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# Diversity of black Aspergilli isolated from raisins in Argentina: Polyphasic approach to species identification and development of SCAR markers for *Aspergillus ibericus*

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ABSTRACT

*Aspergillus* section *Nigri* is a heterogeneous fungal group including some ochratoxin A producer species that usually contaminate raisins. The section contains the Series *Carbonaria* which includes the toxigenic species *Aspergillus carbonarius* and nontoxigenic *Aspergillus ibericus* that are phenotypically undistinguishable. The aim of this study was to examine the diversity of black aspergilli isolated from raisins and to develop a specific genetic marker to distinguish *A. ibericus* from *A. carbonarius*. The species most frequently found in raisins in this study were *Aspergillus tubingensis* (35.4%) and *A. carbonarius* (32.3%), followed by *Aspergillus luchuensis* (10.7%), *Aspergillus japonicus* (7.7%), *Aspergillus niger* (6.2%), *Aspergillus welwitschiae* (4.6%) and *A. ibericus* (3.1%). Based on inter-simple sequence repeat (ISSR) fingerprinting profiles of major *Aspergillus* section *Nigri* members, a sequence-characterized amplified region (SCAR) marker was identified. Primers were designed based on the conserved regions of the SCAR marker and were utilized in a PCR for simultaneous identification of *A. carbonarius* and *A. ibericus*. The detection level of the SCAR-PCR was found to be 0.01 ng of purified DNA. The present SCAR-PCR is rapid and less cumbersome than conventional identification techniques and could be a supplementary strategy and a reliable tool for high-throughput sample analysis.



## 1. Introduction

Several studies have shown that ochratoxin A (OTA) is produced during infection of grapes in vineyards mainly by mycotoxigenic strains of black aspergilli (section *Nigri*), in particular *Aspergillus carbonarius* and species belonging to the *Aspergillus niger* aggregate. OTA has been reported worldwide in grapes and derived products (Cabañes et al., 2002; Da Rocha Rosa et al., 2002; Magnoli et al., 2004; Ponsone et al., 2010; Visconti et al., 2008).

Black aspergilli comprise one of the more difficult groups to classify and identify and several taxonomic schemes for them have been proposed (Al-Musallam, 1980; Hong et al., 2013; Kozakiewicz, 1989; Raper and Fennell, 1965; Samson et al., 2004, 2007; Varga et al., 2011). New molecular approaches have shown that there is a high biodiversity within this group, which makes identification based solely on their phenotypic characters difficult (Perrone et al., 2007; Samson et al., 2007).

OTA production by black aspergilli occurring on grapes and derived products has been widely studied in recent years with sometimes ambiguous reports on the toxigenicity and the percentage of toxigenic strains among the species. OTA producing strains of *A. carbonarius* ranged between 80 and 100%, whereas the range was around 0–45% for *A. niger* aggregate (Bau et al., 2005; Magnoli et al., 2003, 2004; Perrone et al., 2006a; Romero et al., 2005). Some reports claimed the production of OTA also by *Aspergillus japonicus* but this has not been confirmed yet (Battilani et al., 2003; Dalcero et al., 2002; Ponsone et al., 2007). The variability in the results may be due to an incorrect delimitation of species, since most of the species belonging to *A. niger* aggregate can be distinguished only by molecular methods (Samson et al., 2007). Also, the differences in the ratio of nontoxigenic to toxigenic *A. carbonarius* are probably due to misidentification of the non-OTA producer *Aspergillus ibericus* (Serra et al., 2006). Furthermore, the variability also can be attributed to the different number of strains evaluated and production conditions including synthetic medium, temperature, incubation time, or other factors tested to evaluate the OTA production.

*A. carbonarius*, although less common than other black aspergilli, is considered to be the predominant species responsible for OTA

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contamination in grapes and wine, because of the common ability of the strains to produce high levels of the toxin (Astoreca et al., 2010; Mateo et al., 2007). Due to the variations in the toxin production potency of different aspergilli, accurate identification of *Aspergillus* species is of great importance, in order to define potential toxicological risks at an early stage (Magan, 2006). The only report of three isolates of *A. carbonarius* unambiguously identified and characterized, unable to produce OTA was recently published by Cabañes et al. (2013).

The difficulties in species recognition within the *Aspergillus* section *Nigri* and the fact that most of the studies carried out on black aspergilli occurring on food in Argentina lack molecular characterization (Dalcero et al., 2002; Magnoli et al., 2003; Ponsone et al., 2007) have caused confusion in evaluation of the species distribution on food and therefore on the ability to evaluate potential OTA contamination. Although the data are limited, recently Chiotta et al. (2010) and Barberis et al. (2013) evaluated strains isolated from grapes and vineyard soils in Argentina using different molecular markers. The authors found *Aspergillus tubingensis* (*A. niger* aggregate) to be the most frequent species with percentages of 82.9 and 89%, respectively; followed by *A. carbonarius* and uniseriate *Aspergillus* species. In addition, Chiotta et al. (2011) reported the production of $FB_2$ and $FB_4$ by 5 (35%) *A. niger* strains isolated from grapes in Argentina.

Molecular identification has not been carried out in most of the studies dealing with the mycobiota of raisins in Argentina, which could lead to misidentification of some closely related species. Therefore, the objective of this study was to identify the black aspergilli isolated from raisins in Argentina using a polyphasic approach including morphological examination, mycotoxin analysis (OTA and $FB_2$), single inter simple sequence repeat (ISSR) markers and calmodulin gene sequence analysis. The other goal of the study was to identify an ISSR profile-based sequence-characterized amplified region (SCAR) marker that was *A. ibericus*-specific and to develop diagnostic PCR primers for the discrimination of *A. ibericus* from *A. carbonarius*.

## 2. Materials and methods

### 2.1. Fungal isolates

Fifty sun-dried raisin samples were obtained from San Juan, Argentina, during 2010. Each sample, containing one hundred raisins, was maintained at 4 °C until analysis. The samples were surface sterilized, and placed on dichloran-rose bengal (DRBC) medium (Pitt and Hocking, 1997). A representative number of black aspergilli from each sample were isolated from DRBC and sub-cultured on Malt Extract Agar (MEA) for further identification at species level. Other isolates of black aspergilli maintained in the culture collection of the Department of Microbiology and Immunology, Universidad Nacional de Río Cuarto were included in the study. The initial identification of the different strains of *Aspergillus* section *Nigri* was performed using macroscopic and microscopic morphological criteria in accordance with appropriate keys (Klich, 2002). In total sixty-five *Aspergillus* section *Nigri* strains were evaluated: *A. carbonarius* (23), *A. niger* aggregate (37), and uniseriate *Aspergillus* (5) isolated from raisins (Table 1). In addition, the study included nine reference strains, *A. japonicus* ITEM 7034, *Aspergillus aculeatus* ITEM 7046, *Aspergillus uvarum* ITEM 4834, *A. ibericus* ITEM 4776, *A. tubingensis* ANRC 452, *A. niger* FRR5695, *Aspergillus welwitschiae* ITEM 4502, *A. carbonarius* A2034 and *Aspergillus luchuensis* ANRC 703 which had been identified in previous studies in our laboratory (Giaj Merlera, 2014). The reference strains were kindly provided by Dr. Giancarlo Perrone from Institute of Sciences of Food Production, CNR (Bari, Italy). All strains were kept in 15% glycerol and frozen at −80 °C in the culture collection of the Department of Microbiology and Immunology, Universidad Nacional de Río Cuarto, Cordoba, Argentina.

### 2.2. DNA extraction

Total genomic DNA was extracted by using the cetyltrimethylammonium bromide (CTAB) method as described by Leslie and Summerell (2006). In brief, all the strains were grown in Wickerham medium, containing 40 g glucose, 5 g peptone, 3 g yeast extract, 3 g malt extract and water up to 1000 mL and incubated on an orbital shaker (150 rpm) for at least three days at 25 ± 1 °C. The resulting mycelia were harvested by filtration through non-gauze milk filters (Ken AG, Ashland, Ohio). Excess water was removed by blotting mycelia between clean paper towels, and the dried mycelia were stored frozen at −20 °C until ground and extracted with CTAB. The quality of genomic DNA was determined by electrophoresis and quantified using a fluorometer (Qubit™-Invitrogen, Buenos Aires, Argentina).

### 2.3. Species-specific PCR assay

All isolates were subjected to species-specific PCR using species-specific primers for *A. japonicus*/*A. aculeatus* (JAPO1/2) and *A. carbonarius* (CARBO 1/2) (Perrone et al., 2004), and *A. niger* (NIG1/2) and *A. tubingensis* (TUB1/2) (Susca et al., 2007). All amplification reactions were carried out in a final volume of 20 μL containing 10 ng template DNA, 0.5 μM of each primer (JAPO1/2 and CARBO 1/2) and 0.1 μM of each primer (NIG1/2 and TUB 1/2), 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1.25 units *Taq* DNA polymerase (Invitrogen, Buenos Aires, Argentina).

PCR was performed in a PTC-2000 Thermal Cycler (MJ Research Inc., Watertown, MA). Amplification conditions were: denaturation at 94 °C for 5 min; 30 (NIG1/2 and TUB 1/2) and 35 (JAPO1/2 and CARBO 1/2) cycles of denaturation at 94 °C for 50 s; annealing at 60 °C (NIG1/2 and TUB1/2) and 58 °C (JAPO1/2 and CARBO 1/2) for 50 s; extension at 72 °C for 1 min; final extension at 72 °C for 7 min, followed by cooling at 4 °C until recovery of the samples. All amplification products generated were resolved on 1.5% agarose gels. The gels were stained with ethidium bromide (5 μg/mL) and visualized under UV light. The size of the DNA fragments was estimated by comparing the DNA bands with a 100 bp DNA ladder (Invitrogen, Buenos Aires, Argentina).

### 2.4. ISSR analysis

Two primers, CTC $(GT)_8$ and AG $(CTC)_5$, were used to assess diversity of *Aspergillus* section *Nigri* from raisins. These primers were previously selected based on the amplification profile considering the ability to consistently amplify the same fragment(s) from a given isolate (Giaj Merlera, 2014). Amplification was conducted in a PTC-2000 Thermal Cycler (MJ Research Inc., Watertown, MA) according to Giaj Merlera (2014). All the amplicons generated were resolved on 1.5% agarose gels. The gels were stained with ethidium bromide (5 μg/mL) and visualized under UV light. The size of the DNA fragments was estimated by comparing the DNA bands with a 100 bp DNA ladder (Invitrogen, Buenos Aires, Argentina). Gel images were photographed using a gel documentation system (MiniBIS Pro, DNR).

The presence or absence of ISSR bands was manually scored, and recorded in a binary format. All bands were scored, including those that were monomorphic. Bands migrating at the same position were assumed to be homologous and to represent the same allele and locus. Bands of differing mobility were treated as independent loci with two alleles (present or absent). Unresolvable bands and missing data were treated as missing data.

To test the reproducibility of the amplification patterns, all DNA amplifications were repeated at least twice to ensure reproducibility of primers. The bands were considered reproducible and scorable only after observing and comparing them in two separate amplifications for each primer. Clear and intense bands were scored while faint

**Table 1**
Strains of *Aspergillus* section *Nigri* species included in the study.

| Morphological identification | Source | Molecular identification | | | Micotoxins | |
|---|---|---|---|---|---|---|
| | | Primer specific | ISSR | Calmodulin (Accession no) | OTA | FB2 |
| *Aspergillus uniseriate* | | | | | | |
| ANRC 528 | Mendoza | JAPO1/2 | *A. japonicus* | KR020698 | nd | ND |
| ANRC 529 | San Juan | JAPO1/2 | *A. japonicus* | KF225507.1 | nd | ND |
| ANRC 530 | San Juan | JAPO1/2 | *A. japonicus* | | nd | ND |
| ANRC 545 | San Juan | JAPO1/2 | *A. japonicus* | KR020699 | nd | ND |
| ANRC 549 | San Juan | JAPO1/2 | *A. japonicus* | KR020700 | nd | ND |
| *A. niger aggregate* | | | | | | |
| ANRC 539 | San Juan | NIG1/2; TUB1/2: — | *A. luchuensis* | KR020710 | nd | ND |
| ANRC 540 | San Juan | NIG1/2; TUB1/2: — | *A. luchuensis* | KR020707 | nd | ND |
| ANRC 544 | San Juan | NIG1/2; TUB1/2: — | *A. luchuensis* | KR020711 | nd | ND |
| ANRC 548 | San Juan | NIG1/2; TUB1/2: — | *A. luchuensis* | KR020712 | nd | ND |
| ANRC 553 | San Juan | NIG1/2; TUB1/2: — | *A. luchuensis* | | nd | ND |
| ANRC 554 | San Juan | NIG1/2; TUB1/2: — | *A. luchuensis* | KR020708 | nd | ND |
| ANRC 555 | San Juan | NIG1/2; TUB1/2: — | *A. luchuensis* | KR020709 | nd | ND |
| ANRC 516 | Mendoza | NIG1/2 | *A. niger*/*A. awamori* | KR020704 | nd | nd |
| ANRC 517 | Mendoza | NIG1/2 | *A. niger*/*A. awamori* | KR020705 | nd | nd |
| ANRC 519 | Mendoza | NIG1/2 | *A. niger*/*A. awamori* | KR020702 | nd | nd |
| ANRC 521 | Mendoza | NIG1/2 | *A. niger*/*A. awamori* | KR020701 | nd | nd |
| ANRC 524 | San Juan | NIG1/2 | *A. niger*/*A. awamori* | KF225503.1 | nd | + |
| ANRC 552 | San Juan | NIG1/2 | *A. niger*/*A. awamori* | KR020706 | nd | nd |
| ANRC 556 | San Juan | NIG1/2 | *A. niger*/*A. awamori* | KR020703 | nd | nd |
| ANRC 518 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 520 | Mendoza | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 522 | Mendoza | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 523 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 525 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 526 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 527 | San Juan | TUB1/2 | *A. tubingensis* | KF225512.1 | nd | ND |
| ANRC 563 | Unknown | TUB1/2 | *A. tubingensis* | KR020713 | nd | ND |
| ANRC 531 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 532 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 533 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 534 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 535 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 536 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 537 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 538 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 541 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 542 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 543 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 546 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 550 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 551 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| ANRC 557 | San Juan | TUB1/2 | *A. tubingensis* | | nd | ND |
| Series *Carbonaria* | | | | | | |
| ANRC 501 | San Juan | CARBO1/2 | *A. carbonarius* | | + | ND |
| ANRC 502 | San Juan | CARBO1/2 | *A. carbonarius* | KF225500.1 | + | ND |
| ANRC 503 | San Juan | CARBO1/2 | *A. carbonarius* | | + | ND |
| ANRC 504 | San Juan | CARBO1/2 | *A. carbonarius* | | + | ND |
| ANRC 505 | San Juan | CARBO1/2 | *A. carbonarius* | | + | ND |
| ANRC 506 | San Juan | CARBO1/2 | *A. carbonarius* | | + | ND |
| ANRC 507 | San Juan | CARBO1/2 | *A. carbonarius* | | + | ND |
| ANRC 508 | San Juan | CARBO1/2 | *A. carbonarius* | | + | ND |
| ANRC 509 | San Juan | CARBO1/2 | *A. carbonarius* | | + | ND |
| ANRC 510 | San Juan | CARBO1/2 | *A. carbonarius* | | + | ND |
| ANRC 511 | San Juan | CARBO1/2 | *A. carbonarius* | | + | ND |
| ANRC 512 | San Juan | CARBO1/2 | *A. carbonarius* | | + | ND |
| ANRC 513 | San Juan | CARBO1/2 | *A. carbonarius* | | + | ND |
| ANRC 514 | San Juan | CARBO1/2 | *A. carbonarius* | KF225502.1 | + | ND |
| ANRC 515 | San Juan | CARBO1/2 | *A. carbonarius* | | + | ND |
| ANRC 558 | Unknown | CARBO1/2 | *A. carbonarius* | | + | ND |
| ANRC 559 | Unknown | CARBO1/2 | *A. carbonarius* | KR020714 | + | ND |
| ANRC 560 | Unknown | CARBO1/2 | *A. carbonarius* | | + | ND |
| ANRC 561 | Unknown | CARBO1/2 | *A. carbonarius* | | + | ND |
| ANRC 562 | Unknown | CARBO1/2 | *A. carbonarius* | | + | ND |
| ANRC 564 | Unknown | CARBO1/2 | *A. carbonarius* | | + | ND |
| ANRC 500 | San Juan | CARBO1/2: — | *A. ibericus* | KF225499.1 | nd | ND |
| ANRC 547 | San Juan | CARBO1/2: — | *A. ibericus* | KR020715 | nd | ND |

bands against background smear were not considered for the further analysis.

### 2.5. Banding pattern analysis

To estimate the genetic distance between individuals, a similarity coefficient ($S$) was calculated using the formula: $S = 2N_{xy} / (N_x + N_y)$, where $N_x$ and $N_y$ are the number of fragments amplified in isolates $x$ and $y$, respectively, and $N_{xy}$ is the number of fragments shared by the two isolates (Nei and Li, 1979). Genetic distance (D) was derived from similarity coefficients as $D = 1 - S$. Genetic distance matrices were constructed for isolates from the compiled ISSR data. Dendrograms were prepared by using the UPGMA (unweighted pair-group method using arithmetic averages) algorithm in NTSYSpc 2.0 (Numerical Taxonomy System) software package (Rohlf, 1990). The ISSR data were subjected to bootstrap analysis with 1000 replications with PAUP* version 4.0 (Swofford, 2001) to determine if there was significant genetic substructure or clustering among isolates as they were resolved by the ISSR. The cophenetic correlation coefficient (CCC) was chosen to indicate the level of distortion between the similarity matrix and cluster analysis. NTSYSpc version 2.0 was used to perform these analyses (Rohlf, 1987).

### 2.6. Fungal DNA amplification and calmodulin analysis

DNA sequencing approach was utilized to confirm the identities of the black-spored isolates. DNA fragment of the partial calmodulin gene was amplified and sequenced using CL1 and CL2A primers, as described by O'Donnell et al. (2000).

Both DNA strands were sequenced used an ABI 3730XL sequencer (Applied Biosystems). Sequencing errors were detected and corrected using BioEdit sofware version 7.0.9.0 (Thompson et al., 1994). Nucleotide sequence comparisons were performed using the Basic Local Alignment Search Tool (BLAST) network services of the National Center for Biotechnology Information (NCBI) database. DNA sequences generated in this study have been deposited in GenBank under accession numbers KF225499–KF225512 and KR020698–KR020715 (Table 1).

Multiple sequence alignment of the partial calmodulin gene was obtained using the clustal (Katoh and Standley, 2013). Calmodulin sequences representing previously described species of the *Aspergillus* section *Nigri* obtained from GenBank were included in our analyses (Varga et al., 2011). Based on this alignment phylogenetic analysis was performed using TNT version 1.1 (Goloboff et al., 2008) by maximum parsimony method. The analyses were performed using the heuristic search option with 1000 random addition sequences with tree-bisection reconnection (TBR) branch swapping, saving 10 trees per replicate. All the most parsimonious trees found in the replicates were subject to a final round of TBR. Gaps were treated as "fifth state". The robustness of the tree obtained was evaluated by 1000 bootstrap replications. *Aspergillus flavus* CBS 100927 was used as outgroup.

### 2.7. Ochratoxin A production by Aspergillus section Nigri isolates

OTA production was tested in all 65 strains belonging to section *Nigri* (Table 1). OTA was determined following the methodology described by Téren et al. (1996), with some modifications. Briefly, the strains were grown in stationary cultures in 1 mL quantities of YES (2% yeast extract, 15% sucrose) medium at 25 °C for 7 days in the dark. After incubation, a portion of these culture media (0.5 mL) was mixed with 0.5 mL of chloroform and centrifuged at 4000 *g* for 10 min. The chloroform phase was transferred to a clean tube, evaporated to dryness and redissolved in 0.5 mL of mobile phase. The HPLC apparatus used for determination of OTA was a Hewlett-Packard Series 1100 (Hewlett-Packard Company, Palo Alto, CA, USA) chromatograph with a loop of 50 μL, equipped with a fluorescence detector (λexc = 330 nm and λem = 460) and a C18 column (150 × 4.6 mm, 5 μm particle size; Supelcosil LC-ABZ, Supelco, Bellefonte, PA, USA), connected to a pre-column (20 × 4.6 mm, 5 μm particle size; Security Guard, Phenomenex). The mobile phase was pumped at 1.0 mL/min, and consisted of an isocratic system composed: acetonitrile, water, acetic acid (99:99:2, v/v). OTA was quantified based on HPLC fluorometric response compared with the OTA standard solution using data module Hewlett Packard Kayak XA (HP ChemStation Rev. A.06.01). The retention time of OTA was at 5.7 min. Detection limits (LOD) were 10 ng/mL OTA.

### 2.8. Fumonisin $B_2$ production by A. niger/A. welwitschiae

Fumonisin production was tested in six strains identified by molecular tools as *A. niger*/*A. welwitschiae*. *A. welwitschiae* ITEM 4502, a well-known fumonisin producer (Susca et al., 2010) was used as positive control. $FB_2$ was analyzed following the methodology described by Frisvad et al. (2007) with HPLC detection following the procedure described by Shephard et al. (1990).

All $FB_2$ positive strains were confirmed by LC–MS/MS analysis, using a Waters 2695 Alliance HPLC (Waters Corporation, Milford, MA, USA) equipped with a Waters Alliance 2685 pump, a Waters Alliance 2695 autosampler, a diode array detector Waters 2996 PDA interfaced to a Quattro Ultima Platinum tandem quadrupole mass spectrometer with electrospray ionization (ESI) source. The column used was a 2.1 mm × 150 mm i.d., 3.5 μm, XBridge C18 with a 2.1 mm × 10 mm i.d., guard column of the same material (Waters, Milford, MA). The interfaces were operated in a positive ion mode. Nebulizer and desolvation gases were nitrogen heated to 150 and 200 °C, respectively. The capillary voltage was 3.00 kV. The nitrogen flows were adjusted to 104 and 678 L/h for cone and desolvation gases, respectively. Multiple-reaction monitoring (MRM) was used for toxin determination. The precursor peak [M + H] + of $FB_2$ (m/z 706.3) and two products peaks (m/z 318.5 and m/z 336.3) were monitored to accomplish both quantitation and qualification criteria. Data acquisition and processing were performed using Mass Lynx V.4.1, Waters INC software. Multiple reaction monitoring trace m/z 706 > 336 was used for quantitation.

The mobile phase of the chromatographic procedure was a gradient of aqueous 1% acetic acid/5 mM ammonium acetate (solvent A) and methanol 1% acetic acid/5 mM ammonium acetate (solvent B). At the initial time, the eluent was 10.5% of solvent B. This composition was kept over 2 min, after which a linear gradient to 97.5% of solvent B was performed for 12 min, and then the eluent was kept at this composition for 3 min in order to clean the column. The initial conditions were stabilized over 5 min before the next injection. The flow rate was 0.2 mL/min. The column temperature was maintained at 22 C. Aliquots of 30 μL of sample extracts were injected into the HPLC unit. Four points of identification were used to identify $FB_2$, i.e. retention time of 17.6 min, the precursor [M + H] + of m/z 706.3 and both product ions (m/z 318.5 and 336.3). A calibration curve was obtained injecting 10 μL of different $FB_2$ solutions (0.25, 0.5, 1.0, and 2.0 μg/mL) in acetonitrile:water (1:1). Good linearity with a correlation coefficient higher than 0.996 was obtained for the calibration range. The calculated instrumental LOD (S/N = 3) for $FB_2$ was 0.01 ng/g and LOQ (S/N = 5) was 0.05 ng/g, and the relative within-day and between-day standard deviations (% RSD) were 6.5.

### 2.9. Cloning and sequencing of fragment unique from A. ibericus

The target DNA fragment in ISSR profile was extracted from agarose gel and the DNA fragment was recovered by adding molecular biology-grade water for 10 min at 37 °C. An aliquot of the recovered DNA fragment was re-amplified using the corresponding primer to verify that only a single band was excised. The fragment recovered was cloned into the pGEM-T easy vector (Promega Corporation, USA). *Escherichia coli* JM109 competent cells were transformed with the recombinant vector and were then plated onto LB agar–ampicillin–IPTG–X-gal plates. Positive colonies were determined by blue/white screening. Plasmids from randomly selected white colonies were extracted using alkaline

lysis method (Sambrook and Russell 2001). The size of cloned fragment was verified by using the M13F/M13R primer and digested by restriction enzymes in multiple clone site of vector (EcoRI).

The fragment cloned was sequenced by an automated ABI 3730XL sequencer (Applied Biosystems) using M13 specific primer. Sequencing errors were detected and corrected using BioEdit sofware version 7.0.9.0 (Thompson et al., 1994). Nucleotide sequence comparisons were performed using BLAST network services of the NCBI database.

### 2.10. SCAR primers and PCR conditions

Species-specific primers (Fibe1/Ribe2) for *A. ibericus* were designed, based on the obtained sequences of the cloned ISSR fragment, using primer 3 (Rozen and Skaletsky, 2000) and tested for optimal PCR conditions. Amplification reactions were carried out in a final volume of 20 μL containing 10 ng template DNA, 0.2 μM of each primer (Fibe/Ribe), 1× PCR buffer (50 mM KCl; 10 mM Tris–HCl a pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1.25 units *Taq* DNA polymerase (Invitrogen, Buenos Aires, Argentina). PCRs were performed in a PTC-2000 Thermal Cycler (MJ Research Inc., Watertown, MA). Amplification conditions were: denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 1 min; annealing at 57 °C for 50 s; extension at 72 °C for 1 min; final extension at 72 °C for 5 min, followed by cooling at 4 °C until recovery of the samples. All amplification products generated were resolved on 1.5% agarose gels. The gels were stained with ethidium bromide (5 μg/mL) and visualized under UV light. The size of the DNA fragments was estimated by comparing the DNA bands with a 100 bp DNA ladder (Invitrogen, Buenos Aires, Argentina).

The specificity of the PCR primer pairs was determined individually for genomic DNA extracted from pure cultures of *A. ibericus*, *A. carbonarius*, *A. japonicus*, *A. aculeatus*, *A. uvarum*, *A. niger*, *A. tubingensis*, *A. welwitschiae*, *Aspergillus brasiliensis*, *A. luchuensis*, and other species isolated from raisins such as *Alternaria* spp., *Fusarium* spp., *Aspergillus* spp. PCR was tested using different concentrations of *A. ibericus* DNA (ITEM 4776 and ARNC 500 F strains) from 10 ng to 0.01 pg per microliter in a 20-μl PCR.

### 2.11. Multiplex PCR for A. carbonarius and A. ibericus discrimination

To identify simultaneously both *A. carbonarius* and *A. ibericus* in a single reaction, amplifications were carried out in a final volume of 20 μL containing 10 ng template DNA, 0.2 μM of each primer (Fibe1/Ribe2) and 0.5 μM of each primer (CARBO1/2), 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1.25 units Taq DNA polymerase (Invitrogen, Buenos Aires, Argentina). PCR was performed in a PTC-2000 Thermal Cycler (MJ Research Inc., Watertown, MA). Amplification conditions were: denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 1 min; annealing at 57 °C for 30 s; extension at 72 °C for 1 min; final extension at 72 °C for 5 min, followed by cooling at 4 °C until recovery of the samples. All amplification products generated were resolved on 1.5% agarose gels. The gels were stained with ethidium bromide (5 μg/mL) and visualized under UV light. The size of the DNA fragments was estimated by comparing the DNA bands with a 100 bp DNA ladder (Invitrogen, Buenos Aires, Argentina).

## 3. Results

### 3.1. Diversity of black Aspergilli isolated from raisins

Mycological examination of raisins indicated that *Aspergillus* section *Nigri* species were presented in 100% of the analyzed samples. Initially, black aspergilli isolated from raisins were classified morphologically into three subgroups (according to Abarca et al., 2004), *A. niger* aggregate (56.9%), *A. carbonarius* (35.4%) and *Aspergillus* "uniseriate" (7.7%), and thereafter a total of 65 isolates were molecularly identified by species specific primers.

### 3.2. OTA and FB2 production by isolates

Of the strains morphologically classified as *A. carbonarius*, 91.3% produced OTA. The levels of toxin produced ranged from 42 to 389 ng/mL on YES medium. In contrast, none of the isolates belonging to the *A. niger* aggregate and uniseriate *Aspergillus* produced detectable levels of OTA. Of the 6 strains that were classified as *A. niger*/*A. welwitschiae* by molecular characterization, only 16.6% were able to produce detectable levels of $FB_2$.

### 3.3. Molecular characterization of Aspergillus isolates by ISSR and calmodulin analysis

In order to clarify the species diversity reported in raisins, a partial molecular characterization of the 65 isolates previously characterized by morphological criteria was analyzed at the species level by the use of ISSR-PCR using CTC $(GT)_8$ and AG $(CTC)_5$ primers. The profile of the reference strains was used for comparison with the individual isolate profiles. Fig. 1 shows ISSR profile of 18 isolates of *Aspergillus* section *Nigri* obtained using CTC $(GT)_8$ primers. Polymorphisms both within and between species were obtained for both primers evaluated. A total of 78 fragments with different sizes (200 to 2500 bp) were clearly amplified and allowed the construction of a data matrix, which was used to produce a dendrogram (Fig. 2). The dendrogram calculated from the similarity matrix showed clear separation of the *Aspergillus* Uniseriates, *Aspergillus* series *Carbonaria* and *A. niger* aggregate (Fig. 2).

Cluster "*Aspergillus* series *Carbonaria*" included species belonging to *Aspergillus* series *Carbonaria* (*A. carbonarius* and *A. ibericus*) and was resolved on 2 subclades (A and B). The subclade A contained the ANRC 547 and ANRC 500 strains, identified morphologically as *A. carbonarius* but negative for CARBO specific primer and non-OTA-producers, being associated with *A. ibericus* (support value 100%). The taxonomic categorization, as *A. ibericus*, was confirmed by calmodulin sequence analysis (Fig. 3). The subclade B included 21 strains identified morphologically as *A. carbonarius*, positives for CARBO1/CARBO2 primers. These strains were able to produce detectable levels of OTA (42 a 389 ng/mL; mean 161 ng/mL).

The cluster "*Aspergillus* uniseriate" included *A. japonicus* (support value 100%) and *A. aculeatus* (support value 77%). The five isolates from raisins amplified with JAPO1/JAPO2 primers and showed 93% similarity with the *A. japonicus* ITEM 7034 (support values 100%). Selected

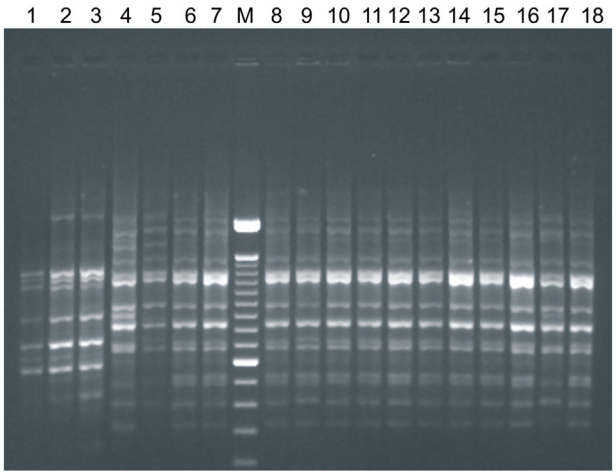


**Fig. 1.** Amplification patterns of *Aspergillus* section *Nigri* with primers $GAC(CGCA)_4$. M: Lane 1: *A. ibericus* ITEM 4776; Lane 2: ANRC 500; Lane 3: ANRC 547; Lane 4: ANRC 501; Lane 5: ANRC 502; Lane 6: ANRC 503; Lane 7: ANRC 504; Lane M: Molecular size marker (100 bp DNA Ladder Marker Invitrogen); Lane 8: ANRC 505; Lane 9: ANRC 506; Lane 10: ANRC 507; Lane 11: ANRC 508; Lane 12: ANRC 509; Lane 13: ANRC 510; Lane 14: ANRC 511; Lane 15: ANRC 512; Lane 16: ANRC 513; Lane 17: ANRC 514; and Lane 18: ANRC 515.

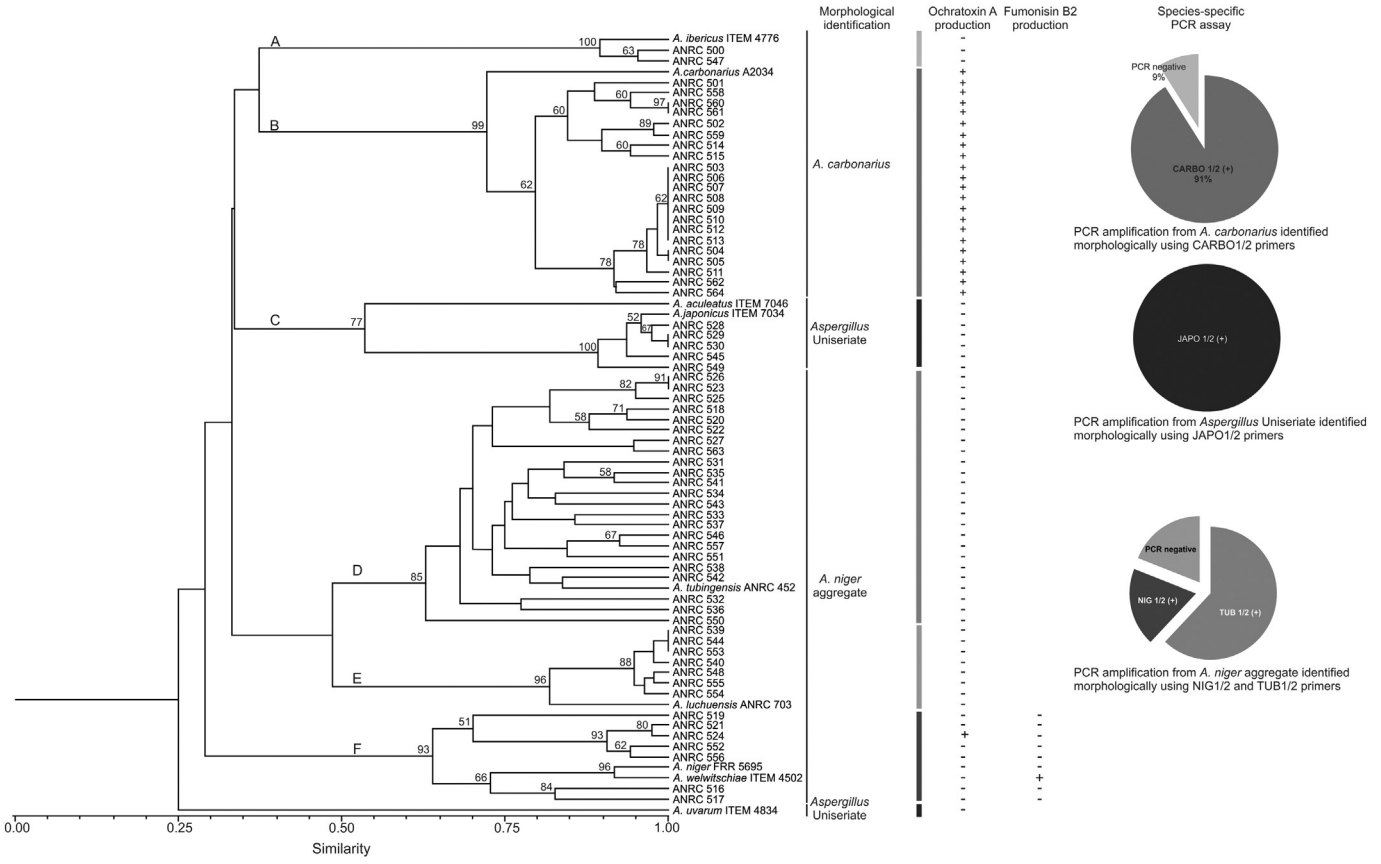


**Fig. 2.** Cluster analysis dendrogram of the 74 *Aspergillus* section *Nigri* using ISSR data. Numbers above branches are bootstrap values. Only values above 50% are indicated. −:not detectable; +: positive micotoxin production. CARBO1/2(+), TUB1/2(+), NIG1/2(+), and JAPO1/2(+): specific amplification. PCR negative: not amplification.

isolates from these five were confirmed by calmodulin sequence analysis as *A. japonicus* (Fig. 3).

The cluster "*A. niger aggregate*" included *A. tubingensis* (support value 85%), *A. luchuensis* (support value 96%) and *A. niger*/*A. welwitschiae* (support value 93%) (Fig. 2). The subclade D included 23 strains that showed 72% similarity with *A. tubingensis* ANRC 452 with high values of support (85%) and, amplified with specific TUB1/TUB2 primers. The seven isolates that were not amplified by the species-specific primers NIG1/NIG2 and TUB1/TUB2 were included in subcluster E, these isolates showed 82% similarity with the *A. luchuensis* ANRC 703 with high values of support (96%). The taxonomic categorization as *A. luchuensis* was confirmed by calmodulin sequence analysis (Fig. 3).

The subcluster F included *A. welwitschiae* and *A. niger* species (support value 93%). Within this subcluster the 7 strains positive for *A. niger*/*A. welwitschiae* specific primers (NIG1/NIG2) were located. These strains showed a similarity of 70% and 67% with the *A. niger* FRR 5695 and *A. welwitschiae* ITEM 4502, respectively, with high values of support (93%) (Fig. 2). The taxonomic categorization of these isolates were confirmed by calmodulin sequence analysis, allowing differentiation of *A. niger* from *A. welwitschiae* (Fig. 3). The clusters generated by ISSR band profiles did not allow discrimination between *A. niger* and *A. welwitschiae*.

### 3.4. SCAR identification, primer design and PCR optimization

Comparison of ISSR band patterns derived from different *Aspergillus* section *Nigri* revealed several characteristic bands for all species evaluated, however we focused on *A. ibericus* and the rapid differentiation from *A. carbonarius*, taking into account its impact as an OTA producer. After DNA recovery all bands produced by *A. ibericus* were evaluated. Only the minor bands (approximately 400 bp) showed a unique PCR product in the re-amplification. This product was cloned and subsequently sequenced. Nucleotide sequence comparison showed a significant match with FAD binding domain protein (XM_001399734.2) from *Aspergillus*, but with a query cover sequence of 54%, which allowed primers design. Based on the sequence obtained, one set of specific primers for *A. ibericus* named Fibe/Ribe was designed: 5′-CCAACGGT ATCACTGGATCA-3′ and 5′-GACGCAGACGAGTACGAAGA-3′. The size of the expected product was 189 pb.

After PCR optimization, the specificity of the primers was confirmed using *Aspergillus* section *Nigri* species and other fungal species. Under optimum conditions, the Fibe/Ribe primers only amplified a unique product of the expected size in *A. ibericus* strains and they did not amplify with other fungal species (Fig. 4). The sensitivity of the marker revealed that as little as 10 pg of template is sufficient for PCR diagnosis (Fig. 5).

The differences in the lengths of the species specific products generated with Fibe/Ribe (189 pb) and CARBO1/2 (375 pb) and, the absence of primer–dimer formation, allowed the design of a multiplex PCR. This PCR technique allowed, in a simple amplification, the discrimination of both species (Fig. 6).

## 4. Discussion

The species distribution of *Aspergillus* section *Nigri* in grapes and raisins is an important issue for risk evaluation of OTA contamination. A number of differences have been reported in the analysis of grapes and raisins from Argentina and other parts of the world, perhaps because of misidentifications or because the changes in taxonomy have confused the natural incidence and species distribution. The taxonomy of *Aspergillus* section *Nigri* is widely studied but although identification at section level is quite easy, at species level it is much

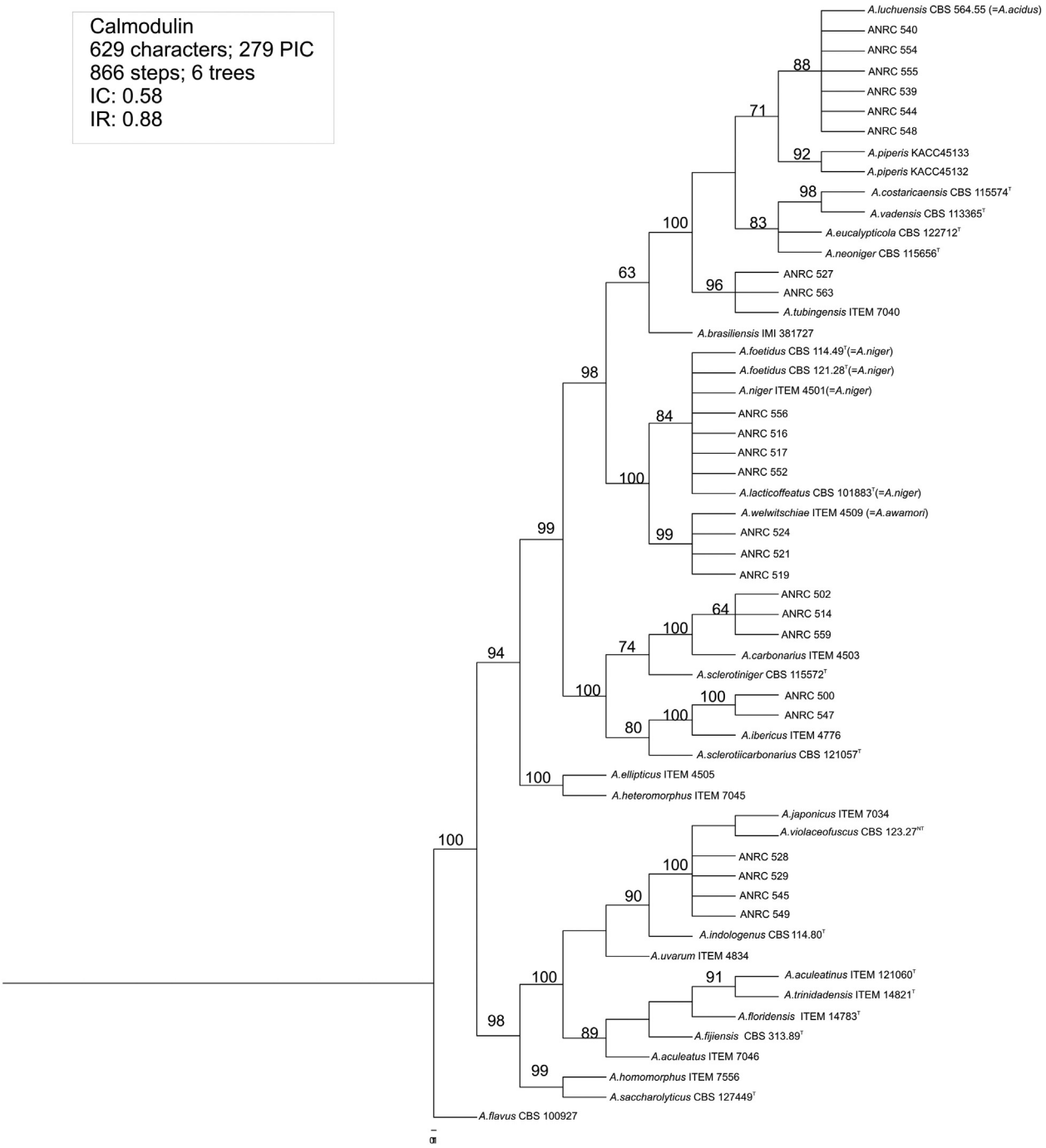


**Fig. 3.** One of the MP trees obtained based on phylogenetic analysis of calmodulin sequence data of *Aspergillus* section *Nigri*. Numbers above branches are bootstrap values. Only values above 70% are indicated.

more complex since morphological differences in taxa are very subtle, requiring taxonomic expertise. Species can be identified by micromorphological analysis of the fungal structures by light microscopy. Scanning Electron Microscopy (SEM) can be helpful for vesicle observation which is necessary for distinguishing between uniseriate and biseriate species, and conidia ornamentation which enables distinction between the *A. niger* aggregate, *A. carbonarius* and *A. ibericus* (Serra et al., 2006; Varga et al., 2000). Molecular methods are commonly used today for species identification among fungi. However, in accordance with the polyphasic species concept, other criteria are also included (Samson et al., 2007; Varga et al., 2011).

In the present study, we developed an ISSR marker that allowed evaluation of species diversity of *Aspergillus* section *Nigri* isolates from raisins, the ISSR data analysis was consistent with the identity of the isolates based on classical morphology, specific primers and OTA production. Although this marker does not allow the taxonomic categorization of the species it could be a very useful tool for screening when working with a large number of isolates.

In addition, this marker enabled the main species belonging to the *Aspergillus* section *Nigri* such as *A. japonicus*, *A. carbonarius*, *A. ibericus*, *A. niger*/*A. welwistchiae*, *A. tubingensis*, *and A. luchuensis* to be distinguished.

The results of *Aspergillus* section *Nigri* diversity obtained by ISSR assay were consistent with various studies of populations of black aspergilli from European and Argentinean vineyards using AFLP analysis (Chiotta et al., 2010; Perrone et al., 2006b; Serra et al., 2006;). Also,

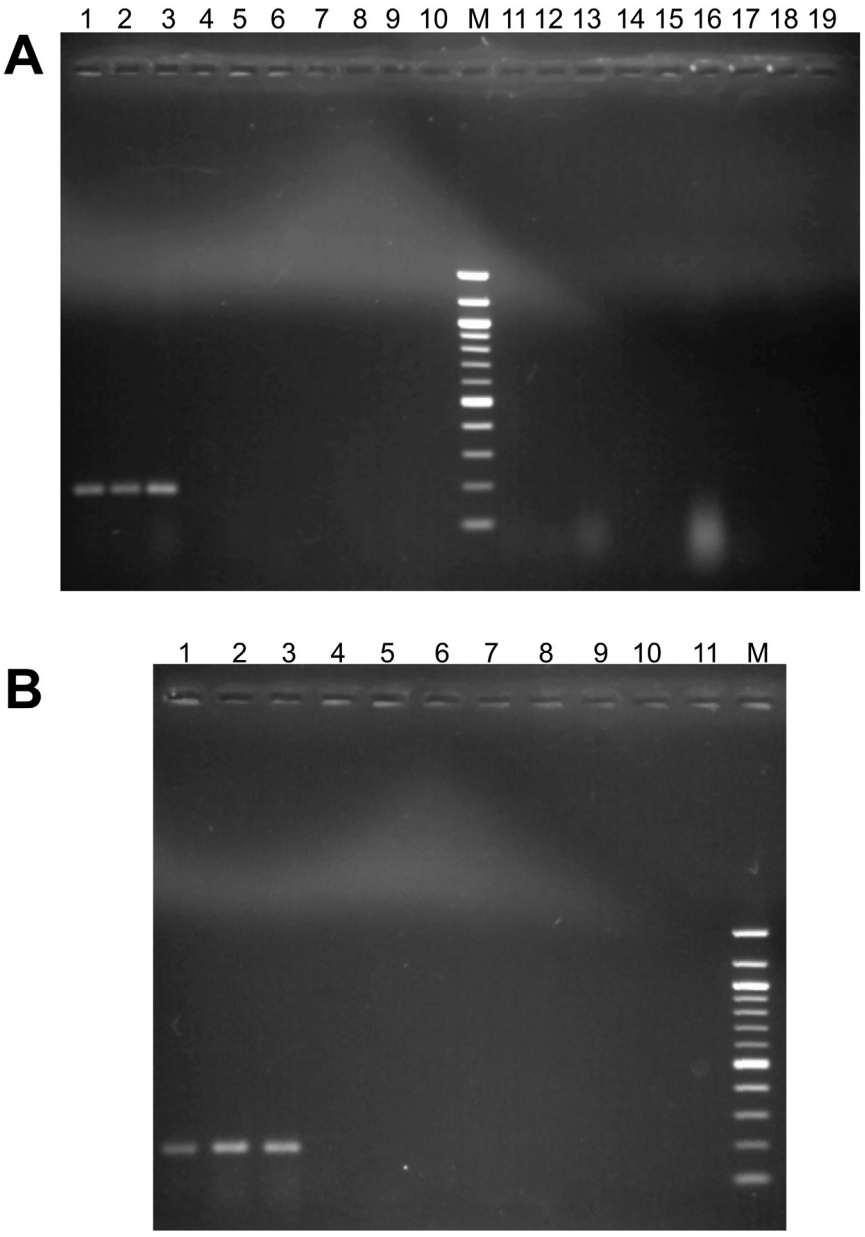


**Fig. 4.** Agarose gel electrophoresis of PCR-amplified products using specific primers Fibe1/Ribe2. (A) Lanes 1–3: *A. ibericus* ITEM 4776, ANRC 500, ANRC 547; Lanes 4–10: *A. carbonarius* ANRC 501, 502, 514, 558, 559, 601, A2034; M: 100-bp DNA ladder; Lane 11: *A. japonicus* ITEM 7034; Lane 12: *A. aculeatus* ITEM; Lane 13: *A. uvarum* ITEM 4834; Lane 14: *A. niger* ANRC 704; Lane 15: *A. tubingensis* ANRC 452; Lane 16: *A. welwitschiae* ANRC 524; Lane 17: *A. brasiliensis* ANRC 412; Lane 18: *A. luchuensis* ANRC 703; and Lane 19: negative control. (B) Lanes 1–3: *A. ibericus* ITEM 4776, ANRC 500, ANRC 547; Lanes 4–6: *Alternaria oregonensis* A70, *Alternaria infectoria* 128, *Alternaria alternata* 158; Lanes 7–8: *Fusarium graminearum* RCFG6001, *Fusarium proliferatum* ITEM 16395; Lanes 9–10: *Aspergillus flavus* FA213, *Aspergillus parasitus* LV3 84; Lane 10: negative control; and M: 100-bp DNA ladder.

similar data were obtained using other molecular markers such as rep-PCR (Palencia et al., 2009), ITS-RFLP (Martinez-Culebras and Ramon, 2007) and RFLP (Bau et al., 2006).

The species most frequently found in raisins in this study were *A. tubingensis* (35.4%) and *A. carbonarius* (32.3%), followed by *A. luchuensis* (10.7%), *A. japonicus* (7.7%), *A. niger* (6.2%), *A. welwitschiae* (4.6%) and *A. ibericus* (3.1%). These results partially agree with those found by Chiotta et al. (2011) and Barberis et al. (2013) who evaluated strains isolated from grapes and vineyard soil from Argentina, although the occurrence levels of *A. carbonarius* in the later were lower (11% and 2%). Gómez et al. (2006) conducted a study in three typical grape-growing regions in Spain, demonstrating that after sun drying the occurrence of *A. carbonarius* was higher than that recorded at harvesting time. Dekanea (2005) and Magan and Aldred (2005) reported that at the end of the drying period, before processing and packaging, *A. carbonarius* can occur with a contamination rate of 50%. This is a possible explanation of the high percentage of this species in raisins compared with grapes, raisins being a substrate with higher risk of OTA contamination.

This is the first report in Argentina where the species belonging to *Aspergillus* section *Nigri* isolated from raisins were characterized by a genetic study. ISSR markers were useful for the identification of strains of black aspergilli. For example, *A. tubingensis* and *A. luchuensis*, difficult to differentiate based only on morphology, can be differentiated into the *A. niger* aggregate. Also, the ISSR marker differentiated *A. ibericus* from *A. carbonarius*, phenotypically very similar. Both species have black biseriate *Aspergillus* heads with long stipes and relatively large conidia (*A. carbonarius* 7–9 mm and *A ibericus* 5–7 mm), but the main difference is that *A. ibericus* strains are unable to produce detectable OTA, unlike *A. carbonarius*, in which all strains are strong OTA producers (Serra et al., 2006). Many studies conducted in Argentina that did not use molecular identification, described *A. carbonarius* which did not produce

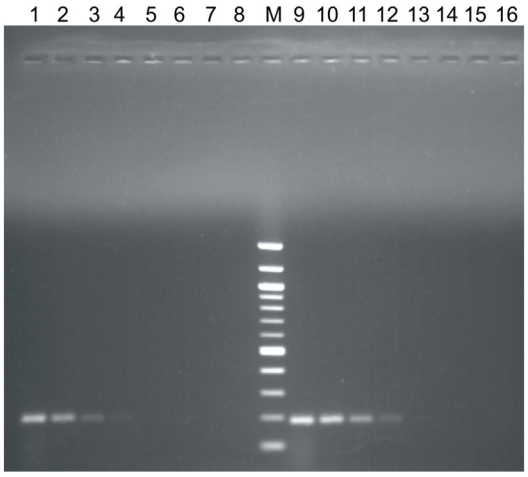


**Fig. 5.** Sensitivity of PCR with primers Fibe1/Ribe2 using different concentrations of *A. ibericus* DNA. Lanes 1–7, ITEM 4776 reference strain DNA at concentrations 10 ng, 1 ng, 0.1 ng, 0.01 ng, 0.001 ng, 0.0001 ng, and 0.00001 ng; lane 8, negative control; Lane M, 100-bp DNA ladder; lanes 9–15, ANRC 500 strain DNA at concentrations 10 ng, 1 ng, 0.1 ng, 0.01 ng, 0.001 ng, 0.0001 ng, and 0.00001 ng; and lane 16, negative control.

OTA (Chiotta et al., 2009; Dalcero et al., 2002; Magnoli et al., 2003; Ponsone et al., 2007; Romero et al., 2005). It is possible that these isolates were misidentified or confused with the closely related species *A. ibericus*, which has not been reported previously in Argentina. Recently, Cabañes et al. (2013) discovered three non-ochratoxigenic wild strains of *A. carbonarius*, unambiguously identified, characterized in depth. One purpose of this study was to demonstrate the efficacy of ISSR-PCR analysis to differentiate *A. ibericus* from *A. carbonarius*, these species being very difficult to differentiate by classical morphological criteria. This differentiation is very important to avoid overestimating toxicological contamination and related risks. A wide variety of specific primers for *A. carbonarius* detection has been developed (Mulé et al., 2006; Perrone et al., 2004; Schmidt et al., 2004), since *A. carbonarius* is considered the main species contributing to OTA contamination in the food chain. Strains morphologically identified as *A. carbonarius* that do not produce OTA could be true non-toxigenic strains or the closely related species *A. ibericus*. For this reason, we developed a SCAR marker from a single band of ISSR profiles specific for *A. ibericus* strains that allowed the development of Fibe/Ribe primers. The resulting PCR diagnostic allowed clear distinction of *A. ibericus* from the closely related species *A. carbonarius* quickly, reliably and inexpensively. We believe that this is the first study describing the development of SCAR markers from ISSR profiles in *Aspergillus* section *Nigri*.

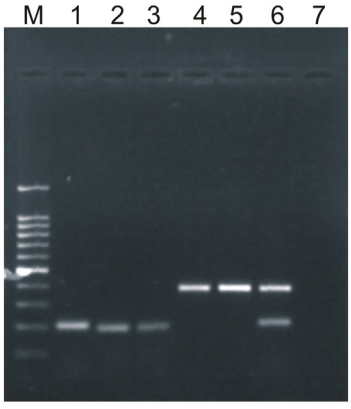


**Fig. 6.** Amplification products to evaluate the specificity of multiple PCR method for specific detection of *A. ibericus* (189 pb) and *A. carbonarius* (375 pb). M: 100 bp DNA Ladder; Lane 1: *A. ibericus* ITEM 4776; Lane 2: *A. ibericus* ANRC 500, Lane 3: *A. ibericus* ANRC 547; Lane 4: *A. carbonarius* 514; Lane 5: *A. carbonarius* A2034; Lane 6: Combination of DNA *A. carbonarius* A2034 and *A. ibericus* ITEM 4776; and Lane 7: negative control.

## Acknowledgments

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