

Aspergillus sect. *Aeni* sect. nov., a new section of the genus for *A. karnatakaensis* sp. nov. and some allied fungi

János Varga^{1,2}, Jens C. Frisvad³ and Robert A. Samson¹

¹CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, NL-3584 CT Utrecht, The Netherlands

²Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Közép fasor 52, H-6726 Szeged, Hungary; corresponding author e-mail: jvarga@bio.u-szeged.hu

³Department of Systems Biology, Building 221, Søtofts Plads, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

Abstract: The new species *Aspergillus karnatakaensis* sp. nov. is described and illustrated. All three isolates of this species were isolated from Indian soil; two from soil under a coconut palm in a coffee plantation in Karnataka, and one from soil in the Machrar river bed in Bansa district. This species is closely related to, but clearly distinct, from *A. aeneus* based on β -tubulin or calmodulin sequence data. Sequences of the ITS region of these two species are identical. *Aspergillus karnatakaensis* produced terrein, gregatins, asteltoxin, karnatakafurans A and B and the unknown metabolite, provisionally named NIDU. *Aspergillus karnatakaensis* belongs to a well-defined clade within *Aspergillus* subgenus *Nidulantes* together with eight other species including *A. aeneus*, *A. crustosus*, *A. eburneocremeus*, *A. heyangensis*, and the teleomorph producing-species *Emericella bicolor*, *E. discophora*, *E. spectabilis*, and *E. foeniculicola*. This clade is placed in a new section, *Aspergillus* sect. *Aeni* sect. nov. All teleomorph species assigned to this section are able to produce sterigmatocystin.

Key words:

Aspergillus subgen. *Nidulantes*
 β -tubulin
 calmodulin
Eurotiales
 extrolites
 ITS
 polyphasic taxonomy

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INTRODUCTION

Aspergillus subgenus *Nidulantes* is one of the largest subgenera of the genus *Aspergillus*, including about 80 species (Peterson 2008, Peterson *et al.* 2008). Several species of this subgenus have a teleomorph assigned to *Emericella* (Pitt *et al.* 2000, Samson 2000, Frisvad & Samson 2004). Species of subgenus *Nidulantes* are important as opportunistic human pathogens (Verweij *et al.* 2008, Varga *et al.* 2008), as producers of various secondary metabolites which are useful for the pharmaceutical industry (e.g. penicillin, echinocandins, ophiobolins), and mycotoxins which are harmful to animals and humans (e.g. aflatoxins, sterigmatocystin; Frisvad *et al.* 2004, 2005, Frisvad & Samson 2004, Zalar *et al.* 2008).

During surveys of *Aspergillus* isolates from soil samples from subtropical regions, two interesting isolates were recovered which did not match any known species of the genus. We used the polyphasic approach, including sequence analysis of parts of the β -tubulin and calmodulin genes and the ITS nrDNA region, macro- and micromorphological analyses, and examination of the extrolite profiles of the isolates to differentiate the new species *Aspergillus karnatakaensis* sp. nov. We also analysed strains of species which appeared to be closely related to the new species for the production

of extrolites and found sterigmatocystin in all species with a teleomorphic state studied and also in *Aspergillus eburneocremeus*.

MATERIALS AND METHODS

Isolates

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

Morphological analysis

For macromorphological observations, Czapek yeast autolysate (CYA), malt extract autolysate (MEA) agar, Yeast Extract Sucrose agar (YES), creatine sucrose agar (CREA), and oatmeal agar (OA) were used (Samson *et al.* 2010). 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, microscopic mounts were made in lactic acid from MEA and OA colonies and a drop of alcohol was added to remove air bubbles and excess conidia.

Extrolite analysis

The isolates were grown on CYA and YES at 25 °C for 7 d. Extrolites were extracted after incubation. Five 6 mm plugs

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Table 1. Isolates of *Aspergillus* and *Emericella* spp. examined in this study.

Species	Strain No. ^a	Substratum, country, location	GenBank No.		
			β -tubulin	calmodulin	ITS
<i>A. aeneus</i>	CBS 128.54 ^T = NRRL 4769	Forest soil, Modilen, Somalia	EF652298	EF652386	EF652474
<i>A. crustosus</i>	CBS 478.65 ^T = NRRL 4988	Skin scrapings, man, Kankakee, Illinois, USA	EF652313	EF652401	EF652489
<i>A. eburneocremeus</i>	CBS 130.54 ^T = NRRL 4773	Forest soil, Modien Forest, Somalia	EF652300	EF652388	EF652476
<i>A. heyangensis</i>	CBS 101751 ^T	Placentae of <i>Gossypium</i> sp., Heyang, Shaanxi Province, China	FJ491520	FJ491521	FJ491522
<i>A. karnatakaensis</i>	CBS 102800 ^T = IBT 22153	Soil under coconut palm in coffee plantation, India, Karnataka	EU482438	EU482431	EU482441
<i>A. karnatakaensis</i>	CBS 102799 = IBT 22154	Soil under coconut palm in coffee plantation, India, Karnataka	EU482436	EU482430	EU482443
<i>A. karnatakaensis</i>	NRRL 4649	Soil in the Machrar river bed located in district Bansa, state Madhya Pradesh, India	EF652292	EF652380	EF652468
<i>E. bicolor</i>	CBS 425.77 ^T	Soil from Artemisia grassland, USA, Wyoming, Teton Basin	EF652335	EF652423	EF652511
<i>E. discophora</i>	CBS 469.88 ^T = IBT 21910	Soil, Spain	AY339999	EU443970	EU448272
<i>E. discophora</i>	CBS 470.88 = IBT 21911	Forest soil, Spain	AY340000	EU443969	EU448266
<i>E. foeniculicola</i>	CBS 156.80 ^T	<i>Foeniculum vulgare</i> seed, China	EU443990	EU443968	EU448274
<i>E. heterothallica</i>	CBS 489.65 ^T	Soil, Costa Rica	EU076369	EU076361	AB248987
<i>E. spectabilis</i>	CBS 429.77 ^T	Coal mine spoil material, Wyoming, USA, Seminole no. 1 mine	EU482437	EU482429	EU482442

^aCultures are deposited in/were obtained from the following collections: CBS, CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands; IBT, Culture Collection of Fungi, Mycology Group, BioCentrum-DTU, Technical University of Denmark, Lyngby, Denmark; NRRL, Agricultural Research Service Culture Collection, Peoria, IL, USA.

of each agar medium were taken and pooled together into the 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 and analyzed by HPLC using alkylphenone retention indices and diode array UV-VIS detection as described by Frisvad & Thrane (1987, 1993), with minor modifications as described by Smedsgaard (1997). The column used was a 50 × 2 mm Luna C-18 (II) reversed phase column (Phenomenex, CA, USA) fitted with a 2 × 2 mm guard column.

Genotypic analysis

The cultures used for the molecular studies were grown on malt peptone (MP) broth using 1 % (w/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 Biotechnology.) 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).

Data analysis

The sequence data was optimised using the software package Seqman from DNASTar Inc. Sequence alignments were performed by MEGA v. 4.0 (Tamura et al. 2007) and improved

manually. For parsimony analysis, PAUP v. 4.0b10 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 individually 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). *Eurotium heterothallica* was used as outgroup in these analyses (Houbraken et al. 2007). The alignments were deposited in TreeBASE (<treebase.org/treebase-web/home.html>) under accession number S11027.

RESULTS AND DISCUSSION

Phylogeny

Of the aligned β -tubulin sequences, a portion with 438 positions, including 107 parsimony informative characters, was selected for the analysis; MP analysis of the sequence data resulted in two similar, equally most parsimonious trees (tree length = 289 steps, consistency index = 0.7855, retention index = 0.7919), one of which is shown in Fig. 1. The calmodulin data set consisted of 492 characters, including 188

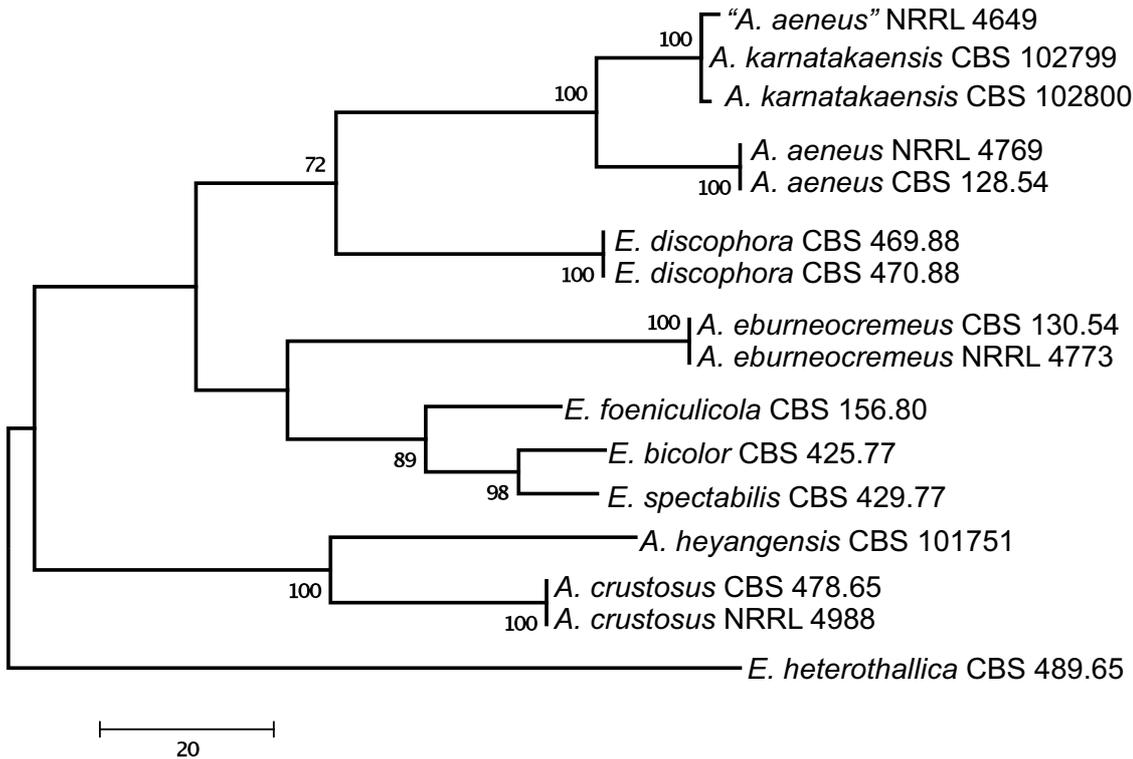


Fig. 1. One of the two equally MP trees obtained based on phylogenetic analysis of β -tubulin sequence data of *Aspergillus* sect. *Aenei*. Numbers above branches are bootstrap support values. Only values above 70 % are indicated.

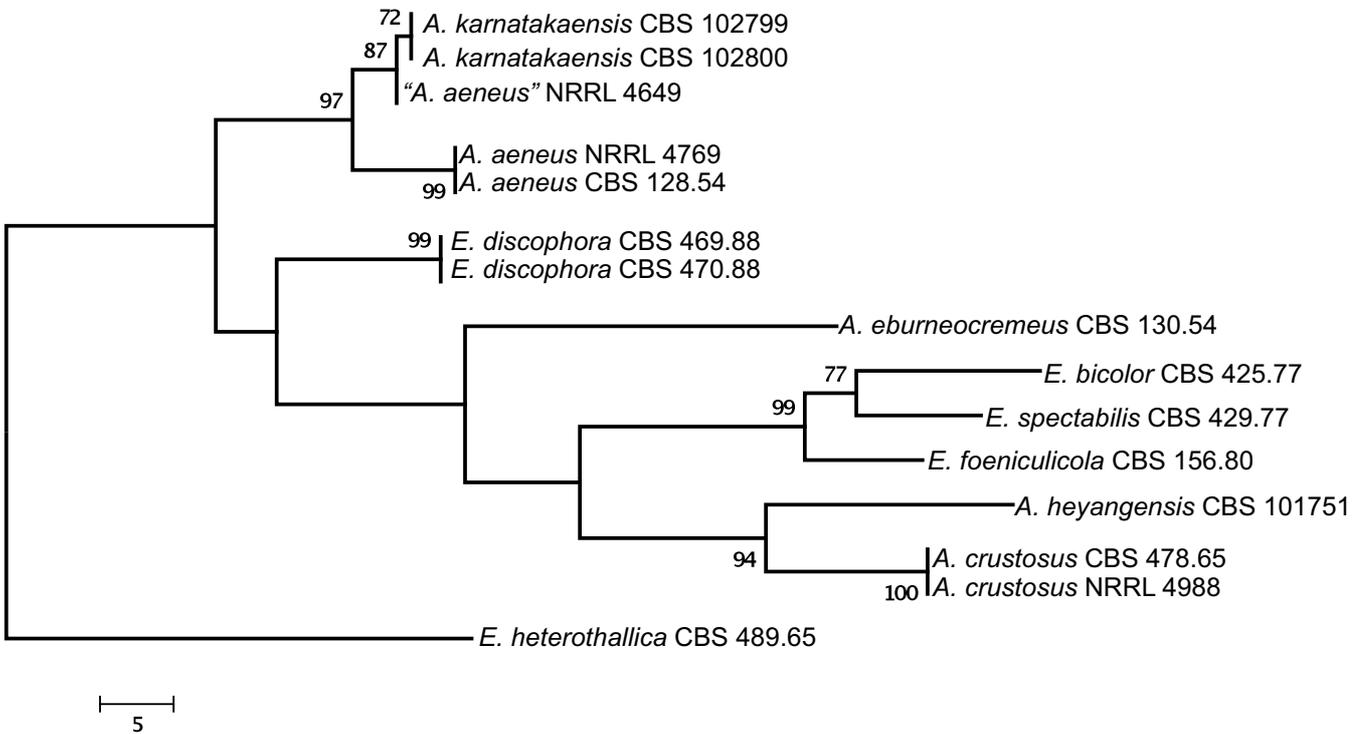


Fig. 2. The single MP tree obtained based on phylogenetic analysis of calmodulin sequence data of *Aspergillus* sect. *Aenei*. Numbers above branches are bootstrap support values. Only values above 70 % are indicated.

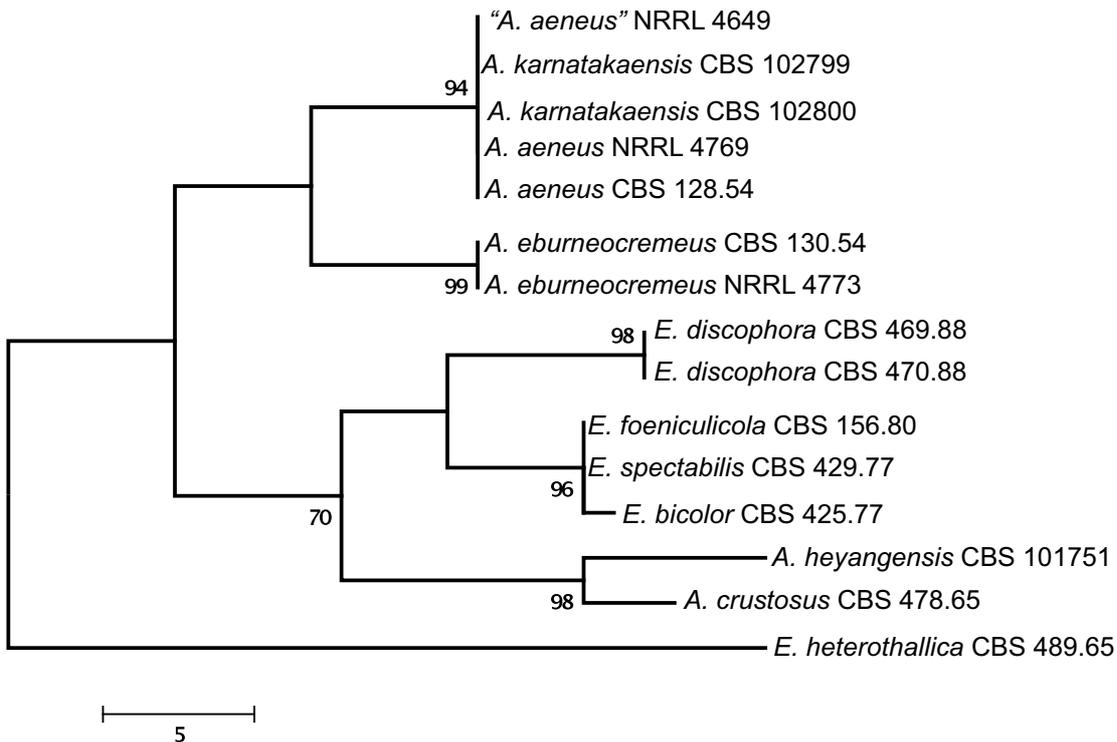


Fig. 3. One of four equally MP trees obtained based on phylogenetic analysis of ITS sequence data of *Aspergillus* sect. *Aenei*. Numbers above branches are bootstrap support values. Only values above 70 % are indicated.

parsimony informative sites; MP analysis resulted in a single most parsimonious tree (length = 485, consistency index = 0.7402, retention index = 0.8040), which is presented in Fig. 2. The ITS data set consisted of 451 characters, including 43 parsimony informative sites; MP analysis resulted in four equally most parsimonious trees (length = 105, consistency index = 0.8190, retention index = 0.8541), one of which is presented in Fig. 3.

The two isolates from Karnataka, India were found to be closely related to *Aspergillus aeneus* based on phylogenetic analysis of protein coding sequences (Figs 1, 2), and had identical ITS sequences to *A. aeneus* (Fig. 3). One additional isolate also from India, "*A. aeneus*" NRRL 4649 (= IMI 086833) was found to be conspecific with these two isolates. This isolate was obtained from soil of the Machrar river bed in the district of Bansa, Madhya Pradesh (Rai *et al.* 1964), and is morphologically similar to the other two Indian isolates. The three isolates are described here as a new taxon, *A. karnatakaensis* sp. nov. A typical characteristic is the formation of a crust of Hülle cells. The strains were incubated on various media for ascoma production, but in none of the strains were ascomata or ascospores found. Also, a mating experiment with the three strains did not induce ascoma production.

Aspergillus karnatakaensis formed a well-supported clade together with four *Emericella* species, *E. foeniculicola*, *E. bicolor*, *E. spectabilis* and *E. discophora*, and four species known to reproduce only asexually, including *A. aeneus*, *A. eburneocremeus*, *A. crustosus* and *A. heyangensis* on the trees based on calmodulin (Fig. 4), β -tubulin, and ITS sequence data (data not shown). Based on these

observations, we describe *Aspergillus* sect. *Aenei* sect. nov. to accommodate these species within subgenus *Nidulantes*. This group of species was originally assigned to section *Nidulantes* (Raper & Fennell 1965, Christensen *et al.* 1978, Samson 1979, Udagawa & Muroi 1979, Sun & Qi 1994).

Extrolites

Aspergillus karnatakaensis isolates were found to produce karnatakafurans A and B (Manniche *et al.* 2004), terrein, gregatins, asteltoxin (until now only detected in CBS 102799) and the partially characterised metabolite NIDU. Both gregatins and NIDU are also produced by *A. granulosis*, while karnatakafurans are produced in common with *A. aeneus* and *A. multicolor*. However, phylogenetic analysis of sequence data of *A. multicolor* (Peterson 2008) and *A. granulosis* (Houbraken *et al.* 2007) indicated that they are not closely related to *A. karnatakaensis*, while *A. aeneus* is.

Among the other species found to belong to the same clade as *A. karnatakaensis*, *Emericella bicolor* produces sterigmatocystin, versicolorins, some anthraquinones, and a polar extrolite with end-absorption; *E. foeniculicola* produces sterigmatocystin (and many other sterigmatocystin and versicolorin-related compounds), xanthocillin derivatives, and the partially characterised (but common) metabolite DRI; *E. spectabilis* produces two members of the shamixanthone biosynthetic family (both more polar than shamixanthone itself) and a member of the sterigmatocystin biosynthetic family; *A. heyangensis* produces a decaturin in common with *A. aeneus* and *A. karnatakaensis* and NIDU, while *E. discophora* produces sterigmatocystin and versicolorins (Zalar *et al.*

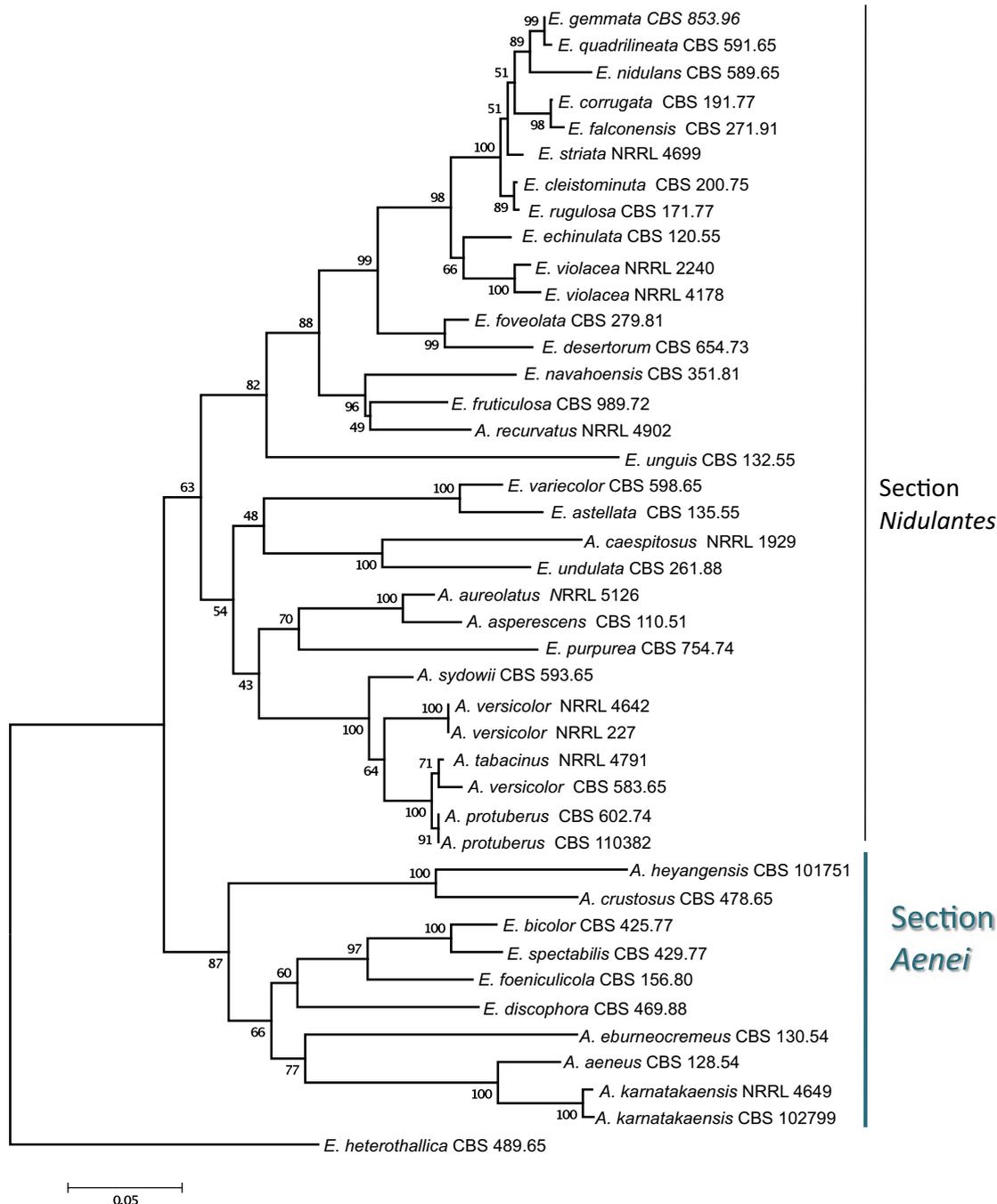


Fig. 4. Phylogenetic affinities of *Aspergillus* section *Aenei* to section *Nidulantes* based on neighbor-joining analysis of calmodulin sequence data of selected species assigned to these sections. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

2008). Decaturins are antiinsectan metabolites which have previously been identified in *Penicillium* species including *P. thiersii* and *P. decaturense* (Zhang *et al.* 2003, Li *et al.* 2005). *Aspergillus eburneocremeus* has both sterigmatocystin and mer NF-8054X in common with *E. heterothallica*. *Aspergillus crustosus* is different from all these species in producing only PR-toxin and related mycotoxins, and has no extrolites in common with the other species in sect. *Aenei*. All *Emericella* species in sect. *Aenei* produce sterigmatocystin, while the *Aspergillus* species without a known teleomorph apparently cannot produce it, with the exception of *A. eburneocremeus*. However, sterigmatocystin is common throughout the different

sections of subgenus *Nidulantes*, and has even been found in sections *Ochraceorosei* and *Flavi* (Frisvad *et al.* 2005). Other extrolites such as shamixanthones, mer NF-8054X and the related emesterones, and terrein have also been found in other species in section *Nidulantes*. *Aspergillus heyangensis* is only known from ex-type cultures and re-examination of the cultures showed that the taxon has great similarities with the species mentioned above, including its inability to grow at 37 °C, and the shape of the conidial heads and vesicles, although this species does not produce Hülle cells (Fig. 5). That species also produces the unknown metabolite NIDU, as do *A. karnatakaensis* and *E. discophora* (Table 2).

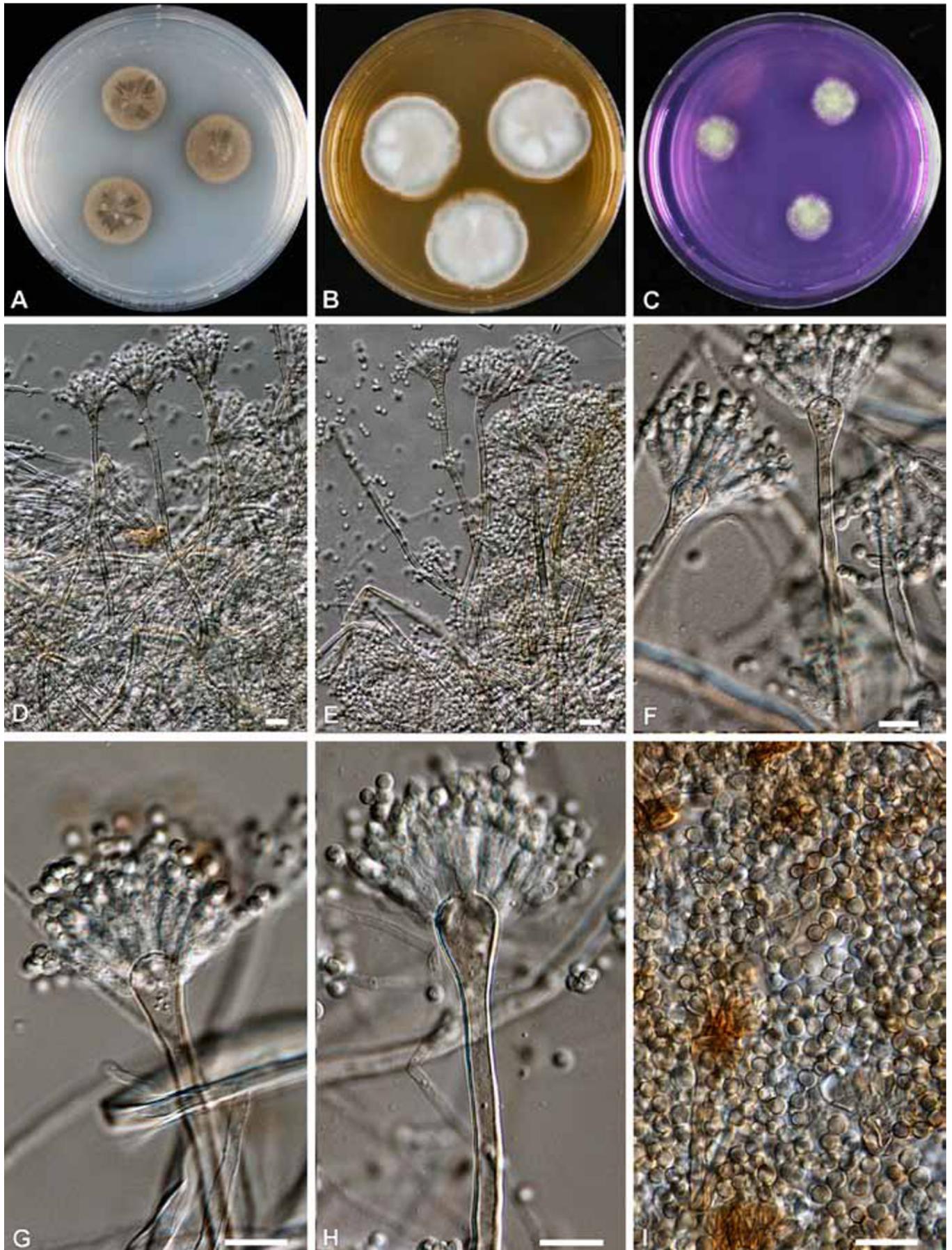


Fig. 5. *Aspergillus heyangensis* (CBS 101751). A–C. Colonies incubated at 25 °C for 7 d; A on CYA, B on MEA, C on CREA. D–I. Conidiophores and conidia. Bars = 10 μm.

Table 2. Extrolites produced by members of *Aspergillus* section *Aenei*.

Species	Culture collection number	Extrolites
<i>A. aeneus</i>	IMI 069855ii = CBS 128.54	astelt toxin, fumitremorgin B, karnatakafurans, a decaturin, GUUM*
<i>A. crustosus</i>	IMI 135819 = CBS 478.65	PR-toxin
<i>A. eburneocremeus</i>	IMI 069856 = CBS 130.54	mer-NF 8054X, sterigmatocystin
<i>A. heyangensis</i>	CBS 101751 = IBT 29634	a decaturin, NIDU*
<i>A. karnatakaensis</i>	IBT 22154 = CBS 102799	astelt toxin, gregatins, karnatakafuran A and B, quinolactacin, terrein, NIDU*, GUUM*
<i>A. karnatakaensis</i>	IBT 22153 = CBS 102800	astelt toxin, gregatins, karnatakafuran A and B, physcion, quinolactacin, terrein, NIDU*, GUUM*
<i>A. karnatakaensis</i>	IMI 086833ii = WB 4649	a decaturin, karnatakafuran A and B, terrein, GUUM*
<i>E. bicolor</i>	CBS 425.77 = IBT 22833	sterigmatocystin
<i>E. discophora</i>	CBS 469.88 = IBT 21910	sterigmatocystin
<i>E. discophora</i>	CBS 470.88 = IBT 21911	sterigmatocystin, NIDU*
<i>E. foeniculicola</i>	CBS 156.80 = IBT 22831	DRI, sterigmatocystin, xanthocillin FA
<i>E. heterothallica</i>	WB 5097 = IBT 22604	DRI, emeheteron, sterigmatocystin, mer-NF 8054X, stellatin
<i>E. heterothallica</i>	CBS 489.65 = WB 5096 = IBT 22607	DRI*, NIDU*, versicolorins, mer-NF 8054X
<i>E. heterothallica</i>	WB 4981 = IBT 22605	DRI*, sterigmatocystin, mer-NF 8054X
<i>E. heterothallica</i>	WB 4983 = IBT 22606	DRI*, sterigmatocystin, mer-NF 8054X
<i>E. spectabilis</i>	CBS 429.77 = IBT 22891	extrolites with shamixanthone chromophore, trace of sterigmatocystin

*NIDU, GUUM, and DRI are common extrolites with a characteristic UV chromophore. Their structure has not been elucidated yet.

***Aspergillus karnatakaensis* Varga, Frisvad & Samson, sp. nov.**

MycoBank MB517549

(Fig. 6)

Coloniis *Emericellae* similibus. Conidiophoris cum stipitibus laevibus, conidiis subglobose vel late ellipsoideis. Aggregationibus insignibus cum tegumento ex cellulis globosis efferentibus.

Typus: INDIA: Karnataka, near Chickmagalur, Netraconda Estate, isolated from soil under coconut palm (*Cocos nucifera*) in coffee plantation, 20 Dec. 1996, J.C. Frisvad (CBS H-20502 -- holotypus, culture ex-holotype CBS 102800).

Colonies on CYA, at 25 °C: 31–37 mm diam after 7 d, reverse orange; on MEA, at 25 °C: 12–19 mm, reverse yellow; on YES, at 25 °C: 33–45 mm, reverse pink to raspberry-red reverse; on OAT, at 25 °C: 16–23 mm, Hülle cells present; on CYA, at 37 °C: no growth to micro-colony (<1 mm); on CREA: weak to moderate growth, no acid production. **Conidial heads** reddish brown, yellow exudate droplets on CYA colonies. **Conidiophores** biserial, smooth, light brown stipes, 2.5–4 µm wide; vesicles subglobose to subclavate, 5–8 µm diam. **Conidiogenous cells** (phialides) 2–2.5 × 4–5 µm, metulae, 2–3 × 4–6 µm. **Conidia** globose or rarely subglobose, smooth to finely roughened, Hülle cells produced in crusts, globose to ellipsoidal, thick-walled, hyaline, 75–200 µm diam.

Diagnostic features: Apart from producing gregatins, terrein, and karnatakafuran A and B, isolates of this species produce a series of fluorescing extrolites (more than 33) with characteristic UV spectra. Also distinguished by producing Hülle cells in crusts.

***Aspergillus* sect. *Aenei* Varga & Samson, sect. nov.**

MycoBank MB517672

Sectionis *Nidulantium* similis, sed taxis cum conidiophoris brunneolis, vesiculis ampulliformibus et capitulis conidiorum biserialibus; statu anamorphoso cum ascosporis laevibus, convexis, aequatorialiter bicristatis; in cultura ad 40 °C haud crescenti.

Typus: *Aspergillus aeneus* Sappa. 1954.

Species assigned to *Aspergillus* sect. *Aenei* form a well-supported clade, basal to section *Nidulantes sensu* Peterson (2008) based on ITS, β-tubulin, calmodulin, and RNA polymerase 2 sequences (see Fig. 11 in Peterson 2008). The section includes four species able to reproduce both sexually and asexually (*Emericella discophora*, *E. bicolor*, *E. spectabilis*, *E. foeniculicola*), and five species for which the teleomorph is unknown (*A. aeneus*, *A. eburneocremeus*, *A. crustosus*, *A. heyangensis*, and *A. karnatakaensis*). All species are characterised by brownish conidiophores, flask-shaped vesicles, and biserial conidial heads. Several species produce Hülle cells abundantly in masses (except for *A. heyangensis*, which does not produce Hülle cells at all). The teleomorph-producing species assigned to this section all have smooth convex ascospores with two equatorial crests. None of the species assigned to this section are able to grow at or above 40 °C. All teleomorph species, together with *A. eburneocremeus*, are able to produce sterigmatocystin. The relationship of *E. spectabilis* to *A. crustosus* has already been suggested by Christensen *et al.* (1978), while *E. discophora* was found to be related to *E. foeniculicola* (Zalar *et al.* 2008).

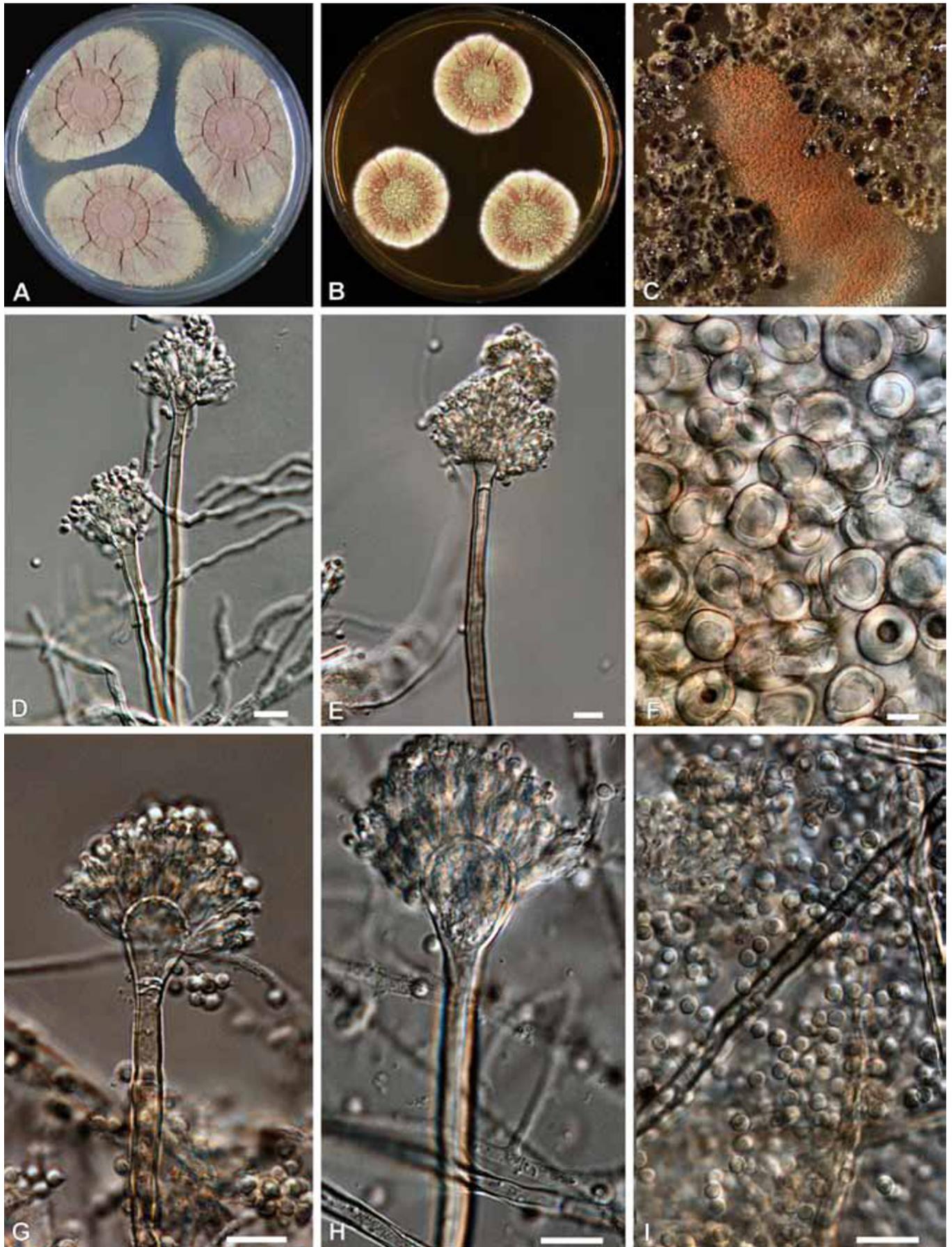


Fig. 6. *Aspergillus karnatakaensis* (CBS 102800). **A, B.** Colonies incubated at 25 °C for 7 d, A on CYA, B on MEA. **C.** Crusts of Hülle cells. **D, E, and G–I.** Conidiophores and conidia. **F.** Hülle cells. Bars = 10 µm, except F = 100 µm.

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