Plant Pathology (2013) Doi: 10.1111/ppa.12110

Species of the *Colletotrichum gloeosporioides* and *C. boninense* complexes associated with olive anthracnose

L. Schena[a]*, S. Mosca[a], S. O. Cacciola[b], R. Faedda[b], S. M. Sanzani[c], G. E. Agosteo[a], V. Sergeeva[d] and G. Magnano di San Lio[a]

[a]*Dipartimento di Agraria, Università degli Studi Mediterranea, Località Feo di Vito, 89122 Reggio Calabria;* [b]*Dipartimento di Gestione dei Sistemi Agroalimentari e Ambientali, Università degli Studi, Via Santa Sofia, 100, 95123 Catania;* [c]*Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti, Università degli Studi 'Aldo Moro', Via Amendola 165/A, 70126 Bari, Italy; and* [d]*School of Natural Sciences, University of Western Sydney, Locked bag 1797, Penrith South DC, NSW 2751, Australia*

The taxonomic status of *Colletotrichum gloeosporioides sensu lato* (*s.l.*) associated with olive anthracnose is still undetermined and the pathogenic ability of this species complex is controversial. In the present study, isolates obtained from olive and provisionally identified as *C. gloeosporioides s.l.* on the basis of morphological and cultural features were reclassified using ITS and *TUB2* as DNA barcode markers and referred to seven distinct species, recently separated within *C. gloeosporioides* (*C. aenigma*, *C. gloeosporioides sensu stricto* (*s.s.*), *C. kahawae*, *C. queenslandicum*, *C. siamense* and *C. theobromicola*) and *C. boninense* (*C. karstii*) species complexes. Furthermore, isolates of *C. kahawae* were ascribed to the subspecies *ciggaro* by analysing the *GS* gene. A single isolate, not in either of these two species complexes, was not identified at the species level. In pathogenicity tests on detached olive drupes some of these species, including *C. aenigma*, *C. kahawae* subsp. *ciggaro*, *C. queenslandicum*, *C. siamense* and *C. karstii*, were shown to be weakly pathogenic. Moreover, they were found very sporadically on olive. In contrast, some isolates of *C. gloeosporioides s.s.* and isolates of *C. theobromicola* proved to be virulent on both green and ripening olives. This study gives a better insight into both the aetiology and the epidemiology of olive anthracnose and might have implications for biosecurity and quarantine because *C. theobromicola* has never been reported in major European olive-producing countries.

Keywords: *Colletotrichum boninense*, *Colletotrichum gloeosporioides*, *Colletotrichum theobromicola*, DNA barcode markers, olive anthracnose

Introduction

Olive anthracnose, caused by different species of the genus *Colletotrichum*, is the most damaging disease of olive fruit worldwide and severely affects both fruit yield and quality of oil (Cacciola *et al.*, 2012). In recent years, progress has been made in understanding the complex aetiology and epidemiology of this disease, but several aspects still remain unclear, mainly because the taxonomy of *Colletotrichum* has been under revision for a long time and a comprehensive taxonomic definition of the species included in this genus has only recently been provided (Cannon *et al.*, 2012). Species of *Colletotrichum* have traditionally been identified using morphological and cultural characteristics along with host and geographic distribution. However, it has become progressively clear that the use of these approaches without molecular support is inappropriate because morphological and cultural characters can change under different growth conditions, or can be lost or change with repeated subculturing. Moreover, only the anamorphic stage of many species is known, and the host criterion is inadequate because most *Colletotrichum* species are polyphagous. Recently, significant advances in the understanding of the diversity within the genus have been made by combining conventional analyses with phylogenetic data retrieved from six different genes of a comprehensive collection of species and isolates of worldwide origin (Cannon *et al.*, 2012; Damm *et al.*, 2012a,b; Weir *et al.*, 2012). This study has redefined the taxonomy of the genus and identified several new species, most of which grouped within nine different former complexes of species.

The causal agent of olive anthracnose, originally described in Portugal as *Gloeosporium olivarum*, was subsequently reclassified as *Colletotrichum gloeosporioides*. The sexual stage, *Glomerella cingulata*, has been observed both in axenic cultures and on artificially inoculated olive trees. More recently, two complexes of species showing high phenotypic and genotypic diversity, *C. gloeosporioides sensu lato* (*s.l.*) and *C. acutatum s.l.*, have been associated with olive anthracnose, the latter being prevalent in most olive-growing areas where the disease occurs epidemically (Talhinhas *et al.*, 2005, 2011; Moral *et al.*, 2008; Cacciola *et al.*, 2011).

Molecular investigations on the *C. acutatum s.l.* complex have revealed five different groups (A2, A3, A4, A5 and A6) associated with olive anthracnose (Martín &

*E-mail: lschena@unirc.it

García-Figueres, 1999; Sreenivasaprasad & Talhinhas, 2005). These groups have subsequently been elevated to new species, defined on the basis of molecular, morphological and cultural characters, as *C. simmondsii*, *C. fioriniae*, *C. clavatum* (syn. *C. godetiae*), *C. acutatum*, (*C. acutatum s.s.*) and *C. rhombiforme*, respectively (Shivas & Tan, 2009; Faedda *et al.*, 2011; Damm *et al.*, 2012b). Furthermore, according to a more recent study, group A2 comprises 11 different species in addition to the formerly described *C. simmondsii* (Shivas & Tan, 2009; Damm *et al.*, 2012b). Currently available data indicate that *C. simmondsii*, *C. fioriniae*, *C. clavatum* and *C. acutatum s.s.* are the most important pathogens associated with olive anthracnose worldwide (Cacciola *et al.*, 2012).

Whilst the classification of species previously referred to collectively as *C. acutatum s. l.* and their role as causal agents of olive anthracnose have been clarified, the taxonomic status of *C. gloeosporioides s.l.* associated with olive anthracnose is still undetermined and the pathogenic ability of this species complex is debated. Twenty-two species plus one subspecies have recently been identified within this complex (Weir *et al.*, 2012) but very little information is currently available on the identity of isolates obtained from olive drupes. In many cases, *C. gloeosporioides s.l.* has been shown to be a causal agent of olive anthracnose, although it has rarely been associated with epidemic explosions of the disease (Agosteo *et al.*, 2002; Talhinhas *et al.*, 2009; Faedda *et al.*, 2011). Talhinhas *et al.* (2009, 2011) isolated *C. gloeosporioides* in different olive-growing areas in Portugal, but observed that *C. acutatum* was highly prevalent (97% of the isolates). Moreover, these authors speculated that infections by the former species, which they considered as a less aggressive pathogen, are a consequence of favourable environmental conditions, high inoculum pressure and high host susceptibility. In agreement with previous observations, pathogenicity tests conducted in Spain by Martín *et al.* (2002) using detached drupes showed weaker virulence of *C. gloeosporioides* isolates compared to isolates of the *C. acutatum* species complex. Furthermore, *C. gloeosporioides* was the sole species found in olive orchards in Sicily (southern Italy), a region where epidemic explosions of olive anthracnose have never been recorded and rot of olive drupes has been observed occasionally in late autumn only on mature fruits (Cacciola *et al.*, 2012). These observations would suggest that *C. gloeosporioides* behaves as a weak opportunistic pathogen of olive fruit. However, this conclusion is contradicted by other reports. Talhinhas *et al.* (2005) found that, although *C. gloeosporioides* was sporadic in its occurrence in Portugal, symptoms on infected olives were indistinguishable from those of *C. acutatum* and pathogenicity tests revealed *C. gloeosporioides* isolates to be as virulent as those of *C. simmondsii*, a species of the latter complex. Furthermore, *C. gloeosporioides* has been reported as the causal agent of olive anthracnose in Tunisia (Rhouma *et al.*, 2010) and seems to be as widespread as *C. acutatum* on rotten olives in Iran (Sanei & Razavi, 2012). Finally, on the basis of preliminary observations it can be inferred that in Australia, in contrast to European olive-growing regions, *C. gloeosporioides* is widespread in areas where severe outbreaks of olive anthracnose have been recorded (Sergeeva & Spooner-Hart, 2010; V. Sergeeva, unpublished results).

The aim of the present study was to investigate the involvement of isolates of *Colletotrichum* spp., provisionally identified as *C. gloeosporioides s.l.* on the basis of their morphology, in the aetiology and epidemiology of olive anthracnose. To this aim a collection of isolates obtained from olive drupes with symptoms of anthracnose was characterized molecularly using internal transcribed spacers (ITS) and β-tubulin 2 (*TUB2*) genes as DNA barcode markers. Furthermore, the virulence of isolates was assessed on both green and ripening olive drupes.

Materials and methods

Fungal isolates

Isolates of *Colletotrichum* spp. provisionally identified as *C. gloeosporioides s.l.* on the basis of morphological and cultural features were collected between 1992 and 2011 from olive drupes with symptoms of anthracnose in Italy, Australia, China and Montenegro (Table 1). Additional isolates from other hosts, mainly sourced from citrus in Italy, were used as references. All isolates were obtained from monoconidial or single-hypha cultures and stock cultures were maintained on potato dextrose agar (PDA; Oxoid Ltd) slants under mineral oil at 5°C.

Amplification and sequencing of target genes

Genomic DNA was extracted from *Colletotrichum* isolates following the procedure of Schena & Cooke (2006). The ITS1–5·8S–ITS2 region and the fragment of the β-tubulin 2 gene (*TUB2*) between exons 2 and 6 were amplified and sequenced from the complete panel of isolates as described by Faedda *et al.* (2011). Furthermore, a 900-bp intron of the glutamine synthetase (*GS*) gene was amplified and sequenced from isolates F317 and Isol.53 using primers GSF1 and GSR1 (Guerber *et al.*, 2003).

Amplified products were analysed by electrophoresis and single bands of the expected size were purified with the QIAquick PCR purification kit (QIAGEN) and sequenced with both forward and reverse primers by Macrogen Europe (Amsterdam, The Netherlands). The ChromasPro v. 1.5 software (http://www.technelysium.com.au/) was used to evaluate reliability of sequences and to create consensus sequences. Unreliable sequences in which either forward or reverse sequences contained doubtful bases were resequenced.

Molecular identification of isolates

Internal transcribed spacer and *TUB2* sequences obtained in the present study, and validated sequences representative of all species identified within the *C. gloeosporioides* (Weir *et al.*, 2012) and *C. boninense* (Damm *et al.*, 2012a) species complexes, were phylogenetically analysed to determine the relationship between different olive isolates and define their taxonomic status in the light of the new molecular criteria for classification.

Table 1 Isolates and species of *Colletotrichum* spp. characterized in the study. Origin and accession number for ITS and *TUB2* genes are reported for each isolate

Species	Isolate code	Origin			GenBank accession no.	
		Host	Country	Year	ITS	*TUB2*
C. aenigma	C53	*Pyrus communis*	Italy	1995	KC425658	KC425705
	F318	*Citrus sinensis*	Italy	2012	KC425657	KC425706
	OLF23	*Olea europaea*	Italy	1993	KC425656	KC425704
C. alienum	F263	*Nerium oleander*	Australia	2011	KC425654	KC425707
C. gloeosporioides s.s.	A1	*Annona cherimoya*	Italy	1992	KC425636	KC425692
	ACI2	*O. europaea*	Italy	2000	KC425632	KC425674
	C1	*Citrus limon*	Italy	1992	KC425644	KC425686
	C2	*C. sinensis*	Italy	1992	KC425627	KC425691
	C42	*Pistacia vera*	Italy	1995	KC425625	KC425688
	C43	*Prunus cerasifera*	Italy	2005	KC425624	KC425689
	CAMP34	*O. europaea*	Italy	2005	KC425626	KC425687
	CAMP7	*O. europaea*	Italy	2005	KC425639	KC425693
	CINA	*O. europaea*	China	1993	KC425638	KC425684
	F108	*O. europaea*	Italy	1992	KC425623	KC425681
	F114	*O. europaea*	Italy	1992	KC425628	KC425673
	F169	*C. sinensis*	Italy	2011	KC425629	KC425679
	F170	*O. europaea*	Italy	2011	KC425631	KC425697
	F171	*O. europaea*	Italy	2010	KC425635	KC425698
	F172	*C. sinensis*	Italy	2011	KC425633	KC425699
	F191	*O. europaea*	Italy	2011	KC425622	KC425682
	F225	*C. sinensis*	Italy	2011	KC425637	KC425676
	F226	*C. sinensis*	Italy	2011	KC425640	KC425678
	F227	*O. europaea*	Italy	2011	KC425641	KC425680
	F229	*O. europaea*	Italy	2011	KC425642	KC425675
	F239	*C. sinensis*	Italy	2011	KC425643	KC425677
	F264	*C. limon*	Australia	2011	KC425621	KC425696
	F265	*C. limon*	Australia	2011	KC425619	KC425695
	F266	*C. limon*	Australia	2011	KC425620	KC425694
	F96	*Capsicum annuum*	Italy	1992	KC425645	KC425672
	Isol.1765	*Citrus* sp.	California	1999	KC425647	KC425690
	Isol.8	*C. sinensis*	California	1999	KC425648	KC425671
	OL15	*O. europaea*	Italy	1992	KC425630	KC425700
	PEP	*Capsicum annuum*	Italy	1992	KC425646	KC425685
S1/S2	*O. europaea*	Italy	1992	KC425634	KC425683	
C. kahawae	F317	*O. europaea*	Italy	1995	KC425663	KC425711
	Isol.53	*O. europaea*	Italy	1995	KC425662	KC425710
C. karstii	C18	*Citrus* sp.	Italy	1992	KC425667	KC425721
	C47	*Pistacia vera*	Italy	1995	KC425665	KC425719
	CAM	*Camellia* sp.	Italy	1990	KC425664	KC425716
	OLF38	*O. europaea*	Italy	1992	KC425666	KC425718
	OLF39	*O. europaea*	Italy	1992	KC425669	KC425720
	OLF41	*O. europaea*	Italy	1992	KC425668	KC425717
C. musae	F15	*Musa* × *paradisiaca*	Italy	1992	KC425661	KC425708
C. queenslandicum	VMIN	*O. europaea*	Montenegro	1997	KC425655	KC425709
C. siamense	F272	*Hibiscus* sp.	Australia	2011	KC425649	KC425703
	F28	*O. europaea*	Australia	2007	KC425659	KC425702
	F30	*O. europaea*	Australia	2007	KC425660	KC425701
C. theobromicola	F27	*O. europaea*	Australia	2007	KC425653	KC425712
	F315	*O. europaea*	Australia	2011	KC425652	KC425713
	F316	*O. europaea*	Australia	2011	KC425650	KC425714
	F319	*O. europaea*	Australia	2011	KC425651	KC425715
Colletotrichum sp.	OLF24	*O. europaea*	Italy	1992	KC425670	KC425722

Three additional sequences of *Colletotrichum* spp., one deposited in GenBank as *C. crassipes* (CBS159.75; Farr *et al.*, 2006) and two as *Colletotrichum* sp. (3386 and 4801; Rojas *et al.*, 2010), were included in the analysis because they are closely related to a single isolate from olive (OLF24) that did not belong either to the *C. gloeosporioides* or *C. boninense* species complexes. Where available, sequences from ex-type or authentic culture were preferentially included in the analysis as a reference. Phylogenetic analysis was conducted for the ITS and *TUB2* sequences as well as for the combined data set of the two

markers using maximum likelihood and Bayesian methods. TOPALi v. 2 (http://www.topali.org/) was used to determine the substitution model that best fitted the data. The model HKY + I + G was selected for the Bayesian and maximum likelihood phylogenetic analysis using MrBayes v. 3.1.1 and PhyML v. 2.4.5, respectively, implemented in TOPALi. Bayesian analysis was performed with four runs conducted simultaneously for 500 000 generations with 10% sampling frequency and burn in of 30%. Maximum likelihood was performed with 100 bootstrap replicates.

An additional phylogenetic analysis was performed using the *GS* gene, to identify isolates of *C. kahawae* (F317 and Isol.53) at the subspecies level. Analysis was performed as described above using sequences of the two isolates and validated sequences representative of *C. kahawae* subsp. *kahawae* and *C. kahawae* subsp. *ciggaro* (Weir *et al.*, 2012).

Pathogenicity tests

Pathogenicity tests were conducted using detached olive drupes of cv. Coratina collected in Apulia (southern Italy). Isolates of *Colletotrichum* spp. were mainly sourced from olive and included representatives of the different species identified by molecular analysis. Two isolates from *Citrus* and one isolate from oleander (*Nerium oleander*) were also included in the tests along with reference virulent strains of *C. clavatum*, *C. simmondsii* and *C. acutatum s.s.* Pathogenicity tests were repeated twice using green olives harvested in October or olives at the colour-changing stage (ripening olives) harvested in early December. In both trials, drupes of uniform size and ripeness were surface sterilized by immersion in a 1% sodium hypochlorite solution for 1 min, washed twice with tap water, air-dried and wounded in the equatorial zone with a pin (0·5 mm diameter). Olives were inoculated by placing a PDA agar plug (5 mm diameter) containing actively growing mycelium on each wound. A sterile agar plug was placed on olive drupes used as a negative control. Inoculated drupes (15 per isolate) were placed in plastic boxes to maintain high relative humidity and incubated at 20°C. Starting from 5 days after inoculation, the extension of decaying tissues was recorded every 3 days, using the following empirical scale: 0 = symptomless olives; 1, 2, 3 and 4 = 1–25, 26–50, 51–75 and 76–100% of rotten olive surface, respectively. The same scale was used to evaluate the sporulation, i.e. the presence of orange gelatinous matrices embedding conidia emerging from acervuli. The scale enabled the calculation of the McKinney index (McKinney, 1923) expressing the weighted average of disease severity as percentage of the highest level of disease. The index was calculated using the formula: $M_i = [\Sigma(d \times f)/T_n \times D] \times 100$ where d is the value of the class within the empirical scale, f is the frequency of each class, T_n is the total number of olives examined and D the highest degree of the empirical scale. An arcsine transformation was applied to the data prior to analysis of variance (ANOVA) and mean values were compared using Tukey's HSD test using SPSS v. 19.

All fungal isolates included in pathogenicity tests were reisolated from a representative number of inoculated olives to confirm their identity.

Results

Molecular identification of isolates

Reliable DNA sequences of the ITS and *TUB2* regions were obtained for all isolates included in the present study and sequences were deposited in GenBank with accession numbers from KC425619 to KC425670 (ITS region) and from KC425671 to KC425722 (*TUB2*) (Table 1). Similarly, high quality sequences of the *GS* gene were obtained for isolates F317 and Isol.53 (KF170408 and KF170407, respectively).

The phylogenetic analysis of the combined data set of sequences from ITS and *TUB2* regions using MrBayes and PhyML produced trees with a similar topology and high concordance with those reported for the *C. gloeosporioides* (Weir *et al.*, 2012) and *C. boninense* (Damm *et al.*, 2012a) species complexes (Fig. 1). A similar grouping of isolates was also observed when the two regions were analysed separately, although discrimination between isolates was less accurate with the ITS sequences alone (data not shown).

According to the cluster analysis, isolates from olive drupes were identified as *C. gloeosporioides s.s.* (13 isolates), *C. theobromicola* (four isolates), *C. kahawae* (two isolates), *C. siamense* (two isolates), *C. aenigma* (one isolate) and *C. queenslandicum* (one isolate) of the *C. gloeosporioides* species complex, and as *C. karstii* (three isolates) of the *C. boninense* species complex (Fig. 1). A single isolate from olive (OLF24) did not cluster in either of the *C. gloeosporioides* and *C. boninense* species complexes, and showed high similarity although not identity with isolates deposited in GenBank as *C. crassipes* (Farr *et al.*, 2006) and *Colletotrichum* sp. (Rojas *et al.*, 2010). Isolates from other hosts were mainly identified as *C. gloeosporioides s.s.* (primarily sourced from *Citrus* sp. but also from *Capsicum* sp., *Prunus* sp., *Pistacia* sp. and *Annona* sp.) and as *C. siamense* (from *Hybiscus* sp.), *C. aenigma* (from *Pyrus* sp.), *C. alienum* (from *N. oleander*), *C. musae* (from *Musa* sp.) and *C. karstii* (from *Citrus* sp., *Pistacia* sp. and *Camellia* sp.) (Fig. 1).

The phylogenetic analysis of isolates F317 and Isol.53 along with reference isolates of *C. kahawae* subsp. *kahawae* and *C. kahawae* subsp. *ciggaro* allowed the two Italian isolates to be ascribed to the subspecies *ciggaro* (Fig. 2).

Figure 1 Phylogenetic tree including isolates of *Colletotrichum* collected from olive drupes with anthracnose symptoms and other hosts (Table 1) along with isolates representative of species of the *C. gloeosporioides* and *C. boninense* species complex as defined by Weir *et al.* (2012) and Damm *et al.* (2012a), respectively. Three additional isolates of *Colletotrichum* spp. (Farr *et al.*, 2006; Rojas *et al.*, 2010) were included in the analysis because they were closely related to an isolate from olive (OLF24) characterized in the present study. The tree was built using concatenated sequences of the *TUB2* and ITS sequences; numbers on nodes represent the statistical support for the Bayesian method. Culture accession numbers are listed along with host plant genus and country of origin. Species delimitations are indicated with brackets. Isolates characterized in the present study are indicated with arrows; the asterisks (*) indicate isolates from olive drupes.


CBS128504, Citrus, New Zealand, C. constrictum
CBS130241, Dacrycarpus, New Zealand, C. dacrycarpi
CBS125377, Hippeastrum, China, C. hippeastri
CBS128501, Passiflora, Brazil, C. brasiliense
CBS128525, Parsonsia, New Zealand, C. parsonsiae
CBS128505, Capsicum, New Zealand, C. novae-zelandiae
CBS101059, Brassica, New Zealand, C. brassicicola
CBS128527, Brachyglottis, New Zealand, C. beeveri
CBS129818, Passiflora, Colombia, C. colombiense
CBS128544, Solanum, New Zealand, C. torulosum
IMI347923, Cymbidium, Australia, C. cymbidiicola
CBS129828, Oncidium, Germany, C. oncidii
CBS123775, Crinum, Japan, C. boninense s.s.
CBS378.94, Dracaena, Italy, C. petchii
CBS129826, Hevea, Colombia, C. annellatum
CBS175.67, Phyllanthus, India, C. phyllanthi
C. boninense s.l.
C18, Citrus, Italy
C47, Pistacia, Italy
CAM, Camelia, Italy
OLF38, Olea, Italy
OLF39, Olea, Italy
OLF41, Olea, Italy
CBS129815, Solanum, Colombia, C. karstii
CBS129830, Eucalyptus, South, Africa, C. karstii
CBS861.72, Bombax, Brazil, C. karstii
CBS129824, Musa, Colombia, C. karstii
CBS129833, Musa, Mexico, C. karstii
C. karstii
3386, Theobroma, Panama, Colletotrichum sp.
4801, Theobroma, Panama, Colletotrichum sp.
OLF24, Olea, Italy
CBS159.75, Stored grain, India, C. crassipes
Colletotrichum spp.
CBS127831, Xanthorrhoea, Australia, C. xanthorrhoeae
ICMP10492, Diospyros, Japan, C. horii
ICMP18565, Olea, Australia, C. theobromicola
F27, Olea, Australia
F315, Olea, Australia
F316, Olea, Australia
F319, Olea, Australia
ICMP18567, Olea, Australia, C. theobromicola
CBS124251, Stylosanthes, Australia, C. theobromicola
CBS124945, Theobroma, Panama, C. theobromicola
CBS142.31, Fragaria × ananassa, USA, C. theobromicola
C. theobromicola
ICMP4832, Cordyline, New Zealand, C. ti
ICMP18579, Cordyline, Thailand, C. cordylinicola
ICMP18537, Coprosma, New Zealand, C. aotearoa
ICMP18658, Clidemia, USA, Hawaii, C. clidemiae
ICMP10643, Camellia × williamsii, UK, G. cingulata
CBS135.30, Coffea, Kenya, C. kahawae subsp. kahawae
CBS237.49, Hypericum, Germany, C. kahawae subsp. ciggaro
ICMP18539, Olea, Australia, C. kahawae subsp. ciggaro
Isol.53, Olea, Italy
F317, Olea, Italy
IMI319418, Coffea, Kenya, C. kahawae subsp. kahawae
C. kahawae
CBS304.67, Dioscorea, India, C. alatae
CBS130418, Coffea, Thailand, C. asianum
ICMP1778, Malus, Brasil, C. queenslandicum
VMIN, Olea, former Yugoslavia
ICMP18705, Coffea, Fiji, C. queenslandicum
C. queenslandicum
ICMP19051, Salsola, Hungary, C. salsolae
ATCC201874, Aeschynomene, USA, C. aeschynomenes
CBS124949, Theobroma, Panama, C. tropicale
ICMP18574, Pistacia, Australia, C. siamense
CBS125378, Hymenocallis, China, C. siamense
ICMP12567, Persea, Australia, C. siamense
ICMP18121, Dioscorea, Nigeria, C. siamense
F28, Olea, Australia
F30, Olea, Australia
F272, Hibiscus, Australia
CBS130417, Coffea, Thailand, C. siamense
C. siamense
IMI52264, Musa, Kenya, C. musae
F15, Musa, Italy
C. musae
ICMP12071, Malus, New Zealand, C. alienum
F263, Nerium, Australia
C. alienum
CBS470.96, Nuphar, USA, C. nupharicola
CBS238.49, Ficus, Germany, C. fructicola
CBS125397, Tetragastris, Panama, C. fructicola
ICMP18608, Persea, Israel, C. aenigma
ICMP18686, Pyrus, Japan, C. aenigma
C53, Pyrus, Italy
OLF23, Olea, Italy
F318, Olea, Italy
C. aenigma
F265, Citrus, Australia
F266, Citrus, Australia
F264, Citrus, Australia
IMI356878, Citrus, Italy, C. gloeosporioides s.s.
F96, Capsicum, Italy
F239, Citrus, Italy
F229, Olea, Italy
F227, Olea, Italy
F226, Citrus, Italy
F225, Citrus, Italy
F191, Olea, Italy
F172, Citrus, Italy
F171, Olea, Italy
F170, Olea, Italy
F169, Citrus, Italy
F114, Olea, Italy
F108, Olea, Italy
CINA, Olea, China
CAMP7, Olea, Italy
CAMP34, Olea, Italy
C1, Citrus, Italy
ACI2, Olea, Italy
A1, Annona, Italy
C43, Prunus, Italy
C42, Pistacia, Italy
C2, Citrus, Italy
S1/S2, Olea, Italy
PEP, Capsicum, Italy
Isol.1765, Citrus, Italy
OL15, Olea, Italy
Isol.8, Citrus, California
C. gloeosporioides s.s.
C. gloeosporioides s.l.
0·3

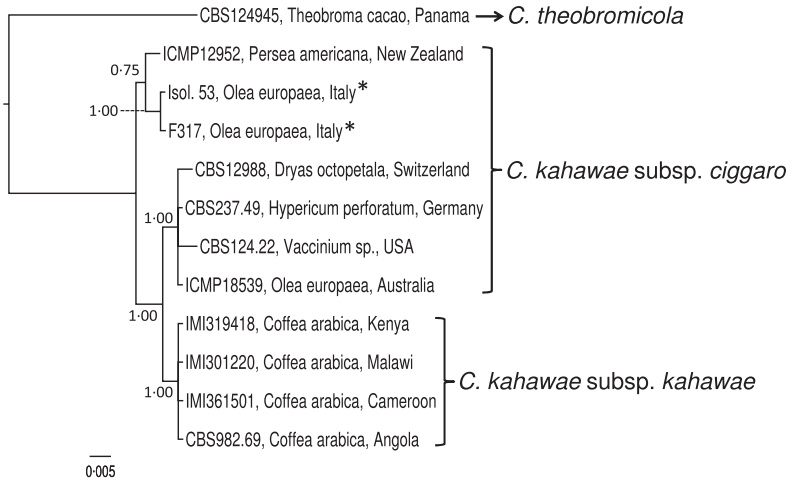

Figure 2 Phylogenetic tree including isolates of *Colletotrichum kahawae* sourced from olives in the present study (Isol.53 and F317) along with representative isolates of *C. kahawae* subsp. *kahawae* and *C. kahawae* subsp. *ciggaro*, as defined by Weir *et al.* (2012). The tree was built using sequences of a 900-bp intron of the glutamine synthetase (*GS*) gene (Guerber *et al.*, 2003); numbers on nodes represent the statistical support for the Bayesian method. Culture accession numbers are listed along with host plant species and country of origin. Species delimitations are indicated with brackets. The asterisks (*) indicate isolates obtained in the present study.

Pathogenicity tests

Pathogenicity tests revealed a markedly higher sensitivity to anthracnose in ripening olives as compared to green drupes (Fig. 3). Both the diameter of lesions and the sporulation were significantly lower on green olives than on ripening olives. Many isolates did not cause rot on green olives (Fig. 3a). On green olives, isolates of *C. gloeosporioides s.s.* showed a great range of virulence, from the complete absence of symptoms (isolate CAMP34) to extensive decay (isolates S1/S2 and F191) not significantly different from that caused by the reference strains of *C. acutatum s.s.*, *C. clavatum* and *C. simmondsii* (Figs 3a & S1). However, while isolate S1/S2 sporulated rarely, isolate F191 produced more spores, comparable to that of *C. clavatum* and significantly greater than the sporulation of *C. acutatum s.s.* and *C. simmondsii*. An intermediate level of virulence was observed for two isolates (F266 and Isol.1765) sourced from citrus leaves, with one of these (F266) producing conspicuous sporulation. Conversely, a significantly lower level of virulence was determined for four other isolates from olive drupes (F171, CAMP7 and ACI2) and, among these, only isolate F171 produced masses of spores after 21 days of incubation (Fig. 3a).

A high level of virulence in terms of both extension of rot and sporulation was also revealed for the two isolates of *C. theobromicola* analysed in the present study (F27 and F316; Figs 3a & S1). In particular the level of sporulation of these two isolates was significantly higher than that of the reference isolate of *C. simmondsii*.

Other isolates from olive drupes belonging to the *C. gloeosporioides* (*C. siamense*, *C. aenigma*, *C. queenslandicum* and *C. kahawae* subsp. *ciggaro*) and *C. boninense* (*C. karstii*) species complexes did not cause any significant rots on inoculated green olives. Restricted necrosis of the tissues was occasionally observed for some isolates around the inoculation site. Similar behaviour was also recorded for the unidentified isolate OLF24 and for the isolate of *C. alienum* from oleander.

When tests were performed on ripening olives, the differences between isolates in terms of extension of necrotic lesion and sporulation ability were reduced, as a result of higher sensitivity of drupes to anthracnose. However, results concerning the virulence of different isolates largely confirmed those obtained on green olives (Fig. 3b). Interestingly, on ripening olives a noticeable development of rot was also induced by isolates of *C. siamense*, *C. aenigma*, *C. alienum*, *C. queenslandicum* and *C. kahawae* subsp. *ciggaro* that were not pathogenic on green olives. The three isolates of *C. karstii* and isolate OFL24 produced restricted lesions around the inoculation site in a limited number of fruits, without any sporulation.

In general, the pattern observed for the sporulation ability of the isolates was similar on both green and ripening olives, i.e. isolates characterized by a higher production of spores on green olives sporulated even more on ripening drupes. Furthermore, significant sporulation occurred on ripening olives inoculated with an isolate of *C. gloeosporioides s.s.* (ACI2) and an isolate of *C. siamense* (F28) that sporulated poorly on green olives.

Discussion

In the present study, seven distinct species of *Colletotrichum* spp. were identified by analysing a collection of isolates obtained from olive drupes with symptoms of anthracnose and provisionally identified as *C. gloeosporioides s.l.* on the basis of their morphological and cultural characters. The species identified were recently separated within the *C. gloeosporioides* (*C. gloeosporio-*

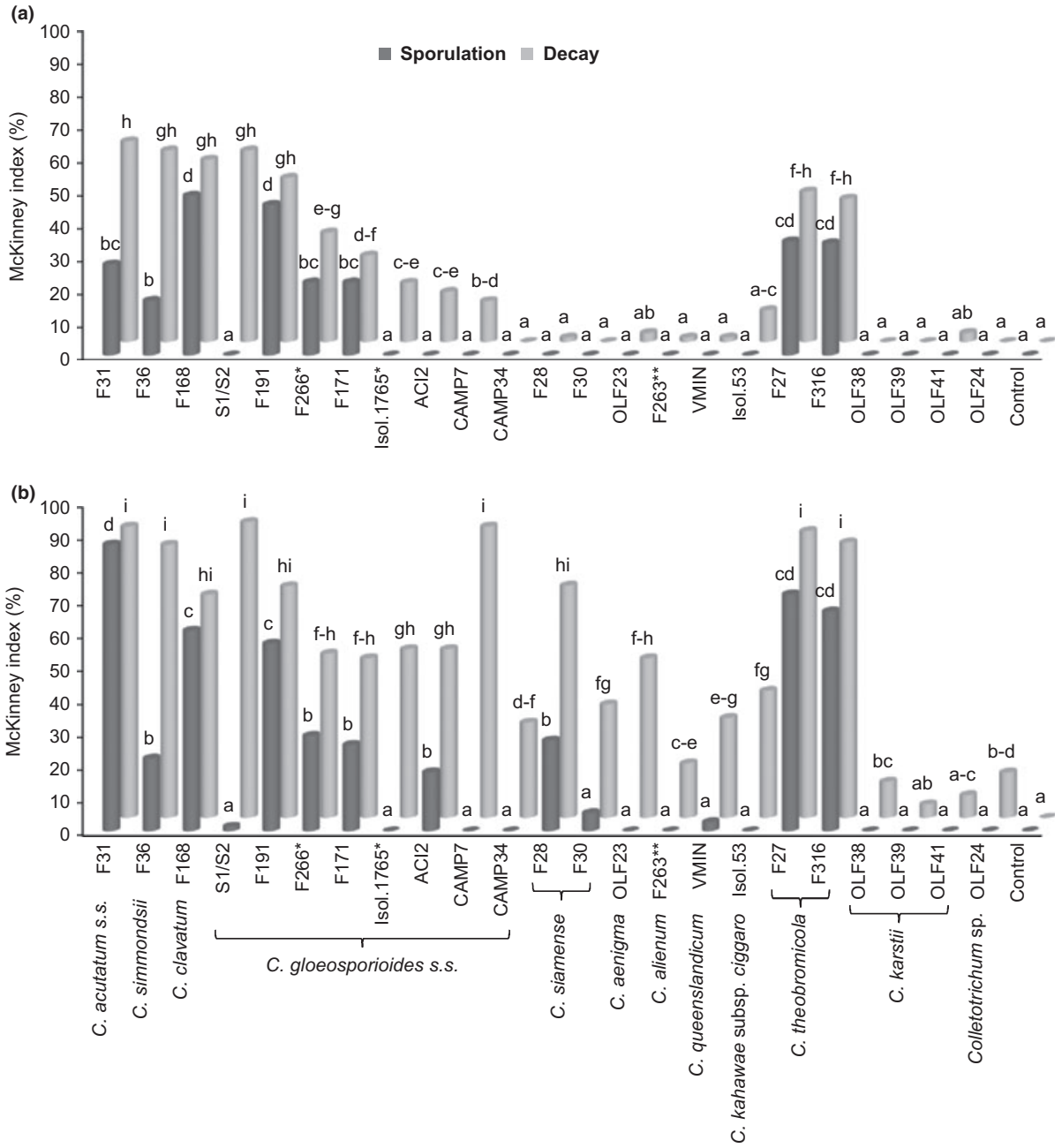

Figure 3 Extension of sporulation and decay (rotten areas) on green (a) and ripening (b) olive drupes inoculated with *Colletotrichum* isolates representative of the different species identified by molecular analyses. Isolates were mainly sourced from olive drupes but also from *Citrus* (*) and *Nerium* (**) leaves. Virulent isolates of *C. acutatum s.s.*, *C. simmondsii* and *C. clavatum* were used as a reference; olive drupes inoculated with an agar plug without mycelium were used as a negative control. In both (a) and (b) histograms, values of the same variable (sporulation and decay) with the same letters did not differ significantly according to Tukey's test ($P \leq 0{\cdot}05$).

ides s.s., *C. siamense*, *C. aenigma*, *C. queenslandicum*, *C. theobromicola* and *C. kahawae*) and the *C. boninense* (*C. karstii*) complexes, and their identification was greatly facilitated by the release of validated sequences for all accepted *Colletotrichum* species (Cannon *et al.*, 2012; Damm *et al.*, 2012a,b; Weir *et al.*, 2012). Both ITS and *TUB2* sequences were used to construct a phylogenetic tree for species identification in order to exploit the robustness of ITS regions and accuracy of *TUB2* sequences. The ITS regions have been proposed as the official fungal DNA barcode marker because they can be easily amplified and sequenced and, compared to alternative genes, they have the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter- and intraspecific variation (Schoch *et al.*, 2012). However,

the ITS regions are not universally accepted as the preferential DNA barcode marker, mainly because they are not always discriminant as phylogenetically closely related species may have sequences identical or differing only by a few nucleotide positions (Kiss, 2012). For instance, only 10 of the 22 accepted species within the *C. gloeosporioides* complex can be identified using the ITS regions (Weir *et al.*, 2012), whereas the fragment of the *TUB2* gene amplified in the present study, comprising a region between exons 2 and 6, is amongst the most effective at distinguishing single species (Damm *et al.*, 2012b; Weir *et al.*, 2012). In fact, the tree constructed in the present study largely confirmed those of the *C. gloeosporioides* and *C. boninense* complexes as defined by Weir *et al.* (2012) and Damm *et al.* (2012a), respectively, and was detailed enough to enable the identification of almost all isolates. One exception was represented by *C. kahawae*: according to Weir *et al.* (2012) this comprises two subspecies, *kahawae* and *ciggaro*, which can be molecularly distinguished only using sequences from the *GS* gene. Indeed, the analysis of this gene enabled the identification of the two Italian isolates as *C. kahawae* subsp. *ciggaro*, confirming that the subspecies *kahawae* is specifically ascribed to *Colletotrichum* isolates causing coffee berry disease in Africa (Varzea *et al.*, 2002; Silva *et al.*, 2012).

The molecular identification of a single isolate (OLF24) that was not a member of either the *C. gloeosporioides* or *C. boninense* complexes was not possible because sequences of this isolate did not match any of those from accepted species (Cannon *et al.*, 2012; Damm *et al.*, 2012a,b; Weir *et al.*, 2012). GenBank BLAST analysis with ITS and *TUB2* sequences revealed, among other things, a high similarity with sequences from an isolate sourced from stored grain in India and reported as *C. crassipes* (CBS 159.75; Farr *et al.*, 2006), and with two isolates sourced from *Theobroma* sp. in Panama and reported as *Colletotrichum* sp. clade C (3386 and 4801; Rojas *et al.*, 2010). Identification of isolate OLF24 is further complicated by the unresolved identity of *C. crassipes*.

Thirty out of 51 isolates analysed in the present study were identified as *C. gloeosporioides s.s.*, 13 of which were sourced from decayed olive drupes. Sequences of these 51 isolates were identical except for a single nucleotide deletion at position 32 of the ITS gene which characterized three isolates (F264, F265 and F266) collected in Australia from lemon. All other isolates had sequences identical to those of the CG-2 group of *C. gloeosporioides* identified by Talhinhas *et al.* (2005, 2009). This group represented 8% of the *Colletotrichum* population in the Algarve region (Portugal) and was differentiated from a CG-1 (9% of the population) by a single nucleotide (Talhinhas *et al.*, 2009). Interestingly, isolates belonging to groups CG-1 and CG-2 were evenly distributed across the Algarve region, over different years and hosts but, whereas all CG-1 isolates were associated with low disease severity, some CG-2 isolates were obtained from plants with up to 100% disease severity (Talhinhas *et al.*, 2009).

Despite the high genetic uniformity of *C. gloeosporioides s.s.* isolates analysed in the present study, pathogenicity tests on both green and ripening olives revealed very high variability in terms of the extension of rotten surface. Furthermore, some isolates caused a rapid development of decay but did not produce masses of conidia, while other isolates sporulated on drupes as profusely as the reference isolates of *C. clavatum*, *C. simmondsii* and *C. acutatum s.s.* These results might explain contrasting reports concerning the pathogenicity of *C. gloeosporioides* isolates from olive. In Spain, Martín *et al.* (2002) observed that *C. gloeosporioides* was less virulent than *C. acutatum* on detached olive drupes whilst Talhinhas *et al.* (2005) found that symptoms caused by *C. gloeosporioides* on infected olives were indistinguishable from those of *C. acutatum*, and on inoculated olives *C. gloeosporioides* isolates were as aggressive as the most virulent *C. simmondsii* isolates; however, more recently the same authors indicated that *C. gloeosporioides* was a less aggressive pathogen (Talhinhas *et al.*, 2011). Although *C. gloeosporioides* is less widespread than species of the *C. acutatum* complex, such as *C. acutatum s.s.*, *C. clavatum* and *C. simmondsii* (Cacciola *et al.*, 2011, 2012) in olive-growing areas of Italy, Portugal and Spain where anthracnose of drupes occurs epidemically, Talhinhas *et al.* (2011) noticed that in the Algarve region (Portugal) the proportion of *C. gloeosporioides* was relatively high (17%). Therefore it cannot be ruled out that *C. gloeosporioides* is a major causal agent of olive anthracnose in some olive-growing regions. The high virulence shown in this study by some isolates of *C. gloeosporioides s.s.* on both green and ripening olives confirms this species as a severe pathogen of olive. Interestingly, two isolates of *C. gloeosporioides s.s.* from citrus leaves (F266 and Isol.1765) included in pathogenicity tests also showed quite a high level of virulence on olive drupes, suggesting that heterologous isolates of this species could be potentially pathogenic to olive drupes. Unfortunately, isolates of *C. gloeosporioides* from olive have been identified, even recently, using conventional morphological criteria (Rhouma *et al.*, 2010; Sanei & Razavi, 2012). On the other hand, most identifications based on molecular methods are also imprecise and, in a few cases, even unreliable. Fundamentally, they suffered from the lack of a comprehensive classification system of the species based on adequately discriminatory genetic markers. Two isolates from olive (GLOCOL-08.1 and GLOCOL-16) were identified as *C. gloeosporioides* by PCR-RFLP of the ITS1–5·8S–ITS2 region, but actual ITS sequences were not available (Martín & García-Figueres, 1999). In recent field surveys, Talhinhas *et al.* (2005, 2011) identified the isolates they obtained from olive by using a PCR primer (TBCG) matching an identical sequence in the great majority of species of the *C. gloeosporioides s.l.* complex. As a consequence it cannot be excluded that some of the isolates they identified were not actually *C. gloeosporioides s.s.*

The four isolates of *C. theobromicola* from olive drupes included in the present study had identical ITS

and *TUB2* sequences and were identified because they clustered within a specific group containing the ex-epitype culture of *C. fragariae*, the ex-holotype culture of *C. gloeosporioides* f. *stylosanthis* and the ex-neotype culture of *C. theobromicola*, an earlier name for these species (Weir *et al.*, 2012). The *C. theobromicola* clade, as defined by Weir *et al.* (2012), is broadly distributed in tropical and subtropical regions on a wide range of hosts. The species contains several putatively specialized pathogens, including the pathogen of strawberry runners described by Brooks (1931) as *C. fragariae*, and the pathogen of *Stylosanthes* referred to as *C. gloeosporioides* f. *stylosanthis* (Munaut *et al.*, 2002). According to Weir *et al.* (2012), *C. theobromicola* is genetically heterogeneous and could be segregated into distinct species. Indeed, in this study significant genetic variation has been detected within the *TUB2* gene among isolates clustering in the *C. theobromicola* clade: for instance at least three nucleotides differentiated the four Australian isolates from olive from other isolates of the same clade, such as the deposited cultures ICMP18556, ICMP18565 and ICMP18567 that share with them both the host (olive) and geographic origin (Australia). Although isolates referred to as *C. theobromicola* have been previously reported on olive, in this study it has been demonstrated for the first time that this species is a very aggressive pathogen on olive drupes. The isolates in this study were collected from olive orchards in Australia during severe epidemics of anthracnose of drupes (V. Sergeeva, data not shown), and caused large necrotic lesions and sporulated profusely on both green and ripe drupes. Interestingly, all isolates of this species from olive available so far, including the four isolates characterized in this study and the cultures deposited in recognized collections, have been collected in Australia. It can be speculated that in this country *C. theobromicola* is responsible for epidemic olive anthracnose infections previously attributed to *C. gloeosporioides s.l.* (Sergeeva & Spooner-Hart, 2010). In contrast, *C. theobromicola* has never been reported in Europe to date (http://www.q-bank.eu/Fungi/BiolomicsInfo.aspx).

The remaining isolates of *C. gloeosporioides s.l.* obtained from olive drupes and characterized in this study were identified as *C. kahawae* subsp. *ciggaro*, *C. queenslandicum*, *C. siamense* and *C. aenigma*. *Colletotrichum kahawae* subsp. *ciggaro* has a broad geographical distribution, including Australia, Germany, New Zealand and South Africa (Weir *et al.*, 2012) but, to the best of the authors' knowledge, it has never been reported in Italy. Its holotype (UWS124) was collected in 1989 in Australia from *Olea europaea* by V. Sergeeva. The only confirmed reports of *C. queenslandicum* (basionym: *Colletotrichum gloeosporioides* var. *minus*) are from Australia on *Carica papaya* and *Persea americana* and from Fiji on coffee berries (Simmonds, 1968). *Colletotrichum siamense* was originally described from coffee in Thailand, but according to Weir *et al.* (2012) this species is widely found on many hosts across several tropical and subtropical regions. Finally, *C. aenigma* is only known from two collections, one from *Pyrus pyrifolia* in Japan, the other from *P. americana* in Israel (Weir *et al.*, 2012). In pathogenicity tests isolates of these four species were all characterized by very low virulence and did not cause decay of green olives.

A very low level of virulence was also shown by the isolates of the *C. boninense* species complex identified as *C. karstii* and by the isolate OLF24 of *Colletotrichum* sp. *Colletotrichum karstii* is known as a pathogen of orchids (Yang *et al.*, 2011). According to Damm *et al.* (2012a), it is the most common and geographically diverse species in the *C. boninense* complex and occurs on many host plants including *P. americana*, *Cucurbita* spp. and *Passiflora edulis*. On the basis of the results of pathogenicity tests, it can be inferred that this polyphagous and widespread species does not constitute a serious threat for olive production.

In conclusion, in this study several different species of the *C. gloeosporioides* complex have been associated with olive anthracnose, but according to pathogenicity tests not all these species are primary pathogens and some, such as *C. aenigma*, *C. kahawae* subsp. *ciggaro*, *C. queenslandicum* and *C. siamense*, occur very sporadically on olive. In contrast, the role of *C. gloeosporioides s.s.* and *C. theobromicola* as possible causal agents of olive anthracnose seems worthy of further investigation. In pathogenicity tests, isolates of these species rapidly colonized olive drupes and showed a great ability to sporulate on infected tissues. In previous studies, Moral *et al.* (2008) clearly demonstrated a correlation between the susceptibility of detached olive drupes artificially inoculated with *C. acutatum* and field susceptibility of olive cultivars to anthracnose disease. This correlation was closer when green olives were used in pathogenicity tests. Accordingly, differences in virulence among tested isolates were more evident on green olives compared to mature olive drupes. Extensive field surveys in olive-growing countries where epidemic outbreaks of olive anthracnose occur frequently have revealed that a different *Colletotrichum* species usually dominates in a particular area, thus suggesting an adaptive potential to different host genetic background and environmental factors (Sreenivasaprasad & Talhinhas, 2005; Talhinhas *et al.*, 2011). Optimum and cardinal temperatures and the ability to sporulate are among the factors that can influence the fitness of *Colletorichum* species and their adaptive capability to a particular environment (Cacciola *et al.*, 2011; Moral *et al.*, 2012).

A precise and universally recognized definition of *Colletotrichum* species associated with olive anthracnose will contribute to a better understanding of both its aetiology and epidemiology and might have practical implications for biosecurity and quarantine. For instance, *C. theobromicola* has been demonstrated to be an aggressive pathogen of olive drupes and has never been reported in the most important European olive-producing countries bordering the Mediterranean Sea. As the Mediterranean basin is expected to be particularly vulnerable to climate changes, the emergence of new olive diseases is likely to

occur (Graniti *et al.*, 2011). In this context, pathogens typical of tropical and subtropical regions, such as *C. theobromicola*, could play a major role.

Acknowledgements

This work was partially funded by MIUR-FIRB 2010 'Metagenomic strategies to assess genetic diversity in soilborne *Phytophthora* species'.

References

Agosteo GE, Magnano di San Lio G, Frisullo S, Cacciola SO, 2002. Characterisation of the causal agent of olive anthracnose in southern Italy. *Acta Horticulturae* **586**, 713–6.

Brooks AN, 1931. Anthracnose of strawberry caused by *Colletotrichum fragariae* n. sp. *Phytopathology* **21**, 739–44.

Cacciola SO, Faedda R, Agosteo GE, 2011. Anthracnose. In: Schena L, Agosteo GE, Cacciola SO, eds. *Olive Diseases and Disorders*. Transworld Research Network: Kerala, India, 223–46.

Cacciola SO, Faedda R, Sinatra F *et al.*, 2012. Olive anthracnose. *Journal of Plant Pathology* **94**, 29–44.

Cannon PF, Damm U, Johnston PR, Weir BS, 2012. *Colletotrichum* – current status and future directions. *Studies in Mycology* **73**, 181–213.

Damm U, Cannon PF, Woudenberg JHC *et al.*, 2012a. The *Colletotrichum boninense* species complex. *Studies in Mycology* **73**, 1–36.

Damm U, Cannon PF, Woudenberg JHC, Crous PW, 2012b. The *Colletotrichum acutatum* species complex. *Studies in Mycology* **73**, 37–114.

Faedda R, Agosteo GE, Schena L *et al.*, 2011. *Colletotrichum clavatum* sp. nov. identified as the causal agent of olive anthracnose in Italy. *Phytopathologia Mediterranea* **50**, 283–302.

Farr DF, Aime MC, Rossman AY, Palm ME, 2006. Species of *Colletotrichum* on *Agavaceae*. *Mycological Research* **110**, 1395–408.

Graniti A, Faedda R, Cacciola SO, Magnano di San Lio G, 2011. Olive diseases in a changing ecosystem. In: Schena L, Agosteo GE, Cacciola SO, eds. *Olive Diseases and Disorders*. Transworld Research Network: Kerala, India, 403–33.

Guerber JC, Liu B, Correll JC, Johnston PR, 2003. Characterization of diversity in *Colletotrichum acutatum sensu lato* by sequence analysis of two gene introns, mtDNA and intron RFLPs, and mating compatibility. *Mycologia* **95**, 872–95.

Kiss L, 2012. Limits of nuclear ribosomal DNA internal transcribed spacer (ITS) sequences as species barcodes for *Fungi*. *Proceedings of the National Academy of Sciences, USA* **109**, E1811.

Martín MP, García-Figueres F, 1999. *Colletotrichum acutatum* and *C. gloeosporioides* cause anthracnose on olives. *European Journal of Plant Pathology* **105**, 733–41.

Martín MP, García-Figueres F, Trapero A, 2002. Iniciadores específicos para detectar las especies de *Colletotrichum* causantes de la antracnosis de los olivos. *Boletín de Sanidad Vegetal Plagas* **28**, 43–50.

McKinney HH, 1923. Influence of soil temperature and moisture on infection on wheat seedling by *Helminthosporium sativum*. *Journal of Agricultural Research* **26**, 195–217.

Moral J, Bouhmidi K, Trapero A, 2008. Influence of fruit maturity, cultivar susceptibility, and inoculation method on infection of olive fruit by *Colletotrichum acutatum*. *Plant Disease* **92**, 1421–6.

Moral J, Jurado-Bello J, Sánchez MI, de Oliveira R, Trapero A, 2012. Effect of temperature, wetness duration, and planting density on olive anthracnose caused by *Colletotrichum* spp. *Phytopathology* **102**, 974–81.

Munaut F, Hamaide N, Maraite H, 2002. Genomic and pathogenic diversity in *Colletotrichum gloeosporioides* from wild native Mexican *Stylosanthes* spp., and taxonomic implications. *Mycological Research* **106**, 579–93.

Rhouma A, Triki MA, Msallem M, 2010. First report of olive anthracnose caused by *Colletotrichum gloeosporioides* in Tunisia. *Phytopathologia Mediterranea* **49**, 95–8.

Rojas EI, Rehner SA, Samuels GJ *et al.*, 2010. *Colletotrichum gloeosporioides* s.l. associated with *Theobroma cacao* and other plants in Panamá: multilocus phylogenies distinguish host-associated pathogens from asymptomatic endophytes. *Mycologia* **102**, 1318–38.

Sanei SJ, Razavi SE, 2012. Survey of olive fungal disease in north of Iran. *Annual Review & Research in Biology* **2**, 27–36.

Schena L, Cooke DEL, 2006. Assessing the potential of regions of the nuclear and mitochondrial genome to develop a 'molecular tool box' for the detection and characterization of *Phytophthora* species. *Journal of Microbiological Methods* **67**, 70–85.

Schoch CL, Seifert KA, Huhndorf S *et al.*, 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for *Fungi*. *Proceedings of the National Academy of Sciences, USA* **109**, 6241–6.

Sergeeva V, Spooner-Hart R, 2010. Anthracnose and Queensland fruit fly in olives. *The Olive Press* **16**, 23–4.

Shivas RG, Tan YP, 2009. A taxonomic re-assessment of *Colletotrichum acutatum* introducing *C. fioriniae* comb. et stat. nov. and *C. simmondsii* sp. nov. *Fungal Diversity* **39**, 111–22.

Silva DN, Talhinhas P, Cai L *et al.*, 2012. Host-jump drives rapid and recent ecological speciation of the emergent fungal pathogen *Colletotrichum kahawae*. *Molecular Ecology* **21**, 2655–70.

Simmonds JH, 1968. Type specimens of *Colletotrichum gloeosporioides* var. *minor* and *Colletotrichum acutatum*. *Queensland Journal of Agricultural and Animal Science* **25**, 178A.

Sreenivasaprasad S, Talhinhas P, 2005. Genotypic and phenotypic diversity in *Colletotrichum acutatum*, a cosmopolitan pathogen causing anthracnose on a wide range of hosts. *Molecular Plant Pathology* **6**, 361–78.

Talhinhas P, Sreenivasaprasad S, Neves-Martins J, Oliveira H, 2005. Molecular and phenotypic analyses reveal association of diverse *Colletotrichum acutatum* groups and a low level of *C. gloeosporioides* with olive anthracnose. *Applied and Environmental Microbiology* **71**, 2987–98.

Talhinhas P, Neves-Martins J, Oliveira H, Sreenivasaprasad S, 2009. The distinctive population structure of *Colletotrichum* species associated with olive anthracnose in the Algarve region of Portugal reflects a host-pathogen diversity hot spot. *FEMS Microbiology Letters* **296**, 31–8.

Talhinhas P, Mota-Capitão C, Martins S *et al.*, 2011. Epidemiology, histopathology and aetiology of olive anthracnose caused by *Colletotrichum acutatum* and *C. gloeosporioides* in Portugal. *Plant Pathology* **60**, 483–95.

Varzea VMP, Rodrigues CJ Jr, Lewis BG, 2002. Distinguishing characteristics and vegetative compatibility of *Colletotrichum kahawe* in comparison with other related species from coffee. *Plant Pathology* **51**, 202–7.

Weir BS, Johnston PR, Damm U, 2012. The *Colletotrichum gloeosporioides* species complex. *Studies in Mycology* **73**, 115–80.

Yang YL, Cai L, Yu ZN, Liu ZY, Hyde KD, 2011. *Colletotrichum* species on *Orchidaceae* in southwest China. *Cryptogamie* **32**, 229–53.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. Examples of symptoms on olive drupes inoculated with different isolates of *Colletotrichum gloeosporioides sensu stricto* or with *C. theobromicola*. Drupes were uniformly green at the time of inoculation.