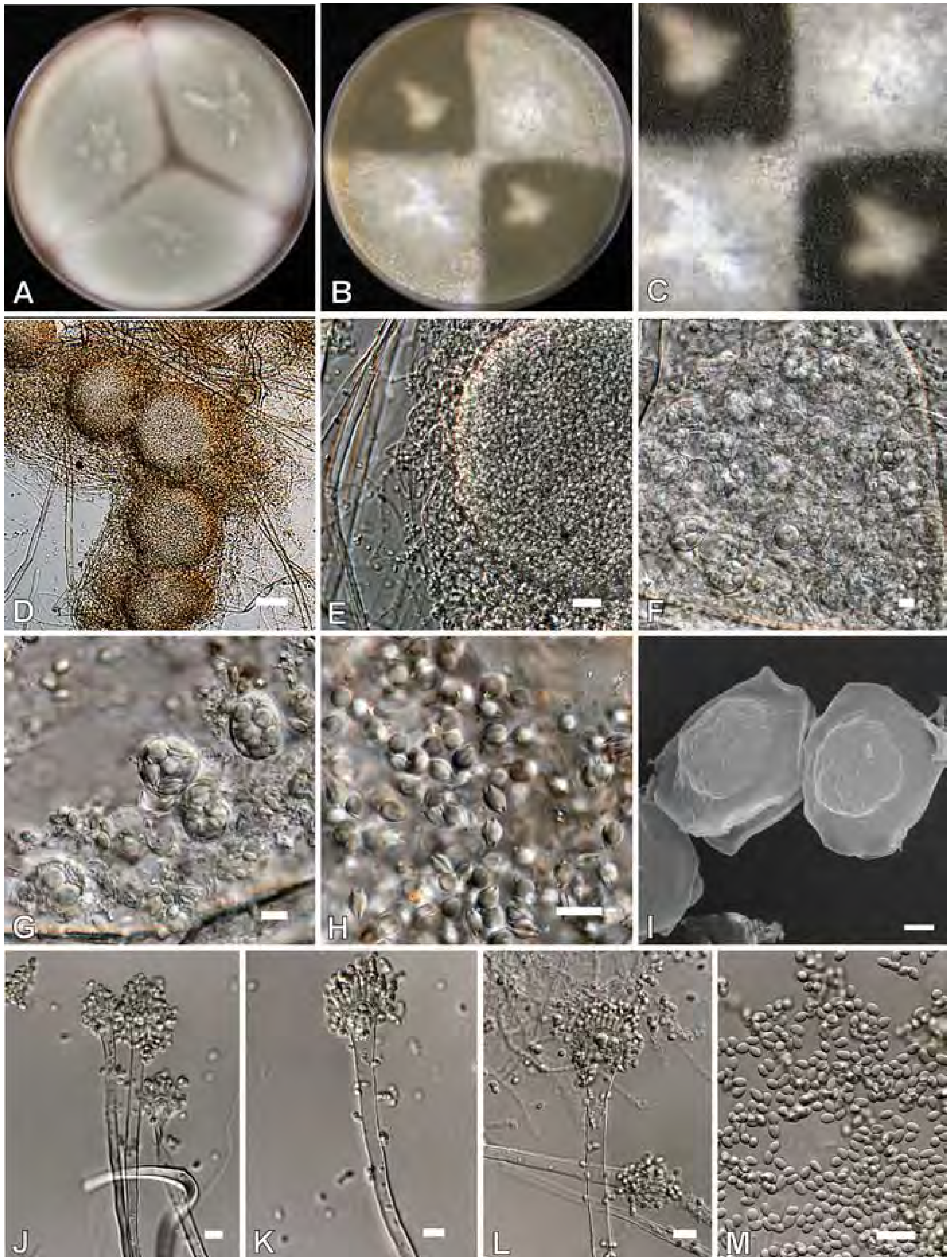

Fig. 33. *Neosartorya spathulata*. A–B. Colonies 14 d 25 °C. A. MEA. B–C. Crossing of mating types on MEA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 1 µm.

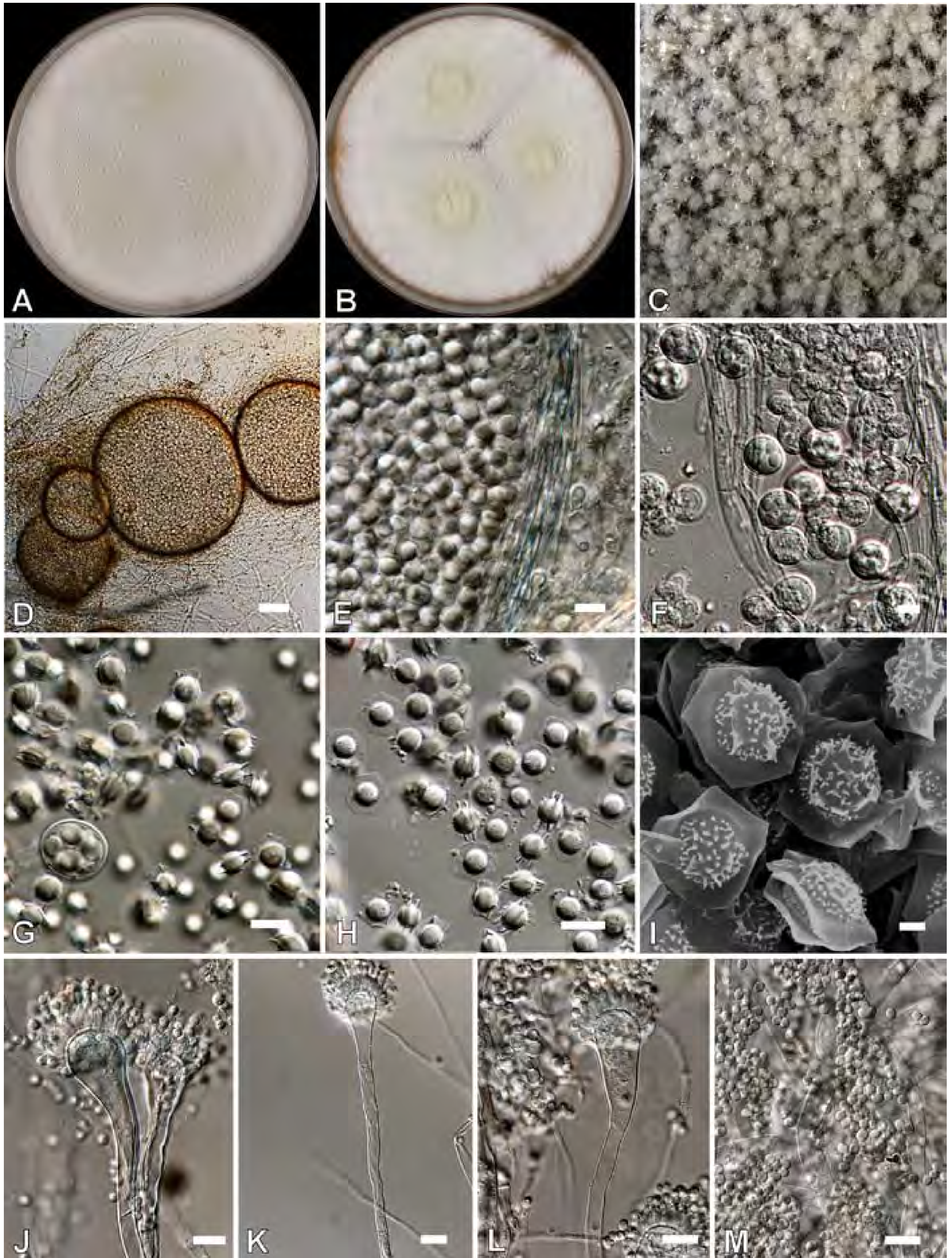

Fig. 34. *Neosartorya spinosa*. A–B. Colonies 14 d 25 °C. A. OA. B. MEA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 1 µm.

Colony colour (CZA): mustard-yellow
Conidiation: sparse
Reverse colour (CZA): yellow-orange
Colony texture: granulose
Conidial head: loosely columnar
Stipe: 80–140 × 3.5–5.5 µm, heavy walled, septate, coloured in terminal areas
Vesicle diam, shape: 10–12 µm, flask shaped to globose
Conidium size, shape, surface texture: 2.5–3 µm, globose, microverrucose
Homothallic
Cleistothecia: 50–175 µm, cartridge buff
Ascospores: 4.5–5.5 µm, with two widely separated flexuous equatorial crests, convex surfaces finely echinulate

Cultures examined: CBS 498.65; IFO 31358

Diagnostic features: faster growth rate and pronounced echinulate ascospore ornamentation distinguishes this species from *N. aurata*

Similar species: *N. aurata*

Distribution: U.S.A., Argentina

Ecology and habitats: Soil, salt grass (*Distichlis scoparia*)

Extrolites: quinolactacin, avenaciolide

Pathogenicity: not reported

Neosartorya tatenoi Horie, Miyaji, Yokoyama, Udagawa & Campos-Takagi [anamorph: *A. tatenoi* Y. Horie, M. Miyaji, K. Yokoy., Udagawa & Campos-Takagi], Trans. Mycol. Soc. Japan 33: 395. 1992. Fig. 36.
= *Neosartorya delicata* H.Z. Kong [anamorph: *A. delicatus* H.Z. Kong]

Type: CBM FA 0022, from soil, Brazil

Other no. of the type: CBS 407.93; IBT 21589

Morphological characteristics
Colony diam (7 d): CYA25: 35–39 mm mm; MEA25: 31–39 mm; YES25: 57–74 mm; OA25: 50–55 mm; CYA37: 72–78 mm; CREA: poor gowth and no acid production
Colony colour: pale yellow to yellowish white
Conidiation: sparse
Reverse colour (CZA): orange white to pale orange
Colony texture: velutinous to floccose
Conidial head: short columnar
Stipe: 270 × 4–7.5 µm
Vesicle diam, shape: 10–20 µm, hemispherical to flask-shaped
Conidium size, shape, surface texture: 2–3(–3.5) µm, globose to ovoid, smooth
Homothallic
Cleistothecia: 140–360 × 140–310 µm, hyaline to pale yellowish brown
Ascospores: 5–5.5 µm, lenticular, with two equatorial crests, convex surfaces with distinctly and nerrowly reticulate ridges

Cultures examined: CBS 407.93; NRRL 4584

Diagnostic features: distinct narrowly reticulate ascospore ornamentation

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

Distribution: Brazil, Dominican Republic

Ecology and habitats: soil

Extrolites: aszonalenins

Pathogenicity: not reported

Neosartorya udagawae Horie, Miyaji & Nishim. [anamorph: *A. udagawae* Horie, Miyaji & Nishim.], Mycoscience 36: 199. 1995. Fig. 37.

Type: CBM FA-0703 & CBM FA-0702, from soil, Brazil

Other no. of the type: CBS 114217 & CBS 114218

Morphological characteristics
Colony diam (7 d): CYA25: 33–36 mm; MEA25: 63–68 mm; YES25: 64–68 mm; OA25: 51–55 mm; CYA37: 61–65mm; CREA: poor growth and no acid production
Colony colour (CZA): dull green
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 x 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 114217, CBS 114218

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

Extrolites: fumigatin, fumagillin, tryptoquivaline, tryptoquivalone

Pathogenicity: pathogenic to humans (Balajee *et al.* 2006; Moragues *et al.* 2006)

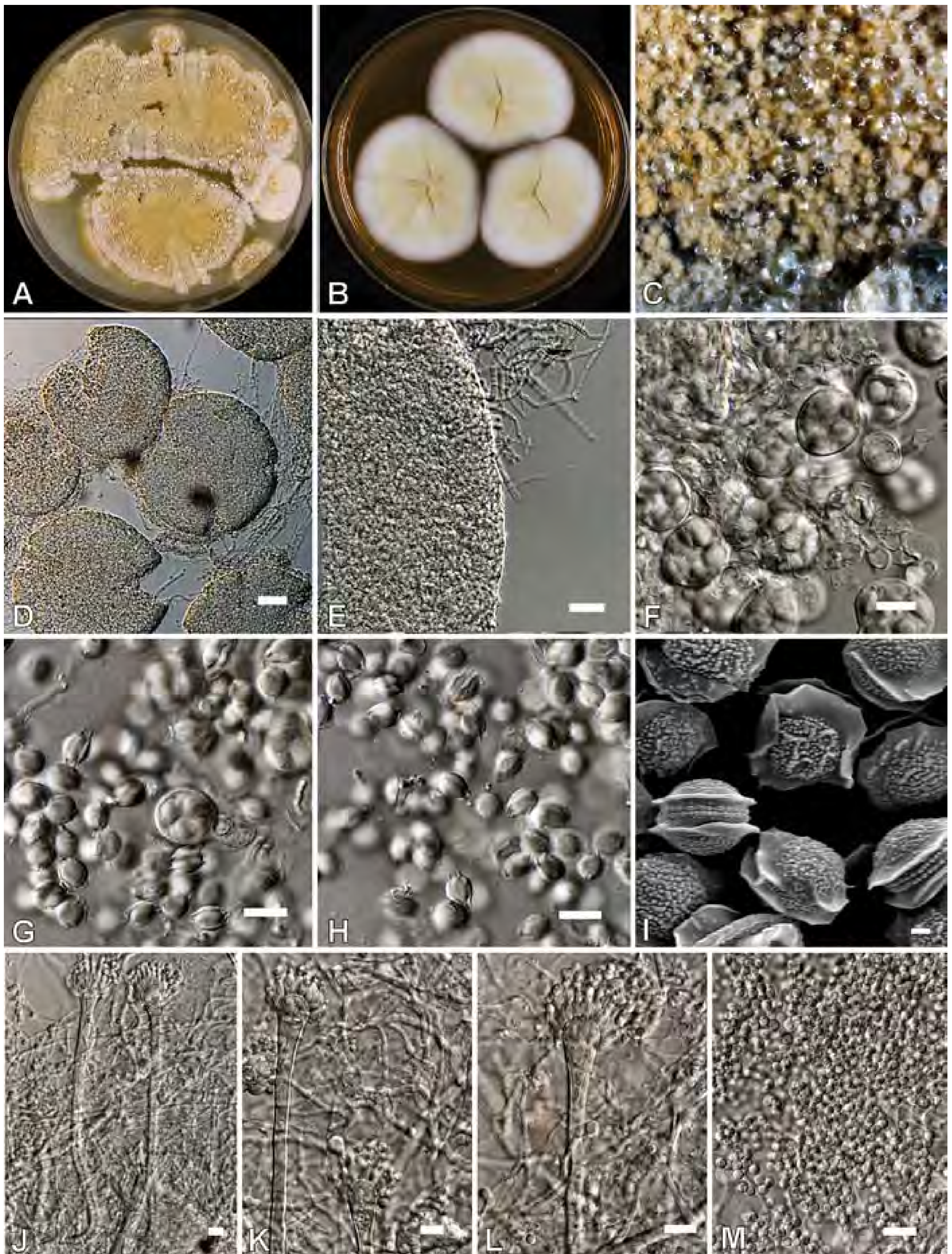

Fig. 35. *Neosartorya stramenia*. A–B. Colonies 14 d 25 °C. A. OA. B. MEA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 1 µm.

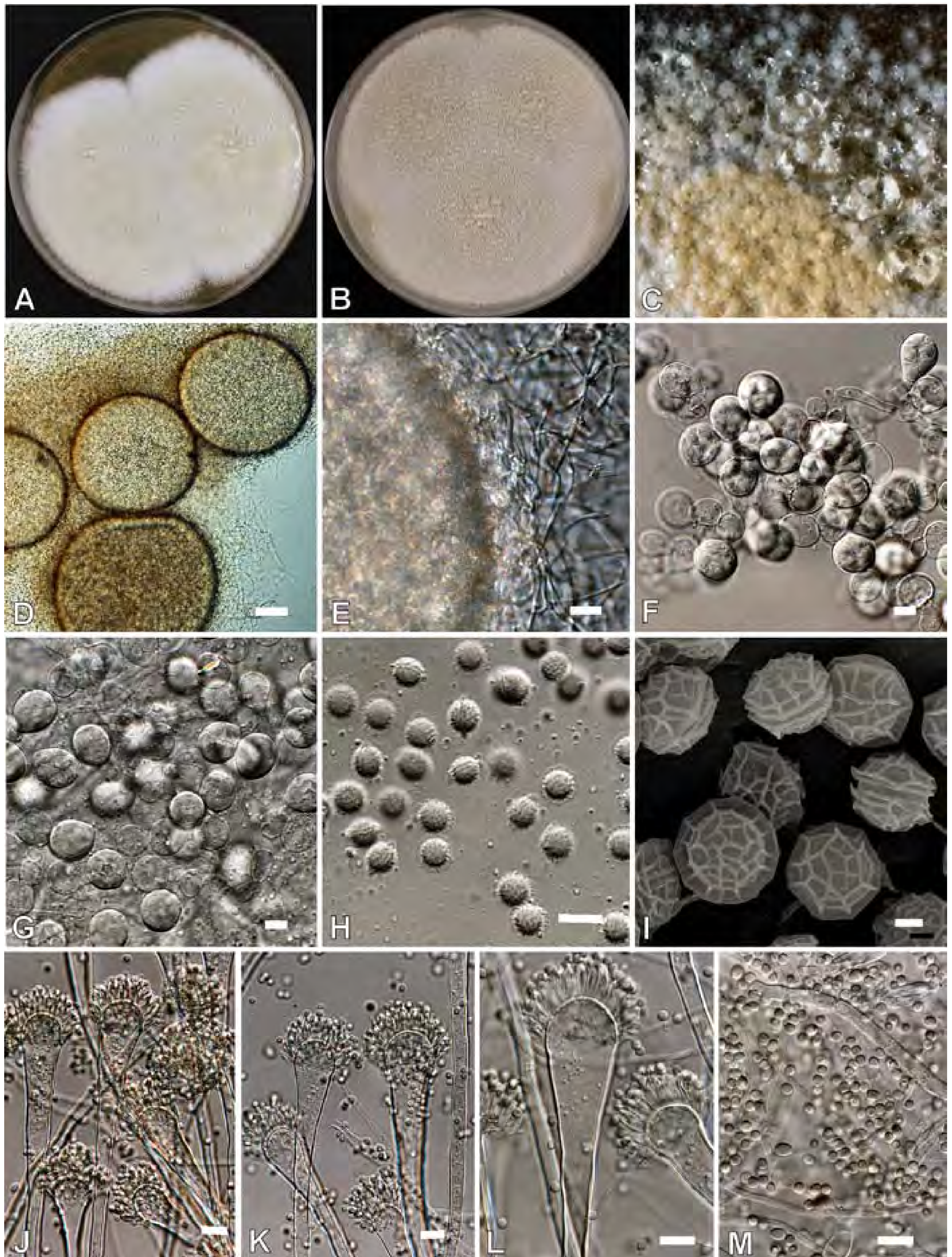

Fig. 36. *Neosartorya tatenoi*. A–B. Colonies 14 d 25 °C. A. MEA. B. OA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm¸ I = 1 µm.

Fig. 37. *Neosartorya udagawae*. A–B. Colonies 14 d 25 °C. A. MEA. B. Crossing of mating types on MEA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 1 µm.

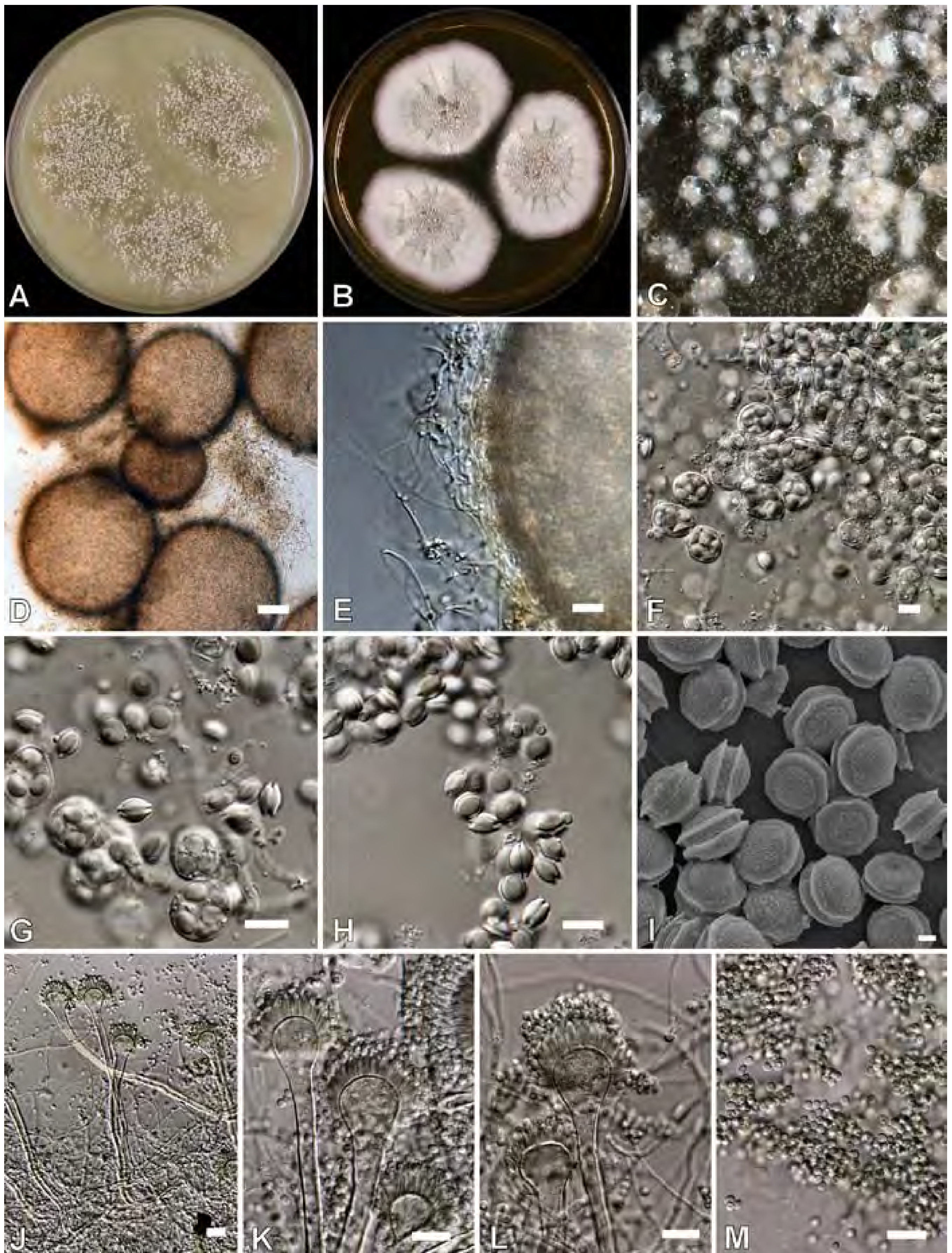

Fig. 38. *Neosartorya warcupii*. A–B. Colonies 14 d 25 °C. A. OA. B. MEA. C–E. Ascomata. F–G. Asci and ascospores. H. Ascospores. I. SEM of ascospores. J–L. Conidiophores. M. Conidia. Scale bars = 10 µm, except D = 30 µm, E = 15 µm, I = 1 µm.

Neosartorya warcupii Peterson, Varga & Samson, **sp. nov.** (Fig. 38) – MycoBank MB505572.

Homothallica; cleistothecia superficialia, alba vel dilute lutea, globosa vel subglobosa, 200–350 µm diam, in hyphis hyalinis vel luteoalbis laxe obtextis. Asci octospori, globosi vel subglobosi, 4.5–7 µm diam, evanescentes. Ascosporae 5.5–7 µm diam, cristis angustis, aequatoriis binis, pagina convexa sublaevigata. Mycelium ex hyphis hyalinis, ramosis, septatis, laeviparietinis constans. Capitula conidialia curta, columnaria. Conidiophora ex hyphis aeriis exorientia, uniseriata, stipitibus 100–150 x 4–5 µm; vesiculae ampulliformes, 10–25 µm diam; phialides 7.5–9 x 2–3 µm, dimidium supernum vesiculae obtegentes. Conidia subglobosa vel ellipsoidea, laevia, 1.8–1.5 µm diam. Coloniae in agaro MEA in 7 dieibus et 25 °C celeriter crescentes, 35–40 mm diam, albae, capitulis conidialibus paucis. Coloniae in agaro CYA in 7 dieibus et 25 °C 20–30 mm diam, cremeoalbae, centro ab hyphis aerialibus laxe obtecto; capitula conidialia pauca; colonia reversa luteobrunnea vel atrobrunnea.

Holotype of *Neosartorya warcupii,* here designated as NRRL 35723^T (dried culture), isolated from soil, Finder"s Range, Australia.

Homothallic, cleistothecia superficial, 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 shapedm 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 × 4–6 µm; vesicles subclavate 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.

Extrolites: wortmannin-like, aszonalenin-like, chromanol-like, tryptoquivaline-like and tryptoquivalone-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

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