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ORIGINAL ARTICLE

Biodiversity of species of *Aspergillus* section *Fumigati* in semi-desert soils in Argentina

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Soil

Abstract The distribution of *Aspergillus* species in soil has been widely studied all over the world. The aim of this study was the phenotypic and genotypic characterization of species *Aspergillus* belonging to section *Fumigati* present in soils from two Argentinian semi-desert areas having different geological conditions. Altogether, 23 isolates belonging to *Aspergillus* section *Fumigati* were recovered and identified using a polyphasic approach including phenotypic and molecular identifications. *Aspergillus fumigatus sensu stricto* and *Aspergillus fumigatiaffinis* had the highest frequency, of occurrence while isolates closely related to *Aspergillus udagawae* and *Aspergillus felis* were rarely observed. *A. fumigatiaffinis* and isolates closer to *A. udagawae* were isolated for the first time from Argentinian soils and this is the first report on the occurrence of species belonging to the *A. felis* clade in South America. Recent scientific interests in biodiversity, as well as the increasing importance of aspergilli as causative agents of human and animal diseases increase the need to understand the diversity and occurrence of these fungi in nature.

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PALABRAS CLAVE
Aspergillus felis;
Aspergillus udagawae;
Aspergillus fumigatiaffinis;
Aspergillus fumigatus;
Suelo

Biodiversidad de especies de *Aspergillus* de la sección *Fumigati* en suelos semidesérticos de Argentina

Resumen La distribución de especies de *Aspergillus* en el suelo se ha estudiado ampliamente en todo el mundo. El objetivo de este trabajo fue caracterizar fenotípica y genotípicamente las especies pertenecientes a la sección *Fumigati* presentes en los suelos de dos zonas semidesérticas de Argentina con diferentes geologías. En total, 23 *Aspergillus* de la sección *Fumigati* fueron aislados e identificados utilizando un enfoque polifásico incluyendo identificaciones fenotípicas y moleculares. *Aspergillus fumigatus sensu stricto* y *Aspergillus fumigatiaffinis* aparecieron con mayor frecuencia, mientras que los aislamientos relacionados a *Aspergillus udagawae* y a *Aspergillus felis* se observaron raramente. Este es el primer informe de *A. fumigatiaffinis* y de aislamientos estrechamente relacionados a *A. udagawae* en suelos argentinos; también el primero sobre la ocurrencia de especies pertenecientes al clado *A. felis* en Sudamérica. El emergente interés científico en la biodiversidad, así como la creciente importancia de *Aspergillus* como agentes causales de enfermedades humanas y animales, aumentan la necesidad de conocer la diversidad y la ocurrencia de estos hongos en la naturaleza.

Introduction

In the 21st century, *Aspergillus* and its teleomorphs have been investigated with polyphasic methods to examine variability among species. Currently, according to the polyphasic taxonomy, Houbraken et al.[24] and Hubka et al.[25] proposed that the genus *Aspergillus* is classified into four subgenera (*Aspergillus, Circumdati, Fumigati and Nidulantes*) and 20 sections and each includes a number of related species.

Section *Fumigati* is one of the most species-rich sections in the genus *Aspergillus* and includes species with overall significance for medicine, pharmacology, biotechnology, food and soil mycology. At present, the section consists of 51 taxa: 21 strictly anamorphic *Aspergillus* species and 30 *Neosartorya* species[43].

The distribution of *Aspergillus* species in soil has been widely studied all over the world[30]. Information on the diversity of *Aspergillus* species was reported in more than 270 studies of microfungi from soil between 0 and 46 degrees North or South (N/S) and concluded that the relative percentage of *Aspergillus* species is greatest in 25–35 degrees N/S. Many rare species and most new species of *Aspergillus* have been reported only from tropical and subtropical soils[19,22,29].

The distribution of *Aspergillus* species in Argentinian soils has been treated in several studies, but there are only limited data available regarding section *Fumigati*[14,31,32]. The aim of this study was the phenotypic and genotypic characterization of species belonging to section *Fumigati* present in soils from two Argentinian semi-desert areas having different geological conditions.

Materials and methods

Areas of study

In winter (July) 2011 soil samples from Talampaya National Park and Pampa de Achala were collected.

Talampaya National Park is in the south west of La Rioja province (29°46′S and 67°54′O). The park covers an area of 2150 square kilometers, at an altitude of 1300 meters above mean sea level. This National Park is in a semiarid continental zone with 150–170 mm annual rainfall. The climate is hot in summer, with temperatures exceeding 50 °C, and going down to −7 to −9 °C on winter nights. On the characteristic sandy and stony soils of the place, the vegetation is represented by xerophilous bushes and cactuses[2,3].

Pampa de Achala is a hydrologic natural reserve located in the north west of Córdoba province (31°41′S and 64°50′O). It is a *pampa* (plain) of 146 000 square kilometers with gentle slope and steep gorge located at an altitude between 1500 and 2790 m above mean sea level. The climate of the region is temperate-cold; maximum temperatures are generally 30 °C in the summer, falling below −20 °C during the winter. Rainfall takes place from October to April and is about 800 mm annually. The Pampa's vegetation is characterized by scrub and grasslands[1,3].

Sampling

Soil samples were collected in Talampaya National Park in a 55 km-section of national road 76. Soil samples were collected in Pampa de Achala in a 30 km-section of the old

provincial route 14. In both cases, sampling was carried out every 5 km, moving away 200 or 300 m from the main road. Five samples were collected randomly in a 20 m^2 radius. A pool of about 250 g was made. The samples were gathered with a sterilized spoon from the superficial layer (2–3 cm). In cases in which the soil was hard, a knife tip sterilized *in situ* using iodized alcohol was used. The soil samples were stored in sterilized paper bags at 4 °C until they were analyzed.

Soil processing

Dilution technique: One gram of each sample was diluted in 10 ml sterilized water. From this 1:10 dilution a 1:100 dilution was made. Of each dilution (1:10 and 1:100), 0.2 ml were transferred to Petri dishes containing potato-dextrose-agar (PDA) with chloramphenicol (0.25 g/l). Each dilution was cultured twice. Culture plates were incubated for 7 days at 37 °C in order to inhibit the growth of mesophilous molds. The plates were kept for 20 days waiting for the possible growth of associated teleomorphs.

The pH of each soil sample collected (10 g) was measured immediately using a pH meter (Jenco 6230), after dilution in 125 ml sterile distilled water with 5 min of agitation.

Isolation and morphological diagnosis

Primary culture plates were examined under a stereoscopic microscope and every *Aspergillus* and its teleomorphs were counted and sub-cultured on specific media. Each species was counted once in each sample, even if it appeared twice in the same plate or in the duplicate. Species frequency was calculated by its presence in total samples.

Isolates were subcultured on Czapek yeast autolysate agar (CYA), oatmeal agar (OA) and malt extract agar (MEA) and incubated for a week at 25 °C. Identification was done by macroscopic and microscopic morphology criteria according to general taxonomic keys. To distinguish growing maximum temperature range, isolates were inoculated on CYA and incubated for 7 days at 10-37-42-45-48 and 50 °C[30,37,38,40].

Molecular identification

Cultures were grown on 2 ml malt peptone broth [10% (v/v) malt extract (Brix 10) and 0.1% (w/v) bacto peptone (Difco)], in 15 ml tubes. The cultures were incubated at 25 °C for 7 days[37].

DNA was extracted from mycelia using the Masterpure™ yeast DNA purification kit (Epicenter Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions.

Random amplified polymorphic DNA (RAPD)-PCRs under the conditions described by Hong[18] was performed to identify *Aspergillus fumigatus sensu stricto* (*A. fumigatus s.s.*) and to detect other different band patterns. Primers PELF (5′-ATATCATCGAAGCCGC-3′) and URP1F (5′-ATCCAAGGTCCGAGACAACC-3′) were used and the *A. fumigatus* type strain CBS 133.61 was used as pattern.

Isolates genetically different from type strain *A. fumigatus* were sent to the Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Hungary for molecular identification. DNA was extracted as mentioned above. Amplification of the partial calmodulin gene (*calM*) and β-tubulin (*benA*) was carried out using primers cmd5 (5′-CCGAGTACAAGGAGGCCTTC-3′), cmd6 (5′-CCGATAGAGGTCATAACGTGG-3′)[18], and Bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′), Bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′)[15], respectively. The amplified DNA fragments were purified by the QIAquick PCR purification kit (Qiagene, Hilden, Germany). Sequence analyses were performed with the BigDye Terminator 3.1 Cycle Sequencing Ready Reaction Kit (ABI 0401041, Foster City, California) for both strands. Sequences were analyzed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). Alignment of partial *calM* and *benA* sequences was done using MAFFT v7.149b with the L-INS-i option[28]. Phylogenetic reconstruction was conducted using Maximum Likelihood analyses in raxmlGUI v1.3.1 under the GTR + Γ model[41]. The analysis was run in 1000 bootstrap replicates. The *calM* and *benA* sequences of type isolates of section *Fumigati* species were obtained from the database of the National Center for Biotechnology Information (NCBI) (Table 1).

Nucleotide sequence accession numbers

Sequences of *calM* of the 14 *Aspergillus* section *Fumigati* isolates were submitted to the European Nucleotide Archive (ENA) and assigned accession numbers KP824727–KP824740. Sequences of *benA* of the isolates related to *A. udagawae* and to *A. felis* were submitted to the European Nucleotide Archive (ENA) and assigned accession numbers LT674551–LT674557.

Results

A total of 17 soil samples was collected, 11 in Talampaya National Park and 6 in Pampa de Achala. The pH values of Talampaya National Park soil samples were alkaline, ranging from 8.00 to 9.83. In contrast, the pH values of Pampa de Achala soil samples were acid, ranging from 4.91 to 5.65 (Table 2).

From these 17 samples, 39 *Aspergillus* isolates were obtained. According to their phenotypic features, 23/39 belong to subgenus *Fumigati*, 8/39 to subgenus *Nidulantes* and 8/39 to subgenus *Circumdati*.

Within subgenus *Fumigati* only isolates belonging to section *Fumigati* were found, which were present in all the 17 samples analyzed.

The RAPD-PCR for 23 isolates of *Aspergillus* section *Fumigati* was conducted. Nine (9/17, 52.94%) isolates were genetically similar to type strain *A. fumigatus s.s.* (CBS 133.61) (data not shown) and the 14 genetically different isolates were identified by sequencing parts of the *calM* gene (Fig. 1) and the *benA* gene (Fig. 2). Nine (9/17, 52.94%) isolates were clearly identified as *A. fumigatiaffinis* using the *calM* gene. Two (2/17, 11.76%) isolates were closely related to *A. udagawae* and 3/17 (17.65%) isolates belong to the *A. felis* clade sequencing the *calM* and *benA* genes. Table 2 shows the *Aspergillus* section *Fumigati* identified and the areas where they were found.

A. fumigatus s.s. were identified by morphological and growth characteristics: dark blue-green color and velutinous

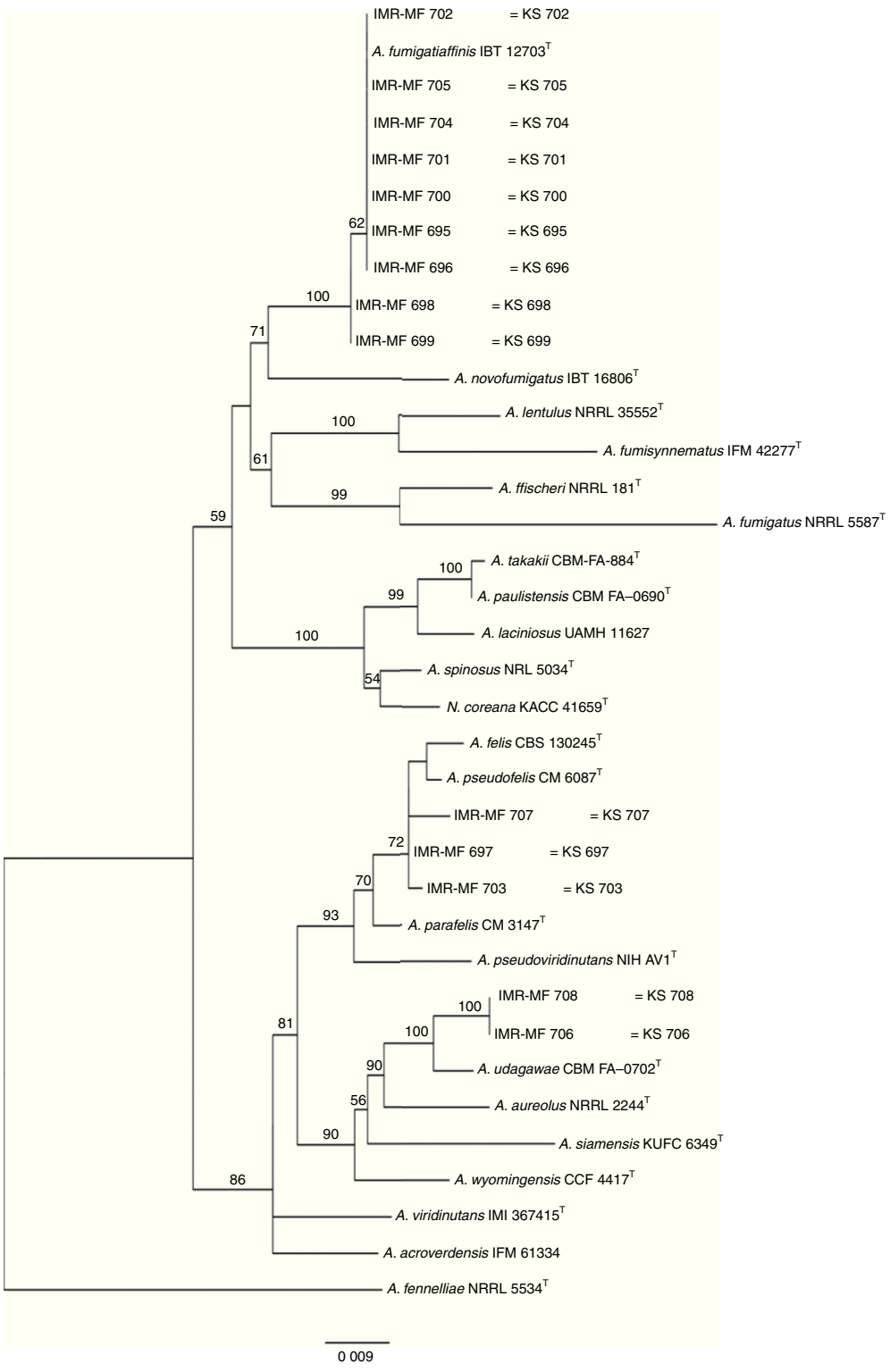

Figure 1 Taxonomic position of the strains of *Aspergillus* section *Fumigati* isolated from Argentinian soils based on partial Calmodulin gene phylogeny. Phylogenetic tree inferred from maximum likelihood analysis. Only bootstrap values ≥50% are shown.

colony, fast and abundant sporulation on MEA and CYA. Most isolates had subclavate vesicles (13-26 μm) and the conidiophore stipe diameter ranged from 5 to 9 μm. All *A. fumigatus s.s.* did not grow at 10 °C and grew at 50 °C on CYA, as was expected according to the literature[18,20,38,40].

A. fumigatiaffinis, and the isolates closely related to *A. felis* and *A. udagawae* showed less sporulation on MEA and CYA than *A. fumigatus s.s.* and the colonies were white, with a dull green center. Most isolates of *A. fumigatiaffinis* showed (sub)globose vesicles (15–23 μm),

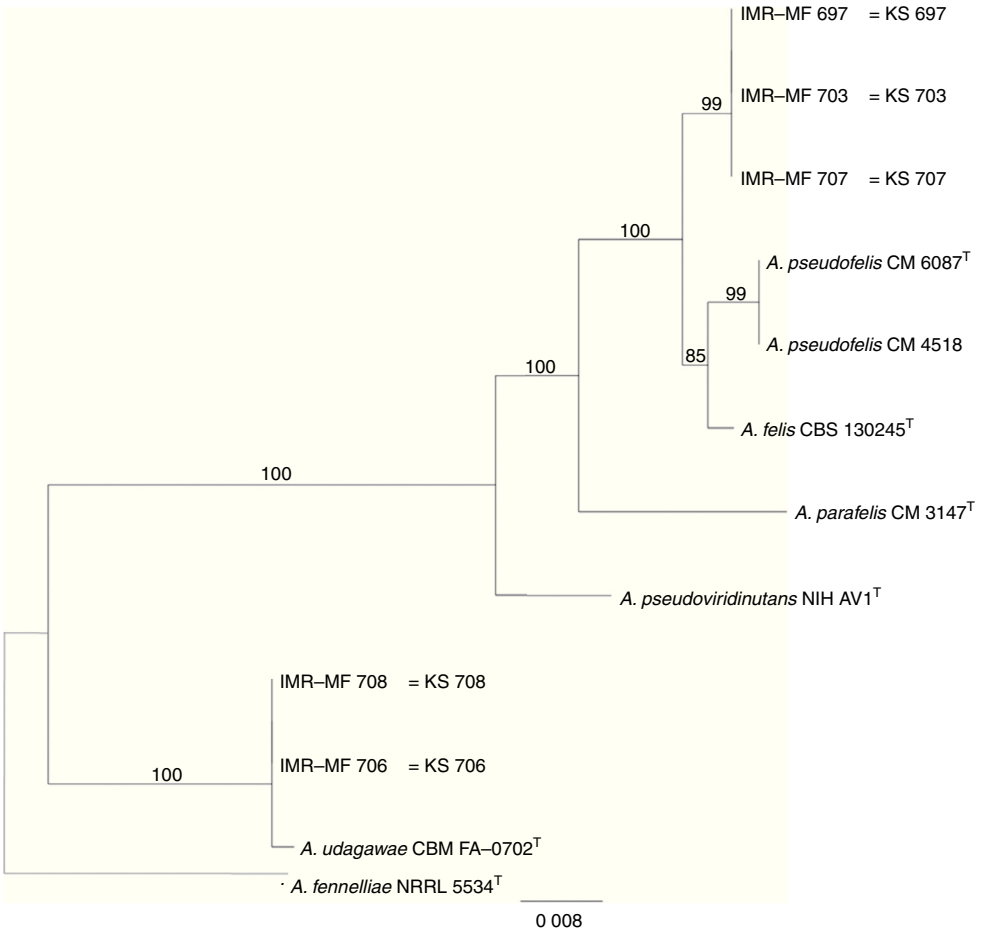

Figure 2 Taxonomic position of the isolates related to *Aspergillus udagawae* and *Aspergillus felis* based on partial β-tubulin gene phylogeny. Phylogenetic tree inferred from Maximum Likelihood analysis. Only bootstrap values ≥50% are shown.

while vesicles of isolates belonging to the *A. felis* clade were subclavate (15–17 μm) and isolates closely related to *A. udagawae* exhibited hemispherical to flask-shaped vesicles (11–16 μm). The width of conidiophore stipes in *A. fumigatiaffinis* ranged from 6 to 8 μm, while in isolates belonging to the *A. felis* clade from 5 to 9 μm and in the isolates closely related to *A. udagawae* from 4 to 6 μm. All these isolates were able to grow at 10 °C and unable to grow at 50 °C.

All isolates were deposited at Szeged Microbiological Collection of the Department of Microbiology, Faculty of Science and Informatics, University of Szeged, Hungary under the assigned numbers KS695–KS708.

Discussion

In a vast compilation of studies on *Aspergillus* species in soil it was found that *Aspergillus* species most frequently occur in subtropical zones, between 25 and 35 degrees N/S[22,23,29]. Considering that the area studied is included in those latitudes, the *Aspergillus* spp. frequencies found probably ratify that assertion.

Section *Fumigati* was represented in both eco-regions studied, although with different species distributions. *A. fumigatus s.s.* and *A. fumigatiaffinis* were the most frequent species, followed by isolates closely related to *A. felis* and *A. udagawae*. However, unlike the other species, *A. fumigatus s.s.* occurred in both areas, confirming its capability of adaptation and ubiquity. *A. fumigatiaffinis* and isolates closely related to *A. udagawae* and belonging to the *A. felis* clade were isolated for the first time from Argentinian soils.

A. fumigatiaffinis was only isolated in Talampaya Park. The climatic characteristics and height above mean sea level of Talampaya Park are similar to those of Socorro city (USA), where *A. fumigatiaffinis* was reported for the first time[18].

The presence of two isolates closely related to *A. udagawae* only in Pampa de Achala provides more data about the plasticity of this species because, even though it was described in Brazil in a humid subtropical area, it has also been identified as infecting humans and domestic animals in areas with extreme environments[23,26,27,42].

A. felis is an important species in *Aspergillus* section *Fumigati* described recently[10]. *A. felis* (neosartorya-morph) is phenotypically similar to *A. viridinutans*; however, it differs by its ability to grow at 45 °C and is phylogenetically related to *A. aureolus* and *A. udagawae*[27]. In our study, the isolates belonging to the *A. felis* clade were only isolated in Pampa de Achala. Although *A. felis* was isolated in soils of Wyoming, USA, this is the first report on the ambient occurrence of a species of this clade in the South American continent[34].

Table 1 *calM* and *benA* sequences of type isolates of section *Fumigati* used for phylogenetic analysis

Species[a]	Reference strain	*calM*[b]	*benA*[b]	Species	Reference strain	*calM*[b]	*benA*[b]
A. udagawae	CBM FA-070^T	AB748566.1	AF132226.1	*A. parafelis*	CM 3147^T	KJ914702.1	KJ914692.1
A. aureolus	NRRL 224^T	EF669877.1	–	*A. pseudofelis*	CM 4518	–	KJ914696.1
A. wyomingensis	CCF 441^T	HF933397.1	–	*A. pseudofelis*	CM 6087^T	KJ914705.1	KJ914697.1
A. felis	CBS 13024^T	JX021715.1	JX021700.1	*A. siamensis*	KUFC 634^T	AB776704.1	–
A. viridinutans	IMI 36741^T	DQ534162.1	–	*A. novofumigatus*	IBT 1680^T	DQ094893.1	–
A. arcoverdensis	IFM 6133^T	AB818856.1	–	*A. fumigatiaffinis*	IBT 1270^T	DQ094891.1	–
A. takakii	CBM FA-88^T	AB787566.1	–	*A. fumisynnematus*	IFM 4227^T	AB259968.1	–
N. paulistensis	CBM FA-069^T	AB488766.1	–	*A. lentulus*	NRRL 3555^T	EF669895.1	–
A. laciniosus	UAMH 11627^T	JX845619.1	–	*A. fischeri*	NRRL 18^T	EF669865.1	–
N. coreana	KACC 4165^T	AY870718.1	–	*A. fumigatus*	NRRL 5587^T	EF669922.1	–
A. spinosus	NRRL 503^T	EF669914.1	–	*A. fennelliae*[*]	NRRL 553^T	EF669920.1	EU014108.1
A. pseudoviridinutans	NIH AV 1^T	KJ914708.1	KJ914690.1				

[a] Obtained from the database National Center for Biotechnology Information.
[b] GenBank accession number for calmodulin (*calM*) and β-tubulin (*benA*) gene.
[*] Selected species as outgroup. A.: *Aspergillus*; N.: *Neosartorya*.

Table 2 Frequency of *Aspergillus* section *Fumigati* isolated and pH of soil samples

Soil samples	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	t	P1	P2	P3	P4	P5	P6	*t*
pH	9.64	9.06	9.02	8.78	8.94	8.30	8.00	9.27	9.52	9.52	9.83		5.65	5.61	5.36	5.04	4.91	5.30	
Section *Fumigati*																			
Aspergillus felis clade												0	X				X	X	3
Aspergillus fumigatiaffinis	X	X	X	X	X		X	X	X	X		9							0
Aspergillus fumigatus s. s.		X	X			X					X	4		X	X	X	X	X	5
Aspergillus udagawae												0				X		X	2
Total species												13							10

T: Talampaya National Park; P: Pampa de Achala; t: total.

Aspergillus is a large genus of ubiquitous and cosmopolitan fungi. Their high adaptability allows them to survive at different temperatures, low water activity and variations of pH and O_2 concentration in soil[29,39,45]. This could be an explanation of the isolation of these strains in the extreme environments studied.

Fungi can tolerate a wide pH range[39]. The available evidence for the fungal growth–pH relationship thus indicates a significantly weaker direct connection with pH than in the case of bacteria, although pure culture studies have shown preference for certain pH values for different taxa of soil fungi[12,39,45]. In our study, *A. fumigatiaffinis* was isolated only from alkaline soils of Talampaya Park. In contrast, the isolates closely related to *A. felis* and *A. udagawae* were isolated only from Pampa de Achala where the soil pH was slightly acid. Further investigations are essential for correlating the preference for certain pH values for these taxa in order to allow definitive conclusions.

Even though *A. fumigatus* is the most prevalent agent of aspergillosis, several other species of section *Fumigati* have also been reported from clinical samples as causative agents of disease: *A. lentulus*[7], *A. udagawae*[16,26,42], *A. felis*[9,10,44] and *A. fumigatiaffinis*[4]. In Argentina, a recent work with clinical isolates shows the circulating *Aspergillus* section Fumigati species. *A. fumigatus* s.s. was the most frequent followed by *A. udagawae*[17].

Furthermore, these species show high *in vitro* MICs of azole drugs and amphotericin B, therefore they are frequently refractory to standard antifungal therapy[4,8].

In this work, morphological and physiological characteristics were useful for differentiating isolates belonging to section *Fumigati* from those belonging to other sections. However, sequence-based methods are needed to assign isolates of section *Fumigati* at the species level. In this study, the species identification of isolates related to *A. udagawae* (IMR-MF 706 and IMR-MF 708) and to *A. felis* (IMR-MF 697, IMR-MF 703, IMR-MF 707) was not clear using only the partial *calM* sequences. The results of the analysis of partial *benA* sequences were necessary to suggest that IMR-MF 706 and 708 isolates were closer to *A. udagawae* and to the others isolate belonging to *A. felis* clade.

Identification of environmental and clinical isolates by molecular techniques is common practice in European countries and in the USA[4,6,18,20–22,34,40,42,43,46] but there is scant information in South America[5,11,13,17,33,35,36].

In medical mycology, as molecular methods become more available, they will allow the accurate identification of fungal infectious agents. The correct identification of species within section *Fumigati* could help to predict the severity of the disease and guide antifungal therapy.

The present work was carried out as a contribution to the knowledge of the ecology of section *Fumigati*, to understand where this section occurs in nature due to the increasing importance of these opportunistic pathogens.

Ethical responsibilities

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interest

The authors have no conflict of interest to declare.

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