Paraconiothyrium fuckelii, *Diaporthe eres* and *Neocosmospora parceramosa* causing cane blight of red raspberry in Northern Italy

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Abstract

Red raspberry (*Rubus idaeus* L.) represents a relevant crop which production has largely increased worldwide during the last decade. Cane blight is one of the most common diseases of red raspberry and it can produce considerable losses. During 2019–2021, surveys were conducted in red raspberry orchards in Cuneo province, Northern Italy, to investigate the etiology and pathogen diversity in association with cane blight. Eleven isolates were collected from symptomatic plants of the cultivars ‘Diamond Jubilee’ and ‘Grandeur’, and were identified through multi-locus phylogenies and morphological features. Three fungal species were identified: *Paraconiothyrium fuckelii*, *Diaporthe eres* and *Neocosmospora parceramosa*. Four different genomic regions were included in the molecular analyses: ITS, *tef1*, *tub2* and *rpb2*. The species pathogenicity was confirmed and *P. fuckelii* was revealed as the most aggressive. This study provides an insight into raspberry cane blight in Italy and the genomic loci sequencing lay the basis to develop specific diagnostic protocols to monitor the presence of pathogens and to prevent disease spread, adopting effective integrated control strategies.

Keywords *Rubus idaeus* · Multi-locus typing · Etiology · Pathogenicity · Berry fruit

Introduction

Red raspberry (*Rubus idaeus* L., Rosaceae) is a deciduous shrub with a biennial cane habit. Raspberry cultivars can be primocane and floricanes based on their fruiting habit (Martin et al. 2017). Primocane raspberries produce fruit during the fall of the first year and during the summer of the second year after plantation. Whilst, floricanes fruiting raspberries produce berries only on the second year of growth. Primocane cultivars offer the convenience to extend the cropping season, with reduction of costs and higher fruit quality. Thus, different breeding programmes were conducted worldwide to promote the use of these cultivars and to increase yield, fruit

quality and biotic and abiotic stress tolerance (Graham and Brennan 2018).

Red raspberry is considered a crop of global significance for its production and economic value and, due to the provided health benefits, its cultivation has increased worldwide (Graham and Brennan 2018; Retamales and Hancock 2012; Zhang et al. 2018). Europe is a leading region for raspberry production with more than 103,000 ha cultivated mainly in the northern and eastern regions (FAOSTAT 2020). In Italy, during the last decade, an increasing interest was observed in berry fruit cultivation and raspberry production achieved 2,280 tonnes with 360 ha cultivated in 2020 (FAOSTAT 2020). The rising importance of this crop led to more intensive cultivation systems (Graham and Brennan 2018). All berry fruit crops represent an expanding sector within Italian agriculture; they are included among the major fresh fruit exported during the last decade (FAOSTAT 2020). Orchards are usually established under plastic nets and fibre cover or in plastic tunnels to increase the temperature and to extend the growing season of the crop (Graham and Brennan 2018; Retamales and Hancock 2012). However, the intensive production system, the introduction of plant material from foreign countries as well as new cultivars, can promote

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the introduction of emerging or new pathogens (Dolan et al. 2018). Cane blight represents one of the most relevant diseases caused by fungal pathogens on raspberry plants (Martin et al. 2017). The disease affects canes that were wounded during their vegetative year causing xylem infections, bud failure, lateral shoot wilt and, in severe cases, cane death. Lesions are usually represented by dark red to purple areas around wounds with irregular dark purple borders (Graham and Brennan 2018). The fungal species *Paraconiothyrium fuckelii* is reported as the causal agent of raspberry cane blight (Martin et al. 2017). Synonyms of this species are *Leptosphaeria coniothyrium*, *Coniothyrium fuckelii*, *Microsphaeropsis fuckelii* and *Kalmusia coniothyrium* (Damm et al. 2008; De Gruyter et al. 2013; Mikulic-Petkovsek et al. 2014; Verkley et al. 2004). Verkley et al. (2014) renamed it after a reassessment within the Montagnulaceae family. This pathogen is also known to cause stem canker of *Rosa* sp. (Horst and Cloyd 2007; Jamali 2020) and it was reported in association with other woody hosts species in the genera *Malus*, *Prunus*, *Ribes* and *Vaccinium* (Bérubé and Nicolas 2015; Chen et al. 2017; Farr and Rossman 2021). *Paraconiothyrium fuckelii* usually overwinters on dead floricanes as pseudothecia that produce ascospores or as pycnidia that produce conidia. Thus, old floricanes represent the primary source of inoculum in *Rubus* orchards (Martin et al. 2017). Little information is known about the infectivity of ascospores, whilst conidia could be dispersed by splashing rains from early spring to late autumn and could enter through cane wounds causing infection and considerable losses in raspberry production (Dolan et al. 2018).

Girichev et al. (2018) reported raspberry cane disease as caused by a complex of different phytopathogenic fungi. Moreover, several fungal species can survive as saprophytes on canes with a role in disease development (Girichev et al. 2018). Thus, it is necessary to clearly understand the etiology of raspberry cane blight to identify the fungal species associated with symptomatic or asymptomatic plant materials and their role in the disease development. Previous studies reported the influence of the raspberry genotype on the resistance to cane diseases caused by *Botrytis cinerea*, *Didymella applanata* and *Leptosphaeria coniothyrium* (White et al. 1990). Therefore, etiological studies on pathogens related to different raspberry cultivars could set the basis for new breeding programs.

Several berry fruit orchards were monitored over a three years period (2019–2021) in Piedmont, Northern Italy.

Etiological and pathogenic studies were conducted to (i) assess the fungal species diversity associated with raspberry cane blight using molecular and phylogenetic tools, (ii) to characterize the species phenotypes, and (iii) to test the pathogenicity of the species found on different cultivars, fulfilling Koch's postulates.

Materials and methods

Field sampling and fungal isolation

During the period May 2019 to April 2021, surveys were conducted in three raspberry plantations in Falicetto, Lagnasco and Manta (Cuneo, Piedmont, Italy) (Table 1). Samples from whole symptomatic plants of the primocane cultivars 'Grandeur' and 'Diamond Jubilee' were collected from the part showing stripe lesions on canes, and transferred in the laboratory. Disease incidence (DI) was assessed on 100 observed plants in each of the three orchards, and the average percentage of symptomatic plants was determined. Twenty symptomatic plants were collected from each orchard. The wood samples (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 the margin between healthy and necrotic tissues and plated on potato dextrose agar (PDA, Oxoid) 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, 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 11 isolates were obtained and 11 of these were selected based on colony aspect and used for molecular characterization (Table 2). 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

Genomic 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

Table 1 Coordinates and location of the orchards monitored in this study
DI=Disease Index assigned at the beginning of the surveys

N° orchard	Site (City, Region)	Coordinates	<i>Rubus idaeus</i> cultivar	DI
1	Verzuolo (Cuneo, Piedmont)	44°35'49.1"N 7°30'10.2"E	'Grandeur'	80%
2	Lagnasco (Cuneo, Piedmont)	44°37'20.9"N 7°33'05.6"E	'Diamond jubilee'	70%
3	Verzuolo (Cuneo, Piedmont)	44°35'51.8"N 7°30'02.4"E	'Grandeur'	80%

Table 2 Collection details and GenBank accession numbers of isolates included in this study

Species	Culture No. ^a	Locality	Host	GenBank No. ^b			
				ITS	<i>tef1</i>	<i>tub2</i>	<i>rpb2</i>
<i>Diaporthe acaciigena</i>	CBS 129,521	Australia	<i>Acacia retinoges</i>	KC343005	KC343731	KC343973	-
<i>D. ampelina</i>	CBS 114016 ^T	France	<i>Vitis vinifera</i>	AF230751	GQ250351	JX275452	-
<i>D. amygdali</i>	CBS 126679 ^T	Portugal	<i>Prunus dulcis</i>	KC343022	KC343748	KC343990	-
<i>D. arecae</i>	CBS 535.75	Suriname	<i>Citrus</i> sp.	KC343032	KC343758	KC344000	-
<i>D. australfricana</i>	CBS 111886	Australia	<i>Vitis vinifera</i>	KC343038	KC343764	KC344006	-
<i>D. baccae</i>	CBS 136972 ^T	Italy	<i>Vaccinium corymbosum</i>	KJ160565	KJ160597	MF418509	-
<i>D. carpini</i>	CBS 114437	Sweden	<i>Carpinus betulus</i>	KC343044	KC343770	KC344012	-
<i>D. citri</i>	CBS 135422	USA	<i>Citrus</i> sp.	KC843311	KC843071	KC843187	-
<i>D. eres</i>	CBS 101742	Netherlands	<i>Fraxinus</i> sp.	KC343073	KC343799	KC344041	-
	CBS 116953	New Zeland	<i>Pyrus pyrifolia</i>	KC343147	KC343873	KC344115	-
	CBS 138594	Germany	<i>Ulmus laevis</i>	KJ210529	KJ210550	KJ420799	-
	CBS 143344	Czech Republic	<i>Vitis vinifera</i>	MG281020	MG281541	MG281193	-
	CVG473 [*]	Italy	<i>Rubus idaeus</i> cv. 'Diamond Jubilee'	MZ712970	MZ717163	MZ717170	-
	CVG474	Italy	<i>Rubus idaeus</i> cv. 'Diamond Jubilee'	MZ712971	MZ717164	MZ717171	-
<i>D. notophagi</i>	BRIP54801 ^T	Australia	<i>Notophagus cunninghamii</i>	JX862530	JX862536	KF170922	-
<i>D. perijuncta</i>	CBS 109745 ^T	Austria	<i>Ulmus glabra</i>	KC343172	KC343898	KC344140	-
<i>D. rudis</i>	CBS 113201	Portugal	<i>Vitis vinifera</i>	KC343234	KC343960	KC344202	-
<i>D. rudis</i>	CBS 266.85	Netherlands	<i>Rosa rugosa</i>	KC343237	KC343963	KC344205	-
<i>D. sterilis</i>	CBS 136969 ^T	Italy	<i>Vaccinium corymbosum</i>	KJ160579	KJ160611	KJ160528	-
<i>D. toxica</i>	CBS 594.93 ^T	Australia	<i>Lupinus angustifolius</i>	KC343220	KC343946	KC344188	-
<i>D. vaccinii</i>	CBS 118571	USA	<i>Vaccinium corymbosum</i>	KC343223	KC343949	KC344191	-
	CBS 160.32 ^T	USA	<i>Vaccinium macrocarpon</i>	AF317578	GQ250326	KC344196	-
<i>Diaporthella corylina</i>	CBS 121124 ^T	China	<i>Corylus</i> sp.	KC343004	KC343730	KC343972	-
<i>Geejayesia cicatricum</i>	CBS 125552	Slovenia	Dead twig	HQ728145	HM626644	-	HQ728153
<i>Helminthosporium solani</i>	CBS 640.85	The Netherlands	<i>Solanum tuberosum</i>	MH861907	-	-	KY984403
<i>Neocosmospora acutispora</i>	CBS 145461 ^T	Guatemala	<i>Coffea arabica</i>	LR583700	LR583593	-	LR583814
<i>N. bostrycoides</i>	CBS 392.66	Unknown	<i>Bertholletia excelsa</i>	LR583705	LR583598	-	LR583819
<i>N. brevicona</i>	CBS 204.31 ^{ET}	Indonesia	<i>Gladiolus</i> sp.	LR583707	LR583600	-	LR583821
<i>N. brevis</i>	CBS 144387 ^T	Belgium	Polluted soilwater	LR583708	LR583601	-	LR583822
<i>N. catenata</i>	CBS 143228	USA	<i>Stegostoma fasciatum</i>	KC808255	KC808213	-	KC808354
<i>N. cyanescens</i>	CBS 518.82 ^T	Netherlands	Human foot	AB190389	LR583605	-	LR583826
<i>N. diminuta</i>	CBS 144390 ^T	Unknown	<i>Coelocaryon preusii</i>	LR583713	LR583607	-	LR583828
<i>N. falciformis</i>	CBS 141593 ^T (ex-type of <i>F. paranaense</i>)	Brazil	<i>Glycine max</i>	MG787463	KF597797	-	KF680011
<i>N. gamtoosensis</i>	CBS 146502 ^T	South Africa	<i>Citrus sinensis</i>	MW173063	MW248762	-	MW218116
<i>N. hypohenemi</i>	CBS 145464 ^T	Benin	<i>Hypothenemus hampei</i>	LR583715	JF740850	-	JF741176
	CBS 145466	Uganda	<i>Hypothenemus hampei</i>	LR597067	JF740851	-	JF741177
<i>N. keratoplastica</i>	CBS 490.63 ^T	Japan	Human	LR583721	LT906670	-	LT960562
<i>N. lerouxii</i>	CBS 146514 ^T	South Africa	<i>Citrus sinensis</i>	MW173069	MW248768	-	MW218123
<i>N. liriodendri</i>	CBS 117481 ^T	USA	<i>Liriodendron tulipifera</i>	AF178404	AF178340	-	EU329506
<i>N. longissima</i>	CBS 126407 ^T	New Zealand	Tree bark	LR583731	LR583621	-	LR583846
<i>N. macrospora</i>	CBS 142424 ^T	Italy	<i>Citrus sinensis</i>	LT746266	LT746218	-	LT746331
	CPC 28193	Italy	<i>Citrus sinensis</i>	LT746268	LT746220	-	LT746333

Table 2 (continued)

Species	Culture No. ^a	Locality	Host	GenBank No. ^b			
				ITS	<i>tefl</i>	<i>tub2</i>	<i>rpb2</i>
<i>N. martii</i>	CBS 115659 ^{ET}	Germany	<i>Solanum tuberosum</i>	JX435206	JX435156	-	JX435256
<i>N. metavorans</i>	CBS 135789 ^T	Greece	Human pleural effusion	LR583738	LR583627	-	LR583849
<i>N. parceramosa</i>	CBS 115695 ^T	South Africa	Soil	JX435199	JX435149	-	JX435249
	NRRL 31158	USA	Human wound	DQ094389	DQ246916	-	EU329559
	NRRL 32301	USA	Human eye	EU329677	DQ246929	-	EU329567
	CVG461	Italy	<i>Rubus idaeus</i> cv. 'Diamond Jubilee'	MZ712976	MZ717165	-	MZ717180
	CVG462*	Italy	<i>Rubus idaeus</i> cv. 'Diamond Jubilee'	MZ712977	MZ717166	-	MZ717181
	CVG682	Italy	<i>Rubus idaeus</i> cv. 'Grandeur'	MZ712978	MZ717167	-	MZ717182
	CVG684	Italy	<i>Rubus idaeus</i> cv. 'Grandeur'	MZ712979	MZ717168	-	MZ717183
	CVG685	Italy	<i>Rubus idaeus</i> cv. 'Grandeur'	MZ712980	MZ717169	-	MZ717184
	CBS 144142 ^T	Italy	<i>Persea americana</i>	LT991940	LT991902	-	LT991909
	CBS 144143	Italy	<i>Persea americana</i>	LT991941	LT991903	-	LT991910
<i>N. petroliphila</i>	CBS 203.32	South Africa	<i>Pelargonium</i> sp.	DQ094320	DQ246835	-	LR583857
	CBS 398.66	Brazil	<i>Saccharum officinarum</i>	LR583749	LR583633	-	LR583859
	NRRL 22141	New Zealand	<i>Cucurbita</i> sp.	DQ094307	AF178329	-	EU329491
	NRRL 22142	USA	<i>Cucurbita</i> sp.	AF178411	AF178347	-	FJ240379
<i>N. pisi</i>	CBS 178.47	The Netherlands	<i>Pisum sativum</i>	LR583751	LR583634	-	LR583860
<i>N. pseudoradicicola</i>	CBS 145472 ^T	Papua New Guinea	Diseased cocoa pods	JF740899	JF740757	-	JF741084
	NRRL 25138	Papua New Guinea	Diseased cocoa pods	JF740900	JF740758	-	JF741085
<i>N. solani</i>	CBS 101018 ^T (ex-type of <i>N. rubicola</i>)	Italy	<i>Rubus idaeus</i>	LR583770	LR583651	-	LR583878
<i>N. spathulata</i>	CBS145474 ^T	USA	Human synovial fluid	EU329674	DQ246882	-	EU329542
<i>N. vasinfesta</i>	CBS 446.93	Japan	Soil	LR583791	LR583670	-	LR583898
<i>Paraconiothyrium africanum</i>	CBS 121,166	South Africa	<i>Prunus persica</i>	EU295650	-	JX496368	-
<i>P. archidendri</i>	CBS 168.77	Myanmar	<i>Archidendron bigeminum</i>	JX496049	-	JX496388	-
<i>P. brasiliense</i>	CBS 100299	Brazil	Culture from holotype	MH862698	-	JX496350	-
	STE-U 6300	South Africa	<i>Prunus persica</i>	EU295632	-	-	-
<i>P. cyclothyrioides</i>	CBS 972.95	Papua New Guinea	Unknown	JX496119	-	JX496458	-
<i>P. estuarinum</i>	CBS 109,850	Brazil	Culture from holotype	MH862842	-	JX496355	LT854937
<i>P. fuckelii</i>	CBS 508.94	-	<i>Rosa</i> sp.	JX496096	-	JX496435	-
	CBS 584.69	-	Gymnosperm	JX496098	-	JX496437	-
	CBS 764.71B	The Netherlands	Unknown	JX496112	-	JX496451	-
	CBS 797.95	The Netherlands	Unknown	JX496113	-	JX496452	-
	CVG482*	Italy	<i>Rubus idaeus</i> cv. 'Diamond Jubilee'	MZ712973	-	MZ717173	MZ717177
	CVG969	Italy	<i>Rubus idaeus</i> cv. 'Grandeur'	MZ712974	-	MZ717174	MZ717178
	CVG970	Italy	<i>Rubus idaeus</i> cv. 'Grandeur'	MZ712975	-	MZ717175	MZ717179
	CVG1017	Italy	<i>Rubus idaeus</i> cv. 'Grandeur'	MZ712972	-	MZ717172	MZ717176
	CBS 113269	Albania	Resupinate polypore fungus	JX496020	-	JX496359	-

Table 2 (continued)

Species	Culture No. ^a	Locality	Host	GenBank No. ^b			
				ITS	<i>tef1</i>	<i>tub2</i>	<i>rpb2</i>
<i>P. hawaiiense</i>	CBS 120025 ^T	USA	<i>Sophora chrysophylla</i>	DQ885897	-	JX496366	-
<i>P. sporulosum</i>	CBS 358.75A	The Netherlands	Unknown	AJ293815	-	-	-
<i>P. variabile</i>	CBS 120014	Italy	<i>Actinidia chinensis</i>	JX496026	-	JX496365	-
	CBS 119486	Italy	<i>Actinidia chinensis</i>	EF055359	-	JX496362	-
<i>Paraphaeosphaeria minitans</i>	CBS 860.71	Germany	Unknown	JX496117	-	JX496456	-

^aBRIP: Plant Pathology Herbarium, Department of Primary Industries, Dutton Park, Queensland, Australia; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; CVG: AGROINNOVA, Grugliasco, Torino, Italy; NRRL: Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, US Department of Agriculture, Peoria, IL, USA; STEU: University of Stellenbosch, Stellenbosch, South Africa. Ex-type and ex-epitype cultures are indicated with ^T

^bITS: internal transcribed spacers 1 and 2 together with 5.8S nrDNA; *tub2*: beta-tubulin gene; *tef1*: translation elongation factor 1-alpha gene; *rpb2*: RNA polymerase second largest subunit. Sequences generated in this study indicated in red. * Isolates used for phenotypic characterization and pathogenicity test

identification was achieved through DNA amplification and sequencing of a combined dataset of genes: the nuclear ribosomal internal transcribed spacer (ITS) region, partial regions of the β -tubulin (*tub2*), translation elongation factor-1 α (*tef1*) and RNA polymerase second largest subunit (*rpb2*) genes. The primers used for each locus found are reported in Table 3. The PCR amplification mixtures and cycling conditions adopted for the analyses of ITS, *tub2*, *tef1* conducted on *Paraconiothyrium* isolates were followed as described in Aveskamp et al. (2009) and Zhang et al. (2009). The ITS, *tub2*, *tef1* regions analysed for *Diaporthe* isolates were amplified according to PCR protocols reported by Guarnaccia and Crous (2018). For the isolates identified as *Neocosmospora* isolates, the protocols were adopted according to Guarnaccia et al. (2021) for the ITS, *tef1* and first part of *rpb2* loci. For the part of *rpb2* region amplified with the primers RPB2-7cf and RPB2-11ar, the PCR protocol by Yilmaz et al. (2020) was optimized as follows: 95 °C 1 min; 40 cycles of 95 °C 45 s, 52 °C 90 s, 72 °C

2 min; 72 °C 10 min and adding BSA to the mixture (1.5 μ l as final volume in a total volume of 25 μ l per sample). An amount of 5 μ l of PCR product for each PCR reaction was examined by electrophoresis at 100 V on 1% agarose (VWR Life Science AMRESCO^R biochemicals) gels stained with GelRedTM. PCR products were purified and 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; Kearse et al. 2012).

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

Table 3 Primers used in this study for molecular analyses

Locus	Primer name	Primer sequence 5'->3'	Reference
ITS	ITS1	TCCGTAGGTGAACCTGCGG	White et al. (1990)
	ITS4	TCCTCCGCTTATTGATATGC	
<i>rpb2</i>	RPB2-5f2	GGGGWGAYCAGAAGAAGGC	Liu et al. (1999)
	RPB2-7cr	CCCATRGCTTGYYTTRCCCAT	
	RPB2-7cf	ATGGGYAARCAAGCYATGGG	Sung et al. (2007)
	RPB2-11ar	GCRTGGATCTTRTCRTCSACC	
<i>tub2</i>	TUB2Rd	GTBCACCTYCARACCGGYCARTG	Aveskamp et al. (2009)
	TUB4Fd	CCRGAYTGRCCRAARACRAAGTTGTC	
<i>tef1</i>	EF1-728f	CATCGAGAAGTTTCGAGAAGG	Carbone and Kohn (1999)
	EF1-986r	TACTTGAAGGAACCCCTTACC	
	EF1-983f	GCYCCYGGHCAYCGTGAYTTYAT	Rehner and Buckley (2005)
	EF1-2218r	ACHGTRCCRATACCACCRATCTT	

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). After an initial analysis of the ITS locus (data not shown), a subset of representative isolates was selected to clearly distinguish the isolates at species level. Phylogeny was processed through different analyses conducted individually for each locus (data not shown) and as multilocus sequence analyses, as described above. The phylogenies were based on Bayesian 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 and Sullivan 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, consistency index, retention index and rescaled consistence index 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 2).

Phenotypic characterization

Agar plugs (5 mm diam.) of representative strains were taken from the edges of actively growing cultures on PDA-S and transferred onto the centre of 9 cm diam. Petri dishes containing PDA or malt extract agar (MEA; Oxoid). *Diaporthe eres* representative strain was also transferred on 1% water agar supplemented with sterile pine needles (PNA; Smith et al. 1996). All the plates were 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. Conidia 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 $\times 40$ magnification. The average and standard deviations of conidia dimensions were calculated.

Pathogenicity tests

Pathogenicity tests with representative isolates of the isolated species (*P. fuckelii*, *D. eres* and *N. parceramosa*) were performed to fulfil Koch's postulates. Representative isolates of each species (Table 2) were selected and inoculated on healthy raspberry plants (cv 'Grandeur' and 'Diamond Jubilee'). Each of the three fungal pathogens was inoculated onto 8 raspberry plants of each cultivar, with a total of 24 plants inoculated with pathogens. A sterile scalpel was used to remove the outer bark of the cane at 3–4 cm from the crown. Mycelium plugs (4 mm diam.) were taken from 10-day-old cultures on PDA and placed with the mycelium in contact with the internal plant tissues. The inoculation points were wrapped with Parafilm^R. A further 8 plants of each cultivar were treated with sterile PDA plugs as inoculation controls. All the plants were placed in a growth chamber at 24 °C for 6 weeks. After this period, the lengths of lesions developed on the internal cane tissues were measured after peeling off the bark. Fifteen days after inoculation, the symptom severity (SS) associated with each inoculated species was evaluated as the length of vascular discoloration which were measured upward and downward from the inoculation points. Small portions (0.3 cm) of symptomatic tissue from the edge of cane lesions were placed onto PDA-S 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 cane blight were observed in all raspberry orchards surveyed. All the investigated crops were covered by anti-hail nets and presented relative humidity condition up to 78%. Brown stripe lesions in correspondence of wounds were detected in the early stage of the disease. Later, the lesions progressively extended longitudinally through the canes, showing cracking and causing the death of buds and lateral shoots. In several cases, lesions affected the vascular tissues with consequent cane decay and blight. Considerable vascular damage and cane death occurred in all the orchards and on both the cultivars investigated (Fig. 1). DI was assessed

Fig. 1 Natural cane blight of *Rubus idaeus* 'Grandeur' in the field with wilt (**a**) and defoliation (**b**). External lesions (**c**, **e**), cracking (**d**) and vascular discoloration of the canes (**f**, **g**)

as reported in Table 1. Based on colony morphology, phylogenetic analyses, and conidia characteristics (see below), *D. eres* was isolated from plant of the cultivar ‘Diamond Jubilee’, whilst *P. fuckelii* and *N. parceramosa* were isolated from both ‘Diamond Jubilee’ and ‘Grandeur’ plants.

Phylogenetic analyses

The combined-locus phylogeny of *Paraconiothyrium* consisted of 21 sequences, *Diaporthe* consisted of 23 sequences and *Neocosmospora* of 40, including outgroups. A total of 1,457 characters (ITS: 1–569, *tub2*: 576–990 and *rpb2*: 997–1457) were included in the *Paraconiothyrium* phylogenetic analyses. A total of 1,400 characters (ITS: 1–554, *tub2*: 561–955 and *tefl*: 962–1400) were included in the *Diaporthe* phylogenetic analyses. The analyses for the *Neocosmospora* group consisted of 2,641 nucleotides (ITS: 1–467, *tefl*: 474–1131 and *rpb2*: 1138–2641). 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 4. Bootstrap support values from the parsimony analysis were plotted on the Bayesian phylogenies presented in Figs. 2, 3 and 4. Unique site patterns for each partition and all the parameters of the Bayesian analyses are reported in Table 4. For both of the Bayesian analyses, MrModeltest recommended the models reported in Table 5. In the *Paraconiothyrium* species analysis four isolates from symptomatic raspberry plants grouped

with four reference strains of *P. fuckelii* (Fig. 2), whilst two isolates of *Diaporthe* clustered with four reference strains of *D. eres* (Fig. 3). The final tree generated for *Neocosmospora* species showed that five isolates clustered with the type specimen and two reference strains of *N. parceramosa* (Fig. 4).

Phenotypic characterization

Morphological observations, supported by phylogenetic inference, were described for three known species (Fig. 5).

Paraconiothyrium fuckelii

Ten-day-old colonies on PDA and MEA were woolly to floccose with pale grey aerial mycelium. Reverse bay, fading over sienna in the centre to pale luteous at the margin on MEA and bay to whitish at the margin on PDA. Colony diameter: $3.57 \pm 0.06 \times 3.54 \pm 0.06$ µm on PDA and $3.54 \pm 0.15 \times 3.23 \pm 0.15$ µm on MEA. Conidia variable in shape, subglobose to ellipsoid or obovoid, olivaceous-brown, 0-septate, $4.2\text{--}2.7 \times 3\text{--}2.2$ µm, mean $\pm$ SD = $3.28 \pm 0.4 \times 2.62 \pm 0.2$

Diaporthe eres

Ten-day-old colonies on PDA and MEA flat with pale grey to white moderate aerial mycelium, reverse pale grey on PDA and with black patches in the centre on MEA.

Table 4 Parsimony and Bayesian analyses characteristics in this study

Phylogenetic analysis	Locus(i)	<i>Paraconiothyrium</i>	<i>Diaporthe</i>	<i>Neocosmospora</i>
		ITS + <i>tub2</i> + <i>rpb2</i>	TS + <i>tub2</i> + <i>tefl</i>	ITS + <i>tefl</i> + <i>rpb2</i>
Maximum Parsimony	Total sites	1445	1388	2529
	Constant sites	899	607	1783
	Variable sites	319	325	427
	Parsimony Informative sites	227	456	319
	Tree length	900	1910	1333
	Consistency index	0,791	0,66	0,685
	Retention index	0,784	0,708	0,708
	Rescaled consistence index	0,621	0,467	0,485
Bayesian inference	Unique site patterns of ITS	150	149	134
	Unique site patterns of <i>tefl</i>	-	296	177
	Unique site patterns of <i>tub2</i>	152	186	-
	Unique site patterns of <i>rpb2</i>	43	-	241
	Generation ran	655,000	216,000	175,000
	Generated trees	656	221	352
	Sampled trees	492	77	259

Fig. 2 Consensus phylogram of 656 trees resulting from a Bayesian analysis of the combined ITS, *tub2* and *rpb2* sequences of *Paraconiothyrium* strains. Bayesian posterior probability values and bootstrap

support values are indicated at the nodes. The tree was rooted to *Helminthosporium solani* (CBS 640.85). Type specimens are indicated in bold

Colony diameter: $8.5 \pm 0.05 \times 6.24 \pm 1.99$ μm on PDA and $5.87 \pm 0.20 \times 5.24 \pm 1.19$ μm on MEA. Conidial mass globose to ellipsoidal, conidiomata pycnidial, sporulating, globose to conical, scattered, dark brown to black, superficial to embedded. Alpha conidia aseptate, hyaline,

smooth, oblong to ellipsoid, apex bluntly rounded, guttulate $7.2\text{--}3.2 \times 3.0\text{--}1.9$ μm , mean $\pm$ SD = $5.8 \pm 1.0 \times 2.46 \pm 0.3$. Beta conidia hyaline, aseptate, smooth, spindle shaped, slightly curved $30.9\text{--}21.3 \times 1.4\text{--}0.8$ μm , mean $\pm$ SD = $26.5 \pm 2.2 \times 1.1 \pm 0.2$.

Fig. 3 Consensus phylogram of 221 trees resulting from a Bayesian analysis of the combined ITS, *tub2* and *tef1* sequences of *Diaporthe* strains. Bayesian posterior probability values and bootstrap support

values are indicated at the nodes. The tree was rooted to *Diaporthe corylina* (CBS 121124). Type specimens are indicated in bold

Fig. 4 Consensus phylogram of 352 trees resulting from a Bayesian analysis of the combined ITS, *tef1* and *rpb2* sequences of *Neocosmospora* strains. Bayesian posterior probability values and bootstrap

support values are indicated at the nodes. The tree was rooted to *Geejaysesia cicatricum* (CBS 125552). Type specimens are indicated in bold

Table 5 Evolutionary models as determined by MrModeltest (Nylander 2004)

Genus	Locus	Evolutionary model ^a
<i>Paraconiothyrium</i>	ITS	GTR+I+G
	<i>tub2</i>	K80+G
	<i>rpb2</i>	SYM
<i>Diaporthe</i>	ITS	SYM+I+G
	<i>tub2</i>	HKY+G
	<i>tef1</i>	GTR+I+G
<i>Neocosmospora</i>	ITS	SYM+G
	<i>tef1</i>	GTR+G
	<i>rpb2</i>	SYM+I+G

^aG: Gamma distributed rate variation among sites; GTR: Generalised time-reversible; HKY: Hasegawa-Kishino-Yano; I: Proportion of invariable sites; K80: Kimura's two parameter model; SYM: Symmetrical model

Neocosmospora parceramosa

Ten-day-old colonies on PDA flat, cottony to felty with white to pale straw aerial mycelium and entire margin, reverse straw to honey. Colony diameter: $5.43 \pm 0.06 \times 5.50 \pm 0.10$ µm. Colonies produced microconidia ellipsoidal, subcylindrical to clavate, straight, hyaline, $7.8\text{--}4.2 \times 3.4\text{--}2.3$ µm, mean $\pm$ SD = $5.75 \pm 0.98 \times 2.88 \pm 0.35$ and macroconidia gently curved, 3–5-septate, hyaline, $45.3\text{--}31.9 \times 4.9\text{--}3.8$ µm, mean $\pm$ SD = $40.39 \pm 3.44 \times 4.50 \pm 0.36$.

Pathogenicity tests

After 60 days, all the tested isolates caused lesions and internal discolouration on the inoculated plants. In some cases, a severe dieback was observed and plant death occurred. Symptoms were similar to those found in the field-grown plants (Fig. 6). Re-isolation of pathogens was obtained with a frequency of grown colonies from small portions of affected tissues placed onto PDA-S between 90 and 95%. The identification of the re-isolated species was confirmed using culture and molecular features, fulfilling Koch's postulates. *Paraconiothyrium fuckelii* has revealed to be the most aggressive species on both the inoculated cultivars causing vascular lesion of average length 5.1 and 4.0 cm on 'Grandeur' and 'Diamond Jubilee', respectively. Moreover, *P. fuckelii* caused the death of 2 of the inoculated 'Grandeur' and 1 of 'Diamond Jubilee' plants. *Diaporthe eres* and *N. parceramosa* caused less severe symptoms with mean vascular lesions length of 3.0 and 2.7 cm on 'Grandeur' and 'Diamond Jubilee', respectively, and 1.3 cm on both tested cultivars. The pairwise comparison

obtained from the Kruskal–Wallis test, showed significant differences ($P < 0.05$) among the three isolates (Fig. 7).

Discussion

The increasing interest in berry fruit production promoted the development of new and improved commercial cultivars through several breeding programmes (Foster et al. 2019). Moreover, an intensification of the production systems through agronomic practices, use of irrigation systems and use of fertilizers, occurred to supply the consumers demand (Graham and Brennan 2018; Mezzetti 2014). However, the consequent consistent movement of plant materials and fruit favoured the spread of emerging diseases in different production areas (Dolan et al. 2018; Guarnaccia et al. 2020).

The present study was conducted in the most important fruit production area of Piedmont where a strong interest for raspberry cultivation is present. The study provides an investigation of the fungal pathogens associated with raspberry cane blight observed during the conducted surveys. *Paraconiothyrium fuckelii*, generally considered the causal agent of cane blight (Martin et al. 2017), was consistently isolated from symptomatic raspberry canes. Moreover, *D. eres* and *N. parceramosa* were found in association with cane blight disease and produced symptoms on artificially inoculated raspberry plants. The occurrence of different fungal species demonstrated the presence of a complex of fungi involved in the development of raspberry cane diseases as described by Girichev et al. (2018). Thus, considering the difficulty to distinguish different pathogens from symptoms or based on morphological traits, it becomes necessary the use of molecular tools to provide a correct identification (Girichev et al. 2018). The multi-locus analyses conducted in this work identified the three fungal pathogens. *Paraconiothyrium fuckelii* is known worldwide as the only causal agent of raspberry cane blight disease. It has been reported, with its previous taxonomic synonyms, on *R. idaeus* in Australia, Bulgaria, Canada, New Zealand, Poland, Russia and USA (Farr and Rossman 2021). In Italy, it has been found in association with withered grapes (Lorenzini et al. 2016).

Diaporthe spp. are important plant pathogens reported in association with stem bight and cankers of different fruit trees (Guarnaccia et al. 2020; Hilário et al. 2020; Yang et al. 2018). Their identification, mainly based on morphological traits and plant hosts, has resulted in a high number of described species. Thus, during the last years, multilocus phylogenies were adopted to reassess species within *Diaporthe* genus (Santos et al. 2017). The genomic loci sequencing provided in this study demonstrated the identification of

Fig. 5 Morphological characteristics of the different fungal species grown for 10 d on PDA. **a, b** and **c** *Paraconiothyrium fuckelii*; **d, e** and **f** *Diaporthe eres*; **g, h** and **i** *Neocosmospora parceramosa*. Scale bars = 10 μ m

the isolated strains as *D. eres*. This species has been reported in association with raspberry cane disease in Germany and on other *Rubus* spp. in Austria, Croatia, France and Ireland (Farr and Rossman 2021). In Italy it has been isolated in association with stem blight on other fruit trees such as *Castanea vesca*, *Ficus carica*, *Juglans regia*, *Prunus persica*, *Pyrus communis*, *Sambucus nigra* and *Vitis vinifera* (Bertetti et al. 2018; Dissanayake et al. 2017; Prencipe et al. 2017).

Several *Neocosmospora* spp. have been reported in Italy as pathogenic on fruit crops such as *Citrus* spp., *Ficus carica* and *Persea americana* (Guarnaccia et al. 2018, 2019; Sandoval-Denis et al. 2018), however, *N. parceramosa* (synonym: *Fusarium parceramosum*) has been mainly isolated as presumed human pathogen and as present in plumbing systems, whilst the type specimen (CBS 115,695) was obtained from a soil sample collected in South Africa

Fig. 6 Death of raspberry plant cv ‘Diamond Jubilee’ inoculated with *Paraconiothyrium fuckelii* compared with healthy control plant (a). Internal discoloration of plant cv ‘Grandeur’ with *Diaporthe eres* (b) and *P. fuckelii* (c)

(Sandoval-Denis et al. 2019). The correct identification of this species was achieved by adding the second part of the *rpb2* region to the multi-locus sequence alignments analysed. Thus, demonstrating the importance of molecular diagnostic tools as reliable, objective, and necessary for correct species analysis. To our knowledge, this work represents the first report of *N. parceramosa* on *R. idaeus* in Italy and worldwide, and the first report of *P. fuckelii* and *D. eres* on raspberry in Italy.

The species collected during the surveys were confirmed as pathogenic. However, a different susceptibility among the tested cultivars was registered. Plants of the cv ‘Grandeur’ were consistently affected by the fungal pathogens and cane death occurred abundantly, whilst lower lesion length was observed on ‘Diamond Jubilee’ plants cv. Varietal resistance is a fundamental point to consider before orchard establishment, along with the use of certified plant materials. Favourable temperatures and high levels of relative humidity can also play a major role, thus, the interaction between found pathogens and environment temperature should be assayed. Moreover, good agronomic practices should be adopted to minimize the occurrence of wounds that can facilitate the pathogens entry within the vascular tissues. Providing correct distances among plants and regular cane thinning, reducing wound formation during berry harvesting, removing pruning debris and infected canes could be useful methods to reduce fungal pathogen spread, thus should be investigated.

Furthermore, it should be considered that weather conditions could promote pathogen and disease development.

Studies conducted on *D. eres* in Greece showed that this pathogen has an optimal growth at 25 °C, however, considerable mycelial growth was assessed within the range 20–30 °C (Thomidis and Michailides 2009). Other analyses on water availability and relative humidity confirmed the influence of these abiotic factors on *D. eres* mycelial growth and conidia germination (Arciuolo et al. 2021). Wet and humid conditions have been reported as favourable also for *Paraconiothyrium fuckelii* development (Martin et al. 2017). Thus, periods with high temperature and high relative humidity could contribute to the spread of this pathogen. Moreover, cross infections within different plant hosts might occur, concurring to the diffusion of the disease among different orchards (Guarnaccia and Crous 2018). Further studies are needed to understand the epidemiology of raspberry cane blight with aim of monitoring disease occurrence and to develop prediction models for the adoption of effective management strategies.

In conclusion, the present work is the first insight into raspberry cane blight in Italy. The provided phylogenetic analyses and genomic loci sequencing set the basis for the development of species-specific diagnostic protocols to detect pathogen presence early in nursery and orchard environment. Further analyses are necessary to establish the role of abiotic factors and the relationships within the fungal species found in disease development and spread. The investigation of these aspects could permit the adoption of more sustainable management strategies and contribute to the constant reduction of chemical fungicides.

Fig. 7 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 three representative isolates. Significant difference was accepted for $P < 0.05$. ° Outliers. *: Extreme values

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request. The data that support the findings of this study are available from.

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