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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.

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

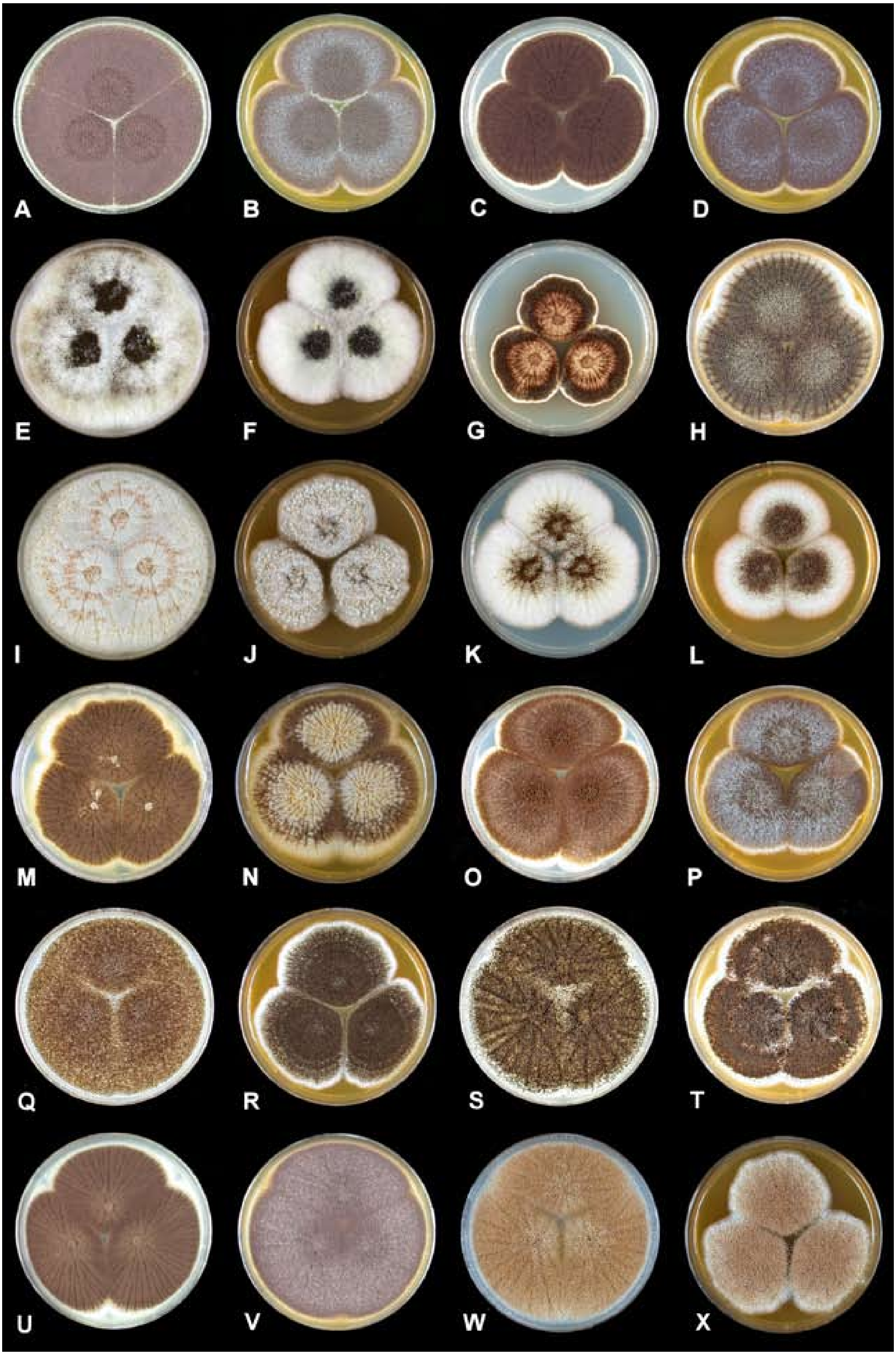


Fig. 1. Colony morphologies of type strains of species assigned to *Aspergillus* section *Nigri* grown on CYA and MEA plates at 25 °C for 7 d. (A–B) *A. aculeatinus*, (C–D) *A. aculeatus*, (E–F) *A. brasiliensis*, (G–H) *A. carbonarius,* (I–J) *A. costaricaensis*, (K–L) *A. ellipticus*, (M–N) *A. foetidus*,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

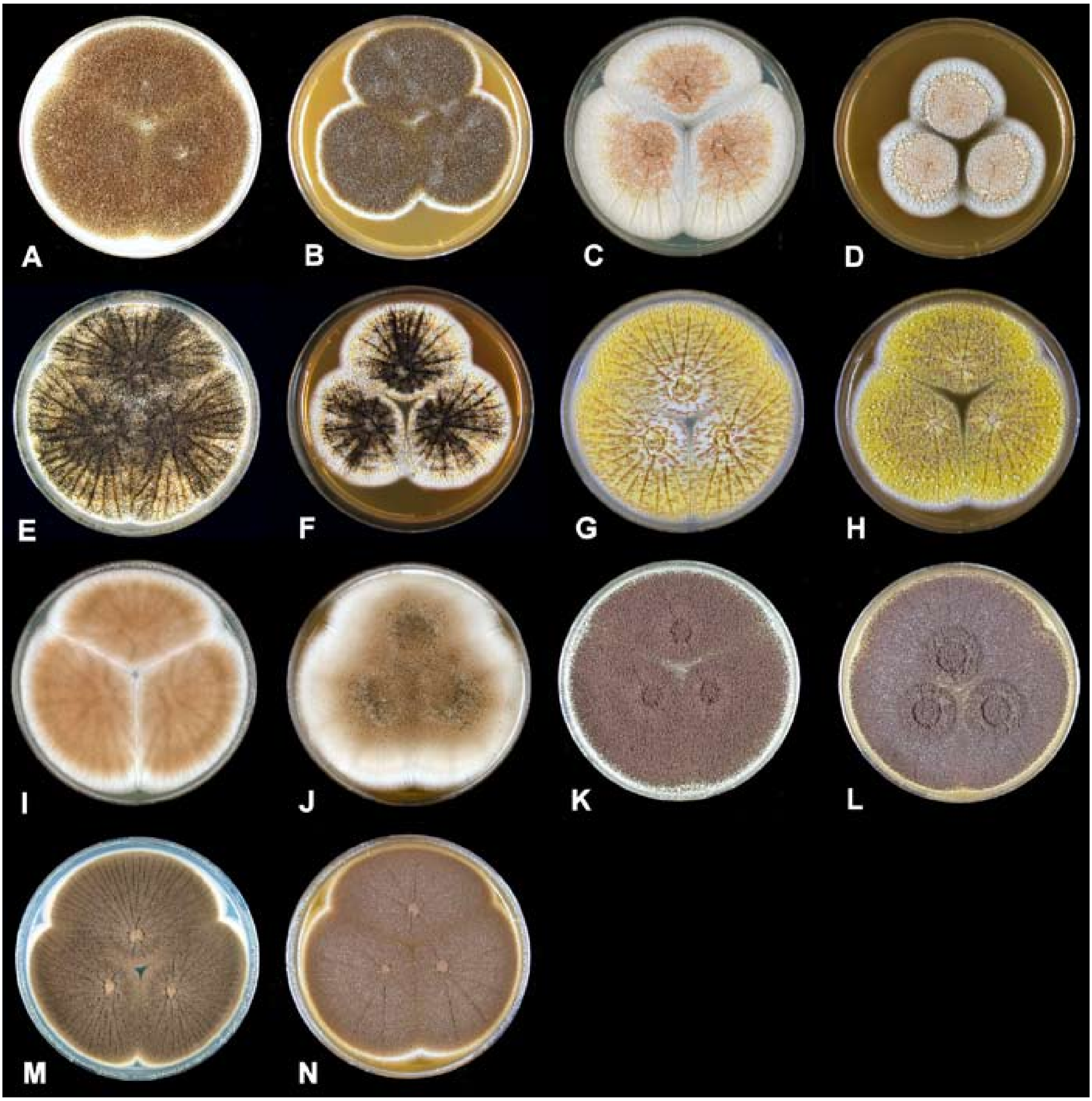


Fig. 2. Colony morphologies of type strains of species assigned to *Aspergillus* section *Nigri* grown on CYA and MEA plates at 25 °C for 7 d. (A–B) *A. niger*, (C–D) *A. piperis*, (E–F) *A. sclerotiicarbonarius*, (G–H) *A. sclerotioniger*, (I–J) *A. tubingensis*, (K–L) *A. uvarum,* (M–N) *A. vadensis*.

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^{o}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

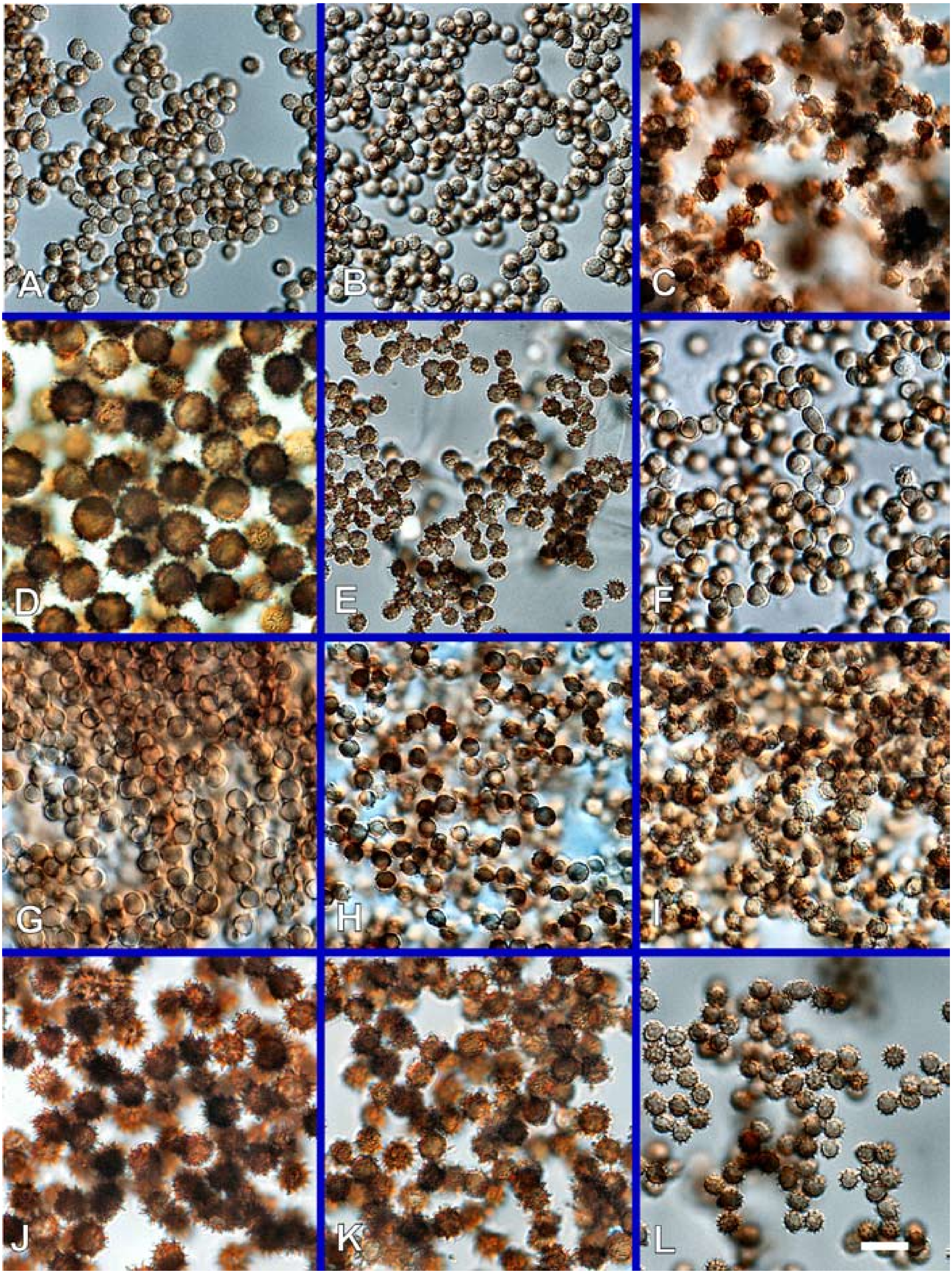


Fig. 3. Light microscopic pictures of conidia of type strains and representative isolates of *Aspergillus* section *Nigri*. (A) *A. aculeatinus*^{T} (2.5–4.5 µm), (B) *A. aculeatus*^{T} (3.5–5 µm), (C) *A. brasiliensis*^{T} (3.5–4.5 µm), (D) *A. carbonarius*^{T} (7–9 µm), (E) *A. costaricaensis*^{T} (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.

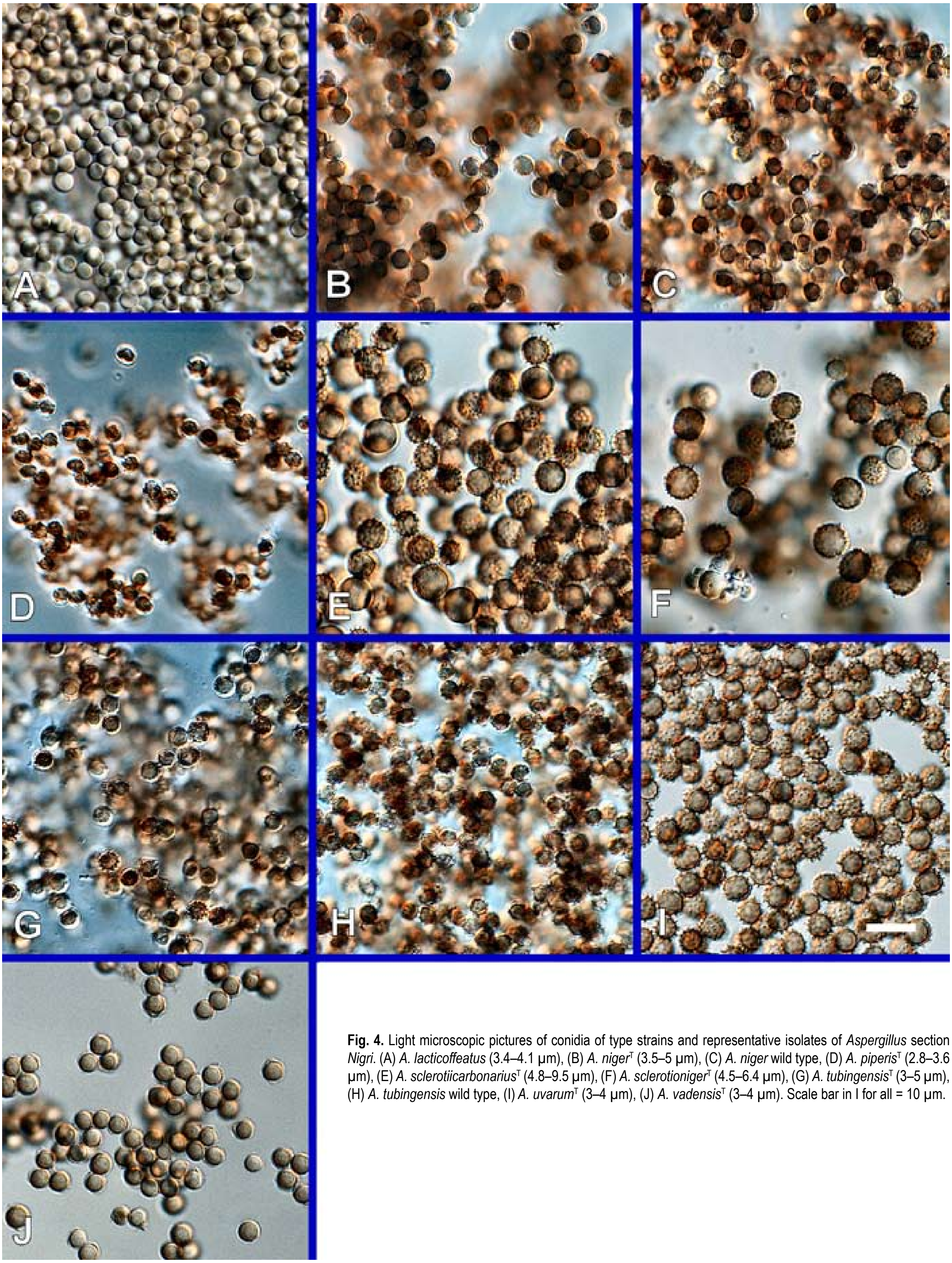
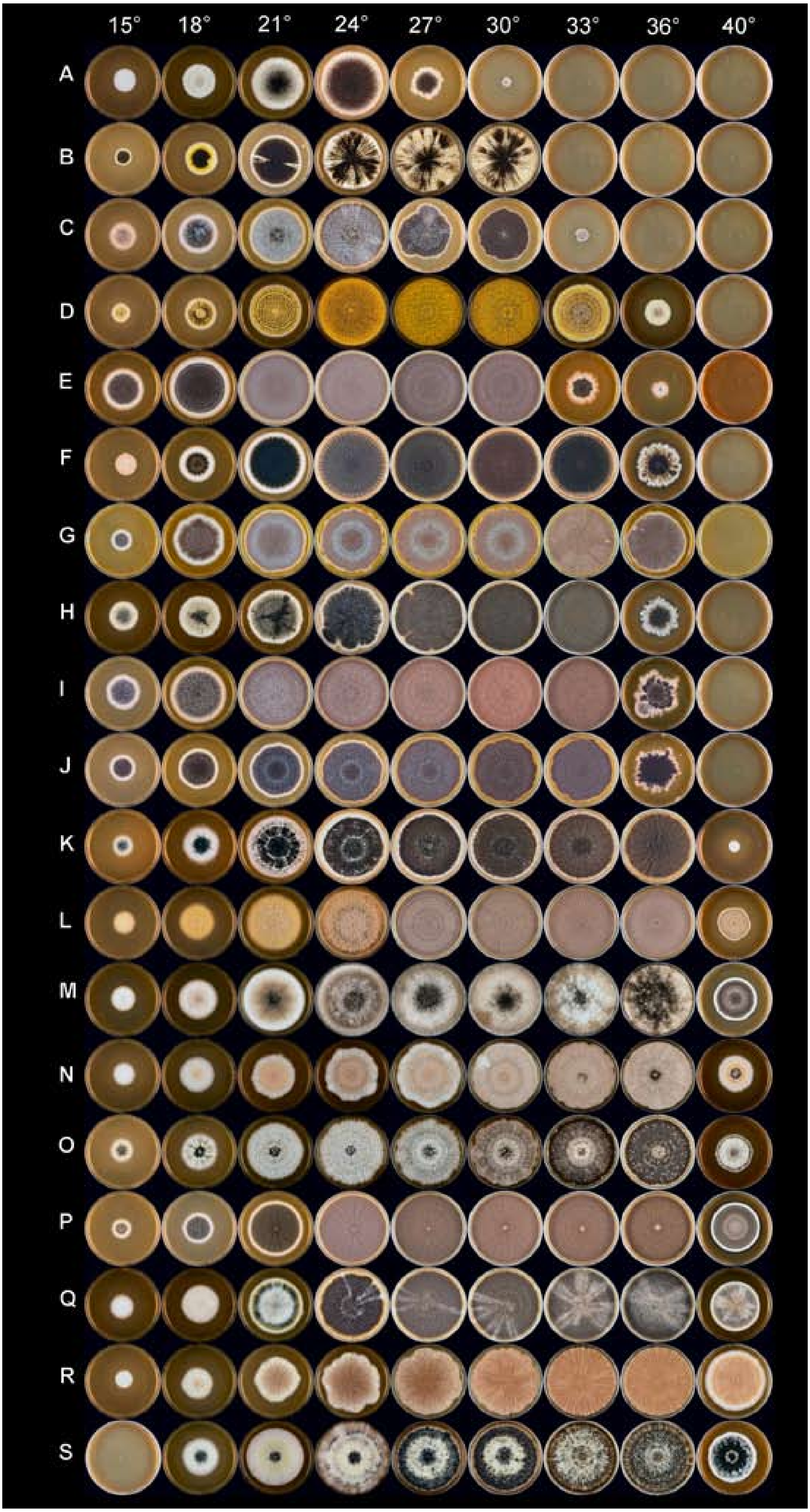


Fig. 4. Light microscopic pictures of conidia of type strains and representative isolates of *Aspergillus* section *Nigri*. (A) *A. lacticoffeatus* (3.4–4.1 µm), (B) *A. niger*^{T} (3.5–5 µm), (C) *A. niger* wild type, (D) *A. piperis*^{T} (2.8–3.6 µm), (E) *A. sclerotiicarbonarius*^{T} (4.8–9.5 µm), (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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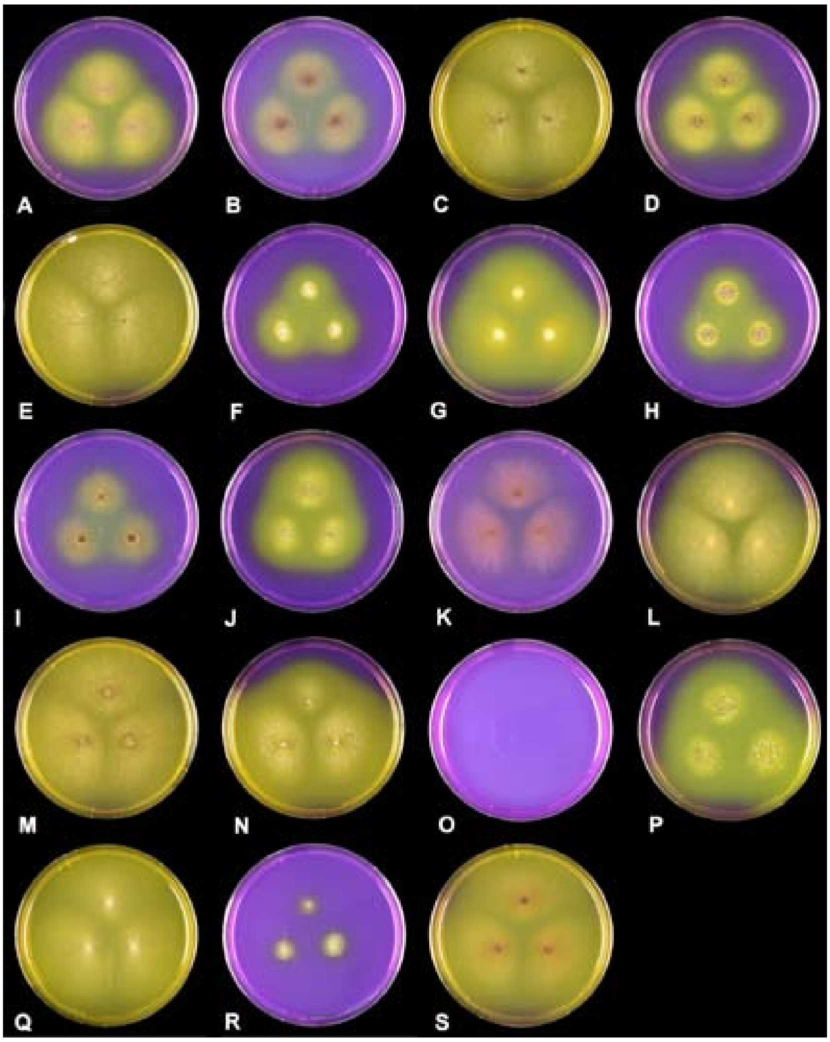

Fig. 6. Growth of type strains of *Aspergillus* section *Nigri* on creatine sucrose agar (CREA) plates. (A) *A. aculeatinus*^{T}; (B) *A. aculeatus*^{T}; (C) *A. brasiliensis*^{T}; (D) *A. carbonarius*^{T}; (E) *A. costaricaensis*^{T}; (F) *A. ellipticus*^{T}; (G) *A. foetidus*^{T}; (H) *A. heteromorphus*^{T}; (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}.

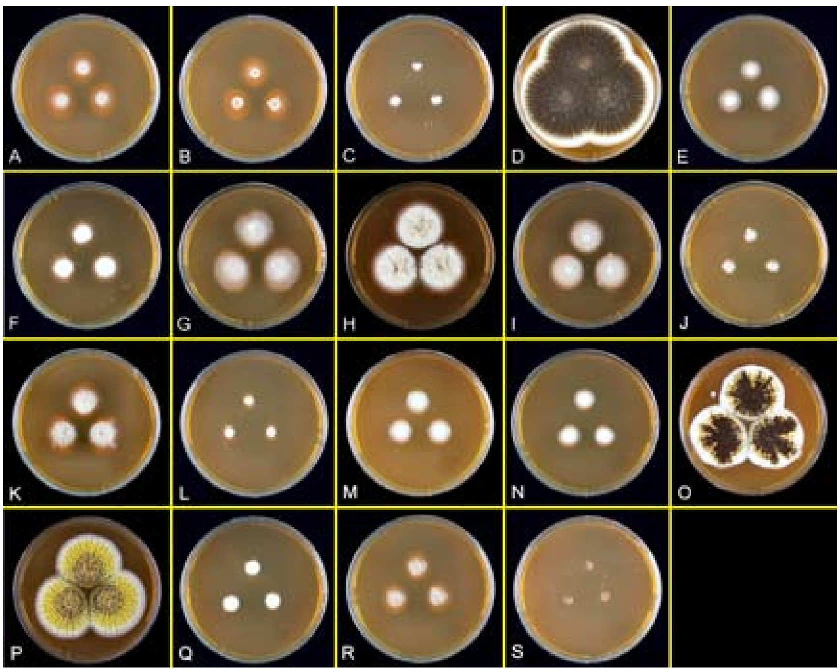

Fig. 7. Growth and sporulation of type strains of *Aspergillus* section *Nigri* on malt extract agar with Boscolid (MEA-B) after 7 d incubation. (A) *A. aculeatinus*; (B) *A. aculeatus*; (C) *A. brasiliensis*; (D) *A. carbonarius*; (E) *A. costaricaensis*; (F) *A. ellipticus*; (G) *A. foetidus*; (H) *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*.

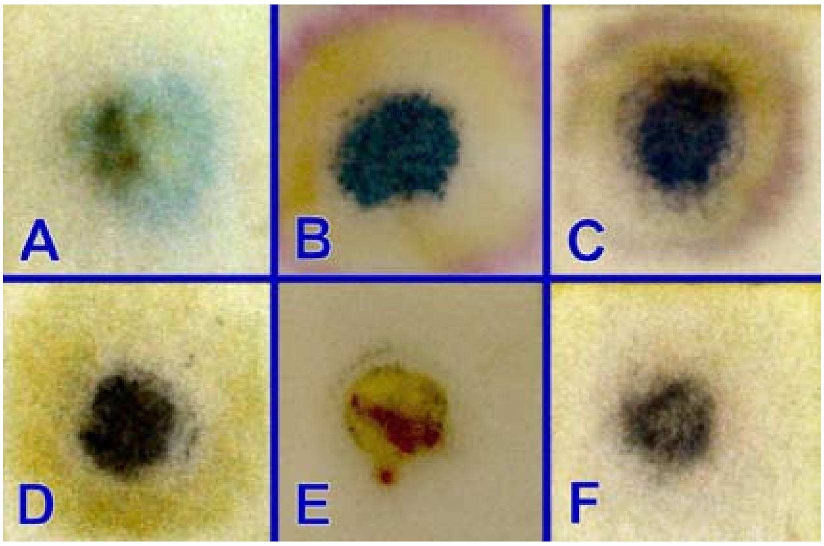

Fig. 8. Ehrlich colour reaction of some *Aspergillus* species in section *Nigri*. (A) blue in *A. costaricaensis*, (B) purple ring in *A. heteromorphus*, (C) purple ring in *A. homomorphus*, (D) yellow-green in *A. niger*, (E) purple-red at sclerotial area of *A. sclerotioniger* and (F) no reaction in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

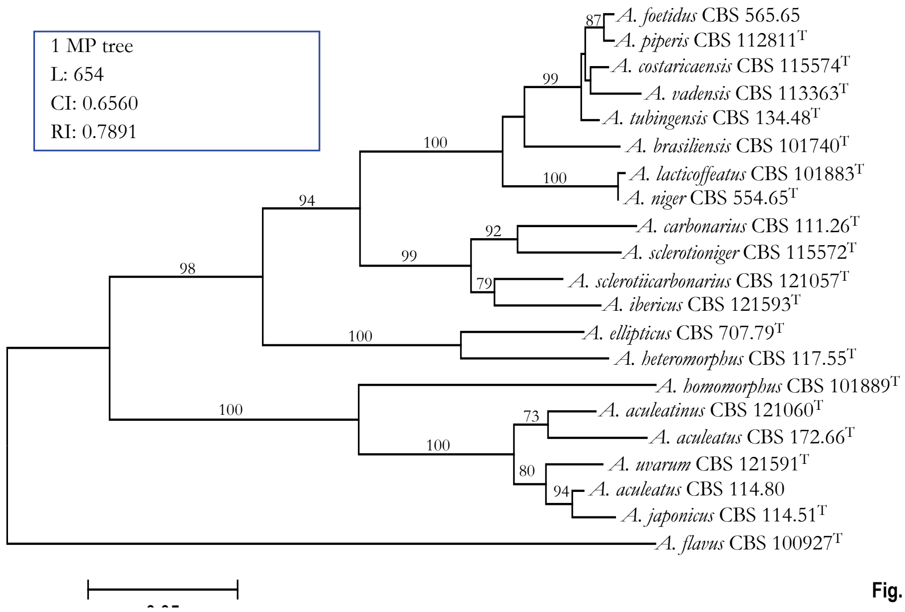

Fig. 9A.

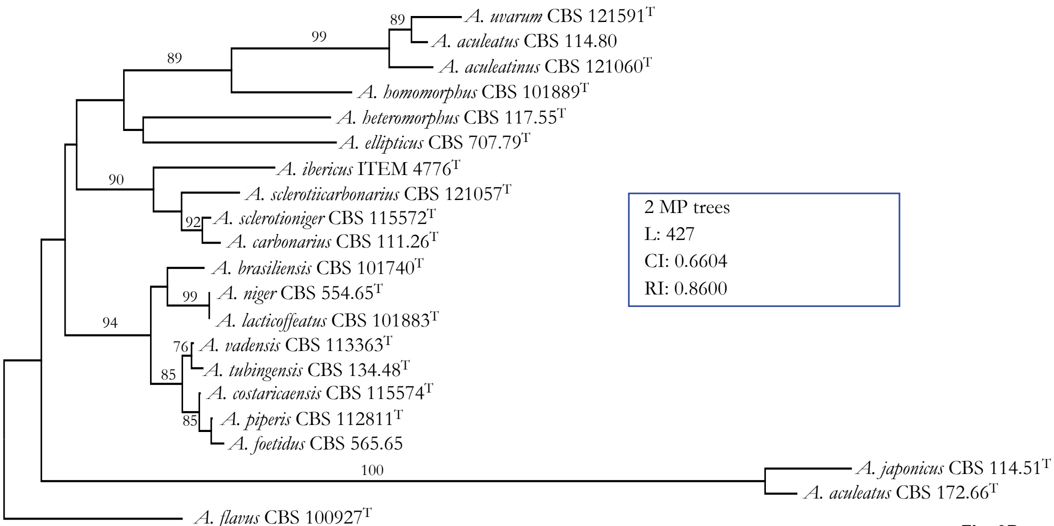

Fig. 9B.

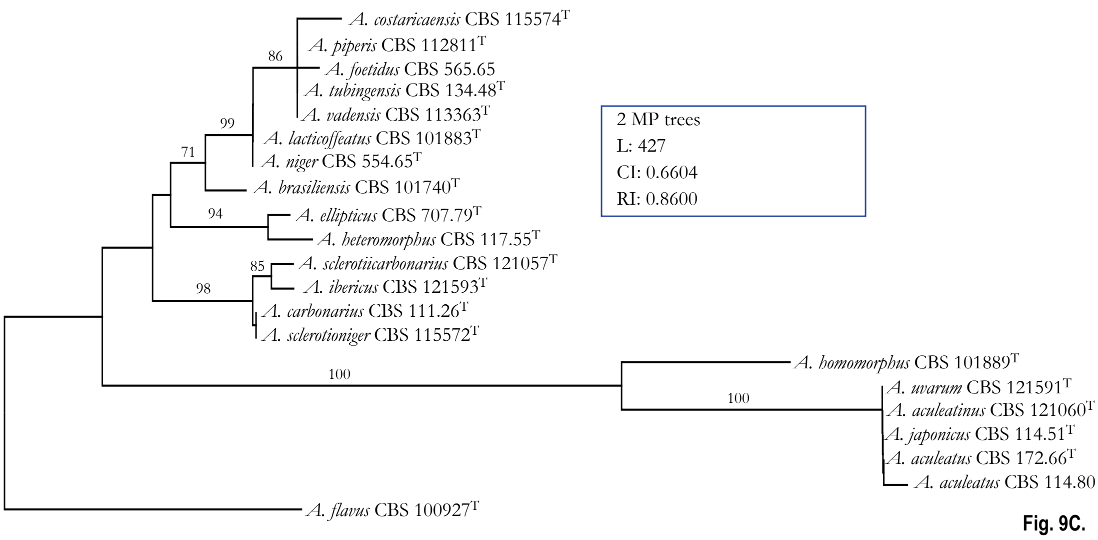

Fig. 9C.

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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