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Fungal pathogens associated with stem blight and dieback of blueberry in northern Italy

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Summary. *Vaccinium* spp. are cultivated worldwide due to their important commercial value and fruit health benefits. However, the increasing global trade of berries and plants has resulted in major incidence of the diseases related to this crop. Stem blight and dieback associated with different fungal pathogens are the most common symptoms observed, and represent serious threats to blueberry production. Surveys were conducted in highbush blueberry orchards in Cuneo province, Northern Italy, to assess the fungal species diversity associated with stem blight and dieback. A total of 38 isolates were collected from symptomatic plants of the cultivars 'Last Call', 'Blue Ribbon' and 'Top Shelf'. Four fungal species were identified through multi-locus typing and morphological characters: *Neofusicoccum parvum*, *Diaporthe rudis*, *Cadophora luteo-olivacea* and *Peroneutypa scoparia*. Molecular analyses included three different genomic regions: ITS, *tub2*, and *tef1*. Pathogenicity tests showed that all four species were pathogenic to blueberry plants. *Neofusicoccum parvum* was the most aggressive species. The present study increases understanding of the fungi associated with blueberry stem blight and dieback, providing preliminary knowledge for further studies on disease epidemiology and management strategies. This is the first report worldwide of *P. scoparia* and *C. luteo-olivacea* on *Vaccinium corymbosum*, as well as the first report of *D. rudis* on blueberry in Italy.

Keywords. *Vaccinium corymbosum,* multi-locus typing, *Neofusicoccum*, *Diaporthe*, *Cadophora*, *Peroneutypa*.

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INTRODUCTION

Cultivated highbush blueberry (*Vaccinium corymbosum* L., *Ericaceae*) is a woody deciduous shrub native to North America. Blueberry is an economically important crop, grown in Argentina, Australia, Canada, Chile, China, Europe, Mexico, Morocco, New Zealand, Peru, and USA (FAOSTAT 2019). In Europe, the first plantation of this crop was reported in The Netherlands,

and this region is currently one of the major producers together with Spain, Portugal, Poland, Germany, UK, France and Italy (Retamales and Hancock, 2018). In Italy, blueberry production has been considerably increased since 2010 to 2018, due to adoption of modern agricultural practices and new cultivars able to adapt to diverse pedoclimatic conditions. Total annual production in Italy is 1,675 tonnes of berries (FAOSTAT 2019). The most important blueberry production areas in this country are Trentino Alto Adige, Veneto and Piedmont (Brazelton, 2011). In total, 1,143 ha are cultivated with blueberry, and more than half of this amount (633 ha) are in Piedmont (FAOSTAT 2019).

The berries are highly considered in the food and pharmaceutical industries due to their content of beneficial nutrients (Nestby *et al*., 2011) and bioactive compounds such as antioxidants (Norberto *et al*., 2013). Thus, this crop has received high attention (Ma *et al*., 2018) leading to intercontinental movement of plant material associated with human activities and global changes, resulting in an increased spread of diseases in new cultivation areas (Polashock *et al*., 2017; Hilário *et al*., 2020a,b).

The most common disease symptoms observed in blueberry plantations consist of stem blight and dieback, associated with the presence of fungal pathogens (Lombard *et al*., 2014; Pérez *et al*., 2014; Xu *et al*., 2015; Cardinaals *et al*., 2018; Tennakoon *et al*., 2018; Scarlett *et al*., 2019; Hilário *et al*., 2020b), which limit the longevity of blueberry plants and reduce their fruit yields (Elfar *et al*., 2013). These blight and dieback symptoms have been associated with several fungal species belonging to *Botryosphaeria, Diaporthe*, *Neofusicoccum, Neopestalotiopsis,* and *Phoma*-like genera (McDonald and Eskalen, 2011; Lombard *et al*., 2014; Pérez *et al.,* 2014; Xu *et al*., 2015; Cardinaals *et al*., 2018; Tennakoon *et al*., 2018; Scarlett *et al*., 2019; Hilário *et al*., 2020b).

Blueberry stem blight and dieback can be divided into three groups: Botryosphaeria, Fusicoccum and Phomopsis cankers. Botryosphaeria stem canker is mainly related to *Botryosphaeria corticis*, while *B. dothidea* is commonly known as the causal agent of Botryosphaeria stem blight (Polashock *et al*., 2017). However, among the *Botryosphaeriaceae*, different species have been reported in association with highbush blueberry stem cankers and dieback. These include *Botryosphaeria corticis, B. dothidea*, *Diplodia seriata, Lasiodiplodia theobromae*, *Neofusicoccum arbuti, N .australe, N. luteum, N. nonquaesitum*, *N. parvum,* and *N. ribis* (Phillips *et al*., 2006; Wright and Harmon, 2010; Pérez *et al*., 2014; Xu *et al*., 2015). Fusicoccum canker, also known as Godronia canker, is caused by *Godronia cassandrae* f. sp. *vaccinii* (synonym *Fusicoccum putrefaciens*), which has been reported to cause severe losses in North America and Europe (Strømeng and Stensvand, 2011).

Phomopsis canker is caused by *Phomopsis vaccinii*, known as *Diaporthe vaccinii* after the abolition of the dual nomenclature (Schoch *et a*l., 2012; Lombard *et al*., 2014). In Europe, *D. vaccinii* has been reported in Lithuania, Romania, United Kingdom, Latvia and the Netherlands and is included in the EPPO A2 list (Lombard *et al*., 2014; Cardinaals *et al*., 2018). However, several other *Diaporthe* spp. have been detected on blueberry bushes, causing twig blight, stem cankers and fruit rot. *Diaporthe eres* has been reported in Croatia (Ivić *et al*., 2018), and in the Netherlands with *D. rudis* (Lombard *et al*., 2014), while *D. ambigua*, *D. australafricana*, *D. foeniculina* and *D. passiflorae* have been found in Chile (Latorre *et al*., 2012; Elfar *et al*., 2013). Lombard *et al.* (2014) described *D. asheicola*, *D. baccae*, and *D. sterilis* as new species associated with wood diseases of blueberry plants from Chile and Italy.

Fungal species in *Botryosphaeriaceae* and *Diaporthe* are well known as endophytic, latent and woody pathogens on a wide range of host plants (Xu *et al*., 2015; Marsberg *et al*., 2017; Marin-Felix *et al*., 2017). The endophytic phase could persist until stressful conditions for plant growth arise, after which symptoms can occur (Slippers and Wingfield, 2007). Due to this behaviour, these pathogens are often not detected by quarantine systems (Marsberg *et al*., 2017). Different fungal species could also coexist on the same host, so disease diagnoses based on symptoms or on fungal morphological traits are not reliable (Michalecka *et al*., 2017). A multigene approach to phylogenetic analyses is needed to obtain a high level of confidence in pathogen identifications (Elfar *et al*., 2013; Lombard *et al*., 2014).

During the spring of 2019, stem blight and dieback symptoms were observed in several blueberry orchards in Piedmont, Northern Italy. The present study aimed to: (i) assess the fungal species diversity associated with stem blight and dieback using molecular and phylogenetic tools; (ii) establish morphological analyses of the species identified and determine the average growth rate at different temperatures; and (iii) test the pathogenicity of the species obtained to compare their virulence and to fulfil Koch's postulates.

MATERIALS AND METHODS

Field sampling and fungal isolation

Surveys were carried out from March to September 2019, in four blueberry plantations in Lagnasco (CN),

Piedmont, Northern Italy. Samples from symptomatic plants of 'Last Call', 'Blue Ribbon' and 'Top Shelf' were collected from wilted stems and branches and canker lesions. Disease incidence (DI) was assessed on approx. 60 plants in each of the four orchards in June, and the average percentage of symptomatic and dead plants was determined. Ten symptomatic plants were collected from each orchard. All the investigated plantations were covered by anti-hail nets, and represented high relative humidity levels up to 78%. The wood samples (5–10 mm) were surface sterilised in 70% ethanol for 1 min, rinsed in sterile distilled water (SDW) for 1 min, and then dried on sterile absorbent paper. Small fragments (2–3 mm) were cut from the edges of healthy and necrotic tissues and plated on potato dextrose agar (PDA, Oxoid) amended with 100 μg mL^{-1} 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, mycelium plugs from the margins of resulting colonies were placed on new PDA plates. After 5 d, pure cultures were established from single hyphal tip transfers.

A total of 38 isolates were obtained and used for molecular characterization (Table 1). Stock cultures of these isolates are kept at -80°C in the AGROINNOVA Centre of Competence (University of Torino) culture collection, Torino, Italy.

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

For all fungal isolates, total DNA was extracted from 0.1 g of mycelium grown on PDA, using the E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek), following the manufacturer's instructions. Species identification was achieved through DNA amplification and sequencing of a combined dataset of genes: the nuclear ribosomal internal transcribed spacer (ITS) region, and partial regions of the β-tubulin (*tub2*) and translation elongation factor-1α (*tef1*) genes. ITS region of each isolate was amplified using the universal primers ITS1 and ITS4 (White *et al*., 1990). The primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) were used to amplify part of the *tef1* gene in isolates identified as *Neofusicoccum* spp. (Marin-Felix *et al*., 2017), *Diaporthe* spp., and *Cadophora* spp. (Marin-Felix *et al*., 2019). The primers T1 and Bt2b (Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997) were used to amplify the partial *tub2* gene in isolates identified as *Neofusicoccum* spp. (Marin-Felix *et al*., 2017), *Diaporthe* spp. (Guarnaccia *et al*., 2018; Marin-Felix *et al*., 2019), and *Peroneutypa* spp. (Carmarán *et al*., 2006). The PCR amplification mixtures and cycling conditions adopted for all three loci were followed as described in each of the cited references (above). An amount of 5 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 GelRedTM. PCR products were sequenced in both directions by Eurofins Genomics Service (Ebersberg). The DNA sequences generated 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).

An initial phylogenetic analysis was conducted using 38 ITS sequences of isolates collected in this study and 38 reference strains deposited in GenBank (Table 1), to give an overview of genus identification. The analysis included sequences from 76 isolates spanning the different genera selected on the preliminary results provided by BLAST analysis and one outgroup taxon (*Colletotrichum gloeosporioides* ICMP 17821). To then distinguish the isolates at species level, a subset of representative isolates was selected based on the results of the overview ITS analysis, and was processed through different phylogenetic analyses conducted individually for each locus (data not shown) and as multilocus sequence analyses using the following locus combinations: ITS, *tef1* and *tub2* for members of *Neofusicoccum* and *Diaporthe* (Marin-Felix *et al*., 2017; 2019); ITS and *tub2* for members of *Peroneutypa* and close species of the *Diatrypaceae* (Trouillas *et al*., 2010; 2011); and ITS and *tef1* for isolates related to *Cadophora* (Travadon *et al*., 2015). The ex-type strain of *Diplodia seriata* (CBS 110875; Marin-Felix *et al*., 2017) was used as the outgroup for the analysis of *Neofusicoccum* spp., and *Diaporthella corilina* (CBS 121124; Marin-Felix *et al*., 2019) was used as outgroup for *Diaporthe* spp. *Liberomyces pistaciae* (CBS 144225; Vitale *et al*., 2018), was used as the outgroup for *Diatrypaceae*, and *Neofusicoccum parvum* (CBS 110301; Travadon *et al*., 2015) were used as the outgroup for *Cadophora* spp. The phylogenies were based on Bayesian

Table 1. Collection details and GenBank accession numbers of isolates included in this study.

Species	Isolate code[1]	Country	Host	Genbank accession number[2]		
				ITS	*tub2*	*tef1*
Cadophora fastigiata	CBS 307.49^T	Sweden	-	AY249073	KM497131	KM497087
Cadophora finlandica	CBS 444.86^T	Finland	-	AF486119	KM497130	KM497086
Cadophora luteo-olivacea	CBS 128571	Spain	*Vitis vinifera*	HQ661085	-	HQ661070
	CBS 128576	Spain	*Vitis vinifera*	HQ661092	-	HQ661077
	CBS 141.41^T	Sweden	-	AY249066	KM497133	KM497089
	CVG 650*	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	*MT261874*	-	MT293544
	CVG 651	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	*MT261875*	-	-
	CVG 652	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	*MT261876*	-	*MT293545*
	CVG 653	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	MT261877	-	-
	CVG 654	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	*MT261878*	-	*MT293546*
	CVG 655	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	*MT261879*	-	-
Cadophora malorum	CBS 165.42^T	Netherlands	*Amblystoma mexicanum*	AY249059	KM497134	KM497090
Cadophora novi eboraci	CBS 101359	Italy	*Actinidia chinensis*	DQ404350	KM497135	KM497092
	NYC13	USA	*Vitis vulpina*	KM497036	KM497117	KM497073
Cadophora melini	CBS 268.33^T	Sweden	-	AY249072	KM497132	KM497088
	U11	USA	*Vitis vinifera* 'Sangiovese'	KM497032	KM497113	KM497069
Cadophora orientoamericana	NYC11	USA	*Vitis vinifera* 'Chardonnay'	KM497024	KM497105	KM497061
	NYC3	USA	*Vitis labruscana* 'Concord'	KM497021	KM497102	KM497058
Cadophora spadicis	CBS111743	Italy	*Actinidia chinensis*	DQ404351	KM497136	KM497091
Cryptosphaeria subcutanea	CBS 240.87	Norway	*Salix borealis*	KT425232	KT425167	-
Diaporthella corylina	CBS 121124^T	China	*Corylus*	KC343004	KC343972	KC343730
Diaporthe acaciigena	CBS 129521	Australia	*Acacia retinoges*	KC343005	KC343973	KC343731
Diaporthe ampelina	CBS 114016^T	France	*Vitis vinifera*	AF230751	JX275452	GQ250351
Diaporthe amygdali	CBS 126679^T	Portugal	*Prunu dulcis*	KC343022	KC343990	KC343748
Diaporthe arecae	CBS 535.75	Suriname	*Citrus* sp.	KC343032	KC344000	KC343758
Diaporthe australfricana	CBS 111886	Australia	*Vits vinifera*	KC343038	KC344006	KC343764
Diaporthe baccae	CBS 136972^T	Italy	*Vaccinium corymbosum*	KJ160565	MF418509	KJ160597
Diaporthe carpini	CBS 114437	Sweden	*Carpinus betulus*	KC343044	KC344012	KC343770
Diaporthe citri	CBS 135422	USA	*Citrus* sp.	KC843311	KC843187	KC843071
Diaporthe eres	CBS 116953	New Zeland	*Pyrus pyrifolia*	KC343147	KC344115	KC343873
	CBS 138594	Germany	*Ulmus laevis*	KJ210529	KJ420799	KJ210550
Diaporthe notophagi	BRIP 54801^T	Australia	*Notophagus cunninghamii*	JX862530	KF170922	JX862536
Diaporthe perjuncta	CBS 109745^T	Austria	*Ulmus glabra*	KC343172	KC344140	KC343898
Diaporthe phaseolorum	CBS 127465	New Zeland	*Actinidia chinensis*	KC343174	KC344142	KC343900
Diaporthe rudis	CBS 113201	Portugal	*Vits vinifera*	KC343234	KC344202	KC343960
	CBS 114436	Sweden	*Sambucus* cf. *racemosa*	KC343236	KC344204	KC343962
	CBS 114011	Portugal	*Vitis vinifera*	KC343235	KC344203	KC343961
	CBS 266.85	Netherlands	*Rosa rugosa*	KC343237	KC344205	KC343963
	CVG 658*	Italy	*Vaccinium corymbosum* 'Last Call'	*MT261894*	-	-
	CVG 659	Italy	*Vaccinium corymbosum* 'Last Call'	*MT261895*	*MT293528*	*MT293536*
	CVG 660	Italy	*Vaccinium corymbosum* 'Last Call'	*MT261896*	*MT293529*	*MT293537*
	CVG 661	Italy	*Vaccinium corymbosum* 'Last Call'	*MT261897*	*MT293530*	*MT293538*
	CVG 662	Italy	*Vaccinium corymbosum* 'Last Call'	*MT261898*	*MT293531*	*MT293539*
Diaporthe sterilis	CBS 136969^T	Italy	*Vaccinium corymbosum*	KJ160579	KJ160528	KJ160611
Diaporthe toxica	CBS 594.93^T	Australia	*Lupinus angustifolis*	KC343220	KC344188	KC343946
Diaporthe vaccinii	CBS 160.32^T	USA	*Vaccinium macrocarpon*	AF317578	KC344196	GQ250326

(Continued)

Table 1. (Continued).

Species	Isolate code[1]	Country	Host	Genbank accession number[2]		
				ITS	*tub2*	*tef1*
	CBS 118571	USA	*Vaccinium corymbosum*	KC343223	KC344191	KC343949
	CBS 122114	USA	*Vaccinium corymbosum*	KC343225	KC344193	KC343951
Diatrype stigma	DCASH200	USA	*Quercus* sp.	GQ294003	GQ293947	-
Diatrypella atlantica	HUEFS 194228	Brazil	-	KR363998	KM396615	-
Diatrypella pulvinata	CBS 181.97	Netherlands	*Quercus robur*	-	AJ302443	-
Diplodia seriata	CBS 110875	South Africa	*Vitis vinifera*	AY343456	KX464827	KX464592
Eutypa cremea	CBS 120837	South Africa	*Prunus salicina*	KY752762	KY752791	-
Eutypa lata	ADSC300	Australia	*Schinus molle* var. *areira*	HQ692610	HQ692493	-
	CBS 121430	South Africa	*Prunus armeniaca*	KY752766	KY752794	-
	EP18	New South Wales	*Vitis vinifera*	HQ692611	HQ692501	-
	SACEA01	Australia	*Ceanothus*sp.	HQ692615	HQ692499	-
Eutypa leptoplaca	ADFIC100	Australia	*Ficus macrophylla*	HQ692608	HQ692485	-
Eutypa maura	CBS 219.87	Switzerland	*Acer pseudoplatanus*	AY684224	DQ006967	-
Eutypa tetragona	CBS 284.87	France	*Sarothamnus scoparius*	DQ006923	DQ006960	-
Eutypella cerviculata	CBS 221.87	Switzerland	*Alnus glutinosa*	AJ302468	-	-
Eutypella citricola	STEU 8098	South Africa	*Vitis vinifera*	KY111634	KY111588	-
Eutypella microtheca	STEU 8107	South Africa	*Vitis vinifera*	KY111629	KY111608	-
Eutypella vitis	MSUELM13	USA	*Vitis vinifera*	DQ006943	DQ006999	-
Liberomyces pistaciae	CBS 144225	Italy	*Pistacia vera*	MH797562	MH797697	-
Neofusicoccum algeriense	CBS 137504^{T}	Mexico	*Rubus idaeus*	KJ657702	-	KJ657715
Neofusicoccum arbuti	CBS 116131^{T}	USA: Washington	*Arbutus menziesii*	AY819720	KF531793	KF531792
Neofusicoccum australe	CBS 139662^{T}	Australia	*Acacia* sp.	AY339262	AY339254	AY339270
	CBS 121115	South Africa	*Prunus persica*	EF445355	KX464948	EF445386
Neofusicoccum batangarum	CBS 124924^{T}	Cameroon	*Terminalia catappa*	FJ900607	FJ900634	FJ900653
Neofusicoccum cryptoaustrale	CBS 122813^{T}	South Africa	*Eucalyptus* sp.	FJ752742	FJ752756	FJ752713
Neofusicoccum italicum	MFLUCC 15-0900^{T}	Italy	*Vitis vinifera*	KY856755	-	KY856754
Neofusicoccum kwambonambiense	CBS 102.17^{T}	USA: Florida	*Carya illinoensis*	KX464169	KX464964	KX464686
Neofusicoccum luteum	CBS 562.92^{T}	New Zealand	*Actinidia deliciosa*	KX464170	KX464968	KX464690
Neofusicoccum mangiferae	CBS 118532	Australia	*Mangifera indica*	AY615186	AY615173	DQ093220
Neofusicoccum mediterraneum	CBS 121718^{T}	Greece	*Eucalyptus* sp.	GU251176	-	GU251308
Neofusicoccum parvum	CBS 123650	South Africa	*Syzygium cordatum*	KX464182	KX464994	KX464708
	CMW 9081^{T}	New Zealand	*Populus nigra*	AY236943	AY236917	AY236888
	CVG 444*	Italy	*Vaccinium corymbosum* 'Top Shelf'	*MT261980*	*MT293532*	*MT293540*
	CVG 445	Italy	*Vaccinium corymbosum* 'Top Shelf'	*MT261981*	-	-
	CVG 446	Italy	*Vaccinium corymbosum* 'Top Shelf'	*MT261982*	-	-
	CVG 642	Italy	*Vaccinium corymbosum* 'Top Shelf'	*MT261983*	*MT293533*	*MT293541*
	CVG 643	Italy	*Vaccinium corymbosum* 'Top Shelf'	*MT261984*	-	-
	CVG 644	Italy	*Vaccinium corymbosum* 'Top Shelf'	*MT261985*	-	-
	CVG 645	Italy	*Vaccinium corymbosum* 'Top Shelf'	*MT261986*	-	-
	CVG 646	Italy	*Vaccinium corymbosum* 'Top Shelf'	*MT261987*	-	-
	CVG 647	Italy	*Vaccinium corymbosum* 'Top Shelf'	*MT261988*	*MT293534*	*MT293542*
	CVG 648	Italy	*Vaccinium corymbosum* 'Top Shelf'	*MT261989*	-	-
	CVG 649	Italy	*Vaccinium corymbosum* 'Top Shelf'	MT261990	-	-

(Continued)

Table 1. (Continued).

Species	Isolate code[1]	Country	Host	Genbank accession number[2]		
				ITS	*tub2*	*tef1*
	CVG 656	Italy	*Vaccinium corymbosum* 'Top Shelf'	*MT261991*	-	-
	CVG 657	Italy	*Vaccinium corymbosum* 'Top Shelf'	*MT261992*	*MT293535*	*MT293543*
Neofusicoccum pistaciarum	CBS 113083^T	USA, California	*Pistacia vera*	KX464186	KX464998	KX464712
Neofusicoccum protearum	CBS 114176	South Africa	*Leucadendron laureolum*	AF452539	KX465006	KX464720
Neofusicoccum stellenboschiana	CBS 110864^T	South Africa	*Vitis vinifera*	AY343407	KX465047	AY343348
	CBS 121116	South Africa	*Prunus armeniaca*	EF445356	KX465049	EF445387
Neofusicoccum terminaliae	CBS 125264	South Africa	*Terminalia sericea*	GQ471804	KX465053	GQ471782
Neofusicoccum vitifusiforme	CBS 110887^T	South Africa	*Vitis vinifera*	AY343383	KX465061	AY343343
Peroneutypa alsophila	CBS 250.87	France	*Arthrocnemum fruticosum*	AJ302467	-	-
Peroneutypa curvispora	HUEFS 136877	Brazil	-	KM396646	-	-
Peroneutypa diminutiasca	MFLUCC 17-2144	Thailand	-	MG873479	MH316765	-
Peroneutypa diminutispora	HUEFS 192196	Brazil	-	KM396647	-	-
Peroneutypa kochiana	F-092, 373	Spain	*Atriplex halimus*	AJ302462	-	-
Peroneutypa longiasca	MFLUCC 17-0371	Thailand	-	MF959502	-	-
Peroneutypa mackenziei	MFLUCC 16-0072	Thailand	-	KY283083	KY706363	-
Peroneutypa rubiformis	MFLUCC 17-2142	Thailand	-	MG873477	MH316763	-
Peroneutypa scoparia	CBS 242.87	France	*Robinia pseudoacacia*	AJ302465	-	-
	DFMAL100	France	*Robinia pseudoacacia*	GQ293962	GQ294029	-
	MFLUCC 17-2143	Thailand	-	MG873478	MH316764	-
	CVG 561*	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	*MT261914*	*MT293522*	-
	CVG 562	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	*MT261915*	*MT293523*	-
	CVG 563	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	*MT261916*	MT293524	-
	CVG 564	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	*MT261917*	-	-
	CVG 565	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	*MT261918*	-	-
	CVG 566	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	MT261919	-	-
	CVG 567	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	*MT261920*	-	-
	CVG 568	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	*MT261921*	-	-
	CVG 569	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	*MT261922*	-	-
	CVG 570	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	*MT261923*	*MT293525*	-
	CVG 571	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	*MT261924*	*MT293526*	-
	CVG 572	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	*MT261925*	*MT293527*	-
	CVG 580	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	*MT261926*	-	-
	CVG 581	Italy	*Vaccinium corymbosum* 'Blue Ribbon'	*MT261927*	-	-

[1] BRIP: Plant Pathology Herbarium, Department of Primary Industries, Dutton Park, Queensland, Australia; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; CMW: Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; CVG: AGROINNOVA, Grugliasco, Torino, Italy; HUEFS Herbarium of the State University of Feira de Santana; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; STEU: University of Stellenbosch, Stellenbosch, South Africa. The strains named ADSC300, DCASH200, DFMAL100, EP18, MSUELM13, NYCs, SACEA01 and U11 were reported on further studies (Trouillas *et al.*, 2010, 2011; Travadon *et al.*, 2015; Moyo *et al.*, 2018). Ex-type and ex-epitype cultures are indicated with ^{T}.
[2] ITS: internal transcribed spacers 1 and 2 together with 5.8S nrDNA; *tub2*: beta-tubulin gene; *tef1*: translation elongation factor 1-α gene. Sequences generated in this study indicated in *italics*.
* Isolates used for phenotypic characterization and pathogenicity test.

Inference (BI) and Maximum Parsimony (MP) for the multi-locus analyses. 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 1,000 generations. Analyses stopped when the average standard deviation of split frequencies was below 0.01. 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 1,000 replications. Sequences generated in this study were deposited in GenBank (Table 1).

Phenotypic characterization

Agar plugs (5 mm diam.) of representative strains (CVG 444 – *N. parvum*, CVG 658 – *D. rudis*, CVG 561 – *P. scoparia*, and CVG 650 – *C. luteo-olivacea*) were taken from the edges of actively growing cultures on PDA-S and transferred onto the centre of 9 cm diam. Petri dishes containing 2% water agar supplemented with sterile pine needles (PNA; Smith *et al*., 1996), PDA, malt extract agar (MEA; Oxoid), or synthetic nutrient-poor agar (SNA, Leslie and Summerell, 2006), then incubated at 20–21°C under a 12 h photoperiod to induce sporulation. Colony characters were observed after 10 d, and culture colours were determined (Rayner, 1970). Cultures were examined periodically for the development of conidiomata. Conidium characteristics were examined by mounting fungal structures in SDW, and the lengths and widths of 30 conidia were measured for each isolate using a light microscope at ×400 magnification. The average and standard deviations of conidium dimensions were calculated. Isolate growth rates were determined on PDA plates incubated in the dark at 25°C. Three plates were used for each isolate. After 5 d, radii of colonies were measured, and mean radii were calculated to determine the growth rates for each isolate.

Pathogenicity tests

Pathogenicity tests with four species (*N. parvum*, *D. rudis*, *C. luteo-olivacea* and *P. scoparia*) were performed to evaluate Koch's postulates. Four representative strains of each species (Table 1) were used to inoculate potted 1-y-old healthy blueberry plants ('Duke'). Three plants were inoculated with one isolate for each species. For each inoculation, a sterile scalpel was used to cut a piece of the bark tissue exposing the cambium. Each plant was wounded at five points. Mycelium plugs (5 mm diam.) were taken from 10-d-old cultures on PDA and placed with the mycelium in contact with the internal plant tissues. Each inoculation point was wrapped with Parafilm®. The same number of plants were treated with sterile PDA discs as inoculation controls.

The plants were placed in a growth chamber at 24°C for 3 weeks. After this period, the lengths of lesions developed on the internal woody tissues were measured after removing the bark. Fifteen d after inoculation, the symptom severity (SS) associated with each inoculated species was evaluated as the length of wood lesions induced. The shoots were cut and the bark peeled off and the lengths of vascular discolouration were measured upward and downward from the inoculation points. Small portions (0.5 cm) of symptomatic tissue from the edge of shoot lesions were placed onto PDA to re-isolate the fungal species, and these were identified based on their colony characteristics and ITS sequencing. This trial was conducted twice and each trial was considered a replicate. Because no normal distribution was observed in the lesion dimension data, the Kruskal-Wallis non-parametric test (at $P = 0.05$) was performed to determine significant differences among the strains. The data analysis was conducted using SPSS software 26 (IBM Corporate).

RESULTS

Field sampling and fungal isolation

Symptoms of stem blight were observed on all the surveyed blueberry plants, and cultivars, and in all the orchards surveyed (Table 2). Plant stems with necrotic internal tissues were observed. Dieback and death of the plants occurred in all the orchards investigated (Figure 1). Symptoms were detected on young and mature plants. DI of symptomatic plants was between 20% and 30% in the four sites. The proportions of dead plants averaged 10%. Based on colony morphology, phylogenetic analyses, and conidium characteristics (see below), *Neofusicoccum parvum* was isolated from 'Top Shelf' plants, *Diaporthe rudis* was isolated from 'Last Call', and

Table 2. Origin of fungal isolates obtained from *Vaccinium corymbosum*.

Species	Code	*Vaccinium corymbosum* cv.	Orchard/Locality
Cadophora luteo-olivacea	CVG 650–655	Blue Ribbon	Orchard 1/ Lagnasco (CN)
Diaporthe rudis	CVG 658–662	Last Call	Orchard 2/ Lagnasco (CN)
Neofusicoccum parvum	CVG 444–446	Top Shelf	Orchard 3/ Lagnasco (CN)
	CVG 642–649	Top Shelf	Orchard 4/ Lagnasco (CN)
	CVG 656, CVG 657	Top Shelf	Orchard 4/ Lagnasco (CN)
Peroneutypa scoparia	CVG 561–572	Blue Ribbon	Orchard 1/ Lagnasco (CN)
	CVG 580, CVG 581	Blue Ribbon	Orchard 1/ Lagnasco (CN)

Cadophora luteo-olivacea and *Peroneutypa scoparia* were isolated from 'Blue Ribbon'.

Phylogenetic analysis

The overview phylogenetic analysis of the 38 isolates obtained from blueberry plants showed that six isolates belonged to *Cadophora*, 13 isolates clustered in a clade with *Neofusicoccum* spp., five formed a distinct, well-supported, lineage with *Diaporthe* spp. The remaining 14 isolates grouped with *Peroneutypa* spp. in the clade with other strains belonging to the *Diatrypaceae* (Figure 2). The combined locus phylogeny of *Neofusicoccum* consisted of 24 sequences, *Diaporthe* consisted of 28 sequences, *Cadophora* of 18, and the *Diatrypaceae* of 32 sequences, including outgroups. A total of 1,139 characters (ITS: 1–521, *tef1*: 528–732 and *tub2*: 739–1136) were included in the *Neofusicoccum* phylogenetic analyses. A total of 1,373 characters (ITS: 1–563, *tef1*: 570–975 and *tub2*: 982–1373) were included in the *Diaporthe* phylogenetic analyses. The analyses for the *Cadophora* group consisted of 819 nucleotides (ITS: 1–499 and *tef1*: 506–819), and the analyses of *Diatrypaceae* were based on a total of 974 characters (ITS: 1–593 and *tub2*: 600–974). A maximum of 1,000 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 presented in Figures 2 to 6. For both of the Bayesian analyses, MrModeltest recommended the models reported in Table 4. Unique site patterns for each partition and all the parameters of the Bayesian analyses are reported in Table 3. In the *Neofusicoccum* species analysis, four isolates from symptomatic *Vaccinium* plants clustered with the ex-type and one reference strain of *N. parvum* (Figure 3), while the final tree generated for *Diaporthe* showed that four isolates grouped with four reference strains of *D. rudis* (Figure 4). Three isolates clustered with the ex-type and two reference strains of *C. luteo-olivacea* (Figure 5), and six isolates clustered as *P. scoparia* in the phylogenetic tree from the analyses of the *Diatrypaceae* (Figure 6).

Phenotypic characterization

Morphological observations, supported by phylogenetic inference, were described for four known species.

Neofusicoccum parvum

Ten-d-old colonies on PDA (Figure 7A), and MEA, were cottony with entire margins and dark to pale grey aerial mycelium. On SNA, the colonies were powdery, with dark to pale grey aerial mycelium. Colony reverse sides were black to light grey and whitish on PDA (Figure 7B) and MEA, and greyish to white on SNA. Conidia were hyaline, ellipsoidal with rounded apices and flat bases, with dimensions of 17–19 × 4.2–6.4 µm, mean ± SD = 17.9 ± 0.6 × 5.3 ± 0.6 µm. The mean daily colony growth rate at 25°C was 4.7 mm.

Diaporthe rudis

Ten-d-old colonies on PDA (Figure 7C), MEA and SNA were flat and fluffy with entire margins and aerial, white to pale grey mycelium. Colony reverse sides were light buff with greyish to brown halos on PDA (Figure 7D), honey buff on MEA and white on SNA. Alpha-conidia were hyaline, aseptate, smooth, biguttulate and ellipsoidal, with subtruncate bases, with dimensions of 6.5–8.4 × 2–2.5 µm, mean ± SD = 7.5 ± 0.6 × 2.3 ± 0.2 µm. The mean daily colony growth rate at 25°C was 4.9 mm.

Cadophora luteo-olivacea

Ten-d-old colonies on PDA were flat and velvety with smooth margins, and the mycelium was grey to white

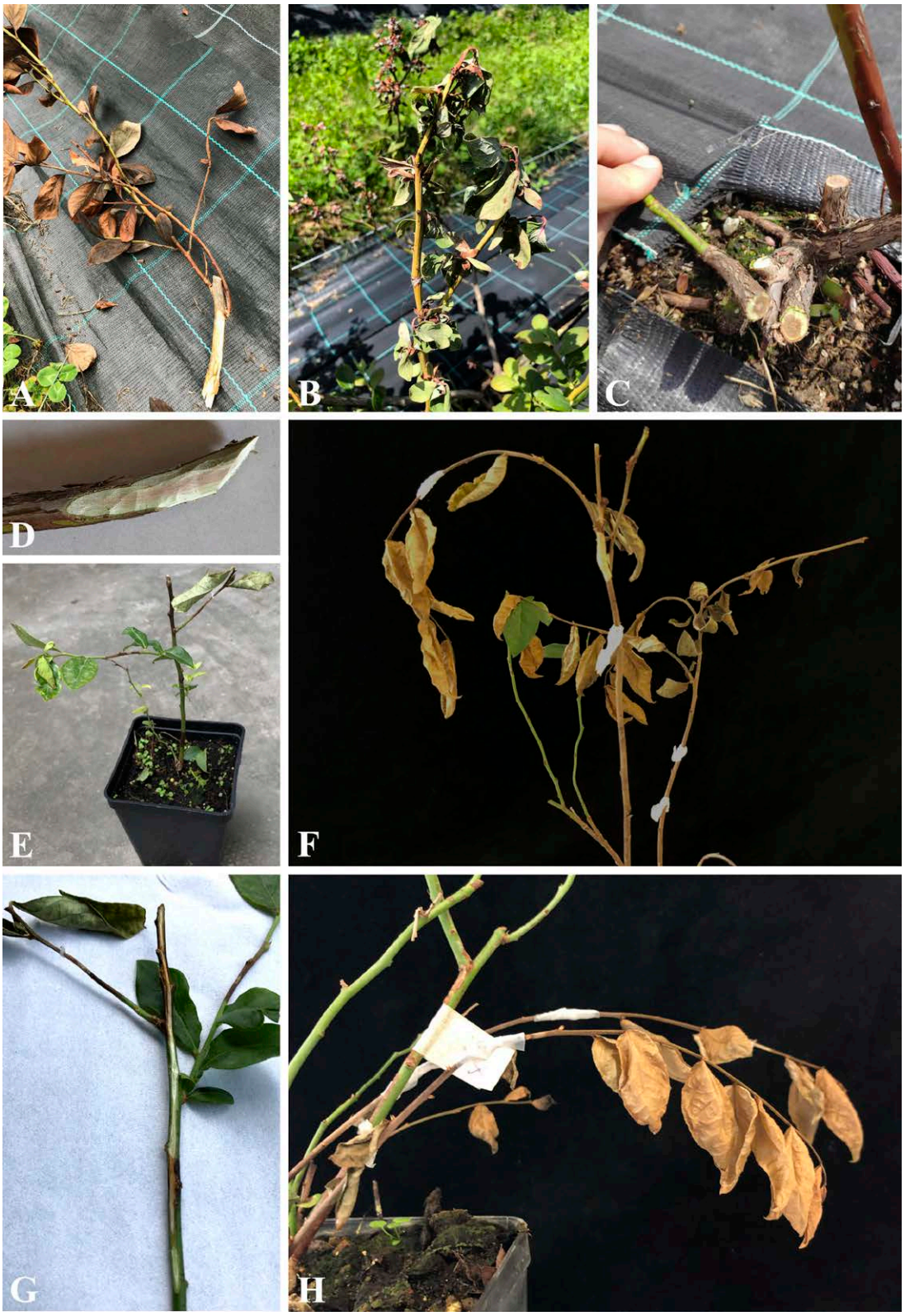

Figure 1. Natural dieback (A) and stem blight (B) of *Vaccinium corymbosum* 'Blue Ribbon' in the field, and a stem showing internal necrosis in the wood of a cultivated plant of 'Top Shelf' (C, D). Wilting and internal discolouration caused by inoculation of *Diaporthe rudis* (E, G), and death of leaves and stems caused by *Neofusicoccum parvum* inoculation (F, H).

Table 3. Parsimony and Bayesian analyses characteristics in this study.

Analysis		Overview tree	*Neofusicoccum*	*Diaporthe*	*Cadophora*	*Peroneutypa*
	Locus(i)	ITS	ITS+*tef1*+*tub2*	ITS+*tef1*+*tub2*	ITS+*tef1*	ITS+*tub2*
Parsimony analysis	Total sites	737	1,124	1,361	847	1,078
	Constant sites	271	854	594	360	425
	Variable sites	76	186	289	204	182
	Parsimony Informative sites	390	84	478	283	471
	Tree length	1,716	368	2,049	779	1,881
	Consistency index	0.579	0.834	0.638	0.883	0.625
	Retention index	0.924	0.850	0.757	0.934	0.809
	Rescaled consistence index	0.535	0.709	0.483	0.825	0.505
Bayesian analysis	Unique site patterns of ITS	474	79	168	137	314
	Unique site patterns of *tef1*	-	79	286	176	-
	Unique site patterns of *tub2*	-	54	184	-	251
	Generation ran	770,000	490,000	1,150,000	1,620,000	390,000
	Generated trees	1542	982	2302	3242	782
	Sampled trees	1158	738	1728	976	588

(Figure 7G). Colonies on MEA were velvety, harbouring fissures, with smooth margins, and grey mycelium. Colonies on SNA were powdery, with light olivaceous mycelium. Colony reverse sides were dark grey to white on PDA (Figure 7H), buff honey on MEA and light olivaceous on SNA. Conidia were hyaline, with up to three guttules, ovoid or oblong ellipsoidal, with dimensions of 4–6.4 × 2–3 µm, mean ± SD = 5.4 ± 0.9 × 2.4 ± 0.4 µm. The mean daily colony growth rate at 25°C was 0.9 mm.

Peroneutypa scoparia

Ten-d-old colonies on PDA (Figure 7E) and MEA were flat and fluffy with entire margins, and white to grey aerial mycelium. Conidiomata were black on PDA and MEA. Colony reverse sides were dark to light grey on PDA (Figure 7F), dark brown to buff honey on MEA, and white on SNA. Conidia were filiform and curved, with dimensions of 10.5–13.8 × 1–1.5 µm, mean ± SD = 12.4 ± 1.3 × 1.2 ± 0.2 µm. The mean daily colony growth rate at 25°C was 1.8 mm.

Pathogenicity

After 30 d, all of the isolates caused lesions on inoculated plants (Figure 8), which were similar to those detected on the field-grown plants, and all the inoculated fungi were re-isolated from plants on which they were inoculated. The frequency of re-isolation was between 90 and 95%. The identities of the respective inoculated and re-isolated species were confirmed using culture and molecular features, fulfilling Koch's postulates. Lesions and internal discolouration were observed in correspondence to the inoculation points (Figure 7). *Neofusicoccum parvum* was the most aggressive pathogen, causing necrotic lesions of average length 5.8 cm. *Diaporthe rudis* caused less severe symptoms on shoots (mean lesions length = 1.5 cm) than *N. parvum*, but with lesion lengths significantly greater than the control plants. *Cadophora luteo-olivacea* and *Peroneutypa scoparia* isolates were the least aggressive with mean lesion lengths, respectively, of 0.9 cm and 0.8 cm. Weak symptoms were observed on control plants (mean lesion length = 0.2 cm), probably due to reaction to wounding. The pairwise comparison obtained from the Kruskal-Wallis test, showed significant differences ($P < 0.05$) between the four isolates and the control. Comparison of the isolates showed significant differences ($P < 0.05$), except for the isolates CVG561 and CVG650 ($P = 0.798$).

DISCUSSION

Blueberry has been cultivated In Italy since the 1970, and is considered as a niche crop (Retamales and Hancock, 2018). Blueberries are included in daily fresh fruit consumption, with annual production of 1,675 tonnes (FAOSTAT 2019). Stem blight and dieback could represent serious threats to this crop. The present study is the first to investigate the species diversity of fungal woody pathogens associated with stem blight and dieback of highbush blueberry in a major production area in Italy. During

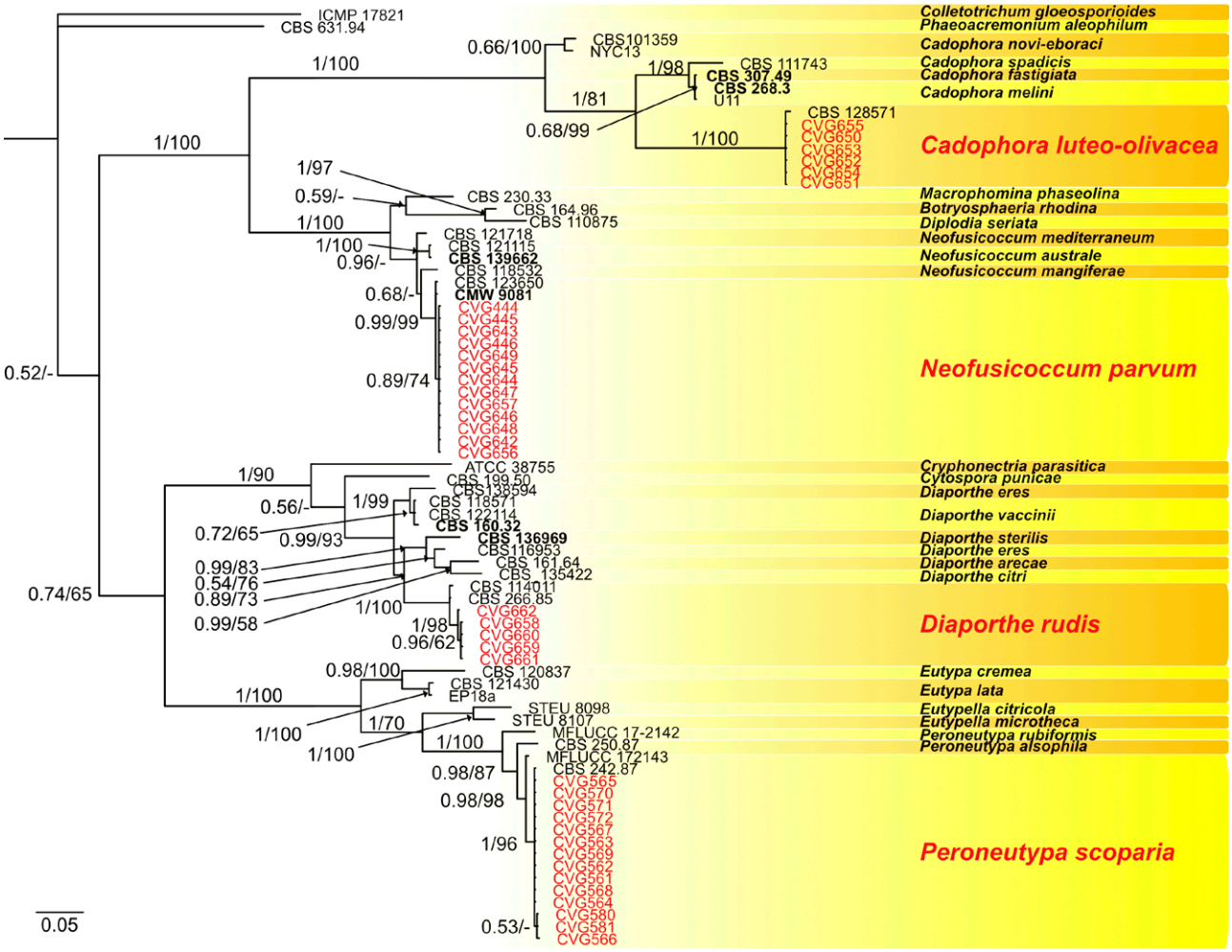

Figure 2. Consensus phylogram of 1,542 trees resulting from a Bayesian analysis of the ITS sequences of 38 fungus isolates collected in this study (in red) and further reference strains. Bayesian posterior probability values and bootstrap support values are indicated at the nodes. The tree was rooted to *Colletotrichum gloeosporioides* (ICMP 17821).

the surveys, the four fungi *Neofusicoccum parvum*, *Diaporthe rudis*, *Cadophora luteo-olivacea* and *Peroneutypa scoparia* were found in association with blueberry stem blight and dieback in four different orchards.

Neofusicoccum parvum has been found in association with blueberry canker and dieback in Asia, Europe, South and North America, (Espinoza *et al.*, 2009; Koike *et al.*, 2014; Xu *et al.*, 2015; Scarlett *et al.*, 2019; Hilário *et al.*, 2020b). In Italy, this fungus has been detected on several fruit plant hosts, as an endophyte and latent pathogen (Carlucci *et al.*, 2013; Guarnaccia *et al.*, 2016; Riccioni *et al.*, 2017; Aiello *et al.*, 2020). In the present study, *N. parvum* was the most aggressive when inoculated on the blueberry 'Duke', which was used for the pathogenicity tests as representative cultivar, because it is the most cultivated worldwide. *Neofusicoccum parvum* produced significant necrotic lesions, progressive discolouration of the internal tissues, and death of inoculated branches This confirms the high level of aggressiveness of this fungus on blueberry, as has been reported previously (Espinoza *et al.*, 2009; Xu *et al.*, 2015; Scarlett *et al.*, 2019; Hilário *et al.*, 2020b).

Diaporthe spp. are well known pathogens occurring as pathogenic or harmless endophytes on blueberry (Latorre *et al.*, 2012; Elfar *et al.*, 2013; Cardinaals *et al.*, 2018; Hilário *et al.*, 2020a), and on other host plants (Guarnaccia *et al.*, 2016; Battilani *et al.*, 2018; Guarnaccia *et al.*, 2018; Guarnaccia and Crous, 2018; Yang *et al.*, 2018). According to Santos *et al.* (2017), molecular tools and multilocus approaches should be used for accurate resolution of species within *Diaporthe*. The present study analysed three sequence

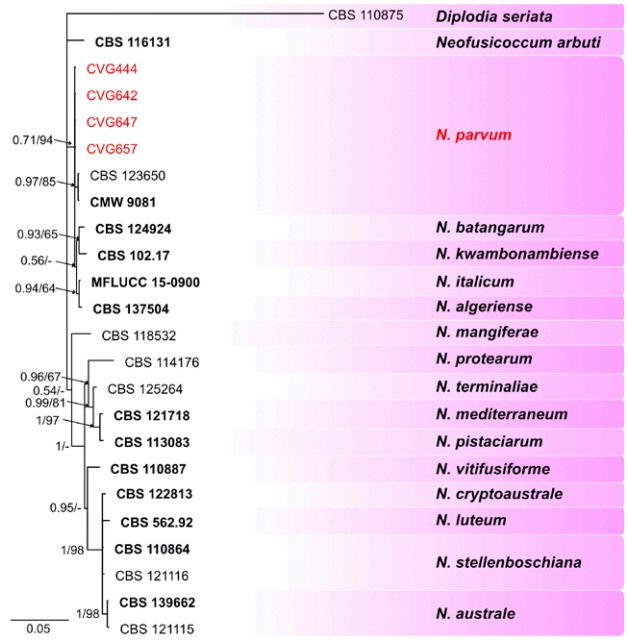

Figure 3. Consensus phylogram of 982 trees resulting from a Bayesian analysis of the combined ITS, *tef1* and *tub2* sequences of *Neofusicoccum* strains. Bayesian posterior probability values and bootstrap support values are indicated at the nodes. The tree was rooted to *Diplodia seriata* (CBS 110875).

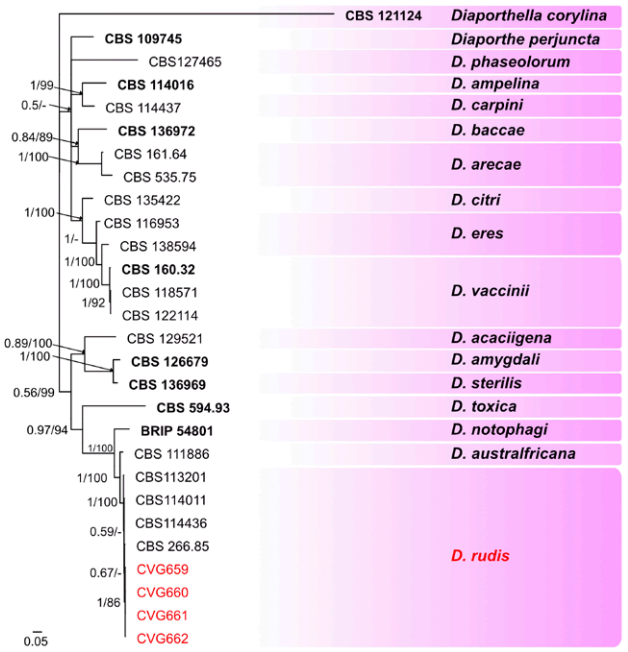

Figure 4. Consensus phylogram of 2,302 trees resulting from a Bayesian analysis of the combined ITS, *tef1* and *tub2* sequences of *Diaporthe* strains. Bayesian posterior probability values and bootstrap support values are indicated at the nodes. The tree was rooted to *Diaporthella corylina* (CBS 121124).

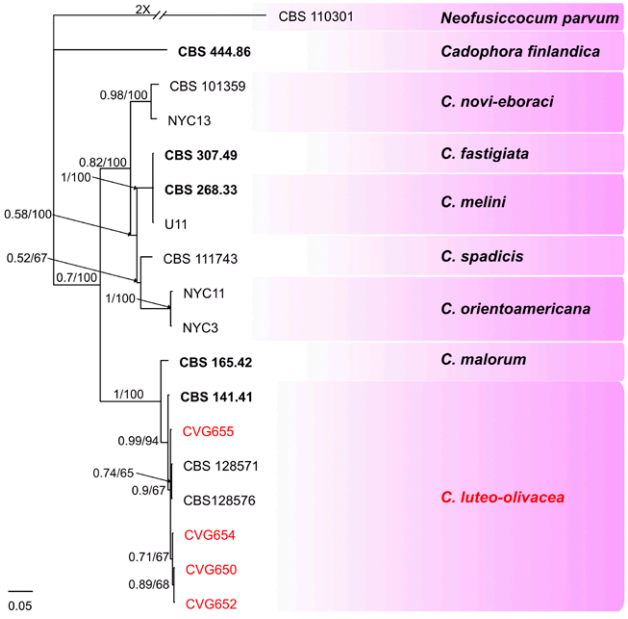

Figure 5. Consensus phylogram of 3,242 trees resulting from a Bayesian analysis of the combined ITS and *tef1* sequences of *Cadophora* strains. Bayesian posterior probability values and bootstrap support values are indicated at the nodes. The tree was rooted to *Neofusicoccum parvum* (CBS 110301).

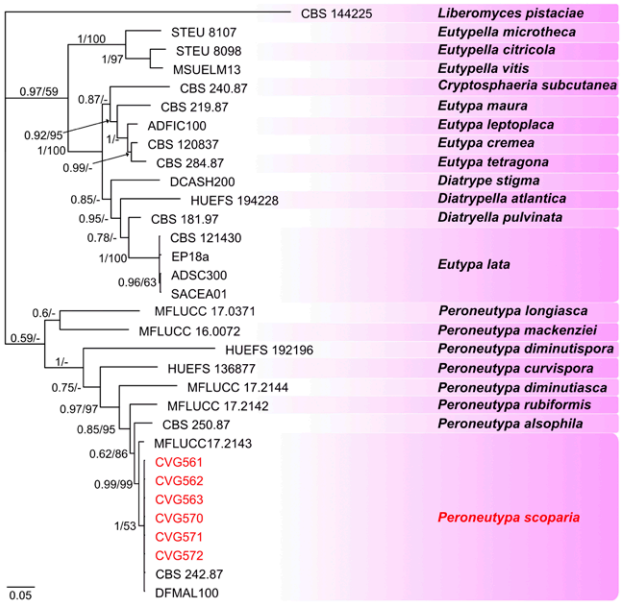

Figure 6. Consensus phylogram of 782 trees resulting from a Bayesian analysis of the combined ITS and *tub2* sequences of *Peroneutypa* and other strains of the Diatrypaceae. Bayesian posterior probability values and bootstrap support values are indicated at the nodes. The tree was rooted to *Liberomyces pistaciae* (CBS 110301).

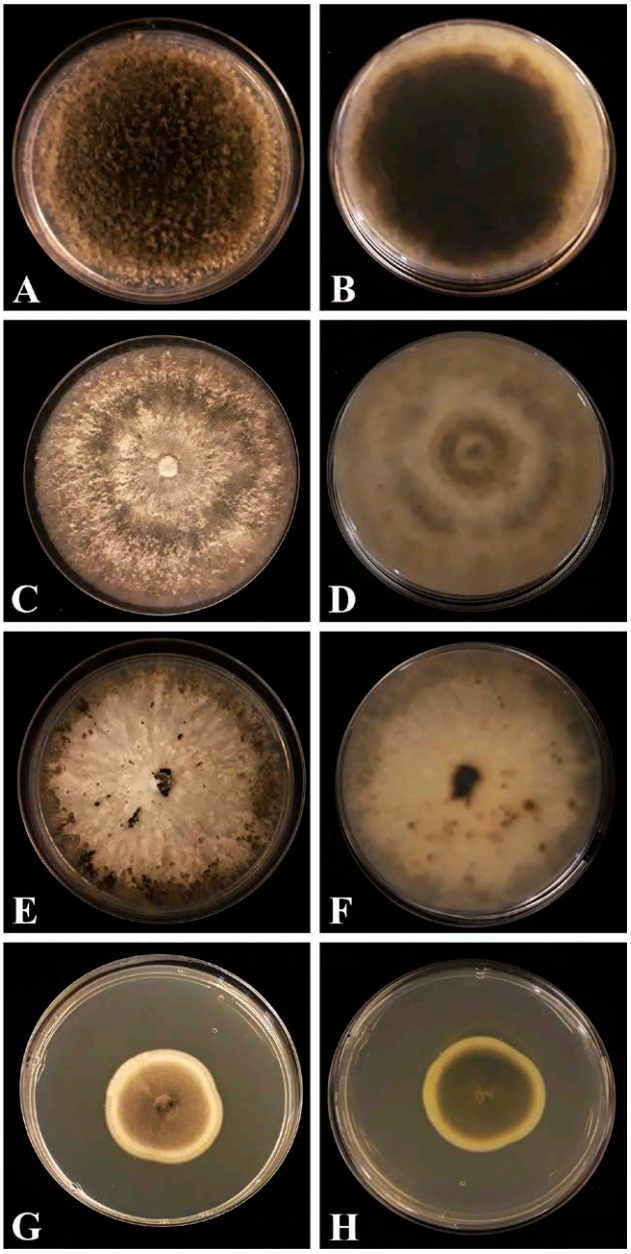

Figure 7. Morphological characteristics of the front and reverse sides of colonies of the different fungal species grown 10 d on PDA. A and B *Neofusicoccum parvum*. C and D *Diaporthe rudis*. E and F. *Peroneutypa scoparia*. G and H *Cadophora luteo-olivacea*.

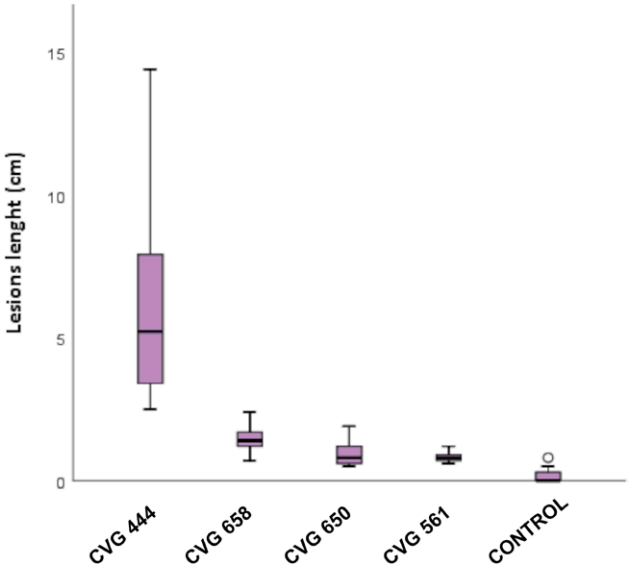

Figure 8. Box plot showing the results of the pathogenicity tests. Boxes represent the interquartile range, while the horizontal line within each box indicates the average value. The Kruskal-Wallis test was carried out to compare the mean lesion lengths (cm) from inoculation with four representative isolates, and significant difference was accepted for $P<0.05$.

Table 4. Evolutionary models as determined by MrModeltest (Nylander 2004).

Genus	Locus	Evolutionary model[1]
Overview tree	ITS	GTR+I+G
Neofusicoccum	ITS	SYM+I+G
	tef1	GTR+I+G
	tub2	HKY+G
Diaporthe	ITS	SYM+I+G
	tef1	GTR+I+G
	tub2	HKY+G
Peroneutypa	ITS	GTR+I+G
	tub2	GTR+G
Cadophora	ITS	SYM+G
	tef1	HKY+G

1 G: Gamma distributed rate variation among sites; GTR: Generalised time-reversible; HKY: Hasegawa-Kishino-Yano; I: Proportion of invariable sites; SYM: Symmetrical model.

datasets (ITS, *tub2*, and *tef1*). *Diaporthe rudis* has been recognized as having a broad host range, and is distributed in Australia, Canada, Chile, Europe, New Zealand and South Africa (Marin-Felix *et al.*, 2019). The present study is the first to report *D. rudis* on *V. corymbosum* in Italy. The pathogenicity tests showed that this species was of intermediate aggressiveness on highbush blueberry plants.

Cadophora luteo-olivacea has been reported in association with grapevine trunk diseases (Gramaje *et al.*, 2011; Travadon *et al.*, 2015; Raimondo *et al.*, 2019), and on several woody hosts (Bien and Damm, 2020; Farr and Rossman, 2020). This fungus also causes postharvest diseases on kiwifruit, pear, and apple (Spadaro *et al.*, 2010; Köhl *et al.*, 2018; Eichmeier *et al.*, 2020). Gramaje *et al.* (2011) reported the high variability in *C. luteo-oli-*

vacea colony pigmentation and the critical role of DNA sequencing to clearly identify this pathogen.

Peroneutypa scoparia belongs to the *Diatrypaceae*, members of which are commonly classified as saprophytes, endophytes, and pathogens on a wide range of woody hosts such as grapevine, pistachio and *Prunus* spp. (Trouillas *et al.*, 2011; Shang *et al.* 2017; Aiello *et al.*, 2019; Mehrabi *et al.*, 2019). Due to the morphological similarities among the *Diatrypaceae*, molecular analyses on ITS and partial β-tubulin regions have been established to resolve and clarify taxonomy within this family (Trouillas *et al.*, 2010, 2011; Shang *et al.* 2017; Mehrabi *et al.*, 2019; Moyo *et al.*, 2019). Carmarán *et al.* (2006) included the *Peroneutypa* within the *Diatrypaceae*, and *P. scoparia* is the currently accepted, with the synonyms *Eutypa scoparia*, *Eutypella scoparia*, *Peroneutypella scoparia*, *Sphaeria scoparia*, and *Valsa scoparia*.

In the present study, *C. luteo-olivacea* and *P. scoparia* were isolated from diseased cv. 'Blue Ribbon' blueberry plants. This is the first report of these species in association with blueberry plants in Italy, as well as worldwide. Furthermore, both these fungi were weakly aggressive on the 'Duke'.

All the fungi pathogens detected during this survey in association with severe losses in blueberry orchards were confirmed as pathogenic. Because pathogenicity was tested only on 'Duke', further pathogenicity tests are planned to assess pathogenicity of these fungi on other blueberry cultivars such as 'Top Shelf', 'Blue Ribbon', 'Last Call' and 'Cargo'. Good agronomic practices such as removing pruning debris, and wound protection after pruning and chilling, could reduce inoculum sources and the disease severity. Healthy propagation material from certified nurseries should be adopted, because these pathogens can survive as endophytes or as latent infections. Favourable climatic conditions and high temperatures during the blueberry production cycle could also play a major role in disease development. Further investigation of climate effects on the fungal pathogen species, and epidemiology of stem blight and dieback diseases of blueberry, should be conducted to develop specific protocols for effective disease management.

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