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Colletotrichum clavatum sp. nov. identified as the causal agent of olive anthracnose in Italy

Roberto FAEDDA[1], Giovanni Enrico AGOSTEO[2], Leonardo SCHENA[2], Saveria MOSCA[2], Salvatore FRISULLO[3], Gaetano MAGNANO DI SAN LIO[2] and Santa Olga CACCIOLA[4]

[1]Dipartimento di Gestione dei Sistemi Agroalimentari e Ambientali, Università degli Studi di Catania, Via S. Sofia 100, 95123 Catania, Italia
[2]Dipartimento di Gestione dei Sistemi Agrari e Forestali, Università Mediterranea di Reggio Calabria, Località Feo di Vito, 89122 Reggio Calabria, Italia
[3]Dipartimento di Scienze Agroambientali, Chimica e Difesa Vegetale, Università degli Studi di Foggia, Via Napoli 25, 7100 Foggia, Italia
[4]Dipartimento di Scienze del Farmaco, Università degli Studi di Catania, Viale A. Doria 6, 95125 Catania, Italia

Summary. Molecular analyses of a large population of isolates, previously identified as group B or genetic group A4 of the *Colletotrichum acutatum* species complex, mainly of Italian origin from olive, but also from other hosts collected since 1992, confirmed a well-resolved phylogenetic lineage with distinctive phenotypic characters which can be recognized as a separate species. Based on RAPD genomic fingerprinting, ITS and β-tubulin DNA sequences, this species was clearly distinct from *C. acutatum sensu stricto, C. fioriniae* and *C. simmondsii* as well as from the genetic groups A1, A6, A7 and A8, all previously referred to as *C. acutatum sensu lato.* Group A4 is widespread in Europe, being responsible for olive anthracnose epidemics in some Mediterranean countries, including Greece, Italy, Montenegro, Portugal and Spain; moreover, it causes anthracnose diseases on a wide range of other hosts including about 20 different genera of woody and herbaceous plants, ornamentals and fruit trees. This new anamorphic taxon is described as *Colletotrichum clavatum* sp. nov.

Key words: molecular phylogeny, RAPD-PCR, β-tubulin, ITS region.

Introduction

C*olletotrichum acutatum sensu lato* (*s. l.*) is a species group as it includes variants with distinct morphological and molecular characters (Sreenivasaprasad and Talhinhas, 2005; Hyde *et al*., 2009). It comprises important fungal pathogens which are responsible for economically significant diseases of temperate, subtropical and tropical crops, commonly recognized as anthracnoses (Peres *et al.*, 2005; Sreenivasaprasad and Talhinhas, 2005; Johnston *et al*., 2005). *Colletotrichum acutatum* J.H. Simmonds ex J.H. Simmonds was first reported as a distinct species on pawpaw (*Carica papaya*) in Queensland (Australia) by Simmonds (1965), who validated the species later with a broad concept and designated a holotype and six paratypes varying considerably in morphological and molecular characteristics (Simmonds, 1968). Subsequently, *C. acutatum* was referred to as causal agent of anthracnose diseases on a large number of crops and non-cultivated plant species (Shi *et al.*, 1996; Zulfiqar *et al.*, 1996; Johnston and Jones, 1997; Freeman *et al.*, 1998). The teleomorph of *C. acutatum*, *Glomerella acutata* J.C. Guerber & J.C. Correll, was first obtained *in vitro* by crossing different self-sterile monoconidial strains of *C. acutatum* (Guerber and Correll, 2001) and it was subsequently observed on naturally infected fruits of highbush blueberry in Norway (Talgø *et al.*, 2007).

The main morphological characters adopted to differentiate *C. acutatum* from other species

Corresponding author: S.O. Cacciola
Fax: +39 095 7384220
E-mail: olgacacciola@unict.it

of *Colletotrichum* have been the shape of conidia, which are fusiform and pointed at both ends, and the slow growth rate in culture. However, as *C. acutatum* in a broad sense shows a high degree of phenotypic and genetic diversity, it has been difficult to discriminate from other species of *Colletotrichum,* including *C. gloeosporioides* which exhibits a few overlapping morphological traits and host range (Talhinhas *et al*., 2002).

Colletotrichum acutatum s. l. was first introduced by Johnston and Jones (1997) to accommodate isolates that clustered with *C. acutatum sensu* Simmonds and others that showed a wide range of morphological and genetic diversity. Later, Lardner *et al.* (1999) used RAPD analyses and morphological and cultural features to split *C. acutatum s. l.* into seven distinct taxa, including five morphologically and genetically uniform groups, designated as A, B, C, D, E, and two species, *Glomerella miyabeana* (Fukushi) Arx and *C. acutatum* f. sp. *pineum* Dingley & J.W. Gilmour. Subsequently, the analysis of the Internal Transcribed Spacer (ITS) regions of the ribosomal DNA (rDNA) and a fragment of the β-tubulin-2 gene enabled the rearrangement of a global *C. acutatum* population into eight different molecular groups (from A1 to A8). These showed some degree of correlation with phenotypic characteristics, host association patterns and geographical distribution (Sreenivasaprasad and Talhinhas, 2005). The same genes have been more recently utilized to reassess three different genetic groups within *C. acutatum s. l.* and describe two new species, *C. fioriniae* (Marcelino & S. Gouli) R.G. Shivas & Y.P. Tan and *C. simmondsii* R.G. Shivas & Y.P. Tan (Shivas and Tan, 2009). The new species corresponded to group C (also known as A3) and group D (also known as A2), respectively, whereas a third group was defined as *C. acutatum* (*sensu* Simmonds) and corresponded to group A (also known as A5).

Colletotrichum acutatum group B or genetic group A4, as identified, respectively, by Lardner *et al.* (1999) and Sreenivasaprasad and Talhinhas (2005), is a cosmopolitan pathogen on a wide host range, including *Olea europaea*, *Fragaria* × *ananassa, Lycopersicon esculentum*, *Malus domestica, Ficus carica*, *Eriobotrya japonica, Feijoa sellowiana, Hepatica acutiloba, Sambucus nigra, Prunus dulcis, Rhododendron* spp., *Rubus* sp., *Ceanothus* sp., *Vitis* sp., *Juglans* sp., *Primula* sp., *Camellia* sp. and *Bergenia* sp. This group has been demonstrated to be responsible for epidemic outbreaks of fruit anthracnose of olive (*O. europaea*) in Greece, Italy, Andalucia (southern Spain) and Montenegro, while in Portugal, South Africa and Australia other *C. acutatum* groups are predominant as causal agents of this disease (Cacciola *et al*., 2007; Talhinhas *et al*., 2009; Sergeeva *et al*., 2010). Anthracnose is the most important disease of olive fruit worldwide, causing significant yield losses and poor olive oil quality (Bompeix *et al*., 1988; Graniti *et al*., 1993; Moral *et al*., 2008). In previous studies it has been supposed that this variant (group B or genetic group A4), like other groups within *C. acutatum s. l.*, may represent a distinct *Colletrotrichum* species, because it can be separated on the basis of morphological traits and multiple genetic markers. These include isozymes, random amplified polymorphic DNA (RAPD)-polymerase chain reaction (PCR), restriction fragment length polymorphisms (RFLPs) of repetitive elements of nuclear DNA or A+T rich mitochondrial DNA (mtDNA), RFLPs of 1-kb intron of the glutamine synthetase (*GS*) gene and sequences of ITS-rDNA, β-tubulin (*tub 2*) gene and intron 2 of both glutaraldehyde-3-phosphate dehydrogenase (*G3PD*) and *GS* genes (Vinnere *et al*., 2002; Guerber *et al*., 2003; Sreenivasaprasad and Talhinhas, 2005; Peres *et al*., 2008; MacKenzie *et al*., 2009; Shivas and Tan, 2009; Sergeeva *et al*., 2010). However, the hypothesis that group A4 can be regarded as a well-defined taxon has not been definitely demonstrated.

In the present study, a large population of isolates of *C. acutatum* A4, mostly obtained from olive in Italian regions where olive anthracnose is endemic, have been collected since 1992 and analyzed with a polyphasic approach to discern both the taxonomic status and the phylogenetic position of this genetic group within *C. acutatum s. l.* ITS and β-tubulin 2 sequences of this collection of isolates were compared with GenBank deposited sequences of isolates of *Colletotrichum,* including *C. acutatum* A4 and *C. acutatum sensu stricto* (*s. s.*) of worldwide origin and from various hosts.

Materials and methods

Fungal isolates

Colletotrichum isolates examined in this study are listed in Table 1. The majority were obtained

Table 1. Isolates of *Colletotrichum* spp. examined in this study.

Species/Isolate code	Host	Geographical origin	Collection date	GenBank Accession No.	
				ITS-rDNA	ß-tubulin 2
C. clavatum (formerly *C. acutatum* group B[a] or A4[b])					
CBS 193.32[c]	*Olea europaea*	Greece	1932[c]	AJ749688	AJ748612
IMI 398854 (= OL10)	*O. europaea*	Rizziconi (RC), Calabria, Italy	1992	JN121126	JN121213
OL1	*O. europaea*	Gioia Tauro (RC), Calabria, Italy	1992	JN121127	JN121214
OL5	*O. europaea*	Gioia Tauro (RC), Calabria, Italy	1992	JN121128	JN121215
OL9	*O. europaea*	Gioia Tauro (RC), Calabria, Italy	1992	JN121129	JN121216
OL11	*O. europaea*	Rizziconi (RC), Calabria, Italy	1992	JN121130	JN121217
OL16	*O. europaea*	Francica (RC), Calabria, Italy	1992	JN121131	JN121218
ITRANA1	*O. europaea*	Calabria, Italy	1998	JN121132	JN121219
OLP2	*O. europaea*	Taurianova (RC), Calabria, Italy	1998	JN121133	JN121220
OLP8	*O. europaea*	Taurianova (RC), Calabria, Italy	1998	JN121134	JN121221
OLP10	*O. europaea*	Rizziconi (RC), Calabria, Italy	1999	JN121135	JN121222
OLP11	*O. europaea*	Rizziconi (RC), Calabria, Italy	1999	JN121136	JN121223
OLP12	*O. europaea*	Rizziconi (RC), Calabria, Italy	1999	JN121137	JN121224
OLP13	*O. europaea*	Rizziconi (RC), Calabria, Italy	1999	JN121138	JN121225
OLP14	*O. europaea*	Rizziconi (RC), Calabria, Italy	1999	JN121139	JN121226
OLP16	*O. europaea*	Rizziconi (RC), Calabria, Italy	1999	JN121140	JN121227
CAMP35	*O. europaea*	Rizziconi (RC), Calabria, Italy	2005	JN121141	JN121228
CAMP36	*O. europaea*	S. Giorgio Morgeto (RC), Calabria, Italy	2005	JN121142	JN121229
SCA	*O. europaea*	S. Cristina in Aspromonte (RC), Calabria, Italy	1999	JN121143	JN121230
SEL	*O. europaea*	S. Eufemia di Lamezia (CZ), Calabria, Italy	1999	JN121144	JN121231
VAR1	*O. europaea*	Varapodio (RC), Calabria, Italy	1999	JN121145	JN121232
RI1	*O. europaea*	Rizziconi (RC), Calabria, Italy	1999	JN121146	JN121233
IMI 398855 (= OL20)	*O. europaea*	Ruffano (LE), Apulia, Italy	1992	JN121147	JN121234
CAMP27	*O. europaea*	Bosco Belvedere (LE), Apulia, Italy	2005	JN121148	JN121235
CAMP30	*O. europaea*	Montesano Salentino (LE), Apulia, Italy	2005	JN121149	JN121236
CAMP37	*O. europaea*	Torricella (LE), Apulia, Italy	2005	JN121150	JN121237
CAMP40	*O. europaea*	Bosco Grande (LE), Apulia, Italy	2005	JN121151	JN121238
OL12	*O. europaea*	Ruffano (LE), Apulia, Italy	1992	JN121152	JN121239
OL14	*O. europaea*	Bari, Apulia, Italy	1992	JN121153	JN121240
OL15	*O. europaea*	Bari, Apulia, Italy	1992	JN121154	JN121241
OL17	*O. europaea*	Ruffano (LE), Apulia, Italy	1992	JN121155	JN121242
OL21	*O. europaea*	Ruffano (LE), Apulia, Italy	1992	JN121156	JN121243
OL22	*O. europaea*	Ruffano (LE), Apulia, Italy	1992	JN121157	JN121244
OL23	*O. europaea*	Ruffano (LE), Apulia, Italy	1992	JN121158	JN121245
OLF21	*O. europaea*	Ruffano (LE), Apulia, Italy	1992	JN121159	JN121246
OLF22	*O. europaea*	Ruffano (LE), Apulia, Italy	1992	JN121160	JN121247
OLF40	*O. europaea*	Ruffano (LE), Apulia, Italy	1992	JN121161	JN121248

continues

Table 1. *continued*

Species/Isolate code	Host	Geographical origin	Collection date	GenBank Accession No.	
				ITS-rDNA	ß-tubulin 2
OLF48	*O. europaea*	Ruffano (LE), Apulia, Italy	1992	JN121162	JN121249
OLF97E	*O. europaea*	Ruffano (LE), Apulia, Italy	1992	JN121163	JN121250
CIRRI	*O. europaea*	Ruffano (LE), Apulia, Italy	1998	JN121164	JN121251
18	*O. europaea*	Tricase (LE), Apulia, Italy	2010	JN121165	JN121252
31	*O. europaea*	San Michele Salentino (LE), Apulia, Italy	2010	JN121166	JN121253
42	*O. europaea*	Lecce, Apulia, Italy	2010	JN121167	JN121254
64	*O. europaea*	Matino (LE), Apulia, Italy	2010	JN121168	JN121255
75	*O. europaea*	Supersano (LE), Apulia, Italy	2010	JN121169	JN121256
79	*O. europaea*	Ostuni (BR), Apulia, Italy	2010	JN121170	JN121257
81	*O. europaea*	Ugento (LE), Apulia, Italy	2010	JN121171	JN121258
86	*O. europaea*	Tuglie (LE), Apulia, Italy	2010	JN121172	JN121259
97	*O. europaea*	Parabita (LE), Apulia, Italy	2010	JN121173	JN121260
ORA1	*O. europaea*	Sardinia, Italy	1995	JN121174	JN121261
ORA2	*O. europaea*	Sardinia, Italy	1995	JN121175	JN121262
UMB1A	*O. europaea*	Umbria, Italy	2003	JN121176	JN121263
UMB1B	*O. europaea*	Umbria, Italy	2003	JN121177	JN121264
UMB1C	*O. europaea*	Umbria, Italy	2003	JN121178	JN121265
CGMUL	*O. europaea*	Montenegro	1994	JN121179	JN121266
MELA	*Malus domestica*	Sicily, Italy	2000	JN121180	JN121267
AZJ	*Azalea japonica*	Biella, Piedmont, Italy	2005[d]	JN121181	JN121268
8689	*Prunus avium*	Norway	2005[d]	JN121182	JN121269
C. acutatum					
UWS 14	*O. europaea*	Menangle, NSW, Australia	2007	JN121183	JN121270
UWS 103	*O. europaea*	Hunter Valley Pokolbin, NSW, Australia	2008	JN121184	JN121271
UWS 147	*O. europaea*	Hopelands, Australia	2008	JN121185	JN121272
UWS 149	*O. europaea*	Agonis Ridge WA, Australia	2008	JN121186	JN121273
UWS 166	*O. europaea*	Australia	2011	JN121187	JN121274
67	*P. dulcis*	NSW, Australia	2001[d]	JN121188	JN121275
OLE	*Nerium oleander*	Milazzo (ME), Sicily, Italy[h]	2001	JN121189	JN121276
C. fioriniae					
OLPUGLIA	*O. oleaster*	Smirne, Turkey	1998	JN121190	JN121277
ACUVA	*Vitis vinifera*	Conversano (BA), Apulia, Italy	1998	JN121191	JN121278
1409	*Carica papaya*	Hawaii	1999[d]	JN121192	JN121279
1491	*Vaccinium myrtillus*	Latium, Italy	2002	JN121193	JN121280
C. simmondsii					
725	*Fragaria* × *ananassa*	Yolo, CA, United States	1999[d,e]	JN121194	JN121281
SPL100	*Fragaria* × *ananassa*	Calabria, Italy	1998	JN121195	JN121282

continues

Table 1. *continued*

Species/Isolate code	Host	Geographical origin	Collection date	GenBank Accession No.	
				ITS-rDNA	ß-tubulin 2
SPL103	*Fragaria* × *ananassa*	Lamezia Terme (CZ), Calabria, Italy	1998	JN121196	JN121283
FRA	*Fragaria* × *ananassa*	Lamezia Terme (CZ), Calabria, Italy	1998	JN121197	JN121284
CAF	*Fragaria* × *ananassa*	Apulia, Italy	1998	JN121198	JN121285
UWS 68	*O. europaea*	Hunter Valley Laguna, NSW, Australia	2007	JN121199	JN121286
UWS 137	*O. europaea*	Springbrook, QL, Australia	2008	JN121200	JN121287
CBS 231.49[f]	*O. europaea*	Portugal	1949	JN121201	JN121288
1566	*O. europaea*	Tavira, Algarve, Portugal	2006	JN121202	JN121289
1567	*O. europaea*	Tavira, Algarve, Portugal	2006	JN121203	JN121290
1568	*O. europaea*	Evora, Alto Alentejo, Portugal	2006	JN121204	JN121291
1568/B	*O. europaea*	Evora, Alto Alentejo, Portugal	2006	JN121205	JN121292
1572	*O. europaea*	Estremoz, Alto Alentejo, Portugal	2006	JN121206	JN121293
1570	*O. europaea*	Santarem, Ribatejo, Portugal	2006	JN121207	JN121294
1036	*Cyclamen persicum*	Latium, Italy	2002	JN121208	JN121295
C. gloeosporioides					
8	*Citrus sinensis*	Yolo (CA), United States	1999[d,e]	JN121209	JN121296
1765	*Citrus* sp.	United States	1999[d]	JN121210	JN121297
C2	*C. limon*	Lamezia Terme (CZ), Calabria, Italy	1992	JN121211	JN121298
C. musae					
MUSAE	*Musa* × *paradisiaca*	Sicily, Italy[g]	1992	JN121212	JN121299
C. circinans					
CIRCI	*Allium cepa*	Tropea (VV), Calabria, Italy	1992	-	-

[a] Group as described by Lardner *et al.* (1999).
[b] Group as defined by Sreenivasaprasad and Talhinhas (2005).
[c] Isolate deposited by L. Petri in 1932 and supplied as *C. gloeosporioides* from Centraalbureau voor Schimmelcultures (CBS), The Netherlands.
[d] Year isolates were received.
[e] Isolates cited in Förster and Adaskaveg, 1999.
[f] Isolate deposited by G.J.M.A. Gorter in 1949 and supplied as *C. gloeosporioides* by CBS.
[g] Fruit imported from Central America.
[h] First record of *C. acutatum sensu stricto* in Italy.

from drupes and leaves of olive with symptoms of anthracnose, collected between 1992 and 2011 in various regions of southern and central Italy, including Apulia, Calabria, Sardinia and Umbria. The bulk of isolates came from the Calabria and Apulia regions (southern Italy). All isolates were obtained from monoconidial cultures and stock cultures were maintained on potato dextrose agar (PDA, Oxoid Ltd, Basingstoke, UK) slants under mineral oil at 10–12°C in the collections of the Dipartimento di Gestione dei Sistemi Agroalimentari e Ambientali, University of Catania (Italy) and the Dipartimento di Gestione dei Sistemi Agrari e Forestali, Mediterranean University of Reggio Calabria (Italy). Isolates of *C. gloeosporioides*, *C. musae,* and *C. circinans* were used as outgroups for comparisons (Table 1).

Morphology of conidia and appressoria

Isolates of *Colletotrichum* were inoculated in

the center of 9-cm-diameter Petri dishes containing PDA with a 5-mm-diameter plug, each taken from the margin of a 5-day-old actively growing colony kept at 24(±1)°C, and incubated under fluorescent light for at least 7 days at 24(±1)°C to stimulate the conidiogenesis. Conidia were mounted in water and observed microscopically at ×1000 magnification. For each isolate 100 conidia were randomly selected, and length, width and shape were recorded.

The observation of the morphology of conidia by scanning electron microscopy (SEM) was carried out by growing isolates on PDA at 24(±1)°C under continuous fluorescent light as described above. After 7–10 days of incubation, plugs of 4×4 mm were fixed in 2% glutaraldehyde in 0.1 M sodium-cacodylate buffer (EMS), pH 7.2, for 1 h at 4°C and then post-fixed in 1% osmium tetroxide (EMS) for 1 h at 4°C. After dehydration in graded ethanol and critical point drying using CO_2 (Emscope-CPD 750), the samples were attached by CCC carbon adhesive directly on the microscope stubs, coated with vacuum evaporated gold (Emscope-SM 300) and observed using a Field Emission Scanning Electron Microscope (FESEM).

Appressoria were produced in slide cultures on potato-carrot-agar (PCA, Smith and Onions, 1984) grown at 25°C for 7 days alternating natural light and darkness (Sutton, 1968) and on PDA in the dark (Cai *et al.*, 2009).

Cultural characterization of isolates

To determine cardinal growth temperatures for isolates, 5-mm-diameter mycelium plugs taken from the margins of 5-day-old actively growing colonies at 24(±1)°C were transferred onto PDA and incubated at 5, 10, 15, 20, 24, 27, 30 or 35°C both in the dark and under continuous fluorescent light. Colony diameters were measured daily for 7 days. Three replicates of each isolate were evaluated, and the experiments were repeated twice. Growth rate was calculated after a 7-day-incubation period as mean daily growth rate (mm day^{-1}) for each temperature.

Molecular characterization

RAPD-PCR analyses

PCR reactions were performed using 16 decamer oligonucleotides (OPB-01, OPB-03, OPB-07, OPB-09, OPB-14, OPB-19, OPF-01, OPF-03, OPF-04, OPF-06, OPF-09, OPF-10, OPF-11, OPF-13, OPF-15 and OPF-20) selected during preliminary investigations with a restricted number of isolates (data not shown). All primers were purchased from Operon Technologies Inc. (Alameda, CA, USA). Total DNA was extracted from fresh mycelium scraped from cultures grown on PDA for 7 days at 24°C using DNeasy Plant Mini Kit according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). RAPD-PCR was carried out in 25 µl of reaction mixture containing 20 mM Tris HCl, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 100 µM each dNTP, 0.2 µM primer, 5 ng genomic DNA and 1 U *Taq* DNA Polymerase (Invitrogen, Life Technologies, Carlsbad, CA, USA). Amplification was performed in a Perkin-Elmer Cetus (Norwalk, CT, USA) GeneAmp PCR System 9600, starting with 2.5 min at 94°C, followed by 45 cycles consisting of 30 s at 94°C, 1 min at 36°C, 2 min at 72°C, and a final step of 5 min at 72°C. Amplicons were analyzed by electrophoresis in 1.5% agarose gels containing SYBR Safe DNA gel stain (Invitrogen, Life Technology Corporation, Carlsbad, CA, USA) or ethidium bromide (0.5 µg ml^{-1}) in Tris-acetate-EDTA (TAE) buffer. After separation, the bands were visualized on a UV transilluminator and the gels photographed using a digital camera. RAPD analyses were repeated at least twice per isolate and primer. A negative control using water instead of template DNA was included in all amplifications.

ITS-rDNA and β-tubulin analyses

Genomic DNA was extracted from *Colletotrichum* isolates following the procedure described by Schena and Cooke (2006). The ITS1-5.8S-ITS2 region and a fragment of the β-tubulin 2 gene comprised between exons 2 and 6 (Glass and Donaldson, 1995) were amplified with primers ITS5 and ITS4 (White *et al.*, 1990), and primers T1 (O'Donnell and Cigelink, 1997) and βt2b (Glass and Donaldson, 1995), respectively. Amplifications were performed in a 25-µl reaction volume containing 1× PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each deoxyribonucleotide triphosphate, 0.5 µM of each primer, 10 ng template DNA, and one unit of *Taq* DNA Polymerase (Invitrogen). A negative control using water instead of template DNA was included in all PCR reactions. PCR reactions were performed in an automated thermal cycler (GeneAmp PCR

System 9600, Perkin-Elmer Cetus) programmed to perform 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 50 s at 58°C (ITS) or 60°C (β-tubulin) and 1 min at 72°C. All reactions ended with 10 min at 72°C. Amplified products were analyzed by electrophoresis as described above, 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 version 1.5' software (http://www.technelysium.com.au/) was utilized to evaluate reliability of sequences and to create consensus sequences. Non-reliable sequences in which either forward or reverse sequences contained doubtful bases were sequenced a second time.

Phylogenetic analyses

Cluster analysis to compare associations between bands of RAPD patterns obtained from *Colletotrichum* isolates was performed by using the PAST software ver. 2.09 (Hammer *et al*., 2001). The genetic relatedness between all isolates was represented as a dendrogram generated using the Dice similarity index (Dice, 1945) and the unweighted pair-group method with arithmetical averages (UPGMA) algorithm (Sneath and Sokal, 1973). Nodal support was assessed using bootstrap analysis from 1,000 replicates (Felsenstein, 1985).

ITS and β-tubulin 2 sequences obtained in the present study (Table 1) and GenBank deposited sequences, selected as representative taxa of *Colletotrichum* (Table 2), were utilized to determine phylogenetic relationships between *C. acutatum* genetic group A4 and other species and genetic groups of *C. acutatum s. l.*

Multiple alignment of both ITS and β-tubulin 2 sequences was carried out by CLUSTALW (Thompson *et al*., 1994) with a total of 109 and 101 nucleotide sequences, respectively. Molecular phylogeny estimation was performed by UPGMA distance-based, Maximum Likelihood (ML) and Bayesian character-based methods. Pair-wise genetic distance was estimated using the Kimura two-parameter (K2P) model (Kimura, 1980) with complete deletion option to treat gaps. The degree of statistical support for the nodes of the phylograms generated by UPGMA clustering was evaluated in 1,000 resample trees by the bootstrap interior-branch test (Sitnikova *et al*., 1995). ML analysis was conducted using K2P substitution model and Nearest-Neighbor-Interchange method with 1,000 bootstrap replicates. Analyses were performed using MEGA software ver. 5 (Tamura *et al*., 2011). Bayesian analysis was performed using MrBayes ver. 3.1.1 as implemented in TOPALi v2.5 (Milne *et al*., 2009). Four runs were conducted simultaneously for 200,000 generations with 10% sampling frequency and burn-in of 25%. Multi-alignment of datasets are accessible through TreeBase under the reference number S11685 (http://purl.org/phylo/treebase/phylows/study/TB2:S11685).

Results

RAPD-PCR analyses

Reliable amplification products were obtained from all isolates. A total of 376 different RAPD loci were detected consistently with 16 random primers in all isolates of *Colletotrichum*. The size of fragments ranged from 100 and 3,000 bp and each primer generated between two and 12 bands. Only major bands ranging from 200 to 2,000 bp amplifiable with high reproducibility in different PCR reactions were scored for consistency. All selected decamer primers generated polymorphic bands among the different species or molecular groups of *Colletotrichum* isolates (overall polymorphism of 98.72%) and monomorphic banding patterns within each of these groups (average monomorphism per group of 85.63%). The genetic distance based on the similarity index of Dice (1945) demonstrated sufficient genetic divergence to discriminate the isolates analyzed in this study into different *Colletotrichum* species (Figure 1). RAPD patterns of Italian isolates from olive (including IMI 398854 and IMI 398855), rhododendron (AZJ), apple (MELA), olive isolates from Greece (CBS 193.32) and Montenegro (CGMUL), and the sweet cherry isolate from Norway (8689), which can be referred to the genetic group A4 of *C. acutatum*, were different from all other *Colletotrichum* isolates examined. They clustered together into a distinct group that was very well supported by bootstrap analysis (100% of generated trees), and clearly differed from the reference isolates of *C. acutatum, C. simmondsii* and *C. fioriniae,* as well as from the isolates of *C. gloeosporioides*

Table 2. GenBank ITS and β-tubulin sequences of *Colletotrichum* isolates used as references for phylogenetic analyses.

Isolate code	Host	Geographical origin	GenBank Accession No.		Reference
			ITS-rDNA	ß-tubulin	
C. clavatum					
JG05	*Ceanothus* sp.	France	AJ300557	AJ409302	Talhinhas *et al.*, 2002
CBS 193.32	*Olea europaea*	Greece	AJ749688	AJ748612	Talhinhas *et al.*, 2005
PT169	*O. europaea*	Portugal	AJ749685	AJ748609	Talhinhas *et al.*, 2005
C. acutatum					
IMI 117617 (holotype)	*Carica papaya*	Australia	AF411700		Vinnere *et al.*, 2002
IMI 117620 (ex-paratype)	*C. papaya*	Australia	FJ788417	FJ788419	Shivas and Tan, 2009
C. simmondsii					
BRIP 28519 (ex-holotype)	*C. papaya*	Australia	GU183331	GU183289	Shivas and Tan, 2009
STE-U 4452	*Protea magnifica*	South Africa	AY376503	AY376551	Lubbe *et al.*, 2004
PT135	*O. europaea*	Portugal	AJ749683	AJ748607	Talhinhas *et al.*, 2005
C. fioriniae					
EHS58 (ex-holotype)	*Fiorinia externa*	USA	EF464594	EF593325	Marcelino *et al.*, 2008
STE-U 5287	*Malus* sp.	USA	AY376509	AY376557	Lubbe *et al.*, 2004
C. acutatum A1[a]					
IMI 350308	*Lupinus* sp.	Unknown	AJ300561		Talhinhas *et al.*, 2002
CA546	*Lupinus* sp.	Unknown	AJ749674	AJ748631	Talhinhas *et al.*, 2005
C. acutatum A6[a]					
PT250	*O. europaea*	Portugal	AJ749700	AJ748624	Talhinhas *et al.*, 2005
S2	*Rhododendron* × *orbiculare*	Sweden	AF411719		Vinnere *et al.*, 2002
C. acutatum A7[a]					
IMI 345581	*Fragaria* × *ananassa*	New Zealand	AJ536212		Martinez-Culebras *et al.*, 2003
BBA 65797	*Syringa vulgaris*	Germany	AJ301925		Nirenberg *et al.*, 2002
C. acutatum A8[a]					
TOM-9	*Cyphomandra betacea*	Colombia	AF521205		Afanador-Kafuri *et al.*, 2003
TOM-21	*C. betacea*	Colombia	AF521196		Afanador-Kafuri *et al.*, 2003
C. gloeosporioides					
IMI 356878 (ex-epitype)	*Citrus sinensis*	Italy	EU371022		Cannon *et al.*, 2008
STE-U 4295	*Citrus* sp.	Italy	AY376532	AY376580	Lubbe *et al.*, 2004
C. musae					
CBS 116870 (ex-epitype)	*Musa* sp.	North America	HQ596292	HQ596280	Su *et al.*, 2011
B15	*Musa* sp.	Thailand	DQ453986	DQ454038	Shenoy *et al.*, 2007

[a] Group as defined by Sreenivasaprasad and Talhinhas (2005)

and *C. musae* used as outgroups. In turn, this cluster exhibited a small genetic variation and encompassed two different subgroups of isolates (86 and 49% bootstrap support values, respectively). The first subgroup included an isolate from rhododendron (AZJ) sourced in Piedmont (Italy), an isolate from sweet cherry (8689) sourced in Norway and all olive isolates from Apulia (Italy). The second subgroup comprised an Italian isolate from apple (MELA), an olive isolate from Greece

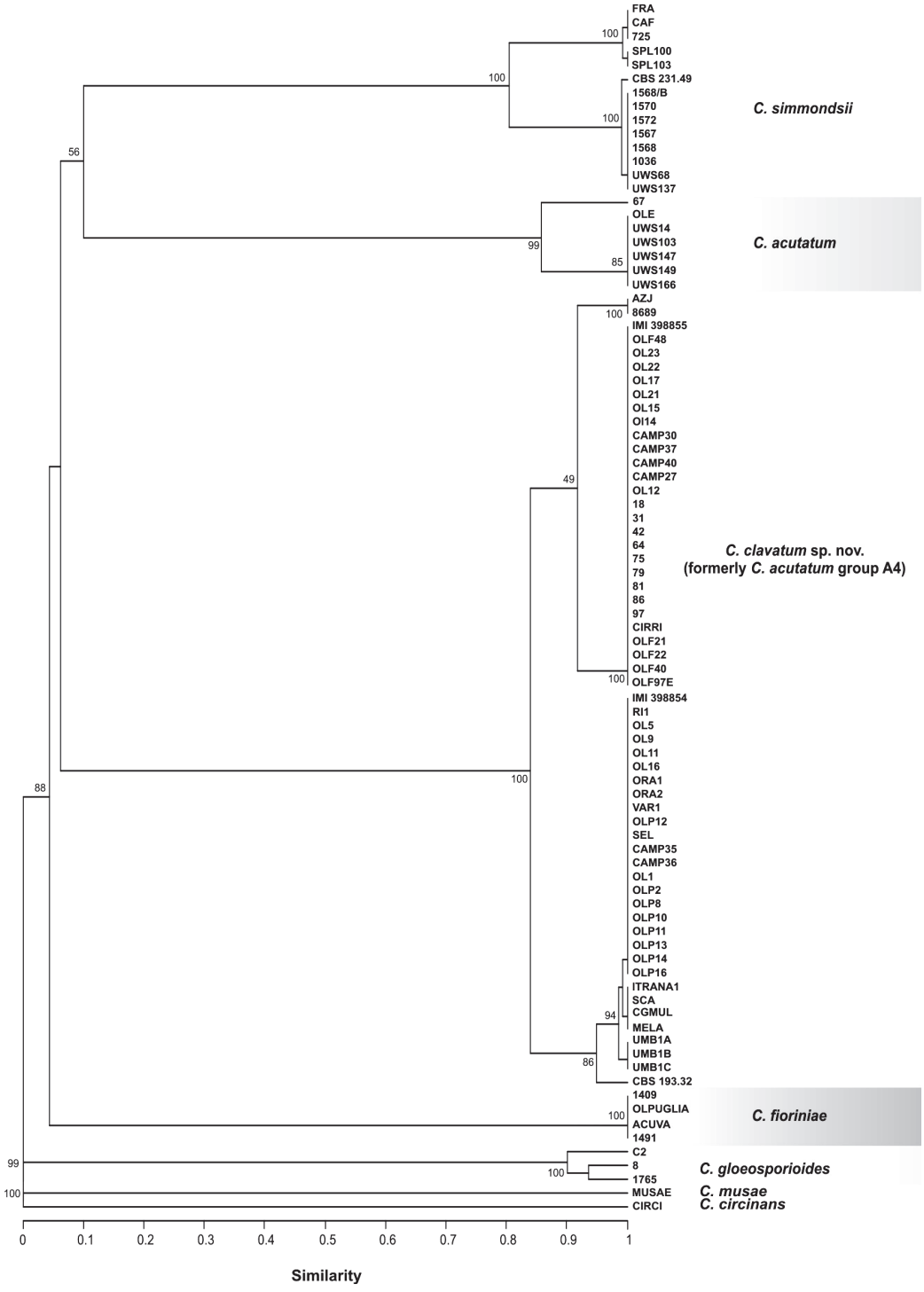

Figure 1. Dendrogram obtained by cluster analysis of RAPD profiles of *Colletotrichum* isolates listed in Table 1. Figures on branches are the bootstrap values as percentage of bootstrap replication from 1,000 replicate analyses. The dendrogram clearly differentiates all isolates of *C. clavatum* (formerly *C. acutatum* molecular group A4) from isolates of *C. acutatum*, *C. simmondsii* and *C. fioriniae* (formerly *C. acutatum* molecular groups A5, A2 and A3, respectively), as well as from isolates of *C. gloeosporioides*, *C. musae* and *C. circinans* used as outgroups.

(CBS 193.32) and Montenegro (CGMUL) as well as the olive isolates from Calabria, Sardinia and Umbria.

Phylogenetic analysis

The identity of all isolates investigated in this study was confirmed using ITS and β-tubulin sequence data. The ITS dataset used for phylogenetic analysis included 501 sites with 68 (13.57%) potentially informative sites and a final expected transition/transversion ratio (TI/TV) of 1.81. As for the β-tubulin 2 gene, 766 sites containing 308 (40.21%) variable sites with a TI/TV ratio of 4.62 were identified.

The ITS-based UPGMA phylogenetic tree of *Colletotrichum* isolates investigated in the present study revealed eight phylogenetic groups within *C. acutatum s. l.* (Figure 2) which were congruent with those of previously reported studies by Sreenivasaprasad and Talhinhas (2005) and Shivas and Tan (2009). Italian isolates from olive (e.g. IMI 398854 and IMI 398855), rhododendron (AZJ) and apple (MELA), as well as isolates from sweet cherry sourced in Norway (8689) and olive sourced in Montenegro (CGMUL) had ITS sequences identical to those of reference isolates (CBS 193.32, AJ748612; JG05, AJ409302; PT169, AJ748609). These isolates, ascribable to *C. acutatum* group A4 *sensu* Sreenivasaprasad and Talhinhas, formed a well-defined clade distinctly separated from the clades encompassing isolates of *C. acutatum s. s., C. simmondsii, C. fioriniae* and *C. acutatum* genetic groups A1, A6, A7 and A8, as well as from *C. gloeosporioides* and *C. musae* (Figure 2). The mean genetic distance of *C. acutatum* group A4 from *C. acutatum s. s., C. simmondsii, C. fioriniae* and *C. acutatum* genetic groups A1, A6, A7 and A8 was 0.009. The most closely related clade was the genetic group A7 of *C. acutatum* (68% bootstrap support) with a genetic distance of 0.003. The genetic distance of *C. acutatum* group A4 from *C. gloeosporioides* and *C. musae*, used as outgroups, was 0.113 and 0.103, respectively (99% bootstrap support). In terms of sequence divergence, *C. acutatum* group A4 showed the least divergence (0.28%) as compared to *C. acutatum* A7 and a divergence of 1.12% with *C. acutatum*, *C. fioriniae* and *C. acutatum* A6 (Table 3). Higher levels of divergence were observed between *C. acutatum* group A4 and *C. simmondsii, C. acutatum* group A1, and *C. acutatum* group A8. On the whole, these levels of divergence were generally greater if compared with those differentiating the newly described species *C. fioriniae* and *C. simmondsii* from other *C. acutatum s. l.* (Table 3).

The topology of the β-tubulin 2 tree generated with UPGMA was similar to that of ITS (Figure 3). However, this gene showed greater polymorphism with a mean genetic distance of 0.053, enabling a major resolution for isolates clustering in *C. simmondsii* and *C. fioriniae* clades. The genetic distance of *C. acutatum* group A4 from both *C. gloeosporioides* and *C. musae* was 0.29. All isolates of the genetic group A4 clustered together (100% bootstrap support) in a monophyletic clade clearly separate from other species of *Colletotrichum*, including *C. acutatum s. s., C. simmondsii, C. fioriniae* and *C. acutatum* groups A1 and A6 (Figure 3). Mean pair-wise percent divergence values for β-tubulin 2 gene were greater compared to those of the ITS regions, whereas patterns were very similar to those of the above described ITS sequences (Table 4).

Phylogenetic groups largely congruent with those of the above described UPGMA analysis were also obtained with the Maximum Likelihood and Bayesian analyses (data not shown).

Taxonomy

Analysis of ITS and β-tubulin sequences supports the group A4 of *C. acutatum* as a separate species within the *C. acutatum* complex. This species corresponds also to group B of Lardner *et al.* (1999). The allocation and the formal description of this genetic group to the species rank besides the taxonomic significance could also have epidemiological implications as well as quarantine relevance.

Colletotrichum clavatum G.E. Agosteo, R. Faedda & S.O. Cacciola, **sp. nov.** (Figure 4)

Etymology: *clavatum* refers to club-shaped conidia that are the dominant form for this species.

MycoBank number: MB561749

Coloniae in PDA cinereae et lanuginosae cum margine regulari albicante, mycelio aerio denso, sparsis floccis albicantibus mycelii et luteis massis conidiorum sparsis sed saepius in media colonia. Aversum coloniae pallide cinereum ad pallide roseum. In agaro PDA post septem dies ad 24°C

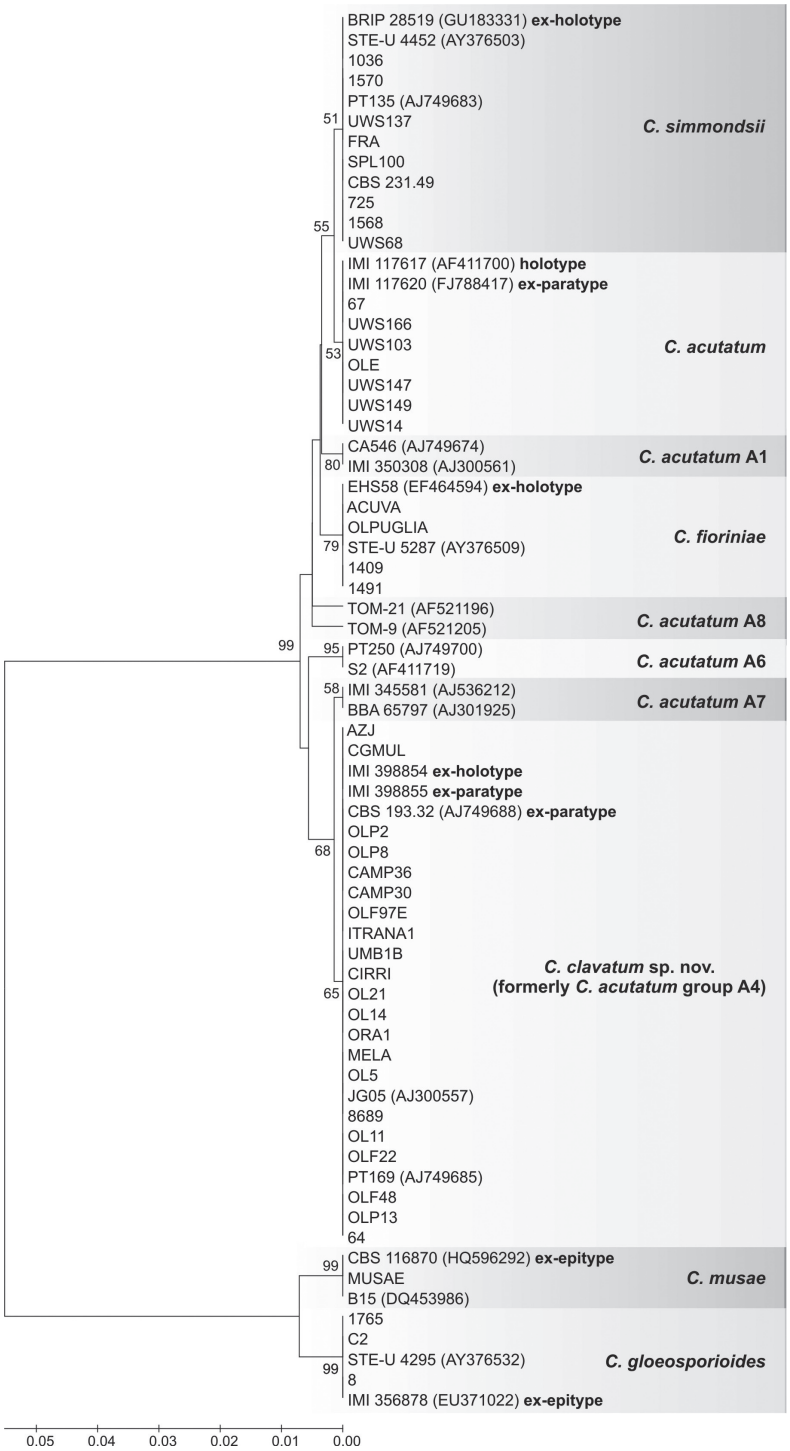

Figure 2. UPGMA phylogenetic tree of *Colletotrichum* based on ITS1-5.8S-ITS2 of ribosomal DNA, showing the position of *C. clavatum* sp. nov. in relation to other known *Colletotrichum* species and genetic groups of *C. acutatum*. Bootstrap interior-branch values (1,000 replicates) ≥50% are displayed between the nodes. The GenBank accession numbers of the reference sequences are in brackets preceded by the corresponding isolate codes. The scale bar indicates a 0.001 U of genetic distance from Kimura's two-parameter model.

Table 3. Average pairwise percent sequence divergence among *Colletotrichum* spp. based on 109 nucleotide sequences of the ITS-rDNA region.

	Species/Group	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
1.	*C. clavatum*[a]									
2.	*C. acutatum*	1.12								
3.	*C. fioriniae*	1.12	0.56							
4.	*C. simmondsii*	1.40	0.28	0.84						
5.	*C. acutatum* A1	1.96	0.84	0.84	0.56					
6.	*C. acutatum* A6	1.12	1.68	1.68	1.96	2.52				
7.	*C. acutatum* A7	0.28	0.84	0.84	1.12	1.68	0.84			
8.	*C. acutatum* A8	2.24	1.12	1.12	0.84	0.84	2.80	1.96		
9.	*C. gloeosporioides*	10.36	10.36	10.36	10.64	11.20	9.80	10.08	11.06	
10.	*C. musae*	9.52	9.52	10.08	9.80	10.36	10.08	9.80	10.22	1.40

[a] Formerly *C. acutatum* genetic group A4.

colonia est fere 40 mm diametro. Sclerotia et setae absunt. Conidia unicellularia, subcylindrata vel saepius subclavata, 10–(14.9)–17 × 4.0–(4.6)–5.0 μm, *saepe in medio constricta, hyalina, levia, apice obtuso, basi angusta, brevi projectione annelli forma. Appressoria fusca unicellularia, levia, plerumque clavata vel lobata, saepe complexa 8–(11.5)–19 × 4.5–(5.5)–7.5* μm. *Haec species differt ab aliis taxis generics aspectu coloniae, conidiis saepe clavatis et ITS et β-tubulina ordine.*

Isolates of *C. clavatum* on PDA show uniform colony morphology white to greyish pale salmon or light brown, dense aerial mycelium. In reverse, dull beige to pale salmon. Mycelium may produce pink conidial masses mainly in the centre of the colony (Figure 4). The conidiogenesis is stimulated by light, and under natural light colonies develop greyish concentric rings. Conidiophores are septate and bear enteroblastic, phialidic, hyaline conidiogenous cells and, in culture, conidia are produced after 7 days of incubation (Figure 5 A, B, C). Conidia developing also in the aerial mycelium are hyaline and unicellular, subcylindrical or more frequently clavate, smooth and thin-walled, measuring 10–(15) –17×4.0–(4.6)–5.0 μm, with a mean length/breadth (l/b) ratio 3.3 (±0.9). They have a rounded distal apex and often a light median constriction, a funnel-shaped base ending with a short ring-like projection (Figure 5 D). In slide cultures on PCA, hyphal appressoria are melanized, mostly regular in shape, brown, ovate to long clavate, sometimes lobate, often complex, that is, producing columns of several closely connected appressoria (Sutton, 1992), measuring 8–(11.5)–19×4.5–(5.5)–7.5 μm (Figure 4 D). Sclerotia and setae obsente. On PDA, mycelium grew between 10 and 30°C with an optimum at 24°C, whereas at 5 and 35°C no growth was observed. Radial growth rate at 24°C was between 3.8 and 8.2 mm per day, with an average (± S.D) of 5.9±1.0 mm day^{-1}.

Typus: Italy: Rizziconi, Reggio Calabria (southern Italy), isolated from rotten olive fruit (*Olea europaea* L.), collected in an olive orchard (*O. europaea*) in the Gioia Tauro plain, Rizziconi, Reggio Calabria, October 1992, G. E. Agosteo and G. Magnano di San Lio. Holotype: OLDC10 (dried culture on PDA, herbarium of the Dipartimento di Gestione dei Sistemi Agrari e Forestali, Mediterranean University of Reggio Calabria). Ex-type living culture OL10. This strain is available at IMI, CABI Bioscience, Egham, Surrey, UK (IMI 398854) and the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, (CBS 130251), ITS sequence GenBank JN121126, β-tubulin 2 sequence GenBank JN121213, DIGESA Culture Collection, Catania (Italy) and in the Culture Collection of the Dipartimento di Gestione dei Sistemi Agrari e Forestali, Mediterranean University of Reggio Calabria, Italy (F49).

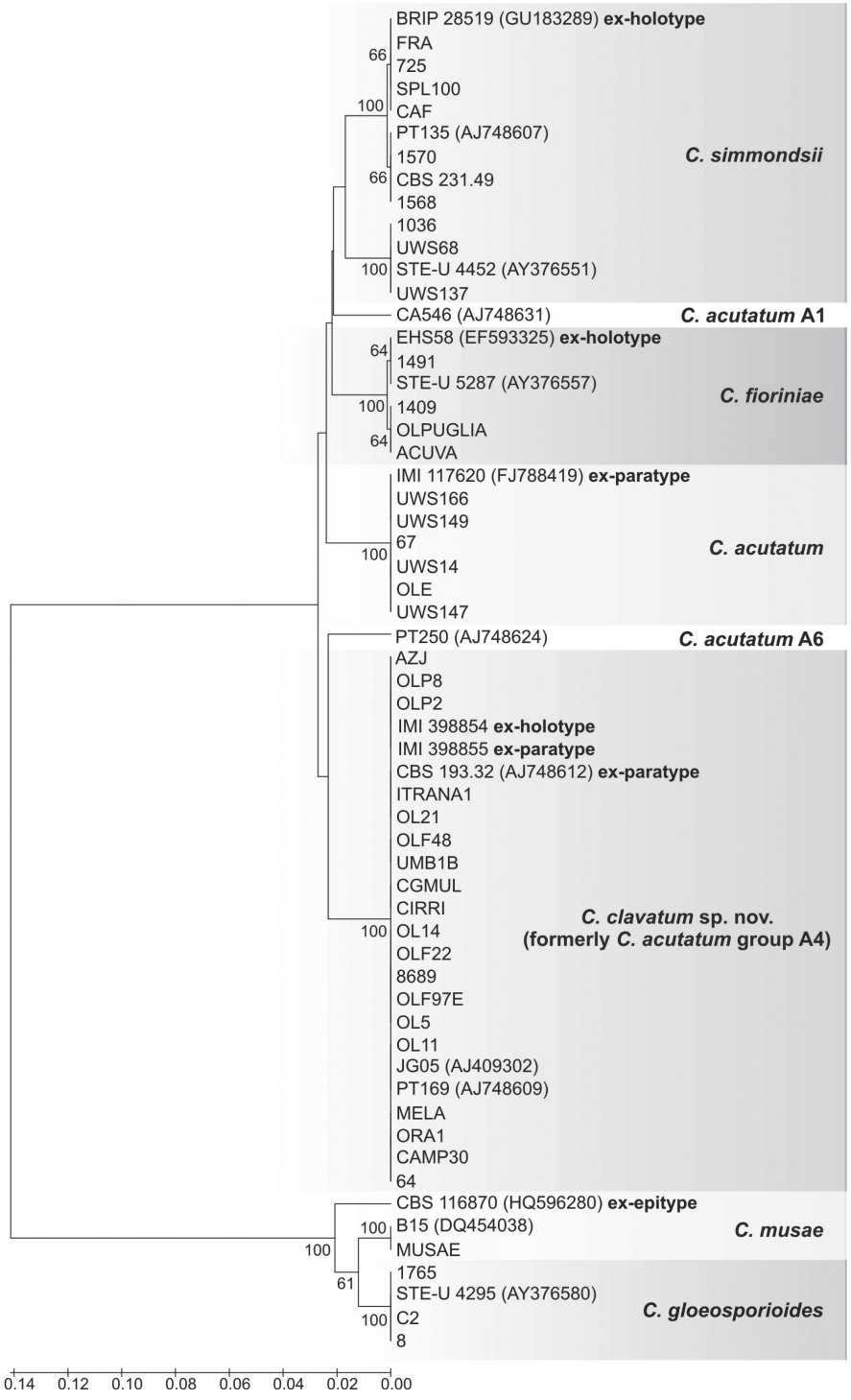

Figure 3. UPGMA phylogram of *Colletotrichum* inferred from partial -tubulin-2 gene DNA sequences, showing the position of *C. clavatum* sp. nov. in relation to other known *Colletotrichum* species and genetic groups of *C. acutatum*. Bootstrap interior-branch values (1,000 replicates) ≥50% are displayed between the nodes. The GenBank accession numbers of the reference sequences are in brackets preceded by the corresponding isolate codes. The scale bar indicates a 0.001 U of genetic distance from Kimura's two-parameter model.

Table 4. Average pairwise percent sequence divergence among *Colletotrichum* spp. based on 101 nucleotide sequences of the β-tubulin 2 partial gene.

	Species/Group	*1*	*2*	*3*	*4*	*5*	*6*	*7*
1.	*C. clavatum* sp. nov.[a]							
2.	*C. acutatum*	5.26						
3.	*C. fioriniae*	5.92	4.87					
4.	*C. simmondsii*	4.79	4.52	4.19				
5.	*C. acutatum* A1	5.00	4.47	4.87	4.13			
6.	*C. acutatum* A6	4.47	4.47	5.66	4.92	4.47		
7.	*C. gloeosporioides*	23.95	22.89	23.82	22.30	23.68	23.16	
8.	*C. musae*	23.68	22.63	23.82	22.04	23.42	23.16	3.16

[a] Formerly *C. acutatum* genetic group A4.

Additional specimens examined (see Table 1). The earliest collection of isolates of *C. clavatum* examined was obtained from *Olea europaea* of the Calabria and Apulia regions in southern Italy starting from 1992. Additional isolates of the same species were then collected from other Italian regions and European countries and different hosts. Paratypes of *C. clavatum* deposited in International Collections are OL20 (=IMI 398855, CBS 130252) and CBS 193.32 (Figure 6).

Habitat: the list of all known hosts of *C. clavatum* includes *Olea europaea*, *Fragaria* × *ananassa, Lycopersicon esculentum*, *Malus domestica, Ficus*

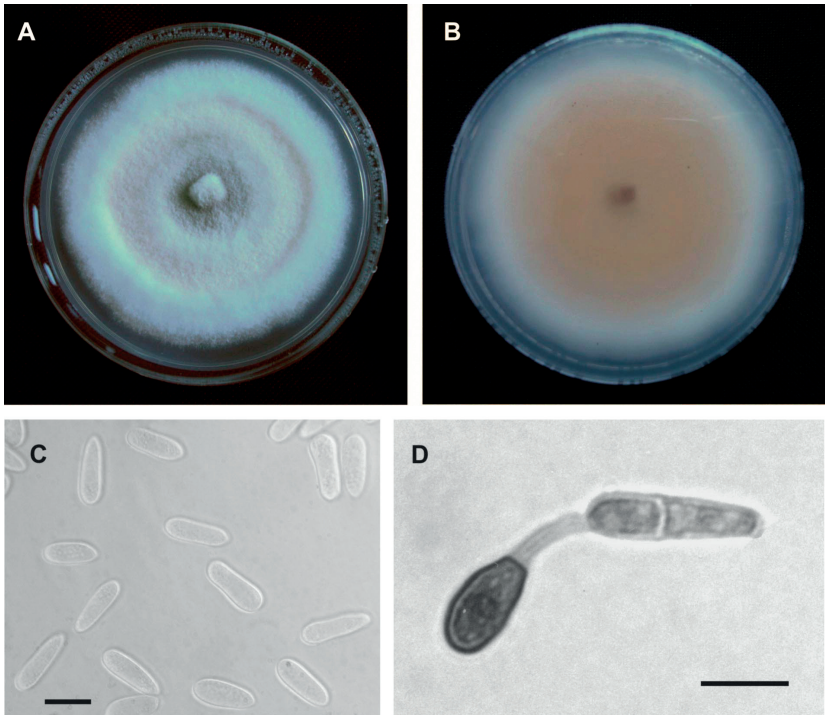

Figure 4. Colony morphology of *Colletotrichum clavatum* IMI 398854 (ex-type). Plate in front (A) and reverse (B) grown for 14 days on PDA at 24(±1)°C. Light micrographs showing the characteristic clavate conidia (C) and a germinating conidium with a clavate, melanized appressorium at the apex of the germ tube (D). Scale bars = 10 μm.

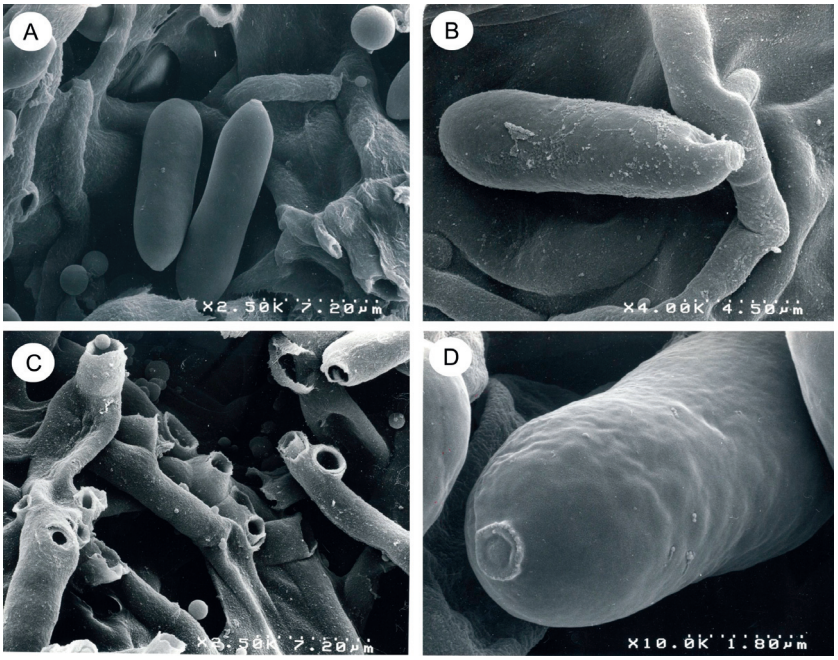

Figure 5. *Colletotrichum clavatum* IMI 398854 (ex-type). SEM view of clavate conidia (A and B), phialides with conidiogenous loci (C) and ring-like basal peduncle of a conidium (D).

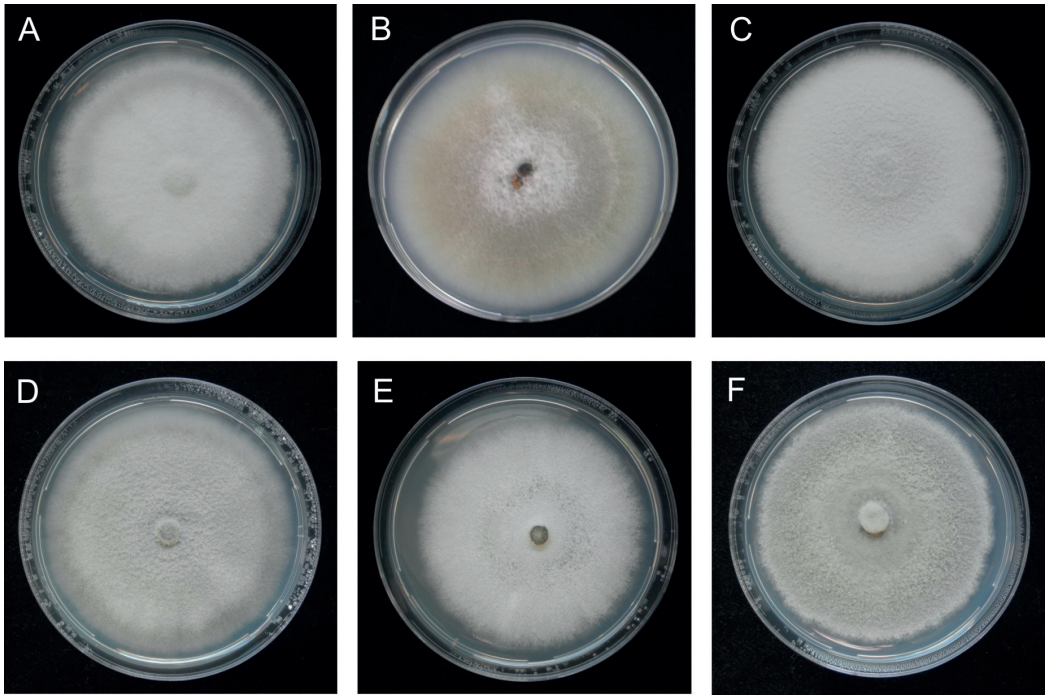

Figure 6. Colony morphology of isolates of *Colletotrichum clavatum* sp. nov. grown for 14 days on PDA at 24(±1)°C. A, IMI 398855, ex-paratype (olive isolate from Apulia, Italy); B, CBS 193.32, ex-paratype (olive isolate from Greece); C, AZJ (rhododendron isolate from Piedmont, Italy); D, ORA1 (olive isolate from Sardinia, Italy); E, CIRRI (olive isolate from Apulia, Italy); F, OLF97E (olive isolate from Apulia, Italy).

carica, *Eriobotrya japonica, Feijoa sellowiana, Hepatica acutiloba, Sambucus nigra, Prunus dulcis, Rhododendron* spp., *Rubus* sp., *Ceanothus* sp., *Vitis* sp., *Juglans* sp., *Primula* sp., *Camellia* sp. and *Bergenia* sp. (Sreenivasaprasad and Talhinhas, 2005)

Teleomorph: Unknown

Notes: *Colletotrichum clavatum* is distinct from other species of *Colletotrichum* in its colony morphology, shape and l/b ratio of conidia, ITS and β-tubulin 2 sequences, and RAPD patterns. This new species is characterized by its club-shaped conidia with rounded distal apices and median constriction, funnel shaped base with a short cylindrical projection. In other species of *Colletotrichum*, conidia are mostly cylindrical with both ends rounded. *Colletotrichum clavatum* has conidia with a greater l/b ratio with respect to other species of this genus. Moreover, growth of isolates of *C. clavatum* is slower in comparison with that of isolates of other species of *Colletotrichum*.

Discussion

In this study, a polyphasic approach was used to confirm that *Colletotricum clavatum*, formerly identified as *C. acutatum* group B or A4, can be considered a distinct species within *C. acutatum s. l.* as hypothesized in previous studies (Sreenivasaprasad and Talhinhas 2005; Cacciola *et al.*, 2007; Shivas and Tan, 2009; Sergeeva *et al.*, 2010). The characterization of the causal agent of olive fruit anthracnose in Italy prompted us to allocate a large population of isolates collected over almost 20 years to the appropriate taxonomic status.

RAPD genomic fingerprinting and phylogenetic analysis of both ITS and β-tubulin DNA sequences showed that this species constitutes a strongly supported monophyletic lineage clearly distinct from *C. acutatum s. s.* and other species or genetic groups previously referred to as *C. acutatum s. l.* These analyses are directly comparable with those of Sreenivasaprasad and Talhinhas (2005), who identified eight phylogenetic groupings of the *C. acutatum* species complex, as well as with those of Shivas and Tan (2009) who recently revised the taxonomy of *C. acutatum* introducing two new species, *C. fioriniae* and *C. simmondsii*.

Even though a multi-gene phylogenetic analysis has been suggested by several authors to give a better understanding of the relationships within *Colletotrichum* (Cai *et al.*, 2009), RAPD-PCR is a method that provides a better overview of the entire genome (Laroche *et al.*, 1995) and, as also demonstrated by Yang and Sweetingham (1998), this allowed better resolution for *Colletotrichum* than ITS sequences. RAPD analysis enabled us to highlight greater genetic intraspecific variability and to give better interspecific resolving power with respect to ITS and β-tubulin 2 sequences. In fact, RAPD patterns of *Colletotrichum* isolates examined in this study, besides showing a very high interspecific polymorphism, made it possible to differentiate *C. clavatum* isolates into two well-defined subgroups. Italian isolates of *C. clavatum* from olive correlated with their geographic origin as all isolates collected in the Apulia region clustered into the same subgroup, which was distinct from the subgroup comprising isolates from other regions, including Calabria, Sardinia and Umbria. Thus, it could be speculated that populations of *C. clavatum* from olive established in the Calabria and Apulia regions, respectively, are undergoing an allotropic speciation process, or otherwise they have originated from different introductions, very probably from Greece or Albania (Ciccarone, 1950; Agosteo, 2010). To the best of our knowledge, the oldest living culture of this new species, an isolate from olive indicated here as a paratype (CBS 193.32), dates back to 1930 and was deposited at CBS by Lionello Petri who had received it from Jean Serejanni of the Benaki Phytopathological Institute of Kiphissia, Athens, Greece, (Petri, 1930; Biraghi, 1934).

Biometric characteristics of *C. clavatum* overlap with those of other *Colletotrichum* species, such as *C. acutatum*, *C. fioriniae* and *C. simmondsii;* however, even though this new taxon was identified primarily on the basis of molecular analyses, it shows morphological and physiological traits, including the shape of conidia and the colony morphology, which discriminate it from all other *Colletotrichum* species, confirming that in this genus conidial morphology and cultural characters reflect phylogeny more than host association (Than *et al.*, 2008). Although, according to Sreenivasaprasad and Talhinhas (2005), group A4 fits into group B of Lardner *et al.* (1999), iso-

lates of *C. clavatum* examined in the present study did not produce perithecia *in vitro* and the perfect stage has never been found in plants with natural infections. In contracts, isolates of group B were described as differentiating perithecia in culture.

Colletotrichum clavatum is a polyphagous and widespread pathogen in Europe, and is common as causal agent of anthracnose of olive in the Mediterranean basin (Agosteo *et al*., 2002; Talhinhas *et al*., 2002, 2005; Cacciola *et al*., 2007; Talhinhas *et al*., 2009; Sergeeva *et al*., 2010; Talhinhas *et al*., 2011). Recently, this pathogen was reported as causal agent of strawberry anthracnose in northern Europe (Damm *et al*., 2010; Van Hemelrijck *et al*., 2010). Moreover, our results confirmed the previous study of Sreenivasaprasad and Talhinhas (2005) inferring that *C. clavatum* is associated with azalea anthracnose, an emerging disease of this ornamental flower plant in Europe (Vinnere *et al*., 2002; Bertetti *et al*., 2008), as well as cherry anthracnose in Norway (Børve and Stensvand, 2006; Cacciola *et al*., 2007). *Colletotrichum clavatum* has also been reported to occur in New Zealand and the USA (Johnston and Jones, 1997; Lardner *et al*., 1999; Sreenivasaprasad and Talhinhas, 2005). Furthermore, other ITS sequences accessioned by GenBank matching those of *C. clavatum* demonstrate that this species is present in other countries and in different hosts; however, these records are not associated to any publication and should be carefully verified.

The re-assessment of the systematics of *C. acutatum*, which has led to the introduction of three novel species as well as to a better definition of *C. acutatum s. s.*, has practical implications for biosecurity, quarantine, plant breeding and disease control (Shivas and Tan, 2009; Hyde *et al.,* 2010). *C. acutatum* (teleomorph *G. acutata*) has, for example, been classifed as an organism of quarantine significance in the European Community since 1993 and was included in the list A2 of the European and Mediterranean Plant Protection Organization (EPPO) as causal agent of anthracnose of strawberry. The causal agent of olive anthracnose has never been on the EPPO lists, as according to von Arx (1957) it has been referred to as *G. cingulata* (Bompeix *et al*., 1988; Graniti *et al*., 1993) or its anamorph *C. gloeosporioides* which is improperly considered a ubiquitous species (Phoulivong *et al*., 2010). By contrast, results of the present study show that in Italy the prevailing causal agents of strawberry and olive anthracnoses are *C. simmondsii* and *C. clavatum*, respectively, while *C. acutatum s. s.*, which is widespread and causes anthracnose diseases of important crops in the Austral hemisphere, has been found only occasionally on potted oleander plants grown under greenhouse conditions in a nursery of ornamentals in Sicily (southern Italy). A precise and stable definition of species previously referred to collectively as *C. acutatum s. l.* will ultimately contribute to improve diagnosis and control of these important plant pathogens.

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