

Characterisation and epitypification of *Pseudocercospora cladosporioides*, the causal organism of Cercospora leaf spot of olives

Arantxa ÁVILA¹, Johannes Z. GROENEWALD², A. TRAPERO¹ and Pedro W. CROUS^{1*}

¹ Department of Agronomy, ETSIAM, University of Córdoba, Apdo. 3048, ES-14080 Córdoba, Spain.

² Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Uppsalaalaan 8, 3584 CT Utrecht, The Netherlands.

E-mail: crousw@cbs.knaw.nl

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Cercospora leaf spot of olives is a serious defoliating disease attributed to *Pseudocercospora cladosporioides*. Although the disease is well distributed throughout olive growing regions of the world, its epidemiology and population structure remains unknown. The aim of this study was to establish the genetic variability of Spanish isolates of *P. cladosporioides* using DNA sequence data from the ITS region, as well as two protein-coding genes, actin and calmodulin. Phylogenetic data obtained here support *P. cladosporioides* as closely related to other *Pseudocercospora* species that cluster within *Mycosphaerella*. Spanish isolates clustered in two clades: isolates from Catalonia were different from those collected in Andalusia. However, isolates appeared to be genetically relatively homogeneous, suggesting that chemical control of this disease via a managed spraying programme may prove a viable option for controlling the disease in Spain.

INTRODUCTION

Cercospora leaf spot is a serious disease of olives. It is caused by *Pseudocercospora cladosporioides*. This disease, which is usually associated with a high level of defoliation, can cause a delay in fruit ripening and a decrease in oil yield (González Fragoso 1927, García Figueres 1991). It is more prominent in years with high humidity and moderate temperatures. Even though it is widely distributed in most olive growing regions in the world where susceptible cultivars are grown, it has remained largely unstudied (Trapero & Blanco 2004).

Disease symptoms differ on the adaxial and the abaxial leaf surfaces. On the adaxial surface, irregular, chlorotic areas become brown and necrotic with age. The abaxial leaf surface shows areas turned leaden-grey by the presence of asexual fruiting structures (Del Moral & Medina 1985, Trapero & Blanco 2004). These disease symptoms are non-specific, however, and are frequently confused with those caused by other pathogens such as *Fuscladium oleagineum* (syn.) (*Spilocaea oleaginea*), and *Colletotrichum* spp., as well as symptoms caused by abiotic factors.

Currently, non-mutational mechanisms for introducing genetic variation in *P. cladosporioides* are unknown, as neither a sexual state nor parosexuality

has been demonstrated. Although it is commonly accepted that *Pseudocercospora* species have teleomorphs in *Mycosphaerella* (Stewart *et al.* 1999, Crous *et al.* 2000), this has not yet been documented for *P. cladosporioides*. Spermatogonia bearing spermatia, which are indicative of the sexual cycle, have been observed in diseased leaves in close proximity to the anamorph. However, whether these structures are connected to *P. cladosporioides*, remains to be proven, as they have not been observed in culture (Del Moral & Medina 1985).

Knowledge of the population structure of a pathogen in agricultural ecosystems is important as it can provide information about the pathogen's speciation and evolutionary history. It can suggest the potential for the development of new races, or provide an indication of the ability of the fungus to adapt to fungicides (Robbertse & Crous 2000, Robbertse *et al.* 2000, 2001). The success of disease management practices frequently depends on these factors, knowledge of which may also help to optimize management of resistance genes, fungicide regimes and cultivation practices (McDonald & Linde 2002).

Several studies have been conducted to determine the physiological and morphological variation that exists among isolates of *P. cladosporioides* (Ávila, Benali & Trapero 2004). Although these studies applied criteria that have been widely used in taxonomy (Crous 1998), the traits documented were not polymorphic

* Corresponding author.

Table 1. List of isolates studied.

Accession no.	Cultivar	Location	GenBank accession number (ITS, ACT, CAL)
PC2, 40	Picudo	Priego de Córdoba, Córdoba ^b	PC2: AY438248, AY438240, AY438257
PC11	Hojiblanca	Priego de Córdoba, Córdoba ^b	
PC19	Picudo	Priego de Córdoba, Córdoba ^b	AY438256, AY438247, AY438264
PC20, 21	Hojiblanca	Baena, Córdoba ^b	
PC3, 12	Hojiblanca	Monturque, Córdoba ^b	PC3: AY438253, AY438245, AY438262
PC31, 32	Cornicabra	Córdoba ^b	
PC96=CBS 113866	Blanqueta de Elvas	Córdoba ^b	AY438252, AY438244, AY438261
PC107	Manzanilla de Jaén	Córdoba ^b	
PC27	Verdial de Vélez	Córdoba ^b	
PC121	Arbequina	Constantí ^c	
PC52	Picual	Reus, Tarragona ^c	
PC115	Corbella	Reus, Tarragona ^c	
PC118	Arbequina	Reus, Tarragona ^c	
PC54, 109=CBS 114079	Morrut	Aldover, Tarragona ^c	PC54: AY438249, AY438241, AY438258 PC109: AY438250, AY438242, AY438259 PC58: AY438251, AY438243, AY438260
PC56, 58	Morrut	Aldover, Tarragona ^c	
PC71	Sevillanca	Aldover, Tarragona ^c	
PC61, 63, 64, 67	Sevillanca	Camarles, Tarragona ^c	
PC 114	Farga	St.bárbara, Tarragona ^c	
PC65	Sevillanca	St.bárbara, Tarragona ^c	
PC 91	Farga	Godell, Tarragona ^c	
PC93	Sevillanca	Mas de barberas, Tarragona ^c	
PC129, 124, 128	Hojiblanca	Antequera, Málaga ^b	PC124: AY438255, –, –
PC122=CBS 113867	Lechin de Granada	Cullar vega, Sevilla ^b	AY438254, AY438246, AY438263
CBS 117482 ^a	–	Tunisia	DQ008122, DQ008123, DQ008124

^a Ex-epitype isolate from Tunisia.^{b,c} All other isolates from Spain: ^b Andalucia, ^c Catalonia.

enough to allow distinction of individuals within a population. The application of molecular markers has facilitated studies on fungal systematics, population biology, evolution, and detection. DNA sequence analysis of the ITS1, 5.8S, and ITS2, as well as some protein-coding genes has been widely used in systematic studies of *Mycosphaerella* at the species level (Crous *et al.* 2000, 2001, 2004b, Tessmann *et al.* 2001). To examine variation at the population level, other molecular approaches have also been used in *Mycosphaerella*, including isozymes (Boshoff *et al.* 1996), RAPDs (Campbell *et al.* 1996, Kema *et al.* 2000), microsatellite markers (Adhikari, Wallwork & Goodwin 2004), RFLPs (Inglis *et al.* 2001), AFLPs (Kema *et al.* 2002), and combinations of two or more of these techniques (Groenewald *et al.* 2005).

The aims of the present study were: (1) to study the genetic variability of isolates of *P. cladosporioides* obtained from different geographic locations and cultivars in Spain, using partial ITS sequences, as well as sequences of two protein-coding genes, actin and calmodulin; and (2) to determine the phylogenetic position of *P. cladosporioides* within *Mycosphaerella* based on ITS sequence data.

MATERIALS AND METHODS

Isolates

Symptomatic leaves were collected from different locations and varieties of olive trees in Spain, and 35

isolates were selected for molecular studies (Table 1). Isolates used in this study were obtained via direct transfer of single conidia by plating them onto potato-dextrose agar (PDA; Gams *et al.* 1998) and incubation at 24 °C under a 12 h cool fluorescent white light/darkness regime for 2–3 wk.

DNA phylogeny

Genomic DNA was extracted from mycelium of all isolates (Table 1) using the protocol of the FastDNA® Kit (Bio101 System) as recommended by the manufacturer. The DNA concentration was estimated by comparing the intensity of ethidium bromide fluorescence of the DNA sample to a known concentration of Smartladder™ DNA marker (Eurogentec®, Seraing, Belgium) on 2% (W/V) agarose gels using an ImageMaster™ VDS system (Amersham Pharmacia Biotech, Little Chalfont).

Three genomic areas, namely the ITS region (ITS) and portions of the actin (ACT) and calmodulin (CAL) genes were amplified and sequenced for each *Pseudocercospora cladosporioides* isolate using the primers ITS1/ITS4 (White *et al.* 1990), ACT-512F/ACT-783R (Carbone & Kohn 1999) and CAL-228F/CAL 737R (Carbone & Kohn 1999), respectively. PCR amplification of the loci as well as the subsequent alignment and phylogenetic analysis of the sequences were treated as described by Crous *et al.* (2004a). The GenBank sequences of *Cladosporium herbarum* (AY251078) and *Cladosporium cladosporioides* (AY251074) were

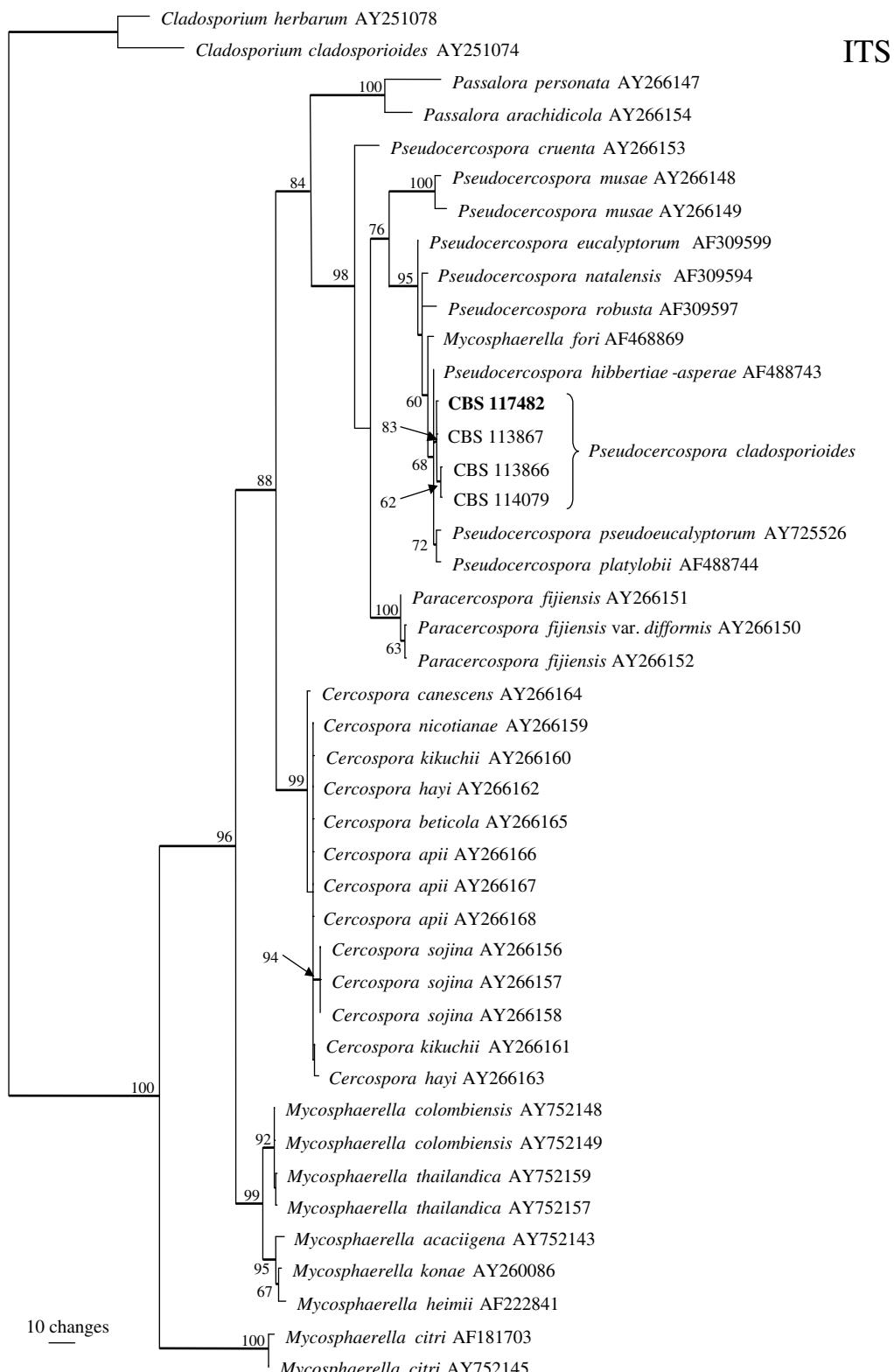


Fig. 1. One of 102 most parsimonious trees obtained from the ITS sequence alignment ($TL = 465$ steps, $CI = 0.761$, $RI = 0.910$, $RC = 0.693$). The scale bar indicates 10 changes and the numbers at the nodes represent bootstrap support values based on 1000 resamplings. Branches that appear in the strict consensus tree are indicated by thickened lines and the type strain of *Pseudocercospora cladosporioides* is indicated in bold print. The GenBank sequences of *Cladosporium herbarum* (AY251078) and *C. cladosporioides* (AY251074) were included as outgroups.

included as outgroups for the ITS alignment and that of *Mycosphaerella thailandica* (AY752159, AY752220 and AY752251) and *Mycosphaerella colombiensis*

(AY752149, AY752211 and AY752242) for the combined analyses (ITS, ACT and CAL). Representatives of sequences generated in this study were submitted

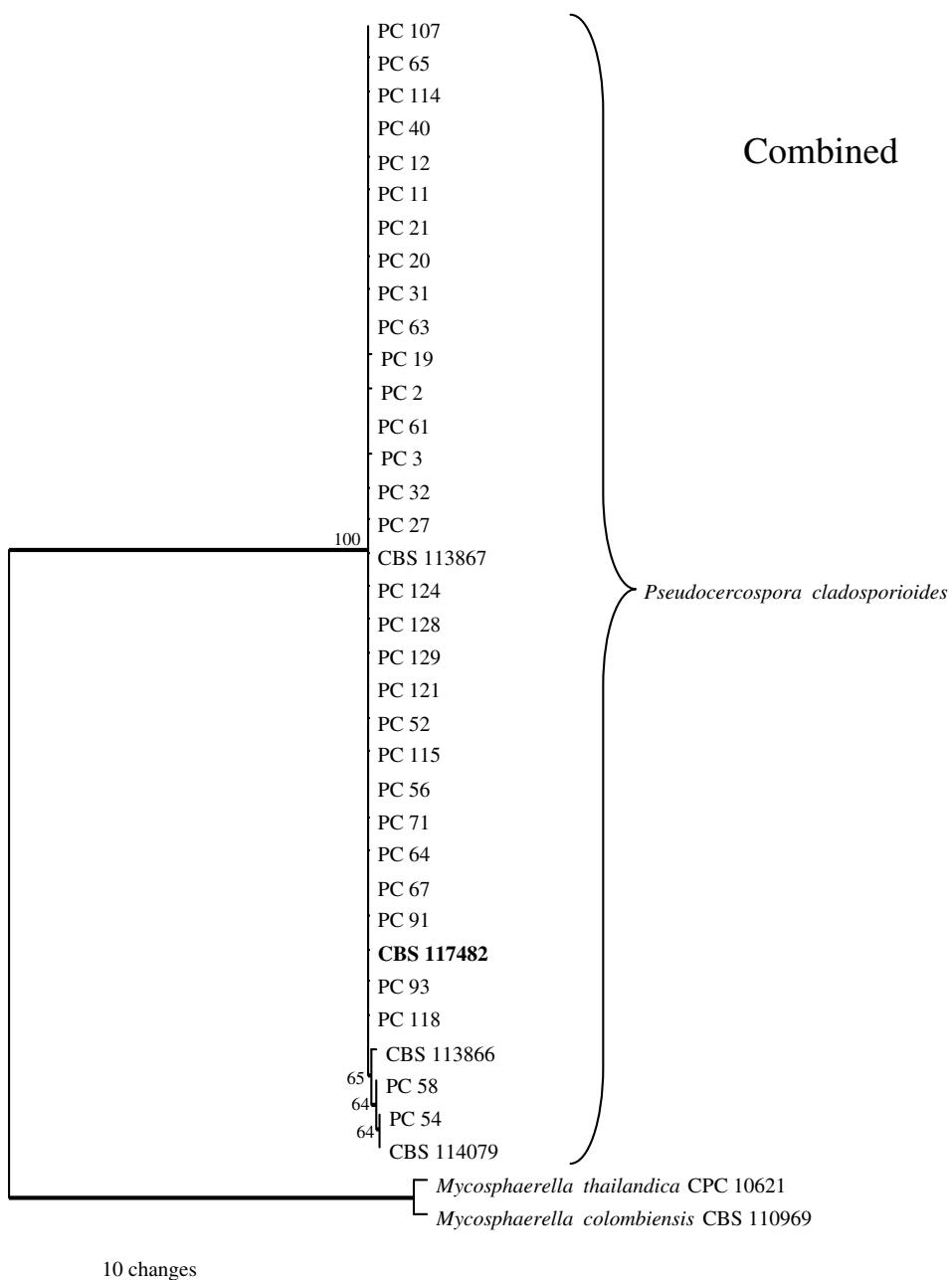


Fig. 2. One of two most parsimonious trees obtained from the combined ITS, actin and calmodulin sequence alignment (TL = 196 steps, CI = 1.000, RI = 1.000, RC = 1.000). The scale bar indicates 10 changes and the numbers at the nodes represent bootstrap support values based on 1000 resamplings. Branches that appear in the strict consensus tree are indicated by thickened lines and the type strain of *Pseudocercospora cladosporioides* is indicated in bold print. The GenBank sequences of *Mycosphaerella thailandica* (AY752159, AY752220 and AY752251) and *M. colombiensis* (AY752149, AY752211 and AY752242) were included as outgroups.

to GenBank (Table 1) and alignments to TreeBASE (accession no. SN2302).

Morphology

Isolates were inoculated onto PDA plates, and incubated under continuous near-ultraviolet light at 25 ° for 6 d. Microscopic observations were made from colonies

on host material, as well as cultures on PDA, and preparations mounted in lactic acid. The 95% confidence intervals of conidial measurements were derived from 30 observations. Cultural characteristics were determined from colonies cultivated on PDA using the colour charts of Rayner (1970). Reference cultures and specimens were deposited at the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, the Netherlands.

Table 2. Nucleotide differences observed for Spanish *P. cladosporioides* isolates sequenced in this study. The nucleotide positions indicated were taken from the position of the character in the GenBank sequence of the ex-epitype strain CBS 117482 (DQ008122, DQ008123 and DQ008124 for ITS, actin and calmodulin respectively).

Isolate	Gene areas and nucleotide positions							
	ITS				Actin		Calmodulin	
	ITS1	ITS2	ITS2	ITS2	Intron	Exon	Exon	Exon
Isolate	130	364	369	399	53	212	282	
CBS 117482	T	T	T	T	C	G	C	
PC58	C ^a	C ^a						
CBS 114079	C ^a	C ^a			T ^a		T ^a	
PC54	C ^a	C ^a			T ^a		T ^a	
CBS 113866	C ^a		C ^a					
PC3				G ^b				
PC2							T ^a	
PC19					T ^b			

^a Transition.

^b Transversion.

RESULTS

Phylogenetic analyses

To determine the taxonomic position of *Pseudocercospora cladosporioides*, ITS sequences of four strains were added to an alignment of sequences obtained from GenBank. The data matrix contained 43 taxa (including the two outgroups) and 502 characters were included in the analysis (including alignment gaps). Of these 502 characters, 277 were constant, 35 were parsimony-uninformative and 190 were parsimony-informative. The same overall topology was found irrespective of whether MP or NJ analyses were performed or which substitution model was used. Based on the DNA sequence data derived from the ITS region, *P. cladosporioides* was shown to be closely related to other species of *Pseudocercospora* (98% bootstrap support) (Fig. 1). Although *Pseudocercospora* presents a monophyletic clade in the current analysis, this is not the case once more diverse isolates are added (Crous *et al.* 2004a). Isolates of *P. cladosporioides* (83% bootstrap support) are found within a larger clade (95% support) which also contains isolates of *P. platylobii*, *P. pseudoeucalyptorum*, *P. hibbertiae-asperae*, *P. robusta*, *P. natalensis*, *P. eucalyptorum* and *Mycosphaerella fori*.

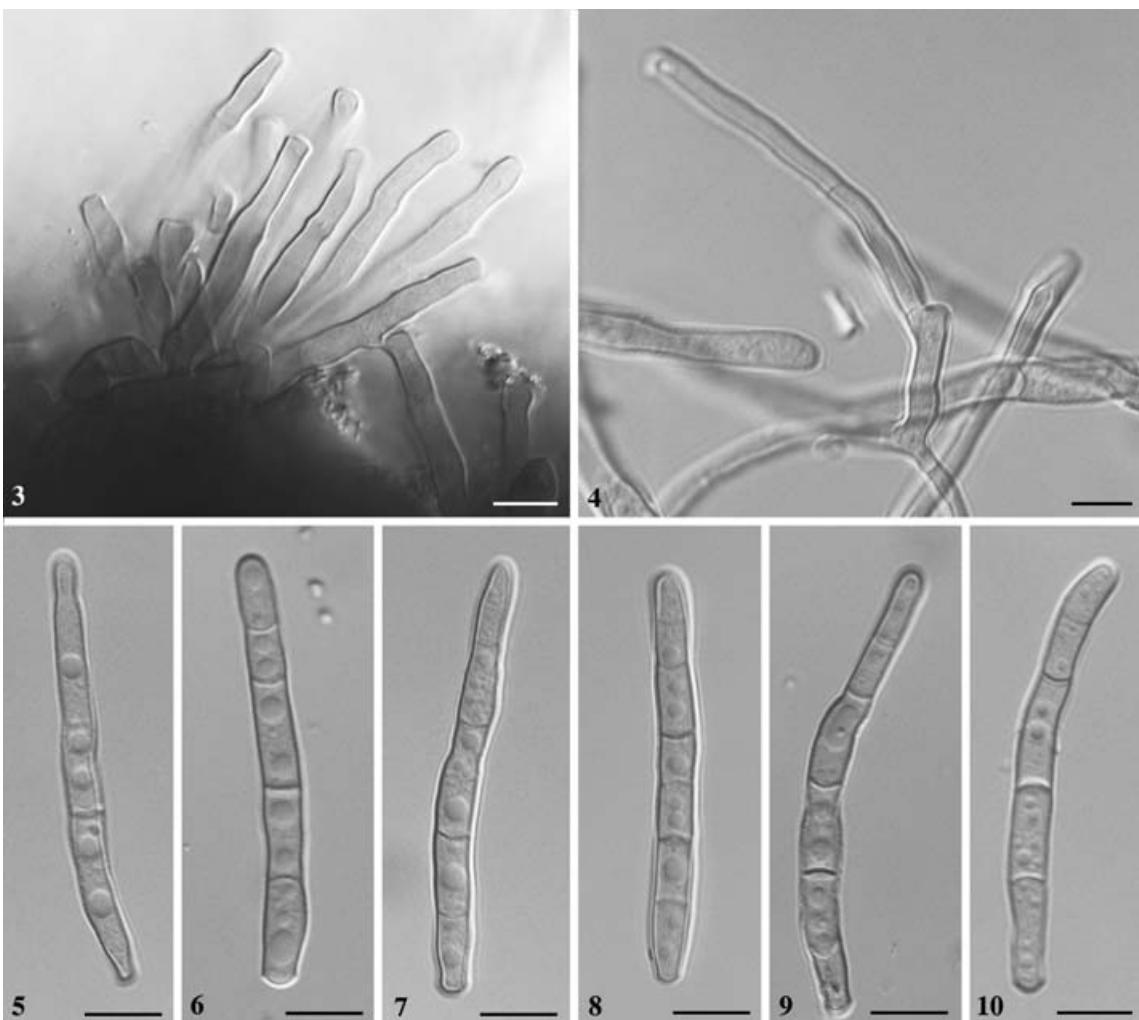
The multi-locus data matrix contained 37 taxa (including the two outgroups and the epitype reference strain from Tunisia) and 975 characters (475, 212 and 288 characters for ITS, ACT and CAL respectively) including alignment gaps. The partition homogeneity test did not detect any incongruence between the datasets ($P=1.000$) and the three datasets

were therefore combined. Of the 975 characters, 782 were constant, 9 were parsimony-uninformative and 184 were parsimony-informative. Maximum parsimony analysis produced two equally parsimonious trees, one of which is shown in Fig. 2. The MP consensus tree had a topology similar to that of the NJ tree. However, in the NJ analyses, all of the isolates were grouped with a basal polytomy, except for isolate PC19, which formed a sister taxon to the grouped isolates (data not shown). This isolate is included in the basal polytomy in the parsimony trees (Fig. 2). In both the NJ and MP analyses, four isolates formed a subclade (bootstrap support value of 65%) within the basal polytomy, namely CBS 113866, CBS 114079, PC54 and PC58.

At sequence level, only a few polymorphic sites were found among the *P. cladosporioides* isolates sequenced (Table 2). Of the 34 Spanish isolates sequenced, 27 had exactly the same sequence for all regions. The sequence of the ex-epitype strain (CBS 117483) was selected as reference, and the seven variable isolates were compared to it (Table 2). Actin showed the lowest variation, with a C/T change at position 53 ($1/214=0.467\%$ difference). Two changes were found in calmodulin ($2/305=0.656\%$ difference): a G/T change at position 212 and a C/T change at position 282. Four base changes ($4/474=0.844\%$) were present in the ITS sequence. The first was a T/C change at position 130 (in the first internal transcribed spacer, ITS1); the second and third T/C changes at positions 364 and 369, and the fourth was a T/G change at position 399. The last three changes occurred in ITS2 (the second internal transcribed spacer), which was more polymorphic than ITS1. Therefore, seven nucleotides changed among the three genes (encompassing 993 nucleotides in total) in the 34 Spanish isolates. The changes included five transitions and two transversions. In three of the isolates, PC2, PC3 and PC19, just a single mutation was observed. Two isolates, PC54 and CBS 114079, shared the highest number of mutations seen, namely two in ITS and two in calmodulin. That these two isolates shared the same four mutations is not surprising, as they were isolated from the same locality and may have been ramets of the same genet.

TAXONOMY

As no holotype specimen was preserved for *Pseudocercospora cladosporioides*, Braun (1993) selected another specimen from Saccardo's herbarium (from Tunisia) to serve as neotype. No cultures were available, however, and one of us (PWC) undertook to recollect and culture the fungus from its type locality. This collection (herb. CBS 14507), which closely resembles that of the neotype (PAD), is designated as epitype below.



Figs 3–10. *Pseudocercospora cladosporioides* (epitype). **Fig. 3.** Fascicle of conidiophores on leaf surface. **Fig. 4.** Secondary mycelium with conidiophore and conidiogenous scars. **Fig. 5–10.** Medium brown, subcylindrical conidia *in vivo*. Bars = 10 µm.

Pseudocercospora cladosporioides (Sacc.) U. Braun,
Mycotaxon **48**: 282 (1993). (Figs 3–10)

Basionym: *Cercospora cladosporioides* Sacc., *Michelia* **2**: 556 (1882).

Synonyms: *Mycocentrospora cladosporioides* (Sacc.) P. Costa, *Publ. Pat. Veg. Verissimo de Almeida Lisbon* **27**: 611 (1976); comb. inval.

Mycocentrospora cladosporioides (Sacc.) Deighton, *Micol. Pap.* **151**: 6 (1983).

Leaf spots amphigenous, irregular to subcircular, frequently associated with tip blight, medium brown on adaxial surface, dirty grey on abaxial surface, with distinct borders and brown to chlorotic margins. *Mycelium* internal and external; superficial hyphae branched, 3–4 µm wide, septate, pale brown, smooth. *Conidiomata* sporodochial to fasciculate on adaxial surface, grey, initially appearing as shiny blisters when bursting through the waxy cuticle, up to 200 µm wide and 70 µm high; stromata up to 150 µm wide

and 50 µm high. *Conidiophores* aggregated in dense sporodochia on adaxial surface, or in loose fascicles, or on superficial mycelium on abaxial surface; conidiophores brown, smooth to finely verruculose, 1–4-septate, subcylindrical, straight to geniculate-sinuous, mostly unbranched or branched above, 20–50 × 3–5 µm. *Conidiogenous cells* terminal, unbranched, medium brown, smooth to finely verruculose, tapering to bluntly rounded apices, proliferating sympodially, or several times percurrently near apex, 10–30 × 3–4 µm. *Conidia* solitary, pale to medium brown, smooth to finely verruculose, guttulate, subcylindrical, apex obtuse, base long obconically subtruncate, straight to slightly curved, 3–7-septate, (30–)50–65(–95) × (3.5–)4(–4.5) µm; hila inconspicuous, unthickened, not darkened nor refractive.

Cultures: Colonies 10–15 mm diam on PDA after 14 d under near-UV at 25 °. Colonies erumpent, spreading, with smooth, regular margins and moderate aerial mycelium; surface on PDA smoke-grey to pale olivaceous-grey; reverse olivaceous-grey to iron-grey.

Host range and distribution: *Olea dioica*, *O. europaea*, *Olea* sp. (Oleaceae), Algeria, Argentina, Australia, Chile, China, Germany, Greece, India, Italy, Netherlands Antilles, New Zealand, Portugal, Spain, Tanzania, Tunisia, USA (CA, LA, TX), Yugoslavia (Crous & Braun 2003).

Typus: on *Olea europaea*, **Tunisia:** 20 Nov. 1926, *M. Chabeolin* (PAD-neotype, designated in Braun 1993); Dec. 2003, *P.W. Crous* (herb. CBS 14507-epitypus hic designatus; cultures ex-epitype, CBS 117482=CPC 10913, CPC 10914–10915).

DISCUSSION

Previous taxonomic evaluations of *Pseudocercospora cladosporioides* have been based on its morphology *in planta* (*Olea europaea*) (Braun 1993). The present study, however, provides new insight into the phylogenetic relationship of *P. cladosporioides* to other *Mycosphaerella* species, as well as into the variation existing in isolates from different parts of Spain.

The phylogenetic data provide strong support for *P. cladosporioides* and other *Pseudocercospora* species as a monophyletic group. Short branch lengths among species within the *Pseudocercospora* cluster indicate a recently shared common ancestor. The phenetic relationship among phylogenetically closely related *Pseudocercospora* spp. (Beilharz & Cunningham 2003) and *P. cladosporioides* remains unclear based on the current data.

Analysis of the three independent loci studied showed few nucleotide substitutions. Most of the variation was found in the ITS region, with the ITS1 being less polymorphic than the ITS2. Thus, the ITS region provided more phylogenetic information at the population level than other protein-coding genes such as calmodulin and actin. This is in contrast to what has been observed in species of *Cercospora*, where the ITS region again tended to be more conserved than other protein coding genes (Crous *et al.* 2004b, Groenewald *et al.* 2005), suggesting that the same genes evolved at a different rate in various anamorph genera in *Mycosphaerella*.

Isolates of *P. cladosporioides* examined in this study clustered in two clades in both the NJ and MP analysis based on combined sequence analyses. However, only one clade was well supported by bootstrap. Although the second clade did not achieve a high bootstrap value, the isolates from Catalonia were clearly different from those collected in Andalusia. It appears that two phylogenetic groups of *P. cladosporioides* co-exist in Spain, although this could not be correlated with any demonstrable morphological or pathogenic differences. Using a molecular approach, however, it is possible to detect species-level or significant infraspecific changes long before changes in behaviour or morphology became evident (Taylor *et al.* 2000).

Genetic variability and population size are considered important factors for the survival of plant pathogens, particularly in a changing environment. Because we have demonstrated that Spanish isolates of *P. cladosporioides* have a low genetic diversity, chemical control of this disease via a managed spraying programme may prove a viable option to controlling Cercospora leaf spot disease of olives in Spain.

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