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Morphology and phylogeny of *Cladosporium subuliforme*, causing yellow leaf spot of pepper in Cuba

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Abstract—*Cladosporium subuliforme* is redescribed and illustrated from a specimen collected from yellow leaf spots on pepper (*Capsicum annuum*) cultivated in high tunnels in Cuba. Molecular phylogenetic analyses of morphologically close *Cladosporium* species are provided.

Key words—*Capnodiales*, hyphomycetes, plant disease, taxonomy, tropics

Introduction

Cladosporium Link (Bensch et al. 2010, 2012; Crous et al. 2007; Dugan et al. 2004; Ellis 1971, 1976; Ho et al. 1999; Schubert et al. 2007) typified by *C. herbarum* is a large cosmopolitan hyphomycete genus. IndexFungorum

(2016) lists 777 names for *Cladosporium*, although several species have been transferred to other genera (Bensch et al. 2010, 2012; Crous et al. 2007). Most species are saprobes and can be found in almost all ecological niches and substrates for easy spread through the air where they are common (Ho et al. 1999, Mullins 2001). Some species are pathogenic on cultivated plants (Ellis 1976; McKemy & Morgan-Jones 1990, 1992; Morgan-Jones & McKemy 1992; Schubert & Braun 2004) and a few species are causal agents of human diseases or disorders, mostly as opportunists (Hoog et al. 2011, Sandoval-Denis et al. 2015).

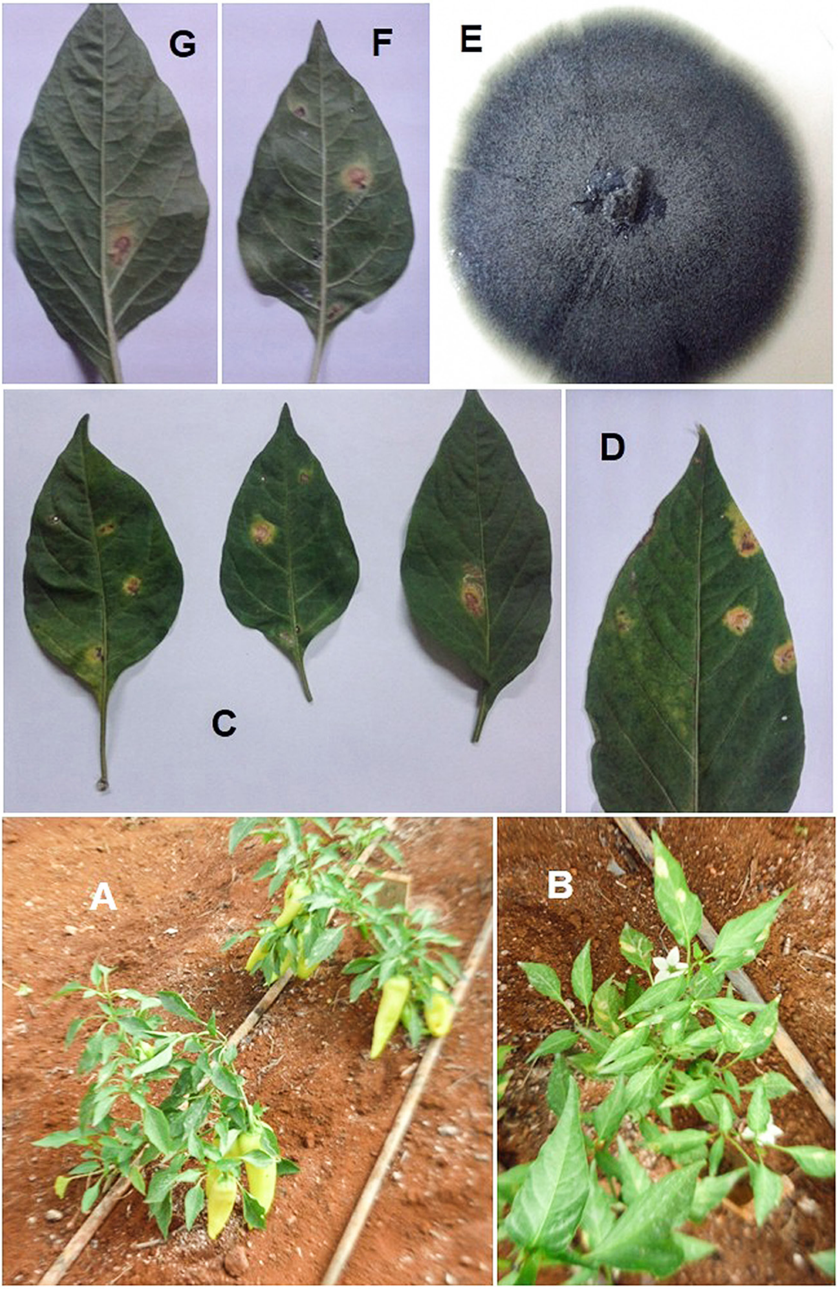
During an inspection of cultivated plants of *Capsicum annuum* (pepper) in high tunnels at the INIFAT Campus, numerous yellow leaf-spotted plants were found associated with a fungus identified as *Cladosporium subuliforme*. The species is redescribed and illustrated below, and phylogenetic analysis of closely related species is provided.

Material & methods

Collection, isolation, and morphology— Samples of leafspots of pepper were collected in high tunnels at the cultivated areas of the INIFAT Campus, Santiago de Las Vegas, Cuba, and placed in paper bags, taken to the laboratory, and processed according to Waller et al. (2002). The fungus was isolated from the substrate using a single conidium isolation technique (Castañeda-Ruiz et al. 2000), grown on oatmeal agar with adjusted pH 6.4, and incubated at 25 °C under alternating cycles of 12 h of daylight and 12 h darkness. Mounts were prepared in PVL (polyvinyl alcohol) and lactic acid and measurements were taken at ×1000. Photomicrographs were obtained with a Zeiss Axio Imager M1 light microscope (Carl Zeiss Meditec, Germany). The pathogenicity test (Koch's postulates) of *C. subuliforme* on pepper plants was confirmed after inoculation of healthy plants with *C. subuliforme* (INIFAT C13/100) following the methodology proposed by Waller et al. (2002). Cultures obtained were deposited in the INIFAT fungus collection, Santiago de Las Vegas, Cuba (INIFAT), in the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands (CBS), and in the Faculty of Medicine, Rovira i Virgili University, Reus, Spain (FMR).

DNA extraction, sequencing, and phylogenetic analyses — Total genomic DNA was extracted from mycelia obtained from colonies growing on PDA using FastPrep (MP Biomedicals, Santa Ana, CA) according to the manufacturer's protocol. DNA was quantified using the NanoDrop 3000 (Thermo Scientific, Madrid, Spain). The primers ITS5 and ITS4 (White et al. 1990) were used to amplify the complete ITS

Fig 1. *Cladosporium subuliforme*. A, B. Infected pepper plant growing in a high tunnel; C, D. Collected yellow leafspots on pepper;. E. INIFAT C13/100 colony on oatmeal agar after 10 days; F, G. Yellow leafspots on pepper after pathogenicity test inoculation.


G
F
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D
C
A
B

(ITS1+5.8S gene+ITS2) rDNA region; the primer pair LR0R/LR5 (Vilgalys & Hester 1990, Rehner & Samuels 1994) was used to amplify a fragment of the large subunit (LSU) rDNA gene; the EF-728F/EF-986R primer pair was used for the translation elongation factor 1α gene (EF-1α) and the ACT-512F/ACT-783R primer pair for the actin gene (Act) (Carbone & Kohn 1999).

Sequencing was performed in both directions using the same PCR primers at Macrogen Europe (Macrogen, Inc., Amsterdam). Consensus sequences were obtained using SeqMan version 7.0.0 (DNASTAR Lasergene, Madison, WI). An initial presumptive generic identification of the isolates was performed based on BLAST searches of ITS and LSU sequences in the GenBank (2016) and CBS (2016) databases. Multiple sequence alignments of each locus were performed in MEGA version6 (Tamura et al. 2013) using the ClustalW application (Thompson et al. 1994), refined with MUSCLE (Edgar 2004), and manually adjusted where necessary. The alignments were complemented with a total of 57 sequences from ex-type or reference strains obtained from GenBank and selected on the basis of the sequence similarity results.

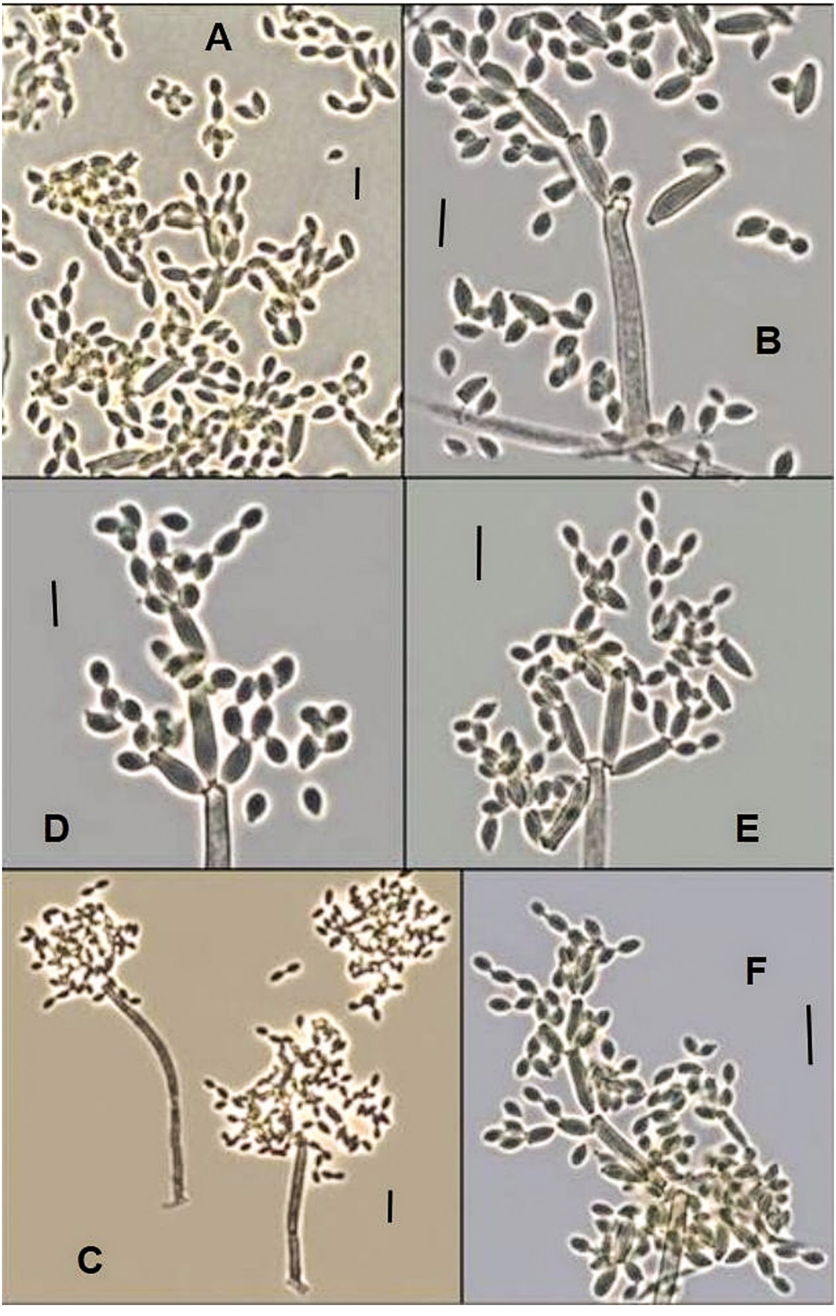
Phylogenetic reconstructions were made using maximum-likelihood (ML; MEGA version 6) and Bayesian Inference (BI; MrBayes version 3.1.2) (Huelsenbeck & Ronquist 2001). The best nucleotide substitution model (generalized time-reversible model with gamma distribution and a portion of invariable sites [GTR+G+I] for the three independent data sets) was estimated using MrModelTest version 2.3 (Nylander 2004) following the Akaike criterion. Phylogenetic analyses using ML were at first made individually for ITS, EF-1α, and Act and compared in order to assess for any incongruent results between nodes with high statistical support. As no incongruences were observed, the three loci were combined. For the ML analysis, nearest-neighbor interchange (NNI) was used as the heuristic method for tree inference. Support for the internal branches was assessed by a search of 1000 bootstrapped sets of data. A bootstrap support value (BS) of ≥70 was considered significant. For BI analysis, two simultaneous runs of 10,000,000 generations were performed, with samples stored every 1000 generations. The 50% majority-rule consensus tree and posterior probability values (PP) were calculated after discarding the first 25% of the samples. A PP value of ≥0.95 was considered significant.

Taxonomy

Cladosporium subuliforme Bensch, Crous & U. Braun, Stud. Mycol. 67: 77. (2010). FIGS 1–3

SYMPTOMS ON HOST: subcircular, angular or irregular yellow leaf spot, amphigenous, scattered, slightly necrotic at the centre. COLONIES on oatmeal agar floccose, greenish gray to dark greenish olivaceous, margins

FIG 2. *Cladosporium subuliforme* (INIFAT C13/100). A. Ramoconidia and conidia; B, C. Conidiophores, conidiogenous cells, ramoconidia, and conidia; D–F. Conidiogenous cells, ramoconidia, and conidia. Scale bars = 10 µm.


A
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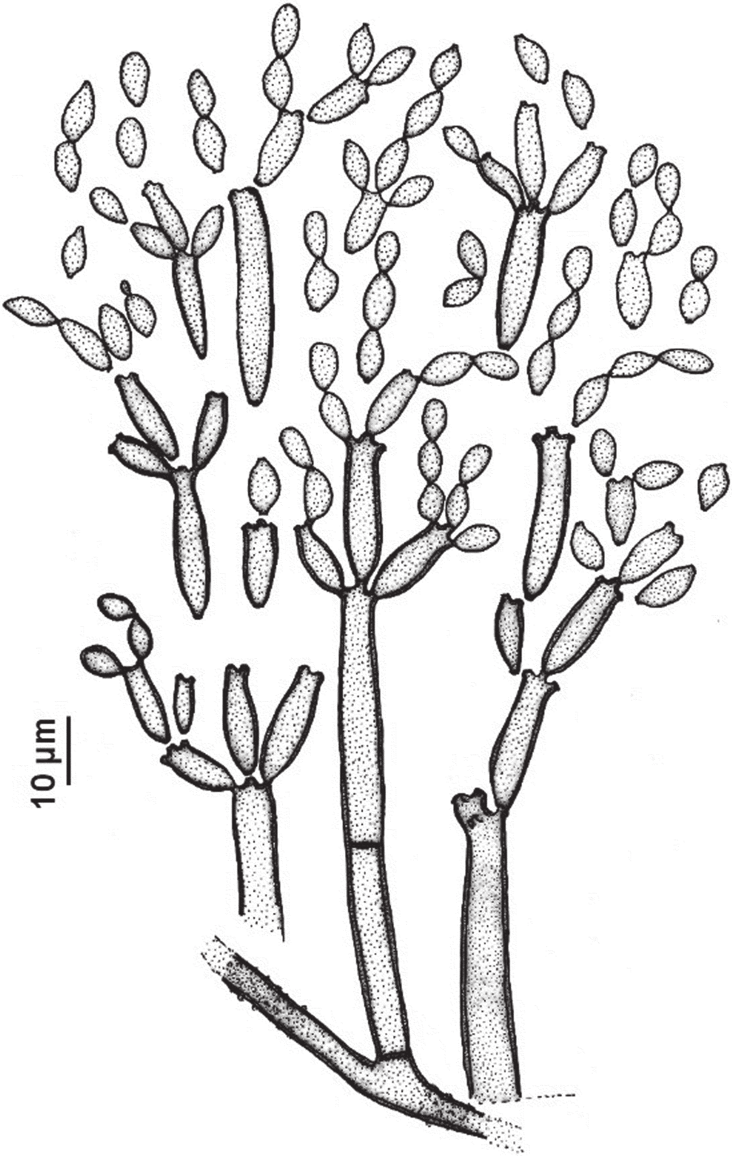

Fig 3. *Cladosporium subuliforme* (INIFAT C13/100).
Conidiophore, conidiogenous cells, ramoconidia, and conidia.

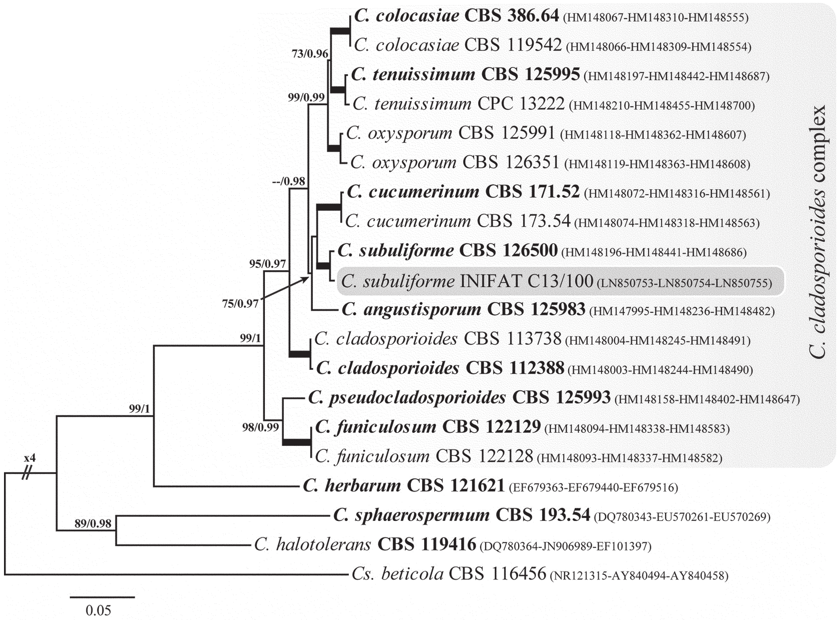

FIG 4. Maximum likelihood tree inferred from combined ITS, EF-1α, and Act sequences showing the phylogenetic relationships between the isolate INIFAT C13/100 and representatives of *Cladosporium* spp. Numbers above the branches are bootstrap values (BS ≥70%) followed by posterior probability values (PP ≥0.95). Fully supported branches (BS 100%/PP 1) are shown in bold. Numbers between parentheses indicate the GenBank accession numbers for the ITS, EF-1α, and Act gene sequences. Sequences from type specimens marked in bold. The scale bar indicates the number of nucleotide substitutions per site. The tree was rooted with *Cercospora beticola* CBS 116456.

pale olivaceous-brown or dull green, after 10 days or pulvinate, scattered, amphigenous, yellow, 300–550 µm diam. Mycelium superficial and immersed, composed of septate, branched, verruculose, pale olivaceous brown hyphae, 2–4 µm diam. CONIDIOPHORES macronematous, branched, erect, straight or flexuous, cylindrical, unbranched or slightly branched toward the apex, septate, olivaceous brown, smooth or verruculose near the base, 40–220 × 2–4 µm. CONIDIOGENOUS CELLS polyblastic, terminal, sometimes intercalary, integrated, cylindrical or oblong, somewhat subnavicular, 12–35 × 4 µm with several thickened and refractive conidiogenous loci, slightly denticulate. Conidial secession schizolytic. RAMOCONIDIA primary and secondary, cuneiform, subcylindrical, subnavicular, truncated at the base, 15–30 × 2–3.5

µm, smooth, olivaceous brown, dry. CONIDIA blastocatenate, ellipsoidal, ovoid, obovoid to subglobose, forming branched chains, unicellular, smooth, pale olivaceous brown, dry, 3–7 × 3–5 µm, truncated, slightly thickened and refractive at the base or both ends.

SPECIMEN EXAMINED: **CUBA. LA HABANA, Santiago de Las Vegas**, INIFAT Campus, in high tunnels, 22°58′N 82°20′W, on subcircular, angular, or irregular yellow leafspots on *Capsicum annuum* L. (*Solanaceae*), 12-VI-2013, coll. T. Shagarodsky (Live cultures: INIFAT C13/100 = CBS 140073 = FMR 14409; GenBank LN850753, LN850754, LN850755, LN850756).

NOTE: The Cuban material has shorter ramoconidia and longer conidia than those of the type material (ramoconidia 14–35 µm long; conidia 2.5–12 µm long; Bensch et al. 2012), but otherwise fits well the original description.

Phylogenetic analyses FIG. 4

A preliminary identification of the isolate INIFAT C13/100 based on sequence comparison of the ITS and LSU loci showed that it was related with members of the *Cladosporium cladosporioides* complex. However, a species identification was not possible using this approach given the poor resolution of these loci.

A further phylogenetic analysis was performed based on the combined ITS, EF-1α, and Act sequences. The final alignment consisted of a total 973 characters (ITS 443; EF-1α 325; Act 205), of which 378 were variable (ITS 89; EF-1α 186; Act 103) and 182 were informative (ITS 13; EF-1α 105; Act 64). The resulting phylogenetic tree (FIG. 4) showed that INIFAT C13/100 formed a strongly supported monophyletic group with the ex-type strain of *C. subuliforme* (CBS 126500) (BS 100%, PP 1) with a sequence similarity of 100% (ITS), 98.8% (EF-1α), and 98.8% (Act). The clade representing *C. subuliforme* clustered closely related with several species included in the *C. cladosporioides* complex of *Cladosporium*: *C. angustisporum*, *C. cladosporioides*, *C. colocasiae*, *C. cucumerinum*, *C. oxysporum*, and *C. tenuissimum*.

Therefore, the molecular analyses confirmed INIFAT C13/100 identification as *C. subuliforme*. Our phylogeny agrees with previous work on *Cladosporium* (Bensch et al. 2010, 2012); *C. subuliforme* clusters as a phylogenetically well-defined species sister to *C. angustisporum* and *C. cucumerinum*. (*Cladosporium cucumerinum* has also been reported from *Capsicum annuum* in South America; Mendes et al. 1998). However, despite their genetic closeness, the three species are easily differentiated based on the shape and length of their conidiophores and conidia (Bensch et al. 2012). Two other species, *C. cladosporioides* and *C. oxysporum*, also described from *Capsicum* species in South America and Asia (Farr et al. 2016), are also related morphologically and phylogenetically with *C. subuliforme*. Nevertheless, *C. subuliforme* differs from *C. cladosporioides* by its long and narrow subuliform (subulate) conidiophores, and its narrower conidiogenous loci and conidial hila, and can be distinguished from *C. oxysporum* by its non-nodulose non-geniculate conidiophores (Bensch et al. 2012).

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Literature cited

Bensch K, Groenewald JZ, Dijksterhuis J, Starink-Willemse M, Andersen B, Summerell BA, Shin HD, Dugan FM, Schroers HJ, Braun U, Crous PW. 2010. Species and ecological diversity within the *Cladosporium cladosporioides* complex (*Davidiellaceae*, *Capnodiales*). Studies in Mycology. 67: 1–94. http://dx.doi.org/10.3114/sim.2010.67.01

Bensch K, Braun U, Groenewald JZ, Crous PW. 2012. The genus *Cladosporium*. Studies in Mycology. 72: 1–401. http://dx.doi.org/10.3114/sim0003

Carbone I, Kohn LM. 1999. A method for designing primer set for speciation studies in filamentous ascomycetes. Mycologia 91: 553–556.

Castañeda-Ruiz RF, Iturriaga T, Decock C. 2000. *Bulbocatenospora*, a new hyphomycete genus from Venezuela. Mycological Research 104: 107–109. http://dx.doi.org/10.1017/S095375629900101X

CBS. 2016. http://www.cbs.knaw.nl/ [accessed 16 May 2016].

Crous PW, Braun U, Schubert K, Groenewald JZ. 2007. Delimiting *Cladosporium* from morphologically similar genera. Studies in Mycology. 58: 33–56. http://dx.doi.org/10.3114/sim.2007.58.02

Dugan FM, Schubert K, Braun U. 2004. Check-list of *Cladosporium* names. Schlechtendalia 11: 1–103.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accurancy and high throughput. Nucleic Acids Research 32: 4673–4680. http//dx.doi.org/10.1093/nar/gkh340.

Ellis MB. 1971. Dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey.

Ellis MB. 1976. More dematiaceous hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey.

Farr DF, Rossman AY. 2016. Fungal databases, Systematic Mycology and Microbiology Laboratory, ARS, USDA. http://nt.ars-grin.gov/fungaldatabases [accessed 25 May 2016]

GenBank. 2016. http://www.ncbi.nlm.nih.gov/ [accessed 16 May 2016]

Ho MHM, Castañeda RF, Dugan FM, Jong SC. 1999. *Cladosporium* and *Cladophialophora* in culture: description and an expanded key. Mycotaxon. 72: 115–157.

Hoog GS de, Guarro J, Gené J, Figueras MJ. 2011. Atlas of clinical fungi, electronic version 3.1. CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands.

Huelsenbeck JP, Ronquist F. 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754–755. http://dx.doi.org/10.1093/bioinformatics/17.8.754

Index Fungorum. 2016 Fungal names search. http://www.indexfungorum.org/names/Names.asp [accessed 16 May 2016]

McKemy JM, Morgan-Jones G. 1990. Studies in the genus *Cladosporium* sensu lato. II. Concerning *Heterosporium gracile*, the causal organism of leaf spot disease of iris species. Mycotaxon. 39: 425–440.

McKemy JM, Morgan-Jones G. 1992. Studies in the genus *Cladosporium* sensu lato. VII. Concerning *Cladosporium cucumerinum*, causal organism of crown blight and scab or gummosis of cucurbits. Mycotaxon. 43: 163–170.

Mendes MAS, da Silva VL, Dianese JC, Ferreira MASV, Santos CEN, Neto EG, Urben AF, Castro C. 1998. Fungos em plants no Brasil. Embrapa-SPI/Embrapa-Cenargen, Brasilia. 555 p.

Morgan-Jones G, McKemy JM. 1992. Studies in the genus *Cladosporium* sensu lato VI. Concerning *Cladosporium vignae*, causal organism of leaf and pod spot of cowpea (*Vignaunguiculata*) and leaf blight of *Lespedeza bicolor*. Mycotaxon. 43: 9–20.

Mullins J. 2001. Microorganisms in outdoor air. 3–16, in: B Flannigan et al. (eds). Microoganisms in home and indoor work environments: diversity, health impacts, investigation and control. Taylor & Francis, London.

Nylander JA. 2004. MrModeltest v2. Evolutionary biology centre, Uppsala University, Uppsala, Sweden.

Rehner SA, Samuels GJ. 1994. Taxonomy and phylogeny of *Gliocladium* analyzed from nuclear large subunit ribosomal DNA sequences. Mycological Research 98: 625–634. http://dx.doi.org/10.1016/S0953-7562(09)80409-7

Sandoval-Denis M, Sutton DA, Martin-Vicente A, Cano-Lira JF, Wiederhold N, Guarro J, Gené J. 2015. *Cladosporium* species recovered from clinical samples in the United States. Journal of Clinical Microbiology 53: 2990–3000. http://dx.doi.org/10.1128/JCM.01482-15

Schubert K, Braun U. 2004. Taxonomic revision of the genus *Cladosporium* s. lat. 2. *Cladosporium* species occurring on hosts of the families *Bignoniaceae* and *Orchidaceae*. Sydowia 56: 296–317.

Schubert K, Groenewald JZ, Braun U, Dijksterhius J, Starink MS, Hill CF, Zalar P, Hoog GS de, Crous PW. 2007. Biodiversity in the *Cladosporium herbarum* complex (*Davidiella*, *Capnodiales*), with standardization of methods for *Cladosporium* taxonomy and diagnostics. Studies in Mycology 58: 105–156. http://dx.doi.org/10.3114/sim.2007.58.05

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0 Molecular Biology Evolution 30: 2725–2719. http://dx.doi.org/10.1093/molbev/mst197

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673–4680. http://dx.doi.org/10.1093/nar/22.22.4673

Vilgalys R, Hester M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. Journal of Bacteriology 172: 4238–4246.

Waller JM, Lenné JM, Waller SJ. 2002. Plant pathologist´s pocketbook (3rd edition). CABI Publishing, Wallingford, UK.

White TJ, Bruns T, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. p 315–322, in: MA Innis et al. (eds). PCR protocols: a guide to methods and applications. Academic Press, New York. http://dx.doi.org/10.1016/B978-0-12-372180-8.50042-1