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Ochratoxin A Production and Amplified Fragment Length Polymorphism Analysis of *Aspergillus carbonarius*, *Aspergillus tubingensis*, and *Aspergillus niger* Strains Isolated from Grapes in Italy

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Ochratoxin A is a potent nephrotoxin and a possible human carcinogen that can contaminate various agricultural products, including grapes and wine. The capabilities of species other than *Aspergillus carbonarius* within *Aspergillus* section *Nigri* to produce ochratoxin A from grapes are uncertain, since strain identification is based primarily on morphological traits. We used amplified fragment length polymorphisms (AFLPs) and genomic DNA sequences (rRNA, calmodulin, and β-tubulin genes) to identify 77 black aspergilli isolated from grape berries collected in a 2-year survey in 16 vineyards throughout Italy. Four main clusters were distinguished, and they shared an AFLP similarity of <25%. Twenty-two of 23 strains of *A. carbonarius* produced ochratoxin A (6 to 7,500 μg/liter), 5 of 20 strains of *A. tubingensis* produced ochratoxin A (4 to 130 μg/liter), 3 of 15 strains of *A. niger* produced ochratoxin A (250 to 360 μg/liter), and none of the 19 strains of *Aspergillus* "uniseriate" produced ochratoxin A above the level of detection (4 μg/liter). These findings indicate that *A. tubingensis* is able to produce ochratoxin and that, together with *A. carbonarius* and *A. niger*, it may be responsible for the ochratoxin contamination of wine in Italy.

Ochratoxin A (OTA) is an important mycotoxin; is considered to be nephrotoxic, immunotoxic, genotoxic, and teratogenic; and has been classified by the International Agency for Research on Cancer as a possible human carcinogen (group 2B) (10). Ochratoxin A is produced by a small number of species in the genera *Aspergillus*, *Petromyces*, *Neopetromyces*, and *Penicillium* (14) and can contaminate various agricultural products, including grapes and wine (13, 34, 36). Accurate identification of ochratoxigenic fungi is of great importance because the toxin profiles of individual species vary and because the fungi that are present limit and define the potential toxicological risks (40). Unfortunately, the taxonomy of *Aspergillus* section *Nigri* is not completely resolved, especially within the *Aspergillus niger* aggregate (5, 20, 35, 38). The *A. niger* aggregate of Al Musallam (5) is currently described as two species, *A. foetidus* and *A. niger*, that are subdivided further into seven varieties, based on morphological and cultural criteria (5, 18, 38). Molecular studies support the division of the *A. niger* aggregate into two morphologically indistinguishable species, *A. niger* and *A. tubingensis* (20, 32, 48).

The presence of ochratoxin A in wine is a relatively recent mycotoxicological problem (31, 33, 44, 49) that is due to contamination by black aspergilli, primarily strains of *A. carbonarius* and others belonging to the *A. niger* species aggregate (8, 21, 46). These reports are all based on morphological identifications, which have limited ability to distinguish species in the *A. niger* aggregate. Amplified fragment length polymorphism (AFLP) analysis, described by Vos et al. (50), can be used for strain identification, especially at low taxonomic ranks (41, 42). Recent studies suggest that these markers can be used to evaluate genetic relatedness among fungal species (23, 51) and to clarify relationships within or between closely related groups or species (24, 47). The advantages of this technique are its high discriminatory power, reproducibility, and robustness. It also can be easily automated (4, 45), and numerous independent polymorphisms can be identified with relatively little change in the protocol.

Our objective in this study was to determine which strain types of the black aspergilli isolated from grapes in Italy, characterized by different molecular approaches (AFLP analysis and DNA sequencing of 28S, internal transcribed spacer [ITS], calmodulin, and β-tubulin genes), can produce ochratoxin A. This report is the first to combine a toxicological characterization of black aspergilli isolated from grapes with different molecular techniques of genetic identification.

MATERIALS AND METHODS

Strains. Sixteen standard strains from species in the *Aspergillus* section *Nigri* were used for AFLP and sequence comparison; they were *A. carbonarius* IMI 016136 (*ex*-type strain, ITEM 4503, CBS 111.26) and IMI 41875 (ITEM 4504; CBS 420.64); *A. niger* IMI 50566 (*ex*-type strain, ITEM 4501, CBS 554.65), IMI 091881 (ITEM 4502; CBS 555.65), and IMI 015954 (ITEM 4506; CBS 126.48; also reported as *A. foetidus*); *A. tubingensis* CBS 134.48 (*ex*-type strain, ITEM 7040), IMI 172296 (ITEM 4500; CBS 115.29), and IMI 211395 (ITEM 4498; CBS 136.52; also reported as *A. phoenicis*); *A. awamori* IMI 211394 (*ex*-type strain, ITEM 4509, CBS 557.65); *A. foetidus* var. *pallidus* IMI 175963 (*ex*-type

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strain, ITEM 4508, CBS 565.65); *A. japonicus* CBS 114.51 (*ex*-type strain, ITEM 7034) and IMI 211387 (ITEM 4497; CBS 568.65); *A. aculeatus* IMI 211388 (*ex*-type strain, ITEM 7046, CBS 172.66); *A. pulverulentus* IMI 211396 (ITEM 4510; CBS 558.65); *A. helicothrix* IMI 278383 (*ex*-type strain, ITEM 4499, CBS 677.79). *A. ellipticus* IMI 172283 (*ex*-type strain, ITEM 4505, CBS 482.65); and *A. heteromorphus* IMI 172288a (*ex*-type strain, ITEM 7045, CBS 117.55). Seventy-seven *Aspergillus* strains isolated from grapes in several vineyards throughout Italy were characterized by molecular analysis and tested for ochratoxin A production.

Field sampling and fungal isolation. Aspergilli were selected during a survey carried out in 2000 and 2001 in 16 vineyards. Five grape berries were taken from a single bunch of grapes collected from each of 10 plants along a diagonal transect in each vineyard. The berries were placed in moist chambers, i.e., petri dishes (9 cm in diameter) containing disks of blotting paper (8 cm in diameter) wetted with 2 ml of sterile water, that were then sealed with Parafilm and incubated at 25 ± 2°C for 7 days. Fungal colonies were transferred to standard identification media, Czapek agar, Czapek yeast agar, and malt extract agar (39); incubated at 25 ± 2°C in the dark for 7 days; and identified according to standard morphological criteria (17). Seventy-seven strains derived from single-conidial-head subcultures were assigned ITEM numbers and deposited in the culture collection of the Institute of Sciences of Food Production (http://www.ispa.cnr.it/Collection).

AFLP analysis. Fungal strains were grown in shake cultures (150 rpm; 25°C; 2 days) in Wickerham's medium (glucose, 40 g; peptone, 5 g; yeast extract, 3 g; malt extract, 3 g; and distilled water to 1 liter). Genomic DNA was extracted with the E.Z.N.A. Fungal DNA Miniprep Kit (Omega Bio-tek, Doraville, GA) according to the manufacturer's protocol. DNA was dissolved in sterile water, diluted to 20 ng/μl, and stored at −20°C.

We used the AFLP Microbial Fingerprinting kit (Applied Biosystems–Perkin-Elmer Corporation, Foster City, CA) according to the manufacturer's instructions. Approximately 10 ng of genomic DNA from each isolate was cut with EcoRI and MseI (New England Biolabs, Hitchin, Hertfordshire, United Kingdom), and the DNA fragments were ligated to double-stranded restriction site-specific adaptors from the kit. A preselective PCR (72°C for 2 min; 20 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min; and then holding at 4°C) was carried out in a 20-μl (final volume) mixture. For the selective PCR, 1.5 μl of a 1:20 dilution of the first PCR product was amplified in a 10-μl (final volume) mixture using selective primers. Four separate primer combinations were utilized for the selective amplification: EcoRI+AC and MseI+CC; EcoRI+AT and MseI+CG; EcoRI+AC and MseI+CA; and EcoRI+G and MseI+CT. The EcoRI primers were labeled with fluorescent dye (Applied Biosystems). The PCR program for selective AFLP amplification was one cycle of 94°C for 2 min and one cycle of 94°C for 20 s, 66°C for 30 s, and 72°C for 2 min; this cycle was followed by nine cycles in which the annealing temperature was lowered each cycle by 1°C from 65°C to 57°C. After that, 20 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min were performed, followed by a final extension step at 60°C for 30 min, and then holding at 4°C indefinitely by using a model 9700 GeneAmp PCR system.

After amplification, 1 μl of reaction product was mixed with 20 μl formamide and 0.5 μl GeneScan-500 (ROX) size standard (Applied Biosystems), ranging from 35 to 500 bp in length. The mixture was heated for 2 min at 95°C and snap cooled on ice. The product was separated by capillary electrophoresis on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). After electrophoresis, the pattern was extracted with GeneScan collection software version 3.1.2 (Applied Biosystems), and the fingerprints were analyzed with Genotyper software (Applied Biosystems). DNA samples from five strains were tested in triplicate, and DNA samples from the other strains were tested in duplicate. DNAs from three replicate cultures of five strains each were also tested.

Peak height thresholds were set at 200. The Genotyper software (Applied Biosystems) was set to medium smoothing. Bands of the same size in different individuals were assumed to be homologous and to represent the same allele. Bands of different sizes were treated as independent loci with two alleles (present and absent). Data were analyzed with an AFLP manager database developed by ACGT BioInformatica S.r.l. (Bari, Italy) and were exported in a binary format with "1" for the presence of a band/peak and "0" for its absence. For clustering, two different analyses were performed. Fragments between 100 and 500 bp and between 200 and 500 bp were analyzed with NTSYS software by using the Dice similarity coefficient and clustered by the unweighted pair group method (27); the clusters obtained were also found by neighbor-joining analysis and maximum-parsimony networks using MEGA version 3.0, with a bootstrap analysis of 1,000 repetitions (19).

Fungal-DNA amplification and sequencing. The identities of the ochratoxigenic species (*A. carbonarius*, *A. niger*, and *A. tubingensis*) also were confirmed by DNA sequencing. The nuclear rRNA gene containing the ITS region and domains D1 and D2 at the 5′ end of the 28S rRNA gene were amplified with primers F65/R635 and ITS5/ITS4, respectively (28). The calmodulin and β-tubulin genes were amplified by using the conditions and primers described by O'Donnell et al. (29).

After amplification, the PCR products were purified by agarose gel electrophoresis and excised from the agarose gel using spin columns (DNA Gel Extraction Kit; Millipore Corporation, Bedford, MA). Sequence analysis was performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit for both strands, and the sequences were aligned with the MT Navigator software (Applied Biosystems). All the sequencing reaction mixtures were purified by gel filtration through Sephadex G-50 (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in double-distilled water and analyzed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The resulting sequences of all the isolates were aligned by the Clustal method with the DNAMAN program (Lynnon Corporation, Quebec, Canada).

Ochratoxin A production. Strains were grown in triplicate in 100-ml stationary cultures of enriched Czapek yeast broth (10) in 500-ml Erlenmeyer flasks for 14 days at 25 ± 2°C in the dark. The cultures were homogenized for 30 min (Ultra-Turrax; IKA, Staufen, Germany) and filtered through folded filter paper (S&S 595½; Schleicher & Schuell, Dassel, Germany). One hundred microliters of the filtrate was diluted with 900 μl of the high-performance liquid chromatography (HPLC) mobile phase (acetonitrile-2% acetic acid, 41:59) and then passed through a 0.45-μm nylon syringe filter (Micron Separations Inc., Westborough, Mass.) prior to HPLC analysis.

HPLC analyses. An ochratoxin A standard was purchased from Sigma (St. Louis, MO). A solution of ochratoxin A (40 mg/ml in benzene-acetic acid, 99:1 [vol/vol]) was calibrated spectrophotometrically (Lambda 2; Perkin-Elmer Corp., Norwalk, Conn.) at 333 nm with 5,550 for the extinction coefficient (6) and stored at −20°C. Working standards were prepared by evaporating an exact volume of the calibrated solution under a stream of nitrogen and redissolving the residue in the mobile phase.

The HPLC system consisted of a Perkin-Elmer 200 instrument equipped with an ISS 200 sampling system (loop volume, 150 μl) and a Jasco FP-920 fluorescence detector set at 333-nm excitation and 470-nm emission. The system was controlled by Perkin-Elmer Turbochrom PC software. A Select B RP-8 column (5-μm particle size; 150- by 4-mm inside diameter; Merck, Darmstadt, Germany) was employed at ambient temperature (20 to 25°C), with a mobile phase of acetonitrile-2% acetic acid (41:59 for ochratoxin A and 55:45 for ochratoxin A methyl ester) at 1.2 ml/min. The injection volume was 30 μl. Ochratoxin A standards of 2 to 60 pg were injected. Peak areas were quantified with the Turbochrom PC software. Ochratoxin A in the extracts was methylated, and the extracts were reanalyzed by HPLC for qualitative confirmation of positive samples (52). The detection limit was 4 μg/liter. All analyses were run in triplicate, and the mean values were reported; standard deviations were <5% of the reported values.

Nucleotide sequence accession numbers. The calmodulin sequences were deposited in the EMBL nucleotide sequence database (Table 1).

RESULTS

We examined 77 *Aspergillus* strains (Table 2) selected arbitrarily from 692 isolates of black aspergilli isolated from grapes in Italy during a 2-year survey. Clear polymorphisms both within and between species were obtained by AFLP analysis for each of the four primer pairs. Each primer combination consistently distinguished the taxa in section *Nigri*, as evidenced by the distribution of the *ex*-type strains in the neighbor-joining dendrogram (Fig. 1). Unweighted pair group method and maximum-parsimony analyses (data not shown) also divided the isolates into the same clusters, with a bootstrap support of 89 to 100% and 100%, respectively. The 77 strains analyzed clearly separated into four clusters: *A. tubingensis*, *Aspergillus* "uniseriate," *A. carbonarius*, and *A. niger* (Fig. 1), with a similarity of <20% for strains in different clusters. Strains were assigned to a species if they shared more than 50% of the bands present in an *ex*-type strain. All strains of *A. carbonarius* and *A. tubingensis* had high similarity (more

TABLE 1. Fungal type cultures and sequence accession numbers

Taxon name[c]	Accession no.			
	ITS[a]	28S[a]	β-Tubulin[a]	Calmodulin[b]
A. carbonarius				
CBS 111.26[T], ITEM 4503, ATCC 1025, IMI 016136, NRRL 369, WB 369, QM 331	AJ280011	AJ280011	AY585532	AJ964873
A. niger				
CBS 554.65[T], ITEM 4501, ATCC 16888, IMI 050566, NRRL 326, WB 326	AJ223852 AY656630 AY373852	AY656630 AY373852	AY585536	AJ964872
A. tubingensis				
CBS 134.48[T], ITEM 7040	AJ223853	1[d]	AY 820007	AJ964876
A. awamori				
CBS 557.65[T], ITEM 4509, ATCC 16877, IMI 211394, IOC 230, WB 4948	AM087614	2[d]	AY 820001	AJ964874
A. japonicus				
CBS 114.51[T], ITEM 7034	AJ279985	3[d]	AY585542	AJ964875
A. aculeatus				
CBS 172.66[T], ITEM 7046, ATCC 16872, IMI 211388, WB 5094	AJ279988	U28813	AY585540	AJ964877

[a] GenBank.
[b] EMBL.
[c] Strain designation and pseudonyms. T, *ex*-type culture.
[d] 100% homology compared with other strains in databank: 1, AJ280008; 2, U03519; 3, AJ876880.

TABLE 2. OTA production by *Aspergillus* section *Nigri* strains isolated from grapes in different locations throughout Italy

ITEM	Origin[a]	OTA[b] (μg/liter)
A. carbonarius		
4722	Brindisi	110 ± 34
4723	Lecce	100 ± 21
4724	Brindisi	24 ± 9
4729	Bari	64 ± 8
4849	Piacenza	100 ± 30
4854	Ravenna	130 ± 11
4864	Ragusa	6 ± 4
4865	Ragusa	200 ± 53
5000	Cuneo	130 ± 11
5002	Reggio Emilia	120 ± 85
5003	Reggio Emilia	48 ± 7
5005	Imola	10 ± 5
5006	Pesaro	20 ± 5
5007	Pesaro	43 ± 15
5008	Chieti	1,100 ± 58
5009	Chieti	45 ± 16
5010	Brindisi	7,500 ± 390
5011	Brindisi	17 ± 9
5012	Sassari	2,100 ± 960
4838	Ragusa	82 ± 29
4839	Ragusa	60 ± 22
4841	Trapani	210 ± 260
A. tubingensis		
4496	Lecce	130 ± 59
4709	Bari	2 ± 1.6
4861	Brindisi	7 ± 4
5014	Brindisi	7 ± 4
5016	Ragusa	21 ± 5
A. niger		
7096	Brindisi	310 ± 14
7097	Piacenza	360 ± 29
7098	Verona	250 ± 34

[a] Locality in Italy (province).
[b] Mean of three repetitions ± standard error.

than 50%) with respect to their *ex*-type strains. The *A. niger* cluster strains were more similar to the *ex*-type strains of *A. awamori* (58%) than to the *A. niger ex*-type strain (40 to 45%). The *Aspergillus* "uniseriate" strains from grapes were at best distantly related to the *ex*-type strains of *A. japonicus* and *A. aculeatus*, with a similarity of >20%. We termed this cluster *Aspergillus* "uniseriate" because we could not assign the strains to any of the presently accepted uniseriate taxa in the *Nigri* section (Fig. 1). Both the AFLP analyses at fragment cutoffs of 100 and 200 bp gave the same grouping and similarity among the four clusters and their related *ex*-type strains.

***Aspergillus carbonarius*.** The *A. carbonarius* cluster contained all 23 strains identified morphologically as *A. carbonarius*. All the isolates clustered at a similarity of 68 to 78% with the *A. carbonarius ex*-type, CBS 111.26 (Fig. 1), while, among them, most of the members of the population shared ≥78% of the bands. *Aspergillus carbonarius* was the most toxigenic species, with 22/23 strains positive for ochratoxin A production and with ITEM 5001 the only strain in the species that did not produce OTA. The amount of ochratoxin A produced varied widely by strain, from 6 μg/liter by ITEM 4864 to 7,500 μg/liter by ITEM 5010 (Table 2). Sequences of rRNA, calmodulin, and β-tubulin genes from the *ex*-type strain, CBS 111.26, and the 23 *Aspergillus* strains characterized as *A. carbonarius* by AFLP analysis were identical.

***Aspergillus tubingensis*.** Twenty strains shared 62 to 85% of their bands with the *ex*-type strain of *A. tubingensis*, CBS 134.48. The *A. phoenicis ex*-type strain, CBS 136.52, molecularly reidentified as *A. tubingensis*, and the *A. pulverulentus ex*-type strain, CBS 558.65, also belonged to this cluster (Fig. 1). Strains in this cluster could not be clearly identified as *A. tubingensis* by morphological criteria. Five of the 20 strains produced detectable levels of ochratoxin A (4 to 130 μg/liter) (Table 2). The strains that did not produce OTA were ITEM 4208, 4210, 4720, 4721, 4725, 4726, 4728, 4837, 4840, 4845, 4852, 4855, 4860, 5015, and 5017. The sequences of the rRNA,

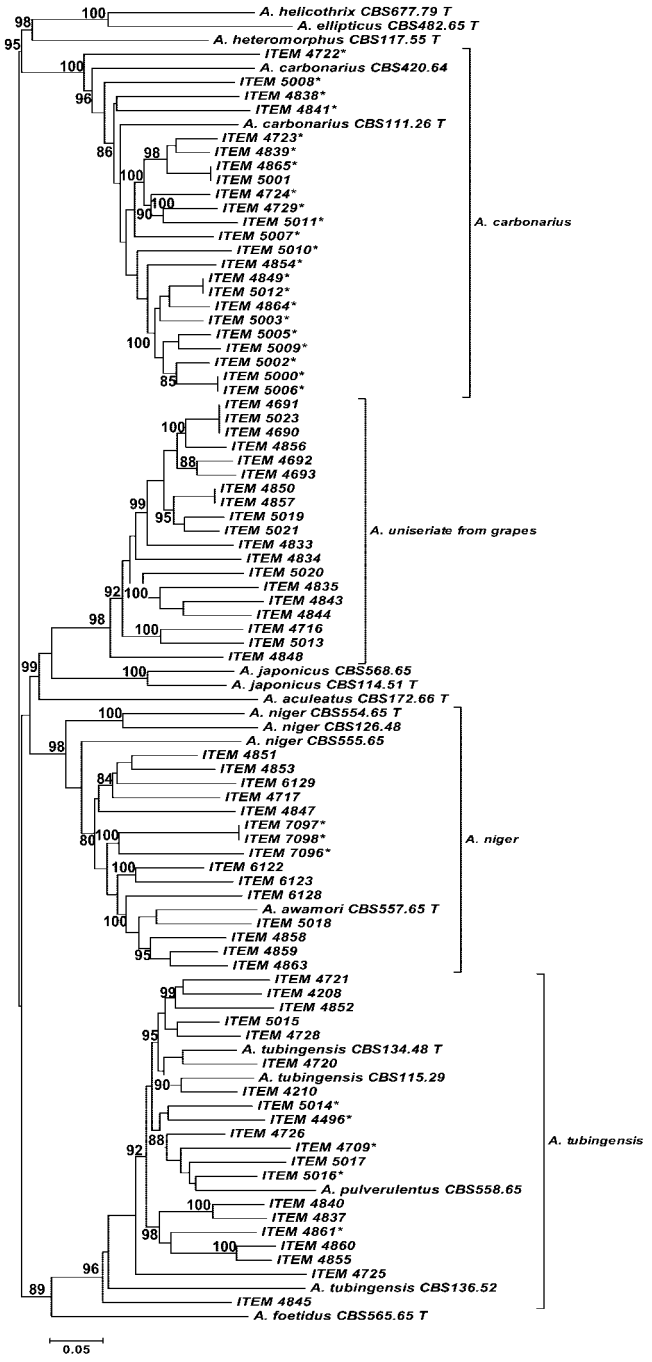

FIG. 1. Dendrogram of 94 isolates of *Aspergillus* section *Nigri* based on cluster analysis with the neighbor-joining method using the Dice genetic distance coefficient on AFLP data obtained with four primer pairs generated by MEGA 3 software. The number at each node indicates the percentage bootstrap support (out of 1,000) for clusters with ≥80% support. Strains labeled with an asterisk are ochratoxin A producers.

calmodulin, and β-tubulin genes of *ex*-type strain CBS 134.48 and the 20 *Aspergillus* strains characterized as *A. tubingensis* by AFLP analysis were identical. The *ex*-type strains of *A. phoenicis* and *A. pulverulentus* had the same sequences for the rRNA, calmodulin, and β-tubulin genes as did the *A. tubingensis* type strain.

***Aspergillus niger*.** Fifteen strains were grouped in the *A. niger* cluster with an AFLP similarity of 40 to 46% to the *ex*-type of *A. niger*, CBS 554.65, and with a similarity of 58 to 75% to the *ex*-type strain of *A. awamori*, CBS 557.65, also identified as *A. niger* by β-tubulin analysis (39). However, the AFLP analysis at a cutoff of 200 bp revealed a greater distance between the *ex*-type of *A. niger*, CBS 554.65, and the 15 strains (35% similarity). The sequences of the rRNA genes for the *ex*-type strains and the 15 field strains in this group were identical. The calmodulin and β-tubulin genes of the 15 strains had identities of 98% and 100% with those from the *A. niger* and *A. awamori* type strains, respectively. Three of the 15 strains in this group produced detectable ochratoxin A (250 to 360 μg/liter) (Table 2). The OTA-nonproducing strains were ITEM 4717, 4847, 4851, 4853, 4858, 4859, 4863, 5018, 6122, 6123, 6128, and 6129. All 15 strains were morphologically indistinguishable from *A. tubingensis*.

***Aspergillus* "uniseriate."** All 19 strains morphologically identified as *A. japonicus* clustered together and averaged 68% similarity to one another. This cluster was genetically different from the *A. japonicus ex*-type strain, CBS 114.51, and the *A. aculeatus ex*-type strain (similarity, <20%) (Fig. 1). None of the 19 strains tested produced detectable ochratoxin A, and they are available as ITEM 4690, 4691, 4692, 4693, 4716, 4833, 4834, 4835, 4843, 4844, 4848, 4850, 4856, 4857, 5013, 5019, 5020, 5021, and 5023.

DISCUSSION

Aspergillus carbonarius contained both the highest proportion of toxigenic strains (95%) and the highest producers of ochratoxin A (up to 7.5 μg/ml). Previous reports of toxin production by *A. carbonarius* found that 25 to 100% of the strains examined produced ochratoxin A in amounts ranging from 0.3 to 234 μg/g when cultured in an agar medium (12, 37). Further study is needed to determine the cause of this variation. *A. carbonarius* is a source of ochratoxin A in crops such as coffee and dried vine fruits (1, 16), but its incidence probably is underreported, since all of the black aspergilli are commonly reported only as "*A. niger*" (2). This is the first report of ochratoxin A production by molecularly characterized strains of *A. carbonarius* from grapes. Although *A. carbonarius* has distinctive morphological traits that are easily identified in a microscopic evaluation, molecular tools can speed the identification process and make it more objective. In this respect, AFLP analysis also can be used for identification purposes (42) and for the development of species-specific PCR primers based on unique AFLP fragments (43).

The other ochratoxin-A-producing *Aspergillus* species we found were *A. tubingensis* and *A. niger*. These species are difficult to differentiate on the basis of morphology, with reliable characteristics for distinguishing *A. niger*, *A. foetidus*, and *A. tubingensis* remaining to be identified (40). *Aspergillus tubingensis* was first described by Mosseray (25) and then by Raper and Fennel (35) as a species in the *A. niger* group. However, its morphological characteristics are very similar to those of other species in the *A. niger* species aggregate. Al Musallam (5) in her revision of the *A. niger* aggregate suggested that "*A. niger*" was composed of two species, namely, *A. foetidus* and *A. niger* sensu stricto, and that *A. tubingensis* was one of the six varieties

in *A. niger*. Molecular studies (2, 20, 32, 40), however, strongly support our results, which are consistent with the hypothesis that *A. tubingensis* is clearly distinct from *A. niger*. Of special importance is our determination that *A. tubingensis* can produce ochratoxin A. It is not clear what proportion of the strains of "*A. niger*" previously reported to produce this mycotoxin and recovered from coffee beans (26), vine fruits (15, 21), and animal feed (11), or available from culture collections (3, 30, 46), are *A. tubingensis*. The identity of the strains from grapes as *A. tubingensis* is consistent with DNA sequence comparisons made with four previously analyzed diagnostic genes (3, 20, 32, 40). In contrast with our results, Accensi et al. (3) evaluated isolates of the *A. niger* aggregate (none from grapes) and found that "N-type" *A. niger* strains produced ochratoxin A but that *A. tubingensis* strains (termed the "T type") could not.

The strains of *A. tubingensis* and *A. niger* differ from those of *A. carbonarius* both in the percentages of ochratoxin A-producing isolates recovered (25%, 20%, and 95%, respectively) and in the amounts of ochratoxin produced in liquid cultures (4 to 130, 255 to 357, and 6 to 7,500 μg/liter, respectively). These results confirm that *A. carbonarius* is an important ochratoxin-producing fungus in wine and that, together with *A. tubingensis* and *A. niger*, it may be responsible for the ochratoxin A contamination of wine in Italy.

Although two recent studies (9, 11) reported the production of ochratoxin A by *A. japonicus* strains isolated from grapes and animal feed, none of our *Aspergillus* "uniseriate" strains produced detectable ochratoxin A. The genetic distance, based on AFLPs, of our strains from the *ex*-type strains, however, leaves the taxonomic status of our cultures in need of further definition and resolution, as they could represent a new population or an as-yet-undescribed *Aspergillus* species.

AFLP analysis is a useful and powerful tool for studying the genetic diversity of black aspergilli. Our AFLP results, combined with DNA sequence analysis of diagnostic genes, confirm the validity and utility of the AFLP technique for evaluating genetic relatedness among fungal species (23, 51), and in particular, for resolving relationships within or between closely related groups or species (24, 47). Previous studies have shown that this technique is suitable for differentiating fungal strains at and below the species level (7, 22, 41, 42). Our study suggests that AFLPs have utility for this purpose in this group of *Aspergillus* species as well. A combination of biochemical and molecular methods is needed to correctly evaluate the potential toxicological risk in grapes caused by these fungi.

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