

Multilocus molecular identification and phylogenetic analysis of *Colletotrichum tamarilloi* as the causal agent of Tamarillo (*Solanum betaceum*) anthracnose in the Ecuadorian highlands

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Abstract Anthracnose caused by *Colletotrichum* spp., is one of the most serious diseases affecting tamarillo (*Solanum betaceum*) production in the Ecuadorian highlands. The objective of this study was to characterise *Colletotrichum* isolates obtained from tamarillo to clarify its taxonomic and phylogenetic position. Based on phenotypic and morphologic characterisation, the isolates of this study were consistently grouped within the *Colletotrichum acutatum* complex. Multilocus molecular phylogenetic analysis based on combined sequences of actin (ACT), β -tubulin (TUB2), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sequences using Bayesian probabilities, indicated that 16 of 20 isolates from Imbabura, Cotopaxi, Tungurahua, Bolívar, Chimborazo, Azuay and Loja provinces, belonged to *C. tamarilloi*. This study represents the first reported case of anthracnose of *S. betaceum* caused by *C. tamarilloi* in the Ecuadorian highlands. Interestingly, pathogenicity tests and multilocus molecular analysis revealed a new infraspecific and more aggressive species formed by four isolates obtained from the Pichincha province. These findings probably

provided interesting information related to mutant isolates and a possible description of a new pathogenic species affecting tamarillo fruits.

Keywords *Colletotrichum tamarilloi* · Anthracnose · Actin · β -tubulin · Glyceraldehyde 3-phosphate dehydrogenase

Introduction

Tamarillo (*Solanum betaceum*), is native to Ecuador, Colombia, Peru, Chile and Bolivia (Prohens and Nuez 2001). In Ecuador, tamarillo is cultivated in warmer regions in small orchards for local production and consumption, as well as in larger areas for commercial purposes. Commercially, 14,748 ha are cultivated in the provinces of Carchi, Imbabura, Pichincha, Cotopaxi, Tungurahua, Chimborazo, Bolívar, Cañar, Azuay and Loja, Ecuador (CICO-CORPEI 2009). However, tamarillo production is seriously affected by anthracnose caused by *Colletotrichum* spp., among other pathogens (Santillán 2001). Mainly *Colletotrichum* species affect fruits at different developmental stages, causing productivity losses up to 100% in areas of high incidence (León et al. 2004). The same authors mentioned that this disease can be controlled up to 80% with the application of protectant fungicides such as mancozeb, chlorothalonil and captan, and with systemic benzimidazole fungicides such as benlate and carbendazim. Nevertheless, the constant and prolonged use of the latter has induced resistance in *Colletotrichum*, limiting the effectiveness of

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these products under field conditions apart from high environmental pollution (Kongtragoul et al. 2011). Furthermore, Blank et al. (1987) indicated that the excessive use of systemic fungicides can generate contamination that remains on tamarillo fruits destined for human consumption, thus limiting seriously the exportation, due to strict regulations of importing countries of this fruit. According to Than et al. (2008), for effective disease control, the first approach is the accurate identification of the causal agent in a particular region to design environmentally friendly control strategies. Formerly, the identification of *Colletotrichum* spp. has been based on the use of morphological and phenotypic features (Brown et al. 1996), as well as host range (Freeman et al. 1998; Afanador-Kafuri et al. 2003). The most used morphological character is the shape of the conidia and appressoria (Simmonds 1965). Based on those features, it is difficult to differentiate between isolates from *C. acutatum* and *C. gloeosporioides* and has shown unreliable to separate species within the genus (Freeman and Rodríguez 1995; Sreenivasaprasad and Talhinas 2005). Currently, molecular tools are widely used to complement identification and differentiation of *Colletotrichum* spp. in several important crops (Photita et al. 2005; Damm et al. 2012; Weir et al. 2012). In Ecuador and Colombia, previous works based on sequences of the ITS region (internal transcribed spacer) of 16S rRNA and morphological characters, *Colletotrichum acutatum* was reported as the causal agent of anthracnose of tamarillo (Afanador-Kafuri et al. 2003; Falconi et al. 2013). However, investigations on *Colletotrichum* spp. carried out by Crouch et al. (2009), indicated that the ITS region alone is often unreliable and not enough to resolve the relationships and differences among species. Within the *C. acutatum* populations, Sreenivasaprasad and Talhinas (2005) differentiated infraspecific groups, designated as clades A1 to A8 based on rDNA ITS and beta-tubulin DNA (TUB2) sequences, because of their sequence data. A recent study using multilocus molecular sequence data of actin (ACT), β -tubulin (TUB2), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), histone 3 (HIS 3), chitin synthase 1 (CHS-1) and ITS from strains of the *C. acutatum* species complex, combined with Bayesian inference, led to recognize and identify thirty-one species within this complex, of which 21 had not previously been known (Damm et al. 2012), including *C. tamarilloi*, specific for tamarillo.

The aim of this study was to amplify and clarify the taxonomy and genetic position of *Colletotrichum* species that cause tamarillo anthracnose in the Ecuadorian highlands. We used a multi-disciplinary approach including a phenotypic, a morphologic and a multilocus molecular characterization of isolates of *Colletotrichum* spp.

Materials and methods

Sample collection

Tamarillo orchards located in the Ecuadorian highlands, were surveyed for common symptoms of anthracnose such as black lesions on fruits accompanied by erumpent pink conidial masses (Fig. 1). A total of 35 infected fruits were collected from January to June 2015, in eight different provinces of Ecuadorian highlands (Imbabura: 4 samples, Pichincha: 12 samples, Cotopaxi: two samples, Tungurahua: 11 samples, Chimborazo: one sample, Bolivar: two samples, Azuay: two samples and Loja: one sample) (Fig. 2). Depending on the geographical location, 20 of the 35 samples (1–5 per province) were selected for complete identification and DNA analysis (Table 1). A code was assigned to each sample that included location and province.

Fungal isolation and purification

The infected fruits were cut into 5-mm pieces with a sterile razor blade (2.5 mm of lesion and 2.5 mm of healthy fruit) surface-sterilized in 1% sodium hypochlorite for 2 min and in 70% ethanol for 1 min and washed three times in sterilized distilled water. All *Colletotrichum* isolates obtained from infected fruits were grown on acidulated potato dextrose agar (PDAA) (Difco Laboratories, Detroit, U.S.A.) for 7 days at 21 ± 2 °C (Gunnell and Gubler 1992). A single-spore protocol was used to obtain pure *Colletotrichum* cultures (Choi et al. 1999). These cultures from each isolate were stored on dry filter paper (Whatman No. 42) at 4 °C, according to the protocol proposed by Michaelsen et al. (2013).

Phenotypic and morphological analysis

Macrometric characteristics of fungal colonies considered were: colony color, margin and sporulation. From each *Colletotrichum* isolate, a total of 25 conidia and 10 appressoria were examined for morphological

Fig. 1 Common anthracnose lesions observed on tamarillo fruits. Samples were collected at San José de Minas (Pichincha province) in Ecuador



characterization. Conidia description included: size, shape, color and surface. Appressoria description included: size, color and shape, single or in groups, wall and edge characteristics. Conidia and appressoria sizes

were measured using a microscope (Olympus Confocal Fluoview 1000 DIC with Differential Interference Contrast) with the software FV10_ASW 3.0, available at Laboratory of Plant Pathology of the “Agencia

Fig. 2 Ecuadorian highland provinces sampled for tamarillo anthracnose

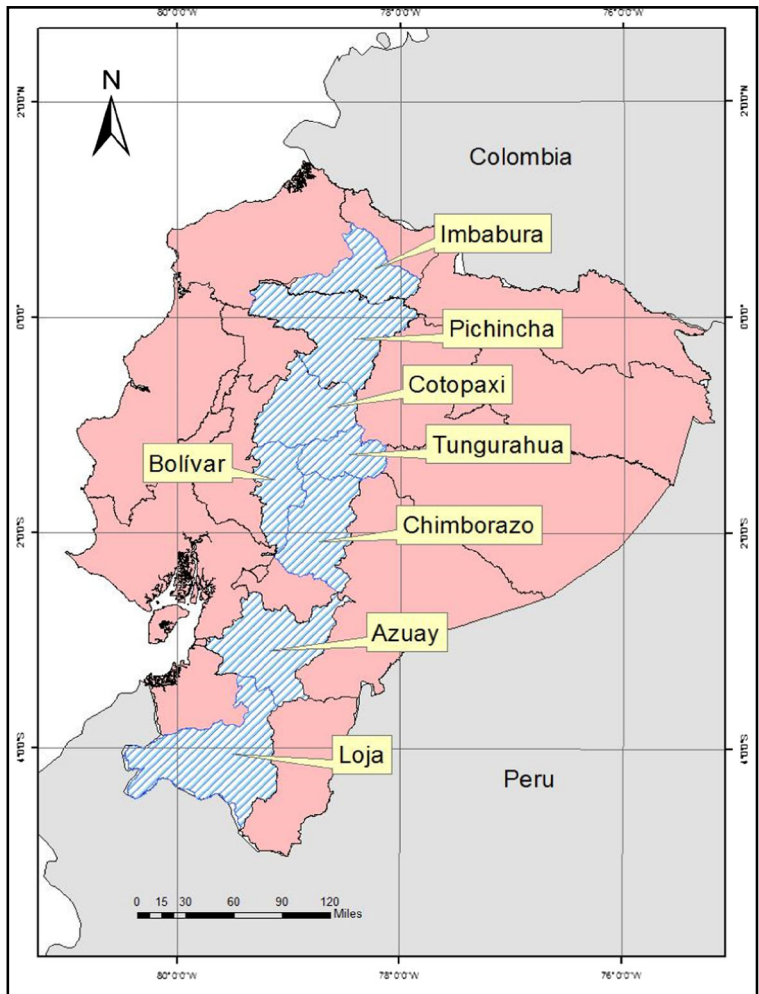


Table 1 Phenotypic characterisation of *Colletotrichum* spp. isolates from tamarillo in Ecuadorian highlands

Isolate Code	Location/Province	Phenotypic characteristics		
		Colony center	Margin	Conidial masses
Sb-Im	San Blas/Imbabura	Olivaceous gray	White	Presence
Ct-Im	Cotacahi/Imbabura	Olivaceous gray	White	Presence
Ur-Im	Urcuquí/Imbabura	Olivaceous gray	White	Presence
Cu-Pi	Cuendina/Pichincha	Greenish gray	White	Presence
Ta-Pi	Tambillo/Pichincha	Greenish gray	White	Presence
Az-Pi	Azcáubi/Pichincha	Iron gray	White	Presence
Mi-Pi	Minas/Pichincha	Dark gray	White	Absence
Pi-Pi	Pinguilla/Pichincha	White cream	White	Presence
Pz-Co	Panzaleo/Cotopaxi	Olivaceous gray	White	Presence
Mu-Co	Mulliquindil/Cotopaxi	Olivaceous gray	White	Presence
Sa-Th	San Andrés/Tungurahua	Olivaceous gray	White	Presence
Pr-Th	Pelileo viejo/Tungurahua	Olivaceous gray	White	Presence
Ur-Th	Urbina/Tungurahua	Olivaceous gray	White	Presence
Pa-Th	Patate/Tungurahua	Olivaceous gray	White	Presence
Ma-Ch	Penipe/Chimborazo	Olivaceous gray	White cream	Presence
Sp-Bo	San Pablo/Bolívar	Olivaceous gray	White	Presence
Ch-Bo	Chillanes/Bolívar	Olivaceous gray	White	Presence
Lo-Lj	Valle de Loja/Loja	Olivaceous gray	White	Presence
Pa-Az	Paute/Azuay	Olivaceous gray	White	Presence
Zh-Az	Zhimbrug/Azuay	Olivaceous gray	White	Presence

¹ Fungal colonies were grown for 14 days at 21 ± 2 °C on acidulated potato dextrose agar

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For statistical analysis of conidia and appressoria measurements, the mean, standard error and range of length and width, were calculated using the software SPSS v.20 (<http://www.ibm.com/analytics/us/en/technology/spss/>).

Fungal DNA isolation

Fungal mycelium of each isolate was grown for 5 days in 100 ml of dextrose-potato-yeast extract broth (pH 5.5) (Difco Laboratories, Detroit, U.S.A.) at 20 °C under constant agitation (120 rpm), according to the protocol of Castellanos and Mosquera (2011). After that, the mycelium was collected and dried in 4-in. sterile gauze pads, and grounded in a mortar with 1600 µl of 2X CTAB buffer. The DNA was extracted using a CTAB protocol for plants (Shagai-Marouf et al. 1984) and re-suspended in 100 µl of TE buffer, and the working solution was diluted with ultrapure distilled water (Invitrogen, California, U.S.A.) up to a final

concentration of 20 ng/µl. Total nucleic acid concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, U.S.A.), available in the Laboratory of Molecular Biology of the International Zoonosis Center (IZC) at Central University of Ecuador.

PCR amplification, product purification and sequencing

ACT, TUB2 and GAPDH genes were amplified using the following primers: ACT-512F/ACT-783R (Carbone and Kohn 1999); T1/T2 (O’Donnell and Cigelnik 1997) and GDF1/GDR1 (Guerber et al. 2003), respectively. PCR reactions were performed in a total volume of 10 µl, containing 1 µl of Buffer (10X), 0.2 µl (10 mM) each of dATP, dCTP, dGTP, and dTTP, 0.3 µl of MgCl₂ (50 mM), 0.2 µl of each primer (10 µM), 0.2 µl of Platinum® *Taq*DNA Polymerase (5 U/µl) (Invitrogen, California, U.S.A.) and 1 µl of DNA (20 ng/µl). All PCR reactions were carried out on a Techne DNA Thermal Cycler FFG02HSD (Techne,

Staffordshire, UK) starting with 3 min of initial denaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, 30 s at 55 °C (TUB2) and 58 °C (ACT and GAPDH), extension for 45 s at 72 °C and a final extension for 4 min at 72 °C. PCR products were purified using the Pure Link PCR Purification Kit (Invitrogen, California, U.S.A.) following the manufacturer's instructions and sequenced using the commercial facilities at GENWIZ (New Jersey, U.S.A.). All sequences obtained were edited and submitted to GenBank database (Table 3).

Phylogenetic analysis

For each gene DNA sequences were assembled and edited using BioEdit (version 7.1.9) (<http://www.mbio.ncsu.edu/BioEdit/BioEdit.html>). The multiple sequence alignment was based on the MUSCLE function in Geneious program (version 9.0.5) (<http://www.geneious.com/>) (Kearse et al. 2012). Alignment gaps were treated as missing data (including the letter N), reaching an equal weight in all sequences analysed. A preliminary phylogenetic analysis of each gene was conducted using 52 sequences including 20 sequences from this study, and 32 retrieved from the GenBank, employing the maximum likelihood method and Tamura Nei Model with the software package MEGA 6.06 (<http://www.megasoftware.net/>) (Tamura et al. 2013). For multilocus molecular analysis, a Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities using MrBayes v. 3.2.6 (<http://mrbayes.sourceforge.net/manual.php>) (Ronquist and Huelsenbeck 2003) for the combined sequence datasets. The analyses of two MCMC chains were run from random trees for 1000 000 generations and sampled every 1000 generations (to achieve a probability of 0.02). Using Tree Annotator program (version 1.8.2) (<http://beast.bio.ed.ac.uk/treeannotator>) (Rambaut and Drummond 2010), 20 % of total trees generated were discarded as the burn-in phase and the posterior probabilities from the remaining trees determined. The posterior sample of trees generated a maximum clade credibility tree, with the highest estimations (posterior probability limit). To visualize the consensus phylogenetic tree, a FigTree program (version 1.4.2) (<http://beast.bio.ed.ac.uk/figtree>) was used. In addition, DNA sequences of 92 representative *Colletotrichum* spp. isolates belonging to *C. acutatum*

complex obtained from GenBank were included in the multilocus molecular phylogenetic analysis.

Pathogenicity tests

Twenty isolates of *Colletotrichum* spp. were tested for a pathogenic differential behavior in humid chambers in the laboratory (18 °C and 85–90% of relative humidity). Pathogenicity was assessed on wounded tamarillo fruits (susceptible cultivar “Gigante común”) with inoculum of *Colletotrichum*. For surface disinfection, the fruits were immersed in a 70% ethanol solution, for 60 s and then in a 5% sodium hypochlorite solution for 2 min, and immediately after washed in sterile distilled water for 1 min. The inoculum was prepared from 21-day-old colonies on Green Bean Agar (GBA) (Chull et al. 2008) and consisted of conidia suspensions of each isolate adjusted to 1×10^6 conidia/ml in sterile water using a Neubauer Counting chamber (Santos et al. 2013). Tamarillo fruits were wounded and inoculated by injecting (10 mm deep) the conidia suspensions (0.5 ml). Two fruits were inoculated with each one isolate. Negative controls were injected with sterile water. The results were evaluated 21 days after inoculation. In order to confirm the presence of the causal agent, the fungus was recovered from these lesions, and then grown in pure culture on GBA until new sporulation was observed. The conidia were visualised under optical microscope.

The lesion diameters measured with a graduated ruler (mm) and growth rates on GBA medium were analysed by a completely randomized experimental design with three replications. Furthermore, to group *Colletotrichum* isolates, based on growth rate on GBA (mm) and lesion diameter (mm), a cluster analysis with a Euclidean distance and average linkage was conducted. All measurements and the dendrogram were calculated with statistical program SPSS v.20 (<http://www.ibm.com/analytics/us/en/technology/spss/>).

Results

Symptomatology

Common symptomatology observed on infected fruits was black lesions accompanied by erumpent pink conidial masses as described by Falconí et al. (2013) (Fig. 1). Disease incidence in all sampled locations ranged from 60 to 80%.

Phenotypic characterisation

On PDAa medium, the colony color of the majority of the isolates changed with time from pale white to olivaceous gray in the front and back surface with abundant conidial masses of pink-salmon color near the center and with entire white margin. These phenotypic features were consistent with observations made by Afanador-Kafuri et al. (2003), Damm et al. (2012) and Falconí et al. (2013), placing those isolates within *C. acutatum* complex. These characteristics included isolates from Imbabura, Cotopaxi, Tungurahua, Chimborazo, Bolívar, Loja and Azuay. *Colletotrichum* isolates belonging to the Pichincha province, showed different shades of gray (dark gray, greenish gray, iron gray and white cream), indicating a phenotypic variability of isolates from this province (Fig. 3; Table 1).

Morphological characterisation

Conidia of most of the isolates were hyaline, smooth-walled, aseptate, cylindrical in shape and acute at both ends, except of those of isolate Mu-Co that showed a fusiform shape and acute at both ends (Fig. 3). Morphometric analysis indicated that the length and width of conidia ranged from 10.00 to 14.18 μm and 3.69 to 4.67 μm , respectively (Table 2). These dimensions are in accordance with ranges reported by Afanador-Kafuri et al. (2003) and Damm et al. (2012) for *C. acutatum* and *C. tamarilloi*, respectively. Appressoria were single, smooth-walled, subglobose and clavate in shape, arising from vegetative hyphae and not from germinating conidia. Appressoria length and width size ranged from 5.49 to 10.25 μm and 4.57 to 7.19 μm , respectively (Table 2). In the same way, these dimensions are in agreement with reports by Du et al. (2005) and Damm et al. (2012) for *C. acutatum* and *C. tamarilloi*, respectively (Table 3).

PCR amplification of taxonomic genes fragments

All isolates produced amplicons of expected size for *C. acutatum* complex for the following genes: ACT (316 pb), GAPDH (308 bp) and TUB2 (716 bp) (Damm et al. 2012). No amplifications were observed in negative control samples (water).

Blast searches

Based on DNA sequences of the three genes studied, 16 of the 20 isolates belonged to *C. tamarilloi*. DNA sequences had 100% homology with sequences from GenBank (Accession nos. ACT: JQ949505, TUB2: JQ949835 and GAPDH: JQ948514). We were not able to identify the remaining 4 isolates (Az-Pi, Mi-Pi, Ta-Pi and Cu-Pi) because their sequences showed 98 to 99% of identity with different *Colletotrichum* spp. These were *C. lupini* CBS 109225 (GenBank accession number: JQ949476, JQ949806 and JQ948485), *C. costaricense* CBS 330.75 (GenBank accession number: JQ949501), *C. limeticola* CBS 114.14 (GenBank accession number: JQ949514; JQ949844 and JQ948523), *C. tamarilloi* CBS 129814 (GenBank accession number: JQ949505; JQ948514), *C. melonis* CBS 159.84 (GenBank accession number: JQ949845 and JQ948524) and *C. cuscutae* IMI 304802 (GenBank accession number: JQ948525).

Multilocus molecular phylogenetic analysis

Preliminary phylogenetic trees were constructed for each individual gene studied (ACT, TUB2 and GAPDH) to a total of 52 sequences including 20 sequences from this study. All three phylogenetic trees showed that 16 of our sequences consistently grouped with *C. tamarilloi*, differentiating them from others species such as *C. lupini*, *C. limeticola*, *C. costaricense* and *C. cuscutae* (data not shown). The sequences of the remaining 4 isolates obtained from Pichincha province (Az-Pi, Mi-Pi, Ta-Pi and Cu-Pi) formed a different clade.

For the multigene analyses, a total of 92 sequences of the *C. acutatum* complex were analyzed including the outgroup (*C. orchidophilum* strains: CBS 631.80, CBS 632.80 and CBS 119291) using Bayesian probabilities. The total number of characters processed was 1049 including the alignment gaps. A multilocus molecular phylogenetic tree generated in MrBayes, showed that all concatenated sequences obtained in this investigation were grouped in the Clade 1 (classification proposed by Damm et al. 2012). This group was conforming by *C. lupini*, *C. tamarilloi*, *C. costaricense*, *C. cuscutae*, *C. limeticola* and *C. melonis*. Additionally, 16 of the 20 sequences grouped within the *C. tamarilloi* subclade (formally classified as infraspecific group “A8” by Sreenivasaprasad and Talhinas 2005). The four remaining isolates corresponding to Az-Pi, Mi-Pi, Ta-Pi

Fig. 3 Colonies, conidia and appressoria of representative *Colletotrichum* spp. isolates from tamarillo in Ecuadorian highlands grown for 14 days at 21 ± 2 °C on acidulated potato dextrose agar (PDAA). **a, b** and **c** represent to isolate Mu-Co (Mulliquindil/Cotopaxi); **d, e** and **f** represent to isolate Ta-Pi (Tambillo/Pichincha); **g, h** and **i** represent to isolate Az-Pi (Azcázubi/Pichincha); **j, k** and **l** represent to isolate Cu-Pi (Cuendina/Pichincha); **m, n** and **o** represent to isolate Mi-Pi (Minas/Pichincha); **p, q** and **r** represent to isolate Pi-Pi (Pinguilla/Pichincha). All scale bars represents 10 μ m

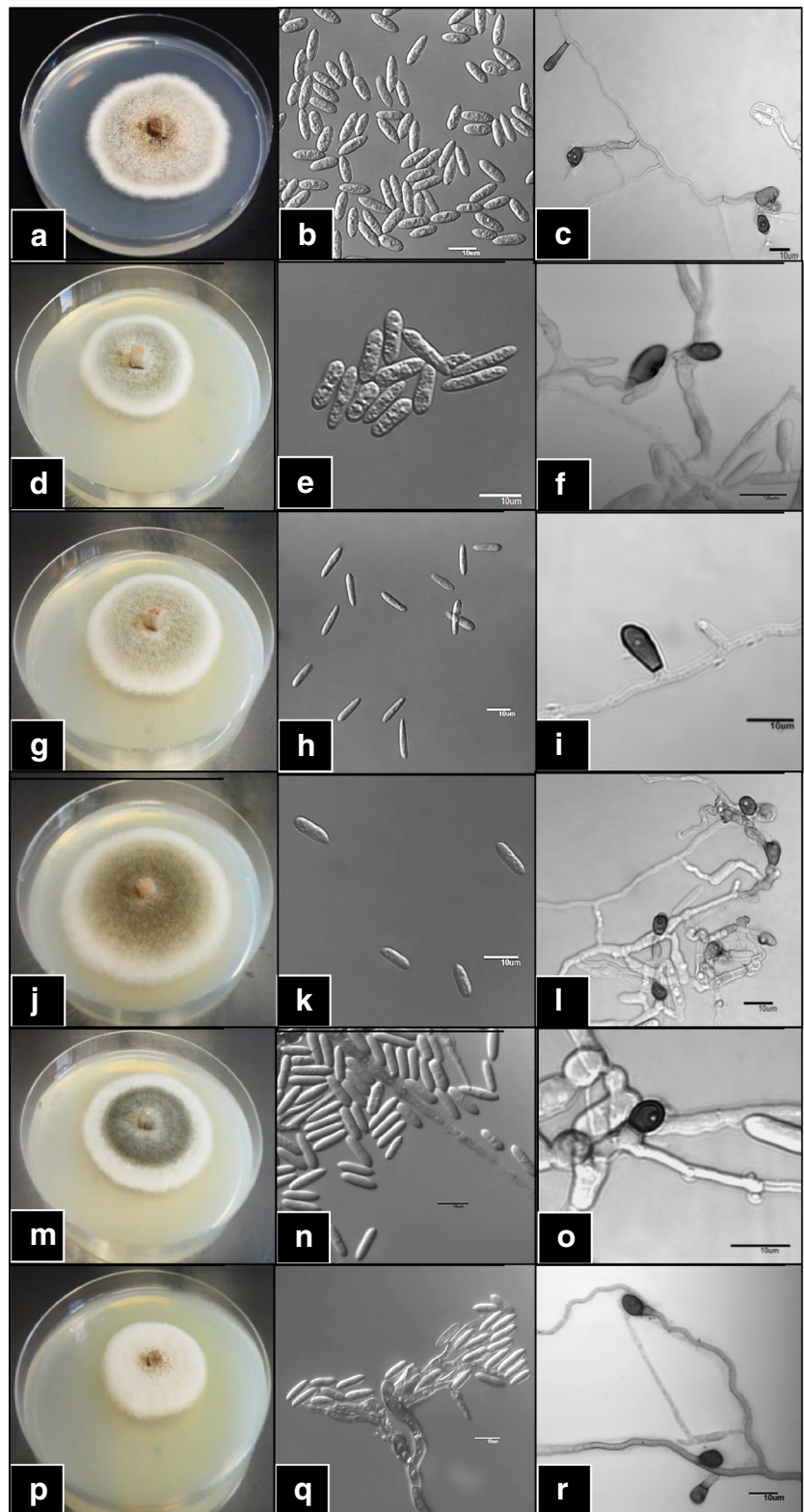


Table 2 Conidia and appressoria (size and shape) of *Colletotrichum* spp. isolated from tamarillo in Ecuadorian highlands

Isolate Code	Conidia			Appressoria		
	Length (μm)	Width (μm)	Shape	Length (μm)	Width (μm)	Shape
Sb-Im	12.1	4.02	C ^b	8.58	5.05	Cl ^d
Ct-Im	11.1	4.44	C	7.54	6.85	Cl
Ur-Im	12.11	3.75	C	9.54	5.61	Cl
Cu-Pi	14.18	4.53	C	9.88	6.08	Cl
Ta-Pi	12.75	4.23	C	6.84	4.95	Cl
Az-Pi	14.01	4.56	C	8.91	7.19	Cl-Sb ^e
Mi-Pi	12.31	3.91	C	7.29	5.97	Cl-Sb
Pi-Pi	11.83	4.3	C	10.25	6.3	Cl
Pz-Co	11.03	3.81	C	9.45	6.16	Cl
Mu-Co	12.79	4.23	F ^c	9.71	5.42	Cl
Sa-Th	12.3	4.09	C	8.83	5.55	Cl
Pr-Th	12.05	4.67	C	6.67	4.9	Cl
Ur-Th	12.85	4.21	C	10.16	4.87	Sb ^f
Pa-Th	11.12	4.02	C	5.49	6	Cl
Ma-Ch	13.39	4.46	C	7.09	4.88	Cl-Sb
Sp-Bo	10.45	4.11	C	8.71	4.57	Cl
Ch-Bo	11.01	4.07	C	10.17	5.76	Cl
Lo-Lj	12.69	4.21	C	8.15	5.58	Sb
Pa-Az	10	4.06	C	7.73	6.01	Cl
Zh-Az	12.17	3.69	C	7.7	5.58	Cl
Mean	12.11	4.17		8.54	5.66	
St. Dev.:	1.1	0.27		1.52	0.68	
Range	10.00–14.18	3.69–4.67		5.49–11.40	4.57–7.19	
Reported range ^a	8.5–15.00	2.5–4.5		4.00–16.00	3.5–8.00	

^a Range reported by Du et al. (2005) and Damm et al. (2012)

^b Cylindrical shape with acute ends

^c Fusiform shape acute ends

^d Clavate shape

^e Clavate and subglobose shape

^f Subglobose shape

and Cu-Pi codes, formed a different unrecognised group within the same Clade 1, suggesting a potential new species (Fig. 4). In general, topology of the phylogenetic tree constructed in this investigation was similar with a previous tree of several species belonging to *C. acutatum* complex published by Damm et al. (2012).

Bootstrapping and the Bayesian posterior probability value observed in the multilocus phylogenetic tree for the Clade 1, showed that the 48 sequences achieved a bootstrap of 99 (1/1), indicating a strong association between the concatenated sequences from this study and the *C. tamarilloi* sequences of the same three genes

obtained from GenBank. Bootstrapping and the Bayesian posterior probability value of the unrecognised infraspecific group (unreported previously in the literature), showed a bootstrap value of 99 (1/1), indicating a strong association between sequences from these isolates (Az-Pi, Mi-Pi, Ta-Pi and Cu-Pi), but different from the other infraspecific groups described previously by Damm et al. (2012).

In the four isolates above mentioned, differences in the nucleotides sequence were observed in the introns (non-coding sections of a DNA) of the three genes examined compared with the remaining 16 isolates. In the actin gene

Table 3 Accession numbers of DNA sequences of three genes from 20 *Colletotrichum tamarilloi* isolates from Ecuadorian highlands submitted to the GenBank

Isolate Code	GenBank accession number		
	actin	β -tubulin	GAPDH
Sb-Im	KU315496	KU315512	KU315528
Ct-Im	KU315497	KU315513	KU315529
Ur-Im	KU315498	KU315514	KU315530
Pi-Pi	KU315499	KU315515	KU315531
Pz-Co	KU315500	KU315516	KU315532
Mu-Co	KU315501	KU315517	KU315533
Sa-Th	KU315502	KU315518	KU315534
Pr-Th	KU315503	KU315519	KU315535
Ur-Th	KU315504	KU315520	KU315536
Pa-Th	KU315505	KU315521	KU315537
Ma-Ch	KU315506	KU315522	KU315538
Sp-Bo	KU315507	KU315523	KU315539
Ch-Bo	KU315508	KU315524	KU315540
Lo-Lj	KU315509	KU315525	KU315541
Pa-Az	KU315510	KU315526	KU315542
Zh-Az	KU315511	KU315527	KU315543
Cu-Pi	KX002094	KX002098	KX002102
Ta-Pi	KX002095	KX002099	KX002103
Az-Pi	KX002096	KX002100	KX002104
Mi-Pi	KX002097	KX002101	KX002105

a total of 4 bp differences were observed (one deletion and three transitions), in the β -tubulin gene a total of 6 bp differences were observed (six transitions), and in the GAPDH gene a total of 4 bp differences were observed (four transitions). In the three genes the most transitions observed were changes from guanine (G) to adenine (A) and from thymine (T) to cytosine (C), indicating mutations probably induced by constant fungicide applications (Yarden and Katan 1993). Furthermore, phenotypically these four isolates exhibited a different macrometric appearance in terms of their colonies color on PDAa medium from dark gray, greenish gray, orange gray and white cream, when compared to the remaining *C. tamarilloi* isolates that were white pale to olivaceous gray.

Pathogenicity tests

All wounded tamarillo fruits inoculated with the 20 *Colletotrichum* isolates showed anthracnose symptoms (Fig. 5). The analyses of variance for the lesion diameter

(p -value <0.0001) and colony diameter on GBA (p -value 0.003), indicated significant differences between the twenty isolates evaluated. Sixteen of twenty isolates recorded a diameter of lesion of 35 to 48 mm, forming a common pathogenic group; however, the diameter of lesions produced by the four remaining isolates (codes Cu-Pi, Mi-Pi, Ta-Pi and Az-Pi) were of 53 to 65 mm, indicating that these latter isolates were more aggressive both in growth rate as well as in necrosis production on fruits compared to the sixteen isolates of *C. tamarilloi* (Fig. 5).

Furthermore, the results of the growth rate on GBA medium 28 days after inoculation, indicated that the more aggressive isolates had a higher colony diameter (73–83 mm) than the remaining sixteen isolates analysed (30–52 mm) (data not shown).

Regarding to the relationship between lesion diameter and colony features of all isolates, the cluster analysis confirmed the results of the phylogenetic analysis, where two different clusters were identified in this investigation; the first cluster formed by the more aggressive isolates: Cu-Pi, Mi-Pi, Ta-Pi and Az-Pi; and the second cluster formed by the common pathogenic isolates: Zh-AZ, Ur-Th, Pa-Th, Ma-Ch, Ct-Im, Pr-Th, Mu-Co, Pz-Co, Pa-Az, Lo-Lj, Ch-Bo, Sb-Im, Pi-Pi, Sa-Th, Ur-Im and Sp-Bo (Fig. 6). The cophenetic correlation was 0.90 (90%), indicating that the dendrogram preserved faithfully the pairwise distances between the original un-modeled data points.

Discussion

This study aimed to amplify and clarify the taxonomy and genetic position of the *Colletotrichum* species causing anthracnose of tamarillo in the Ecuadorian highlands by phenotypic, morphologic, pathogenic and multilocus molecular phylogenetic analyses.

The symptomatology observed in the field during sample collection, was consistent with the description of this disease given by Afanador-Kafuri et al. (2003) and Falconí et al. (2013), who mentioned symptoms such as depressed black lesions on fruit accompanied by erupting pink spore masses in lesions (Fig. 1). Pathogenetically, Bailey et al. (1992) mentioned that *Colletotrichum* produces four hydrolytic enzymes (polygalacturonases, pectinases, proteinases and cutinases), which are capable of degrading the carbohydrates and proteins constituents of the pericarp of tamarillo fruit and produce big necrotic lesions.

Fig. 4 A Bayesian inference of multilocus phylogenetic tree of 92 isolates in the *Colletotrichum acutatum* species complex. The tree was built using concatenated sequences of actin (ACT), β -tubulin (TUB2) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes. Bayesian probability values (>0.9) are shown in nodes. Delimitations in boxes indicated the interest clade (Clade 1), infraspecific group belonging to *C. tamarilloi* species and infraspecific group unidentified. Names in bold with asterisk represent the isolates characterised in this study. *Colletotrichum orchidophilum* (strains: CBS 631.80, CBS 632.80 and CBS 119291) sequences were used as outgroups. The scale bar indicates the number of expected changes per site

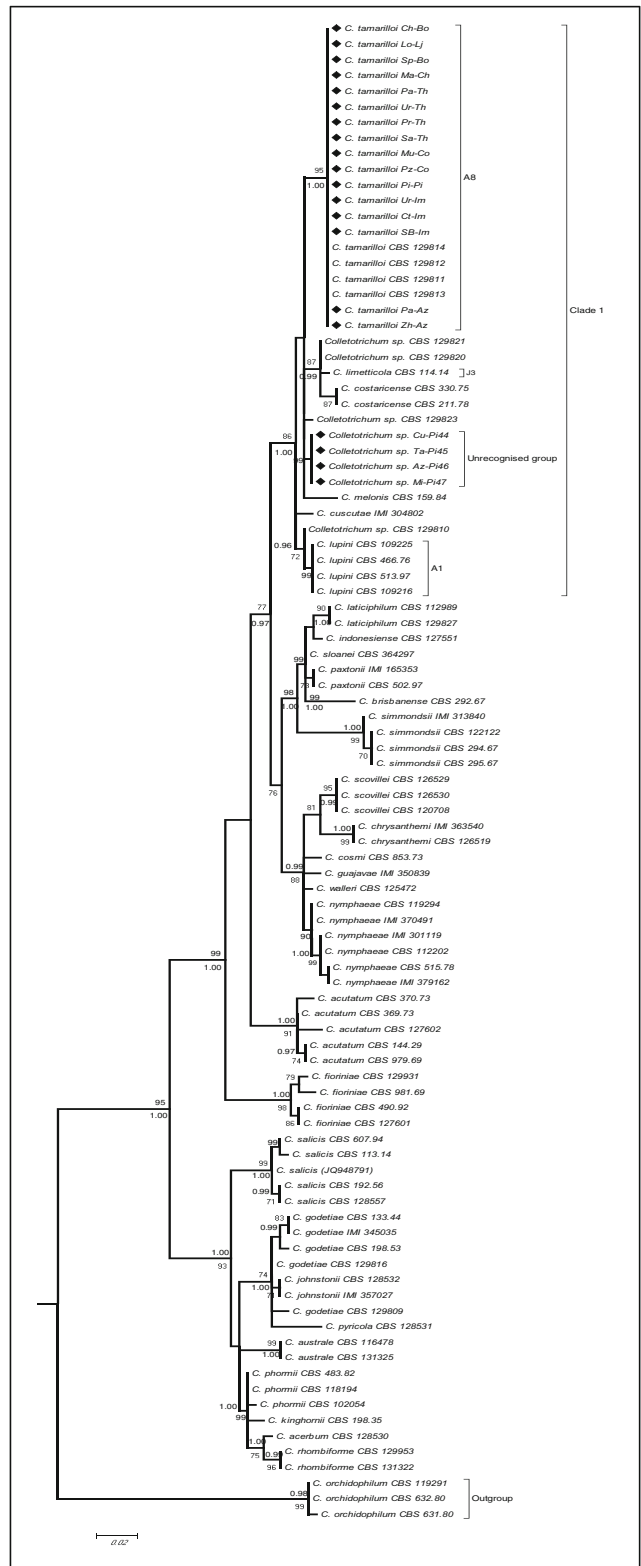




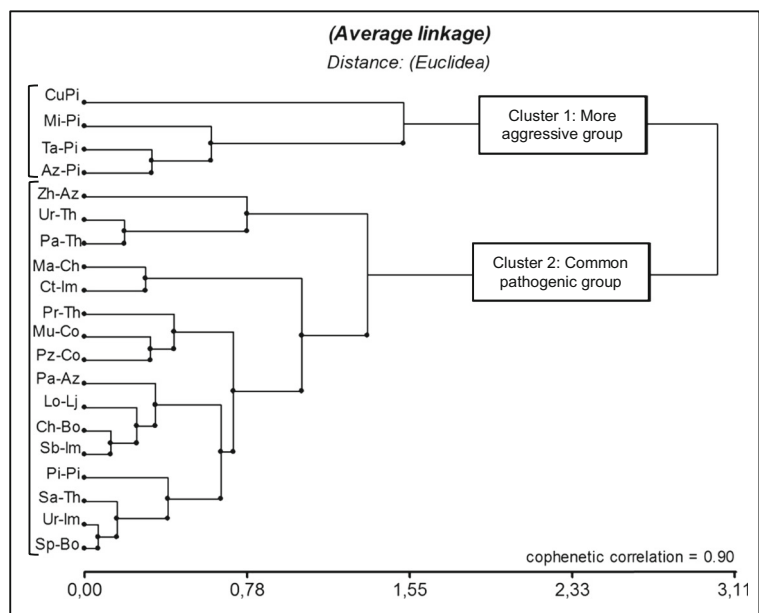
Fig. 5 Tamarillo fruits cv. Gigante común inoculated with twenty *Colletotrichum* isolates. Pictures from 1 to 16 show the common pathogenic group causing anthracnose symptoms; and pictures from 17 to 20 show the more aggressive group causing

anthracnose symptoms, corresponding to the codes Cu-Pi, Mi-Pi, Ta-Pi and Az-Pi. The advance lesions of anthracnose appeared from 18 to 21 days after inoculation

Phenotypic characterisation showed that 75% (15 isolates from Imbabura, Cotopaxi, Tungurahua, Chimborazo, Bolívar, Loja and Azuay provinces) of the colonies evaluated in this study were olivaceous gray in the front and back surface with conidia masses of pink-salmon color, in agreement with the description of Afanador-Kafuri et al. (2003) and Falconí et al. (2013). They mentioned that *C. acutatum* isolates from tamarillo changed in time from white to olivaceous gray with conidia consistently pink-salmon (Fig. 3). Damm et al. (2012) expanded the specific phenotypic colony features for the *Colletotrichum* species that affects tamarillo, such as entire white margin with an olivaceous

gray surface, almost entirely covered by felty-white and with a pale olivaceous. The remaining 25% of the isolates (five isolates from Pichincha province), showed several shades of gray from dark gray, greenish gray, iron gray to white cream (Fig. 3), indicating phenotypic variability around the Ecuadorian highlands. In addition, Nirenberg et al. (2002) mentioned that a possible cause for this phenotypic diversity within the complex is species preferences to specific hosts or geographical regions, and by possible selective pressure due to the continuous systemic fungicide application. Interestingly, in the Pichincha province (in Cuendina, Tambillo, Azcázubi and Minas) tamarillo growers

Fig. 6 Dendrogram based on the diameter of lesion and colony, indicating a cluster 1 constituted for the more aggressive group and a cluster 2 constituted for the common pathogenic isolates. The Euclidean distance and average linkage were used for this analysis



mentioned that disease prevention required constant systemic fungicide applications (every 5 days during winter and every 10 days during summer), to reduce field losses that can reach 100% (pers. Comm.).

Most of the *Colletotrichum* isolates (19 of 20) produced predominantly cylindrical conidia with both ends acute but one isolate (Mu-Co) showed fusiform conidia with both ends acute (Fig. 3). Afanador-Kafuri et al. (2003), used this morphological feature to describe to *C. acutatum* in tamarillo, indicating that the primary diagnostic is the presence of cylindrical or fusiform conidia with acute ends; however, Sreenivasaprasad and Talhinhas (2005), indicated that this characteristic is not absolute nor reliable to identify specific differences within the *C. acutatum* populations. In this investigation, we found a low variation in conidial shape (cylindrical) in the species affecting tamarillo fruits. Buddie et al. (1999) explained that the variation of conidial shape in some circumstances is due to older strains, especially if they have been frequently subcultured, may have conidia that are more variable in appearance than those derived from recent subcultures. Another cause of variation in the shape of conidia is the formation of conidia in acervuli or in the aerial mycelium (Nirenberg et al. 2002). Length and width range of conidia were consistent with those reported by Afanador-Kafuri et al. (2003) and Damm et al. (2012) for *C. acutatum* and *C. tamarilloi*, respectively (Fig. 3). Similarly, most appressoria showed a clavate and clavate-subglobose shape, nevertheless, two isolates (Ur-Th and Lo-Lj) showed appressoria of subglobose shape generated from the vegetative hyphae. According to Du et al. (2005), the appressorial shape can be used for taxonomic studies in *Colletotrichum* species, because if the appressoria are formed directly from conidia, the variation is little and highly conserved within each species. Length and width range of appressoria from this study were consistent with results achieved by Damm et al. (2012) (Fig. 3). According to the same authors, other *Colletotrichum* species belonging to the *C. acutatum* complex such as *C. lupini*, *C. melonis* and *C. costaricense* have conidial shapes and dimensions similar to *C. tamarilloi*. In this investigation, the results from the morphological characterisation does not provided reliable characters for *C. tamarilloi* recognition and seemed to depend on host/origin of the isolate or the growth medium. Phylogenetic analysis of each gene and the multilocus molecular analysis (Fig. 4), indicated that 16 of 20 isolates collected and analysed in this study belonged to *C. tamarilloi* within the *C. acutatum*

complex. This is the first report of this species as the causal agent of anthracnose of tamarillo in Ecuador. Our findings are in agreement with Damm et al. (2012), who suggested that to reliably identify *C. acutatum* species CHS-1 (chitin synthase 1), HIS3, TUB2 or GAPDH genes can be used and most effectively GAPDH gene. Until 2013 previous reports about tamarillo anthracnose in Colombia (Afanador-Kafuri et al. 2003) and Ecuador (Falconi et al. 2013), using phenotypic, morphological and molecular information sequencing the ITS region, indicated that the causal agent of this disease was *C. acutatum*. Nevertheless, Crouch et al. (2009) and Seifert (2009), mentioned that the phylogenetic trees generated with the ITS sequences from *Colletotrichum* isolates, were poorly supported by bootstrap values, and the 10% of the individual isolates were incorrectly placed. The same authors concluded that the ITS sequences may yield unreliable species diagnosis, particularly if sequence similarity alone is the only criterion applied. For the above reason, we decided to use actin, β -tubulin and glyceraldehyde 3-phosphate dehydrogenase genes to study the taxonomic position of *Colletotrichum* isolates obtained from infected tamarillo fruits in the Ecuadorian highlands. Formerly, Sreenivasaprasad and Talhinhas (2005) identified eight distinct molecular groups (A1–A8), based on rDNA ITS and β -tubulin DNA (TUB2) sequences and showed some degree of correlation with the morphological characteristics and varying patterns of host association and geographical distribution were used as basis for and incorporated in the study of Damm et al. (2012), where were named 31 species previously recognized and 21 species have not been recognised. This last information helped to this investigation to compare and confirm the results obtained in the multilocus molecular phylogenetic tree (Fig. 4). Our analysis recognised a new undescribed *Colletotrichum* group, collected in the Pichincha province in the locations corresponding to Cuendina, Tambillo, Azcáubi and Minas. In these locations we observed a high incidence of tamarillo anthracnose (95–100%) plus a constant application of high doses of fungicides (benzimidazole), probably generating *Colletotrichum* resistance to these products with no efficient control of this pathogen in short time periods. León et al. (2004) indicated that the fungicides mostly used to control tamarillo anthracnose in the Ecuadorian highlands were systemic benzimidazole fungicides (benlate and carbendazim). Their mode of action is to inhibit microtubule assembly by binding to the β -subunit of β -tubulin and interfering with microtubule formation during

mitosis cell division. The continuous applications of these products can generate mutations, and new and more virulent *Colletotrichum* isolates (Chung et al. 2006). In this investigation, the transitions observed in the β -tubulin gene (TUB2) (change a guanine by adenine and thymine by cytosine), suggested that the fungi transitions induced by constant fungicide applications can generate highly pathogenic resistant mutants.

Additionally, the differences observed in the multilocus molecular analysis were confirmed by the pathogenicity tests and cluster analysis, where the isolates Cu-Pi, Mi-Pi, Ta-Pi and Az-Pi, showed to be more aggressive and recorded the higher diameter of colony in GBA medium.

In conclusion, using phenotypic, molecular and pathogenic analysis, we described for the first time *C. tamarilloi* as the causal agent of tamarillo anthracnose in Ecuadorian highlands. Furthermore, the remaining four isolates that showed molecular differences (in the introns for three genes), also showed to be more aggressive both in growth rate as necrosis production on fruits than the other sixteen isolates. In the phylogenetic analysis, these four isolates formed a new intraspecific group undescribed previously and a more aggressive group identified by the cluster analysis. Fungicide sensitivity tests using benzimidazoles (belante and carbendazim) are needed to support the results found in this investigation. Additional conserved genes corresponding to histone (HIS3) and chitin synthase 1 (CHS-1) need to be examined to complement molecular characterisation and confirm our findings regarding the previously undescribed intraspecific group. This study can provide guidance for establishing more effective and environmentally friendly control measures for this disease, that in some cases can devastate entire tamarillo plantations.

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