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Short communication

Molecular characterization of *Aspergillus fumigatus* isolated from raw cow milk in Argentina
Molecular typing of *A. fumigatus* from raw cow milk

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ABSTRACT

Aspergillus fumigatus, the major etiological agent of human and animal aspergillosis, is a gliotoxinogenic species into section *Fumigati* commonly found in contaminated animal environments. In dairy herds, exposed areas of lactating cows, as mammalian glandule, can be easily contaminated by them. This study was aimed to identify *A. fumigatus* sensu lato strains (identified based on morphology) isolated from raw cow milk at species level, by morphological and molecular techniques, and to estimate their genetic variability. Forty-five *A. fumigatus* strains showed similar RAPD profiles (generated with PELF and URP1F primers) to each other and to *A. fumigatus* sensu stricto reference strains; also, they were almost identical to clinical human and feed-borne *A. fumigatus* strains included in the assay, since their similarity coefficient ranged from 0.7 to 1.00. Therefore, all strains were characterized as belonging to *A. fumigatus* sensu stricto species. This result was supported by sequencing the *ben*A gene of selected strains and by maximum parsimony analysis. In addition, RAPD fingerprinting demonstrated intra-specific genetic variability into the *A. fumigatus* sensu stricto cluster. The results found in this study strengthen the fact that *A. fumigatus* sensu stricto is the predominant species in the *Aspergillus* section *Fumigati* found in animal environments such as dairy herd environments, while other species such as *A. novofumigatus*, *A. fumigatiaffinis*, *A. udagawae* and *A. lentulus* may be rarely isolated. Since no differences between animal and human strains were observed they can become pathogenic also for farm handlers'. Moreover, the presence of *A. fumigatus* sensu stricto in raw cow milk is probably a very important risk factor since milk and its by-products are generally indented for human consumption, then gliotoxin could be transferred to them.

1. Introduction

Aspergillus fumigatus, the major etiological agent of human and animal aspergillosis, is commonly found in contaminated animal environments. It is frequently isolated from sorghum and maize silages and other contaminated animal feeds (Keller et al., 2012; Pena et al., 2010; Pereyra et al., 2008). *Aspergillus fumigatus* is able to produce gliotoxin and other tremorgenic compounds that could induce neurological syndromes to farm workers who manipulate moldy feed containing it (Amitani et al., 1995; Pena et al., 2010, 2015, 2015). Large number of spores is easily spread in the air indicating high risk of exposure both for animals and humans. Several infectious diseases caused by *A. fumigatus* in animals, such as respiratory infection in chicks, ducks and horses, have been reported (Arné et al., 2011; Beernaert et al., 2010; Girma et al., 2016; Guida et al., 2005; Kendall et al., 2008). In ruminants, this fungus causes respiratory infections, abortion, mastitis and other clinical cases (Gourreau et al., 1988; Smith, 1989).

Species including in *Aspergillus* section *Fumigati* are characterized by uniseriate and columnar conidial heads in shades of green and flask shaped vesicles (Raper and Fennell, 1965). *Aspergillus fumigatus* is identified by macroscopic and microscopic features and, despite some variations in its phenotype, such as vesicle size and shape and stipe length, has been largely regarded as a single species. However, molecular studies have demonstrated that several morphologically identified *A. fumigatus* strains might be genetically distinct (Hong et al., 2005; Montenegro et al., 2009). Therefore, molecular identification is highly recommended to the correct identification of the species within species complex, because they could share morphological characteristics that

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may do difficult their identification to species level. Into the "*A. fumigatus* complex" group, molecular studies have demonstrated that several strains morphologically identified as *A. fumigatus* belonged to *A. lentulus*, *A. viridinutans*, *A. fumigatiaffinis*, *A. fumisynnematus*, *Neosartorya pseudofischeri*, *N. hiratsukae* or *N. udagawae* species (Balajee et al., 2005a, 2005b, 2006; Hong et al., 2008; Samson et al., 2007). Some of them have been described as human pathogens and reported to be resistant in vitro to azole antifungals (Alcazar-Fuoli et al., 2008; Yaguchi et al., 2007). In order to resolve this issue, different approaches have been applied to identify the species belonging to the section *Fumigati*. Among them, Hong et al. (2005, 2010) re-identified 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. In addition, Pena et al. (2015) and Soleiro et al. (2013) characterized a set of feed-borne and clinical *A. fumigatus* sensu lato strains isolated from Argentina and Brazil applying PCR-restriction length fragment polymorphisms (PCR-RFLP) and RAPD (with PELF and URP1F). They found that the molecular identification of *A. fumigatus* sensu lato strains resulted in a unique species into the *Aspergillus* section *Fumigati*, which was *A. fumigatus* sensu stricto; also, their genetic profiles were identical to human/animal clinical strains. 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 raw cow milk samples in Argentina, applying neutral molecular markers. The study of the prevalence of *A. fumigatus* sensu stricto in dairy herd environment could help in predicting the epidemiological risk that milking cows and farm handlers' are exposed. Moreover, the presence of *A. fumigatus* in milk intended to human consumption is probably a very important risk factor in the transmission of gliotoxin and other mycotoxins to humans. This work was aimed i) to apply the RAPD molecular marker with primers PELF and URP1F to confirm the identification at species level of *A. fumigatus* sensu lato strains isolated from cow milk samples in Argentina and ii) to determine and compare their genetic variability.

2. Materials and methods

2.1. Strains isolation

Forty five *A. fumigatus* sensu lato strains isolated from raw cow milk were randomly selected and used for their identification at species level (by morphological and molecular characterization) and for estimation of its genetic variability.

The *A. fumigatus* strains came from a sampling carried out on 44 dairy establishments located in Cordoba province, in the central region of Argentina on March to September 2007. Each one of them owned between 100 and 250 cows in milking and daily average milk productions of 10–20 l/cow. A total of 901 individual milking cows were sampled following the random systemic method of Thrusfield (2007). The udders were disinfected with ethanol 70%, dried with individual paper towel and the first jet of milk discarded. Fungal isolation were performed on petri plates containing dichloran rose bengal chloramphenicol agar (DRBC) and dichloran 18% glycerol agar (DG18) (Pitt and Hocking, 1997). Colonies resembled *A. fumigatus* were taken and sub-cultured in Malt Extract Agar (MEA) that was subsequently incubated at 25 °C for 7 days in darkness.

2.2. Morphological identification

For the morphological analysis, all *A. fumigatus* sensu lato strains were identified by using the morphological criteria (Klich and Pitt, 2002; Samson et al., 2007). Briefly, the strains were grown for 7 days as 3-point inoculations on Czapek agar, Czapek yeast extract agar (CYA), Czapek yeast extract agar with 20% sucrose (CY20S) and malt extract agar (MEA) plates at 25 °C, and on CYA at 37 °C.

Morphological data were analyzed through analysis of variance (ANOVA). The Fisher's test of Least-Significant Difference (LSD) was used to determine the significant differences between means.

2.3. Molecular identification

For the molecular identification of the strains and for estimation of its genetic variability, the RAPD molecular marker (using primers PELF and URP1F, according to the methods described in Pena et al., 2015) was applied. The reference strains *A. fumigatus* sensu stricto CBS 127.801, *A. fumigatus* sensu stricto CBS 127.278, *A. lentulus* CBS 117.180, *A. novofumigatus* CBS 117.519, *A. novofumigatus* DTO 249-H5 and *A. udagawae* CBS 114.218 were also included as standards on each gel. Genetic similarity between them is shown in Fig. S1 (supplementary file). Moreover, nine *A. fumigatus* sensu lato strains isolated from sputum of patients suffering invasive pulmonary aspergillosis (coming from clinic centers of Santa Fé and Tucumán provinces) which had not been identified by molecular criteria until now, and other three *A. fumigatus* sensu stricto strains isolated from animal feeds previously identified (Pena et al., 2015) were included in the analysis for comparison of their DNA profiles with the assayed strains. Summarizing, a total of 57 *A. fumigatus* strains were analyzed by RAPD markers.

2.3.1. Fungal biomass production and DNA extraction

From each *A. fumigatus* strain grown on malt extract agar (MEA), spores were inoculated in Erlenmeyer flasks of 250 ml containing 50 ml of sterile Wickerham medium (Mulé et al., 2006) and then, incubated on an orbital shaker at 1.26 *g* at 25 ± 1 °C for 3 days. Mycelia were harvested by filtration, dried and frozen at −20 °C until ground. Fungal DNA was extracted using a hexadecyltrimethylammonium bromide (CTAB) procedure (Leslie and Summerell, 2006). Total DNA extracted was quantified subjecting samples to electrophoresis on 0.8% agarose gel stained with ethidium bromide (0.5 μg/ml). After the run, DNA dilutions from de total DNA were quantified by UV spectrophotometry using a NanoDrop 2000 Spectrophotometer (ThermoFischer Scientific, Waltham, Massachusetts, USA). Also, its purity was determined. After that, work dilutions were made to obtain fungal DNA at 20–25 ng/μl for RAPD reactions.

2.3.2. RAPD-based characterization

PCR amplifications with primers URP1F and PELF were made, separately, as stated in Pena et al. (2015). Briefly, 25 μl PCR reactions containing 1 X 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^{-1} of each primer were performed. A negative control was included in every set of reactions. PCR amplifications were conducted as follows: 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 indefinitely. The products were electrophoresed on a 1.5% agarose gel stained with 0.5 μg/ ml ethidium bromide and photographed using a MiniBIS Pro DNR Bio-imaging systems photoanalyzer. The fragment sizes were measured by comparison with DNA 100-bp ladder (Invitrogen by Life Technologies) (with reference bands between 100 and 2072 bp). The presence or absence of polymorphic RAPD bands was scored manually and the data recorded in a binary format. Each scored band of differing electrophoretic migration was treated as a single independent locus with two alleles (present or absent).

2.3.2.1. Genetic distance and cluster analysis of RAPD data. To estimate genetic distances between individuals, similarity coefficients (S) were calculated using the formula: $S = 2\ Nxy/(Nx + Ny)$, where Nx represents the number of fragments amplified in isolated x and y,

respectively; and Nxy 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 and 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 (Numerical Taxonomy System; Applied Biostatistics Inc., New York, NY, USA) software package (Rohlf, 1990). The RAPD data were subject to bootstrap analysis with 10,000 replications using the program PAUP* 4.0a152 (Swofford, 2001) to determine whether there was significant genetic substructure or clustering among isolates as resolved by RAPD data.

2.3.3. Confirmation of RAPD fingerprinting results by sequencing of benA regions

Based on the RAPD profiles, 10 strains were selected according to their position on RAPD dendrogram for sequencing of *ben*A regions for definitively confirm its taxonomic state at species level. Amplifications were made using the primers Bt2a (5′-GGTAACCAAATCGGTGCTGCT TTC-3′) and Bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) as stated in Pena et al. (2015). These primers, described for Glass and Donaldson (1995), yielded a 550 bp-size amplicon. PCR reactions were made with 10–20 ng of fungal DNA in a total volume of 50 µl of 1 X 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. 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 indefinitely. For DNA sequencing of both strands, templates DNA were sent to Macrogen Inc. Korea. Sequencing errors were detected and corrected using BioEdit software version 7.0.9.0 (Thompson et al., 1994). Nucleotide sequence comparisons were performed using the Basic Local Alignment Search Tool (BLAST) network services of the National Centre for Biotechnology Information (NCBI) database with *benA* sequences as the query.

Multiple sequence alignment of the partial *ben*A sequences was performed using the Web-based program MAFFT (Katoh and Standley, 2013). Also, Partial *ben*A sequences from reference strains of species into section *Fumigati* obtained from GenBank were included in the analysis. (Table S1, supplementary file). Based on this alignment, maximum parsimony analysis was performed with heuristic search with random addition sequences, branch swapping by tree bisection-reconnection (TBR) and MAXTREES set at 10000, using TNT v1.1 (Goloboff et al., 2008). Relative robustness of the individual branches was estimated by bootstrapping, using 10,000 replicates. *Aspergillus clavatus* strain H522 (GenBank accession AF057212.1) was used as outgroup.

The strains used in this study were deposited in the culture collection at the Department of Microbiology and Immunology, National University of Rio Cuarto (UNRC) as RC followed by the isolation number (for example RC2210) and kept cryopreserved in sterile 15% glycerol.

3. Results

3.1. Morphological characterization

The morphologic characterization of strains showed typical features of the *A. fumigatus* (sensu lato) species (Table 1). Although, the morphologic characterization of ten isolates is shown in Table 1, all strains were analyzed. All strains showed similarity between them in both macroscopic and microscopic characteristics, i.e. colony texture was velutinose for all isolates (data not shown), but some of them showed limited conidiation in MEA. However, no clear statistics differences in

Table 1
Macroscopic and microscopic characterization of some *Aspergillus fumigatus* strains isolated from raw cow milk.

Macroscopic features									Microscopic features (media ± SD)	
Strains[a]	Colony diameter (media ± SD) mm)					Conidiation on MEA	Colony color on MEA		Stipe (µm)	Vesicle diameter (µm)
	MEA	CYA25	CYA37	CY20S	Czapec-Dox		Front	Reverse	Length	Wide	
RC2210	52.0 ± 0.0	51.4 ± 2.3	Nd	52.7 ± 4.2	52.0 ± 2.0	Abundant	Green grey dull	Pale yellow	356.7 ± 98.9	6.6 ± 1.2	18.2 ± 2.7
RC2213	63.3 ± 3.1	52.7 ± 1.1	62.7 ± 1.1	57.3 ± 1.1	50.0 ± 2.0	Limited	Green grey dull	Dull yellow	283.2 ± 68.6	5.4 ± 1.2	17.3 ± 2.0
RC2229	70.0 ± 10.0	66.7 ± 3.1	65.3 ± 6.1	67.3 ± 2.3	56.0 ± 2.0	Abundant	Green grey dull	Pale yellow	270.0 ± 36.0	4.4 ± 0.7	15.0 ± 2.3
RC2240	64.0 ± 4.0	60.7 ± 2.3	72.0 ± 10.6	61.3 ± 2.3	53.3 ± 1.1	Abundant	Green grey dull	Pale yellow	288.0 ± 48.0	7.2 ± 0.0	16.8 ± 2.7
RC2243	70.0 ± 2.0	57.3 ± 1.1	67.3 ± 6.4	62.0 ± 2.0	56.7 ± 1.1	Limited	Green grey dull	Pale yellow	241.4 ± 22.9	5.4 ± 1.2	17.4 ± 2.6
RC2249	58.0 ± 2.0	66.7 ± 1.1	60.5 ± 11.0	55.3 ± 4.6	45.3 ± 3.0	Limited	Green grey dull	Pale yellow	294.0 ± 40.9	6.5 ± 2.0	18.0 ± 3.8
RC2251	60.0 ± 0.0	57.3 ± 3.1	62.7 ± 6.4	59.3 ± 5.0	48.7 ± 2.3	Abundant	Green grey dull	Pale yellow	336.0 ± 96.0	4.8 ± 0.0	18.6 ± 1.2
RC2257	54.7 ± 5.0	53.3 ± 1.1	60.0 ± 0.0	54.0 ± 3.6	46.7 ± 3.0	Abundant	Green grey dull	Dull yellow	324.0 ± 93.7	4.8 ± 2.4	15.0 ± 2.1
RC2261	70.0 ± 8.7	57.3 ± 2.3	69.3 ± 10.1	61.3 ± 3.1	47.3 ± 1.1	Abundant	Green grey dull	Pale yellow	232.0 ± 13.9	6.0 ± 1.4	17.5 ± 3.3
RC2263	64.7 ± 2.3	57.3 ± 2.3	66.7 ± 7.6	60.0 ± 0.0	48.7 ± 2.3	Abundant	Green grey dull	Pale yellow	225.0 ± 22.7	6.0 ± 1.2	18.2 ± 2.1

Nd: not determined.
[a] *Aspergillus fumigatus* strains isolated from raw milk samples.

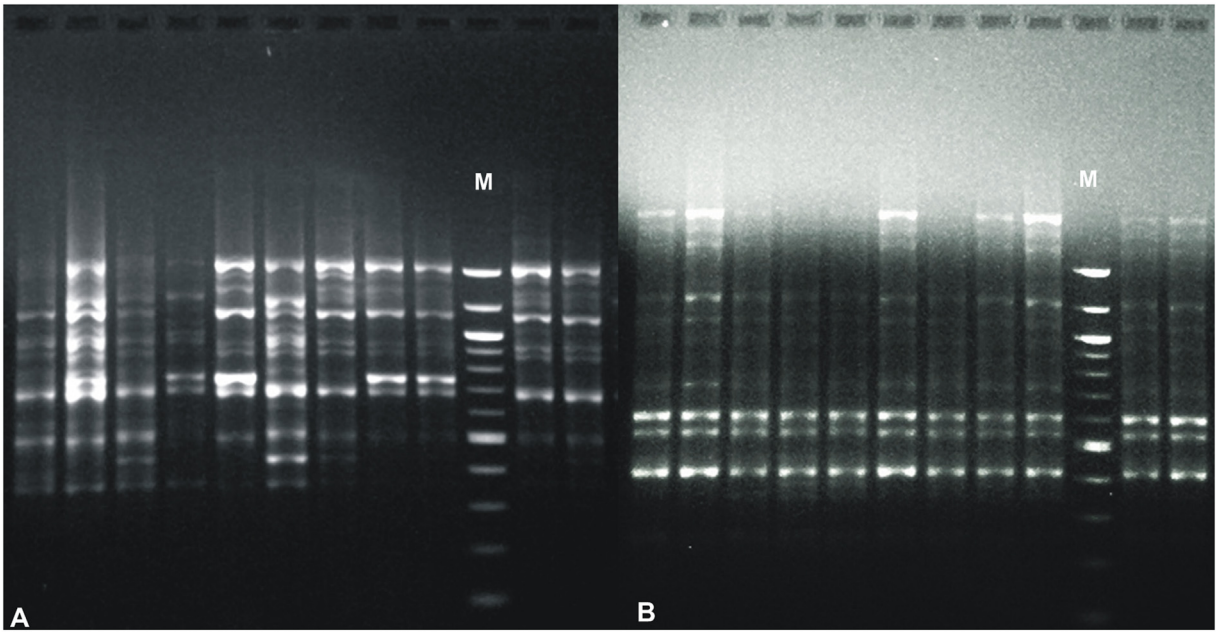

Fig. 1. *Aspergillus fumigatus* RAPD profiles of strains isolated from cow milk obtained with PELF (A) and URP1F (B) primers. M: 100-bp DNA ladder (Invitrogen by Life Technologies).

the morphometric features such as colony diameter in different culture media, stipe length and wide and diameter of the fungal vesicle were found, since some strains were statistically different between them and not others, and since these differences were not observed for all features measured.

3.2. RAPD-based fingerprinting

Band patterns resulting from the RAPD fingerprinting with primers PELF and URP1F of some *A. fumigatus* strains of milk samples are shown in Fig. 1; even though, all strains were assayed with both primers. The ability to produce polymorphic and reproducible band patterns among the *Aspergillus* section *Fumigati* isolates were previously tested (Pena et al., 2015). More polymorphic haplotypes were obtained with PELF than with URP1F primer. For this reason, band reproducibility was checked, at least twice, and identical results were obtained in all experiences. Forty-five isolates of *A. fumigatus* from milk samples showed similar band patterns to each other and to *A. fumigatus* sensu stricto CBS 127.801 and *A. fumigatus* sensu stricto CBS 127.27 reference strains, although some isolates showed differences in minor bands. Moreover, its band patterns were almost identical to the clinical human *A. fumigatus* strains included in the assay. Fifty-four distinct and scoreable bands were obtained with both primers and allowed the construction of 63 isolates × 54 loci data matrix, which was analyzed and used to produce a UPGMA dendrogram (Fig. 2). It allowed the comparison among the haplotypes and showed a clear separation of the four *type*-strains into section *Fumigati* with similarities < 50%. The studied strains were grouped in a consistent group including all *A. fumigatus* strains isolated from cow milk and the reference strains *A. fumigatus* sensu stricto CBS 127.801 and CBS 127.278 (83% of statistical support). Also, the isolates placed in this group showed 70% of genetic similarly among them. The strains were assigned to a species if they shared more than 50% of the bands with the reference strain (Perrone et al., 2006). Therefore, all *A. fumigatus* assayed strains were typified as belonging to *A. fumigatus* sensu stricto species. Moreover, RAPD fingerprinting also allowed demonstrating intra-specific genetic variability in the *A. fumigatus* sensu stricto group, since genetic similarity coefficients among these isolates ranged from 0.70 to 1.00.

Furthermore, for definitively confirmation of the RAPD results, representative strains were subjected to sequencing of *benA* regions and the obtained sequences compared to sequences from the Genbank for results verification. 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 100% (February 2018, http://blast.ncbi.nlm. nih.gov/Blast.cgi). Therefore, it confirmed its identification as *A. fumigatus* sensu stricto species. The obtained *benA* sequences have been deposited in GenBank under accession numbers KY464099–KY464107 (http://www.ncbi.nlm.nih.gov/nucleotide).

Further, a maximum parsimony analysis was performed with the sequences obtained during *ben*A sequencing of selected strains together with sequences of other *Aspergillus* section *Fumigati* species retrieved from GenBank. As it is shown in Fig. 3, all strains formed a separate clade with *A. fumigatus* sensu stricto reference strains (bootstraping 100%) that was different from the other *Aspergillus* section *Fumigati* species included in the analysis.

4. Discussion

In this study, a group of *A. fumigatus* sensu lato strains isolated from raw cow milk samples taken from different dairy herds in Argentina were typified by its morphologic and genotypic (RAPD marker and *ben*A sequencing) features. Also the genetic variability of them was estimated. The prevalence of *A. fumigatus* in dairy environment was previously studied. Pellegrino et al. (2013) conducted a mycological survey of the milk samples from which the strains of the current study were isolated and found that *A. fumigatus* was the second more frequently isolated species of *Aspergillus* after *A. flavus*. Moreover, they demonstrated the gliotoxinogenic ability of the strains and its association with high somatic cell counts in milk samples. Alonso et al. (2009) reported the mycobiota isolated from raw materials and cow feed intended to milking cows in the same dairy herd establishments sampled and found that *Aspergillus* and *Fusarium* were the main fungi genera isolated. Fungi are frequent in the cow's environment, whether hygiene of farm is poor contamination of milk by toxinogenic fungi can occur. Morphological characterization showed some differences between the *A. fumigatus* sensu lato strains analyzed but no clear association between strains or variables were found when statistical analysis of the data was applied.

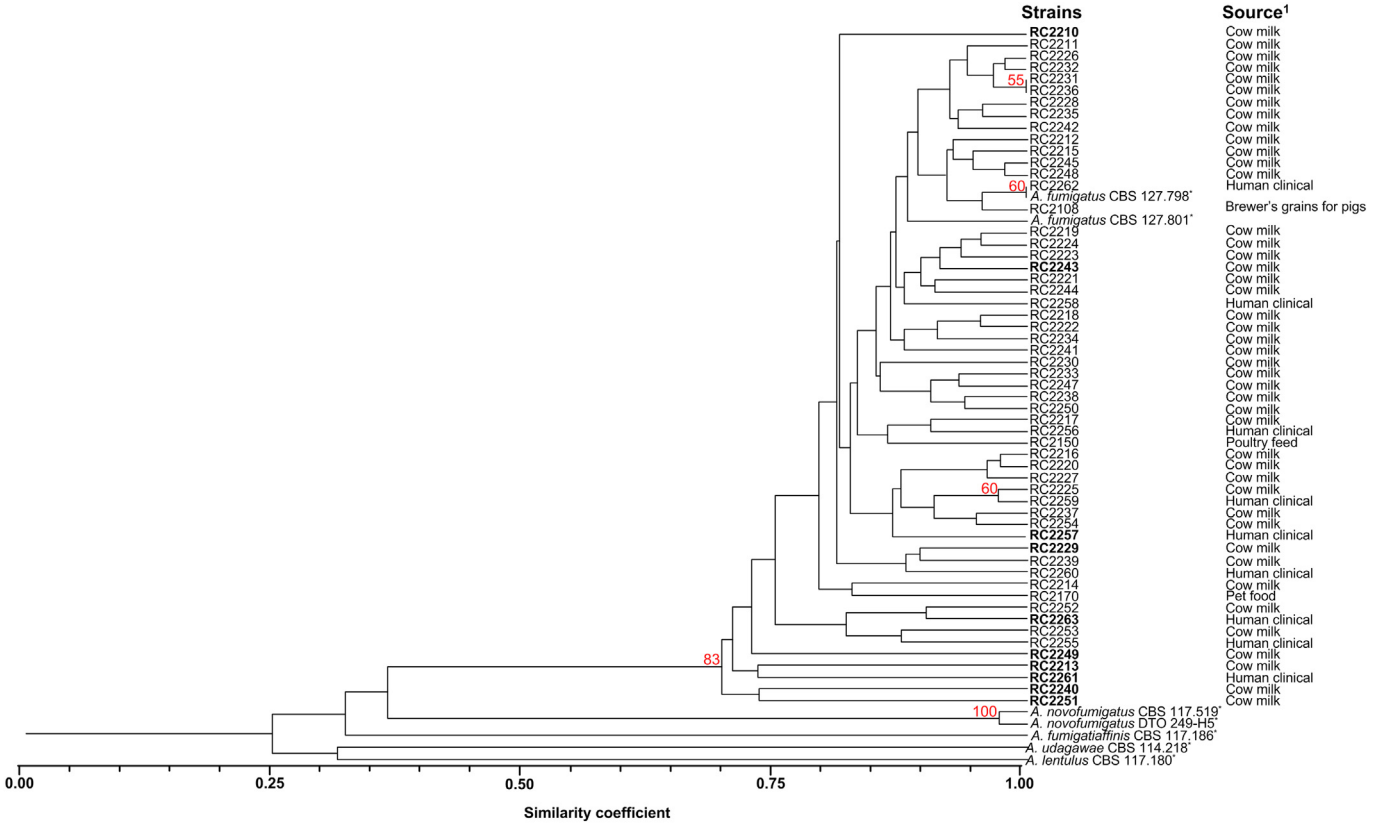

Fig. 2. UPGMA dendrogram of 63 *A. fumigatus* sensu lato isolates and reference strains into the *Aspergillus* section *Fumigati* (*) generated with the NTSYS software. Bootstrap values higher than 50% are shown above the branches. Sequenced strains at β-tubulin gene are shown in bold.[1] Source of strains isolation.

This suggested that the variability observed in macroscopic and microscopic features was given by intra-specific variability. Therefore, the molecular analysis was consequently carried out to clarify this result. Although the RAPD analysis was applied identically as Pena et al. (2015), greater number of polymorphic bands was obtained. This fact could be assigned to a better gel resolution that allowed increasing the zone of the gel for reading bands. All isolates of *A. fumigatus* from raw cow milk samples showed almost the same band patterns to each other and to *A. fumigatus* sensu stricto reference strains, clinical human *A. fumigatus* and those isolated from animal feeds. Therefore, by applying

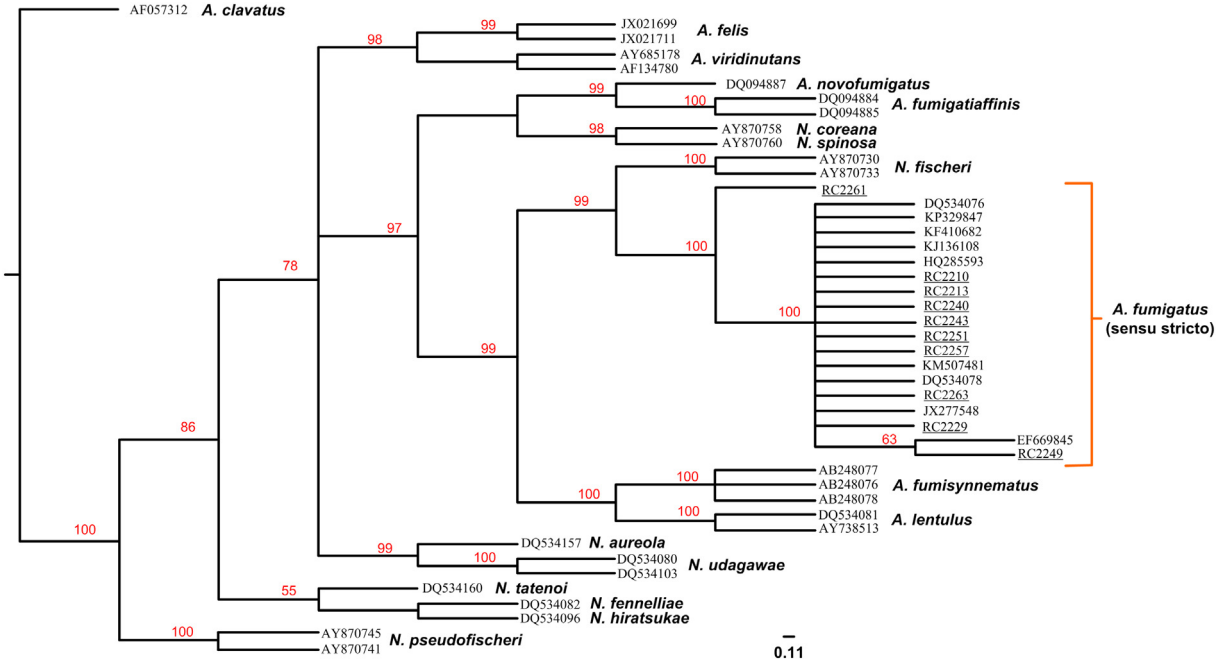

Fig. 3. "Maximum parsimony tree inferred from *ben*A sequences of selected *A. fumigatus* sensu stricto strains. Bootstrap values higher than 50% are shown above the branches. *Aspergillus clavatus* strain H522 (GenBank accession AF057212.1) was used as outgroup."

the RAPD molecular markers all *A. fumigatus* assayed strains were typified as *A. fumigatus* sensu stricto species. Moreover, this result was supported by sequencing the *ben*A gene of selected strains and by maximum parsimony analysis. These results suggest that *A. fumigatus* sensu stricto is a predominant species in *Aspergillus* section *Fumigati* found in animal environments such as dairy herd environments as well as in human clinical cases, while other species including *A. novofumigatus*, *A. fumigatiaffinis*, *A. udagawae* and *A. lentulus* may be rarely isolated. Using the RAPD approach applied here, Hong et al. (2010) evaluated 146 worldwide clinical and environmental (soil) strains of *A. fumigatus* sensu lato and found that 140 (95.8%) of them were identified as *A. fumigatus* sensu stricto whereas 3 (2.1%) were *A. lentulus*, and the remaining three (3) strains were typified as *A. viridinutans* complex, *Neosartorya udagawae* and *N.* cf. *nishimurae*. Primers PELF and URP1F, previously selected by Hong et al. (2005) were considered as a rapid and reliable method to identify *A. fumigatus* and *A. lentulus*. In the current study, closely related species to *A. fumigatus* sensu stricto such as *A. novofumigatus*, *A. fumigatiaffinis*, *A. udagawae* and *A. lentulus* were clearly separated. Moreover, genetic variability was found at intraspecific level in the *A. fumigatus* sensu stricto cluster and isolates seemed to form a homogeneous group with a high degree of similarity among them. This fact could also support the phenotypic variability observed between *A. fumigatus* isolates during its morphological characterization.

Exposed areas of lactating cows as mammalian glandule can be easily contaminated by *A. fumigatus* sensu stricto, due to it is frequently found in the dairy herd environment. Different researchers have studied the role of azoles as an antifungal therapy for aspergillosis (Seyedmousavi et al., 2015; Verweij et al., 2016). Studies related to azole resistance, heat resistance and UHT/pasteurization treatments should be carried out with our strains in order to design future strategies for the prevention and control of Aspergillosis. Since no differences between animal and human strains from cases of Aspergillosis were observed, they may become pathogenic to cows and also for the farm handlers'. Moreover, the presence of the gliotoxinogenic *A. fumigatus* sensu stricto in raw cow milk samples could be a very important risk factor since milk and its by-products are indented for human consumption.

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Conflicts of interest

None.

References

Alcazar-Fuoli, L., Mellado, E., Alastruey-Izquierdo, A., Cuenca-Estrella, M., Rodriguez-Tudela, J.L., 2008. *Aspergillus* section *Fumigati*: antifungal susceptibility patterns and sequence-based identification. Antimicrob. Agents Chemother. 52, 1244–1251.

Alonso, V.A., Monge, M.P., Dalcero, A.M., Keller, K.M., Rosa, C.A. da R., Cavaglieri, L.R., Chiacchiera, S.M., 2009. Contribution of raw materials on dairy cattle feedstuff aflatoxin contamination in Central Argentina. Rev. Bras. Med. Vet. 31, 92–99.

Amitani, R., Taylor, G., Elezis, E., Llewellyn-Jones, C., Mitchell, J., Kuze, F., Cole, P.J., Wilson, R., 1995. Purification and characterization of factors produced by *Aspergillus fumigatus* which affect human ciliated respiratory epithelium. Infect. Immun. 63, 3266–3271.

Arné, P., Thierry, S., Wang, D., Deville, M., Le Loc'h, G., Desoutter, A., Féménia, F., Nieguitsila, Adélaïde, Huang, W., Chermette, R., Guillot, J., 2011. *Aspergillus fumigatus* in poultry. Int. J. Microbiol. http://dx.doi.org/10.1155/2011/746356.

Balajee, S.A., Gribskov, J., Brandt, M., Fothergill, A., Marr, K.A., 2005a. Mistaken identity: *Neosartorya pseudofischeri* and its anamorph masquerading as *Aspergillus fumigatus*. J. Clin. Microbiol. 43, 5996–5999.

Balajee, S.A., Gribskov, J.L., Hanley, E., Nickle, D., Marr, K.A., 2005b. *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. Eukaryot. Cell 4, 625–632.

Balajee, S.A., Nickle, D., Varga, J., Marr, K.A., 2006. Molecular studies reveal frequent misidentification of *Aspergillus fumigatus* by morphotyping. Eukaryot. Cell 5, 1705–1712.

Beernaert, L.A., Pasmans, F., Van Waeyenberghe, L., Haesebrouck, F., Martel, A., 2010. *Aspergillus* infections in birds: a review. Avian Pathol. 39, 325–331.

Girma, G., Abebaw, M., Zemene, M., Mamuye, Y., Getaneh, G., 2016. A review on aspergillosis in poultry. J. Vet. Sci. Technol. 7, 382. http://dx.doi.org/10.4172/2157-7579.1000382.

Glass, N.L., Donaldson, G., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microbiol. 61, 1323–1330.

Goloboff, P.A., Farris, J.S., Nixon, K.C., 2008. TNT, a free program for phylogenetic analysis. Cladistics 24, 774–786.

Gourreau, J.M., Laval, A., Badinand, F., 1988. Les aspergilloses des bovins. Point Vét. 20, 589–608.

Guida, N., Mesplet, M., Di Gennaro, E., Digilio, P., Vicente Moras, E., 2005. *Aspergillus fumigatus* in nasopharyngeal cavity of horses. Rev. Iberoam. Micol. 22, 160–162 (Presencia de *Aspergillus fumigatus* en la cavidad nasofaríngea de equinos).

Hong, S.B., Go, S.J., Shin, H.D., Frisvad, J.C., Samson, R.A., 2005. Polyphasic taxonomy of *Aspergillus fumigatus* and related species. Mycologia 97, 1316–1329.

Hong, S.B., Kim, D.H., Park, I.C., Choi, Y.J., Shin, H.D., Samson, R., 2010. Re-identification of *Aspergillus fumigatus* sensu lato based on a new concept of species delimitation. J. Microbiol. 48, 607–615.

Hong, S.B., Shin, H.D., Hong, J.B., Frisvad, J.C., Nielsen, P.V., Varga, J., Samson, R.A., 2008. New taxa of *Neosartorya* and *Aspergillus* in *Aspergillus* section *Fumigati*. Antonie Van Leeuwenhoek 93, 87–98.

Katoh, Standley, 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability (outlines version 7). Mol. Biol. Evol. 30, 772–780.

Keller, L.A.M., Keller, K.M., Monge, M.P., Pereyra, C.M., Alonso, V.A., Cavaglieri, L.R., et al., 2012. Gliotoxin contamination in and pre- and postfermented corn, sorghum and wet brewer's grains silage in Sao Paulo and Rio de Janeiro State, Brazil. J. Appl. Microbiol. 112, 865–873.

Kendall, A., Bröjer, J., Karlstam, E., Pringle, J., 2008. Enilconazole treatment of horses with superficial *Aspergillus* Spp. rhinitis. J. Vet. Intern. Med. 22, 1239–1242.

Klich, M.A., Pitt, J., 2002. A Laboratory Guide to Common *Aspergillus* Species and Their Teleomorphs. CSIRO, Division of Food Research Sydney, Academic Press, Australia.

Leslie, J.F., Summerell, B.A., 2006. The Fusarium Laboratory Manual. Blackwell, Ames, IA, pp. 57–59.

Montenegro, G., Sánchez Puch, S., Jewtuchowicz, V.M., Pinoni, M.V., Relloso, S., Temporitti, E., et al., 2009. Phenotypic and genotypic characterization of *Aspergillus lentulus* and *Aspergillus fumigatus* isolates in a patient with probable invasive aspergillosis. J. Med. Microbiol. 58, 391–395.

Mulé, G., Susca, A., Logrieco, A., Stea, G., Visconti, A., 2006. Development of a quantitative real-time PCR assay for the detection of *Aspergillus carbonarius* in grapes. Int. J. Food Microbiol. 111, 28–34.

Nei, M., Li, W.H., 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. U. S. A. 76, 5269–5273.

Pellegrino, M., Alonso, V., Vissio, C., Larriestra, A., Chiacchiera, S.M., Bogni, C., Cavaglieri, L., 2013. Gliotoxinogenic *Aspergillus fumigatus* in the dairy herd environment. Mycotoxin Res. 29, 71–78.

Pena, G.A., Coelho, I., Reynoso, M.M., Soleiro, C., Cavaglieri, L.R., 2015. Characterization and genetic variability of feed-borne and clinical animal/human *Aspergillus fumigatus* strains using molecular markers. Med. Mycol. 53, 699–708.

Pena, G.A., Monge, M.P., González Pereyra, M.L., Dalcero, A.M., Rosa, C.A., Chiacchiera, S.M., Cavaglieri, L.R., 2015. Gliotoxin production by *Aspergillus fumigatus* strains from animal environment. Micro-analytical sample treatment combined with a LC-MS/MS method for gliotoxin determination. Mycotoxin Res. 31, 145–150.

Pena, G.A., Pereyra, C.M., Armando, M.R., Chiacchiera, S.M., Magnoli, C.E., Orlando, J.L., Dalcero, A.M., Rosa, C.A., Cavaglieri, L.R., 2010. *Aspergillus fumigatus* toxicity and gliotoxin levels in feed stuff for domestic animals and pets in Argentina. Lett. Appl. Microbiol. 50, 77–81.

Pereyra, C.M., Alonso, V.A., Rosa, C.A.R., Chiacchiera, S.M., Dalcero, A.M., Cavaglieri, L.R., 2008. Gliotoxin natural incidence of *Aspergillus fumigatus* isolated from corn silage and ready dairy cattle feed. World Mycotoxin J. 1, 457–462.

Perrone, G., Mulè, G., Susca, A., Battilani, B., Pietri, A., Logrieco, A., 2006. Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius*, *Aspergillus tubingensis*, and *Aspergillus niger* strains isolated from grapes in Italy. Appl. Environ. Microbiol. 72, 680–685.

Pitt, J.I., Hocking, A.D., 1997. Fungi and Food Spoilage, 2nd edn. Blackie Academic Press, London.

Raper, K.B., Fennell, D.I., 1965. The genus *Aspergillus*. Williams & Wilkins, Baltimore.

Rohlf, F.J., 1990. Fitting curves to outlines. In: Rohlf, F.J., Bookstein, F.L. (Eds.), Proceedings of the Michigan Morphometrics. Workshop Edn. Museum of Zoology Special Publication No. 2 University of Michigan, Michigan, USA, pp. 167–177.

Samson, R.A., Hong, S., Peterson, S.W., Frisvad, J.C., Varga, J., 2007. Polyphasic taxonomy of *Aspergillus* section *Fumigati* and its teleomorph *Neosartorya*. Stud. Mycol. 59, 147–203. http://dx.doi.org/10.3114/sim.2007.59.14.

Seyedmousavi, S., Guillot, J., Arné, P., de Hoog, G.S., Mouton, J.W., Melchers, W.J., Verweij, P.E., 2015. *Aspergillus* and aspergilloses in wild and domestic animals: a global health concern with parallels to human disease. Med. Mycol. 53, 765–797. http://dx.doi.org/10.1093/mmy/myv067.

Smith, J.M., 1989. Opportunistic Mycoses of Man and Other Animals. C.A.B. International Mycological Institute ed. Wallingford, Oxon, pp. 251 (ISBN 0-85198-638-2).
Soleiro, C.A., Pena, G.A., Cavaglieri, L.R., Coelho, I., Keller, L.M., Dalcero, A.M., et al., 2013. Typing clinical and animal environment *Aspergillus fumigatus* gliotoxin producer strains isolated from Brazil by PCR-RFLP markers. Lett. Appl. Microbiol. 57, 484–491.
Swofford, D.L., 2001. PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4.0*. Sinauer Associates, Underland, MA, USA.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
Thrusfield, M., 2007. Veterinary Epidemiology, 3rd Edn. Blackwell Science, Oxford, USA, pp. 1–593.
Verweij, P.E., Chowdhary, A., Melchers, W.J.G., Meis, J.F., 2016. Azole resistance in *Aspergillus fumigatus*: can we retain the clinical use of mold-active antifungal azoles? Clin. Infect. Dis. 62, 362–368. http://dx.doi.org/10.1093/cid/civ885.
Yaguchi, T., Horie, Y., Tanaka, R., Matsuzawa, T., Ito, J., Nishimura, K., 2007. Molecular phylogenetics of multiple genes on *Aspergillus* section *Fumigati* isolated from clinical specimens in Japan. Nihon Ishinkin Gakkai Zasshi. 48, 37–46.