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A new *Cytospora* species pathogenic on *Carpobrotus edulis* in its native habitat

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Abstract: *Carpobrotus edulis* (*Aizoaceae*) is a fleshy creeper, native to South Africa and commonly found growing on coastal seashores. Recently this plant has been observed dying in large patches in areas close to Cape Town. Symptoms include a wilting of the leaves associated with death of the woody stems. The aim of this study was to identify the probable cause of this disease. Dead and dying stem tissues were found to be colonised by a species of *Cytospora*. Isolates of this fungus were identified based on DNA sequence data from the rDNA-ITS, translation elongation factor 1-α, β-tubulin and large subunit rDNA loci. Analyses of the data showed that the fungus is a new species of *Cytospora,* described here as *Cytospora carpobroti sp. nov.* Pathogenicity tests showed that *C. carpobroti* resulted in distinct lesions on inoculated stems but not the fleshy leaves. The origin of *C. carpobroti* is unknown and there is concern that it could be an introduced pathogen threatening the health of this important native plant.

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INTRODUCTION

Carpobrotus edulis (*Aizoaceae*), commonly known as sour fig, is an indigenous succulent species in South Africa. It occurs as a creeper growing along the east coast of the country and has been moved to many parts of the world where it is a popular garden plant. Ecologically, it plays an important role in binding sands in coastal areas (Wisura & Glen 1993, Chiban *et al.* 2011). *Carpobrotus edulis* is also used in traditional medicine because it contains antimicrobial compounds and its fruits are edible (Rood 1994, van der Watt & Pretorius 2001, Springfield *et al.* 2003).

Little is known regarding the diseases of *C. edulis*. It was believed to be free of pathogens when it was imported into California from South Africa for stabilising soil in the early 1900's (McDonald *et al*. 1984). However, in 1980 many *C. edulis* plants were reported dying in southern California and McDonald *et al.* (1984) confirmed *Pythium aphanidermatum*, *Phytophthora cryptogea* and *Verticillium dahlia* causing the disease in that area. Other fungal pathogens identified on *C. edulis* include *Albugo trianthemae* causing white rust in New Zealand (McKenzie & Johnston 1999) and *Anthostomella spartii* on dead stems of *C. edulis* in Portugal (Francis 1975). There is only one reported endophyte, a *Coniothyrium* sp. known from this plant on La Gomera, one of the Canary Islands (Kock *et al.* 2007).

The genus *Cytospora* (*Diaporthales*, *Cytosporaceae*) includes fungal species that occur mostly on woody plants including angiosperms and gymnosperms. Species of *Cytospora* (sexual morphs: *Leucostoma, Valsa, Valsella and Valseutypella*) have a cosmopolitan distribution and are frequent endophytes in healthy plant tissues. They can also be important pathogens when their hosts are subjected to stress (Schoeneweiss 1981, 1983). In this pathogenic phase, most *Cytospora* species cause canker and die-back diseases (Sinclair *et al.* 1987, Farr *et al.* 1989). The conidiomata of these fungi are then found on the dying wood associated with these tissues.

Many *Cytospora* species are known to cause plant diseases; some of these are quite host specific while others have broad host ranges. For example, *C. chrysosperma* has been recorded on more than 80 plant hosts (Farr & Rossman 2016), while *C. eucalypticola* is a well-known pathogen specifically on *Eucalyptus* (Adams *et al.* 2005). Although the most aggressive *Cytospora* spp. occur on angiosperms, there are exceptions such as *C. kunzei* (= *Valsa kunzei*), which is a pathogen of *Pinus elderica* in plantations (Kavak 2005).

The aim of this study was to identify the *Cytospora* sp. closely associated with the death of *C. edulis* in various areas of the Cape Peninsula of South Africa. In addition, pathogenicity tests were conducted to assess the possible role of the fungus in causing disease.

MATERIALS AND METHODS

Collection of samples and isolations

Samples of dying plants (Fig. 1) were collected at various sites along the Cape Peninsula, in the Western Cape Province of South Africa, in August 2016. The diseased stems were placed in paper bags and transferred to the laboratory for further study. Symptomatic tissues were examined under a dissection microscope where it was possible to observe conidiomata typical of a *Cytospora* sp. The conidial masses were exposed by removing the apices of the conidiomata with a sharp scalpel blade. Spore masses were removed from these structures with

Fig. 1. A. *Carpobrotus edulis* plants. **B.** Dying *C. edulis*.

a sterile needle, and conidia were spread onto the surface of malt extract agar (MEA: 2 % Biolab malt extract, 2 % Difco agar) supplemented with streptomycin (400 mg/L). Specimens from this study were lodged in South African National Collection of Fungi (PREM), Roodeplaat, Pretoria, South Africa. Living cultures were preserved in the collection (CMW) of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa, and the live culture collection (PPRI) of the South African National Collection of Fungi, Roodeplaat, Pretoria, South Africa.

DNA extraction, sequencing and phylogenetic analyses

DNA was extracted using the PrepMan® Ultra kit (Applied Biosystems) from mycelium of 5-d-old axenic cultures. DNA sequences were generated for the internal transcribed spacer region of the ribosomal RNA (rRNA) operon amplified with primers ITS-1F (Gardes & Bruns 1993) and ITS-4 (White *et al*. 1990), the translation elongation factor 1-α (*TEF1-α*) gene amplified with primers EF1-728F and EF1-986R (Carbone & Kohn 1999), the β-tubulin (*TUB2*) gene amplified with primers BT2a and BT2b (Glass & Donaldson 1995) and large ribosomal subunit (LSU) gene region amplified with primers LR0R and LR5F (Vilgalys & Hester 1990).

The 25 µL PCR reaction mixtures contained 1 µL DNA, 17.7 µL molecular distilled water, 0.3 µL MyTaq DNA Polymerase, 5 µL MyTaq Buffer ($MgCl_2$, dNTPs), 0.5 µL of each primer. The amplification conditions were as follows; initial denaturation of 5 min at 95 °C, followed by 25 cycles of 30 s at 95 °C, 30 s at 55 °C for ITS and LSU, 53 °C for *TEF1-α* and 54 °C for *TUB2*, and 1 min at 72 °C, and a final extension of 7 min at 72 °C. The PCR amplicons were visualized separately on a 1 % agarose gel with GelRed™. The conditions for the PCR sequencing were the same as those described by Begoude *et al*. (2010).

The sequences were compared with our unpublished sequence dataset for the phylogenetic analyses. The datasets were aligned online using MAFFT v. 7.0 (https://mafft.cbrc.jp/alignment/server/) (Katoh *et al.* (2017) and checked manually for alignment errors. The phylogenetic analyses for all the datasets were performed using Maximum Likelihood (ML). The best nucleotide substitution models for each dataset were found separately with jModelTest v. 3.7 (Posada & Buckley 2004). The model for GTR was chosen for the combined datasets of ITS, *TEF1*-α, *TUB2* and LSU. The ML analyses were performed in PAUP v. 4.0b10 with the heuristic search option with 100 random additions of taxa, tree bisection and reconstruction (TBR) branch swapping and 1000 bootstrap replications in PAUP. The consensus trees were constructed in MEGA v. 7 and posterior probabilities were assigned to branches after a 60 % majority rule.

Morphological characteristics

Purified cultures were incubated on 2 % MEA, at 25 °C for 2 wk after which fruiting structures began to develop on MEA. Fungal structures from both naturally infected tissue and culture were mounted on slides in sterile water that was later replaced with 85 % lactic acid and all the measurements and images were captured using these specimens. Up to fifty measurements were made for conidia and other morphologically characteristic structures where these were available. Naturally infected tissues bearing fungal structures were cut into small pieces and boiled for 1 min. The saturated pieces were mounted in Tissue Freezing Medium® (Leica, Germany) and cut into sections (12–16 µm thick) using a Leica Cryo-microtome (Leica, Germany). The sections were mounted on microscope slides in 85 % lactic acid for further observation. Observations were made using Nikon Eclipse Ni compound and SMZ18 dissection microscopes (Nikon, Japan). Images were captured with a Nikon DS-Ri camera and with the imaging software NIS-Elements BR, and all the measurements were also captured with this software.

Pathogenicity trials

Two inoculation techniques were used in this study. In one trial, the fleshy leaves of *C. edulis* were inoculated and in a second trial, inoculations were made on the woody stem tissues. In each case, 20 plants were inoculated with one of two isolates (CMW 48981 and CMW 48983) of the *Cytospora* sp. isolated from dying plants. An additional 20 plants were inoculated as controls with sterile toothpicks or uncolonised agar plugs.

The inoculum for the leaf inoculations was in the form of sterile tooth picks that had been placed on the surface of MEA for colonisation by the fungal isolate. The inoculated plates were incubated at 24 °C for 2 wk to ensure that the *Cytospora* sp. had fully penetrated the tooth picks. The colonized as well as the sterile tooth picks as control were then inserted firmly into the fleshy leaves of *C. edulis* plants grown in a greenhouse. These plants were observed for the appearance of symptoms for 6 wk.

For stem inoculations, the two *Cytospora* isolates were allowed to grow on MEA for 5 d at 24 °C. Discs (5 mm diam) of agar were cut from the actively growing margins of the cultures and these were placed into wounds of the same size on the *C. edulis* stems. In the case of the controls, inoculations were made with sterile MEA discs. The inoculated stems were sealed with parafilm to reduce desiccation and the chance of contamination. These plants were maintained in a greenhouse at 24 °C under natural light conditions and observed for the appearance of symptoms for 6 wk. Following this period, the trial was terminated, lesion sizes were measured and re-isolations were made on MEA. The variation in lesion length was analysed using Kruskal-Wallis Rank Sum test in R v. 3.4.3. A representative set of isolates were identified based on morphological characters in order to ensure that the isolated fungi were the same as those that had been inoculated into the plants.

RESULTS

Isolates, sequencing and phylogenetic analyses

Five isolates were obtained from symptomatic tissues and these were all included in the DNA sequence analyses (Table 1). The sequence datasets for the ITS, *TEF1-α, TUB2* and LSU regions were analysed individually and in combination. The ITS sequence dataset contained 515, the *TEF1-α* dataset 361, the *TUB2* dataset 545, the LSU dataset 482 and the combined dataset 1903 characters (TreeBASE Accession No. 22682).

A distinct clade, which clustered as sister to *Cytospora austromontana* was revealed in all the analyses (Fig. 2). These isolates differed from *C. austromontana* by unique fixed alleles in ITS (7 bp), *TEF1-α* (35 bp), *TUB2* (29 bp) and LSU (7 bp). The topology of the trees obtained from the ML analyses were similar for all loci as well as in the combined analyses.

Morphological characteristics

The isolates in the unknown fungus had pale grey colonies with moderate aerial mycelium. The cultures produced conidiomata with yellow oozing conidia and hyaline allantoid conidia after approximately 10 d.

Taxonomy

A new species in the *Cytosporaceae* (= *Valsaceae*) is described here for the isolates from *Carpobrotus edulis* that reside in a unique clade amongst other species of *Cytospora*. No sexual morph was found on the host or in culture, and the description is based on morphological characteristics of the asexual morph only.

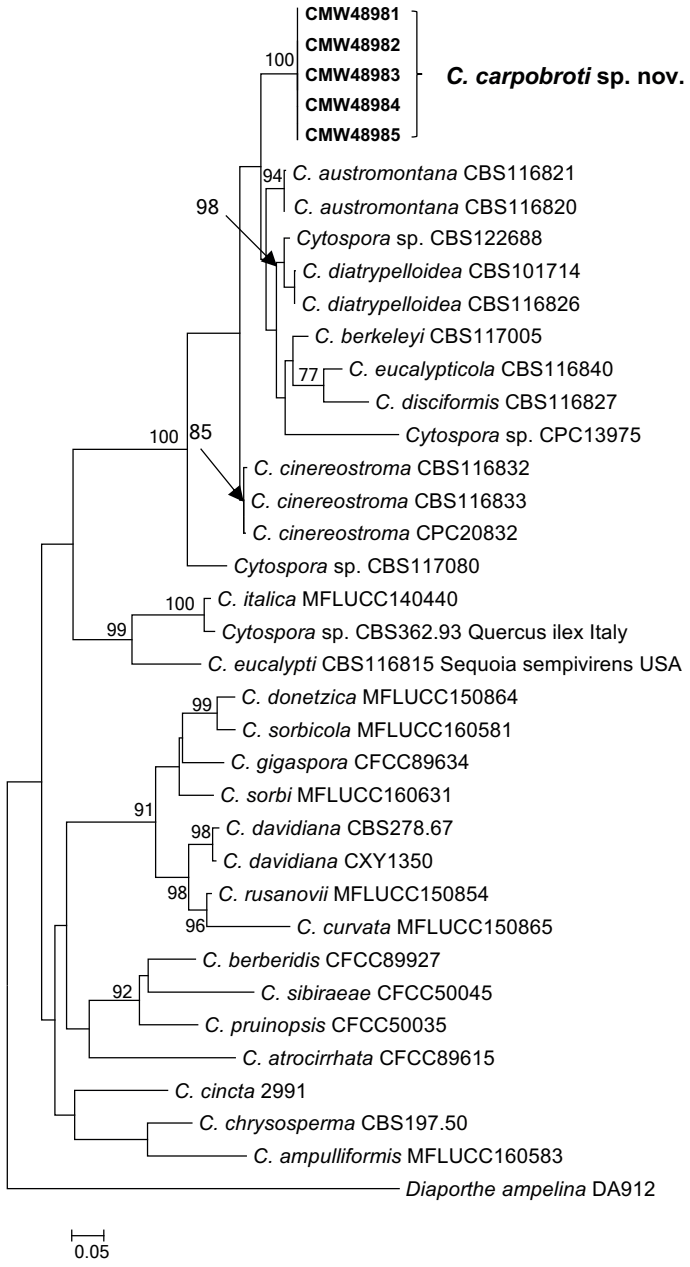

Fig. 2. Maximum Likelihood (ML) tree of the combined data set of ITS r-DNA, β-tubulin, *TEF1-α* and LSU loci sequences. Bootstrap values above 70 % are given at the nodes. The tree was rooted to *Diaporthe ampelina*. Isolates of this study are indicated as bold.

Cytospora carpobroti Jami, Marinc. & M.J. Wingf., **sp. nov.** MycoBank MB825251. Fig. 3.

Etymology: Name refers to the host genus *Carpobrotus*.

Conidiostromata in vivo subepidermal, immersed, subglobose to ellipsoidal, uni- or multiloculate, convoluted, 150–330 µm long, 130–315 µm wide, with ostiolar neck reaching the surface of the substrate. *Stromatic tissue* eustromatic, *textura angularis*. *Conidiomatal walls* composed of 4–6 layers of thick-walled, moderately compressed, pigmented cells, 10.5–27 µm thick. *Conidiomata in vitro* on toothpick in 2 % MEA, stromata 0.3–0.4 mm diam, multilocular, subdivided by invaginations, giving rise to up to 5 elongated black necks, with obtusely rounded apex, exuding a yellow conidial cirrhus or globoid conidial mass.

Fig. 3. Micrographs of *Cytospora carpobroti sp. nov.* (holotype PREM 62170, ex-holotype CMW 48981 = PPRI 29136). **A.** Symptomatic stem. **B.** Vertical section of conidiomata immersed in stem. **C.** Close-up of conidiomatal wall showing conidiophores lining along the locule. **D.** Conidiomata formed on toothpick in 2 % MEA. **E.** Vertical section of conidiomata *in vitro*. **F, G.** Conidiophores and conidiogenous cells. **H.** Conidia. **I.** Culture morphology of 5 d and 50 d old on 2 % MEA in the dark (reverse in lower half). Scale bars: A = 1 mm; B, E = 100 µm; C = 50 µm; D = 250 µm; F–H = 5 µm.

Table 1. The *Cytospora carpobroti* isolates from *Carpobrotus edulis* of this study used in the phylogenetic analyses. Type isolate is indicated in bold.

Isolate No.	Identity	Location	Collector	GenBank			
				ITS	*TEF1*-α	*TUB2*	LSU
CMW 48981	*Cytospora carpobroti*	Cape Town, South Africa	M.J. Wingfield	MH382812	MH411212	MH411207	MH411216
CMW 48982	*Cytospora carpobroti*	Cape Town, South Africa	M.J. Wingfield	MH382813	MH411213	MH411208	-
CMW 48983	*Cytospora carpobroti*	Cape Town, South Africa	M.J. Wingfield	MH382814	-	MH411209	MH411217
CMW 48984	*Cytospora carpobroti*	Cape Town, South Africa	M.J. Wingfield	MH382815	MH411214	MH411210	MH411218
CMW 48985	*Cytospora carpobroti*	Cape Town, South Africa	M.J. Wingfield	MH382816	MH411215	MH411211	MH411219

Fig. 4. Inoculations of *Carpobrotus edulis* stem and leaf tissue with *Cytospora carpobroti sp. nov.* after six weeks. **A, B.** Stems inoculated with isolates CMW 48983 and CMW 48981 respectively showing distinct lesion development. **C.** Leaf inoculated with isolate CMW 48983 with no lesion development. **D.** Control inoculation on stem showing absence of lesion. Scale bar = 5 mm.

Conidiophores borne along the locules, hyaline, smooth, branched, aseptate, 8.5–11 × 2.5–3.5 µm, embedded in a gelatinous layer. *Conidiogenous cells* phialidic, sub-cylindrical, tapering towards apices, collarettes minute, 4.5–12 × 1–2 µm; arranged in rosettes. *Conidia* hyaline, smooth, guttulate, allantoid, apex sub-obtusely rounded, aseptate, 3.5–6 × 1 µm (avg. 5.0 × 1 µm).

Culture characteristics: Colony on 2 % MEA showing optimum growth at 25 °C, reaching 69.2 mm in dark in 5 d, followed by at 30 °C reaching 55.6 mm and at 20 °C reaching 50.7 mm, no growth at 35 °C, showing circular growth with smooth margin, mycelium mostly aerial, flat and fluffy, at lower temperature mycelium mostly submerged, above and reverse olivaceous buff with inner circle greenish olivaceous.

Host: *Carpobrotus edulis.*

Distribution: Cape Town, South Africa.

Specimen examined: **South Africa**, Western Cape Province, Cape Town, *Carpobrotus edulis*, Aug. 2016, *M.J. Wingfield* (holotype PREM 62170, culture ex-type CMW 48981 = PPRI 29136).

Additional materials examined: Isolates CMW 48982 = PPRI 29137, CMW 48983 = PPRI 29138, CMW 48985.

Note: *Cytospora carpobroti* is morphologically similar to *C. austromontana* except for the dimensions of the conidiogenous cells of *C. austromontana* (7–12 × 1.5), which are larger than those of *C. carpobroti.*

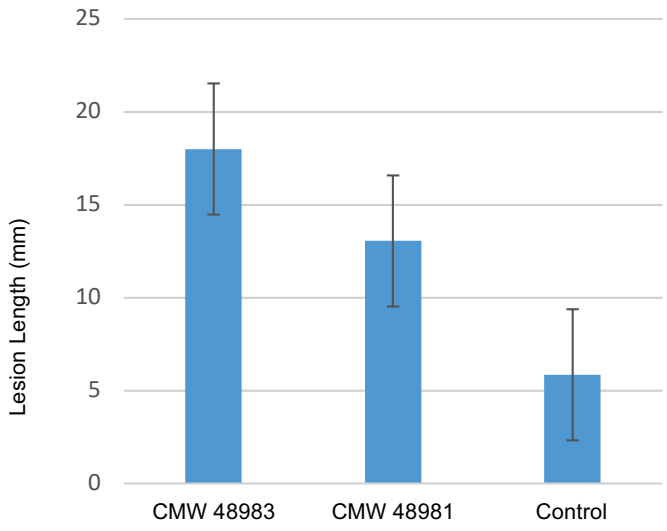

Fig. 5. Mean lesion length (mm) for isolates of *Cytospora carpobroti* 6 wk after inoculation on *Carpobrotus edulis.*

Pathogenicity trials

The two isolates of *C. carpobroti* produced lesions in the cambium of inoculated *C. edulis* stems within 6 wk. In contrast, there was no lesion development in the inoculated fleshy leaves or in any of the control inoculations (Fig. 4). Statistical analyses showed that lesion size (Fig. 5) for the new species were significant (P-value < 0.05). *Cytospora carpobroti* was consistently re-isolated from lesions. The fungus was never re-isolated from the control inoculations.

DISCUSSION

This study reports a new disease of *C. edulis* occurring in South Africa. The fungus isolated from diseased plants was found to represent a new species of *Cytospora* for which the name *C. carpobroti* is here established. Pathogenicity tests with two isolates of *C. carpobroti* showed that the fungus was able to cause disease on inoculated stems of *C. edulis*. Interestingly, inoculations on the fleshy leaves of plants failed to induce lesions.

Cytospora spp. commonly have wide host ranges (Adams *et al.* 2005) and occur mostly on woody plants. It was consequently interesting to encounter a disease caused by a *Cytospora* sp. on a succulent plant in this study. However, the stems of *C. edulis* are woody and the fact that pathogenicity tests were positive only on the woody stems is consistent with the known biology of *Cytospora* spp.

Based on phylogenetic analyses, the newly described *C. carpobroti* resides in a sister clade with *C. austromontana*. *Cytospora carpobroti* is morphologically similar to *C. austromontana* other than in the dimensions of conidiogenous cells. *Cytospora austromontana* was first isolated from a cankered branch of *Eucalyptus pauciflora* in Australia (Adams *et al.* 2005) and it was remarkable to find a closely related species from a very different ecological niche in the present study.

Relatively few studies have been conducted on the pathogenicity of *Cytospora* species. Generally, these fungi are considered endophytes and opportunistic or weak secondary pathogens (Adams *et al*. 2005). Some species such as *C. mali* are well-known pathogens on pear and apple trees, which are able to induce canker formation in pathogenicity tests (Ke *et al.* 2013). Although *C. carpobroti* was able to cause lesions on inoculated plants in this study, it is possible that these plants were subjected to some stress factor and that the fungus was acting as an opportunistic pathogen. This would be consistent with the biology of most *Cytospora* spp. causing plant diseases.

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