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New and revisited species in Aspergillus section Nigri

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Abstract: Four new species, Aspergillus eucalypticola, A. neoniger, A. fijiensis and A. indologenus are described and illustrated. Aspergillus eucalypticola was isolated from Eucalyptus leaf from Australia, and is related to A. tubingensis and A. costaricanaeis, but could clearly be distinguished from them based on either β-tubulin or calmodulin sequence data. Aspergillus eucalypticola produced pyranonigrin A, funalenone, aurasperone B and other naphtho-γ-pyrones. Aspergillus neoniger is also a biseriate species isolated from desert sand in Namibia, and mangrove water in Venezuela, which produces aurasperone B and pyranonigrin A. Aspergillus fijiensis is a uniseriate species related to A. aculeatinus, and was isolated from soil in Fiji, and from Lactuca sativa in Indonesia. This species is able to grow at 37 °C, and produces asperparalines and okaramins. Aspergillus indologenus was isolated from soil, India. This species also belongs to the uniseriate group of black aspergilli, and was found to be related to, but clearly distinguishable from A. uvarum based on β-tubulin, calmodulin and ITS sequence data. Aspergillus indologenus produced the insecticidal compounds okaramins A, B, H, and two types of indol-alkaloids which have not been structure elucidated. Two other species, A. violaceofuscus and A. acidus, are revalidated based on molecular and extrolite data. Aspergillus violaceofuscus was found to be related to A. japonicus, and produced some of the same indol-alkaloids as A. indologenus, and also produced several families of partially characterised extrolites that were also found in A. heteromorphus. Aspergillus acidus (previously known as A. foetidus var. pallidus and A. foetidus var. acidus) is also a valid species, while A. foetidus is a synonym of A. niger based on molecular and physiological data. Two other species described previously, A. coreanus and A. lactocefeus, were found to be colour mutants of A. acidus and A. niger, respectively. Methods which could be used to distinguish the two closely related and economically important species A. niger and A. awarnori are also detailed. Although these species differ in their occurrence and several physiological means (elastase activities, abilities to utilise 2-deoxy-D-glucose as sole carbon source), our data indicate that only molecular approaches including sequence analysis of calmodulin or β-tubulin genes, AFLP analysis, UP-PCR analysis or mtDNA RFLP analysis can be used reliably to distinguish these sibling species. Aspergillus section Nigri now includes 26 taxa.

Key words: Aspergillus section Nigri, phylogeny, polyphasic taxonomy, extrolites.

Taxonomic novelties: Aspergillus eucalypticola Varga, Frisvad & Samson sp. nov., Aspergillus fijiensis Varga, Frisvad & Samson sp. nov., Aspergillus indologenus Frisvad, Varga & Samson sp. nov., Aspergillus neoniger Varga, Frisvad & Samson sp. nov.

INTRODUCTION

The black aspergilli (Aspergillus section Nigri; Gams et al. 1985) are 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. 2004b, Ferracin et al. 2009). 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 species are occasionally difficult to recognise based solely on their phenotypic characters (Samson et al. 2007).

During a study of the genetic relationships among black aspergilli collected worldwide, four isolates have been identified which did not fit to any of the currently accepted 19 species of Aspergillus section Nigri (Samson et al. 2007, Noonim et al. 2008, Perrone et al. 2008). We used a polyphasic approach including sequence analysis of parts of the β-tubulin and calmodulin genes and the ITS region, macro- and micromorphologcal analyses and examination of extrolite profiles of the isolates to describe four new species in this section. Besides, the applicability of various approaches for distinguishing the two closely related species A. niger and A. awarnori has also been examined. The methods tested include morphological, physiological, ecological and molecular approaches.

MATERIALS AND METHODS

Isolates

The strains used in this study are listed in Table 1.

Morphological analysis

For macromorphological observations, Czapek Yeast Autolysate (CYA) agar, Malt Extract Autolysate (MEA) agar, Yeast Extract SucroseAgar (YES), Creatine Agar (CREA), and OatmealAgar (OA) were used (Samson et al. 2004a). The isolates were inoculated at three points on each plate of each medium and incubated at 25 °C and 37 °C in the dark for 7 d. For micromorphological observations,
Extrolite analysis

The isolates were grown on CYA and YES at 25 °C for 7 d. Extrites were extracted after incubation. Five plucks of each agar medium were taken and pooled together into same vial for extraction with 0.75 mL of a mixture of ethyl acetate/dichloromethane/methanol (3:2:1) (v/v/v) with 1 % (v/v) formic acid. The extracts were filtered

| Accession No. | Species | Origin and information (abbreviation) | β-tubulin | calmodulin | ITS |
|---------------|---------|----------------------------------------|-----------|------------|-----|
| CBS 564.65^7 | A. acidum | Unknown substratum, Japan | AY585533 | AY585533 | AJ280009 |
| CBS 121060^7 | A. aculeatus | Arabica green coffee bean, Thailand | EU159220 | EU159241 | EU159211 |
| CBS 172.66^7 | A. aculeatus | Tropical soil, unknown origin | AY585540 | AY564877 | AJ27988 |
| CBS 557.65^7 | A. awamori | NRRL 4948 = WB 4948 | AY820001 | AY564874 | AM086714 |
| CBS 101740^7 | A. brasiliensis | Soil, Sao Paulo, Pedreira, Brazil | AY820006 | AM295175 | AJ280010 |
| CBS 111.26^7 | A. carbonarius | Paper, origin unknown | AY585532 | AY564873 | DQ900605 |
| CBS 119384^7 | A. coreanus | Nuruk, Boun-up, Bounkun, Chungbuk Prov., Korea | FJ491693 | FJ4916702 | FJ491684 |
| CBS 115574^7 | A. costaricaensis | Soil, Taboga Island, Guaguin garden, Costa Rica | AY820014 | EU163268 | DQ900602 |
| CBS 707.79^7 | A. everti | Soil, Costa Rica | AY585530 | AM117809 | AJ280014 |
| CBS 122712^7 | A. eucalypticola | Eucalyptus leaves, Australia | EU482435 | EU482433 | EU482439 |
| CBS 119.49 | A. fijensis | Lactuca sativa, Palembang, Indonesia | FJ491689 | FJ491701 | FJ491679 |
| CBS 313.89^7 | A. fijensis | Soil, Fiji | FJ491688 | FJ491695 | FJ491680 |
| CBS 121.28^NT | A. foetidus | Awamori-koji alcoholic beverage, Ryuku island, Japan | FJ491690 | FJ491694 | FJ491683 |
| CBS 114.49^7 | A. foetidus | NRRL 341, Thom 5135.17; K. Yakoyama, Japan | EF661090 | EF661155 | EF661187 |
| CBS 117.55^7 | A. heteromorphus | Culture contaminant, Brazil | AY585559 | AM241461 | AJ280013 |
| CBS 101889^7 | A. homomorphus | Soil, near Dead Sea, Israel | AY820015 | AM878665 | EF166063 |
| CBS 121593^7 | A. ibenicus | Grapes, Portugal | AM197478 | AJ971805 | AY566525 |
| CBS 114.80^7 | A. indologenus | Soil, India | AY585539 | AM197470 | AJ280005 |
| CBS 114.51^7 | A. japonicus | Saito 5087, origin unknown | AY585542 | AY564875 | AJ279895 |
| CBS 101883^7 | A. lacticoffeatus | Coffee bean, South Sumatra, Indonesia | AY19998 | EU163270 | DQ900604 |
| CBS 115657 | A. neoniger | Desert sand, Namibia | FJ491692 | FJ491699 | FJ491681 |
| CBS 115656^7 | A. neoniger | Mangrove water, Mochima Bay, Venezuela | FJ491691 | FJ491700 | FJ491682 |
| CBS 554.65^7 | A. niger | Soil, near Dead Sea, Israel | AY585536 | AY564872 | AJ223852 |
| CBS 112811^7 | A. piperis | Black pepper, Denmark | AY820013 | EU163267 | DQ900603 |
| CBS 121057^7 | A. scleroticonarbonarius | Robusta coffee bean, Thailand | EU159229 | EU159235 | EU159216 |
| CBS 127449^7 | A. saccharolyticus | under a toilet seat made of treated oak wood, Gentofte, Denmark | HM953535 | HM953554 | HM953552 |
| CBS 115572^7 | A. sclerotiorngan | Coffee bean, Kamataka, India | AY819996 | EU163271 | DQ900606 |
| CBS 134.48^7 | A. tubingensis | Origin unknown | AY820007 | AY564876 | AJ223853 |
| CBS 121591^7 | A. uvarum | Healthy Cisternino grape, Italy | AM745751 | AM745755 | AM745751 |
| CBS 113365^7 | A. vadensis | Origin unknown | AY585531 | EU163269 | AY565549 |
| CBS 11577 | A. violaceofuscus | Marine environment, Bahamas | EU482434 | EU482432 | EU482440 |
| CBS 123.27^NT | A. violaceofuscus | Soil, Puerto Rico; Thom 3522.30 | FJ491685 | FJ491698 | FJ491678 |
| CBS 122.35 | A. violaceofuscus mut. grisea | WB 4880 | FJ491687 | FJ491696 | FJ491676 |
| CBS 102.23^7 | A. violaceofuscus | Received from D. Borrel, Strassbourg, France | FJ491686 | FJ491697 | FJ491677 |

Table 1. Isolates examined in this study.

^T = Type, ^NT = Neotype

microscopic mounts were made in lactic acid from MEA colonies and a drop of alcohol was added to remove air bubbles and excess conidia.

**Physiological analysis**

Aspergillus niger and A. awamori isolates were analysed using various methods. Elastase activity was studied on Czapek Dox minimal medium without NaNO₃, with 0.05 % elastin (Sigma, St Louis, MO, USA) and 0.05 % Rose Bengal (Sigma, St Louis, MO, USA), buffered to pH 7.6 with NaOH (modified after Blanco et al. 2002). Conidial suspensions of A. niger and A. awamori strains were prepared from cultures grown on YPD agar slants. The suspensions were diluted in 1 mL bidistilled water. Plates were inoculated in a central spot with 20 μL of the conidium suspensions and were incubated for 7 d at 37 °C. The diameters of the halo of elastin lysis were measured. The experiment was repeated three times, and the average diameters were calculated for each strain. Statistical analysis was made using the R software package (http://

of Systems Biology-DTU were used to confirm the identity of the detected extrolites.
www.r-project.org)). The assumptions of ANOVA were tested using the diagnostic plots in R. According to Quantile-Quantile (QQ) Plot the data were not normally distributed, thus the Kruskal-Wallis test was applied to compare the average diameters between the two species.

Carbon source assimilation tests were performed on minimal medium (MM: 0.5 % (NH₄)₂SO₄, 0.1 % KH₂PO₄, 0.05 % MgSO₄, 2 % agar) with 0.2 % single carbon source. Conidial suspensions of eight *A. niger* and eight *A. awamori* strains were prepared from 5-d-old cultures grown on YPD agar slants. The suspensions were diluted in bidistilled water and conidia were filtered. An YPD plate was inoculated in 16 points with 15 µL of the conidium suspensions and was incubated for 3 d at 25 °C. Strains were replicated to the MM plates, which contained single carbon sources using a 16-pronged replicator. Plates were incubated for 7 d at 25 °C. The experiment was repeated twice and control series was made on MM plates without carbon source and YPD plates.

Thirty carbon sources were tested, which were selected based on previous carbon source utilisation experiments (Varga *et al.* 2000): glucose, D-xylose, galactose, D-lyxose, L-sorbose, L-rhamnose, lactose, eritri, galacct, L-valine, L-β-phenilalanine, L-triptophane, L-trycone, L-serine, L-cysteine, L-asparagine acid, L-tyrosine, L-synase, L-histidine, L-citrulline, cis-aconitic acid, vanillin, vanillin acid, L-ascorbic acid, D-glucoseamine, glycylglycine, salicin, pectin, melezitose, α-ketogluatric acid. Different growth patterns of the strains belonging to the two species were observed simply in the case of L-sorbose, so the test was extended to 2-deoxy-D-glucose because of the structural similarity of these two compounds.

**Genotypic analysis**

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. The ITS region and parts of the β-tubulin and calmodulin genes were amplified and sequenced as described previously (Varga *et al.* 2007a–c).

Part of the FUM8 gene was amplified using primers vnF1 and vnR3 as described by Susca *et al.* (2010). Primer sets were also designed to target part of the chloroperoxidase gene of black aspergilli presumably taking part in ochratoxin biosynthesis. Construction of the primers was carried out by using the homologous sequences identified in the genomic sequences of *Aspergillus niger* CBS 513.88 and *Aspergillus carbonarius* ITEM 5010 isolates. The designed chloroperoxidase specific PCR primers were BCPOF (5’- CTGGGCCGACTGCTCCAC-3’) and BCPOR (5’- TCTATCGTACGGCAGACGCT-3’) which generated specific amplicons of about 250 base pairs. Amplifications were performed on a PTC-0148 Mini48 thermocycler (BioRad, USA), using the following amplification steps: 4 min of initial denaturation at 94 °C followed by 35 amplification cycles of 20 s at 94 °C, 15 s at 62 °C and 30 s at 2 °C and a final extension step for 1 min at 72 °C.

DNA sequences were edited with the DNAStar computer package and an alignment of the sequences and neighbour joining analyses were performed using the MEGA v. 4 software (Tamura *et al.* 2007). To determine the support for each clade, a bootstrap analysis was performed with 1 000 replications. *Aspergillus flavus* CBS 100927® was used as outgroup in these analyses.

Phylogenetic analysis of sequence data was also performed using PAUP* v. 4.0b10 (Swofford 2000). Alignment gaps were treated as fifth character state, parsimony uninformative characters were excluded and all characters were unordered and equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option. To assess the robustness of the topology, 1 000 bootstrap replicates were run by maximum parsimony (Hillis & Bull 1993). Other measures including tree length, consistency index and retention index were also calculated. Sequences were deposited at GenBank under accession numbers listed in Table 1.

UP-PCR analyses were carried out according to Bulat *et al.* (2000). DNA was isolated as described in the literature (Leach *et al.* 1986). The primers used were L45, AS15inv, L15/AS19, AA2M2, L21, 3-2, AS4, AS15 (Lübeck et al. 1998, Bulat *et al.* 2000). The amplification process consisted of a predenaturation step for 1 min at 94 °C, followed by 35 cycles (30 s at 94 °C, 45 s at 55 °C, and 1 min at 72 °C), plus a final extension of 2 min at 72 °C. The amplification products were separated by electrophoresis in 1 % agarose gels, stained with ethidium bromide, and visualised under UV light. All amplifications were repeated at least two times. The faint bands which did not appear in all repeated experiments were not counted during cluster analysis.

Altogether 88 fragments were noted and a binomial matrix was created so that presence and absence of DNA fragments were scored as 1 or 0, respectively. Cluster analysis was carried out by using PHYLIP v. 3.67 software package (Felsenstein 2007). Phylogenetical tree was created by using neighbor-joining method (Saito *et al.* 1987) with the program NEIGHBOR from the PHYLIP program package.

**RESULTS AND DISCUSSION**

**Phylogenetic analysis of sequence data**

The calmodulin data set consisted of 478 characters including 218 parsimony informative sites; MP analysis resulted in 33 most parsimonious trees (length = 621, consistency index = 0.620787, retention index = 0.873655), one of which is presented in Fig. 1. Of the aligned β-tubulin sequences, a portion of 468 positions including 221 parsimony informative characters was selected for the analysis; MP analysis of the sequence data resulted in 29 similar, equally most parsimonious trees (tree length = 464 steps, consistency index = 0.622172, retention index = 0.882394), one of which is shown in Fig. 2. The ITS data set consisted of 478 characters including 81 parsimony informative sites; MP analysis resulted in 211 equally most parsimonious trees (length = 143, consistency index = 0.837989, retention index = 0.957910), one of which is presented in Fig. 3.

All four species could be distinguished from the currently accepted species of *Aspergillus* section Nigri (Samson *et al.* 2007) based on either calmodulin or β-tubulin sequence data, and isolate CBS 114.80 also exhibited unique ITS sequences (Fig. 3). *Aspergillus eucalypticola* was found to be related to *A. aculeatinus* section Nigri (Samson *et al.* 2007) based on either calmodulin or β-tubulin sequence data, and isolate CBS 114.80 also exhibited unique ITS sequences (Fig. 3). *Aspergillus violaceofuscus* and *A. indologenus* belonged to a clade including *A. uvarum* and *A. aculeatinus* on the tree based on β-tubulin sequence data (Fig. 1), and to a clade including *A. uvarum* and *A. japonicus* on the tree based on calmodulin sequence data (Fig. 2).
Sequence data also indicate that both the A. foetidus ex-type strain (CBS 114.49 = NRRL 341) and the neotype strain (CBS 121.28), and A. lactofoetidus are synonyms of A. niger, while A. coreanus Yu et al. (2004) is a synonym of A. foetidus var. acidus (validly named A. acidus by Kozakiewicz 1989). Aspergillus lactofoetidus and A. coreanus differ from other black aspergilli in producing brownish conidia, and not producing naphtho-γ-pyrones. There appears to be a link between the black pigment and the naphtho-γ-pyrones via the gene PksA (Jørgensen et al. 2011, Chiang et al. 2011) Besides, A. coreanus also does not produce antafumicins, produced by some A. acidus isolates. Aspergillus lactofoetidus also carries the fumonisin biosynthetic genes, similarly to A. niger (Meijer et al., unpubl. data). Based on these observations, we consider A. foetidus and A. lactofoetidus as synonyms of A. niger, and A. coreanus of A. acidus, respectively.

Our data indicate that Aspergillus section Nigri comprises 26 species including the new species A. saccharolyticus Sørensen et al. (2011). These taxa can be divided into five main clades (called series in Frisvad et al. 2007a; Fig. 1). The A. niger clade includes 10 biserial species, and was divided into three subclades based on β-tubulin and calmodulin sequence data: the A. tubingensis, A. niger and A. brasilensis subclades. Only A. niger is known to be able to produce ochratoxin A and fumonisins from this clade (Samson et al. 2004b, Frisvad et al. 2007b). Another main clade includes relatives of A. carbonarius (A. ibericus, A. sclerotioniger and A. sclerotiocarbonarius). These species series are characterised by relatively large conidia and two of them, A. carbonarius and A. sclerotioniger are able to produce ochratoxin A. Aspergillus ellipticus and A. heteromorphus form another clade, while the biserial A. homomorphus forms a distinct clade. All uniseriate species belong to the A. aculeatus clade, involving seven species (Fig. 1).

**Extrolites**

The extrolites produce by black Aspergillus species have been reviewed by Samson et al. (2007) and Nielsen et al. (2009). The new species described here produce a series of bioactive extrolites, especially the uniseriate species A. indologenus and A. fijiensis.

Aspergillus eucalypticola CBS 122712 produced pyranonigrin A, funalenone, aurasperone B and other naphtho-γ-pyrones, and an unknown extrolite ("MYC") in common with A. neoniger (CBS 115656 = IBT 20973 and CBS 115657 = IBT 23434). Aspergillus neoniger produced funalenone, naphtho-γ-pyrones, pyranonigrin
A and “MYC”, and it is chemically very closely related to A. eucalypticola.

Aspergillus indologenus CBS 114.80 = IBT 3679 produced the insecticidal compounds okaramins A, B, H earlier also reported from an A. aculeatus isolate (Hayashi et al. 1999), partially characterised polar alkaloids, a series of very apolar sclerotial indol-alkaloids (related to aflavinins) and unique indol-alkaloids with similar UV spectra as the fumitremorgins. Aspergillus violaceofuscus produced some indol-alkaloids also found in A. indologenus, but it also produced several families of partially characterised extrolites that have also been found in A. heteromorphus (“SMIF”, “PON”, “SENLAB” (a pyranonigrin-related compound), and yellow compounds with characteristic UV spectra). Aspergillus fijienis CBS 313.89 and CBS 119.49 produced asperparalins, secalonic acid D, F and the partially characterised “BAM”, “PON” = “FIB1” & “FIB2”, “GLABRINOL”, “SENLAB”, and “YE1”. CBS 313.89 in addition produced “DERH” and “YE2” and CBS 119.49 additionally produced neoxaline, and “TRU”. Asperparaline A (= aspergillimide = VM55598), asperparaline B and C have earlier been reported from Aspergillus japonicus ATCC 204480 (Hayashi et al. 1997, 2000) and asperparaline A, 16-keto aspergillimide, VM54159, SB203105 and SB 200437 have been isolated from “a black Aspergillus with pink sclerotia” IMI 337664 (Banks et al. 1997) and neoxaline has been isolated from A. japonicus (Hirano et al. 1979). Based on the extrolite data, ATCC 204480 and IMI 337664 may indeed belong to A. fijiensis, but we have not examined these cultures yet.

Species related to A. niger, such as A. eucalypticola and A. neoiger and the well known species A. carbonarius and A. tubingensis, produce different combinations of pyranonigrins, tensidols, ketonins, fumonisins, funalenones, naphtho-γ-pyrones, ochratoxins, asperazines, and pyrophen (Samson et al. 2004b, 2007), while species related to A. aculeatus, A. aculeatinus, A. japonicus, A. uvarum, and the new species described here, A. indologenus, A. fijienis and the revived A. violaceofuscus produce different combinations of asperparalins, okaramins, sclerotial indol-alkaloids, and secalonic acids (Parenicova et al. 2001, Samson et al. 2004b, Samson et al. 2007, Noonim et al. 2008).

Fig. 2. The single MP tree obtained based on phylogenetic analysis of β-tubulin sequence data of Aspergillus section Nigri. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

![Tree Diagram](image-url)
Approaches to distinguish between isolates of the sibling species *A. niger* and *A. awamori*

*Aspergillus awamori* has recently been revalidated as a cryptic species within the *A. niger* species (Perrone et al. 2011). These species cannot be reliably separated from each other using either morphological or extrolite data. However, molecular data including sequence-based approaches using either β-tubulin, calmodulin or translation elongation factor a sequences and AFLP analysis were found to be useful for distinguishing these species (Perrone et al. 2011).

*Aspergillus niger* and *A. awamori* are economically important as isolates of both species are able to produce fumonisins and/or ochratoxins (Varga et al. 2010, Perrone et al. 2011). In view of the importance of these species in mycotoxin contamination of various agricultural products (see below), we examined other possibilities which could be used for the easy identification of these species.
**Fig. 5.** One of the 73 MP trees obtained based on phylogenetic analysis of FUM8 sequence data of *A. niger* and *A. awamori* isolates (tree length: 411, consistency index: 0.961240, retention index: 0.980843). Numbers above branches are bootstrap values. Only values above 70% are indicated.

**Fig. 6.** One of the 153 MP trees obtained based on phylogenetic analysis of chloroperoxidase sequence data of *A. niger* and *A. awamori* isolates (tree length: 113, consistency index: 0.767677, retention index: 0.886700). Numbers above branches are bootstrap values. Only values above 70% are indicated.
Molecular approaches

UP-PCR analysis (Bulat et al. 2000) was found to be also useful for species delineation (Fig. 4). This technique is similar to RAPD, but is more reliable as it uses higher annealing temperatures and longer primers (Bulat et al. 2000). Besides, UP-PCR analysis is easier to perform than AFLP analysis. Similarly to AFLP analysis (Perrone et al. 2011), this technique could also be used successfully to separate the examined A. niger and A. awamori isolates into two clusters (Fig. 4).

Another possibility is the application of mitochondrial DNA RFLP analyses. This technique was previously used to assign isolates of the A. niger species aggregate to different haplotypes (Varga et al. 1993, 1994). Our study revealed that one of these types previously called mtDNA type 1c actually corresponds to A. awamori (data not shown).

Attempts have also been made to use sequences of mycotoxin biosynthetic genes for distinguishing A. niger from A. awamori. Susca et al. (2010) examined the presence of FUM8 encoding an a-oxoamine synthase in black aspergilli came from grapes. They found no strict correlation between the phylogenetic trees based on sequences of partial calmodulin gene and FUM8 (Fig. 5). Similar results were found in our laboratory using sequences of either FUM8, or another fumonisin biosynthetic gene, FUM1, encoding for a polyketide synthase taking part in fumonisin biosynthesis (Varga et al., unpubl. data). It was suggested that, similarly to that observed in the trichothecene biosynthesis gene cluster of the Fusarium graminearum species complex, balancing selection could be responsible for maintaining sequence polymorphisms within the fumonisin gene cluster (Ward et al. 2002, Susca et al. 2010).

The applicability of another mycotoxin biosynthetic gene, a chloroperoxidase gene presumably taking part in ochratoxin biosynthesis was also examined for distinguishing A. niger and A. awamori. This gene has been found to take part in ochratoxin biosynthesis in Penicillium verrucosum and P. nordicum (Geisen 2007). Homologues of these genes were identified in the full genome sequences of A. niger and A. carbonarius, and primers were designed to amplify orthologous in species assigned to the A. niger species complex. Phylogenetic analysis of the sequence data indicate that sequences of a chloroperoxidase gene are useful for species delineation in the A. niger species aggregate (Fig. 6).

Aspergillus niger and A. awamori could also be distinguished based on their chloroperoxidase sequences.

Morphological and physiological approaches

Molecular methods are commonly used today for species identification among fungi. However, in accordance with the polyphasic species concept, other criteria have also been searched for. Aspergillus niger and A. awamori cannot be distinguished based on morphology alone. Regarding extrorhiz production, isolates of both species produce several metabolites in common including the mycotoxins ochratoxin A and fumonisin B₁, and they also share the production of pyranonigrin A, tensidol B, funalenone, malformins and naphtho-γ-pyrones. The growth rates of the isolates of these species are also similar at different temperatures (Varga et al., unpubl. data).

Carbon source utilisation tests revealed that A. niger and A. awamori has very similar utilisation spectra (data not shown). Different growth of the strains belonging to the two species was observed only in the case of L-sorbose: A. awamori strains grew less intensively on this sugar than A. niger strains. Consequently, the test was extended to 2-deoxy-D-glucose because of the structural similarity of these two compounds. Of the 30 isolates examined, 13 of the examined A. niger isolates grew well, while 13 of the 15 examined A. awamori isolates failed to grow on 2-deoxy-D-glucose as sole carbon source (data not shown). Microscopical analysis of the colonies indicated that conidial germination was inhibited in the case of A. awamori isolates (data not shown). Furthermore, 2-deoxy-D-glucose was earlier found to inhibit conidium germination in Penicillium expansum (Kazi et al. 1997).

Antifungal susceptibilities of the isolates has also been examined using five antifungal drugs including amphotericin B, fluconazole, itraconazole, ketoconazole and terbinafine (Szegeti et al. 2011). Species-specific differences were not observed between A. niger and A. awamori isolates. All isolates were highly susceptible to terbinafine, while exhibited moderate susceptibilities against amphotericin B, fluconazole and ketoconazole. However, in general, A. niger and A. awamori were found to have higher MICs for azoles than A. tubingensis (Szegeti et al. 2011).

Elastase production is treated as a virulence factor in Aspergillus fumigatus, contributing to the invasiveness of the fungus during infection (Denning et al. 1993, Kolattukudy et al. 1993, Blanco et al. 2002, Garcia et al. 2006). Elastase activities of the isolates were tested according to the method of Blanco et al. (2002). The data revealed that A. awamori isolates in general exhibit higher elastase activities in a much narrower range than A. niger (Fig. 7). We found significant difference between the elastase activities of the isolates belonging to the two species (\(\chi^2 = 8.017; p=0.0046;\) Kruskal-Wallis test). In general, A. awamori isolates showed more intensive elastin lysis than A. niger isolates, although an outlier was found in the case of an A. niger strain, which exhibited very high elastase activities (Fig. 7). The high elastase activity of this isolate could be due to simple mutations as has been proposed for A. fumigatus recently (Alvarez-Perez et al. 2010).

Ecology

Several differences have been found recently regarding the distribution of A. niger and A. awamori is various habitats. Both species have been found in various ratios in indoor air in various buildings in Southern Hungary and Serbia, although A. awamori was more frequently encountered in Serbia than in Hungary (Varga et al. 2010).
Aspergillus eucalypticola

Varga, Frisvad & Samson, sp. nov. MycoBank MB560387. Fig. 9.

Aspergillus eucalypticola was isolated from an Eucalyptus leaf from Australia, and resembles morphologically A. tubingensis and A. costaricaensis. It can be distinguished from these two taxa by the β-tubulin or calmodulin sequence data. Aspergillus eucalypticola produces pyranonigrin A, funalenone, aurasperone B and other naphtho-γ-pyrones.

Aspergillus fijiensis

Varga, Frisvad & Samson, sp. nov. MycoBank MB560388. Fig. 10.

Aspergillus fijiensis was isolated from an Eucalyptus leaf from Australia, and resembles morphologically A. tubingensis and A. costaricaensis. It can be distinguished from these two taxa by the β-tubulin or calmodulin sequence data. Aspergillus eucalypticola produces pyranonigrin A, funalenone, aurasperone B and other naphtho-γ-pyrones.

Aspergillus indologenus

Frisvad, Varga & Samson, sp. nov. MycoBank MB560389. Fig. 11.

Aspergillus indologenus was isolated from soil in Fiji, and from Lactuca sativa in Indonesia. This species is able to grow at 37 °C, and produces asperparalines and okaramins.

Species descriptions

Aspergillus acidus

Kozak. Mycol. Pap. 161: 110 (1989) Fig. 8.

Culture ex-type: IMI 104688 = CBS 564.65, Japan, Nakazawa, 1936.

Aspergillus acidus produces pyranonigrin A, funalenone, aurasperone B and other naphtho-γ-pyrones.

Aspergillus eucalypticola

Varga, Frisvad & Samson, sp. nov. MycoBank MB560387. Fig. 9.

Aspergillus eucalypticola was isolated from an Eucalyptus leaf from Australia, and resembles morphologically A. tubingensis and A. costaricaensis. It can be distinguished from these two taxa by the β-tubulin or calmodulin sequence data. Aspergillus eucalypticola produces pyranonigrin A, funalenone, aurasperone B and other naphtho-γ-pyrones.

Aspergillus fijiensis

Varga, Frisvad & Samson, sp. nov. MycoBank MB560388. Fig. 10.

Aspergillus fijiensis was isolated from a soil sample in Fiji, and from Lactuca sativa in Indonesia. This species is able to grow at 37 °C, and produces asperparalines and okaramins.

Aspergillus indologenus

Frisvad, Varga & Samson, sp. nov. MycoBank MB560389. Fig. 11.

Aspergillus indologenus was isolated from soil in Fiji, and from Lactuca sativa in Indonesia. This species is able to grow at 37 °C, and produces asperparalines and okaramins.
Fig. 8. Aspergillus acidus A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.
New and revisited species in *Aspergillus* section *Nigri*

Fig. 9. *Aspergillus eucalypticola* sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.
Aspergillus fijiensis sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA. D–I. Conidiophores and conidia. Scale bars = 10 µm.
Fig. 11. Aspergillus indologenus sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.
Fig. 12. Aspergillus neoniger sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 μm.
Fig. 13. Aspergillus violaceofuscus A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.
Conidiophores uniseriate with globose vesicles 10–18 µm wide; stipe sometimes bent, smooth-walled to finely roughened, hyaline, 2.5–5 µm. Conidia ellipsoidal to slightly fusiform, 3.5–4 × 4–5.5 µm, brown, coarsely roughened to echinulate. Sclerotia not observed.

Aspergillus violaceofuscus has uniserial conidial heads and is related to, but clearly distinguishable from A. uvarum based on β-tubulin, calmodulin and ITS sequence data. Aspergillus violaceofuscus has typical ellipsoidal to fusiform conidia, which are coarsely roughened to echinulate.

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