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Molecular characterization of the black *Aspergillus* isolates responsible for ochratoxin A contamination in grapes and wine in relation to taxonomy of *Aspergillus* section *Nigri*

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ABSTRACT

This work examines ochratoxigenic mycobiota in grapes by ap-PCR analysis sequence analysis of the ITS and IGS regions and ability to produce OTA. A comparison was also made with many reference strains of *Aspergillus* section *Nigri*. Based on ap-PCR profiles, derived from two microsatelite primers, three main groups were obtained by UPGMA cluster analysis corresponding to *A. carbonarius*, *A. niger* and *A. tubingensis*. The cophenetic correlation values corresponding to ap-PCR UPGMA analysis revealed a higher genetic variability in *A. niger* and *A. tubingensis* than in *A. carbonarius*. In addition, no genotypical differences could be established between OTA producers and nonproducers in all species analysed. Phylogenetic relationships inferred from ITS and IGS sequences are, mostly, congruent with earlier works. *A. niger* and *A. tubingensis* strains were closely related, but not identical, and they clustered into two distinct groups within the *A. niger* aggregate. Sequence analysis also showed genetic divergences between strains of *A. foetidus* and the rest of the *Aspergillus* section *Nigri*. Additionally, the phylogenetic analysis was consistent in separating a new group of ochratoxigenic strains, frequently isolated from grapes, named *A. tubingensis*-like. All strains of *A. carbonarius* analysed by sequence analysis had identical ITS and IGS sequences confirming the lack of significant genetic variability within this important ochratoxigenic species.

1. Introduction

Black *Aspergillus* species have gained importance since they were described to be the main source of ochratoxin A (OTA) contamination in grapes and wine. As a result, several surveys have been published dealing with epidemiology, ecology and distribution of black aspergilli occurring on grapes worldwide (Battilani and Pietri, 2002; Da Rocha Rosa et al., 2002; Sage et al., 2002; Abarca et al., 2003; Serra et al., 2003; Bellí et al., 2004; Leong et al., 2004, 2007; Magnoli et al., 2007). These studies have clarified that the main ochratoxigenic black *Aspergillus* species occurring on grapes are the biseriate *Aspergillus niger* aggregate and *Aspergillus carbonarius*. In general, the reported percentages of OTA-producing strains in *A. carbonarius* are higher than those reported for members of the *A. niger* aggregate (Battilani et al., 2006). By contrast, there is a higher incidence of species belonging to the *A. niger* aggregate, mainly *A. niger* and *Aspergillus tubingensis*, although other species have also been reported. For instance, *Aspergillus foetidus* strains were isolated from grapes in studies carried out in South America; however, their identity has not been confirmed by molecular data (Chulze et al., 2006; Ponsone et al., 2007). Furthermore, a new group of ochratoxigenic strains, closely related to *A. tubingensis*, has been identified in Spain by ITS-RFLP (Martínez-Culebras and Ramón, 2007). As well as the biseriate species, the ability of the uniseriate *Aspergillus aculeatus* and *Aspergillus japonicus* to produce OTA has also been reported (Dalcero et al., 2002; Ponsone et al., 2007).

It is important to accurately identify and the assign black aspergilli occurring on grapes to taxonomic ranks because the toxin profiles of individual strains vary and the fungi present in the field represent and define potential toxicological risks. However, the taxonomy of *Aspergillus* section *Nigri* is complicated and so far has not been fully resolved, especially within the *A. niger* aggregate. Al-Musallam (1980) described *A. niger* as an aggregate of two species, *A. foetidus* and *A. niger*, that are subdivided further into seven varieties, based on morphological and cultural criteria. Molecular studies are now providing useful data, which help clarify the identification and taxonomy of black *Aspergillus*. Such studies involve RFLPs of both nuclear and mitochondrial DNA, PCR-based

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Table 1
Black *Aspergillus* strains used in this study.

Strains	No.	Source of isolation	OTA	Accession number	
				ITS	IGS
A. aculeatus CECT 2968		Soil, India	−	EU821331	EU821252
A. awamori CECT 2907		Bran	+	EU821299	EU821243
A. brasiliensis CBS 101740		Soil, Brazil	−	AJ280010	EU821256
A. carbonarius CBS 111.26		Unknown	+	AY585550	EU821231
CBS 11380		Cocoa, Nigeria	+	EU821322	EU821232
W04-13, 20, 30, 35, 43, 46, 54, 48, 52, 92, 102, 108, 140, 145, 147, 155, 166, 167, 168, 169, 171	21	Grape, Spain	+		
W04-07		Grape, Spain	−		
W04-02		Grape, Spain	+	EU821326	EU821258
W04-08		Grape, Spain	−	EU821327	EU821259
W04-32		Grape, Spain	+	EU821324	EU821257
W04-40		Grape, Spain	+	EU821325	EU821261
W04-49		Grape, Spain	+	EU821323	EU821262
W04-86		Grape, Spain	+	EU821328	EU821260
A. costariciensis CBS 115574		Soil, Costa Rica	−	EU821311	EU821237
A. ellipticus CBS 482.65		Soil, Costa Rica	−	EU821329	EU821247
A. ficuum CBS 555.65		Unknown	+	EU821312	EU821255
A. foetidus CBS 565.65		Unknown	−	EU821309	EU821233
A. foetidus var *acidus* CBS 128.528		Unknown	+	EU821310	EU821234
A. foetidus var *pallidus* CBS 114.52		Unknown	+	EU821284	EU821235
A. helicotrix CBS 677.79		*A. ellipticus* culture	−	EU821314	EU821248
A. henneberguii CECT 2801		Unknown	−	EU821313	EU821246
A. heteromorphus CBS 117.55		Trichophyton culture	−	EU821305	EU821249
A. homomorphus CBS 101889		Soil, Israel	−	EU821330	EU821250
A. japonicus CBS 114.51		Unknown	−	EU821306	EU821253
W04-104		Grape, Spain	−	EU821307	EU821254
A. lacticoffeatus CBS101883^T		Coffea robusta, Indonesia	+	EU821315	EU821251
A. niger CECT 2090		Unknown	−	EU821298	EU821245
W04-136		Grape, Spain	+		
W04-4, 9, 59, 82, 124, 150, 153, 152, 154, 157	10	Grape, Spain	−		
W04-05		Grape, Spain	−	EU821301	EU821266
W04-06		Grape, Spain	−	EU821302	EU821267
W04-148		Grape, Spain	−	EU821303	EU821264
W04-149		Grape, Spain	−	EU821304	EU821265
W04-151		Grape, Spain	−		
A. piperis CBS 112811^T		Pepper, Denmark	−	EU821316	EU821236
A. phoenicis CBS 136.52		Kuro Koji, Japan	−	EU821308	EU821238
A. pulverulentus CBS 558.65		Unknown	+	EU821317	EU821242
A. sclerotioniger CBS 115572		Unknown	−	EU821318	EU821283
A. tubingensis CBS 134.48,		Unknown	−	AJ223853	EU821239
CBS 115.29		Unknown	−	EU821285	EU821240
W04-67, 73, 93, 106,	4	Grape, Spain	+		
W04-3, 14, 29, 45, 47, 55, 56, 60, 63, 65, 66, 68, 76, 78, 81, 95, 103, 107, 109, 111, 112, 115, 116, 117, 118, 123, 125, 128, 129, 137, 141, 143, 144, 146, 160, 161, 162, 163, 164, 173, 175	41	Grape, Spain	−		
W04-50, 97, 120	3	Grape, Spain	nt		
W04-34		Grape, Spain	−	EU821319	EU821241
W04-36		Grape, Spain	−	EU821320	EU821268
W04-38		Grape, Spain	−	EU821321	EU821269
W04-53		Grape, Spain	−	EU821286	EU821270
W04-57		Grape, Spain	−	EU821287	EU821271
W04-67		Grape, Spain	+	EU821288	EU821273
W04-84		Grape, Spain	−	EU821292	EU821272
W04-110		Grape, Spain	−	EU821289	EU821274
W04-120		Grape, Spain	−	EU821290	EU821275
W04-130		Grape, Spain	−	EU821291	EU821276
A. tubingensis-like					
W04-41, 44, 64, 71, 72, 77, 79, 105, 131, 132, 135, 165	12		−		
W04-17		Grape, Spain	+	EU821296	EU821279
W04-80		Grape, Spain	+	EU821293	EU821277
W04-83		Grape, Spain	+	EU821294	EU821278
W04-158		Grape, Spain	−	EU821295	EU821280
W04-159		Grape, Spain	−	EU821297	EU821281
A. usamii CBS 191700		Unknown	+	EU821300	EU821244
A. vadensis CBS 113365		Plant tissue	−	AY585549	EU821282

techniques and phylogenetic analysis (for overview see Abarca et al., 2004; Geiser et al., 2007; Perrone et al., 2007; Samson et al., 2007). In a previous taxonomical review, Samson et al. (2004) suggested a provisional revision of the section based on phenotypic and genotypic features using typical strains of each taxon. Fifteen species were accepted, of which four were newly described (*Aspergillus costariciensis*, *Aspergillus lacticoffeatus*, *Aspergillus piperis* and *Aspergillus sclerotioniger*). Additionally, several black *Aspergillus* species have been described recently, such as *Aspergillus brasiliensis* (Varga et al., 2007), *Aspergillus ibericus* (Serra et al., 2006), *Aspergillus uvarum* (Perrone et al., 2008) and *Aspergillus vadensis* (de Vries et al., 2005). Of these *A. brasiliensis*, *A. ibericus* and *A. uvarum* have also occasionally been found on grapes but they did not produce OTA.

In this study, black aspergilli from grapes were characterized by combining arbitrarily primed PCR (ap-PCR) analysis (Welsh and McClelland, 1990) with ITS and IGS sequencing and ability to produce OTA. A comparison was made with reference strains of *Aspergillus* section *Nigri*.

2. Materials and methods

2.1. Strains

The strains examined are listed in Table 1. Black *Aspergillus* strains were isolated from Spanish vineyards and held in the Institute of Agrochemistry and Food Technology of the National Spanish Research Council (IATA-CSIC). They were previously identified by ITS-RFLP profiles (Martínez-Culebras and Ramón, 2007). Reference strains were provided by Centralbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands) and the Spanish Type Culture Collection (CECT, Valencia, Spain). The reference strains used in the present study were previously tested for OTA production (Samson et al., 2004; Martínez Culebras et al., in press).

2.2. DNA preparation

All strains were grown on MEA medium at 28 °C for 6–8 days. Mycelium was collected from the plates, frozen in liquid nitrogen and ground to a fine powder. DNA extractions were performed using 100 mg of powder and the commercial EZNA Fungal DNA kit (Omega bio-teck, Doraville, USA) according to the manufacturer's instructions.

2.3. Ap-PCR amplification and analysis

For ap-PCR reactions, primers were derived from minisatellite or repeat sequences as follows: GACGACGACGACGAC and GACAGACA-GACAGACA. In the text, these primers have been designated (GAC)5 and (GACA)4, respectively. In order to guarantee the reproducibility of ap-PCR technique, two monosporic cultures of each strain were used as controls. DNA amplifications were performed in a total volume of 25 µl containing 20–40 ng of genomic DNA, 0.2 µM primer, 0.1 mM dNTPs and 1 U of DNA polymerase (Netzyme, Molecular Netline Bioproducts, N.E.E.D, SL, Spain). The reaction mixtures were performed in a thermalcycler Techne TC-512 (N.E.E.D, SL, Spain) starting with 3 min of denaturation at 95 °C followed by 35 cycles consisting of