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Research Paper

Toxigenic profile and AFLP variability of A*lternaria alternata* and *Alternaria infectoria* occurring on wheat

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Abstract

The objectives of this study were to evaluate the ability to produce alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid (TA) by *A. alternat*a and *A. infectoria* strains recovered from wheat kernels obtained from one of the main production area in Argentina; to confirm using AFLPs molecular markers the identify of the isolates up to species level, and to evaluate the intra and inter-specific genetic diversity of these two *Alternaria* species. Among all the *Alternaria* strains tested (254), 84% of them were able to produce mycotoxins. The most frequent profile of toxin production found was the co-production of AOH and AME in both species tested. TA was only produced by strains of *A. alternata.* Amplified fragment polymorphism (AFLPs) analysis was applied to a set of 89 isolates of *Alternaria* spp (40 were *A. infectoria* and 49 were *A. alternata*) in order to confirm the morphological identification. The results showed that AFLPs are powerful diagnostic tool for differentiating between *A. alternata* and *A. infectoria*. Indeed, in the current study the outgroup strains, *A. tenuissima* was consistently classified. Characteristic polymorphic bands separated these two species regardless of the primer combination used. Related to intraspecific variability, *A. alternata* and *A. infectoria* isolates evaluated seemed to form and homogeneous group with a high degree of similarity among the isolates within each species. However, there was more scoreable polymorphism within *A. alternata* than within *A. infectoria* isolates. There was a concordance between morphological identification and separation up to species level using molecular markers. Clear polymorphism both within and between species showed that AFLP can be used to asses genetic variation in *A. alternata* and *A. infectoria*. The most important finding of the present study was the report on AOH and AME production by *A. infectoria* strains isolated from wheat kernels in Argentina on a semisynthetic media for the first time. Also, specific bands for *A. alternata* and *A. infectoria* have been identified; these may be useful for the design of specific PCR primers in order to differentiate these species and to detect them in cereals.

Key words: *: Alternaria alternata*, *Alternaria infectoria*, alternariol, alternariol monomethyl ether, tenuazonic acid, AFLPs, wheat, genetic variation.

Introduction

Wheat production in Argentina covers about 5.21 millions hectares. The production reaches 12.5 million tons during the 2005/2006 harvest season ranking Argentina 12th as wheat producer in the world. Most of the production (60%) is exported mainly to Latin-American countries as seeds and wheat flour (MAGPyA 2011)

Alternaria, *Aspergillus* and *Fusarium* species can be found as pre-harvest fungal contaminants in wheat. This contamination affects the wheat milling industry due to low quality of wheat by products and the potential risk of mycotoxin contamination. Ripening ears of wheat are colo-

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nized by *A. alternata* soon after emergence, and this specie is reported to be the most common subepidermal fungus of wheat grains. *A. alternata* alone or with another fungus can cause a conspicuous black or brown discoloration of wheat kernels called black-point disease (Logrieco *et al.*, 2003)

In Argentina, previous studies have shown that *Alternaria* was the predominant genera found on wheat cultivated in different agroecological regions (Gonzalez *et al.*, 1996; 1999, Broggi *et al.*, 2007; Ramirez *et al.*, 2005). The most prevalent *Alternaria* specie found was *A. alternat*a but also *A. infectoria* was isolated. Recently, Perello *et al.* (2008) have observed an increase on the incidence levels of *A. infectoria* on wheat probably due to changes in cropping systems in most of the different agroclimatic zones in Argentina. Also, these authors have associated *A. infectoria* as the ethiological agent of black point in wheat grains in Argentina.

Species of *Alternaria* are well known for the production of toxic secondary metabolites, some of which are powerful mycotoxins that have been implicated in the development of cancer in mammals (Thomma, 2003). Among these metabolites with mammalian toxicity are the dibenzo-α-pyrones altenuene (AE), alternariol (AOH), alternariol monomethyl ether (AME) and a derivate of tetramic acid, tenuazonic acid (TA) (Logrieco *et al.*, 2003; Ostry, 2008). Some or all of these mycotoxins have been demonstrated to be produced by *Alternaria* species on wheat, tomato, sorghum, pecans, sunflower and cotton (Scott, 2001; Ostry, 2008).

Most *Alternaria* species, including *A. alternata*, exhibit considerable morphological plasticity that is dependent upon cultural conditions, substrate, temperature, light, and humidity. In addition, within a culture, there is a considerable range of variation in conidium morphology regarding to size, shape, septation, color, and ornamentation that is dependent upon conidium age (Andersen *et al.*, 2001; Simmons, 2007). Moreover, there are several small-spored catenulate *Alternaria* spp. with morphological characteristics that overlap those of *A. alternata*; the most commonly known are *A. tenuissima* and *A. infectoria*. Further complication on the taxonomy of this group of fungi is the presence of numerous isolates with intermediate characteristics that do not clearly segregate into recognized species (Simmons and Roberts, 1993; Simmons, 2007). Thus, differentiation of these fungi can be difficult for those not familiar with the specific morphological characteristics that separate these species, and it has been suggested that these fungi, in particular *A. alternata*, are frequently misidentified (Roberts *et al.*, 2000; Simmons, 2007)

With the advancement of molecular techniques, several studies have examined taxonomic relationships among small-spored catenulate *Alternaria* spp. using a variety of methods, including RAPD-PCR, RFLP, PCR-RFLP in an attempt to establish consensuses with contemporary morphological-based species (Kusaba and Tsunge, 1994; Weir *et al.*, 1998; Roberts *et al.*, 2000; Pryor and Michailides, 2002; Peever *et al.*, 2002).

Other fingerprinting method commonly used to study closely related taxa includes amplified fragment length polymorphisms (AFLP). This technique developed by Vos *et al.* (1995) represents a powerful highly reproducible, PCR-based DNA-fingerprinting technique for DNA of any origin and complexity. Because a large number of polymorphic loci can be investigated in a single experiment the AFLP technique has become one of the major methods of choice for studies of genetic diversity, particularly in species where markers requiring genomic sequence are not available. The highly polymorphic nature of AFLP markers makes them especially useful for differentiating clonal lineages of fungi that reproduce asexually (McDonald, 1997). AFLP markers have been used to study genetic diversity and taxonomic relatedness within and between isolates of diverse *Alternaria* species (Bock *et al.*, 2002; Pérez Martínez *et al.*, 2004; Gannibal *et al.*, 2007)

The objectives of the present work were (i) to evaluate the ability to produce AOH, AME and TA of *A. alternat*a and *A. infectoria* strains recovered from wheat kernels obtained from one of the main production area in Argentina and ii) to confirm using AFLPs molecular markers the identify of the isolates up to species level, and (iii) to evaluate the intra and inter-specific genetic diversity of these two *Alternaria* species.

Materials and Methods

Fungal strains

Two hundred and fifty four single-conidial strains of small spored catenulate *Alternaria* taxa (129 *A. alternata* and 125 *A. infectoria*) isolated from wheat kernels harvested in Cordoba province (localities), Argentina, were used in the present study. All the localities belong to the Region V within the major wheat production area of Argentina. These isolates have been morphologically characterized according to Simmons (1992, 2007) given heed mostly to three-dimensional sporulation patterns. The strains are deposited in the culture collection at the Department of Microbiology and Immunology, Universidad Nacional de Rio Cuarto, Cordoba, Argentina.

Type or representative cultures of *A. alternata*, *A. infectoria* and *A. tenuissima* (EGS 34-016, EGS 27-193, and EGS 34-015, respectively) were included for comparative purposes and as reference for each morphological group.

Mycotoxin analyses

Petri plates containing ground rice-corn steep liquor medium (GRCS; ground rice 50 g, corn steep liquor 5 g, agar 15 g, 1000 mL distilled water) were inoculated centrally with a 4 mm diameter agar disk taken from the margin of a 7-day-old colony of each *Alternaria* isolate grown on synthetic nutrient agar (SNA) (Gerlach and Nirenberg,

1982). The plates were incubated for 14 d at 25 °C in darkness (Chulze *et al.*, 1994).

The extraction method used was based on a microescale extraction (Smedsgaard, 1997) modified into a three step extraction procedure suited for *Alternaria* metobolites by Andersen *et al.* (2001). After the incubation, 3 agar plugs (4 mm diameter) were cut from the edge of a colony from each Petri plate and placed in a 4 mL screw-cap vial. The plugs were extracted in 1.5 mL chloroform/methanol (2:1 v/v) for 60 min in an ultrasonic bath. The extract was transferred to clean 4 mL amber vials and evaporated to dryness (N_2, 50 °C). The same plugs were then extracted ultrasonically for 60 min in 1.3 mL ethyl acetate containing 1% formic acid. The second extract was transferred to the amber vial containing the first dried extract and evaporated. The plugs were then extracted ultrasonically for 60 min with 1.5 mL of 2-propanol and the extract transferred to the amber vial with the two previous extracts and evaporated. The pooled, dried extract was re-dissolved ultrasonically in 1 mL methanol and 1 mL of acetonitrile:water (25:75 v/v), filtered through a 0.45 μm filter and transferred to a clean 1.5 mL amber vial prior to HPLC analysis.

The HPLC system consisted of a Hewlett Packard model 1100 pump (Palo Alto, CA, USA) connected to a Hewlett Packard 1100 Series variable wavelength detector and a data module Hewlett Packard Kayak XA (HP ChemStation Rev. A.06.01). Chromatographic separations were performed on a Symmetry C_{18} (100 x 4.6 mm i.d., 5 μm particle size) connected to a guard column SecurityGuard (20 x 4.6 mm i.d.) filled with the same phase. The mobile phase consisted of two consecutive isocratic mobile phase mixture containing acetonitrile:0.027 M sodium dihydrogen phosphate solution (25:75, v/v Sn A) acetonitrile:0.027 M sodium dihydrogen phosphate solution (50:50, v/v Sn B). Solvent A pumped for 3.5 min at 1.0 mL min^{-1} followed by solvent B pumped for 16.5 min at 1.0 mL min^{-1}. The addition of sodium hydrogen phosphate to the mobile phase and the use of two consecutive compositions allowed the elution of TA as a sharp and symmetric peak. The detector was set at 256 nm for AOH and AME and 279 nm for TA detection. Injection volume was 50 μL and the retention time of AOH, AME and TA were 11.8, 17.5, 7.0 min respectively. Quantification was relative to external standards of 0.5, 1.0, 2.0 and 3 μg mL^{-1} in acetonitrile:0.027 M sodium dihydrogen phosphate solution (25:75, v/v).

Recovery experiment was performed on GRCS medium at levels of 0.1 to 10 μg/g with AOH, AME and TA, respectively. Mean recovery and repeatability (relative standard deviation) ranged from 85 to 98% (0.2 to 1.4%), from 88 to 97% (0.1 to 2%), from to 86% to 92% (0.5 to 2.5%) for AOH, AME and TA, respectively. Limit of detection (signal-to-noise ratio 3) was 0.01 μg/g for the three toxins and the quantification limit was established as three times the detection limit

DNA isolation

A suspension of spores from each isolate was obtained from synthetic low nutrient agar (SNA) (Gerlach and Nirenberg 1982) and used to inoculate Erlenmeyer flasks containing Wikerman medium (Mulé *et al.*, 2004). The flasks were incubated on an orbital shaker (150 rpm) for at least three days at 25 ± 1 °C. Mycelia were harvested by filtration through non-gauze milk filters (Ken AG, Ashland, Ohio, 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 (CTAB) method (Leslie and Summerell, 2006).

AFLP protocol

AFLPs reactions were performed as described by Vos *et al.* (1995), as modified by Leslie and Summerell (2006) 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 digested to completion with *Eco*RI and *Mse*I and ligated to AFLP adapters in a single overnight at room temperature (21 to 24 °C) were used. The digested and ligated templates were diluted in 9 volumes of Tris-EDTA buffer prior to preamplification. Samples were preamplified with the following cycling 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 used (*Eco*RI+TT/*Mse*I+G and *Eco*RI+TG/*Mse*I+G) were used. *Eco*RI primers for specific amplification were end-labeled with [γ-^{33}P] ATP. For final specific AFLP reactions, 1.3 μL of diluted preamplification reactions were used and the final volume was 5 μL. The PCR program for the AFLP amplification was: 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 °C 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) 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) marker had run approximately 22 cm. After that, the gels were transferred to 3 MM gel blotting paper (Midwest Scientific, Valley Park, MO) and dried before exposure to X-ray film at room temperature (Classic Blue Sensitive,

Midwest Scientific) for 3 to 7 days to resolve banding patterns. Bands sizes were estimated on polyacrylamide gels against [γ-^{33}P] ATP labeled BRL low-mass ladder (Life Technologies, Rockville, MD). 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 distances between individuals, similarity coefficients (*S*) were calculated using the formula: $S = 2N_{xy} / (N_x + N_y)$, where N_x are 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 prepared using the UPGMA (unweighted pair-group method using arithmetic averages) clustering strategy of the NTSYSpc 2.0 (Numerical Taxonomy System) software package (Rohlf, 1990). The AFLP data were subjected to bootstrap analysis with 1000 replications using the program PAUP* version 4.0 (Swofford, 1999) in order to solve whether there was significant genetic substructure or clustering among isolates as resolved by AFLP data.

Results

Mycotoxin production

The profile of mycotoxin production on ground rice-corn steep liquor medium of 129 and 125 isolates of *A. alternata* and *A. infectoria* is reported Table 1. Among all the *Alternaria s*trains tested (254), 84% of them were able to produce mycotoxins. The most frequent profile of toxin production found was the co-production of AOH and AME in both species tested. TA was only produced by strains of *A. alternata* (Table 2).

Table 2 - Frequency of different toxigenic profiles found in *Alternaria alternata* and *Alternaria infectoria* strains isolated from wheat kernels.

	Nº of strains	Toxigenic profile	Frequency
A. alternata	19	AOH-AME-TA	15%
	63	AOH-AME	50%
	23	AOH	17%
	3	AOH-TA	2%
	1	TA	1%
	20	Non producers	15%
A. infectoria	79	AOH-AME	63%
	22	AOH	18%
	2	AME	1%
	22	Non producers	18%

TA: Tenuazonic acid; AOH: alternariol; AME: alternariol monomethyl ether.

The percentage of strains that did not produce any of the tested toxins was similar for both species, while AME yield the highest concentration. Also, the percentage of producing isolates and the range of toxin production were similar for both species.

The maximum amount of AME (6600 μg/g) was produced by an *A. alternata* isolate, and also the maximum amount of AOH (520 μg/g) was also produced by other *A. alternata* strains.

With regard to the coproduction of toxins in *A. alternata* strains, 19 were positive for all three toxins, 63 for both AOH and AME, and 3 for AOH and TA. The correlation coefficient (*r*) between the concentration of AOH and AME was 0.5 ($p < 0.005$).

In relation to the coproduction of toxins in *A. infectoria* strains, 79 were positive for AOH and AME production and the correlation coefficient (*r*) between the concentration of AOH and AME was 0.55 ($p < 0.005$).

AFLPs analysis

Forty isolates of *A. infectoria* and 49 isolates of *A. alternata* were selected among all the isolates studied for toxigenic capability for further AFLPs analysis in order to

Table 1 - Toxin production by strains of *Alternaia alternata* and *Alternaria infectoria* isolated from wheat kernels.

Specie	Nº of isolates	Mycotoxin production			
		Mycotoxin	Nº of positive	Average[a] (μg/g)	Range[a] (μg/g)
A. alternata	109 (129)	TA	22 (20%)	246.4	156.7-470.0
		AOH	108 (99%)	54.5	3.7-520.0
		AME	82 (79%)	223.7	3.1-6600.0
A. infectoria	103 (125)	AOH	101 (98%)	62.2	1.8-433.3
		AME	81 (78%)	303.0	2.75-4714.3

TA: Tenuazonic acid; AOH: alternariol; AME: alternariol monomethyl ether.
[a]Average and range of toxin production for positive isolates.

confirm they identity. Two selective primer pair combinations (EcoRI+TG/MseI+G and EcoRI+TT/MseI+G) turned out to produce a complex well resolved fingerprint pattern. A total of 135 bands for all species, obtained from two primer sets, were scored, of which 117 were present in *A. alternata* and 102 were present in *A. infectoria*. Clear polymorphism both within and between species showed that AFLP can be used to asses genetic variation in *A. alternata* and *A. infectoria*.

Overall, the primer combination EcoRI+TG/MseI+G produced 69 polymorphic of 73 total bands (94.5%) and the primer combinations EcoRI+TT/MseI+G produced 59 polymorphic of 62 total bands (94%). Within *A. alternata*, 95 loci (81.2%) were polymorphic while that within *A. infectoria*, 50 loci (49%) were polymorphic (Table 3). It is evident, that there were more scoreable polymorphisms within *A. alternata* than within *A. infectoria* isolates, using the two primer-pair combinations.

The identification of 135 distinct and scoreable bands allowed the construction of a 91 isolates x 135 loci data matrix, which was analyzed and used to produce a dendrogram (Figure 1). The resultant UPGMA dendrogram allowed the comparison among the haplotypes and showed a clear separation of two groups (A and B) with a similarity < 50% for strains in different clusters. Isolates placed in the group A contained all 49 isolates identified morphologically as *A. alternata* and the reference strain *A. alternata* EGS 34-016, whereas the group B included all 40 isolates identified morphologically as *A. infectoria* and the references strains *A. infectoria* EGS 27-193. On this basis, we identified candidate AFLP markers able to differentiate between these groups. For the 49 *A. alternata* isolates, 13 markers were present exclusively in this specie, while for *A. infectoria* isolates 23 markers provided 100% differentiation between the two groups. The results of UPGMA showed that *A. infectoria* group was tightly clustered, but separate from that of *A. alternata*.

Genetic distance (*D*) was calculated for paired comparison of all isolates based on the normalized identity of each locus in each of the analyzed species. Genetic similarity coefficients between isolates averaged 0.74 (range from 0.60 to 1.00) for *A. alternata* isolates and 0.88 (range from 0.77 to 1.00) for *A. infectoria* isolates.

Within the clade A, *A. alternata* EGS 34-016 showed a 75% similarity to the 49 *A. alternata* isolates included in this study. Within the clade B, *A. infectoria* EGS 27-193 showed an 87% similarity to the 40 *A. infectoria* examined. Representative isolate of *A. tenuissima* (EGS 34-015) clustered separately form isolates of *A. alternaria* and *A. infectoria* showing a 55% and 43% similarity respectively.

There was no clear separation of the isolates from different geographic location, demonstrated by the bootstrap values of the non-terminal branches that generally were < 50%.

Discussion

Our finding indicates that isolates of *A. alternaria* and *A. infectoria* isolated from wheat kernels are able to produce mycotoxins on GRCS medium. In the present study we use GRCS medium to evaluate toxin production, because this media have been described as the most suitable for *Alternaria* mycotoxins screening according to Chulze *et al.* (1984). It is a simple media that support a good production of *Alternaria* toxins and also these toxins can be easily extracted.

There is a previous work done in Argentina that have determine the toxigenic profile of *Alternaria* (mainly *A. alternata* and *A. tenuissima*) from wheat grown in Argentina but from different provinces (La Pampa and Buenos Aires) (Patriarca *et al.*, 2007).The toxigenic profile was demonstrated on autoclaved polished rice al 40% of moisture. The levels of mycotoxins production of *A. alternata* isolates (61) were similar to our results. They do not report the presence of *A. infectoria* on wheat kernels.

The levels of AOH and AME produced by *A. alternata* in this study are similar to levels reported elserwere for strains isolated from wheat, although TA production was lower in the present study. Logrieco *et al.* (2003) reported on 14 isolates from Italy, Yugoslavia, Greece, Lebanon, Egypt and Turkey, and showed that 100% produced TA (up to 6000 μg/g), and 13/14 produced AOH (up to 120 μg/g) and AME (59 g/g). Li *et al.* (2001) evaluate the ability of 22 strains of *A. alternata* isolated from Chinese weathered wheat kernels on autoclaved polished rice and durum wheat kernels (40% moisture). They found that all strains were able to produce AOH and AME, and only few also produce TA. Also, they suggest that polish rice seems to support a bit more production of *Alternaria* metabolites than wheat.

Table 3 - AFLP fragments amplified in isolates of *Alternaria alternata* and *Alternaria infectoria* with two primer pairs combinations.

Primer combination	Overall isolates (n = 89)		*A. alternata* (*n* = 49)		*A. infectoria* (*n* = 40)	
	Total/polymorphic bands	% of polymorphic bands	Total/polymorphic bands	% of polymorphic bands	Total/polymorphic bands	% of polymorphic bands
EcoRI+TG/MseI+G	73/69	94.5	63/46	73.0	45/15	33.3
EcoRI+TT/MseI+G	62/59	95	54/49	91.0	57/35	61.4
Total	135/128	94.8	117/95	73.0	102/50	49.0

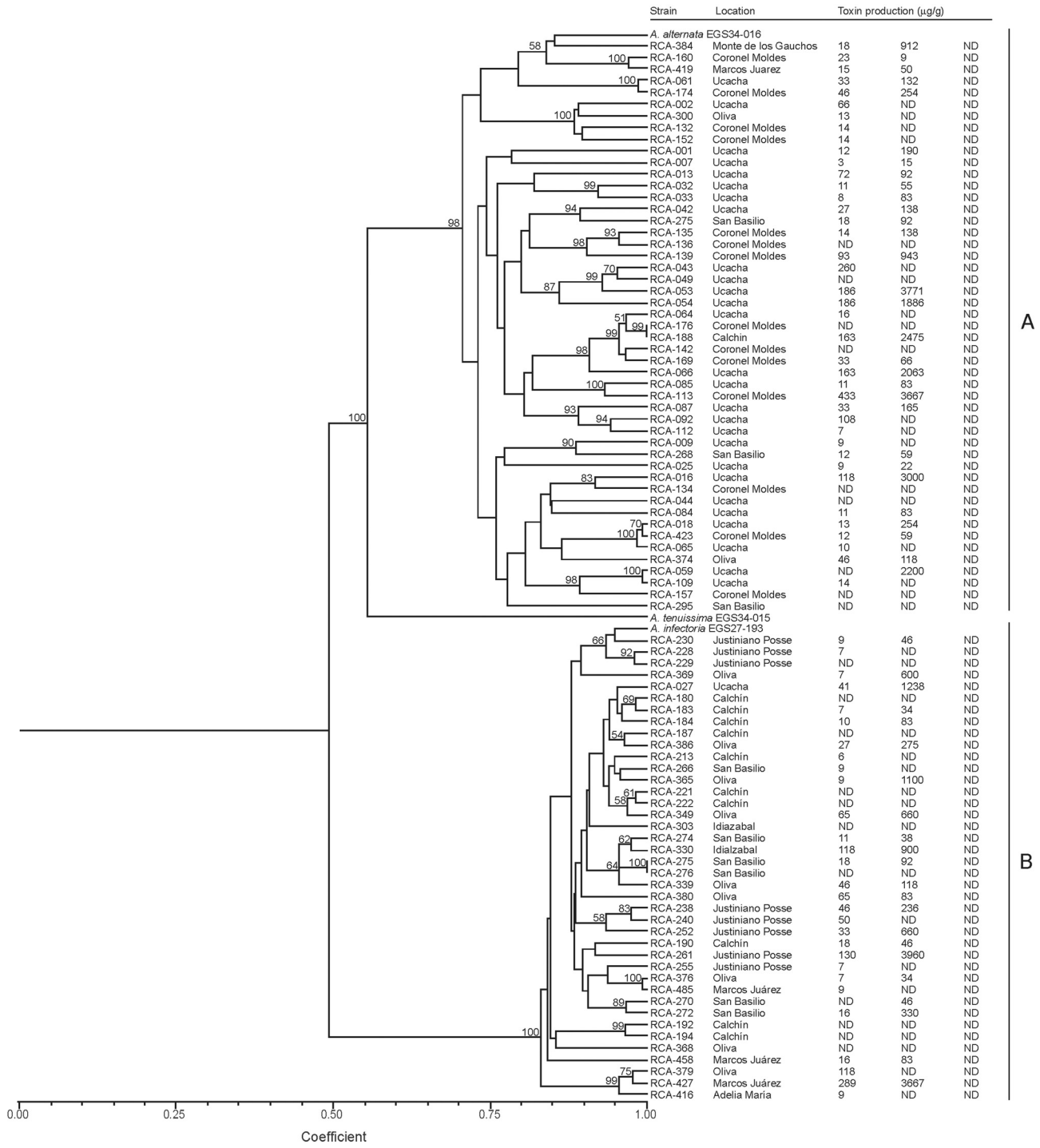

Figure 1 - Dendogram showing genetic relatedness of the 89 *Alternaria alternata*/ *A. infectoria* isolates and reference strains based on cluster analysis. Numbers above the branches indicate booststrap values of 1000 replicates. Only values > 50% are showed.

We were able to demonstrate for the first time that isolates of *A. infectoria* from wheat in Argentina are able to produce AOH and AME. Few data are available in the literature on the toxigenic profile of *Alternaria* species other than *A. alternata*. According to Andersen *et al.* (2009) chemically the *A. infectoria* species-group is very different from other *Alternaria* species, producing metabolites that are not found in other species-group. None of the taxa in *A. infectoria* species-group has ever been shown to produce alternariols or tenuazonic acid, which are common in some small-spored *Alternaria.* This affirmation in not completely true, considering that Bruce *et al.* (1984) have evaluated the ability to produce AOH, AME and TA of 3 isolates of *Pleospora infectoria* from wheat samples on

USA. Two out of 3 isolates of *P. infectoria* were able to produce one the three toxins, and the other produce just TA on autoclaved polish rice. Also we need to remember that *A. infectoria* was commonly known as the perfect stage of *Pleospora infectoria* until Simmons (1986) provided a binomial name *A. infectoria*. He showed that its telemorph does not belong in *Plesospora,* and had been widely accepted and erected as new genus, *Lewia* Barr and Simmons (Simmons, 1986).

Maybe the reason for no mycotoxins production, until now, can be the media used by researchers for this purpose (*i.e.* DRYES, DG18, PDA + DN). Another reason can be the reduced number of strains studied (39) and just only 3 isolates from wheat.

Members of the *A. infectoria* (morphological) group all belong to the infectoria species-group, which is genetically distinct and phylogenetically distant from other species-group (brassicola species-group and the alternata species-group). The *A. infectoria* group comprises at least 10 known species (Andersen *et al.*, 2009). Morphologically the *A. infectoria* group differs from others *Alternaria* species-groups in the three dimensional sporulation pattern (Simmons and Roberts, 1993). Characteristic for the *A. infectoria* group is the production of small conidia in branched chains with long, geniculate multilocus secondary conidiophores between conidia (Simmons, 2007).

Due to our results on mycotoxin production by *A. infectoria* strains, we decide to confirm the morphological identification of this specie and also the *A. alternata* strains by using AFLPs and also evaluate the intra and interspecific genetic diversity of these two *Alternaria* species present in wheat kernels in Argentina.

There is a regular need for identification of *Alternaria* isolates, because they have acquired different abilities in nature, which affect us negatively. Artificially identification systems based on any stable differentiation characters eg AFLP, metabolite profile, sporulation patterns; obtained under standarized conditions still play and important role in taxonomy. Strains of *A. infectoria* species-group show characteristics phenotypical traits, which can be recognized, and used for identification.

Identification of many *Alternaria* species is particularly difficult due to the variation and plasticity of colony and morphological characteristics. The results showed that AFLP markers are powerful diagnostic tool for differentiating between *A. alternata* and *A. infectoria*. Indeed, in the current study the outgroup strains, *A. tenuissima* was consistently separated from the others species. Characteristic polymorphic bands separated these two species regardless of the primer combination used.

A number of studies have employed DNA fingerprinting for analysis of relationship among morphologically distinct taxa or groups within the alternata species-group. The most common techniques used for this purpose have been RAPD-PCR, RFLP, PCR-RFLP (Kusaba and Tsunge 1994; Weir *et al.*, 1998; Roberts *et al.*, 2000; Peever *et al.*, 2002; Pryor and Michailides 2002) and recently by AFLP analysis (Hong *et al.*, 2006; Gannibal *et al.*, 2007). RAPD analysis of isolates recovered from pear and cherry, primarily, support segregation of *A. alternata*, *A. tenuissima, A. arborescens*, *A. gaisen* and *A. infectoria* based upon morphology (Roberts *et al.*, 2000). However, studies using RAPD and PCR-RFLP data from species recovered from pistachio only supported segregation of the *A. arborescens* and *A. infectoria,* but isolates of *A. alternata* and *A. tenuissima* were resolved as a single clade with no segregation of morphological types (Pryor and Michailides, 2002). Also base upon AFLP data, Hong *et al.* (2006) fail to resolve the segregation between *A. alternata* and *A tenuissima* strains isolated from hazelnut and walnut in Europe.

In the present study AFLP markers were able to discriminate as separated clades strains of *A. alternata*, *A. tenusissima* and *A. infectoria*, species which have distinct sporulation patterns. Similar results have been also reported in a previous study using AFLP analysis of Russian *A. tenuissima* strains from wheat kernels (Gannibal *et al.*, 2007).

Related to intraspecific variability, *A. alternata* and *A. infectoria* isolates evaluated seemed to form and homogeneous group with high degree of similarity among the isolates within each species. However, there was more scoreable polymorphism within *A. alternata* than within *A. infectoria* isolates.

Previous studies on *A. alternata* using morphology approach (Simmons, 1978), isozyme analysis (Petrunak and Christ, 1992), RAPD-PCR markers, PCR-RFLP markers (Weir *et al.*, 1998; Pryor and Michailides, 2002), RFLP in the rDNA (Aradhya *et al.*, 2001) and using AFLP (Bock *et al.*, 2002) have found considerable variation among isolates.

The source of variation (mutation, somatic hybridization and heterokariosis, gene flow or balancing selection) (Burdon and Silk, 1997) in many apparently asexual fungi is unknown, although the level of recombination can be typical of a sexual system (Burt *et al.*, 1996; Geiser *et al.*, 1998; McDonald *et al.*, 1999). The relative importance of the mentioned evolutionary forces in *A. alternata* and *A. infectoria* populations need to be investigated in the future. Due to the lack of data, only general comments can be made here. Mutation can putatively play and important rol given the large amounts of propagules produced during an epidemic on a small spatial scale.

There are potential long distance dispersal of propagules of *Alternaria* gene flow may play an important rol in enhancing genotypic variation. However, the pathogen's life style alternate between saprophytic growth and a parasitic cycle when a susceptible host is available. This may result in a balancing selection for saprophytic *vs.* parasitic

fitness components and thus maintain or generate a high degree of polymorphism.

Despite teleomorphs (*Lewia* spp.) being identified for same *Alternaria* spp, (like *A. infectoria*) the sexual stage for most either do not exist or remain unidentified (Simmons, 2007). Although, *A. infectoria* (perfect state: *Lewia infectoria*) isolates have the ability to reproduce both sexually and asexually, and, the relative proportion of sexual and asexual reproduction is not known. Ascomata can be observed frequently in nature, but are not know to have been produced in axenic culture (Simmons, 2007). *A. infectoria* is homothallic; therefore, individual ascomata can yield homozygous progenies from selfing. The latter will not result in segregating progenies in haploid organisms. Thus the explanation of genetic diversity is somewhat difficult.

The genetic variability may therefore arise from asexual genetic recombination (parasexuality). However, evidence of natural parasexualism has not been obtained to date in this species (Salamiah *et al.*, 2001). A heterokarion state has been suggested for various *Alternaria* species (Tsuge *et al.*, 1987) and stable fusants have been purified (Salamiah *et al.*, 2001). Many Alternarias are therefore likely to be haploid fungi existing in a vegetative phase, reproducing asexually, and would be expected to have a high level of clonality (Vogler *et al.*, 1991).

This study has identified polymorphic and specific bands for *A. alternata*, *A. infectoria* and *A. tenuissima*, which could be useful to establish a PCR diagnostic assay. This could be particularly important in the correct identification of these three species; also a vast number of isolates can be screened in a short time. Also this study demonstrates the suitability of the AFLP technique for detailed analysis of genetic variation in this two *Alternaria* species.

This is the first report on AOH and AME production by *A. infectoria* strains isolated from wheat kernels in Argentina and the second in the world. Also, we found concordance between morphological identification and separation up to species level using molecular markers. Considering the association between *A. infectoria* and wheat as the ethiological agent of black point in wheat grains in Argentina, this specie can be responsible of natural contamination of AOH and AME on wheat.

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