Diversity of airborne Cladosporium *species isolated from agricultural environments of northern Argentinean Patagonia: molecular characterization and plant pathogenicity*

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Diversity of airborne *Cladosporium* species isolated from agricultural environments of northern Argentinean Patagonia: molecular characterization and plant pathogenicity

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Abstract Fungal spores are normal components of external environments. They have been reported to be associated with human, animal and plant diseases causing primary and opportunistic infections. *Cladosporium* is commonly the most frequently isolated genus from air samples, and its species are considered mainly saprophytic, but there is a wide variety of taxa that can cause adverse effects on human and animal health and also on plants. In this work, we aimed to record *Cladosporium* spores frequency of isolation in northern Argentinean Patagonia and to perform a molecular characterization based on actin gene complemented with EF1α and ITS genes. The ability of the pathogens to cause disease in pip fruits of Packham's Triumph and Abate Fetel pears and red delicious apples was determined. Results confirmed *Cladosporium* spores as the main genera isolated from air samples, and the molecular characterization revealed the existence of 11 species in this region grouped in *C. cladosporioides* and *C. herbarum* complexes. Pathogenicity tests revealed that *Cladosporium* sp. cause disease on fruit. Abate Fetel was the most susceptible to infection. These results compose the first study in Argentina in respect of identification at species level of airborne *Cladosporium* spores, and furthermore, it is the first report in northern Patagonia and the high valley of Río Negro productive region. This knowledge can help farmers to take preventive measures in order to avoid biological damage.

Keywords *Cladosporium* · Airborne · Molecular characterization · Northern Patagonia

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1 Introduction

Argentina is mainly an agricultural country, and the province of Río Negro, in northern Patagonia, is the first producer and exporter of pip fruits in the nation. This province possesses a valley which begins at the confluence of the rivers Limay and Neuquén where the Río Negro River originates. This valley splits in three different zones through its path: high valley, middle valley and lower valley. The high valley region is situated between 38°40′S and 39°20′S and 65°50′W and 68°20′W (Rodríguez and Muñoz 2006).

Fungi are one of the most common microorganisms in air. They are considered to be related to air pollution

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and have been proposed to cause adverse effects to human, animal (Robbins et al. 2000; Lee et al. 2006) and plant health (Cvetnic and Pepelnjak 1997; Fang et al. 2005; Abdel Hameed et al. 2009). Aeromycological studies in our country are scarce, being none existent with respect to the high valley region. Besides, most of the studies in Argentina have been carried out in external and internal environments in relation to human health, but few have focused on agricultural environments in relation to possible plant diseases to our knowledge.

Cladosporium is usually the most frequently isolated genus of aerial samples in many parts of the world, reaching values between 30 and 85% of isolated spores (Mullins 2001; Sindt et al. 2016). *Cladosporium* spores have been investigated in several countries including Spain and Portugal (Kulko and Marfenina 2001; Mitakakis and Guest 2001; Shelton et al. 2002; Grinn-Gofron et al. 2011; Aira et al. 2012), Turkey (Erkara et al. 2008; Artac et al. 2009), France (Sindt et al. 2016); and Saudi Arabia (Alhussaini et al. 2015), but *Cladosporium* species in air samples in Argentina have not been investigated.

This genus is one of the largest and most heterogeneous both morphologically and phylogenetically (Sandoval-Denis et al. 2015), and it has been reported to have over 700 different taxa (Dugan et al. 2004). Therefore, it is necessary to examine its diversity, phylogeny and taxonomy in order to achieve an updated and modern revision. Bensch et al. (2012) carried out a taxonomic study of this genus based on morphological and molecular analysis. As a result 169 taxa were included in *Cladosporium* genus after a reexamination of 993 names under study repositioning the rest in other genera.

On the other hand, several preliminary studies led to the introduction of subgenera due to the discovery of three heterogeneous complexes: *C. herbarum* (Schubert et al. 2007), *C. sphaerospermum* (Zalar et al. 2007; Dugan et al. 2008) and *C. cladosporioides* (Bensch et al. 2010), each of them composed by species that are distinguishable morphologically and genetically. Species showing clear morphological differences possibly belong to different species complexes and even genetically they are usually distinct and form separate clusters (Braun 2003; Bensch et al. 2012). In order to identify and distinguish closely related species with similar morphological characteristics, molecular sequence analysis seems to be a useful method. However, ITS analysis is often not sufficient to differentiate taxa closely related and morphologically similar within complexes. In fact, a few authors choose to perform a multilocus DNA sequence approach based on ITS, actin, calmodulin, translation elongation factor 1-α and histone H3 because they provide a higher resolution and a better discrimination of closely allied species (Schubert et al. 2007; Bensch et al. 2010).

Cladosporium includes many saprophytic species isolated from soil and plant materials, and some others are plant pathogens affecting mainly post-harvest processes (Tournas 2005; Aira et al. 2012) and animals, including humans causing allergies and opportunistic infections (Sandoval-Denis et al. 2015). There are also some species that can biodegrade some aromatic compounds in industry (Alhussaini et al. 2015).

In this study, due to the fact that fruit culture and its exportation are the main economic activity we focus on the ability of *Cladosporium* species to produce plant pathogenicity in pip fruits. Some researchers remark the ability of *C. herbarum* complex species to cause side rots that become problematic in pears stored for long periods (Sugar and Powers 1986; Benbow and Sugar 1999). A local study carried out in the high valley region reported decays by *Alternaria* spp. and *Cladosporium* spp. on "Bosc" pear fruits during cold storage (Lutz et al. 2016). In Korea, a high frequency of *Cladosporium* from diseased tissues of Asian pears showing skin sooty dapple disease was reported, forming a microbial complex with other microorganisms (Park et al. 2008).

Considering the absence of aeromycological studies in the high valley producing region of Argentinean northern Patagonia, the aim of this work was to identify and determine the pathogenicity of *Cladosporium* species in agricultural environments of this zone in order to take preventive measures and thus avoid biological damage and economical losses.

2 Materials and methods

2.1 Sampling area

Eight rural establishments were selected for sampling. Four of them are located in the eastern part of the valley and the other four in the central zone. All of

them present the espalier production system for pip fruits, currently the most widespread in the region. Within each establishment, samples were taken by duplicate at the geographical points that make up the vertices of a triangle, once for each season of the year during the period 2014–2016.

2.2 Sampling method

Sampling was carried out using a microflow air sampler (Microflow α 90 Aquaria version 3.0.0 cod. G.1015). In this equipment, the air is drawn through a perforated autoclavable anodized aluminum head, with 380 holes (Ø 1 mm), at constant velocity (30–60–90–100–120 L/min) and for a specific period of time depending on the volume to be sampled (1–2000 L). The inflow air impacts in the surface of the culture media placed in 90-mm disposable Petri dishes containing potato dextrose agar (PDA) medium supplemented with Chloramphenicol (0.1 g/L) to inhibit bacteria development (Bueno et al. 2003; Muhsin and Adlan 2012) and dichloran (2 mg/L) to limit fungal colonies growth. Two volumes were used to take samples: 50 and 100 L at the lowest sample rate, 30 L/min. These parameters were chosen after several trials on field which threw fungal counts between 10 and 100 CFU/plate suitable for counting and further isolation and identification. The sampler was placed at a height of 1.50 m over ground level (Al-Doory and Domson 1984; Aríngoli et al. 2008), on a photographic tripod and the head of the equipment was cleaned with iso-propanol between each sampling. A positive hole conversion table was not used to obtain data with this microflow air sample.

Isolation and morphological identification: plates obtained after sampling were incubated at 25 °C for 5 days (Verhoeff et al. 1990; Aríngoli et al. 2008) and were used for differential count of fungal genera after microscopic observation which was carried out according to Samson et al. (2000) and Pitt and Hocking (2009). Concentrations were calculated as colony-forming units per cubic meter of air (CFU/m^3). To obtain pure cultures of *Cladosporium* isolates for further tests, pieces of mycelia were aseptically taken and placed on individual 60-mm plates with malt extract agar (MEA) and then incubated as previously mentioned. For specie identification, 397 isolates were randomly selected and grouped according to the morphological characteristics of the colonies based on mycelia color and diameter, texture, furcation and other general observations of the colony appearance. Moreover, they were observed microscopically in order to classify them between the three major complexes. Then, 59 isolates representative of all the morphological groups obtained were proportionally and randomly chosen to perform molecular tests.

2.3 Molecular methods

2.3.1 Mycelia harvest

Pieces of mycelium from each isolate were aseptically taken and placed into a penicillin vial containing 5 mL of YES medium (sucrose 15% and yeast extract 2%). Vials were incubated in an orbital shaker at 100 rpm at 25 °C for 48–96 h until observation of a mycelium layer free of spores. Then, mycelia were dried with Whatman No. 1 filter paper, weighed and stored in Eppendorf tubes at − 75 °C until use.

2.3.2 DNA extraction

DNA extraction was performed with the DNeasy Plant Mini Kit following manufacturer's instructions (Qiagen, Intl) with a previous rupture with liquid nitrogen. Genomic DNA was quantified with the fluorimeter Qubit 2.0 (Life Technologies, Intl.).

2.3.3 PCR amplifications and sequencing

To obtain resolution at species level, partial gene sequences of the actin gene (ACT) were obtained through PCR amplification and sequenced to identify the isolates. This gene was supplemented with the translation elongation factor 1-α gene (EF1α) and the internal transcribed spacer (ITS) of nuclear ribosomal DNA.

PCR amplifications for ACT gene and EF1α gene were performed using primers ACT-512F: ATGTGCAAGGCCGGTTTCGC and ACT-783R: TACGAGTCCTTCTGGCCCAT for actin gene and primers EF1-728F: CATCGAGAAGTTCGAGAAGG and EF1-986R: TACTTGAAGGAACCCTTACC for EF1α gene (Carbone and Kohn 1999; Bensch et al. 2012). Reactions contained a total volume of 40 µL with 20 ng of DNA template, 1× PCR buffer, 0.5 µM of each primer (GBT Oligos, Buenos Aires), 0.2 µM of each dNTP, 2 mM MgCl2,

1%, DMSO and 0.5 U of Taq DNA polymerase (Thermo Fischer Scientific, Invitrogen, Argentina S.A.). Reaction tubes were placed in a Multigene Thermal Cycler (Labnet International Cycler) with the following running conditions: initial denaturation step at 95 °C for 8 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C for 30 s and extension at 72 °C for 1 min, with a final extension step at 72 °C for 5 min. Water was used as negative control.

PCR amplifications for internal transcribed spacer (ITS) of nuclear ribosomal DNA were performed with ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4: 5′-TCCTCCGCTTATTGATATGC-3′ primers (White et al. 1990). Reactions contained a total volume of 40 µL with 20 ng of DNA template, 1× PCR buffer, 1 µM of each primer (GBT Oligos, Buenos Aires), 0.2 µM of each dNTP, 1.5 mM $MgCl_2$, 1% DMSO and 1 U of Taq DNA polymerase (Thermo Fischer Scientific, Invitrogen Argentina S.A.). Reaction tubes were placed in a Multigene Thermal Cycler (Labnet International Cycler) with the following running conditions: initial denaturation step at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min, with a final extension step at 72 °C for 5 min. Water was used as negative control.

Amplicons were visualized under UV light after running 10 µL of each reaction on 1% agarose gel with Gel Red. Sequencing of fragments was done with ACT-512F primer EF1-728F primer and ITS1 primer by Macrogen Inc. (Seoul, Korea).

2.3.4 Phylogenetic analysis

The resulting sequences were compared with other fungal DNA sequences from NCBI's GenBank sequence database using a blast search to identify species. Phylogenetic analyses were run using the MEGA 7 program package (Kumar et al. 2016), and *Cladosporium* strain reference sequences used were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The species strain numbers, and the ACT, EF1α and ITS sequences with their respective GenBank accession numbers are indicated in Table 1. The sequence of *Cercospora beticola*, strain CBS116456, was used as outgroup in the analyses to root the trees (Bensch et al. 2012). The isolates nucleotide sequences were aligned all together with the reference sequences using the Muscle method (Edgar 2004), and when necessary they were adjusted manually. The phylogenetic study was performed using the maximum likelihood analyses with Kimura 2 parameters using gamma distribution model and treating gaps, and missing data as partial deletion (Greco et al. 2015). The bootstrap values were generated with 1000 replicates, and they were considered significant when they were higher than 85%. The ACT alignment consisted of 161 and the concatenated alignment of ACT, EF1α and ITS of 736 nucleotide positions.

2.3.5 Storage of isolates/strains

Pure isolates of each *Cladosporium* species were maintained on solid MEA at 4 °C and stored in glycerol 18% v/v at − 75 °C for long-term storage.

2.3.6 Pathogenicity tests

Twenty-six molecularly identified isolates were used to perform pathogenicity tests: 3 *C. cladosporioides*, 3 *C. pseudocladosporioides*, 3 *C. macrocarpum*, 3 *C. asperulatum*, 3 *C. limoniforme*, 3 *C. aggregatocicatricatum*, 2 *C. tenellum*, 1 *C. ramotenellum*, 1 *C. subtilissimum* and 4 *C.* sp. The pathogenicity and host specificity for each of the 26 isolates were determined on the following fruit types and varieties: Packham's Triumph and Abate Fetel pears and red delicious apples. The tests were performed immediately after harvest, using the toothpicks technique (Andersen et al. 2002). Toothpicks split into halves were autoclaved five times in distilled water and once in MEA broth. Once sterile, they were placed in pairs in 90-mm-diameter Petri dishes containing medium MEA supplemented with chloramphenicol (0.1 g/L). Plates were inoculated at the center of each pair with each of the strains to be evaluated and incubated at 25 °C for 15–21 days. Fruits were disinfected superficially submerging them in a hypochlorite solution (10%) for 5 min and rinsed twice with sterile water in laminar flow. Sterile fruits were inoculated by introducing three toothpicks into the fruit at a depth of approximately 8 mm, two of them colonized by each *Cladosporium* isolate and one free of fungal growth, as

Table 1 Actin, EF1α and ITS sequences used in the phylogenetic analyses

Species	Strain	GenBank nucleotide accession number		
		Actin	EF1α	ITS
Cercospora beticola	CBS:116456	AY840458	AY840494	NR121315
Cladosporium aggregatocicatricatum	CPC:14709	KT600645		
	CBS:113151	KT600646		
	14ACT	MG680498		
	36ACT	MG680499		
	37ACT	MG680500		
Cladosporium allicinum	CBS:121624	EF679502	EF679425	EF679350
	CPC:11840	EF679497	EF679420	EF679345
	21ACT	MG680501		
Cladosporium asperulatum	CBS:126340	HM148485		
	5ACT	MG680502		
	15ACT	MG680503		
	16ACT	MG680504		
	27ACT	MG680505		
	39ACT	MG680506		
	50ACT	MG680507		
	51ACT	MG680508		
	58ACT	MG680509		
	59ACT	MG680510		
Cladosporium cladosporioides	CPC:11121	HM148505		
	CPC:11123	HM148507		
	CPC:13978	HM148551		
	MUT ITA 4985	KU315007		
	MUT ITA 1729	KU935615		
	UTHSC DI-13-209	LN834543		
	1ACT	MG680511		
	19ACT	MG680512		
	20ACT	MG680513		
	23ACT	MG680514		
	25ACT	MG680515		
	26ACT	MG680516		
	29ACT	MG680517		
	42ACT	MG680518		
	43ACT	MG680519		
	46ACT	MG680520		
	47ACT	MG680521		
	52ACT	MG680522		
	56ACT	MG680523		

Table 1 continued

Species	Strain	GenBank nucleotide accession number		
		Actin	EF1α	ITS
Cladosporium limoniforme	CBS:113737	KT600591		
	CPC:12039	KT600592		
	18ACT	MG680524		
	24ACT	MG680525		
	40ACT	MG680526		
	41ACT	MG680527		
	45ACT	MG680528		
	49ACT	MG680529		
	55ACT	MG680530		
Cladosporium macrocarpum	CPC:12757	EF679532		
	UTHSC DI-13-191	LN834563		
	6ACT	MG680531		
	11ACT	MG680532		
	12ACT	MG680533		
Cladosporium pseudocladosporioides	CPC:11841	HM148657		
	CPC:14010	HM148671		
	CPC:14020	HM148674		
	2ACT	MG680534		
	3ACT	MG680535		
	4ACT	MG680536		
	22ACT	MG680537		
	30ACT	MG680538		
Cladosporium ramotenellum	CBS:109031	KT600615		
	CPC:13789	KT600629		
	17ACT	MG680539		
Cladosporium sinuosum	CBS:121629	EF679450	EF679464	EF679386
	CPC:17632	KT600642	KT600544	KT600445
Cladosporium sphaerospermum	CPC:13995	EU570273		
	CPC:14016	EU570274		
Cladosporium subtilissimum	CBS:17252	EF679544		
	CBS:113741	EF679545		
	7ACT	MG680540		
	13ACT	MG680541		
	28ACT	MG680542		
	44ACT	MG680543		
	48ACT	MG680544		
	53ACT	MG680545		
Cladosporium tenellum	CPC:11813	EF679553		
	CPC:12051	EF679554		
	31ACT	MG680546		
	33ACT	MG680547		

Table 1 continued

Species	Strain	GenBank nucleotide accession number		
		Actin	EF1α	ITS
Cladosporium sp.	8ACT	MG680548	MG686620	MG702494
	9ACT	MG680549	MG686621	MG702495
	32ACT	MG680550	MG686622	MG702496
	57ACT	MG680551	MG686623	MG702497
	34ACT	MG680552	MG686624	MG702498
	35ACT	MG680553	MG686625	MG702499
	10ACT	MG680554	MG686626	MG702500
	54ACT	MG680555	MG686627	MG702501
	38ACT	MG680556	MG686628	MG702502

control. Then, they were individually placed in transparent plastic bags and incubated at 25 °C for 14 days. After the incubation period, the external and internal lesions of the fruits were observed. Pathogenicity was determined according to the diameter of the lesion and confirmed according to the Koch postulates. Host specificity was determined according to the ability of the strain to produce the lesion or not.

3 Results

3.1 Relative frequencies of isolation

Total fungal counts for each season of both years of sampling were considered to calculate relative frequencies of the genus. In the first year of sampling, total fungal count was 2.41×10^4 CFU/m^3 in autumn, 1.79×10^4 CFU/m^3 in winter, 2.45×10^4 CFU/m^3 in spring and 1.01×10^5 CFU/m^3 in summer. *Cladosporium* isolates count for each season and their relative frequencies of isolation were as follows: 1.67×10^4 CFU/m^3 (69.13%) in autumn, 1.37×10^4 CFU/m^3 (76.61%) in winter, 1.89×10^4 CFU/m^3 (77.19%) in spring and 8.72×10^4 CFU/m^3 (85.98%) in summer. Total fungal count on the second year of sampling was 5.84×10^4 CFU/m^3 in autumn, 2.33×10^4 CFU/m^3 in winter, 8.58×10^3 CFU/m^3 in spring and 4.39×10^4 CFU/m^3 in summer. *Cladosporium* isolates count for each season and their relative frequencies of isolation were as follows: 4.46×10^4 CFU/m^3 (76.42%) in autumn, 1.72×10^4 CFU/m^3 (74.03%) in winter, 5.62×10^3 CFU/m^3 (65.5%) in spring and 2.81×10^4 CFU/m^3 (64.0%) in summer. Average and SD values are shown in Table 2.

3.1.1 *Molecular characterization and phylogenetic analysis*

The sequences of the studied isolates were 100% identical to GenBank type strain reference sequences except for 9 isolates (8 ACT, 9 ACT, 10 ACT, 32 ACT, 34 ACT, 35 ACT, 38 ACT, 54 ACT and 57 ACT) which shared at most 94% of homology with *C. allicinum* and/or *C. sinuosum* type strain reference sequences. The phylogenetic analysis based on the actin gene fragment resulted in discrimination between the isolates included in this study, and the three major *Cladosporium* complexes and all the terminal species clades were strongly supported having bootstrap values higher than 85. As a result, 45.76% of the isolates were nested into the *C. cladosporioides* complex, where 13 isolates were identified as *C. cladosporioides*, 5 isolates as *C. pseudocladosporioides* and 9 isolates as *C. asperulatum*. The remaining 54.24% of the isolates belonged to the *C. herbarum* complex where 8 isolates were identified as *C. limoniforme*, 5 isolates as *C. subtilissimum*, 3 isolates as *C. aggregatocicatricatum*, 3 isolates as *C. macrocarpum*, 2 isolates as *C. tenellum*, 1 isolate as *C. allicinum* and 1 isolate as *C. ramotenellum*. The lasting 9 isolates formed part of three terminal clades which were distant phylogenetically from any species of the genus currently known (Fig. 1). These nine species were then tested with EF1α and ITS genes in order to achieve taxa identification. However, blast results for each gene threw a 100% of homology with several type strain reference sequences and phylogenetic trees based on each gene did not show species resolution (data not shown).

Table 2 Average and standard deviation values for total fungal counts and *Cladosporium* isolates counts of the first and second year of sampling

Season	1st year of sampling				2nd year of sampling			
	Total fungal count (CFU/m^3)		*Cladosporium* isolates count (CFU/m^3)		Total fungal count (CFU/m^3)		*Cladosporium* isolates count (CFU/m^3)	
	Average	SD	Average	SD	Average	SD	Average	SD
Autumn	2.41×10^4	$\pm 1.70 \times 10^2$	1.67×10^4	$\pm 4.79 \times 10^2$	5.84×10^4	$\pm 8.34 \times 10^2$	4.46×10^4	$\pm 8.77 \times 10^2$
Winter	1.79×10^4	$\pm 6.08 \times 10^2$	1.37×10^4	$\pm 1.26 \times 10^2$	2.33×10^4	$\pm 8.49 \times 10^2$	1.72×10^4	$\pm 1.06 \times 10^3$
Spring	2.45×10^4	$\pm 8.63 \times 10^2$	1.89×10^4	$\pm 8.20 \times 10^2$	8.58×10^3	$\pm 1.72 \times 10^2$	5.62×10^3	$\pm 5.37 \times 10^2$
Summer	1.01×10^5	$\pm 9.31 \times 10^2$	8.72×10^4	$\pm 1.09 \times 10^3$	4.39×10^4	$\pm 8.63 \times 10^2$	2.81×10^4	$\pm 8.34 \times 10^2$

Therefore, we proceeded to carry out a concatenated study using the sequences from the three genes and results aided to support those obtained with actin gene phylogenetic analysis (Fig. 2). Thus, these isolates are outlined to be part of three different new species. Nevertheless, further phenotypical and molecular analysis must be conducted in order to confirm their identity.

3.1.2 *Morphological characterization and Cladosporium species frequencies of isolation*

Prior to molecular identification, characterization based on macroscopical features of the colonies grouped the 397 isolates in 25 different morphological groups which after the molecular analysis were reduced into 13 groups (Fig. 3). This morphological identification was carried out according to the key proposed by Bensch et al. (2012). The 397 isolates were identified at species level according to their morphological group belonging and respective molecular characterization. As a result, 58.68% of the species belonged to the *C. cladosporioides* complex in which isolates were identified as *C. cladosporioides* (35.01%), *C. asperulatum* (17.88%) and *C. pseudocladosporioides* (5.79%). The remaining 41.32% were nested into the *C. herbarum* complex where isolates were identified as *C. limoniforme* (25.44%), *C. subtilissimum* (6.05%), *C. aggregatocicatricatum* (2.02%), *C. tenellum* (1.51%), *C. macrocarpum* (1.51%), *C. allicinum* (1.01%), *C. ramotenellum* (0.25%) and *C.* sp. (3.53%).

3.1.3 *Pathogenicity*

On pears of Packham's Triumph variety, only 4 isolates caused lesion on healthy fruits (15.4%); 2 of them belonged to *C. pseudocladosporioides* species (7.7%) and the other two to *C. asperulatum* species (7.7%). The external appearance of lesions is difficult to appreciate due to the absence of a necrotic area on the tissue and the lack of a color different from the natural skin color of the fruit. A necrotic point at the inoculation site with a maximum extent of 0.5 cm could only be observed. Only one isolate of *C. asperulatum* showed fungal growth on the external tissue and caused a big necrotic patch which turned brown to blackish (Fig. 4a). Internal injuries consisted of a black or brownish necrosis which extended from 0.5 to 1.8 cm from the inoculation point. It also produced a hardening of the tissue at the necrotic patch. One isolate of *C. pseudocladosporioides* and one of *C. asperulatum* caused hollowing of the inner tissue (Fig. 4b).

On pears of Abate Fetel variety, six fruits did not exhibit any lesion in the external or internal tissues (15.6%). Seventeen fruits presented external symptoms (65.4%) with diameters ranging from 0.6 to 1.5 cm caused by isolates of *C. cladosporioides*, *C. pseudocladosporioides*, *C. asperulatum*, *C. aggregatocicatricatum* and *C. ramotenellum*. In all of them, a drying zone covering the diameter of the lesion was detected and it was generally colorless except for one of *C. asperulatum* isolate which caused a brown necrosis halo and for *C. cladosporioides*, *C. pseudocladosporioides* and *C. asperulatum* isolates which presented black necrosis in 4 fruits at the inoculation point (Fig. 4c). Moreover, *C. cladosporioides*, *C. pseudocladosporioides*, *C. asperulatum*, *C.*

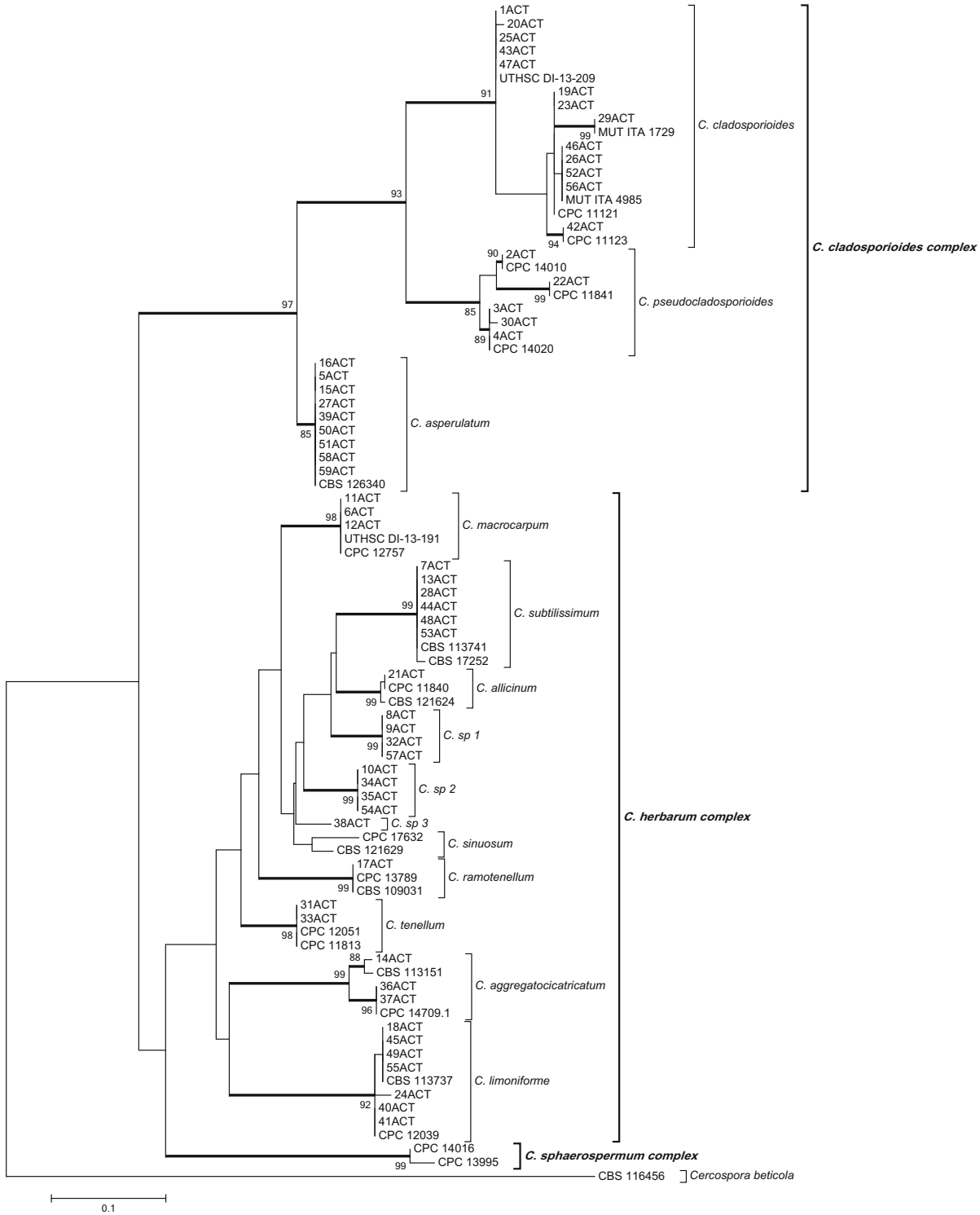

Fig. 1 Maximum likelihood phylogenetic tree based on the ACT gene displaying the analysis of the 59 *Cladosporium* isolates obtained from air samples altogether with type strain reference sequences. *Cercospora beticola* (CBS:116456) was used as outgroup to root the tree. The thickened lines represent lineages with > 85% bootstrap values

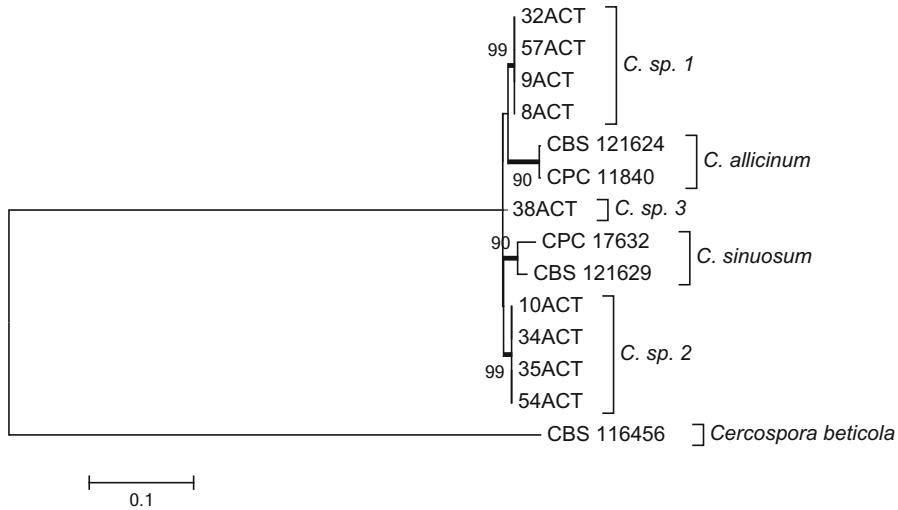

Fig. 2 Maximum likelihood phylogenetic tree based on the concatenated analysis of ACT, EF1α and ITS genes displaying the analysis of the 59 *Cladosporium* isolates obtained from air samples altogether with type strain reference sequences. *Cercospora beticola* (CBS:116456) was used as outgroup to root the tree. The thickened lines represent lineages with > 85% bootstrap values

aggregatocicatricatum and *C. ramotenellum* isolates showed mycelial development in six fruits. Three of the fruits tested did not show any kind of external symptoms (11.54%). On the other hand, 16 fruits presented inner lesions consisting of a black necrosis caused by isolates of all of the species tested (61.54%) (Fig. 4d). Only one *Cladosporium* sp. isolate showed a brown necrosis of the inner tissue (3.85%). The diameters of lesions ranged from 0.8 to 3 cm of injury. Only two isolates of *C. cladosporioides* and *C. pseudocladosporioides* presented mycelium development with hollowing of the tissue. Every lesion observed whether it was necrotic or hollowed, exhibited drying characteristics of the compromised area and hardening of the tissue. Nine fruits did not exhibit any kind of injury (34.61%).

On the other hand, red delicious apple was resistant to the 26 isolates tested.

4 Discussion

These results compose the first study in Argentina in respect of identification at species level of airborne *Cladosporium* spores. Furthermore, it is the first report in northern Patagonia and high valley of Río Negro productive region. Fungal counts obtained along the 2 years of sampling showed and confirmed *Cladosporium* spores as the main genera isolated from air samples, as other authors have previously cited (Mullins 2001; Sindt et al. 2016).

Sandoval-Denis claims that morphological identification of *Cladosporium* species is difficult due to the high morphological similarity between closely related species (Sandoval-Denis et al. 2015). This was confirmed while morphological group classification was carried out. Moreover, we have also observed morphological variability within each species. This is reflected by the reduction in species number from the 25 morphological different groups obtained by the macroscopical analysis to the 11 species confirmed by molecular techniques.

On the other hand, while this genus has been reported to be phylogenetically very heterogeneous (Sandoval-Denis et al. 2015), the phylogenetic analysis carried out in this work based on the actin gene proved its efficiency to discriminate between *Cladosporium* species as other authors reported (Schubert et al. 2007; Bensch et al. 2010; Sandoval-Denis et al. 2015). However, it was not possible to identify 15% of the isolates tested. These were grouped in two clades and one monotypic lineage and they may probably represent three new species. However, this must be confirmed by further studies combining morphological with molecular studies.

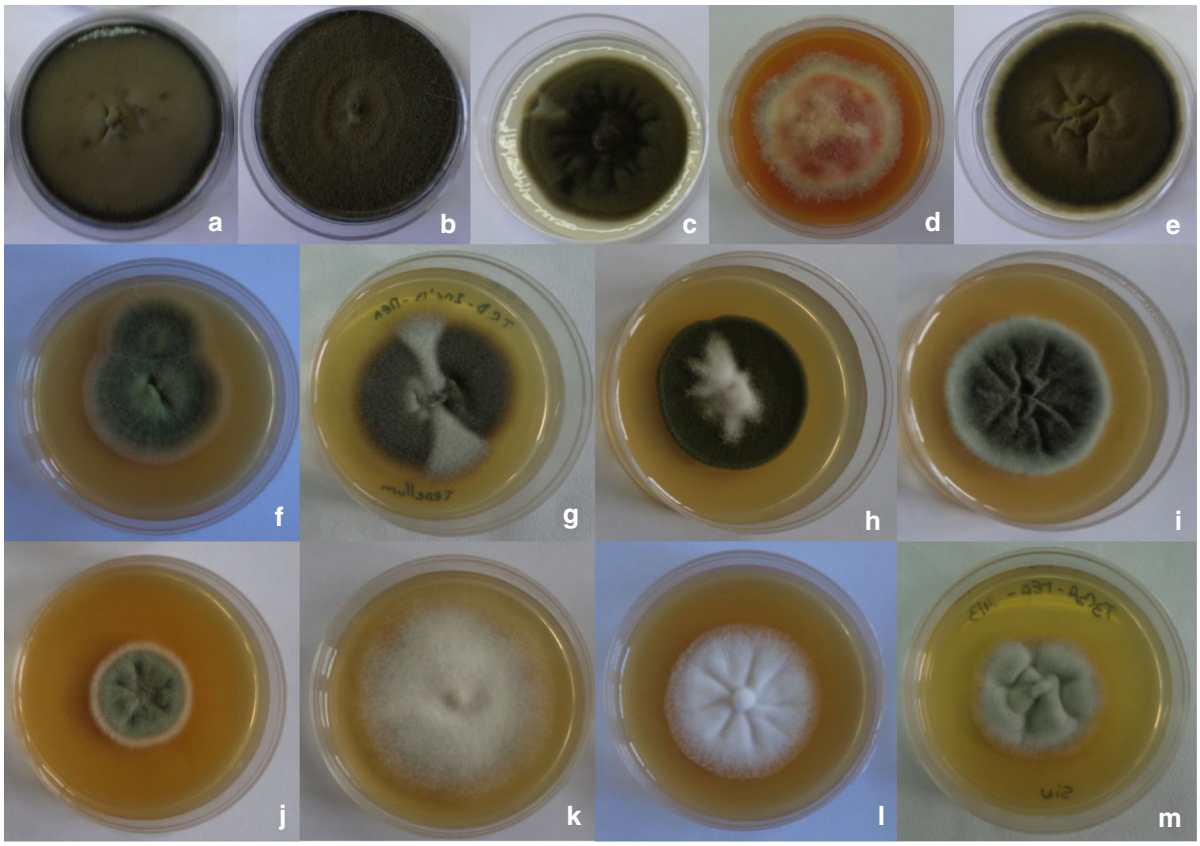

Fig. 3 *Cladosporium* species morphological classification: The figure shows macroscopical characteristics of the 13 species group found after the molecular analysis. **a** *C. cladosporioides*; **b** *C. pseudocladosporioides*; **c** *C. asperulatum*; **d** *C. macrocarpum*; **e** *C. limoniforme*; **f** *C. ramotenellum*; **g** *C. tenellum*; **h** *C. aggregatocicatricatum*; **i** *C. allicinum*; **j** *C. subtilissimum*; **k** *C.* sp. 1; **l** *C.* sp. 2; **m** *C.* sp. 3

Molecular characterization thus revealed the existence of eleven identified species in this region. Isolates from *C. cladosporioides* and *C. herbarum* complexes coexist in similar proportions, though *C. cladosporioides* complex species seem to be slightly more abundant and with a more restricted variety of species. However, none of the isolates identified in this study belonged to the *C. sphaerospermum* complex. Considering that the northern Patagonia region exhibits continental climatological characteristics (Rodríguez and Muñoz 2006), the absence of isolates belonging to this complex is consistent with Schubert et al. (2007) and Bensch et al. (2012) who associate the coexistence of these species to extreme ecological environments.

Regarding species predominance, spores of *C. cladosporioides* species are among the most ubiquitous ones found in outdoor samples (Domsch et al. 1980; Mullins 2001; Park et al. 2004; Bensch et al. 2012). Our results confirm *C. cladosporioides* as the main species found in environmental samples followed closely by *C. limoniforme*, a new taxon recently described by Bensch et al. (2012).

Respect to *Cladosporium* species pathogenicity, it has been proven their ability to cause lesions in healthy pears in many parts of the world (Sugar and Powers 1986; Benbow and Sugar 1999; Park et al. 2008; Lutz et al. 2016). We could demonstrate the host specificity not only for *C. herbarum* complex species but also for *C. cladosporioides* complex species. Moreover, among pears variety, Abate Fetel was the most susceptible to infection. The external lesions observed were not generally identical to those visualized in pears naturally infected, but this could be related to the experimental laboratory conditions which certainly did not match to those given in cold storage for long

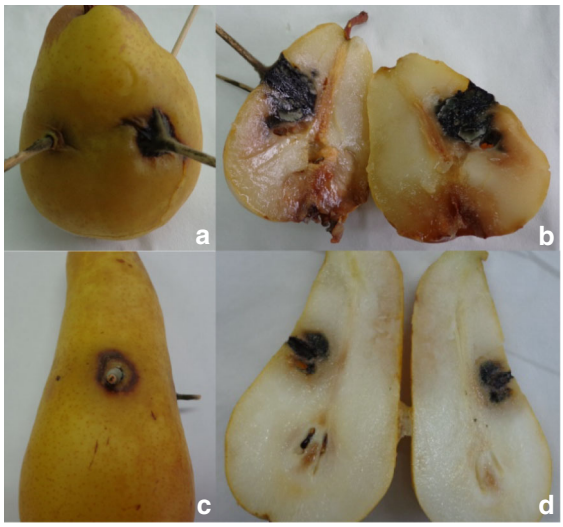

Fig. 4 Lesions caused by *Cladosporium asperulatum*. **a** External symptomatology on Packham's Triumph pear in which mycelium development covering a black necrotic lesion can be observed. **b** Internal injury of the same pear which consists of a big necrotic patch and hollowing of the tissue. **c** External symptomatology on Abate Fetel pear which shows a brown necrotic patch around the inoculation site. **d** Internal symptomatology of the same pear exhibiting black necrosis and hollowing of the tissue

time periods (Lutz et al. 2016). These data are relevant to the production sector due to the fact that Abate Fetel is the fourth pear variety with more abundance in this region and acknowledging the presence of this pathogenic species could help producers to take appropriate preventive measures.

In conclusion, we have conducted a study which has brought in light knowledge about *Cladosporium* species and their occurrence in rural environments of northern Argentinean Patagonia based on molecular and morphological characterization. Moreover, studies carried out to test their pathogenicity were successful and revealed *Cladosporium* species were able to cause injury on healthy fruits. This becomes important when taking into account the fact that this is the main genus present in air which can contaminate fruits on fields, such as Abate Fetel pears, that later are stored under refrigeration conditions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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