Identification and Characterization of *Alternaria* Species Associated with Moldy Core of Apple in Chile

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Abstract

Moldy core (MC) of apple is an important disease in Chile, with prevalence observed between 4 and 46% in Fuji, Oregon Spur Red Chief, and Scarlet apple in the 2014–15 and 2015–16 growing seasons. However, there is no information on the identity of the causal agents associated with MC in Chile. The analysis of 653 MC fruit revealed the presence of several genera of filamentous fungi. However, species of *Alternaria* (67.7%) were by far the most frequently fungi isolated. In total, 41 *Alternaria* isolates were characterized morphologically and molecularly using *Alternaria* major allergen Alt a1, calmodulin, and plasma membrane ATPase gene regions. Six small-spored *Alternaria* spp. were identified; namely, in order of importance, *Alternaria tenuissima*, *A. arborescens*, *A. alternata*, and *A. dumosa* in sect. *Alternaria*; *A. frumentii* in sect. *Infectoriae*;

and *A. kordkuyana* in sect. *Pseudoalternaria*. MC symptoms were reproducible and consisted of a light gray to dark olive-green mycelium over the carpel and seed of immature and mature fruit, confirming that the isolates of these *Alternaria* spp. were pathogenic. These isolates caused brown necrotic lesions with concentric rings on wounded detached apple leaves. This study demonstrated that at least six *Alternaria* spp. are the cause of MC of apple in Chile. These *Alternaria* spp. were isolated alone, or with two or more species coexisting in the same fruit. This is the first report of *A. tenuissima*, *A. arborescens*, *A. frumentii*, *A. dumosa*, and *A. kordkuyana* associated with MC of apple in Chile and the first report of *A. frumentii*, *A. kordkuyana*, and *A. dumosa* causing MC of apple worldwide.

Chile is one of the largest apple producers in the southern hemisphere, with more than 37,200 ha planted and a total production of approximately 765,000 tons in 2016 (<https://www.odepa.gob.cl/>). Moldy core and core rot are considered two important diseases in apple with an open sinus from the calyx to the core region (McLeod 2014). Core rot usually follows a moldy core condition. This study primarily focuses on moldy core, which considerably affects apple production in Chile. A disease prevalence between 4 and 29% was estimated in Oregon Spur (= Red King Oregon) apple at harvest in Chile late in the 1980s (Latorre 1986; Morales 1986). Recently, a moldy core prevalence between 4 and 46% was determined at harvest in new apple cultivars (Fuji, Red Chief, and Scarlet) planted in Chile. Similarly, prevalences varying from 5 to 12% have been reported in susceptible cultivars in Australia, Israel, and South Africa (Kupferman 1992; Reuveni 2006; Serdani et al. 1998). A moldy core prevalence exceeding 40% was reported in Israel in 2003 (Reuveni 2006), and 38 to 65% of fruit with moldy core were estimated in Ohio in 1980 and 1981 (Ellis and Barrat 1983).

Moldy core infection occurs in the orchard but it can also occur in the packing house (Spotts et al. 1988). It is characterized by fungal mycelial growth that is restricted to the carpel without necessarily affecting the fruit flesh. Infected fruit ripen earlier, are often smaller and slightly deformed, and fall prematurely compared with healthy fruit (Latorre 1983; McLeod 2014; Niem et al. 2007). Because of the importance of moldy core, apple packing houses commonly estimate disease prevalence at the reception of each apple lot in Chile. Only those lots with no evidence of moldy core are kept in controlled atmosphere chambers.

The open-sinus condition that characterizes most Red Delicious apple, unlike Granny Smith apple, can explain the relatively high susceptibility to moldy core exhibited by this cultivar (Pinilla et al. 1996; Serdani et al. 1998). However, other factors such as mesoderm pH and locule sensitivity to colonization can affect apple susceptibility to moldy core in open-sinus apple (Niem et al. 2007).

Species of *Alternaria* have been frequently associated with moldy core (Ellis and Barrat 1983; McLeod 2014; Reuveni et al. 2002). However, other genera of filamentous fungi such as species of *Aspergillus*, *Cladosporium*, *Coniothyrium*, *Epicoccum*, *Phoma*, and *Stemphylium* have also been associated with this syndrome; nonetheless, species of *Alternaria* are the most frequently isolated fungi (Ellis and Barrat 1983; Gao et al. 2013; McLeod 2014; Serdani et al. 2002).

At least two small-spored *Alternaria* spp., *Alternaria alternata* and *A. tenuissima*, have been associated with moldy core of apple (McLeod 2014). Nevertheless, recent studies suggest that a complex of small-spored *Alternaria* spp., including *A. alternata*, *A. arborescens*, *A. infectoria*, and *A. tenuissima*, may be associated with moldy core of apple (Gao et al. 2013; Ntasiou et al. 2015). A similar situation has been reported for core rot in pomegranate (Luo et al. 2017; Michailides et al. 2008).

The identification of the small-spored species of the genus *Alternaria* based on taxonomic characteristics is challenging due to a high diversity and few characteristics that allow their unambiguous identification (Andrew et al. 2009). At present, molecular and phylogenetic analysis are essential for recognizing species within small-spored *Alternaria* spp. Different loci have been used, such as nuclear ribosomal DNA (rDNA), internal transcribed spacer (ITS) regions, mitochondrial small subunit, and many protein-coding genes (Andrew et al. 2009; Hong et al. 2005; Lawrence et al. 2013, 2016; Pryor and Gilbertson 2000; Woudenberg et al. 2013, 2015). A multilocus study was carried out by Lawrence et al. (2013), who showed that the most informative protein-coding genes to identify *Alternaria* spp., in order of importance, are plasma membrane ATPase (*ATPase*), calmodulin (*CAL*), *Alternaria* major allergen Alt a1 (*Alt a1*), glyceraldehyde-3-phosphate dehydrogenase, and actin. However, *ATPase* and *CAL* have been suggested as the most appropriate markers for molecular identification within small-spored *Alternaria* spp. Other genes such as β -tubulin and translation elongation factor 1- α were the least informative genes for the identification of *Alternaria* spp. (Lawrence et al. 2013, 2016). Furthermore, the histone 3 gene (*HIS3*) has been used to separate *A. alternata* from *A. tenuissima* (Rang et al. 2002; Sun and Huang 2017).

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In Chile, the etiology of moldy core remains to be determined, particularly for cultivars that are planted at present. Therefore, the objective of this work was to characterize and study the role of *Alternaria* spp. associated with moldy core of apple in Chile.

Materials and Methods

Sampling locations and fungal isolation. In total, 2,000 apparently healthy fruit of apple cultivars Fuji, Red Chief, Oregon Spur, and Scarlet, with open-sinus fruit, were sampled randomly from three commercial orchards located in Graneros (34°0.40' latitude S), Río Claro (35°15' latitude S), and Colbún (35°43' latitude S) in 2015. Eight additional orchards, between Pirque (33°0.40' latitude S), and Traiguén (38°0.34' latitude S), were surveyed in 2016 (Fig. 1). In addition, apparently healthy fruit ($n = 200$) of Granny Smith apple, with a closed sinus, were sampled in one orchard in Graneros. Fruit were sampled 2 to 3 weeks before harvest, transported to the laboratory, stored at 5°C, and evaluated within 2 weeks. The apple fruit were washed under running tap water and surface disinfected (75% ethanol, 5 min) before sectioning each fruit longitudinally. The carpels and flesh were examined and classified as healthy or diseased (Gao et al. 2013). The later fruit were classified as (i) brown core, with brown necrotic lesions of the carpel walls and absence of mycelium growth; (ii) moldy core (Fig. 2a), with carpels colonized by gray to dark olive-green mycelium; (iii) dry rot, with dry and corky brown rot of the flesh, in addition to the presence of mycelium in the carpels; or (iv) wet rot, with wet and soft brown rot of the flesh, in addition to mycelium growth in the carpels.

Isolations were attempted from small pieces (2 to 5 mm in length) of mesocarp (flesh), carpel walls, and seed on potato dextrose agar (PDA) acidified with 92% lactic acid at 0.5 ml/liter (APDA) plus 0.1% Igepal CO-630 (Sigma-Aldrich, St. Louis), which was used as a colony growth restrictor (Elfars et al. 2013). The cultures were incubated for 7 to 10 days at room temperature (20 to 22°C) in the dark prior to the determination of the total number of *Alternaria*-like colonies under a stereoscopic microscope. Fungal colonies were tentatively identified as *Alternaria* spp. by the colony morphology (color and texture) and by the conidiophore and conidial characteristics. Colonies of other filamentous fungi were counted and identified to genus level on the basis of colony morphology (color and texture) and conidiophore and conidial characteristics.

Mycelia from *Alternaria*-like colonies of 330 isolates were transferred to APDA, and pure cultures were then obtained by transferring a single hyphal tip to fresh APDA. Isolates were kept on APDA at 5°C until used.

Molecular characterization. In total, 33 *Alternaria* isolates from moldy core and eight isolates from leaf spot of apple (Elfars et al. 2018), representing each of six morphological groups differentiated on the basis of colony morphology and characteristics of their conidiophores and conidia, were selected for molecular studies (Table 1). Fourteen of these isolates were initially examined molecularly by the ITS and *HIS3* sequence analysis. However, these analyses were not very informative. Therefore, the 41 isolates were examined for *Alt a1*, *ATPase*, and *CAL*.

DNA was extracted from the isolates from 7- to 10-day-old mycelia cultured on APDA incubated at 20 to 22°C. Mycelia were carefully separated from the agar medium with the aid of a sterile scalpel and ground in liquid nitrogen in a porcelain mortar. Genomic DNA was extracted using a DNA extraction kit (Wizard Genomic DNA Purification Kit; Promega Corp., Madison, WI). The DNA yield was estimated by gel electrophoresis and GelRed (Biotium Inc., Alameda, CA) staining and visualized by UV transillumination at 320 nm.

The ITS, *Alt a1*, *ATPase*, *CAL*, and *HIS3* genes were amplified using primer pairs ITS5/ITS4 (White et al. 1990), ATPDF1/ATPDRI, CALDF1/CALDR1 (Lawrence et al. 2013), Alt-for/Alt-rev (Hong et al. 2005), and H3-1a/H3-1b (Glass and Donaldson 1995), respectively. Polymerase chain reaction (PCR) was conducted in a thermal cycler (Veriti 96-Well; Applied Biosystems, Foster City, CA) in a final volume of 25 µl containing 5 µl of 10× PCR buffer, 1 µl of 25 mM MgCl₂, 0.2 µl of 10 mM dNTP, 1 µl of a 15-mM solution of each primer, 0.13 µl containing 5 U of *Taq* DNA polymerase (GoTaq Flexi DNA Polymerase, Promega Corp.), and 2 µl of

template DNA. The amplification protocol included preheating for 3 min at 95°C; followed by 35 cycles of denaturation at 95°C for 30 s, annealing for 30 s at 59°C for the ITS, 60°C for *ATPase*, 57°C for *CAL*, 58°C for *Alt a1*, and 64.5°C for *HIS3*, and extension at 72°C for 90 s; with a final extension for 7 min at 72°C.

The PCR products were separated on 2% agarose gels in 1.0× Tris-acetate-EDTA buffer and stained with GelRed, and the DNA fragments were visualized by UV transillumination at 320 nm.

The PCR-amplified products were purified and sequenced from both directions by Macrogen Inc. (Seoul, South Korea). The sequences were edited using Proseq v.2.91 (Filatov 2002) and aligned using ClustalX v.2.1 (Larkin et al. 2007). A BLASTn search analysis was performed against reference sequences in the GenBank (<https://www.ncbi.nlm.nih.gov/>) database.

A phylogenetic analysis was performed for a maximum parsimony (MP) analysis with the aid of MEGA v.7.0 (Kumar et al. 2016). Gaps were treated as missing data. The MP tree was obtained using the tree-bisection-reconnection branch-swapping algorithm and 1,000 random sequence additions. Branch stability was estimated with a bootstrap analysis with 1,000 replicates. The alignment included sequences from the 33 *Alternaria* isolates obtained from moldy core of apple and eight *Alternaria* isolates obtained from leaf spot of apple in Chile (Table 1). Based on the BLASTn results, *Alternaria* sequences deposited in GenBank mainly more *Alternaria* spp. included in sections; *Alternaria*, *Infectoriae*, and *Pseudoalternaria*, including ex-types, were used in phylogenetic analysis. Sequences of *Stemphylium botryosum*, *S. callistephi*, and *S. vesicarium* were included as the out-group (Table 2). The phylogenetic analysis was performed independently for each gene. The congruence of the evolution between the *ATPase* and *CAL* genes and between *ATPase*, *CAL*, and *Alt a1* was studied using the partition homogeneity tests with the aid of PAUP, v.4.0b10 (Swofford 2002).

Morphological characterization. Colony morphology of 19 *Alternaria* isolates from apple was characterized in 9-cm-diameter plastic Petri dishes containing APDA, potato carrot agar (PCA; containing 20 g of potato, 20 g of carrot, and 20 g of agar per liter of distilled water) (Simmons 2007), and dichloran rose Bengal yeast extract sucrose agar (DRYES; containing 2 mg of dichloran, 25 mg of rose Bengal, 20 g of yeast extract, and 150 g of sucrose per liter of distilled water) (Frisvad 1983). APDA and PCA plates were incubated for 7 days at 22°C with photoperiods of 8 h of light and 16 h

Fig. 1. Map of Chile showing the geographic distribution of apple orchards. Administrative Regions of Chile: MR = Metropolitan Region and regions VI, VII, VIII, and IX.

of darkness. Light was provided by daylight candles (TL-D 36W/54-765; Philips, Amsterdam, Netherlands) placed 40 cm above the plates. The DRYES plates were incubated for 7 days at 22°C under either daylight or darkness conditions. The color, texture, and margins of the colonies and the development of pigments on APDA were determined. The color of the colonies on DRYES was determined. The color was established with the aid of the Pantone color chart (www.pantone-colours.com). Conidia and conidiophores from three PCA plates per isolate were mounted on colorless adhesive tape and placed on top of a drop of Shear's mounting medium (10 g of potassium acetate, 200 ml of glycerin, 300 ml of 95% ethanol, and 500 ml of distilled water) before being observed under a light microscope. Conidiophore ($n = 15$ per isolate) length and width, cell numbers, and branching were determined. Conidial ($n = 50$) shape, length, width, number of transepta, and beak length were measured and compared with published descriptions (Gannibal and Lawrence 2016; Poursafar et al. 2018; Pryor and Michailides 2002; Simmons 2007).

Vegetative compatibility tests. The vegetative compatibility within *A. alternata* ($n = 3$ isolates), *A. arborescens* ($n = 3$ isolates), *A. dumosa* ($n = 1$ isolate), *A. frumenti* ($n = 2$ isolates), *A. kordkuyana* ($n = 1$ isolate), and *A. tenuissima* ($n = 3$ isolates) were conducted based on the work of van der Waals et al. (2004). Two isolates were paired on APDA on 90-mm Petri dishes. Two mycelial plugs (<4 mm in diameter) were cut from actively growing APDA cultures and placed 2 cm apart. Reactions were scored after 7 days of incubation in the dark at

20°C. All isolates were paired with themselves to ensure self-compatibility. Pairings were repeated to confirm the results. Pairings were examined macroscopically for the presence of an antagonism zone in the region of mycelial contact (van der Waals et al. 2004).

Effect of temperature on mycelial growth. The effect of temperature on the mycelial growth of *A. alternata* ($n = 5$ isolates), *A. arborescens* ($n = 5$ isolates), *A. dumosa* ($n = 1$ isolate), *A. frumenti* ($n = 2$ isolates), *A. kordkuyana* ($n = 1$ isolate), and *A. tenuissima* ($n = 5$ isolates) was studied. For this purpose, a 4-mm-diameter mycelial plug was placed in the center of each Petri dish (9 cm) containing 15 ml of APDA and incubated in thermal chambers (Velp Scientifica, Milan, Italy) adjusted to 0 to 40°C ($\pm 1^\circ\text{C}$) at 5°C intervals. Temperatures were checked with a HOBO PRO temperature sensor (Onset Computer Corp., Bourne, MA) with two terminals; one was outside the Petri dish and the second was placed inside the Petri dish on the agar medium surface. Petri dishes were incubated for at least 12 h at the desired temperature before use. The growth of two perpendicular mycelial radials was determined after 2, 4, and 6 days of incubation. Isolates showing no growth after 6 days at 0 or 40°C were reincubated at 22°C for 5 days to determine their viability. This experiment was conducted twice.

Pathogenicity tests. To stimulate sporulation, isolates were cultivated in 0.05× PDA (Pryor and Michailides 2002) for 7 days at 22°C with cycles of 10 h of light and 14 h of darkness. Inoculum suspensions were prepared with 10- to 14-day-old cultures on 0.05× PDA. Dishes were flooded with approximately 20 ml of 0.05% of Tween 80

Fig. 2. Symptoms and pathogenicity of moldy core of Red Chief apple caused by *Alternaria tenuissima*. **a**, naturally infected fruit; **b**, artificially inoculated immature fruit; **c**, colonization and sporulation of *A. tenuissima* on the apple seed surface; and **d**, necrotic lesions on apple leaves inoculated with conidia of *A. tenuissima*.

and the surface of the medium was scraped with a sterile scalpel. The conidial suspension was filtered through two layers gauze and the final concentration was adjusted to 1×10^6 conidia/ml using a hemacytometer. Isolates of *A. alternata* ($n = 5$ isolates), *A. arborescens* ($n = 5$ isolates), *A. dumosa* ($n = 1$ isolate), *A. frumenti* ($n = 2$ isolates), *A. kordkuyana* ($n = 1$ isolate), and *A. tenuissima* ($n = 5$ isolates) were evaluated for pathogenicity.

Apple fruit. Immature fruit (mean equatorial diameter of 31.6 mm) of Red Chief apple ($n = 4$) were surface disinfected in 75% ethanol for 5 min and air dried before sectioning transversally. One half of each fruit were inoculated with $10 \mu\text{l}$ of conidial suspensions that were placed in each of the five carpels and then covered with the other half of the fruit to avoid rapid dehydration. Fruit were incubated

at 20°C in a humid chamber for 10 days prior to determination of the proportion of carpels with visible mycelial growth. An equal number of fruit treated with sterile water served as the controls. Reisolations from carpels were performed on APDA. This experiment was repeated as described above using mature fruit (mean total soluble solids 13.1%), except that the fruit were inoculated with $30 \mu\text{l}$ of conidial suspensions placed in each of the five carpels.

Immature fruit and mature fruit of Red Chief apple ($n = 4$) were surface disinfected in 75% ethanol for 5 min and air dried. Each fruit was inoculated with $10 \mu\text{l}$ of conidial suspension that was deposited on top of four punctures made with the aid of a sterile hypodermic needle. Fruit were incubated in humid chambers at 20°C for 10 days prior to determining the necrotic lesions. An equal number of fruit,

Table 1. Source of isolates of *Alternaria* spp. from apple obtained in commercial orchard in Central Chile and GenBank accession numbers for sequences of three genes (*Alternaria* major allergen Alt a1, plasma membrane ATPase, and Calmodulin) of *Alternaria* isolates studied

Species	Symptom	Locality ^x	Latitude ^y	Apple cultivar	GenBank accession number ^w		
					<i>Alt a1</i>	<i>ATPase</i>	<i>CAL</i>
<i>Alternaria alternata</i>							
7MC ^z	Brown core	Río Claro, VII	35°15′	Red Chief	MG925174	MG740627	MG925132
10LS	Leaf spot	Pirque, MR	33°40′	Scarlet	MG925165	MG740618	MG925123
12LS ^z	Leaf spot	Morza, VII	34°50′	Scarlet	MG925166	MG740619	MG925124
16MC ^z	Moldy core	Pirque, MR	33°40′	Scarlet	MG925175	MG740628	MG925133
17MC	Moldy core	Graneros, VI	34°4′	Oregon Spur	MG925176	MG740629	MG925134
30MC ^z	Moldy core	Graneros, VI	34°4′	Oregon Spur	MG925177	MG740630	MG925135
36MC ^z	Moldy core	Los Niches, VII	35°3′	Scarlet	MG925178	MG740631	MG925136
<i>A. arborescens</i>							
8MC ^z	Brown core	Colbún, VII	35°43′	Red Chief	MG925179	MG740632	MG925137
13LS ^z	Leaf spot	Los Ángeles, VIII	37°33′	Scarlet	MG925167	MG740621	MG925126
14LS	Leaf spot	Los Ángeles, VIII	37°33′	Scarlet	MG925168	MG740620	MG925125
19MC ^z	Moldy core	Pirque, MR	33°40′	Scarlet	MG925180	MG740633	MG925138
23MC	Moldy core	Longaví, VII	36°12′	Scarlet	MG925181	MG740637	MG925142
31MC	Moldy core	Los Ángeles, VIII	37°33′	Scarlet	MG925182	MG740634	MG925139
35MC	Moldy core	Río Claro, VII	35°15′	Red Chief	MG925183	MG740636	MG925141
38MC ^z	Moldy core	Traiguén, IX	38°21′	Fuji	MG925184	MG740638	MG925143
39MC	Moldy core	Morza, VII	34°50′	Red Chief	MG925185	MG740639	MG925144
40MC ^z	Moldy core	Graneros, VI	34°4′	Oregon Spur	MG925186	MG740635	MG925140
<i>A. dumosa</i>							
28MC ^z	Moldy core	Morza, VII	34°50′	Red Chief	MG925187	MG740640	MG925145
<i>A. frumenti</i>							
2MC ^z	Brown core	Colbún, VII	35°43′	Red Chief	MG925188	MG740641	MG925146
37MC ^z	Moldy core	Traiguén, IX	38°21′	Red Chief	MG925189	MG740642	MG925147
<i>A. kordkuyana</i>							
34MC ^z	Moldy core	Longaví, VII	36°12′	Scarlet	MG925190	MG740643	MG925148
<i>A. tenuissima</i>							
1MC	Brown core	Río Claro, VII	35°15′	Red Chief	MG925191	MG740644	MG925149
3MC	Dry rot	Graneros, VI	34°4′	Oregon Spur	MG925192	MG740645	MG925150
4LS	Leaf spot	Morza, VII	34°50′	Scarlet	MG925170	MG740623	MG925128
5LS	Leaf spot	Pirque, MR	33°40′	Scarlet	MG925171	MG740625	MG925129
6LS	Leaf spot	Colbún, VII	35°43′	Red Chief	MG925172	MG740624	MG925130
11LS ^z	Leaf spot	Morza, VII	34°50′	Scarlet	MG925173	MG740626	MG925131
15MC ^z	Brown core	Río Claro, VII	35°15′	Red Chief	MG925193	MG740646	MG925151
18MC ^z	Moldy core	Longaví, VII	36°12′	Scarlet	MG925194	MG740647	MG925152
20MC	Moldy core	Longaví, VII	36°0′	Scarlet	MG925195	MG740648	MG925153
21MC	Moldy core	Morza, VII	34°50′	Scarlet	MG925196	MG740649	MG925154
22MC	Moldy core	Río Claro, VII	35°15′	Red Chief	MG925197	MG740650	MG925155
24MC	Moldy core	Los Niches, VII	35°3′	Red Chief	MG925198	MG740651	MG925156
25MC	Moldy core	Río Claro, VII	35°15′	Red Chief	MG925199	MG740652	MG925157
26MC	Moldy core	Longaví, VII	36°12′	Red Chief	MG925200	MG740653	MG925158
27MC	Moldy core	Longaví, VII	36°12′	Red Chief	MG925201	MG740654	MG925159
29MC	Moldy core	Longaví, VII	36°12′	Scarlet	MG925202	MG740655	MG925160
32MC	Moldy core	Río Claro, VII	35°15′	Red Chief	MG925203	MG740656	MG925161
33MC ^z	Moldy core	Los Ángeles, VIII	37°33′	Scarlet	MG925204	MG740657	MG925162
41MC	Moldy core	Graneros, VI	34°4′	Oregon Spur	MG925205	MG740658	MG925163
42MC ^z	Moldy core	Graneros, VI	34°4′	Oregon Spur	MG925206	MG740659	MG925164

^w Genes: *Alt a1* = *Alternaria* major allergen Alt a1, *ATPase* = plasma membrane ATPase, and *CAL* = calmodulin.

^x Administrative Regions of Chile: MR = Metropolitan Region and regions VI, VII, VIII, and IX.

^y Southern latitude.

^z Isolates used for morphological characterization, effect of temperature on mycelial growth test, and pathogenicity tests.

injured but not inoculated, treated with sterile solution were left as the negative control.

Apple seed. Seed from immature and mature Red Chief apple were surface disinfected with 75% ethanol for 30 s. Five seeds were aseptically placed in humid chambers ($n = 4$), inoculated with 10 μ l of conidial suspension per seed, and incubated at 20°C for 7 days before examining each seed for the presence of mycelial growth, sporulation, and necrotic lesions. An equal number of seed treated with

sterile water served as the negative controls. Reisolations from surface mycelial growth and necrotic lesions were attempted on APDA.

Apple leaves. Detached leaves of Red Chief apple ($n = 4$), taken from the newest leaves of the shoots, were surface disinfected in 1% NaOCl for 1 min followed by sterile distilled water for 1 min and air dried. Then, the leaves were wounded with three punctures made with a sterile hypodermic needle in the apical, basal, and middle regions of each leaf blade. Leaves were inoculated by delivering 15 μ l of conidial suspension per wound site. Leaves were incubated at 20°C in a humid chamber for 7 days before the diameters of the lesions were determined. An equal number of wounded leaves treated with sterile water served as the negative controls. Reisolations from the necrotic lesions on APDA were attempted, and isolates were identified on the basis of the conidial morphology. This experiment was conducted twice. This experiment was repeated as described above using unwounded leaves.

Design and statistical analysis. All experimental treatments were performed in a completely randomized design with four replicates. Means were separated according to Fisher's least significant difference test ($P < 0.05$). All analyses were performed with the aid of SigmaStat 3.1 (Systat Software Inc., San Jose, CA). Data percentages were arcsine $\sqrt{(x/100)}$ transformed and necrotic lesion length data were transformed to $\ln(x + 1)$ prior to analysis; however, nontransformed data are presented. The mean daily radial growth rates were estimated by linear regression analysis between y = mycelial growth and x = time.

Results

Sampling locations and fungal isolation. Of 2,200 fruit sampled, 653 (29.7%) were diseased fruit, characterized by the presence of a gray to dark olive-green cottony mycelium (moldy core). In addition, 158 (7.2%) fruit showed a brown core, 31 (1.4%) fruit showed dry rot, and 4 (0.2%) fruit showed wet rot. A moldy core was most prevalent, varying from 16 to 46% in Oregon Spur, Red Chief, and Scarlet apple and between 4 and 13% in Fuji apple. The brown core prevalence varied from 0.1 to 69%. A low prevalence of core rot was determined in Oregon Spur, Red Chief, and Scarlet apple (0.3 to 3.0%). Granny Smith apple fruit were symptomless (Table 3).

Species of *Alternaria* were the most frequently and consistently isolated fungi, found in 56.2% of the apple samples in 2014–15 and 79.2% in 2015–16, followed by (in order of species by frequency) *Cladosporium*, *Penicillium*, *Stemphylium*, *Epicoccum*, *Botrytis*, and *Fusarium* (Table 3). In total, 330 isolates of *Alternaria* spp. were obtained from moldy core apple fruit (Table 3).

Molecular characterization. Sequences of *ATPase* of the *Alternaria* isolates from apple ranged from 1,188 to 1,197 bp. MP analysis produced a consensus tree (Fig. 3) inferred from the two most parsimonious trees (tree length [TL] = 567, consistency index [CI] = 0.747, retention index [RI] = 0.953, and rescaled consistency index [RC] = 0.713). The phylogenetic tree obtained with *ATPase* sequences delimited six main clades (groups A to F) (Fig. 3). Clade I (group A), with 97% bootstrap support, contained sequences of isolates of *A. alternata* from Chilean apple together with the *A. alternata* ex-type (JQ671874) and *A. destruens* ex-type (JQ671873). Clade II (group B), with 88% bootstrap support, contained isolates of *A. tenuissima*, including the sequence of the *A. tenuissima* ex-type (JQ811989). Clade III (group F), with 70% bootstrap support, grouped isolates of *A. dumosa* together with the *A. dumosa* ex-type (JQ671877). Clade IV (group C), with 100% bootstrap support, contained isolates of *A. arborescens*, including the *A. arborescens* ex-type (JQ671880). Clade V (group D), with 91% bootstrap support, grouped isolates from moldy core of Chilean apple together with *ATPase* sequences of *A. frumenti* (JQ671823) in sect. *Infectoriae*. Clade VI (group E), with 99% bootstrap support, contained one isolate from Chilean apple that was grouped together with *A. kordkuyana* ex-type (MF033860) in sect. *Pseudoalternaria* (Fig. 3).

Sequences of *CAL* of the *Alternaria* spp. from apple ranged from 775 to 794 nucleotides. MP analysis produced one parsimonious trees (TL = 355, CI = 0.785, RI = 0.977, and RC = 0.768). The phylogenetic tree obtained with *CAL* sequences delimited four main clades. Clade I, with 82% bootstrap support, contained isolates of the *A. tenuissima*, *A. destruens* ex-type (JQ646207), and *A. limoniasperae* ex-type (JQ646213). Clade II, with 79% bootstrap support, grouped

Table 2. Accession numbers for reference sequences of three genes of *Alternaria* isolates in GenBank used for phylogenetic analysis in this study

Species	Isolates ^y	GenBank accession number ^x		
		<i>Alt a1</i>	<i>ATPase</i>	<i>CAL</i>
<i>Alternaria alternata</i>	EGS 34-016 ^z	KP275691	JQ671874	JQ646208
<i>A. alternata</i>	X1191	KJ920991	KJ908234	KJ920956
<i>A. alternata</i>	X1048	KJ920997	KJ908230	KJ920957
<i>A. alternata</i>	BMP0463	na	JQ811981	na
<i>A. angustiovoidea</i>	EGS 36-172 ^z	JQ646398	JQ671869	JQ646203
<i>A. arborescens</i>	EGS 39-128 ^z	AY563303	JQ671880	JQ646214
<i>A. arborescens</i>	BMP0462	na	JQ811990	na
<i>A. arborescens</i>	3.J24	KJ921023	KJ908244	KJ920979
<i>A. arborescens</i>	X1013	KJ920987	KJ908247	KJ920949
<i>A. arborescens</i>	X1312	KJ921008	KJ908242	KJ920948
<i>A. argyroxiphii</i>	EGS 35-122 ^z	JQ646434	JQ671926	JQ646260
<i>A. arrhenatheri</i>	56492-12FD ^z	na	JQ693603	na
<i>A. californica</i>	EGS 52-082 ^z	JQ646373	JQ671813	JQ646147
<i>A. cerialis</i>	EGS 43-072 ^z	JQ646405	JQ671883	JQ646217
<i>A. conjuncta</i>	EGS 37-139 ^z	AY563281	JQ671824	JQ646158
<i>A. daucicaulis</i>	EGS 36-1947 ^z	na	JQ671822	JQ646156
<i>A. destruens</i>	EGS 46-069 ^z	JQ646402	JQ671873	JQ646207
<i>A. dumosa</i>	EGS 45-007 ^z	na	JQ671877	JQ646211
<i>A. ethzedia</i>	EGS 37-143 ^z	AY563284	JQ671805	JQ646139
<i>A. frumenti</i>	EGS 44-001 ^z	JQ646378	JQ671823	JQ646157
<i>A. graminicola</i>	EGS 41-139 ^z	na	JQ671819	JQ646153
<i>A. herbiphorbicola</i>	EGS 40-140 ^z	JQ646410	JQ671888	JQ646222
<i>A. hordeicaustralica</i>	EGS 44-200 ^z	na	JQ671811	JQ646145
<i>A. hordeicola</i>	EGS 50-184 ^z	JQ646372	JQ671812	JQ646146
<i>A. humuli</i>	EGS 47-140 ^z	na	JQ671821	JQ646155
<i>A. incomplexa</i>	EGS 17-103 ^z	JQ646374	JQ671815	JQ646149
<i>A. infectoria</i>	EGS 27-193 ^z	FJ266502	JQ671804	JQ646138
<i>A. infectoria</i>	3.J14	KJ921017	KJ908219	KJ920977
<i>A. infectoria</i>	X1273	KJ920982	KJ908218	KJ920953
<i>A. intercepta</i>	EGS 49-137 ^z	JQ646380	JQ671826	JQ646160
<i>A. kordkuyana</i>	KQ-20 ^z	na	MF033860	na
<i>A. limoniasperae</i>	EGS 45-100 ^z	JQ646370	JQ671879	JQ646213
<i>A. malorum</i>	EGS 52.153 ^z	JQ646369	JQ671800	JQ646134
<i>A. merytae</i>	EGS 46-153 ^z	na	JQ671820	JQ646154
<i>A. metachromatica</i>	EGS 38-132 ^z	AY563285	JQ671809	JQ646143
<i>A. novae-zelandiae</i>	EGS 48-092 ^z	JQ646379	JQ671825	JQ646159
<i>A. oregonensis</i>	EGS 29-194 ^z	AY563283	JQ671827	JQ646161
<i>A. parvicaespitosa</i>	X1272 ^z	KJ920981	KJ908217	KJ920952
<i>A. rosae</i>	EGS 41-130 ^z	JQ646370	JQ671803	JQ646137
<i>A. tenuissima</i>	EGS 34.015 ^z	KP275690	JQ811989	JQ646209
<i>A. tenuissima</i>	BMP2256	na	JQ811983	na
<i>A. tenuissima</i>	BMP1254	na	JQ811988	na
<i>A. triticimaculans</i>	EGS 41-050 ^z	na	JQ671806	JQ646140
<i>A. tritici</i>	EGS 17-061 ^z	JQ646371	JQ671808	JQ646142
<i>A. ventricosa</i>	EGS 52-075 ^z	JQ646377	JQ671818	JQ646152
<i>A. viburni</i>	EGS 49-147 ^z	JQ646375	JQ671816	JQ646150
<i>Stemphylium botryosum</i>	BMP 0360	AY563274	JQ671767	JQ646101
<i>S. callistephi</i>	BMP 0377	AY563276	JQ671769	JQ646103
<i>S. vesicarium</i>	BMP 0374	AY563275	JQ671768	JQ646102

^x Genes: *Alt a1* = *Alternaria* major allergen Alt a1, *ATPase* = plasma membrane ATPase, and *CAL* = calmodulin; na = sequences not available in GenBank.

^y BMP = B. M. Pryor, Division of Plant Pathology, Department of Plant Sciences; EGS = E. G. Simmons, Mycological Services, Crawfordsville, IN 47933; 3.J = Pryor and Michailides 2002; and X = Zhu and Xiao 2015.

^z Ex-type.

isolates of *A. dumosa* together with the *A. dumosa* ex-type (JQ646211). Clade III, with 99% bootstrap support, contained sequences of isolates of *A. arborescens*, including the sequences of the *A. arborescens* ex-type (AY563303), *A. cerealis* (JQ646217), and *A. angustioidea* (JQ646203). Clade IV, with 96% bootstrap support, grouped isolates *A. alternata* and *A. tenuissima* together with the ex-types (JQ646208 and JQ646209). Clade V, with 97% bootstrap support, contained isolate from Chilean apple, including the *A. parvicaespitosa* ex-type (KJ920952) of sect. *Pseudoalternaria*. A DNA sequence of *A. kordkuyana* ex-type for the *CAL* gene is not available. Clade VII, with 100% bootstrap support, contained sequences of isolates from Chilean apple, including the ex-type of *A. infectoria*, *A. frumenti*, and other *Alternaria* spp. of sect. *Infectoriae*.

Sequences of *Alt a1* of the *Alternaria* spp. from apple ranged from 483 to 485 nucleotides. MP analysis yielded four most parsimonious trees (TL = 200, CI = 0.700, RI = 0.940, and RC = 0.658). The phylogenetic tree obtained with *Alt a1* sequences delimited five main clades. Clade I, with 100% bootstrap support, contained sequences of isolates of *A. alternata* and *A. tenuissima* from Chilean apple that were grouped together with *Alt a1* sequences of the *A. alternata* and *A. tenuissima* ex-types (KP275691 and KP275690). Clade II, with 100% bootstrap support, contained isolates of *A. arborescens* and *A. frumenti*, including sequences of the *A. arborescens* ex-type (AY563303). Clade III, with 100% bootstrap support, grouped isolates *A. alternata*, *A. dumosa*, and *A. tenuissima* together with the *A. destruens* and *A. herbiphorbicola* ex-types. Clade IV, with 70% bootstrap support, contained the ex-type of *A. infectoria* and other *Alternaria* spp. of sect. *Infectoriae* but none of the Chilean isolates were grouped in clade IV. Clade V, with 98% bootstrap support, contained one isolate from Chilean apple, including the ex-type *A. rosae* and *A. parvicaespitosa* of sect. *Pseudoalternaria*. A DNA sequence of *A. kordkuyana* ex-type for *Alt a1* gene is not available.

The partitioned homogeneity test showed significant differences ($P < 0.05$) between the *Alt a1*, *ATPase*, and *CAL* genes and between *ATPase* and *CAL*. Therefore, the concatenated analysis of these genes was not performed.

The MP analysis of the rDNA ITS sequence was not informative to clearly delimit the small-spored species of *Alternaria* associated with moldy core of apple in the present study. Similarly, the phylogenetic analysis of the partial coding sequence of the *HIS* gene was not useful to distinguish isolates of *Alternaria* spp. from moldy core of apple.

Morphological characterization. All *Alternaria* isolates produced single conidiophores and catenulate muriform, brown to golden-brown conidia. However, six groups (A, B, C, D, E, and F) of isolates were differentiated based on the colony morphology and

characteristics of their conidiophores and conidia. Isolates in group A were identified as *A. alternata* in sect. *Alternaria* and produced cottony, gray to green (Pantone number 5777-5763) colonies on APDA and gray-green colonies on DRYES medium. Isolates produced conidial chains with numerous secondary chains branching on short conidiophores (mean $49.8 \pm 11.5 \times 4.0 \pm 0.5 \mu\text{m}$) of 3 to 6 cells. Conidia were ovoid to ellipsoid (mean $28.3 \pm 4.4 \times 11.7 \pm 1.6 \mu\text{m}$) with 3 to 7 transepta and 0 to 1 longisepta (Fig. 4; Table 4).

Isolates in group B were identified as *A. tenuissima* in sect. *Alternaria* and produced cottony, olive brown (Pantone number 5815-5825) colonies with slight concentric growth rings and prominent white margins on APDA and gray-green to yellow-green colonies on DRYES medium. The isolates produced conidial chains of 4 to 12 conidia, rarely with a lateral branch, on conidiophores of $41.1 \pm 9.7 \times 4.2 \pm 0.5 \mu\text{m}$ of 4 to 5 cells. Conidia were ovoid to obclavate with a narrow tapered upper half (mean $34.7 \pm 5.9 \times 11 \pm 1.3 \mu\text{m}$) with 3 to 6 transepta, with the majority of the conidia exhibiting a dark transverse median septa, with 0 to 2 longisepta and a long beak (Fig. 4; Table 4).

Isolates in group C were identified as *A. arborescens* in sect. *Alternaria* and produced cottony, olive-brown (Pantone number 5815-5825) colonies with prominent concentric growth rings and narrow white margins on APDA and dark yellow-green colonies on DRYES medium. Isolates produced conidial chains of 2 to 7 conidia, with a very distinctive long conidiophores (mean $225.4 \pm 80 \times 3.8 \pm 0.5 \mu\text{m}$) of 7 to 10 cells and extended secondary conidiophores. Sporulation was concentrated near the apex of secondary, tertiary, and even quaternary conidiophores. Conidia were ovoid to ellipsoid (mean $23.2 \pm 4.2 \times 10.1 \pm 1.3 \mu\text{m}$) with 1 to 4 transepta and no longisepta or, rarely, 1 longisepta (Fig. 4; Table 4).

Isolates in group D were identified as *A. frumenti* in sect. *Infectoriae* and produced floccules, gray to yellow-green (Pantone number 5777-5783) colonies with slight concentric growth rings on APDA and white colonies on DRYES medium. Primary conidiophores were relatively short (mean $63.8 \pm 16.4 \times 4.2 \pm 0.7 \mu\text{m}$) of 3 to 6 cells. Secondary conidiophores were branched and geniculated. Conidia were ovoid to ellipsoid (mean $33.7 \pm 6 \times 10.2 \pm 1.6 \mu\text{m}$) with 2 to 5 transepta and 0 to 1 longisepta in 0 to 3 transverse segments, produced on secondary and tertiary conidiophores (Fig. 4; Table 4).

The isolate in group E was identified as *A. kordkuyana* in sect. *Pseudoalternaria*. This isolate produced gray to dark-green (Pantone number 5763-5743) colonies with slight concentric growth rings on APDA and white colonies on DRYES medium. Isolates produced short conidial chains of 1 to 6 conidia with lateral chains branching on short conidiophores (mean $56.1 \pm 10.8 \times 3.8 \pm 0.5 \mu\text{m}$) of 4 to

Table 3. Frequency of isolation of *Alternaria* spp. and other filamentous fungi obtained from apple fruit ($n = 2,200$) collected and harvested in commercial orchards in Central Valley of Chile

Growing season, cultivar	Prevalence (%) ^y				Isolates (%) ^z							
	MC	BC	DR	WR	Alt	Bot	Cla	Epi	Fus	Pen	Ste	Others
2014–15												
Open-sinus												
Fuji ($n = 200$)	4.0	4.5	0.5	0.0	6.4	0.0	0.5	0.0	0.0	0.0	1.1	1.1
Oregon Spur ($n = 100$)	16.0	69.0	3.0	1.0	23.0	0.0	7.0	4.8	0.5	8.0	0.5	3.7
Red Chief ($n = 200$)	26.5	19.0	1.0	0.0	26.8	0.0	8.0	0.0	0.0	1.1	1.1	6.4
Total (%)	...	...	...	...	56.2	0.0	15.5	4.8	0.5	9.1	2.7	11.2
Closed-sinus												
Granny Smith ($n = 100$)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2015–16												
Open-sinus												
Fuji ($n = 100$)	13.0	7.0	0.0	0.0	3.5	0.0	0.3	0.0	0.0	0.0	0.0	0.0
Oregon Spur ($n = 100$)	46.0	24.0	2.0	1.0	16.0	0.3	2.4	0.0	0.0	3.1	3.1	4.9
Red Chief ($n = 700$)	38.9	0.1	0.9	0.3	30.9	0.3	0.0	0.0	0.3	0.3	0.0	2.1
Scarlett ($n = 600$)	40.8	1.7	2.8	0.0	28.8	0.0	0.3	0.0	0.0	0.7	0.7	1.4
Total (%)	...	...	...	...	79.2	0.7	3.1	0.0	0.3	4.2	3.8	8.4
Closed-sinus												
Granny Smith ($n = 100$)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

^y Percentages based on total number of fruit per apple cultivar. MC = moldy core, BC = brown core, DR = dry rot, and WR = wet rot.

^z Percentages were calculated from the total fungal isolates, $n = 187$ and $n = 287$ obtained in 2014–15 and 2015–16 growing seasons, respectively. Alt = *Alternaria* spp., Bot = *Botrytis* spp., Cla = *Cladosporium* spp., Epi = *Epicoccum* spp., Fus = *Fusarium* spp., Pen = *Penicillium* spp., and Ste = *Stemphylium* spp.

6 cells. Conidia were ovoid to elliptical (mean $24.7 \pm 6.8 \times 9.7 \pm 2.2 \mu\text{m}$) with 1 to 4 transepta and 0 to 1 longisepta (Fig. 4; Table 4).

The isolate in group F was identified as *A. dumosa* in sect. *Alternaria*. This isolate produced cottony, light brown-green (Pantone number 5803-5783) colonies with slight concentric growth rings on APDA and gray-green colonies on DRYES medium. Isolates produced conidial chains with numerous secondary chains branching on

short conidiophores (mean $60.2 \pm 19.9 \times 3.9 \pm 0.4 \mu\text{m}$) of 4 to 5 cells. Conidia were ovoid (mean $18.3 \pm 3 \times 8.9 \pm 1.6 \mu\text{m}$) with 1 to 4 transepta and 0 to 1 longisepta (Fig. 4; Table 4).

Vegetative compatibility tests. Of 169 pairings studied, 150 pairings were consistently compatible, developing a clear nonantagonistic line at the contact zone; 1 pairing was incompatible; and 18 pairings were uncertain (Table 5).

Fig. 3. Phylogenetic tree obtained from maximum-parsimony analysis of the plasma membrane ATPase gene from sequences of *Alternaria* spp. from Chilean apple and from sequences of ex-types in GenBank. The consensus tree inferred from the two most parsimonious trees and bootstrap values are shown. The tree was rooted with *Stemphylium botryosum*, *S. callistephi*, and *S. vesicarium*. Tree length = 567, consistency index = 0.747, retention index = 0.953, and rescaled consistency index = 0.713. Numbers and MC or LS are *Alternaria* isolates from apple in Chile; other codes are isolates from GenBank.

Effect of temperature on mycelial growth. The radial mycelial growth of the *Alternaria* isolates was significantly ($P < 0.001$) affected by temperature and isolate, with a significant ($P < 0.001$) temperature–isolate interaction. All *Alternaria* isolates were able to grow between 5 and 35°C (Fig. 5). However, some isolates showed very

slight growth (<1 mm per 6 days) at 0°C. All isolates regained normal growth at 22°C. Optimal radial mycelial growth was obtained at 25°C. No growth occurred at 40°C in 8 days. Only some isolates of *A. arborescens* (isolate 38MC), *A. dumosa* (isolate 28MC), and *A. tenuissima* (isolate 33 MC) were able to regain growth after 5 days

Fig. 4. Morphological characteristics of species of *Alternaria* isolated from apple moldy core. **a** to **f**, Colony morphology on acidified potato dextrose agar plates incubated for 7 days at 22°C in cycles of 8 h of light and 16 h of darkness; and **g** to **r**, sporulation pattern on potato carrot agar plates incubated for 7 days at 22°C in cycles of 8 h of light and 16 h of darkness. **a**, **g**, and **m**, *Alternaria alternata*; **b**, **h**, and **n**, *A. arborescens*; **c**, **i**, and **o**, *A. frumentii*; **d**, **j**, and **p**, *A. tenuissima*; **e**, **k**, and **q**, *A. dumosa*; and **f**, **l**, and **r**, *A. kordkuyana*. Scale bars: **g** to **l** = 50 µm and **m** to **r** = 10 µm.

Table 4. Morphometric characterization of conidiophores and conidia of *Alternaria* spp. isolated from moldy core of apple fruit in Chile

Isolates (group)	Colony ^w	Conidiophores ^{x,y}		Conidia ^{y,z}		
	Growth (mm/day)	Length (µm)	Width (µm)	Length (µm)	Width (µm)	L/W
<i>Alternaria alternata</i> (A)						
7MC	7.3 ± 0.3	36.4 ± 7.5	4.3 ± 0.5	32.3 ± 5.2	13.1 ± 1.7	1.8 ± 0.3
12LS	8.5 ± 0.4	56.3 ± 11.9	3.9 ± 0.4	23.4 ± 3.4	10.0 ± 1.3	2.4 ± 0.4
16MC	8.3 ± 0.2	54.2 ± 13.1	3.9 ± 0.5	35.4 ± 6.1	13.3 ± 2.1	2.7 ± 0.5
30MC	9.0 ± 0.1	57.9 ± 14.9	4.2 ± 0.5	26.2 ± 4.2	11.6 ± 1.6	2.3 ± 0.4
36MC	7.5 ± 0.2	44.3 ± 10.3	3.9 ± 0.4	24.3 ± 3.2	10.7 ± 1.2	2.3 ± 0.3
<i>A. arborescens</i> (C)						
8MC	6.3 ± 0.3	239.0 ± 104.3	4.1 ± 0.5	29.1 ± 5.8	10.6 ± 1.6	2.8 ± 0.6
13LS	6.7 ± 0.3	226.7 ± 94.5	3.5 ± 0.5	21.6 ± 3.3	9.2 ± 1.1	2.4 ± 0.4
19MC	8.1 ± 0.2	208.9 ± 73.6	3.8 ± 0.6	22.2 ± 3.6	11.1 ± 1.2	2.1 ± 0.3
38MC	7.6 ± 0.3	236.5 ± 62.9	3.5 ± 0.5	19.9 ± 4.1	9.3 ± 1.2	2.2 ± 0.5
40MC	7.3 ± 0.3	216.1 ± 64.6	4.0 ± 0.3	23.4 ± 4.2	10.4 ± 1.6	2.3 ± 0.4
<i>A. dumosa</i> (F)						
28MC	9.8 ± 0.2	60.2 ± 13.9	3.9 ± 0.4	18.3 ± 3.0	8.9 ± 1.6	2.1 ± 0.4
<i>A. frumentii</i> (D)						
2MC	9.0 ± 0.3	56.6 ± 18.6	4.5 ± 0.8	28.8 ± 4.8	9.3 ± 1.0	2.2 ± 0.6
37MC	8.9 ± 0.1	70.9 ± 14.1	3.9 ± 0.6	38.6 ± 7.1	11.0 ± 2.1	3.6 ± 0.9
<i>A. kordkuyana</i> (E)						
34MC	12.0 ± 0.2	56.1 ± 10.8	3.8 ± 0.5	24.7 ± 6.8	9.7 ± 2.2	2.6 ± 0.5
<i>A. tenuissima</i> (B)						
11LS	7.9 ± 0.3	43.1 ± 7.4	4.3 ± 0.4	31.0 ± 5.3	11.9 ± 1.6	2.6 ± 0.5
15MC	6.0 ± 0.3	39.3 ± 7.9	3.9 ± 0.5	35.6 ± 5.7	10.7 ± 1.3	2.4 ± 0.5
18MC	6.6 ± 0.2	41.6 ± 11.2	4.3 ± 0.5	36.5 ± 5.9	11.5 ± 1.4	3.2 ± 0.7
33MC	8.2 ± 0.2	49.1 ± 10.9	4.4 ± 0.7	31.2 ± 6.7	9.1 ± 1.0	2.3 ± 0.6
42MC	8.6 ± 0.3	32.2 ± 11.1	4.0 ± 0.5	39.0 ± 6.1	11.8 ± 1.1	3.3 ± 0.6

^w Growth rate: means of four plates ± standard error. Incubated on acidified potato dextrose agar for 6 days at 25°C in the dark.

^x Means of at least 15 primary conidiophores per isolates ± standard deviation.

^y Incubated on potato carrot agar for 7 days at 22°C in cycles of 8 h of light and 16 h of darkness.

^z Means of at least 50 conidia per isolate ± standard deviation. L/W = length/width ratio.

at 22°C. The differences in the mycelial growth rate among the *Alternaria* isolates were significant ($P < 0.001$). The highest growth rate was obtained for *A. kordkuyana* (12.0 mm/day), followed by *A. dumosa* (9.8 mm/day), *A. frumentii* (8.9 mm/day), *A. alternata* (8.1 mm/day), *A. tenuissima* (7.6 mm/day), and *A. arborescens* (7.2 mm/day) (Fig. 6).

Pathogenicity tests. All *Alternaria* spp. isolates developed moldy core characterized by the presence of a gray to dark olive-green mycelium with or without sporulation restricted to the carpels (Fig. 2b) in immature and mature apple fruit (Table 6). Reisolations were accomplished for 100% of the inoculated fruit. Noninoculated fruit remained asymptomatic.

All *Alternaria* isolates affected 100% of the inoculated seed, developing a superficial gray to dark olive-green mycelium and rotted 100% of the immature seed. Isolates of *A. arborescens*, *A. tenuissima*, and *A. kordkuyana* and one isolate of *A. alternata* sporulated on the seed after 5 days at 20°C.

Independently of the *Alternaria* spp., none of the isolates were able to cause necrotic lesions on the epidermis and flesh of immature and mature fruit after conidial inoculations, except for a very superficial oxidation and minimal but not measurable necrosis.

Independently of the *Alternaria* isolates, seed from mature fruit were superficially colonized, developing a gray to dark olive-green mycelium and abundant dark sporulation (Fig. 2c). However, the seed were not rotted, comprising only the testa without evidence of internal seed invasion after 10 days at 20°C. Significant differences ($P < 0.001$) in the percentage of seed colonization were obtained among isolates and *Alternaria* spp.

Without exception, all *Alternaria* isolates were pathogenic on detached apple leaves, developing brown necrotic lesions of 0.4 to 5.7 mm in diameter, with concentric rings and a chlorotic halo (Fig. 2d), after 5 days at 20°C. Significant differences ($P < 0.001$) in the lesion diameters were found among isolates and *Alternaria* spp. Differences in virulence were consistently obtained, with isolates of *A. tenuissima* being the most virulent (mean of 4.3 mm), followed by isolates of *A. alternata* (mean of 2.9 mm), *A. arborescens* (mean of 2.5 mm), *A. dumosa* (2.1 mm), *A. frumentii* (mean of 2.0 mm), and *A. kordkuyana*, which was the least virulent isolate (0.4 mm) (Table 6). Reisolations from the margins of the necrotic lesions were successful from 100% of the inoculated leaves. The identification of reisolated fungi was confirmed morphologically. Unwounded but inoculated leaves remained asymptomatic.

Discussion

The analysis of 653 fruit showing moldy core revealed the presence of several genera of filamentous fungi; however, species of *Alternaria* (67.7%) were by far the most commonly and consistently isolated causal agents. Moldy core was only found on apple cultivars having open-sinus fruit but was absent in Granny Smith, a cultivar with closed-sinus fruit.

Based on the results of this study, at least six species of small-spored *Alternaria* were found: namely, in order of importance, *A. tenuissima*, *A. arborescens*, *A. alternata*, *A. dumosa* in sect. *Alternaria*, *A. frumentii*, in sect. *Infectariae*, and *A. kordkuyana* in sect. *Pseudoalternaria*. These species were identified morphologically and molecularly according to Gannibal and Lawrence (2016), Lawrence et al. (2013), Poursafar et al. (2018), Pryor and Michailides (2002), Simmons (2007), and Zhu and Xiao (2015). The identification of *A. kordkuyana* should be further studied using additional gene sequences. A concatenated analysis using DNA sequences of *ATPase*, *CAL*, and *Alt a1* genes would be advisable. However, DNA sequences of *CAL* and *Alt a1* genes are not available for *A. kordkuyana* ex-type.

These *Alternaria* spp. were isolated alone, or two or more species coexisted in the same fruit. To some extent, the vegetative compatibility exhibited by most species and isolates explained the coexistence of more than one species on the same fruit carpel. Complexes of small-spored *Alternaria* spp. have been previously identified causing diseases in different fruit crops such as core rot of apple (Serdani et al. 2002), *Alternaria* leaf blotch and fruit spot (Hartveld et al. 2013; Rotondo et al. 2012), heart rot of pomegranate (Luo et al. 2017; Michailides et al. 2008), *Alternaria* fruit rot of blueberry (Zhu and Xiao 2015), and *Alternaria* late blight of pistachio (Pryor and Michailides 2002).

All *Alternaria* isolates obtained from moldy core of apple were small-spored *Alternaria* spp. with single conidiophores, catenulate, and muriform conidia (Simmons 2007). There is a general agreement that the identification of *Alternaria* spp. is taxonomically difficult, particularly the small-spored *Alternaria* spp., because they share

Fig. 5. Effect of temperature on the mean radial mycelial growth of isolates of *Alternaria* spp. determined after 6 days of incubation on acidified potato dextrose in the dark. Vertical bars = standard errors.

Table 5. Vegetative compatibility tests among isolates of *Alternaria* spp. obtained from moldy core of apple^a

Isolates														Isolates	Species
7 MC	12 LS	16 MC	8 MC	13 LS	38 MC	28 MC	2 MC	37 MC	34 MC	11 LS	15 MC	18 MC			
C	C	C	C	C	C	C	C	C	U	C	C	C	7 MC	<i>Alternaria alternata</i>	
...	C	C	U	C	C	C	C	C	C	C	C	C	12LS	...	
...	...	C	C	C	C	C	C	C	C	U	U	U	16 MC	...	
...	...	...	C	C	C	C	C	U	U	U	C	U	8 MC	<i>A. arborescens</i>	
...	...	...	...	C	C	C	C	U	I	U	C	C	13 LS	...	
...	...	...	...	...	C	C	C	C	U	C	C	C	38 MC	...	
...	...	...	...	...	...	C	C	C	U	C	C	C	28 MC	<i>A. dumosa</i>	
...	...	...	...	...	...	...	C	C	U	C	C	C	2 MC	<i>A. frumentii</i>	
...	...	...	...	...	...	...	...	C	C	U	C	C	37 MC	...	
...	...	...	...	...	...	...	...	...	C	U	U	C	34 MC	<i>A. kordkuyana</i>	
...	...	...	...	...	...	...	...	...	...	C	U	C	11 LS	<i>A. tenuissima</i>	
...	...	...	...	...	...	...	...	...	...	...	C	C	15 MC	...	
...	...	...	...	...	...	...	...	...	...	...	...	C	18 MC	...	

^a Symbols: compatible (C), uncertain (U), and incompatible (I) *Alternaria* isolates.

and have overlapping morphological characteristics (Andrew et al. 2009, Serdani et al. 2002). Colonies of isolates in sect. *Alternaria* varied between gray-green to yellow-green. Isolates of *A. frumenti* and *A. kordkuyana*, as expected for *Alternaria* isolates of sect. *Infectoriae* and *Pseudoalternaria*, developed white and distinctive colonies on DRYES medium (Andersen et al. 2002; Lawrence et al. 2014; Poursafar et al. 2018).

In contrast to previous works (Rang et al. 2002; Sun and Huang 2017; Zheng et al. 2015), the phylogenetic analysis of the partial coding sequence of the *HIS* gene was inadequate to distinguish isolates of *Alternaria* spp. from moldy core of apple in this study. Similarly to previous studies (Lawrence et al. 2013, 2016; Luo et al. 2017; Zheng et al. 2015), the MP analysis of the rDNA ITS sequence allowed us to clearly delimit the small-spored species of *Alternaria* within sections *Alternaria*, *Infectoriae*, and *Pseudoalternaria*. However, it was not informative enough to segregate the species within each section.

Phylogenetic analysis using the *ATPase*, *CAL*, and *Alt a1* genes has been proposed to differentiate species of *Alternaria* in previous works (Lawrence et al. 2013; Zhu and Xiao 2015). In the present study, the *Alt a1* gene provides sufficient information to clearly distinguish *Alternaria* isolates in sect. *Pseudoalternaria* from sect. *Alternaria* and sect. *Infectoriae*. The *CAL* gene allowed the differentiation of *A. arborescens*, *A. dumosa*, and isolates of *Alternaria* sect. *Infectoriae* and sect. *Pseudoalternaria*, placing them in four separate clusters, but the information provided by this gene was not sufficient to distinguish *A. tenuissima* and *A. alternata* as separate species. These last two species were clearly separated using the *ATPase* gene. However, in agreement with Zhu and Xiao (2015), *A. destruens* was clustered into the same group with *A. alternata* isolates from apple and the reference isolates of *A. alternata* but *A. alternata* and *A. destruens* could be differentiated by their sporulation patterns, which widely differ.

In Greece, Ntasiou et al. (2015) determined the presence of *A. tenuissima* and *A. arborescens* as the cause of core rot of apple. Gao et al. (2013) reported an association of *A. alternata*, *A. arborescens*,

A. infectoria, and *A. tenuissima* with moldy core in Fuji apple in China. Similarly, Serdani et al. (2002), studying the *Alternaria* spp. associated with dry core rot of apple in South Africa, demonstrated the presence of *A. arborescens*, *A. infectoria*, and *A. tenuissima*. However, isolates of *A. infectoria* from China and South Africa were not pathogenic. In contrast to results from Gao et al. (2013) and Serdani et al. (2002), isolates of *A. frumenti* in sect. *Infectoriae* from Chile were pathogenic on apple fruit and leaves. Additionally, in this study, two other pathogenic *Alternaria* spp., *A. dumosa* and *A. kordkuyana*, were found.

Independently of the *Alternaria* spp., all isolates were pathogenic, colonizing the carpels and rotting seed from immature fruit, but the seed from mature fruit were only surface colonized, without affecting the seed internally. All isolates induced small necrotic lesions on detached wounded leaves but were unable to infect nonwounded leaves, suggesting that leaf injuries are needed for infection under field conditions. However, significant ($P < 0.001$) differences in lesion diameter were obtained, suggesting differences in virulence among *Alternaria* isolates, with *A. tenuissima* being the most virulent species, followed by *A. alternata*, *A. arborescens*, *A. dumosa*, *A. frumenti*, and *A. kordkuyana*. Independently of the virulence on leaf assays and the lack of pathogenicity on immature and mature fruit, all *Alternaria* spp. showed a high capacity to colonize the carpels of both immature and mature fruit, which is the main concern regarding moldy core of apple fruit. Furthermore, differences in virulence between *Alternaria* spp. have been detected on other hosts. For example, on blueberry and pistachio, isolates of *A. infectoria* were less virulent than *A. alternata*, *A. arborescens*, and *A. tenuissima* (Pryor and Michailides 2002; Zhu and Xiao 2015).

Alternaria isolates were not able to produce fruit rot lesions in this study, as was reported previously for some isolates of *A. alternata*, *A. arborescens*, and *A. tenuissima* (Harteveld et al. 2014; Rotondo et al. 2012). These differences in the pathogenic capacities suggest the presence of different pathotypes. Nevertheless, differences in the susceptibility of apple cultivars used in the pathogenicity tests can also

Fig. 6. Mycelial growth rate of isolates *Alternaria* spp. estimated on acidified potato dextrose in the dark at 25°C. Vertical bars = standard errors.

explain these results. Large differences in susceptibility have been reported; Golden Delicious was very susceptible, Top Red was moderately resistant, and Jonathan was resistant (Gur et al. 2017). In addition, the inoculated tissue should be considered, because it has been shown that the mesoderm close to the skin is highly resistant to *Alternaria* rot, increasing in susceptibility toward the carpels (Gur et al. 2017). Interestingly, no evidence was obtained in this study of *A. mali*, which has been associated mainly with fruit rot of apple (Gur et al. 2017; Harteveld et al. 2013; Rotondo et al. 2012).

All of the reference isolates obtained from necrotic leaf spot of apple were equally able to colonize carpels internally, developing a cottony gray to olive-green mycelium that characterized apple moldy core. Therefore, there was no evidence for apple tissue specificity among isolates of *Alternaria* spp. attacking apple. Similar to findings by Rang et al. (2002), our results suggested that moldy core resulted from opportunistic and favorable conditions rather than true specific pathogenic abilities exhibited by the isolates of *Alternaria* spp.

Moldy core prevalence between 4 and 46% was estimated in this study, reinforcing the economic importance of moldy core in the Chilean apple industry. As has been reported in other apple-producing countries (Ellis and Barrat 1983, Kupferman 1992; Miller 1959; Reuveni 2006; Serdani et al. 1998), moldy core has been associated with the open-sinus condition of Delicious cultivars. In this study, 40 and 53% of Oregon Spur and Red Chief apple with an open sinus were very susceptible, showing the highest moldy core prevalence (16 to 46%). In the present study, Fuji (Red Delicious × Ralls Janet) had a high proportion of open-sinus fruit at harvest (42%).

However, a relatively low (<13%) proportion of moldy core was determined. This finding can be explained by factors other than the sinus condition of the fruit (Shtienberg 2012). Differences in the mesoderm and locule sensitivity of each apple cultivar to colonization by moldy core fungi could also explain the substantial variation in prevalence obtained among open-sinus apple cultivars in this study (Niem et al. 2007).

The apparent resistance of Granny Smith apple to moldy core appeared to be associated with the closed-sinus condition characterizing Granny Smith fruit (Serdani et al. 1998).

In summary, this study demonstrates that moldy core of apple is caused by many small-spored *Alternaria* spp. The most frequently isolated species was *A. tenuissima*, which was virulent on apple leaf assays and showed a high capacity to colonize apple carpels. However, *A. tenuissima* and the other *Alternaria* spp. show no capacity to cause lesion on the epidermis and flesh of apple fruit. This species coexisted with one or more *Alternaria* spp. This is the first report of *A. arborescens*, *A. dumosa*, *A. frumenti*, *A. tenuissima*, and *A. kordkuyana* associated with moldy core of apple in Chile and the first report of *A. dumosa*, *A. frumenti*, and *A. kordkuyana* causing moldy core of apple worldwide. The results of this study do not exclude the possibility that other species may be involved in the *Alternaria* complex associated with moldy core of apple. The presence of different species of *Alternaria* may have implications to consider when establishing control strategies, because *Alternaria* spp. may have different sensitivities to fungicides. More information on moldy core epidemiology and sensitivity to fungicides of *Alternaria* spp. is needed in order to implement successful control strategies.

Table 6. Pathogenicity of *Alternaria* spp. isolates on fruit and leaves of Red Chief apple, producing colonization of carpels and seed and causing necrotic lesions on leaves^x

Isolates	Immature fruit ^y			Mature fruit ^y			Leaves ^z
	CM (%)	SR (%)	DR (mm)	CM (%)	SM (%)	DR (mm)	NL (mm)
<i>Alternaria alternata</i>							
7MC	85 a–d	100	0.0	100 ns	90 b	0.0	4.7 a–c
12LS	65 d	100	0.0	70	95 ab	0.0	2.5 c–g
16MC	80 a–d	100	0.0	80	90 b	0.0	1.3 g
30MC	95 ab	100	0.0	90	100 a	0.0	4.0 a–d
36MC	75 cd	100	0.0	80	100 a	0.0	2.0 d–g
Mean	80	100	0.0	84	95	0.0	2.9
<i>A. arborescens</i>							
8MC	80 b–d	100	0.0	100	100 a	0.0	2.9 b–f
13LS	85 a–d	100	0.0	90	100 a	0.0	2.8 b–f
19MC	85 a–d	100	0.0	90	100 a	0.0	3.0 a–e
38MC	95 ab	100	0.0	95	100 a	0.0	1.9 d–g
40MC	90 a–c	100	0.0	90	100 a	0.0	2.1 e–g
Mean	87	100	0.0	93	100	0.0	2.5
<i>A. dumosa</i>							
28MC	100 a	100	0.0	95	100 a	0.0	2.1 e–g
<i>A. frumenti</i>							
2MC	80 a–d	100	0.0	85	45 c	0.0	2.2 c–g
37MC	90 a–c	100	0.0	80	50 c	0.0	1.5 fg
Mean	95	100	0.0	83	48	0.0	2.0
<i>A. kordkuyana</i>							
34MC	95 ab	100	0.0	95	90 b	0.0	0.4 h
<i>A. tenuissima</i>							
11LS	95 ab	100	0.0	95	100 a	0.0	5.7 a
15MC	100 a	100	0.0	95	100 a	0.0	3.4 a–e
18MC	90 a–c	100	0.0	85	100 a	0.0	4.9 ab
33MC	95 ab	100	0.0	90	100 a	0.0	5.3 ab
42MC	95 ab	100	0.0	90	100 a	0.0	2.3 d–g
Mean	95	100	0.0	91	100	0.0	4.3

^x Abbreviations: CM = carpel moldy, SR = seed rot, DR = dry rot, SM = seed moldy, and NL = necrotic lesions. Noninoculated controls remained symptomless and those data were excluded from the statistical analysis. Means of four replicates followed by the same letter in each column did not differ significantly, separated according to the Fisher's least significant difference test ($P < 0.05$); ns = not significant at $P = 0.05$.

^y Five carpels per fruit ($n = 4$) and five seeds per replicate ($n = 4$) were inoculated with a conidial suspension (10^6 conidia/ml) incubated in humid chambers at 20°C for 5 days for immature fruit and for 10 days for mature fruit. Data percentages were $\arcsin\sqrt{(x/100)}$ transformed but nontransformed data are presented.

^z Leaves were inoculated with conidial suspension (10^6 conidia/ml), and lesion diameters were determined after 4 days at 20°C in humid chambers. Data were transformed to $\ln(x + 1)$ prior to analysis but nontransformed data are presented.

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