Ochratoxin A (OTA) was discovered in 1965 as a secondary metabolite of a strain of *Aspergillus ochraceus* (61). OTA exhibits intestinal fragility, nephrotoxicity, immunosuppression, teratogenicity, carcinogenicity (11, 19, 24, 27, 34), and cytotoxicity in hepatic cell lines (11) and induces iron deficiency anemia (23). OTA could be responsible for Balkan endemic nephropathy. It has been reported that OTA concentration in the blood serum of Balkan endemic nephropathy patients was 10-fold higher than in the blood serum of people from other regions (4, 57). The International Agency for Research on Cancer classifies OTA in group 2B (possibly carcinogenic to humans) (25).

Fungi from two genera are known to produce ochratoxins. In genus *Penicillium*, OTA is produced by *P. verrucosum* (53) and *P. nordicum* (33) and in genus *Aspergillus* by *A. ochraceus*, *A. melleus*, *A. auricomus*, *A. ostianus*, *A. petrakii*, *A. sclerotiorum*, and *A. sulphureus*, all in section *Circumdati* (formerly the *A. ochraceus* group) (3, 10, 22, 62). *Aspergillus alius capitum* and *Aspergillus albietensis*, formerly placed in section *Circumdati*, but recently shown to be more closely related to section *Flavi*, have also been described as OTA producers (50). In recent years, some members of *Aspergillus* section *Nigri* (formerly the *A. ochraceus* group) such as *Aspergillus ochraceus* var. *niger* and *Aspergillus carbonarius* have been reported as ochratoxigenic fungi (1, 6, 15, 21, 40). More recently, the ability of the uniseriate species of black aspergilli *Aspergillus japonicus* to produce OTA has been mentioned (9, 17). In the *A. niger* aggregate, it has always been difficult to distinguish one taxon from another by morphological means because the differences are very subtle. The division of this *A. niger* aggregate into two species, namely *A. niger* and *Aspergillus tubingensis*, according to restriction fragment length polymorphism (RFLP) analysis of total DNA was proposed by Kusters van Someren et al. (32). Studies involving a molecular approach followed and substantially confirmed these results (7, 37, 43, 63, 64). Although the ability of *A. niger* to produce OTA has been previously described (1), the species *A. tubingensis* has not been reported to be an OTA producer (2).

Ochratoxin A has been detected in human blood (12, 16, 49, 59) and food and drinks such as cereals (mainly wheat, barley, corn, and oats), seeds, beans, pulses, peanuts, dried fruits, coffee, milk, and beer (30, 54, 56, 58, 60); in recent years, it has been detected in wine (13, 38, 48, 66). Due to the presence of...
OTA in food and drinks typical in the human diet, the study of OTA has become increasingly important. The Joint Food and Agriculture Organization of the United Nations/World Health Organization Expert Committee on Food Additives has discussed the imposition of a maximum tolerable weekly intake of 100 ng of toxin/kg of body weight (28) and a maximum level of 5 to 20 μg OTA/kg in cereals, both processed and unprocessed (29). The Office International de la Vigne et du Vin fixed 2 μg/liter as a maximum level of OTA in wine (46).

Wine is a product of great economic relevance around the world, especially in wine-producing countries. Recently, it has been shown that OTA is stable in wine for at least 1 year (38).

There are differences between northern and southern European regions regarding OTA levels in wines. Several surveys carried out in different countries have reported OTA levels in grape products and wine ranging from 0.01 to 3.5 μg/liter. These levels were higher in products from southern regions of Europe than in northern regions (9, 40, 41, 47, 48, 51). The fungal mycobiota on ripe grape is very critical in assessing the risk of OTA presence in wine. Therefore, some researchers have recently studied the grape mycobiota in different countries (5, 14, 55), but no attention has been paid to the study of ochratoxigenic fungi cocurrence in different grape varieties.

The aims of the present study were (i) assessment of the native mycobiota in different grape varieties grown in Spain, (ii) morphological and molecular characterization of potential OTA-producing isolates from each variety by PCR-RFLPs of the rRNA genes internal transcribed spacer (ITS) region, and (iii) identification of ochratoxinogenic isolates among these fungal species.

### MATERIALS AND METHODS

**Samples.** In the present study, a total of 52 grape samples (44 red grapes and 8 white grapes) (Vitis vinifera) were analyzed. Table 1 shows the studied grape varieties, the geographical origin of the vineyards, and the number of samples of each grape variety.

The criteria for farm selection within each sample area were size and the quality of wines derived from their grapes. Samples were harvested in late September during the grape harvest, and plants were chosen along diagonal transects to obtain random sampling. Grape bunches (each about 1 kg) were taken and placed in previously sterilized bags, which were kept at about 4°C until analysis, which was carried out within 24 h of harvest.

Fifty berries were picked from all the parts of bunches and homogenized in a stomacher (IUL Instruments, Barcelona, Spain). From the homogenate, decimal seriate dilutions were made under sterile conditions. These solutions were used to inoculate petri dishes containing malt extract agar (Cultimed; Panrec Quimica S.A., Barcelona, Spain). Petri dishes were then incubated at 28°C for 5 to 7 days in the dark. After incubation, the number of CFU of filamentous fungi per milliliter of berry homogenate was evaluated.

**Identification of fungi.** Taxonomic identification of all isolates was achieved through macroscopic and microscopic observation with the aid of guidelines published for each genus or general guidelines (8, 31, 52). *A. carbonarius* was identified through microscopic observation, and *Aspergillus niger* aggregate (*A. niger* and *A. tubingenensis*) was identified on the basis of the determination of restriction patterns of PCR-amplified rRNA gene products. Fungal DNA was isolated according to the method described by Lee and Taylor (35). The ITS1-5.8S-rRNA gene-ITS2 region was amplified by PCR. Two oligonucleotide fungal primers (ITSI and ITSI2) described by White et al. (67) were used for amplification. Random amplified products were digested overnight at 37°C with restriction endonuclease Rsal (Boehringer Mannheim). PCR products and restriction fragments were separated by electrophoresis in 1% and 2% agarose gels, respectively, with 0.5% Tris-borate-EDTA buffer. After electrophoresis, gels were stained with ethidium bromide (0.5 μg/ml), and the DNA bands were visualized with a UV transilluminator. DNA sizes were estimated by comparison with a DNA length standard (100-bp molecular marker; Gibco BRL Life Technologies, Inc., Rockville, Md.). The restriction patterns obtained for the different isolates from grape samples were compared with those obtained under the same conditions from two type strains (*A. niger* CECT 2807 and *A. tubingenensis* CECT 20393) held at the Spanish Collection of Type Cultures (Valencia University, Burjassot, Valencia, Spain). Strain CECT 20393 corresponds to IMI 172296 (International Mycological Institute, Surrey, United Kingdom) and CBS 115.29 (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands).

To perform DNA sequencing, PCR products were cleaned with the Gene Clean II Purification kit (Bio 101, La Jolla, Calif.). Then, PCR products were sequenced using the Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) and an Applied Biosystems automated DNA sequencer (model 373A) according to the manufacturer's instructions. The primers ITS1 and ITS4 were also used to obtain the sequence of both strands. The National Center for Biotechnology Information (NCBI) Nucleotide Database was used to compare nucleotide sequences.

All isolates assayed for OTA production are held lophylized at the fungal collection of the Fungi and Mycoxins in Food Group (Department of Microbiology and Ecology, Valencia University). Due to the originality of the results obtained in the present study on OTA production by *A. tubingenensis*, producing isolates Bo56, Bo66, and Mm24 of this species were deposited in the Spanish Collection of Type Cultures under reference numbers CECT 20543, CECT 20544, and CECT 20545, respectively.

**Characterization of OTA-producing isolates.** Characterization of ochratoxinogenic isolates was carried out by inoculation of Erlenmeyer flasks containing 50 ml of yeast extract-sucrose broth (YES; 2% yeast extract, 15% sucrose) supplemented with 5% bee pollen to increase OTA production (40) with 1 ml of a spore suspension (10^7 spores of each isolate/ml). Bee pollen used as an ingredient was mainly from *Citrus* spp. and, secondarily, from *Eucalyptus* spp.; it was a gift of a Valencian company of bee products. Bee pollen was previously assayed to ensure it contained undetectable OTA levels. Before inoculation, culture media was autoclaved for 30 min at 111°C. Spero suspensions used for inoculation were prepared from single-sporo cultures made in potato-dextrose agar and grown for 7 days at 25°C. Erlenmeyer flasks containing inoculated media were incubated for 28 days at 25°C in the dark.

OTA extraction from YES-5% bee pollen cultures was accomplished as follows. The content of each flask was filtered through Whatman no. 4 filter paper, acidified to pH 2.8 to 3.0 with 0.1 M phosphoric acid, and extracted in a separatory funnel with chloroform (three times; each extraction, 5 ml). The organic extracts were combined, evaporated to dryness in a rotary evaporator, and suspended in 100 μl of acetonitrile-water-acetic acid (99:99:2 [vol/vol]) for further analysis.

OTA separation and detection were performed by liquid chromatography (LC) according to the method of Visconti et al. (66) with some modifications. The liquid chromatographic system used for OTA analysis consisted of a Waters 600 pump connected to a Waters 474 fluorescence detector. System control and signal treatment were carried out with Millennium 32 software, version 3.01.05 (Waters, Milford, Mass.). Separation was performed with a stainless steel LiChroper 100 C18 reversed-phase column (250 by 4 mm; 5-μm particle size) connected to a guard column (4 by 4 mm; 5-μm particle size) filled with the same phase (Agilent Technologies, Waldbronn, Germany). The mobile phase was acetonitrile-water-acetic acid (99:99:2 [vol/vol]) at a flow rate of 1.0 ml/min. It was filtered through a 0.45-μm nylon membrane filter and degassed by an

<table>
<thead>
<tr>
<th>TABLE 1. Characteristics of the grape samples used in the study</th>
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<tbody>
<tr>
<td><strong>Color of berries</strong></td>
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<tr>
<td><strong>Red</strong></td>
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<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td><strong>White</strong></td>
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<td></td>
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</tbody>
</table>

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### RESULTS

**Fungal contamination of grapes.** Eight fungal genera were isolated from the grape samples (*Aspergillus*, *Alternaria*, *Acremonium*, *Penicillium*, *Cladosporium*, *Fusarium*, *Rhizopus*, and *Phoma*). Table 2 shows the contamination levels of the five grape varieties by these fungal genera and the total counts.

Tempranillo was the most contaminated grape variety, with $1.6 \times 10^5$ CFU/ml. The genus *Phoma* was isolated quite frequently in this variety while this genus was not found in the remaining varieties. The Moscatel variety showed the lowest level of fungal contamination ($6.6 \times 10^3$ CFU/ml). The remaining grape varieties (Bobal, Monastrell and Garnacha) showed very similar contamination levels, which were $1.2 \times 10^4$, $1.0 \times 10^5$, and $9.2 \times 10^3$ CFU/ml, respectively.

Generally speaking and considering all the grape varieties included in this study, the most frequently isolated fungi were *Alternaria* spp. and *Cladosporium* spp. In these varieties, the number of CFU/ml ranged from $2.9 \times 10^2$ to $7 \times 10^3$ for the first genus, and from $34$ to $9.3 \times 10^3$ for the second genus, depending on the variety. Statistical analysis (analysis of variance) using all data confirmed that these two genera were dominant among the mycobiota of the studied grape samples. The $P$ value was $0.0000$, which indicates that there are very significant differences between these two genera and the remaining found genera with regard to isolation frequency.

When considering only the *A. niger* aggregate (*A. niger* and *A. tubingensis*) and *A. carbonarius*, the analysis of variance showed that there were significant differences ($P = 0.000$) in contamination levels among the different grape varieties. The most contaminated grape varieties by *Aspergillus* section *Nigri* were Monastrell, Moscatel, and Bobal, where average *A. carbonarius* contamination levels were $4 \times 10^2$, $2.8 \times 10^3$, and $1.6 \times 10^5$ CFU/ml, respectively. The number of CFU of *A. carbonarius/ml* in Garnacha and Tempranillo varieties was signif-

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**Table 2.** Fungal contamination levels in five grape varieties grown in Spain

<table>
<thead>
<tr>
<th>Grape Variety (no. of samples analyzed)</th>
<th>Bobal (10)</th>
<th>Tempranillo (18)</th>
<th>Garnacha (7)</th>
<th>Monastrell (9)</th>
<th>Moscatel (8)</th>
</tr>
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<tbody>
<tr>
<td>Total (CFU/ml)</td>
<td>10 CFU/ml</td>
<td>1.6 $\times 10^5$</td>
<td>1.3 $\times 10^4$</td>
<td>1.4 $\times 10^3$</td>
<td>2.6 $\times 10^4$</td>
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<tr>
<td>CFU/ml</td>
<td>1.6 $\times 10^5$</td>
<td>1.3 $\times 10^4$</td>
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*Number of samples showing fungal contamination.

**Percentage of CFU of each fungus/ml with respect to the total CFU/ml count in each grape variety.
OTA production, 92 (44.9%) produced this toxin. The production levels ranged from 1.2 to 3,530 ng/ml of culture medium. Eighty-nine of the ochratoxigenic isolates were classified as *A. carbonarius* (74.2% of the 120 tested isolates), while the remaining 3 were classified as *A. tubingensis* (14.3% of the 21 tested isolates) on the basis of RFLP. OTA was detected in cultures of isolates Bo56, Bo66, and Mn24 but not in cultures of the remaining isolates of the *A. niger* aggregate. Their OTA production levels varied from 46.4 to 111.5 ng/ml of culture medium. OTA was not detected in cultures of the 64 assayed isolates of *A. niger*. Comparison of the ITS1-5.8S rRNA gene-ITS2 sequences from the amplified regions of isolates Bo56, Bo66, and Mn24 with those available in the NCBI Nucleotide Database showed that the ITS sequences of both isolates Bo56 and Bo66 were identical to the sequences of *A. tubingensis* CBS 643.92 and CBS 127.49 (EMBL accession numbers for the sequenced region are AJ280008 and AJ280007, respectively), except for a single nucleotide (G instead of T) at position 532. Thus, they showed 99% of identity (592 out of 593 bp). Isolate Mn24 showed 100% of identity (593 out of 593 bp) with the same CBS strains.

The distribution of the 205 isolates and the grape varieties from which they came was as follows: 37 isolates from Bobal, 53 isolates from Garnacha, 36 isolates from Tempranillo, 31 isolates from Monastrell, and 48 isolates from Moscatel.

Analysis by LC-ion trap MS of the YES–5% bee pollen extracts confirmed the identity of OTA in cultures. The peaks produced by the \([M + H]^+\) ion \((m/z\ 404)\) and the \([M + H]^+ - \text{HCOOH}\) ion \((m/z\ 358)\) were observed in the mass spectra of the OTA standard and the cultures where OTA had been detected by LC with fluorescence detection.

Although the occurrence levels of *A. carbonarius* were significantly different with regard to the grape varieties studied in the present report, the percentages of OTA-producing isolates from the different grape varieties were very similar (74%, 85%, 80%, 63%, and 69% for Bobal, Garnacha, Monastrell, Tempranillo, and Moscatel, respectively).

**DISCUSSION**

After analysis of the occurrence data of fungi in grapes from other varieties and geographic locations reported by other authors (5, 9, 14, 39, 55), it can be observed that the contaminant mycobiota differs from the results found in this work. Abrunhosa et al. (5) did not find *Aspergillus* in samples of grapes grown in Portugal. Battilani et al. (9) found *Aspergillus*
spp. in grapes grown in Italy, with the Nigri section largely predominating. The most abundant were molds with biseriate conidial heads (A. niger and A. tubingensis), followed by Aspergillus with uniseriate conidial heads (Aspergillus aculeatus and A. japonicus) and A. carbonarius, in that order. Cabanes et al. (14) did not find molds with uniseriate conidial heads in grapes grown in Spain. Magnoli et al. (39) found a clear dominance of the genus Alternaria in grapes grown in Argentina. Their results are similar to those found in the present report. However, the levels of Cladosporium were much lower in Argentinean samples. Although Sage et al. (55) did not evaluate the occurrence of the different fungi in French grapes, they found black fungi with uniseriate and biseriate conidial heads, including A. carbonarius. No Aspergillus section Nigri isolates with uniseriate conidial heads were detected in the samples analyzed in the present work, in agreement with Cabanes et al. (14). It should be noted that despite the differences in geographic location, the varieties studied by the different authors were different as well, which could explain the disagreement of the results found among the samples. Our results show that grape variety has a strong influence on the occurrence of Aspergillus section Nigri. Cabanes et al. (14) found Penicillium purpureogenum in all samples of the white Garnacha grape variety that they studied. Their samples were from Tarragona, an area that is near to Cuenca and Valencia, sampled in the present study. However, the occurrence of Penicillium spp. in our samples was generally low (Table 2), and P. purpureogenum was not isolated in any sample. Obviously, there is a great complexity with regard to the native mycobiota in different grape varieties.

It is interesting that there are not significant differences in ochratoxinogenic fungi occurrence on samples of the same grape variety when grown in vineyards in different areas. Studies of OTA incidence in wines carried out in various countries point out that the number of samples contaminated with this toxin and their levels are higher in wines from southern than from northern regions of Europe. These levels ranged from 0.01 to 3.5 μg/liter (9, 40, 41, 47, 48, 51). However, the grape variety used to produce wines might have a strong influence on these differences. It has been reported (9, 40, 41, 47, 48, 51) that wines from red berries exhibit higher OTA levels than wines from pink and white ones, in this sequence. Four of the grape varieties analyzed by us (Bobal, Garnacha, Tempranillo, and Monastrell) have red berries (Table 1), and there were significant differences among them in the occurrence of ochratoxinogenic fungi. This result is very compatible with the idea that OTA levels in wines depend on the grape variety from which they are produced, regardless of the color of berries. The higher OTA content of red wines might be associated with the grape variety used to produce them and with the winemaking process, especially with the contact time between grape juice (must) and berry skins. However, more studies are needed to assure differences in grape varieties with regard to susceptibility to fungal contamination.

The taxonomy of black aspergilli is far from clear. It has long been studied by means of morphological and cultural criteria. Whereas A. carbonarius can be microscopically distinguished by conidial size and ornamentation, all the taxa in the A. niger aggregate are morphologically indistinguishable. This problem has been the origin of misidentifications and discrepancies in the physiological characteristics of the species included in this aggregate. The results from the present study agree with those from Accensi et al. (7). A target for endonuclease RsaI was detected in the rRNA gene ITS1 of A. niger. It does not exist in the sequence of A. tubingensis. The PCR-amplified-5.8S rRNA gene of A. niger was digested into two fragments of 519 and 76 bp. The 76-bp fragment was too small to remain on the gel (Fig. 1).

Before this report, A. tubingensis had not been found to be able to produce OTA (2, 9, 14, 56). However, in this work, three OTA-producing isolates (two from the Bobal and one from the Monastrell grape varieties) were found. The classification of these isolates as A. tubingensis based on RFLP were confirmed by sequencing the ITS1-5.8S rRNA gene-ITS2 region. Two of the isolates (Bo56 and Bo66) matched at 99% (592 of 593 bp) with A. tubingensis CBS 643.92 and A. tubingensis CBS 127.49; 100% identity was found for the third isolate (Mn24). According to the NCBI Nucleotide Database, the difference in the sequence of this region between type strains of A. tubingensis CBS 643.92 and CBS 134.48 (EMBL accession number AJ223853.1) is one nucleotide (the last strain has C instead of G) at position 16 (99% identity). There are various possible reasons for the disagreement in results from different researchers. The difficult differentiation between A. niger and A. tubingensis based on morphological characteristics may have provided misidentifications. In other cases (2, 14), failing to detect OTA in cultures of A. tubingensis might be due to culture medium and/or incubation time. In these reports, the authors used YES broth, incubation time was only 7 days, and sampling sizes for LC analysis were small. This accumulation of events could have led to negative results. Current research is being carried out in our laboratory to study the influence of culture medium on OTA production by Aspergillus section Nigri isolates (unpublished data). It has been shown that accumulations of OTA in YES broth and YES broth supplemented with 5% grape must are 80% and 92%, respectively, of the levels reached with YES broth supplemented with 5% bee pollen. The capacity of bee pollen to stimulate biosynthesis of OTA by A. ochraceus has been previously reported (42), but it has not been studied until now in Aspergillus section Nigri.

P. verrucosum was not detected in any sample. According to several authors (10, 26, 55), ochratoxins in southern Europe seem to be connected with the presence of Aspergillus, while in Germany and in Scandinavia, they would be connected with the presence of penicillia. Owing to their different temperature needs (26) ochratoxinogenic penicillia grow well over a range of temperatures (4 to 31°C), whereas aspergilli that produce OTA require higher temperatures (12 to 39°C). Temperatures in the Spanish grape crop areas are generally high, especially during summer when berry ripening and harvest take place.

The high occurrence of Alternaria spp. in the analyzed samples is noteworthy. Species of this genus have been reported as producers of some mycotoxins such as alternariol, alternariol monomethyl ether, tenuazonic acid, and zinniol, among others (18, 20, 65). According to Miller (45), the cooccurrence of several mycotoxins can influence each other’s production levels, as well as the toxicity of contaminated material. To date, research on this topic has not been carried out either with grapes or, consequently, with wine. In any case, grape selection avoiding the use of decaying berries constitutes a critical con-
trol point that should be exhaustively monitored. A suitable selection of clusters rejecting the rotten ones could reduce the OTA content of wines by 98% (36, 44).

On the basis of our results, it can be deduced that the fungi responsible for OTA occurrence in wines made with the five grape varieties studied here and grown in Spain belong mainly to the species A. carbonarius and to a lesser extent to species from the A. niger aggregate, specifically, A. tubingenensis, a species never previously reported as an OTA producer.

ACKNOWLEDGMENTS

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