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Colletotrichum acutatum and *C. gloeosporioides* cause anthracnose on olives

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Abstract

Morphological and cultural features and restriction fragment length polymorphism analysis of ITS regions, including 5.8S rDNA, from 26 isolates of *Colletotrichum* species revealed that isolates from olive fruits, previously identified as *C. gloeosporioides,* belong to two taxa: *C. acutatum* and *C. gloeosporioides*. Comparison of both ITS sequence data with reference isolates confirmed the presence of both species in olives affected by anthracnose disease.

Introduction

Olive trees (*Olea europaea* L.) in the south of Catalonia (Spain), are affected by anthracnose disease. The causal agent of this disease has been described as *Gloeosporium olivarum* Alm. However, von Arx (1970) and Sutton (1980, 1992) included it in the species complex *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., anamorph of *Glomerella cingulata* (Ston.) Sp. & Schr., which has been considered the cause of anthracnose on a wide range of plants. Based on conidial morphology and cultural characters, García et al. (1996) established two groups from the isolates of *Colletotrichum* from olives in Spain: type N (strains with grey to brown cultures and fast growth) and type T (strains with whitish to salmon cultures and slow growth). These two groups cause the same anthracnose symptoms.

The main objective of this study was to establish an easy and reliable method to identify the causal agents of anthracnose in olives. To achieve this, isolates from olive fruits, previously identified as *C. gloeosporioides*, were compared with those derived from different hosts, using not only morphological and cultural characters, but also molecular tools (RFLP and sequencing of ITS rDNA). Molecular techniques, such as arbitrarily primed PCR (Freeman and Rodriguez, 1995), RFLPs and sequence analyses, have been used to resolve the systematic problems in other *Colletotrichum* species (Sherriff et al., 1995).

Materials and methods

Fungal strains

Field-collected material was cultured on potato dextrose agar (PDA, ADSA micro, Ref. 1–438) prior to DNA isolation to confirm the identity of the taxa. Original identification, host and source of the isolates examined are given in Table 1. In pathogenicity tests all the isolates from olive were able to cause anthracnose symptoms on olive fruits (García, 1994). Reference isolates of *C. acutatum*, *C. fragariae* and *C. gloeosporioides* from the 'Colección Española de Cultivos Tipos' (CECT) were also included in this study. Mycelium of each isolate was grown on three aqueous media at 28 °C on casein hydrolysis medium (CHM) prepared according to Paterson and Bridge (1994) (this medium become opaque when cold) for 72 h to determine protease activity and on PDA and Czapek-Dox medium (BIOLIFE S.r.l., code 1360) to assess growth rate.

Table 1. Original identifications and origins of *Colletotrichum* isolates and identifications based on molecular data

Isolate code	Supplied as	Supplier's code[1]	Geographical origin	Original host	Molecular identification
ACUCOL-R	*C. acutatum*	CECT 20120, IMI 345028	?	*Fragaria x ananassa*	*C. acutatum*
CIRCOL-01	*C. circinans*	CC-270211	Baix Camp, Tarragona, S	*Allium cepa* (bulb)	*C. circinans*
COCCOL-01	*C. coccodes*	CC-9712 V	València, S	*Solanum lycopersicum* (root)	*C. coccodes*
CRACOL-01	*C. crassipes*	CCra-9712 V	València, S	*Epidendrum fragans* (leaf)	*C. crassipes*
FRACOL-R	*C. fragariae*	CECT-20121, IMI 345047	?	*Fragaria x ananassa*	*C. fragariae*
GLOCOL-01	*C. gloeosporioides*	GL-118-9411	Montsià, Tarragona, S	*Olea europaea* (fruit)	*C. acutatum*
GLOCOL-02	*C. gloeosporioides*	GL-117-9411	Montsià, Tarragona, S	*Olea europaea* (fruit)	*C. acutatum*
GLOCOL-03	*C. gloeosporioides*	GL-9411	Montsià, Tarragona, S	*Olea europaea* (fruit)	*C. acutatum*
GLOCOL-04	*C. gloeosporioides*	CG-9712 V	València, S	*Citrus aurantium* (stem)	*C. gloeosporioides*
GLOCOL-05	*C. gloeosporioides*	GL-98205	Montsià, Tarragona, S	*Olea europaea* (fruit)	*C. acutatum*
GLOCOL-06	*C. gloeosporioides*	GL-98206	Baix Ebre, Tarragona, S	*Olea europaea* (fruit)	ND
GLOCOL-07	*C. gloeosporioides*	GL-98207	Baix Ebre, Tarragona, S	*Olea europaea* (fruit)	*C. acutatum*
GLOCOL-08	*C. gloeosporioides*	GL-98208	Montsià, Tarragona, S	*Olea europaea* (fruit)	*C. acutatum/ C. gloeosporioides*
GLOCOL-09	*C. gloeosporioides*	GL-98209	Montsià, Tarragona, S	*Olea europaea* (fruit)	*C. acutatum*
GLOCOL-10	*C. gloeosporioides*	GL-98210	Montsià, Tarragona, S	*Olea europaea* (fruit)	*C. acutatum*
GLOCOL-11	*C. gloeosporioides*	GL-98211	Montsià, Tarragona, S	*Olea europaea* (leaf)	*C. acutatum*
GLOCOL-12	*C. gloeosporioides*	CD-27486	Maresme, Barcelona, S	*Dieffenbachia* sp. (stem)	*Colletotrichum* sp.
GLOCOL-13	*C. gloeosporioides*	GL-21424	Montsià, Barcelona, S	*Citrus aurantium* (stem)	*C. gloeosporioides*
GLOCOL-14	*C. gloeosporioides*	CG-JGJ-A1	València, S	*Citrus aurantium* (stem)	*C. gloeosporioides*
GLOCOL-15	*C. gloeosporioides*	CG-26389	Menorca, S	*Citrus aurantium* (stem)	*C. gloeosporioides*
GLOCOL-16	*C. gloeosporioides*	GL-EP9801	Montsià, Tarragona, S	*Olea europaea* (fruit)	*C. gloeosporioides*
GLOCOL-R	*C. gloeosporioides*	CECT 21073, IMI 356878	?	*Citrus* sp. (leaf)	*C. gloeosporioides*
SPCOL-01	*Colletotrichum* sp.	CF-9511-T	Maresme, Barcelona, S	*Fragaria vesca* (fruit)	*C. acutatum*
SPCOL-02	*Colletotrichum* sp.	CF-9511-A	Maresme, Barcelona, S	*Fragaria vesca* (fruit)	*C. acutatum*
SPCOL-03	*Colletotrichum* sp.	CF-22555-B	Huelva, S	*Fragaria vesca* (fruit)	*C. acutatum*
TRICOL-01	*C. trichellum*	CT-27621	Vallès Oriental, Barcelona, S	*Hedera helix* (leaf)	*C. trichellum*

[1]The reference isolates were sent by CECT (Univ. València, Spain); remaining isolates are deposited at the Laboratori de Sanitat Agrària (Dept. Agricultura, Ramaderia i Pesca, Generalitat de Catalunya, Barcelona, Spain).

DNA extraction

DNA was extracted using the protocols of Whiting et al. (1997) and/or EZNA Fungi DNA miniprep kit (Omega Biotek). EZNA kits combine the reversible nucleic acid-binding properties of HiBind® matrix with the speed and versatility of spin columns to eliminate polysacchrarides, polyphenols and other enzyme inhibitors from lysed fungal hyphae. Twenty mg of fresh mycelium from PDA were used in both of DNA isolation protocols, except for the three reference isolates for which over 80 μg of lyophilized mycelium was required. DNA was re-suspended in pre-warmed, sterile milli-Q water (approximate final concentration: 1 ng μl^{-1}).

PCR amplification

Primers ITS1F (Gardes and Bruns, 1993) and ITS4 (White et al., 1990) were used for PCR amplifications of the ITS regions, including the 5.8S rDNA. The amplification reactions were done using two protocols: (a) standard procedure described in White et al. (1990) in a total reaction volume of 20 μl, and/or (b) individual reactions to a final volume of 25 μl with Ready-To-Go® PCR Beads (Amersham-Pharmacia

Biotech) as described in Winka et al. (1998). PCR Beads are provided dry and are stable at room temperature; each bead contains all necessary reagents, except primer and template, for performing a 25 μl PCR amplification reaction.

The PCR was performed by using a Techne DNA thermocycler (model Progene). In both protocols, the cycling parameters were 5 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, followed by 33 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 30 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. Before initiating the cycling parameters an initial denaturation at 94 °C for 5 min was done. Controls, lacking fungal DNA, were run for each experiment to check for DNA contamination of the reagents. Amplification products were separated by electrophoresis in agarose gels containing 1.2–2% Agarose (SERVA) run in TAE buffer for 30 min at 5 V cm^{-1}. The DNA was stained with ethidium bromide present in the agarose gel (0.4 μg ml^{-1}). Gels were recorded on Polaroid 667 black and white film. The lengths of the amplification products were estimated by comparison to a kb-ladder (Gibco, BRL).

RFLP analysis

Aliquots (5 μl) of amplified DNA were digested separately with 2 units of each enzyme according to the manufacturers' recommendations in 20 μl total volume by reaction: *Alu I* (Promega), *Hae III*, *Msp I* and *Taq I* (GeneCraft). The enzymes were chosen on the basis of economic criteria and after screening previously published sequences of the ITS region (Latunde-Dada et al., 1996; Mills et al., 1994; Sherriff et al., 1994, 1995). The restriction fragments were separated using 2% agarose gels by electrophoresis in TAE buffer (Separide™ Gel matrix, Gibco BRL) for 1 h at 5 V cm^{-1}.

RFLP data evaluation

For data analysis, only the reproducible and distinct strongly-stained bands were taken into account, whereas faint or less reproducible bands were ignored. RFLP patterns were transformed into a binary character matrix (1 for presence, 0 for absence of a band at a particular position). Data were analysed using the programme TREECON (Van de Peer and De Wachter, 1994). The genetic distance (GDxy) or genetic dissimilarity was calculated according to Nei and Li (1979) as $Gdxy = 1 - (2Nxy/(Nx + Ny))$ where Nxy is the number of fragments (bands) shared in lines 'x' and 'y', and Nx is the number of fragments in line 'x', and Ny is the number of fragments in line 'y'. *Fusarium oxysporum* (Foxy 10037) was used as outgroup. Bootstrap analysis used 1000 resamples of the data.

Sequencing

Amplification products were cleaned using the QIAquick PCR purification kit (QIAGEN Inc., Chatsworth, CA, USA) and both strands were sequenced separately using primers ITS1 or ITS1F and ITS4 with an ABI Prism 310 Genetic Analyzer and the ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq®DNA Polymerase (Perkin Elmer Applied Biosystem). Sequence Navigator™ Sequence Comparison software (Perkin Elmer) was used to identify the consensus sequence from the two strands of each isolate. The new sequences have been lodged in the EMBL database with the accession numbers indicated in Table 2.

Alignment and phylogenetic analysis

Sequences obtained in this study were compared with homologous sequences retrieved from the EMBL GenBank (Table 2). Sequence Navigator™ Sequence Comparison for pairwise comparisons and SEQAPP software for multiple sequences were used to search for the best alignment. Where ambiguities in the alignment

Table 2. GenBank accession number and voucher information for nucleotide sequences of *Colletotrichum* isolates

Code	Collection	ITS1	ITS2
ACU-397	*C. acutatum*, 397	Z32915	Z32914
ACU-NI90	*C. acutatum*, NI90	Z32928	Z32927
ACUCOL-R*	*C. acutatum*, CECT 20120	AF090853	
COC-527.77	*C. coccodes*, 527.77	Z32930	Z32929
COC-NFT	*C. coccodes*, NFT	Z32933	Z32932
FRA-63-1	*C. fragariae*, 63-1	Z32943	Z32942
FRACOL-R*	*C. fragariae*, CECT 20121	AF090854	
GLOCOL-1*	*C. gloeosporioides*, GL-118-9411	AF081292	
GLOCOL-R*	*C. gloeosporioides*, CECT 21073	AF090855	
MUC-CMG	*C. musae*, CMG	Z32995	Z22994
MUS-4Q	*C. musae*, 4Q	Z32997	Z32996

*New sequences obtained in this study.

occurred, the alignment chosen was the one generating the fewest potentially informative characters. Alignment gaps were marked '–' and unresolved nucleotides or unknown sequences were indicated with 'N'. Parsimony analysis was performed using the computer program PAUP 3.0s (Phylogenetic Program Using Parsimony) of Swofford (1991). One isolate of *C. coccodes* (COC-NFT) was used as the outgroup. Branch robustness was estimated by bootstrap analysis (Felsentein, 1985) of 1000 heuristic replicates.

Results

Morphological/cultural studies

The protease activity on CHM for each isolate, as well as the growth rate on PDA and Czapek-Dox Agar are shown in Table 3. Protease activity was indicated by a clear zone produced around the fungal colony within a growth period of 72–96 h on CHM. On PDA, the reference isolate of *C. acutatum* (ACUCOL-R), which gave a positive reaction in CHM, produced whitish mycelium and low relative growth rate (6.3 mm day^{-1}). The reference isolates of *C. gloeosporioides* (GLOCOL-R) and *C. fragariae* (FRACOL-R) showed greyish mycelia on PDA (growth rate: 11.7 mm day^{-1} and 13.7 mm day^{-1}, respectively), with some orange zones due to the high rate of conidiogenesis, and a negative reaction on CHM. The isolates of *C. gloeosporioides* from citrus showed the same cultural features as the reference isolate of this species. However, the three *Colletotrichum* spp. isolates from *Fragaria* produced reactions with CHM that were similar to *C. acutatum* and not to *C. fragariae.*

Within the isolates from olives, two main morphocultures were obtained. Most of the strains showed an PDA white-orange to grey-brown mycelia, positive reactions on CHM and a slow growth rate on PDA (<8.0 mm day^{-1}) and Czapek-Dox Agar (<5.0 mm day^{-1}). Two strains, GLOCOL-8.1 and GLOCOL-16, produced a sparse grey mycelium with some orange zones due to the presence of abundant conidia; negative reaction on CHM and fast growth (PDA > 10 mm day^{-1}; Czapek-Dox Agar >7.0 mm day^{-1}).

Table 3. Reaction on CHM for each isolate of *Colletotrichum* shown in Table 1 and growth rate on PDA and Czapek-Dox media

Isolate code	CHM[1]	PDA mm day^{-1}	Czapek mm day^{-1}
ACUCOL-R	++	6.3	3.9
CIRCOL-01	–	6.1	5.6
COCCOL-01	–	7.2	7.7
CRACOL-01	–	10.3	NG[2]
FRACOL-R	–	13.7	9.0
GLOCOL-01	+	6.2	1.3
GLOCOL-02	+	6.4	1.3
GLOCOL-03	+	6.1	1.9
GLOCOL-04	–	11.6	8.0
GLOCOL-05	+	7.2	1.6
GLOCOL-07	+	7.1	1.7
GLOCOL-08.1	–	11.2	8.2
GLOCOL-08.2	+	7.4	1.9
GLOCOL-09	+	7.2	2.0
GLOCOL-10	+	6.9	1.9
GLOCOL-11	+	6.6	2.2
GLOCOL-12	–	11.8	8.2
GLOCOL-13	–	11.7	7.9
GLOCOL-14	–	11.5	8.1
GLOCOL-15	–	11.6	8.0
GLOCOL-16	–	11.5	8.1
GLOCOL-R	–	11.7	9.7
SPCOL-01	++	6.6	3.2
SPCOL-02	++	ND	2.9
SPCOL-03	++	7.9	3.1
TRICOL-01	+	9.7	5.2

[1]Level of protease activity at 72 h: '–' not activity; '+' protease activity indicated by a clear zone of 2 mm produced around the fungal colony; '++' protease activity indicated by a clear zone of 4 mm produced around the fungal colony.
[2]NG: no growth.

Amplification and RFLP analysis

The ITS regions and the 5.8S rDNA of all samples were successfully amplified using both protocols and primers ITS1F and ITS4. With the standard amplification protocol, diluting the DNA 1 : 100 gave amplification (data not shown). However, with individual PCR-beads, stronger amplifications were obtained using the DNA without dilution (data not shown). In all the amplifications a single DNA fragment of over 603 bp was obtained for all of the 26 *Colletotrichum* strains. All amplimers were digested with each of the enzymes, except with *Alu* I (Table 4). This enzyme did not cut either the DNA fragments of 9 strains from olive, the reference isolate of *C. acutatum*, or the three *Colletotrichum* spp. from *Fragaria*. Samples of DNA digested with *Alu* I, *Hae* III, *Msp* I and *Taq* I showed, in general, good resolution with characteristic patterns, although with *Hae* III and *Msp* I some partial digestions were obtained. Restriction fragments less than 50 bp were not taken into consideration because they were not clearly resolved by electrophoresis in 2% Separide

Table 4. Fragment lengths (bp) after endonuclease digestion with *Alu* I, *Hae* III, *Msp* I and *Taq* I of the ITS regions including the 5.8S rDNA of *Colletotrichum* spp. shown in Table 1

Isolates code	*Alu* I		*Hae III*			*Msp* I					*Taq* I			
ACUCOL-R, GLOCOL-01, GLOCOL-02, GLOCOL-03, GLOCOL-05, GLOCOL-07, GLOCOL-08.2, GLOCOL-09, GLOCOL-10, GLOCOL-11	680		280	170	140	298	154				298	230	75	
CIRCOL-01, COCCOL-01	396	220	280	170		450	154				298	230	75	
CRACOL-01	405	220	280	170	130	298	154	100			298	170	75	
FRACOL-R	396	190	280	170	140	298	154	100			285	230		
GLOCOL-R, GLOCOL-04, GLOCOL-08.1, GLOCOL-13, GLOCOL-14, GLOCOL-15, GLOCOL-16	396	220	280	170	140	298	154	100			285	230	75	
GLOCOL-12	680		420	170	75	298	154	130	100	75	250	201	75	50

agarose gels. Thus, the size of the PCR products estimated by adding the sizes of the restriction fragments was slightly less than the size estimated for the undigested PCR products.

Polymorphism was observed with all the enzymes tested. With *Alu* I, two main patterns where produced. One was shared by cultured isolates from olive and the isolate GLOCOL-12 from *Dieffenbachia* spp., and the other one was shared by the reference isolate of *C. gloeosporioides* (GLOCOL-R) and the isolates from citrus. Within the olive isolates there were 2 exceptions; GLOCOL-8.1 and GLOCOL-16 gave the same pattern as isolates from citrus. In addition, the majority pattern obtained from the olive isolates was shared by the reference isolate of *C. acutatum* (ACUCOL-R) and the three unidentified *Colletotrichum* cultures, all isolated from *Fragaria* spp. *Msp* I and *Taq* I also produced two main patterns, giving the same results mentioned above, except for isolate GLOCOL-12, which showed a different pattern with each enzyme. Isolates from olive and citrus trees were monomorphic to *Hae* III and the pattern was different from the isolate GLOCOL-12.

To estimate the level of divergence among PCR-amplified rDNA fragments, a matrix of pairwise genetic distances was calculated from the binary matrix (data not shown). The dendrogram constructed from the distance matrix with the neighbour-joining method is shown in Figure 1. All the *Colletrotrichum* species in this study clustered together and the bootstrap value of 100% highly supports the monophyly for the genus. The dendrogram revealed two major groups within the isolates studied. In group A, the reference isolate of *C. acutatum* clustered together with the isolates from olives (except GLOCOL-08.1 and GLOCOL-16) and the three unidentified isolates from *Fragaria vesca*; the bootstrap of 99% strongly supports the validity of this group. In group B, GLOCOL-8.1 and GLOCOL-16 from olives clustered together with the reference isolate of *C. gloeosporioides* from citrus. The low bootstrap value of 57% shows that this grouping is not firmly established. On the other hand, the isolates of *C. coccodes* (COCCOL-01) and *C. circinans* (CIRCOL-01) showed identical RFLP patterns (Table 4), and in Figure 1 they clustered in a well-supported group (99% bootstrap).

Sequence data

The alignment of the ITS sequences showed ITS1 as a more variable region than ITS2. From the 348 aligned characters, including both regions, 32 were informative in the ITS1 and 17 in the ITS2. The alignment file can be accessed through WWW at http://www.ctv.es/USERS/fgfigueres/MariPaz/mpmartín.htm. Table 5 shows the distance matrix obtained from these sequences. Fifteen most parsimonious trees (MPTs) were obtained by heuristic methods (tree length = 79; consistency index (CI) = 0.823; retention index (RI) = 0.898; re-scaled consistency index = 0.739). Two main lineages, marked 'A' and 'B' in the phylogram (Figure 2), were detected, both branches with very high bootstrap support (98% and 100%). Lineage A comprises the isolates of *C. acutatum* and

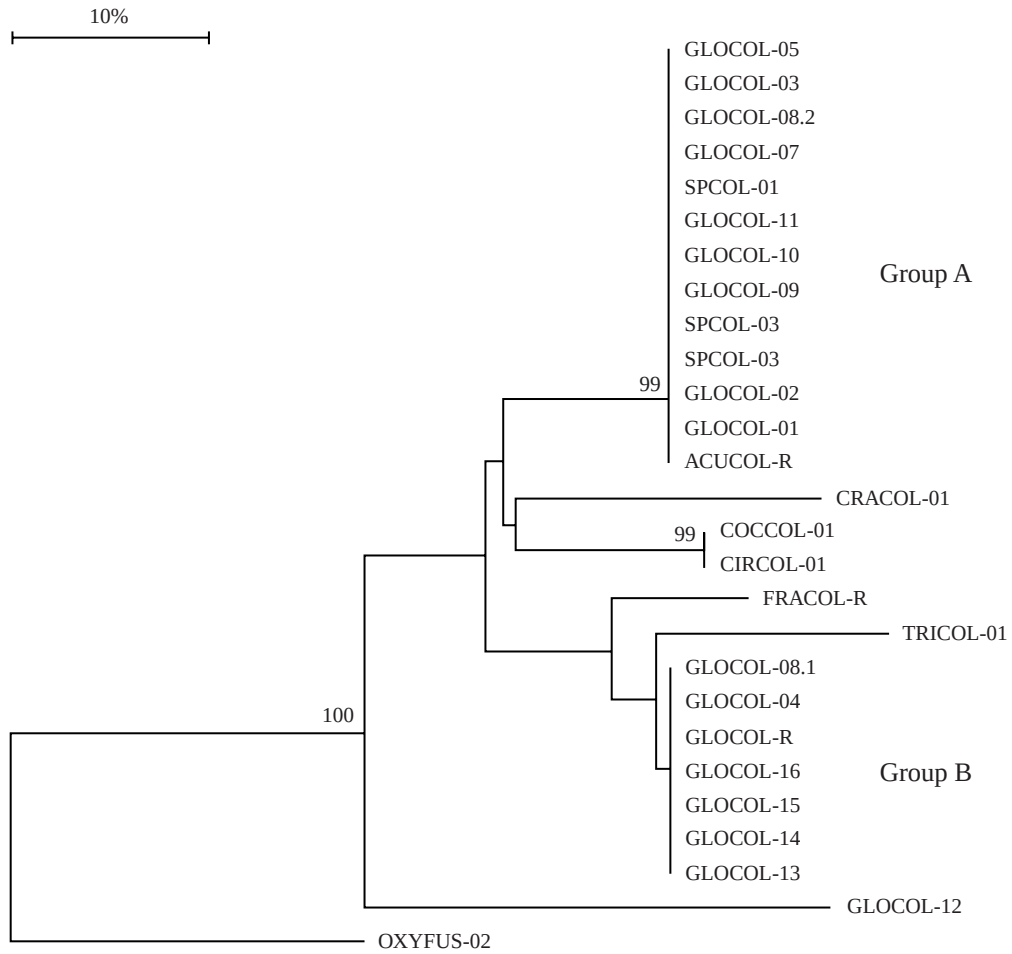

Figure 1. Dendrogram constructed from PCR-RFLP data of ITS1–5.8S–ITS2 of *Colletotrichum* isolates using the neighbour-joining method from the distance matrix calculated according to Nei and Li (1979). Data were analysed using the programme TREECON (Van de Peer and De Watcher, 1994) (original and molecular identifications and source of the isolates are given in Table 1).

Table 5. Pairwise distances from ITS1–5.8 S–ITS2 nucleotide sequence data between taxa (absolute distance) of *Colletotrichum* spp. shown in Table 2

ACU-397	—									
ACU-NI90	0.035	—								
ACUCOL-R	0.000	0.035	—							
COC-527.77	0.076	0.064	0.077	—						
COC-NFT	0.076	0.064	0.077	0.000	—					
FRA-63-1	0.108	0.105	0.115	0.075	0.075	—				
FRACOL.-R	0.112	0.114	0.115	0.085	0.085	0.019				
GLOCOL-01	0.003	0.038	0.003	0.080	0.080	0.111	0.115	—		
GLOCOL-R	0.115	0.114	0.118	0.094	0.094	0.025	0.035	0.118	—	
MUC-CMG	0.118	0.117	0.116	0.097	0.097	0.032	0.044	0.122	0.044	—
MUC-4Q	0.112	0.110	0.109	0.091	0.091	0.029	0.041	0.116	0.041	0.006

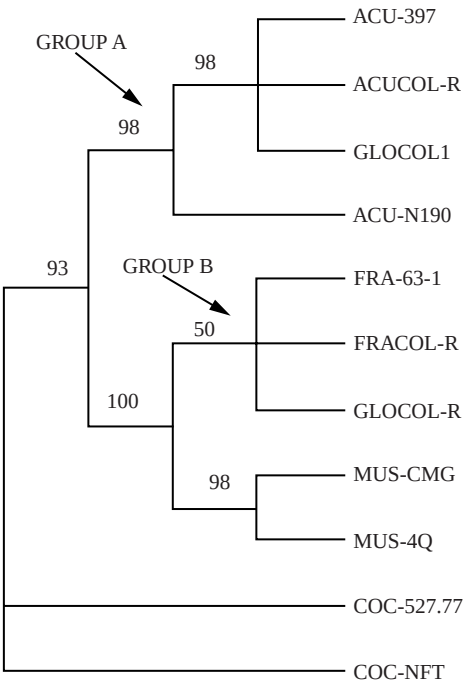

Figure 2. Consensus tree of the 15 most parsimonious trees obtained from ITS1–5.8S–ITS2 nucleotide sequence data of *Colletotrichum* isolates by heuristic methods. Percentages of 1000 bootstrap replication are above branches (identification of isolates are given in Table 2).

GLOCOL-1 from olive. Lineage B, includes the isolates of *C. fragariae*, *C. gloeosporioides* and *C. musae*.

Discussion

On the basis of the definition of a 'species', i.e. groups of isolates with highly conserved DNA with correlated morphological and biochemical characters (Bailey et al., 1996), we can conclude that there are two distinct species causing anthracnose in olive, *C. acutatum* and *C. gloeosporiodes*.

The two main groups of *Colletotrichum* from olive 'A' and 'B', obtained in the neighbour-joining analysis of RFLP data, agree with the behaviour on CHM and growth rates on PDA and Czapek-Dox agar (Figure 1). Agostini et al. (1992) defined three culture types, based on morphology, growth rate and colony characteristics, from 17 isolates of *Citrus* from Florida. On the basis of DNA variation at several loci, as well as on cultural morphology and growth, Liyanage et al. (1992) reported two distinct populations of this fungus from sweet orange (*Citrus sinensus*) and Tahiti lime (*Citrus latifolia*): slow-growing orange (SGO) strains and fast-growing grey (FGG) strains. This agrees with our data if we consider the SGO strains as belonging to group A and the FGG to group B. Brown et al. (1996), comparing 10 isolates belonging to the three growth forms of *C. gloeosporioides* from *Citrus* mentioned in Agostini et al. (1992), and five isolates of *C. acutatum*, concluded that the SGO strains and the Key lime anthracnose (KLA) isolates belong to the species *C. acutatum* and not to *C. gloeosporioides*. In our RFLP-ITS analysis we included reference isolates of *C. acutatum*, *C. fragariae* and *C. gloeosporioides*. Identical RFLP patterns, as well as similar cultural characteristics, were found within 9 isolates from olives and the reference isolate of *C. acutatum*. This indicated that isolates of olive fruit included in group A belong to the species *C. acutatum*, whilst those in group B are *C. gloeosporioides*. Johnston and Jones (1997) reported the presence of these two species as the agents of bitter rot disease of apples in New Zealand; the symptoms caused by the two fungi were apparently indistinguishable. According to our data, *C. acutatum* seems to be the main *Colletotrichum* species present in olives in Catalonia, although more isolates have to be studied to confirm this. Higgins (1926) considered *C. gloeosporioides* an opportunistic species. This view is also mentioned in Agostini and Timmer (1994) who state that the SGO anthracnose strains (*C. acutatum)* cause post-bloom fruit drop disease of *Citrus*, whereas the FGG strain (*C. gloeosporioides*) is primarily saprophytic on senescent and dead tissue.

It is interesting to note that GLOCOL-08.1 and GLOCOL-08.2, which were isolated from the same olive fruit, clustered in the two different groups, GLOCOL-08.1 in group B (*C. gloeosporioides*) and GLOCOL-08.2 in group A (*C. acutatum*).

Moreover, the RFLP data suggest that *C. circinans* and *C. coccodes* are identical and could belong to the same taxa. However, it would be necessary to study more collections to clearly state the taxonomic boundaries around these two *Colletotrichum* species that were isolated from bulbs and roots.

Sizes of the ITS1 and ITS2 regions were in accordance with those published by previous authors. Mills et al. (1992) mentioned 171 bp in the ITS1 region of isolates identified as *C. gloeosporioides*; however Sreenivasaprasad et al. (1994) found 180–181 bp in 12 isolates of *Colletotrichum*, originally identified as either *C. frutigenum*, *C. gloeosporioides* or *C. musae*

and which showed 97–100% homology to the reference *C. acutatum* isolates used in their studies. Sreenivasaprasad et al. (1996) reported that the size of the ITS1 region from 93 isolates representing 18 *Colletotrichum* species varied from 159 to 185 bp. For the ITS2 region Sherriff et al. (1994) obtained 159 bp from 27 isolates of different *Colletotrichum* species and Bailey et al. (1996) found one base more. According to Sreenivasaprasad et al. (1996), the size of this region for *C. acutatum*, *C. coccodes*, *C. fragariae*, *C. gloeosporioides* and *C. musae* ranged from 152 to 153 bp. From the similarity matrix of ITS sequence data and the corresponding tree and associated bootstrap values, it is clear that isolate GLOCOL-1 is 98% similar to *C. acutatum*.

No data have previously been published on digestion of the ITS regions of the rDNA with *Alu* I and *Taq* I in *Colletotrichum* species. However, patterns obtained with *Hae* III and *Msp* I from the reference isolate of *C. gloeosporioides* agree with those published in Bailey et al. (1996). This study has shown that RFLP data can be used to distinguish between species of *Colletotrichum*, obtained from the same host. The digestion of amplified ITS-rDNA, is a cheap, fast and reliable method to distinguish isolates of different *Colletotrichum* species without using specific primers. It should be remembered, however, that as mentioned in Sutton (1992), morphology will always form part of the separation between species or groups of species.

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