available online at www.studiesinmycology.org

STUDIES IN MYCOLOGY 79: 49–84.

The *Colletotrichum destructivum* species complex – hemibiotrophic pathogens of forage and field crops

U. Damm[1*], R.J. O'Connell[2], J.Z. Groenewald[1], and P.W. Crous[1,3,4]

[1]*CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands;* [2]*UMR1290 BIOGER-CPP, INRA-AgroParisTech, 78850 Thiverval-Grignon, France;* [3]*Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa;* [4]*Wageningen University and Research Centre (WUR), Laboratory of Phytopathology, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands*

*Correspondence: U. Damm, ulrike.damm@senckenberg.de, Present address: Senckenberg Museum of Natural History Görlitz, PF 300 154, 02806 Görlitz, Germany.

Abstract: *Colletotrichum destructivum* is an important plant pathogen, mainly of forage and grain legumes including clover, alfalfa, cowpea and lentil, but has also been reported as an anthracnose pathogen of many other plants worldwide. Several *Colletotrichum* isolates, previously reported as closely related to *C. destructivum*, are known to establish hemibiotrophic infections in different hosts. The inconsistent application of names to those isolates based on outdated species concepts has caused much taxonomic confusion, particularly in the plant pathology literature. A multilocus DNA sequence analysis (ITS, GAPDH, CHS-1, HIS3, ACT, TUB2) of 83 isolates of *C. destructivum* and related species revealed 16 clades that are recognised as separate species in the *C. destructivum* complex, which includes *C. destructivum*, *C. fuscum*, *C. higginsianum*, *C. lini* and *C. tabacum*. Each of these species is lecto-, epi- or neotypified in this study. Additionally, eight species, namely *C. americae-borealis*, *C. antirrhinicola*, *C. bryoniicola*, *C. lentis*, *C. ocimi*, *C. pisicola*, *C. utrechtense* and *C. vignae* are newly described.

Key words: Anthracnose, *Ascomycota*, *Glomerella*, Phylogenetics, Systematics.

Taxonomic novelties: New species: *Colletotrichum americae-borealis* Damm, *C. antirrhinicola* Damm, *C. bryoniicola* Damm, *C. lentis* Damm, *C. ocimi* Damm, *C. pisicola* Damm, *C. utrechtense* Damm, *C. vignae* Damm; **Typifications: Epitypifications (basionyms):** *C. destructivum* O'Gara, *C. fuscum* Laubert, *C. higginsianum* Sacc., *Gloeosporium lini* Westerd; **Lectotypifications (basionyms):** *C. fuscum* Laubert, *Gm. lini* Westerd., *C. pisi* Pat; **Neotypification (basionym):** *C. tabacum* Böning.

Published online 28 October 2014; http://dx.doi.org/10.1016/j.simyco.2014.09.003. Hard copy: September 2014.

INTRODUCTION

Colletotrichum destructivum was originally described as the causal organism of a disease of clover (*Trifolium pratense* and *T. hybridum*) in the western USA (O'Gara 1915). To date this species has been reported from more than 30 hosts belonging to at least 11 plant families, the majority of them being *Fabaceae* (especially *Trifolium*, *Medicago*, *Glycine*), but also including several reports from *Poaceae* (especially *Phalaris*, *Triticum*) and a few reports from *Asteraceae* (*Chrysanthemum*), *Convolvulaceae* (*Cuscuta*), *Magnoliaceae* (*Michelia*), *Menispermaceae* (*Cocculus*), *Polygonaceae* (*Rumex*), *Solanaceae* (*Nicotiana*), *Lamiaceae* (*Perilla*), *Scophulariaceae* (*Antirrhinum*, *Sutera*) and *Orchidaceae* (*Bletilla*). These reports originate from 18 countries, mainly in North America, Asia and Africa; with comparatively few reports from Europe, South America and Oceania (Kawaradani *et al.* 2008, Tomioka *et al.* 2011, 2012, Farr & Rossman 2014).

According to Sutton (1992), conidia of *C. destructivum* measure 10–22 × 4–6 µm, are straight to slightly curved, abruptly tapered to an obtuse apex and a truncate base, while according to Baxter *et al.* (1983) they are much narrower, measuring 16–18 × 3 µm, mostly straight and have tapered ends.

Since many other *Colletotrichum* species are also known from the host plants listed above, there is confusion about the names applied to different collections. For example, Cannon *et al.* (2012) found that half of the ITS sequences of *C. trifolii* submitted to GenBank prior to their study, were based on misidentified strains that actually belonged to the *C. destructivum* complex. Many isolates assigned to the *C. destructivum* species complex in a preliminary phylogeny based on ITS and included in this study for further analysis, had previously been identified as *C. coccodes*, *C. lindemuthianum*, *C. trifolii*, *C. truncatum*, *C. gloeosporioides* or *Glomerella cingulata* var. *cingulata*. Further confusion was caused by connecting *C. destructivum* to the sexual morph *Ga. glycines* (Tiffany & Gilman 1954, Manandhar *et al.* 1986), which was originally described by Lehman & Wolf (1926) from soybean stems as the sexual morph of *C. glycines*. In contrast, von Arx & Müller (1954) treated *Ga. glycines* as a form of *Ga. cingulata* with large ascospores.

A number of species were observed to have a similar morphology to *C. destructivum* and were considered to be closely related to that species. In the study of Moriwaki *et al.* (2002), Japanese *Colletotrichum* isolates clustered into 20 groups based on ITS1 sequences, which correlated with their morphology; isolates of *C. destructivum*, *C. fuscum*, *C. higginsianum* and *C. linicola* belonged to the same ribosomal group and were considered as possibly conspecific. Based on D2 and ITS2 rDNA sequences, Latunde-Dada & Lucas (2007) found a close relationship among *C. destructivum* isolates from *Vigna unguiculata* and *Medicago sativa*, *C. linicola* isolates from *Linum* and *C. truncatum* isolates from *Pisum sativum*, *Vicia faba*

Peer review under responsibility of CBS-KNAW Fungal Biodiversity Centre.

and *Lens culinaris*, which clustered with *C. higginsianum* isolates in their phylogeny. Based on multilocus phylogenies, *C. destructivum* was recently delineated as a species complex with *C. fuscum*, *C. higginsianum*, *C. tabacum*, *C. linicola* and *Ga. truncata* (Cannon *et al.* 2012, O'Connell *et al.* 2012). However, only a few isolates were included in those studies.

The infection strategy of isolates from several hosts of *C. destructivum* and related species has been reported as hemibiotrophic (Bailey *et al.* 1992, O'Connell *et al.* 1993, Shen *et al.* 2001) and several genes involved in plant infection have been studied (Huser *et al.* 2009, Kleemann *et al.* 2012, Liu *et al.* 2013b). To better understand the molecular basis of the infection process, O'Connell *et al.* (2012) compared genome and transcriptome sequence data of *C. higginsianum* with those of *C. graminicola*, a hemibiotrophic species from a different *Colletotrichum* species complex. This study revealed that both species possessed unusually large sets of pathogenicity-related genes, combining features of both biotrophic and necrotrophic pathogens. In particular, genes encoding plant cell wall-degrading enzymes, proteases and secondary metabolism enzymes are all expanded, similar to necrotrophs, but these fungi also encode large numbers of effector proteins for host manipulation, more similar to biotrophs. Transcriptome sequencing showed that expression of these genes is highly stage-specific, with most effector and secondary metabolism genes expressed early during appressorial penetration and biotrophy, and most plant cell wall-degrading enzymes, proteases and nutrient uptake transporters induced later at the switch to necrotrophy.

Prior to this study, the phylogenetic relationships of species in the *C. destructivum* complex have been studied inadequately using modern molecular methods. Many species names in this complex have been applied inconsistently or incorrectly, as there have been no recent studies of type specimens and few ex-type cultures are available for sequence analyses. Preliminary results based on multilocus DNA sequences of a small dataset indicated that isolates from different hosts belonged to several closely related species. The aim of our study was to recollect, delineate, typify and characterise the species within the *C. destructivum* complex, based on multilocus DNA sequence and morphological data.

MATERIALS AND METHODS

Isolates

A total of 83 isolates from the CBS-KNAW Fungal Biodiversity Centre (CBS), Utrecht, the Netherlands, and other culture collections was studied, most of which had been previously identified as *C. destructivum*. Type specimens (holo-, lecto-, epi- and neotypes) of the species studied are located in the fungaria of the CBS, the US National Fungus Collections (BPI), Beltsville, Maryland, USA, the Royal Botanic Gardens, Kew, UK, (IMI and K(M)), and the Botanic Garden and Botanical Museum Berlin-Dahlem, Freie Universität Berlin (B), Germany. All descriptions are based on ex-holotype, ex-epitype or ex-neotype cultures as applicable. Features of other isolates or specimens are included if they deviate from the ex-type cultures. Subcultures of the holo-, epi- and neotypes as well as all other isolates used for morphological and sequence analyses are maintained in the culture collections listed in Table 1.

Morphological analysis

To enhance sporulation, autoclaved filter paper and double-autoclaved stems of *Anthriscus sylvestris* were placed onto the surface of synthetic nutrient-poor agar medium (SNA; Nirenberg 1976). SNA and OA (oatmeal agar; Crous *et al.* 2009) cultures were incubated at 20 °C under near-UV light with a 12 h photoperiod for 10 d. Measurements and photomicrographs of characteristic structures were made according to Damm *et al.* (2007). Appressoria were observed on the reverse side of SNA plates. Microscopic preparations were made in clear lactic acid, with 30 measurements per structure and observed with a Nikon SMZ1000 dissecting microscope (DM) or with a Nikon Eclipse 80i microscope using differential interference contrast (DIC) illumination.

Colony characters and pigment production on SNA and OA cultures incubated at 20 °C under near-UV light with a 12 h photoperiod were noted after 10 d. Colony colours were rated according to Rayner (1970). Growth rates were measured after 7 and 10 d.

Phylogenetic analysis

Genomic DNA of the isolates was extracted using the method of Damm *et al.* (2008). The ITS, GAPDH, and partial sequences of the chitin synthase 1 (CHS-1), histone H3 (HIS3), actin (ACT) and beta-tubulin (TUB2) genes were amplified and sequenced using the primer pairs ITS-1F (Gardes and Bruns 1993) + ITS-4 (White *et al.* 1990), GDF1 + GDR1 (Guerber *et al.* 2003), CHS-354R + CHS-79F (Carbone & Kohn 1999), CYLH3F + CYLH3R (Crous *et al.* 2004b), ACT-512F + ACT-783R (Carbone & Kohn 1999) and T1 (O'Donnell & Cigelnik 1997) + Bt-2b (Glass & Donaldson 1995) or T1 + BT4R (Woudenberg *et al.* 2009), respectively. The PCRs were performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, California) in a total volume of 12.5 µL. The GAPDH, CHS-1, HIS3, ACT and TUB2 PCR mixture contained 1 µL 20× diluted genomic DNA, 0.2 µM of each primer, 1× PCR buffer (Bioline, Luckenwalde, Germany), 2 mM $MgCl_2$, 20 µM of each dNTP, 0.7 µL DMSO and 0.25 U Taq DNA polymerase (Bioline). Conditions for PCR of these genes constituted an initial denaturation step of 5 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 52 °C and 30 s at 72 °C, and a final denaturation step of 7 min at 72 °C, while the ITS PCR was performed as described by Woudenberg *et al.* (2009). The DNA sequences generated with forward and reverse primers were used to obtain consensus sequences using Bionumerics v. 4.60 (Applied Maths, St-Marthens-Lathem, Belgium), and the alignment assembled and manually adjusted using Sequence Alignment Editor v. 2.0a11 (Rambaut 2002).

To determine whether the six sequence datasets were congruent and combinable, tree topologies of 70 % reciprocal Neighbour-Joining bootstrap with Maximum Likelihood distances (10 000 replicates) with substitution models determined separately for each partition using MrModeltest v. 2.3 (Nylander 2004) were compared visually (Mason-Gamer and Kellogg 1996). A maximum parsimony analysis was performed on the multilocus alignment (ITS, GAPDH, CHS-1, HIS3, ACT, TUB2) as well as for each gene separately with PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2003) using the heuristic search option with 100 random sequence additions and tree bisection and reconstruction (TBR) as the branch-swapping