



# High species diversity in *Colletotrichum* associated with citrus diseases in Europe

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## Key words

Anthraxnose  
*Citrus*  
multi-locus sequence typing  
pathogenicity

**Abstract** Species of *Colletotrichum* are considered important plant pathogens, saprobes, and endophytes on a wide range of plant hosts. Several species are well-known on citrus, either as agents of pre- or post-harvest infections, such as anthracnose, postbloom fruit drop, tear stain and stem-end rot on fruit, or as wither-tip of twigs. In this study we explored the occurrence, diversity and pathogenicity of *Colletotrichum* spp. associated with *Citrus* and allied genera in European orchards, nurseries and gardens. Surveys were carried out during 2015 and 2016 in Greece, Italy, Malta, Portugal and Spain. A total of 174 *Colletotrichum* strains were isolated from symptomatic leaves, fruits, petals and twigs. A multi-locus phylogeny was established based on seven genomic loci (ITS, *GAPDH*, *ACT*, *CAL*, *CHS-1*, *HIS3* and *TUB2*), and the morphological characters of the isolates determined. Preliminary pathogenicity tests were performed on orange fruits with representative isolates. *Colletotrichum* strains were identified as members of three major species complexes. *Colletotrichum gloeosporioides* s.str. and two novel species (*C. helleniense* and *C. hystricis*) were identified in the *C. gloeosporioides* species complex. *Colletotrichum karstii*, *C. novae-zelandiae* and two novel species (*C. catinaense* and *C. limonicola*) in the *C. boninense* species complex, and *C. acutatum* s.str. was also isolated as member of *C. acutatum* species complex. *Colletotrichum gloeosporioides* and *C. karstii* were the predominant species of *Colletotrichum* isolated. This study represents the first report of *C. acutatum* on citrus in Europe, and the first detection of *C. novae-zelandiae* from outside New Zealand. Pathogenicity tests revealed *C. gloeosporioides* s.str. to be the most virulent species on fruits. The present study improves our understanding of species associated with several disease symptoms on citrus fruits and plants, and provides useful information for effective disease management.

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## INTRODUCTION

*Colletotrichum* is one of the most important genera of plant pathogenic fungi, responsible for several diseases in many crops worldwide (Sutton 1992, Cannon et al. 2000, 2012, Cai et al. 2009, Udayanga et al. 2013). *Colletotrichum* spp. were recently included in the list of the 10 most important plant pathogenic fungi in the world, based on perceived scientific and economic importance (Dean et al. 2012). Agricultural production losses caused by *Colletotrichum* spp. involve important staple food crops grown in developing countries throughout the tropics and subtropics (Dean et al. 2012). *Colletotrichum* species can infect more than 30 plant genera (Perfect et al. 1999, Farr et al. 2006, Damm et al. 2012a, b, Farr & Rossman 2017), causing anthracnose disease and postharvest decay on a wide range of tropical, subtropical and temperate fruits, grasses, vegetable crops and ornamental plants (Bailey & Jeger 1992, Bernstein et al. 1995, Freeman & Shabi 1996, Crouch et al. 2009, Lima et al. 2011, Damm et al. 2012a, b, Anderson et al. 2013, Crous et al. 2016b, Guarnaccia et al. 2016, De Silva et al. 2017). Moreover, many *Colletotrichum* species are latent plant pathogens, endophytes, epiphytes or saprobes, switching to a pathogenic lifestyle when host plants are subjected to

stress conditions, or placed in postharvest storage (Crous et al. 2016a). Appressoria that develop from germinating spores, start plant infection by penetration of the cuticle (Deising et al. 2000) and occasionally also of the epidermal cells via fungal hyphae (Bailey & Jeger 1992).

The taxonomy of *Colletotrichum* species has recently been reviewed in several impactful studies (Cannon et al. 2008, Cai et al. 2009, Damm et al. 2009, 2012a, b, 2013, 2014, Weir et al. 2012, Liu et al. 2014, 2015, 2016). Before the molecular era, morphological characters such as size and shape of conidia and appressoria, presence or absence of setae, aspect, colour and growth rate of the colonies, formed the basis to study and compare the taxonomy of *Colletotrichum* species (Von Arx 1957, Sutton 1980, 1992). Modern studies demonstrated that these characters are not reliable for species level identification due to their variability under changing environmental conditions (Cai et al. 2009, Liu et al. 2016).

Following adoption of the use of multi-gene phylogenetic analysis, the polyphasic protocols for studying the genus *Colletotrichum* significantly changed the classification and species concepts in *Colletotrichum* (Cannon et al. 2012, Damm et al. 2012a, b, 2013, 2014, Weir et al. 2012). Several systematic studies of nearly all acknowledged species have led to the identification of 11 *Colletotrichum* species complexes, and more than 20 singleton species (Cannon et al. 2012, Liu et al. 2014, 2016, Marin-Felix et al. 2017). In plant pathology the most important species are members of the *C. gloeosporioides* (Cannon et al. 2008, Phoulivong et al. 2010, Weir et al. 2012), *C. acutatum* (Marcelino et al. 2008, Shivas & Tan 2009, Damm et al. 2012a, Baroncelli et al. 2015), *C. boninense* (Moriwaki et al. 2003, Yang et al. 2009, Damm et al. 2012b) and *C. truncatum*

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(Damm et al. 2009, Cannon et al. 2012) complexes. The use of multi-locus phylogenetic analyses revealed many cases, in which certain *Colletotrichum* spp. that were historically considered to be causal agents of economically important plant disease, were then revealed to be different species, such as *C. alienum* which seems to be the most important species in *Proteaceae* cultivation (Liu et al. 2013), and not *C. gloeosporioides* s.str. as previously assumed (Lubbe et al. 2004).

The citrus industry is one of the most important fruit industries worldwide. The Mediterranean countries are second only to China for fruit production, and are the largest fruit exporter after South Africa (FAO 2016). Therefore, the study and knowledge of all the pathogens affecting this crop is imperative. The use of a polyphasic approach in the past revealed many cryptic and new *Colletotrichum* species associated with citrus, belonging to four species complexes, namely: the *C. boninense* species complex (*C. boninense*, *C. citricola*, *C. constrictum*, *C. karstii* and *C. novae-zelandiae*) (Damm et al. 2012b, Huang et al. 2013); the *C. acutatum* species complex (*C. abscissum*, *C. acutatum*, *C. citri*, *C. godetiae*, *C. johnstonii*, *C. limeticola* and *C. simmondsii*) (Damm et al. 2012a, Huang et al. 2013, Crous et al. 2015); the *C. truncatum* species complex (*C. truncatum*) (Damm et al. 2009) and the *C. gloeosporioides* species complex (*C. fructicola*, *C. gloeosporioides*, *C. kahawae* subsp. *ciggaro* and *C. siamense*) (Weir et al. 2012, Huang et al. 2013, Perrone et al. 2016, Liu et al. 2016). Further *Colletotrichum* species such as *C. brevisporum* and *C. tropicicola* have been reported in association with citrus (Huang et al. 2013).

Several major diseases of citrus are caused internationally by *Colletotrichum* species (Timmer et al. 2000, Lima et al. 2011). According to several reports published before the main *Colletotrichum* revisions (Damm et al. 2009, 2012a, b, 2013, 2014, Weir et al. 2012), *C. gloeosporioides* and *C. abscissum* (previously known as *C. acutatum*) are the causal agents of postbloom fruit drop (PFD) in Brazil (Peres et al. 2008, Lima et al. 2011, Crous et al. 2015) and Bermuda (McGovern et al. 2012), causing petal necrosis, abscission of developing fruit and the formation of persistent calyces of various citrus species. A recent extensive investigation in citrus orchards of São Paulo state (Brazil), revealed only *C. abscissum* and *C. gloeosporioides* s.str. associated with PFD disease (Silva et al. 2016). Key lime anthracnose (KLA), a disease complex relating to leaves, flowers and fruits of Key lime, was initially reported to be caused by *C. acutatum* (Brown et al. 1996, Peres et al. 2008, MacKenzie et al. 2009), but later classified as *C. limeticola* (Damm et al. 2012a). *Colletotrichum gloeosporioides* was previously thought to be the only *Colletotrichum* species causing post-harvest anthracnose (Brown 1975, Sutton 1980, Freeman & Shabi 1996), but recent works showed that several species of *Colletotrichum* are associated with fruit decay worldwide (Peng et al. 2012, Damm et al. 2012a, b, Weir et al. 2012). Huang et al. (2013) demonstrated the ability of *C. fructicola* and *C. truncatum* to cause anthracnose on citrus fruits. Moreover, *C. gloeosporioides* s.lat. was also reported to cause pre-harvest symptoms such as wither-tip on twigs, tear-stain (Klotz 1961, Benyahia et al. 2003) and stem-end rot on fruit (Kaur et al. 2007).

Recently, various infections caused by *Colletotrichum* spp. strongly compromised citrus production in different Mediterranean countries: heavy pre-harvest anthracnose symptoms appeared on orange fruits and lesions on leaves of mandarins in Italy (Aiello et al. 2015, Perrone et al. 2016), twig wither-tip symptoms were observed on cultivated orange trees in Tunisia (Rhaïem & Taylor 2016), and severe anthracnose symptoms on unripe and ripe lemon fruits were recorded in Portugal (Ramos

et al. 2016). In these studies, *Colletotrichum* species belonging to the *C. acutatum* species complex were never found associated with citrus. However, *C. acutatum* s.lat. was reported in Mediterranean countries causing diseases on several hosts such as *Fragaria* × *ananassa* (Garrido et al. 2008), *Arbutus unedo* (Polizzi et al. 2011) and *Olea europaea* (Talhinhas et al. 2011). Because of the commercial yield losses in citrus orchards caused by *Colletotrichum* infections, the recent findings and the changes in the species concepts, new surveys are required to study the *Colletotrichum* species diversity related to citrus and their occurrence and association with foliar and fruit diseases.

The current study aimed to investigate the major citrus production areas in Europe by large-scale sampling, and to identify isolates via morphology and multi-locus phylogeny based on modern taxonomic concepts. In 2015 and 2016 several surveys were conducted in commercial nurseries, citrus orchards, gardens, backyards and plant collections to determine the occurrence of *Colletotrichum* spp. associated with *Citrus* and allied genera (*Atlantia*, *Fortunella*, *Microcitrus*, *Murraya*, *Poncirus*). In particular the objectives of the present study were:

- i) to conduct extensive surveys for sampling fresh plant materials;
- ii) to cultivate as many *Colletotrichum* isolates as possible;
- iii) to subject those isolates to DNA sequence analyses combined with morphological characterisation;
- iv) to compare the obtained results with the data from other phylogenetic studies on the genus; and
- v) to evaluate the pathogenicity of *Colletotrichum* species to citrus fruit.

## MATERIALS AND METHODS

### Sampling and isolation

During 2015 and 2016 several surveys were conducted in many of the main citrus-producing regions of Europe. Fruits and leaves with lesions and typical anthracnose symptoms and twigs showing wither-tip were collected from more than 70 sites in Andalusia, Valencia, Balearic Islands (Spain), Apulia, Calabria, Sicily, Eolian Islands (Italy), Algarve (Portugal), Misolonghi, Nafplio, Arta, Crete (Greece) and Malta and Gozo (Malta). Investigated species of *Citrus* and allied genera such as *Atlantia*, *Fortunella*, *Microcitrus*, *Murraya* and *Poncirus* (*Rutaceae*) included Australasian lime, citranges, citrons, kumquat, mandarins, oranges, pummelo, grapefruit, limes, lemons and ornamental brushes.

Fungal isolates were obtained following two procedures. In the first, leaf, fruit and twig fragments (5 × 5 mm) were surface-sterilised in a sodium hypochlorite solution (10 %) for 20 s, followed by 70 % ethanol for 30 s, and rinsed three times in sterilised water. The fragments were dried in sterilised tissue paper, placed on malt extract agar (MEA; Crous et al. 2009) amended with 100 µg/mL penicillin and 100 µg/mL streptomycin (MEA-PS) and incubated at 25 °C until characteristic *Colletotrichum* colonies were observed. In the second procedure, plant material was incubated in moist chambers at room temperature (25 °C ± 3 °C) for up to 10 d and inspected daily for fungal sporulation. Sporulating conidiomata obtained through both procedures were collected and crushed in a drop of sterile water and then spread over the surface of MEA-PS plates. After 24 h germinating spores were individually transferred onto MEA plates. The isolates used in this study are maintained in the culture collection of the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, The Netherlands, and in the working collection of Pedro Crous (CPC), housed at the Westerdijk Institute.

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

Species	Culture no. <sup>1</sup>	Host	Locality	Associated symptoms	ITS	GAPDH	ACT	CAL	CHS-1	TUB2	HIS3
<i>Colletotrichum abscissum</i>	COAD 1876	<i>Citrus sinensis</i>	Brazil	–	KP843124	KP843127	KP843139	–	KP843130	KP843133	KP843136
	COAD 1877	<i>Citrus sinensis</i>	Brazil	–	KP843126	KP843129	KP843141	–	KP843132	KP843135	KP843138
<i>C. acutatum</i>	CBS 112759	<i>Hakea seneca</i>	South Africa	–	JQ948391	JQ948722	JQ949712	–	JQ949052	JQ950040	JQ950382
	CBS 112996	<i>Carica papaya</i>	Australia	–	JQ005776	JQ948677	JQ005839	–	JQ005797	JQ005860	JQ005818
<i>C. alienum</i>	CBS 129952	<i>Olea europaea</i>	Portugal	–	JQ948364	JQ948695	JQ949685	–	JQ949025	JQ950015	JQ949355
	CBS 142407 = CPC 27005	<i>Citrus sinensis</i>	Italy, Messina	Leaf lesion	KY856397	KY856221	KY855968	–	KY856133	KY856479	KY856303
<i>C. annelatum</i>	ICMP 12071	<i>Citrus limon</i>	Italy, Messina	Leaf lesion	KY856398	KY856222	KY855969	–	KY856134	KY856480	KY856304
<i>C. asiaticum</i>	CBS 129826	<i>Malus domestica</i>	New Zealand	–	JX010251	JX010228	JX009572	JX009654	JX009882	JX010411	–
	CBS 130418	<i>Hevea brasiliensis</i>	Colombia	–	JQ005222	JQ005309	JQ005570	JQ005743	JQ005396	JQ005656	JQ005483
<i>C. boninense</i>	CBS 123755	<i>Coffea arabica</i>	Thailand	–	FJ972612	JX010053	JX009584	FJ917506	JX009867	JX010406	KY856305
	GZAAS5.09505	<i>Citrus asiaticum</i> 'Sinicum'	Japan	–	JQ005153	JQ005240	JQ005501	JQ005674	JQ005327	JQ005588	JQ005414
<i>C. brevisporum</i>	GZAAS5.09545	<i>Citrus medica</i>	China	–	JQ247622	JQ247598	JQ247646	–	–	JQ247634	–
	ICMP 10643	<i>Citrus medica</i>	China	–	JQ247623	JQ247599	JQ247647	JQ247589	–	JQ247635	–
<i>C. carmeliae</i>	CBS 142416 = CPC 28019	<i>Camellia x williamsii</i>	UK	–	JX010224	JX009908	JX009540	JX009630	JX008891	JX010436	–
	CBS 142417 = CPC 27978	<i>Citrus sinensis</i>	Portugal, Mesquita	Fruit tear stain	KY856399	KY856223	KY855970	KY856052	KY856135	KY856481	KY856306
<i>C. calinaense</i>	CBS 134233	<i>Citrus reticulata</i>	Italy, Catania	Leaf lesion	KY856400	KY856224	KY855971	KY856053	KY856136	KY856482	KY856307
	CPC 28149	<i>Citrus aurantifolia</i>	Italy, Catania	Twigs wither-tip	KY856401	KY856225	KY855972	KY856054	KY856137	KY856483	KY856308
<i>C. citri</i>	CBS 134233	<i>Citrus aurantifolia</i>	China	–	KC293581	KC293741	KY855973	KC293701	KY856138	KC293661	KY856309
	CBS 134234	<i>Citrus aurantifolia</i>	China	–	KC293582	KC293742	KY855974	KC293702	KY856139	KC293662	KY856310
<i>C. citrifolia</i>	CBS 134228	<i>Citrus unchui</i>	China	–	KC293576	KC293736	KC293616	KC293696	KY856140	KC293656	KY856311
	CBS 134229	<i>Citrus unchui</i>	China	–	KC293577	KC293737	KC293617	KC293697	KY856141	KC293657	KY856312
<i>C. constrictum</i>	CBS 128504	<i>Citrus limon</i>	New Zealand	–	JQ005238	JQ005325	JQ005586	JQ005759	JQ005412	JQ005672	KY856313
	CBS 238.49	<i>Ficus edulis</i>	Germany	–	JX010181	JX009923	JX009495	JX009671	JX008839	JX010400	KY856314
<i>C. fructicola</i>	CBS 125397	<i>Tetragastris panamensis</i>	Panama	–	JX010173	JX010032	JX009581	JX009674	JX009874	JX010409	KY856315
	ICMP 18581	<i>Coffea arabica</i>	Thailand	–	JX010165	JX010033	FJ907426	FJ917508	JX009866	JX010405	–
<i>C. gloeosporioides</i>	CBS 112999	<i>Citrus sinensis</i>	Italy	–	JX010152	JX010056	JX009531	JX009731	JX009818	JX010445	–
	CBS 142408 = CPC 28059	<i>Citrus sinensis</i> 'Lanelate'	Spain, Moncada	Petal lesions	KY856402	KY856226	KY855975	KY856055	KY856142	KY856484	KY856316
<i>C. gloeosporioides</i>	CPC 26172	<i>Citrus sinensis</i> 'Tarocco Tapi'	Italy, Catania	Twigs wither-tip	KY856403	KY856227	KY855976	KY856056	KY856143	KY856485	KY856317
	CPC 26178	<i>Citrus sinensis</i> 'Tarocco Tapi'	Italy, Catania	Leaf lesion	KY856404	KY856228	KY855977	KY856057	KY856144	KY856486	KY856318
<i>C. gloeosporioides</i>	CPC 26371	<i>Citrus sinensis</i> 'Valencia'	Italy, Catania	Twigs wither-tip	KY856405	KY856229	KY855978	KY856058	KY856145	KY856487	KY856319
	CPC 26372	<i>Citrus sinensis</i> 'Valencia'	Italy, Catania	Twigs wither-tip	KY856406	KY856230	KY855979	KY856059	KY856146	KY856488	KY856321
<i>C. gloeosporioides</i>	CPC 26376	<i>Citrus paradisi</i>	Italy, Catania	Twigs wither-tip	KY856407	KY856231	KY855980	KY856060	KY856147	KY856489	KY856322
	CPC 26381	<i>Citrus limon</i>	Italy, Catania	Twigs wither-tip	KY856408	KY856232	KY855981	KY856061	KY856148	KY856490	KY856323
<i>C. gloeosporioides</i>	CPC 26479	<i>Citrus sinensis</i>	Italy, Enna	Fruit lesion	KY856409	KY856233	KY855982	KY856062	KY856149	KY856491	KY856324
	CPC 26486	<i>Citrus sinensis</i>	Italy, Enna	Fruit lesion	KY856410	KY856234	KY855983	KY856063	KY856150	KY856492	KY856325
<i>C. gloeosporioides</i>	CPC 26488	<i>Citrus sinensis</i>	Italy, Catania	Fruit lesion	KY856411	KY856235	KY855984	KY856064	KY856151	KY856493	KY856326
	CPC 26515	<i>Citrus medica</i>	Italy, Catania	Leaf lesion	KY856412	KY856236	KY855985	KY856065	KY856152	KY856494	KY856327
<i>C. gloeosporioides</i>	CPC 26803	<i>Citrus sinensis</i> 'Tarocco Meli'	Italy, Catania	Twigs wither-tip	KY856413	KY856237	KY855986	KY856066	KY856153	KY856495	KY856328
	CPC 26809	<i>Citrus limon</i>	Spain, Malaga	Leaf lesion	KY856414	KY856238	KY855987	KY856067	KY856154	KY856496	KY856329
<i>C. gloeosporioides</i>	CPC 26823	<i>Citrus paradisi</i>	Spain, Malaga	Leaf lesion	KY856415	KY856239	KY855988	KY856068	KY856155	KY856497	KY856330
	CPC 26937	<i>Citrus paradisi</i>	Spain, Malaga	Twigs wither-tip	KY856416	KY856240	KY855989	KY856069	KY856156	KY856498	KY856331
<i>C. gloeosporioides</i>	CPC 26957	<i>Citrus reticulata</i> 'Nova'	Greece, Naipilo	Leaf lesion	KY856417	KY856241	KY855990	KY856070	KY856157	KY856499	KY856332
	CPC 26965	<i>Citrus sinensis</i>	Italy, Vibo Valentia	Fruit lesion	KY856418	KY856242	KY855991	KY856071	KY856158	KY856500	KY856333
<i>C. gloeosporioides</i>	CPC 26975	<i>Citrus paradisi</i>	Italy, Vibo Valentia	Twigs wither-tip	KY856419	KY856243	KY855992	KY856072	KY856159	KY856501	KY856334
	CPC 26985	<i>Citrus reticulata</i> 'Nova'	Italy, Vibo Valentia	Leaf lesion	KY856420	KY856244	KY855993	KY856073	KY856160	KY856502	KY856335
<i>C. gloeosporioides</i>	CPC 27019	<i>Citrus limon</i>	Italy, Cosenza	Twigs wither-tip	KY856421	KY856245	KY855994	KY856074	KY856161	KY856494	KY856336
	CPC 27021	<i>Fortunella margarita</i>	Italy, Vibo Valentia	Twigs wither-tip	KY856422	KY856246	KY855995	KY856075	KY856162	KY856504	KY856337
<i>C. gloeosporioides</i>	CPC 27088	<i>Citrus reticulata</i>	Greece, Missolonghi	Leaf lesion	KY856423	KY856247	KY855996	KY856076	KY856163	KY856505	KY856338
	CPC 27127	<i>Citrus maxima</i>	Greece, Missolonghi	Twigs wither-tip	KY856424	KY856248	KY855997	KY856077	KY856164	KY856506	KY856339
<i>C. gloeosporioides</i>	CPC 27129	<i>Citrus bergamia</i>	Greece, Missolonghi	Fruit lesion	KY856425	KY856249	KY855998	KY856078	KY856165	KY856507	KY856340
	CPC 27839	<i>Citrus sinensis</i>	Italy, Catania	Leaf lesion	KY856426	KY856250	KY855999	KY856079	KY856166	KY856508	KY856341
<i>C. gloeosporioides</i>	CPC 27841	<i>Citrus sinensis</i>	Italy, Catania	Leaf lesion	KY856427	KY856251	KY856000	KY856080	KY856167	KY856509	KY856342
	CPC 27905	<i>Citrus limon</i>	Malta, Gozo	Twigs wither-tip	KY856428	KY856252	KY856001	KY856081	KY856168	KY856510	KY856343
<i>C. gloeosporioides</i>	CPC 27923	<i>Citrus sinensis</i>	Malta, Gozo	Leaf litter	KY856429	KY856253	KY856002	KY856082	KY856169	KY856511	KY856344

C. <i>gloeosporioides</i> (cont.)	CPC 27939	<i>Citrus limon</i>	Portugal, Faro	Leaf lesion	KY856430	KY856254	KY856003	KY856083	KY856170	KY856512	KY856345
	CPC 27941	<i>Citrus sinensis</i>	Portugal, Silves	Twigs wither-tip	KY856431	KY856255	KY856004	KY856084	KY856171	KY856513	KY856346
	CPC 27971	<i>Citrus sinensis</i> 'Valencia'	Portugal, Mesquita	Fruit lesion	KY856432	KY856256	KY856005	KY856085	KY856172	KY856514	KY856347
	CPC 27991	<i>Citrus sinensis</i> 'Valencia'	Portugal, Mesquita	Fruit tear stain	KY856433	KY856257	KY856006	KY856086	KY856173	KY856515	KY856348
	CPC 28001	<i>Citrus paradisi</i>	Portugal, Faro	Leaf lesion	KY856434	KY856258	KY856007	KY856087	KY856174	KY856516	KY856349
	CPC 28021	<i>Citrus sinensis</i>	Portugal, Mesquita	Twigs wither-tip	KY856435	KY856259	KY856008	KY856088	KY856175	KY856517	KY856350
	CPC 28023	<i>Citrus limon</i>	Portugal, Monchique	Leaf lesion	KY856436	KY856260	KY856009	KY856089	KY856176	KY856518	KY856351
	CPC 28029	<i>Citrus sinensis</i>	Portugal, Silves	Twigs wither-tip	KY856437	KY856261	KY856010	KY856090	KY856177	KY856519	KY856352
	CPC 28052	<i>Citrus reticulata</i>	Spain, Algemesi	Twigs wither-tip	KY856438	KY856262	KY856011	KY856091	KY856178	KY856520	KY856353
	CPC 28056	<i>Citrus sinensis</i> 'Lanelate'	Spain, Moncada	Petal lesions	KY856439	KY856263	KY856012	KY856092	KY856179	KY856521	KY856354
	CPC 28061	<i>Citrus sinensis</i>	Spain, Castelló	Leaf lesion	KY856440	KY856264	KY856013	KY856093	KY856180	KY856522	KY856355
	CPC 28063	<i>Citrus sinensis</i>	Spain, Castelló	Leaf lesion	KY856441	KY856265	KY856014	KY856094	KY856181	KY856523	KY856356
	CPC 28155	<i>Citrus floridana</i>	Italy, Catania	Fruit lesion	KY856442	KY856266	KY856015	KY856095	KY856182	KY856524	KY856357
	CPC 28159	<i>Citrus digitata</i>	Italy, Catania	Leaf lesion	KY856443	KY856267	KY856016	KY856096	KY856183	KY856525	KY856358
	CPC 28196	<i>Alitalia citroides</i>	Spain, Solier	Leaf lesion	KY856444	KY856268	KY856017	KY856097	KY856184	KY856526	KY856359
	CPC 28197	<i>Microcitrus australasica</i>	Spain, Solier	Twigs wither-tip	KY856445	KY856269	KY856018	KY856098	KY856185	KY856527	KY856360
	ICMP 12938	<i>Citrus sinensis</i>	New Zealand	—	JX009935	JX009935	JX009935	JX009732	JX009746	—	—
	ICMP 18695	<i>Citrus</i> sp.	USA	—	JX010153	JX009979	JX009494	JX009735	JX009779	—	—
	ICMP 18730	<i>Citrus</i> sp.	New Zealand	—	JX010157	JX009981	JX009548	JX009737	JX009861	—	—
	ICMP 18738	<i>Carya illinoensis</i>	Australia	—	JX010151	JX009976	JX009542	JX009730	JX009797	—	—
	<b>CBS 133.44</b>	<i>Clarkia hybrida</i>	Denmark	—	JQ948733	JQ948733	JQ949723	—	JQ949063	JQ950053	JQ949393
C. <i>gondelae</i>	<b>CBS 142418 = CPC 26844</b>	<i>Poncirus trifoliata</i>	Greece, Atria	Twigs wither-tip	KY856446	KY856270	KY856019	KY856099	KY856186	KY856528	KY856361
	<b>CBS 142419 = CPC 27107</b>	<i>Citrus reticulata</i>	Greece, Atria	Fruit lesion	KY856447	KY856271	KY856020	KY856100	KY856187	KY856529	KY856362
	CPC 26845	<i>Poncirus trifoliata</i>	Greece, Atria	Twigs wither-tip	KY856448	KY856272	KY856021	KY856101	KY856188	KY856530	KY856363
	CPC 27108	<i>Citrus reticulata</i>	Greece, Atria	Fruit lesion	KY856449	KY856273	KY856022	KY856102	KY856189	KY856531	KY856364
C. <i>hystricis</i>	<b>CBS 142411 = CPC 28153</b>	<i>Citrus hystrix</i>	Italy, Catania	Leaf lesion	KY856450	KY856274	KY856023	KY856103	KY856190	KY856532	KY856365
	<b>CBS 142412 = CPC 28154</b>	<i>Citrus hystrix</i>	Italy, Catania	Leaf lesion	KY856451	KY856275	KY856024	KY856104	KY856191	KY856533	KY856366
C. <i>johnstonii</i>	<b>CBS 128532</b>	<i>Citrus</i> sp.	New Zealand	—	JQ948443	JQ948774	JQ949764	—	JQ949104	JQ950094	JQ949434
C. <i>kahawae</i> subsp. <i>kahawae</i>	<b>ICMP 17816</b>	<i>Coffea arabica</i>	Kenya	—	JX010231	JX010012	JX009452	JX009642	JX009813	JX010444	—
C. <i>kahawae</i> subsp. <i>ciggaro</i>	<b>ICMP 18539</b>	<i>Olea europaea</i>	Australia	—	JX010230	JX009966	JX009523	JX009635	JX009800	JX010434	—
	CBS 126532	<i>Citrus</i> sp.	South Africa	—	JQ005209	JQ005296	JQ005557	JQ005730	JQ005383	JQ005643	JQ005470
	CBS 127597	<i>Diospyros australis</i>	Australia	—	JQ005204	JQ005291	JQ005552	JQ005725	JQ005378	JQ005638	JQ005465
	CBS 128551	<i>Citrus</i> sp.	New Zealand	—	JQ005189	JQ005276	JQ005537	JQ005710	JQ005382	JQ005623	JQ005469
	CBS 129829	<i>Gossypium hirsutum</i>	Germany	—	JQ005175	JQ005282	JQ005523	JQ005696	JQ005349	JQ005609	JQ005436
	CBS 129833	<i>Musa</i> sp.	Mexico	—	KC293570	KC293610	KC293610	KC293690	KY856192	KC293650	KY856367
	CBS 134226	<i>Citrus limon</i>	China	—	KY856452	KY856276	KY856025	KY856105	KY856193	KY856534	KY856368
	<b>CBS 142415 = CPC 26379</b>	<i>Fortunella margarita</i>	Italy, Catania	Fruit tear stain	KY856453	KY856277	KY856026	KY856106	KY856194	KY856535	KY856369
	CPC 26375	<i>Citrus paradisi</i>	Italy, Catania	Twigs wither-tip	KY856454	KY856278	KY856027	KY856107	KY856195	KY856536	KY856370
	CPC 27023	<i>Citrus sinensis</i>	Italy, Cosenza	Leaf lesion	KY856455	KY856279	KY856028	KY856108	KY856196	KY856537	KY856371
	CPC 27035	<i>Citrus paradisi</i>	Spain, Almeria	Leaf lesion	KY856456	KY856280	KY856029	KY856109	KY856197	KY856538	KY856372
	CPC 27063	<i>Fortunella margarita</i>	Italy, Vibo Valentia	Leaf lesion	KY856457	KY856281	KY856030	KY856110	KY856198	KY856539	KY856373
	CPC 27065	<i>Citrus sinensis</i>	Spain, Almeria	Leaf lesion	KY856458	KY856282	KY856031	KY856111	KY856199	KY856540	KY856374
	CPC 27077	<i>Citrus reticulata</i> 'Nova'	Italy, Catania	Twigs wither-tip	KY856459	KY856283	KY856032	KY856112	KY856200	KY856541	KY856375
	CPC 27817	<i>Citrus paradisi</i>	Italy, Catania	Twigs wither-tip	KY856460	KY856284	KY856033	KY856113	KY856201	KY856542	KY856376
	CPC 27845	<i>Citrus sinensis</i>	Italy, Catania	Twigs wither-tip	KY856461	KY856285	KY856034	KY856114	KY856202	KY856543	KY856377
	CPC 27979	<i>Citrus reticulata</i>	Italy, Catania	Leaf lesion	KY856462	KY856286	KY856035	KY856115	KY856203	KY856544	KY856378
	CPC 27989	<i>Citrus sinensis</i>	Portugal, Mesquita	Twigs wither-tip	KY856463	KY856287	KY856036	KY856116	KY856204	KY856545	KY856379
	CPC 28065	<i>Citrus limon</i>	Portugal, Faro	Twigs wither-tip	KY856464	KY856288	KY856037	KY856117	KY856205	KY856546	KY856380
	CPC 28142	<i>Citrus limon</i>	Spain, Castelló	Leaf lesion	KY856465	KY856289	KY856038	KY856118	KY856206	KY856547	KY856381
	CPC 31139	<i>Citrus sinensis</i>	Italy, Catania	Fruit lesion	KY856466	KY856290	KY856039	KY856119	KY856207	KY856548	KY856382
	CPC 31143	<i>Citrus sinensis</i>	Italy, Catania	Leaf lesion	KY856467	KY856291	KY856040	KY856120	KY856208	KY856549	KY856383
	CPC 31144	<i>Citrus sinensis</i>	Malta, Zurrieq	Twigs wither-tip	KY856468	KY856292	KY856041	KY856121	KY856209	KY856550	KY856384
	CPC 31144	<i>Citrus sinensis</i>	Malta, Zurrieq	Twigs wither-tip	KY856469	KY856293	KY856042	KY856122	KY856210	KY856551	KY856385
	CPC 31196	<i>Murraya paniculata</i>	Italy, Catania	Leaf lesion	KY856470	KY856294	KY856043	KY856123	KY856211	KY856552	KY856386
	<b>CBS 114.14</b>	<i>Citrus aurantiifolia</i>	USA, Florida	—	JQ948523	JQ948523	JQ949514	—	JQ948854	JQ949844	JQ949184
	<b>CBS 142409 = CPC 27861</b>	<i>Citrus limon</i>	Malta, Gozo	Leaf lesion	KY856471	KY856295	KY856044	KY856124	KY856212	KY856553	KY856387
	<b>CBS 142410 = CPC 31141</b>	<i>Citrus limon</i>	Malta, Gozo	Leaf lesion	KY856472	KY856296	KY856045	KY856125	KY856213	KY856554	KY856388
	CPC 27862	<i>Citrus limon</i>	Malta, Gozo	Leaf lesion	KY856473	KY856297	KY856046	KY856126	KY856214	KY856555	KY856389

Table 1 (cont.)

Species	Culture no. <sup>1</sup>	Host	Locality	Associated symptoms	GenBank no. <sup>2</sup>							
					ITS	GAPDH	ACT	CAL	CHS-1	TUB2	HIS3	
<i>C. musae</i>	<b>CBS 116870</b>	<i>Musa</i> sp.	USA	–	JX010146	JX010050	JX009433	JX009742	JX009896	HQ596280	–	
<i>C. novae-zelandiae</i>	<b>CBS 128505</b>	<i>Capisicum annuum</i>	New Zealand	–	JQ005228	JQ005315	JQ005576	JQ005749	JQ005402	JQ005862	JQ005489	
	CBS 130240	<i>Citrus medica</i>	New Zealand	–	JQ005229	JQ005316	JQ005577	JQ005750	JQ005403	JQ005863	JQ005490	
	CBS 142413 = CPC 26949	<i>Citrus paradisi</i>	Greece, Missolonghi	Leaf lesion	KY856474	KY856298	KY856047	KY856127	KY856215	KY856556	KY856390	
	CBS 142414 = CPC 27888	<i>Citrus sinensis</i>	Malta, Gozo	Twigs wither-tip	KY856475	KY856300	KY856048	KY856128	KY856216	KY856557	KY856391	
	CPC 27864	<i>Citrus limon</i>	Malta, Gozo	Twigs wither-tip	KY856476	KY856300	KY856049	KY856129	KY856217	KY856558	KY856392	
	CPC 27890	<i>Citrus sinensis</i>	Malta, Gozo	Twigs wither-tip	KY856477	KY856301	KY856050	KY856130	KY856218	KY856559	KY856393	
	CPC 27957	<i>Citrus limon</i>	Malta, Gozo	Leaf lesion	KY856478	KY856302	KY856051	KY856131	KY856219	KY856560	KY856394	
<i>C. siamense</i>	<b>GZAA55.09506</b>	<i>Murraya</i> sp.	China	–	JQ247633	JQ247609	JQ247657	JQ247596	–	JQ247644	–	
<i>C. simmondsii</i>	<b>CBS 122122</b>	<i>Carica papaya</i>	Australia	–	JQ948276	JQ948606	JQ949597	–	JQ948937	JQ949927	JQ949267	
	GZAA55.09510	<i>Murraya</i> sp.	China	–	JQ247631	JQ247607	JQ247655	JQ247595	–	JQ247643	–	
<i>C. ti</i>	<b>ICMP 4832</b>	<i>Cordyline</i> sp.	New Zealand	–	JX010269	JX009952	JX009520	JX009649	JX009898	JX010442	–	
<i>C. tropicale</i>	<b>CBS 124949</b>	<i>Theobroma cacao</i>	Panama	–	JX010264	JX010007	JX009489	JX009719	JX009870	JX010407	KY856395	
<i>C. tropicicola</i>	<b>BCC 38877</b>	<i>Citrus maxima</i>	Thailand	–	JN050240	JN050229	JN050218	–	JN050246	–	–	
<i>C. truncatum</i>	<b>CBS 151.35</b>	<i>Phaseolus lunatus</i>	USA	–	GU227862	GU228254	GU227960	KY856132	GU228352	GU228156	GU228058	
	CBS 134232	<i>Citrus limon</i>	China	–	KC293580	KC293740	KC293620	KC293700	KY856220	KC293660	KY856396	
<i>Monilochaetes infuscans</i>	<b>CBS 869.96</b>	<i>Ipomoea batatas</i>	South Africa	–	JQ005780	JX546612	JQ005843	–	JQ005801	JQ005864	JQ005822	

<sup>1</sup> BCC: Culture Collection, National Center for Genetic Engineering and Biotechnology (BIOTEC), Khlong Luang, Pathumthani, Thailand; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; COAD: Coleção Octávio Almeida Drummond, Viçosa, Brazil; CPC: Culture collection of P.W. Crous, housed at the Westerdijk Institute; GZAA55: Guizhou Academy of Agricultural Science herbarium, Guizhou Province, China; ICMP: International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand.

<sup>2</sup> ITS: internal transcribed spacers 1 and 2 together with 5.8S rDNA; GAPDH: partial glyceraldehyde-3-phosphate dehydrogenase gene; CHS-1: partial chitin synthase 1 gene; TUB2: partial beta-tubulin gene; HIS3: histone3. Sequences generated in this study are indicated in *italics*.

### DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted using a Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, USA) following the manufacturer's instructions. Partial regions of seven loci were amplified. The primers ITS5 and ITS4 (White et al. 1990) were used to amplify the internal transcribed spacer region (ITS) of the nuclear ribosomal RNA operon, including the 3' end of the 18S rRNA, the first internal transcribed spacer region, the 5.8S rRNA gene; the second internal transcribed spacer region and the 5' end of the 28S rRNA gene. The primers CL1 and CL2 (O'Donnell et al. 2000) were used to amplify part of the calmodulin (*CAL*) gene. The partial glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was amplified using primers GDF1 + GDR1 (Guerber et al. 2003). The primers ACT-512F and ACT-783R (Carbone & Kohn 1999) were used to amplify part of the actin gene (*ACT*). The partial beta-tubulin (*TUB2*) gene was amplified with primers T1 (Glass & Donaldson 1995) and Bt-2b (O'Donnell & Cigelnik 1997). The primers CHS-79F and CHS-345R (Carbone & Kohn 1999) were used to amplify part of the chitin synthase 1 (*CHS-1*) gene. The partial histone3 (*HIS3*) gene was amplified with primers CYLH3F and CYLH3R (Crous et al. 2004b).

The PCR amplification mixtures and cycling conditions for all seven loci were followed as described by Damm et al. (2012b). The PCR products were sequenced in both directions using the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems Life Technologies, Carlsbad, CA, USA), after which amplicons were purified through Sephadex G-50 Fine columns (GE Healthcare, Freiburg, Germany) in MultiScreen HV plates (Millipore, Billerica, MA). Purified sequence reactions were analysed on an Applied Biosystems 3730xl DNA Analyzer (Life Technologies, Carlsbad, CA, USA). The DNA sequences generated were analysed and consensus sequences were computed using the program SeqMan Pro (DNASTAR, Madison, WI, USA).

### Phylogenetic analyses

Novel sequences generated 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 by using the MAFFT v. 7 online server (<http://mafft.cbrc.jp/alignment/server/index.html>) (Kato & Standley 2013), and then manually adjusted in MEGA v. 6.06 (Tamura et al. 2013). To establish the identity of isolates at species level, phylogenetic analyses were conducted first individually for each locus (data not shown) and then as concatenated analyses of seven loci. Two separate analyses were conducted for the *C. boninense* species complex and the remainder of the *Colletotrichum* spp. included in this study. Additional reference sequences were selected based on recent studies on *Colletotrichum* species (Damm et al. 2012a, b, Weir et al. 2012, Huang et al. 2013). Phylogenetic analyses were based on Maximum Parsimony (MP) for all the individual loci and on both MP and Bayesian Inference (BI) for the multilocus 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 to 0.2 and trees were sampled every 1 000 generations. Analyses stopped once the average standard deviation of split frequencies was below 0.01. The MP analyses were done using PAUP (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' 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 for parsimony and the bootstrap analyses (Hillis & Bull 1993) were based on 1 000 replications. Sequences generated in this study were deposited in GenBank (Table 1) and alignments and phylogenetic trees in TreeBASE (www.treebase.org).

### Morphology

Agar plugs (6-mm-diam) were taken from the edge of actively growing cultures on PDA and transferred to the centre of 9-cm-diam Petri dishes containing PDA and synthetic nutrient-poor agar medium (SNA; Nirenberg 1976) as described in recent studies (Huang et al. 2013, Diao et al. 2017). Cultures were incubated at 25 °C with a 12/12 h fluorescent light/dark cycle for 10 d. Colony characters and pigment production on PDA and SNA were noted after 10 d. Colony colours were rated according to Rayner (1970). Cultures were examined periodically for the development of ascomata, conidiomata and setae. Colony diameters were measured after 7 and 10 d. The morphological characteristics were examined by mounting fungal structures in clear lactic acid and 30 measurements at  $\times 1\,000$  magnification were determined for each isolate using a Zeiss AxioScope 2 microscope with interference contrast (DIC) optics. Descriptions and illustrations of taxonomic novelties were deposited in MycoBank (www.Mycobank.org; Crous et al. 2004a).

### Pathogenicity

Fruits of two sweet orange (*Citrus sinensis*) clones ('Tarocco Scirè' and 'Tarocco Nucellare') were collected in Sicily during the veraison stage and used for artificial inoculation. A subset of 13 isolates representing the *Colletotrichum* species isolated from specimens collected in Europe (Table 2) were inoculated following the adapted wound/drop method (Cai et al. 2009, Aiello et al. 2015, Cristóbal-Martínez et al. 2017). Eight fruits for each isolate/clone combination were inoculated. Fruits were washed and surface-disinfected by immersion in 70 % ethanol for 10 min, and rinsed twice in sterilised water. Six inoculation points per fruit were labelled with a dot made with a permanent marker and were injured using a sterile pipette tip (wounds of 2 mm diam). A spore suspension ( $1.0 \times 10^5$  conidia/mL) was obtained from cultures grown on PDA for 15 d at 27 °C, and 10  $\mu$ L were injected into each inoculation point. Control fruits

were inoculated with sterile water. The inoculated oranges were placed in plastic containers, covered with plastic bags and incubated in a growth chamber with 100 % relative humidity at 25 °C under a lighting rig providing a 12 h photoperiod. Symptom development was evaluated 10 d after inoculation and the percentage of infected inoculation points was calculated per each isolate/clone combination. This percentage value was calculated by the formula [(%) = (infected inoculation points / inoculated inoculation points)  $\times$  100 %].

The inoculated fungi were re-isolated from the obtained lesions and the identity of the re-isolated fungi confirmed by sequencing the loci *ACT* and *GAPDH*, thus fulfilling Koch's postulates.

## RESULTS

### Sampling and isolation

Symptoms of anthracnose caused by *Colletotrichum* spp. were frequently observed on several *Citrus* species in all countries investigated. The leaves presented necrotic, more or less circular spots. These lesions appeared with a pale brown to purple margin and produced the fruiting bodies of the fungus (Fig. 1a–b). Different symptoms appeared on fruits. Irregular and sunken lesions, of variable size, from purple-brown to black with acervuli (Fig. 1d–g), were observed. Further, fruits showed tear stain (Fig. 1h), as superficial, reddish brown streaks or bands (down) along the fruit. Moreover, a dark-brown to black rot, with a well-defined margin at the stem-end was occasionally detected (Fig. 1i). Mummified fruits were occasionally observed in association with affected tips (Fig. 1c). Twigs showed typical dieback and wither-tip (Fig. 1k). Under high moisture conditions, pink masses of spores appeared sporulating in acervuli on dead twigs. A total of 174 monosporic isolates resembling those of the genus *Colletotrichum* were collected. The *Colletotrichum* isolates were recovered from 17 species of *Citrus* at 44 different sites in multiple locations of Greece, Italy, Malta, Spain and Portugal. Among them, 67 isolates were obtained from leaves, 72 were associated with twigs, 28 from fruits and seven were isolated from petals. Based on initial ITS and *GAPDH* sequencing, 82 representative isolates were selected (Table 1) for phylogenetic analysis and further taxonomic study.

### Phylogenetic analyses

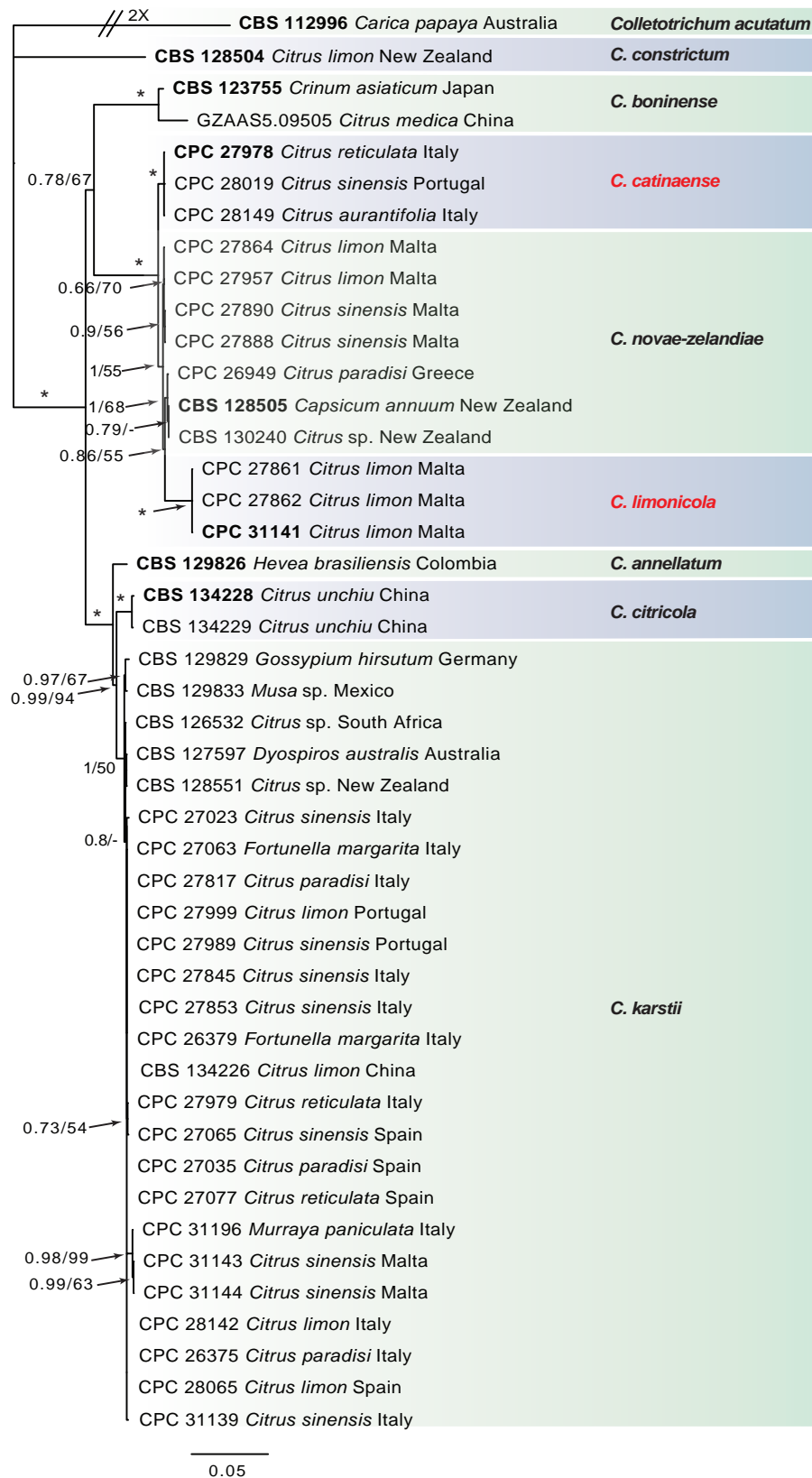
The 14 MP trees derived from the single gene sequence alignments (ITS, *GAPDH*, *ACT*, *CAL*, *CHS-1*, *HIS3* and *TUB2*) for both the *C. boninense* species complex and the remainder of the *Colletotrichum* spp., produced topologically similar trees, and confirmed that 30 isolates recovered in this study belong to the *C. boninense* species complex, 50 to *C. gloeosporioides* species complex and two to *C. acutatum* species complex. The combined species phylogeny of the *C. boninense* species complex consisted of 45 sequences, including the outgroup sequences of *C. acutatum* (culture CBS 112996). All the species belonging to the *C. gloeosporioides* and *C. acutatum* species complexes were included in a combined phylogeny consisting of 86 sequences, including the outgroup sequences of *Moniolochaetes infuscans* (CBS 896.96). A total of 3 149 characters (ITS: 1–549, *GAPDH*: 556–863, *ACT*: 870–1166, *CAL*: 1173–1946, *CHS-1*: 1953–2237, *TUB2*: 2244–2754, *HIS3*: 2761–3149) were included in both phylogenetic analyses. For the phylogeny of the *C. boninense* species complex, 411 characters were parsimony-informative, 454 were variable and parsimony-uninformative and 2 248 characters were constant. A maximum of 1 000 equally most parsimonious trees were saved (Tree length = 1 236, CI = 0.871, RI = 0.947 and RC = 0.825). Regarding the *C. gloeosporioides* and *C. acutatum* species complexes, 1 171 characters were parsimony-informative, 319 were variable and parsimony-uninformative and

**Table 2** Pathogenicity testing of *Colletotrichum* species: percentage of infected inoculation points of citrus fruits.

Species	Isolates	Infected inoculation points (%)	
		Tarocco 'Scirè'	Tarocco 'Nucellare'
<i>Colletotrichum acutatum</i>	CBS 142407 = CPC 27005	0	0
<i>C. catinaense</i>	CBS 142417 = CPC 27978	12.5	4.1
<i>C. catinaense</i>	CBS 142416 = CPC 28019	18.75	6.2
<i>C. gloeosporioides</i>	CBS 142408 = CPC 28059	87.5	83.3
<i>C. helleniense</i>	CBS 142418 = CPC 26844	14.6	8.3
<i>C. helleniense</i>	CBS 142419 = CPC 27107	31.2	16.6
<i>C. hystricis</i>	CBS 142411 = CPC 28153	20.8	8.3
<i>C. hystricis</i>	CBS 142412 = CPC 28154	16.6	10.4
<i>C. karstii</i>	CBS 142415 = CPC 26379	8.3	6.2
<i>C. limonicola</i>	CBS 142409 = CPC 27861	25	8.3
<i>C. limonicola</i>	CBS 142410 = CPC 31141	16.6	12.5
<i>C. novae-zelandiae</i>	CBS 142413 = CPC 26949	20.8	16.6
<i>C. novae-zelandiae</i>	CBS 142414 = CPC 27888	10.4	4.1

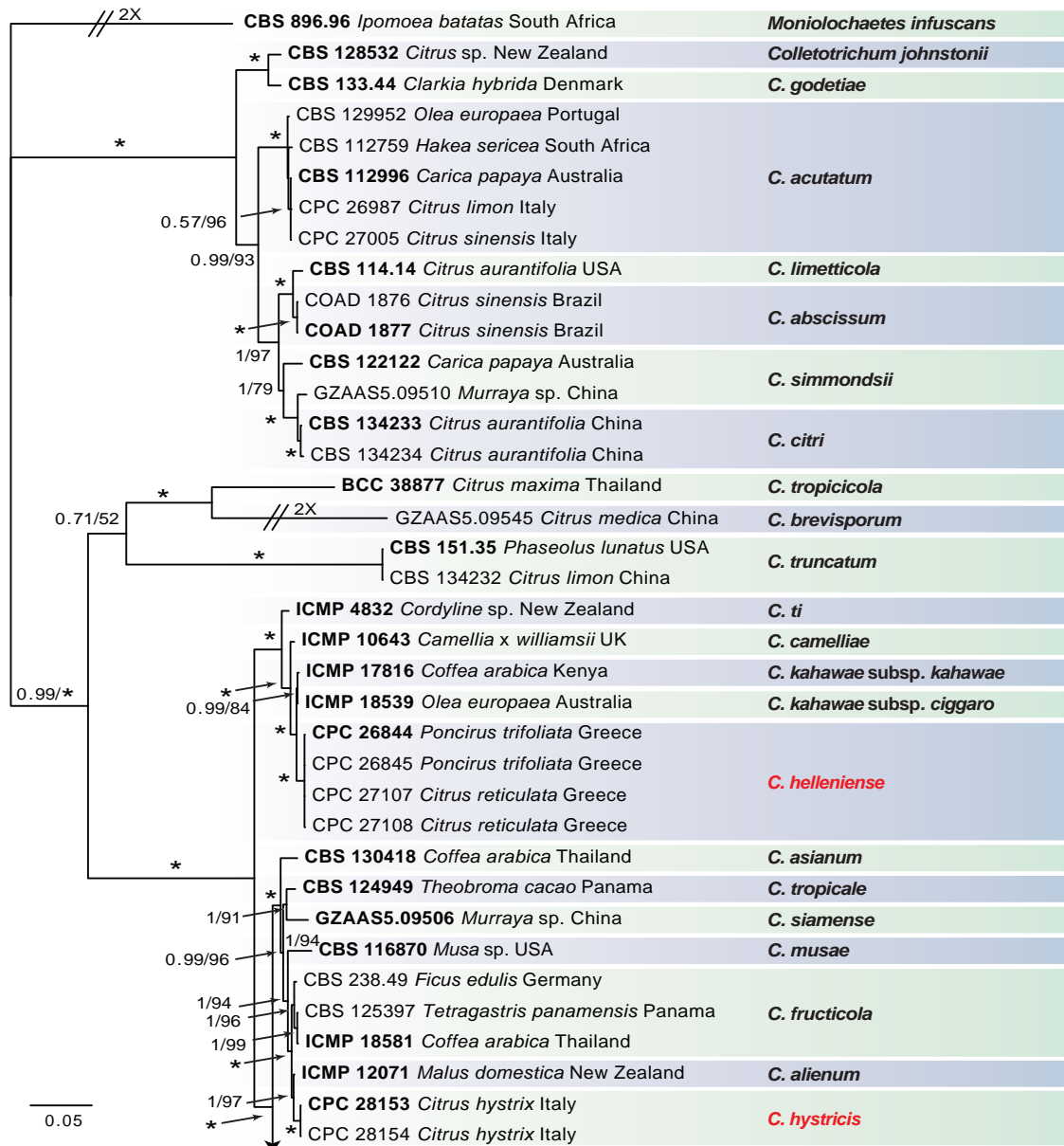


**Fig. 1** Symptoms on citrus tissues with associated *Colletotrichum* spp. a–b. Anthracnose symptoms on leaves of naturally infected: a. *Citrus bergamia* and b. *Fortunella margarita*; c. mummified fruit of *Citrus limon*; d–g. various symptoms on fruits: d. diverse lesions and e–f. sunken lesions on orange and g. on mandarin; h. tear stain on grapefruit; i. stem-end rot on orange; j. typical anthracnose on fallen orange fruits; k. wither-tip of *Citrus sinensis* tree.



**Fig. 2** Consensus phylogram of 4 522 trees resulting from a Bayesian analysis of the combined ITS, CAL, GAPDH, ACT, TUB2, CHS-1 and HIS3 sequence alignments of the *Colletotrichum boninense* species complex. Bootstrap support values and Bayesian posterior probability values are indicated at the nodes. The asterisk symbol (\*) represents full support (1/100). Substrate and country of origin, where known, are listed next to the strain numbers. In red the novel species. The tree was rooted to *Colletotrichum acutatum* (CBS 112996).





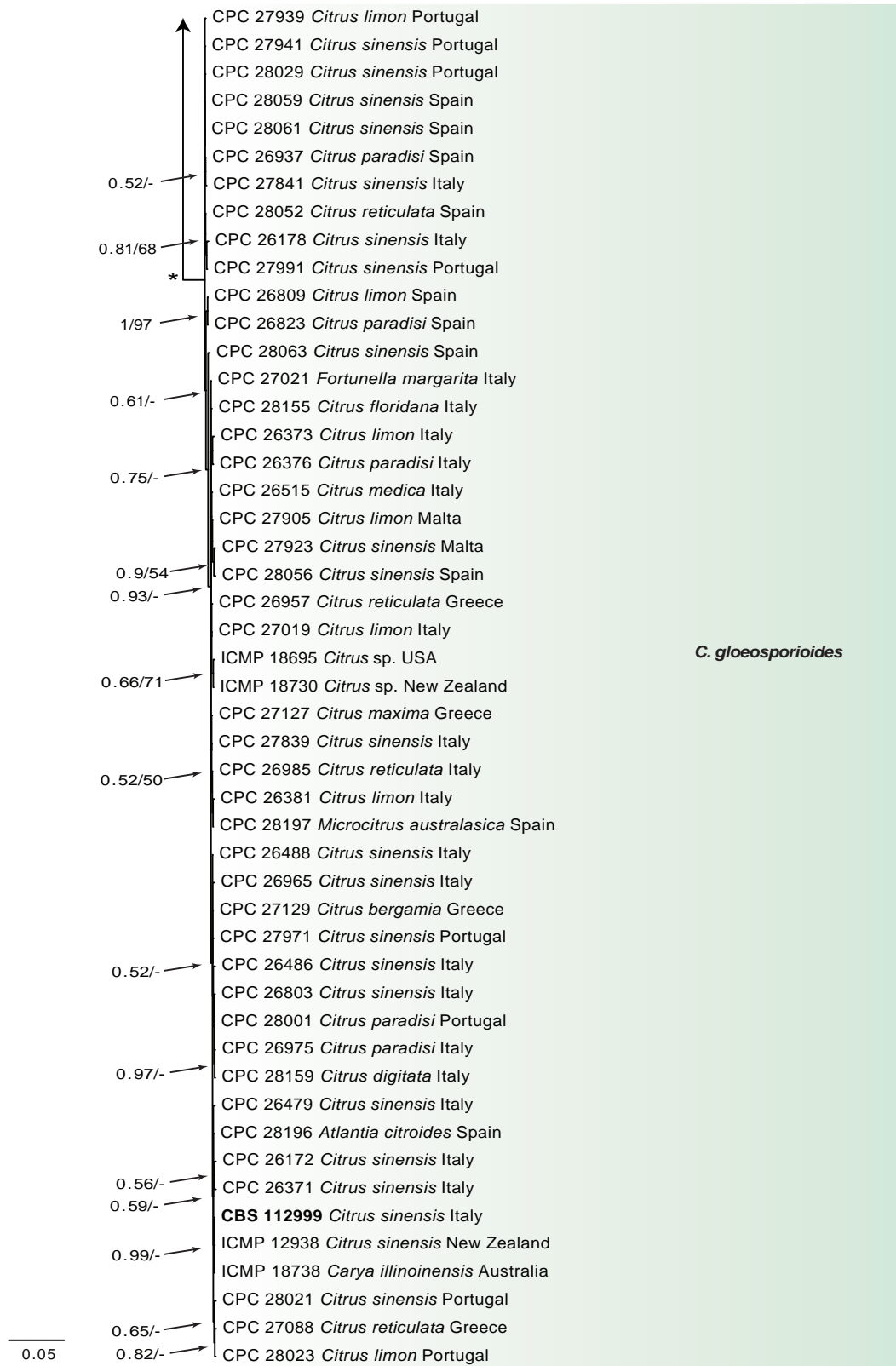
**Fig. 3** Consensus phylogram of 9 782 trees resulting from a Bayesian analysis of the combined ITS, *CAL*, *GAPDH*, *ACT*, *TUB2*, *CHS-1* and *HIS3* sequence alignments of *Colletotrichum acutatum* and *C. gloeosporioides* species complex. Bootstrap support values and Bayesian posterior probability values are indicated at the nodes. The asterisk symbol (\*) represents full support (1/100). Substrate and country of origin, where known, are indicated next to the strain numbers. The tree was rooted to *Moniolochaetes infuscans* (CBS 896.96).

1 623 characters were constant. A maximum of 1 000 equally most parsimonious trees were saved (Tree length = 3 238, CI = 0.736, RI = 0.921 and RC = 0.678). Bootstrap support values from the parsimony analysis were plotted on the Bayesian phylogenies presented in Fig. 2, 3. For both of the Bayesian analyses, MrModeltest suggested that all partitions should be analysed with dirichlet state frequency distributions, except for the *CHS-1* partition, which was analysed with a fixed state frequency distribution. The following models were recommended by MrModeltest and used: GTR+I+G for ITS, *CAL* and *HIS3*, HKY+I+G for *GAPDH* and *TUB2*, HKY+G for *ACT* and SYM+I+G for *CHS-1*. In the Bayesian analysis of the *C. boninense* species complex, the ITS partition had 68 unique site patterns, the *GAPDH* partition 147, the *ACT* partition 108, the *CAL* partition 144, the *CHS-1* partition 51, the *TUB2* partition 146, the *HIS3* partition 72 and the analysis ran for 2 260 000 generations, resulting in 4 522 trees of which 3 392 trees were used to calculate the posterior probabilities. Regarding the *C. gloeosporioides* and *C. acutatum* species complex, the ITS

partition had 167 unique site patterns, the *GAPDH* partition 247, the *ACT* partition 183, the *CAL* partition 304, the *CHS-1* partition 81, the *TUB2* partition 257, the *HIS3* partition 123 and the analysis ran for 4 890 000 generations, resulting in 9 782 trees of which 7 338 trees were used to calculate the posterior probabilities.

In the *C. boninense* species complex analysis 19 *Citrus* isolates clustered with six reference strains of *C. karstii*, whilst five isolates clustered with the ex-type of *C. novae-zelandiae*. Moreover, three isolates were identified as *C. catinaense* and a further three as *C. limonicola*, forming two highly supported subclades (1.00/100) which are embedded in the same clade with *C. novae-zelandiae*. In the other analyses two isolates clustered with the ex-type strain of *C. acutatum* s.str. and 44 isolates with the ex-type strain and other reference strains of *C. gloeosporioides* s.str. Furthermore, two isolates were identified as *C. hystrix* (closely related to *C. alienum*) and four as *C. helleniense* (close to *C. kahawae* subspecies) within the *C. gloeosporioides* species complex.

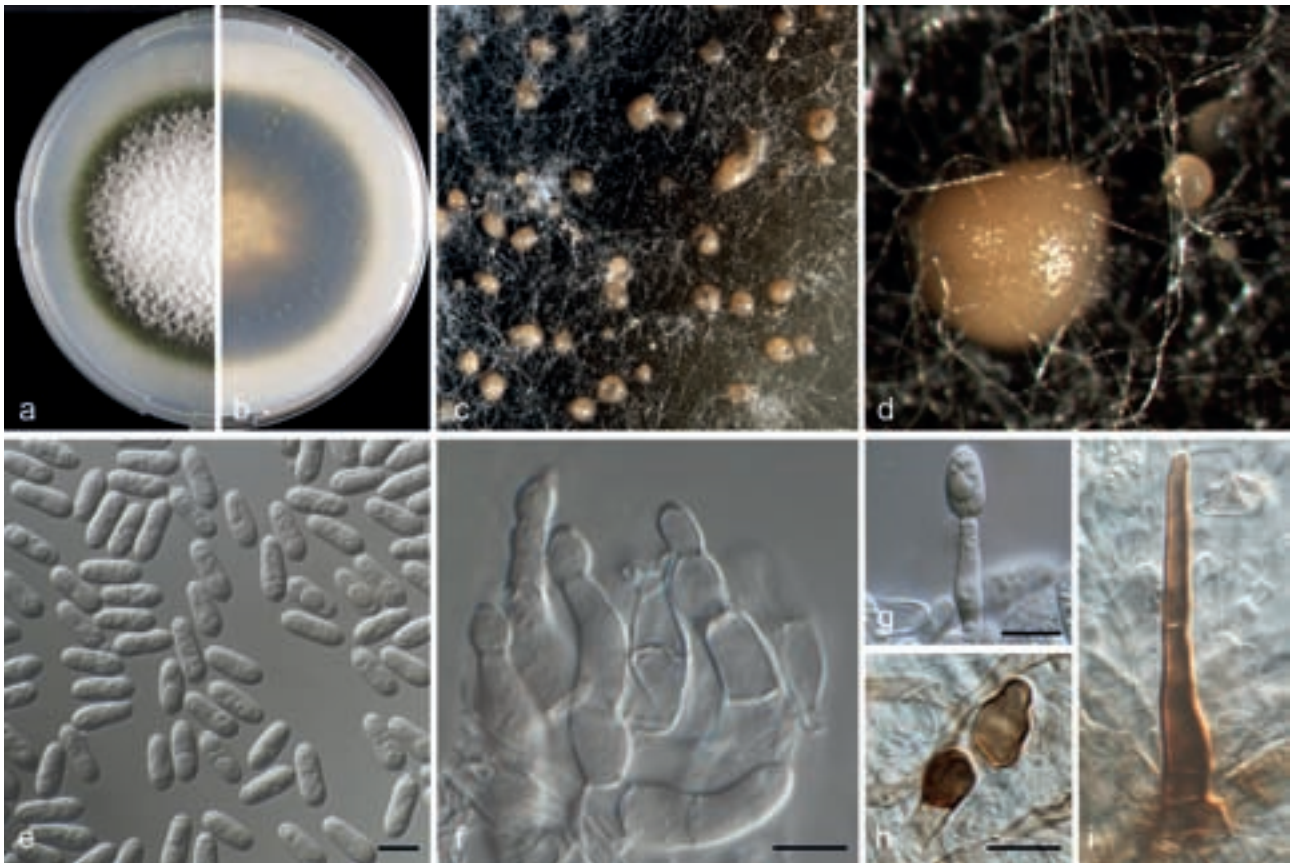
Fig. 3 (cont.)



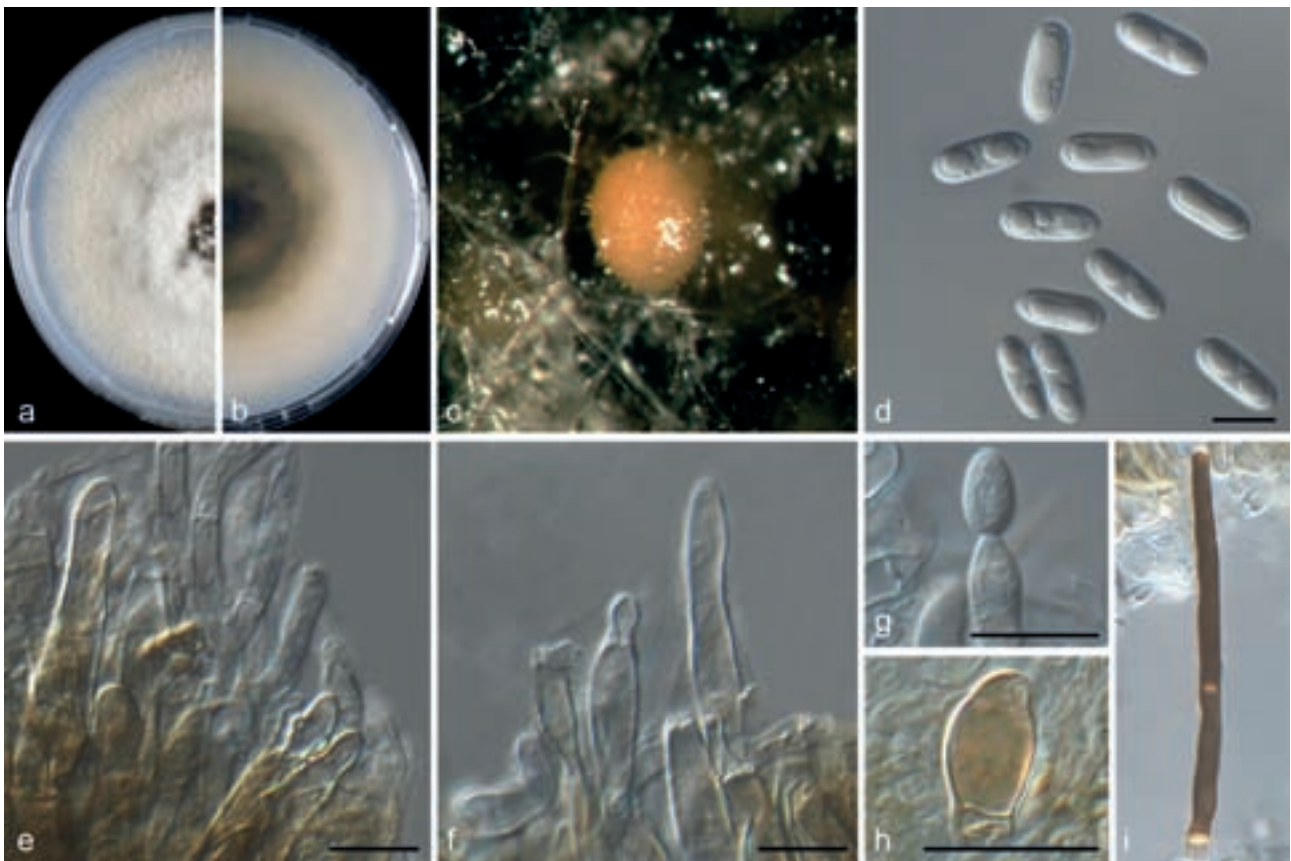
The individual alignments and trees of the seven single genes in both analyses, were compared as well with respect to their performance in species recognition. In the *C. boninense* species complex analysis, *TUB2* differentiated all the taxa. Moreover, the single loci *CAL* and *GAPDH*, clearly separated *C. catinaense* and *C. limonicola*, respectively. In the other analyses, all the *Colletotrichum* species collected from citrus in this study differed in *GAPDH* sequences. Furthermore, *C. helleniense* was separated also by *CAL* and *TUB2*, whilst *ACT* and *CHS-1* distinguished *C. hystricis*.

### Taxonomy

Morphological observations, supported by phylogenetic inference, were used to identify four known species (*C. gloeosporioides*, *C. novae-zelandiae*, *C. karstii* and *C. acutatum*) and to describe four novel species. Culture characteristics were assessed, and the colour of upper and lower surfaces of Petri dishes determined as shown in Fig. 4–7. Hyphal appressoria were abundantly observed on the reverse side of colonies growing on SNA plates. Based on the results of both the phylogenetic and morphological analyses, the four distinct novel species are described below.



**Fig. 4** *Colletotrichum catinaense* (CBS 142417). a–b. Colonies on PDA above and below; c–d. conidiomata; e. conidia; f–g. conidiophores; h. appressoria; i. seta (a–g, i from PDA; h from SNA). — Scale bars = 10 µm.



**Fig. 5** *Colletotrichum helleniense* (CBS 142418). a–b. Colonies on PDA above and below; c. conidiomata; d. conidia; e–g. conidiophores; h. appressoria; i. seta (a–f, i from PDA; g–h from SNA). — Scale bars = 10 µm.

***Colletotrichum catinaense*** Guarnaccia & Crous, sp. nov. — MycoBank MB820247; Fig. 4

*Etymology.* Named after the city where the first strain was collected, Catania (ancient Latin name, *Catina*).

*Asexual morph on SNA.* Vegetative hyphae hyaline, septate, branched, 1–9 µm diam. Conidiomata, chlamydospores and setae absent. Conidiophores hyaline, smooth-walled, septate, branched, to 40 µm long, formed from hyphae. Conidiogenous cells hyaline, smooth-walled, cylindrical to inflated, 5–18 × 4–5 µm. Conidia hyaline, smooth-walled, aseptate, cylindrical, rounded apex and base, contents granular and guttulate, 11.5–15 × 4–5.5 µm, mean ± SD = 13.5 ± 0.9 × 4.8 ± 0.5 µm, L/W ratio = 2.7. Appressoria medium to dark brown, roundish with an undulate margin, single, 3.5–6 × 3–5.5 µm, mean ± SD = 4.8 ± 0.9 × 4.2 ± 0.5 µm, L/W ratio = 1.2.

*Asexual morph on PDA.* Conidiomata acervular, conidiophores and setae formed on a cushion of pale brown, thick-walled, angular cells, 3.5–7 µm diam. Setae brown, smooth, 2–3-septate, 50–120 µm long, base conical or inflated, dark brown, tip rounded. Conidiophores hyaline, smooth-walled, septate and branched, to 40 µm long. Conidiogenous cells hyaline, smooth-walled, cylindrical, 5–16 × 4–5 µm. Conidia hyaline, smooth-walled, aseptate, cylindrical, rounded apex and base, contents granular and guttulate, 13–16 × 4.5–6 µm, mean ± SD = 14.3 ± 1 × 5.5 ± 0.5 µm, L/W ratio = 2.6.

Culture characteristics — Colonies on SNA flat with entire margin, hyaline, 35–37 mm diam in 7 d (49–52 mm in 10 d). Colonies on PDA flat with entire margin, buff honey in the centre to green olivaceous at the margin, partly covered with floccose white aerial mycelium and with black conidiomata. Conidia present in orange to pale brown mass. Reverse buff, pale luteous to isabelline, dark green in the margin, 66–68 mm diam in 7 d (75–76 mm diam in 10 d).

*Materials examined.* ITALY, Mineo, Catania, from leaf lesion of *Citrus reticulata* (mandarin), 23 Sept. 2015, V. Guarnaccia (CBS H-23024 holotype, culture ex-type CBS 142417 = CPC 27978). — PORTUGAL, Mesquita, from fruit tear-stain of *Citrus sinensis* (orange), 7 Oct. 2015, V. Guarnaccia (culture CBS 142416 = CPC 28019).

*Notes* — *Colletotrichum catinaense* was isolated from several hosts in Italy and Portugal. The isolation of this species from multiple combinations of organ/host demonstrates its ability to colonise different citrus tissues. This species is phylogenetically close to but clearly differentiated from *C. novaezelandiae* in *CAL* and *TUB* sequences. *Colletotrichum novaezelandiae* formed a separate lineage/cluster in all single-gene phylogenies (Damm et al. 2012b) before this study. Based on multi-locus phylogenetic analyses performed in this study (Fig. 2), *C. catinaense* together with *C. limonicola* (described below) are new species belonging to the same clade of *C. novaezelandiae* within the *C. boninense* species complex. This species is morphologically indistinguishable from the other two species of the same clade.

***Colletotrichum helleniense*** Guarnaccia & Crous, sp. nov. — MycoBank MB820249; Fig. 5

*Etymology.* Named after the country where it was collected, Greece (ancient name, *Hellas*).

*Asexual morph on SNA.* Vegetative hyphae hyaline, septate, branched, 1–8 µm diam. Conidiomata, chlamydospores and setae absent. Conidiophores formed from hyphae, hyaline, smooth to finely verruculose, septate, branched, to 50 µm long. Conidiogenous cells are hyaline, smooth-walled, cylindrical to inflated, 5–15 × 4–5 µm. Conidia hyaline, smooth-walled, aseptate, straight, cylindrical, rounded apex and base, contents granular or guttulate, 11–14.5 × 4–5.5 µm, mean ± SD = 12.2

± 0.7 × 4.7 ± 0.5 µm, L/W ratio = 2.6. Appressoria medium to dark brown, roundish or irregular in shape, single or in small groups, 5–10 × 7–15 µm.

*Asexual morph on PDA.* Conidiomata acervular, conidiophores and setae formed on a cushion of pale brown, thick-walled, angular cells, 3.5–7 µm diam. Setae brown, smooth, 2-septate, 55–90 µm long, base conical or inflated, dark brown, tip rounded. Conidiophores hyaline, smooth to undulate walled, septate and branched, to 35 µm long. Conidiogenous cells hyaline, smooth-walled, cylindrical, 5–15 × 4–5.5 µm. Conidia hyaline, smooth-walled, aseptate, cylindrical, rounded apex and base, contents granular or guttulate, 11.5–14.5 × 4–5.5 µm, mean ± SD = 12.7 ± 0.8 × 4.7 ± 0.5 µm, L/W ratio = 2.7.

Culture characteristics — Colonies on SNA flat with entire margin, hyaline, 40–46 mm diam in 7 d (54–59 mm diam in 10 d). Colonies on PDA with entire margin, green to grey in the centre and white to pale buff in the margin, entirely covered with floccose to dense, white to grey aerial mycelium and with black conidiomata. Conidia present in pinkish orange mass. Reverse grey to buff, pale luteous, 59–62 mm diam in 7 d (72–75 mm diam in 10 d).

*Materials examined.* GREECE, Arta, from wither-tip twigs of *Poncirus trifoliata* (citrumelo), 20 May 2015, V. Guarnaccia (CBS H-23025 holotype, culture ex-type CBS 142418 = CPC 26844); from fruit lesions of *C. reticulata* (mandarin), 20 May 2015, V. Guarnaccia (culture CBS 142419 = CPC 27107).

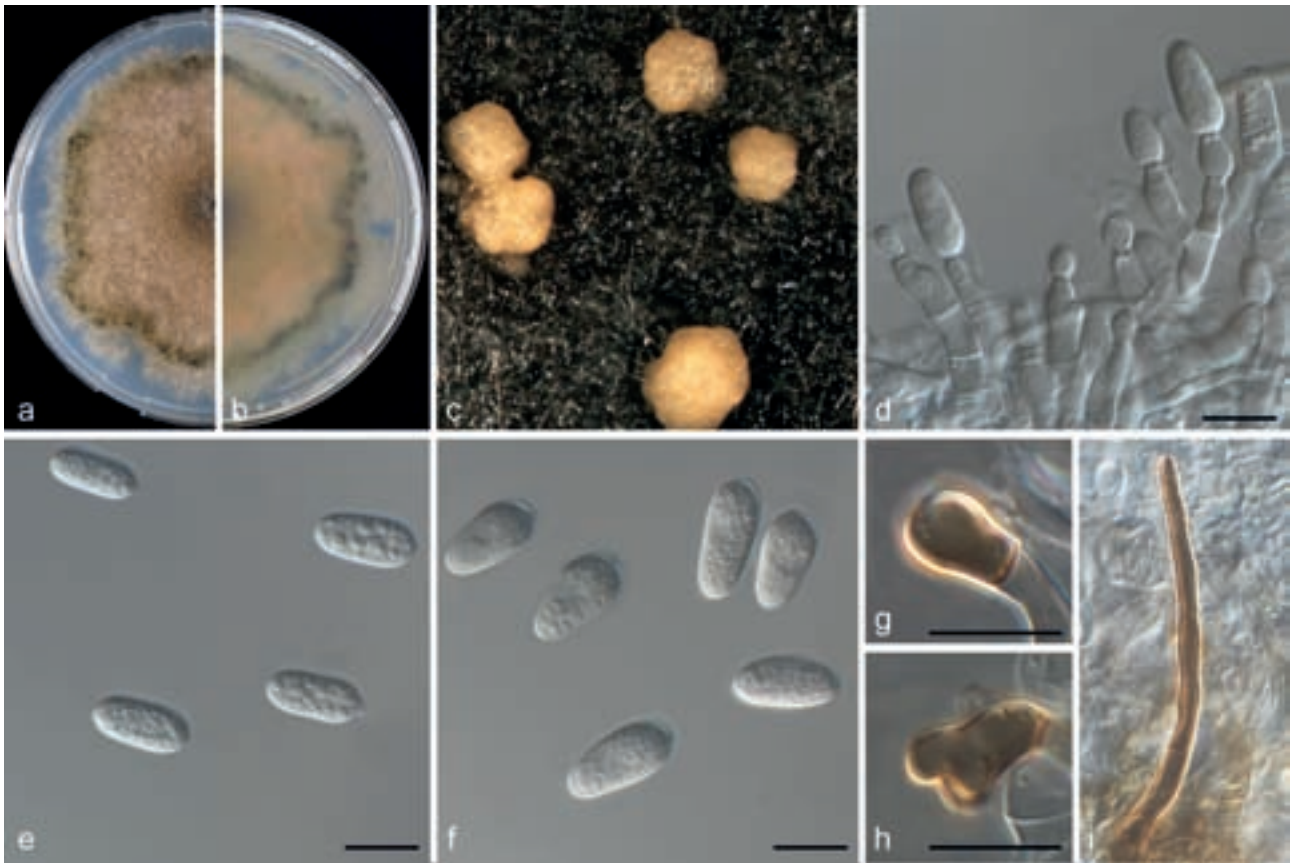
*Notes* — *Colletotrichum helleniense* was isolated from *Citrus reticulata* fruit lesions and from *Poncirus trifoliata* wither-tip twigs in Greece. *Poncirus* is an allied genus of *Citrus*, in the *Rutaceae*, containing species mostly used as rootstock for citrus. These results show the ability of *C. helleniense* to colonise tissues of different genera within the *Rutaceae*. This species is phylogenetically close to but clearly differentiated from *C. kahawae* based on *GAPDH*, *CAL* and *TUB2*. Two subspecies of *C. kahawae* were described in the past; *C. kahawae* subsp. *kahawae* and *C. kahawae* subsp. *ciggaro* (Weir et al. 2012). Recently, the legitimacy of this distinction has been supported by Batista et al. (2016), who accepted the two subspp. as two cryptic species. *Colletotrichum helleniense* is clearly separate from both *C. kahawae* subspecies and from further species such as *C. aotearoa*, *C. clidemiae*, *C. cordylinicola*, *C. jiangxiense*, *C. psidii*, *C. rhexiae* (data not shown) belonging to the same clade (Diao et al. 2017, Weir et al. 2012). Therefore, *C. helleniense* represents a new species in the *C. kahawae* clade, belonging to the *C. gloeosporioides* species complex.

***Colletotrichum hystrixis*** Guarnaccia & Crous, sp. nov. — MycoBank MB820252; Fig. 6

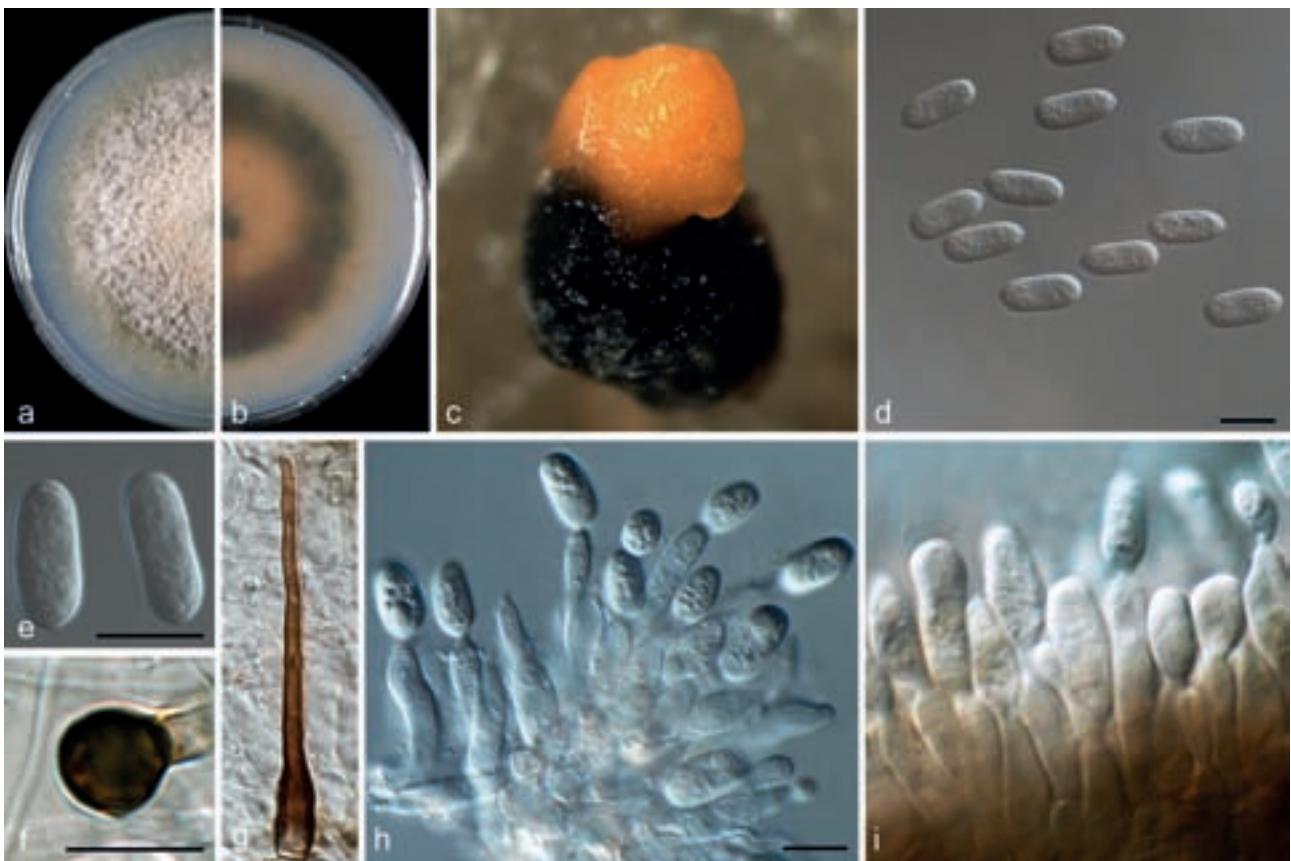
*Etymology.* In reference to its occurrence on *Citrus hystrix*.

*Asexual morph on SNA.* Vegetative hyphae hyaline, septate, 1–7 µm diam. Conidiomata, chlamydospores and setae absent. Conidiophores formed from hyphae, hyaline, smooth-walled, septate, branched, to 40 µm long. Conidiogenous cells hyaline, smooth-walled, cylindrical, 5–10 × 4–5 µm. Conidia hyaline, smooth-walled, aseptate, straight, cylindrical to obovoidal, rounded apex and base, contents granular, 13–15 × 4–5.5 µm, mean ± SD = 14 ± 1.3 × 4.8 ± 0.5 µm, L/W ratio = 2.8. Appressoria dark brown, globose to irregular shape, single, with irregular lobes, 3.5–8 × 3–5.5 µm, mean ± SD = 6 ± 0.9 × 4.2 ± 0.5 µm, L/W ratio = 1.4.

*Asexual morph on PDA.* Conidiomata acervular, conidiophores and setae formed on a cushion of pale brown, thick-walled, angular cells, 3.5–7 µm diam. Setae brown, smooth, 2–3-septate, curved, 50–100 µm long, base conical, dark brown, tip rounded. Conidiophores hyaline, smooth-walled, septate, branched, to 50 µm long. Conidiogenous cells hyaline, smooth-walled to



**Fig. 6** *Colletotrichum hystricis* (CBS 142411). a–b. Colonies on PDA above and below; c. conidiomata; d. conidiophores; e–f. conidia; g–h. appressoria; i. seta (a–g, i from PDA; h from SNA). — Scale bars = 10  $\mu$ m.



**Fig. 7** *Colletotrichum limonicola* (CBS 142410). a–b. Colonies on PDA above and below; c. conidiomata; d–e. conidia; f. appressoria; g. seta; h–i. conidiophores (a–e, g–i from PDA; f from SNA). — Scale bars = 10  $\mu$ m.

undulate, cylindrical, 5–20 × 3–5 µm. *Conidia* hyaline, smooth-walled, cylindrical to obovoidal, aseptate, rounded apex and base, contents granular, 14–16 × 4.5–6 µm, mean ± SD = 13.8 ± 1 × 5.1 ± 0.4 µm, L/W ratio = 2.7.

Culture characteristics — Colonies on SNA flat with entire margin, hyaline, 60–61 mm diam in 7 d (72–75 mm diam in 10 d). Colonies on PDA flat with entire margin, buff honey to pinkish, green to grey in the margin, entirely covered with white aerial mycelium and with black conidiomata. Conidia present in orange mass. Reverse buff, pale luteous to dark green, 69–71 mm diam in 7 d (80–82 mm diam in 10 d).

*Materials examined.* ITALY, Mascali, Catania, from leaf lesion of *Citrus hystrix*, 30 Jan. 2016, V. Guarnaccia (CBS H-23026 holotype, culture ex-type CBS 142411 = CPC 28153); *ibid.*, (culture CBS 142412 = CPC 28154).

Notes — *Colletotrichum hystricis* was isolated from *Citrus hystrix* leaf lesions in Sicily, Italy. This species differs from closely related species in *GAPDH*, *ACT* and *CHS-1* sequences. *Colletotrichum hystricis* is similar to *C. alienum* and other species such as *C. aenigma*, *C. conoides* and *C. nupharicola* (Weir et al. 2012, Diao et al. 2017) but represents a distinct taxon, supported also by morphological differences. *Colletotrichum hystricis* differs from *C. alienum* in having obovoidal conidia (also on SNA) and a slower growth rate.

***Colletotrichum limonicola*** Guarnaccia & Crous, *sp. nov.* — MycoBank MB820254; Fig. 7

*Etymology.* In reference to its occurrence on *Citrus limon*.

*Asexual morph on SNA.* Vegetative hyphae hyaline, septate, branched, 1–10 µm diam. *Conidiomata*, *chlamydospores* and *setae* absent. *Conidiophores* formed from hyphae, hyaline, smooth-walled, septate, branched, to 50 µm long. *Conidigenous cells* are hyaline, smooth-walled, cylindrical to inflated, 5–20 × 4–5 µm. *Conidia* hyaline, smooth-walled, aseptate, straight, cylindrical, rounded apex and base, contents granular, 9–15 × 4–6 µm, mean ± SD = 12.2 ± 1.3 × 6 ± 0.5 µm, L/W ratio = 2.5. *Appressoria* medium to dark brown, roundish, single, 3–6 × 3–5.5 µm, mean ± SD = 4.5 ± 0.9 × 4.2 ± 0.5 µm, L/W ratio = 1.1.

*Asexual morph on PDA.* *Conidiomata* acervular, *conidiophores* and *setae* formed on a cushion of pale brown, thick-walled, angular cells, 3.5–7 µm diam. *Setae* brown, smooth, 2–3-septate, 45–100 µm long, base conical or inflated, dark brown, tip rounded. *Conidiophores* hyaline, smooth walled, septate and branched, to 50 µm long. *Conidigenous cells* hyaline, smooth-walled, cylindrical, 7–16 × 4–5.5 µm. *Conidia* hyaline, smooth-walled, aseptate, cylindrical, rounded apex and base, contents granular, 9.5–15.5 × 4–6 µm, mean ± SD = 12.7 ± 1.3 × 5 ± 0.5 µm, L/W ratio = 2.5.

Culture characteristics — Colonies on SNA flat with entire margin, hyaline, 44–46 mm diam in 7 d (58–60 mm diam in 10 d). Colonies on PDA flat with entire margin, buff honey in the centre to green olivaceous in the margin, entirely covered with floccose white aerial mycelium and with black conidiomata. Conidia present in orange to pale brown mass. Reverse buff, pale luteous to dark green, 64–66 mm diam in 7 d (75–76 mm diam in 10 d).

*Materials examined.* MALTA, Gozo, from wither-tip twigs of *Citrus limon* (lemon), 11 July 2016, V. Guarnaccia (CBS H-23027 holotype, culture ex-type CBS 142410 = CPC 31141); from leaf lesions of *C. limon*, 22 Sept. 2015, V. Guarnaccia (culture CBS 142409 = CPC 27861).

Notes — *Colletotrichum limonicola* was isolated from leaf lesions and twigs with wither-tip symptoms on *Citrus limon* in Malta. This species is phylogenetically close to but clearly differentiated from *C. novae-zelandiae* based on *GAPDH* and *TUB*. *Colletotrichum limonicola* and *C. catinaense* (described above)

are new species belonging to the same clade of *C. novae-zelandiae* in the *C. boninense* species complex.

### Pathogenicity

All tested isolates except that of *C. acutatum* were pathogenic to most of the detached orange fruits (Table 2). Both *Citrus sinensis* clones tested developed typical brown lesions around the fruit wounds after 10 d (Fig. 8). *Colletotrichum gloeosporioides* and *C. karstii*, respectively, showed the highest and the weakest aggressiveness among the eight species inoculated. Clone ‘Tarocco Scirè’ was more susceptible. The inoculated *Colletotrichum* isolates were re-isolated from the symptomatic tissues, fulfilling Koch’s postulates. No symptoms developed on the negative controls.

### DISCUSSION

Recent studies of phylogenetic analyses in the genus *Colletotrichum* revealed species to cluster in 11 major clades, as well as a number of small clusters and isolated species (Cannon et al. 2012, Marin-Felix et al. 2017). Four of these major clades represent important species complexes (*C. acutatum*, *C. boninense*, *C. gloeosporioides* and *C. truncatum*) (Damm et al. 2009, 2012a, b, Weir et al. 2012), which include very important plant pathogenic species. In these studies, a large number of taxa were differentiated and described. The recent revision and epitypification of the main *Colletotrichum* species complexes (Damm et al. 2009, 2012a, b, 2013, 2014, Weir et al. 2012, Liu et al. 2014), as well as several studies that focussed on citrus diseases (Peng et al. 2012, Huang et al. 2013, Aiello et al. 2015, Perrone et al. 2016), facilitated the description of several new species on *Citrus* and allied genera in this study (Table 3).

*Colletotrichum* spp. are frequently associated with several citrus diseases worldwide (Timmer et al. 2000), such as PFD on sweet orange, KLA on lime and wither-tip, leaf spot, pre- and post-harvest anthracnose on different hosts (Brown et al. 1996, Timmer et al. 2000, Peres et al. 2008, Lima et al. 2011, McGovern et al. 2012). Before the multi-gene analysis era, *C. acutatum* was identified as the only species responsible for PFD (Peres et al. 2008) and KLA (Brown et al. 1996). Similarly, *C. gloeosporioides* was reported as the only *Colletotrichum* species to cause citrus fruit anthracnose (Brown 1975, Timmer et al. 2000). During the last decade a polyphasic approach was used in several *Colletotrichum* studies, revealing new species involved with citrus diseases, such as *C. abscissum* and *C. gloeosporioides* associated with PFD (Lima et al. 2011, Crous et al. 2015, Silva et al. 2016).

During the last years *Colletotrichum* spp. affected several commercial orchards in the main citrus producing areas of Mediterranean, causing a broad variety of symptoms and, consequently, losses of marketable fruits (Aiello et al. 2015, Ramos et al. 2016, Rhaïem & Taylor 2016). Therefore, the need for a large-scale investigation of *Colletotrichum* spp. associated with citrus infections in Europe was needed. This study provides the first molecular characterisation of *Colletotrichum* diversity related to citrus production in Europe, combined with morphological characterisation.

We performed single gene and multilocus DNA sequence analyses combining seven loci (*ITS*, *CAL*, *GAPDH*, *ACT*, *TUB2*, *CHS-1* and *HIS3*) commonly used in previous phylogenetic studies of the *C. gloeosporioides*, *C. acutatum* and *C. boninense* species complexes (Damm et al. 2012a, b, Weir et al. 2012, Bragança et al. 2016). These species complexes incorporate several taxa (Damm et al. 2012a, b, Weir et al. 2012). However, only the closest taxa to the eight *Colletotrichum* species recovered in



**Fig. 8** Pathogenicity test of selected *Colletotrichum* isolates on *Citrus sinensis* fruits after 10 d. Fruits inoculated with: a–d. *C. gloeosporioides* (CBS 142408); e. *C. catinaense* (CBS 142417); f. *C. limonicola* (CBS 142410); g. *C. novae-zelandiae* (CBS 142414); h. *C. hystricis* (CBS 142411); i. *C. helleniense* (CBS 142418); j. *C. karstii* (CBS 142415).

this study, were selected based on BLAST searches of NCBI's GenBank nucleotide database and included in the analyses. The final phylogenetic trees clearly distinguished each of these eight species.

We surveyed several citrus orchards, plant nurseries, private gardens and collections in five Mediterranean European countries. We further investigated host plant members of *Citrus*-allied genera, also economically important as ornamental (*Atlantia*, *Murraya*) or rootstock plants (*Poncirus*), and also for fruit production (*Fortunella*, *Microcitrus*). We obtained 174 *Colletotrichum* single spore strains from symptomatic tissues. Based on multi-locus data we found species allocated in three species complexes. *Colletotrichum gloeosporioides* in the *C. gloeosporioides* species complex, and *C. karstii* in the *C. boninense* species complex were the predominant species. However, *C. gloeosporioides* was found in all the countries investigated, whereas *C. karstii* was not isolated from samples collected in Greece. Moreover, *C. acutatum* s.str., part of the

*C. acutatum* species complex, was recovered only on the Aeolian Islands (Italy), a volcanic archipelago to the north of Sicily. *Colletotrichum novae-zelandiae* was recovered in association with leaf spot on grapefruit in Greece and with twig cankers in orange and lemon trees in Malta. In addition, four new species were detected and described. *Colletotrichum catinaense* was associated with multiple symptoms on different hosts in Italy and Portugal. *Colletotrichum helleniense* was isolated from *Citrus reticulata* fruit anthracnose and from leaf lesions on *Poncirus trifoliata* in Greece. *Colletotrichum hystricis* was associated with leaf lesions of young plants of *Citrus hystrix* cultivated in a greenhouse located on Sicily and *C. limonicola* was recovered on Malta from leaf lesions on lemon plants.

Pathogenicity of all the species isolated from citrus samples collected in Europe was preliminarily tested on two clones of *Citrus sinensis*. Representative isolates were selected and artificially inoculated on orange fruits of clones 'Tarocco Scirè' and 'Tarocco Nucellare' (Rapisarda & Russo 2003). All of the

**Table 3** Global distribution of *Colletotrichum* species occurring in *Citrus* hosts and allied genera.

Species complex	Species	Host	Organ	Geographical distribution	Reference(s)
<i>C. acutatum</i>	<i>C. abscissum</i>	<i>Citrus sinensis</i>	Flower	Brazil, USA	Crous et al. (2015), Bragança et al. (2016)
	<i>C. acutatum</i>	<i>Citrus limon</i>	Leaf	Italy	This study
		<i>Citrus sinensis</i>	Leaf		
	<i>C. citri</i>	<i>Citrus aurantiifolia</i>	Twig	China	Huang et al. (2013)
	<i>C. godetiae</i>	<i>Citrus aurantium</i>	Fruit	Unknown	Damm et al. (2012a)
	<i>C. johnstonii</i>	<i>Citrus</i> sp.	Fruit	New Zealand	Damm et al. (2012a)
	<i>C. limetticola</i>	<i>Citrus aurantiifolia</i>	Twig	Cuba, USA	Clausen (1912), Damm et al. (2012a)
	<i>C. simmondsii</i>	<i>Citrus reticulata</i>	Fruit	China	Peng et al. (2012), Phouivong et al. (2012)
		<i>Murraya</i> sp.	Leaf		
	<i>C. boninense</i>	<i>C. boninense</i>	<i>Citrus medica</i>	Leaf	China
<i>C. catinaense</i>		<i>Citrus aurantiifolia</i>	Twig	Italy, Malta, Portugal	This study
		<i>Citrus reticulata</i>	Leaf		
		<i>Citrus sinensis</i>	Fruit		
<i>C. citricola</i>		<i>Citrus unchiu</i>	Leaf	China	Huang et al. (2013)
<i>C. constrictum</i>		<i>Citrus limon</i>	Fruit	New Zealand	Damm et al. (2012b)
<i>C. karstii</i>		<i>Citrus grandis</i>	Leaf, twig	China, Europe, New Zealand, South Africa	Damm et al. (2012b), Peng et al. (2012), Huang et al. (2013), This study
		<i>Citrus limon</i>	Fruit, leaf, twig		
		<i>Citrus paradisi</i>	Twig		
		<i>Citrus reticulata</i>	Leaf, twig		
		<i>Citrus sinensis</i>	Fruit, leaf, twig		
		<i>Fortunella margarita</i>	Fruit		
		<i>Murraya paniculata</i>	Leaf		
<i>C. limoncola</i>		<i>Citrus limon</i>	Leaf	Malta	This study
<i>C. novae-zelandiae</i>		<i>Citrus medica</i>	Fruit	Greece, Malta, New Zealand	Damm et al. (2012b), This study
	<i>Citrus limon</i>	Leaf, twig			
	<i>Citrus paradisi</i>	Leaf			
	<i>Citrus sinensis</i>	Twig			
<i>C. gloeosporioides</i>	<i>C. fructicola</i>	<i>Citrus reticulata</i>	Leaf	China	Huang et al. (2013)
		<i>Fortunella margarita</i>	Branch		
	<i>C. gloeosporioides</i>	<i>Atlantia citroides</i>	Leaf	Brazil, China, Ethiopia, Ghana, Greece, Italy, Malta, Portugal, Spain, New Zealand, Tunisia, USA	Lima et al. (2011), Weir et al. (2012), Huang et al. (2013), Honger et al. (2016), Moges et al. (2016), Rhaeim & Taylor (2016), This study
		<i>Citrus bergamia</i>	Fruit		
		<i>Citrus digitata</i>	Leaf		
		<i>Citrus floridana</i>	Fruit		
		<i>Citrus grandis</i>	Leaf		
		<i>Citrus limon</i>	Fruit, leaf, twig		
		<i>Citrus maxima</i>	Twig		
		<i>Citrus medica</i>	Leaf		
		<i>Citrus paradisi</i>	Leaf, twig		
		<i>Citrus reticulata</i>	Fruit, leaf, twig		
		<i>Citrus sinensis</i>	Flower, fruit, leaf, twig		
		<i>Citrus unchiu</i>	Branch, leaf		
		<i>Fortunella margarita</i>	Twig		
		<i>Microcitrus australasica</i>	Twig		
	<i>C. helleniense</i>	<i>Citrus reticulata</i>	Fruit	Greece	This study
		<i>Poncirus trifoliata</i>	Twig		
	<i>C. hystricis</i>	<i>Citrus hystrix</i>	Leaf	Italy	This study
	<i>C. kahawae</i> subsp. <i>ciggaro</i>	<i>Citrus reticulata</i>	Leaf	Italy	Perrone et al. (2016)
<i>C. siamense</i>	<i>Murraya</i> sp.	Leaf	China	Liu et al. (2016)	
<i>C. truncatum</i>	<i>C. truncatum</i>	<i>Citrus flamea</i>	Twig	China	Huang et al. (2013)
		<i>Citrus limon</i>	Leaf		
–	<i>C. brevisporum</i>	<i>Citrus medica</i>	Leaf	China	Peng et al. (2012)
–	<i>C. tropicicola</i>	<i>Citrus maxima</i>	Leaf	Thailand	Liu et al. (2014)

*Colletotrichum* species tested, except *C. acutatum*, developed lesions on fruits. These results demonstrated a cross-infection potential between multiple species on fruits of two clones of species as already reported by a previous study on *Colletotrichum* (Freeman et al. 1998). However, our pathogenicity experiments were conducted under extreme conditions commonly applied in artificial inoculations, and it remains to be seen how easily the symptoms development will happen under natural conditions. The pathogenicity test performed in this study confirmed that *C. acutatum* is not able to cause symptoms on citrus fruits.

However, the establishment of the PFD disease caused by *C. acutatum* in Europe should be a focus in future surveys. *Colletotrichum gloeosporioides* was the most aggressive species, causing typical brown lesions that involved the skin and the albedo tissues. Although *C. karstii* showed the lowest aggressiveness, the pathogenicity test demonstrated its ability to cause lesions on fruits, which was also true for the remaining species, *C. catinaense*, *C. helleniense*, *C. hystricis*, *C. limoncola* and *C. novae-zelandiae*. The clone 'Tarocco Scirè' appeared more susceptible than 'Tarocco Nucellare' as Aiello et al. (2015) recently demonstrated for *C. gloeosporioides* and *C. karstii*.



*Colletotrichum acutatum* s.lat. is a common pathogen of several crops, including citrus, worldwide (Damm et al. 2012a). In Europe it has been detected on different hosts such as strawberry (Garrido et al. 2008), strawberry tree (Polizzi et al. 2011), olives (Moral et al. 2008), but never on citrus. Furthermore, *C. novae-zelandiae* was previously recovered from grapefruit and chili in New Zealand (Damm et al. 2012b). Thus, this study represents the first report of *C. acutatum* associated with citrus in Europe and the first detection of *C. novae-zelandiae* outside of New Zealand. *Colletotrichum karstii*, a member of the *C. boninense* species complex, has been reported on many host plants with a wide geographical distribution (Damm et al. 2012b). This species has been reported on citrus in South Africa, New Zealand and China (Damm et al. 2012b, Peng et al. 2012, Huang et al. 2013) as well as in Europe, where it was reported as citrus pathogen in Italy and Portugal (Aiello et al. 2015, Ramos et al. 2016). In Europe, *C. karstii* has been detected also on other hosts such as tropical fruits, cotton and lupine plants (Damm et al. 2012b, Ismail et al. 2015). *Colletotrichum gloeosporioides* was largely dominant in our investigation, in agreement with recent global results (Lima et al. 2011, Huang et al. 2013, Aiello et al. 2015, Honger et al. 2016, Ramos et al. 2016, Rhaiem & Taylor 2016). *Colletotrichum gloeosporioides* was isolated from all the citrus organs sampled (leaves, flowers, fruit and twigs), and proved to be the most aggressive *Colletotrichum* species. This species is reported as pathogen of the main cultivated citrus species worldwide (Huang et al. 2013) and to our knowledge the present study represents the first report of *C. gloeosporioides* associated with citrus flower disease in Europe, previously reported in Brazil (Lima et al. 2011). *Colletotrichum catinaense* and *C. limonicola* represent new species in the *C. novae-zelandiae* clade within the *C. boninense* species complex. *Colletotrichum catinaense* was recovered associated with infections of diverse *Citrus* species, whereas *C. limonicola* has been isolated only from lemon leaf lesions. Thus, more surveys are needed to investigate distribution and host specificity of this new species. *Colletotrichum helleniense* was isolated from *Citrus reticulata* and from *Poncirus trifoliata*, a member of the *Rutaceae* family largely cultivated in nurseries as citrus rootstock due to its economically useful traits, including cold temperature and poor soil tolerance, and Citrus Tristeza Virus resistance (Garnsey & Barrett 1987). This report shows the ability of *C. helleniense* to colonise tissues of different genera within the *Rutaceae*. Recently, Batista et al. (2016) supported the distinction of two *C. kahawae* subsp. as two cryptic species. *Colletotrichum kahawae* subsp. *ciggaro*, one of these subspecies, has also recently been recorded by Perrone et al. (2016) as a pathogen of mandarin (*Citrus reticulata*). However, *C. helleniense* is phylogenetically close to both *C. kahawae* subspecies, but clearly differentiated based on multi-locus phylogenetic analyses. As such it thus represents a new species in the *C. kahawae* clade in the *C. gloeosporioides* species complex. *Colletotrichum hystricis* was isolated from lesions on leaves of *Citrus hystrix*. This *Citrus* species is commonly cultivated, has a pleasant smell, and is referred to as medicinal lime (Yaacob & Subhadrabandhu 1995). The fruit is not appreciated, but is economically important for the extraction of essential oil used for cooking and cosmetics (Allen 1967). *Colletotrichum hystricis* is close to but clearly differentiated from *C. alienum*, which is commonly associated with cultivated fruits (Weir et al. 2012). In the present study it is described as a distinct taxon, supported also by morphological differences such as having obovoidal conidia and a slower growth rate in culture. Moreover, *C. alienum* is characterised by the development of perithecia in culture, whereas the two strains of *C. hystricis* did not produce perithecia on artificial media in this study.

The present study provides the first overview of *Colletotrichum* diversity associated with several disease symptoms on citrus fruits and plants in Europe, and provides useful information for pathogenicity evaluation and effective disease control. Preliminary inoculations also demonstrated the ability of all the *Colletotrichum* spp. found in Europe to cause infections on orange fruits. Further studies are thus required to resolve the host range and pathogenicity of the *Colletotrichum* species reported on other *Citrus* spp. and different plant organs.

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