Morphological, Pathogenic, and Molecular Characterization of *Colletotrichum acutatum* Isolates Causing Almond Anthracnose in Spain

Ana López-Moral, Maria Carmen Raya-Ortega, Carlos Agustí-Brisach, and Luis F. Roca, Departamento de Agronomía, ETSIAM, Universidad de Córdoba, Campus de Rabanales, Edif. C4, 14071 Córdoba, Spain; Maria Lovera, Departamento de Fruticultura Mediterránea, IFAPA, Alameda del Obispo, 14004 Córdoba, Spain; Francisca Luque, Departamento de Agronomía, ETSIAM, Universidad de Córdoba, Campus de Rabanales; Octavio Arquero, Departamento de Fruticultura Mediterránea, IFAPA; and Antonio Trapero,[†] Departamento de Agronomía, ETSIAM, Universidad de Córdoba, Campus de Rabanales

Abstract

Almond anthracnose is a serious and emerging disease in several countries. All isolates causing almond anthracnose have been assigned to the *Colletotrichum acutatum* species complex, of which only *C. fioriniae* and *C. godetiae* have been associated with the disease to date. Here, we characterized *Colletotrichum* isolates from almond fruit affected by anthracnose in the Andalusia region. Two *Colletotrichum* isolates causing olive anthracnose were included for comparison. Morphological characteristics were useful for separating the isolates into groups based on colony morphology. Pathogenicity tests in almond, olive, and apple fruit showed differences in virulence and some degree of pathogenic specialization

among isolates. Molecular characterization allowed clear identification of the *Colletotrichum* isolates tested. The olive isolates were identified as *C. godetiae* and *C. nymphaeae*, both previously identified in Andalusian olive orchards. Two phylogenetic species were identified among the almond isolates: *C. godetiae*, with gray colonies, which is well known in other countries, and *C. acutatum*, with pink-orange colonies. This species identification differs from those of pink-colony subpopulations described in other countries, which are *C. fioriniae*. Therefore, this study is also the first report of a new species of *Colletotrichum* causing almond anthracnose within the *C. acutatum* species complex.

Almond (*Prunus dulcis* L.) is a traditional and characteristic crop in the Mediterranean Basin, with great social and economic importance due to its large acreage and its demarcation, mostly in areas with unfavorable climatic and orographic conditions (Arquero et al. 2013). In Spain, it is the fruit crop second in acreage after the cultivated olive (*Olea europaea* subsp. *europaea* L.). The almond crop currently occupies approximately 500,000 ha in Spain, of which 150,000 ha are located in the Andalusia region (southern Spain). The average crop yield of almond kernels in Spain is less than 150 kg ha⁻¹ whereas, in California (San Joaquin Valley), it is approximately 2,500 kg ha⁻¹ (Ollero-Lara et al. 2016). During the last few years, almond crops have undergone a significant change in Spain. The current economic impact of almond kernels all over the world as well as the necessity to find extensive alternative crops in this country have led growers in the Iberian Peninsula to establish new almond plantings in nontraditional areas for this crop, with optimum soil and climatic conditions. However, in these new plantings, the incidence of foliar diseases has been higher than that in traditional fields located in areas with unfavorable climatic and orographic conditions, which is limiting its profitability (Arquero et al. 2013; Ollero-Lara et al. 2016).

In spring 2014, a serious fruit disease showing typical anthracnose symptoms was observed in almond orchards in the provinces of Huelva and Sevilla (Andalusia region), where it is considered an emerging disease (López-Moral et al. 2016). Almond anthracnose was described for the first time in Italy in 1896. Between the beginning and the middle of the twentieth century, the disease was detected in California, South Africa, Tunisia, and France (Förster and Adaskaveg 1999; Teviotdale et al. 2002). However, it was considered a secondary disease in the Mediterranean Basin until the 1960s. In Spain, symptoms of the disease were first observed in 1900 on the island of Mallorca (Ballester 1916) but the causal agent of the disease was not

identified until 1965 in Huesca (northeastern Spain). Four years later, it was identified in Valencia, Castellón, and Tarragona (eastern Spain) (Palazón and Palazón 1979). Since then, it has been detected regularly, especially in orchards on the Mediterranean coast, becoming a clearly endemic disease. Currently, almond anthracnose is widely distributed in the main almond-growing areas worldwide (Teviotdale et al. 2002).

The pathogen mainly infects fruit, causing depressed, sunken, round, and orange lesions from 5 to 12 mm in diameter. In the incipient lesions, the coloration of affected areas does not clearly differ from the normal epidermis. However, a whitish mycelium that produces abundant orange masses of conidia appears when the symptoms progress. Finally, the affected fruit mummify, and most of them fall to the soil. The mummies that remain in the tree canopy during autumn and winter serve as the main inoculum source for the spring crop the following year. Almond trees with large numbers of diseased fruit also show defoliation and dieback of shoots and branches due to the toxins produced by the pathogen in the infected fruit (Adaskaveg et al. 2012; Förster and Adaskaveg 1999). This disease may cause important economic losses due to the premature loss of fruit, which could represent up to 80% of the harvest (López-Moral et al. 2016). Even the leaves may show symptoms in the tips and margins, giving rise to irregular lesions which conclude in total necrosis of the leaf (Supplementary Fig. S1) (Palazón and Palazón 1979).

The causal agent of almond anthracnose was described for the first time in 1896 in Italy as *Gloeosporium amygdalinum* Brizi. Later, studies confirmed that this species was indistinguishable from other *Gloeosporium* spp., being reclassified by von Arx (1970) in the *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. species complex. In 1965, the species *C. acutatum* J. H. Simmonds, close to *C. gloeosporioides*, was identified. These two *Colletotrichum* spp. differ in morphology, mainly in the ends of their conidia, which are sharp in *C. acutatum* and rounded in *C. gloeosporioides* (Simmonds 1965; Sutton 1980, 1992). Consequently, based on these morphological characteristics, all isolates of *C. gloeosporioides* causing almond anthracnose were reclassified as *C. acutatum* (most of the isolates) and *C. gloeosporioides* (a few isolates recovered in Israel) (Freeman et al. 1998). After that time, based on molecular characteristics, all isolates causing almond anthracnose have been assigned to the species *C. acutatum* (Adaskaveg and Hartin 1997; Freeman et al. 2000; Guerber et al. 2003; McKay et al. 2009). Nevertheless, because *C. acutatum* is an extremely genetically variable species, it has recently

[†]Corresponding author: A. Trapero; E-mail: trapero@uco.es

*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary figures are published online.

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been considered as a species complex, within which 31 phylogenetic species have been described (Damm et al. 2012). Among them, only two species, *C. fiorinae* Marcelino & Gouli ex R. G. Shivas & Y. P. Tan and *C. godetiae* Neerg., have been associated with almond anthracnose. These two species coincide with the two subpopulations established on the basis of morphological characteristics, mainly pink (*C. fiorinae*) or gray (*C. godetiae*) colony color, which have been described for the causal agents of almond anthracnose (Damm et al. 2012; Guerber et al. 2003; Teviotdale et al. 2002).

In Andalusia, almond and olive crops coexist in the same agroecosystem. Olive is the most important perennial crop in Spain, leading the world production of olive oil and olive fruit, and it is also seriously affected by anthracnose (Moral et al. 2014). Olive anthracnose has been plentifully studied all over the world (Cacciola et al. 2012; Moral et al. 2014; Talhinhos et al. 2011). In Spain, the disease's symptoms have been mainly associated with the fruit at ripening, causing fruit rot, mummification, and fruit drop (Moral et al. 2009; Moral et al. 2014). The causal agents belong to the species complex of *C. acutatum* and *C. gloeosporioides* (Cacciola et al. 2012; Moral et al. 2014; Talhinhos et al. 2011). Within the *C. acutatum* species complex, *C. godetiae* is the dominant species in Spain (Moral et al. 2014). Nevertheless, the interactions between the *Colletotrichum* spp. associated with anthracnose in the almond and olive crops have not yet been studied.

In Spain, little attention has been given to almond anthracnose. Nevertheless, it is considered a reemerging disease, becoming endemic. It has increasingly become a cause for concern among growers in the almond-producing areas of the Andalusia region. For these reasons, diagnosis and control of almond anthracnose have gained great importance. However, although several studies about the identification and characterization of *Colletotrichum* isolates obtained from almond have been conducted in different areas of the world (Adaskaveg and Hartin 1997; Förster and Adaskaveg 1999; Freeman et al. 2000; Guerber et al. 2003; McKay et al. 2009), the etiology, epidemiology, and control of this disease in Spain is still uncertain. Identification and characterization of the causal agent is essential to establish the basis of studies on epidemiology and disease control. Thus, the main objective of this study was to characterize *Colletotrichum* isolates obtained from almond affected by anthracnose in Andalusia, in relation to morphological, pathogenic, and genetic characteristics. At the same time, the almond isolates were compared with *Colletotrichum* isolates causing olive anthracnose in the same region.

Material and Methods

Collection of fungal isolates. From 2014 to 2016, almond fruit showing typical anthracnose symptoms were collected from 10 commercial orchards located in the municipalities of Alcalá del Río, Lebrija,

Maribáñez and Villamanrique de la Condesa (Seville province); Gibraleón (Huelva province); Santa Cruz (Córdoba province), and Badajoz (Badajoz province), all in southwestern Spain. One commercial orchard was surveyed per location, with the exception of Lebrija, Gibraleón, and Villamanrique municipalities, in which two commercial orchards were surveyed. Affected fruit were surface disinfected with commercial bleach (Cl at 50 g liter⁻¹) at 10% (vol/vol) in sterile water for 1 min, and air dried on sterile filter paper for 30 min. Affected tissues were cut with a sterile scalpel and plated on potato dextrose agar (PDA) (Difco Laboratories) acidified with lactic acid (2.5 ml of 25% [vol/vol] per liter of medium) to minimize bacterial growth (APDA). When the affected fruit tissues showed abundant pathogen sporulation, masses of conidia were removed using a sterile needle and cultured in petri dishes on APDA. Petri dishes were incubated at 25 ± 2°C under a 12-h diurnal photoperiod of cool fluorescent light (350 µmol m⁻² s⁻¹) for 5 days, and hyphal tips from the colonies were transferred to PDA and incubated as described above. In total, 420 *Colletotrichum* isolates were obtained and divided into two different groups or subpopulations according to the morphology of the colony, especially colony color (gray and pink-orange). In all, 12 *Colletotrichum* isolates (9 isolates of the gray subpopulation and 3 isolates of the pink-orange subpopulation) were selected for further analysis in this study (Table 1). In addition, two *Colletotrichum* isolates, Col-506 and Col-508, representatives of the two subpopulations causing anthracnose in olive orchards in the Andalusia region, were also included (Table 1). Single-spore isolates were prepared prior to use by means of the serial dilution method (Dhingra and Sinclair 1995). All isolates were used for molecular characterization. Representative isolates from each almond subpopulation—gray (Col-522, Col-548, and Col-555) or pink-orange (Col-536, Col-537, and Col-538)—and from olive (Col-506 and Col-508) were selected for morphological characterization and pathogenicity tests on detached almond, olive, and apple fruit (Table 1). All isolates are maintained in the collection of the Departamento de Agronomía at the Universidad de Córdoba (Spain).

Colony and conidium morphology. Four representative isolates of the two subpopulations from almond (Col-522 and Col-536) and the two subpopulations from olive (Col-506 and Col-508) (Table 1) were used to study conidium morphology growing in two different media, PDA, and artificially inoculated detached Guara almond fruit (moderately susceptible) (López-Moral et al. 2016). Healthy fruit were collected from the network of trials of almond orchards belonging to the Andalusian Institute for Research and Formation in Agriculture and Fishery (IFAPA in Spanish) located in Córdoba province. For the evaluation on culture medium, the isolates were grown on PDA for 7 days, as described above. There were three replicate Petri dishes per isolate. For the evaluation on artificially inoculated detached almond fruit, each fruit was punctured superficially with a needle (0.8 mm in diameter and

Table 1. Isolates of *Colletotrichum* spp. used in this study

Isolate ^w	Fungal species	Host	Subpopulation (colony type)	Affected tissue	Origin (location, province, orchard) ^x
Col-506 ^{y,z}	<i>Colletotrichum nymphaeae</i>	<i>Olea europaea</i>	Light gray	Fruit	Hornachuelos, Córdoba
Col-508 ^{y,z}	<i>C. godetiae</i>	<i>O. europaea</i>	Dark gray	Fruit	Hornachuelos, Córdoba
Col-536 ^{y,z}	<i>Colletotrichum</i> sp.	<i>Prunus dulcis</i>	Pink-orange	Fruit	Lebrija, Sevilla, orchard 1
Col-537 ^y	<i>Colletotrichum</i> sp.	<i>P. dulcis</i>	Pink-orange	Fruit	Lebrija, Sevilla, orchard 1
Col-538 ^y	<i>Colletotrichum</i> sp.	<i>P. dulcis</i>	Pink-orange	Fruit	Lebrija, Sevilla, orchard 2
Col-522 ^{y,z}	<i>Colletotrichum</i> sp.	<i>P. dulcis</i>	Gray	Fruit	Lebrija, Sevilla, orchard 2
Col-524	<i>Colletotrichum</i> sp.	<i>P. dulcis</i>	Gray	Fruit	Lebrija, Sevilla, orchard 3
Col-525	<i>Colletotrichum</i> sp.	<i>P. dulcis</i>	Gray	Fruit	Lebrija, Sevilla, orchard 3
Col-540	<i>Colletotrichum</i> sp.	<i>P. dulcis</i>	Gray	Fruit	Gibraleón, Huelva
Col-542	<i>Colletotrichum</i> sp.	<i>P. dulcis</i>	Gray	Fruit	Villamanrique, Sevilla
Col-544	<i>Colletotrichum</i> sp.	<i>P. dulcis</i>	Gray	Fruit	Villamanrique, Sevilla
Col-548 ^x	<i>Colletotrichum</i> sp.	<i>P. dulcis</i>	Gray	Fruit	Maribáñez, Sevilla
Col-555 ^x	<i>Colletotrichum</i> sp.	<i>P. dulcis</i>	Gray	Fruit	Gibraleón, Huelva
Col-613	<i>Colletotrichum</i> sp.	<i>P. dulcis</i>	Gray	Fruit	Santa Cruz, Córdoba

^w All isolates were used for molecular characterization.

^x All locations belong to the Andalusia region, southern Spain. Number of the orchard is indicated when several orchards were surveyed in the same location.

^y *Colletotrichum* isolates used for pathogenicity tests to almond.

^z *Colletotrichum* isolates selected as representative of each host and fungal subpopulation (colony type) used for morphological characterization and for pathogenicity tests to olive and apple fruit.

25 mm long) before inoculation. Fruit were placed in humid chambers (plastic containers, 22 by 16 by 10 cm), inoculated with a 20- μ l drop of a conidial suspension adjusted with a hemocytometer to 10^6 conidia ml^{-1} deposited on the wound, and incubated at $23 \pm 2^\circ\text{C}$ with a 12-h photoperiod and 100% relative humidity (RH) for 14 days. A completely randomized design, with fungal isolates as the independent variable and humid chambers as replications, was performed. There were three replicate humid chambers per isolate or control, and 15 fruit per humid chamber and per isolate or control were used (225 almond fruit in total). The experiment was conducted twice.

For each isolate and growth medium (PDA or almond fruit), the size and the shape of 100 conidia were measured by using a Nikon Eclipse 80i microscope (Nikon Corp.) at $\times 400$ magnification. Culture characteristics (texture, density, and color) were recorded on 7-day-old colonies grown on PDA. The size of the conidia was determined by measuring their length and width, and their volume was calculated (considering conidia as cylindrical structures) by the equation $V = \pi \cdot r^2 \cdot h$, where r = conidium width/2 and h = conidium length. According to their shape, conidia were classified into three categories: (i) conidia with two rounded ends (ellipsoid), (ii) conidia with one rounded end and the other acute (clavate), and (iii) conidia with two acute ends (fusoid). Data were expressed as a percentage of each category to determine the predominant conidium type for each isolate.

Data from the two replicates of the experiment were combined after checking for homogeneity of the experimental error variances with the F test. For the statistical analysis, length, width, and volume variables were analyzed using the Kruskal-Wallis nonparametric method, due to the lack of homogeneity of variances. Mean values were compared by Dunn's test at $P = 0.05$. All data of this study were analyzed using Statistix 10 (Analytical Software 2013).

Conidium production. Mycelial plugs (7 mm in diameter) obtained from the margins of 7-day-old actively growing colonies on PDA of the four representative isolates (Col-506, Col-508, Col-522, and Col-536) were placed in the center of Petri dishes (9 cm in diameter, one plug per plate) with oatmeal agar (OA) (Crous et al. 2009) or Spezieller Nährstoffarmer agar (SNA) (Leslie and Summerell 2006). Petri dishes were incubated at $23 \pm 2^\circ\text{C}$ with a 12-h photoperiod. After 21 days, four plugs of agar (7 mm in diameter), with mycelia and spores, were cut from the growing edge of each colony from each culture media. The plugs of each plate were introduced into a glass tube containing 25 ml of distilled and deionized sterile water (DDSW). Glass tubes with plugs were shaken for 5 s, and the number of conidia per milliliter was counted using a hemocytometer. There were three replicate Petri dishes per isolate and culture medium combination, and the experiment was conducted twice.

A randomized complete block design was used, with experiments as blocks, isolates and media as the independent variables, and Petri dishes as replications. The two runs of the experiment were combined after checking for homogeneity of the experimental error variances by the F test. Analysis of variance (ANOVA) was applied considering each medium (OA and SNA) separately, because there were large differences between the media. Means were compared according to Fisher's protected least significant difference (LSD) test at $P = 0.05$ (Steel and Torrie 1985).

Effect of temperature on mycelial growth. Mycelial plugs of the four representative isolates (Col-506, Col-508, Col-522, and Col-536) were placed on PDA, as described above. Petri dishes were incubated at 0, 5, 10, 15, 20, 25, 30, and 35°C in darkness. After 8 days of incubation, the largest and smallest diameters of colonies were measured using a digital scale ruler. Mean data were converted to radial growth rate (millimeters per day). There were three replicate Petri dishes per isolate and temperature combination, and the experiment was conducted twice.

For each isolate, to analyze the variation of the mycelial growth rate over temperature, a nonlinear adjustment of the data were applied using the generalized Analytis Beta model, which is expressed by the following equation (Moral et al. 2012; Viruega et al. 2011):

$$Y = c \times t^a \times (1 - t)^b \quad (1)$$

In this equation, Y = standardized growth rate; t = standardized temperature obtained by the formula $t = (T - T_{\min}) / (T_{\max} - T_{\min})$, where $T_{\min}$ and $T_{\max}$ are the minimum and maximum growth temperatures, respectively; and a , b , and c are unknown parameters. The above equation may be rewritten as

$$Y = d \times (T - T_{\min})^a \times (T_{\max} - T)^b \quad (2)$$

Subsequently, the optimum growth temperature (T_{opt}) and its corresponding maximum growth rate were calculated for each isolate. For this, the values of a and b obtained from equation 2 in each repetition of each isolate were replaced in equation 3, which was obtained by equating to 0 the derivative of equation 2:

$$T_{\text{opt}} = [(a \times T_{\max}) + (b \times T_{\min})] / (a + b) \quad (3)$$

ANOVA was performed on the estimated optimum temperature and maximum growth rate data obtained from the fitted model. General ANOVA were applied to the pooled data of both variables using experimental runs as blocks, because variances of the experimental error were homogeneous. Mean values of optimum temperature and maximum growth rate were compared using Fisher's protected LSD test at $P = 0.05$ (Steel and Torrie 1985).

Pathogenicity tests. *Pathogenicity on detached almond fruit.* Detached, healthy and unwounded Guara and Lauranne almond fruit, which are moderately susceptible (López-Moral et al. 2016) and representative of the most used cultivars in the new almond plantings in Spain (Arquero et al. 2013), were collected in the almond orchards of the IFAPA trials network in the province of Córdoba. Fruit were harvested at their first stages of maturity, when they had not yet reached their final size and were highly susceptible to the disease. They were inoculated with six *Colletotrichum* isolates from almond (Col-522, Col-536, Col-537, Col-538, Col-548, and Col-555) and two from olive (Col-506 and Col-508) (Table 1). Almond fruit were washed with 0.02% Tween-20 solution in tap water for 1 min, surface sterilized by immersing them in 10% solution of commercial bleach (Cl at 50 g liter^{-1}) for 1 min, and double washed with tap water. A second disinfection was carried out with 70% ethanol solution for 1 min, and the fruit were allowed to air dry on sterile filter paper for 30 min. Disinfected almond fruit were placed in humid chambers, wounded, inoculated, and incubated as described above. Noninoculated almond fruit with a 20- μ l drop of DDSW deposited in the wound were included as controls. A factorial randomized complete-block design with three replicate humid chambers per cultivar and isolate or control was performed. In all, 15 fruit per humid chamber, per cultivar, and per isolate or control were used (810 almond fruit in total). The experiment was conducted twice.

Disease severity in inoculated fruit was evaluated every 4 days until most of the fruit reached 90 to 100% of their surface affected (20 days; five evaluations). The average of the major and minor diameters of the lesion produced in each fruit was used to determine the percentage of affected fruit surface for each isolate, cultivar, and evaluation time. For each cultivar-isolate combination, the area under the disease progress curve (AUDPC) was calculated by trapezoidal integration of the percentage of affected fruit surface over time. Subsequently, relative AUDPC (RAUDPC) was obtained by dividing each AUDPC by the highest AUDPC value obtained in the experiment. RAUDPC data from both experiments were subjected to a factorial ANOVA, and the treatment means were compared according to Tukey's honestly significant difference (HSD) test at $P = 0.05$ because there were more than five means (Steel and Torrie 1985). Because interaction between fungal isolate and almond cultivar was not significant, isolates were compared by combining the data from both cultivars.

Pathogenicity on detached olive fruit. Unwounded violet (color class 3) (Barranco and Rallo 2005), moderately susceptible Arbequina olive fruit (Moral et al. 2008) were collected from healthy olive fruit growing in the World Olive Germplasm Bank belonging to the IFAPA, located in Córdoba province. The four isolates selected for inoculation were Col-506 and Col-508 from olive and Col-522 and Col-536 from almond. Before inoculation, the olive fruit were washed and surface

disinfected, as described by Moral et al. (2008). Fruit were placed in humid chambers as described above and inoculated by spraying them until the run-off point with a conidial suspension adjusted with a hemocytometer to 10^5 conidia ml^{-1} . After inoculation, the humid chambers were incubated at $23 \pm 2^\circ\text{C}$, with a 12-h photoperiod. In addition, non-inoculated olive fruit sprayed with DDSW were included as controls. A completely randomized design with three replicate humid chambers per isolate and control was used, with 20 fruit per humid chamber and per isolate or control (300 olive fruit in total). The experiment was conducted twice.

Disease severity in inoculated fruit was evaluated weekly until most of the fruit achieved the maximum value (100%, in approximately 28 days). Disease severity was assessed using a 0-to-5 rating scale, where 0 = no symptoms and 1 = 1 to 25, 2 = 26 to 50, 3 = 51 to 75, 4 = >75, and 5 = 100% of the fruit surface affected (Moral et al. 2008). A disease severity index (DSI) was calculated in each replication using the formula $\text{DSI} = [(\sum n_i \times i) / (N \times 5)] \times 100$, where i represents a severity (0 to 5), n_i is the number of fruit with severity i , N is the total number of fruit, and 5 is the highest value of the severity rating scale. RAUDPC was calculated by trapezoidal integration of DSI values over time. RAUDPC data from the two runs of the experiment were subjected to ANOVA, and means were compared according to Fisher's protected LSD test at $P = 0.05$ because there were fewer than five means (Steel and Torrie 1985).

Pathogenicity on apple fruit. Inoculation of apple fruit (*Malus domestica* Borkh) was carried out to study the pathogenicity of *Colletotrichum* isolates in a different host. Healthy and unwounded Golden Delicious apple fruit obtained from a commercial market were used. Four isolates of *Colletotrichum* selected as representative (Col-506 and Col-508 from olive and Col-522 and Col-536 from almond) were used for inoculation. Fruit were washed under running tap water, surface sterilized by immersion in 70% ethanol solution, and allowed to air dry on a laboratory bench. Subsequently, four holes (7 mm in diameter and 3 mm deep) were made in the widest part of the fruit using a cork borer, with equal distances between them. Inoculation was carried out by depositing inside each hole a 40- μl drop of a conidial suspension adjusted with a hemocytometer to 10^4 conidia ml^{-1} . Noninoculated apple fruit with a 40- μl drop of DDSW deposited into each hole were included as controls. Inoculated and noninoculated apple fruit were placed in humid chambers (closed plastic containers of 30 by 45 by 15 cm, filled with sterile perlite at the bottom, with 100% RH obtained by adding 500 ml of water) and incubated as described above. A factorial randomized complete-block design with three replicate humid chambers per isolate and control was used, with apple fruit per humid chamber and per isolate or control (60 fruit in total). The experiment was conducted twice.

Disease severity was assessed by measuring the major and minor diameters of each lesion in each fruit. The first evaluation was carried out 10 days after inoculation, and subsequent evaluations were performed every 3 days until the lesions reached the maximum diameter (16 days; three evaluations). The average diameter was expressed in millimeters of real growth by deducting the 7 mm corresponding to the hole diameter. Subsequently,

RAUDPC was calculated by trapezoidal integration of the mean diameter of the lesion over time, and a one-way ANOVA was applied to the RAUDPC data. Comparison of means was performed according to Fisher's protected LSD test at $P = 0.05$ (Steel and Torrie 1985).

Molecular characterization. DNA extraction. All isolates of *Colletotrichum* spp. shown in Table 1 were characterized molecularly. For genomic DNA, fungal mycelium and conidia from single-spore cultures grown on PDA at $23 \pm 2^\circ\text{C}$ with a 12-h photoperiod for 7 days were used. Total DNA was extracted using the EZNA Fungal DNA Kit (OMEGA Bio-Tek) following the manufacturer's instructions. The concentration and purity of the extracted DNA were determined with a MaestroNano spectrophotometer (MaestroGen).

PCR analysis and sequencing. Six genomic areas were amplified and sequenced, including the 5.8S nuclear ribosomal gene with two flanking internal transcribed spacers (ITS), a 200-bp intron of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and partial sequences of the chitin synthase 1 (CHS-1), histone 3 (HIS3), actin (ACT), and β -tubulin (TUB2) genes. The primer pairs used for each genomic area, as well as their sequences, are shown in Table 2.

Polymerase chain reaction (PCR) amplifications were performed in a MyCycler Thermal Cycler (Bio-Rad) in a total volume of 25 μl . All PCR mixtures contained 20 ng of genomic DNA, 5 μl of $5\times$ My Taq reaction buffer, and 0.13 μl of My Taq DNA polymerase (BioLine). Additionally, for GAPDH, CHS-1, HIS3, and ACT PCR, 0.2 μM each primer was used, and the reaction conditions were an initial denaturation step of 5 min at 94°C ; followed by 40 cycles of 30 s at 94°C , 30 s at 52°C , and 30 s at 72°C ; and a final step of 7 min at 72°C (Damm et al. 2012). For the ITS region PCR, 0.4 μM each primer was used, and the conditions were an initial denaturation step of 3 min at 94°C ; followed by 35 cycles of 15 s at 94°C , 20 s at 48°C , and 1 min at 72°C ; and a final step of 7 min at 72°C . A negative control was included in all PCR, for which ultrapure water was used instead of DNA. Amplification products were checked by electrophoresis in 1.5% (wt/vol) agarose gel stained with RedSafe (Intron Biotechnology) and visualized under UV. As a molecular-weight marker, a 100-bp DNA Ladder-GTP (gTPbio) was used. Subsequently, the PCR products were purified using MEGAquick-spin Total Fragment DNA Purification kit (INTRON Biotechnology) following the manufacturer's instructions, and they were sent to the Central Service Support Research of the University of Córdoba (Spain) for sequencing.

Phylogenetic analysis. The nucleotide sequences generated by the forward and reverse primers, after being checked and corrected where necessary, were used to obtain consensus sequences using DNASTART LaserGen SeqMan v. 7.0.0. In addition, sequences from the 45 main species of *Colletotrichum* and from *C. orchidophilum* Damm, P. F. Cannon & Crous as outgroup (Damm et al. 2012) were obtained from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) and included in the analysis (Table 3).

Previous studies conducted by Damm et al. (2012) indicated that all six genomic areas under study were consistent and combinable, because they showed no conflict in the tree topologies obtained.

Table 2. Primers used to amplify the six genomic areas studied by DNA analysis

Primer ^y	Primer pairs	Genomic sequence (5'–3')	Size (bp) ^z	Reference
ITS	ITS4	TCCTCCGCTTATTGATATGC	540	White et al. 1990
	ITS5	GGAAGTAAAAGTCGTAACAAAG	...	...
TUB2	BT2A	GGTAACCAAATCGGTGCTGCTTTC	429	Glass and Donaldson 1995
	BT2B	ACCCTCAGTGTAGTGACCCTTGGC	...	...
ACT	ACT-512F	ATGTGCAAGGCCGGTTTCGC	246	Carbone and Kohn 1999
	ACT783R	TACGAGTCCTTCTGCCCCAT	...	...
CHS-1	CHS-79F	TGGGGCAAGGATGCTTGAAGAAG	282	Carbone and Kohn 1999
	CHS-354R	TGGAAGAACCATCTGTGAGAGTTC	...	...
HIS3	CYLH3F	AGGTCCACTGGTGGCAAG	382	Crous et al. 2004
	CYLH3R	AGCTGGATGTCCTTGACTG	...	...
GAPDH	GDF1	GCCGTCAACGACCCCTTCATTGA	250	Guerber et al. 2003
	GDF1	GGGTGGAGTCGTACTTGAGCATGT	...	...

^y ITS = internal transcribed spacer, TUB2 = β -tubulin, ACT = actin, CHS-1 = chitin synthase 1, HIS3 = histone 3, and GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

^z Expected polymerase chain reaction product.

Therefore, multilocus sequences obtained from the union of individual genes were used for phylogenetic analysis. Each of the individual genes, as well as a combined data set, was aligned and analyzed using MEGA, v.7 (Kumar et al. 2016). Phylogenetic Data Editor (PhyDE-1) software was used to concatenate the genes and to form a supermatrix. First, a maximum parsimony (MP) analysis was performed for multilocus alignment using the tree-bisection-reconnection algorithm, in which initial trees (10 replicates) were obtained by random addition of sequences. Alignment gaps were treated as missing data. The robustness of the trees obtained was measured by 2,000 bootstrap replications. Tree length (L), consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI) were also calculated. Multilocus sequences were also analyzed by Bayesian Inference using the Markov Chain Monte Carlo algorithm.

MrBayes, v.3.2.2 was used to generate phylogenetic trees with Bayesian probabilities (Ronquist et al. 2012). MEGA, v.7 (Kumar et al. 2016) was used to determine the models of nucleotide substitution used for each gene partition. The analysis was initiated with a random tree and default parameters. Two analyses in parallel were performed with four Markov chains, evaluating 10^7 generations and saving the search results once every 100 generations. Sequences derived in this study have been deposited at GenBank (Table 3).

Results

Collection of fungal isolates. In total, 420 *Colletotrichum* spp. isolates from affected almond fruit with symptoms of anthracnose were recovered. Based on the morphology of the colonies on PDA, especially the color and abundance of mycelium and spore masses,

Table 3. Isolates of *Colletotrichum* spp. used in this study, with collection details and GenBank accessions

Species, isolate ^a	Substrate (host)	GenBank accession number ^y					
		ITS	TUB2	ACT	CHS-1	HIS3	GAPDH
<i>Colletotrichum acerbum</i>							
CBS 128530, ICMP 12921, PRJ 1199.3*	<i>Malus domestica</i>	JQ948459	JQ950110	JQ949780	JQ949120	JQ949450	JQ948790
<i>C. acutatum</i>							
CBS 112996, ATCC 56816, STE-U 5292*	<i>Carica papaya</i>	JQ005776	JQ005860	JQ005839	JQ005797	JQ005818	JQ948677
CBS 129952, PT227, RB015	<i>Olea europaea</i>	JQ948364	JQ950015	JQ949685	JQ949025	JQ949355	JQ948695
CBS 127598, 223/09	<i>O. europaea</i>	JQ948363	JQ950014	JQ949684	JQ949024	JQ949354	JQ948694
Col-536	<i>Prunus dulcis</i>	KY171894	KY171902	KY171910	KY171918	KY171926	KY171934
Col-537	<i>P. dulcis</i>	KY171895	KY171903	KY171911	KY171919	KY171927	KY171935
Col-538	<i>P. dulcis</i>	KY171896	KY171904	KY171912	KY171920	KY171928	KY171936
<i>C. australe</i>							
CBS 116478, HKUCC 2616*	<i>Trachycarpus fortunei</i>	JQ948455	JQ950106	JQ949776	JQ949116	JQ949446	JQ948786
<i>C. brisbanense</i>							
CBS 292.67, DPI 11711*	<i>Capsicum annuum</i>	JQ948291	JQ949942	JQ949612	JQ948952	JQ949282	JQ948621
<i>C. chrysanthemi</i>							
IMI 364540, CPC 18930	<i>Chrysanthemum coronarium</i>	JQ948273	JQ949924	JQ949594	JQ948934	JQ949264	JQ948603
<i>C. cosmi</i>							
CBS 853.73, PD 73/856*	<i>Cosmos</i> sp.	JQ948274	JQ949925	JQ949595	JQ948935	JQ949265	JQ948604
<i>C. costaricense</i>							
CBS 330.75*	<i>Coffea arabica</i>	JQ948180	JQ949831	JQ949501	JQ949120	JQ949450	JQ948790
<i>C. cuscuteae</i>							
IMI 304802, CPC 18873*	<i>Cuscuta</i> sp.	JQ948195	JQ949846	JQ949516	JQ949025	JQ949355	JQ948695
<i>C. fioriniae</i>							
IMI 345583, CPC 18889	<i>Fragaria × ananassa</i>	JQ948333	JQ949984	JQ949654	JQ005797	JQ005818	JQ948677
IMI 345575, CPC 18888	<i>Fragaria × ananassa</i>	JQ948332	JQ949983	JQ949653	JQ949116	JQ949446	JQ948786
CBS 125396 GJS 08-140A	<i>Malus domestica</i>	JQ948299	JQ949950	JQ949620	JQ948952	JQ949282	JQ948621
CBS 129946, PT170, RB021	<i>O. europaea</i>	JQ948342	JQ949993	JQ949663	JQ949024	JQ949354	JQ948694
CBS 293.67, DPI 13120	<i>Persea americana</i>	JQ948310	JQ949961	JQ949631	JQ948934	JQ949264	JQ948603
CBS 127537, STE-U 5289	<i>Vaccinium</i> sp.	JQ948318	JQ949969	JQ949639	JQ948935	JQ949265	JQ948604
<i>C. godetiae</i>							
CBS 133.44*	<i>Clarkia hybrida</i>	JQ948402	JQ950053	JQ949723	JQ949063	JQ949393	JQ948733
CBS 130251, OL 10, IMI 398854	<i>O. europea</i>	JQ948413	JQ950064	JQ949734	JQ949074	JQ949404	JQ948744
CBS 193.32	<i>O. europea</i>	JQ948415	JQ950066	JQ949736	JQ949076	JQ949406	JQ948746
CBS 130252, IMI 398855, OL 20	<i>O. europaea</i>	JQ948414	JQ950065	JQ949735	JQ949075	JQ949405	JQ948745
Col-508	<i>O. europaea</i>	KY171892	KY171900	KY171908	KY171916	KY171924	KY171932
CBS 126527, PD 93/1748	<i>Prunus avium</i>	JQ948408	JQ950059	JQ949729	JQ949069	JQ949399	JQ948739
CBS 126522, PD 88/472, BBA 70345	<i>P. cerasus</i>	JQ948411	JQ950062	JQ949732	JQ949072	JQ949402	JQ948742
CBS 129934, ALM-IKS-7Q	<i>P. dulcis</i>	JQ948431	JQ950082	JQ949752	JQ949072	JQ949422	JQ948762
Col-522	<i>P. dulcis</i>	KY171893	KY171901	KY171909	KY171917	KY171925	KY171933
Col-524	<i>P. dulcis</i>	KY644521	KY644527	KY644533	KY644539	KY644545	KY644551
Col-525	<i>P. dulcis</i>	KY644522	KY644528	KY644534	KY644540	KY644546	KY644552
Col-540	<i>P. dulcis</i>	KY644523	KY644529	KY644535	KY644541	KY644547	KY644553

(continued on next page)

^y ITS = internal transcribed spacer, TUB2 = β -tubulin, ACT = actin, CHS-1 = chitin synthase 1, HIS3 = histone 3, and GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

^a ATCC = American Type Culture Collection, Virginia; CBS = Culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; IMI = Culture collection of CABI Europe UK Centre, Egham, UK; BRIP = Plant Pathology Herbarium, Department of Employment, Economic, Development and Innovation, Queensland, Australia; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand; STE-U = Culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa; HKUCC = The University of Hong Kong Culture Collection, Hong Kong, China; PD = Plantenziektenkundige Dienst Wageningen, The Netherlands; and STE-U = Culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa. An asterisk (*) indicates ex-holotype or ex-epitype cultures. Sequences from GenBank used in the phylogenetic analysis are indicated in bold (Damm et al. 2012).

the *Colletotrichum* isolates were divided into two different groups: (i) gray colonies with abundant aerial mycelium and few spore masses (gray type) and (ii) pink-orange colonies with very scarce aerial mycelium and abundant spore masses (pink-orange type) (Table 1). Of the 420 isolates, 375 were classified as gray type and 45 as pink-orange type, which represent 89.3 and 10.7% of the fungal collection, respectively. Pink-orange isolates were recovered only from two commercial orchards located in Lebrija (Sevilla province). In these fields, both colony types were recovered, indicating that both fungal populations can coexist in the same field or growing area.

Colony and conidium morphology. The growth pattern of all colonies was radial with concentric circles. The colonies of the olive isolates Col-506 and Col-508 had similar appearances, with formation of abundant aerial mycelium, few conidial masses, and light-

to dark-gray color. However, colonies of the Col-508 isolate could be distinguished from those of the Col-506 isolate by their darker gray color, more irregular edges, and higher production of conidial masses. The colonies of the almond isolates Col-536 and Col-522 differed greatly from each other. The Col-536 isolate showed a very characteristic and specific colony morphology, with an intense pink-orange color, very scarce aerial mycelium, regular edges, and abundant conidial masses. In contrast, colonies of the Col-522 isolate were gray, with abundant and dense aerial mycelium, irregular edges, and little production of conidial masses. Comparing the colonies of the olive isolates with those of the almond isolates, the most similar colonies were those of isolates Col-508 (from olive) and Col-522 (from almond). Even in this case, there were slight differences between them that allowed differentiation, such as the less dense and

Table 3. (continued from preceding page)

Species, isolate ^z	Substrate (host)	GenBank accession number ^y					
		ITS	TUB2	ACT	CHS-1	HIS3	GAPDH
Col-542	<i>P. dulcis</i>	KY644524	KY644530	KY644536	KY644542	KY644548	KY644554
Col-544	<i>P. dulcis</i>	KY644525	KY644531	KY644537	KY644543	KY644549	KY644555
Col-548	<i>P. dulcis</i>	KY171897	KY171905	KY171913	KY171921	KY171929	KY171937
Col-555	<i>P. dulcis</i>	KY171898	KY171906	KY171914	KY171922	KY171930	KY171938
Col-613	<i>P. dulcis</i>	KY644526	KY644532	KY644538	KY644544	KY644550	KY644556
IMI 376331, CPC 18933*	<i>Prunus</i> sp.	JQ948409	JQ950060	JQ949730	JQ949070	JQ949400	JQ948740
<i>C. guajavae</i>							
IMI 350839, CPC 18893*	<i>Psidium guajava</i>	JQ948270	JQ949921	JQ949591	JQ948931	JQ949261	JQ948600
<i>C. indonesiense</i>							
CBS 127551, CPC 14986	<i>Eucalyptus</i> sp.	JQ948288	JQ949939	JQ949609	JQ948949	JQ949279	JQ948618
<i>C. johnstonii</i>							
CBS 128532, ICMP 12926, PRJ 1139.3*	<i>Solanum lycopersicum</i>	JQ948444	JQ950095	JQ949435	JQ949105	JQ949105	JQ948775
<i>C. kinghornii</i>							
CBS 198.35*	<i>Phormium</i> sp.	JQ948454	JQ950105	JQ949775	JQ949115	JQ949445	JQ948785
<i>C. laticiphiolum</i>							
CBS 112989, IMI 383015, STE-U 5303*	<i>Hevea basiliensis</i>	JQ948289	JQ949940	JQ949610	JQ948950	JQ949280	JQ948619
<i>C. limetticola</i>							
CBS 114.14*	<i>Citrus aurantifolia</i>	JQ948193	JQ949844	JQ949514	JQ948854	JQ949184	JQ948523
<i>C. lupini</i>							
CBS 109225, BBA 70884*	<i>Lupinus albus</i>	JQ948155	JQ949806	JQ949476	JQ948816	JQ949146	JQ948485
<i>C. melonis</i>							
CBS 159.84*	<i>Cucumis melo</i>	JQ948194	JQ949845	JQ949515	JQ948855	JQ949185	JQ948524
<i>C. nymphaeae</i>							
CBS 515.78*	<i>Nymphaea alba</i>	JQ948197	JQ949848	JQ949518	JQ948858	JQ949188	JQ948527
CBS 231.49	<i>O. europaea</i>	JQ948202	JQ949853	JQ949523	JQ948863	JQ949193	JQ948532
CBS 129945, PT135, RB012	<i>O. europaea</i>	JQ948201	JQ949852	JQ949522	JQ948862	JQ949192	JQ948531
Col-506	<i>O. europaea</i>	KY171891	KY171899	KY171907	KY171915	KY171923	KY171931
<i>C. orchidophilum</i>							
CBS 119291, MEP 1545	<i>Cynoches aureum</i>	JQ948154	JQ949805	JQ949475	JQ948815	JQ949145	JQ948484
CBS 632.80*	<i>Dendrobium</i> sp.	JQ948151	JQ949802	JQ949472	JQ948812	JQ949142	JQ948481
<i>C. paxtonii</i>							
IMI 165753, CPC 18868*	<i>Musa</i> sp.	JQ948285	JQ949936	JQ949606	JQ948946	JQ949276	JQ948615
<i>C. phormii</i>							
CBS 118194, AR 3546*	<i>Phormium</i> sp.	JQ948446	JQ950097	JQ949767	JQ949107	JQ949437	JQ948777
<i>C. pyricola</i>							
CBS 128531, ICMP 12924, PRJ 977.1*	<i>Pyrus communis</i>	JQ948445	JQ950096	JQ949766	JQ949106	JQ949436	JQ948776
<i>C. rhombiforme</i>							
CBS 129953, PT250, RB011*	<i>O. europaea</i>	JQ948457	JQ950108	JQ949778	JQ949115	JQ949448	JQ948788
<i>C. salicis</i>							
CBS 607.94*	<i>Salix</i> sp.	JQ948460	JQ950111	JQ949781	JQ949121	JQ949451	JQ948791
<i>C. scovillei</i>							
CBS 126529, PD 94/921-3, BBA 70349*	<i>Capsicum</i> sp.	JQ978267	JQ949918	JQ949588	JQ948928	JQ948928	JQ948597
<i>C. simmondsii</i>							
CBS 122122, BRIP 28519*	<i>Carica papaya</i>	JQ948276	JQ949927	JQ949597	JQ948937	JQ949267	JQ948606
<i>C. sloanei</i>							
IMI 364297, CPC 18929*	<i>Theobroma cacao</i>	JQ948287	JQ949938	JQ949608	JQ948948	JQ949278	JQ948617
<i>C. tamarilloi</i>							
CBS 129814, T.A.6*	<i>Solanum betaceum</i>	JQ948184	JQ949835	JQ949505	JQ948845	JQ949175	JQ948514
<i>C. walleri</i>							
CBS 125472, BMT(HL)19*	<i>Coffea</i> sp.	JQ948275	JQ949926	JQ949596	JQ948936	JQ949266	JQ948605

darker gray mycelium in the colonies of the olive isolate Col-508. Interestingly, the color of the colonies that developed on almond fruit artificially inoculated with isolates Col-522 and Col-536 from almond coincided with the colors of the colonies grown on PDA, being gray or pink-orange for isolates Col-522 or Col-536, respectively (Supplementary Fig. S2).

Concerning conidium morphology, the pathogen showed unicellular, hyaline, clavate to fusoid conidia. There were significant differences in the size and volume of conidia between the media, with higher sizes found on PDA than on artificially inoculated almond fruit. The mean $\pm$ standard error conidium length and width on PDA varied significantly between isolates ($P < 0.0001$), being $14.4 \pm 1.25 \times 3.8 \pm 0.41$ and $10.4 \pm 1.19 \times 3.2 \pm 0.62$ μm for isolates Col-508 and Col-536, respectively. Data analysis using the nonparametric method of Kruskal-Wallis showed significant differences among *Colletotrichum* isolates regarding the length and width, and comparison of means allowed the separation of two clear homogeneous groups according to the host of origin (almond or olive). Concerning conidium volume, four groups corresponding to each of the *Colletotrichum* isolates studied were established, and the following diminishing volume order was observed: Col-508 > Col-522 > Col-506 > Col-536 (Table 4). Concerning conidium shape, 65% of conidia from olive were clavate (category 2), whereas 60% of conidia from almond were fusoid (category 3).

On the other hand, the conidium length data obtained from artificially inoculated almond fruit showed significant differences between isolates ($P < 0.0001$), being 13.2 ± 1.20 and 10.8 ± 1.18 μm for isolates Col-508 and Col-536, respectively. However, no significant differences in width ($P = 0.0623$) were observed between them. Two homogeneous groups were clearly separated by conidium volume. Conidia produced by the isolate from olive Col-508 were significantly larger ($P < 0.0001$) than those produced by the rest of the isolates evaluated, which did not differ from each other (Table 4).

Conidium production. The isolates of *Colletotrichum* spp. studied were able to produce conidia in the two culture media tested (OA and SNA). All isolates showed higher conidium production in SNA than in OA, and there were significant differences ($P < 0.0001$) among all isolates in each culture medium. On OA, the highest conidium production was for isolate Col-536 (130,938 conidia ml^{-1}), followed by Col-508 (45,875 conidia ml^{-1}) and Col-506 (23,625 conidia ml^{-1}), with the smallest value for Col-522 (4,375 conidia ml^{-1}).

Table 4. Conidial measures (length, width, and volume) of the representative *Colletotrichum* spp. isolates grown on potato dextrose agar (PDA) or on inoculated almond fruit^x

Medium, isolate	Length (μm)	Width (μm)	Volume (μm^3)
PDA ^y			
Col-508	14.4 ± 1.25 a	3.8 ± 0.41 a	165.7 ± 39.51 a
Col-522	12.8 ± 1.29 b	3.7 ± 0.33 a	140.4 ± 29.66 b
Col-506	12.1 ± 1.44 c	3.4 ± 0.62 b	114.0 ± 44.47 c
Col-536	10.4 ± 1.19 d	3.2 ± 0.62 b	87.2 ± 35.04 d
Average	12.4 ± 1.93 A	3.5 ± 0.57 A	126.8 ± 47.55 A
Almond fruit ^z			
Col-508	13.2 ± 1.20 a	3.5 ± 0.51 a	128.6 ± 35.37 a
Col-522	11.3 ± 1.11 b	3.5 ± 0.53 a	109.6 ± 32.12 b
Col-506	11.6 ± 1.34 b	3.3 ± 0.60 a	103.1 ± 37.83 b
Col-536	10.8 ± 1.18 c	3.4 ± 0.58 a	99.4 ± 34.57 b
Average	11.7 ± 1.50 B	3.4 ± 0.56 B	110.2 ± 36.67 B

^x Each measure represents the average of 100 conidia $\pm$ standard error. Means in a column followed by the same lowercase letter do not differ significantly according to Dunn's test at $P = 0.05$. Uppercase letters indicate the different homogeneous groups for the mean values of each parameter (length, width, and volume) in both tested media (PDA and almond fruit) according to Dunn's test at $P = 0.05$.

^y Conidia were obtained from colonies grown on PDA at $23 \pm 2^\circ\text{C}$ with a 12-h photoperiod of fluorescent light ($350 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 7 days.

^z Conidia were obtained from colonies developed on inoculated almond fruit incubated in humid chamber at $23 \pm 2^\circ\text{C}$ with a 12-h photoperiod for 14 days.

On SNA, the highest conidium production was for isolate Col-536 (492,500 conidia ml^{-1}), followed by Col-508 (269,688 conidia ml^{-1}) and Col-506 (214,213 conidia ml^{-1}), with the smallest value for Col-522 (135,000 conidia ml^{-1}).

Effect of temperature on mycelial growth. All isolates of *Colletotrichum* spp. tested had a similar growth pattern in PDA at the different temperatures evaluated, with the exception of Col-522 from almond. Optimum growth temperatures ranged from 22.4 to 23.7°C and the maximum growth rate from 4.8 to 6.8 mm day^{-1} for the isolates Col-522 and Col-536, respectively. ANOVA showed that isolate Col-522 had an optimum growth temperature (22.4°C) significantly lower ($P < 0.0001$) than the rest of the isolates, which did not differ among themselves. Only one isolate (Col-506, from olive) grew at the maximum temperature tested (35°C) but this temperature was not lethal for the other isolates, because mycelial plugs incubated at this temperature were able to grow when they were replated on PDA and incubated at 23°C for 7 days. Isolate Col-522 was the only one to grow at 0°C . However, isolates Col-506 and Col-508 from olive and Col-536 from almond were not able to grow below 4 to 5°C , showing limited growth at low temperatures (Fig. 1; Table 5). With respect to the maximum growth rate, the comparison of means allowed the establishment of three homogeneous groups. The isolate Col-536 showed a faster growth rate (6.8 mm day^{-1}), followed by Col-508 (6.5 mm day^{-1}) and Col-506 (6.4 mm day^{-1}), among which no significant differences were found. Finally, isolate Col-522 showed the smallest growth rate (4.8 mm day^{-1}).

Pathogenicity tests. *Pathogenicity on detached almond fruit.* The eight *Colletotrichum* isolates tested (six from almond and two from olive), representative of the two colony types, were pathogenic to almond fruit, causing depressed, round, and necrotic lesions that extended radially to reach the whole fruit. The factorial ANOVA showed significant differences in virulence between isolates from the two hosts of origin ($P = 0.0062$) (Fig. 2A) and between almond cultivars ($P < 0.0001$) but not for the interaction between isolate and cultivar ($P = 0.5144$). For all isolates tested, Lauranne proved to be significantly more susceptible (RAUDPC = 81.4%) than Guara (RAUDPC = 74.0%).

Because the interaction between isolate and cultivar was not significant, data from both cultivars were combined for further statistical analysis. Tukey's HSD contrasts showed that there were two clear homogenous groups: one major group comprising the pink isolates from almond (Col-536, Col-537, and Col-538) and the two isolates from olive (Col-506 and Col-508) and a second group with the gray isolates from almond (Col-522, Col-548, and Col-555), with the first group the most virulent (Fig. 2A).

Pathogenicity on detached olive fruit. All *Colletotrichum* isolates tested, coming from olive or from almond, were pathogenic to olive fruit, causing fruit rot. ANOVA showed significant differences in virulence ($P < 0.0001$) between isolates from the two hosts of origin. Comparison of means using Fisher's protected LSD test showed three homogeneous groups. The two isolates from olive (Col-506 and Col-510) grouped together with 84.6 and 87.6% RAUDPC values, respectively, and were significantly more virulent than those from almond (Col-522 and Col-536). Finally, significant differences were also detected between isolates from almond, with the pink-orange isolate Col-536 (RAUDPC = 45.2%) being more virulent than the gray isolate Col-522 (RAUDPC = 5.6%) (Fig. 2B).

Pathogenicity on apple fruit. All isolates of *Colletotrichum* spp. from olive and almond origin and from both colony types were pathogenic to apple fruit, causing circular necrotic lesions around wounds. ANOVA showed significant differences in virulence between isolates ($P < 0.0001$). Comparison of means by Fisher's protected LSD test showed two homogeneous groups. The major group was formed by isolates Col-506 (RAUDPC = 91.1%) and Col-508 (RAUDPC = 85.2%) from olive and the pink-orange isolate Col-536 from almond (RAUDPC = 88.6%), which were significantly more virulent than the gray isolate Col-522 from almond (RAUDPC = 36.9%) (Fig. 2C).

Phylogenetic analysis. Six gene sequences from a total of 61 isolates of *Colletotrichum* spp., including the outgroup *C. orchidophilum*,

were studied in this work (Table 3). In total, 1,974 characters including alignment gaps were processed, of which 352 characters were parsimony-informative sites and 1,496 were constant. The gene boundaries in the combined matrix were: ITS = 1-417; TUB2 = 418-794; ACT = 795-1,041; CHS-1 = 1,042-1,349; HIS3 = 1,320-1,705; and GAPDH = 1,706-1,974.

MP analysis showed three equally parsimonious trees ($L = 738$ steps, $CI = 0.662$, $RI = 0.935$, $RC = 0.668$, $HI = 0.338$), one of which is shown in Figure 3. For Bayesian analysis, model JC+G was selected for the genomic region ITS; model K2+G for the regions TUB2, ACT, CHS-1, and GAPDH; and model TN93+G for the region HIS3. The consensus tree determined by Bayesian inference confirmed the topology obtained by MP. Bayesian posterior probability values agreed with bootstrap supports (Fig. 3).

The isolates of *Colletotrichum* spp. under study were classified into three well-supported clades with a bootstrap support of 100% and Bayesian posterior probability value of 1.00. The pink-orange isolates from almond (Col-536, Col-537, and Col-538) were grouped

together in clade 4, *C. acutatum* (bootstrap support: 100%, Bayesian posterior probability value = 1.00). However, the nine gray isolates from almond (Col-522, Col-524, Col-525, Col-540, Col-542, Col-544, Col-548, Col-555, and Col-613), as well as the dark-gray isolate from olive (Col-508), were included in the subclade of *C. godetiae*, in clade 5 (100%, 1.00). Finally, the isolate from olive belonging to the light-gray subpopulation (Col-506) clustered together with isolates of *C. nymphaeae* (100%, 1.00) in a subclade belonging to clade 2.

Discussion

The cultivation of almond in Spain has undergone a major expansion during recent years. The incidence of foliar diseases in these new plantings is much higher than in traditional orchards. Therefore, diagnosis and control of fungal diseases of this crop have gained great importance. However, there are significant gaps in the knowledge of some of these diseases (i.e., almond anthracnose). Because anthracnose is an emerging disease in Spain, and because the taxonomy of *Colletotrichum* spp. has been recently revised (Cannon et al. 2012;

Fig. 1. Effect of temperature on mycelial growth rate (millimeters per day) of the four representative *Colletotrichum* isolates from olive (Col-506 and Col-508) and almond (Col-522 and Col-536) grown on potato dextrose agar at 0, 5, 10, 15, 20, 25, 30, and 35°C in darkness for 8 days. For each *Colletotrichum* isolate, average growth rates over temperature were adjusted to a nonlinear regression curve using the Analytis Beta model. Data points are the means of two independent sets (experiments) of three replicated Petri dishes. Vertical bars are the standard error of the means.

Table 5. Effect of temperature on mycelial growth of the representative *Colletotrichum* spp. isolates from olive (Col-506 and Col-508) and almond (Col-522 and Col-536) grown at 0, 5, 10, 15, 20, 25, 30, and 35°C in darkness for 8 days^w

Isolate	Analytis Beta model ^x			Temperature (°C) ^y			MGR (mm day ⁻¹) ^z
	R^2	a	b	Optimum	Minimum	Maximum	
Col-506	0.957	2.10	1.41	23.6 a	5	36	6.4 b
Col-508	0.999	1.98	0.06	23.5 a	5	31	6.5 b
Col-522	0.986	2.26	0.76	22.4 b	0	30	4.8 c
Col-536	0.960	3.70	2.14	23.7 a	4	35	6.8 a

^w Means in a column followed by the same letter do not differ significantly according to the Fisher's protected least significant difference test at $P = 0.05$.

^x Analytis Beta model, where R^2 = coefficient of determination, and a and b = coefficients of regression.

^y For each *Colletotrichum* isolate, temperature average growth rates were adjusted to a regression curve to estimate the optimum growth temperature.

^z Maximum growth rate.

Damm et al. 2012), the correct identification of the causal agent is one of the most important aspects to elucidate. Indeed, this work represents the first study elucidating the etiology of almond anthracnose in Spain, providing valuable information about the causal agents associated with the disease in this country. Moreover, this study is the

first report of a new species of *Colletotrichum* causing almond anthracnose within the *C. acutatum* species complex. Taxonomic studies on *Colletotrichum* have mainly been focused on the identification of specific or intraspecific taxa, based on phenotypic differences, mainly characteristics of colony morphology,

Fig. 2. Relative area under the disease progress curve (RAUDPC) on fruit of **A**, Guara and Lauranne almond; **B**, Arbequina olive; and **C**, Golden Delicious apple inoculated with *Colletotrichum* spp. RAUDPC on almond fruit (A) represents the pooled values for Guara and Lauranne cultivars. Columns are the means of two independent sets (experiments) of three replicated (humid chambers) in each host inoculation, with 15 almond, 20 olive, and 4 apple fruit per humid chamber. Vertical bars are the standard error of the means. Columns with the same letter do not differ significantly according to the Tukey's honestly significant difference test (A) or to the Fisher's protected least significant difference test (B and C), both at $P = 0.05$.

optimum growth temperature and growth rate, as well as conidium shape and size (Freeman et al. 1998; 2000). Because environmental factors have a great influence on the stability of morphological traits and the existence of intermediate forms, morphological criteria are not always adequate to provide a correct identification of *Colletotrichum* spp. (Freeman et al. 1998). For this reason, molecular techniques, such as phylogenetic analyses of ribosomal genes (i.e., ITS, 28S, etc.) and functional protein regions (i.e., actin, β -tubulin, calmodulin, etc.), have been incorporated in recent years as fundamental aspects of the identification of species within this genus (Cannon et al. 2012; Damm et al. 2012; Guerber et al. 2003; McKay et al. 2009). Therefore, in the present work, isolates of *Colletotrichum* spp. collected from almond and olive trees infected by anthracnose were characterized by morphological and molecular methods and by pathogenicity tests. All of these characteristics were useful for identifying the species of *Colletotrichum* causing almond anthracnose in the Andalusia region.

For morphological characterization, the curvature of the ends of the conidia is one of the most important morphological characteristics in the taxonomy of *Colletotrichum* spp. (Sutton 1980, 1992; von Arx 1970) and is particularly used to separate the two main species associated with almond anthracnose, which are *C. acutatum* (sharp conidium ends) and *C. gloeosporioides* (rounded conidium ends) (Freeman et al. 1998; Simmonds 1965). In this study, all isolates from almond showed more than 60% of the conidia having two sharp ends. Thus, according to traditional criteria, these isolates could be classified within the *C. acutatum* species complex (Damm et al. 2012). Meanwhile, the two isolates from olive (Col-506 and Col-508) presented more than 65% of conidia with a single sharp end; thus, their classification under one species or the other is arbitrary. Therefore, our results reveal that it is not possible to identify all isolates of *Colletotrichum* based exclusively on this morphological characteristic. The conidium size, another morphological characteristic of taxonomic importance (Sutton 1980, 1992), was not helpful to differentiate the species in this work. Although there were significant differences in the volumes of conidia among isolates, they did not help us to classify them clearly. In fact, the four isolates studied showed significant differences for this variable. Although the size of the conidia has been used to differentiate species of *Colletotrichum*, this characteristic is very variable depending on the culture media and environmental conditions. Thus, it has not had a great importance in the taxonomy of *Colletotrichum* spp. (Sutton 1992; von Arx 1970). In fact, differences in the size and volume of conidia produced on PDA and on inoculated almond fruit were observed, showing larger sizes on PDA. This could be because the synthetic PDA provides more nutrients and optimum conditions for fungal development than plant material, in which several plant physiological factors could be affecting pathogen development. In addition, differences in conidium shape and size were observed between both of the *C. godetiae* isolates, Col-522 and Col-508 from almond and olive, respectively. However, in this last case, these morphological differences could also express genetic differences between the two isolates that could not be detected by the analysis of the six genomic areas used in phylogenetic characterization.

With respect to conidium production, there were significant differences between the four isolates tested and between the two culture media used to induce sporulation. It is interesting to note that *C. acutatum* isolate Col-536 (pink-orange subpopulation) from almond was able to produce markedly more conidia than *C. godetiae* isolate Col-522 (gray subpopulation) from almond in both media tested. In addition, conidium production of *C. godetiae* Col-522 was lower than that of *C. godetiae* Col-508 from olive. For the size of the conidia, this characteristic is very variable and has little importance in the taxonomy of genus *Colletotrichum*. However, it has been used to characterize some isolates or species (von Arx 1970).

The growth pattern at different temperatures has traditionally been used as a criterion to differentiate species of the genus *Colletotrichum* (Bernstein et al. 1995; Smith and Black 1990). All isolates tested showed a similar pattern of mycelial growth on PDA at the different temperatures evaluated, with the exception of *C. godetiae* isolate Col-522 from almond, which showed an optimum growth temperature

significantly lower than the rest of the isolates tested. In addition, this isolate was able to grow at temperatures below 5°C, with 0°C being the minimum growth temperature, whereas isolates *C. nymphaeae* Col-506 and *C. godetiae* Col-508 from olive and isolate *C. acutatum* Col-536 from almond were not able to grow below 4 to 5°C. However, the maximum growth rate presented a greater variation among isolates. Significant differences between the two isolates from olive were not observed, whereas there were significant differences between the

Fig. 3. One of three most parsimonious trees (length= 738 steps, consistency index = 0.662, retention index = 0.935, rescaled consistency index = 0.668, and homoplasy index = 0.338) obtained from a heuristic search of the combined internal transcribed spacer, β -tubulin, actin, chitin synthase 1, histone 3, and glyceraldehyde-3-phosphate dehydrogenase sequence alignment of the *Colletotrichum acutatum* species complex. Bootstrap support values above 70% and Bayesian posterior probability values above 0.95 are shown at the nodes. *Colletotrichum orchidophilum* CBS 632.80 and CBS 119291 were used as outgroup. *Colletotrichum* isolates identified in this study are emphasized in bold and their strain numbers are followed by substrate (host species).

two isolates from almond and between them and the olive isolates. These results suggest a greater adaptability of *Colletotrichum* isolates from almond at different temperatures. In fact, although *C. godetiae* isolate Col-522 from almond has a lower growth rate than *C. godetiae* isolate Col-508 from olive, the activity of *C. godetiae* isolate Col-522 from almond at low temperatures could be an adaptive advantage in its life cycle. This temperature adaptability could be essential to increase the inoculum of *C. godetiae* isolates from almond during the epiphytic phase (winter) as well as for primary infections at fruit setting (late winter to early spring), mainly in almond-growing areas with hot spring and summer seasons. According to this latter conclusion, in this study, we also present a life cycle of *Colletotrichum* spp. causing almond anthracnose (Fig. 4) that has been slightly modified from the one proposed by Peres et al. (2005). The life cycle presented in this study was adapted to hot, dry areas such those in southern Spain, where climatic conditions during spring and summer are not favorable for the pathogen's activity. This putative climatic adaptability of isolates of *C. godetiae* associated with almond anthracnose has been confirmed during the disease surveys conducted in this current work. Moreover, these surveys revealed that the frequency of *C. godetiae* isolated from affected almond fruit was markedly higher (375 isolates; 89.3%) than that of *C. acutatum* (45 isolates; 10.7%). Our results are in concordance with those observed for olive anthracnose, in which *C. godetiae* is also the most dominant species in the Andalusia region (Moral et al. 2014). However, further surveys are needed to confirm the frequency of each of the species associated with almond anthracnose in the Andalusia region.

Pathogenicity or pathogenic specialization of isolates of *Colletotrichum* spp. on their hosts of origin has also been a characteristic traditionally used in the identification of specific or intraspecific taxa in this genus (Sutton 1980; von Arx 1970). Pathogenicity tests showed significant differences among *Colletotrichum* isolates and host plants. Pathogenicity tests in olive fruit showed that both *Colletotrichum* isolates from olive (Col-506 and Col-508) were more virulent than the *Colletotrichum* isolates from almond (Col-522 and Col-536). However, this difference was not observed in pathogenicity tests conducted using almond and apple fruit because, in these hosts, the *C. acutatum* isolates (pink-orange subpopulation) from almond did not differ in virulence from the olive isolates. In the three hosts tested, the *C. godetiae* isolates (gray subpopulation) from almond were the least virulent. Thus, our results suggest that *Colletotrichum* isolates from olive showed little or no pathogenic specialization, because they were able to infect and develop symptoms in other hosts with a degree of virulence similar to that in its host of origin. These

results do not coincide with those obtained in previous studies conducted by Moral et al. (2007), which showed a remarkable pathogenic specialization for isolates from olive. However, the isolates from almond showed some degree of specialization, because the isolate *C. acutatum* Col-536 was more virulent in almond and apple fruit than in olive fruit. Concerning cultivar resistance in almond, Lauranne turned out to be more susceptible to anthracnose than Guara. However, the cultivar-isolate interaction was not significant according to ANOVA, because none of the eight *Colletotrichum* isolates showed any preference for either of the two almond cultivars. These results might vary if more and different almond cultivars were inoculated.

Phylogenetic analysis showed five main clades, in agreement with the taxonomical studies of genus *Colletotrichum* recently performed by Damm et al. (2012). Our results concluded that isolates of the pink-orange subpopulation from almond (Col-536, Col-537, and Col-538) belong to the species *C. acutatum* and isolates of the gray subpopulation from almond (Col-522, Col-524, Col-525, Col-540, Col-542, Col-544, Col-548, Col-555, and Col-613) belong to *C. godetiae*. Regarding the isolates from olive, the isolate belonging to the light-gray subpopulation (Col-506) was identified as *C. nymphaeae*, while the isolate belonging to the dark-gray subpopulation (Col-508) was identified as *C. godetiae*. The use of molecular techniques based on the analysis of different genomic areas was the key to correctly identifying the *Colletotrichum* isolates included in this study. However, in this study, morphological characteristics also allowed separation of the fungal isolates into different groups. For instance, *C. godetiae* isolates from different hosts, almond (Col-522) and olive (Col-508), were clearly separated by different morphological characteristics, whereas this difference was not detected by molecular methods. Finally, the two phylogenetic species identified among isolates from olive, *C. godetiae* and *C. nymphaeae*, had been previously identified and are representative isolates of *C. acutatum* species complex present in the olive-growing areas in the Andalusia region, with *C. godetiae* being the most frequent species (Moral et al. 2014). This work represents the first identification of phylogenetic species between populations of the *C. acutatum* species complex causing almond anthracnose in Spain. Of the two identified species, *C. godetiae* coincides with the gray subpopulation, which is well known in other countries (Damm et al. 2012; Guerber et al. 2003). However, the species *C. acutatum*, which presents pink-orange colonies, does not coincide with the pink subpopulations described in other countries, which have been identified as *C. fioriniae* (Damm et al. 2012; Guerber et al. 2003). Thus, to our knowledge, this is the first report

Fig. 4. Life cycle of almond anthracnose caused by *Colletotrichum* spp. adapted to the environmental conditions in Andalusia region (southern Spain), characterized by a Mediterranean climate with a long dry and hot period during the late spring and summer.

of a new identified species, *C. acutatum*, within the *C. acutatum* species complex, causing almond anthracnose.

C. acutatum has been described in major almond-growing areas worldwide, such as Australia (McKay et al. 2014) and California (Adaskaveg et al. 1997), as causing fruit rot in several crops other than the almond tree (Damm et al. 2012) and, in Portugal, as associated mainly with olive anthracnose (Sreenivasaprasad and Talhinhos 2005). Our work is relevant because we give new etiological information about *C. acutatum*, reporting this species for the first time as a causal agent of almond anthracnose. Moreover, important epidemiological traits have been described in this study. In this sense, we have demonstrated that *C. acutatum* isolates from almond had a higher capacity to produce conidia and a higher growth rate at high temperatures (35°C) than the other tested isolates. This information is relevant because it suggests that these isolates are especially dangerous for areas with warm spring seasons, particularly when rains occur, facilitating infection and disease development. Concerning *C. godetiae* isolates from almond, our results show that these isolates have a lower capacity to produce conidia, lower growth rate, and lower virulence than the other tested isolates. However, the ability of these isolates to grow at low temperatures is an important advantage for causing infection during the short infection period of their life cycle (late winter to early spring) in most years. This could explain why *C. godetiae* is markedly more frequent in affected almond orchards in the Andalusia region than *C. acutatum*. The information generated in the current study will be highly advantageous for developing future epidemiological and control studies of the disease. Finally, the life cycle of almond anthracnose in southern Spain as modified in this study (Fig. 4) should be used to determine the critical stages for effective management strategies against the disease.

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