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Biodiversity of *Aspergillus* species in some important agricultural products

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Abstract: The genus *Aspergillus* is one of the most important filamentous fungal genera. *Aspergillus* species are used in the fermentation industry, but they are also responsible of various plant and food secondary rot, with the consequence of possible accumulation of mycotoxins. The aflatoxin producing *A. flavus* and *A. parasiticus,* and ochratoxinogenic *A. niger*, *A. ochraceus* and *A. carbonarius* species are frequently encountered in agricultural products. Studies on the biodiversity of toxigenic *Aspergillus* species is useful to clarify molecular, ecological and biochemical characteristics of the different species in relation to their different adaptation to environmental and geographical conditions, and to their potential toxigenicity. Here we analyzed the biodiversity of ochratoxin producing species occurring on two important crops: grapes and coffee, and the genetic diversity of *A. flavus* populations occurring in agricultural fields. Altogether nine different black *Aspergillus* species can be found on grapes which are often difficult to identify with classical methods. The polyphasic approach used in our studies led to the identification of three new species occurring on grapes: *A. brasiliensis*, *A. ibericus*, and *A. uvarum.* Similar studies on the *Aspergillus* species occurring on coffee beans have evidenced in the last five years that *A. carbonarius* is an important source of ochratoxin A in coffee. Four new species within the black aspergilli were also identified in coffee beans: *A. sclerotioniger*, *A. lacticoffeatus*, *A. sclerotiicarbonarius*, and *A. aculeatinus*. The genetic diversity within *A. flavus* populations has been widely studied in relation to their potential aflatoxigenicity and morphological variants L- and S-strains. Within *A. flavus* and other *Aspergillus* species capable of aflatoxin production, considerable diversity is found. We summarise the main recent achievements in the diversity of the aflatoxin gene cluster in *A. flavus* populations, *A. parasiticus* and the non-toxigenic *A. oryzae*. Studies are needed in order to characterise the aflatoxin biosynthetic genes in the new related taxa *A. minisclerotigenes* and *A. arachidicola*.

Key words: aflatoxins, *Aspergillus* Sect. *Nigri*, Sect. *Flavi*, grapes, ochratoxin A, polyphasic identification coffee beans.

INTRODUCTION

Although they are not considered to be major cause of plant disease, *Aspergillus* species are responsible for several disorders in various plant and plant products. The most common species are *A. niger* and *A. flavus*, followed by *A. parasiticus*, *A. ochraceus*, *A. carbonarius*, and *A. alliaceus*. They can contaminate agricultural products at different stages including pre-harvest, harvest, processing and handling. Changes due to spoilage by *Aspergillus* species can be of sensorial, nutritional and qualitative nature like: pigmentation, discoloration, rotting, development of off-odors and off-flavors. However, the most notable consequence of their presence is mycotoxins contamination of foods and feeds. Because they are opportunistic pathogens, most of them are encountered as storage moulds on plant products (Kozakiewicz 1989). Various mycotoxins have been identified in foods and feeds contaminated by *Aspergillus* species, the most important are the aflatoxins and ochratoxin A (Varga *et al.* 2004). Aflatoxins B_1, B_2, G_1, G_2 are the most toxic and carcinogenic naturally occurring mycotoxins. Due to their extreme hepatocarcinogenicity, extensive research has been carried out on the natural occurrence, identification, characterisation, biosynthesis, and genetic regulation of aflatoxins (Payne & Brown 1998; Bennett & Klich 2003; Yu *et al.* 2004). Aflatoxins pose a risk to human health because of their extensive pre-harvest contamination of corn, cotton, soybean, peanuts and tree nuts, and because residues from contaminated feed may appear in milk. The most important aflatoxin producing species belong to *Aspergillus* section *Flavi*, including *A. flavus, A. parasiticus* and several other species (Bennett & Klich 2003). Extensive research has examined the role of the environment in fostering aflatoxin contamination episodes in corn and cottonseed (Cotty 2006; Cleveland *et al.* 2003). However, there is still no firm understanding of why contamination occurs during certain years, but not in others. In this regard, the conflicting involvements of insect damage to the crop, drought, and natural microbiological competition in creating favorable conditions for aflatoxin contamination complicate research efforts.

Ochratoxin A (OTA) is a potent nephrotoxin which may contaminate various food and feed products (grains, legumes, coffee, dried fruits, beer and wine, and meat). It also exhibits carcinogenic, teratogenic and immunotoxic properties in rats and possibly in humans (IARC 1993). The genotoxicity of OTA remains controversial (EFSA 2006). OTA is receiving increasing attention worldwide because of its wide distribution in food and feed and human exposure that most likely comes from low level of OTA contamination of a wide range of different foods (Petzinger & Weidenbach 2002). The economically most important OTA producers belong to *Aspergillus* sections *Circumdati* and *Nigri* (Samson *et al.* 2004; Frisvad *et al.* 2004).

In this review we briefly analyze the biodiversity and the phylogenetic relationships within two of the most important sections: *Flavi* and *Nigri* occurring in some important agricultural products including grapes and derived products, coffee beans and other agricultural products. We find that, while *A. flavus* is involved

Table 1. Species concepts of black aspergilli according to different authors.

Raper and Fennell (1965)	Al-Musallam (1980)	Kozakiewicz (1989)	RLFP* analysis	Samson *et al.* (2004)
A. japonicus	*A. japonicus* var. *japonicus*	*A. japonicus*	*A. japonicus*	*A. japonicus*
A. aculeatus	*A. japonicus* var. *aculeatus*	*A. atroviolaceus*	*A. aculeatus*	*A. aculeatus*
A. carbonarius	*A. carbonarius*	*A. carbonarius*	*A. carbonarius*	*A. carbonarius*
		A. fonsecaeus		
A. heteromorphus	*A. heteromorphus*	*A. heteromorphus*	*A. heteromorphus*	*A. heteromorphus*
A. ellipticus	*A. ellipticus*	*A. ellipticus*	*A. ellipticus*	*A. ellipticus*
	A. helicothrix	*A. helicothrix*		*A. sclerotioniger*
				A. homomorphus
***A. niger* aggregate:**				
A. niger	*A. niger* var. *niger*	*A. niger* var. *niger*	*A. niger*	*A. niger*
A. tubingensis	*A. niger* var. *niger f. hennebergii*	*A. niger* var. *tubingensis*	*A. tubingensis*	*A. tubingensis*
A. phoenicis	*A. niger* var. *phoenicis*	*A .niger* var. *phoenicis*	*A. foetidus*	*A. foetidus*
A. polverulentus	*A. niger* var. *phoenicis f. pulverulentus*	*A.niger* var. *polverulentus*	*A. brasiliensis*	*A. brasiliensis* (Varga *et al.* 2007)
A. awamori	*A. niger* var. *awamori*	*A. niger* var. *awamori*		*A. costaricaensis*
A. ficuum	*A. niger* var. *nanus*			*A. lacticoffeatus*
A. foetidus	*A. niger* var. *usamii*	*A. niger* var. *ficuum*		*A. piperis*
A. foetidus var. *pallidus*	*A. niger* var. *intermedius*	*A. citrus* var. *citrus*		*A. vadensis*
A. foetidus var. *acidus*	*A. foetidus*	*A. acidus*		*A. ibericus* (Serra *et al.* 2006)
		A. citrus var. *pallidus*		*A. uvarum* (Perrone *et al.* 2007)

*Results of various RLFP analysis by different authors: Kusters-van Someren *et al.* (1991); Megnegneu *et al.* (1993); Varga *et al.* (1993, 1994); Accensi *et al.* (1999); Parenicova *et al.* (1997, 2001)

Table 2. Morphological and biochemical diversity of black aspergilli occurring on grapes.

Species	Conidial size (µm)	Color and size of sclerotia (mm)	Source	OTA	Extrolites produced
Biseriates					
A. brasiliensis (Varga *et al.* 2007)	3.5–4.5	Found only in some strain, white, 1–1.5	Soil, grape	-	Naphtho-γ-pyrones (including aurasperone B), pyrophen, tensidol A & B, dihydrocarolic acid, aflavinine
A. carbonarius (Bainier) (Thom 1916)	7–9	Pink to brown, 1	Grape, cocoa, coffee,spices, palm oil, soil, air	+	Pyranonigrin A, naphtho-γ-pyrones
A. foetidus (Thom & Raper 1945)	3.5–4.5	Found only in some strain, white, 1–1.5	Tomato, grape, bottled fruits	-	Antafumicins, asperazine, funalenone, naphtho-γ-pyrones, pyranonigrin A
A. ibericus (Serra *et al.* 2006)	5–7	-	Grape	-	Naptho-γ-pyrones, pyranonigrin A
A. niger (Tieghem 1867)	3.5–5	-	Grape, cocoa, coffee, cereals, soil, paper, date palm	+/-	Funalenone, kotanins, naphtho-γ-pyrones, pyranonigrin A, pyrophen, tensidol A and B
A. tubingensis ((Schober) Mosseray 1934)	3–5	White to pink, 0.5–0.8	Grape, cocoa, coffee, soil, cereals	+/-	Asperazine, funalenone, naphtho-γ-pyrones, pyranonigrin A, tensidol A & B
Uniseriates					
A. aculeatus (Iizuka 1953)	4–5	-	Grape, papaya, pistachio, rice, tomato	-	Secalonic acid D& F
A. japonicus (Saito 1906)	4–5	white to cream, 0.5	Grape, green coffee berries, pineapple, sesame seed	-	Secalonic acid D& F
A. uvarum (Perrone *et al.* submitted)	3–4	dark brown to black	Grape	-	Secalonic acid D, geodin, erdin, asterric acid

in the majority of the agricultural contamination episodes, at least in the United States, the specific role of the S-strain and L-strain *A. flavus* has not yet been established.

Biodiversity of black aspergilli on grapes from Europe

Black aspergilli, which comprises species belonging to *Aspergillus* section *Nigri*, are worldwide distributed and have a significant impact on modern society. Many species cause food spoilage, and several are used in the fermentation industry (Bennett & Klich 1992), or candidate in the biotechnology industries. *A. niger* has even been granted the GRAS (Generally Regarded As Safe) status in certain industrial production processes by the Food and Drug Administration of the US government. Although the main source of black aspergilli is soil, they are among the most common fungi causing food spoilage and biodeterioration of other material. Various reports evidenced that members of the *A. niger* species complex, together with *A. carbonarius* and *A. japonicus/aculeatus* are frequently responsible for post-harvest decay of fresh fruit (apples, pears, peaches, citrus, grapes, figs, strawberries, tomatoes, melons, etc.) and some vegetables (especially onions, garlic, and yams); furthermore it is also among the commonest fungi isolated from dried fruit, beans, oil seeds and nuts (peanuts, pecans, pistachios, hazelnuts, almonds, walnuts etc.) (JECFA 2001). Recently, the significance of these species has completely changed since some of them, in particular *A. carbonarius,* is considered as the main source of OTA in grape and wine (Cabanes *et al.* 2002; Da Rocha Rosa *et al.* 2002; Battilani & Pietri, 2002; Magnoli *et al.* 2003, Leong *et al.* 2007a). Over the past five years several surveys and reports were published dealing with the epidemiology, ecology and distribution of black aspergilli occurring in wine grape and dried grape vineyards. Most of the surveys were from Mediterranean and South American countries and Australia. These studies clarified that the biseriate species *A. niger* "aggregate" and *Aspergillus carbonarius*, and the uniseriate species *A. aculeatus* and *A. japonicus* are the prevalent species occurring on grapes (Da Rocha Rosa *et al.* 2002; Battilani *et al.* 2003; Serra *et al.* 2005; Leong *et al.* 2006; Ponsone *et al.* 2007). In general species of the *A. niger* aggregate appear to be the dominant black *Aspergillus* species in all the countries studied, although some vineyards and years showed higher incidence of *A. carbonarius* isolates (Cabanes *et al.* 2002; Tjamos *et al.* 2004). In particular, the occurrence and frequency of ochratoxigenic strains in *A. carbonarius* and *A. niger* "aggregate" on grape proved to be similar in the Mediterranean countries and in Australia. On the contrary, *A. niger* was reported as the main ochratoxigenic species occurring on grapes in South America, while *A. carbonarius* occurred in Argentina mainly on retailed dried vine fruits with a low capacity to produce OTA (Chulze *et al.* 2006).

Ochratoxin A production of black aspergilli occurring on grapes was widely studied in the last years with sometimes ambiguous reports on the toxigenicity and the percentage of toxigenic strains among the species. The OTA producing strains of *A. carbonarius* ranged between 70 and 100 % when grown *in vitro* and tested using HPLC, while the range of producing strains was around 2–20 % for *A. niger* and *A. tubingensis* (Battilani *et al.* 2006; Perrone *et al.* 2006a). Some reports claimed the production of OTA also by *A. japonicus* but it has not yet been confirmed (Dalcero *et al.* 2002; Battilani *et al.* 2003). Recently, Ponsone *et al.* (2007) studying the occurrence and toxigenicity of *Aspergillus* species in Argentinean vineyards found that *A. niger* aggregate was the most frequent species on grapes with 27 % of the isolates producing OTA. The authors also confirmed the production of OTA by *A. japonicus* and *A. aculeatus* strains, but this work lacks molecular identification of the strains.

Black aspergilli are one of the more difficult groups concerning classification and identification. The taxonomy of *Aspergillus* section *Nigri* has been studied by many taxonomists, leading to various species concepts (Table 1). The difficulties in species recognition within the *Aspergillus niger* "aggregate" and the fact that most of the studies carried out on black aspergilli occurring on grapes lack molecular characterisation of the strains perplexed the extent of their natural occurrence and species distribution on grapes and food. In this respect, in 2001–2002 a large survey of black aspergilli occurring on grape from 107 vineyards in different European countries was performed within the EU project Wine-Ochra Risk (QLK1-CT-2001-01761) in order to characterise the species diversity and the potential toxigenic strains in the Mediterranean basin. This survey led to the identification of four main populations separated molecularly using AFLP, RFLP and sequence analyses (Bau *et al.* 2006; Perrone *et al.* 2006a, 2006b). These populations included *A. carbonarius*, *A. tubingensis*, *A. niger*, and a group of *Aspergillus* "uniseriate" isolates morphologically indistinguishable from *A. japonicus* and *A. aculeatus* but clearly separated by molecular techniques (Fig. 1). The genetic variability of these four populations observed by AFLP polymorphisms ranged from 15 to 35 % in *A. carbonarius*, *A. tubingensis* and the *Aspergillus* "uniseriate" group and 45–55 % in the *A. niger* group. The higher genetic diversity encountered in *A. niger* reflect the complexity of this taxon/group and the difficulties of identification at species level. The main OTA producer was *A. carbonarius* (95–100 % of strains), while the production of OTA was limited to a smaller proportion of strains in *A. niger* and *A. tubingensis* (10–15 % of the strains). No OTA production was observed in strains belonging to *Aspergillus* "uniseriate" group.

This species diversity was also revealed by sequence analyses of partial calmodulin (660 bp) and β-tubulin (1360 bp) genes which confirmed a significant molecular divergence of *Aspergillus* "uniseriate" group from other *Aspergillus* species. The description of a new species named *A. uvarum* isolated only from grape has been recently submitted (Perrone *et al.* 2007). Furthermore, during these surveys *A. ibericus,* a new species closely related to *A. carbonarius* and unable to produce OTA, was also described (Serra *et al.* 2006). Recently, a further characterisation of five atypical *A. niger* strains (Fig. 1) collected from Portugal grapes evidenced their similarity with other black *Aspergillus* isolates collected worldwide, which did not fit into any species of *Aspergillus* section *Nigri*. This new species called *A. brasiliensis* has recently been described and characterised by a polyphasic taxonomic approach by Varga *et al.* (2007) using macro- and micromorphology, secondary metabolite profiles, partial sequences of the β-tubulin, calmodulin and ITS genes, and AFLP analysis.

The morphological and biochemical diversity of black aspergilli occurring on grapes is being summarised in Table 2. They differ both in their micromorphology and in extrolite profiles, but for some species like *A. niger*, *A. tubingensis*, *A. foetidus* and *A. brasiliensis* molecular data (chemical or DNA based) are needed for their correct identification. The most frequently occurring species, as underlined above, are the "biseriate" *A. niger*, *A. tubingensis* and *A. carbonarius*, together with the "uniseriate" *A. japonicus*, *A. aculeatus* and the new species *A. uvarum* currently found only on European grapes (Perrone *et al.* 2006b). The other three species *A. brasiliensis*, *A. ibericus* and *A. foetidus* are occasionally found on grapes; in particular *A. ibericus* and *A. brasiliensis* were found only in some

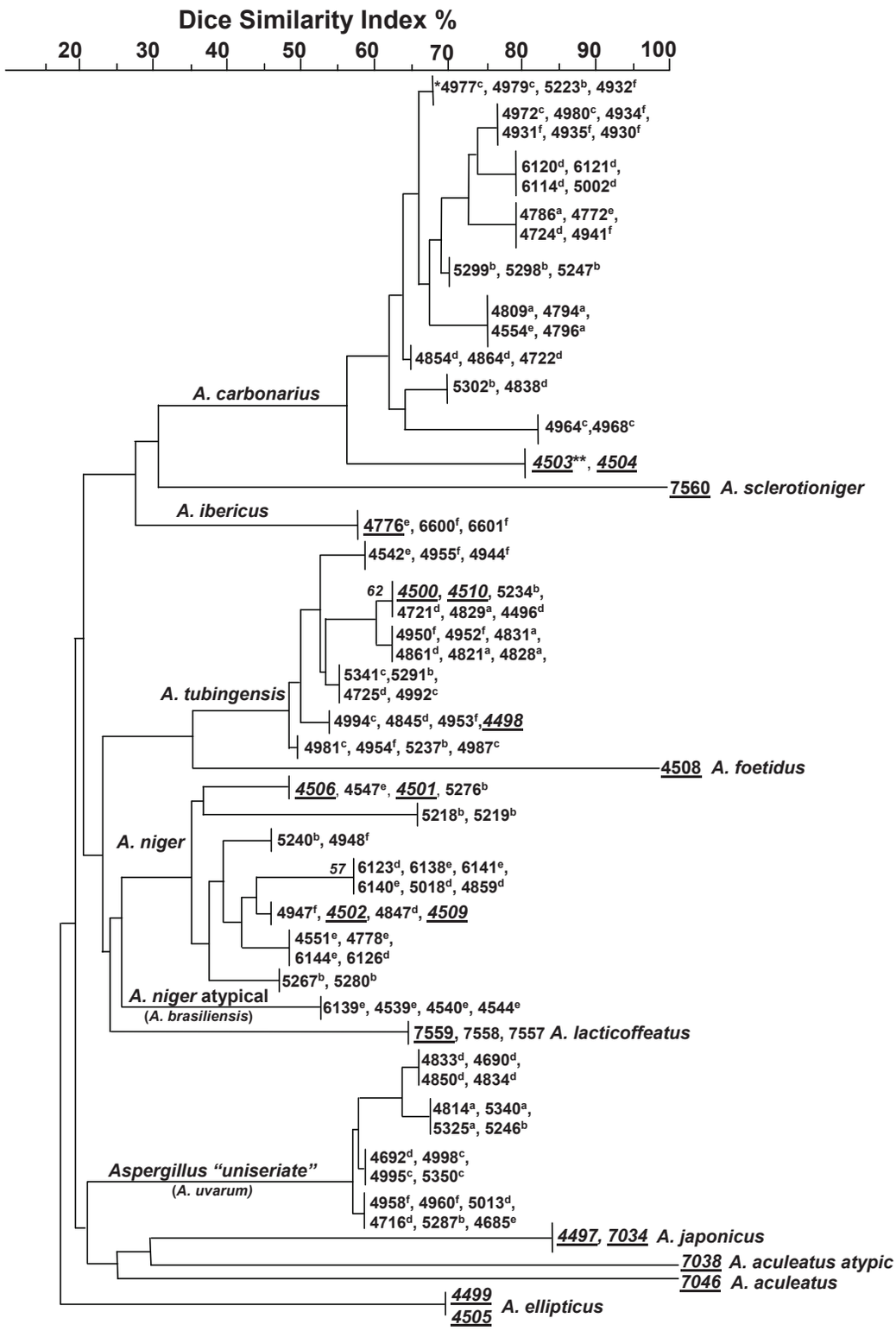

Fig. 1. AFLP dendrogram evidencing molecular biodiversity of representative black aspergilli isolated from grape in Europe.

grape samples from the Iberian Peninsula. *A. foetidus* was only found on grapes in South American surveys, but its identity has not been confirmed by molecular data (Chulze *et al.* 2006; Ponsone *et al.* 2007). The molecular diversity of the species within section *Nigri* is shown in Figs 1 and 2. The AFLP dendrogram (Fig. 1) summarises the data obtained using four different primer combinations for strains isolated from grapes in Europe in comparison with the type-strains of section *Nigri*. The same grouping was obtained by phylogenetic analysis of partial calmodulin sequence data (Fig. 2) and part of the β-tubulin gene (data not shown). These data indicate the need for molecular characterisation of these populations for a better and comprehensive identification of the complex of species involved in the *Aspergillus* black rot disease of grapes. In this respect, the molecular diversity of black aspergilli using partial calmodulin gene sequence data was widely exploited in the last three years and led to the development of primer pairs and SSCP tools for the rapid

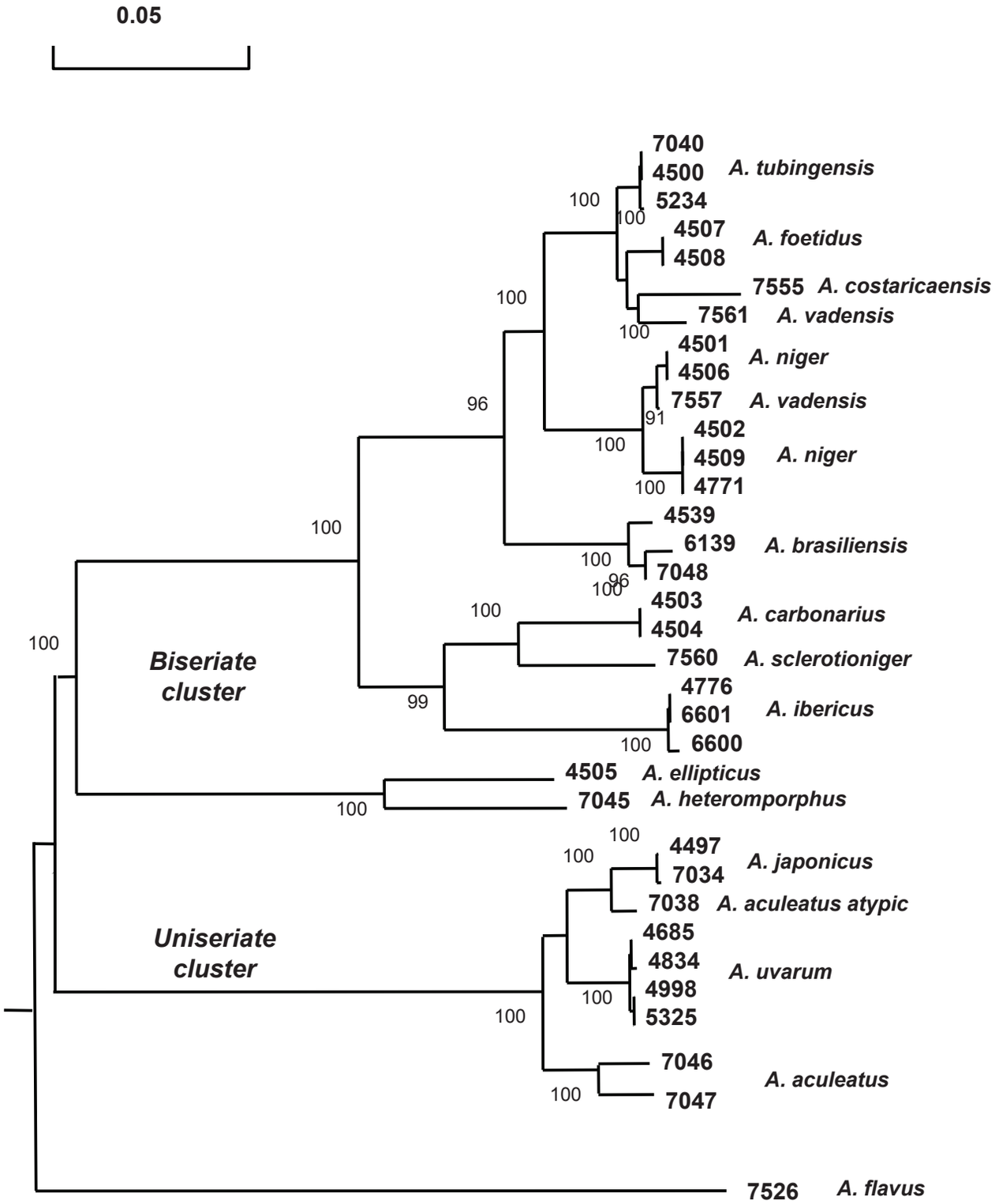

Fig. 2. Phylogenetic tree based on calmodulin sequence data of *Aspergillus* section *Nigri.* Numbers above branches are bootstrap values**.** Only values above 70 % are indicated.
***** Strains were labelled using accession number of ITEM**,** Culture Collection of Agri-Food Important Toxigenic Fungi, ISPA-CNR, Bari, Italy.

and robust identification of the main species within the section (Perrone *et al.* 2004; Susca *et al.* 2007a, 2007b). In particular the SSCP analysis was successfully used to detect sequence variations contained in an about 180 bp-region of the calmodulin gene in order to identify species of *Aspergillus* section *Nigri*. The method developed allows discrimination between 11 *Aspergillus* species belonging to section *Nigri*: *A. aculeatus, A. japonicus, A. uvarum*, *A. ellipticus, A. heteromorphus, A. carbonarius, A. ibericus, A. brasiliensis, A. niger*, *A. foetidus,* and *A. tubingensis*.

Furthermore, the distribution and species diversity of black aspergilli has recently been studied in 8 vineyards of Primitivo and Negroamaro varieties in Apulia (a region with high risk of OTA contamination in wine) during three grape growing seasons (2004–2006) within the Work supported by MIUR Project 12818 – SIVINA (D.M. n. 593/2000). *Aspergillus niger* "aggregate" was predominant from early veraison to ripening representing 80–85 % of contamination. *A. carbonarius* increased from veraison reaching 15–20 % at ripening stage, while the *Aspergillus* "uniseriate" were only found from early veraison to ripening decreasing from 15–20 % to 0–5 % of the population. About 600 strains of black aspergilli, representative of the sampling were isolated, identified and characterised for OTA production. Five percent of *A. niger* aggregate strains (360) resulted produced OTA, while all *A. carbonarius* strains (200) and none of the *Aspergillus* "uniseriate" strains (50) were positive to OTA production (Cozzi *et al.* 2007). Studies are in progress to characterise the *A. niger* "aggregate" strains to identify the percentage of *A. niger*, *A. tubingensis* and *A. brasiliensis* strains presence on this south Italian population from grapes. In order to establish a fully correct relationship between species and OTA production, the reported producing isolates and the chemical

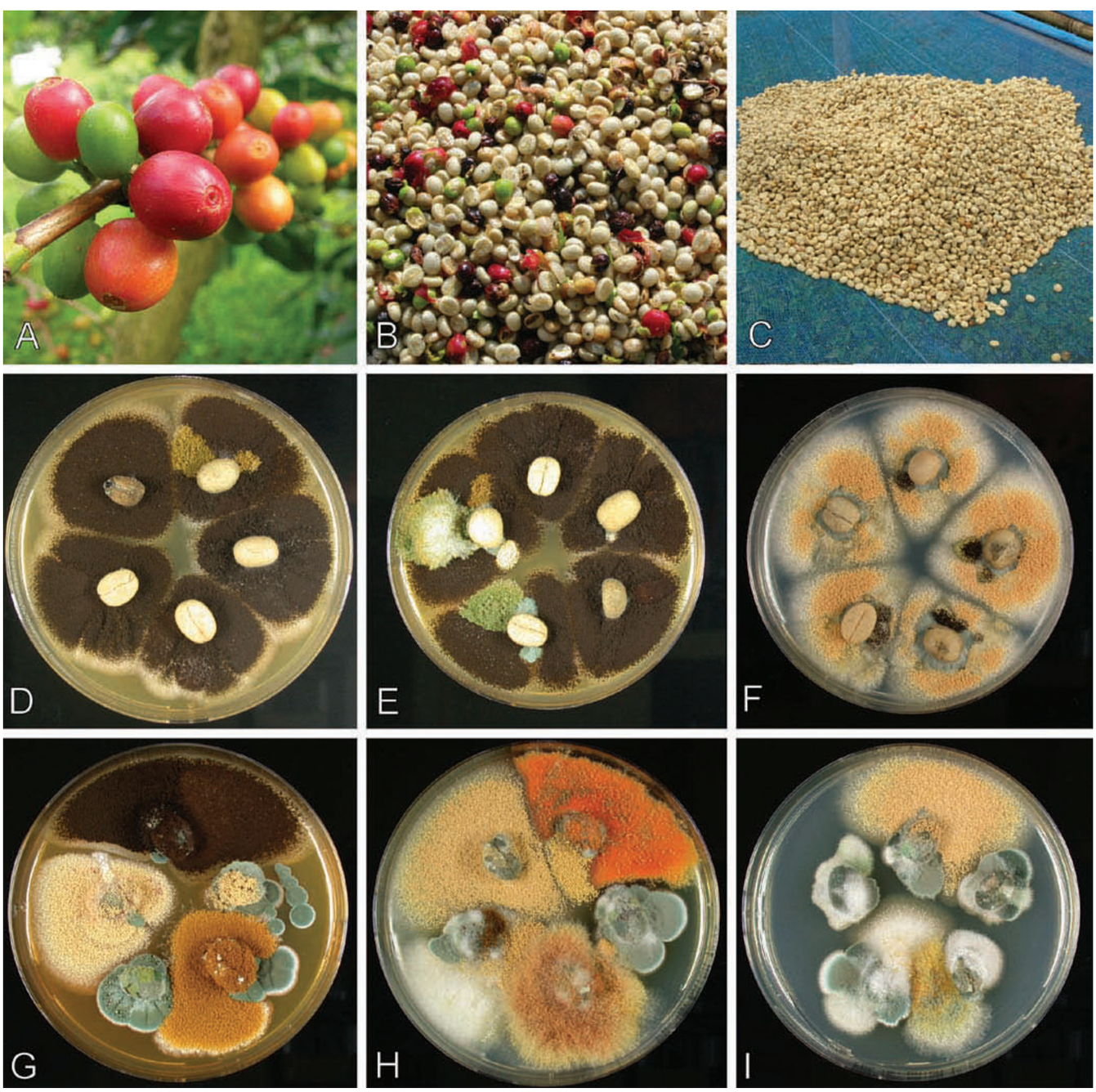

Fig. 3. A. Arabica coffee. Ripen cherries on tree. B. depulped cherries. C. dried parchment coffee beans in drying yard. D–F. direct plating of parchment coffee beans on MEA and DG18. G–I. direct plating of green coffee beans on MEA and DG18.

evidence need to be further confirmed as recommended by Frisvad *et al*. (2006).

In conclusion, a different species distribution of black aspergilli may occur in Europe in relation with metereological conditions (Battilani *et al.* 2006) and geographical areas: *A. tubingensis* and *A. niger* proved to be the dominant species in all countries, while *A. carbonarius* appears to be prevalent in southern Mediterranean areas (south of France, Southern Italy, Portugal and Greece). The distribution of *A. ibericus* is limited to Spain and Portugal, while *A. uvarum* occurs more frequently in Italy, France, Greece and Israel.

Biodiversity of black aspergilli on Thai coffee beans

Ochratoxin A contamination of coffee is a worldwide problem. The presence of OTA in green coffee bean has been reported by several authors in wide concentration ranging between 0.2 and 360 µg/kg (Levi *et al.* 1974; Taniwaki 2006). Extensive sampling of green coffee beans of both Arabica and Robusta types worldwide indicated that although OTA contamination is more frequent in some areas including mainly African countries, no producing country was found to be free of contamination (Taniwaki 2006). Although previously *A. ochraceus* was suggested to be sole source of OTA contamination on coffee (Stack *et al.* 1983), recent studies indicated that other species, including *A. steynii*, *A. westerdijkiae, A. carbonarius, A. lacticoffeatus, A. sclerotioniger* and *A. niger* are also able to produce OTA on coffee (Téren *et al.* 1997; Samson *et al.* 2004; Frisvad *et al.* 2004). Different types of black aspergilli were reported in coffee bean from different countries. *A. niger* and *A. carbonarius* occured most frequently. Extensive studies have been carried out on the mycobiota of Brazilian coffee recently. From the study of arabica coffee beans by Taniwaki *et al*. (2003), the results showed that *A. niger* was the species found most commonly (63 % of potential OTA producers), but only 3 % of them produced OTA. *A. ochraceus* also occurred commonly (31 % of isolates), and 75 % of those studied were capable of OTA production, a much higher percentage than reported elsewhere. *A. carbonarius* was found (6 % of isolates) only in the hottest region sampled, and only from beans in the drying yard or in storage. However, 77 % of the *A.carbonarius* isolates were capable of producing OTA. Other studies reported similar species distribution on Brazilian coffee beans. Martins *et al*. (2003) used a conventional method to identify fungal flora in coffee bean. The predominant fungal genus was *Aspergillus*, including *A. niger* (83.3 %), *A. ochraceus* (53.3 %) and *A. flavus* (25 %). The incidence of other genera was substantially lower than that of aspergilli. Magnani *et al.* (2005) isolated and identified *Aspergillus* spp. that contaminate coffee beans by sequencing the ITS region of the isolates. The incidence of potentially ochratoxigenic species was 82 % with *A. niger* being found most frequently, followed by *A. ochraceus* and *A. carbonarius*. However, the mycobiota of coffee beans in other countries or different type of coffee beans can be significantly different, e.g. in Ilic *et al.* (2007), Vietnamese Robusta coffee beans were studied, and *A. niger* was the only ochratoxigenic species recovered. However, in another study carried out by Leong *et al*. (2007b) *A. carbonarius* isolates have also been recovered from Vietnamese Robusta and Arabica coffee bean samples.

We examined the mycobiota of coffee beans came from Thailand to clarify which species could be responsible for OTA contamination in this region. Different types of coffee varieties are cultivated in Thailand. *Coffea arabica* is the one grown in the Northern mountain area with elevation of more than 2 500 feet above sea level and average temperature of 18–25 °C. *Coffea canephora* var. *robusta* is grown in the Southern region of Thailand characterised by a totally different geography and climate, with elevation of not more than 500 feet above sea level, much more rain fall and average temperature of 25–35 °C.

Molecular identifications have not been carried out in most studies dealing with the mycobiota of coffee beans, which could lead to mis-identification of some closely-related species. In this study we analyzed the black aspergilli isolated from coffee beans using a polyphasic approach including morphological examinations, analysis of extrolite profiles and sequence analysis.

For Arabica coffee bean samples from the North, two types of samples, parchment coffee bean and green coffee beans were examined. Overall results showed that approximately 75 % of the samples were contaminated by black aspergilli, and similar levels of contamination were observed for isolates belonging to *Aspergillus* section *Circumdati*. (Fig. 3) *A. niger* was the predominant species but there were sometimes more than two species colonising the same beans. The related species *A. tubingensis* and *A. foetidus* were also common. Discrimination between *A. niger* and related species could be easily archeived by partial β-tubulin gene sequencing (Fig. 4). All three species were clustered in separate clade. Compared to the molecular method using sequencing of the ITS regions and with RFLP analysis of rRNA by Magnani *et al.* (2005), β-tubulin gene sequencing is more applicable and proved to be more efficient for species identification. Surprisingly, *A. carbonarius* was not detected, possibly as a result of climate selection as *A. carbonarius* occurs more frequently in hot regions. So species belonging to both sections *Circumdati* and *Nigri* could be responsible for OTA contamination in this region.

Two types of Robusta coffee beans, dried coffee cherries and green coffee beans from the South were also studied. Black aspergilli were the predominant in the mycobiota, with 100 % contamination in coffee cherry samples and approximately 98 % contamination in green coffee bean samples (Fig. 5), much higher than those reported in Brazilian coffee beans (Taniwaki 2003). Both *A. carbonarius* and *A. niger* were common and predominant in both types of coffee bean. *Aspergillus* spp. belonging to section *Circumdati* (*A. westerdijkiae*) was detected only in one sample. These results confirm a previous study of Joosten *et al.* (2001), who found that most of the examined 14 green coffee samples came from Southern Thailand were contaminated by black aspergilli, half of them by *A. carbonarius.* Based on these data, we presume that black aspergilli, especially *A. carbonarius* may play an important role in OTA contamination of coffee beans in Southern Thailand.

As a result of the survey of ochratoxin-producing aspergilli in Thai coffee beans, we also identified 2 new black *Aspergillus* species. One of them (*A. aculeatinus)* is related to *A. aculeatus* and other uniseriate black aspergilli and could be recovered from both regions, while the other one (*A. sclerotiicarbonarius)* is related to *A. carbonarius* and *A. ibericus*, and was found only in the Southern region of Thailand. Formal description of these species is in progress.

The diversity of black aspergilli recovered from Thai coffee beans is summarised in Table 3. Comparing the occurrence of black aspergilli from different parts of Thailand, remarkable differences were observed. *A. carbonarius* and *A. sclerotiicarbonarius* were found only in Southern Thailand while *A. foetidus* was found only in the Northern region. These differences could be due to differences in the geography, climate and methods used for coffee processing in the two regions. The so-called wet method is used for Arabica coffee processing while the dry method is used for Robusta coffee processing. Principally, the dry method has three basic steps:

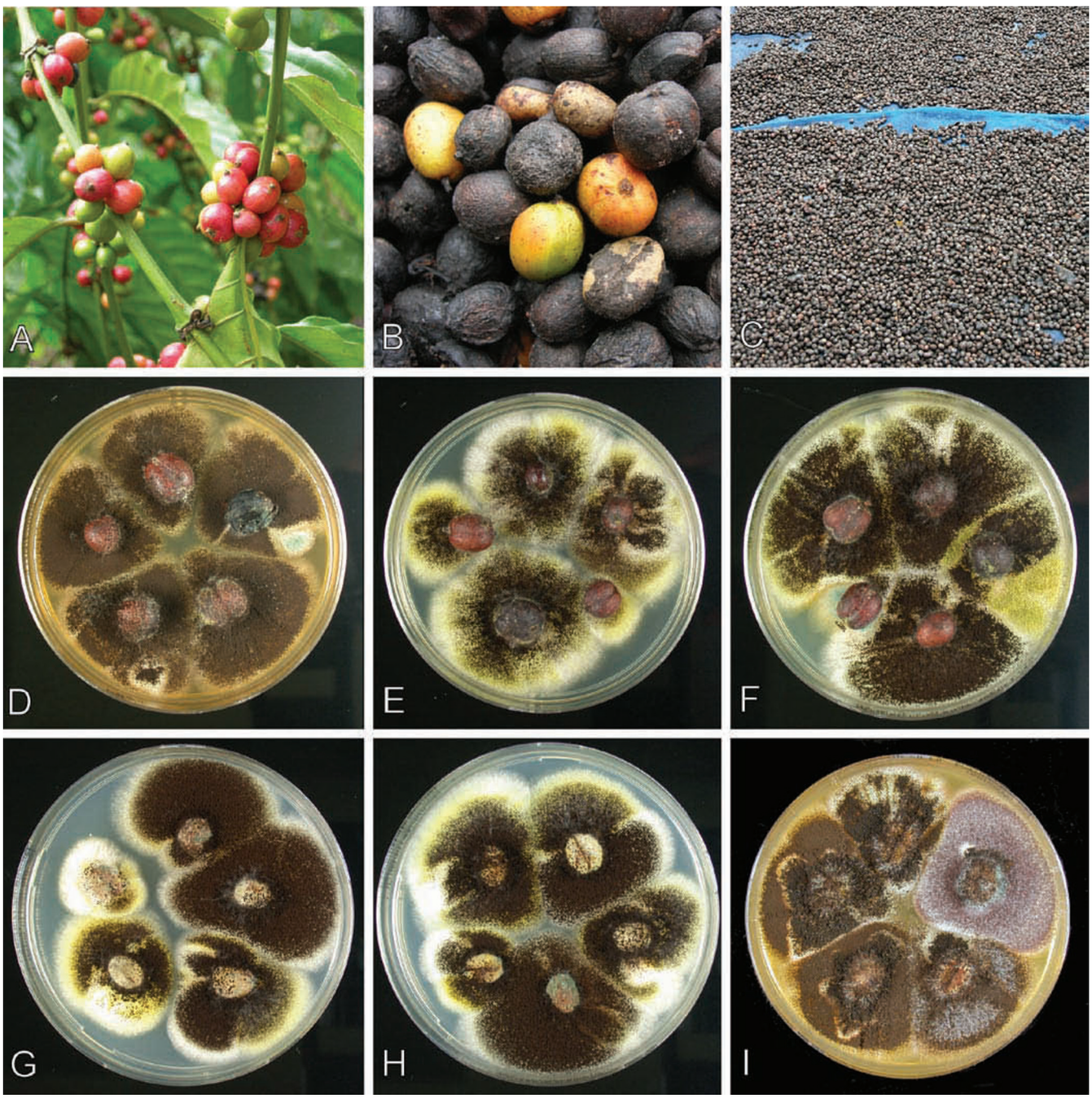

Fig. 4. Robusta coffee. A. Ripe cherries on tree. B. dried cherries. C. dried coffee beans in drying yard. D–F. direct plating of coffee cherries on MEA and DG18. G–I. direct plating of green coffee beans on MEA and DG18.

Table 3. Distribution and ochratoxin producing abilities of black aspergilli in Thai coffee beans.

Arabica (Northern Thailand)	**Robusta (Southern Thailand)**	**Ochratoxin A production**	**Ochratoxin B production**
A. niger (44 %)	*A. niger* (28 %)	++	++
A. tubingensis (19 %)	*A. tubingensis* (17 %)	-	-
A. foetidus (28 %)	-	-	-
A. aculeatinus (9 %)	*A. aculeatinus* (15 %)	-	-
-	*A. carbonarius* (35 %)	+++++	-
-	*A. sclerotiicarbonarius* (5 %)	-	-

In brackets = percent of isolates identified from each type of Thai coffee beans.

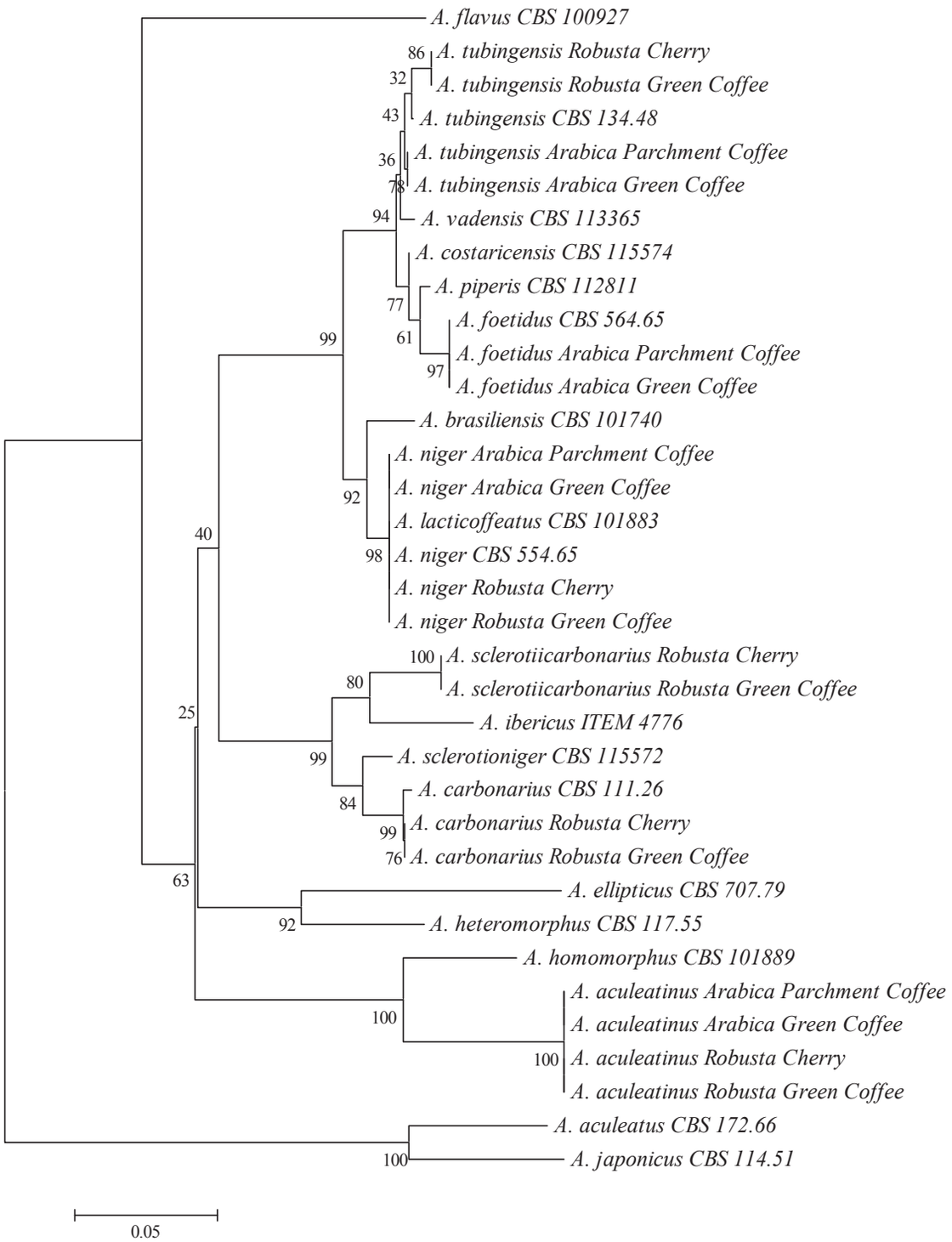

Fig. 5. Neighbour-joining tree based on phylogenic analysis of the partial β-tubulin gene sequences of black aspergilli recovered from Thai coffee.

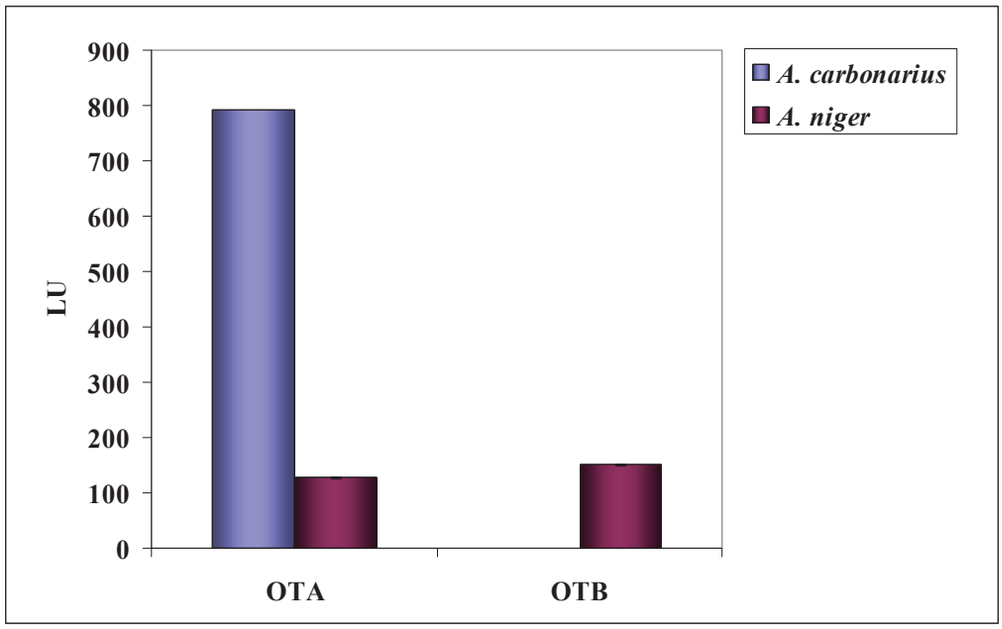

Fig. 6. Comparison of relative average abilities (expressed as Luminescence Unit from Fluorescence detector: LU) to produce ochratoxins of *A. carbonarius* and *A. niger*.

cleaning, drying and hulling. In Thailand, the whole Robusta cherry is directly dried with sun drying. Suarez-Quiroz *et al*. (2004) also reported that the dry method seemed to increase the presence of *A. niger* on the coffee beans. The wet method involves one more processing step: a fermentation step followed by cleaning to separate the beans from the pulp. This may cause changes in the natural substrate leading to changes in the species composition of the fungi colonising the beans. Differences in contact surfaces during processing may also play an important role in fungal contamination.

Ochratoxin producing abilities of black aspergilli isolated from Thai coffee beans were examined by the agar plug method of Smedsgaard (1997). OTA production was analysed by high performance liquid chromatography. A total of 83 isolates representing 6 species, *A. carbonarius*, *A. niger*, *A. tubingensis*, *A. foetidus*, *A. aculeatinus* and *A. sclerotiicarbonarius*, were analyzed. The results confirmed former studies, only *A. carbonarius* and *A. niger* could produce ochratoxins. In this study, 100 % of the *A. carbonarius* isolates tested could produce large amounts of OTA but none of them produced ochratoxin B (Table 3). This is in agreement with Joosten *et al*. (2001), who reported that all *A. carbonarius* strains isolated from Thai coffee produced a significant amount of OTA. Similarly, Pardo *et al.* (2004) found that all *A. carbonarius* isolates came from coffee beans from various countries produced OTA, and Leong *et al.* (2007b) also observed that almost all (110/113) of the examined *A. carbonarius* isolates came from Vietnamese coffee beans could produce OTA. However, Taniwaki *et al.* (2003) observed that only 77 % of the *A. carbonarius* isolates came from Brazilian coffee beans produced OTA. Differences in the ratio of *A. carbonarius* isolates able to produce OTA could be due to misidentification of the non-OTA producer *A. ibericus* as *A. carbonarius* in previous studies. In contrast with previous reports, where 2–3 % of *A.niger* isolates isolated from coffee beans could produce ochratoxins (Heenan *et al*. 1998, Taniwaki *et al*. 2003), 13 % of the *A. niger* strains came from Thai coffee could produce both OTA and ochratoxin B but in rather small amounts compared to *A. carbonarius* (Fig. 6). It is more likely that *A. carbonarius* is the source of OTA contamination in Thai coffee beans.

In conclusion, diversity of black aspergilli in coffee beans occurring in Thailand depends on a combination of various factors including coffee variety, geographic region, climate and processing method. Significantly, more Robusta than Arabica beans were infected by black aspergilli, in agreement with the findings of Leong *et al.* (2007b) and Pardo *et al.* (2004). *A. niger* and related species are more important as contaminants of Arabica coffee beans in Northern Thailand, while *A. carbonarius* is responsible for OTA contamination of Robusta coffee beans in Southern parts of Thailand.

Genetic diversity in *A. flavus* and implications for agriculture

Aspergillus flavus is the most common species associated with aflatoxin contamination of agricultural crops (Cotty *et al.* 1994, Cotty 1997) (Fig. 7). *A. flavus* populations are highly diverse and their stability in the soil and on the plant is not well understood. An atoxigenic relative of *A. flavus*, *A. oryzae*, is widely used in Asian fermentation processes. It is now increasingly clear that *A. oryzae* is not a separate species, but actually is only one many examples of atoxigenic variants of *A. flavus* (Geiser *et al.* 2000). As much as 40 % of the soil isolates of *A. flavus* are incapable of producing aflatoxins (Cotty *et al.* 1994). Addition of atoxigenic strains of *A. flavus* to the soil of susceptible crops to dilute out toxin-producing strains is being used to remediate aflatoxin contamination of cotton and peanuts (Cotty and Bayman 1993, Horn *et al.* 2000, Horn and Dorner 2002).

As with other haploid fungal species, genetic isolation in *A. flavus* may be maintained by a vegetative compatibility system (Leslie 1993). A typical soil population is usually composed of isolates from hundreds of different vegetative compatibility groups (VCGs) (Bayman and Cotty 1991). No genetic exchange was found among *A. flavus* atoxigenic VCG isolates and toxin-producing isolates collected from six geographically separated regions, suggesting that recombination among VCGs is rare (Ehrlich *et al.* 2007b).

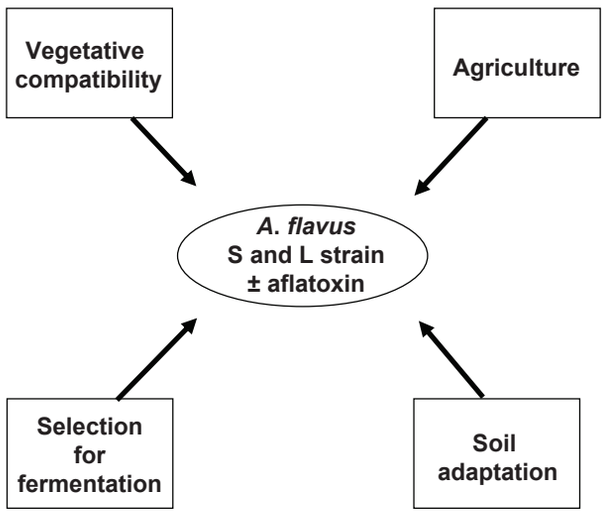

Fig. 7. Causes of *A. flavus* diversity.

A. flavus soil populations also contain isolates from two morphologically distinct sclerotial size variants, termed the L-strain for isolates with average sclerotial size greater than 400 µm and the S-strain for isolates with sclerotial size less that 400 µm (Cotty 1997). On typical laboratory growth media S-strain isolates produce higher levels of aflatoxins, more abundant sclerotia, and generally fewer conidia. Atoxigenic S-strain isolates are very rarely found in natural environments. Another consistent difference between S- and L-strain isolates is the size of deletion of portions of the genes, *norB* and *cypA* in the aflatoxin cluster. The size of the deletion in the *norB-cypA* gene was 1.5 kb for S-strain isolates and 0.8 kb for L-strain isolates. The gene *cypA* encodes a P450 monooxygenase that is necessary for formation of G aflatoxins. The deletion, therefore, is the reason why *A. flavus* is incapable of producing G aflatoxins. (Ehrlich *et al.* 2004). Most interestingly, *A. oryzae* isolates have an S-strain type deletion even though they morphologically resemble L strain *A. flavus* and make abundant conidia. When this gap size is included in a phylogenetic dataset that includes polymorphisms in the omtA gene region of the aflatoxin cluster, a clade was distinguished that contained members of both aflatoxin-producing S strain isolates and L strain isolates incapable of AF production. Another clade was distinguished that contained both *A. oryzae* and L-strain isolates incapable of AF production. From this data we reasoned that the L-strain is the ancestral species and that *A. oryzae* derived from an atoxigenic L-strain ancestor, whereas S-strain isolates derived from an aflatoxin-producing L-strain ancestor (Chang 2006).

The adaptation of *A. flavus* to the carbon-rich environment of certain agricultural communities is perhaps conducive to gene loss. Many of the isolates incapable of aflatoxin production have multiple mutations in their aflatoxin cluster genes. A careful study of deletion patterns in different L-strain *A. flavus* isolates from peanut fields found that, in these isolates, part or most of the aflatoxin biosynthesis gene cluster is missing (Chang *et al.* 2005). Isolates of *A. oryzae* also have large deletions of the aflatoxin gene cluster (Lee *et al.* 2006). In some of these isolates the remaining aflatoxin biosynthesis genes Neighboured the telomere. Proximity to the telomere may make the cluster more unstable. In *A. parasiticus* when normal development is thwarted, by forced repeated mycelial transfer, the resulting isolate permanently loses some of its normal developmental functions (Kale *et al.* 2003). It does not form conidia properly or make aflatoxins. The defects in these isolates remain to be determined.

Production of aflatoxin and its precursor metabolites is associated with increased production of conidia (Wilkinson *et al.* 2004), but so far, unlike the protective role of melanin, no evidence has been found that the conidia are protected by making the aflatoxin cluster metabolites. It is thought that the red pigmented dothistromin may be a virulence factor for *D. septosporum* responsible for its pathogenicity to pine (Bradshaw *et al.* 2002). Like dothistromin, most of the aflatoxin precursor metabolites are red or orange. Because of their color, the metabolites could have helped to foster dispersal. In addition, since section Flavi isolates are normally saprophytic, polyketide metabolites may increase fungal survival in soil. Such a benefit may be unnecessary in carbon-rich agricultural environments. In such environments, the ability to make aflatoxins could be a vestigial function. To support this conjecture, when section *Flavi* isolates are collected from non-agricultural soils, almost all of the isolates examined were capable of producing aflatoxins (Ehrlich *et al.* 2007a). Furthermore, in some soils, *A. flavus* was not the most prominent species. Understanding the role of aflatoxin production and in general secondary metabolite production may only be possible if attempts are made to duplicate in the laboratory the conditions of the natural environment in which these aspergilli evolved.

CONCLUSIONS

Complexes of pathogenic and opportunistic species of *Aspergillus* can colonise and induce disease symptoms in various plants and plant products, and produce toxic secondary metabolites (mycotoxins) in the infected tissue. In this chapter we evidenced how environmental conditions, geographical areas and crops can influence both fungal populations associated and production of mycotoxins. In this respect, the studies on economically important *Aspergillus* species by a polyphasic approach are innovative, strategic and helpful in assessing the biodiversity of the population/species and the potential risk of mycotoxin contamination of the agricultural products. In particular, the phylogenetic analysis of sequences of

β-tubulin and calmodulin genes, AFLP polymorphisms and extrolite profiles together with morphological analysis have led to reconsider in the last five years the taxonomy within the *Aspergillus* section *Nigri* (about seven new species has been described). Also the reports on the occurrence of black aspergilli in agricultural products and their potential toxigenicity must be reconsidered on the basis of the wide molecular biodiversity found within morphologically undistinguishable strains of this section. Furthermore, there is a need of molecular studies on South-American black *Aspergillus* populations occurring on grapes and other agricultural products in order to ascertain the species composition and potential toxigenicity. Finally, the presence of an *Aspergillus* uniseriate population typical of grapes in Europe, named *A. uvarum*, is an interesting finding that needs further investigation in grapevine areas outside Europe in order to evaluate the distribution of this new species at a global level.

Within *A. flavus* and other *Aspergillus* species capable of aflatoxin production, considerable diversity is found. Such diversity makes it more difficult to assign firm taxonomic identity to isolates from such populations. For example, should all *A. flavus* that are incapable of producing aflatoxins be considered to be *A. oryzae*? Such isolates are routinely found in agricultural fields, but only some are now classified as *A. oryzae*. We now know that loss of G-aflatoxin formation in *A. flavus* is a result of deletions in three genes encoding enzymes required for conv. of O-methylsterigmatocystin to aflatoxin G1 and G2, namely the cytochrome P450, *cypA*, and the reductases, *nadA* and *norB*. The aflatoxin clusters of *A. parasiticus* and the recently described related taxon, *A. minisclerotigenes* from Australia, West Africa, and Argentina that produces both B and G aflatoxins contain functional v.s of these genes (Pildain *et al.* 2007). Further studies are neded to clarify if the other newly described species, *A. arachidicola*, which is closely related to *A. parasiticus*, also carry these genes. The separation between *A. parasiticus* and *A. flavus* is estimated to have occurred more than 8 Mya. The conidia of *A. minisclerotigenes* resemble those of *A. flavus* while those of *A. parasiticus* are distinctly different in appearance. Further studies need to be done to sort out what selective factors, both environmental and genetic affect cluster gene stability in these related organisms. In this regard, we need to know if agricultural interactions play a role in causing gene instability? We expect that comparisons of different fungal genomes and developing a better understanding of regulatory relationships may help in answering some of these questions.

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