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ORIGINAL ARTICLE

Colletotrichum spp. causing anthracnose on ornamental plants in northern Italy

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Abstract

Species of *Colletotrichum* are considered among the most important plant pathogens, saprobes and endophytes on a wide range of ornamentals, fruits and vegetables. Several *Colletotrichum* species have been reported in nurseries and public or private gardens in northern Italy. In this study, the occurrence, diversity and pathogenicity of *Colletotrichum* spp. associated with several ornamental hosts was explored. Survey were carried out during the 2013–2019 period in Piedmont, Italy. A total of 22 *Colletotrichum* isolates were collected from symptomatic leaves and stems of two *Campanula* spp., *Ceanothus thyrsiflorus*, *Coreopsis lanceolata*, *Cyclamen persicum*, *Hydrangea paniculata*, *Liquidambar styraciflua*, *Mahonia aquifolium* and *Rhyncospermum jasminoides*. A multi-locus phylogeny was established based on the basis of three genomic loci (*gapdh*, *act* and *tub2*). The pathogenicity of selected, representative isolates was tested. *Colletotrichum* isolates were identified as members of four important species complexes: *Acutatum*, *Gloeosporioides*, *Dematium* and *Destructivum*. *Colletotrichum fioriniae*, *C. nymphaeae* and *C. fuscum* were found in association with leaf lesions of *Mahonia aquifolium*, *Campanula rapunculoides* and *Coreopsis lanceolata*, respectively. *Colletotrichum lineola*, *C. grossum* and *C. cigarro* were isolated from *Campanula trachelium*, *Rhyncospermum jasminoides* and *Liquidambar styraciflua*, respectively. *Colletotrichum fructicola* was found to be responsible of anthracnose of *Ceanothus thyrsiflorus*, *Hydrangea paniculata*, *Cyclamen persicum* and *Liquidambar styraciflua*. All the tested isolates were pathogenic and reproduced identical symptoms to those observed in private gardens and nurseries. The present study improves our understanding of *Colletotrichum* spp. associated with different ornamental hosts and provides useful information for an effective disease management programme.

Keywords Leaf spot · Multi-locus sequence typing · Pathogenicity · Species complex

Introduction

The genus *Colletotrichum* has been reported as one of the ten most important plant pathogens in the world based on economic importance (Dean et al. 2012). Anthracnose disease caused by species members of this genus could affect several plants from woody to herbaceous ones, producing significant economic losses in tropical, subtropical and temperate regions. They can develop on fruit, leaves, stems, tubers and seedlings in the field or in greenhouse environment and it is also important as causing post-harvest disease on fruit and vegetables (Cannon et al. 2012; Damm et al. 2012a, b; Udayanga et al. 2013). *Colletotrichum* spp. show different lifestyles which can be generally classified as necrotrophic, hemibiotrophic, latent and endophytic (De Silva et al. 2017). Detection and control of diseases caused by *Colletotrichum* spp. could be difficult due to the complex life cycle of many species, to the ability to change lifestyle and to the potential cross infection of different plant hosts (O'Connell et al. 2012).

After the advent of the molecular era, the adoption of multigene phylogenetic analyses, combined with the traditional morphology-based identification methods, led to a deep revision within the taxonomy of this genus. Several major studies (Cannon et al. 2008; Cai et al. 2009; Damm et al. 2009; 2012a, b; 2013; 2014; Weir et al. 2012) significantly changed the classification and species concepts in *Colletotrichum*. Currently, 14 *Colletotrichum* species complexes (SC) and

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more than 24 singleton species have been identified (Cannon et al. 2012; Marin-Felix et al. 2017). In plant pathology, the most relevant species are part of the *C. gloeosporioides* (Cannon et al. 2008; Weir et al. 2012), *C. acutatum* (Shivas and Tan 2009; Damm et al. 2012a), *C. boninense* (Damm et al. 2012b) and *C. truncatum* (Damm et al. 2009; Cannon et al. 2012) species complexes. Moreover, the *C. destructivum* (Damm et al. 2009) and *C. dematium* (Damm et al. 2014) SC includes important plant pathogens.

Ornamental plants include flowering, bedding potted and garden plants, evergreen and deciduous trees, foliage plants, woody ornamentals, shrubs, herbaceous perennials and cut flowers along with nursery crops, cut cultivated greens and propagation material (Gullino and Garibaldi 2007; Chase et al. 2018). Ornamental plants are usually cultivated in gardens and for commercial purpose, which has increased globally over the past few decades. In Italy, the value of floriculture amounts to more than 2.5 billion euros, half of which comes from the production of flowers and potted plants. There are 27 thousand companies involved in the sector, distributed in almost 29,000 hectares of agricultural land overall (Croci 2018). During the last several decades, this sector has passed through different technological changes, such as environmental and nutrition controls, which have pushed plants to their limits of growth and productivity. Thus, new limiting factors have been generated related to disease occurrence and management, and ornamental plants are more conducive to several pathogens and diseases (Guarnaccia et al. 2014; Gullino et al. 2015).

Several *Colletotrichum* spp. have been detected on ornamental plants from southern to northern Italy (Polizzi et al. 2011; Camele et al. 2018; Guarnaccia et al. 2019; Gilardi et al. 2015; Garibaldi et al. 2016) and particular environmental conditions, such as high humidity and temperature, play a major role in development and sporulation of these pathogens (Cannon et al. 2012).

The present study refers to anthracnose symptoms detected on nine different ornamental host plants: *Liquidambar styraciflua* (American sweetgum), *Ceanothus thyrsiflorus* (blue blossom), *Hydrangea paniculata* (hortensia), *Cyclamen persicum* (cyclamen), *Rhyncospermum jasminoides* (star jasmine), *Mahonia aquifolium* (Oregon grape), *Coreopsis lanceolata* (lanceleaf coreopsis) and two *Campanula* spp. (campanula).

Cyclamen persicum is a flowering herbaceous perennial, appreciated as an indoor plant cultivated for its attractive white to pink to deep red flowers (Takamura 2007). American sweetgum (*L. styraciflua*) is a deciduous tree, native to North America and popular as ornamental tree in temperate climates (Brand and Lineberger 1992). *Coreopsis lanceolata* and *Campanula* spp. are perennial plants, largely cultivated in borders or gardens and usually planted in mass or groups, known as bedding plants (Lee 2012; Scariot et al. 2012). *Rhyncospermum jasminoides* is an evergreen woody, climbing liana (Weaver and Anderson 2012). Hortensia is a deciduous shrub native to China and Japan (Lancaster and Wesley 2008), whilst, *C. thyrsiflorus* and *M. aquifolium* are evergreen shrubs. These bushes are used in mixed shrub borders or in open woodland gardens (Schmidt 2006; Garibaldi et al. 2020).

Thus, considering the important value of ornamental crops, the relevance of this agriculture sector in Italy and the impact of *Colletotrichum* spp. on these hosts, surveys were conducted over a 5-year period in Northern Italy. The aims of this study were: (i) to characterize species of *Colletotrichum* isolated from ornamentals hosts combining multi-locus phylogenetic analysis with morphological features and (ii) to evaluate the pathogenicity of *Colletotrichum* species on the host plants from which they were isolated.

Material and methods

Field surveys and fungal isolation

Samples were obtained from symptomatic plants grown in a private garden near Biella in Northern Italy (45°36'N 8°03'E) and in a nursery specialized in the production and sale of young ornamentals in northern Italy, with appropriate permissions. The garden is subjected to the continuous survey as a representative site exposed to the introduction of new plant pests, being cultivated several exotic plants.

Anthracnose symptoms consisting of brown to black lesions were detected on leaves of two *Campanula* spp., *Ce. thyrsiflorus* (blue blossom), *Cor. lanceolata*, *Cy. persicum*, *H. paniculata* (hortensia), *L. styraciflua* (American sweetgum), *M. aquifolium* (Oregon grape), *R. jasminoides* and on stem and leaves of *Cy. persicum* between 2013–2019 period. During the field survey, the disease incidence was recorded.

Samples from around 20 plants per species which showed anthracnose were randomly collected. Small sections (0.2–0.5 cm long) from the lesion margin were surface disinfected with 1% sodium hypochlorite for 1 min, then rinsed once in sterile distilled water (SDW), dried on sterile paper and placed on potato dextrose agar (PDA, Oxoid, Basingstoke, England) plates amended with 25 ppm streptomycin sulphate (PDA + A. Sigma-Aldrich, St. Louis, MO, USA). The plates were incubated at 25 ± 1 °C under a 12 h photoperiod. After an incubation of 48 to 72 h, hyphae from the margin of the colonies resembling *Colletotrichum* spp. were placed on PDA plates. Five days later, single spores were transferred into PDA plates to establish pure cultures.

A total of 22 isolates were obtained and used for molecular characterization (Table 1). Stock cultures are maintained in

15% glycerol solution at − 80 °C in the Agroinnova (University of Torino) culture collection, Torino, Italy.

DNA extraction, PCR amplification and sequencing

Total DNA was extracted for all *Colletotrichum* isolates using the E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek, Darmstadt, Germany), following the manufacturer's instructions. Partial regions of three loci were amplified. The partial glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), actin (*act*) and beta-tubulin (*tub2*) genes were amplified using GDF1 and GDR1 (Guerber et al. 2003), ACT-512F and ACT-783R (Carbone and Kohn 1999), and T1 (Glass and Donaldson 1995) and Bt-2b (O'Donnell and Cigelnik 1997) primers, respectively. The amplification mixtures of polymerase chain reaction (PCR) and relative cycling conditions were adopted for all three loci according to Guarnaccia et al. (2019). PCR products were sequenced by Eurofins Genomics Service (Ebersberg, Germany). The generated sequences were analysed and assembled with the program Geneious v. 11.1.5 (Auckland, New Zealand).

Phylogenetic analyses

The sequences obtained in this study were analysed using the NCBIs GenBank database through the "BLAST" command, then compared with reference sequences downloaded from GenBank, aligned by using the MAFFT v. 7 online servers (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 analyses were conducted for individual locus (data not shown) and as concatenated analyses of three loci with the aim to identify the isolates to the species level. Additional reference sequences were selected based on recent studies on *Colletotrichum* species (Damm et al. 2009; 2012a, b; Weir et al. 2012; Guarnaccia et al. 2017). The phylogeny was developed based on Maximum Parsimony (MP) for all individual loci, and based on both MP and Bayesian Inference (BI) for the combined multilocus analyses. For BI, the best evolutionary model was suggested by MrModeltest v. 2.3 (Nylander 2004) for each partition. MrBayes v. 3.2.5 (Ronquist et al. 2012) was used to obtain phylogenetic trees with 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 established at 0.2 and trees were sampled every 1000 generations. The analyses were considered done when the average standard deviation of split frequencies was less than 0.01. The MP analyses were conducted using PAUP (Swofford 2003). Phylogenetic relationships were estimated by heuristic searches with 100 random additional sequences. Tree bisection-reconnection was adopted, with the branch swapping option set at 'best trees' only with all characters weighted equally and alignment gaps treated as fifth state. Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) were calculated to set the 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).

Morphology

Agar plugs (5-mm-diam) of representative strains were taken from the edge of 10-day-old cultures and transferred to the centre of 9-cm-diam Petri dishes containing PDA. Plates were incubated at 25 ± 1 °C under 12 h photoperiod for 10 d. Three cultures plates of each isolate were investigated. Colony characters, colors and diameter were observed/measured after 10 d. Cultures were examined over time for ascomata, conidiomata and setae development. The morphological characteristics were examined by mounting fungal structures in water and 30 measurements at 400X magnification were determined for each isolate using a Nikon Eclipse 55i microscope.

Pathogenicity

One representative isolate of each *Colletotrichum* species identified was selected and inoculated on the same host that was isolated. The isolates were grown on PDA with streptomycin sulphate (25 mg/L) and kept at 25 °C with a 12 h photoperiod for 7 days.

Five 1-year old plants for each plant species were used. Leaves of the plant hosts were sprayed with conidia suspensions (final concentration of 10^6 conidia/ml). The pathogenicity trials were established both on wounded and unwounded leaves. Leaves were wounded at the centre of the adaxial surface using a sterile needle. SDW and sterile PDA plugs (5 mm diam) were used for control plants. The plants were covered with a transparent plastic film to keep a high level of relative humidity (RH) and kept in a growth chamber at 25 °C with a 12 h photoperiod. The plastic film was removed three days post-inoculation (dpi). The trial was repeated once. A disease severity (DS) index was adopted to rank the plants after 7 to 10 dpi according to these values: 0 indicated healthy plants, 25, low virulent lesions and slight leaf chlorosis, 50, moderate presence of typical anthracnose on leaves, 100, high presence of necrotic spots and dead plants. The pathogen aggressiveness for each host was classificated as = Low L (disease index 0–25); Average = M (disease index 26–50); High H (disease index > 51) (Guarnaccia et al. 2019). The inoculated fungi were re-isolated and identified by sequencing the *gapdh* locus, thus fulfilling Koch's postulates.

Table 1 Collection details of *Colletotrichum* isolates, aggressiveness expressed after the pathogenicity test and GenBank accession numbers of other *Colletotrichum* isolates included in this study

Species	Culture No.[1]	Host	Locality	Aggressiveness	GenBank No.[2]		
					gapdh	*tub2*	*act*
C. abscissum	COAD 1877^T	*Citrus sinensis*	Brazil	_	KP843129	KP843135	KP843141
C. acutatum	CBS 112996^T	*Carica papaya*	Australia	_	JQ948677	JQ005860	JQ005839
C. antirrhinicola	CBS 102189^T	*Antirrhinum majus*	New Zeland	_	KM105531	KM105460	KM105390
C. boninense	CBS 123755^T	*Crinum asiaticum* var *sinicum*	Japan	_	JQ005240	JQ005588	JQ005501
C. brisbanense	CBS 292.67^T	*Capsicum annuum*	Australia	_	JQ948621	JQ949942	JQ949612
C. bryoniicola	CBS 109849	*Bryonia dioica*	Netherlands	_	KM105532	KM105461	KM105391
C. chrysanthemi	CBS 126519	*Chrysanthemum coronarium*	Netherlands	_	JQ948602	JQ949923	JQ949593
C. cigarro	ICMP 18539^T	*Olea europaea*	Australia	_	JX009966	JX010434	JX009523
	CVG217*	*Liquidambar styraciflua*	Italy	M	*MT292042*	*MT292081*	*MT292062*
C. circinans	CBS 221.81^T	*Allium cepa*	Serbia	_	GU228247	GU228149	GU227953
C. citri	CBS 134233^T	*Citurs aurantiifolia*	China	_	KC293741	KC293661	KC293621
C. coccodes	CBS 126378	*Solanum tuberosum*	South Africa	_	JX546739	JX546882	JX546643
C. conoides	CGMCC 3.17615^T	*Capsicum annuum*	China	_	KP890162	KP890174	KP890144
C. dematium	IMI 350847	*Solanum tuberosum*	Australia	_	GU228217	GU228119	GU227923
	CBS 125340	*Apiaceae*	Czech Republic	_	GU228212	GU228114	GU227918
	CBS 125.5	*Eryngium campestre*	France	_	GU228211	GU228113	GU227917
C. destructivum	CBS 136228	*Crupina vulgaris*	Greece	_	KM105574	KM105499	KM105429
C. fioriniae	ATCC 28992	*Malus domestica*	USA	_	JQ948627	JQ949948	JQ949618
	CBS 129916	*Vaccinium* sp.	USA	_	JQ948647	JQ949968	JQ949638
	CBS 293.67	*Persea americana*	Australia	_	JQ948640	JQ949961	JQ949631
	19/18*	*Mahonia aquifolium*	Italy	M	*MT292057*	*MN520416*	*MN520417*
C. fructicola	ICMP 18581, CBS 130416^T	*Coffea arabica*	Thailand	_	JX010033	JX010405	FJ907426
	LC 2923	*Camellia sinensis*	China	_	KJ954784	KJ955232	KJ954365
	CBS 238.49	*Ficus edulis*	Germany	_	JX009923	JX010400	JX009495
	CVG 212*	*Ceanothus thrysiflorus*	Italy	H	*MT292041*	*MT292080*	*MT292061*
	CVG 218	*Liquidambar styraciflua*	Italy	M	*MT292043*	*MT292082*	*MT292063*
	CVG 220	*Liquidambar styraciflua*	Italy	_	*MT292044*	*MT292083*	*MT292064*
	CVG 221*	*Liquidambar styraciflua*	Italy	_	*MT292045*	*MT292084*	*MT292065*
	CVG 224*	*Hydrangea paniculata*	Italy	L	*MT292046*	*MT292085*	*MT292066*
	CVG 225	*Hydrangea paniculata*	Italy		*MT292047*	*MT292086*	*MT292067*
	CVG 270*	*Cyclamen persicum*	Italy	M	*MT292048*	*MT292087*	*MT292068*
	CVG 271	*Cyclamen persicum*	Italy	_	*MT292049*	*MT292088*	*MT292069*
	CVG 272	*Cyclamen persicum*	Italy	_	*MT292050*	*MT292089*	*MT292070*
	CVG 273	*Cyclamen persicum*	Italy	_	*MT292051*	*MT292090*	*MT292071*
	CVG 274	*Cyclamen persicum*	Italy	_	*MT292052*	*MT292091*	*MT292072*
	CVG 275	*Cyclamen persicum*	Italy	_	*MT292053*	*MT292092*	*MT292073*
	CVG 276	*Cyclamen persicum*	Italy	_	*MT292054*	*MT292093*	*MT292074*
C. fuscum	CBS 133701^T	*Digitalis lutea*	Germany	_	KM105524	KM105454	KM105384
	62-1*	*Coreopsis lanceolata*	Italy	M	*MT013215*	*MN615837*	*MN615838*
C. gloeosporioides	ICMP17821, CBS 112999	*Citrus sinensis*	Italy	_	JX010056	JX010445	JX009531
C. godetiae	CBS 133.44	*Clarkia hybrida*	Denmark	_	JQ948733	JQ950053	JQ949723
C. grevilleae	CBSB 132879^T	*Grevillea* sp.	Italy	_	KC297010	KC297102	KC296941
C. grossum	CGMCC 3.17614^T	*Capsicum annuum*	China	_	KP890159	KP890171	KP890141
	CVG437*		Italy	L	*MT292055*	*MT292094*	*MT292075*

Table 1 (continued)

Species	Culture No.[1]	Host	Locality	Aggressiveness	GenBank No.[2] *gapdh*	*tub2*	*act*
		Rhyncospermum jasminoides					
	CVG443	*Rhyncospermum jasminoides*	Italy		*MT292056*	_	*MT292076*
C. helleniense	CBS 142418, CPC 26844	*Pncirus trifoliata*	Greece	_	KY856270	KY856528	KY856019
C. hemerocallidis	CBS 130642^{T}	*Hemerocallis fulva var fulva*	China	_	JQ400012	JQ400019	JQ399991
C. jiangxiense	CGMCC 3.17363^{T}	*Camellia sinensis*	China	_	KJ954902	KJ955348	KJ95447
C. kahawae	ICMP 17816	*Coffea arabica*	Kenya	_	JX010012	JX010444	JX009452
C. lineola	CBS 125337^{T}	*Apiaceae*	Czech Republic	_	GU228221	GU228123	GU227927
	CVG207*	*Campanula trachelium*	Italy	L	*MT292038*	*MT292077*	*MT292058*
	CVG208	*Campanula trachelium*	Italy	_	*MT292039*	*MT292078*	*MT292059*
	CVG209	*Campanula trachelium*	Italy	_	*MT292040*	*MT292079*	*MT292060*
C. lini	CBS 172.51^{T}	*Linum usitatissimum*	Netherlands	_	KM105581	JQ005849	JQ005828
C. nigrum	CBS 127562	*Cichorium intybus*	Chile	_	JX546746	JX546889	JX546650
C. nupharicola	CBS 470.96, ICMP 18187	*Nuphar lutea* subsp. *polysepala*	USA	_	JX009972	JX010398	JX009437
C. nymphaeae	CBS 119294	*Leucaena* sp.	Mexico	_	JQ948535	JQ949856	JQ949526
	CBS 515.78	*Nymphaeae alba*	Netherlands	_	JQ948527	JQ949848	JQ949518
	19/27*	*Campanula rapunculoides*	Italy	M	*MN551611*	*MN551609*	*MN551610*
C. ocimi	CBS 298.94	*Ocimum basilicum*	Italy	_	KM105577	KM105502	KM105432
C. theobromicola	ICMP 18649^{T}	*Theobroma cacao*	Panama	_	JX010006	JX010447	JX009444
C. vignae	CBS 501.97	*Vigna unguiculata*	Nigeria	_	KM105534	KM105463	KM105393
Moniolochaetes infuscans	CBS 869.96	*Ipomoea batatas*	South Africa	_	JX546612	JQ005864	JQ005843

[1] ATCC: American Type Culture Collection, Virginia, USA; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; CGMCC: China General Microbiological Culture Collection Center, Beijing, China; CVG: Agroinnova, Grugliasco, Torino, Italy; COAD: Coleção Octávio Almeida Drummond, Viçosa, Brazil; 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. Ex-type and ex-epitype cultures are indicated with T.

[2] *gapdh*: glyceraldehyde-3-phosphate dehydrogenase gene; *tub2*: beta-tubulin gene; *act*: actin gene. Sequences generated in this study indicated in *italics*.

*Isolates used for pathogenicity experiments and morphology description.

Results

Field surveys and fungal isolation

Typical anthracnose symptoms caused by *Colletotrichum* spp. were found on different ornamental hosts (Table 1) and were identified as those caused by *Colletotrichum* spp. Disease incidence was established considering the percentage of affected leaves and varied from 15 to 40%, depending on environmental conditions and the species of the host plants.

Symptoms were detected on leaves of *Ca. rapunculoides*, *Cor. lanceolata M. aquifolium Ca. trachelium*, *Ce. thyrsiflorus*, *H. paniculata*, *L. styraciflua* on plants grown outdoors in a private garden, and on different cultivars of *Cy. persicum* and *R. jasminoide*s grown in nursery at temperature from 18 to 28 °C.

Brown to black necrotic lesions were observed on leaves on all the investigated host species. On *Ca. trachelium*, the first symptoms consisted of as small, circular spots, subsequently becoming irregular, expanded to dark lesions surrounded by a chlorotic halo. Chlorotic leaves with light brown, irregular necrotic spots were detected, along with affected petioles on 7–8 month-old plants of *Ca. rapunculoides*. All the affected tissues rotted and became dry. Leaf blight with black, irregular necrosis with a well-defined margin that expanded up was observed on *Co. lanceolata*. Whilst, circular brown spots with chlorotic halo developing at margin were observed on cyclamen, as well as on blueblossom plants.

Brown necrotic lesions, 10–60 mm in diameter, with dark irregular margin were found on 30 years old plants of *L. styraciflua*. Warm temperature and HR promoted the development and the spread of the lesions. Regarding *H. paniculata*, initial pale brown irregular lesions were observed on the apical leaf margin that subsequently spread covering the entire leaf surface. On *M. aquifolium* were observed irregularly circular, brown, slightly sunken, necrotic lesions surrounded by a chlorotic halo. Lesions enlarged up to 10 mm in diameter and eventually coalesced. Small dark brown necrotic spots were detected on leaves of 2-year-old plants of *R. jasminoides* cultivated in commercial nursery.

Phylogenetic analyses

Four alignments were analysed representing single gene analyses of *act*, *gapdh*, *tub2* and a combined alignment of the three genes. The alignments produced topologically similar trees. The combined species phylogeny of the *Colletotrichum* isolates consisted of 63 sequences, including the outgroup sequences of *Moniolochaetes infuscans* (CBS 896.96).

A total of 1065 characters (*gapdh*: 1–308, *act*: 315–611, *tub2*: 618–1065) were included in the phylogenetic analysis, 521 characters resulted as parsimony-informative, 148 as variable and parsimony-uninformative, and 384 were constant. A maximum number of 1000 equally most parsimonious trees were saved (Tree length = 1846, CI = 0.660, RI = 0.933 and RC = 0.616). Bootstrap support values obtained with the parsimony analysis are showed on the Bayesian phylogenies in Fig. 1. For the Bayesian analyses, the dirichlet state frequency distributions were suggested by MrModeltest for analysing all the partitions. The following models, recommended by MrModeltest, were used: HKY + G for *act*, and GTR + G for *gapdh* and *tub2*. In the Bayesian analysis, the *gapdh* partition had 268 unique site patterns, the *act* partition had 179 unique site patterns, the *tub2* partition had 216 unique site patterns and the analysis ran for 1.295.000 generations, resulting in 2592 trees of which 1944 trees were used to calculate the posterior probabilities. In the combined analyses twelve isolates (seven from *Cy. persicum*, three from *L. styraciflua* and two from *H. paniculata*) clustered with two reference strains and the ex-type of *Col. fructicola*, whilst one isolate from *L. styraciflua* and two isolates from *R. jaminoides* were identified as *Col. cigarro* and *Col. grossum*, respectively. Three isolates from *Ca. trachelium* clustered with the epitype of *Col. lineola*. Three isolates from *M. aquifolium*, *Ca. rapunculoides* and *Cor. lanceolata* were identified as *Col. fioriniae*, *Col. nymphaeae* and *Col. fuscum*, respectively.

Morphology

Morphological observations, supported by phylogenetic inference, were used to characterize the six known species described below. Culture characteristics were observed and the colour of upper and lower surfaces of Petri dishes assessed.

Colletotrichum fioriniae

Asexual morph on PDA. Conidiomata acervular. Hyphae hyaline, septate and branched. Setae not observed. Conidia hyaline, ellipsoid with rounded ends, with guttulae, produced in abundance after 10 days growing on media. Conidia size (mean ± SD): 13.03 ± 1 × 4.5 ± 0.6 μm. Culture characteristics — colonies velvety to cottony and flat with salmon-like coloured in the centre to pale grey and white at the margin mycelium. Orange spore masses. Reverse orange – pale brown to white at the margin. Colony diameter: 68–68 mm in 10 d.

Colletotrichum fructicola

Asexual morph on PDA. Conidiomata acervular. Hyphae hyaline, septate and branched. Setae not observed. Conidia hyaline, ellipsoid with rounded ends, with guttulae, produced in abundance after 10 days growing on media. Conidia size (mean ± SD): 16.3 ± 1.6 × 4.9 ± 0.6 μm. Culture characteristics — flat colonies cottony to floccose with entire margin, white - light grey mycelium. Reverse black with a pale circle at the margin. Colony diameter: 85–85 mm in 10 d.

Colletotrichum fuscum

Asexual morph on PDA. Conidiomata acervular. Hyphae hyaline, septate and branched. Setae not observed. Conidia hyaline, cylindrical to ellipsoid with rounded ends, with guttulae, produced in abundance after 10 days growing on media. Conidia size (mean ± SD): 16.8 ± 2.5 × 4.0 ± 0.4 μm. Culture characteristics — colonies velvety to powdery and flat black colored in the center to pale grey and with white margin. Black spore masses. Reverse dark grey to light gray at the margin. Colony diameter: 64–63 mm in 10 d.

Colletotrichum grossum

Asexual morph on PDA. Conidiomata acervular. Hyphae hyaline, septate and branched. Setae not observed. Conidia hyaline, cylindrical to ellipsoid with rounded ends, with guttulae, produced in abundance after 10 days growing on media. Conidia size (mean ± SD): 17.3 ± 1.5 × 4.6 ± 0.6 μm. Culture characteristics — colonies velvety to cottony and flat with dark to pale grey mycelium. Orange spore masses. Reverse black to pale grey. Colony diameter: 90–90 mm in 10 d.

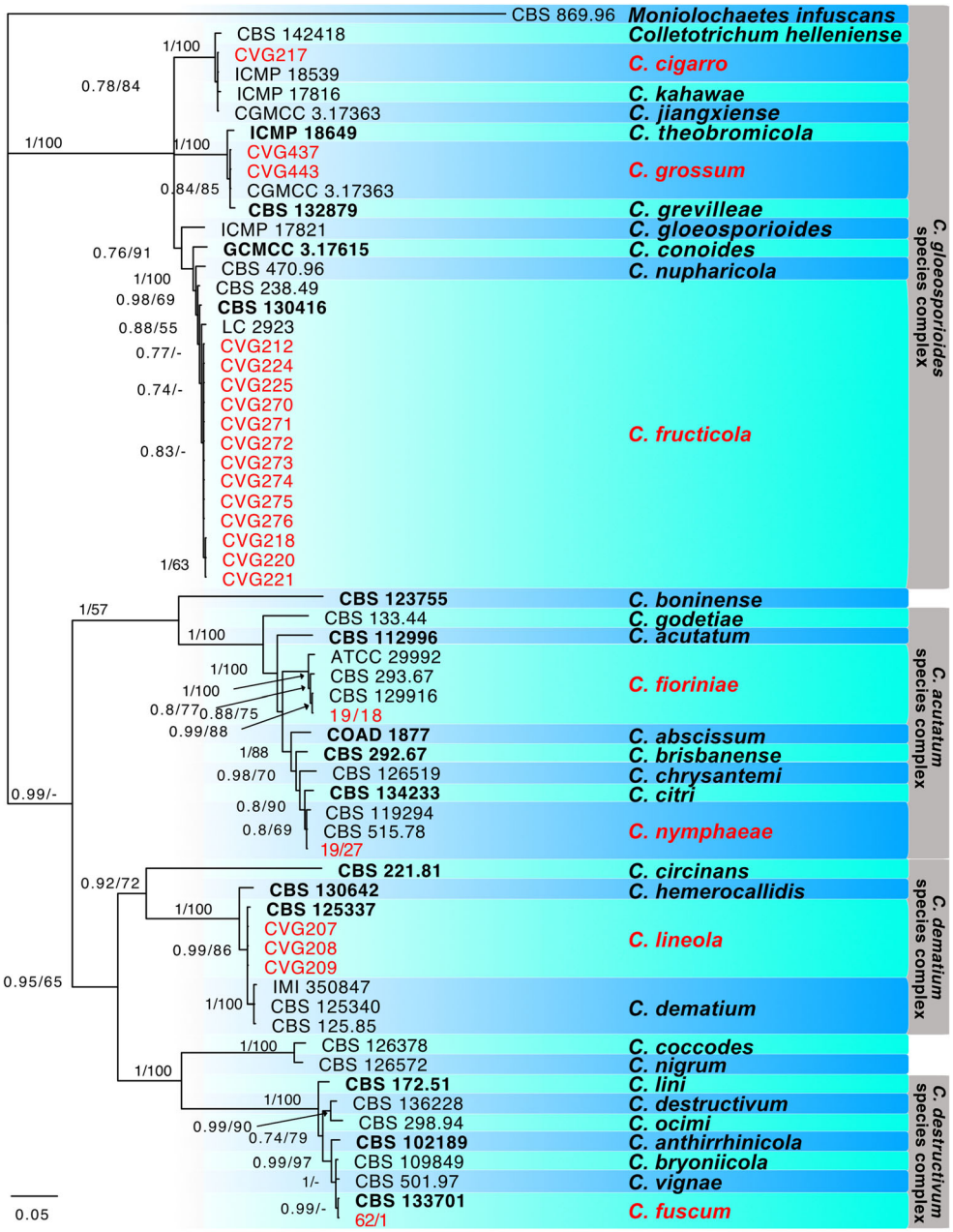

Fig. 1 Consensus phylogram resulting from a Bayesian analysis of the combined *gapdh*, *act* and *tub2* sequences of *Colletotrichum* spp. Bayesian posterior probability values and Bootstrap support values are indicated at the nodes. *Colletotrichum* species complexes are on the left. The strains collected and species found in this study are in red. The tree was rooted to *Moniolochaetes infuscans* (CBS 869.96)

Colletotrichum cigarro

Asexual morph on PDA. Conidiomata acervular. Hyphae hyaline, septate and branched. Setae not observed. Conidia hyaline, ellipsoid with rounded ends, with guttulae, produced in abundance after 10 days growing on media. Conidia size (mean ± SD): 15.4 ± 1 × 6.0 ± 0.6 µm. Culture characteristics — colonies velvety to cottony and flat with grey mycelium. Orange spore masses. Reverse pale grey. Colony diameter: 90–90 mm in 10 d.

Colletotrichum lineola

Asexual morph on PDA. Conidiomata acervular. Hyphae hyaline, septate and branched. Setae not observed. Conidia hyaline, curved with ends, with guttulae, produced in abundance after 7 days growing on media. Conidia size (mean ± SD): 27.0 ± 1.7 × 3.6 ± 0.4 µm. Culture characteristics — colonies velvety and flat with dark to pale grey and yellowish at the margin mycelium. Reverse pale brown with yellow halo at the margin. Colony diameter: 55–45 mm in 10 d.

Colletotrichum nymphaeae

Asexual morph on PDA. Conidiomata acervular. Hyphae hyaline, septate and branched. Setae not observed. Conidia hyaline, ellipsoid with rounded ends, with guttulae, produced in abundance after 7 days growing on media. Conidia size (mean ± SD): 13.7 ± 1 × 4.5 ± 0.6 μm. Culture characteristics — colonies velvety and flat with salmon-like coloured in the centre to pale grey and black at the margin mycelium. Orange spore masses. Reverse orange to pale grey. Colony diameter: 67–67 mm in 10 d.

Pathogenicity

All the tested isolates revealed to be pathogenic to the original hosts inoculated only through simulation of wounded leaves conditions. The disease symptoms were comparable to those observed in the field at the beginning of this survey (Fig. 2), they consisting of dark brown to black necrotic spots and lesions. The *Colletotrichum* species aggressiveness varied based on the pathogen/host association (Table 1).

The identity of the re-isolated fungi was confirmed by sequencing the *gapdh* locus and Koch's postulates were fulfilled. No symptoms have developed on the control and unwounded plants.

Discussion

Species of *Colletotrichum* were recovered with a frequency of 70% from leaf tissues with typical symptoms of anthracnose of the different host species. The diversity of *Colletotrichum* spp. associated with ornamental plants in northern Italy and their aggressiveness were investigated in this study. Fungal species were identified based on the polyphasic approach promoted by Cai et al. (2009), which demonstrated that traditional morphological characters alone are considered not reliable anymore for the identification of *Colletotrichum* species. Thus, multilocus sequence analyses, combined with morphological and pathogenic data, have become the most used characterization tool.

This study is based on a robust multi-locus phylogeny established on three genomic loci. Currently, the ITS region is the most widely sequenced region, however, according to Crouch et al. (2009), the currently available ITS sequence data of *Colletotrichum* spp. could lead to unreliable identifications. Thus, ITS locus was not considered in the present work and the loci *gapdh*, *act* and *tub2* were combined and analysed with previous phylogenetic analyses of the genus *Colletotrichum* (Damm et al. 2012a, b; 2014; Guarnaccia et al. 2017). The characterization revealed a diversity in the composition of *Colletotrichum* spp. associated with ornamental host plants. Twenty-two strains were characterized as seven different species: *Col. fioriniae* and *Col. nymphaeae* belonging to the *Col. acutatum* SC, *Col. fuscum* belonging to the *Col. destructivum* SC, *Col. lineola* within the *Col. dematium* SC and *Col. fructicola*, *Col. cigarro* and *Col. grossum* into the *Col. gloeosporioides* SC.

Col. fioriniae and *Col. nymphaeae* were found in association with *M. aquifolium* and *Ca. rapunculoides*, respectively. *Col. fuscum* and *Col. lineola* were isolated from leaf spots on *Cor. lanceolata* and *Ca. trachelium*, respectively. Whilst, *Col. cigarro* and *Col. grossum* caused necrotic lesions on *L. styraciflua* and *R. jasminoides*, respectively. Moreover, *Col. fructicola* was isolated from leaves of *L. styraciflua* and *H. paniculata* and stems and leaves of *Cy. persicum*. The association between *Col. fioriniae* and *M. aquifolium* and *Col. cigarro* and *L. styraciflua*, were reported on the base of a minor number of locus-sequencing (Garibaldi et al. 2016; 2020), thus this study confirms the identification of *Col. fioriniae* as pathogen of *M. aquifolium* and clarifies the identity of *Col. cigarro* as pathogen of *L. styraciflua*. Necrotic spots, round to oval spots with a purple margin irregularly spreading to the entire leaf, brownish anthracnose lesions or streaks often interesting the margins of the leaves and premature leaf fall, were observed.

Col. fioriniae and *Col. nymphaeae* belong to the same SC and they can be separated by analysis of the tub2 gene (Damm et al. 2012b). *Colletotrichum fioriniae* was described on *Fiorinia externa* (a scale insect) and widely reported as an endophyte (Marcelino et al. 2008) and as a worldwide distributed pathogen causing rot on several fruit such as almond, apple, avocado, blueberry, cranberry, mango, nectarine and pepper (Damm et al. 2012a; Freeman and Shabi 1996). Recently, *Col. fioriniae* has been found in association with *Origanum vulgare* and *Salvia leucantha* in Italy (Guarnaccia et al. 2019), demonstrating its established presence in this territory and the ability to cause disease on diverse hosts. *Col. nymphaeae* is known as pathogen on different hosts, such as *Anemone* spp., *Capsicum* spp., *Fragaria* × *ananassa*, *Malus domestica* and *Vitis vinifera* (Jayawardena et al. 2016), in Italy is known as a major pathogen of olive (Antelmi et al. 2019), however it was never reported on *Campanula* spp.

Colletotrichum fuscum can be distinguished from other species in the *Col. destructivum* SC by *gapdh* sequence data and it was found in association with *Digitalis* sp. in Germany and The Netherlands (Damm et al. 2014). *Col. lineola*, the type species of the genus *Colletotrichum*, is widespread on temperate regions, mostly found on cereals (Damm et al. 2009). To our knowledge, this is the first report of *Col. lineola* in Italy and as pathogen of *Campanula* spp. *Colletotrichum kahawae* subsp. *cigarro* has been described as a species with a wide host range and distribution by Weir et al. (2012). Batista et al. (2016) considered this species, along with *Col. kahawae*

Fig. 2 Symptoms caused by *Colletotrichum* spp. observed in the field (**a**, **b**, **c**, **d**, **e**, **f**, **g**) and after pathogenicity trials (**h**, **i**, **j**, **k**) on leaves of different ornamental plants. (**a**) *Cyclamen persicum*, (**b**) *Coreopsis lanceolata*, (**c**, **j**) *Liquidambar styraciflua*, (**d**) *Campanula rapunculoides*, (**e**) *Mahonia aquifolium*, (**f**, **g**, **h**) *Hydrangea paniculata*, (**i**) *Rhyncospermum jasminoides*, (**k**) *Ceanothus thyrsiflorus*

subsp. *kahawae*, as cryptic. However, the analysis conducted in this study permitted to identify the strain as *Col. cigarro* through the comparison of nucleotide sequences with other species belonging to the same clade, according with the recent description of new combination by Cabral et al. (2020). This species was reported in Italy on mandarin (Perrone et al. 2016) and in other European countries on *Camellia* and *Olea* plants (Cabral et al. 2020).

Colletotrichum grossum is reported as pathogen on *Capsicum annuum* var. *grossum* in China (Diao et al. 2017) and it was detected on mango leaves in Cuba (Manzano León et al. 2018). It can be distinguished with the use of *gapdh*, *act* and *tub2* sequence data from *Col. theobromicola* (Jayawardena et al. 2016). This study represents the first founding of this species in Italy. Isolates of *Col. fructicola* worldwide distributed are biologically different. They were recovered from *Coffea*, *Pyrus pyrifolia*, *Limonium*, *Malus domestica* and *Fragaria* x *ananassa*, *Persea Americana*, *Ficus*, *Dioscorea*, *Theobroma* and *Tetragastris* in Thailand, Japan, Israel, USA, Australia, Germany, Nigeria and Panama, respectively (Weir et al. 2012). Recently, it was reported in Italy as causing fruit rot of avocado (Guarnaccia et al. 2016) and leaf spots on *Salvia greggii* (Guarnaccia et al. 2019). Moreover, it was identified among the new pathogens of *Malus domestica* by the EPPO (2018) and it was inserted in the EPPO alert list within pests with high economic importance and more likely to transfer.

Although with different levels of aggressiveness, all the tested strains revealed to be pathogenic. For example, *Col. fructicola* showed variability in severity of anthracnose

causing on different host, producing relevant infection on *Ce. thyrsiflorus* and *Cy. persicum*, but not on *H. paniculata*. It is imperative at this stage to consider the pathogenicity results as preliminary, and further studies should be conducted in future to assess the putative cross pathogenicity and the role of plant tissue wounds for the symptoms development. Moreover, other pathogens were reported after several surveys in the same territory of northern Italy on the host plants considered in this study, demonstrating that several emerging and well-established pathogens can represent a threat for the cultivation of these plants (Gullino et al. 2015; Garibaldi et al. 2017).

Greenhouse and nursery environments could induce suitable conditions for the development of pathogens and, particularly, of diseases caused by *Colletotrichum* which sporulation and spread are easily promoted with high temperature and humidity (Washington et al. 2006). Thus, considering the relevance of this sector and the susceptibility of these crops to several pathogens, it is important to provide a correct pest diagnosis for an accurate and effective disease management. Since classification of pests are the basis to any further studies on plant and fruit disease as they represent a key-role in pathogen control, this work could provide useful tools to analyse target loci for a fast detection of species within *Colletotrichum* genus. Future studies will focus on detailed experiments to deeply investigate pathogenicity and epidemiological aspects.

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Compliance with ethical standards

Conflict of interest The Authors declare that they have no conflict of interest. This article does not contain any studies with animals performed by any of the authors. This article does not contain any studies with human participants or animals performed by any of the authors.

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