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Molecular characterization and toxigenic profile of *Aspergillus* section *Nigri* populations isolated from the main grape-growing regions in Argentina

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Abstract

Aims: The objective of this study was to evaluate the biodiversity of *Aspergillus* section *Nigri* populations from Argentinean vineyards by morphological, toxigenic and AFLP analysis.

Materials and methods: Five hundred and thirty-eight strains were isolated from grapes during 2006/07 and 2007/08 vintages. The morphological identification and toxigenic profile for all strains isolated were performed. Eighty-eight strains were selected for characterization at species level by AFLP markers. Cluster analysis showed a clear separation into four main groups: *A. carbonarius*, *A. tubingensis*, *A. niger* 'aggregate' and *Aspergillus* 'uniseriate'. *A. carbonarius* strains constituted a homogeneous group, while a high degree of genetic diversity was found within the *A. niger* 'aggregate' and '*A. uniseriate*' clusters. The *A. tubingensis* cluster was the most prevalent group and was clearly separated from *A. niger* 'aggregate'. Ten strains showed 45% homology with *A. tubingensis* FRR 5720 *ex*-type strain and were considered as 'atypical' or a closely related species. AFLP results indicate that no genotypical differences can be established between ochratoxigenic and nonochratoxigenic strains.

Conclusions: *Aspergillus* section *Nigri* populations on grapes were represented mainly by four groups. *A. tubingensis* species were separated from *A. niger* 'aggregate' group and some of their strains produced OTA.

Significance and Impact of the Study: This study provides new data on molecular characterization of *Aspergillus* section *Nigri* populations in Argentina.

Introduction

Aspergillus section *Nigri* species have gained importance in the last decade as they are described as the main source of ochratoxin A (OTA) contamination in grapes and wine worldwide (Zimmerli and Dick 1996; Visconti *et al.* 2008). OTA is a nephrotoxic mycotoxin and also exhibits carcinogenic, teratogenic and immunotoxic properties in rats and possibly in humans (IARC 1993), while its genotoxicity remains controversial (EFSA 2006). Different surveys have analysed the epidemiology, ecology and distribution of black aspergilli occurring in vineyards from different regions (Da Rocha Rosa *et al.* 2002; Serra *et al.* 2003; Bellí *et al.* 2004; Sage *et al.* 2004; Battilani *et al.* 2006a; Leong *et al.* 2006). These studies have shown that the main ochratoxigenic black aspergilli species occurring on grapes are *Aspergillus niger* 'aggregate' and *Aspergillus carbonarius*. These species differ in their OTA-producing ability, because the percentage of *A. carbonarius* OTA-producing strains and the levels produced are higher than those reported for strains belonging to *A. niger* 'aggregate'. However, in the vineyards, *A. niger* 'aggregate' appears to be a dominant species within the section *Nigri*, and its presence can also contribute to

OTA contamination in a varying degree (Bau *et al.* 2005; Bellí *et al.* 2006; Battilani *et al.* 2006b; Leong 2007).

Argentina ranks fifth among wine producers in the world. The grape-growing area is located in Andina region (latitude 40–22°S). Previous studies carried out in Argentinean vineyards revealed that species included within the *A. niger* 'aggregate' were the dominant species isolated from Mendoza wine-growing region (Magnoli *et al.* 2003; Ponsone *et al.* 2007). *A carbonarius* species was mainly isolated from other regions such as La Rioja and San Juan (Chiotta *et al.* 2009). These species were identified using only morphological criteria. However, the difficulties in their recognition suggest that it is necessary to carry out an adequate molecular characterization because the toxin profiles of individual strains vary and to define potential toxicological risks. Amplified fragment length polymorphisms (AFLPs) can be a useful tool to clarify the identification of black aspergilli occurring on grapes. It is a genotyping method based on the selective amplification of a subset of DNA fragments generated by restriction enzymes digestion (Vos *et al.* 1995). AFLPs allow for differentiation of a large number of polymorphisms with only little variation of the protocol and with a high power of discrimination, reproducibility and robustness. This technique has proved useful for the classification at the species level allowing for the evaluation of the genetic relatedness between them and closely related species (Treadway *et al.* 1999; Marasas *et al.* 2001; Zeller *et al.* 2003). Previous studies suggested the presence of four groups assigned to *Aspergillus* section *Nigri* by AFLPs, and that AFLPs could separate species very closely related morphologically and phylogenetically, such as *A. tubingensis* from *A. niger* 'aggregate' (Perrone *et al.* 2006a,b). Also, these markers have been used to study fungal populations from other mycotoxigenic genera such as *Fusarium* (Chulze *et al.* 2000; Zeller *et al.* 2004; Reynoso *et al.* 2009), *Aspergillus* (Montiel *et al.* 2003; Schmidt *et al.* 2004; Barros *et al.* 2007) and *Penicillium* (Castella *et al.* 2002; Frisvad *et al.* 2005).

The aims of this study were as follows: (i) to evaluate the biodiversity within *Aspergillus* section *Nigri* populations isolated from Argentinean vineyards using AFLP molecular markers; (ii) to determine the genetic diversity and relatedness among the species isolated; and (iii) to associate the genetic diversity with the toxigenic profile of the strains.

Materials and methods

Strain identification

A total of 538 *Aspergillus* section *Nigri* strains were isolated and collected from 50 vineyards belonging to seven grape-growing regions in Argentina, during 2006/07 and 2007/08 vintages. The areas sampled included the following: La Rioja – Chilecito, San Juan – Tulum Valley, Mendoza – Uco Valley, Mendoza North-East, Mendoza ZARM (high area near Mendoza River), Mendoza South and Neuquén-Río Negro. The species identification was carried out using morphological criteria according to Klich (2002). The strains within *Aspergillus* section *Nigri* were classified according to the morphology of their spores and conidial heads into three groups: those with uniseriate conidial heads as *Aspergillus* 'uniseriate' and, among those with biseriate conidial heads, as *Aspergillus niger* 'aggregate' and *Aspergillus carbonarius*.

Ochratoxigenic profile

OTA production in culture medium

The OTA production ability of the strains was evaluated as described previously by Bragulat *et al.* (2001). Strains were inoculated in CYA medium (containing 1 g of K_2HPO_4, 10 ml of Czapek concentrate, 1 ml of trace metal solution, 5 g of yeast extract, 30 g of sucrose, 15 g of agar and water up to 1 l) and incubated at 25°C for 7 days. Three agar plugs (3 mm) of the fungal colony grown in the medium were taken along the radius from the inoculum point and placed in a vial with 1 ml of methanol. After 60 min, the extract was filtered through a membrane filter (Syringe filters, 17 mm, 0·45 μm, nylon membranes, TITAN) and submitted to a high-performance liquid chromatography (HPLC) analysis. The assay was carried out by duplicate.

OTA detection and quantification

The HPLC apparatus used for determination of OTA was a Hewlett-Packard (Hewlett-Packard Co., 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 precolumn (20 × 4·6 mm, 5 μm particle size; Supelguard LC-ABZ). The mobile phase was pumped at 1·0 ml min^{-1} and consisted of an isocratic system composed: acetonitrile, water, and acetic acid (99 : 99 : 2). OTA was quantified on the basis of HPLC fluorometric response compared with the OTA standard.

AFLP analysis

Fungal strains

Eighty-eight strains were selected at random for identification at species level by amplified fragment length polymorphism (AFLP) technique. The strains examined are listed in Table 2. *Aspergillus niger* FRR 5722, *Aspergillus awamori* FRR 5804, *Aspergillus tubingensis* FRR 5720, *Aspergillus carbonarius* FRR 5690, *Aspergillus japonicus*

ITEM 7034 and *Aspergillus aculeatus* ITEM 7046 were included as *ex*-type strains.

Nucleic acid extraction

A suspension of spores from each fungal strain was grown in Wickerham medium containing 40 g of glucose, 5 g of peptone, 3 g of yeast extract, 3 g of malt extract and water up to 1 l. The Erlenmeyer flasks were incubated on an orbital shaker at 1·26 g at 25 ± 1°C. Mycelia from 3-day-old cultures were harvested by filtration through nongauze milk filters (Ken AG, Ashland, OH, USA), excess water was removed by blotting mycelia between clean paper towels, and dried mycelia were stored frozen at −20°C until ground. Fungal DNA was extracted with the cetyltrimethylammonium bromide method developed by Murray and Thompson (1980) and modified by Kerényi *et al.* (1999).

AFLP reactions

AFLP reactions were performed as described by Vos *et al.* (1995) and modified by Zeller *et al.* (2000) in a PTC-2000 Thermal Cycler (MJ Research Inc., Watertown, MA, USA). All buffers and DNA-modifying enzymes were used following either the manufacturer's instructions or standard protocols (Sambrook *et al.* 1989). Genomic DNAs were digested to completion with *Eco*RI and *Mse*I and ligated to AFLP adapters overnight at room temperature (21–24°C). The digested and ligated templates were diluted in nine volumes of Tris–EDTA buffer prior to preamplification. Samples were preamplified under the following cyclic conditions: initial denaturation at 94°C for 60 s, followed by 20 cycles consisting of 30 s at 94°C, 60 s at 56°C and 60 s at 72°C, and a final extension step of 72°C for 5 min, and then held at 4°C indefinitely. Preamplified reactions were diluted 1 : 50 with H_2O prior to final specific AFLP amplification. Two primer pair combinations (*Eco*RI + TG/*Mse*I + CG; *Eco*RI + AT/*Mse*I + CG) were used. *Eco*RI primers for specific amplification were end-labelled [γ^{-33} P] ATP. For final specific AFLP reactions, 1·3 μl of diluted preamplification reactions was used, and the final volume was 5 μl. The PCR program for the AFLP amplification was as follows: one cycle of 94°C for 30 s, 65°C for 30 s and 72°C for 30 s; then, this cycle was followed by a 12-cycle step-down protocol in which the annealing temperature was lowered each cycle by 0·7°C from 65 to 56°C. After that, 23 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 60 s were performed, followed by a final extension step of 72°C for 5 min and then held at 4°C.

AFLP fragments were separated in denaturing 6% polyacrylamide gels (Long Ranger gel solution; BMA, Rockland, ME, USA) with 1× Tris-borate EDTA buffer (pH 8·0) in both the gels and the running buffer. Gels were run at a constant power of 60 W until the xylene cyanol (Sigma, St Louis, MO, USA) marker had run approximately 22 cm. After that, the gels were transferred to a 3-mm gel-blotting paper (Midwest Scientific, Valley Park, MO, USA) and dried before exposure to X-ray film at room temperature (Classic Blue Sensitive; Midwest Scientific) for 3–7 days to resolve banding patterns. Band sizes were estimated on polyacrylamide gels against [γ^{-33} P] ATP-labeled BRL low-mass ladder (Life Technologies, Rockville, MD, USA). The presence or absence of polymorphic AFLP bands was scored manually and the data recorded in a binary format. All polymorphic bands in this size range were scored, including those assumed to be homologous and to represent the same allele and locus. Each scored band of differing mobility was treated as a single independent locus with two alleles (present or absent).

Genetic distance and cluster analysis of AFLP data

To estimate the genetic distance between individuals, similarity coefficients (S) were calculated using the formula: $S = 2N_{xy}/(N_x + N_y)$, where N_x represents the number of fragments amplified in isolated 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 follows: $D = 1 - S$. Genetic distance matrices were constructed for isolates using the compiled AFLP data. Dendrograms were obtained using the unweighted pair-group method using arithmetic averages (UPGMA) clustering strategy of the NTSYSPC 2.0 (Numerical Taxonomy System; Applied Biostatistics Inc., New York, NY, USA) software package (Rohlf 1990). The AFLP data were subject to bootstrap analysis with 1000 replications using the program PAUP* ver. 4.0 (Swofford 1999), to determine whether there was significant genetic substructure or clustering among isolates as resolved by AFLP data.

Results

Strain identification and ochratoxigenic profile

The morphological characterization of 538 *Aspergillus* section *Nigri* strains showed that *A. niger* 'aggregate' represented the most common species (81% in 2006/07 and 87% in 2007/08), followed by *Aspergillus carbonarius* (12% in 2006/07 and 8% in 2007/08). The *Aspergillus* 'uniseriate' species were isolated in lower percentages than those of the other species during both vintages (8% in 2006/07 and 6% in 2007/08).

From the total strains evaluated (n = 275) during the 2006/07 vintage, 32% were OTA producers with levels ranging from 0·5 to 1,285 μg kg^{-1} of Czapek yeast extract agar (CYA) medium. Sixty-nine per cent of the strains was identified as *A. niger* 'aggregate', and the 31%

remained as *A. carbonarius*. From 263 strains isolated during the 2007/08 vintage, a low percentage was toxigenic (19%), but the OTA levels produced were higher than those produced in the previous vintage and ranged from 0·5 to 7583 μg kg^{-1}. Fifty-nine per cent of the OTA-producer strains was identified as *A. niger* 'aggregate' and the remaining 41% was *A. carbonarius*. The 'uniseriate' *Aspergillus* strains isolated in both periods evaluated were not OTA producers (Table 1).

AFLP analysis

A subset of 88 representative strains selected arbitrarily from the 538 isolates of *Aspergillus* section *Nigri* was eval-

Table 1 Ochratoxin A (OTA) production by *Aspergillus* section *Nigri* strains

Vintages	Regions	Species	Number of strains OTA-positives/total	OTA range* (μg kg^{-1})	Mean*
2006/07	La Rioja – Chilecito	*A. niger* aggregate	9/38	0·5–8·4	2·9
		A. carbonarius	19/21	0·5–1,285	207·0
		A. uniseriate	0/15	ND	–
	San Juan – Tulum Valley	*A. niger* aggregate	8/36	0·5–6·7	1·5
		A. carbonarius	6/6	1·2–770·0	196·9
		A. uniseriate	0/3	ND	–
	Mendoza – Uco Valley	*A. niger* aggregate	5/7	0·1–17·5	4·6
		A. carbonarius	–	–	–
		A. uniseriate	–	–	–
	Mendoza North-East	*A. niger* aggregate	4/24	0·5–17·8	5·1
		A. carbonarius	2/2	37·0–501·3	269·2
		A. uniseriate	0/2	ND	–
	Mendoza ZARM†	*A. niger* aggregate	11/22	0·5–4·2	1·8
		A. carbonarius	1/1	2·9	2·9
		A. uniseriate	0/1	ND	–
	Mendoza South	*A. niger* aggregate	7/20	0·5–3·6	2·2
		A. carbonarius	0/1	ND	–
		A. uniseriate	–	–	–
	Neuquén – Río Negro	*A. niger* aggregate	14/75	0·5–12·1	2·4
		A. carbonarius	–	–	–
		A. uniseriate	0/1	ND	–
2007/08	La Rioja – Chilecito	*A. niger* aggregate	9/26	0·7–2590	310·0
		A. carbonarius	9/9	30·1–3214	1139
		A. uniseriate	0/11	ND	–
	San Juan – Tulum Valley	*A. niger* aggregate	2/31	5·0–60·5	32·8
		A. carbonarius	8/8	10·0–7583	1605
		A. uniseriate	–	–	–
	Mendoza – Uco Valley	*A. niger* aggregate	1/20	3·9	3·9
		A. carbonarius	–	–	–
		A. uniseriate	–	–	–
	Mendoza North-East	*A. niger* aggregate	4/40	0·3–50·8	15·1
		A. carbonarius	–	–	–
		A. uniseriate	–	–	–
	Mendoza ZARM†	*A. niger* aggregate	6/34	0·3–5·4	1·4
		A. carbonarius	–	–	–
		A. uniseriate	0/1	ND	–
	Mendoza South	*A. niger* aggregate	3/47	11·3–20·5	15·2
		A. carbonarius	1/1	7356	7356
		A. uniseriate	0/1	ND	–
	Neuquén – Río Negro	*A. niger* aggregate	1/34	36·6	36·6
		A. carbonarius	–	–	–
		A. uniseriate	–	–	–

ND, not detected.
Detection limit: 0·05 μg kg^{-1}.
*Range and mean of OTA-positive strains data.
†ZARM: high area near the Mendoza river.

uated using AFLP markers. Two selective primer pair combinations (*Eco*RI+TG/*Mse*I+CG and *Eco*RI+AT/*Mse*I+CG) turned out to produce a complex, well-resolved fingerprint pattern. The region of approximately 100–400 pb was considered for similarity calculation and subsequent graphical visualization. Polymorphisms both within and between species were obtained for each of the two primer pairs. The identification of 107 distinct and scoreable bands allowed the construction of 88 isolates × 107 loci data matrix, which was analysed and used to produce a dendrogram (Fig. 1). The resultant UPGMA dendrogram allowed the comparison among the haplotypes and showed a clear separation of four groups: *A. carbonarius*, *A. tubingensis*, *A. niger* 'aggregate' and *Aspergillus* 'uniseriate'. The similarity among strains from different clusters was <25%. The strains were assigned to a species if they shared more than 50% of the bands with the *ex*-type strain. Out of the total strains analysed, 16 belonged to *A. carbonarius*, 44 to *A. tubingensis*, 12 to *A. niger* 'aggregate' and 6 to *Aspergillus* 'uniseriate'. Ten strains were not assigned readily to any of the *ex*-type strains included in the study. However, these strains were grouped and showed a higher degree of similarity with *A. tubingensis ex*-type strain (45%), so they were considered as *A. tubingensis* 'atypical' or a closely related species. The description of the four cluster identified is reported later and showed in Fig. 1 and Table 2.

Aspergillus 'uniseriate'
This cluster showed low homology (42%) and high internal heterogeneity. Five strains clustered with *Aspergillus japonicus* ITEM 7034, 3 showed a similarity of 37% and in particular, ASNRC 84 strain a similarity of 52%. Two strains were grouped with *A. aculeatus* ITEM 7046 *ex*-type with a similarity of 61%. None of the strains evaluated produced detectable OTA levels.

Aspergillus tubingensis
The largest number of the strains ($n = 38$) showed a similarity of 68% with *A. tubingensis* FRR 5720 *ex*-type strain. Six strains formed other branches, three were not grouped with other strains, and the rest formed one smaller group with a similarity of 60% among them. The strains included in this cluster were not previously identified as *A. tubingensis* by morphological criteria. Twelve of the 46 strains produced detectable levels of ochratoxin A.

Aspergillus carbonarius
The *A. carbonarius* cluster included the 16 strains identified morphologically as *A. carbonarius*. All the strains clustered at a similarity value of 81% with the *A. carbonarius* FRR 5690 *ex*-type strain, while, among them, shared 90% of the bands. In particular, six strains were grouped with the *ex*-type strain with 100% of homology. All the strains were OTA producers with variable production levels.

Aspergillus niger 'aggregate'
Twelve strains clustered with a similarity value ranging from from 42 to 90% with *A. niger* FRR 5722 *ex*-type strain, and from 36 to 82% with *A. awamori* FRR 5804 *ex*-type strain. This group was considered as *A. niger* 'aggregate' cluster as there was no distinctive homology with any of the two ex-types previously mentioned. All strains analysed were morphologically undistinguished from the strains included in *A. tubingensis* cluster. Two of the 12 strains in this group produced detectable OTA levels.

Discussion

In a previous study, we have found that species within *Aspergillus* section *Nigri* were frequently isolated in the vineyards of the different grape-growing regions in Argentina (Chiotta *et al.* 2009). The morphological identification showed that *A. niger* 'aggregate' species were the most prevalent, followed by *A. carbonarius* and *Aspergillus* 'uniseriate'. However, at present there are no studies on molecular characterization of the black aspergilli populations from wine grapes in Argentina and AFLP analysis provided a useful tool to determine the diversity of the strains isolated.

The AFLP results showed that *Aspergillus* section *Nigri* populations were heterogeneous and represented mainly by four well-defined groups including *A. carbonarius*, *A. tubingensis*, *A. niger* 'aggregate' and *A.* 'uniseriate'. *A. carbonarius* species was distinguished morphologically, and by molecular analysis could be clearly separated forming a homogeneous group. In contrast, the strains included in *A. niger* 'aggregate' group were not differentiated using morphological criteria and the AFLP markers allowed for the separation into *A. niger* 'aggregate' and *A. tubingensis* clusters. The strains included in *A. niger* 'aggregate' cluster showed a high degree of genetic diversity and had no distinctive homology with *A. awamori* or *A. niger* ex-type strains. However, the *A. tubingensis* cluster could be clearly differentiated from *A. niger* 'aggregate' cluster and was represented by the most of the strains isolated. In addition, ten strains were included in this cluster and showed low homology with *A. tubingensis* FRR 5720 *ex*-type strain, thus supporting the possible presence of atypical strains or a closely related species within *A. tubingensis* group. Previously, *A. tubingensis* species have not been described in vineyards from Argentina, and considering its OTA profile, the characterization of this species is relevant.

Although AFLP markers allowed the separation of uniseriate species from the other clusters, these markers

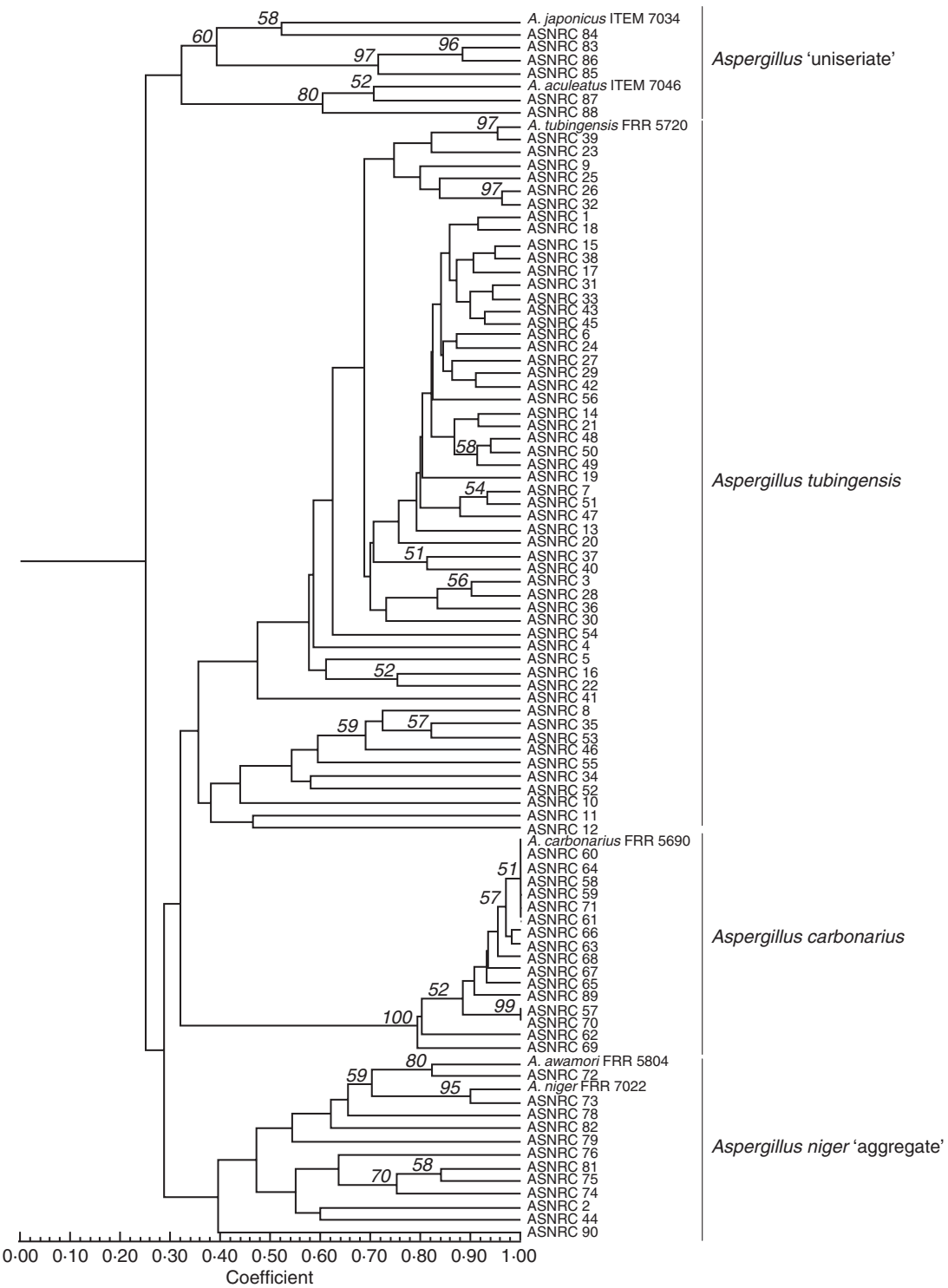

Figure 1 Dendrogram showing genetic relatedness of the *Aspergillus* section *Nigri* populations isolated from Argentinean vineyards based on UPGMA analysis.

were not useful to discriminate the strains included in the 'uniseriate' cluster at species level. The high diversity in this cluster is possibly because of the presence of other species such as *A. uvarum* or *A. aculeatinus*. Therefore, further studies will be needed to clarify the results.

Table 2 Origin and ochratoxin A (OTA) production of *Aspergillus* section *Nigri* strains included in AFLP analysis

Strain	Species	Origin	OTA	Vintage
ASNRC 84	*A.* 'uniseriate'	La Rioja	nd	2006/07
ASNRC 83	*A.* 'uniseriate'	La Rioja	nd	2006/07
ASNRC 86	*A.* 'uniseriate'	La Rioja	nd	2007/08
ASNRC 85	*A.* 'uniseriate'	La Rioja	nd	2006/07
ASNRC 87	*A.* 'uniseriate'	Mendoza – ZARM	nd	2006/07
ASNRC 88	*A.* 'uniseriate'	Mendoza – ZARM	nd	2006/07
ASNRC 39	*A. tubingensis*	Mendoza – South	nd	2006/07
ASNRC 23	*A. tubingensis*	Mendoza – North-East	nd	2007/08
ASNRC 9	*A. tubingensis*	La Rioja	nd	2006/07
ASNRC 25	*A. tubingensis*	Mendoza – North-East	nd	2007/08
ASNRC 26	*A. tubingensis*	Mendoza – North-East	nd	2007/08
ASNRC 32	*A. tubingensis*	Neuquén-Río negro	nd	2007/08
ASNRC 1	*A. tubingensis*	Mendoza – North-East	nd	2006/07
ASNRC 18	*A. tubingensis*	Mendoza – Uco Valley	nd	2007/08
ASNRC 15	*A. tubingensis*	Mendoza – Uco Valley	+	2006/07
ASNRC 38	*A. tubingensis*	Neuquén – Río negro	nd	2006/07
ASNRC 17	*A. tubingensis*	Neuquén – Río negro	nd	2006/07
ASNRC 31	*A. tubingensis*	Neuquén – Río negro	nd	2006/07
ASNRC 33	*A. tubingensis*	Neuquén – Río negro	+	2006/07
ASNRC 43	*A. tubingensis*	Neuquén – Río negro	LD	2007/08
ASNRC 45	*A. tubingensis*	Neuquén – Río negro	nd	2007/08
ASNRC 6	*A. tubingensis*	Mendoza – ZARM	nd	2007/08
ASNRC 24	*A. tubingensis*	Mendoza – North-East	nd	2007/08
ASNRC 27	*A. tubingensis*	Mendoza – North-East	nd	2007/08
ASNRC 29	*A. tubingensis*	Neuquén – Río negro	nd	2006/07
ASNRC 42	*A. tubingensis*	Neuquén – Río negro	nd	2007/08
ASNRC 56	*A. tubingensis*	San Juan	+	2007/08
ASNRC 14	*A. tubingensis*	La Rioja	nd	2006/07
ASNRC 21	*A. tubingensis*	Mendoza – North-East	nd	2007/08
ASNRC 48	*A. tubingensis*	San Juan	nd	2006/07
ASNRC 50	*A. tubingensis*	San Juan	nd	2006/07
ASNRC 49	*A. tubingensis*	San Juan	+	2006/07
ASNRC 19	*A. tubingensis*	Neuquén – Río negro	+	2006/07
ASNRC 7	*A. tubingensis*	Mendoza – ZARM	nd	2007/08
ASNRC 51	*A. tubingensis*	San Juan	nd	2006/07
ASNRC 47	*A. tubingensis*	La Rioja	nd	2007/08
ASNRC 13	*A. tubingensis*	La Rioja	nd	2006/07
ASNRC 20	*A. tubingensis*	Mendoza – North-East	nd	2007/08
ASNRC 37	*A. tubingensis*	La Rioja	nd	2007/08
ASNRC 40	*A. tubingensis*	Mendoza – South	nd	2007/08
ASNRC 3	*A. tubingensis*	Mendoza – North-East	+	2006/07
ASNRC 28	*A. tubingensis*	Neuquén-Río negro	nd	2006/07
ASNRC 36	*A. tubingensis*	La Rioja	nd	2007/08
ASNRC 30	*A. tubingensis*	Neuquén-Río negro	+	2006/07
ASNRC 54	*A. tubingensis*	San Juan	nd	2007/08
ASNRC 4	*A. tubingensis*	Mendoza – ZARM	+	2007/08
ASNRC 5	*A. tubingensis*	Mendoza – ZARM	nd	2007/08
ASNRC 16	*A. tubingensis*	La Rioja	+	2006/07
ASNRC 22	*A. tubingensis*	Neuquén-Río negro	+	2006/07
ASNRC 41	*A. tubingensis*	Neuquén-Río negro	nd	2007/08
ASNRC 8	*A. tubingensis* 'atypical'	La Rioja	nd	2006/07
ASNRC 35	*A. tubingensis* 'atypical'	San Juan	nd	2006/07
ASNRC 53	*A. tubingensis* 'atypical'	La Rioja	nd	2007/08
ASNRC 46	*A. tubingensis* 'atypical'	San Juan	+	2006/07
ASNRC 55	*A. tubingensis* 'atypical'	San Juan	nd	2007/08
ASNRC 34	*A. tubingensis* 'atypical'	La Rioja	nd	2007/08
ASNRC 52	*A. tubingensis* 'atypical'	San Juan	+	2006/07

Table 2 (*Continued*)

Strain	Species	Origin	OTA	Vintage
ASNRC 10	*A. tubingensis* 'atypical'	La Rioja	nd	2006/07
ASNRC 11	*A. tubingensis* 'atypical'	La Rioja	nd	2006/07
ASNRC 12	*A. tubingensis* 'atypical'	La Rioja	nd	2007/08
ASNRC 60	*A. carbonarius*	San Juan	+	2007/08
ASNRC 64	*A. carbonarius*	La Rioja	++	2006/07
ASNRC 58	*A. carbonarius*	San Juan	++	2006/07
ASNRC 59	*A. carbonarius*	San Juan	++	2006/07
ASNRC 71	*A. carbonarius*	La Rioja	++	2007/08
ASNRC 89	*A. carbonarius*	San Juan	+	2007/08
ASNRC 66	*A. carbonarius*	La Rioja	+++	2006/07
ASNRC 63	*A. carbonarius*	San Juan	++++	2007/08
ASNRC 68	*A. carbonarius*	La Rioja	+++	2007/08
ASNRC 67	*A. carbonarius*	La Rioja	++	2006/07
ASNRC 65	*A. carbonarius*	La Rioja	+	2006/07
ASNRC 89	*A. carbonarius*	La Rioja	+	2006/07
ASNRC 57	*A. carbonarius*	San Juan	+++	2006/07
ASNRC 70	*A. carbonarius*	La Rioja	+	2007/08
ASNRC 62	*A. carbonarius*	San Juan	++++	2007/08
ASNRC 69	*A. carbonarius*	La Rioja	+	2007/08
ASNRC 72	*A. niger* 'aggregate'	Mendoza – ZARM	+	2006/07
ASNRC 73	*A. niger* 'aggregate'	Mendoza – ZARM	nd	2006/07
ASNRC 78	*A. niger* 'aggregate'	Neuquén – Río Negro	nd	2007/08
ASNRC 82	*A. niger* 'aggregate'	Mendoza – Uco Valley	nd	2007/08
ASNRC 79	*A. niger* 'aggregate'	Mendoza – Noeste	nd	2006/07
ASNRC 76	*A. niger* 'aggregate'	Mendoza – ZARM	nd	2007/08
ASNRC 81	*A. niger* 'aggregate'	Mendoza – Uco Valley	nd	2007/08
ASNRC 75	*A. niger* 'aggregate'	Mendoza – ZARM	nd	2006/07
ASNRC 74	*A. niger* 'aggregate'	Mendoza – ZARM	nd	2006/07
ASNRC 2	*A. niger* 'aggregate'	Mendoza – ZARM	nd	2007/08
ASNRC 44	*A. niger* 'aggregate'	Neuquén-Río negro	nd	2007/08
ASNRC 90	*A. niger* 'aggregate'	San Juan	+	2006/07
Reference strains				
FRR 7022	*A. niger*			
FRR 5804	*Aspergillus awamori*			
FRR 5720	*Aspergillus tubingensis*			
FRR 5690	*Aspergillus carbonarius*			
ITEM 7034	*Aspergillus japonicus*			
ITEM 7046	*Aspergillus aculeatus*			

ND, not detected.
Detection limit: 0·05 μg kg^{-1}.
OTA levels: +, <100; ++, 100–1000; +++, 1000–5000; ++++, >5000 μg kg^{-1}.

In summary, our results are consistent with Perrone *et al.* (2006b) who studied the black aspergilli populations from European vineyards using AFLP analysis. In addition, similar data were obtained using other molecular markers such as rep-PCR (Palencia *et al.* 2009), ITS-RFLP (Martínez-Culebras and Ramón 2007) and RFLP (Bau *et al.* 2006).

Several molecular methods were employed in an attempt to distinguish between ochratoxin producer and nonproducer strains within *Aspergillus* section *Nigri* (Geiser *et al.* 2007). In our study, AFLP analysis was ineffective in separating toxigenic from nontoxigenic strains, because both types of strains were intermixed within the groups. This result agrees with previous studies using AFLPs (Schmidt *et al.* 2004; Perrone *et al.* 2006b; Oliveri *et al.* 2008), RAPD-PCR (Dachoupakan *et al.* 2009) and ap-PCR (Martínez-Culebras *et al.* 2009). In contrast, Bau *et al.* (2006) using RFLP analysis grouped *A. niger* aggregate strains in type *N* and *T* and showed that all the OTA producer strains were *N*-RFLP type.

This is the first report in South America where the species belonging to *Aspergillus* section *Nigri* isolated from grapes were characterized by a genetic study. Molecular markers were useful for the identification of black aspergilli strains and particularly *A. tubingensis* species could be differentiated from *A. niger* 'aggregate' group.

However, a sequence-based analysis will be necessary to widen these results and furthermore to characterize the population belonging to the group of strains assigned as atypical of closely related species.

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