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Molecular diversity of agriculturally important *Aspergillus* species

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Abstract

Although *Aspergillus* species are not usually considered as serious plant pathogens, Aspergilli are frequently encountered in plant products. The most important consequence of their presence is mycotoxin contamination. The main mycotoxins produced by Aspergilli are the aflatoxins, ochratoxin A and patulin, which are produced by a variety of *Aspergillus* species in different plant commodities. Phylogenetic analysis of sequences of the ribosomal RNA gene cluster is useful for clarifying taxonomic relationships among toxigenic Aspergilli causing pre- and postharvest contamination of agricultural products. Molecular data has enabled us to clarify the taxonomy of black Aspergilli, *A. flavus* and its relatives, and sections *Circumdati* and *Clavati*, which include ochratoxin and patulin-producing species. Phylogenetically unrelated species were found to produce the same mycotoxins, indicating that mycotoxin-producing abilities of the isolates have been lost (or gained) several times during the evolution of the genus. The data also indicate that biosynthetic gene-based probes are necessary for molecular detection of these mycotoxin-producing organisms. The organisation of the biosynthetic genes of patulin and ochratoxins is unknown, although experiments are in progress in several laboratories to clarify the genetic background of biosynthesis of these mycotoxins. Identification of biosynthetic genes responsible for mycotoxin production is essential for clarifying the evolution of mycotoxin biosynthesis in Aspergilli, and to develop specific gene probes for the detection of mycotoxin-producing Aspergilli in agricultural products.

Introduction

Aspergillus is among the most economically important of the fungal genera. Isolates of *Aspergillus* are used for the production of soy sauce, several organic acids and enzymes, and biologically active metabolites such as lovastatin (Campbell-Platt and Cook, 1989; Bennett and Klich, 1992; Pariza and Johnson, 2001; Manzoni and Rollini, 2002). Although not considered to be a major cause of plant disease, *Aspergillus* species are responsible for several disorders in various plants (Table 1). The most common plant pathogens are *Aspergillus niger* and *A. flavus* (Table 1). In contrast to specialised plant pathogens such as the powdery mildews, rusts and some *Fusarium* species, *Aspergillus* species are opportunistic pathogens without host specialisation (St Leger et al., 2000). While only a limited number of *Aspergillus* species can invade living plant tissues, several are encountered as storage moulds on plant products (Raper and Fennell, 1965; Kozakiewicz, 1989). *Aspergillus* species can contaminate foods and feeds at different stages including harvest, processing and handling. Changes due to spoilage by *Aspergillus* species can be of a sensory nature, e.g., pigmented growth, discolouration, rotting and the development of off-odours and off-flavours. The most important aspect of food spoilage is, however, the formation of mycotoxins, which may have harmful effects on human and animal health. Several *Aspergillus* mycotoxins have been identified as contaminants in foods and feeds, the economically most important of which are the

Table 1. *Aspergillus* species involved in plant pathogenesis (compiled from Raper and Fennell, 1965; Kozakiewicz, 1989; Michailides et al., 2002; www.apsnet.org)

Plant	Disease	*Aspergillus* sp. involved
Almond	Kernel decay	*A. niger*, *A. flavus*, *A. parasiticus*
	Chlorosis	*A. niger*
Apricot, peach	Ripe fruit rot	*A. niger*
Caladium sp.	Corm rot	*A. niger*
Carrot	Sooty rot	*A. niger*
Cereals	Storage molds	*Aspergillus* sp.
Chickpea	Seedling or seed rot	*A. flavus*
Citrus (*Citrus* spp.)	Albinism	*A. flavus*
	Black mold rot	*A. niger*
Corn	*Aspergillus* ear-kernel rot	*A. flavus*, *A. glaucus*, *A. niger*
	Minor ear rots	*A. niger*, *Aspergillus* sp.
Cotton	Lint contamination	*A. flavus*
Date Palm	Fruit rots	*Aspergillus* sp.
Dracaena sp.	Stem rot	*A. niger*
Fig	Fig smut	*A. niger*
Geranium	Leaf mold	*A. fischerianus*
Grape	*Aspergillus* vine canker	*A. niger*
	Bunch rot (sour rot)	*A. niger*
	Berry rots, raisin molds	*A. aculeatus*, *Aspergillus* sp
House Plants	Stem rot	*A. niger*
Mango	Black mold rot	*A. niger*
Onion, garlic	Black rot	*A. niger*, *A. alliaceus*
Peanut	Crown rot	*A. niger*
Pigeonpea	Seedling or seed rot	*A. flavus*, *A. niger*
Pineapple	*Aspergillus* rot	*A. flavus*
Pistachio	*Aspergillus* fruit rot	*A. niger*
Sansevieria sp.	*Aspergillus* rhizome rot	*A. niger*
Sisal	Bole rot	*A. niger*
Sorghum	Damping-off and seed rot	*Aspergillus* sp.
Strawberry	Fruit rots	*A. niger*

aflatoxins, ochratoxins and patulin (Table 2). These toxins were first identified in *A. flavus, A. ochraceus* and *A. clavatus* (Smith and Moss, 1985). However, recent studies indicate that these compounds can be produced by a number of other *Aspergillus* species. Only a few of these mycotoxin producers are regarded as potential health hazards because most produce only traces of the given mycotoxin (e.g., small amounts of aflatoxins by *A. ruber*, or ochratoxins by *A. wentii* and *A. terreus*), or they are encountered rarely if at all in food products (e.g., aflatoxin producing *A. ochraceoroseus*, *Emericella venezuelensis* and *E. acristata* and ochratoxin-producing isolates of *A. auricomus*) (Pitt, 2000). However, new data indicate that some species recently reported to be mycotoxin producers can be regarded as sources of mycotoxin contamination in various food products (Pitt, 2000; Abarca et al., 2001; Bayman et al., 2002). For example, although ochratoxin-producing abilities of black Aspergilli have only recently been discovered, these fungi are now considered as major sources of ochratoxin contamination in wine, raisins and coffee (Pitt, 2000). Our aim was to examine the molecular diversity within, and evolutionary relationships between, these mycotoxin-producing species.

Taxonomic outline of the *Aspergillus* genus

Because of its economic importance, the genus *Aspergillus* has one of the better-described taxonomies among filamentous fungi. Raper and Fennell (1965) described 18 species groups within this genus based mainly on cultural and morphological features, which were treated as sections belonging to six subgenera by Gams et al. (1985).

Table 2. Some economically important mycotoxins produced by *Aspergillus* species in various agricultural products (Larsen et al., 2001; Bayman et al., 2002; Varga et al., 2003a, b)

Mycotoxins	Agricultural product	Species
Aflatoxins	Peanut, corn, cotton	*A. flavus*, *A. parasiticus*, *A. nomius*
Ochratoxins	Cereals	*P. verrucosum*
	Meat, cheese	*P. nordicum*
	Grape, wine	*A. niger, A. carbonarius*
	Coffee, spices	*A. ochraceus*, *A. niger*, *A. carbonarius*
	Figs	*A. alliaceus*
Patulin	Cereals, malt	*P. expansum*, *A. clavatus*
	Apple, pear	*P. expansum*

Phylogenetic studies of ribosomal RNA gene sequences led to the acceptance of three subgenera with a total of 15 sections and the so-called '*Warcupiella* group', a treatment currently accepted by most *Aspergillus* researchers (Peterson, 2000). This review, gives a general overview of the taxonomic relationships among agriculturally important mycotoxin-producing *Aspergillus* species. The molecular techniques applied include PCR-based methods, RFLP techniques, and phylogenetic analysis of β-tubulin and ribosomal RNA gene sequences in comparison with morphological and physiological features. The fungal groups treated involve those sections which include species most frequently encountered in agricultural products including those of *Aspergillus* sections *Nigri*, *Flavi*, *Circumdati* and *Clavati*, with special emphasis on section *Nigri*.

Aspergillus *section* Nigri

Black Aspergilli (*Aspergillus niger* species group, Raper and Fennell, 1965; *Aspergillus* section *Nigri*, Gams et al., 1985) have a significant impact on modern society. Many species cause food spoilage, and several are used in the fermentation industry to produce hydrolytic enzymes, such as amylases or lipases, and organic acids, such as citric acid and gluconic acid (Raper and Fennell, 1965; Kozakiewicz, 1989; Bennett and Klich, 1992; Pariza and Johnson, 2001). They are also candidates for genetic manipulation in the biotechnology industries since *A. niger* has been granted the GRAS (generally regarded as safe) status by the Food and Drug Administration of the US government. Accordingly, genetically modified black *Aspergillus* isolates are used in the fermentation industry (Pariza and Johnson, 2001). Although the main source of black Aspergilli is soil, members of this section have been isolated from other sources (Table 3). Black Aspergilli are the causal agents of several plant diseases and may produce ochratoxins (Tables 1 and 2). Recently it has also been proposed that *A. niger* has an endophytic life style in onions (Tuffley and Lorbeer, 2002).

Black Aspergilli are one of the more problematic groups for identification. Raper and Fennell (1965) described 12 species of the black Aspergilli. Al-Musallam (1980) revised the taxonomy of the *A. niger* group by taking mainly morphological features into account. She recognized seven species within this group (*A. japonicus*, *A. carbonarius*, *A. ellipticus*, *A. helicothrix*, *A. heteromorphus*, *A. foetidus* and *A. niger*), and described *A. niger* itself as an aggregate consisting of seven varieties and two formae. Kozakiewicz (1989) distinguished *A. ellipticus*, *A. heteromorphus*, *A. japonicus*, *A. helicothrix*, *A. atroviolaceus* (treated as *A. aculeatus* or *A. japonicus* var. *aculeatus* in other classifications) and *A. carbonarius*, a species exhibiting echinulate conidial ornamentations, which distinguished it from the rest of black *Aspergillus* strains, which display verrucose conidia. Within the verrucose category, *A. fonsecaeus*, *A. acidus* (*A. foetidus* var. *acidus*), *A. niger* var. *niger*, *A. niger* var. *phoenicis*, *A. niger* var. *ficuum*, *A. niger* var. *tubingensis*, *A. niger* var. *pulverulentus*, *A. niger* var. *awamori*, *A. citricus* (*A. foetidus*) and *A. citricus* var. *pallidus* (*A. foetidus* var. *pallidus*) were recognised. In recent years, several publications have dealt with the application of different phenotypic and genotypic markers for clarifying the taxonomy of black Aspergilli. Among the genotypic approaches, nuclear and mitochondrial DNA (mtDNA) poly-

Table 3. Ecology of black Aspergilli

Species	Product	Country
A. aculeatus	*Allium cepa*	India
	Amaranthus sp.	India
	Anacardium occidentale	Brunei
	Capsicum sp.	Nigeria
	Cupressus sp.	Egypt
	Glycine max	Sri Lanka
	Gossypium sp.	Mozambique
	Grape	Italy, Spain
	Hibiscus sp.	Sierra Leone
	Meytoxylon rumphi	Malaysia
	Papaya	Venezuela
	Pickle-cured fish	Malaysia
	Pinus sp.	Hong Kong
	Pistachio	Iran
	Rice	India
	Sorghum sp.	Papua New Guinea
	Tomato	Nigeria
A. carbonarius	Air	Java, South Africa
	Cocoa	Nigeria
	Coffea arabica	USA
	Dacrydium araucaroides	New Caledonia
	Grape	Portugal, France, Spain, Italy, Australia, Greece, Israel
	Vicia faba	Sri Lanka
A. foetidus	Bottled fruits	India
	Ground nut seedling	Tanzania
	Tomato	Nigeria
A. japonicus	*Anisophyllea laurina* fruit	Sierra Leone
	Archive cellulose	USA
	Glycine max	Bangladesh
	Grape	Portugal, Israel
	Green coffee berries	India
	Hevea brasiliensis	Sri Lanka
	Musical instrument	India
	Pineapple	Nigeria
	Sesame seeds	India
A. niger	Aspergilloma (man)	?
	Chick (caseous lesions)	UK
	Cotton yarn	UK
	Date palm	Iraq
	Diesel fuel	New Zealand
	Garlic	South Africa
	Grapes	Spain, Italy, Portugal, France, Greece, Israel
	Hay	UK
	Mannihot utilissima	Malaysia
	Nails man	?
	Nasal cavity, dog	?
	Paint	UK
	Paper	Ireland
	Polyurethane foam	UK
	Polyurethane footwear	Germany
	Punica granatum fruit	India
	Radio set	Australia

Table 3. (Continued)

Species	Product	Country
	Sisal boles	Tanzania
	Sorghum vulgare	Fiji
	Welwitschia sp.	South Africa
A. pulverulentus	*Capsicum* sp.	Spain
	Maize	Russia
	Mosquitoes	India
	Psidium guajava	India

morphisms and PCR-based techniques led to the recognition of four species within the *A. niger* species complex (*A. niger*, *A. tubingensis*, *A. brasiliensis*, *A. foetidus*) (Kusters-van Someren et al., 1991; Megnegneau et al., 1993; Varga et al., 1993, 1994, 2000a–f; Accensi et al., 2001). Sequence comparisons of nuclear genes encoding various extracellular enzymes supported these results (Bussink et al., 1991; de Graaff et al., 1994; Gielkens et al., 1997). Yokoyama et al. (2001) distinguished two clusters within the *A. niger* species complex based on phylogenetic analyses of sequences of the mitochondrial cytochrome b gene (although they called the cluster involving the

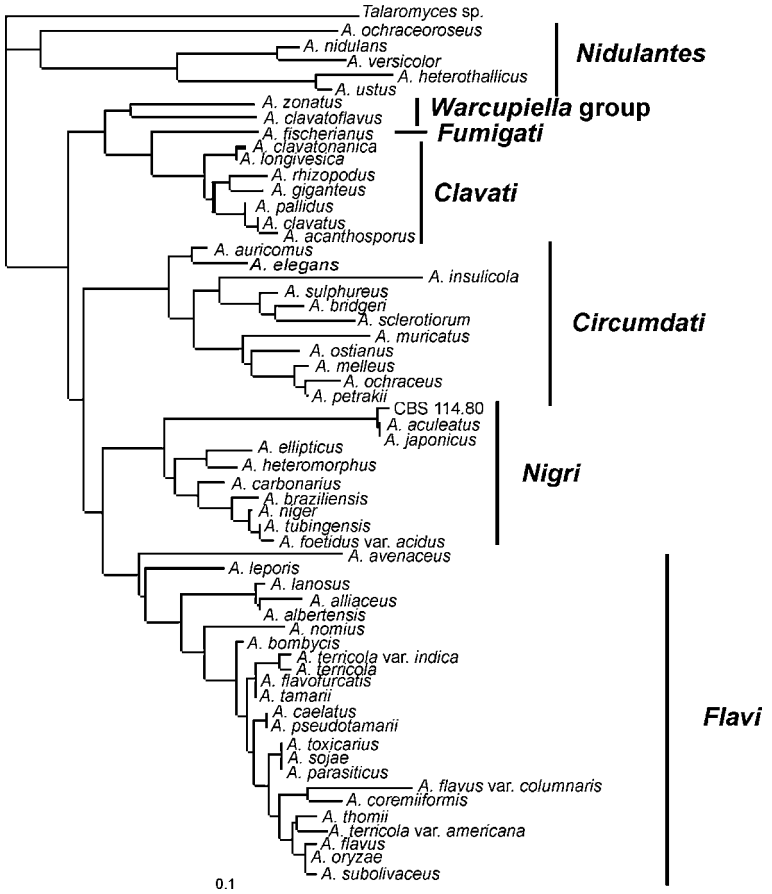

Figure 1. Neighbour-joining tree of the *Aspergillus* species treated in this review based on phylogenetic analysis of their ITS sequences. Sequences were aligned manually and analysed by DNADIST and NEIGHBOR of the PHYLIP software package (Felsenstein, 1995).

A. tubingensis type strain and other black Aspergilli 'the *A. awamori* clade'). Several well-known species names such as *A. awamori*, *A. usamii*, *A. phoenicis* and *A. ficuum* have been reduced to synonymy. Regarding other black *Aspergillus* species, phylogenetic analysis of sequences of the intergenic spacer region and the 5.8 S rRNA gene (ITS region) indicates that, apart from those mentioned earlier, at least five other species belong to this section (*A. heteromorphus*, *A. ellipticus*, *A. carbonarius*, *A. japonicus* and *A. aculeatus*; Figure 1) (Kevei et al., 1996; Hamari et al., 1997; Varga et al., 2000a–f; Parenicova et al., 2001). The uniseriate species *A. japonicus*, *A. aculeatus* and isolate CBS 114.80, which is considered to represent a new species, form one well-defined clade, while the biseriate species are on a separate branch (Parenicova et al., 2001; Figure 1). Although some toxins and other toxic agents, e.g., oxalic acid, nigragillin, malformins and naphtho-γ-pyrones (Schuster et al., 2002), have been reported to be produced by black Aspergilli, the production of ochratoxins is of real economic importance. Ochratoxin production has been observed only in *A. niger* and *A. carbonarius* (Ueno et al., 1991; Abarca et al., 1994, 1997; Horie 1995; Ono et al., 1995; Téren et al., 1996; Wicklow et al., 1996; Accensi et al., 2001). These species are now considered as major sources of ochratoxin contamination in tropical and subtropical foods including dried vine fruits, wines and coffee (Abarca et al., 1997, 2003; Pitt, 2000; Cabanes et al., 2002).

Our research focused on mtDNA variability of black Aspergilli. Wide-ranging variation in mtDNA was observed both among collection strains and in natural populations of the *A. niger* species complex (Varga et al., 1993, 1994). Most isolates were classified as *A. niger* or *A. tubingensis* according to their *Hae*III–*Bgl*II digested mtDNA patterns. *Aspergillus niger* and *A. tubingensis* were grouped into five and six mtDNA types, respectively. Six of the 73 Brazilian isolates exhibited mtDNA and rDNA types different from those of the other strains. These isolates were proposed to represent a new species (*A. brasiliensis*) within *Aspergillus* section *Nigri* (Varga et al., 1994). Physical maps of the different types of mtDNAs have also been determined (Hamari et al., 2003).

We examined the mtDNA haplotypes of 1104 black *Aspergillus* strains collected from soil samples world-wide (Table 4). While some mtDNA types were of universal distribution (*A. niger* mtDNA types 1a–1c and *A. tubingensis* mtDNA types 2a and 2b), others were found in geographically restricted areas. For example, *A. niger* mtDNA types 1d and 1e were only found in Indonesian and Hungarian soil samples, respectively, while *A. brasiliensis* was only detected in Brazil (Table 4). The data also indicate that some populations are very diverse in terms of the mtDNA haplotypes recovered (e.g., Indonesian or African isolates). Interestingly, all black *Aspergillus* isolates collected from Hungarian onions belong to the *A. niger* species (data not shown).

For *A. japonicus* and *A. aculeatus*, the strains were classified into eight different mtDNA RFLP groups based on their *Hae*III-digested mtDNA profiles (Figure 2). Hybridisation data suggest that seven of these mtDNA types have common features in their organisation, while mtDNA type 1, which was exhibited by the *A. aculeatus* type strain and two other strains, probably have quite different mtDNA structures (Figure 2; Hamari et al., 1997). The sizes of *A. japonicus* mtDNAs were in the range of 43–50 kb. Recent results indicate that there is more variability within the mtDNAs of *A. japonicus* (Hamari et al., 2001).

Among the 25 collection strains and field isolates of *Aspergillus carbonarius*, the *Hae*III-digested mtDNA profiles revealed only slight variations, except for one Indian field isolate (IN7), which exhibited completely different mtDNA patterns (data not shown). The mtDNAs of these strains were found to be much larger (45–57 kb) than those found in the *A. niger* aggregate. The physical maps of the mtDNAs of *A. carbonarius* strain IN7 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).

Aspergillus *section* Flavi

Aspergillus section *Flavi* historically includes species with conidial heads in shades of yellow–green to brown, and dark sclerotia. Isolates of the so-called domesticated species, such as *A. oryzae*, *A. sojae* and *A. tamarii* are used in oriental food fermentation processes and as hosts for heterologous gene expression (Campbell-Platt and Cook, 1989). Genetically modified *A. oryzae* strains are used for the production of enzymes including lac-

Table 4. Diversity of black Aspergilli in soil (additional data compiled from Varga et al., 1994; van Diepeningen, 1999)

Country	*Aspergillus niger*					*Aspergillus tubingensis*						*A. brasiliensis*	*A. japonicus*	*A. carbonarius*
	1a[1]	1b	1c	1d	1e	2a	2b	2c	2d	2e	2f			
Australia	–	2	2	–	–	–	–	1	7	2	7	–	–	7
New Zealand	–	7	–	–	–	1	–	–	–	–	–	–	–	–
Australasia total	–	9	2	–	–	1	–	1	7	2	7	–	–	7
Barbados	–	3	1	–	–	3	4	–	–	–	–	–	3	–
Brazil	7	9	35	–	–	8	8	–	–	–	–	6	–	–
Canada	–	–	4	–	–	6	–	–	–	–	–	–	–	–
Panama	–	–	2	–	–	–	1	–	–	–	–	–	16	–
United States	–	2	1	–	–	1	–	–	–	–	–	–	–	–
America total	7	14	43	–	–	18	13	–	–	–	–	6	19	–
Egypt	–	7	7	–	–	10	–	–	–	–	–	–	–	–
Eq. Guinea	–	6	3	–	–	–	–	–	–	–	–	–	–	–
Gabon	3	24	5	–	–	4	7	1	–	1	–	–	40	–
Cameroon	1	16	6	–	–	1	2	6	–	–	–	–	9	1
Morocco	–	–	–	–	–	6	2	–	–	–	–	–	6	1
Tunisia	–	7	6	–	–	12	11	–	–	–	–	–	7	–
Africa total	4	60	27	–	–	33	22	7	–	1	–	–	62	2
France	3	–	1	–	–	–	2	–	–	–	–	–	3	–
Great Britain	2	4	–	–	–	2	2	2	–	1	–	–	2	2
Hungary	2	2	–	–	2	2	–	–	–	–	–	–	5	–
Mallorca	–	7	3	–	–	–	–	–	–	–	–	–	–	–
the Netherlands	6	10	6	–	–	53	7	1	1	–	–	–	–	–
Switzerland	–	–	–	–	–	2	1	1	–	–	–	–	–	–
Europe total	13	23	10	–	2	59	12	4	1	1	–	–	10	2
India	–	–	–	–	–	–	–	–	–	–	–	–	13	1
Indonesia	47	142	112	2	–	82	49	7	9	33	1	–	66	11
Israel	–	1	–	–	–	4	–	–	–	–	–	–	–	2
Malaysia	–	5	–	–	–	1	–	–	–	–	–	–	–	–
Nepal	–	6	–	–	–	–	–	–	–	–	–	–	–	–
Asia total	47	154	112	2	–	87	49	7	9	33	1	–	79	14
World total	71	260	194	2	2	198	96	19	17	37	8	6	170	25

[1] *Aspergillus niger* mtDNA types 1a–1e, and *A. tubingensis* mtDNA types 2a–2f (Varga et al., 1994).

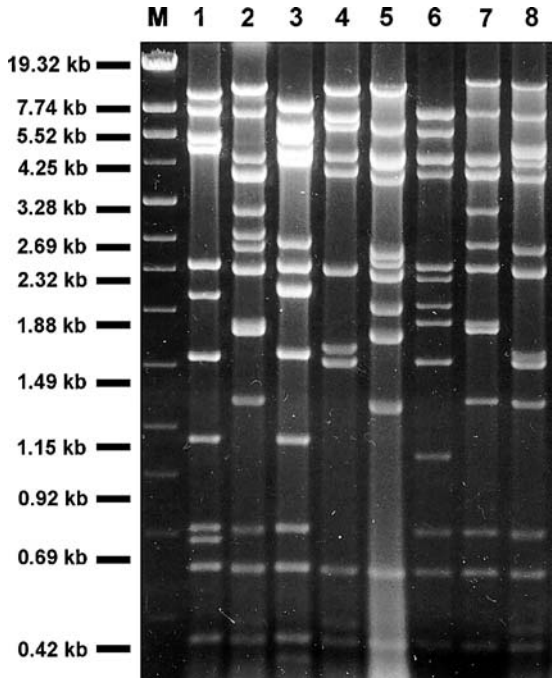

Figure 2. Mitochondrial DNA RFLP patterns of *A. aculeatus* and various *A. japonicus* isolates representing the 7 mtDNA types generated by *Eco*RV (Hamari et al. 2001). Lane M, lambda-pUC mix DNA molecular weight marker (Fermentas); lane 1, *A. aculeatus* CBS 172.66; lane 2, *A. japonicus* IN10; lane 3, *A. japonicus* 440; l; lane 4, *A. japonicus* 564; lane 5, *A. japonicus* 557; lane 6, *A. japonicus* 427; lane 7, *A. japonicus* Fr1.2.1; lane 8, *A. japonicus* 1287.

tase, pectin esterase, lipase, protease and xylanase (Pariza and Johnson, 2001). Several species of section *Flavi* produce aflatoxins, among which aflatoxin B1 is the most toxic of the many naturally occurring secondary metabolites produced by fungi. Aflatoxins are mainly produced by *A. flavus* and *A. parasiticus*, which coexist with and grow on almost any crop or food. However, aflatoxin is a problem because of its extensive preharvest contamination of corn, cotton, soybean, peanuts and tree nuts (Table 1), and because residues from contaminated feed may appear in milk. Because of its extreme toxicity, the presence of even very low amounts of aflatoxins is believed to pose a risk to human health. Thus its presence in the major plant commodities is a perceived consumer food safety issue and has caused severe economic losses to producers. Breeding for corn and peanut cultivars resistant to fungal infection or aflatoxin accumulation have the potential to lower aflatoxin levels in agricultural products. Corn and peanut germplasm lines resistant to aflatoxin accumulation have been developed. Resistance was found to be a polygenic trait (Chen et al., 2001; Gembeh et al., 2001; Tubajika and Damann, 2001). Resistant cultivars differ from susceptible ones in several ways including kernel pericarp wax content and the production of constitutive and inducible kernel proteins e.g. zeamatin (Brown et al., 1999; Tubajika and Damann, 2001). Corn genotypes GT-MS:gk and Yellow Creole, and several inbred lines e.g., Tex6 and MI82 (Hamblin and White, 2000; Naidoo et al., 2002; Windham and Williams, 2002) were proposed as potential sources of resistance, although one of them was found to be heterogeneous (Guo et al., 2002). Biological control of aflatoxin-producing *A. flavus* and *A. parasiticus* isolates by applying nontoxigenic *A. flavus* isolates in peanut fields (the so-called biocompetitive exclusion technique) is also promising (Horn et al., 1994, 2001). The use of nontoxigenic strains of *A. flavus* and *A. parasiticus* in biological control effectively reduces aflatoxin contamination in peanuts. Circle One Global, Inc. (COGI) of Cuthbert, Georgia, USA has applied for an exclusive license for the application of this technique in peanut fields.

Regarding the intra- and interspecific variability of *Aspergillus* section *Flavi*, ITS sequences of type strains or representative isolates of the species and subspecies currently assigned to this section have recently been analysed (Rigó et al., 2002). Phylogenetic analysis of sequence data indicated that species of *Aspergillus* section *Flavi* form distinct clades (Figure 1). The three main clades identified could also be distinguished by colony colour and their ubiquinone systems. The '*A. flavus*' clade includes species characterised with Q-10(H_2) as their main ubiquinone, and conidial colours in shades of green, along with dark sclerotia. Studies on the genetic variability of *A. flavus* indicated that the name is currently applied to a paraphyletic group of isolates that may produce aflatoxins B or G and have large or small sclerotia (Geiser et al., 2000). It was suggested that isolates with small sclerotia, able to produce both aflatoxins B and G (group II), deserve recognition as a new species (Geiser et al., 2000; Ehrlich et al., 2003). The other group (group I) includes isolates producing only aflatoxin B, with large or small sclerotia. This group also includes isolates of *A. oryzae*, and has

previously been described as having a recombining population structure (Geiser et al., 1998). Although several lines of evidence suggest that *A. oryzae* and *A. sojae* are morphological variants of *A. flavus* and *A. parasiticus*, respectively, it was suggested that these taxa should be retained as separate species because of the regulatory confusion that conspecificity might generate in the food industry (Geiser et al., 1998). The '*A. tamarii*' clade contains species with ubiquinone system Q-10(H_2), and conidia in shades of olive to brown, while the '*A. alliaceus*' clade consists of species with the Q-10 ubiquinone system, and conidia in shades of ochre (Kuraishi et al., 1990; Rigó et al., 2002). Two species of this clade, *Petromyces alliaceus* and *P. albertensis,* produce high amounts of ochratoxin (50–300 μg ml^{-1}), and are considered to be responsible for ochratoxin contamination of figs (Varga et al., 1996; Bayman et al., 2002). The recently described aflatoxin-producing species *A. pseudotamarii* and *A. bombycis* are closely related to *A. caelatus* and *A. nomius*, respectively (Ito et al., 2001; Peterson et al., 2001). Physiological properties and mycotoxin-producing abilities of these taxa justify their treatment as separate species (Ito et al., 2001; Peterson et al., 2001). While no evidence of genetic recombination was found in *A. bombycis*, cryptic genetic recombination was observed in *A. nomius* (Peterson et al., 2001). Recent data indicate that *A. nomius* is a paraphyletic group likely to contain several other species (Egel et al., 1994; Feibelman et al., 1998; Cotty and Cardwell, 1999; Ehrlich et al., 2003). Two other species, *A. avenaceus* and *A. leporis*, formed separate lineages not closely related to any of the main clades identified. It is suggested that *A. clavatoflavus* and *A. zonatus* be excluded from *Aspergillus* section *Flavi*, in accordance with previous suggestions (Kozakiewicz, 1989). Phylogenetic analysis of partial 28 S rRNA gene sequences supported these findings (Peterson, 2000). More recently, mtDNA polymorphisms of species assigned to *Aspergillus* section *Flavi* have also been examined (Quirk and Kupinski, 2002). Findings from these studies are also in agreement with those based on nuclear DNA sequence data.

Aflatoxin-producing species (*A. flavus*, *A. parasiticus*, *A. nomius*, *A. bombycis* and *A. pseudotamarii*) are scattered throughout the dendrogram indicating that aflatoxin-producing ability was lost (or gained) several times during evolution. Another aflatoxin-producing species, *A. ochraceoroseus* was not related to any of the species belonging to either sections *Flavi* or *Circumdati* (Klich et al., 2000). This is the only species known to accumulate aflatoxin B_1 and sterigmatocystin simultaneously. This species has previously been assigned to *Aspergillus* sections *Wentii*, *Cremei* or *Circumdati* (Samson, 1979; Christensen, 1981; Kozakiewicz, 1989). However, ITS and f28 S rDNA data indicate that this species is closely related to section *Nidulantes* (Figure 1). Its distant relationship to *Aspergillus* section *Flavi* is supported by the observation that *A. ochraceoroseus* DNA hybridised only weakly if at all to the *A. flavus* and *A. parasiticus* aflatoxin biosynthetic gene probes (Klich et al., 2000). Additionally, the order of genes of the aflatoxin biosynthetic gene cluster of *A. ochraceoroseus* was more similar to that of the sterigmatocystin gene cluster of *A. nidulans* than to that of *A. parasiticus* (Klich et al., 2000). These data indicate that the aflatoxin and sterigmatocystin biosynthetic pathway genes in *A. ochraceoroseus* are different from known pathway genes. Additionally, aflatoxin production has also been observed recently in *Emericella venezuelense* and *E. acristata* isolates, which are also closely related to *A. nidulans* (Klich et al., 2001; J.C. Frisvad and R.A. Samson, personal communication).

Aspergillus *section* Circumdati

Aspergillus section *Circumdati* historically includes species with biseriate conidial heads in shades of yellow to ochre. Species of *Aspergillus* section *Circumdati* are economically important as ochratoxin-producing spoilage organisms. The most frequently encountered species of the section, *A. ochraceus* is predominately isolated from desert and cultivated soil, but is also a postharvest pathogen of several agricultural products including cereals, coffee beans, corn and pecans (Kozakiewicz, 1989). Although different levels of susceptibility were observed among different varieties of various plants, e.g. bean, pearl millet, wheat and rape seed (El Kady et al., 1991; Madhyastha et al., 1993; Ansari and Shrivastava, 1994), no attempts have been made to breed any plants against ochratoxin accumulation. The genetic variability of the most well-known species of the section, *Aspergillus ochraceus*, was examined using genotypic methods (Varga et al., 2000a).

Based on mtDNA restriction profiles, PCR based techniques and ITS sequences, most isolates formed two distinct groups (data not shown). None of the isolates in group 2 produce ochratoxin, so these isolates could be used safely in steroid bioconversions.

Interspecific variability of species assigned to this section was examined using phenotypic features and sequences of the ITS region (Figure 1). Phylogenetic analysis of sequence data indicated that *Aspergillus campestris*, *A. lanosus*, *A. dimorphicus* and *A. sepultus* belong to *Aspergillus* sections *Candidi*, *Flavi* and *Cremei*, respectively (Figure 1; data not shown) (Peterson, 1995; Varga et al., 2000c). Two teleomorphic species previously assigned to this section, *Petromyces alliaceus* and *P. albertensis*, together with the asexual *A. lanosus* were found to belong to *Aspergillus* section *Flavi* (Varga et al., 2000b). These results were also supported by phenotypic data, and by the main ubiquinones observed in these species (Kuraishi et al., 1990). Species of the revised *Aspergillus* section *Circumdati* formed two main clades, which could also be distinguished based on phenotypic methods. A sexually reproducing ochratoxin producing species, *Neopetromyces muricatus* was also found to belong to this section (Frisvad and Samson, 2000; Varga et al., 2000c). All these species are characterised by the Q-10(H_2) ubiquinone system. *Aspergillus auricomus* and *A. elegans* did not belong to any of these clades. Ochratoxin-producing abilities of the isolates examined did not correlate with their taxonomic relationships based on ITS sequence data. However, a clade found not to produce ochratoxins during an earlier study of genetic variability of *A. ochraceus* was also identified in this study (Varga et al., 2000a, c).

Aspergillus *section* Clavati

Species belonging to *Aspergillus* section *Clavati* are characterised by clavate-shaped vesicles and large blue-green uniseriate conidial heads. *Aspergillus clavatus* is the most economically important species of the section, which can be isolated mainly from cultivated soil and dung, but also occurs on stored products (mainly cereals) with high moisture content (Lopez-Diaz and Flannigan, 1997). *Aspergillus clavatus* and its relatives produce a number of mycotoxins including patulin, kojic acid, cytochalasins, and tremorgenic mycotoxins (Flannigan and Pearce, 1994). The genotoxic mycotoxin patulin is receiving world-wide attention due to its frequent occurrence in apple juices (Beretta et al., 2000). Mycotoxin-producing abilities and phylogenetic relationships among isolates representing the six species currently assigned to this section have been examined (Varga et al., 2003a). A phylogenetic analysis of ITS sequence data indicated that most isolates belong to two main clades, which have also been identified previously using 28 S rDNA sequence data (Figure 1) (Peterson, 2000). *Aspergillus pallidus* isolates clustered together with strains of *A. clavatus*. The anamorph of *Hemicarpenteles acanthosporus* was also found to belong to this section. A new genus, *Neocarpenteles* was proposed to accommodate this species as *Neocarpenteles acanthosporum* (Udagawa and Uchiyama, 2002). Correlations were not observed between patulin production and the taxonomic position of the isolates tested, indicating that patulin-producing abilities were lost several times during the evolution of *Aspergillus* section *Clavati*.

Patulin-producing abilities of *Aspergillus* species were examined using analytical (HPLC, TLC) and molecular detection methods. For the latter, a primer pair developed based on iso-epoxydon dehydrogenase (IDH) gene sequences of *Penicillium expansum* by Paterson et al. (2000) was used. This gene encodes for a key enzyme of patulin biosynthesis (Sekiguchi and Gaucher, 1979). A good correlation was observed between patulin production and the presence of an IDH gene fragment in the isolates. *Aspergillus longivesica* was found for the first time to produce patulin (Varga et al., 2003a, b). Further studies are in progress to examine the applicability of this molecular detection method to agricultural products.

The diversity and evolutionary relationships within other *Aspergillus* sections including sections *Fumigati*, *Terrei* and *Aspergillus* have also been examined (Varga et al., 2000d,f; J. Varga et al., unpublished data).

Conclusions and future prospects

Phylogenetic analysis of sequences of the ribosomal RNA gene cluster and the β-tubulin gene clarified the taxonomic relationships of toxigenic Aspergilli causing pre- and postharvest contami-

nation of agricultural products. Molecular data enabled us to clarify the taxonomy of black Aspergilli, *A. flavus* and its relatives, and sections *Circumdati* and *Clavati* including ochratoxin and patulin-producing species, respectively. Phylogenetically unrelated species can produce the same mycotoxins. Aflatoxin and its precursors or intermediates (e.g. sterigmatocystins) are produced by species belonging to at least three different sections, while ochratoxin and patulin are produced by species assigned to seven and eight different sections, respectively. In addition, ochratoxins are produced by some penicillia (Ciegler, 1972; Varga et al., 2001a), while patulin is produced by a variety of other fungal genera (Steiman et al., 1989). Based on these observations, mycotoxin-producing abilities of the isolates appear to have been lost (or gained) several times during the evolution of the genus. The data indicate that it is not possible to develop rDNA based gene probes for the detection of ochratoxin or patulin-producing fungi, an approach applied successfully for fumonisin-producing fusaria. Instead, biosynthetic gene based probes will be necessary for molecular detection of these mycotoxin-producing organisms.

Biosynthetic genes of several mycotoxins including aflatoxin, trichothecenes, fumonisins and ergot alkaloids are clustered in the genome (Keller and Hohn, 1997). This observation led to the speculation that these clusters could be horizontally transferred as a unit to unrelated species, leading to the biosynthesis of the same mycotoxins in phylogenetically unrelated fungi (Walton, 2000). The organisation of the biosynthetic genes of patulin and ochratoxins is not known, although experiments are in progress in several laboratories to clarify the genetic background of biosynthesis of these mycotoxins. Identification of mycotoxin biosynthetic genes is essential for clarifying the evolution of mycotoxin biosynthesis in Aspergilli, and to develop specific gene probes for the detection of mycotoxin-producing Aspergilli in agricultural products.

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