Departament de Sanitat i d'Anatomia Animals
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CARACTERIZACIÓN MOLECULAR DE ESPECIES OCRATOXÍGENAS DEL GÉNERO *Aspergillus* SECCIÓN *Nigri*

Memoria presentada para optar
al grado de doctor

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CERTIFICAN:

que Doña MARTA BAU i PUIG ha realizado el presente trabajo sobre "Caracterización molecular de especies ocratoxígenas del género *Aspergillus* sección *Nigri*" bajo nuestra dirección en el Departament de Sanitat i d'Anatomia Animals de la Universitat Autònoma de Barcelona.

Y para que conste, a efectos de ser presentada como Memoria de Tesis para optar al grado de Doctor en Medicina y Sanidad Animales, firmamos el presente certificado en Bellaterra a 20 de abril de 2005

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- Bau M, Bragulat MR, Abarca ML, Mínguez S, Cabañes FJ. Ochratoxigenic species from Spanish wine grapes. International Journal of Food Microbiology 98 (2005) 125-130.

- Bau M, Bragulat MR, Abarca ML, Mínguez S, Cabañes FJ. Ochratoxin A producing fungi from Spanish vineyards. En: Advances in Food Mycology. Ed. Hocking AD, Pitt JI and Thrane U. Kluwer Academic/Plenum Publishers, New York. 2005 (aceptado para su publicación).

- Bau M, Castellá G, Bragulat MR, Cabañes FJ. DNA based characterization of ochratoxin A-producing and non producing *Aspergillus carbonarius* strains from grapes. Research in Microbiology 156 (2005) 375-381.

- Bau M, Castellá G, Bragulat MR, Cabañes FJ. Molecular characterization of *Aspergillus niger* agreggate species from European wine grapes (enviado para su publicación).

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1. INTRODUCCIÓN

1.1. Importancia de *Aspergillus* sección *Nigri* (Gams *et al*.).

El género *Aspergillus* fue descrito por Micheli en 1729, siendo validado por Link en 1809. Dentro de este género, la sección *Nigri* es una de las más importantes. Sus especies se caracterizan por presentar colonias de coloración oscura, frecuentemente de color negro, y conidióforos uniseriados o biseriados. Su distribución es mundial, siendo especialmente abundantes en suelos de zonas tropicales y subtropicales (51).

1.1.1. Incidencia en alimentos

Las especies de *Aspergillus* sección *Nigri* se aíslan de un gran número de alimentos (Tabla 1). Pueden producir importantes pérdidas económicas, ya que son capaces de deteriorar cereales y otros alimentos almacenados (125).

Tabla 1. Incidencia de *Aspergillus* sección *Nigri* en diversos substratos.

Substrato
Aceite de colza
Café[a]
Carne y derivados: carne, jamón, salami, salchicha, salchicha fermentada
Cereales: arroz, centeno, maíz, trigo
Especias: pimienta negra
Frutas: higo, uva[b]
Frutos secos: almendra, anacardo, avellana, cacahuete, coco, nuez, pacana, pistacho, uva pasa[c]
Leguminosas y oleaginosas: garbanzo, guisante, semilla de girasol, soja
Pescado y derivados: pescado ahumado, pescado en salazón
Verduras: ajo, cebolla

Modificado de Beuchat 1987 (23), Pitt and Hocking 1997 (125) y Samson *et al.* 2000 (139).
[a]32, 77, 105, 160, 163, 166, 173.
[b]20, 37, 135, 137, 147, 170.
[c]2, 63, 100.

1.1.2. Interés biotecnológico

Algunas de las especies de la sección *Nigri*, como *A. niger* y *A. awamori*, tienen una gran importancia en la industria alimentaria, ya que se utilizan como productoras de ácidos orgánicos y enzimas. Los productos de estas especies poseen la categoría GRAS (Generally Recognized as Safe) de la US Food and Drug Administration (25). Desde principios del siglo XX, *A. niger* se ha utilizado como productor de ácido cítrico y ácido glucónico. El ácido cítrico es uno de los principales aditivos de la industria alimentaria, utilizándose, entre otros productos, en refrescos, zumos de frutas, mermeladas, caramelos y vino. Además de los ácidos orgánicos, *A. niger* también es un importante productor de enzimas como: pectinasa, proteasa, amiloglucosidasa, catalasa y glucosa-oxidasa. Actualmente, las técnicas de recombinación del DNA permiten sobreexpresar la producción de estas enzimas y obtener cepas de *A. niger* recombinantes. La seguridad de las cepas productoras, así como la de sus productos, es evaluada antes de salir al mercado (118). Algunas de las especies de la sección *Nigri* se utilizan como fermentos naturales en la elaboración de alimentos y bebidas tradicionales en Oriente (44).

1.1.3. Producción de micotoxinas

Dentro de la sección *Nigri* se encuentran algunas especies productoras de micotoxinas (Tabla 2). *A. niger* tiene la capacidad de producir ocratoxina A (OA) (5), malforminas y naftopironas (56). *A. carbonarius* también produce OA (73) y naftopironas (56), y *A. aculeatus* produce ácido secalónico D (56). Debe destacarse la producción de OA, especialmente debido al uso que tienen algunas de las especies de esta sección, como *A. niger*, en la industria alimentaria, y a la elevada incidencia que presentan estas especies en todo el mundo.

Tabla 2. Micotoxinas y metabolitos secundarios producidos por algunas de las especies de la Sección *Nigri* (56, 116, 140)

Especie	Metabolito
A. aculeatus	Ácido secalónico B, D y F
	Aculeasinas
	Eumodina
	Endocrocina
	Neoxalina
	Okaramina
A. carbonarius	Naftopironas
	Ocratoxina A
	Piranonigrina
A. japonicus	E-64
	Festuclavina
A. niger	Ácido glutacónico
	Ácido 4-hidroximandélico
	Ácido kójico
	Ácido monoglucosiloxioctadecanoico
	Aspergillinas
	Aspereillonas
	Asperrubrol
	Dehidroflavininas
	Flaviolina
	Genisterinas
	Malforminas
	Naftopironas
	Neoequinulina A
	Nigerazinas
	Nigragillina
	Ocratoxina A
	Orlandina
	Piranonigrina
	Tubigensina A y B

1.2. Taxonomía

La sección *Nigri* se encuentra dentro del subgénero *Circumdati* descrito por Gams *et al.* (60). La taxonomía de esta sección es una de las más complejas dentro del género *Aspergillus*, y ha dado lugar a diferentes propuestas taxonómicas (3). Los errores en la clasificación de las especies de este género se deben, básicamente, a que muchas veces las diferencias descritas entre los distintos taxones son muy sutiles. Además, es común identificar todas las colonias de *Aspergillus* de coloración negruzca como *A. niger*.

1.2.1. Antecedentes históricos

El género *Aspergillus* fue descrito por primera vez por Micheli en 1729 en su *Nova Plantarum genera*. En este tratado, una de las nueve especies que describió fue *Aspergillus capitatus capitulo pullo*, un *Aspergillus* de color negro. Posteriormente, otros autores incluyeron especies pertenecientes a la sección *Nigri* en otros géneros, como *Ustilago phoenicis* o *U. ficuum*, que más tarde serían denominadas como *A. phoenicis* o *A. ficuum*, respectivamente. Cramer, en el año 1859 describió el género *Sterigmatocystis* con el fin de agrupar a las especies biseriadas de *Aspergillus*. La especie tipo, *S. antacustica*, se trataba de un *Aspergillus* de la sección *Nigri* y es considerada hoy día como un probable sinónimo de *A. niger*. Pese a que en 1901, Wehmer, en su tratado sobre *Aspergillus*, reúne en un solo grupo a las especies negras de *Sterigmatocystis* y *Aspergillus*, algunos autores continuaron describiendo especies de *Aspergillus* negros dentro del género creado por Cramer. Spegazzini, en el año 1911, describió el género *Aspergillopsis*, dónde incluía las especies negras de *Sterigmatocystis*, si bien esta propuesta no tuvo una gran aceptación (11).

Thom y Church, en 1926, agruparon las especies de *Aspergillus* según sus características morfológicas y describieron 13 especies (tanto uniseriadas como biseriadas) dentro de lo que ellos definieron como el grupo *A. niger*. El 1934, Mosseray describió 35 especies de *Aspergillus* negros, de las que 25 eran nuevas especies o nuevas combinaciones. La clave de identificación que realizó era poco práctica debido a la gran cantidad de características que contempla. En su tratado del año 1945, Thom y Raper redujeron el número de especies a 15 y subdividieron el grupo *A. niger* en tres series según el tamaño de los conidios y la ausencia o presencia de métulas (11).

1.2.2. Clasificación actual

1.2.2.1. Criterios morfológicos

El 1965 Raper y Fennell (131) publicaron un manual que reunía 12 especies y 2 variedades dentro de lo que denominaron el "Grupo *A. niger*". Este grupo englobaba todos los *Aspergillus* con cabezas conidiales oscuras. La diferenciación entre las especies descritas se basaba en el tamaño y coloración de las colonias, la presencia o ausencia de métulas y la forma y ornamentación de los conidios. Samson (138), en su compendio de *Aspergillus* descritos desde la publicación del manual de Raper y Fennell (131), no aceptó ninguna especie nueva dentro de la sección *Nigri*.

Al-Musallam (11) en su revisión de las especies negras del género *Aspergillus*, dividió el grupo en 5 especies fácilmente distinguibles entre sí (*A. carbonarius*, *A. ellipticus*, *A. heteromorphus*, *A. helicotrix* y *A. japonicus*) y el agregado *A. niger*, formado por dos especies: *A. foetidus* y *A. niger*, ésta última dividida a su vez en seis variedades y dos formas. En el manual de Klich y Pitt (82) se aceptaron las modificaciones propuestas por Al-Musallam.

La clasificación en grupos de Raper y Fennell (131) no seguía las normas del Código Internacional de Nomenclatura Botánica. Gams *et al.* (60) reclasificaron el género *Aspergillus* en subgéneros y secciones siguiendo las normas del código, y crearon la sección *Nigri*, dentro del subgénero *Circumdati*.

Kozakiewicz (83) observó que la maduración de los conidios de las especies de la sección *Nigri* es lenta, y que en el caso del agregado *A. niger* pueden ser necesarias 5 semanas de incubación en condiciones óptimas para observar los conidios maduros. Basándose en la ornamentación de los conidios mediante técnicas de microscopía electrónica de barrido (SEM), propuso la división de los *Aspergillus* negros en diez especies y ocho variedades.

Abarca *et al*. (3) presentaron, en su revisión de la sección *Nigri*, la problemática taxonómica y la importancia de las distintas especies de la sección. Estos autores

proponen una sencilla clave para la identificación de las especies más comunes utilizando criterios morfológicos.

Recientemente, Samson *et al*. (140) han descrito 4 nuevas especies dentro de la sección, aceptando 15 taxones: *A. aculeatus, A. brasiliensis, A. carbonarius, A. costaricaensis, A. ellipticus, A. japonicus, A. foetidus, A. heteromorphus, A. homomorphus, A. lacticoffeatus, A. niger, A. piperis, A. sclerotioniger, A. tubingensis* y *A. vadensis*. Estos autores basaron la diferenciación entre las distintas especies en los perfiles de 8 metabolitos secundarios y en algunas características morfológicas: coloración y diámetro de las colonias, presencia y coloración de esclerocios, y tamaño y rugosidad de los conidios. También secuenciaron el gen de la β-tubulina, aunque admitieron que sería necesario un análisis multigénico para aclarar la complejidad de los taxones *A. niger*/*A. tubingensis* y *A. japonicus*/*A. aculeatus*. Sin embargo, el estudio se realizó utilizando un número muy bajo de cepas representativas de las cuatro nuevas especies: 1 cepa en el caso de *A. costaricaensis, A. piperis* y *A. sclerotioniger*, y 3 cepas en el caso de *A. lacticoffeatus*. Además, más de la mitad de las seis cepas pertenecientes a las nuevas especies se aislaron de café. La similitud entre algunas de las especies es muy elevada: este es el caso de *A. lacticoffeatus*, que comparte con *A. niger* la misma secuencia del gen de la β-tubulina, la capacidad de producir OA, pyranonigrinas, kotaninas, y la no producción de esclerocios.

En la tabla 3 se resumen las distintas clasificaciones de las especies de *Aspergillus* sección *Nigri* realizadas fundamentalmente en base a criterios morfológicos.

Tabla 3. Especies de *Aspergillus* sección *Nigri* contempladas en los distintos estudios realizados en base a criterios morfológicos.

Raper y Fennell (1965)	Al-Musallam (1980)	Kozakiewicz (1989)	Samson *et al.* (2004)[a]
A. japonicus Saito	*A. japonicus* var. *japonicus* Saito	*A. japonicus* Saito	*A. japonicus* Saito
A. aculeatus Iizuka	*A. japonicus* var. *aculeatus* (Iizuka) Al-Musallam	*A. atroviolaceus* Moss.	*A. aculeatus* Iizuka
A. carbonarius (Bainier) Thom	*A. carbonarius* (Bainier) Thom	*A. carbonarius* (Bainier) Thom	*A. carbonarius* (Bainier) Thom
			A. sclerotioniger Samson and Frisvad
A. heteromorphus Batista and Maia	*A. heteromorphus* Batista and Maia	*A. heteromorphus* Batista and Maia	*A. heteromorphus* Batista and Maia
A. ellipticus Raper and Fennell	*A. ellipticus* (Raper and Fennell) Al-Musallam	*A. ellipticus* Raper and Fennell	*A. ellipticus* Raper and Fennell
	A. helicothrix Al-Musallam	*A. helicothrix* Al-Musallam	
A. niger van Tieghem	Agregado *A. niger*:	*A. niger* var. *niger* van Tieghem	*A. niger* var. *niger* van Tieghem
A. ficuum (Reichard) Hennings	*A. niger* var. *niger* van Tieghem	*A. niger* var. *tubingensis* (Moss.) Kozakiewicz	*A. vadensis* de Vries *et al.*
A. tubingensis (Schöber) Mosseray	*A. niger* var. *niger* f. *hennebergii* (Blochwitz) Al-	*A. niger* var. *phoenicis* (Corda) Al-Musallam	*A. tubingensis* (Schöber) Mosseray
A. phoenicis (Corda) Thom	Musallam)	*A. niger* var. *pulverulentus* (McAlp)	*A. costaricaensis* Samson and Frisvad
A. pulverulentus (McAlp) Thom	*A. niger* var. *phoenicis* (Corda) Al-Musallam	Kozakiewicz	*A. piperis* Samson and Frisvad
A. awamori Nakazawa	*A. niger* var. *phoenicis* f. *pulverulentus* (McAlp) Al-	*A. niger* var. *awamori* (Nakazawa) Al-Musallam	*A. lacticoffeatus* Samson and Frisvad
A. foetidus (Naka) Thom and Raper	Musallam	*A. niger* var. *ficuum* (Reich.) Kozakiewicz	*"A. brasiliensis"*
A. foetidus var. *pallidus* Naka, Simo	*A. niger* var. *awamori* (Nakazawa) Al-Musallam	*A. citricus* var. *citricus* (Wehmer) Moss.	*A foetidus* Thom and Raper
and Watanabe	*A. niger* var. *usamii* (Sakaguchi *et al.*) Al-Musallam	*A. acidus* Kozakiewicz	
A. foetidus var. *acidus* Naka, Simo	*A. niger* var. *intermedius* (Speg.) Al-Musallam	*A. citricus* var. *pallidus* (Naka, Simo and	
and Watanabe	*A. foetidus* (Tom and Raper)	Watanabe) Kozakiewicz	*A. homomorphus* Steiman, Guiraud, Sage
			and Seigle-Mur. ex Samson and Frisvad[b]

[a]Clasificación en base a criterios morfológicos y quimiotaxonómicos.

[b]Los autores no clarifican la situación de esta especie.

1.2.2.2. Criterios moleculares

Las dificultades que presenta la clasificación morfológica de los integrantes de la sección *Nigri*, especialmente del agregado *A. niger*, propiciaron la aplicación de otros criterios taxonómicos, como los basados en técnicas de biología molecular (Tabla 4).

1.2.2.2.1. Especies biseriadas

1.2.2.2.1.1. Agregado *A. niger*

La diferenciación en base a la morfología de las distintas especies y variedades del agregado *A. niger* es extremadamente difícil. Las distintas propuestas de clasificación de este grupo han generado un gran número de taxones (Tabla 3). Así, los diversos estudios moleculares realizados con cepas del agregado *A. niger* han intentado aclarar su clasificación.

Kusters-van Someren *et al.* (85) analizaron los patrones de RFLP del rDNA obtenidos mediante digestión con la enzima *Sma*I. Observaron 2 patrones distintos (I y II) entre las 23 cepas del agregado *A. niger* analizadas en su estudio. Puesto que la cepa neotipo de *A. niger* mostraba el patrón I, y la cepa tipo de *A. tubingensis* mostraba el patrón II, propusieron denominar al grupo de cepas con patrón I *A. niger*, y al grupo de cepas con patrón II *A. tubingensis*. Estos dos grupos de cepas son indistinguibles morfológicamente.

Posteriormente, en un trabajo dónde se analizaron los RFLPs del DNA total digerido con diferentes enzimas de restricción (*Sma*I, *Eco*RI y *Pst*I), Mégnégneau *et al.* (106) confirmaron los resultados de Kusters-van Someren *et al.* (85). Las cepas de colección del agregado utilizadas en este estudio mostraron 4 patrones distintos (I, I', II y II'), que se podían agrupar en dos grupos (I y II). Estos dos grupos coincidían con los dos grupos observados por Kusters-van Someren *et al.* (85).

Varga *et al.* (179), mediante análisis de RFLP del mtDNA, agruparon las 47 cepas de colección de su estudio en 5 patrones distintos (1a, 1b, 1c, 2a y 2b). Estos 5 patrones se podían agrupar en 2 grupos principales, que coincidían con los grupos propuestos por

Kusters-van Someren *et al.* (85). En un estudio posterior con cepas aisladas de suelo, estos autores evidenciaron el elevado grado de variabilidad del mtDNA dentro del agregado *A. niger* al describir hasta 12 patrones de RFLP del mtDNA. Todos los patrones se pudieron agrupar en 2 grupos principales, coincidiendo con los dos grupos propuestos por Kusters-van Someren *et al.* (85), excepto un patrón. Este tercer patrón se observó solamente en 6 cepas aisladas de suelo de Brasil. Los autores de este estudio apuntaron la posibilidad de que esas cepas, denominadas de manera provisional "*A. brasiliensis*", correspondieran o bien a una subespecie de *A. niger* o bien a una especie nueva dentro del agregado (182).

Parenicova *et al.* (115) describieron un nuevo patrón de RFLP del rDNA digerido con *Pst*I-*Sal*I, representado por cepas tipo de variedades de *A. foetidus*. En este estudio los autores sugirieron la división del agregado en 3 especies morfológicamente idénticas: *A. niger*, *A. tubingensis* y *A. foetidus*. Sin embargo, las cepas que formaban este nuevo grupo se clasificaron dentro del grupo de *A. tubingensis* cuando, en este mismo estudio, se utilizó la técnica descrita por Kusters-van Someren *et al.* (85). Posteriormente, estos mismos autores propusieron la división del agregado en 4 especies morfológicamente idénticas: *A. niger*, *A. tubingensis*, *A. foetidus* y *A. brasiliensis* (116). No obstante, al digerir el DNA total mediante *Pst*I-*Sal*I, técnica utilizada en su trabajo previo mediante la cual separaban las variedades de *A. foetidus* en un nuevo grupo (115), las cepas de *A. niger* y de *A. brasiliensis* mostraron el mismo patrón de RFLP (116). En otro trabajo, Parenicova *et al*. (117) secuenciaron la región ITS-5.8S del rDNA de cepas pertenecientes a la sección *Nigri*, y comprobaron que las diferencias entre las secuencias de las 4 especies eran minúsculas. Las secuencias de *A. niger* y *A. tubingensis* sólo variaban en 3 nucleótidos, mientras que *A. foetidus* y *A. tubingensis* se diferenciaban en 2.

Accensi *et al.* (10) secuenciaron el fragmento ITS-5.8S del rDNA de las cepas tipo de *A. niger* (CBS 554.65) y *A. tubingensis* (CBS 134.48). Comparando las dos secuencias, encontraron una diana para la enzima *Rsa*I en la posición 75 de la secuencia de *A. niger*, no presente en la secuencia de *A. tubingensis*. Así, se describieron 2 patrones de RFLP dentro del agregado *A. niger*: el patrón N, que agrupaba las cepas con el mismo patrón que la cepa tipo de *A. niger*, y el patrón T, que agrupaba las cepas con el mismo patrón

que la cepa tipo de *A. tubingensis*. Entre las cepas analizadas en este estudio se encontraba una cepa identificada como "*A. brasiliensis*", que mostró el patrón N.

En un estudio posterior de los mismos autores se analizaron los patrones de RFLP de 92 cepas del agregado *A. niger* mediante la técnica descrita en el 1999, así como la capacidad ocratoxígena de estas cepas. El 52,2% de las cepas mostraron el patrón T y el 47,8% restante mostraron el patrón N. Al analizar su capacidad de producir la micotoxina, se observó que todas las cepas productoras presentaban el patrón N, mientras que ninguna de las cepas con patrón T producía OA (9). Por el momento, todas las cepas estudiadas productoras de OA presentan el patrón de RFLP tipo N (2, 9, 37).

Yokoyama *et al.* (194) analizaron la secuencia nucleotídica y aminoacídica de un fragmento del gen citocromo *b* mitocondrial de 25 cepas del agregado *A. niger*, y concluyeron que *A. niger* y *A. awamori* eran dos especies distintas.

1.2.2.2.1.2. *Aspergillus carbonarius*

Dentro de la sección *Nigri*, *A. carbonarius* es quizás la especie más distinta al resto. Se puede diferenciar fácilmente por sus conidios de gran tamaño, generalmente con diámetros de 7-9 μm, y ornamentación equinulada.

Las técnicas de RFLP del rDNA y mtDNA, y RAPD, permiten diferenciar claramente *A. carbonarius* del resto de especies del agregado *A. niger* (106, 117, 180).

Kevei *et al*. (81) analizaron la variabilidad intraespecífica de 13 cepas de *A. carbonarius* mediante RFLP del mtDNA y rDNA, y RAPD. Basándose en los patrones observados en la restricción del mtDNA, agruparon las cepas en dos grupos: el grupo 1 -formado por los subgrupos 1a y 1b-, y el grupo 2. Solamente una cepa, la IN7, mostró el patrón de RFLP del mtDNA tipo 2. También se observaron diferencias entre los patrones obtenidos mediante RFLP del rDNA y RAPD de esta cepa y el resto de cepas analizadas. Los autores propusieron que este aislamiento representara una nueva subespecie de *A. carbonarius* (81). El nombre propuesto para esta subespecie fue *'A. carbonarius* var. *indicus'* (180).

Hamari *et al*. (66) realizaron mapas físicos del mtDNA de 3 cepas que representaban los patrones 1a, 1b y 2 descritos por Kevei *et al*. (81). Aunque se observaron diferencias en el tamaño de los distintos tipos de mtDNAs, también se observó que su contenido genético era muy similar.

Parenicova *et al*. (117) separaron fácilmente *A. carbonarius* del resto de especies de la sección mediante secuenciación de los ITS. En este estudio encontraron diferencias de entre 18 y 51 pares de bases entre la secuencias de los ITS de *A. carbonarius* y las otras especies de la sección.

Recientemente, Pelegrinelli-Fungaro *et al*. (121) utilizaron técnicas de RAPD para analizar cepas de *A. carbonarius* aisladas de café. Si bien se desarrolló una técnica de PCR para la detección de *A. carbonarius* en muestras de café, no fue posible asociar los patrones de RAPD obtenidos con la capacidad ocratoxígena de las cepas (121).

1.2.2.2.2. Especies uniseriadas: *A. japonicus*/ *A. aculeatus*

A. japonicus y *A. aculeatus* son las únicas especies uniseriadas de la sección. Algunos autores han considerado estos dos taxones como especies distintas (125, 131), mientras que otros redujeron *A. aculeatus* Iizuka a una variedad de *A. japonicus* : *A. japonicus* var. *aculeatus* Al-Musallam (11, 82). La mayoría de los estudios moleculares realizados con cepas de estos dos taxones se han dirigido a aclarar esta controversia.

Kusters-van Someren *et al*. (85) observaron que los patrones del rDNA digerido con *Sma*I de 5 cepas de *A. japonicus* y *A. aculeatus* eran prácticamente idénticos. Así, consideraron que probablemente se trataba de una misma especie, aunque reconocieron la necesidad de realizar estudios con un mayor número de cepas. No obstante, Mégnégneau *et al*. (106) y Visser *et al*. (190) consiguieron discriminar entre los dos taxones digiriendo el rDNA con *EcoRI* y *Pst*I-*Sal*I. En el estudio de Visser *et al*. (190) la cepa CBS 114.80, considerada *A. aculeatus* según la taxonomía clásica, mostró un patrón distinto al resto de cepas de *A. aculeatus*. Es por ello que los autores apuntaron que se podría tratar de una nueva especie de la sección.

Hamari *et al*. (65) no observaron diferencias entre los patrones de RFLP del rDNA de *A. japonicus* y de *A. aculeatus*. Sin embargo, al analizar el mtDNA obtuvieron 7 patrones de RFLP distintos, siendo el mtDNA de tipo 7 representado sólo por cepas de *A. aculeatus*. Este suceso los llevó a defender la separación de las dos especies.

Los estudios de Parenicova *et al*. (115, 116, 117) permitieron diferenciar los dos taxones mediante RFLPs, dependiendo de las distintas enzimas de restricción utilizadas. Contrariamente, la secuenciación de las regiones ITS1 y 2 (117) y ITS-5.8S del rDNA (116) no permitió distinguir *A. japonicus* de *A. aculeatus*. Aún así, estos autores apoyaron la distinción de estos dos taxones como dos especies distintas. Basándose en la secuenciación de la región ITS-5.8S, los patrones de RFLP y los perfiles de metabolitos secundarios, identificaron un posible tercer taxón uniseriado dentro de la sección, representado por la cepa *A. aculeatus* CBS 114.80 (116), corroborando así los resultados de Visser *et al*. (190).

Yokoyama *et al*. (194) secuenciaron un fragmento del gen mitocondrial del citocromo *b* de 12 especies pertenecientes a la sección *Nigri*, entre ellas *A. japonicus* y *A. aculeatus*. Los autores consideraron que estos dos taxones pertenecían a una misma especie, ya que mostraban la misma secuencia de aminoácidos.

Abarca *et al*. (3) observaron que los dos taxones uniseriados de la sección compartían una secuencia de la región ITS-5.8S del rDNA idéntica, y que por tanto podrían representar una sola especie utilizando este criterio. Los resultados de este trabajo también ponían de manifiesto que las especies uniseriadas se separan claramente de las especies biseriadas de la sección. Esta afirmación también se desprende de los trabajos de Parenicova *et al*. (116) y Varga *et al*. (183).

Tabla 4. Patrones de RFLP de algunas de las especies de la sección *Nigri* según distintos autores.

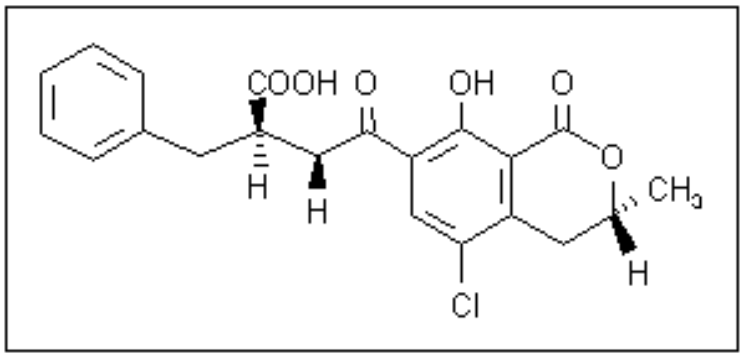
	RFLP rDNA (a)	RFLP rDNA (b)	RFLP mtDNA (c)	RFLP rDNA (d)	RFLP mtDNA (d)	RFLP (*Pst*I-*Sal*I) + Southern 26S (e)	RFLP mtDNA (*Hae*III) (f)	RFLP rDNA (*Sma*I) (g)	RFLP (*Kpn*I+*Xho*I) + Southern *pki*A (g)	RFLP (*Pst*I+*Sal*I) + Southern 28S (g)	RFLP (*Pst*I+*Sal*I) + Southern *pel*A (g)	RFLP ITS-5.8S rDNA (h)	RFLP mtDNA (*Hae*III) (i)	RFLP mtDNA (*Hae*III) (j)	RFLP rDNA (*Sma*I) (j)	RFLP rDNA (*Sma*I) (k)	RFLP (*Kpn*I+*Xho*I) + Southern *pki*A (k)	RFLP (*Kpn*I+*Xho*I) + Southern 28S (k)	RFLP (*Pst*I+*Sal*I) + Southern 28S (k)	RFLP (*Pst*I+*Sal*I) + Southern *pel*A (k)
A. niger	I	I I'	1a 1b 1c	I I'	1a 1b 1c 1d 1e	F		B	B	C	E / F / D	N		1a 1b 1c 1d 1e	I	B	BI	B	B	B
A. tubingensis	II	II II’	2a 2b	II II'	2a 2b 2c 2d 2e 2f	G		C	B / E	B / D	A / B	T		2a 2b 2c 2d 2e 2f	II-II' II	C	BII	A	C	D
A. brasiliensis				III	3							N		3a 3b	III	F	E	D	B	G
A. foetidus								C	B	D	C	T				C	BII	A	D	C
A. carbonarius						E	1a 1b	A	A	A	J			C1a C1b	C1	A	A	C	A	A
A. carbonarius **var. *indicus* (IN7)**							2							C2	C2					
A. japonicus						A		D	C	E	I		1 2 3 4 5 6	J1 J2 J3 J4 J5 J6	J	D	F	A	L/M/N	H/I
A. aculeatus						B/C		D	C	F	H		7	J7	J	D	G/H/I/J	B	F/G/H/I/J/K	J/K/L/M/N/O/P/Q/R/T
A. aculeatus **CBS 114.80**						D										D	K	C	Q	S

a) Kusters van Someren *et al.* 1991; b) Mégnégneau *et al.* 1993; c) Varga *et al*. 1993; d) Varga *et al.* 1994; e) Visser *et al.*1996; f) Kevei *et al.* 1996; g) Parenicova *et al.* 1997; h) Accensi *et al.*1999; i) Hamari *et al.* 1997; j) Varga *et al.* 2000; k) Parenicova *et al.* 2001.

1.3. Ocratoxinas

Las ocratoxinas son micotoxinas producidas por algunas especies de los géneros *Aspergillus* y *Penicillium*. La OA es la más tóxica de ellas, con efectos nefrotóxicos, carcinógenos, teratógenos e inmunosupresores. Está formada por una dihidroisocumarina unida por el grupo 7-carboxilo a una molécula de L-β-fenilalanina mediante un enlace amida. Su estructura química es: (R)-N-[(5-cloro-3,4-dihidro-8-hidroxi-3-metil-1-oxo-1H-2-benzopirano-7-il)carbonil)-L-fenilalanina (Figura 1). La ocratoxina B (OB) es el derivado dicloro de la OA, y la ocratoxina C (OC) es su etil éster. La OA, su metil éster y la 4-hidroxi-OA, junto con la OB, sus metil y etil ésteres y la OC son metabolitos fúngicos. La ocratoxina α (Oα) y la ocratoxina β (Oβ) son productos de la hidrólisis de la OA y la OB, respectivamente, no poseen la molécula de fenilalanina y no son tóxicos (84). La Oα ha sido aislada de la orina de animales de experimentación a los que se administró OA (156). La OA está estructuralmente relacionada con la citrinina (84), otra importante micotoxina que puede aparecer como co-metabolito de la OA en *Penicillium verrucosum* (125).

Figura 1. Estructura química de la OA.



1.3.1. Hongos productores

La OA fue aislada por primera vez en Sudáfrica en 1965 a partir de un cultivo de *A. ochraceus* (175). Posteriormente se demostró la producción de esta toxina por otras especies de la sección *Circumdati* tales como: *A. alliaceus, A. melleus, A. ostianus, A. petrakii, A. sulphureus, A. sclerotiorum* (69), *A. albertensis* y *A. auricomus* (181). Recientemente se han descrito seis nuevas especies de esta sección productoras de OA: *A. cretensis, A. flocculosus, A. pseudoelegans, A. roseoglobulosus, A. steynii* y *A. westerdijkiae* (58). El porcentaje de cepas de *A. ochraceus* productoras de OA varia del 100% a porcentajes menores al 10% (1, 8, 125, 126, 173).

Desde la descripción de la producción de OA por *A. niger* (5), otros autores han descrito la producción de OA en algunas especies pertenecientes a la sección *Nigri*, como en el agregado *A. niger* (167) y *A. carbonarius* (73, 167, 192). La capacidad ocratoxígena de *A. niger* puede suponer un riesgo para la salud humana y animal, ya que esta especie, de distribución muy ubicua, tiene un amplio uso en la industria alimentaria. *A. carbonarius* presenta una menor distribución que *A. niger*, pero un gran porcentaje de sus cepas son productoras de la micotoxina. En el agregado *A. niger* el porcentaje de cepas productoras varia del 0,6% al 50% (3), mientras que en *A. carbonarius* este porcentaje puede llegar al 100% (37, 90, 136, 148). Algunos estudios señalan la producción de OA por parte de las especies uniseriadas de la sección (17, 48, 100), aunque este hecho requiere confirmación, ya que no son consideradas especies productoras (90, 116, 140, 167).

Dentro del género *Aspergillus*, también se han citado como productoras de OA otras especies no pertenecientes a las secciones *Circumdati* o *Nigri* (Tabla 5).

Tabla 1. Especies del género *Aspergillus* citadas como productoras de ocratoxina A.

Especie	Especie
Sección *Aspergillus* (4, 42, 46)	**Sección *Flavi*** (46, 57, 139, 152)
Eurotium amstelodami	*A. flavus*
E. herbariorum	*Petromyces alliaceus* (syn. *P. albertensis*)
A. glaucus	**Sección *Fumigati*** (161, 184)
Sección *Circumdati* (57, 58, 69, 139, 181)	*A. fumigatus*[a]
A. auricomus[a]	*A. viridimutans*
A. cretensis	**Sección *Nigri*** (17, 100, 140)
A. flocculosus	*A. carbonarius*
A. melleus[a]	*A. japonicus*[a]
A. ochraceus	*A. japonicus* var. *aculeatus*[a]
A. ostianus[a]	*A. lacticoffeatus*
A. persii[a]	*A. niger*
A. petrakii[a]	*A. sclerotioniger*
A. pseudoelegans	**Sección *Terrei*** (172)
A. roseoglobulosus	*A. terreus*
A. sclerotiorum	**Sección *Usti*** (172)
A. steynii	*A. ustus*
A. sulphureus	**Sección *Versicolores*** (4, 46, 172)
A. westerdijkiae	*A. versicolor*
Neopetromyces muricatus	*A. sydowii*
	Sección *Wentii* (181)
	A. wentii

[a]Referencias en la que se cita la falta de consistencia y/o la no producción de OA en estas especies: 58, 90, 116, 140, 167.

La producción de OA se detectó también en cepas de *Penicillium viridicatum* (178) y más tarde en otras especies de este género, en algunos casos no siempre correctamente identificadas debido a la complejidad taxonómica que presenta el género *Penicillium*. Tras las revisiones de Ciegler (43) y Pitt (124) se asumió que la producción de OA por *Penicillium* spp. se restringía a *Penicillium verrucosum*. Actualmente se considera la posible división de *P. verrucosum* en 2 especies ocratoxígenas: *P. verrucosum* y *P. nordicum* (87).

Generalmente se considera que *P. verrucosum* es el responsable de la producción de OA en zonas frías, mientras que la contaminación por OA en zonas más cálidas se atribuye a *A. ochraceus* (125). Sin embargo, la detección de la capacidad ocratoxígena de las especies de la sección *Nigri*, *A. niger* var. *niger* y *A. carbonarius*, junto con el hecho que se aíslan con frecuencia de distintos alimentos como uvas, café y pasas, producidos en diversas zonas geográficas (2, 20, 32, 37, 63, 77, 100, 105, 135, 137, 147, 160, 163,

166, 170, 173), hace pensar que dichas especies tienen un papel importante en la contaminación con OA en estas zonas.

1.3.2. Presencia en alimentos

La OA presenta una distribución mundial, debido a la amplia distribución de los hongos que la producen. Se detecta principalmente en substratos vegetales, como cereales y leguminosas (6, 15, 24, 38, 46, 47, 53, 59, 68, 71, 79, 80, 84, 98, 127, 130, 144, 145, 146, 153, 169, 185, 191, 193, 195), y en productos de cerdo y aves (39, 45, 61, 62, 72, 76, 78, 84, 96, 122, 130, 143, 153, 174). Estos animales se contaminan al ser alimentados con piensos que contienen la micotoxina (7, 59, 62, 72, 130). En los últimos años ha adquirido una gran importancia la presencia de OA en otro tipo de substratos, de entre los cuales cabe destacar el vino, el café y la cerveza (Tabla 6). El vino, especialmente el vino tinto, se considera la segunda fuente de OA en la dieta europea, siendo los cereales la primera (12).

Tabla 2. Presencia de OA en distintos alimentos.

Substrato	País	Fuente
Alimentos infantiles a base de cereales	Italia	22, 24
Café	Alemania	29, 67, 114
	Bélgica	152
	Brasil	49, 91, 141, 163, 173
	Canadá	93
	Dinamarca	78
	España	34
	Europa	177
	Holanda	176
	Hungría	53, 166
	Italia	108, 133
	Japón	110, 171, 172
	Portugal	105
	Qatar	6
	Reino Unido	119, 153
	Suiza	26, 31, 33, 77, 128, 129, 159, 186, 196
Cerveza	Alemania	29, 50, 75, 107
	Bélgica	162
	Canadá	142, 154

(sigue)

(Tabla 6, continúa)

Cerveza	Dinamarca	78
	España	88
	Italia	189
	Japón	109, 171
	Sudáfrica	112
	Suiza	196
	Turquia	64
Chocolate y cacao	Alemania	52
	España	36, 149
	Reino Unido	153
Especias	Bélgica	168
	Qatar	6
	Reino Unido	120
Galletas, bizcochos y cereales de desayuno	Alemania	52
	España	27
Pan	España [a]	89
	Italia	187
Pasas	Alemania	52
	Canadá	94
	Qatar	6
	Reino Unido	97, 99, 153
Queso	Alemania	52
Regaliz	Alemania	30
Salsa de soja	Japón	171
Salsas[b]	Alemania	102
Té	Alemania	29
Uva	Italia	16
Vinagres	Alemania	102
	Francia	104
Vino, mosto y zumo de uva	Alemania	102, 103, 113
	Brasil	134
	Canadá	111, 154
	España	19, 28, 35, 95
	Francia	104, 137
	Grecia	155, 158
	Italia	16, 40, 41, 86, 92, 123, 164, 165, 188, 189
	Japón	171
	Marruecos	55
	Portugal	132
	Sudáfrica	150, 157
	Suiza	196, 197
	Reino Unido	153
	Estados Unidos	151
Zumo de grosella	Alemania	102
Zumo de tomate	Alemania	102
Zumo de zanahoria	Alemania	102

[a]También se detectó OA en muestras de pan de Francia, Holanda, Bélgica, Italia, Alemania, Irlanda, Austria, Suiza, Hungría, Estados Unidos, Túnez y Brasil.
[b]Ketchup, mostaza, salsa de pimienta y salsa barbacoa.

Desde la primera descripción de la presencia de OA en vino y zumo de uva (196), se han llevado a cabo numerosos estudios en diferentes países, utilizando distintas variedades de vino (tinto, rosado, blanco, vino de licor), mosto y zumo de uva (19, 21, 28, 35, 41, 54, 55, 70, 86, 95, 104, 123, 132, 134, 150, 151, 154, 155, 157, 158, 165, 171, 188, 189). Los resultados de estos estudios indican que el porcentaje de muestras positivas, así como la cantidad de OA detectada, son generalmente mayores en vinos tintos, seguidos de los rosados y finalmente los blancos (19, 35, 41, 102, 103, 123, 134, 154, 188). Esta diferencia se atribuye al proceso de elaboración del vino tinto, diferente al del vino blanco. En la elaboración del vino tinto se dejan en contacto durante unos días la piel y el jugo de la uva, para extraer los pigmentos naturales. En este período se cree que se produce la contaminación por la toxina presente en la parte externa de la uva, dónde se han desarrollado los hongos (155, 177). Por otra parte, según Ottender y Majerus (113) las elevadas temperaturas y las condiciones aerobias presentes dentro de los tanques, antes del proceso de fermentación, favorecen la formación de OA. Además, los vinos blancos sufren un proceso de clarificación con arcillas, como bentonita o zeolita, que podrían actuar como absorbentes de la OA (74). Los vinos dulces presentan concentraciones de OA mayores incluso que los tintos (35, 123, 197), al realizarse una vendimia más tardía para obtener una uva más dulce.

Los vinos procedentes de países mediterráneos presentan contenidos en OA mayores que los de países del norte de Europa (102, 103, 104, 113, 197). Esta diferencia también existe entre los vinos producidos al norte y al sur de países como Francia, Italia o Grecia (102, 123, 158).

La presencia de OA en mostos y uvas parece ser debida principalmente a *A. carbonarius* (17, 37, 137, 147, 170), contribuyendo también el agregado *A. niger* (101, 135).

En el caso del café, los granos que han sido recolectados directamente de la planta presentan concentraciones de OA mucho más bajas que los granos que han estado en contacto con el suelo (49, 163). Por otra parte, los cafés solubles presentan cantidades de OA mayores que los cafés normales (26, 91, 93, 119, 186). Sin embargo, al ser un producto más concentrado, la OA ingerida en una taza de café preparada a partir de café

normal o a partir de café soluble es similar (153). La contaminación por OA en café parece ser debida principalmente a *A. niger*, *A. carbonarius* y *A. ochraceus* (31, 32, 33, 77, 110, 152, 166, 173).

En el caso de las cervezas, no parecen haber diferencias entre los distintos tipos ni entre los distintos países productores (29, 75, 109). La contaminación por OA en cerveza, al contrario que en el café y en el vino, parece deberse al crecimiento de *P. verrucosum* en la cebada durante el almacenamiento o en la formación de la malta (18).

1.3.3. Legislación

Recientemente, la Unión Europea ha modificado la normativa por la cual se establecen los niveles máximos de OA en distintos productos alimentarios (14). Esta nueva normativa, de cumplimiento obligatorio para todos los estados miembros de la UE, amplía la variedad de substratos con un límite máximo de OA (Tabla 7). Algunos países de la Unión Europea tienen, además, una normativa propia más restrictiva (13)

Tabla 3. Contenido máximo de OA tolerado en alimentos para los estados miembros de la UE (14).

Substrato	Límite OA (µg/kg)
Cereales:	
-cereales en grano sin transformar (incluido arroz sin transformar y alforfón)	5
-productos derivados de los cereales (incluidos productos transformados a base de cereales y los cereales en grano destinados al consumo humano directo)	3
Uvas pasas (pasas de Corinto, sultanas y otras variedades de pasas)	10
Café:	
-café tostado en grano y café tostado molido, con excepción del café soluble	5
-café soluble	10
Vino (tinto, rosado y blanco) y otras bebidas a base de vino y/o mosto de uva	2
Zumo de uva, ingredientes de zumo de uva en otras bebidas, incluido el néctar de fruta y el zumo de uva concentrado reconstituido	2
Mosto de uva y mosto de uva concentrado reconstituido, destinados a consumo humano directo	2
Alimentos elaborados a base de cereales y alimentos infantiles para lactantes y niños de corta edad	0,5
Alimentos dietéticos destinados a usos médicos especiales dirigidos específicamente a lactantes	0,5
Café verde, frutos secos distintos de las uvas pasas, cerveza, cacao y productos del cacao, vinos de licor, productos cárnicos, especias y regaliz	--*

*a determinar antes del 30-06-06

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2. OBJETO DEL ESTUDIO

Los hongos presentan una gran capacidad de adaptación en diversos substratos, entre los que destacan los alimentos destinados al consumo humano y animal. Su importancia deriva no tan sólo por su desarrollo y por el consecuente deterioro de estos productos, sino también porque un elevado número de especies presentan la capacidad de elaborar micotoxinas. En base a estas premisas, cabe destacar las especies de los géneros *Aspergillus*, *Fusarium* y *Penicillium*, ya que son las más abundantes en estos substratos, además de ser potencialmente micotoxígenas.

Una de las micotoxinas que actualmente está recibiendo más atención es la ocratoxina A, ya que además de presentar una elevada toxicidad se encuentra ampliamente distribuida. Es por ello que, recientemente, en Europa se ha ido poniendo en marcha una legislación para su control en diversos productos alimenticios, entre los que cabe destacar el café o el vino. Este último se considera la segunda fuente de ocratoxina A en la dieta europea.

La producción de ocratoxina A se describió por primera vez en la especie *Aspergillus ochraceus*, sin embargo en la actualidad esta capacidad productora se ha ampliado no sólo a especies de la misma sección *Circumdati*, sino a especies de secciones distintas. Entre ellas cabe destacar las especies pertenecientes a la sección *Nigri*, puesto que, además de su amplia y ubicua distribución, algunas de ellas se utilizan en la elaboración de determinados alimentos, y sus productos poseen la categoría GRAS (“Generally recognized as safe”).

Algunos de los miembros incluidos en esta última sección presentan una complejidad taxonómica notable. En algunos casos se debe a la utilización de criterios morfológicos poco consistentes en la descripción de las especies. Este hecho es destacable en las especies que forman parte del agregado *A. niger*, ya que las diferencias descritas entre las distintas especies de este grupo son muy sutiles, siendo en la práctica indiferenciables morfológicamente. Actualmente se están aplicando estudios basados en el análisis del DNA con el objetivo de clarificar la clasificación de estas especies.

Así pues, consideramos de interés conocer la presencia y distribución de las especies del género *Aspergillus* sección *Nigri* en uva, en diversas fases de maduración, y en especial

de las especies productoras de ocratoxina A. Asimismo, caracterizar dichos aislamientos mediante estudios moleculares que faciliten su clasificación y diferenciación.

Por todo ello el objeto del presente trabajo ha sido:

- Estudiar la micobiota presente en uva procedente principalmente de viñedos de la cuenca mediterránea española durante cuatro períodos de maduración.

- Analizar la presencia de las especies pertenecientes a la sección *Nigri* aisladas.

- Detectar la capacidad ocratoxígena de las cepas aisladas pertenecientes a los géneros *Aspergillus* y *Penicillium*.

- Caracterizar mediante estudios moleculares las cepas de la sección *Nigri* aisladas de viñedos españoles.

- Analizar mediante estudios moleculares y estudiar la capacidad ocratoxígena de las cepas de la sección *Nigri* procedentes de viñedos de Europa e Israel.

3. RESUMEN GLOBAL DE LOS RESULTADOS Y DISCUSIÓN

En la presente Memoria de Tesis Doctoral se ha estudiado la micobiota de la uva en diferentes períodos de maduración, con el objetivo de identificar las especies ocratoxígenas presentes. Para ello se han muestreado 7 viñedos distintos, situados principalmente en la costa Mediterránea española, durante los años 2001, 2002 y 2003. Asimismo, se ha analizado la capacidad de producir OA de las cepas potencialmente productoras aisladas en este muestreo. Por otro lado, se han caracterizado a nivel molecular 226 cepas del género *Aspergillus* sección *Nigri* potencialmente ocratoxígenas: 53 cepas pertenecientes a la especie *A. carbonarius* y 173 cepas pertenecientes al agregado *A. niger*. Todas las cepas del estudio molecular son aislamientos de uva de distintos orígenes geográficos: España, Francia, Grecia, Israel, Italia y Portugal.

La micobiota predominante de la uva de los viñedos españoles muestreados perteneció a los géneros *Alternaria*, *Cladosporium* y *Aspergillus*, mientras que el género *Penicillium* representó menos del 3% de los aislamientos. La elevada incidencia de *Alternaria* spp. y *Cladosporium* spp. en los primeros períodos de muestreo decreció con la maduración de la uva, mientras la presencia de *Aspergillus* spp. incrementó significativamente. En todos los casos, se encontraron diferencias significativas en la incidencia de estos géneros en cada período de muestreo. Durante los tres años de muestreo, se aislaron de las uvas un total de 1812 cepas de *Aspergillus* spp., de entre las cuales solamente 13 fueron identificadas como *A. ochraceus*, mientras que 1423 se identificaron como pertenecientes al agregado *A. niger*, y 226 como *A. carbonarius*. El único aislamiento identificado como *P. verrucosum* entre las 214 cepas de *Penicillium* spp. aisladas mostró la capacidad de producir citrinina, pero no OA. Los aislamientos pertenecientes a *Aspergillus* sección *Nigri* representaron alrededor del 90% del total de cepas del género aisladas. Aunque se presentaron en todos los períodos de muestreo, la presencia de las especies del agregado *A. niger* y de *A. carbonarius* fue significativamente superior en la vendimia. Por otra parte, el número de aislamientos en el medio DRBC fue superior que en MEA, si bien estas diferencias no resultaron significativas. Las pepitas de uva analizadas no presentaron contaminación fúngica, por lo tanto se asume que la contaminación con hongos productores de OA en la uva proviene de la superficie del fruto.

En cuanto a los resultados obtenidos en el estudio de producción de OA de las cepas aisladas de viñedos españoles, se ha evidenciado la importante contribución de *A. carbonarius* en la contaminación por OA en uva, y por tanto, en el vino. Esta afirmación se deriva no sólo de la elevada incidencia que presenta esta especie en las uvas, especialmente en la vendimia, sino también del porcentaje de cepas productoras, un 100% en este estudio. El rango de producción de OA en esta especie fue de 0,06-477 μg OA/g de medio de cultivo. En cuanto a las otras especies de *Aspergillus* sección *Nigri* aisladas, solamente el 0,7% de los aislamientos del agregado *A. niger* resultaron ser ocratoxígenos, en un rango de 0,05-231 μg OA/g de medio de cultivo, mientras que ninguno de los aislamientos uniseriados produjeron OA. También se detectó la producción de OA por otras especies de *Aspergillus* no pertenecientes a la sección *Nigri*. Así, 7 de los 13 aislamientos de *A. ochraceus,* y 3 de los 6 de *A. melleus* produjeron OA. También los 3 aislamientos de *A. ostianus* y los 6 de *A. alliaceus* resultaron productores. Algunas de estas especies producen OA en grandes cantidades, pero su baja incidencia en la uva hace que la importancia como fuentes de contaminación sea escasa.

En relación al estudio molecular de *A. carbonarius*, se han caracterizado mediante RAPD y secuenciación de la región ITS-5.8S del rDNA 53 cepas aisladas de uva de diferentes países europeos. Cuarenta y nueve de las cepas presentaron un patrón de RAPD muy similar y la secuencia de la región ITS-5.8S prácticamente idéntica. Todas estas cepas produjeron OA en distintas cantidades. Las 4 cepas restantes mostraron un patrón de RAPD diferente y la secuencia de la región ITS-5.8S distinta a las demás, además de ser las únicas cepas no productoras de OA. Estos aislamientos podrían representar una nueva especie dentro de la sección *Nigri*. Por otra parte, en el estudio molecular del agregado *A. niger* se han caracterizado 173 cepas procedentes de distintos orígenes geográficos, mediante una técnica de RFLP de la región ITS-5.8S del rDNA. Todas las cepas se agruparon en los 2 patrones de RFLP descritos previamente: tipo N (43% de las cepas) y tipo T (57% de las cepas). Se detectó la producción de OA en 20 de las 173 cepas (11,6%). Todas las cepas ocratoxígenas mostraron el patrón tipo N.

4. CONCLUSIONES

1. La micobiota predominante de la uva procedente de viñedos de la cuenca mediterránea española pertenece a los géneros *Alternaria*, *Cladosporium* y *Aspergillus*. El género *Penicillium* tiene una incidencia menor, representando menos del 3% de los aislamientos.

2. Al avanzar el proceso de maduración de la uva la presencia de los géneros *Alternaria* y *Cladosporium* disminuye, mientras que la presencia del género *Aspergillus* aumenta significativamente.

3. Los aislamientos pertenecientes a *Aspergillus* sección *Nigri* representaron alrededor del 90% del total de cepas del género aisladas. La presencia de las especies del agregado *A. niger* y de *A. carbonarius* es significativamente superior en el período de vendimia.

4. Las especies ocratoxígenas *Penicillium verrucosum* y *Aspergillus ochraceus*, generalmente consideradas como las principales fuentes de contaminación por OA en diversos sustratos, han mostrado una presencia muy baja en la uva, y por ello no se consideran responsables de la contaminación por OA en el vino.

5. La principal especie productora de OA en la sección *Nigri* es *A. carbonarius*, ya que todas las cepas de esta especie analizadas han sido capaces de producir esta micotoxina. En el caso del agregado *A. niger*, menos del 12% de las cepas han resultado ocratoxígenas, mientras que ninguna de las cepas uniseriadas de la sección han producido OA.

6. *Aspergillus carbonarius* es el principal responsable de la contaminación de las uvas por OA y por tanto del vino.

7. La contaminación por hongos productores de OA proviene de la superficie del fruto, ya que las pepitas analizadas no presentan contaminación fúngica.

8. El estudio mediante RAPD del DNA genómico de *A. carbonarius* con el cebador ARI 1 no ha permitido correlacionar los grupos obtenidos mediante esta técnica y el origen o el nivel de producción de OA de las cepas.

9. La secuencia del DNA que codifica el gen 5.8S del RNA ribosomal y los ITS1 y 2 ha mostrado una gran uniformidad entre las cepas de *A. carbonarius* procedentes de distintos orígenes geográficos ensayadas.

10. Las cepas tentativamente identificadas como *A. carbonarius* no productoras de OA mostraron un patrón de RAPD y una secuencia de la región ITS-5.8S del rDNA distintos a las demás. Estas cepas podrían representar una nueva especie dentro de la sección *Nigri*.

11. De las cepas del agregado *A. niger* estudiadas, el 43% han mostrado un patrón N y el 57% un patrón T. Sin embargo, la distribución no ha sido tan homogénea teniendo en cuenta el origen geográfico.

12. Todas las cepas del agregado *A. niger* productoras de OA han mostrado el patrón de RFLP tipo N.

5. ARTÍCULOS

5.1. Bau M, Bragulat MR, Abarca ML, Mínguez S, Cabañes FJ. Ochratoxigenic species from Spanish wine grapes. International Journal of Food Microbiology 98 (2005) 125-130.

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Ochratoxigenic species from Spanish wine grapes

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Abstract

The ochratoxigenic mycobiota of grapes belonging to representative wine regions located along the Mediterranean coast of Spain at different developmental stages was identified. During the development of the berries, the occurrence of *Aspergillus* spp. increased while the percentage of berries contaminated by non-ochratoxin A (OTA) producing species such as *Alternaria* spp. and *Cladosporium* spp. decreased. *Penicillium verrucosum*, the only confirmed *Penicillium* spp. that is able to produce OTA, was not isolated. The contamination by OTA-producing species comes from the surface of the berries and not from the inner fruit. Black aspergilli were predominant among the different *Aspergillus* spp. isolated. All the *Aspergillus carbonarius* isolates were able to produce OTA at different concentrations. None of the isolates belonging to *Aspergillus niger* aggregate and to *Aspergillus japonicus* var. *aculeatus* were able to produce OTA. These results are a strong evidence of the contribution of *A. carbonarius* in the OTA contamination in wine grapes, mainly at the last developmental stages of the berries.

Keywords: *Aspergillus carbonarius*; Ochratoxin A; Grapes; Mycobiota

1. Introduction

Ochratoxin A (OTA) is one of the most common naturally occurring mycotoxins and it is receiving increasing attention for its toxic effects and high incidence in a wide range of food commodities. It has been shown to be nephrotoxic, carcinogenic, immunotoxic, genotoxic and teratogenic to all animal species tested. The presence of OTA in blood from healthy humans confirms a continuous and widespread exposure (Anonymous, 1998; Creppy, 1999).

In the European diet, wine (especially red wine) has been identified as the second major source of human exposure to OTA, following cereals (Anonymous, 1998). The European Union has established maximum levels for OTA in cereals and dried vine fruits, and it will decide before December 31, 2003 whether maximum limits for this mycotoxin need to be set for wine and grape juice, among other commodities (Anonymous, 2002). Since the first results were published (Zimmerli and Dick, 1996), the occurrence of OTA in wine and grape juice has been reported in different countries, including Spain (Burdaspal and Legarda, 1999, Lopez de Cerain et al., 2002). Spain is a world class producer of wines, both in quality and in quantity. According to the Office International de la Vigne et du Vin (O.I.V.) (Anonymous, 2000), approximately 15% of the world's total viticulture area and 11% of the world's wine produc-

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tion come from this country. The wines of Spain can be divided into more than 50 recognized wine regions, each of which is very different. It appears that some wines from southern wine-growing regions of Europe and northern Africa, mainly in the Mediterranean basin, have higher OTA contents than those from the northern areas (Zimmerli and Dick, 1996).

Traditionally, *Aspergillus ochraceus* and *Penicillium verrucosum* are considered the main OTA-producing species. It is generally assumed that *P. verrucosum* produces OTA in temperate and cold climates and has been reported almost exclusively in cereal and cereal products while *A. ochraceus* is more commonly associated with different commodities in warmer and tropical climates (Pitt and Hocking, 1997). Recently, black aspergilli, mainly *Aspergillus carbonarius* and members of the *Aspergillus niger* aggregate, have been described as a main possible sources of OTA contamination in grapes from Argentina and Brazil (Da Rocha Rosa et al., 2002), France (Sage et al., 2002) and Italy (Battilani et al., 2003), and also in dried vine fruits from different origin (Abarca et al., 2003; Heenan et al., 1998). Strong evidence of the contribution of *A. carbonarius* to the OTA contamination of wine has been also reported (Cabañes et al., 2002).

The objective of this study was to identify the ochratoxin A producing mycobiota of grapes belonging to representative wine regions located along the Mediterranean coast of Spain (from the northeast to the south of the country), assessing the contribution of the most important fungi in the different developmental stages of the berries.

2. Materials and methods

2.1. Samples

During 2001 season, a total of seven vineyards located mainly along the Mediterranean coast of Spain belonging to five grape-growing regions : Barcelona (two vineyards), Tarragona (two vineyards), Valencia (one vineyard), Murcia (one vineyard) and Cádiz (one vineyard) were studied. Five grape varieties were included: Cabernet Sauvignon (vineyards from Barcelona, Tarragona and Murcia), Garnatxa blanca (Tarragona), Macabeu (Barcelona), Moscatel (Valencia) and Palomino fino (Cádiz). These areas have predominantly Mediterranean climatic influences. Only the vineyard from Cádiz, located in the south of Spain, with a hot dry climate, has Atlantic climatic influences. In every vineyard, from May to September, samples were taken at four different times, coinciding with the following developmental stages of the grape: setting, 1 month after setting, veraison and harvesting. At each sampling time, 10 bunches were obtained from 10 different plants located approximately along two crossing diagonals of the vineyard. Every bunch was collected in a separate paper bag. Samples were sent to the laboratory as soon as they were collected and they were analyzed within 24–48 h maximum.

2.2. Mycological study

From each bunch, five berries were randomly selected and plated directly onto malt extract agar (MEA) (Pitt and Hocking, 1997) supplemented with 100 ppm of chloramphenicol and 50 ppm of streptomycin and five berries more onto dichloran rose bengal chloramphenicol agar (DRBC) (Pitt and Hocking, 1997). In total, 2800 berries were analyzed. Plates were incubated at 25 °C for 7 days. We also studied the presence of fungi in the seeds from a representative number of berries ($n = 280$) of these vineyards. In order to avoid the skin contamination of the berries, they were surface decontaminated using a 5% chlorine solution for 1 min followed by two rinses with sterile-distilled water. After that, seeds were aseptically removed from berries and they were plated directly onto MEA and DRBC.

On the last day of incubation, all fungi considered to represent different species were isolated and transferred to slants and then to plates for identification. Taxonomic identification of different isolates was made using macroscopic and microscopic morphological criteria in accordance with appropriate keys. In order to study the OTA producing mycobiota of grapes, only the isolates belonging to *Aspergillus* spp. and *Penicillium* spp. were identified to species level (Raper and Fennell, 1965; Pitt, 1979; Klich and Pitt, 1988; Pitt and Hocking, 1997) and were preserved at −80 °C. Mucorales were not identified and they were considered as a unique group. The remaining molds were identified to genus level.

2.3. OTA production ability

Isolates belonging to *Aspergillus* spp. and *Penicillium* spp. were evaluated using a previously described HPLC screening method (Bragulat et al., 2001). Briefly, the isolates were grown on Czapek Yeast extract Agar (CYA) and on Yeast extract Sucrose agar (YES) (Pitt and Hocking, 1997) and incubated at 25 °C for 7 days. Isolates identified as *A. carbonarius* were grown on CYA for 10 days at 30 °C because they have been cited as optimal incubation conditions for detecting OTA production in this species (Cabañes et al., 2002; Abarca et al., 2003). From each isolate, three agar plugs were removed from the central area of the colony and extracted with 0.5 ml of methanol. The extracts were filtered and injected into the HPLC.

2.4. Data analysis

Data obtained were analyzed statistically by means of one-way analysis of variance test, Student's *t*-test, and chi-square test. All statistical analyses were performed using SPSS software (version 10.0).

3. Results and discussion

Predominant mycobiota belonged to *Alternaria* spp., *Cladosporium* spp. and *Aspergillus* spp. Taking into account the total number of sampled grapes (n= 2800), these three genera were isolated from 75.6%, 22.5% and 17.3% of plated berries, respectively. *Penicillium* spp. were only isolated from 2.3% of grapes. Mucorales were isolated from 8.3% of berries and other genera such as *Trichoderma, Monilia, Acremonium* and *Botrytis* among others, were isolated at frequencies of less than 0.8%.

Frequency of occurrence of *Alternaria* spp., *Cladosporium* spp., *Aspergillus* spp. and *Penicillium* spp. at each sampling time are shown in Fig. 1. While the incidence of *Penicillium* spp. remains low, there is a clear increase of *Aspergillus* spp. and a decrease in the percentage of grapes contaminated with *Alternaria* spp. and *Cladosporium* spp. with the maturation of the berries. In all cases, statistically significant differences were found in the occurrence of those genera at each sampling time.

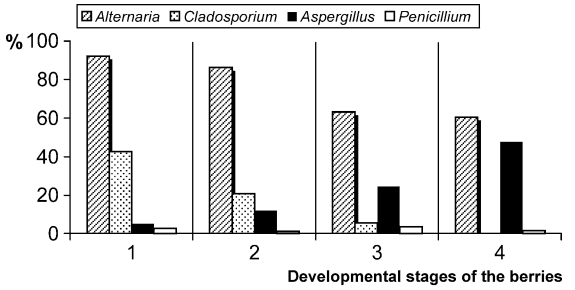

Fig. 1. Occurrence of *Alternaria* spp., *Cladosporium* spp., *Aspergillus* spp. and *Penicillium* spp. at each developmental stages of the berries: (1) setting; (2) 1 month after setting; (3) veraison; (4) harvesting.

No fungal growth was obtained from the seeds removed from berries. This leads to the assumption that the contamination by OTA-producing species comes from the surface of the berries and not from the inner fruit. These fungi are considered secondary rather than primary agents of spoilage because they generally lack the mechanisms to invade and infect plant tissue and only become established after the berry have been infected by a pathogen organism or has been damaged by some physical or physiological cause. On the contrary, the infection of grape by *Botrytis cinerea*, often occurs at bloom time, followed by a period of latency without causing disease symptoms, generally until grape berries begin to ripen. After veraison, *B. cinerea* are distributed throughout the pulp and on steams (Keller et al., 2003).

Table 1 shows the occurrence of *Aspergillus* spp. and *Penicillium* spp. in the 2800 berries plated on the two culture media used. Although the number of isolates recovered in DRBC medium were higher than on MEA, these differences were not statistically significant.

Sixteen *Penicillium* species were identified, but *P. verrucosum*, the OTA-producing species of the genus was not isolated. *Penicillium citrinum, P. glabrum* and *P. chrysogenum* constituted 18.5%, 16.9% and 13.8%, respectively, of the 65 *Penicillium* strains recovered. None of the 65 *Penicillium* isolates was able to produce OTA. Nevertheless, among the *Penicillium* spp. identified, there were some important mycotoxin producers such as *P. citrinum* (citrinin), *P. chrysogenum* and *P. griseofulvum* (roquefortine C) and *P. oxalicum* (secalonic acid D) among others (Pitt and

Table 1
Occurrence and OTA producing ability of *Aspergillus* spp. and *Penicillium* spp. isolated in a total of 2800 berries

Species	*n* (DRBC/MEA)[a]	%	*n* OTA +	Species	*n* (DRBC/MEA)[a]	%
Aspergillus spp.				*Penicillium* spp.[b]		
A. niger aggregate	474 (240/234)	16.93	0	*P. citrinum*	12 (8/4)	0.43
A. carbonarius	101 (60/41)	3.61	101	*P. glabrum*	11 (8/3)	0.39
A. fumigatus	14 (7/7)	0.50	0	*P. chrysogenum*	9 (5/4)	0.32
A. tamarii	8 (4/4)	0.28	0	*P. brevicompactum*	6 (5/1)	0.21
A. ustus	7 (3/4)	0.25	0	*P. sclerotiorum*	6 (6/0)	0.21
A. japonicus var. *aculeatus*	5 (3/2)	0.18	0	*P. variabile*	6 (4/2)	0.21
A. flavus	5 (4/1)	0.18	0	*P. thomii*	5 (1/4)	0.21
E. nidulans	3 (3/0)	0.11	0	*P. oxalicum*	2 (1/1)	0.07
A. terreus	3 (3/0)	0.11	0	*P. canescens*	1 (1/0)	0.04
A. melleus	3 (3/0)	0.11	1	*P. corylophilum*	1 (0/1)	0.04
A. ochraceus	2 (2/0)	0.07	1	*P. decumbens*	1 (1/0)	0.04
A. parasiticus	1 (1/0)	0.04	0	*P. griseofulvum*	1 (1/0)	0.04
A. versicolor	1 (0/1)	0.04	0	*P. italicum*	1 (1/0)	0.04
E. variecolor	1 (0/1)	0.04	0	*P. lividum*	1 (1/0)	0.04
				P. pinophilum	1 (0/1)	0.04
				P. purpurogenum	1 (0/1)	0.04
Total *Aspergillus* spp.	628 (333/295)	22.43	103	Total *Penicillium* spp.	65 (43/22)	2.32

[a] No statistically significant differences were found in the number of isolates recovered in the culture media used (DRBC: dichloran rose bengal chloramphenicol agar; MEA: malt extract agar).
[b] None of *Penicillium* spp. isolates were able to produce OTA.

Hocking, 1997). Due to their low frequency of isolation, the potential for production of mycotoxins by these species is not a cause of concern.

A total of 628 isolates belonging to 15 *Aspergillus* spp. (including *Emericella nidulans* and *E. variecolor*) were identified. Black aspergilli (mainly *A. niger* aggregate and *A. carbonarius*) constituted 92% of the total *Aspergillus* strains isolated. *Aspergillus niger* aggregate were isolated from 16.9% of plated berries, and *A. carbonarius* from 3.6%. The occurrence of the remaining *Aspergillus* spp. ranged from 0.04% to 0.5%.

Some of the *Aspergillus* spp. isolated are well-known potential producers of mycotoxins such as aflatoxins (*A. flavus* and *A. parasiticus*) and sterigmatocystin (*Emericella* spp. and *A. versicolor*) among others (Pitt and Hocking, 1997). Nevertheless, due to their low incidence they do not appear to be a source of mycotoxins in this substrate. The incidence of species of *Aspergillus* section *Circumdati* traditionally considered to be ochratoxigenic was very low, as was their ability to produce OTA. Only one out of the three isolates of *A. melleus* produced OTA (7.28 μg/g) and one of the two isolates of *A. ochraceus* produced this mycotoxin at a level of 73.81 μg/g. These species are probably a relatively unimportant source of OTA in grapes. On the contrary, all *A. carbonarius* isolates ($n = 101$) were able to produce OTA. As shown in Table 2, mean OTA levels produced ranged from 1.92 to 195.46 μg/g. None of the black aspergilli belonging to *A. niger* aggregate and to the uniseriate species *Aspergillus japonicus* var. *aculeatus* were able to produce OTA.

Table 2
OTA production (μg/g of culture medium) by the *A. carbonarius* isolates assayed ($n = 101$) after 10 days of incubation at 30 °C

Range	Mean concentration	Number of isolates (%)
<10	1.92	54 (53.5)
10–100	49.90	42 (41.6)
>100	195.46	5 (4.9)
0–100	31.45	101 (100)

Black aspergilli have been recently reported as the predominant mycobiota of grapes (Cabañes et al., 2002; Da Rocha Rosa et al., 2002; Sage et al., 2002; Battilani et al., 2003). Since the first description of OTA production by *A. niger* var. *niger* (Abarca et al., 1994) and by *A. carbonarius* (Horie, 1995), the reported percentages of ochratoxigenic isolates belonging to *A. niger* aggregate is much lower than in *A.*

Table 3
Distribution of *Aspergilllus* section *Nigri* isolates in the different developmental stages of the berries

Species	Total number	Developmental stage			
		Setting	One month later	Veraison	Harvesting
A. carbonarius	101	1[a]	3[a]	9[a]	88[b]
A. niger aggregate	474	29[a]	71[a,b]	143[b]	231[c]
A. japonicus var. *aculeatus*	5	0	0	0	5
Total	580	30[a]	74[a]	152[b]	324[c]

[a,b,c]Number of isolates that are not followed by the same letter differ significantly ($p < 0.01$).

carbonarius species (Abarca et al., 2001). The OTA producing ability of *A. carbonarius* strains isolated from grapes ranged from 25% to 100% (Cabañes et al., 2002; Da Rocha Rosa et al., 2002; Sage et al., 2002; Battilani et al., 2003) while for isolates belonging to *A. niger* aggregate this percentage was between 5% and 30% (Da Rocha Rosa et al., 2002; Battilani et al., 2003).

Distribution of black aspergilli species in the different developmental stages of the berries are shown in Table 3. Isolates belonging to *A. niger* aggregate were recovered in all the developmental stages surveyed, but a statistically significant increase was observed at the final sampling time. *A. carbonarius*, the most probable source of OTA, achieved also its highest level of isolation at harvesting. Therefore, it is obvious that the risk of OTA production increases sharply with the ripening of berries.

With the only exception of *A. japonicus* var. *aculeatus*, the remaining black aspergilli species were isolated from all the vineyards. When the occurrence of *Aspergillus* section *Nigri* species in the seven vineyards surveyed was studied, no statistically significant differences were observed. As it has been previously reported (Zimmerli and Dick, 1996), the incidence of OTA depends not only on climatic conditions but also on the different practices used in grape cultivation (e.g. use of pesticides) and wine-making (e.g. difference in the way of making white and red wine).

Although the possible participation of different OTA-producing species may occur, our results are a strong evidence of the contribution of *A. carbonarius* in the OTA contamination in grapes, mainly at the last developmental stage of the berries, and consequently in wine. This is supported not only by its important role in the mycobiota of grapes, but also by the extremely high ability to produce OTA and the scarce or null contribution of the typical OTA-producing species *A. ochraceus* and *P. verrucosum*.

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OCHRATOXIN A PRODUCING FUNGI FROM SPANISH VINEYARDS

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1. INTRODUCTION

Ochratoxin A (OTA) is a nephrotoxic mycotoxin naturally occurring in a wide range of food commodities. It has been classified as a possible human renal carcinogen (group 2B) and among other toxic effects, it is teratogenic, immunotoxic, genotoxic, mutagenic and carcinogenic (Creppy, 1999). Following cereals, wine is considered the second major source of OTA in Europe. Since the first report on the occurrence of OTA in wine (Zimmerli and Dick, 1996) its presence in wine and grape juice have been reported in a broad variety of wines from different origins. Up to now, maximum OTA levels have been established for cereals and dried vine fruits in the European Union, but it is possible that other commodities such as wine and grape juices will be regulated before the end of 2003 (Anonymous, 2002).

The occurrence of OTA in wine is due to the presence of fungi on grapes. *Aspergillus ochraceus* and *Penicillium verrucosum* are considered the main OTA-producing species, but *P. verrucosum* is usually found in cool temperate regions and has been reported almost exclusively in cereal and cereal products while *A. ochraceus* is found sporadically in different commodities in warmer and tropical climates (Pitt and Hocking, 1997). The production of OTA by species in *Aspergillus* section *Nigri* has received considerable attention since the first description of OTA production by *Aspergillus niger* var. *niger* (Abarca et al., 1994) and by *Aspergillus carbonarius* (Horie, 1995). Recently, *A. carbonarius* and other black aspergilli belonging to the *A. niger aggregate* have been

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described as a main possible sources of OTA contamination in grapes (Da Rocha Rosa et al., 2002; Sage et al., 2002; Battilani et al., 2003; Magnoli et al., 2003; Serra et al., 2003), wine (Cabañes et al., 2002), and also in dried vine fruits (Heenan et al., 1998; Abarca et al., 2003).

The objective of this study was to identify the ochratoxigenic mycobiota of grapes from vineyards mainly located along the Mediterranean coast of Spain during 2001 and 2002 seasons.

2. MATERIALS AND METHODS

2.1. Samples

During 2001 and 2002 seasons, the ochratoxin A producing fungi of grapes from 7 Spanish vineyards were studied. The vineyards were located mainly along the Mediterranean coast and belonged to 5 winemaking regions : Barcelona (two vineyards), Tarragona (two vineyards), Valencia (one vineyard), Murcia (one vineyard) and Cádiz (one vineyard). In every vineyard, from May to October, samplings were made in four different times, coinciding with the following developmental stages of the grape: setting, one month later of setting, veraison and harvesting. At each sampling time, 10 bunches were collected from 10 different plants located approximately along two crossing diagonals of the vineyard. Every bunch was collected in a separate paper bag and analyzed in the laboratory within 24-48 h of collection.

2.2. Mycological study

Five berries from each bunch were randomly selected and plated directly onto dichloran rose bengal chloramphenicol agar (DRBC) (Pitt and Hocking, 1997) and five berries more onto malt extract agar (MEA) (Pitt and Hocking, 1997) supplemented with 100 ppm of chloramphenicol and 50 ppm of streptomycin. In total, 5,600 berries were analyzed. Plates were incubated at 25ºC for 7 days.

On the last day of incubation, all fungi belonging to *Aspergillus* and *Penicilium* genera were isolated for identification to species level. (Raper and Fennell, 1965; Pitt, 1979; Klich and Pitt, 1988; Pitt and Hocking, 1997).

2.3. Ochratoxigenic ability

Isolates belonging to *Aspergillus* spp. and *Penicillium* spp. were evaluated using a previously described HPLC screening method (Bragulat et al., 2001). Briefly, the isolates were grown on Czapek Yeast extract Agar (CYA) and on Yeast extract Sucrose agar (YES) (Pitt and Hocking, 1977) and incubated at 25ºC for 7 days. Isolates identified as *A.*

carbonarius were grown on CYA for 10 days at 30ºC because they have been cited as optimal incubation conditions for detecting OTA production in this species (Cabañes et al., 2002; Abarca et al., 2003). From each isolate, three agar plugs were removed from different points of the colony and extracted with 0.5 ml of methanol. The extracts were filtered and injected into the HPLC.

2.4. Data analysis

Data obtained were analyzed statistically by means of one-way analysis of variance test and Student's test. All statistical analyses were performed using SPSS software (version 10.0).

3. RESULTS AND DISCUSSION

The occurrence of *Aspergillus* spp. in the 5,600 berries plated on the two culture media used are shown in Table 1. Although the number of isolates recovered in DRBC medium were higher than on MEA, these differences were not statistically significant. A total of 1,061 isolates belonging to twenty *Aspergillus* spp. (including *Emericella* spp. and *Eurotium amstelodami*) were identified. Isolates of *A. carbonarius* and *A. niger* aggregate constituted the 88.7% of the total *Aspergillus* isolates (Figure 1). *Aspergillus niger* aggregate were isolated from 14.2% of plated berries, and *A. carbonarius* from 2.6%. The occurrence of the remaining *Aspergillus* spp. ranged from 0.02% to 0.5%.

The distribution of the *A. niger* aggregate and *A. carbonarius* isolates in 2001 and 2002 seasons during the development of berries are shown in Figure 2. Although they were recovered in all the stages sampled, there was a statistically significant increase at harvesting. The number of isolates recovered in 2002 were lower than in 2001, probably due to different climatic conditions. Nevertheless in both seasons black aspergilli showed the same tendency, achieving their highest level of isolation at harvesting.

A total of 165 isolates belonging to *Penicillium* spp. were identified. The most frequent species were *P. glabrum, P. brevicompactum, P. sclerotiorum, P. citrinum, P. chrysogenum* and *P. thomii*. The occurrence of the remaining *Penicillium* spp. was lower than 0.12%. OTA production was not detected by any of the 165 *Penicillium* isolates. Only one isolate of *P. verrucosum* was identified. This isolates was able to produce citrinin but did not produce OTA.

The ochratoxigenic ability of *Aspergillus* isolates is shown in Table 2. All the *A. carbonarius* isolates (n=144) were able to produce OTA whereas only eight isolates of *A. niger* aggregate (n=797) were ochratoxigenic. None of the *A. japonicus* var. *aculeatus* (n=8) produced OTA.

OTA production was also detected by other *Aspergillus* species outside section *Nigri*. Four of the eight isolates of *A. ochraceus* and two of the four isolates of *A. melleus* produced OTA. All the isolates of *A. ostianus* (n=3) and *A. alliaceus* (n=5) were able to

produce it. Some of these species were able to produce OTA in large quantities, but due to their low occurrence, they are probably a relatively unimportant source of this mycotoxin in grapes.

Although the possible participation of different OTA-producing species may occur, our results are a strong evidence of the contribution of *A. carbonarius* in the OTA contamination in grapes, mainly at the last developmental stage of the berries, and consequently in wine.

4. ACKNOWLEDGEMENTS

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Table 1. Occurrence of *Aspergillus* spp. in the 5,600 berries studied during 2001 and 2002 seasons.

Species	No. (%) of positive plated berries		
	Total (n=5,600)	DRBC[a] (n=2,800)	MEA[a] (n=2,800)
A. niger aggregate	797 (14.23)	438	359
A. carbonarius	144 (2.57)	83	61
A. ustus	26 (0.46)	15	11
A. fumigatus	19 (0.34)	10	9
A. flavus	11 (0.20)	6	5
A. tamarii	9 (0.16)	4	5
A. japonicus var. *aculeatus*	8 (0.14)	3	5
A. ochraceus	8 (0.14)	8	0
E. nidulans	6 (0.11)	4	2
A. alliaceus	5 (0.09)	4	1
A. terreus	5 (0.09)	4	1
A. wentii	5 (0.09)	3	2
A. melleus	4 (0.07)	4	0
A. flavipes	3 (0.05)	2	1
A. ostianus	3 (0.05)	3	0
A. parasiticus	3 (0.05)	2	1
E. amstelodami	2 (0.04)	1	1
E. astellata	1 (0.02)	0	1
E. variecolor	1 (0.02)	0	1
A. versicolor	1 (0.02)	0	1
Total *Aspergillus* spp.	1061	594	467

[a] DRBC: dichloran rose bengal chloramphenicol agar; MEA: malt extract agar.

Table 2. OTA production (μg/g of culture medium) by *Aspergillus* spp.

Species	No.of positive isolates /Total	Mean concentration	Range
A. carbonarius	144 / 144	24.6	0.1 – 378.5
A. niger aggregate	8 / 797	29.2	0.05 – 230.9
A. alliaceus	5 / 5	351.4	197.6 – 715.4
A. ochraceus	4 / 8	440.8	1.3 – 1026.7
A. ostianus	3 / 3	1273.3	245.9 – 2514.1
A. melleus	2 / 4	19.7	7.3 – 32.2

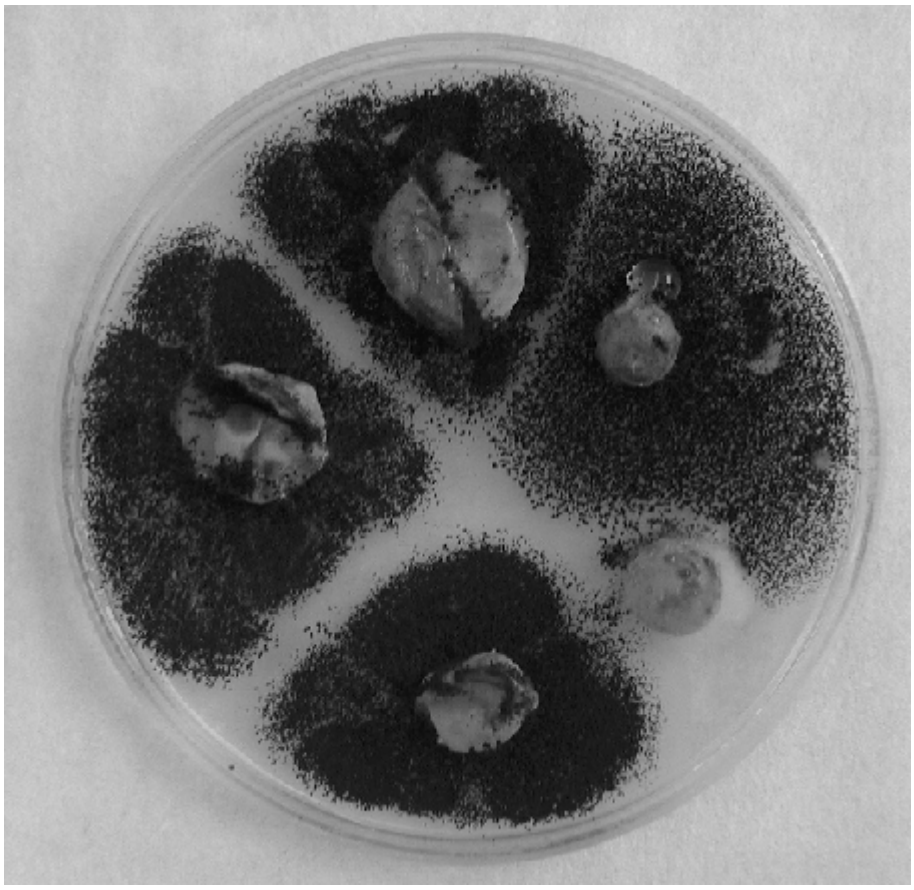

Figure 1. Black aspergilli growing on plated berries from harvesting time. (Note their high occurrence at this sampling time).

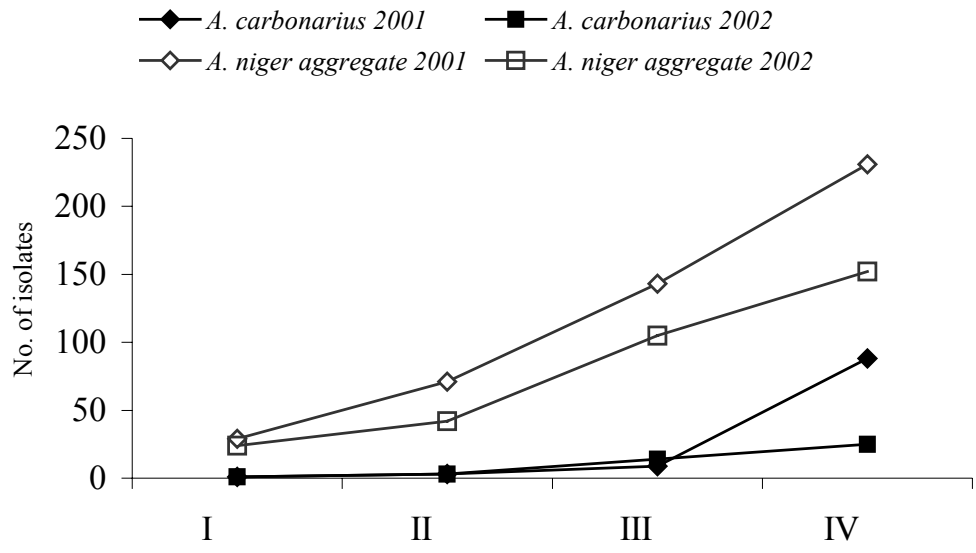

Figure 2. Distribution of *A. carbonarius* and *A. niger* aggregate isolates at each developmental stages of the berries: I, setting; II, one month later of setting; III, veraison; IV, harvesting.

5.3. Bau M, Castellá G, Bragulat MR, Cabañes FJ. DNA based characterization of ochratoxin A-producing and non producing *Aspergillus carbonarius* strains from grapes. Research in Microbiology 156 (2005) 375-381.

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DNA-based characterization of ochratoxin-A-producing and non-producing *Aspergillus carbonarius* strains from grapes

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Abstract

Using molecular methods, a total of 189 strains of black aspergilli, including *Aspergillus carbonarius* and uniseriate species (*Aspergillus aculeatus*, *Aspergillus japonicus*), were studied in order to characterize species responsible for ochratoxin A (OTA) contamination of grapes from Europe and Israel. Sixty-six strains were morphologically identified as belonging to the uniseriate species and 123 as *A. carbonarius*. None of the uniseriate species were able to produce OTA. From the *A. carbonarius* strains, 96.7% were OTA producers (0.1–654.3 μg/g). We characterized 53 strains of *A. carbonarius* from different countries by RAPD and ITS-5.8S rDNA sequencing analysis. Forty-nine strains had a similar RAPD pattern and identical ITS-5.8S rDNA sequences. They produced OTA at different levels. No correlation was observed between the obtained clusters and the OTA production level or origin. Only four strains, morphologically identified as *A. carbonarius*, were unable to produce OTA. These strains showed a different RAPD pattern, and the section of DNA sequenced differed from the sequence of the other 49 strains. These OTA-non-producing strains may represent a new species in the *Aspergillus* section *Nigri*.

Keywords: *Aspergillus carbonarius*; *Aspergillus japonicus*; *Aspergillus aculeatus*; Black aspergilli; Ochratoxin A; RAPD; ITS-5.8S sequences; Wine grapes

1. Introduction

Next to cereals, wine is considered the second major source of ochratoxin A (OTA) in Europe. Up to now, maximum OTA levels have been established for cereals and dried vine fruits in the European Union [6]. Maximum OTA levels for coffee beans, ground roasted coffee, soluble coffee, wine and other wine and/or grape must-based drinks as well as grape juice and grape juice ingredients in other drinks proposed by the European Commission have been recently approved by the Standing Committee on the Food Chain and Animal Health [7].

Although *Aspergillus ochraceus* and *Penicillium verrucosum* are considered the classical OTA-producing species, they are probably unimportant sources of this mycotoxin in grapes. *P. verrucosum* is usually found in cool temperate regions and has been reported almost exclusively in cereals and cereal products, while *A. ochraceus* is found sporadically in different commodities in warmer and tropical climates [30]. Recently, *A. carbonarius* and other black *Aspergillus* species belonging to the *A. niger* aggregate have been described as a main possible source of OTA contamination in grapes worldwide [8,9,12,15,25,26,31,34]. They have also been reported as sources of OTA in coffee [36].

On the other hand, the taxonomy of black aspergilli (*Aspergillus* section *Nigri*) is not clear and many attempts have been made in order to find accurate criteria for species identification [3]. This section has long been studied by means of morphological and cultural criteria. Some species, such as *A. carbonarius* and uniseriate species (*A. japonicus*, *A. aculeatus*) can be easily recognized. Other species, such as *A. helicothrix*, *A. ellipticus* and *A. heteromorphus* are rare. The *A. niger* aggregate assembles a group of closely related morphospecies in this section. Although speciation at the molecular level has been proposed, no clear morphological

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differences can be observed in this group and species identification remains problematic. A RFLP technique has been proposed as a method for screening possible OTA-producing *A. niger* aggregate strains [5]. Following this technique, at the moment all the OTA-producing isolates whose RFLP pattern is known have been classified as type N, whereas none of the type T strains have been able to produce this mycotoxin.

Only some species in the *Aspergillus* section *Nigri* produce OTA. At present, several members belonging to the *A. niger* aggregate [1] and *A. carbonarius* [19] are considered as clearly ochratoxin A-producing species. Reported percentages of ochratoxigenic isolates belonging to the *A. niger* aggregate are much lower than in *A. carbonarius* [2]. The ability of the uniseriate black *Aspergillus* species to produce OTA has been recently mentioned [8,16], but this fact needs to be confirmed, since at present they are not considered as OTA-producing species [25,28,37].

The objectives of this study were to identify potential ochratoxigenic strains belonging to *A. carbonarius* and uniseriate black *Aspergillus* species from grapes of different origins and to characterize, using molecular methods, representative *A. carbonarius* strains from grapes, which are the main responsible fungi involved in OTA contamination of wine.

2. Materials and methods

2.1. Strains

A total of 189 strains of black aspergilli, including *A. carbonarius* and uniseriate species (*A. aculeatus*, *A. japonicus*), were studied. These strains were supplied by different partners and countries involved in the European project QLK1-CT-2001-01761 (Wine-ochra risk). They were isolated during the years 2001 and 2002. Identification of different strains of black aspergilli was made using macroscopic and microscopic morphological criteria in accordance with appropriate keys [3,22,23,30]. They were preserved at −80 °C in the culture collection of the Faculty of Veterinary Science of Barcelona, Spain.

2.2. DNA extraction

Fungal DNA was extracted as described by Accensi et al. [4]. The strains were inoculated in 1.5 ml Eppendorf tubes containing 500 µl of Sabouraud broth (2% glucose, w/v; 1% peptone, w/v) supplemented with chloramphenicol (1 mg l^{-1}), and incubated overnight in an orbital shaker at 300 rpm and 30 °C. Mycelium was recovered after centrifugation and washed with NaCl 0.9% (w/v), frozen in liquid nitrogen and ground to a fine powder with a pipette tip. The powder was incubated for 1 h at 65 °C in 500 µl extraction buffer (50 mM Tris–HCl, 50 mM EDTA, 3% SDS and 1% 2-mercaptoethanol). The lysate was extracted with phenol:chloroform (1:1, v/v), 3 M NaOAc and 1 M NaCl. DNA was recovered by isopropanol precipitation. The pellet was washed with 70% (v/v) ethanol, dried under vacuum and resuspended in TE buffer (Tris–HCl 10 mM, EDTA 1 mM, pH 8). DNA was cleaned with "Geneclean kit II" (BIO 101, Inc., La Jolla, CA), according to the manufacturer's instructions.

2.3. RAPD characterization of A. carbonarius strains

Fifty-three strains that were morphologically identified as *A. carbonarius* were characterized by RAPD. The isolated DNA was used as template DNA for RAPD-PCR. PCRs were performed in a total volume of 25 µl containing 1–10 ng of genomic DNA, 0.5 µM of primer, 0.1 mM dNTPs (Perkin–Elmer, Cerdanyola, Spain) and 1 U of taq DNA polymerase in the buffer provided by the manufacturer (Perkin–Elmer, Cerdanyola, Spain). The primer used was ARI 1 (5′-TGCTTGGCACAGTTGGCTTC-3′) [14].

Amplification reactions were performed in a Perkin–Elmer 2400 DNA thermal cycler which was set to the following conditions: 95 °C, 5 min; 44 cycles consisting of 60 s at 95 °C, 60 s at 36 °C and 4 min at 72 °C. This was followed by a final amplification period of 7 min at 72 °C. At least two separate amplifications were conducted for each isolate. The amplified DNA products were separated by electrophoresis in 2% (w/v) agarose (molecular biology certified agarose, Bio-Rad Laboratories S.A., Barcelona, Spain) in 1× TBE buffer according to Sambrook et al. [33]. Gels were stained in an 1.0 µg/ml ethidium bromide solution and photographed on a gel documentation system (Gel Doc 2000, Bio-Rad Laboratories S.A., Barcelona, Spain). Size marker (100-bp ladder, Bio-Rad Laboratories S.A., Barcelona, Spain) was run on each gel.

The RAPD profiles obtained were used to calculate Dice's coefficient [17] of genetic similarity. A dendrogram was constructed from this coefficient with the UPGMA method [35] of the Bio-Rad Diversity Database Fingerprinting software.

2.4. A. carbonarius ITS-5.8S rDNA sequencing analysis characterization

Fifty-three strains that were morphologically identified as *A. carbonarius* were sequenced. The isolated DNA was used as template DNA. ITS rDNA and 5.8S rDNA were amplified as described by Gené et al. [18], using a Perkin–Elmer 2400 thermal cycler. The primer pairs ITS5 and ITS4 used were described by White et al. [40]. The amplification process consisted of a pre-denaturation step at 94 °C, for 5 min, followed by 35 cycles of denaturation at 95 °C/30 s, annealing at 50 °C/min and extension at 72 °C/min, plus a final extension of 7 min at 72 °C. The molecular masses of the amplified DNA were estimated by comparison with the 100-bp DNA ladder (Bio-Rad Laboratories S.A., Barcelona, Spain) standard lane.

The PCR product was purified with the GFX PCR DNA and gel band purification kit (Amersham Pharmacia Biotech, Uppsala, Sweden), following the supplier's protocol. The purified PCR products were used as a template for sequencing. The protocol "BigDye Terminator v3.1 Cycle Sequencing kit" (Applied Biosystems, Gouda, The Netherlands) was used for sequencing. The primers ITS5 and ITS4 were used in the sequencing reaction. An Applied Biosystems Mod. 3100 sequencer was used to obtain the DNA sequences. The sequences were aligned with Clustal X of multiple sequence alignment computer program [38]. Adjustments for improvement were made by eye where necessary. Cladistic analyses using the Neighbor joining method [32] were performed with the MEGA 2.1 computer program [24] with the Kimura-2 parameter model, including transitions and transversions and with complete deletion for the treatment of the handling gaps/missing data. Confidence values for individual branches were determined by bootstrap analyses (1000 replications) and maximum parsimony.

2.5. OTA production and quantification

OTA production was analyzed in all strains using a previously described high-pressure liquid chromatography (HPLC) screening method [11]. On each sampling occasion, three agar plugs were removed from different points of the colony and extracted with 0.5 ml of methanol. The extracts were filtered and injected into the HPLC. OTA detection and quantification were performed by a Waters LCM1 chromatograph with a Waters 2475 fluorescence detector (excitation wavelength: 330 nm/emission wavelength: 460 nm), and with a column C18 Spherisorb S5 ODS2, 250 × 4.6 mm. Twenty µl of each extract were applied. The mobile phase, with a flow rate of 1 ml/min, consisted of the following linear gradient: acetonitrile, 57%; water, 41%, and acetic acid, 2% [10]. The extracts with the same retention time as OTA (around 6.8 min), were considered positive. Confirmation was made through derivatization of OTA in its methyl-ester [20]. The quantification limit of HPLC technique, with the extraction procedure was 0.01 µg/g for this mycotoxin.

3. Results and discussion

3.1. Phenotypic characterization of the strains

Sixty-six strains were morphologically identified as uniseriate species and 123 as *A. carbonarius* (Tables 1 and 2). Among the black *Aspergillus* species, *A. carbonarius* and the uniseriate species (*A. aculeatus, A. japonicus*) can be microscopically distinguished by vesicle and conidial size and ornamentation [3].

The taxonomic status of the uniseriate species *A. japonicus* and *A. aculeatus* is still in question [23,28]. All the strains analyzed in this study had large vesicles, fitting the description of *A. aculeatus*. This species is usually found in foods [30] whereas *A. japonicus* has been isolated from soils, plant rhizospheres and leaf litter predominantly in tropical zones [23]. However, none of the strains tested in this study were able to produce OTA (Tables 1 and 3) and for this

Table 1
Reference, origin and OTA production (µg/g) in strains identified as uniseriate species of black aspergilli

Strain ref.[a]	Origin	OTA level	Strain ref.	Origin	OTA level	Strain ref.	Origin	OTA level
A-1620	Italy	N.D.	A-1804	Italy	N.D.	A-1832	France	N.D.
A-1621	Italy	N.D.	A-1805	Italy	N.D.	A-1833	France	N.D.
A-1622	Italy	N.D.	A-1806	Italy	N.D.	A-1834	France	N.D.
A-1630	Italy	N.D.	A-1807	Italy	N.D.	A-1835	France	N.D.
A-1631	Italy	N.D.	A-1813	Israel	N.D.	A-1836	France	N.D.
A-1635	Italy	N.D.	A-1814	Israel	N.D.	A-1837	France	N.D.
A-1637	Italy	N.D.	A-1815	Israel	N.D.	A-1850	Greece	N.D.
A-1643	Italy	N.D.	A-1816	Israel	N.D.	A-1871	Greece	N.D.
A-1644	Italy	N.D.	A-1818	France	N.D.	A-1905	Italy	N.D.
A-1671	France	N.D.	A-1819	France	N.D.	A-1906	Italy	N.D.
A-1683	Portugal	N.D.	A-1820	France	N.D.	A-1907	Italy	N.D.
A-1715	Spain	N.D.	A-1821	France	N.D.	A-1908	Italy	N.D.
A-1730	Spain	N.D.	A-1822	France	N.D.	A-1929	Israel	N.D.
A-1731	Spain	N.D.	A-1823	France	N.D.	A-1930	Israel	N.D.
A-1732	Spain	N.D.	A-1824	France	N.D.	A-1931	Israel	N.D.
A-1733	Spain	N.D.	A-1825	France	N.D.	A-1932	Israel	N.D.
A-1734	Spain	N.D.	A-1826	France	N.D.	A-1948	France	N.D.
A-1781	Israel	N.D.	A-1827	France	N.D.	A-1949	France	N.D.
A-1782	Israel	N.D.	A-1828	France	N.D.	A-1950	France	N.D.
A-1783	Israel	N.D.	A-1829	France	N.D.	A-1951	France	N.D.
A-1784	Israel	N.D.	A-1830	France	N.D.	A-1976	Spain	N.D.
A-1785	Israel	N.D.	A-1831	France	N.D.	A-1977	Spain	N.D.

[a] A: Culture Collection of Veterinary Faculty of Barcelona, Spain. N.D: not detected. Detection limit 0.01 µg/g.

Table 2
Reference, origin and OTA production (μg/g) in strains identified as *A. carbonarius*

Strain ref.[a]	Origin	OTA level	Strain ref.	Origin	OTA level	Strain ref.	Origin	OTA level	Strain ref.	Origin	OTA level
A-1604	Spain	N.D.	A-1712	Portugal	5.7	A-1765	Israel	1.7	A-1890	Italy	3.4
A-1616	Portugal	N.D.	A-1716	Spain	51.6	A-1766	Israel	0.3	A-1891	Italy	2.5
A-1617	Portugal	N.D.	A-1717	Spain	1.9	A-1786	Italy	5.5	A-1892	Italy	2.5
A-1618	Portugal	N.D.	A-1718	Spain	12.1	A-1787	Italy	11.2	A-1893	Italy	1.5
A-1625	Italy	8.1	A-1719	Spain	2.4	A-1788	Italy	3.3	A-1894	Italy	1.3
A-1626	Italy	31.6	A-1720	Spain	26.6	A-1789	Italy	20.5	A-1909	Israel	9.9
A-1628	Italy	3.5	A-1721	Spain	33.3	A-1790	Italy	9.2	A-1910	Israel	77.7
A-1636	Italy	4.7	A-1722	Spain	2.7	A-1791	Italy	2.6	A-1911	Israel	3.1
A-1641	Italy	7.8	A-1723	Spain	21.1	A-1792	Italy	4.2	A-1912	Israel	22.8
A-1651	Italy	51.1	A-1724	Spain	28.9	A-1793	Italy	5.3	A-1913	Israel	17.8
A-1652	Italy	6.4	A-1725	Spain	0.8	A-1794	Italy	5.8	A-1914	Israel	2.0
A-1653	France	654.3	A-1726	Spain	58.3	A-1795	Italy	3.6	A-1915	Israel	8.0
A-1654	France	57.7	A-1727	Spain	49.9	A-1796	Italy	1.1	A-1916	Israel	10.5
A-1655	France	215.3	A-1728	Spain	6.0	A-1797	Italy	0.8	A-1917	Israel	15.5
A-1656	France	10.5	A-1729	Spain	63.8	A-1798	Italy	1.3	A-1918	Israel	23.9
A-1657	France	22.8	A-1749	Israel	72.7	A-1841	Greece	8.7	A-1937	Portugal	15.1
A-1658	France	1.9	A-1750	Israel	53.8	A-1842	Greece	113.8	A-1939	Portugal	15.0
A-1659	France	2.9	A-1751	Israel	48.3	A-1851	Greece	15.5	A-1940	Portugal	17.0
A-1660	France	43.4	A-1752	Israel	37.7	A-1867	Greece	32.7	A-1941	Portugal	15.8
A-1661	France	1.1	A-1753	Israel	68.8	A-1876	Greece	0.2	A-1942	Portugal	24.2
A-1662	France	1.5	A-1754	Israel	48.3	A-1877	Greece	48.3	A-1958	France	84.9
A-1665	France	27.8	A-1755	Israel	58.8	A-1878	Greece	56.6	A-1959	France	15.0
A-1687	Portugal	18.3	A-1756	Israel	51.1	A-1879	Greece	25.0	A-1960	France	22.2
A-1688	Portugal	79.9	A-1757	Israel	3.1	A-1880	Greece	15.7	A-1961	France	19.4
A-1693	Portugal	12.8	A-1758	Israel	138.7	A-1881	Greece	24.4	A-1962	France	23.3
A-1696	Portugal	0.1	A-1759	Israel	0.7	A-1883	Greece	25.5	A-1970	Spain	34.9
A-1697	Portugal	0.2	A-1760	Israel	4.7	A-1885	Italy	12.8	A-1971	Spain	41.6
A-1700	Portugal	0.4	A-1761	Israel	0.5	A-1886	Italy	15.0	A-1972	Spain	18.9
A-1701	Portugal	0.1	A-1762	Israel	46.1	A-1887	Italy	0.2	A-1974	Spain	1.9
A-1702	Portugal	30.0	A-1763	Israel	185.4	A-1888	Italy	1.7	A-1975	Spain	36.5
A-1706	Portugal	5.1	A-1764	Israel	42.7	A-1889	Italy	2.5			

[a] A: Culture Collection of Veterinary Faculty of Barcelona, Spain; N.D: not detected. Detection limit 0.01 μg/g.

Table 3
Summary of results obtained with the strains analyzed in this study

Origin	Uniseriate		*A. carbonarius*			
	n	% OTA	*n*	% OTA	OTA range (μg/g)	OTA mean (μg/g)
France	25	0	16	100	1.1–654.3	75.2
Greece	2	0	11	100	0.2–113.8	33.3
Israel	13	0	28	100	0.3–185.4	37.7
Italy	17	0	30	100	0.2–51.1	7.7
Portugal	1	0	18	83.3[a]	0.1–79.9	13.3
Spain	8	0	20	95.0[a]	0.8–63.8	24.7
Total	66	0	123	96.7[a]	0.1–654.3	29.2

[a] Non-OTA-producing strains may represent a new species in the *Aspergillus* section *Nigri*.

reason they were not analyzed using molecular techniques. For the moment neither species is considered to be an OTA-producing species [25,28,37].

3.2. A. carbonarius *molecular characterization and OTA production*

We analyzed a total of 123 *A. carbonarius* strains in order to determine whether they were able to produce OTA. From these strains, 119 (96.7%) were OTA producers. The reported percentages of ochratoxigenic isolates in *A. carbonarius* ranged from 25 to 100% [3]. The concentrations of OTA detected varied from 0.1 to 654.3 μg/g (Tables 2 and 3).

We characterized 53 strains of *A. carbonarius* from different countries by RAPD and ITS-5.8S rDNA sequencing analysis. Results obtained in the RAPD analysis (Fig. 1) showed that 49 strains had a similar banding pattern (4 bands of 1.6, 1.5, 1.0 and 0.8 Kb) (Fig. 2). They produced OTA at different concentrations and no correlation was determined between the obtained clusters and the OTA production level or origin. The ITS-5.8S rDNA sequencing analysis of these strains showed that they had nearly identical sequences and included 612 base pairs (Fig. 3). Only one strain (A-1716) showed a T insertion in the ITS2 region. Moreover, these sequences differed from the sequence of the neotype strain of *A. carbonarius* CBS 111.26 (AJ280011) at a single nucleotide position.

The remaining four strains showed a different RAPD pattern with a common band of 900 bp and the section of DNA sequenced included 614 base pairs, and differed from the sequence of the other 49 strains at 3–5 nucleotide positions.

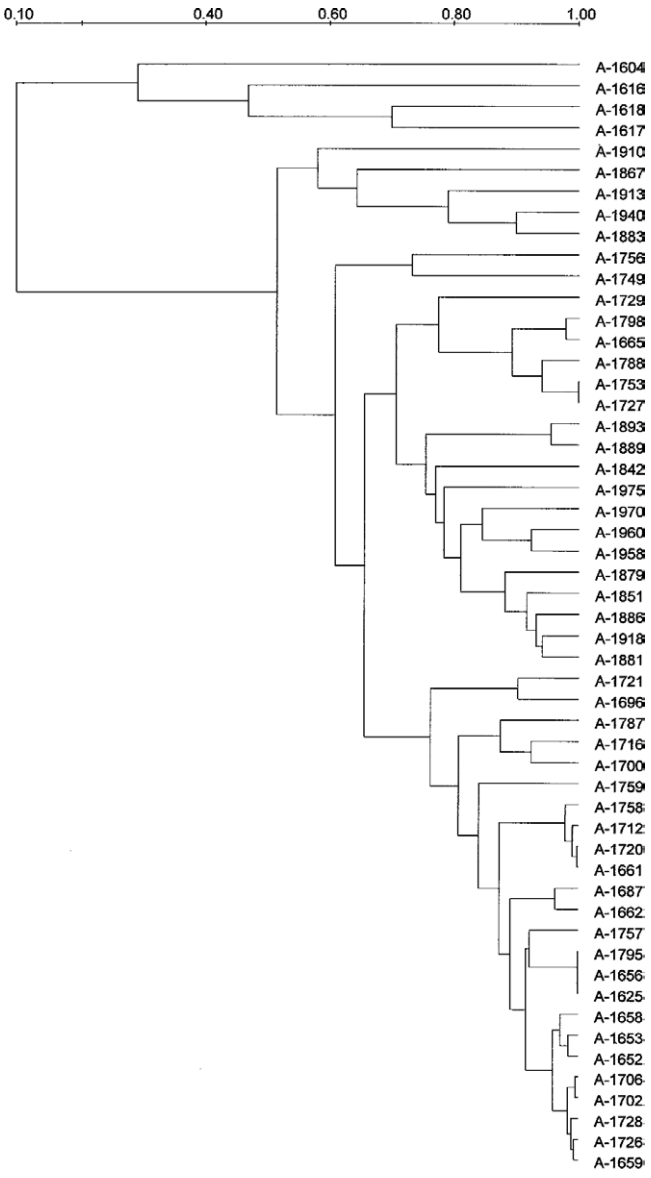

Fig. 1. UPGMA dendrogram of the *A. carbonarius* strains assessed from the comparison of RAPD fingerprints generated with primer ARI1. The scale represents taxonomic distance.

These four strains were the only ones not able to produce OTA.

A. carbonarius forms a separate clade within the biseriate black aspergilli [3] that can be easily separated from other black aspergilli using ITS sequencing [27]. Kevei et al. [21] showed that RFLP analysis of both rDNA and mtDNA revealed only slight intraspecific variations in the morphologically uniform species *A. carbonarius*, but strain-specific characters could be detected by RAPD. Varga et al. [39] analyzed 16 strains of *A. carbonarius* and found identical rDNA patterns, and similar mtDNA and isoenzyme profiles in all strains except for one that was proposed to represent a new subspecies, "*A. carbonarius* var. *indicus*", of the *A. carbonarius* species.

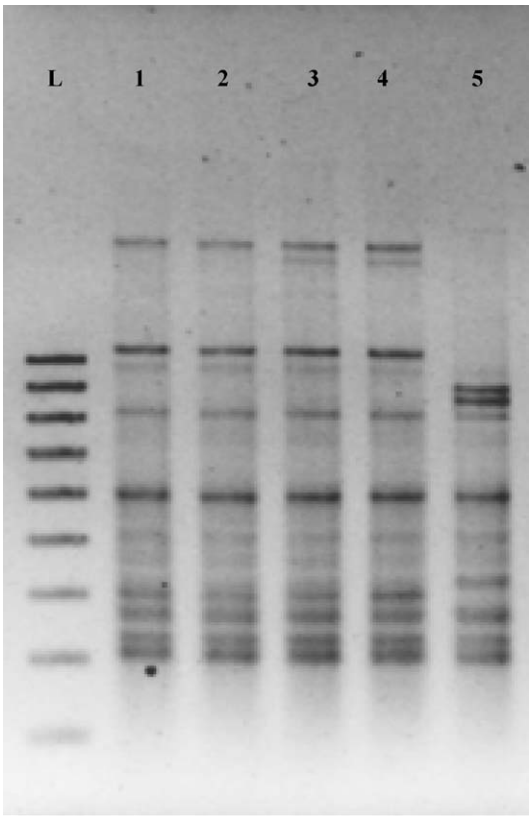

Fig. 2. RAPD generated with primer ARI 1. Lane L is the 100-bp DNA ladder (Bio-Rad Laboratories S.A.). Lanes 1, 2, 3 and 4: banding pattern of OTA-producing *A. carbonarius* strains (A-1958, A-1960, A-1970 and A-1975, respectively); lane 5: banding pattern of a non-OTA-producing strain (A-1604).

Recently, Pelegrinelli-Fungaro et al. [29] compared RAPD patterns of toxigenic and non-toxigenic strains of *A. carbonarius* isolated from coffee beans. Although the PCR-based assay described by the authors was successfully employed to detect *A. carbonarius* in coffee samples, no association was found between the RAPD genotype and the ability to produce OTA of the strains.

In our study only four strains were unable to produce OTA. These strains, three from Portugal (A-1616, A-1617, and A-1618) and one from Spain (A-1604), were tentatively identified as *A. carbonarius*. These non-OTA producing strains may represent a new species. They are now under study because their morphological and genetic characteristics differ from *A. carbonarius* and the rest of the species belonging to the *Aspergillus* section *Nigri* [13].

This study also confirms the high percentage of OTA-producing strains in *A. carbonarius* and the inability of the uniseriate black *Aspergillus* species to produce OTA.

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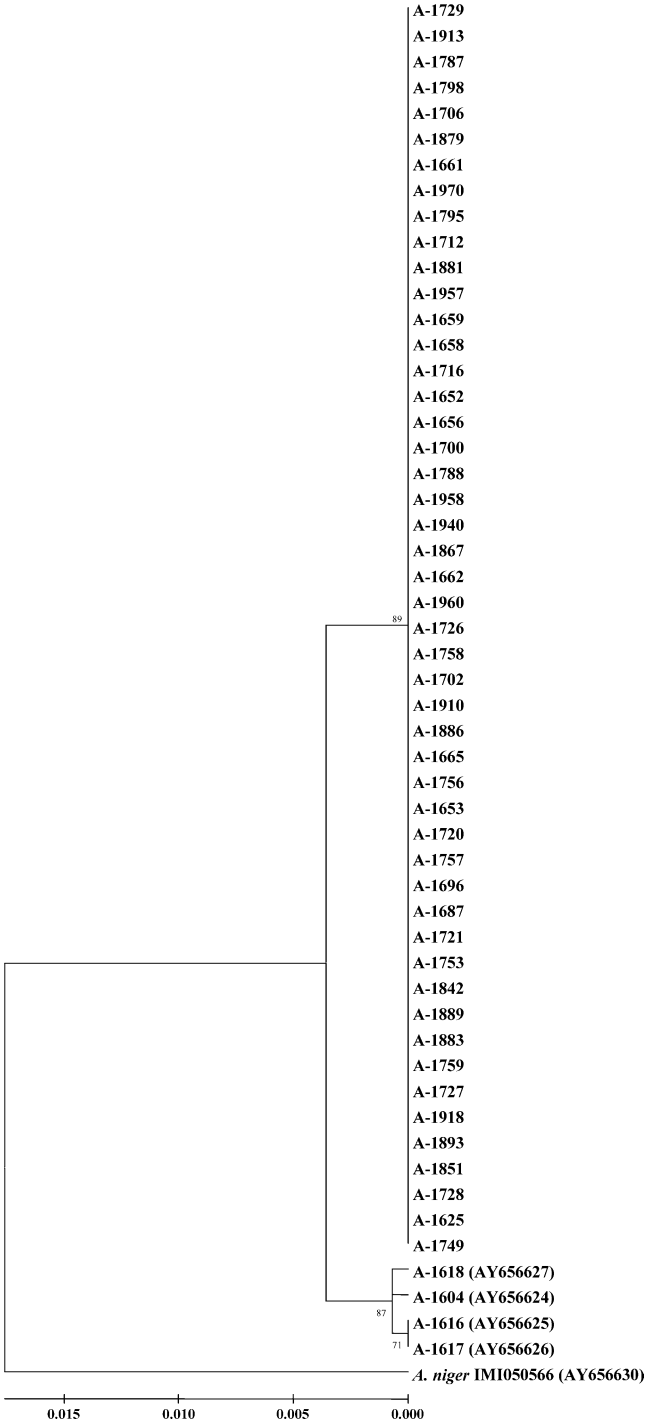

Fig. 3. Neighbor-joining tree based on phylogenetic analysis of the ITS1-5.8S rDNA-ITS2 sequences of the *A. carbonarius* strains. The numbers at branch points are the percentages of 1000 bootstrapped data sets that supported the specific internal branches. Species with GenBank numbers represent sequences deposited at the GenBank.

viding most of the strains used in this study. This research was supported by the European Union project QLK1-CT-2001-01761 (Quality of Life and Management of Living Resources Programme (QoL), Key Action 1 on Food, Nutrition and Health). The financial support of the Ministerio de Ciencia y Tecnología of the Spanish Government (AGL01-2974-C05-03) is also acknowledged.

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Molecular characterization of *Aspergillus niger* aggregate species from European wine grapes.

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ABSTRACT

In order to characterize by molecular methods the *Aspergillus niger* aggregate species involved in the ochratoxin A contamination of European wine grapes, a total of 173 strains were studied. All of the strains were classified into the two RFLP patterns previously defined: type N (43%) and type T (57%). Twenty out of the 173 strains of *A. niger* aggregate produced OTA (0.1 to 10.5 μg/g). All the OTA producing species belonged to the N type.

Keywords: *Aspergillus niger* aggregate, black aspergilli, ochratoxin A, RFLP, wine grapes.

1. Introduction

The great importance of the contribution of the species included in the *Aspergillus* section *Nigri*, mainly *A. carbonarius* and members of the *A. niger* aggregate, in the ochratoxin A (OTA) contamination in winegrapes has been recently cited worldwide (Cabañes *et al.*, 2002; Da Rocha Rosa *et al.*, 2002; Sage *et al.*, 2002; Battilani *et al.*, 2003; Magnoli *et al.*, 2003; Serra *et al.*, 2003; Bau *et al.*, 2004; Leong *et al.*, 2004) and is also presented in this special issue.

Only some species in the *Aspergillus* section *Nigri* produce OTA. At present, several members belonging to the *A. niger* aggregate (Abarca *et al.*, 1994) and *A. carbonarius* (Horie, 1995) are considered as clearly ochratoxin A producing species. Reported percentages of ochratoxigenic isolates belonging to *A. niger* aggregate is much lower than in *A. carbonarius* species (Abarca *et al.*, 2001).

On the other hand, the taxonomy of black aspergilli (*Aspergillus* section *Nigri*) is not clear and many attempts have been made in order to find accurate criteria for species identification (Abarca *et al.*, 2004). This section has been long studied by means of morphological and cultural criteria. Some species, such as *A. carbonarius* and uniseriate species (*A. japonicus*, *A. aculeatus*) can be easily recognised. Other species, such as *A. helicothrix*, *A. ellipticus* and *A. heteromorphus* are rare. *A. niger* aggregate is a group of closely related morphospecies included in this section. Although speciation at molecular level has been proposed, no clear morphological differences can be observed and species identification remains problematic. The division of this *A. niger* aggregate into two phylogenetic species, namely *A. niger* and *A. tubingensis*, according to RFLP analysis of total DNA was proposed (Kusters van Someren *et al.*, 1991). In our laboratory, a method

that clearly differentiate the *A. niger* aggregate isolates into two RFLP patterns, type N and type T, corresponding to the type species of *A. niger* and *A. tubingensis* respectively was described (Accensi *et al.*, 1999).

The objective of this study was to characterize, using molecular methods, *A. niger* aggregate strains involved in the OTA contamination of wine.

2. Materials and methods

2.1. Strains

In total, 173 strains of *A. niger* aggregate were studied. The strains were supplied by different partners and countries involved in the European project QLK1-CT-2001-01761 Wine-ochra risk. They were isolated in the 2001 and 2002 years. Identification of different strains was made using macroscopic and microscopic morphological criteria in accordance with appropriate keys (Klich and Pitt, 1988; Pitt and Hocking, 1997; Klich, 2002; Abarca *et al.*, 2004) and they were preserved at –80ºC in the culture collection of the Faculty of Veterinary of Barcelona, Spain.

2.2. DNA extraction

Fungal DNA was extracted as described by Accensi *et al.* (1999). The strains were inoculated in 1.5 ml Eppendorf tubes containing 500 µl of Sabouraud broth (2% glucose, w/vol; 1% peptone w/vol) supplemented with chloramphenicol (1 mg l^{-1}), and incubated overnight in an orbital shaker at 300 rpm and 30ºC. Mycelium was recovered after centrifugation and washed with NaCl 0.9% (w/vol), frozen in liquid nitrogen and ground to a fine powder with a pipette tip. The powder was incubated for 1 h at 65ºC in 500 µl

extraction buffer (Tris-HCl 50mM, EDTA 50mM, SDS 3% and 2-mercaptoethanol 1%). The lysate was extracted with phenol:chloroform (1:1, vol/vol), 3M NaOAc and 1M NaCl. DNA was recovered by isopropanol precipitation. The pellet was washed with 70% (vol/vol) ethanol, dried under vacuum and resuspended in TE buffer (Tris-HCl 10mM, EDTA 1mM, pH 8). DNA was cleaned with "Geneclean kit II" (BIO 101, inc., La Jolla, CA), according to the manufacturer's instructions.

2.3. RFLP analysis A. niger aggregate

All strains of *A. niger* aggregate (n=173) have been characterized by RFLP. The isolated DNA was used as template DNA for *A. niger* aggregate RFLP analysis characterization and was done as described by Accensi *et al*. (1999). ITS rDNA and 5.8S rDNA were amplified as described by Gené *et al.* (1996), using a Perkin Elmer 2400 thermal cycler. The primer pairs ITS5 and ITS4 used were described by White *et al.* (1990). The amplification process consisted of a pre-denaturation step at 94ºC, for 5 min, followed by 35 cycles of denaturation at 95ºC/30 s, annealing at 50ºC/1 min and extension at 72ºC/1 min, plus a final extension of 7 min at 72ºC. The molecular masses of the amplified DNA were estimated by comparison with the 100-bp DNA ladder (Bio-Rad Laboratories S.A:, Barcelona, Spain) standard lane.

The PCR products were digested overnight with the restriction endonuclease *Rsa*I (GT/AC), following the recommendations of manufacturer (BoehringerManheim). RFLP analysis was performed by loading the product of the digestion reaction onto a 2% agarose gel and stained for 30 min ethidium bromide as described by Sambrook *et al.* (1989).

2.4. OTA production and quantification

OTA production was analyzed using a previously described high-pressure liquid chromatography (HPLC) screening method (Bragulat *et al.*, 2001). On each sampling occasion, three agar plugs were removed from different points of the colony and extracted with 0.5 ml of methanol. The extracts were filtered and injected into the HPLC. OTA detection and quantification was made by a Waters LCM1 chromatograph with a fluorescence detector Waters 2475 (excitation wavelength: 330nm / emission wavelength: 460nm), and with a column C18 Spherisorb S5 ODS2, 250x4,6mm. Twenty μl of each extract were applied. The mobile phase, with a flow rate of 1 ml/minute, consisted of a following linear gradient: acetonitrile, 57%; water, 41% and acetic acid, 2% (Bauer *et al.*, 1987). The extracts with the same retention time as OTA (around 6.8 minutes), were considered positive. Confirmation was made through derivatization of OTA in its methyl-ester (Hunt *et al.*, 1980). The quantification limit of HPLC technique, with the extraction procedure was 0.01 μg/g for this mycotoxin.

3. Results and discussion

Among the black *Aspergillus* species, *A. carbonarius* and the uniseriate species (*A. aculeatus, A. japonicus*) can be microscopically distinguished by vesicle and conidial size and ornamentation. However all the taxa included in the *A. niger* aggregate are morphologically indistinguishable (Abarca *et al.*, 2004).

The ITS-5.8S rDNA fragments of 173 *A. niger* agreggate strains from grapes included in this study were amplified and their PCR amplicons were *Rsa*I digested in order to classify the strains in patterns N or T (Accensi *et al.*, 1999). All of the strains belonging to the *A.*

niger aggregate were classified into the two patterns: type N characterized by two fragments (of 519 and 76 base pairs) and type T characterized by one undigested fragment of 595 base pairs. The results of the *Rsa*I digestions of the PCR amplicons are listed in Table 1. No variation within RFLP patterns N and T in any of the strains used was detected. Lack of variation was previously observed using isolates from other sources including type culture collection strains belonging to this aggregate (Accensi *et al.*, 1999; Accensi *et al.*, 2001). Seventy four isolates belonged to the N type (43%) and 99 isolates (57%) to the T type (Table 2). Taking into account the origin of the strains, the distribution of the strains into the two types of patterns was not similar in most of the countries. With the only exception of strains from Greece, where they were equally distributed (N=18, T=16), in the rest of the countries (France N=1, T=22, Italy N=19, T=13, Israel N=3, T=27, Portugal N=27, T=5 and Spain N=6, T=16) they had not a homogeneous distribution.

The taxonomic value of this method has been corroborated recently using AFLP (special issue Italy). Strains belonging to the type N and T clustered in separate groups.

Twenty out of the 173 strains of *A. niger* aggregate produced OTA (11.6%). The concentrations of OTA detected varied from 0.1 to 10.5 μg/g (Table 2).

Most of the OTA producing strains were from Portugal (40.6%), followed by Greece (11.8%), Spain (9.1%) and Italy (3.1%). No OTA producing strains were detected from France and Israel. The reported percentages of ochratoxigenic isolates in the *A. niger* aggregate range from 0.6% to 50% (Abarca *et al.*, 2004).

All the OTA producing species belonged to the N type. This RFLP characterization has been proposed as a technique for screening possible OTA producing *A. niger* aggregate strains (Accensi *et al.*, 2001), due to the fact that all the OTA-producing isolates whose RFLP pattern is known have been classified as type N, whereas none of the type T strains are able, at the moment, to produce this mycotoxin.

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Table 2. References, RFLP pattern and origin of *A. niger* aggregate strains.

Strain Ref.	IMI Ref.	RFLP pattern	origin	Strain Ref.	IMI Ref.	RFLP pattern	origin	Strain Ref.	IMI Ref.	RFLP pattern	origin	Strain Ref.	IMI Ref.	RFLP pattern	origin
A-1623	388526	N	Italy	A-1694	387232	T	Portugal	A-1799	388550	T	Italy	A-1895	389909	N	Italy
A-1624	388527	T	Italy	A-1695	387233	N	Portugal	A-1800	388551	T	Italy	A-1896	389910	N	Italy
A-1627	388529	N	Italy	A-1698	387238	N	Portugal	A-1802	388657	T	Italy	A-1897	389911	T	Italy
A-1629	388531	N	Italy	A-1699	387239	N	Portugal	A-1808	389168	T	Israel	A-1898	389912	T	Italy
A-1632	388534	T	Italy	A-1703	387243	N	Portugal	A-1809	389169	T	Israel	A-1899	389913	N	Italy
A-1633	388535	N	Italy	A-1704	387244	N	Portugal	A-1810	389170	T	Israel	A-1900	389914	N	Italy
A-1634	388536	N	Italy	A-1705	387245	T	Portugal	A-1811	389171	T	Israel	A-1901	389915	N	Italy
A-1638	388540	N	Italy	A-1707	-	N	Portugal	A-1812	389172	T	Israel	A-1902	389916	N	Italy
A-1639	388541	T	Italy	A-1708	387254	N	Portugal	A-1817	389167	T	Israel	A-1903	389917	N	Italy
A-1640	388542	N	Italy	A-1709	387255	N	Portugal	A-1838	388990	T	Greece	A-1904	389918	T	Italy
A-1642	388544	T	Italy	A-1710	387257	N	Portugal	A-1839	388991	N	Greece	A-1919	390007	T	Israel
A-1645	388547	N	Italy	A-1711	387259	N	Portugal	A-1840	388992	N	Greece	A-1920	390008	T	Israel
A-1646	388548	N	Italy	A-1713	387220	N	Portugal	A-1843	388998	T	Greece	A-1921	390010	T	Israel
A-1647	388547	T	Italy	A-1714	387246	T	Portugal	A-1844	389000	N	Greece	A-1922	390012	T	Israel
A-1648	388552	T	Italy	A-1735	388730	T	Spain	A-1845	389004	T	Greece	A-1923	390009	T	Israel
A-1649	388553	N	Italy	A-1736	388731	T	Spain	A-1846	389007	T	Greece	A-1924	390002	T	Israel
A-1650	388554	N	Italy	A-1737	388732	N	Spain	A-1847	389010	T	Greece	A-1925	390003	T	Israel
A-1663	388498	T	France	A-1738	388733	T	Spain	A-1848	389013	N	Greece	A-1926	390004	T	Israel
A-1664	388499	T	France	A-1739	388700	N	Spain	A-1849	389018	N	Greece	A-1927	390005	N	Israel
A-1666	388501	T	France	A-1740	388702	T	Spain	A-1852	389028	N	Greece	A-1928	390006	N	Israel
A-1667	388502	T	France	A-1741	388701	N	Spain	A-1853	389029	N	Greece	A-1933	390034	N	Portugal
A-1668	388503	T	France	A-1742	388703	T	Spain	A-1854	389030	N	Greece	A-1934	390035	N	Portugal
A-1669	388504	T	France	A-1743	388704	N	Spain	A-1855	-	T	Greece	A-1935	390036	N	Portugal
A-1670	388505	N	France	A-1744	388705	T	Spain	A-1856	389032	N	Greece	A-1936	390037	T	Portugal
A-1672	388507	T	France	A-1745	388706	T	Spain	A-1857	389035	T	Greece	A-1938	390039	N	Portugal
A-1673	388483	T	France	A-1746	388707	T	Spain	A-1858	389040	T	Greece	A-1943	390044	N	Portugal
A-1674	388560	T	France	A-1747	388708	T	Spain	A-1859	389041	N	Greece	A-1944	390045	N	Portugal
A-1675	388561	T	France	A-1748	388709	T	Spain	A-1860	389042	N	Greece	A-1945	390046	N	Portugal
A-1676	388487	T	France	A-1767	388684	T	Israel	A-1861	389043	N	Greece	A-1946	390047	N	Portugal
A-1677	388562	T	France	A-1768	-	N	Israel	A-1862	389047	T	Greece	A-1947	390048	N	Portugal
A-1678	388563	T	France	A-1769	388685	T	Israel	A-1863	389048	N	Greece	A-1952	390089	T	France
A-1679	388565	T	France	A-1770	388683	T	Israel	A-1864	389049	T	Greece	A-1953	390088	T	France
A-1680	388566	T	France	A-1771	388663	T	Israel	A-1865	389052	N	Greece	A-1954	390087	T	France
A-1681	388567	T	France	A-1772	388664	T	Israel	A-1866	389053	N	Greece	A-1955	390086	T	France
A-1682	387208	N	Portugal	A-1773	388691	T	Israel	A-1868	389056	N	Greece	A-1956	390085	T	France
A-1684	387210	N	Portugal	A-1774	388686	T	Israel	A-1869	389058	T	Greece	A-1957	390084	T	France
A-1685	387212	N	Portugal	A-1775	388665	T	Israel	A-1870	389059	N	Greece	A-1963	390409	N	Spain
A-1686	387219	N	Portugal	A-1776	388688	T	Israel	A-1872	389066	N	Greece	A-1964	390410	T	Spain
A-1689	387225	N	Portugal	A-1777	388689	T	Israel	A-1873	389068	T	Greece	A-1965	390411	T	Spain
A-1690	387227	N	Portugal	A-1778	388666	T	Israel	A-1874	389069	T	Greece	A-1966	390412	N	Spain
A-1691	387228	N	Portugal	A-1779	388690	T	Israel	A-1875	389074	T	Greece	A-1967	390413	T	Spain
A-1692	387230	T	Portugal	A-1780	388687	T	Israel	A-1882	389084	T	Greece	A-1968	390414	T	Spain
								A-1884	389089	T	Greece	A-1969	390415	T	Spain

[a] A, Culture collection of Veterinary Faculty of Barcelona, Spain; [b] IMI, CAB International Mycological Institute, Kew, Surrey, UK.

Table 2. Summary of the results obtained with the strains analysed in this study.

Origin		RFLP pattern				
	n	N	T	% OTA [a]	OTA range	OTA mean
France	23	1	22	0	N.D.	N.D.
Greece	34	18	16	11.8	0.2-1.0	0.5
Israel	30	3	27	0	N.D.	N.D.
Italy	32	19	13	3.1	-	5.5
Portugal	32	27	5	40.6	0.2-10.5	4.0
Spain	22	6	16	9.1	0.1-0.8	0.5
Total	**173**	**74**	**99**	**11.6**	**0.1-10.5**	**2.7**

[a] All the OTA producing species belonged to the N type.