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Leaf anthracnose and defoliation of blueberry caused by *Colletotrichum helleniense* in Northern Italy

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Summary. Highbush blueberry is an increasingly important crop due to its economic value and demonstrated health benefits of blueberries. Leaf spots are considered as minor diseases of blueberry plants, but they adversely affect blueberry productivity, causing reduced photosynthetic activity, flower bud formation and berry production. Surveys of blueberry crops were conducted in Piedmont, Northern Italy, during 2019-2020. Fungi isolated from leaf spots of the blueberry cultivar 'Blue Ribbon' were identified as *Colletotrichum helleniense* through a robust multi-locus phylogeny. Eight genomic loci were considered: *tub*, *gapdh*, *act*, *cal*, *his3*, *chs-1*, *ApMat* and *gs*. Morphological characters of a representative strain were assessed. Pathogenicity was confirmed on four blueberry cultivars, although with different levels of aggressiveness to the cultivars. This study shows the importance of a polyphasic approach to investigate species of *Colletotrichum*, and the relevance of molecular tools for the species-level characterization within the 'Kahawae' clade. This is the first report of *Colletotrichum helleniense* causing leaf anthracnose on *Vaccinium corymbosum*.

Keywords. *Vaccinium corymbosum*, multi-locus typing, berry fruit.

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INTRODUCTION

Highbush blueberry (*Vaccinium corymbosum*, *Ericaceae*) is an increasingly important crop in Italy, with Piedmont, Veneto and Trentino Alto Adige as the major production areas (Brazelton, 2011). During the last decade, new cultivars and modern agricultural practices have been adopted, resulting in consistent improvement in berry production in Italy, with 1.675 tons harvested from 172 ha in 2018 (FAOSTAT 2019). Increasing emphasis on healthy life-styles and the recognition of blueberries as natural functional food have favoured berryfruit consumption and influenced the increased world produc-

tion (Polashock *et al*., 2017; Romo-Muñoz *et al*., 2019). However, this led to increased global movement and trade of plant materials, resulting in the spread of pathogens and emergence of new diseases (Polashock *et al*., 2017; Guarnaccia *et al*., 2020; Liu *et al*., 2020).

Anthracnose is one of the most economically important diseases of blueberry (Retamales and Hancock, 2018), and the most frequent and relevant disease of blueberry fruit is anthracnose caused by *Colletotrichum* spp. However, shoot tip blight and leaf spot caused by the same pathogens are included among the additional symptoms, and could develop during growing seasons. Leaf symptoms could reduce photosynthesis, cause premature defoliation and affect subsequent flower bud formation (Polashock *et al*., 2017). Species of *Colletotrichum* have been reported in association with anthracnose on blueberry leaves in different international regions (Farr and Rossman, 2020). Several *Colletotrichum* spp. are important plant pathogens, affecting a wide range of hosts in different climatic regions (Dean *et al*., 2012). Assessment of morphological features of these fungi has been the common method to classify species within *Colletotrichum*. However, adoption of molecular approaches has allowed profound taxonomic revision of the genus (Weir *et al*., 2012; Cabral *et al*., 2020). Previous studies based on morphological features ascribed the species causing blueberry anthracnose mainly to the *Colletotrichum acutatum* species complex (SC), (*C. acutatum sensu stricto*, *C. fioriniae*, *C. nymphaeae*), *C. gloeosporioides* SC (*C. fructicola*, *C. gloeosporioides sensu stricto*, *C. kahawae*, *C. siamense*), *C. boninense* SC (*C. karstii*) and *C. orchidearum* SC (*C. sichuaninese*) (Hartung *et al*., 1981; Barrau *et al*., 2001; Kim *et al*., 2009; Xu *et al*., 2013; Rios *et al*., 2015; Pszczolkowska *et al*., 2016; Ali *et al*., 2019; Liu *et al*. 2020). Liu *et al*. (2020) reported *Colletotrichum fructicola* as the dominant species affecting blueberry leaves in Sichuan Province, China, followed by *Colletotrichum siamense*, *C. kahawae*, *C. karstii*, *C. nymphaeae* and *C. sichuaninese*. Species of *Colletotrichum gloeosporioides* SC were responsible for blueberry leaf spot in China (Xu *et al*., 2013), Korea (Kim *et al*., 2009), Georgia (Ali *et al*., 2019) and the United States of America (Hartung *et al*., 1981). Species of the *Colletotrichum acutatum* SC were found in association with leaf anthracnose in Australia (Shivas *et al*., 2009; 2016), Canada (Verma *et al*., 2006), China (Xu *et al*., 2013), Japan (Yoshida and Tsukiboshi, 2002; Yoshida *et al*., 2007), Korea (Kim *et al*., 2009), New Zealand (Pennycook, 1989; Gadgil *et al*., 2005), Poland (Pszczółkowska *et al*., 2016), Spain (Barrau *et al*., 2001), The Netherlands (Nirenberg *et al*., 2002) and the United States of America (Guerber *et al*., 2003). Rios *et al*. (2015) reported *Colletotrichum karstii* in association with blueberry leaf spots in Brazil. However, since these species could persist as sources of inoculum in the field, affecting orchards and possibly cross-infecting other nearby crops while switching to a pathogenic lifestyles, specific pathogen identification should be provided (Fuentes-Aragón *et al*., 2020). Currently, molecular data are combined with morphological characters and pathogenicity tests in a polyphasic approach to clearly identify species within the *Colletotrichum* SC (Guarnaccia *et al*., 2019, 2021a; Damm *et al*., 2019).

Leaf anthracnose of blueberry bushes was observed in Piedmont, Northern Italy. The aims of the present study were: i) to characterize fungi isolated from this disease using morphological, molecular and phylogenetic tools, and ii) to test their pathogenicity to fulfil Koch's postulates.

MATERIALS AND METHODS

Field sampling and fungus isolation

Surveys were conducted from March to September 2019 in two blueberry plantations in Cuneo, Piedmont, Northern Italy (Orchard 1: 44°37'39.1"N 7°34'14.1"E; Orchard 2: 44°38'20.3"N 7°31'23.8"E). Anthracnose incidence (DI) was assessed based on the percentage of affected plants. Ten symptomatic leaves were randomly collected from about twenty plants. Portions of symptomatic leaves (5–10 mm) were surface sterilised in 1% sodium hypochlorite for 1 min, rinsed in sterile distilled water (SDW) for 1 min and dried on sterile absorbent paper. Small fragments (2–3 mm) were cut from lesion margins and plated on potato dextrose agar (PDA, Merck) amended with 25 ppm of streptomycin sulphate (PDA-S, Sigma-Aldrich). The plates were incubated at 25 ± 1°C under a 12 h photoperiod. Following 48 to 72 h of incubation, sporulating conidiomata obtained were collected and crushed in drops of sterile water and then spread over the surface of PDA-S plates. After 24 h, germinating spores were individually transferred onto PDA plates. Ten isolates were obtained and two of these were used for molecular characterization (Table 1). Stock cultures are maintained at -80°C at the Agroinnova Centre of Competence, University of Torino, Torino, Italy.

DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing

DNA was extracted from two isolates with an E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek,

Table 1. Collection details of *Colletotrichum* isolates, and GenBank accession numbers of other *Colletotrichum* isolates, included in this study.

Species	Culture No.[1]	Host	Locality	GenBank No. [2]							
				gapdh	*tub*	*act*	*his3*	*calm*	*Ap/MAT*	*gs*	*chs-1*
C. acutatum	CBS 112996 [T]	*Carica papaya*	Australia	JQ948677	JQ005860	JQ005839	JQ005818	-	-	-	-
C. aenigma	ICMP 18608 [T]	*Persea americana*	Israel	JX010044	JX010389	JX009443	-	JX009683	KM360143	JX010078	JX009774
C. aeschynomenes	ICMP 17673 [T]	*Aeschynomene virginica*	USA	JX009930	JX010392	JX009483	-	JX009721	KM360145	JX010081	JX009799
C. alienum	ICMP 12071 [T]	*Malus domestica*	New Zealand	JX010028	JX010411	JX009572	-	JX009654	KM360144	JX010101	JX009882
C. aotearoa	ICMP 18537; C1282.4	*Coprosma* sp.	New Zealand	JX010005	JX010420	JX009564	-	JX009611	HE655663	JX010113	JX009853
C. asianum	ICMP 18580; CBS 130418	*Coffea arabica*	Thailand	JX010053	JX010406	JX009584	KY856305	JX009727	-	JX010096	JX009867
C. camelliae	LC1364; CGMCC3.14925	*Camellia sinensis*	China	KJ954782	KJ955230	KJ954363	-	KJ954634	KJ954497	KJ954932	-
C. chrysophilum	CMM4268[T]	*Musa* sp.	Brazil	KX094183	KX094285	KX093982	-	KX094063	KX094325	KX094204	KX094083
C. cigarro	CBS 112984; ICMP17932; STE-U4445; JT1096	*Banksia* sp.	Portugal	KC296989	KC297082	KC296923	KC297048	KC296944	-	KC297014	KC296966
	ICMP 18534; C1252.12	*Kunzea ericoides*	New Zealand	JX009904	JX010427	JX009473	-	JX009634	HE655657	JX010116	JX009765
	ICMP 18539 [T]; C1262.12	*Olea europaea*	Australia	JX009966	JX010434	JX009523	-	JX009635	HE655658	JX010132	JX009800
C. clidemiae	ICMP 18658; C1317.1	*Clidemia hirta*	USA (Hawai)	JX009989	JX010438	JX009537	-	JX009645	KC888929	JX010129	JX009877
C. conoides	CGMCC3.17615	*Capsicum annuum*	China	KP890168	KP890174	-	-	KP890150	-	JX010128	KP890156
C. cordylinicola	MFLUCC 090551 [T]; ICMP 18579	*Cordyline fructicosa*	Thailand	JX009975	JX010440	HM470235	-	HM470238	-	JX010122	JX009864
C. fructicola	ICMP 18581; CBS 130416[T]	*Coffea arabica*	Thailand	JX010033	JX010405	FJ907426	-	FJ917508	-	JX010095	JX009866
C. fructivorum	CBS 124.22[T]; ICMP19122	*Vaccinium* sp.	USA	JX009950	JX010433	JX009536	-	JX009744	JX145278	JX010134	JX009902
C. gloeosporioides	ICMP 17821; CBS 112999	*Citrus sinensis*	Italy	JX010056	JX010445	JX009531	KY856316	JX009731	-	JX010085	JX009818
C. grevilleae	CBS 132879 [T]	*Grevillea* sp.	Italy	KC297010	KC297102	KC296941	KC297056	KC296963	-	KC297033	KC296987
C. grossum	CGMCC 3.17614 [T]	*Capsicum annuum*	China	KP890159	KP890171	KP890141	-	KP890147	-	-	KP890153
C. hebeiense	JZB330028	*Vitis vinifera*	China	KF377495	KF288975	KF377532	-	-	KF377562	-	KF289008
C. helleniense	CBS 142418; CPC 26844	*Poncirus trifoliata*	Greece	KY856270	KY856528	KY856019	KY856361	KY856099	-	-	KY856186
	CVG628	*Vaccinium corymbosum* 'Blue Ribbon'	Italy	**MW368901**	**MW368899**	**MW368903**	**MW368913**	**MW368905**	**MW368907**	**MW368911**	**MW368909**
	CVG629	*Vaccinium corymbosum* 'Blue Ribbon'	Italy	**MW368902**	**MW368900**	**MW368904**	**MW368914**	**MW368906**	**MW368908**	**MW368912**	**MW368910**
C. henanense	LC3030, CGMCC 3.17354, LF238[T]	*Citrus sinensis*	China	KJ954810	KJ955257	KM023257	-	KJ954662	KJ954524	KJ954960	-
C. horii	NBRC 7478 [T]; ICMP 10492	*Diospyros kaki*	Japan	GQ329681	JX010450	JX009438	-	JX009604	JQ807840	JX010137	JX009752
C. hystricis	CBS 142411; CPC 28153	*Citrus hystrix*	Italy	KY856274	KY856532	KY856023	KY856365	KY856103	-	-	-
C. jiangxiense	CGMCC 3.17363 [T]; LC3463; LF687	*Camellia sinensis*	China	KJ954902	KJ955348	KJ954471	-	KJ954752	KJ954607	KJ955051	-

(Continued)

Table 1. (Continued).

Species	Culture No.[1]	Host	Locality	GenBank No. [2]							
				gapdh	*tub*	*act*	*his3*	*calm*	*Ap/MAT*	*gs*	*chs-1*
C. kahawae	CBS 982.69; ICMP17915	*Coffea arabica*	Angola	JX010040	JX010435	JX009474	-	JX009638	-	JX010125	JX009829
	ICMP 17816; IMI 301220	*Coffea arabica*	Kenya	JX010012	JX010444	JX009452	-	JX009642	JQ894579	JX010130	JX009813
	IMI 301220; ICMP17811	*Coffea arabica*	Malawi	JX009970	JX010430	JX009555	-	JX009641	-	JX010131	JX009817
	IMI 361501; ICMP17905	*Coffea arabica*	Cameroon	JX010046	JX010431	JX009561	-	JX009644	-	JX010127	JX009816
C. musae	ICMP 17817	*Musa sapientum*	Kenya	JX010015	JX010395	JX009432	-	JX009689	-	JX010084	JX009815
C. nupharicola	CBS 470.96; ICMP 18187	*Nuphar lutea* subsp. *polysepala*	USA	JX009972	JX010398	JX009437	-	JX009663	-	JX010088	JX009835
C. proteae	CBS 132882	*Protea* sp.	South Africa	KC297009	KC297101	KC296940	KC297045	KC296960	-	KC297032	KC296986
C. psidii	CBS 145.29 [T]	*Psidium* sp.	Italy	JX009967	JX010443	JX009515	-	JX009743	-	JX010133	JX009901
C. queenslandicum	ICMP 1778 [T]	*Carica papaya*	Australia	JX009934	JX010414	JX009447	-	JX009691	KC888928	JX010104	JX009899
C. rhexiae	CBS 133134; Coll1026	*Rhexia virginica*	USA	-	JX145179	-	-	-	JX145290	-	-
C. salsolae	ICMP 19051	*Salsola tragus*	Hungary	JX009916	JX010403	JX009562	-	JX009696	KC888925	JX010093	JX009863
C. siamense	ICMP 18578, CBS 130417[T]	*Coffea arabica*	Thailand	JX009924	JX010404	FJ907423	-	JX009714	JQ899289	JX010094	JX009865
C. temperatum	CBS 133122; Coll883; BPI884100	*Vaccinium macrocarpon*	USA	-	JX145211	-	-	-	JX145298	-	-
C. theobromicola	ICMP 18649 [T]	*Theobroma cacao*	Panama	JX010006	JX010447	JX009444	-	JX009591	-	JX010139	JX009869
C. ti	ICMP 4832 [T]	*Coryline* sp.	New Zealand	JX009952	JX010442	JX009520	-	JX009649	KM360146	JX010123	JX009898
C. tropicale	CBS 124949 [T]	*Theobroma cacao*	Panama	JX010007	JX010407	JX009489	-	JX009719	-	JX010097	JX009870
C. viniferum	GZAAS 5.08601 [T]	*Vitis vinifera* 'Shuijing'	China	JN412798	JN412813	JN412795	-	-	-	JN412787	-
C. wuxiense	JS1A32; CGMCC 3.17894; JS1A44	*Camellia sinensis*	China	KU252046	KU252200	-	-	KU251834	KU251722	KU252101	KU251940
C. xanthorrhoeae	BRIP 45094, ICMP 17903, CBS 127831[T]	*Xanthorrhoea preissii*	Australia	JX009927	JX010448	JX009478		JX009653	KC790689	JX010138	JX009823

[1]ATCC: American Type Culture Collection, Virginia, USA; BRIP: Biosecurity Queensland Plant Pathology Herbarium, Queensland, Australia; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; CGMCC: China General Microbiological Culture Collection Center, Beijing, China; CMM: Culture Collection of Phytopathogenic Fungi Prof. Maria Menezes, Recife, Pernambuco, Brazil; CVG: Agroinnova, Grugliasco, Torino, Italy; ICMP: International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand; IMI: Culture collection of CABI Europe UK Centre, Egham, UK; LC = working collection of Lei Cai, CAS, China; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; NBRC: NBRC Culture Collection, Chiba, Japan. Ex-type and ex-epitype cultures are indicated with [T].

[2] *tub2*: beta-tubulin gene; *gapdh*: glyceraldehyde-3-phosphate dehydrogenase gene; *act*: actin gene; *his3*: histone3; *calm*: calmodulin; *ApMat*: Apn2-Mat1-2 intergenic spacer and partial mating type (*Mat1-2*); *gs*: glutamine synthetase; *chs-1*: chitin synthase 1. Strains recovered and sequences generated in this study are indicated in bold.

Darmstadt, Germany) from 0.1 g of mycelium grown on PDA, following the minikit manufacturer's instructions. Species identification was achieved by DNA sequencing of a combined dataset of eight genomic loci, including beta-tubulin (*tub2*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), actin (*act*), calmodulin (*cal*), histone3 (*his3*), chitin synthase 1 (*chs-1*), Apn2-Mat1-2 intergenic spacer and partial mating type (*Mat1-2*) (*ApMat*), and glutamine synthetase (*gs*). The primers used for each locus shown in Table 2. The amplification mixtures of PCR and respective cycling conditions of Guarnaccia *et al.* (2017) were used for the *tub2*, *gapdh*, *act*, *cal*, *his3* and *chs-1* regions. The *gs* partial gene and *Mat1-2/ApMat* region were amplified using the PCR protocols of Liu *et al.* (2015). Five µL of PCR product for each PCR reaction was examined by electrophoresis at 100V on 1% agarose (VWR Life Science AMRESCO® biochemicals) gels stained with GelRed™. PCR products were sequenced in both directions by Eurofins Genomics Service. The generated sequences were analysed and consensus sequences were computed using the program Geneious v. 11.1.5 (Auckland, New Zealand).

Phylogenetic analyses

New sequences obtained in this study were blasted against the NCBI's GenBank nucleotide database to determine the closest relatives for a taxonomic framework of the studied isolates. Alignments of different gene regions, including sequences obtained from this study and sequences downloaded from GenBank, were initially performed with the MAFFT v. 7 online server (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh and Standley 2013), and then manually adjusted in MEGA v. 7 (Kumar *et al.*, 2016). The phylogenies were based on Maximum Parsimony (MP) and Bayesian Inference (BI) for the multi-locus analyses. The MP analyses were performed using Phylogenetic Analysis Using Parsimony (PAUP) v.4.0b10 (Swofford, 2003). Phylogenetic relationships were estimated by heuristic searches with 100 random addition sequences. Tree bisection-reconnection was used, with the branch swapping option set on 'best trees', with all characters weighted equally and alignment gaps treated as fifth state. Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC) were calculated for parsimony, and the bootstrap analyses (Hillis and Bull, 1993) were based on 1000 replications. Sequences generated in this study were deposited in GenBank (Table 1). For BI, the best evolutionary model for each partition was determined using MrModeltest v. 2.3 (Nylander, 2004) and incorporated into the analyses. MrBayes v. 3.2.5 (Ronquist *et al.*, 2012) was used to generate phylogenetic trees under optimal criteria per partition. The Markov Chain Monte Carlo (MCMC) analysis used four chains and started from a random tree topology. The heating parameter was set at 0.2 and trees were sampled every 1000 generations. Analyses stopped when the average standard deviation of split frequencies was less than 0.01.

Table 2. Primers used in this study.

Locus	Primer name	Primer sequence 5'->3'	Reference
tub	T1	AACATGCGTGAGATTGTAAGT	Glass and Donaldson, 1995
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	O'Donnell and Cigelnik, 1997
gapdh	GDF1	GCCGTCAACGACCCCTTCATTGA	Guerber *et al.* 2003
	GDR1	GGGTGGAGTCGTACTTGAGCATGT	
act	ACT-512F	ATGTGCAAGGCCGGTTTCGC	Carbone and Kohn, 1999
	ACT-783R	TAGGAGTCCTTCTGACCCAT	
cal	CL1	GARTWCAAGGAGGCCTTCTC	O'Donnell *et al.*, 2000
	CL2	TTTTTGCATCATGACCCTTGGC	
his3	CYLH3F	GCAACATCTCGTCCGCTCT	Crous *et al.*, 2004
	CYLH3R	AGCTGGATGTCCTTGGACTG	
chs	CHS-79F	TGGGGCAAGGATGCCTGGAAGAAG	Carbone and Kohn, 1999
	CHS-345R	TGGAAGAACCATCTGTGGGAGTTG	
ApMat	AMF1	TCATTCTACGTATGTGCCCG	Silva *et al.*, 2012
	AMR1	CCAGAAATACACCGAACTTGC	
gs	GSF1	ATGGCCGATACATCTGG	Stephenson *et al.*, 1997
	GSR1	GAACCGTCGAAGTTCCAC	

Isolate morphology

Agar plugs (5 mm diam.) of the strains CVG628 and CVG629 were taken from the edges of actively growing cultures on PDA-S and were transferred onto the centres of Petri dishes (9 cm diam.) containing PDA, then incubated at 25 ± 1°C under 12 h photoperiod for 7 d. Three cultures plates of each strain were investigated. Colony characters and diameters were observed/measured after 7 d. Cultures were examined over time for development of ascomata, conidiomata and setae. The morphological characteristics of the fungi were examined by mounting structures in water, and 30 measurements at 400× magnification were determined for each isolate using a microscope (Nikon Eclipse 55i).

Pathogenicity testing

The pathogenicity of a representative strain CVG629 was tested on detached leaves of each of the four blueberry cultivars 'Blue Ribbon', 'Duke', 'Last Call' and 'Top Shelf'. Thirty leaves of each cultivar were inoculated and 30 leaves were used as controls. Each leaf was disinfested in a 1% sodium hypochlorite solution for 60s followed by three washes of 60s in sterile distilled water and blot dried on sterile absorbent paper before inoculation. The leaves were placed in plastic plates (12.5 × 12.5 × 1.5 cm [length × width × height]) with wet sterile absorbent paper to create a humid chambers. Each leaf was slightly wounded with a sterile needle and then inoculated with 10 µL of a conidium suspension (10^6 conidia mL^{-1}) obtained from a 10d-old culture of the representative isolate grown on PDA. Sterile distilled water was used for the control leaves. The plates were incubated at 25 ± 1°C under 12 h photoperiod for 7 days. The strains CVG628 and CVG629 were also tested *in planta* on attached leaves of the same cultivars. A total of 20 mL of a conidium suspension, obtained as described before, was sprayed on wounded and unwounded leaves. Six plants of each cultivar were used, and three of these were wounded with a sterile needle. A further six plants were similarly treated but spayed with sterile water as experimental controls. The plants were covered with a transparent plastic film to keep high relative humidity, and were transferred to a growth chamber and kept at 25°C with a 12 h photoperiod. The plastic film was removed 3 d post inoculation (dpi). Each trial was repeated once. Data of the replications of the repeated experiments were pooled and analysed together for each trial. Disease severity was evaluated 7 dpi for the trial on detached leaves or 10 dpi for the attached leaves, using a diagrammatic scale for percentage of infected leaf area (Gullino *et al.*, 2017). The scale was: symptomless leaf (0); up to 5% infected leaf area (1); 6 to 10% (2); 11 to 25% (3); 26 to 50% (4); and 51 to 100% (5). Disease severity (DS) was calculated as $DS = \Sigma(\text{no. of leaves} \times X_{0-5})/(\text{no. of leaves})$, where X_{0-5} refers to an approximate class midpoint as follows: $X_0 = 0$; $X_1 = 3\%$; $X_2 = 8\%$; $X_3 = 18\%$; $X_4 = 38\%$; $X_5 = 76\%$. Because no normal distribution was observed, the data were analysed using Kruskal-Wallis non-parametric test (at $P = 0.05$) to determine significant differences among the tested cultivars. The data analyses were conducted using SPSS software 26 (IBM Corporate). The identity of the re-isolated strains was confirmed through sequencing the *gapdh* and *gs* loci, to fulfil Koch's postulates.

RESULTS

Field sampling and fungus isolation

Symptoms identified as those typically caused by *Colletotrichum* spp. were found in two blueberry plantations of the cultivars 'Blue Ribbon' and 'Last Call'. Disease incidence was assessed at 20%, considering the percentage of affected plants. The symptoms observed consisted of brown-to-black, necrotic lesions occurring on mature leaves grown on 1-year-old twigs (Figure 1). Stem defoliation occurred. Based on colony aspect and growth, and conidial morphology characteristics, the fungal strains isolated from symptomatic leaves collected during the surveys were ascribed to *Colletotrichum* spp. and pure fungal cultures were obtained.

Phylogenetic analyses

The combined locus phylogeny of *Colletotrichum* consisted of 46 sequences. A total of 3749 characters (*tub2*: 1–510, *gapdh*: 517–795, *act*: 802–1030, *cal*: 1037–1655, *his3*: 1662–2040, *chs-1*: 2047–2250, *ApMat*: 2257–2927 and *gs*: 2934–3749) were included in the phylogenetic analyses. A maximum of 1000 equally most parsimonious trees were saved, and characteristics of the combined gene partitions used for each phylogenetic analysis are reported in Table 3. Bootstrap support values from the parsimony analysis were plotted on the Bayesian phylogenies (Figure 2). For the Bayesian analyses, MrModel test recommended the models in Table 3. Unique site patterns for each partition and all the parameters of the Bayesian analyses are reported in Table 3. In the generated phylogenetic tree, the strains isolated from *Vaccinium* plants clustered with the ex-type reference strain of *C. helleniense*, within the 'Kaha-

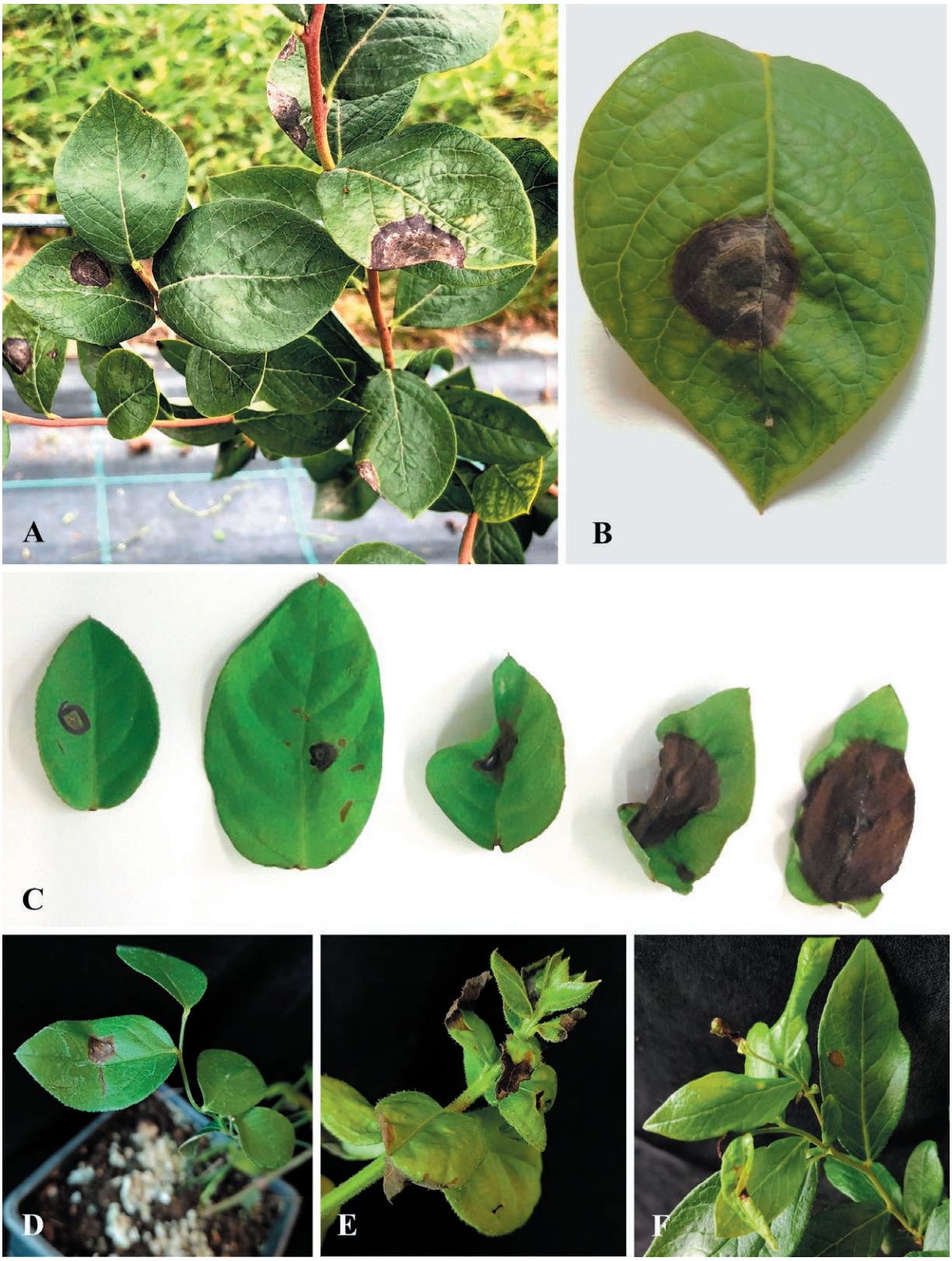

Figure 1. Leaf anthracnose (A and B) of *Vaccinium corymbosum* 'Blue Ribbon' in the field, and leaves of 'Last Call' at different necrotic stages after inoculation (C). Anthracnose developed after inoculations on 'Last Call' wounded (D and F) and unwounded (E) attached leaves.

Table 3. Maximum Parsimony, evolutionary models and Bayesian analysis characteristics of this study.

Parsimony analysis	Total sites	Constant sites	Variable sites	Parsimony informative sites	Tree length	Consistency index	Retention index	Rescaled consistence index	-	-	-
	3707	2066	973	668	2634	0.784	0.865	0.678	-	-	-
Evolutionary model	*tub2*	*gapdh*	*Act*	*cal*	*his3*	*chs-1*	*ApMat*	*gs*	-	-	-
	SYM+G	K80	HKY	SYM+G	HKY+G	K80+G	K80+G	GTR+G	-	-	-
Bayesian analysis	U.S.P.[1] *tub2*	U.S.P. *gapdh*	U.S.P. *act*	U.S.P. *cal*	U.S.P. *his3*	U.S.P. chs-1	U.S.P. *ApMat*	U.S.P. *gs*	Generation ran	Generated trees	Sampled trees
	156	188	97	130	52	42	269	296	8.315.000	8316	6237

[1] U.S.P.: Unique Site Patterns.

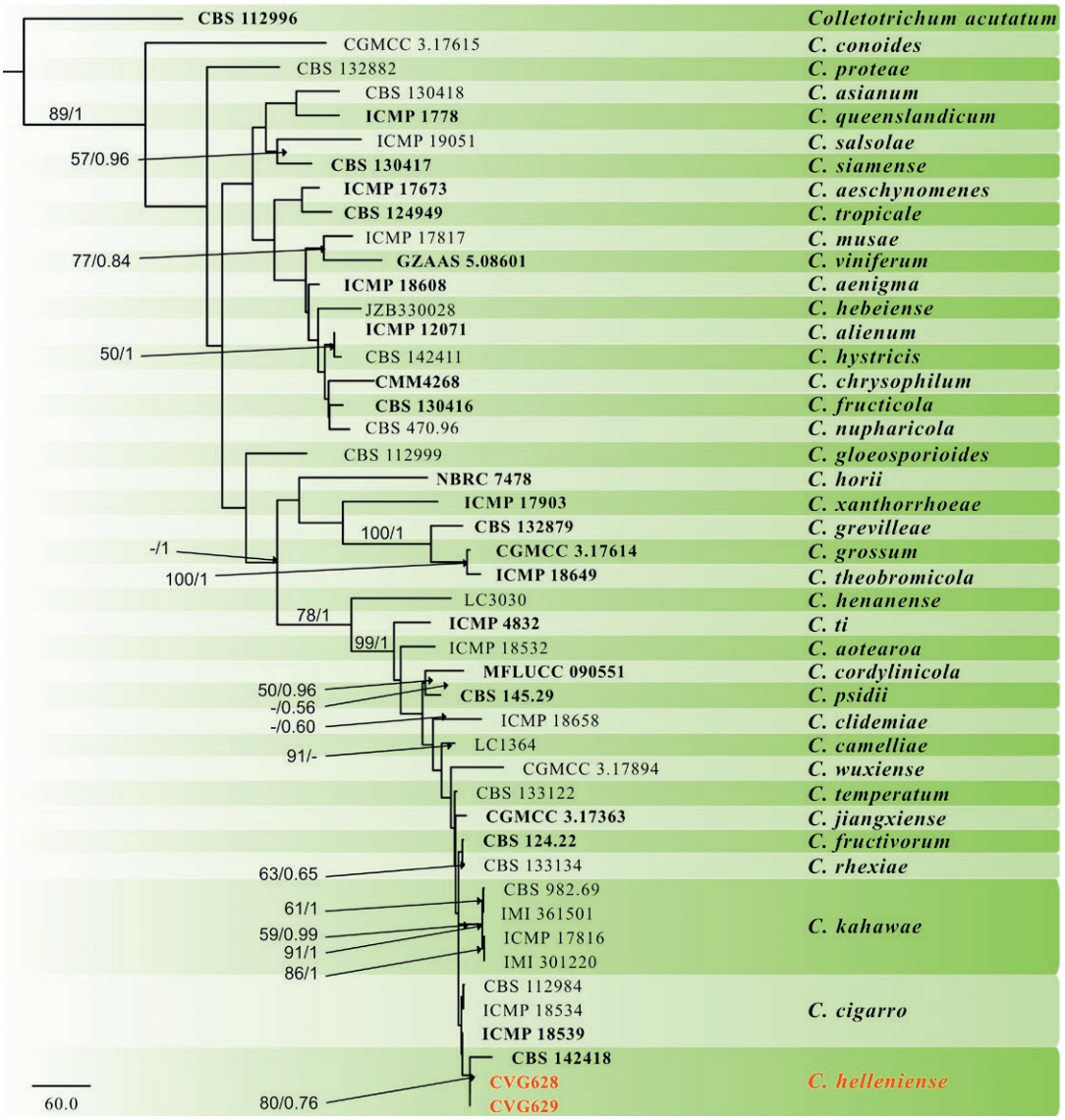

Figure 2. Maximum parsimony (MP) best-tree phylograms obtained from 46 *Colletotrichum gloeosporioides* species complex strains. Numbers on the nodes are MP bootstrap and Bayesian posterior probability values. Isolates obtained from *Vaccinium* in the present study are indicated in red font. Ex-type strains are indicated in bold. The tree was rooted to *Colletotrichum acutatum* (CBS 112996).

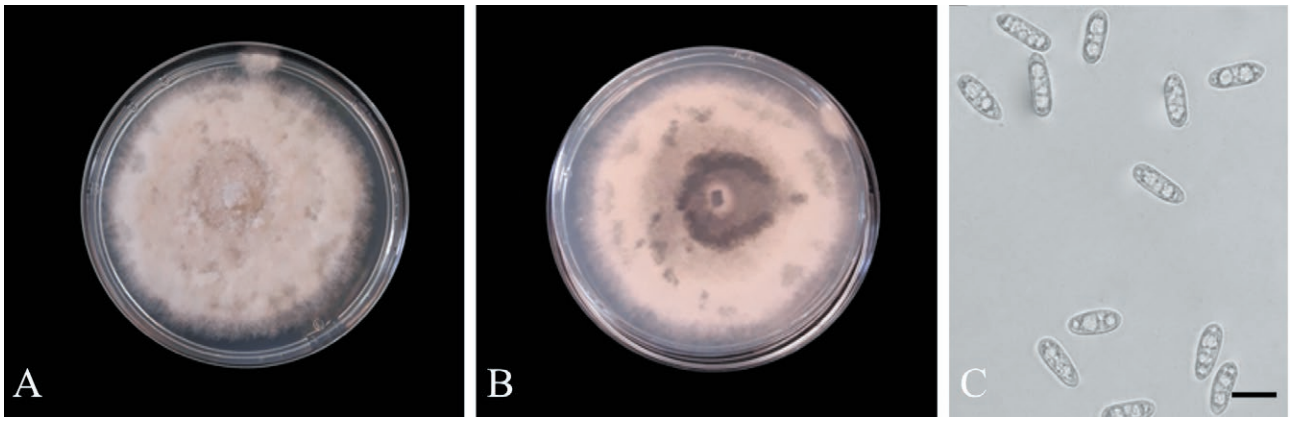

Figure 3. Front (A) and reverse (B) sides of a *Colletotrichum helleniense* colony (CVG629 strain) grown for 7 d on PDA, and conidia (C). Scale bar = 10 µm.

wae' clade and closely related with *C. cigarro*, *C. fructivorum*, *C. jiangxiense*, *C. kahawae*, *C. rhexiae* and *C. temperatum*.

Morphology

Morphological observations, supported by phylogenetic inference, were used to describe the representative strain of *Colletotrichum helleniense* (Figure 3).

Asexual morph on PDA: Conidiomata acervular. Hyphae hyaline, septate and branched. Setae not observed. Conidia hyaline, aseptate, cylindrical with rounded apices and bases, guttulate, mean dimensions 12.2 ± 1.4 × 5.0 ± 0.5 µm for the strain CVG628 and 13.5 ± 1.5 × 4.5 ± 0.5 µm for CVG629. Colonies on PDA with entire margins, grey in the centres and white to pale buff at the margins, entirely covered with floccose to dense, white to grey aerial mycelium, and with black conidiomata. Conidia present in pinkish-salmon-orange masses. Reverse of colonies grey to pale luteous, mean diam. after 7 d = 80 mm for strain CVG628 and 79 mm for CVG629.

Pathogenicity tests

The strains were pathogenic when inoculated on detached leaves, and on wounded and unwounded attached leaves of all the blueberry cultivars tested, although with different levels of aggressiveness (Figures 4 and 5). Symptoms on the leaves consisted of dark brown necrotic spots expanding circularly from the point of inoculation. The pathogenic strains were re-isolated from inoculated leaves, and were identified as described above by blast analysis of the *gapdh* and *gs* loci. This fulfilled Koch's postulates for the two assessed

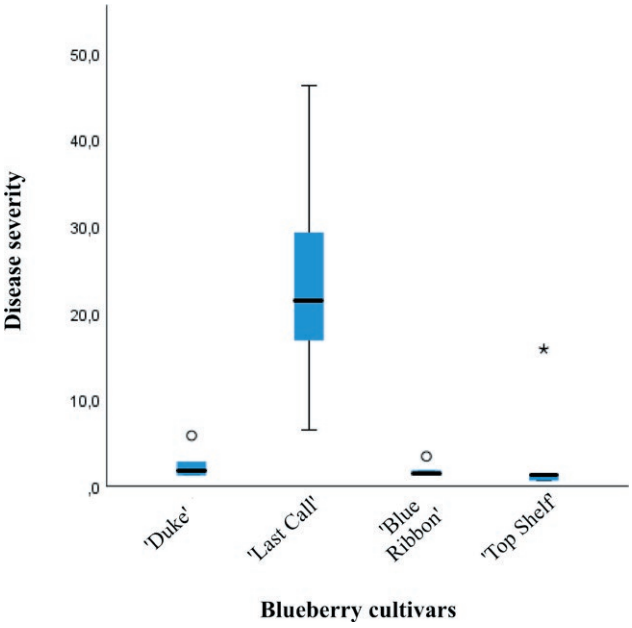

Figure 4. Box plot of results of pathogenicity tests on detached blueberry leaves. Boxes represent the interquartile range, and the horizontal line within each box indicates the average value. The Kruskal-Wallis test was used to compare the mean leaf areas infected after inoculations of four cultivars, and significant differences were accepted at $P < 0.05$.

strains. In test with detached leaves, the pairwise comparison obtained from the Kruskal-Wallis test showed differences ($P < 0.05$) in susceptibility between 'Last Call' and the other three cultivars (Supplementary Table 1). No differences ($P > 0.05$) were observed among 'Blue Ribbon', 'Duke' and 'Top Shelf'. In the test with attached leaves, the pairwise comparison showed differences ($P < 0.05$) only between 'Blue Ribbon' and 'Top Shelf' and

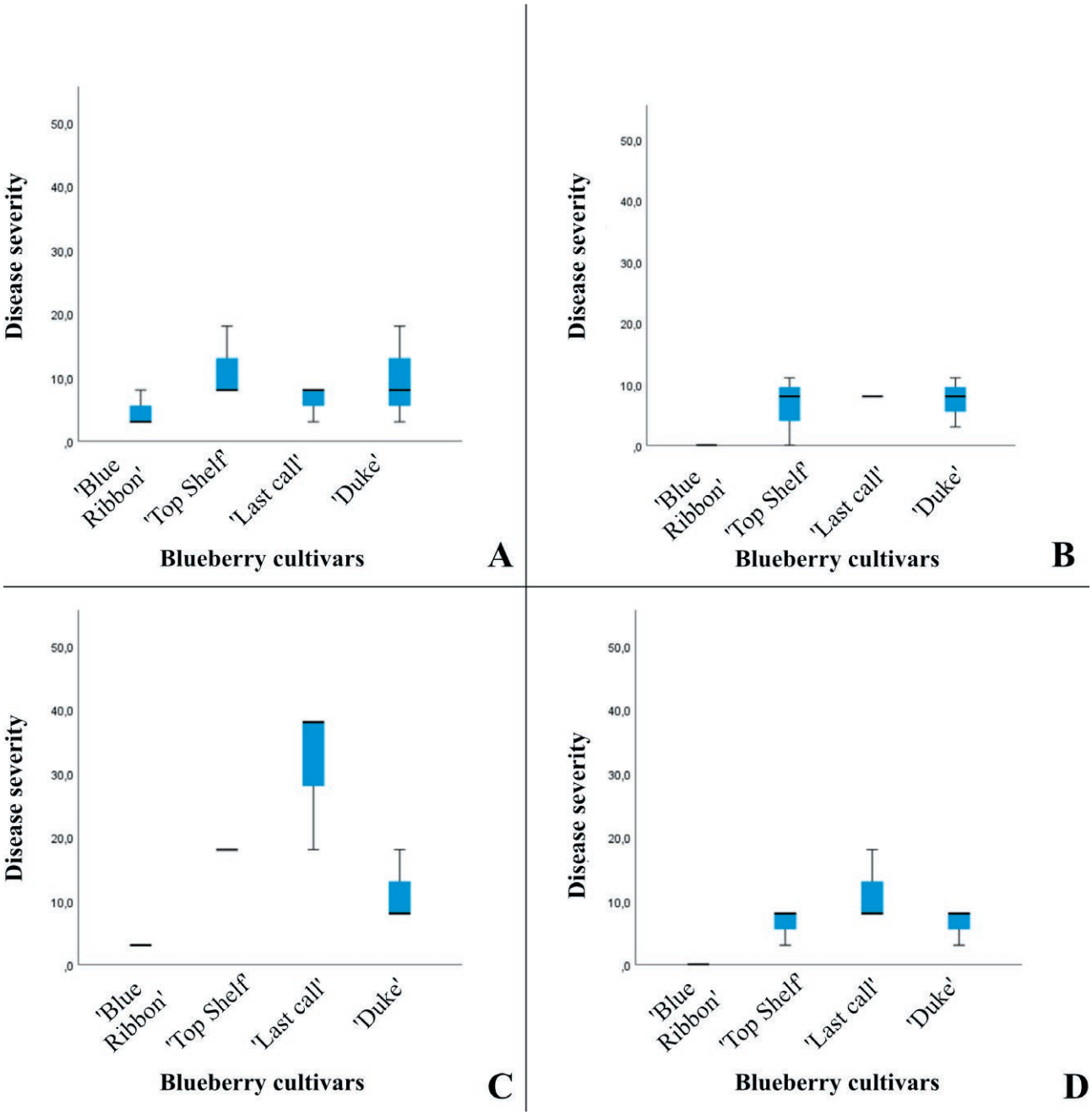

Figure 5. Box plots showing the results of the pathogenicity tests on wounded attached blueberry leaves (A with strain CVG628 and C with CVG629), and on unwounded attached leaves (B with strain CVG628 and D with CVG629). Boxes represent the interquartile range, and the horizontal line within each box indicates the average value. The Kruskal-Wallis test was carried used to compare the mean leaf areas infected after inoculations on four cultivars, and significant differences were accepted at $P < 0.05$.

'Last Call', and between 'Duke' and 'Last Call', and this was only for leaves wounded and inoculated with the strain CVG629 (Supplementary Table 2). The strain CVG628 gave high severity on 'Top Shelf', 'Last Call' and 'Duke' with no difference between wounded and unwounded leaves. The strain CVG629 caused weak symptoms on wounded and unwounded 'Blue Ribbon' leaves, but very severe symptoms on wounded 'Last Call' leaves. No symptoms were observed on control leaves in the detached or attached leaf pathogenicity assays.

DISCUSSION

Different *Colletotrichum* spp. have been reported in association with anthracnose diseases on several fruit crops, where pre- and post-harvest fruit rots are the most common symptoms observed and the most investigated diseases (Dean *et al*., 2012). However, leaf spots also represent a serious threat to fruit production (Benjamin, 2018; Guarnaccia *et al*., 2021b). Necrotic leaf lesions caused by *Colletotrichum* have been reported on blueberry plants, resulting in defoliation and reduced berry yields (Retamales and Hancock, 2018).

The present study is the first investigation of blueberry anthracnose conducted in the major production area of Italy. Since previous *Colletotrichum* studies were based on morphological characterization and ITS region analyses, which were insufficient for species identification (Cai *et al*., 2009), eight different genomic loci have been considered to provide robust multi-locus phylogenies and are useful tools for analyses of target loci for *Colletotrichum* spp. classification. The fungus strains isolated in the present study were identified as *Colletotrichum helleniense*, a species in the *C. kahawae* clade within the *C. gloeosporioides* SC. *Colletotrichum helleniense* has been described by Guarnaccia *et al.* (2017) in association with *Citrus reticulata* fruit lesions and with *Poncirus trifoliata* whither-tip twigs in Greece. The different reported hosts of *C. helleniense* confirms the pathogen's broad host range. This potential cross-infection ability combined with the complex life cycles of *Colletotrichum* spp. increases the difficulties for managing anthracnose diseases (De Silva *et al*., 2017). Cabral *et al.* (2020) provided taxonomic clarification within the 'Kahawae' clade to distinguish *C. kahawae* from *C. cigarro comb. et stat. nov*. Pathological, morphological, cytogenomic and biochemical data were combined with analysis of a nine-locus concatenated dataset including the *ApMat* and *gs* regions. These genomic regions, reported by Cabral *et al.* (2020) as useful to identify *Colletotrichum* spp. within the *C. gloeosporioides* SC, were considered to analyse the isolated *Colletotrichum* spp. investigated in the present study. This is the first report of *Colletotrichum helleniense* on *Vaccinium corymbosum* in Italy, as well as worldwide. However, further studies are required to provide insight on the species members of the 'Kahawae' clade based on genome sequencing, and to ascertain differentiation between *C. cigarro* and *C. helleniense*.

The pathogenicity tests reported here have confirmed the aggressiveness of *C. helleniense,* although the four assessed blueberry cultivars showed different susceptibilities to leaf anthracnose. *Colletotrichum* infections usually start with conidium germination and appressorium production on host tissues. However, direct penetration through stomata or wounds with no appressoria has also been reported, for example, during the infection process of mulberry leaves by *C. gloeosporioides* (De Silva *et al*., 2017). The present study demonstrates the ability of *C. helleniense* to cause anthracnose with and without wounds on attached blueberry leaves. All the cultivars tested were susceptible to the pathogen. However, 'Blue Ribbon' was generally less susceptible, while 'Last Call' developed more severe symptoms, confirming the results obtained from the inoculations on detached leaves. Different climate factors such as hail, rain and freezing, common in the surveyed area of Italy, can induce lesion development on leaves and are abiotic stress factors having potential roles in the disease development. Further studies should be conducted to assess the roles of climate factors and environment conditions on blueberry anthracnose. The pathogenicity results obtained in the present study need to be integrated with further experiments conducted at different temperatures and humidities.

After a first investigation of wood diseases and losses of production caused by several fungal pathogens (Guarnaccia *et al*., 2020), the present study provides new insights on the phytosanitary status of blueberry crops in Northern Italy. Considering the economic relevance of blueberry in this area, further research is required to determine the possible roles of other fungi, the epidemiology of the involved pathogens, and their responses to the currently adopted disease management protocols. This research should aim to establish new effective and sustainable disease management strategies for blueberry crops.

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