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Characterization and genetic variability of feed-borne and clinical animal/human *Aspergillus fumigatus* strains using molecular markers

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Abstract

Aspergillus fumigatus, the major etiological agent of human and animal aspergillosis, is a toxigenic fungus largely regarded as a single species by macroscopic and microscopic features. However, molecular studies have demonstrated that several morphologically identified *A. fumigatus* strains might be genetically distinct. This work was aimed to apply PCR-restriction length fragment polymorphisms (PCR-RFLP) and random amplification of polymorphic DNA (RAPD) molecular markers to characterize a set of feed-borne and clinical *A. fumigatus* sensu lato strains isolated from Argentina and Brazil and to determine and compare their genetic variability. All *A. fumigatus* strains had the same band profile and those typical of *A. fumigatus* sensu stricto positive controls by PCR-RFLP. Moreover, all Argentinian and Brazilian strains typified by RAPD showed similar band patterns to each other and to *A. fumigatus* sensu stricto reference strains regardless of their isolation source (animal feeds or human/animal clinical cases) and geographic origin. Genetic similarity coefficients ranged from 0.61 to 1.00, but almost all isolates showed 78% of genetic similarly suggesting that genetic variability was found at intraspecific level. Finally, *benA* sequencing confirmed its identification as *A. fumigatus* sensu stricto species. These

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results suggest that *A. fumigatus* sensu stricto is a predominant species into *Aspergillus* section *Fumigati* found in animal environments as well as in human/animal clinical cases, while other species may be rarely isolated. The strains involved in human and animal aspergillosis could come from the environment where this fungus is frequently found. Rural workers and animals would be constantly exposed.

Key words: feed-borne *A. fumigatus*, animal feeds, molecular typing, genetic variability estimation.

Introduction

Aspergillus fumigatus, the major etiological agent of human and animal aspergillosis, is a thermophilic fungus commonly found in contaminated animal environments. This fungus is frequently isolated from sorghum and maize silages and other contaminated animal feeds [1–3]. Large number of spores is easily spread in the air indicating its high risk of exposure both for animals and humans. In ruminants, this fungus causes several pulmonary processes, abortion, mastitis, and other clinical cases [4,5]. Moreover, it is able to produce tremorgenic mycotoxins that could induce neurological syndromes to farm workers who manipulate moldy feed containing it [6].

Aspergillus fumigatus is identified by macroscopic and microscopic features and, despite small variations in phenotype, has been largely regarded as a single species. However, molecular studies have demonstrated that several morphologically identified *A. fumigatus* strains might be genetically distinct [7,8]. Species such as *A. lentulus*, *A. viridinutans*, *A. fumigatiaffinis*, *A. fumisynnematus*, *N. pseudofischeri*, *N. hiratsukae* and *N. udagawae* are frequently reported as *A. fumigatus* [9–12]. Some of these species have been recently described as human pathogens, particularly *A. lentulus*, *A. viridinutans*, *N. pseudofischeri* and *N. udagawae* showing *in vitro* resistance to azole antifungals like *A. fumigatus* [13,14]. Therefore, molecular identification is currently recommended to the correct identification of species within the "*A. fumigatus* complex" group since the members of this section have different antifungal susceptibility profiles [14]. In order to resolve this issue, different approaches have been applied to identify the species belonging to the section *Fumigati*. Among them, Staab et al. [15] devised a species identification scheme based on the β-tubulin gene (*benA*) to rapidly identify *A. fumigatus* sensu stricto, *A. lentulus* and *N. udagawae* using polymerase chain reaction (PCR)-restriction length fragment polymorphisms (PCR-RFLPs) with *BccI* restriction enzyme. These authors suggested that *BccI* polymorphisms are genetically stable and characteristic of each those species in the section *Fumigati*. Furthermore, Hong et al. [7,16] reidentified strains of *A. fumigatus* sensu lato (identified based on morphology) obtained from different sources applying random amplification of polymorphic DNA (RAPD) with primers PELF and URP1F. Thus, these species identification schemes based on molecular markers are useful tools for *A. fumigatus* sensu stricto and related species discrimination in the section *Fumigati* and also, for their genetic variability estimation. To our knowledge, this is the first report on the molecular characterization of *A. fumigatus* strains isolated from animal feedstuffs in Argentina, applying neutral molecular markers. Recently, a set of *A. fumigatus* strains isolated from animal feeds and clinical ones isolated from animal and human aspergillosis in Brazil, was typified by PCR-RFLP markers using tree restriction enzymes among which was the *BccI* enzyme. However, no genotypic differences were obtained between them [17]. This work was aimed (i) to apply PCR-RFLP and RAPD, a more polymorphic molecular marker, to confirm characterize feed-borne and clinical *A. fumigatus* sensu lato strains isolated from Argentina and Brazil and (ii) to determine and compare their genetic variability.

Materials and methods

Aspergillus fumigatus strains

A subgroup of 35 strains from a working group of 143 *A. fumigatus* sensu lato strains isolated from animal feeds in Argentina were included in the PCR-RFLP analysis. While 70 strains, including the Argentinian strains typified by PCR-RFLPs, other 18 Argentinian strains (not characterized by this technique) and 17 Brazilian *A. fumigatus* sensu lato strains (typified by PCR-RFLPs by Soleiro - Pena et al. [17]) were used during RAPD fingerprinting. Moreover, based on the molecular fingerprinting results, 17 strains with different band profiles were selected for β-tubulin (*benA*) sequencing (Table 1). All selected strains for molecular analysis as the remaining strains from the working group (143) were previously identified by using the morphological criteria [18,19]. Then, they were assayed for gliotoxin production ability in Yeast Extract Agar (YES) medium [17,20]. The selected strains for molecular analysis were representatively chosen from the different isolation sources studied in this work according to their phenotypic variability and gliotoxin production abilities.

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Table 1. *Aspergillus fumigatus* strains assayed by PCR-RFLP and RAPD markers.

Aspergillus fumigatus isolates no.[1]	Source[2]	Location	Molecular marker[3]
RC2071- RC2072- RC2151-**RC2152**- RC2153- **RC2204**-	Horse feed	Córdoba (Argentina)	PCR-RFLP & RAPD
RC2195- **RC2150**	Poultry feed	Córdoba (Argentina)	PCR-RFLP & RAPD
RC2075- **RC2076**- RC2079- **RC2093**- RC2167- RC2170- RC2171- RC2172-	Pet food	Córdoba (Argentina)	PCR-RFLP & RAPD
RC2063- RC2137- **RC2181**- RC2182- RC2193	Corn silage for cows	Córdoba (Argentina)	PCR-RFLP & RAPD
RC2067- RC2068- RC2107- **RC2108**- RC2113- RC2114-RC2119- RC2124- RC2125	Brewer's grains for pigs	Córdoba (Argentina)	PCR-RFLP & RAPD
RC2069- RC2070- RC2161-**RC2162**- RC2166	Chinchilla feed	Córdoba (Argentina)	PCR-RFLP & RAPD
RC2197- RC2199- RC2201	Poultry feed	Córdoba (Argentina)	RAPD
RC2073- RC2078- RC2082- RC2094	Pet food	Córdoba (Argentina)	RAPD
RC2138- RC2144-RC2186- RC2189	Corn silage for cows	Córdoba (Argentina)	RAPD
RC2102- RC2111-RC2122- RC2134	Brewer's grains for pigs	Córdoba (Argentina)	RAPD
RC2158- RC2205- RC2209	Chinchilla feed	Córdoba (Argentina)	RAPD
AF89c- AF92c- AF122c	Bovine mastitis	Rio do Janeiro (Brazil)	PCR-RFLP (Soleiro-Pena et al., 2013)[4] & RAPD
AF10c- AF17c- **AF23c**- AF32c- AF94c	Poultry feed	Sao Pablo (Brazil)	PCR-RFLP (Soleiro-Pena et al., 2013) & RAPD
AF01c, AF04c	Pig feed	Sao Pablo (Brazil)	PCR-RFLP (Soleiro-Pena et al., 2013) & RAPD
AF108c, **AF123c**	Corn silage for cows	Sao Pablo (Brazil)	PCR-RFLP (Soleiro-Pena et al., 2013) & RAPD
AF105c, AF120c, AF133c	Sorghum silage for cows	Sao Pablo (Brazil)	PCR-RFLP (Soleiro-Pena et al., 2013) & RAPD
AF116c, **AF117c**	Human aspergillosis	Rio do Janeiro (Brazil)	PCR-RFLP (Soleiro-Pena et al., 2013) & RAPD

[1]National University of Río Cuarto, Córdoba, Argentina (RC) Collection Centre. [2]Source of strains isolation. [3]Molecular markers used to typify the *A. fumigatus* strains. PCR-RFLP: PCR-restriction length fragment polymorphisms, RAPD: random amplification of polymorphic DNA. [4]Soleiro-Pena et al., Results of PCR-RFLP previously presented by Soleiro-Pena et al. (2013). Strains sequenced at *benA* regions are shown in bold.

Fungal biomass production and DNA extraction

From each *A. fumigatus* strain grown on Malt Extract Agar (MEA), spores were harvested and used to inoculate Erlenmeyer ffiasks of 250 ml containing 50 ml of sterile Wickerham medium [21]. These ffiasks were incubated on an orbital shaker at 1.26 *g* at 25 ± 1°C for 3 days. Mycelia were harvested by ffiltration through nongauze milk ffilters (Ken AG, Ashland, Ohio, USA) under negative pressure created by vacuum and washed with sterile distilled water. The excess water was removed by blotting mycelia between clean paper towels and finally, dried mycelia were frozen at −20°C until ground. Fungal DNA was extracted using a hexadecyltrimethylammonium bromide (CTAB) procedure [22]. To quantify the total DNA extracted, samples were subjected to electrophoresis on 0.8% agarose gel stained with ethidium bromide (0.5 μg ml^{-1}). After the run, DNA concentration and purity was determined by UV spectrophotometry.

PCR-RFLP

Previous to the *in situ* PCR-RFLP, an *in silico* assay with different restriction enzymes was carried out through NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/) online tool. Before generating restriction maps, *benA* sequences from *A. fumigatus*, *Neosartorya udagawae* and *A. lentulus*, were obtained from GenBank database and aligned using the ClustalW algorithm [23] as implemented in the program

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BioEdit v7.0.9.0 (http://www.mbio.ncsu.edu/RNasaP/info/programs/BIOEDIT/bioedit.html) [24]. Consequently, *BccI* restriction enzyme was chosen due to its availability and because it produces different band patterns that allow distinguishing these closely related species into *Aspergillus* section *Fumigati*.

In situ PCR-RFLP assay was performed using the method proposed by Staab et al. [15] as a reference protocol. However, this method has been modified by using primers Bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and Bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) [25] for *benA* amplification, which yielded a higher size amplicon (550 bp instead of 498 bp, longer at 3′ end). PCR reactions were made with 10–20 ng of fungal DNA in a total volume of 50 μl of 1 × reaction buffer containing 2 mM $MgCl_2$, 1.25 U Taq DNA polymerase (5 U/μl, Invitrogen by Life Technologies, Buenos Aires, Argentina), 0.2 mM of each dNTP and 0.4 μM of each primer. A negative control, containing all reagents without fungal DNA, was included in every set of reactions. Also, *Aspergillus fumigatus* sensu stricto RC2063 (GenBank accession number: JX277549) and *A. fumigatus* sensu stricto RC2108 (GenBank accession number: JX277548), previously identified by *benA* sequencing, were included as positive controls. PCR was conducted according to the following cyclic conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, and a final extension step of 72°C for 7 min, and then held at 4°C indeffinitely. The amplification of *benA* was verified by agarose (1.5%) gel electrophoresis of 5 μl of each reaction mixture with 1× Tris-acetic acid EDTA buffer (pH 8). Digestion of *benA* amplicons was performed in a final volume of 15 μl containing 10 μl of the PCR product, 1× NEBuffer 1 (10 mM Bis-Trispropane-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.0)), 0.15 mg μl^{-1} bovine serum albumin (BSA) and 1 U of *BccI* restriction enzyme (New England Biolabs, Inc., Ipswich, MA, USA). The reactions were incubated at 37°C for 2 h in a water bath. DNA fragments were visualized after electrophoretic run on 1.5% agarose gel stained with 0.5 $\mu g\,ml^{-1}$ ethidium bromide and gels were photographed using a MiniBIS Pro, DNR Bioimaging systems analyzer. The fragment sizes were measured by comparison with DNA 100-bp ladder (Invitrogen by Life Technologies, Buenos Aires, Argentina) whose reference bands vary between 100 and 2072 bp.

RAPD-based characterization

Before typing the selected strains, we checked repeatability and reproducibility of RAPD technique in the Hong et al. [7] protocol. These parameters were assayed using seven reference strains (mentioned below) and other seven *A. fumigatus* sensu lato strains selected according to the following criteria selection: the presence of at least one strain of each isolation source and their gliotoxin production in YES medium (data not shown). Seventy *A. fumigatus* sensu lato strains were selected for their identification at species level and estimation of genetic variability by RAPD technique with PELF and URP1F primers, according to the methods proposed by Hong et al. [7] (Table 1). The reference strains *A. fumigatus* sensu stricto CBS 127.801, *A. fumigatus* sensu stricto CBS 127.278, *A. lentulus* CBS 117.267, *A. novofumigatus* CBS 117.519, *A. novofumigatus* DTO 249-H5; *A. udagawae* CBS 114.217, and *A. udagawae* CBS 114.218 were included as standards on each gel as well as *A. fumigatus* sensu stricto RC2063 and RC2108. Primers PELF (5′-ATATCATCGAAGCCGC-3′) and URP1F (5′-ATCCAAGGTCCGAGACAACC-3′) [26] were previously selected by Hong et al. [7] based on their ability to produce many polymorphic bands among main species into *Aspergillus* section *Fumigati* allowing to separate them. Also, they were able to show differences among inter-species. PCR was performed in 25 μl reactions containing 1 × reaction buffer with 2.5 mM $MgCl_2$, 2.5 μl of template DNA (20–25 ng), 0.2 mM of each dNTP, 1.5 U Taq DNA polymerase (5 U/μl, Invitrogen by Life Technologies, Buenos Aires, Argentina) and 100 pmol/μl of each primer. A negative control was included in every set of reactions. The following parameters were used for every set of PCR amplifications: 4 min at 94°C, followed by 35 steps of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, and a final extension step of 8 min at 72°C and then held at 4°C indeffinitely. PCR products were electrophoresed on agarose gels, photographed and analyzed as was stated above (in the preceding section). The presence or absence of polymorphic RAPD bands was scored manually and the data recorded in a binary format. 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 RAPD data

To estimate the genetic distances between individuals, similarity coefficients (S) were calculated using the formula: $S = 2\,N_{xy}/(N_x + N_y)$, 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 [27]. Genetic distance (D) was derived from similarity coefficients as follows: D = 1-S. Genetic distance matrices were constructed for isolates using the compiled RAPD data obtained from both primers. Dendrograms were obtained using the unweighted pair-group method using arithmetic averages (UPGMA) clustering strategy of the NTSYSpc 2.0

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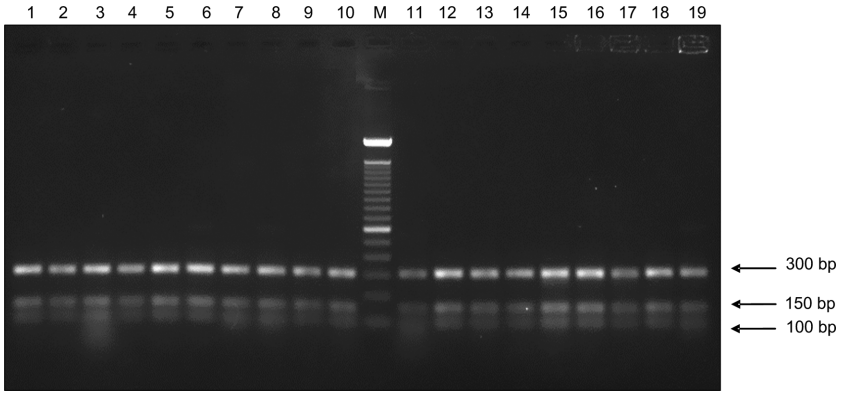

Figure 1. PCR-RFLP profiles of feed-borne *A. fumigatus* strains isolated in Argentina. Strains isolated from horse feed (lines 1–4), poultry feed (lines 5–6), corn silage for cows (7–8), brewer's grains for pigs (lines 9–12), chinchilla feed (lines 13–15) and pet food (lines 16–17). Line 18: positive control *A. fumigatus* sensu stricto RC2108, line 19: positive control *A. fumigatus* sensu stricto RC2063. Line M: 100-bp DNA ladder (Invitrogen by Life Technologies, Buenos Aires, Argentina).

(Numerical Taxonomy System; Applied Biostatistics Inc., New York, NY, USA) software package [28]. The RAPD data were subject to bootstrap analysis with 1000 replications using the program PAUP* 4.0 [29] to determine whether there was significant genetic substructure or clustering among isolates as resolved by RAPD data.

Sequencing of *benA* regions

Based on PCR-RFLP and RAPD fingerprinting results, 17 strains were selected according to their position on RAPD dendrogram for sequencing of *benA* regions for definitively confirm its taxonomic state at species level. Amplifications were performed according to Samson et al. [30]. A negative control, containing all reagents without fungal DNA, was included. *Aspergillus fumigatus* sensu stricto RC2063 (GenBank accession number: JX277549) and *A. fumigatus* sensu stricto RC2108 (GenBank accession number: JX277548), previously identified by *benA* sequencing, were included as positive controls. Amplification was made as follows: 5 min at 94°C followed by 35 cycles of 1 min denaturation at 94°C followed by primer annealing 1 min at 58°C and primer extension 1 min at 72°C and a final 7-min elongation step at 72°C. PCR products were detected after electrophoretic run on 1.5% agarose gels stained with ethidium bromide (0.5 μg/ml). The DNA 100-bp ladder (New England Biolabs, Inc., Ipswich, MA, USA) was used as molecular size marker. For DNA sequencing of both strands, template DNA (45 μl) was directly prepared from PCR products by purifying it through DNA Wizard DNA Clean-Up kit (Promega, Madison, WI, USA) according to the manufacturer's instructions and sequenced by Applied Biosystems Sanger Sequencing Chemistry with the ABI/Hitachi Genetic Analyzer 3130 sequencer. Each sequence was then aligned with Clustal W [23] as implemented in the program BioEdit version 7.0.9.0 [24].

All PCR amplifications were conducted in a MJ Research PTC-200 thermocycler (MJ Research Inc., Watertown, MA, USA).

Results

PCR-RFLP

Primers Bt2a and Bt2b amplified a 550 bp-DNA fragment, which was then used as template DNA in the restriction reaction. Enzymatic digestions of *benA* amplicons with *BccI* showed a 100% of concordance with restriction maps resulted from the *in silico* assay. PCR-RFLP profiles of feed-borne *A. fumigatus* strains isolated in Argentina are shown in Figure 1. Three DNA fragments of 100, 150, and 300 bp were obtained among all assayed strains, all they had the same band profile and the according to *A. fumigatus* sensu stricto RC2063 and RC2108 positive controls.

RAPD-based fingerprinting

Primers PELF and URP1F were tested for their ability to produce polymorphic and reproducible band patters among the *Aspergillus* section *Fumigati* isolates. Band reproducibility was verified at least twice, and identical results were obtained in all the experiences. Seventy isolates of *A. fumigatus* coming from different isolation sources showed similar band patterns to each other and to *A. fumigatus* sensu stricto CBS 127.801 and CBS 127.27 reference strains, although some isolates showed differences in some bands.

Figure 2 shows the RAPD profiles of Argentinian feed-borne *A. fumigatus* isolates acquired with primer PELF. This figure shows only PELF profiles of some Argentinian

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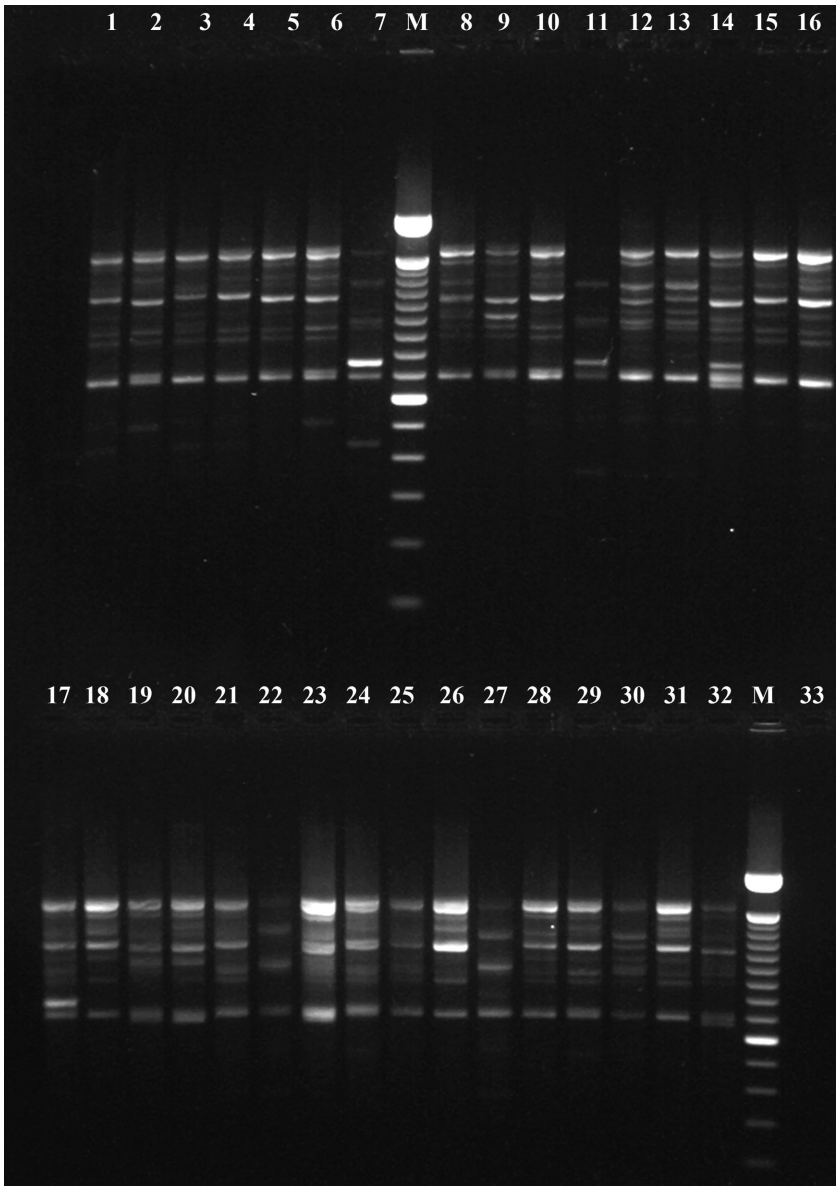

Figure 2. RAPD profiles of Argentinian feed-borne *A. fumigatus* isolates generated with primer PELF. Strains isolated from horse feed (lines 1–5), poultry feed (lines 6–7), pet food (lines 8–15), chinchilla feed (lines 16–19), corn silage (lines 20–24), and brewer's grains for pigs (lines 25–30). Line 31: *A. fumigatus* sensu stricto CBS 127.278 reference strain, line 32: positive control *A. fumigatus* sensu stricto RC2063, line 33: negative control. Lines M: 100-bp DNA ladder (Invitrogen by Life Technologies, Buenos Aires, Argentina). Band profiles of the reference strains were not included.

strains; however, all selected strains for RAPD analysis were tested with both primers. Brazilian strains tested with both primers showed similar band patters among them and to *A. fumigatus* sensu stricto reference strains and to *A. fumigatus* RC2063 and RC2108 strains regardless of their isolation source (animal feeds or human/animal clinical cases). Moreover, after comparing those band patters with the Argentinian isolates both genetic profiles were similar. As were observed during the repeatability and reproducibility assays, primers PELF and URP1F were able to separate *A. fumigatus* sensu stricto, *A. udagawae*, *A. lentulus*, and *A. novofumigatus* reference strains. Forty-seven distinct and scoreable bands were obtained with both primers and allowed the construction of 70 isolates × 47 loci data matrix, which was analyzed and used to produce a dendrogram (Fig. 3).

The resultant UPGMA dendrogram allowed the comparison among the haplotypes and showed a clear separation of these four *type*-strains into section *Fumigati* with similarities <50% (Fig. 3) The strains included in the analysis were grouped in two main clusters (I and II). Cluster I included *A. lentulus* CBS 117.267, and cluster II grouped all the remaining strains although it was sub-divided in other two groups (IIa and IIb, 55% of statistical support). Cluster

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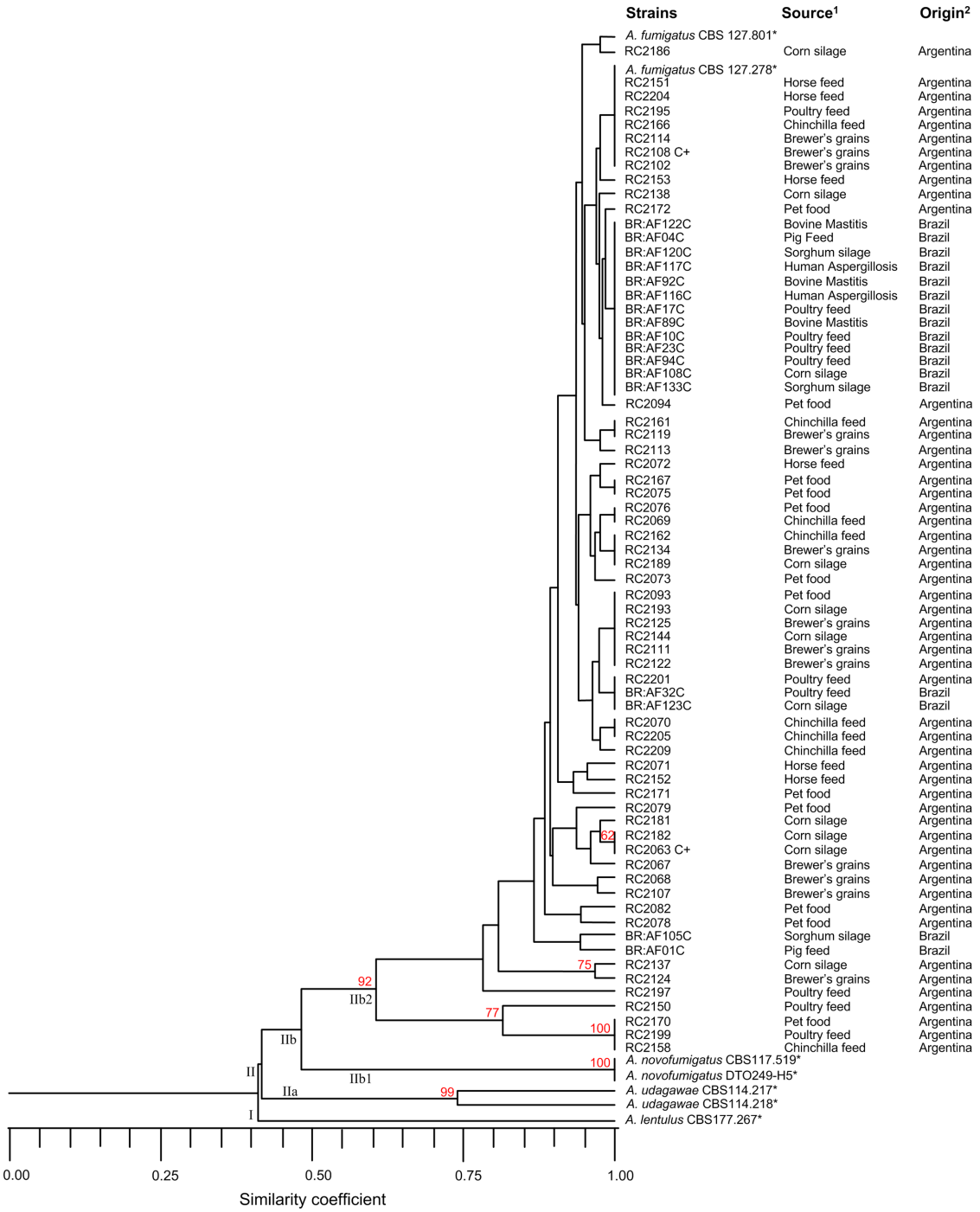

Figure 3. Dendrogram showing genetic relatedness of 70 Argentinian and Brazilian *A. fumigatus* sensu lato isolates and reference strains (*) based on cluster analysis UPGMA generated by NTSYS software. Bootstrap values higher than 60% are show above the branches. [1]Source of strain isolation, [2]Country of strain isolation.

IIa included *A. udagawae* (CBS 114.217 and CBS 114.218) and cluster IIb *A. novofumigatus* (DTO 249-H5 and CBS 117.519), all *A. fumigatus* strains analyzed and *A. fumigatus* sensu stricto (CBS 127.801 and 127.278) reference strains (53% of statistical support). This group was also divided in again two more clusters (IIb1 and IIb2). Cluster IIb1 included both *A. novofumigatus* reference strains and IIb2 grouped the two *A. fumigatus* sensu stricto reference strains and all *A. fumigatus* used in this study (92% of statistical support). Isolates placed in this group included *A. fumigatus* strains from different sources of isolation, which showed 78% of genetic similarly among them. However, it included a subcluster that grouped four strains isolated from pet food, poultry and chinchilla feeds that showed

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less homology with the remaining *A. fumigatus* strains analyzed (77% of statistical support). The strains were assigned to a species if they shared more than 50% of the bands with the reference strain. Therefore, all *A. fumigatus* assayed strains were typified as belonging to *A. fumigatus* sensu stricto species. In addition, RAPD fingerprinting also allowed demonstrating intra-specific genetic variability in the *A. fumigatus* sensu stricto group of assayed strains. Genetic similarity coefficients among these isolates ranged from 0.61 to 1.00.

Furthermore and for definitively confirmation of the results obtained by PCR-RFLPs and RAPD, DNA sequencing of *benA* regions of the selected strains confirmed its identification as *A. fumigatus* sensu stricto species. After conducting BLAST searches of GenBank with *benA* sequences as the query, they had high match with published *A. fumigatus benA* sequences in GenBank showing maximum identities of 99–100% (August 2014, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The obtained *benA* sequences have been deposited in GenBank under accession numbers KF410677-KF410682, JX277548, JX277549, and KM507481-KM507489 (http://www.ncbi.nlm.nih.gov/nucleotide).

Discussion

In this study, during the *in silico* PCR-RFLP analysis, tested enzymes were random selected among those available in NEBcutter online tool and the *BccI* enzyme was chosen due to its ability to distinguish similar morphotypes in the section *Fumigati*. Application of the PCR-RFLP protocol, development by Staab et al. [15] modified by using primers Bt2a and Bt2b (Glass and Donaldson [25]), allowed a "screening" of the feed-borne isolates that were all typified as *A. fumigatus* since they had band profiles according to this species (by *in sílico* and *in situ* analysis). This assay allowed discriminating *A. fumigatus* from *A. lentulus* and *N. udagawae* species based on the high interspecies variability of *benA* gene at intronic regions but is conserved among isolates of the same species. PCR- RFLPs allowed a "screening" of the feed-borne isolates that were all typified as *A. fumigatus* since they had band profiles according to this species (by *in sílico* and *in situ* analysis). This assay allowed discriminating *A. fumigatus* from *A. lentulus* and *N. udagawae* species based on the high interspecies variability of *benA* gene at intronic regions but is conserved among isolates of the same species. Therefore, digestion of *benA* amplicons with *BccI* generated unique banding patterns among these species. However, no polymorphic bands that differentiate *A. fumigatus* sensu stricto from the other related species in the section *Fumigati* (such as *A. fumigattiafinis*, *A. novofumigatus*, *A. viridinutans*, and *A. fumisynnematus*) were obtained. Although, *A. fumigatus* (sensu stricto), *A. lentulus* and *N. udagawae* are considered as the major species into the section *Fumigati* causing disease in human and animals, Katz et al. [31] Yaguchi et al. [13] and Alcazar-Fuoli et al. [14] have recently reported *A. fumigattiafinis*, *A. viridinutans* and *A. fumisynnematus* species as pathogenic to humans. Soleiro-Pena et al. [17] applied a modification on this method based on the *benA* digestion with three restriction enzymes *BccI*, *MspI,* and *Sau3AI* to confirm the taxonomic state at species level of a set of 50 clinical (human and animal) and animal environment *A. fumigatus* isolates from Brazil. Although, in the present study only *BccI* was applied to digest the *benA* amplicons, it was the most efficient enzyme for differentiating *A. fumigatus* (sensu stricto), *A. lentulus,* and *N. udagawae*. The *MspI* enzyme generated similar band patters between *N. udagawae* and *A. lentulus,* whereas *Sau3AI* had the same behavior between *A. fumigatus* and *N. udagawae*. The results obtained by PCR-RFLP are in agreement with Soleiro-Pena et al. [17] since all *A. fumigatus* (sensu lato) were typified as belonging to *A. fumigatus* sensu stricto species regardless of their isolation source. In fact, as in the present work, no differences between clinical and animal environmental isolates were found. The PCR-RFLPs marker is a reliable methodology that has been applied over the years for fingerprinting of several organisms and systematic studies [32–34]. However, in the present study, RAPD markers with primers PELF and URP1F were applied to a greater set of *A. fumigatus* isolates including Argentinian feed-borne strains and Brazilian clinical (human and animal) and animal environment ones [17] to confirm its identification at species level and to determine and compare their genetic variability. All 70 typified strains had similar band patters among them and to *A. fumigatus* sensu stricto reference strains regardless of their isolation source (animal feeds or human/animal clinical cases) and geographic origin (Argentina or Brazil). These results suggest that *A. fumigatus* sensu stricto is a predominant species in *Aspergillus* section *Fumigati* found in animal environments such as animal feedstuffs as well as in human/animal clinical cases, while other species may be rarely isolated. Using the RAPD approach applied here, Hong et al. [16] evaluated 146 worldwide clinical and environmental (soil) strains of *A. fumigatus* sensu lato. Of those strains, 140 (95.8%) were identified as *A. fumigatus* sensu stricto whereas 3 (2.1%) were typified as *A. lentulus*, and the remaining 3 strains as *A. viridinutans* complex, *Neosartorya udagawae* and *N.* cf. *nishimurae*. These authors confirm their result by *benA* sequencing of all typified strains. In the present study, all strains had nearly the same band patterns than *A. fumigatus* sensu stricto reference strains. Therefore, strains with different band patterns and, thus, different position in the RAPD dendrogram were chosen and sequencing at *benA* regions for definitively

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confirmation of the PCR-RFLP and RAPD results. Primers PELF and URP1F, previously selected by Hong et al. [7] were considered as a rapid and reliable method to identify *A. fumigatus* and *A. lentulus*. In this study, besides *A. lentulus*, other related species to *A. fumigatus* sensu stricto such as *A. novofumigatus* and *A. udagawae* were clearly separated from it. Moreover, genetic variability was found at intraspecific level in the *A. fumigatus* sensu stricto group and isolates seemed to form a homogeneous group with a high degree of similarity among them. Also, no genetic differences between clinical and animal environmental isolates from Argentina and/or Brazil were found. These findings support the results of Debeaupuis et al. [35] and Hong et al. [7,16] who demonstrate that *A. fumigatus* sensu stricto strains are genetically homogeneous regardless of their source, and that genetic discrimination in this species was not determined on the basis of the saprophytic or pathogenic origin of the isolates.

In this work, PCR-RFLPs and RAPDs molecular markers were useful to characterize a set of feed-borne and clinical *A. fumigatus* sensu lato strains isolated from Argentina and Brazil. Furthermore, they allowed determining and comparing their genetic variability. These strains, assigned to *A. fumigatus* sensu stricto species, may be involved in human and animal aspergillosis. Also, they could come from the environment where this fungus is frequently found. Rural workers and animals would be constantly exposed.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Supplementary Material

Supplementary material is available at *Medical Mycology* online (http://www.mmy.oxfordjournals.org/).

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