Phytopathol. Mediterr. (2010) 49, 393–405

Amplification of polyketide synthase gene fragments in ochratoxigenic and nonochratoxigenic black aspergilli in grapevine

Michelangelo STORARI[1], Ilaria PERTOT[2], Cesare GESSLER[1] and Giovanni A.L. BROGGINI[1]

[1]Plant Pathology Group, Institute of Integrative Biology, ETH Zurich, 8092 Zurich, Switzerland
[2]IASMA-FEM Research and Innovation Centre, Via E. Mach 1, 38010 San Michele a/A (TN), Italy

Summary. Members of the *Aspergillus* section *Nigri*, also known as black aspergilli produce Ochratoxin A (OTA), a contaminant of wine. Despite potentially severe health effects and economic losses caused by OTA in wine, almost nothing is known about the genetics that lies behind its biosynthesis in black aspergilli in grapevine. In this work, degenerate primer sets were used to amplify 49 polyketide synthase (PKS) gene fragments in reference strains of *A. carbonarius*, *A. niger* and *A. tubingensis*. Deduced amino acid sequences were then compared with those of *aolc35-12* and *aoks1*, two PKS genes involved in OTA biosynthesis in *A. westerdijkiae*. A putative homologue of *aolc35-12* was found in *A. carbonarius* (63% amino acid identity), the main OTA producer on grapes and in an ochratoxigenic *A. niger* strain (58%). In *A. niger* this fragment corresponded to *an15g07920*, a PKS already annotated in the sequenced *A. niger* CBS 513.88 genome as putatively involved in OTA biosynthesis. No *aolc35-12* candidates were found in atoxigenic *A. tubingensis* isolates and no putative homologues of *aoks1* were found in any of the screened strains. A screening of *A. niger* field isolates using specific primers for a*n15g07920* indicated that the absence of this gene is apparently related to a failure to produce OTA. The present work gives a first insight into the genetics of OTA biosynthesis in black aspergilli in grapevine and represents a starting point for further investigation of the OTA biosynthesis pathway and the development of molecular methods to detect the producers in vineyards.

Key words: mycotoxin, ochratoxin A, *A. carbonarius*, *A. niger*, *A. tubingensis*, *A. westerdijkiae*.

Introduction

Ochratoxin A (OTA) is a nephrotoxic, hepatotoxic, immunotoxic, teratogenic and carcinogenic mycotoxin classified by the International Agency for Research on Cancer (IARC) as a possible carcinogen in humans (group 2B). Moreover, OTA is thought to be linked to human diseases including Balkan endemic nephropathy (Pfohl-Leszkowicz and Manderville, 2007). OTA was isolated for the first time from cultures of *Aspergillus ochraceus* in 1965 (van der Merwe *et al.*, 1965). Since then, various species belonging to *Aspergillus* and *Penicillium* have been reported to be potential OTA producers (Bennett and Klich, 2003). OTA contaminates many food commodities including cereals, grapes, grape-derived products, coffee and animal products (Pitt, 2000). The organisms causing contamination vary between crops and locations (Bayman and Baker, 2006).

In wine OTA was first detected in 1996 (Zimmerli and Dick, 1996). Wine is considered the second major source of OTA in the European diet, after cereals. In 2005, EU limited the contamination of wine with OTA, setting a maximum limit of 2 μg L^{-1} [European Commission, 2005]. Agents that cause OTA contamination of grapes, and hence of wine, are members of the *Aspergillus* section *Nigri*, also known as black aspergilli. Black aspergilli isolated from grapes are usually classified as *A. carbonarius*, *A. niger*, *A. tubingensis* and *A. uvarum*.

Corresponding author: C. Gessler
Fax: +41 44 6321572
E-mail: cesare.gessler@agrl.ethz.ch

A. carbonarius is the main OTA producing species in grapes, since 70 to 100% of the strains of this species produce OTA. *A. niger* is less important as an OTA producer than *A. carbonarius*, because only 2–20% of its strains produce OTA (Perrone *et al.*, 2007). However, *A. niger* occurs more often in grapes than *A. carbonarius* and is considered the prime cause of OTA accumulation in Argentinian wine (Chulze *et al.*, 2006; Perrone *et al.*, 2007). *A. tubingensis* is closely related to *A. niger* and can be safely distinguished from it only with molecular techniques (Susca *et al.*, 2007). *A. tubingensis* has been reported as being potentially ochratoxigenic (Medina *et al.*, 2005; Perrone *et al.*, 2006; Martinez-Culebras *et al.*, 2007), but this conclusion has been challenged more recently (Nielsen *et al.*, 2009). Uniseriate strains of *A. uvarum* are generally considered nonochratoxigenic, despite some studies that reported that they had isolated some OTA-producing *A. uvarum* strains (Perrone *et al.*, 2007).

The capacity of black aspergilli to produce OTA has always been evaluated *in vitro* on standard agar media, particularly Yeast Extract Sucrose (YES) and Czapek Yeast Agar (CYA; Bragulat *et al.*, 2001), and only little is known about the genetics underlying OTA production in these species. Moreover, despite the importance of OTA as a mycotoxin, there is also an almost total lack of information about the general biosynthetic pathway.

Chemically, OTA consists of a polyketide derived from a dihydroiso-coumarin group linked to a modified phenylalanine (Niessen *et al.*, 2005). According to one theory the biosynthetic pathway includes the action of a polyketide synthase (PKS), a cyclase, a chloroperoxidase or halogenase, a peptide synthase and an esterase (Huff and Hamilton, 1979).

The first gene involved in OTA biosynthesis, a PKS (*pks*), was identified in *A. ochraceus* using a knock-out mutant (O'Callaghan *et al.*, 2003).

A study of *A. westerdijkiae*, a species recently dismembered from *A. ochraceus* (Frisvad *et al.*, 2004), reported that two different PKSs (*aoks1* and *aolc35-12*, which share more than 98% of identity with *pks* in *A. ochraceus*) are involved in OTA biosynthesis, but they did not give any indication about the chemical reactions that these two enzymes catalyze (Bacha *et al.*, 2009).

A PKS involved in OTA biosynthesis was also found in *P. nordicum* together with other putative OTA biosynthesis genes, including a non-ribosomal peptide synthase and a chloroperoxidase (Karolewiez and Geisen, 2005). A PKS showing a relatively high amino acid identity (63%) to *aolc35-12* was also found in the annotated genome of *A. niger* CBS 513.88 (*an15g07920*; Pel *et al.*, 2007). Atoui *et al.* (2006) identified five PKS gene fragments in *A. carbonarius*, including one (*AcKS10*) with 60% identity to *aolc35-12*. Recently, Gallo *et al.* (2009) described a novel PKS in *A. carbonarius* (*acpks*); the expression of this PKS is correlated with OTA production.

Fungal PKSs (type I PKSs) are multifunction enzymes consisting of different domains, including a β-ketoacyl synthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP) domain which repeatedly catalyze the cndensation of a two-carbon molecule (i.e. a CoA ester) to the growing chain (Kroken *et al.*, 2003). Fungal type I PKSs can be divided into two subgroups: non-reducing PKSs and reducing PKSs. These can be further subdivided into non-reducing subclades I and II, and reducing subclades I, II, III, and IV (Amnuaykanjanasin *et al.*, 2005).

The objective of the present work was to screen reference strains of black aspergilli isolated from grapes in order to detect PKS gene fragments and to identify putative homologues of *aolc35-12* and *aoks1*. Degenerate primers were used to amplify PKS fragments from genomic DNA of reference strains of *A. carbonarius*, *A. niger* and A. *tubingensis*. A DNA mixture of different *A. tubingensis* strains isolated from vineyards in northern Italy (Trentino region) in 2008 was also included in order to increase the number of PKS fragments obtained from this species. Deduced amino acid sequences of the PKS fragments were then compared to the amino acid sequences of Aolc35-12 and Aoks1. Moreover, since only a minority of *A. niger* strains produce OTA, the presence of *an15g07920* was assessed in *A. niger* field isolates and reference strains using specific primers.

Material and methods

Fungal strains, growth conditions, *in vitro* OTA production, DNA extraction and species confirmation

Reference strains were obtained from the ITEM culture collection (http://server.ispa.cnr.it/ITEM/Collection/) and isolates of black aspergilli

were obtained from vineyards in northern Italy (Trentino region) in September 2008. The reference strains included: *A. carbonarius* ITEM 5012, *A. tubingensis* ITEM 4496, *A. niger* ITEM 7096 and *A. niger* ITEM 7098. Isolates from the Trentino region were: 48 *A. niger* isolates (AnT_1 to AnT_48) and five *A. tubingensis* isolates (AtT_1 to AtT_5). OTA production of all strains was evaluated following Bragulat *et al.* (2001) on both CYA and YES agar. The HPLC system was equipped with Jasco PU-980 pumps, a Jasco AS-2055 PLUS sampling system, a C18 column (Spherisorb ODS-2, 250×4.6 mm, 5 μm) and a Jasco 2020 PLUS fluorescence detector (excitation 330 nm, emission 460 nm). The system was controlled by Jasco Chrompass software. The mobile phase consisted of 57% acetonitrile, 41% water and 2% acetic acid (isocratic) pumped at 1.0 mL min^{-1}. The injection volume was 20 μL. The limit of detection, determined by dissolving OTA standards in nonochratoxigenic *A. uvarum* extracts, was about 1 ng mL^{-1}.

For DNA extraction, isolates were grown for 7 d in 10 mL potato dextrose broth (PDB, Difco) at 25°C on a horizontal shaker (150 rpm). Mycelia were then recovered and lyophilized. DNA was extracted using the DNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany) following manufacturer's instructions. The species of each isolate used in this study was identified by sequencing the ITS rDNA and 5.8S rDNA regions using primers (ITS1 and ITS4) and conditions reported by White *et al.* (1990), and using species-specific primers according to Perrone *et al.* (2004) and Susca *et al.* (2007).

Identification of partial PKS sequences

PKS gene fragments were amplified from genomic DNA of *A. niger* ITEM 7096, *A. carbonarius* ITEM 5012 and *A. tubingensis* ITEM 4496 using degenerate primer pairs KAF1/KAR2 (Table 1) designed by Amnuaykanjanasin *et al.* (2005) on the conserved amino acid motifs EA/CHGTGT (KS domains) and FTGQGAQW (AT domains) present on fungal type I PKSs. The same strains were also screened with the primer pair KAOTAF/R (Table 1). Degenerate primer KAOTAF was designed on the amino acid motif EAHGTGT, and KAOTAR on the atypical amino acid motif FTGQGANW present on *aolc35-12*.

The amplification reaction and subsequent cloning steps were similar to those described by Amnuaykanjanasin *et al.* (2005), with some modifications: the 50 μL PCR reactions contained approximately 100 ng genomic DNA, 1e Dream Taq buffer (Fermentas, Hilden, Germany), 3.5 mM Mg^{2+}, 0.1 mM dNTPs, 0.4 μM of each primer and 0.07 U μL^{-1} of Dream Taq (Fermentas). PCR conditions were: 5 min at 95°C; 35 cycles of 0.5 min at 94°C, 1 min at 58°C, and 2 min at 72°C; and 7 min at 72°C. Total PCR products were purified using Illustra™ GFX™ PCR DNA and the Gel Band purification kit (GE Healthcare, München, Germany) and directly cloned into a pJET1.2 vector using a CloneJET™ PCR cloning kit (Fermentas) following manufacturer's instructions. For every transformation, 48 colonies were screened by Colony PCR using the pJET1.2 sequencing

Table 1. Primers used in this study.

Primer name	Sequence (5'-3')	Reference
KAF1	GARKSICAYGGIACIGGIAC	Amnuaykanjanasin *et al.* (2005)
KAR2	CCAYTGIGCICCYTGICCIGTRAA	Amnuaykanjanasin *et al.* (2005)
KAOTAF	GARGCICAYGGCACIGGIAC	This study
KAOTAR	CCARTTIGCICCYTGICCIGTRAA	This study
An_OTA_PKS_1f	TTCTGTCGAGGCAAAGTGTG	This study
An_OTA_PKS_1r	ACTGGGGAGACGAGGTTTTT	This study

primer pair according to manufacturer's instructions. Amplified fragments of the expected length (500–1000 bp) were then sequenced using the ABI PRISM BigDye Terminator v3.0 ready reaction cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. The sequencing reactions were purified through Sephadex G-50 DNA Grade F (Amersham Biosciences, Glattbrugg, Switzerland) and loaded on an ABI 3100 automated sequencer (Applied Biosystems). The test was repeated using a DNA mixture obtained by mixing the same amounts of genomic DNA of the five nonochratoxigenic *A. tubingensis* isolates (AtT_1 to AtT_5) from the Trentino region using KAOTAF/R primers.

Analysis of partial PKS sequences

Sequences of the identified PKS fragments were analyzed with the Sequencher version 4.2 software package (Gene Codes Corporation, Ann Arbor, MI, USA). Putative introns were determined using FSplice (www.softberry.com) and by homology with similar PKSs found in the NCBI database using BLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Deduced amino acid sequences derived from identified PKS gene fragments were aligned using ClustalW (BioEdit software version 7.0.0). EA/CHGTGT and FTGQGAN/QW motifs were eliminated from each sequence. The alignment output was then used in MEGA software version 4.0 (Tamura *et al.*, 2007) to generate phylogenetic trees (bootstrap analysis using Neighbour-Joining with 1,000 replicates and default settings).

Reference PKSs belonging to non-reducing and reducing clades were included in the phylogenetic analysis. These were AnPksST (*A. nidulans* PksST, AAA81586); AnWA (*A. nidulans* WA, 1905375A); ApPksL1 (*A. parasiticus* PksL1, Q12053); AtLovB (*A. terreus* LovB, AAD39830); AtLovF (*A. terreus* LovF, AAD34559); BfPKS3 (*B. fuckeliana* PKS3, AAR90239); BfPKS8 (*B. fuckeliana* PKS8, AAR90244); ChPKS2 (*C. heterostrophus* PKS2, AAR90257); ChPKS12 (*C. heterostrophus* PKS12, AAR90267); ChPKS15 (*C. heterostrophus* PKS15, AAR90269); GmFum1 (*G. moniliformis* Fum1p, AAD43562); GmPKS1 (*G. moniliformis* PKS1, AAR92208); GmPKS2 (*G. moniliformis* PKS2, AAR92209); GmPKS10 (*G. moniliformis* PKS10, AAR92217); PcMlcA (*P. citrinum* MlcA, BAC20564); and PcMlcB (*P. citrinum* MlcB, BAC20566).

ClustalW2 software version 2.0 (http://www.ebi.ac.uk/Tools/clustalw2/index.html; Larkin *et al.*, 2007) was used to compare the deduced amino acid sequences of the identified PKS gene fragments with Aolc35-12 and Aoks1.

Amplification of *an15g07920* fragments in *A. niger* strains

A specific primer pair, An_OTA_PKS_1f/r (440 bp), was designed on the *an15g07920* sequence (Table 1). These primers were used to screen the 48 *A. niger* isolates from vineyards of the Trentino region (AnT_1 to AnT_48) and the 2 *A. niger* reference strains (*A. niger* ITEM 7096 and *A. niger* ITEM 7098) for the occurrence of *an15g07920*. Each PCR reaction (25 μL) contained approximately 5 ng genomic DNA, 1× Dream Taq buffer (Fermentas), 1.5 mM Mg^{2+}, 0.1 mM dNTPs, 0.2 μM of each primer and 0.07 U μL^{-1} of Dream Taq (Fermentas). PCR conditions were: 5 min at 94°C; 25 cycles of 0.5 min at 94°C, 0.5 min at 63°C, and 0.5 min at 72°C; and 7 min at 72°C. The specificity of the reactions was confirmed by sequencing the amplified products.

Results

Fungal isolate identification and determination of OTA production capacity

The sequencing of ITS regions and the amplification with primers specific for *A. carbonarius*, *A. niger* and *A. tubingensis* were used to determine the identity of all black aspergilli isolates used in the study. Each isolate was tested on both CYA and YES agar media for its capacity to produce OTA according to Bragulat *et al.* (2001). The ochratoxigenic capacity of the reference strains was confirmed for *A. niger* ITEM 7096, *A. niger* ITEM 7098 and *A. carbonarius* ITEM 5012. OTA was also detected in colonies of one *A. niger* isolate from the Trentino region (AnT_1). OTA production by these strains ranged from 1 to 20 $\mu g\ g^{-1}$ agar. No OTA was detected in colonies of *A. tubingensis* ITEM 4496, in the five *A. tubingensis* isolates from Trentino (AtT_1 to AtT_5), or in the other 47 *A. niger* isolates (AnT_2 to AnT_48).

Table 2. PKS gene fragments identified in biseriate black aspergilli in grapes.

Species	PKS [a]	DNA source[b]	Degenerate primer pair	Fragment size (bp)	Closest PKS homolog in NCBI database (BlastN) [c]	Identity with Aolc35-12 (%)[d]	Identity with Aoks1 (%)[d]	Accession No.
Aspergillus carbonarius	acpks1	r	KAF1/KAR2	761	*A. niger, an03c0200* (69%)	24	25	HM011515
	acpks2	r	KAF1/KAR2	809		29	37	HM011516
	acpks3	r	KAF1/KAR2	696		29	38	HM011509
	acpks4*	r	KAF1/KAR2	747				HM026486
	acpks5*	r	KAF1/KAR2, KAOTAF/R	683	*A. niger* CBS 513.88, *an04g04340* (72%)			HM026487
	acpks6	r	KAF1/KAR2, KAOTAF/R	672	*A. niger* CBS 513.88, *an03g05440* (75%)	18	23	HM011510
	acpks+	r	KAF1/KAR2	669	*A. carbonarius*, acpks (98%)	32	29	
	acpks7	r	KAF1/KAR2, KAOTAF/R	714		23	24	HM011511
	acpks8*	r	KAF1/KAR2	897				HM026488
	acpks9	r	KAF1/KAR2	681	*A. niger* CBS 513.88, *an09g05730* (79%)	23	22	HM011512
	acpks10	r	KAOTAF/R	809		29	42	HM011517
	acpks11	r	KAOTAF/R	696	*A. niger* CBS 513.88, *an11g09720* (74%)	18	47	HM011513
	acpks12*	r	KAOTAF/R	747				HM026489
	acpks13	r	KAOTAF/R	726	*A. nidulans* FGSC, A4 TPA_reasm (75%)	28	26	HM011514
	acpks14	r	KAOTAF/R	753	*A. niger* CBS 513.88, *an15g07920* (71%)	63	25	GU991531
A. tubingensis	atpks1	r	KAOTAF/R	891	*A. niger*, *an15c0140* (83%)	38	29	HM011518
	atpks2	r	KAOTAF/R	813		32	30	HM011519
	atpks3	r/m	KAF1/KAR2, KAOTAF/R	690	*A. niger* CBS 513.88, *an11g09720* (84%)	32	43	HM011520
	atpks4	r	KAF1/KAR2, KAOTAF/R	684	*A. niger* CBS 513.88, *an13g02960* (94%)	34	37	HM011521
	atpks5	r	KAOTAF/R	666		33	33	HM011522
	atpks6	r	KAOTAF/R	657	*A. niger* CBS 513.88, *an13g02430* (87%)	31	28	HM011523
	atpks7	r	KAOTAF/R	743	*A. niger*, *an14c0090* (82%)	26	37	HM011524
	atpks8	r/m	KAF1/KAR2, KAOTAF/R	676	*A. niger* CBS 513.88, *an03g05440* (87%)	19	22	HM011525
	atpks9	r	KAF1/KAR2	606		31	31	HM011526
	atpks10	r	KAF1/KAR2	774	*A. niger*, *an03c0200* (83%)	25	25	HM011527
	atpks11*	r	KAF1/KAR2	719	*A. niger*, *an11c0160* (85%)			HM026490
	atpks12	r	KAF1/KAR2	798	*A. niger* CBS 513.88, *an15g05090* (81%)	24	27	HM011528
	atpks13*	r	KAF1/KAR2	658				HM026491

continued on the next page

Table 2. (continued)

Species	PKS [a]	DNA source [b]	Degenerate primer pair	Fragment size (bp)	Closest PKS homolog in NCBI database (BlastN) [c]	Identity with Aolc35-12 (%) [d]	Identity with Aoks1 (%) [d]	Accession No.
A. tubingensis	atpks14	r	KAF1/KAR2	802	*A. niger*, *an12c0070* (82%)	26	26	HM011529
	atpks15	r	KAF1/KAR2	696	*A. niger* CBS 513.88, *an14g04850* (85%)	28	26	HM011530
	atpks16	m	KAF1/KAR2	676	*A. niger* CBS 513.88, *an11g07310* (86%)	26	30	HM011531
	atpks17	m	KAF1/KAR2	666		29	41	HM011532
	atpks18	m	KAF1/KAR2	756		30	32	HM011533
	atpks19*	m	KAF1/KAR2	817				HM026492
A. niger	anpks1	r	KAF1/KAR2	725		33	28	HM011534
	an13g02960	r	KAF1/KAR2, KAOTAF/R	684	*A. niger* CBS 513.88, *an13g02960* (98%)	34	38	
	an08c0100	r	KAF1/KAR2	684	*A. niger*, *an08c0100* (97%)	33	41	
	an11c0160*	r	KAF1/KAR2	723	*A. niger*, *an11c0160* (95%)			
	anpks2*	r	KAF1/KAR2	876				HM026493
	an12c0070*	r	KAF1/KAR2	792	*A. niger, an12c0070* (96%)			
	an18g005200	r	KAF1/KAR2	669	*A. niger* CBS 513.88, *an18g00520* (98%)	33	28	
	an03c0200*	r	KAF1/KAR2	782	*A. niger, an03c0200* (96%)			
	an15g07920	r	KAF1/KAR2, KAOTAF/R	756	*A. niger* CBS 513.88, *an15g07920* (97%)	58	32	
	an15c0100*	r	KAF1/KAR2, KAOTAF/R	928	*A. niger*, *an15c0100* (96%)			
	anpks3	r	KAOTAF/R	729		26	37	HM011535
	an14c0090	r	KAOTAF/R	745	*A. niger*, *an14c0090* (97%)	26	39	
	an01g06930	r	KAOTAF/R	741	*A. niger* CBS 513.88, *an01g06930* (94%)	30	24	
	an11c0150*	r	KAOTAF/R	945	*A. niger*, *an11c0150* (96%)			
	an15c0140	r	KAOTAF/R	882	*A. niger*, *an15c0140* (95%)	37	29	

[a] Identified PKS showing more than 90% homology with already deposited sequences in the NCBI database were considered the same and given the original name.
[b] r, reference strains (*A. carbonarius* ITEM 5012, *A. tubingensis* ITEM 4496 and *A. niger* ITEM 7096); m, = DNA mixture of five *A. tubingensis* field isolates.
[c] Only results showing high-scoring database matches are given.
[d] Identity based on deduced amino sequences.
* No deduced amino acid sequence were obtained.
[+] Fragment of the same PKS as those identified by Gallo *et al.* (2008).

Identification of partial PKS sequences

Using KAF1/KAR2 and KAOTAF/R primers, a total of 49 PKS gene fragments, ranging from 600 to 1000 bp, were cloned (Table 2). Some PKS fragments were detected using both primer pairs, whereas the majority of them could only be detected with one of the two primer pairs. For every identified PKS fragment a similarity search was performed using BlastN in NCBI Database, and an accession number was assigned (Table 2).

PKS fragments showing a very high nucleotide identity (>90%) with the already noted PKSs in the sequenced *A. niger* CBS 513.88 genome are shown in Table 2 under their original name. Amino acid sequences could be deduced from the majority of the PKS fragments. For some PKS fragments (indicated with a * in Table 2) it was impossible to define a satisfactory amino acid sequence, usually because of the occurrence of premature stop codons. These PKS fragments were not further analysed.

Each deduced amino acid sequence was compared with the amino acid sequences of *aolc35-12* and *aoks1*. Two PKS fragments having a relatively high identity with *aolc35-12* were identified in the two ochratoxigenic reference strains. In *A. carbonarius* ITEM 5012, a PKS fragment possessing 63% amino acid identity with *aolc35-12* (*acpks14*) was cloned with the KAOTAF/R primer pair. In *A. niger* ITEM 7096, a PKS fragment corresponding to *an15g07920* and showing 58% amino acid identity was cloned with both primer pairs. *an15g07920* was already annotated in the sequenced *A. niger* CBS 513.88 genome as putatively involved in OTA biosynthesis because of its similarity to *aolc35-12*. All the other PKS fragments had an identity not greater than 38% and no putative homologues of *aolc35-12* could be identified in the DNA extracted from *A. tubingensis* ITEM 4496 or the DNA mixture of *A. tubingensis* isolates from Trentino.

The alignment of the deduced amino acid sequences of *acpks14*, *aolc35-12* (63% amino acid identity) and *an15g07920* (72% amino acid identity) is shown in Figure 1.

The amino acid sequence identities found between the PKS fragments and Aoks1 were between 22% and 47%, which makes it difficult to identify possible candidates.

A fragment of *acpks*, a PKS gene putatively involved in OTA biosynthesis in *A. carbonarius* (Gallo *et al.*, 2008), was also detected here (indicated in Table 2 with a +). This PKS fragment had a low degree of identity with both the Aolc35-12 (32%) and the Aoks1 (29%) sequences.

```
Acpks14     PVGDPLEVQAISAALG-MSRDSPLYVGSVKSVVGHLEGGAGMAGLISATMAVESKTIPPV
An15g07920  QVGDPLEVQAILAALG-VARDSPLYVGSVKSVLGHLEGGAGLAGLISATLAVESKMIPPV
Aolc35-12   PVGDPLEVQAIVSALIEQPRDTPLYVGSVKSVIGHLEGGAGLAGLISATLAVESKIIPPV

Acpks14     AGLQTLNPRIPQRPDLKFAKEATPWPREDVRRASINSFGFGGTNAHVVLEDVEGFFSDLF
An15g07920  AGLQSLNPKIPQRDDLKFAREATPWPRWDVRRASINSFGFGGTNAHAVVEDVEGFFADLF
Aolc35-12   AGLKTLNPRIVQREDLKFAQEAMPWPRDDIRRASINSFGFGGTNAHVVLDDVEGFLSEAL

Acpks14     GQQLPGALQLSEVTS-------KALVPSAMKSAVNGIP--ADQPPKESSV--NRLFVISA
An15g07920  GQYIPGALPAPEVDTSLETTPM------LSKPLMSGNASNQ---SVQSWS-TSRLFVISA
Aolc35-12   GPRVIGTLQMTGFSSRMTRRSLRGAEGSNSKAIANGESNGTSRIPLRSWIPENRVFILSA

Acpks14     FDEAGIQRNAASLASHLESMRAITGSDGEERLLNDLCHTLNEKRTRFDWRSYHVADSIDS
An15g07920  FDEAGIQRNTSALAEYLDSKSTTADTDGEDRLLNNLCHTLNEKRTRFDWRSYHVADSIAS
Aolc35-12   FDEAGLDRNAMSMISYLESLKLSGDPDLEEAFMSDLCHTLNAKRTMFDWRSYHVADTIEN

Acpks14     LRNSLQNPRPIRQSPAEKVVRFI
An15g07920  LRESLQHSRAIRQSSAPKPIRFV
Aolc35-12   LKKSLRNIRPYRRSTSSKAVRFI
```

Figure 1. Alignment of deduced amino acid sequences of *aolc35-12* (*Aspergillus westerdijkiae*), *an15g07920* (*A. niger*) and *acpks14* (*A. carbonarius*) PKS gene fragments.

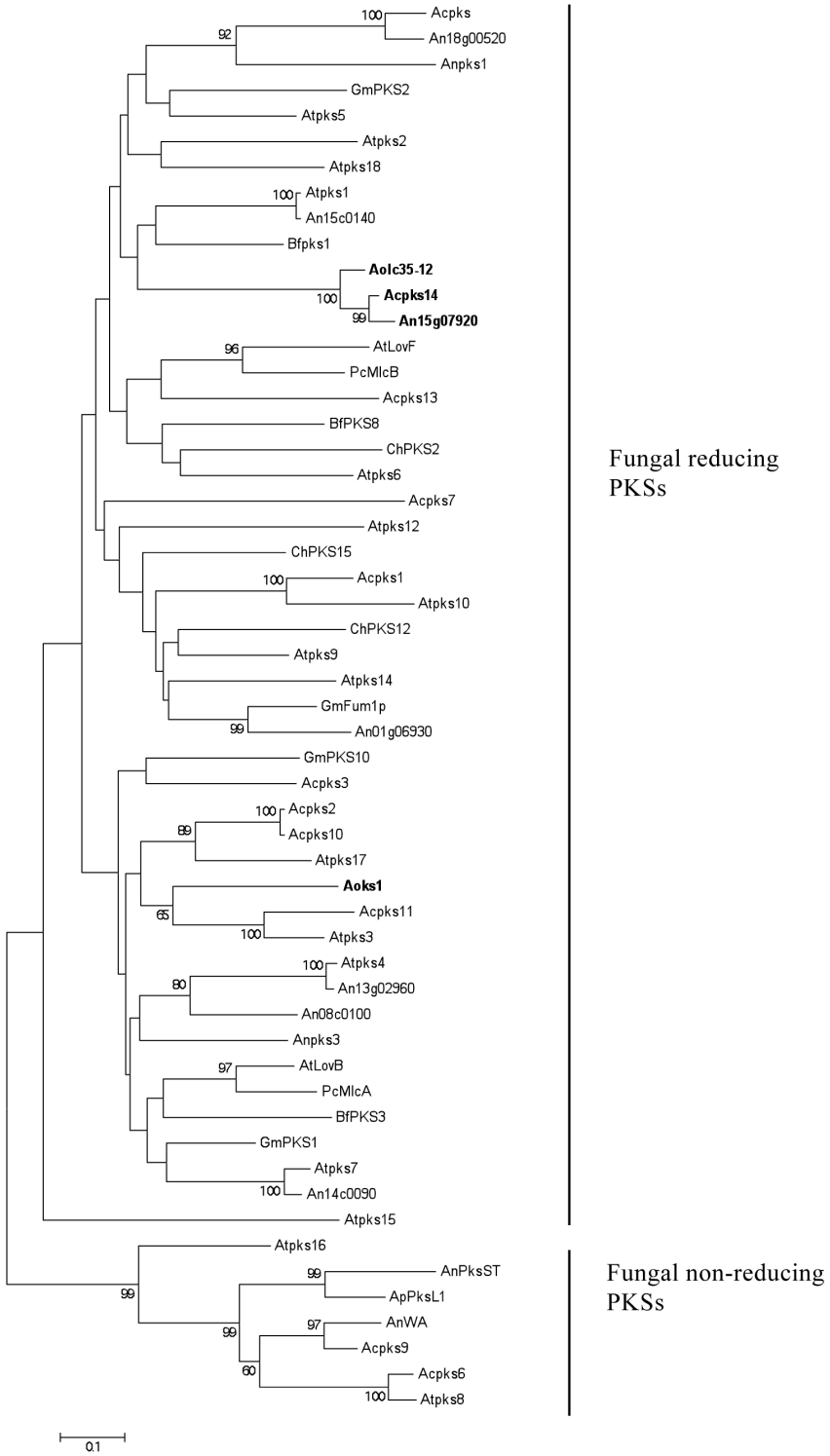

Figure 2. Neighbour-Joining phylogenetic tree (bootstrap analysis conducted using 1,000 replicates) of deduced amino acid sequences of PKS gene fragments identified in black aspergilli in grapes and fragments of fungal non-reducing and reducing reference PKS (from NCBI database). Only bootstrap values ≥50% are shown.

Phylogenetic analysis of PKS fragments

A phylogenetic tree, inferred from Neighbour-Joining (NJ) analysis, was generated using the deduced amino acid sequences of the PKS fragments and reference PKS sequences belonging to various PKS subclades. The deduced amino acid sequences of *aolc35-12* and *aoks1* were also included. Non-reducing PKSs were separated from reducing PKSs in two different clades of the tree (Figure 2). Inside the reducing PKS clade, it was not possible to resolve the reducing subclades I, II, III and IV, even with a Maximum parsimony approach (data not shown), as indicated also by the low bootstrap values. In this tree, all the PKSs involved (*aolc35-12* and *aoks1*) or potentially involved (*an15g07920* and *acpks14*) in OTA production were included in the non-resolved group of reducing PKSs. As expected from the amino acid sequence identities, *aolc35-12*, *an15g07920* and *acpks14* were separated from the other PKSs in a separate subgroup.

Occurrence of *an15g07920* in different *A. niger* strains

The occurrence of *an15g07920* was investigated in 48 *A. niger* isolates recovered from vineyards of the Trentino region (AnT_1 to AnT_48) and two reference *A. niger* strains (ITEM 7096 and ITEM 7098) using specific primers (An_OTA_PKS_1f/r, 440 bp). Amplification of *an15g07920* was seen only in the three ochratoxigenic isolates (ITEM 7096, ITEM 7098 and AnT_1, data not shown), suggesting that this gene is carried only by a small number of *A. niger* field isolates and that its occurrence is apparently limited to the ochratoxigenic strains. The PCR products were cleaned and sequenced to confirm the specificity of the amplification. This primer pair was also tested on *A. carbonarius*, *A. tubingensis* and *A. uvarum* DNA and here too no amplification was seen.

Discussion

A better understanding of OTA production genetics in black aspergilli in grapevine is very important. In the present study, PKS gene fragments in ochratoxigenic and nonochratoxigenic black aspergilli reference strains were amplified using degenerate primers and the occurrence of putative homologues of *aolc35-12* and *aoks1*, two PKS genes involved in OTA biosynthesis in *A. westerdijkiae*, was investigated. Published (Amnuaykanjanasin *et al.*, 2005) and newly designed degenerate primers were chosen according to two conserved domains present in Aolc35-12 (EAHGTGT and FTGQGANW) and Aoks1 (EAHGTGT and FTGQGAQW). The two degenerate primer pairs were applied to reference strains of *A. niger* (ochratoxigenic), *A. carbonarius* (ochratoxigenic) and *A. tubingensis* (nonochratoxigenic). The primer pair KAOTAF/R was also applied to a DNA mixture of nonochratoxigenic *A. tubingensis* field isolates in order to increase the number of cloned PKS fragments for this species.

In total, 49 PKS gene fragments were obtained. The great number of PKS fragments found in each strain supports the validity of the method and of the primers used to screen fungal genomes for PKSs. As expected, many PKS fragments found in *A. niger* ITEM 7096 were already annotated in the sequenced genome of *A. niger* CBS 513.88, whereas others were cloned for the first time.

The deduced PKS amino acid sequences were then compared with the Aolc35-12 and Aoks1 amino acid sequences. With the degenerate primer pairs employed, gene fragments having a relatively high identity to *aolc35-12* were obtained only in ochratoxigenic *A. niger* ITEM 7096 (*an15g07920*, already annotated as a putative homologue in *A. niger* 513.88 genome, 58%) and *A. carbonarius* ITEM 5012 (*acpks14*, 63%). These two PKS gene fragments shared 72% of amino acid identity. The identities displayed by these two PKS gene fragments were satisfactory, since in *A. ochraceoroseus*, genes of the aflatoxin/sterigmatocystin cluster showed an identity of between 57% and 91% with the genes of the *Emericella nidulans* sterigmatocystin cluster and between 35 and 92% with those of *A. flavus* AF gene cluster (Cary *et al.*, 2009). *Acpks14* could be a fragment of the same PKS identified by Atoui *et al.* (2006), since their fragment (*acKS10*) also showed about 60% identity with *aolc35-12* (KS domain). Further studies are needed to fully characterize this gene. All the other 48 PKS gene fragments obtained in this study had an identity with *aolc35-12* of less than 38%.

No putative homologues were determined for *aoks1* in any of the strains, since all PKS fragments had an identity of less than 47% (*acpks11*). This negative result is probably due to the high

variability of the KS-AT interdomain region amplified by these degenerate primers (Amnuaykanjanasin *et al.*, 2009). However, identification based on the amino acid identity of putative homologues of *aolks1* was expected to be difficult also because a similarity search in the NCBI database for this PKS gave different candidates in the *A. niger* genome, whereas for *aolc35-12* only *an15g07920* showed a relatively high degree of similarity. This indicates that the homologues of *aolc35-12* can be distinguished from other PKSs more easily than can homologues of *aoks1*.

In 2009, Gallo *et al.* reported a PKS gene fragment (*acpks*) in the *A. carbonarius* genome whose expression correlated with OTA production. A fragment of this gene was also isolated in the present study from *A. carbonarius* ITEM 5012. This fragment had a relatively low identity with both Aolc35-12 (32%) and Aoks1 (29%) and it is impossible to speculate about its role in OTA biosynthesis based only on a comparison of the amino acids. However, it is interesting to note that in our study a PKS fragment similar to *acpks* was found in ochratoxigenic *A. niger* ITEM 7096 (*an18g00520*, 82% identity), whereas no putative homologue was found in nonochratoxigenic *A. tubingensis*. Further studies on gene expression and knock-out mutants are needed to confirm and characterize the roles of these genes in OTA biosynthesis in black aspergilli.

As in other studies, all PKS gene fragments obtained were classified using a phylogenetic approach (Kroken *et al.*, 2003; Amnuaykanjanasin *et al.*, 2005). Reducing and non-reducing PKS fragments were separated into two clades. However, the PKS gene fragments belonging to reducing PKS subclades could not be clearly separated, probably because the amplified region between the KS and AT domain was more variable than a whole single domain; this was also reported by Mayer *et al.* (2007). As also reported in other studies (Atoui *et al.*, 2006; Bacha *et al.*, 2009), all PKSs involved or potentially involved in OTA biosynthesis were clustered together with the reducing PKSs. However, another tree (NJ, 1000 bootstraps) was constructed with the complete amino acid sequences of the reference PKSs, *aolc35-12*, *aoks1* and *an15g07920* (data not shown). Using longer amino acid sequences it was possible to fully separate these PKSs into each single family. In this tree, *aolc35-12*, *aoks1* and *an15g07920* clustered in the reducing subclade II. Also here, *aolc35-12* and *an15g07920* formed a separate group. This was in accordance with the finding that there was no other PKS having a relatively high identity with these two PKSs in the NCBI database.

The role of the various black aspergilli in OTA production in grapes is still debated. *A. tubingensis* was described by Perrone *et al.* (2006) and Medina *et al.* (2005) as a potential ochratoxigenic species, but the same strains used in those studies did not produce any detectable amount of OTA when tested by Nielsen *et al.* (2009). Moreover, in our laboratory *A. tubingensis* ITEM 4496, one of the strains described as ochratoxigenic by Perrone *et al.* (2006) did not produce any detectable amount of OTA on CYA and YES agar, which are standard growth media commonly used to discriminate between ochratoxigenic and nonochratoxigenic black aspergilli (Bragulat *et al.*, 2001). The misidentification of mycotoxigenic fungi can have different causes, including the wrong characterization of isolates (Frisvad *et al.* 2006) or unspecific chemical analyses (Nielsen *et al.*, 2009). Conflicting results about which fungal strains produce mycotoxins in pure culture may also be due to the detection threshold of the analytical method employed. In this work the detection threshold, determined by dissolving OTA in nonochratoxigenic fungal extract, was about 1 ng mL^{-1} (equivalent to 0.007 μg g^{-1}), which is more than 100 times less than the production range of the ochratoxigenic *A. niger* and *A. carbonarius* isolates found in this study and reported by other authors (Bragulat *et al.*, 2001; Bejaoui *et al.*, 2006). Therefore, OTA production by *A. tubingensis* ITEM 4496 reference strain should be very much less than that of ochratoxigenic *A. niger* and *A. carbonarius* strains. Moreover, fungal isolates as they age can partly or wholly lose their capacity to produce a particular compound (Samson *et al.*, 2007), making comparison between studies even more difficult.

The capacity of a strain or species to produce a mycotoxin can also be determined by assessing the occurrence of biosynthesis genes. It is not unusual to detect mycotoxin biosynthesis genes in atoxigenic, or apparently atoxigenic strains. Many *A. flavus* strains do not produce aflatoxin because of partial deletions in the aflatoxin gene cluster (Cary and Ehrlich, 2006). *A. sojae*, which is closely relat-

ed to *A. flavus* and *A. parasiticus* and which is involved in food fermentation, possesses the aflatoxin biosynthetic gene cluster but does not produce aflatoxin because of a premature stop codon in the pathway-specific aflR regulatory gene and a defect in the PKS gene (Chang *et al.*, 2007). An analogous situation is presented by *A. oryzae* (Chang and Ehrlich, 2010). Discontinuous toxin production, associated with a discontinuous distribution of biosynthesis genes, has also been reported recently for the production of fumonisins in *A. niger* (Susca *et al.*, 2010). Moreover, epigenetic mechanisms may also be involved in the regulation of the expression of secondary metabolites biosynthesis genes (Schwab and Keller, 2008). These findings suggest that PKS gene fragments with a significantly high identity to *aolc35-12* could also be found in DNA from nonochratoxigenic *A. tubingensis* strains. However, degenerate primers applied to DNA from nonochratoxigenic *A. tubingensis* ITEM 4496 and to the DNA mixture from nonochratoxigenic *A. tubingensis* field isolates detected no candidate. Ochratoxigenic ability of *A. tubingensis* was also reported in other works (Martinez-Culebras and Ramón, 2007; Selouane *et al.*, 2009). These degenerate primers could be further used in the ochratoxigenic strains to isolate putative homologues of *aolc35-12*.

In this work *An15g07920* was the best candidate as a homologue of *aolc35-12* in OTA production in *A. niger* species. Since only a minority (2-20%) of *A. niger* strains in grapes are reported to produce OTA (Perrone *et al.*, 2007), it was interesting to have an insight into the frequency of the occurrence of this gene in *A. niger* strains in grapevine. Fifty *A. niger* isolates (48 field isolates and two reference strains) were screened with a primer pair specific for *an15g07920*. Amplification was seen only in the three isolates that produced OTA *in vitro*. This suggests that *an15g07920* is not widespread in populations of *A. niger* in grapes and that the lack of this gene correlates with an inability to produce OTA. However, a larger screening of grape-associated *A. niger* strains in different geographical areas is needed to confirm this correlation and to evaluate the suitability of this gene as a molecular marker for OTA production in *A. niger*.

In conclusion, the study detected several novel PKS gene fragments present in black aspergilli in grapevine, and found some putative homologues of *aolc35-12*, based on their amino acid identity. These findings will serve as a basis for a better understanding of OTA biosynthesis in black aspergilli in grapes and will make it possible to develop molecular markers that will detect and quantify the occurrence of the OTA producers in vineyards.

Acknowledgements

This work was funded by the Autonomous Province of Trento, project ENVIROCHANGE, Call for proposal Major Projects 2006. We thank Renato Guidon from Plant Pathology, ETHZ for valuable technical assistance in microbiological work, Björn Studer from the Institut für Terrestrische Ökosysteme, ETHZ for valuable technical assistance in HPLC analysis, Thomas Paternoster and Stefano Torriani for critically reading the manuscript. We are grateful to Dr. Antonio Logrieco for providing us with the ITEM collection isolates.

Literature cited

Amnuaykanjanasin A., J. Punya, P. Paungmoung, A. Rungrod, A. Tachaleat, S. Pongpattanakitshote, S. Cheevadhanarak and M. Tanticharoen, 2005. Diversity of type I polyketide synthase genes in the wood-decay fungus *Xylaria* spp. BCC 1067. *FEMS Microbiology Letters* 251, 125–136.

Amnuaykanjanasin A., S. Ponghanphot, N. Sengpanich, S. Cheevadhanarak and M. Tanticharoen, 2009. Discovery of insect-specific polyketide synthases, potential PKS-NRPS hybrids, and novel PKS clades in tropical fungi. *Applied and Environmental Microbiology* 75, 3721–3732.

Atoui A., H.P. Dao, F. Mathieu and A. Lebrihi, 2006. Amplification and diversity analysis of ketosynthase domains of putative polyketide synthase genes in *Aspergillus ochraceus* and *Aspergillus carbonarius* producers of ochratoxin A. *Molecular Nutrition & Food Research* 50, 488–493.

Bacha N., A. Atoui, F. Mathieu, T. Liboz and A. Lebrihi, 2009. *Aspergillus westerdijkiae* polyketide synthase gene "aoks1" is involved in the biosynthesis of ochratoxin A. *Fungal Genetics and Biology* 46, 77–84.

Bayman P. and J.L. Baker, 2006. Ochratoxins: a global perspective. *Mycopathologia* 162, 215–223.

Bejaoui H., F. Mathieu, P. Taillandier and A. Lebrihi, 2006. Black aspergilli and ochratoxin A production in French vineyards. *International Journal of Food Microbiology* 111, 46–52.

Bennett J.W. and M. Klich, 2003. Mycotoxins. *Clinical Microbiology Reviews* 16, 497–516.

Bragulat M.R., M.L. Abarca and F.J. Cabañes, 2001. An easy screening method for fungi producing ochratoxin A in pure culture. *International Journal of Food Microbiology* 71, 139–144.

Cary J.W. and K.C. Ehrlich, 2006. Aflatoxigenicity in *Aspergillus*: molecular genetics, phylogenetic relationships and evolutionary implications. *Mycopathologia* 162, 167–177.

Cary J.W., K.C. Ehrlich, S.B. Beltz, P. Harris-Coward and M.A. Klich, 2009. Characterization of the *Aspergillus ochraceoroseus* aflatoxin/sterigmatocystin biosynthetic gene cluster. *Mycologia* 101, 352–362.

Chang P.K. and K.C. Ehrlich, 2010. What does genetic diversity of *Aspergillus flavus* tell us about *Aspergillus oryzae*? *International Journal of Food Microbiology* 138, 189–199.

Chang P.K., K. Matsushima, T. Takahashi, J. Yu, K. Abe, D. Bhatnagar, G.F. Yuan, Y. Koyama and T.E. Cleveland, 2007. Understanding nonaflatoxigenicity of *Aspergillus sojae*: a windfall of aflatoxin biosynthesis research. *Applied Microbiology and Biotechnology* 76, 977–984.

Chulze S.N., C.E. Magnoli and A.M. Dalcero, 2006. Occurrence of ochratoxin A in wine and ochratoxigenic mycoflora in grapes and dried vine fruits in South America. *International Journal of Food Microbiology* 111, Supplement 1, 5–9.

European Commission, 2005. Commission Regulation (EC) No. 123/2005 of 26 January 2005 amending Regulation (EC) No. 466/2001 as regards ochratoxin A. *Official Journal of the European Union* L 25, 3–5. http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2005:025:0003:0005:EN:PDF

Frisvad J.C., J.M. Frank, J.A.M.P. Houbraken, A.F.A. Kuijpers and R.A. Samson, 2004. New ochratoxin A producing species of *Aspergillus section Circumdati*. *Studies in Mycology* 50, 23–43.

Frisvad J.C., K.F. Nielsen and R.A. Samson, 2006. Recommendations concerning the chronic problem of misidentification of mycotoxigenic fungi associated with foods and feeds. *Advance in Experimental Medicine and Biology* 571, 33–46.

Frisvad J.C., J. Smedsgaard, R.A. Samson, T.O. Larsen and U. Thrane, 2007. Fumonisin B2 production by *Aspergillus niger*. *Journal of Agricultural and Food Chemistry* 55, 9727–9732.

Gallo A., G. Perrone, M. Solfrizzo, F. Epifani, A. Abbas, A.D. Dobson and G. Mule, 2009. Characterisation of a pks gene which is expressed during ochratoxin A production by *Aspergillus carbonarius*. *International Journal of Food Microbiology* 129, 8–15.

Huff W.E. and P.B. Hamilton, 1979. Mycotoxins – their biosynthesis in fungi: ochratoxins – metabolites of combined pathways. *Journal of Food Protection* 42, 815–820.

Karolewiez A. and R. Geisen, 2005. Cloning a part of the ochratoxin A biosynthetic gene cluster of *Penicillium nordicum* and characterization of the ochratoxin polyketide synthase gene. *Systematic and Applied Microbiology* 28, 588–595.

Kroken S., N.L. Glass, J.W. Taylor, O.C. Yoder, B.G. Turgeon, 2003. Phylogenomic analysis of type I polyketide synthase genes in pathogenic and saprobic ascomycetes. *Proceedings of the National Academy of Sciences of the United States of America* 100, 15670–15675.

Martínez-Culebras P.V. and D. Ramón, 2007. An ITS-RFLP method to identify black Aspergillus isolates responsible for OTA contamination in grapes and wine. *International Journal of Food Microbiology* 113, 147–153.

Mayer K., J. Ford, G. Macpherson, D. Padgett, B. Volkmann-Kohlmeyer, J. Kohlmeyer, C. Murphy, S. Douglas, J. Wright and J.L.C. Wright, 2007. Exploring the diversity of marine-derived fungal polyketide synthases. *Canadian Journal of Microbiology* 53, 291–302.

Medina A., R. Mateo, L. Lopez-Ocana, F.M. Valle-Algarra and M. Jimenez, 2005. Study of Spanish grape mycobiota and ochratoxin A production by isolates of *Aspergillus tubingensis* and other members of *Aspergillus* section *Nigri*. *Applied and Environmental Microbiology* 71, 4696–4702.

Nielsen K.F., J.M. Mogensen, M. Johansen, T.O. Larsen and J.C. Frisvad, 2009. Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. *Analytical and Bioanalytical Chemistry* 395, 1225–1242.

Niessen L., H. Schmidt, E. Muhlencoert, P. Farber, A. Karolewiez and R. Geisen, 2005. Advances in the molecular diagnosis of ochratoxin A-producing fungi. *Food Additives & Contaminants* 22, 324–334.

O'Callaghan J., M.X. Caddick and A.D. Dobson, 2003. A polyketide synthase gene required for ochratoxin A biosynthesis in *Aspergillus ochraceus*. *Microbiology* 149, 3485–3491.

Pel H.J. *et al.*, 2007. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nature Biotechnology* 25, 221–231.

Perrone G., G. Mule, A. Susca, P. Battilani, A. Pietri and A. Logrieco, 2006. Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius*, *Aspergillus tubingensis*, and *Aspergillus niger* strains isolated from grapes in Italy. *Applied and Environmental Microbiology* 72, 680–685.

Perrone G., A. Susca, G. Cozzi, K. Ehrlich, J. Varga, J.C. Frisvad, M. Meijer, P. Noonim, W. Mahakarnchanakul and R.A. Samson, 2007. Biodiversity of *Aspergillus* species in some important agricultural products. *Studies in Mycology* 59, 53–66.

Perrone G., A. Susca, G. Stea and G. Mule, 2004. PCR assay for identification of *Aspergillus carbonarius* and *Aspergillus japonicus*. *European Journal of Plant Pathology* 110, 641–649.

Pfohl-Leszkowicz, A. and R.A. Manderville, 2007. Ochratoxin A: an overview on toxicity and carcinogenicity in animals and humans. *Molecular Nutrition & Food Research* 51, 61–99.

Pitt J.I., 2000. Toxigenic fungi: which are important? *Medical Mycology* 38 Supplement 1, 17–22.

Samson R.A., S. Hong, S.W. Peterson, J.C. Frisvad and J. Varga, 2007. Polyphasic taxonomy of *Aspergillus* section *Fumigati* and its teleomorph *Neosartorya*. *Studies in Mycology* 59, 147–203.

Schwab E.K. and N.P. Keller, 2008. Regulation of secondary metabolite production in filamentous ascomycetes. *Mycological Research* 112, 225–230.

Selouane A., D. Bouya, A. Lebrihi, C. Decock and A. Bouseta, 2009. Impact of some environmental factors on growth and production of ochratoxin A of/by *Aspergillus tubingensis*, *A. niger*, and *A. carbonarius* isolated from Moroccan grapes. *Journal of Microbiology* 47, 411–419.

Susca A., G. Stea, G. Mule and G. Perrone, 2007. Polymerase chain reaction (PCR) identification of *Aspergillus niger* and *Aspergillus tubingensis* based on the calmodulin gene. *Food Additives & Contaminants* 24, 1154–1160.

Susca A., R.H. Proctor, G. Mulè, G. Stea, A. Ritieni, A. Logrieco and A. Moretti, 2010. Correlation of Mycotoxin Fumonisin B2 production and presence of the fumonisin biosynthetic gene *fum8* in *Aspergillus niger* from grapes. *Journal of Agricultural and Food Chemistry* 58, 9266–9272.

Tamura K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. *Molecular Biology and Evolution* 24, 1596–1599.

van der Merwe K.J., P.S. Steyn, L. Fourie, D.B. Scott and J.J. Theron, 1965. Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus* Wilh. *Nature* 13, 1112–1113.

White T.J., T. Bruns, S. Lee and J. Taylor, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: A guide to Methods and Applications*. *Academic Press*, 315–322.

Zimmerli B. and R. Dick, 1996. Ochratoxin A in table wine and grape-juice: occurrence and risk assessment. *Food Additives & Contaminants* 13, 655–668.

Accepted for publication: November 25, 2010