

Taxonomy and significance of black aspergilli

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Abstract

Members of *Aspergillus* section *Nigri* (formerly *A. niger* group) are distributed worldwide and are regarded as common food spoilage fungi. Some of them are widely used and studied for industrial purposes. They are common sources of extracellular enzymes and organic acids to be used in food processing and are also used in the production of traditional foods, especially in the Orient. Products produced by strains of *Aspergillus niger* hold the GRAS (Generally Recognised As Safe) status from the FDA. However some species in *Aspergillus* section *Nigri* can produce ochratoxin A, a nephrotoxic mycotoxin. In spite of their industrial importance, the taxonomy of black aspergilli (*Aspergillus* section *Nigri*) is not clear and many attempts have been made in order to find suitable taxonomic criteria. The aim of this paper is to provide an overview of the significance of black aspergilli focusing on all the approaches made in the taxonomy of this group of fungi. Some species, such as *A. carbonarius* and uniseriate species can be easily recognised. In the *A. niger* aggregate, although speciation at molecular level has been proposed, no morphological differences can be observed and species identification will therefore remain problematic. Phylogenetic analyses of ITS and 5.8S rDNA gene region of representative black *Aspergillus* species and a simple key to the most common species that can be easily distinguished by morphological criteria are also included.

Introduction

Members of *Aspergillus* section *Nigri* (formerly *A. niger* group) are distributed worldwide, growing upon a wide variety of substrates. They occur in and upon a great variety of substrata, being considered as common food spoilage fungi (Raper and Fennell 1965; Pitt and Hocking 1997).

Although *Aspergillus niger*, the most common species, is a recognized opportunistic pathogen, members of this section are usually regarded as benign fungi. Some of them are a common source of extracellular enzymes and organic acids to be used in food processing. Moreover, *A. niger* products hold the

GRAS (Generally Recognised As Safe) status from the FDA (Bigelis and Lasure 1987), despite the fact that the ability of producing ochratoxin A (OTA) by this species has been reported (Abarca et al. 1994).

It is very common that all *Aspergillus* isolates developing black colonies are identified as *A. niger* and sometimes the same isolate is preserved in culture collections under different species names. Due to their biotechnological importance, the identification of such strains needs to be unambiguous. Up to now, the taxonomy of black aspergilli is far from clear because it is primarily based on morphological criteria and in some cases the differences between the species are very subtle. On the other hand, several taxa

represent only modified industrial strains. However, some efforts have been made in order to clarify their taxonomic position.

The aim of this paper is to provide an overview of the significance of black aspergilli focusing on all the approaches made in the taxonomy of such fungi.

Biotechnological interest

Black aspergilli are widely used and studied for industrial purposes. In Asia, they are widely used, among other fungi, in the manufacture of fermented foods and beverages (Yokotsuka and Sasaki 1998). The primary uses of *A. niger* are for production of enzymes and organic acids by fermentation and it has been used for a long time in food industry without any apparent adverse effects on human health.

From the beginning of last century, *A. niger* has been used for biotechnological production of organic acids such as citric acid and gluconic acid. They remain the only organic acids still produced by mycological processes and used in significant quantities as food additives (Bigelis and Lasure 1987). The production of citric acid has not received much attention in the form of modern methods of molecular biology, presumably because it is considered a mature area. The improvement of strains of *A. niger* used to be done by mutagenesis and selection but metabolic engineering is now possible, in the light of increased knowledge about the regulation of acidogenesis (Moresi and Parente 2000).

A wide-range of fungal enzymes produced by *Aspergillus niger* and other black *Aspergillus* species such as *Aspergillus aculeatus* are used in food processing and today many enzyme products are available on the market from recombinant strains of these species. Each of the enzymes and producing strains has been evaluated for safety before entering the market (Pariza and Jonson 2001). The safety of *A. niger* for industrial use has been reviewed recently (Schuster et al. 2002).

Medical importance

A. niger is the third most common species associated with invasive pulmonary aspergillosis and it is also often a causative agent of aspergilloma (Kwon Chung and Bennett 1992). This species is frequently isolated subclinically or clinically from human external ears

and is repeatedly implicated in human mycoses in different localizations, occasionally in disseminating infections (Kwon Chung and Bennett 1992; De Hoog et al. 2000). It is also a recognized opportunistic pathogen for animals and there have been reports of natural aspergillosis in various species of mammals and birds (Smith 1989).

Nevertheless, reports on clinical implication of black *Aspergillus* species other than *A. niger* are rare. In most papers describing the isolation of such strains, they are presented simply as *A. niger*. However, in such cases it is often difficult to differentiate which species were involved in the pathologic process. *Aspergillus niger* var. *awamori* has been reported as responsible for a case of subcutaneous infection (Paldrok 1965) and *A. aculeatus* was isolated from scrapings of the tongue of a patient with respiratory illness (Williams et al. 1984).

A. niger may be present in high numbers in several occupational situations. The risk of allergic hypersensitivity to inhaled spores can be handled in an industrial environment by minimising the exposure of the workers to spore dust (Schuster et al. 2002). Allergic response is not only caused by inhalation of spores of *A. niger* but also by inhalation of enzyme dust. Thus, inhalation of enzymes derived from *A. niger* present in baking additives have been identified as a causative allergen in baker's asthma (Losada et al. 1986; Quirce et al. 2002).

Toxigenic potential

A second hazard to humans and animals appears to be toxicity associated with the production of mycotoxins. *A. niger* isolates are known to produce several toxic metabolites, such as malformins and naphthopyrones among other secondary metabolites, but they are not considered toxins of concern and up to now they have not been found naturally occurring in cereals. *Aspergillus carbonarius* can also produce naphthopyrones and *A. aculeatus* produces secalonic acid D (Frisvad and Samson 1991). Secalonic acid D has significant animal toxicity, but a role in human or animal disease, especially from this species, has not been shown (Pitt and Hocking 1997).

Nevertheless, the significance of black aspergilli as toxin producing fungi has changed since the evidence that they can produce OTA, a mycotoxin which is receiving increasing attention worldwide because of the hazard it poses to human and animal health. OTA is a

Table 1. Species reported as ochratoxin A producers in *Aspergillus* section *Nigri*

Species	References
<i>A. carbonarius</i>	Abarca et al. (2003); Battilani et al. (2003); Cabañes et al. (2002); Da Rocha et al. (2002); Heenan et al. (1998); Horie (1995); Joosten et al. (2001); Sage et al. (2002); Taniwaki et al. (2003); Téren et al. (1996); Wicklow et al. (1996).
<i>A. niger</i> aggregate	
<i>A. awamori</i>	Téren et al. (1996)
<i>A. awamori</i> var. <i>fumeus</i>	Ono et al. (1995)
<i>A. foetidus</i>	Téren et al. (1996); Ueno et al. (1991); Magnoli et al. (2003)
<i>A. niger</i> group	Nakajima et al. (1997)
<i>A. niger</i>	Da Rocha et al. (2002); Heenan et al. (1998); Taniwaki et al. (2003); Téren et al. (1996, 1997); Urbano et al. (2001)
<i>A. niger</i> var. <i>awamori</i>	Dalcero et al. (2002); Magnoli et al. (2003)
<i>A. niger</i> var. <i>niger</i>	Abarca et al. (1994, 2003); Accensi et al. (2001); Dalcero et al. (2002); Magnoli et al. (2003)
<i>A. usamii</i>	Ono et al. (1995)
<i>A. usamii</i> mut. <i>shirousamii</i>	Ono et al. (1995)
<i>A. japonicus</i>	Battilani et al. (2003)
<i>A. japonicus</i> var. <i>japonicus</i>	Dalcero et al. (2002)

nephrotoxic mycotoxin classified as a possible human renal carcinogen (group 2B) (IARC 1993). It has been implicated in the human disease Balkan endemic nephropathy and the development of urinary tract tumours and renal diseases in Tunisia and Egypt (Creppy 1999; Peraica et al. 1999). It is also teratogenic, carcinogenic and is clearly an immunosuppressive agent. Toxic effects resulting from very low concentrations of OTA in the ng/ml range affect the immune system. It appears that this system is by far the most sensitive among all other sensitive organs (Petzinger and Weidenbach 2002). Human exposure to OTA most likely comes from low level contamination of a wide range of different foods. Elimination of OTA in humans is extremely slow, since the toxin has the longest half-life known for living mammals. Repeated, almost daily uptake of OTA, therefore, will cause toxicologically relevant toxin concentration in blood. As a result of this exposure there is a high incidence of OTA in human blood and breast milk (Creppy 1999; Peraica et al. 1999; Petzinger and Weidenbach 2002; Kuiper Goodman and Scott 1989).

In Europe, the main source of OTA in the diet are cereals and cereal products but other food products can also contribute to the dietary intake, such as coffee, wine, beer, spices, grape juice and also products of animal origin, namely pig kidneys (Codex Alimentarius Commission 1998; Petzinger and Weidenbach 2002). Some countries have established specific plans to control this problem. The Danish control system for OTA in pig kidneys established in 1978 can be regarded as a success because the levels in pigs have

been reduced substantially, and hence the contribution from pig products to the total intake is very small compared with other sources (Jorgensen and Petersen 2002). In March 2002, the European Union established maximum OTA levels for cereals and dried vine fruits. Before the end of 2003, the Commission will possibly include a maximum limit for OTA in other food products (coffee, wine, beer, grape juice, cocoa and spices) (Commission of the European Communities 2002).

OTA was until recently believed to be produced only by *Aspergillus ochraceus* and related species belonging to section *Circumdati* (Hesseltine et al. 1972; Varga et al. 1996) and by *Penicillium verrucosum* (Pitt 1987). It is generally assumed that *P. verrucosum* produces OTA in temperate and cold climates, whereas *A. ochraceus* is more commonly associated with warmer and tropical climates. Nevertheless, such fungi are not always the source of OTA in some commodities.

Although some other *Aspergillus* spp. have been reported as ochratoxigenic (Abarca et al. 2001), the number of reports dealing with the production of OTA by members of section *Nigri* has been increasing since the first description of OTA production by *Aspergillus niger* var. *niger* (Abarca et al. 1994). In this section, the reported OTA-producing species are *A. carbonarius* and those now included in the so-called *A. niger* aggregate (Table 1). The ability of the uniseriate species *Aspergillus japonicus* to produce OTA has been recently mentioned (Dalcero et al. 2002; Battilani et al. 2003), but this fact needs to

be confirmed, since by the moment it is not considered as OTA producing species (Téren et al. 1996; Parenicova et al. 2001). The reported percentages of ochratoxigenic isolates in *A. carbonarius* ranges from 25 to 100%, whereas in the *A. niger* aggregate those percentages are lower, ranging from 0.6% to 50%. Thus, *A. carbonarius* is the major producer of OTA within *Aspergillus* section *Nigri*, but it is very difficult to know the extent of its natural occurrence in foods because all of the black aspergilli are very commonly regarded as '*A. niger*'. In fact, *A. carbonarius* is not described in some currently used identification manuals (Pitt and Hocking 1997; Samson et al. 2000). In order to distinguish between the most common taxa, see the identification key provided below. Recent surveys have shown without any doubt that black Aspergilli are the main source of OTA in products such as wine (Cabañes et al. 2002), grapes (Da Rocha Rosa et al. 2002; Sage et al. 2002; Battilani et al. 2003; Magnoli et al. 2003) and dried vine fruits (Heenan et al. 1998; Abarca et al. 2003), and probably a minor source in coffee (Joosten et al. 2001; Taniwaki et al. 2003; Téren et al. 1997; Urbano et al. 2001).

Taxonomy

History

Raper and Fennell (1965) produced the most comprehensive monograph of this genus and all *Aspergillus* species with conidial heads in some shades of black were included in the same group. Of the many species described, they accepted 12 species and two varieties, distinguished by the uniseriate (single palisade of cells which give rise to conidia) or biseriate (two palisades of cells) structures of the conidiophores (Figure 1), the colour of the colony and the conidial heads, the shape and ornamentation of the conidia, and the growth rate on Czapek agar. Many of the previously described species were synonymous with the species accepted by Raper and Fennell (1965) but in some cases older names are still now in use, especially in culture collections and in reports dealing with industrial strains. Furthermore, Murakami (1979a; 1979b) made a taxonomic study focused only on industrial Japanese strains and classified black aspergilli into two groups: the *A. niger* group and the black-koji mould group. All these proposals contributed to the considerable confusion around species

names in black aspergilli, especially in Japanese literature. In the compilation provided by Samson (1979), none of the species described since Raper and Fennell were accepted.

Al-Musallam (1980) revised the taxonomy of this group of fungi using cluster analysis involving all available morphological and cultural parameters. After both equal and iterative weighting of characters, she established five easily distinguishable species (*A. japonicus* Saito, *A. carbonarius* (Bainier) Thom, *A. helicothrix* Al-Musallam, *A. ellipticus* (Raper and Fennell) Al-Musallam, and *A. heteromorphus* (Batista and Maia) and the *A. niger* aggregate. In Klich and Pitt's Manual (1988) the changes proposed by Al-Musallam were accepted. Kozakiewicz (1989) made a new proposal based upon conidial ornamentation and distinguished 16 taxa.

Raper and Fennell (1965) did not adhere to the rules of the International Code of Botanical Nomenclature. To stabilise the taxonomy, Gams and Samson (1985) provided typification of *Aspergillus* and its associated teleomorphs, while Samson and Gams (1985) typified the species of *Aspergillus* accepted by Raper and Fennell (1965). Gams et al. (1985) formally introduced names by subgenera and sections. Black aspergilli became included in subgenus *Circumdati* and section *Nigri*.

On the other hand, according to the rules of nomenclature about the priority of names, the name *Aspergillus niger* van Tieghem (1867) is predated by other valid names (*A. phoenicis* (Corda) Thom 1840 and *A. ficuum* (Reichardt) Hennings 1867) which have priority under the Botanical Code. Conservation of *A. niger* was proposed (Kozakiewicz et al. 1992) and the proposal accepted by the International Botanical Congress in Tokyo, Japan, in 1993 (Greuter et al. 1994). The formal wording '*nom. cons.*' after the name indicates this protected status (*A. niger* van Tiegh. *nom. cons.*)

Changes in the species concept of black aspergilli according to different authors are shown in Table 2. In all the classifications proposed, the delimitation of some taxa is problematic because they are distinguished by relatively small differences in variable characters. Taxa are rather artificially separated in the diagnostic keys. In the recent years, various biochemical and molecular approaches have been used to develop rapid and reliable methods for identification of black aspergilli.

Figure 2 shows a phylogenetic analysis of ITS and 5.8S rDNA gene region of representative black

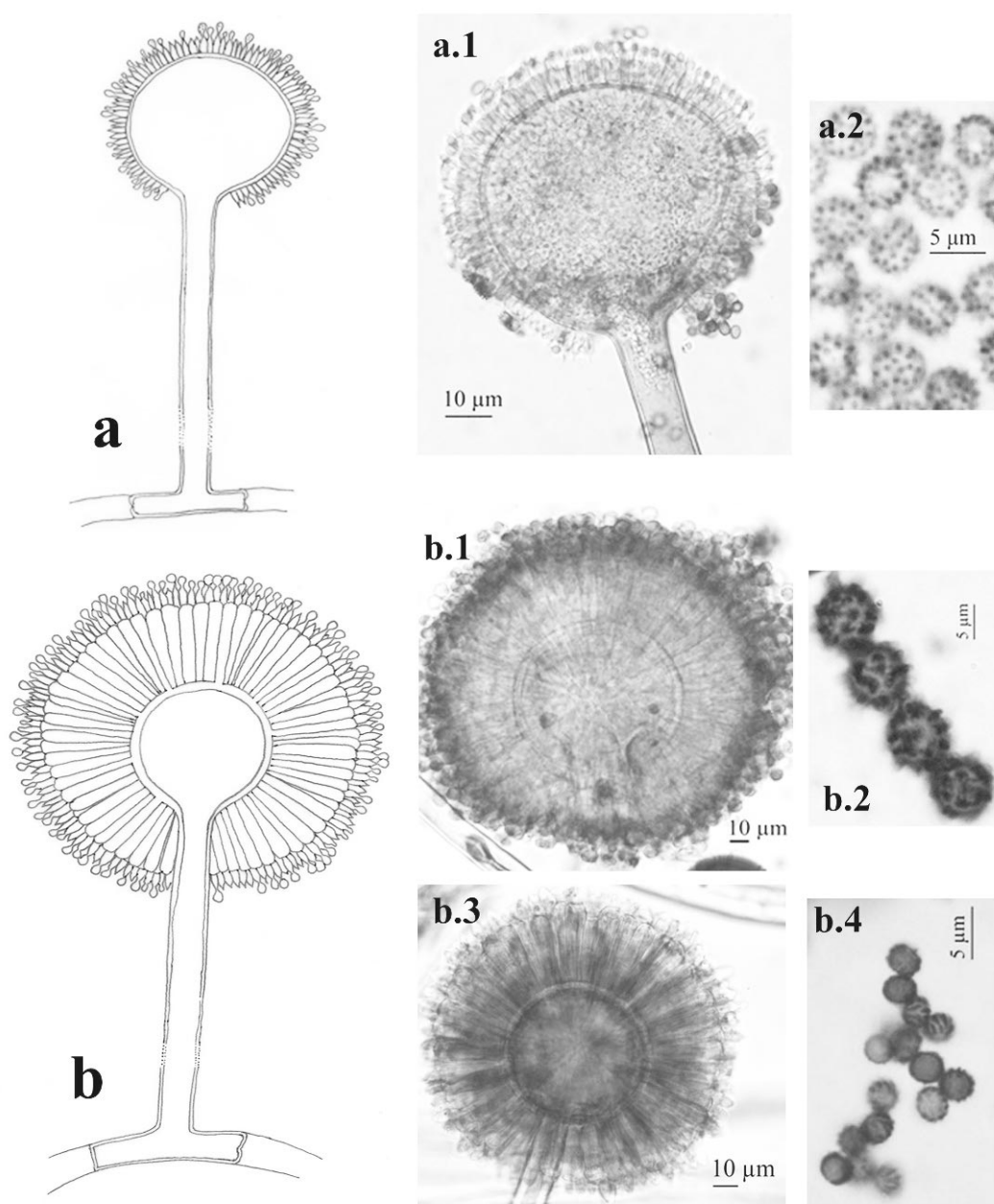


Figure 1. a: Uniseriate *Aspergillus* conidiophore. Note the single palisade of cells (phialides) which give rise to conidia. Conidial head (a.1) and conidia (a.2) of *A. japonicus* var. *aculeatus* (A-969, UAB Mycology group). b: Biseriate *Aspergillus* conidiophore. Note the two palisades of cells (metulae and phialides). *A. carbonarius* (A-1157, UAB Mycology group), conidial head (b.1) and conidia (b.2); *Aspergillus niger* aggregate (A-219, UAB Mycology group), conidial head (b.3) and conidia (b.4).

aspergilli species. Related taxa belonging to *Circumdati* and *Flavi* sections have been also included. *Emericella nidulans* (*Aspergillus* section *Nidulantes*) was used as outgroup. The EMBL accession numbers of the sequences are included in the Figure.

Table 2. Species concepts of black aspergilli according to different authors

Raper and Fennell (1965)	Al-Musallam (1980)	Kozakiewicz (1989)	RFLP* analysis
<i>A. japonicus</i> Saito	<i>A. japonicus</i> var. <i>japonicus</i> Saito	<i>A. japonicus</i> Saito	<i>A. japonicus</i> (1,2,6,9)
<i>A. aculeatus</i> Iizuka	<i>A. japonicus</i> var. <i>aculeatus</i> (Iizuka) Al-Musallam	<i>A. atroviolaceus</i> Moss.	<i>A. aculeatus</i> (6,9)
<i>A. carbonarius</i> (Bainier) Thom	<i>A. carbonarius</i> (Bainier) Thom	<i>A. carbonarius</i> (Bainier) Thom	<i>A. carbonarius</i> (1,2,7,9)
<i>A. heteromorphus</i> Batista and Maia	<i>A. heteromorphus</i> Batista and Maia	<i>A. fonscaeus</i> Thom and Raper <i>A. heteromorphus</i> Batista and Maia	<i>A. heteromorphus</i> (1,2,9)
<i>A. ellipticus</i> Raper and Fennell	<i>A. ellipticus</i> (Raper and Fennell) Al-Musallam	<i>A. ellipticus</i> Raper and Fennell	<i>A. ellipticus</i> (1,2,9)
	<i>A. helicothrix</i> Al-Musallam	<i>A. helicothrix</i> Al-Musallam	
	<i>A. niger</i> aggregate:		<i>A. niger</i> aggregate:
<i>A. niger</i> van Tieghem	<i>A. niger</i> var. <i>niger</i> van Tiegh.	<i>A. niger</i> var. <i>niger</i> van Tiegh.	<i>A. niger</i> or <i>A. tubingensis</i> (1,2,3)
<i>A. ficuum</i> (Reichard) Hennings			
<i>A. tubingensis</i> (Schöber) Mosseray		<i>A. niger</i> var. <i>tubingensis</i> (Moss.) Kozakiewicz	<i>A. niger</i> , <i>A. tubingensis</i> or " <i>A. brasiliensis</i> " (4)
<i>A. phoenicis</i> (Corda) Thom	<i>A. niger</i> var. <i>niger</i> f. <i>hennebergii</i> (Blochwitz) Al-Musallam	<i>A. niger</i> var. <i>phoenicis</i> (Corda) Al-Musallam	<i>A. niger</i> , <i>A. tubingensis</i> , or <i>A. foetidus</i> (5)
<i>A. pulverulentus</i> (McAlp) Thom	<i>A. niger</i> var. <i>phoenicis</i> f. <i>pulverulentus</i> (McAlp) Al-Musallam	<i>A. niger</i> var. <i>pulverulentus</i> (McAlp) Kozakiewicz	Type N or type T (8)
<i>A. awamori</i> Nakazawa	<i>A. niger</i> var. <i>awamori</i> (Nakazawa) Al-Musallam	<i>A. niger</i> var. <i>awamori</i> (Nakazawa) Al-Musallam	
	<i>A. niger</i> var. <i>nanus</i> (Mont.) Al-Musallam		
	<i>A. niger</i> var. <i>usamii</i> (Sakaguchi et al.) Al-Musallam	<i>A. niger</i> var. <i>ficuum</i> (Reich.) Kozakiewicz	<i>A. niger</i> , <i>A. tubingensis</i> , <i>A. foetidus</i> or " <i>A. brasiliensis</i> " (9)
	<i>A. niger</i> var. <i>intermedius</i> (Speg.) Al-Musallam		
<i>A. foetidus</i> (Naka.) Thom and Raper	<i>A. foetidus</i> Thom and Raper	<i>A. citricus</i> var. <i>citricus</i> (Wehmer) Moss. <i>A. acidus</i> Kozakiewicz	
<i>A. foetidus</i> var. <i>pallidus</i> Naka., Simo and Watanabe			
<i>A. foetidus</i> var. <i>acidus</i> Naka., Simo and Watanabe		<i>A. citricus</i> var. <i>pallidus</i> (Naka., Simo and Watanabe) Kozakiewicz	

*Black aspergilli species according to the following studies: 1, Kusters-van Someren et al. (1991); 2, Megnegneau et al. (1993); 3, Varga et al. (1993); 4, Varga et al. (1994); 5, Parenticova et al. (1997); 6, Hamari et al. (1997); 7, Kevei et al. (1997); 8, Accensi et al. (1999); 9, Parenticova et al. (2001).

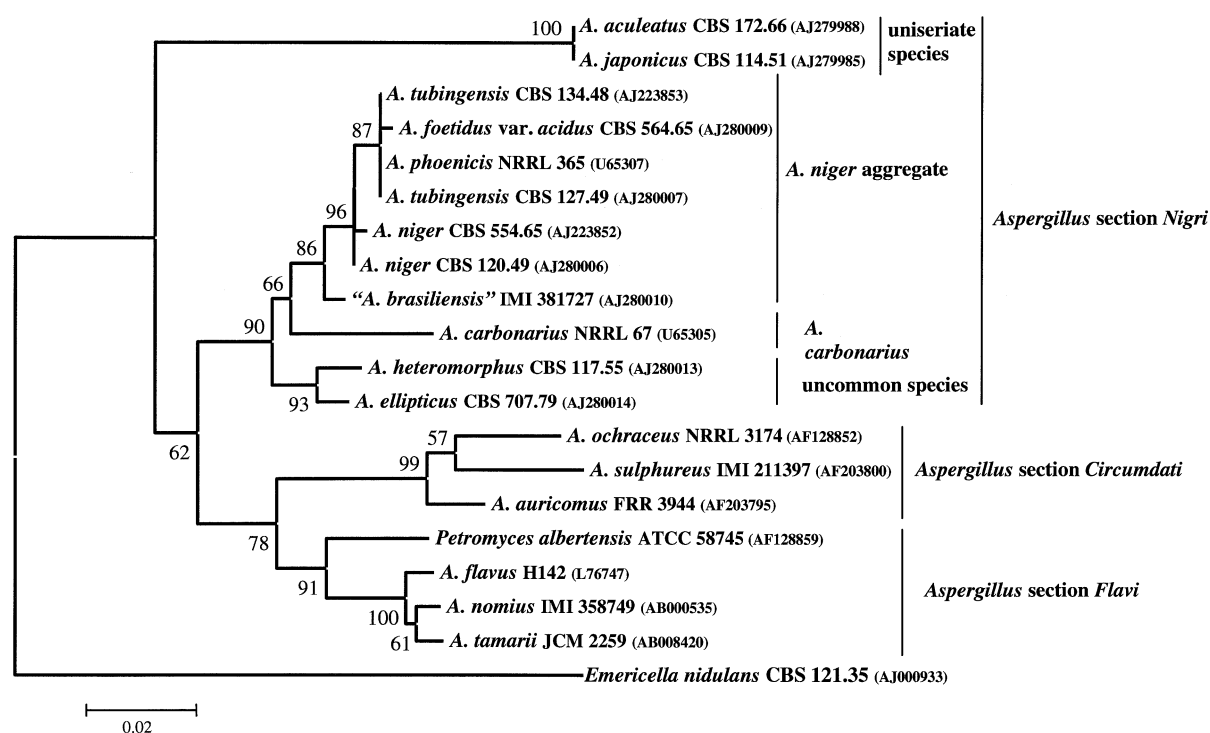


Figure 2. Neighbor-joining tree based on phylogenetic analysis of the ITS1-5.8S rRNA gene-ITS2 sequences. The sequences were aligned with Clustal W (version 1.5) of multiple sequence alignment computer program (Thompson et al. 1994). Adjustments for improvement were made by eye where necessary. Cladistic analyses using the Neighbour-joining method (Saitou and Nei 1987) were performed with the MEGA 2.1 computer program (Kumar et al. 2001) with Kimura 2-parameter model, including transitions and transversions and with pairwise deletion for the treatment of the handling gaps/missing data. Confidence values for individual branches were determined by bootstrap analyses (1000 pseudoreplicates).

Uniseriate species

Aspergillus japonicus / *Aspergillus aculeatus*

A. japonicus and *A. aculeatus* are the only species in this section which are uniseriate (Figure 1). Raper and Fennell (1965) distinguished these two taxa as separate species, but Al-Musallam (1980) reduced *A. aculeatus* Iizuka as a variety of *A. japonicus*. *A. japonicus* var. *aculeatus* (Iizuka) Al-Musallam, is distinguished from *A. japonicus* var. *japonicus* Saito by its larger vesicles (more than 45 μm of diameter) and predominantly ellipsoidal conidia. Nevertheless, sometimes *A. japonicus* var. *japonicus* can also have ellipsoidal conidia. Klich and Pitt (1988) provisionally accepted these varieties. Kozakiewicz (1989) maintained both species separately, but *A. aculeatus* was placed in synonymy with *A. atroviolaceus* Mosseray. Pitt and Hocking (1997) maintained the species separately due to consistent differences in morphology and secondary metabolite production, plus the fact that isolates seen in foods usually fit the

description of *A. aculeatus*. In Klich's manual (2002) only *A. japonicus* is described and it is said that the taxonomic status of *A. aculeatus* is still in question.

In a study of carbon source utilisation patterns, *A. japonicus* and *A. aculeatus* shared the same profile and in the isozyme analysis performed, low levels of variability were observed (Hamari et al. 1997). Kusters-van Someren et al. (1991) reported that the *Sma*I generated rDNA patterns of *A. japonicus* and *A. aculeatus* appeared to be identical. Mégnéneau et al. (1993) and Visser et al. (1996) could distinguish these species by examining their *Eco*RI, and *Pst*I-*Sal*I generated rDNA RFLPs. Hamari et al. (1997) reported that the *Sma*I, *Eco*RI, and *Pst*I-*Sal*I, digested rDNA profiles of the examined *A. japonicus* and *A. aculeatus* strains were invariable but finally they could differentiate both species by RFLPs of the mtDNA. Depending on the restriction enzyme combination used, Parenicova et al. (1997; 2000) could differentiate between both taxa. In a further study, Parenicova et al. (2001) reported that DNA sequence of the internal transcriber spacers (ITS1 and ITS2) and the

5.8S rRNA gene could not be used to distinguish between *A. japonicus* and *A. aculeatus*. Nevertheless, they insisted on their separation and to support this they combined RFLP analysis of nuclear DNA, DNA sequencing, and secondary-metabolite profile analysis. They also reported that one uniseriate isolate (*A. aculeatus* CBS 114.80) should probably represent a third taxon because it showed specific RFLP patterns for all loci examined and a different secondary-metabolic profile from those of *A. japonicus* and *A. aculeatus*.

On the other hand, Yokoyama et al. (2001) studying the section *Nigri* based on a 402-bp segment of the mt cytochrome *b* gene, concluded that *A. japonicus* and *A. aculeatus* belong to a single species because they showed the same amino acid sequences. Strikingly, Hamari et al. (2001) reported transmission of mitochondrial genomes among intraspecific pairs of *A. japonicus* but not when *A. aculeatus* was chosen as the recipient partner.

As shown in Figure 2, *A. japonicus* and *A. aculeatus* form a well-supported clade and, according to ITS1-5.8S rDNA-ITS2 sequences, would represent one species as no differences were observed between the sequences of the two species. Furthermore, the uniseriate black aspergilli are clearly separated from the biseriata black aspergilli. Some other investigations have shown this separation between uni and biseriata species. Croft and Varga (1994) reported that several molecular hybridisation experiments between probes derived from *A. niger* and *A. japonicus* DNA had all given weak hybridisation, suggesting only low homology. They concluded a distant relationship between the *A. niger* aggregate and *A. japonicus*. Parenikova et al. (2001) and Varga et al. (2003) reported that the uniseriate clade was separated from the clade of biseriata black aspergilli. A high bootstrap support (99%) was found for the branch leading to the cluster of all biseriata black aspergilli (Parenikova et al. 2001). Peterson (2000), using the D1 and D2 regions of large subunit rDNA, reported that the uniseriate species form one branch in the section while the biseriata species are on a separate branch. In Figure 2, the biseriata species' clade is in the same branch as that including isolates belonging to sections *Flavi* and *Circumdati*.

Biseriate species

Aspergillus carbonarius

A. carbonarius is possibly the most distinct member of this section. Strains of this species can be easily recognised using light microscopy since their conidia are much larger than those of other black aspergilli and have echinulate conidial ornamentation (Figure 1). Conidia are more than 6 µm (mostly 7-9 µm) of diameter and are multinucleated, containing 5-12 nuclei (Raper and Fennel 1965; Kevei et al. 1996). Strong correlation was observed between the volume of the conidia and the number of nuclei (Kevei et al. 1996). In a carbon source assimilation study, a distinctive pattern for the 13 isolates of *A. carbonarius* studied was reported (Kevei et al. 1996).

RFLP analysis of both rDNA and mtDNA and RAPD analysis clearly differentiates *A. carbonarius* from the other black aspergilli (Kusters-Van Someren et al. 1991; Mégnégneau et al. 1993; Kevei et al. 1996; Parenikova et al. 1997, 2000, 2001; Varga et al. 2000). Furthermore, Kevei et al. (1996) reported a field isolate of *A. carbonarius* which exhibited completely different RAPD, mtDNA and rDNA restriction patterns and proposed that this isolate (IN7) represented a new subspecies of *A. carbonarius*. Physical maps of the mtDNAs of strain IN7 and the other *A. carbonarius* strains were quite different from each other, although the order of the genes on these molecules seemed to be conserved (Hamari et al. 1999; Varga et al. 2000). The name proposed for this isolate was '*A. carbonarius* var. *indicus*'. This isolate showed also a different NADP-dependent glutamate dehydrogenase pattern. Other isozyme profiles were invariable or similar for all the *A. carbonarius* isolates studied (Kevei et al. 1996).

Parenikova et al. (2000) reported that in accordance with the aforementioned data, *A. carbonarius* could easily be separated from the other black aspergilli using ITS sequencing, i.e., there were 18-51 base differences separating *A. carbonarius* from the other taxa. As it is shown in Figure 2, *A. carbonarius* form a separate clade inside the biseriata black aspergilli, in accordance with other reports (Peterson 2000; Parenikova et al. 2001; Varga et al. 2003).

Aspergillus niger aggregate

The taxa included in the so-called *A. niger* aggregate (Al-Musallam 1980) have always been extremely dif-

difficult to distinguish one from each other by morphological means. The differences between the described species and varieties in the proposed classifications are very subtle and the number of taxa varies from one author to another (Table 2).

In an attempt at solving the classification of such important fungi, different taxonomical criteria were applied. Those criteria were, for the most part, based on RFLP analysis techniques. Different authors (Kusters-Van Someren et al. 1991; Mégnéneau et al. 1993; Varga et al. 1993, 1994; Parenicova et al. 1997, 2001) proposed the division of the aggregate into two or more species in accordance with the results obtained (Table 3). The species delimited on the basis of their RFLP patterns received the name of the most representative strain included in the group (i.e., the type or neotype culture). Obviously, this produces a considerable amount of confusion to the non-familiarised mycologist since these species include isolates formerly classified into other taxa by morphological means.

For a better understanding of the subject, we will point out the different authors' contributions to the classification of the taxa included in the *A. niger* aggregate in a chronological way for each kind of study.

Morphological studies

In her revision of black *Aspergillus* species, Al-Musallam (1980) described the *A. niger* aggregate by means of morphological and cultural characteristics. Cultures were grown on Czapek agar and 2% malt agar at 25 °C for 7 and 14 days. Members of the *A. niger* aggregate were characterised by their typically blackish colonies and more or less ornamented conidia, globose to subglobose, and with diameters less than 6 µm (Figure 1). The *A. niger* aggregate was formed by two species, namely *A. foetidus* Thom and Raper and *A. niger*, the latter composed by six varieties and two formae (*A. niger* var. *niger* van Tieghem, *A. niger* var. *niger* f. *hennebergii* (Blochwitz) Al-Musallam, *A. niger* var. *phoenicis* (Corda) Al-Musallam, *A. niger* var. *phoenicis* f. *pulverulentus* (McAlp) Al-Musallam, *A. niger* var. *awamori* (Nakazawa) Al-Musallam, *A. niger* var. *nanus* (Mont.) Al-Musallam, *A. niger* var. *usamii* (Sakaguchi et al.) Al-Musallam, *A. niger* var. *intermedius* (Speg.) Al-Musallam). *A. niger* and *A. foetidus* were only differentiated by their colony diameter on Czapek agar, whereas the varieties of *A. niger* were basically

identified by the roughness and ornamentation of their conidia

Kozakiewicz (1989) asserted that the maturation of conidia of black aspergilli is slow and, in the case of the *A. niger* aggregate, up to 5 weeks of incubation in optimal conditions were needed for the observation of totally matured conidia. Following on from this statement she discarded Al-Musallam's classification (1989) and proposed a new classification based on the conidial ornamentation observed with SEM techniques. Two basic patterns of ornamentation were described: verrucose and echinulate. The black aspergilli included in the aggregate were divided in three species: *A. acidus* Kozakiewicz, *A. citricus* (with two varieties: *A. citricus* var. *citricus* (Wehmer) Moss. and *A. citricus* var. *pallidus* (Naka., Simo and Watanabe) Kozakiewicz) and *A. niger* (with six varieties: *A. niger* var. *niger*, *A. niger* var. *tubingensis* (Moss) Kozakiewicz, *A. niger* var. *phoenicis*, *A. niger* var. *pulverulentus* (McAlp) Kozakiewicz, *A. niger* var. *awamori* and *A. niger* var. *ficuum* (Reich.) Kozakiewicz).

Accensi (2000) carried out a morphological study of 92 isolates (including representative collection strains of the different species) of the *A. niger* aggregate. The strains were grown on different culture media and both macroscopical (diameter, colour, texture, presence of furrows, pigments, and exudates) and microscopical (stipes, vesiculae, metulae, fialides and conidia) characteristics were recorded. The recorded measures of all strains were reasonably consistent from isolate to isolate, but the ranges overlapped, so these characters offered little of value for species differentiation in the *A. niger* aggregate.

Physiological and biochemical studies

The assimilation of seven carbon sources (melicitose, xylitol, vanillic acid, cis-aconitic acid, L-serine and L-tyrosine) produced different patterns in black aspergilli. Nevertheless, no reliable differences were observed between strains of the *A. niger* aggregate, since more than 95% of the more than 400 tested isolates were able to utilise all the seven aforementioned compounds as a unique carbon source, with the exception of L-tyrosine. Consequently, no taxonomical proposal was made based on such criteria in the *A. niger* aggregate (Hamari et al. 1997; Varga et al. 2000).

Isozyme analysis has been also applied to the classification of the *A. niger* aggregate (Mégnéneau et

Table 3. Different RFLP patterns of the *A. niger* aggregate strains according to different authors

	Kusters-van Someren et al. (1991)	Megnagneau et al. (1993)	Varga et al. (1993)	Varga et al. (1994)	Parenticova et al. (1997)	Accensi et al. (1999)	Parenticova et al. (2001)
	RFLP rDNA	RFLP rDNA	RFLP mtDNA	RFLP rDNA	RFLP (PstI-SalI) mtDNA / Southern 28S	RFLP ITS1-5.8SrDNA-ITS2	RFLP (KpnI-XhoI) / Southern 28S / RFLP (PstI-SalI) / Southern 28S
<i>A. niger</i> ^a	I	I	Ia	I	I	N	B
	I'	I'	Ib	I'	C		B
		Ic	Ic		E / F / D		
			Id				
			Ie				
<i>A. tubingensis</i> ^b	II	II	2a	II	B / D	T	A
	II'	II'	2b	II'	B / A		C
			2c				
			2d				
			2e				
			2f				
<i>A. brasiliensis</i> ^{c,e}				III	3	N	D
<i>A. foetidus</i> ^d						T ^f	A ^e
							D ^e
							C ^e
							D ^e

^a, ^b Species delimited by Kusters-van Someren et al. (1991) by means of RFLP analysis only; ^c Species delimited by Varga et al. (1994) by means of RFLP analysis only; ^d Species delimited by Parenticova et al. (1997) by means of RFLP analysis only; ^e Based on strains that were not tested in the previously published studies (Kusters-van Someren et al., 1991; Megnagneau et al., 1993; Varga et al., 1993, 1994); ^f Although in this study there was none of the strains classified by Parenticova et al. (1997) as *A. foetidus* by molecular means, one of those strains (CBS 554.65) has been classified as type T following the method of Accensi et al. (1999).

al. 1993). This technique was only useful for differentiating strains of the *A. niger* aggregate from isolates of the other species of the *Aspergillus* section *Nigri*. A high degree of variability was observed in the tested strains with the analysis of glutamate oxaloacetate transaminase (GOT), 15-hydroxyprostaglandin dehydrogenase (PGDH), dihydrofolate reductase (DHFR) and glucose-6-phosphate dehydrogenase (G6PDH).

The response of 92 strains of the *A. niger* aggregate to different incubation temperatures and NaCl concentrations was studied (Accensi 2000), but no substantial differences were found and no taxonomical proposal was made.

DNA-based studies

The RFLP-based studies of strains of the *A. niger* aggregate carried out are summarised in Table 3.

Kusters-van Someren et al. (1990) by means of Southern blot analysis utilising the *pelD* gene as a probe and Western blot of pectin lyase could not differentiate the tested strains of the *A. niger* aggregate. In a later work, Kusters-van Someren et al. (1991) observed two different *SmaI* digested rDNA RFLP patterns (I and II) among isolates of the *A. niger* aggregate. Because the neotype culture of *A. niger* (CBS 554.65) belonged to the group I and the type culture of *A. tubingensis* (CBS 134.48) to the group II, they proposed to name group I strains *A. niger* and group II strains *A. tubingensis*. These results were confirmed by Southern blot analysis with various pectin lyase genes used as probes. Furthermore, the formation of heterokaryon was not observed among strains of the two proposed species.

Mégnégneau et al. (1993) confirmed the results of Kusters-van Someren et al. (1991) by means of RFLP analysis of total DNA digested with different restriction enzymes (*SmaI*, *EcoRI* and *PstI*). *A. niger* aggregate collection strains showed four different rDNA RFLP patterns (I, I', II, and II'), which could be grouped in two main groups (I and II). These two main groups coincided with the two species delimited by Kusters-van Someren et al. (1991). On the basis of RAPD analysis, Mégnégneau et al. (1993) proved the existence of a high level of intraspecific variability among strains of the *A. niger* aggregate, consistent with the findings obtained by means of RFLP analysis. Karyotype analysis was also performed and showed a high grade of variation among the assayed strains.

Varga et al. (1993), using 47 collection strains of the *A. niger* aggregate, obtained five patterns of mtDNA RFLP (1a, 1b, 1c, 2a and 2b) by means of digestion of the total DNA with different restriction enzymes. These patterns could be grouped in two main groups (1 and 2), that coincided with the two groups proposed by Kusters-van Someren et al. (1991). In a further study with soil isolates, the existence of a high level of variation in mtDNA within *A. niger* aggregate was confirmed by describing up to 12 different mtDNA RFLP patterns (Varga et al. 1994). All those patterns could be grouped in the two main groups 1 and 2, with the exception of one pattern. This RFLP pattern 3 was only shown by six strains isolated from Brazilian soil. Varga et al. (1994) raised the possibility that such Brazilian isolates would form part of a subspecies of *A. niger* or a new species within the *A. niger* aggregate, provisionally called '*A. brasiliensis*'. The results obtained by means of mtDNA RFLP analysis were confirmed by rDNA RFLP analysis. The division of the *A. niger* aggregate into the two species, *A. niger* and *A. tubingensis*, was confirmed again by means of digestion with different restriction enzymes and hybridisation with various probes (Visser et al. 1996).

Kevei et al. (1997) reported the mitochondrial transmission between isolates of the *A. niger* aggregate with different mtDNA RFLP patterns. Such transference was not possible between strains of the *A. niger* aggregate and strains of *A. carbonarius* or *A. japonicus*.

Parenicova et al. (1997) in a study dealing with 23 collection strains of the *A. niger* aggregate, described a new *PstI-SalI* digested rDNA RFLP pattern represented by type strains of *A. foetidus* varieties. Consequently, they proposed the division of the aggregate into three morphologically identical taxa: *A. niger*, *A. tubingensis* and *A. foetidus*. The strains representing the new group had not been tested in the previously published studies but could be classified as *A. tubingensis* when their total DNA was *SmaI* digested following the method of Kusters-van Someren et al. (1991), as noted (Parenicova et al. 1997). In a later work (Parenicova et al. 2001), the division of the *A. niger* aggregate into four morphologically identical species namely *A. niger*, *A. tubingensis*, *A. foetidus* and '*A. brasiliensis*' was proposed. However, as it is shown in Table 3, when following the criteria proposed for segregating some strains of *A. foetidus* as a new group (Parenicova et al. 1997), '*A. brasiliensis*' and *A. niger* shared the same *PstI-SalI* digested

rDNA profile (Parenicova et al. 2001). Moreover, the letters used to refer the obtained RFLP patterns are different from those previously used (Parenicova et al 1997), adding yet more confusion to the proposed classification of the *A. niger* aggregate. An analysis of the sequences of the ITS1-5.8S rDNA-ITS2 region of some black *Aspergillus* strains was included in another survey (Parenicova et al. 2000). The obtained differences between the sequences of the four aforementioned species were strikingly low. Only 3 nucleotides segregated the sequence of *A. niger* from *A. tubingensis* sequence and merely 2 nucleotides separated *A. foetidus* from *A. tubingensis*. There were 5 differences between *A. niger* and *A. foetidus*. In a later study, a neighbour-joining tree based on phylogenetical analysis of such sequences of strains of black aspergilli was presented (Parenicova et al 2001). In that tree, the four proposed species formed a differentiated clade. The sequences of representative strains of *A. foetidus* and *A. tubingensis* were found forming a subclade inside the *A. niger* aggregate clade. The sequence of *A. niger* was very close to the *A. tubingensis*-*A. foetidus* clade (bootstrap value = 99%). The '*A. brasiliensis*' sequence was the most distinct one inside the *A. niger* aggregate's branch (bootstrap value = 94% with the *A. niger*-*A. tubingensis* clade). A similar neighbour-joining tree was presented by Varga et al. (2003). These results are in accordance with those presented in Figure 2. *A. tubingensis* and *A. foetidus* form a distinct clade, which is close to *A. niger* (bootstrap value = 87%). '*A. brasiliensis*' is closely related (bootstrap value = 86%) to the *A. niger* aggregate clade. Similar results are reported by Peterson (2000), who studied the parsimony trees obtained with the sequences of the D1 and D2 regions of the LSU-rDNA. Inside the black aspergilli clade, a clear branch was formed by members of the *A. niger* aggregate, with little variation presented by an inner clade.

Yokohama et al. (2001) analysed the nucleotide and amino acid sequences from the partial mitochondrial cytochrome *b* gene of 25 isolates of the *A. niger* aggregate. The authors proposed a confusing division of the aggregate in two clades: *A. niger* and *A. awamori*.

Accensi et al. (1999) sequenced the ITS1-5.8S rDNA-ITS2 fragment of the type strains of *A. niger* (CBS 554.65) and *A. tubingensis* (CBS 134.48). These sequences were very similar, with only seven differences: five in ITS1 and two in the ITS2. Nevertheless, by comparison of these sequences, a target for

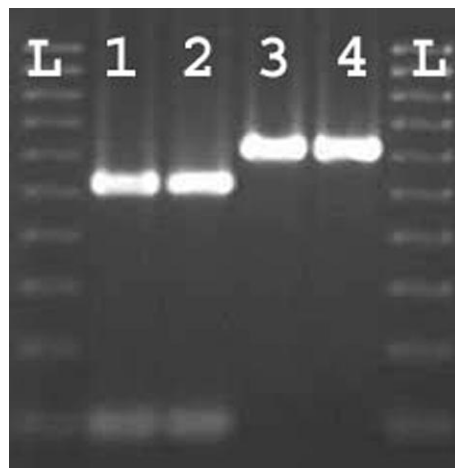


Figure 3. ITS1-5.8S rDNA gene-ITS2 PCR products cleaved by *RsaI* and separated on a 2% agarose gel. Lane L is the 100 bp DNA ladder (Gibco BRL) used as size marker. Pattern N (Lanes 1,2) has two fragments of 519 and 76 bp. Pattern T (Lanes 3,4) has one fragment of 595 bp.

the restriction enzyme *RsaI* was found at position 75 of the sequence of *A. niger* that was not present in the *A. tubingensis* sequence. Thus, two RFLP patterns were described (Figure 3): pattern N (two fragments, 519 and 76 bp) and pattern T (one fragment of 595 bp), which corresponded with the two 'species' proposed by Kusters-van Someren et al. (1991). Due to its simplicity, the patterns N and T are easier to recognise than the RFLP patterns described by other authors (Kusters-Van Someren et al. 1991; Mégnégneau et al. 1993; Varga et al. 1993, 1994; Parenicova et al. 1997, 2001). Furthermore, the lack of variation within the two obtained patterns was not observed in the previously described RFLP methods, which showed high levels of variability, i.e.: up to four patterns in rDNA RFLP patterns (Mégnégneau et al. 1993) to twelve of mtDNA RFLP patterns (Varga et al. 1994). Among the tested strains, there was one '*A. brasiliensis*' isolate, which was classified as a type N strain (Accensi et al 1999). The type strain of *A. foetidus* var. *acidus* (CBS 564.65), included in the *A. foetidus* species as delimited by Parenicova et al. (1997, 2001), can be classified as a type T strain according to the RFLP of the *RsaI* digested ITS1-5.8S rDNA-ITS2. In a latter work, this RFLP method was tested with 92 strains (including both collection and wild isolates). No variation within the two patterns was observed. Forty four isolates were classified as type N isolates and 48 as type T (Accensi et al. 2001). No

morphological differences were observed between the two types of isolates N and T (Accensi 2000). However, when the distribution of the OTA-producing strains of the *A. niger* aggregate amongst the two patterns N and T was studied (Accensi et al. 2001), all the OTA-producing isolates whose RFLP pattern is known were classified as type N, whereas none of the type T strains were able to produce this mycotoxin. Thus, this RFLP technique could be a good way for the screening of possible OTA producing *A. niger* aggregate strains (Accensi et al. 2001; Cabañes et al. 2002; Abarca et al. 2003).

Uncommon species (A. helicothrix, A. ellipticus and A. heteromorphus)

There are three accepted biseriate species that are very uncommon. In fact, there is only one available isolate of each one in the international Culture Collections.

Aspergillus helicothrix (CBS 677.79) was isolated by Al-Musallam (1980) as a contaminant of the original type culture of *Aspergillus ellipticus* (CBS 482.65) and was segregated as a separate species. According to the two available descriptions (Al-Musallam 1980; Kozakiewicz 1989), the distinctive character for this species is the production of sclerotia which are large, brown and covered in coiled setae. Conidia are large (6.5–8.0 µm) and echinulate. *A. ellipticus* (CBS 707.79), isolated by Al-Musallam (1980) from the original mixed contaminated culture of *A. ellipticus* (CBS 482.65) presents conidia ellipsoidal and conspicuously echinulate. The only available strain of *Aspergillus heteromorphus* (CBS 117.55) was isolated from a culture of *Trichophyton* sp. in Brazil by Batista and Maia in 1957. The morphological descriptions of this strain are quite variable. Raper and Fennell (1965) did not observe the heteromorphism emphasised in the original description and used as the basis for the name. Disparities between descriptions were attributed to continuous cultivation of the strain or from differences in incubation temperature and maintenance techniques. Nevertheless, Raper and Fennell (1965) recognised the strain as representing a valid species and the name was retained for it. For Al-Musallam (1980), the echinulate conidia and the heteromorphism observed in few conidial heads were sufficient characters to separate it from the other species, although the description differed from that given by Raper and Fennell and by that of the original authors (Batista

and Maia 1957). In the description given by Kozakiewicz (1989), it is only said that this species shows echinulate conidia and is supposed to exhibit heteromorphism.

Varga et al. (2000) reported that *A. ellipticus* and *A. heteromorphus* could be differentiated from other black aspergilli based on their carbon utilisation profiles. Kusters-van Someren et al. (1991) reported homology between *A. helicothrix* and *A. ellipticus* with respect to their pectin lyase genes. In a further study, *A. helicothrix* was considered as a mutant variant of *A. ellipticus*, since they showed identical rDNA RFLP patterns (Kusters-van Someren et al. 1991). RFLP analysis showed that *A. ellipticus* and *A. heteromorphus* are very closely related (Parenicova et al. 2000, 2002). This relationship is also supported by the ITS sequence data, because only 6 base differences separated these two taxa (Parenicova et al. 2000). As shown in Figure 2, *A. heteromorphus* and *A. ellipticus* are grouped in the same clade with a high bootstrap support (93%). This relationship has been also reported by other authors (Parenicova et al. 2000; Peterson 2000; Varga et al. 2003).

Steiman et al. (1994) reported the isolation of two new species closely resembling *A. heteromorphus* but showing slight morphological differences. Both species were isolated from the same soil sample of Dead Sea area and were named *A. pseudo-heteromorphus* Steiman, Guiraud, Sage and Seigle-Murandi and *A. homomorphus* Steiman, Guiraud, Sage and Seigle-Murandi. We have not had the opportunity of examining these strains but consider it relevant that heteromorphism was also observed in our laboratory when isolates belonging to *A. niger* aggregate were grown on media containing 8% of sodium chloride (Accensi 2000). Both species are considered 'Nom. Inval., Art. 37.4' (Samson 2000).

Identification key

As only some species can be easily distinguished by morphological criteria, the following identification key is the only way to distinguish the most common taxa.

Cultures on Czapek agar, Czapek yeast extract agar (CYA) or Malt extract agar (MEA) (Pitt and Hocking 1997) incubated at 25°C for 7 days may be used.

1.	Aspergilla uniseriate	<i>A. japonicus</i> / <i>A. aculeatus</i> ^a
1.	Aspergilla biseriata	2 ^b
2.	Conidia more than 6 µm diameter	<i>A. carbonarius</i>
2.	Conidia less than 6 µm diameter	<i>A. niger</i> aggregate ^c

^a Whether *A. japonicus* and *A. aculeatus* are one or two species is still in question (see uniseriate species section).

^b *A. helicothrix*, *A. ellipticus* and *A. heteromorphus* are not included in the key because they are very uncommon species (only one available strain of each one).

^c Molecular data indicate that *A. niger* aggregate can be divided in two, three or even four taxa, according to different authors. It is not possible to distinguish between them by morphological means (see *A. niger* aggregate section).

Concluding remarks

This review has shown that morphological classification reflects the genetic relations of strains only in some black *Aspergillus* species. Due to the potential risk of black aspergilli as OTA producing species it is very important to be able to unambiguously identify them. Some species, such as *A. carbonarius* and uniseriate species can be easily recognised. In the *A. niger* aggregate, although speciation at molecular level has been proposed, no morphological differences can be observed and species identification will therefore remain problematic. In the last few years, attempts to simplify the classification of black aspergilli by molecular means have again resulted in a level of complexity similar to that described previously upon morphological criteria. DNA-based techniques show without any doubt the high degree of variability of the members of the *A. niger* aggregate. Such studies are a very valuable tool in the characterization of strains involved in patent applications, epidemiological surveys or in production of interesting secondary metabolites such as ochratoxin A. A balance between taxonomic and practical approaches must be found.

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