Phenotypic, Molecular, and Pathological Characterization of *Colletotrichum acutatum* Associated with Andean Lupine and Tamarillo in the Ecuadorian Andes

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Abstract

Falconí, C. E., Visser, R. G. F., and van Heusden, A. W. 2013. Phenotypic, molecular, and pathological characterization of *Colletotrichum acutatum* associated with Andean lupine and tamarillo in the Ecuadorian Andes. Plant Dis. 97:819-827.

Anthrachnose is a serious problem of both Andean lupine and tamarillo in Ecuador. Morphological features, internal transcribed spacer (ITS) sequences, and host specificity were used to characterize *Colletotrichum* isolates from lupine and tamarillo. Based on phenotypic and molecular characterization, the causal agent of anthrachnose on both hosts was *Colletotrichum acutatum*. All isolates were identified in a *C. acutatum*-specific polymerase chain reaction assay. Colony diameter, conidia shape, and insensitivity to benomyl also placed isolates from both hosts in the *C. acutatum* group. However, a detailed analysis of the ITS sequences placed the lupine and tamarillo isolates from the Ecuadorian Andean zone in two clades, with both lupine and tamarillo

isolates in each clade. *C. acutatum* isolates from Andean lupine were distinct from other *C. acutatum* isolates on lupine around the world. In cross-infection studies, the diameter of lesions produced by isolates from each host was compared on the main stem of two tamarillo and three lupine cultivars. Some isolates produced larger lesions on the host from which they were isolated but others showed similar aggressiveness on their alternate host. Isolates from both hosts were biotrophic on lupine stems, producing little necrosis and abundant sporulation whereas, on tamarillo stems, they produced dark lesions with few conidia. The collection of *C. acutatum* isolates from lupine and tamarillo provides interesting material for the study quantitative host adaptation.

Andean lupine (*Lupinus mutabilis* Sweet) is a native legume that has much to offer in support of nutritional self-sufficiency for the Andean people due to its high levels of protein (>40% in dry grain) (8,18), fat (20%), iron, zinc (8) carbohydrates, minerals, and fibers (20). Lupine, known locally as chocho, plays a role in many production systems in the Ecuadorian highland and is cultivated in intercropping, monoculture, and rotation systems (30).

Tamarillo (*Solanum betaceum* Cav.), another native Ecuadorian plant, grows as a small tree or shrub, bearing edible egg-shaped fruit with a thin skin and soft flesh when ripe (33). Traditionally, lupine has been cultivated in colder areas (5,23) and tamarillo in somewhat warmer areas (33,34). Due to increased demand for lupine, this is changing (43), and host adaptation of anthrachnose will have important epidemiological consequences in areas where two or more potential hosts grow in close proximity.

Worldwide, there are several species of *Colletotrichum* that can cause anthrachnose on legumes, perennial crops, and vegetables (12,13,40). In lupine, anthrachnose causes a typical twisting of petioles and stems or pods, with dark sunken lesions, in the center of which orange conidial masses are produced (Fig. 1A–D). On tamarillo branches, anthrachnose symptoms include dark, depressed lesions varying in size and form (Fig. 1E) and black lesions on fruit accompanied by erumpent pink conidia masses in the lesions. Ultimately, the fruit is deformed and rots completely (Fig. 1F). In Ecuador, yield losses due to anthrachnose can reach up to 100% in lupine (27) and 60% in tamarillo (3,34).

Species identification in *Colletotrichum* has relied mainly on morphology and host range (11,36). The growth of isolates in media amended with benomyl (1) is also a discriminating characteris-

tic. Still, these characteristics are often unreliable to properly determine the species of an isolate (4,36). *Colletotrichum acutatum* can be pathogenic and nonpathogenic and can grow on a number of different hosts (38). Multiple *Colletotrichum* spp. have been implicated as the causal agents of anthrachnose in lupine and tamarillo. The sequence of the internal transcribed spacer (ITS) region (ITS1 to ITS2) of ribosomal DNA (rDNA) shows that anthrachnose on lupine (41) and tamarillo (2) is caused by *C. acutatum* and not *C. gloeosporioides*, which was originally thought to be the causal agent (3,7,19,33,34,51). *C. lupini* has also been designated as the causal agent of lupine anthrachnose (28,45). The objectives of this study were to (i) determine the causal agent of lupine and tamarillo anthrachnose in Ecuador, (ii) analyze lupine and tamarillo *Colletotrichum* isolates from Ecuador based on their ITS sequence, and (iii) assess the pathogenic variability and cross-infection potential. To the best of our knowledge, this is the first study of anthrachnose in Ecuador in which phenotypic and molecular approaches are combined to characterize the population structure of this important pathogen. These findings will help in the development of appropriate disease management and efficient breeding strategies.

Materials and Methods

Fungal isolates. In 2007, 2010, and 2011, symptomatic lupine stems and tamarillo fruit were collected from several production areas in Ecuador. In total, 23 *Colletotrichum* isolates were considered for this study, 18 from lupine and 5 from tamarillo (Table 1). Isolates from lupine were obtained from main stems with dark sunken lesions, in the center of which orange conidial masses were produced (Fig. 1C). Isolates from tamarillo were collected from depressed black lesions on fruit accompanied by erumpent pink conidia in the lesions (Fig. 1F). Not all isolates were used in all the analyses and further details are provided in the appropriate sections. Stock cultures of single-conidia isolates were stored at –10°C in suspensions consisting of 1 g of autoclaved peat + 10% autoclaved sucrose in 2.0-ml cryotubes (Genaxxon). Isolates were propagated by growing small amounts of frozen conidial suspension from cryotubes to petri dishes containing potato dextrose agar (PDA; Difco Laboratories).

Morphological studies. Colony characteristics (transparency, presence of conidial masses, presence and color of concentric

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*The e-Xtra logo stands for “electronic extra” and indicates that Figures 1 and 2 appear in color online.

Accepted for publication 7 January 2013.

bands, pigmentation, and growth pattern) were determined for each isolate. Isolates retrieved from cryopreservation were grown on PDA for 10 days and a conidial suspension in water of 10^6 conidia/ml was prepared. The suspension (15 μ l) was pipetted in the center of a petri dish (PDA amended with Chloramphenicol [Chloromycetin Parke Davis Co.] at 500 mg/liter) and three replications for each isolate were incubated at $17 \pm 2^\circ\text{C}$ in daylight. The colony characteristics listed above were determined after 10 days of incubation.

The growth rate of all isolates on PDA at 15, 20, 25, and 30°C in the dark was measured. Each isolate had three replications which were evaluated daily over 5 days, and the maximum final growth was recorded. The experiments were conducted twice and the means over both replications calculated. The colony radius with three replicates, four measurements per replicate, was analyzed using analysis of variance, least significant differences, and coefficient of variation determined with the Statistic Software Infostat (www.infostat.com.ar).

Twenty-five conidia for each isolate were assessed for morphometric measurements. Conidia size was measured with an ocular micrometric scale (Nikon) at $\times 100$ magnification and conidia shape determined from isolates incubated for 5 days on PDA at $17 \pm 2^\circ\text{C}$ in daylight.

Benomyl sensitivity of the isolates was assessed by comparing colony radius on PDA and on PDA amended with benomyl (Benlate 50WP; DuPont) at 2 mg/liter. Six isolates from lupine and three isolates from tamarillo were tested twice in duplicate. Colony radius mean values of both experiments were compared with isolates grown on PDA and the reference isolate of *C. gloeosporioides* IMI 356878 (GenBank accession AJ536229.1). A colony radius reduction of greater than 30% was considered positive.

DNA preparation. Lupine and tamarillo isolates were grown on PDA at 25°C for 6 days. Mycelium was collected from the plates with a scalpel, frozen in liquid nitrogen, and ground in a mortar to a fine powder. DNA extractions were performed using approximately 100 mg of powdered mycelium with an EZNA Fungal DNA kit (Omega Bio-tek) according to the manufacturer's instructions. DNA samples were diluted to a final concentration of 50 to 100 ng/ μ l.

Taxon-specific polymerase chain reaction amplification. Polymerase chain reaction (PCR) primers for taxon-specific amplification included the ITS4 (49) primer coupled with specific primers for *C. acutatum* (CaInt2) (GGGGAAGCCTCTCGCGG) (4,37) and *C. gloeosporioides* (CgInt) (GGCCTCCCGCCTCCGGGCGG) (4). PCR reactions were performed for 30 cycles (30 s at 95°C , 30 s at 60°C , and 1.5 min at 72°C). Amplification products were separated in 1.5% agarose gels in Tris-acetate-EDTA buffer (32) at 80 V for 2 h. A 100-bp ladder (Invitrogen) was used as molecular standard.

PCR amplification and sequencing of ITS1, 5.8S, and ITS4 regions of the rDNA genes. Universal PCR primers ITS1 and ITS4 were used to amplify the ITS regions between the small and large nuclear rDNA, including the ITS2 and the 5.8S rDNA (49). PCR reactions were carried out on a Techne DNA Thermal Cycler TC-512 in 100 μ l, containing 50 to 100 ng of DNA, 50 mM KCl, 10 mM Tris-HCl (pH 7.5), 80 μ M (each) dNTP, 1 μ M each primer, 2 mM MgCl_2 , and 1 U of DNA polymerase (Invitrogen). The temperature regime of the 40 cycles was 30 s at 95°C , 1 min at 44°C , and 1 min at 72°C . PCR products were purified with the UltraClean PCR Clean-up DNA Purification kit (MoBio) and sequenced using the TaqDyeDeoxy terminator cycle sequencing kit (Applied Biosystems), according to the manufac-

Fig. 1. Anthracnose symptoms caused by natural infection of *Colletotrichum acutatum* on lupine (*Lupinus mutabilis*) **A**, on leaves; **B**, on the apical main stems; **C**, on the main stems; and **D**, on the pods. **E**, Anthracnose symptoms on tamarillo (*Solanum betaceum*) main stem and **F**, symptoms on tamarillo fruit.

turer's instructions, in an Applied Biosystems automatic DNA sequencer model 373A.

Phylogenetic analysis. ITS sequences obtained in this study were compared with ITS sequences of other lupine isolates (*C. acutatum*, *C. lupini* var. *lupini*, and *C. lupini* var. *setosum*), tamarillo (*C. acutatum*), and *C. acutatum* isolates from other hosts from all over the world. Initial comparisons of cognate sequences available in GenBank (www.ncbi.nlm.nih.gov/genbank) were made using the BLAST algorithm. Phylograms based on ITS1 sequence comparisons were constructed using the neighbor-joining (NJ) (31) and maximum parsimony (MP) methods in MEGA 4 (Molecular Evolutionary Genetic Analysis Software version 4 for Windows). NJ analysis was conducted using the MP model for estimating evolutionary distance (d_{ij}) between DNA frequencies. The MP tree was obtained using the close-neighbor-interchange algorithm. Model parameters were empirically determined from the sequences. Bootstrap analysis with 1,000 repetitions was performed to test the significance of the nodes and the robustness of the tree (17). In addition to the sequences generated in this study and other sequences from lupine and tamarillo around the world, interspecific multiple alignments included other ITS1 sequences of *C. fragariae*, *C. gloeosporioides*, and *C. falcatum* obtained from GenBank. *Neurospora crassa* was used as an outgroup (6). The ITS1 sequences of the isolates in this study are deposited in GenBank (www.ncbi.nlm.nih.gov/genbank).

Aggressiveness on alternate host. Differential aggressiveness for five isolates from lupine and five from tamarillo was tested in a cross-inoculation experiment. This study used 1.5-month-old lupine seedlings and 2.0-month-old tamarillo seedlings. Each isolate was evaluated on the host from which it was isolated (host of origin) and on the alternate host. Three susceptible cultivars of lupine ('ECU-2658', 'ECU-7112-1', and 'ECU-722-4') and two susceptible cultivars of tamarillo ('Gigante comun' and 'Comun') were used. Inoculum was prepared by flooding PDA plates on which the isolate had grown for 10 days at 25°C with 4 ml of 0.01% Tween-80 solution and rubbing with a sterile glass rod. The conidial suspensions were diluted to a final concentration of 10^6 conidia/ml of saline solution (0.05% NaCl + 0.01% Tween-80). In preliminary tests, spraying the pathogen reproduced the symptoms in only some individuals of both hosts. A wounding method was chosen to avoid escapes. A hypodermic syringe (0.2 mm in diameter and 0.3 mm in depth) was used to injure lupine or tamarillo

plants at the apical main stem. A 20- μ l droplet of conidia (10^6 conidia/ml) was used for inoculation. The inoculated lupine and tamarillo plants, along with appropriate controls, which were treated with sterile distilled water, were incubated in a tunnel greenhouse with 100% relative humidity at $20 \pm 2^\circ\text{C}$. Lesion size was measured with a ruler parallel to the main stems 20 days after inoculation. Symptoms were compared on both hosts. At the end of the study, the pathogen was reisolated from lupine or tamarillo stems, in order to verify that it was the inoculated pathogen. The significance of the interaction between isolates and hosts was tested with analysis of variance. The experiment was conducted twice and the mean of both assays was reported. Each plant-pathogen combination was represented by three plants of lupine for each pot or one plant of tamarillo per pot and replicated three times. One wound was made in each lupine plant and two wounds per tamarillo plant. The mean lesion length of three lupine plants or two lesions on tamarillo in each pot was the basic unit for analysis. The sources of variance analyzed were the (inoculated) host (lupine or tamarillo), the original host of the isolate (lupine or tamarillo), and the combination original host-inoculated host.

Results

Culture and morphological characteristics. The colony morphology of all isolates (upper and lower surface) was consistent with published descriptions of *C. acutatum* species (21,39,44). All isolates presented either a white-pink or a gray-pink pigmentation on the reverse. The growth of the isolates was regular and progressive on PDA and, after 10 days at $17 \pm 2^\circ\text{C}$ in daylight, the petri dish surface was one-third to two-thirds covered in case of the lupine isolates and almost fully covered by the tamarillo isolates. Three of eight of the lupine and tamarillo isolates formed white to pale aerial mycelium which was cottony and often dense near the center, and the other five developed olive-gray mycelium (Fig. 2A versus B, C, and D). Concentric white and dark-pink circles were seen on the mycelium of lupine isolates. White-pink colonies are generally related to *C. acutatum* and gray-dark ones to *C. gloeosporioides* (39). The tamarillo isolates showed different colony morphology, either with white mycelium slowly turning to a salmon color and without concentric circles (Fig. 2E) or with white mycelium slowly turning gray and producing circles around the center of the culture (Fig. 2F). In general, the colony color of isolates from both lupine and tamarillo changed in time from white to

Table 1. *Colletotrichum* spp. isolates collected in Ecuadorian provinces

Original code	Host species	Affected tissue	Location, province	Collection date
Lup1	Lupine	Stem	Juan Montalvo, Cotopaxi	2007
Lup2	Lupine	Stem	Pangigua grande, Cotopaxi	2007
Lup3	Lupine	Stem	Pusuchusi, Cotopaxi	2007
Lup4	Lupine	Stem	Picualto Alto, Cotopaxi	2007
Lup5	Lupine	Stem	San Juan, Cotopaxi	2007
Tam6	Tamarillo	Fruit	San Pablo, Imbabura	2007
Tam7	Tamarillo	Fruit	Ibarra, Imbabura	2007
Lup8	Lupine	Stem	San Marcos, Cotopaxi	2007
Lup10	Lupine	Stem	El Tejar, Cotopaxi	2007
Lup11	Lupine	Stem	Chinchil, Cotopaxi	2007
Lup12	Lupine	Stem	Chinchil Robayo, Cotopaxi	2007
Lup14	Lupine	Stem	Canchicera, Cotopaxi	2007
Lup16	Lupine	Stem	San Francisco, Cotopaxi	2007
Lup18	Lupine	Stem	Pujili, Cotopaxi	2007
Tam20	Tamarillo	Fruit	Guallabamba, Pichincha	2010
Lup21	Lupine	Stem	Tixan, Chimborazo	2010
Lup24	Lupine	Stem	Guamote, Chimborazo	2010
Lup28	Lupine	Stem	Palmira, Chimborazo	2010
Lup30	Lupine	Stem	Lican, Chimborazo	2010
Lup31	Lupine	Stem	Guazazo, Chimborazo	2010
Lup32	Lupine	Stem	San Andres, Chimborazo	2010
Tam33	Tamarillo	Fruit	Atuntaqui, Imbabura	2011
Tam34	Tamarillo	Fruit	Cotacachi, Imbabura	2011
IMI 356878 ^a	Olive	Fruit	Montsia, Tarragona, Spain	2003

^a Reference isolate IMI 356878 (AJ536229.1) (*Colletotrichum gloeosporioides*) from olive (*Olea europaea* subsp. *europaea*) was kindly provided by Dr. Pedro Martínez-Culebras (IATA, Universidad de Valencia, Spain).

dark but conidia were consistently pink-salmon. *C. acutatum* isolates from lupine release a pink-salmon pigment that differs from a pink-yellow pigment of tamarillo isolates on PDA.

Radial growth of the fungal mycelium showed a high variability between isolates at 15, 20, 25, and 30°C in the dark (Table 2). However, growth rate was more homogeneous between isolates at 25°C. Because of the smaller variation coefficient and the least significant differences at 25°C, the isolates could be divided into various groups. We separated them into three groups: a slow-growing group (Lup4), the fast-growing group (Tam6, Tam7, and *C. gloeosporioides* reference isolate), and an intermediate-growing group (Lup1, Lup14, Lup16, Lup18, Lup28, and Tam20). At 30°C, the growth rate of the fungi reduced. Radial growth of tamarillo isolates was faster than that of the lupine isolates at 25°C. In each group, a higher average of radial growth at four temperatures of incubation was obtained for Tam7 and Lup18. The lower growth at 20 than at either 15 or 25°C of some isolates may be an intrinsic character.

The shape of the conidia varied between lupine isolates at $17 \pm 2^\circ\text{C}$. Conidia of isolates Lup1 and Lup28 were ovoid, with one round and one acute end (Fig. 2A), but conidia of the other lupine isolates were mostly cylindrical, with round ends, resembling *C. gloeosporioides* (Fig. 2B–D). Conidia of tamarillo isolate Tam6 had acute ends typical for *C. acutatum* (Fig. 2E), whereas Tam7 and Tam20 had one round and one acute end (Fig. 2F). Because

isolates Lup14, Lup16, and Lup18 showed cylindrical, rounded-end conidia and gray-olive colonies, they were placed in a separate group (Table 3). Length and width ranges of the conidia of the lupine and tamarillo isolates overlapped (Table 3).

Benomyl test. The benomyl sensitivity assay indicated differences between *C. acutatum* and *C. gloeosporioides*. Lupine and tamarillo isolates showed radial mycelium growth of 30 to 50% on benomyl-amended PDA in comparison with the controls (Table 2). The reference isolate of *C. gloeosporioides* IMI 356878 (GenBank accession AJ536229.1) did not grow in PDA amended with benomyl.

Species-specific primer analyses. DNA from 10 *Colletotrichum* isolates collected from lupine and tamarillo was isolated. A 490-bp DNA fragment was amplified with the *C. acutatum*-specific primers CaInt2 and ITS4 in all lupine and tamarillo isolates. The *C. gloeosporioides*-specific 450-bp DNA fragment was only amplified in the reference isolate *C. gloeosporioides* IMI 356878 (GenBank accession AJ536229.1; Table 2).

Sequencing of rDNA region. *Colletotrichum* isolates from lupine (Lup1, Lup12, Lup14, Lup16, Lup18, Lup24, and Lup28) and tamarillo (Tam6, Tam7, and Tam20) were used for sequence analysis of the ITS region. The results were compared with published *Colletotrichum* ITS sequences from lupine (28,41), tamarillo (2), strawberry (25), olive (40,42), and *C. fioriniae* com et stat nov. and *C. simmondsii* sp. nov. reclassified by Shivas and Tan (35), for-

Fig. 2. Colony and conidial morphological variability of *Colletotrichum acutatum* isolates from lupine (*Lupinus mutabilis*) and tamarillo (*Solanum betaceum*) grown for 10 days at $17 \pm 2^\circ\text{C}$ at day light on potato dextrose agar plates. **A**, Inverse colony surface and conidia shape of isolate Lup1; **B**, inverse colony surface and conidia shape of isolate Lup14; **C**, upper colony surface and conidia shape of isolate Lup16; **D**, upper colony surface and conidia shape of isolate Lup18; **E**, upper colony surface and conidia shape of isolate Tam6; and **F**, upper colony surface and conidia shape of isolate Tam20 (each division line = 10.0 μm).

merly *C. acutatum* in the EMBL database. Sequences of additional representative isolates of the species *C. gloeosporioides*, *C. fragariae*, and *C. falcatum* were also included (25; Table 4). The phylogenetic analyses of 38 taxa of *Colletotrichum* were completed by applying the NJ and MP models (Fig. 3). Phylograms were generated to confirm interspecific separation of *C. acutatum* from lupine and tamarillo with *C. gloeosporioides*, *C. fragariae*, and *C. falcatum*. Removing highly variable positions from the sequence analysis did not affect tree topology. Sequence analysis confirmed that all lupine and tamarillo isolates were *C. acutatum*.

In the phylograms, most Ecuadorian *C. acutatum* lupine isolates were in one subgroup together with Tam6. Other tamarillo isolates (Tam7 and Tam20, two from Colombia) were in subgroup III together with Lup28. Other subgroups in the phylogenetic tree consisted of isolates from around the world. For instance, subgroup I consisted of one *C. acutatum* from lupine (41), one from olive (42), and four *C. lupini* from lupine (28). One *C. acutatum* isolate from lupine (41), one from primula (28), and two *C. fiorinae* com. et stat. nov. of mango and avocado (35) formed subgroup II. Subgroups I, II, and III and the lupine subgroup from Ecuador formed group I. One isolate from strawberry (25) and one from tamarillo, cited as *Cyphomandra betacea* (35), were placed within the separate group II. Two isolates from olive (40,42), one *Colletotrichum* sp. from lupine (41), and two *Colletotrichum simmonsii* from tomato and chili (35) formed a separate clade (group III) (Fig. 3).

In the final dataset, 62 of 460 positions were informative: sequence comparisons gave a homology level of 98.9 to 100% in group I and of 99.6 to 100% in its subgroups. Tree topology was similar for NJ and MP trees with respect to placement of taxa.

Aggressiveness on alternate host. Five *C. acutatum* isolates from lupine and five from tamarillo were tested for their aggressiveness on three lupine and two tamarillo genotypes. The interaction between the inoculated host and the source of the pathogen (original host) was highly significant ($P < 0.0001$) for lesion length (Table 5). With tamarillo as host, 22.00 mm was the maximum lesion length, and this was 24.00 mm with lupine as host (Table 6). Some isolates, such as Tam20, Lup18, and Lup28, caused larger lesions on their original host as on the alternative host. However, there were also cases in which isolates were similarly aggressive. Isolate Lup4 was the most pathogenic on all lupine and tamarillo cultivars.

Symptom expression on lupine was similar for the two populations of *C. acutatum* studied here. Isolates from lupine were highly biotrophic in lupine, producing little or no necrosis on lupine stems after 20 days following infection. Abundant salmon-colored sporulation and intense production of mycelium were also observed 7 days after inoculation. In contrast, isolates from tamarillo sporulated less abundantly on tamarillo and induced dark pigmentation in the lesions, with sporulation appearing 15 days after inoculation. In the host cross test, isolates of tamarillo were also biotrophic on lupine stems, producing little necrosis with sporulation after 7 days, but isolates of lupine produced dark pigmentations with almost no sporulation in tamarillo stems.

Table 2. Cumulative mycelial radial growth of *Colletotrichum* isolates from *Lupinus mutabilis* and *Solanum betaceum* incubated at 25°C for 5 days on potato dextrose agar (PDA) in the dark, relative sensitivity in PDA amended with benomyl, and polymerase chain reaction amplification products using primers CaInt2/internal transcribed spacer (ITS4) and CgInt/ITS4

Isolate ^c	Radial growth for 5 days (mm) ^a				Benomyl sensitivity ^d	Primer reaction ^b	
	15°C	20°C	25°C	30°C		CaInt2	CgInt
Lup1	40.1	41.7	48.6	28.3	+	+	–
Lup4	45.4	45.4	44.0	23.5	+	+	–
Lup14	41.2	36.3	54.4	24.7	+	+	–
Lup16	39.4	37.0	59.3	30.0	+	+	–
Lup18	44.0	44.0	60.6	36.2	+	+	–
Lup28	44.8	44.3	49.9	19.2	+	+	–
Tam6	47.7	35.6	64.8	20.8	+	+	–
Tam7	51.7	48.5	64.6	23.9	+	+	–
Tam20	45.4	43.5	60.0	20.8	+	+	–
IMI 356878	24.5	40.2	64.5	28.2	–	–	+
LSD	1.02	0.46	0.13	0.34	...	...	...
CV	2.07	0.97	0.20	1.18	...	...	...

^a Radial growth of isolates on PDA at selected temperatures in the dark was measured using colonies initiated from 15-μl droplet of 1×10^6 conidia ml⁻¹. Each isolate had three replications, four measurements per replicate, and final maximum growth up to 5 days.

^b Taxon-specific primers CaInt2 (*Colletotrichum acutatum*), and CgInt (*C. gloeosporioides*) were coupled with primer ITS4 for species identification; a positive (+) or negative (–) reaction with fungal DNA of each isolate is designated.

^c Reference isolate IMI 356878 (AJ536229.1) (*C. gloeosporioides*) from olive (*Olea europaea* subsp. *europaea*) was included. LSD = least significant difference and CV = coefficient of variation.

^d Benomyl sensitivity of the isolates was assessed by comparing colony radius on PDA and PDA amended with benomyl (Benlate 50WP, DuPont) at 2 mg liter⁻¹; growth (+) or non-growth (–) mycelial reaction is indicated.

Table 3. Conidia and colony morphology of representative isolates of *Colletotrichum* spp. from lupine and tamarillo species in Ecuador grown for 10 days at 17 ± 2°C in day light on potato dextrose agar (PDA) plates

Isolate	Host	Conidia morphology and size	Colony morphology on PDA
Lup1, Lup28	Lupine	Ovoid, one round and one acute end, 10.0 to 13.0 by 4.5 to 5.0 μm	Transparent, cottony, and low-density colonies with abundant pink-orange conidia masses in the center, transparent concentric bands, pink-salmon pigment covers one-third of the colony surface, no setae or “hair-like structures” present on PDA
Lup14, Lup16, Lup18	Lupine	Cylindrical, rounded-end conidia or one round and one acute end, 12.5 to 18.0 by 5.0 to 5.5 μm	Gray-olive, felty and medium dense colonies with pink conidia masses, dark concentric bands, sclerotia present, pink-salmon pigment covers two-thirds to three-thirds of the colony surface, setae presence or not
Tam6	Tamarillo	Elliptic, pointed at both ends, 12.5 to 18.0 by 2.5 to 4.5 μm	White mycelium, turning salmon with age due to proliferation of conidia masses; no sclerotia present; setae present, no concentric bands presence, yellow-salmon pigment cover two-thirds of the colony surface
Tam7, Tam20	Tamarillo	Elliptic and pointed at one end, 14.0 to 18.5 by 4.5 to 6.0 μm	White mycelium turning gray and powdery with pink conidia masses, salmon in color, produced outward in circles from the center of the culture, sclerotia present, setae present, concentric circles form the center of the colony, yellow-salmon pigment covers two-thirds to three-thirds of the colony surface

Discussion

In this study, we demonstrate that the causal agent of lupine and tamarillo anthracnose in Ecuador is *C. acutatum*. Other species of *Colletotrichum* have been implicated as causes of anthracnose in these hosts, including *C. lupini*. Based on morphological features such as colony color and conidia shape, Nirenberg et al. (28) separated *C. lupini* var. *lupini* from *C. lupini* var. *setosum*. We disagree with the separation of this species into two separate varieties because, in our study, morphological traits of isolates overlapped and it was very difficult to separate them.

Morphological evaluation of isolates from both hosts demonstrated the variability possible in this species. In general, the colony color of isolates both lupine and tamarillo changed in time from white to dark but conidia were consistently pink-salmon. Pink-salmon-colored conidia is a characteristic of *C. acutatum* in formal descriptions (21,39,44). Growth rate and pigment can be considered as parameters to differentiate isolates of lupine and tamarillo. *C. acutatum* lupine isolates grow more slowly than tamarillo isolates and produce a pink-salmon pigment (tamarillo isolates produce a pink-yellow pigment). These morphological characteristics correlated with molecular analysis and lupine and tamarillo isolates clustered in two different groups based on ITS sequences (Fig. 3). In our study, we noticed that some *C. acutatum* isolates from lupine and tamarillo grew faster at 15 and 25 than at 20°C.

This may be an adaption to lower temperatures at night and higher temperatures during the day, with only a short period of intermediate temperatures. Nirenberg et al. (28) and Thomas et al. (45) demonstrated that maximum growth of *Colletotrichum* isolates from lupine was at 25°C; mycelial growth was reduced and conidia not swollen when isolates were grown at 30°C.

Lupine *Colletotrichum* spp. produced conidia that were ovoid, with either one rounded and one pointed end (Fig. 2A) or with a cylindrical shape (Fig. 2B–D). Cylindrical conidia have often been associated with *C. gloeosporioides* (39) and this might be the reason that others have determined *C. gloeosporioides* to be the causal agent of anthracnose in lupine and not *C. acutatum*, although molecular analysis with random amplified polymorphic DNA isolates indicated differently (7,51). In our study, we found cylindrical, pointed at one end or ovoid conidia of *C. acutatum* (Fig. 2A–D). Conidia of tamarillo isolates had either acute ends typical for *C. acutatum* or one round and one acute end (Fig. 2E and F). Morphology of conidia can be affected by differences in artificial conditions but direct identification of isolates coming from the plant is also difficult because conidia morphology varies during the infection process. The use of morphological features to distinguish *Colletotrichum* taxa in general is tricky due to the lack of standardized protocols and international rules and, therefore, taxonomic relationships within the genus are unlikely to be resolved only by the use of traditional morphological characters (39).

Table 4. Internal transcribed spacer 1 and 2 sequences of *Colletotrichum* isolates used in this study

Isolate code	EMBL accession	Determination by the authors as	Host	Country
BBA 70343 PD 93/1373	AJ301915	<i>Colletotrichum acutatum</i> ^a	<i>Primula</i>	The Netherlands
BBA 70344 PD 93/1436	AJ301916	<i>C. lupini</i> var. <i>setosum</i> ^a	<i>Lupinus</i> sp.	The Netherlands
BBA 700073	AJ301927	<i>C. lupini</i> var. <i>setosum</i> ^a	<i>Lupinus polyphyllus</i> hybrid	Germany
BBA 63879	AJ301930.1	<i>C. lupini</i> var. <i>lupini</i> ^a	<i>L. mutabilis</i>	Bolivia
BBA 70884	AJ301948	<i>C. lupini</i> var. <i>lupini</i> ^a	<i>L. albus</i>	Ukraine
	AJ300559	<i>Colletotrichum</i> sp. ^b	<i>L. angustifolius</i>	Portugal
	AJ300563	<i>Colletotrichum</i> sp. ^b	<i>L. mutabilis</i>	Portugal
	AJ311391	<i>C. acutatum</i> ^b	<i>L. albus</i>	Canada
	AJ300558	<i>C. acutatum</i> ^b	<i>L. albus</i>	Portugal
	AJ749674	<i>C. acutatum</i> ^c	<i>Olea europaea</i> subsp. <i>europaea</i>	Portugal
	AJ749679	<i>C. acutatum</i> ^d	<i>O. europaea</i> subsp. <i>europaea</i>	Portugal
	AM991131	<i>Glomerella acutata</i> ^e	<i>O. europaea</i> subsp. <i>europaea</i>	Portugal
	AM991137	<i>G. acutata</i> ^e	<i>O. europaea</i> subsp. <i>europaea</i>	Portugal
Lup1	JN543059 ^f	<i>C. acutatum</i>	<i>L. mutabilis</i>	Ecuador
Tam6	JN543069 ^f	<i>C. acutatum</i>	<i>Solanum betaceum</i>	Ecuador
Tam7	JN543070 ^f	<i>C. acutatum</i>	<i>S. betaceum</i>	Ecuador
Lup12	JN543060 ^f	<i>C. acutatum</i>	<i>L. mutabilis</i>	Ecuador
Lup14	JN543061 ^f	<i>C. acutatum</i>	<i>L. mutabilis</i>	Ecuador
Lup16	JN543062 ^f	<i>C. acutatum</i>	<i>L. mutabilis</i>	Ecuador
Lup18	JN543063 ^f	<i>C. acutatum</i>	<i>L. mutabilis</i>	Ecuador
Tam20	JN543071 ^f	<i>C. acutatum</i>	<i>S. betaceum</i>	Ecuador
Lup21	JN543064 ^f	<i>C. acutatum</i>	<i>L. mutabilis</i>	Ecuador
Lup24	JN543065 ^f	<i>C. acutatum</i>	<i>L. mutabilis</i>	Ecuador
Lup28	JN543066 ^f	<i>C. acutatum</i>	<i>L. mutabilis</i>	Ecuador
Lup30	JN543067 ^f	<i>C. acutatum</i>	<i>L. mutabilis</i>	Ecuador
Lup31	JN543068 ^f	<i>C. acutatum</i>	<i>L. mutabilis</i>	Ecuador
	AF521205	<i>C. acutatum</i> ^g	<i>S. betaceum</i>	Colombia
	AF521210	<i>C. acutatum</i> ^g	<i>S. betaceum</i>	Colombia
345034	AJ536207	<i>C. acutatum</i> ^h	<i>Fragaria</i> × <i>ananassa</i>	Australia
356878	AJ536229	<i>C. gloeosporioides</i> ^h	<i>Fragaria</i> × <i>ananassa</i>	Italy
345047	AJ536223	<i>C. fragariae</i> ^h	<i>Fragaria</i> × <i>ananassa</i>	United States
347765	AJ536231	<i>C. falcatum</i> ^h	<i>Fragaria</i> × <i>ananassa</i>	Bangladesh
20127	GU183320	<i>C. fioriniae</i> ⁱ	<i>Persea americana</i>	Australia
28761	GU183333	<i>C. fioriniae</i> ⁱ	<i>Mangifera indica</i>	Australia
24191	GU183322	<i>C. simmondsii</i> ⁱ	<i>Actinia chinensis</i>	Australia
28420	GU183327	<i>C. simmondsii</i> ⁱ	<i>Cyphomandra betacea</i>	Australia
52657	GU183352	<i>Colletotrichum simmondsii</i> ⁱ	<i>Lycopersicon esculentum</i>	Australia

^a Isolate sequenced by Nirenberg et al. (28).

^b Isolate sequenced by Talhinas et al. (41).

^c Isolate sequenced by Talhinas et al. (2005; EMBL direct submission).

^d Isolate sequenced by Talhinas et al. (42).

^e Isolate sequenced by Talhinas et al. (40).

^f Isolates sequenced by the authors (2011; EMBL accession number).

^g Isolate sequenced by Afanador-Kafuri et al. (2).

^h Isolate sequenced by Martinez-Culebras et al. (25).

ⁱ Isolate sequenced by Shivas and Tan (35).

Primers specific for *C. acutatum* and *C. gloeosporioides* have been used previously to show that *C. acutatum* is the causal agent of lupine anthracnose in Portugal (41), tamarillo anthracnose in Colombia (2), and almond anthracnose in California (9), and that both *C. acutatum* and *C. gloeosporioides* are responsible for anthracnose of olive in Portugal (42). In this study, the use of specific primers supported the identification of *C. acutatum* as the causal agent of lupine and tamarillo anthracnose in the Ecuadorian Andean zone.

C. acutatum can be distinguished based on the ability to grow on PDA with benomyl at 2 mg/liters (Table 2) but differential sensitivity of mixed *Colletotrichum* populations to fungicides such as benomyl can pose problems in disease control (15). In tamarillo, we can speculate that the outbreak of anthracnose in tamarillo is from endemic resistant populations of *C. acutatum*.

Phylogenetic analysis of the ITS sequences divided the *C. acutatum* lupine and tamarillo populations from Ecuador into two sub-

groups and most (one exception) lupine isolates formed one new subgroup (Fig. 3). Earlier studies showed that in strawberry, the pathogen population within a geographical location was very homogeneous and nearly clonal (24–26). This was also found for *C. acutatum* populations on strawberry in a number of locations in the United States (46) and in several locations in Israel (10). In this study, the new subgroup of lupine isolates included one from tamarillo. This isolate was collected in an area where lupine and tamarillo have been cultivated together for about 10 years and it might be a lupine isolate that cross infected a field of tamarillo. Subgroup III grouped tamarillo isolates from Ecuador and Colombia and one lupine isolate (Lup28) (Fig. 3). Based on sequence analysis of the ITS2 region, two subgroups of *C. acutatum* isolates were found on tamarillo (2); in one of these clades, two isolates of lupine known as *C. lupini* var. *lupini* and *C. lupini* var. *setosum* (28) were present. Based on these findings, Afanador-Kafuri et al. (2) suggested that isolates in this clade may not be host specific.

Fig. 3. Phylogram generated from internal transcribed spacer data of *Colletotrichum* isolates from *Lupinus mutabilis*, *Solanum betaceum*, and other hosts around the world using neighbor-joining analysis by using the maximum parsimony model. The optimal tree is shown. Percentage of replicate trees in which associated taxa clustered together in the bootstrap (1,000 replicates) is shown next to the branch nodes. All positions containing gaps and missing data were eliminated from the dataset. Database accession number is provided for reference sequences of Table 1.

Table 5. Analysis of variance of the lesion length using a general linear model^a

Variable	df	Lesion length		
		MS	F value	P value
Inoculated host (I)	1	2.67	0.05	0.8196
Host of origin (H)	1	339.79	6.65	0.0109
I × H	1	1,144.69	22.40	<0.0001

^a Components of variance are the inoculated host (lupine or tamarillo) in this assay, host of origin (lupine or tamarillo) from which isolates were collected, and the combination inoculated host per original host of the isolates; df = degree of freedom, MS = mean square.

Table 6. Diameter (mm) of lesions on three lupine cultivars and two tamarillo cultivars caused by isolates of *Colletotrichum acutatum*

Source isolate	Lupine cultivars				Tamarillo cultivars		
	ECU-2658	ECU-7112-2	ECU- 722-4	Mean	Gigante comun	Comun	Mean
Tamarillo							
Tam6	13.33	18.66	16.00	16.00	22.00	18.33	20.17
Tam7	16.00	16.00	16.00	16.00	22.00	14.66	18.33
Tam20	6.00	8.00	13.33	9.11	22.00	22.00	22.00
Tam33	16.00	5.33	21.33	14.22	22.00	14.66	18.33
Tam34	18.66	10.66	10.66	13.33	18.33	11.00	14.67
Mean	14.00	11.73	15.46	13.73	21.27	16.13	18.70
Lupine							
Lup1	24.00	21.33	21.33	22.22	22.00	22.00	22.00
Lup4	24.00	24.00	24.00	24.00	22.00	22.00	22.00
Lup14	18.66	13.33	24.00	18.66	22.00	14.66	18.33
Lup18	24.00	21.33	24.00	23.11	14.66	0.00	7.33
Lup28	21.33	24.00	24.00	23.11	18.33	3.66	11.00
Mean	22.40	20.80	23.47	22.22	19.80	12.46	16.13

Host range tests clearly showed that lupine and tamarillo are attacked by two separate, host-adapted populations of *C. acutatum* (Table 6). Based on symptoms and lesion size, it is concluded that host adaptation is quantitative rather than qualitative, because isolates were aggressive on their original host but also pathogenic on their alternative host. *C. acutatum* isolates have also been found on tamarillo, lupine, and other hosts (2,41) in other parts of the world. Whitelaw-Wecket et al. (50) showed there was cross infection of *C. acutatum*, *C. fioriniae*, and *C. simmondsii* in different horticultural crops. In our study, lupine isolates were highly biotrophic on lupine but the lupine-adapted isolates caused darkly pigmented lesions on tamarillo stems, which may be classified as a partially necrotrophic reaction. Tamarillo isolates were necrotrophic on tamarillo but the lesions that tamarillo-adapted isolates caused in lupine stems were typical of a biotrophic reaction.

Host adaptation appears to be manifested based on lesion size and sporulation in the alternative host (Table 6). The infection strategy adopted by *C. acutatum* depends on the host being colonized and this fungus may also change its infection strategy when colonizing different tissue, hosts, and cultivars (48). We found early and more sporulation of *C. acutatum* in lupine than in tamarillo. Adaptation to a new host is not always associated with reduced fitness in the original host (22,29,47) and, until more components of aggressiveness (infection efficiency and sporulation in the field, among others) are determined, anthracnose integrated management efforts for both lupine and tamarillo should be coordinated.

Acknowledgments

This research was financially supported by TELFUN project–Wageningen University under the supervision of Wageningen UR Plant Breeding and supported by the Army Polytechnic School (ESPE), Quito-Ecuador. We thank P. V. Martinez-Culebras from IATA, University of Valencia, Spain, for his suggestions and for providing reference isolates; M. Rivera for his help in sampling pathogens; and P. Landazuri and C. Rosas for their help in collecting data.

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