Pseudocercospora opuntiae sp. nov., the causal organism of cactus leaf spot in Mexico

Victoria Ayala-Escobar[1], María de Jesús Yáñez-Morales[1], Uwe Braun[2], Johannes Z. Groenewald[3] and Pedro W. Crous[3*]

[1]Instituto de Fitosanidad, Colegio de Postgraduados, Km 36.5 Carr. México-Texcoco, Montecillo, C.P. 56230, Edo de México, Mexico
[2]Martin-Luther-University, Institute of Geobotany and Botanical Garden, Herbarium, Neuwerk 21, D-06099 Halle (Saale), Germany
[3]Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

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Pseudocercospora opuntiae is newly described from *Opuntia* spp. from Mexico, where it causes a serious disease of this host. Although *P. opuntiae* is morphologically similar to other members of the genus with pigmented conidia and conidiophores, and unthickened, not darkened conidiogenous scars, DNA sequence data of the ITS region revealed that it clusters distant from other species of *Pseudocercospora* within *Mycosphaerella*. These data support the assumption that *Pseudocercospora* is paraphyletic within *Mycosphaerella*.

Key words: mitosporic fungi, *Mycosphaerella* anamorph, North America, *Opuntia*

Introduction

Species of *Opuntia* (cacti) are plants that grow wild in Mexico, where they are native. Various species of this genus are important for the production of prickly pears, or fresh edibles cladodes (pads) "nopalitos". About 42 000 ha of cactus pear have thus far been planted (Mondragon-Jacobo and Pérez González, 1996). *Opuntia fiscus-indica* (L.) Mill. is cultivated throughout the country, whereas *O. megacantha* Salm-Dyck, and *O. streptacantha* Lem. are cultivated only in the Valley of Mexico, where the cv. "Reyna" is the main plant cultivated for its green fruit. Furthermore, *O. lasiacantha* Pfeiff., *O. robusta* H.L. Wendl., and *O. tomentosa* Salm-Dyck are wild cacti in some Valley areas.

In November 2002 a new disease was observed on a cultivated *Opuntia* sp. at "Ailpa Alta" in the Distrito Federal, and later in February 2003 in a stand

*Corresponding author: Pedro Crous; e-mail: crous@cbs.knaw.nl

of a wild *Opuntia* sp. at "El Molino de las Flores" in the municipality of Texcoco of the State of Mexico. Disease symptoms consisted of brown to black, round lesions, approximately 2-4 cm diam, that appeared on the cladodes. At the "Instituto de Fitosanidad, Colegio de Postgraduado" (CP) the new disease was identified as an undescribed member of the genus *Pseudocercospora* Speg. Material was sent to U. Braun at the Martin-Luther-University in Halle, Germany, and P.W. Crous at the Centraalbureau voor Schimmelcultures in Utrecht, The Netherlands, for further identification. The aim of this paper was to elucidate the taxonomy of the causal organism, and to resolve its DNA phylogeny and generic affinity within the *Mycosphaerellaceae*.

Materials and methods

Isolates

Isolates were obtained from symptomatic leaf pieces by placing disinfested necrotic tissue fragments in moisture chambers to enhance sporulation. Monoconidial cultures were subsequently established on water-agar (WA) (20 g agar / 1 L distilled H_2O). Colonies were induced to sporulate on *Opuntia* agar (OPA) (40 g of *Opuntia* cladodes boiled for 10 minutes, and then blended with 20 g agar / 1 L distilled water), oatmeal-agar (OA) (15 g of oatmeal, 20 g agar / 1 L distilled water), and potato-dextrose agar (PDA) (200 g potatoes, 20 g dextrose, 20 g agar / 1 L distilled water) (Gams *et al*., 1998). Dishes of all media were point inoculated and incubated for 4 weeks at ± 24ºC under continuous near-ultraviolet light, and inspected for sporulation at 3 day intervals. Morphological observations *in vitro* were based on sporulating cultures on host material. Thirty observations were made of each structure, with extremes given in parentheses. Descriptions and nomenclatural details were deposited in MycoBank (www.MycoBank.org).

DNA isolation, amplification and phylogeny

The protocol of Lee and Taylor (1990) was used to isolate genomic DNA from fungal mycelium of a monoconidial culture grown on PDA in Petri dishes. The primers ITS1 and ITS4 (White *et al*., 1990) were used to amplify part (ITS) of the nuclear rRNA operon spanning the 3' end of the 18S rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region and the 5' end of the 28S rRNA gene. The PCR conditions, sequence alignment and subsequent phylogenetic analysis followed the methods of Crous *et al*. (2004). Newly generated sequence data were deposited

in GenBank (accession numbers DQ073921–DQ073923), and the alignment in TreeBASE (accession number SN2345).

Koch's postulates

Pathogenicity tests were conducted on four-mo-old healthy cladodes of cultivated *Opuntia*. The experiment consisted of six plants (incl. two controls) that were inoculated (two cladodes per plant) by means of two methods. Cladodes were either unwounded, or wounded by means of a sterile toothpick. Method one consisted of placing colonised OPA agar disks (5 mm diam) on the wound, and covering these with Parafilm. The second method consisted of spraying a suspension of conidia (5000 conidia/mL, emended by means of a haemocytometer) onto the unwounded cladode until run-off. Controls were inoculated with a sterile agar plug, while unwounded leaves were sprayed with sterile water. All the plants were incubated in a moist chamber at ± 90% relative humidity for a period of two months, after which they were placed in a shade house at ambient humidity (± 28ºC) until symptoms appeared. Re-isolations were made from the margins of lesions onto PDA to confirm Koch's postulates.

Results

DNA phylogeny

The sequence alignment consisted of 24 ITS sequences including the outgroup sequence and contained 506 characters (including alignment gaps) which were used in the phylogenetic analyses. Of these characters, 106 were parsimony-informative, 265 were constant and 135 variable characters were parsimony-uninformative. Ten most parsimonious trees (one of which is shown in Fig. 1) were obtained and show three main lineages, namely *Pseudocercospora* (56% bootstrap support), *Cercospora* (100% bootstrap support) and *Passalora* / *Dothistroma* / *Pseudocercospora* (99% bootstrap support). The *Cercospora* and the *Passalora* / *Dothistroma* / *Pseudocercospora* clades are joined with a bootstrap support value of 71%. The tree topologies obtained using neighbour-joining analysis with the uncorrected "p", Kimura-2-parameter and HKY85 substitution models resulted in trees with identical topologies, but these trees differed from the parsimony trees in that they grouped the *Pseudocercospora* and *Cercospora* clades (data not shown). The sequence of *Ps*. *vitis* (Lév.) Speg. (type species of *Pseudocercospora*) is in a well-supported clade (84% bootstrap support)

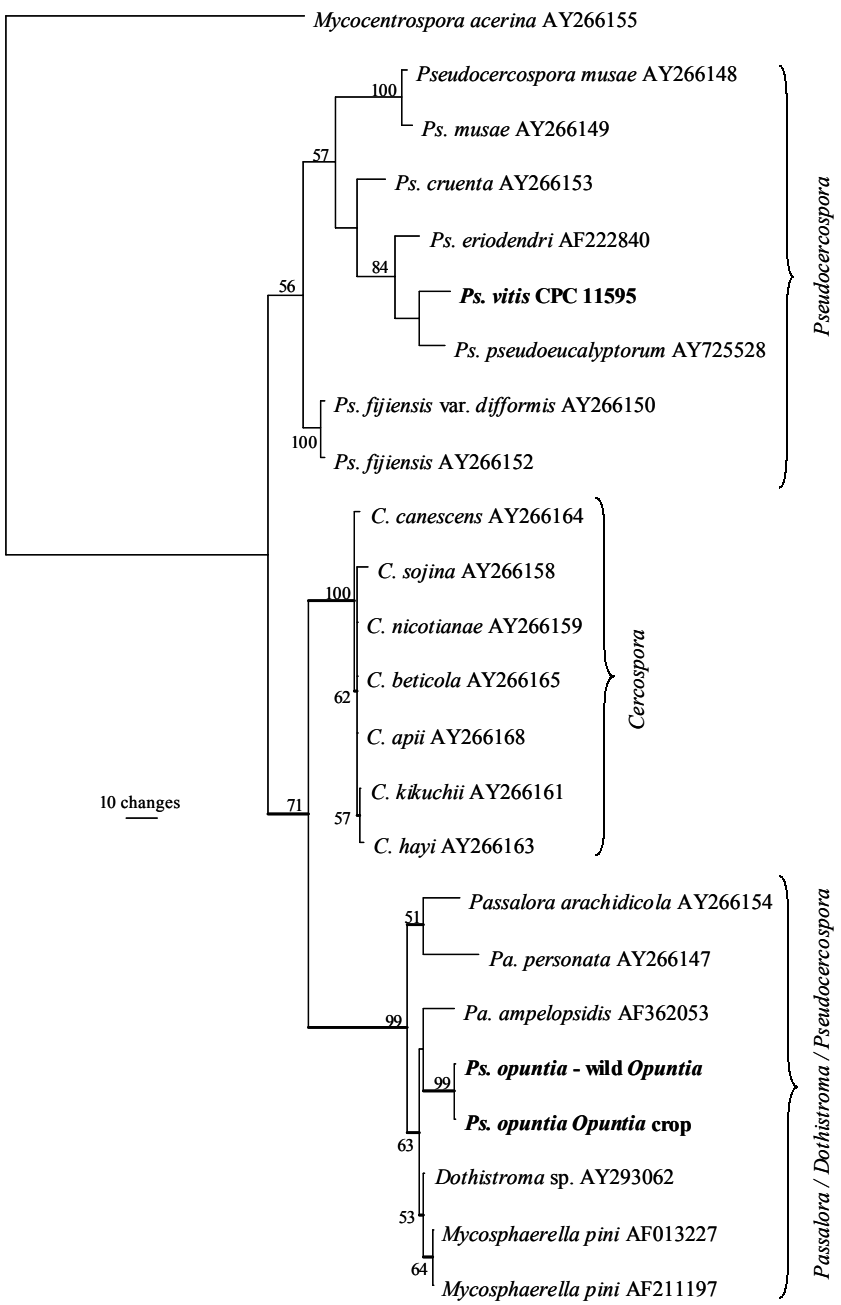


Fig. 1. One of 10 most parsimonious trees obtained from a heuristic search with 100 random taxon additions of the ITS sequence alignment. The scale bar shows 10 changes and bootstrap replicate values from 1000 replicates are shown at the nodes. Newly sequenced isolates are printed in bold face and consensus branches thickened. The tree was rooted to *Mycocentrospora acerina* AY266155.

containing *Ps. pseudoeucalyptorum* Crous and *Ps. eriodendri* (Racib.) U. Braun. The two isolates from *Opuntia* cluster together (99% bootstrap support) in the *Passalora* / *Dothistroma* clade.

Taxonomy

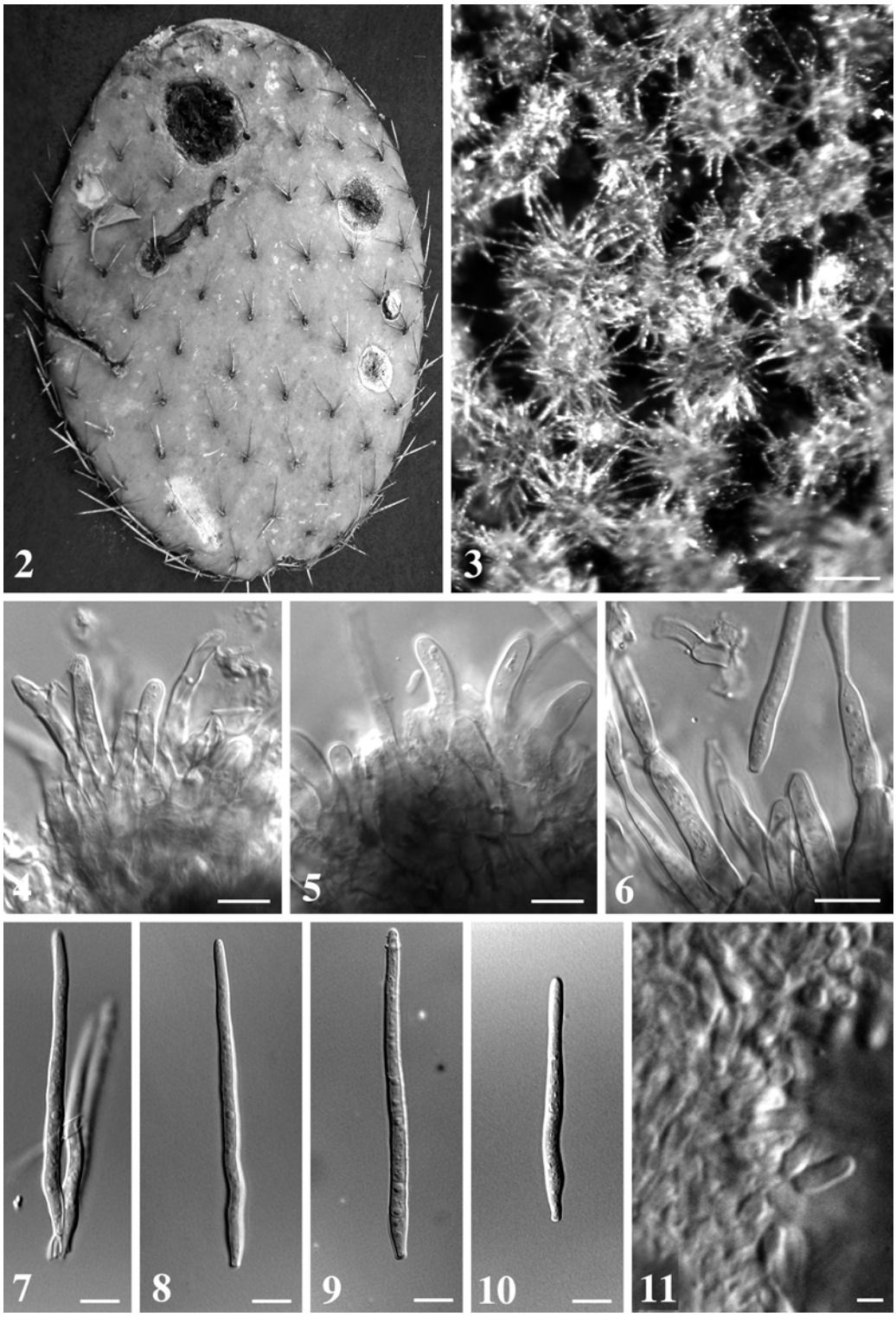
Pseudocercospora opuntiae Ayala-Escobar, Braun & Crous, **sp. nov.**

MycoBank MB500197 (Figs. 2-11)

Etymology: Epithet referring to its host, *Opuntia*.

Maculae subcirculares vel saepe irregulares, 2-4 cm diam, griseo-brunneae, atro-brunneae vel sordide subnigrae. *Coloniae* punctiformes vel pustulatae, atro-brunneae vel nigrae. *Mycelium* immersum (in vivo); hyphae 1-4 µm latae, interdum cellulis inflatis, ad 12 µm latis, pallide olivaceae vel olivaceo-brunneae, tenuitunicatae, leviae. *Stromata* bene evoluta, immersa, 30-150 µm diam, ex cellulis inflatis, 2-10 µm latis, atro-olivaceo-brunneis composita. *Conidiophora* numerosa, dense fasciculata, sporodochiales, erecta, recta, subcylindrica, conica vel leviter geniculata-sinuosa, non-ramosa, 5-40 × 3-7 µm, 0-2-septata, subhyalina, olivacea vel pallide olivaceo-brunnea, tenuitunicata, levia; cellulae conidiogenae integratae, terminales, 5-30 µm longae; cicatrices conidiales inconspicuae, non-incrassatae, non-fuscatae. *Conidia* solitaria, obclavata-cylindrica, 15-80 × 2.5-5 µm, (0-)1-7-septata, subhyalina vel pallide olivaceo-brunnea, tenuitunicata, levia, apice obtuso, basi obconice truncata, hila non-incrassata, non-fuscata.

Lesions subcircular to usually irregular, 2-4 cm diam, greyish-brown, dark brown to dingy blackish (Fig. 2). *Colonies* punctiform to pustulate, dark brown to blackish. *Mycelium* internal (developing superficial hyphae under high humidity in a moist chamber; hyphae 1-4 µm wide, or forming swollen hyphal cells, up to 12 µm diam, sometimes in monilioid sequences, pale olivaceous to olivaceous-brown, thin-walled, smooth). *Stromata* well-developed, immersed, 30-150 µm diam, composed of swollen hyphal cells, 2-10 µm diam, dark olivaceous-brown. *Conidiophores* in large, dense fascicles, forming sporodochial conidiomata, erect, straight, subcylindrical, conic to somewhat geniculate-sinuous, unbranched, 5-40 × 3-7 µm, 0-2-septate (under high humidity in moist chamber up to 300 µm long, strongly branched and pluriseptate), subhyaline, olivaceous to olivaceous-brown, thin-walled, smooth. *Conidiogenous cells* integrated, terminal, 5-30 µm long, proliferating sympodially, but also percurrently; conidiogenous loci inconspicuous, unthickened, not darkened. *Conidia* solitary, obclavate-cylindrical, 15-80 × 2.5-5 µm, (0-)1-7-septate, subhyaline to pale olivaceous-brown, thin-walled, smooth, apex obtuse, base obconically truncate, hila unthickened and not darkened, but sometimes with a minute marginal frill if formed from a percurrently proliferating conidiogenous cells (Figs. 7-10). *Spermatogonia* intermixed with stromata, medium brown, 80-150 µm wide, giving rise to numerous bacilliform to narrowly ellipsoid, straight to slightly curved, hyaline spermatia, 2.5-4 × 1 µm.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Figs. 2-11. *Pseudocercospora opuntiae.* **2.** Symptomatic cladode. **3.** Conidiophore fascicles intermingled with spermatogonia. **4-6.** Conidiophores. **7-10.** Conidia. **11.** Spermatia. Scale bars: 3 = 150 µm, 4-10 = 10 µm, 11 = 1 µm.

Holotype: Mexico, Ailpa Alta, Distrito Federal, on a cultivated *Opuntia* sp. (*Cactaceae*), 24 Nov. 2002, V. Ayala-Escobar and María de J. Yáñez-Morales (CHAPA # 167), culture ex-type CBS 117708 = CPC 11772.

Isotypes: HAL 1837 F and herb. CBS 15601.

Paratypes: Mexico, El Molino de la Flores, Texcoco, Edo. de Mexico, on a wild *Opuntia* sp., Feb. 2003, María de J. Yáñez-Morales and V. Ayala-Escobar (CHAPA # 168, HAL 1838 F).

Cultural characteristics: Colonies in OPA green-grey, reaching 3 cm diam. within 4 weeks at 24ºC; on PDA erumpent, spreading, margins smooth, regular, aerial mycelium moderate, surface grey-olivaceous with a thin white margin, reverse greenish-grey; on OA smoke grey to white due to moderate white aerial mycelium (Rayner, 1970); colonies on PDA reaching 12-16 mm diam. after 2 wks at 24ºC. Sporulation on OPA was first observed after 4 wks. Conidia were straight, cylindrical, subhyaline, 3-5-septate, 39-64 µm long (51 µm av.), base truncate, and apex obtuse (Figs. 7-10).

Koch's postulates

Pseudocercospora opuntiae was successfully re-isolated from the wounded and unwounded cladodes, using both means of inoculation (agar plugs as well as conidial suspension). Control plants remained healthy. Symptomatic cladodes developed stromata and spermatogonia (Fig. 3). Once placed in moist chambers, stromata produced conidiophores within 5 d, and conidia after 8 d.

Discussion

On the lesions in the type material, *Pseudocercospora opuntiae* is associated with a dominant *Asteromella* state that sporulates profusely among the fascicles. *Phyllosticta concave* Seaver has been considered the *Asteromella* spermatial state of *Mycosphaerella opuntiae* (Ellis & Everh.) Dearn. Other names that can be considered for the spermatial state of *Pseudocercospora opuntiae* include *Phyllosticta cacti* (Berk.) Archer, *P. opuntiae* [Sacc. & Speg.] var. *microspora* Cavara and *P. opuntiicola* Bubák, but the taxonomy and generic affinity of these taxa have not yet been resolved (Archer, 1926; Aa and Vanev, 2002), and it remains to be seen if any of them can be applied to the *Asteromella* state of *P. opuntiae*. Although no teleomorph has yet been found,

it seems unlikely that *P. opuntiae* would be related to *M. opuntiae*. The latter fungus was originally described from stems of *Opuntia* in the USA, and von Arx (1984) linked this teleomorph to "*Microdochium*" *lunatum* (Ellis & Everh.) Arx.

On account of the structure of the conidiogenous loci and conidial hila, the new species on *Opuntia* clearly belongs in *Pseudocercospora*, and also represents the first member of the genus described from the *Cactaceae*. DNA sequence data derived from the ITS region of *P. opuntiae* proved interesting, however, as it clearly represented evidence to the fact that *Pseudocercospora* (typified by *P. vitis* (Lév.) Speg.) is paraphyletic within *Mycosphaerella*. Although the genus *Pseudocercospora* is a well-known anamorph of *Mycosphaerella* (Crous *et al*., 2000), some species which are *Pseudocercospora*-like are probably unrelated to *Mycosphaerella*, as for instance *Parapithomyces clitoriae* Alcorn, which has a *Pseudocercospora* synanamorph (Alcorn, 1992).

Although earlier studies have proven that the genus *Mycosphaerella* is monophyletic (Crous *et al*., 1999, 2000, 2001a, 2001b, 2004), it is becoming evident that the same anamorph morphology has evolved more than once within *Mycosphaerella*. The clustering of *P. opuntiae* within a clade consisting of members of *Passalora* Fr. (thickened, darkened, refractive conidial scars), and *Dothistroma* Hulbary (acervuli with percurrent proliferating conidiogenous cells, giving rise to septate, hyaline conidia), clearly reiterates the fact that within *Mycosphaerella*, anamorph morphology is not always phylogenetically informative. These findings question currently accepted anamorph generic concepts in *Mycosphaerella*, which rely heavily upon conidial pigmentation, conidiomatal structure, and the nature of conidial scars (Kirschner *et al*., 2004; Schubert and Braun, 2005), and poses a clear taxonomic challenge for future studies.

Acknowledgements

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References

Aa, H.A. van der and Vanev, A. (2002). *A revision of the species described in Phyllosticta*. Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands.

Alcorn, J.L. (1992). *Parapithomyces clitoriae* sp. nov. (Fungi: Hyphomycetes) and its *Pseudocercospora* anamorph. Australian Systematic Botany 5: 711-715.

Archer, W.A. (1926). Mycological characters of some Sphaeropsidales in culture with reference to classification. Annals of Mycology 24: 1-84.

Arx, J.A. von (1984). Notes on *Monographella* and *Microdochium*. Transactions of the British Mycological Society 82: 373-374.

Crous, P.W., Hong, L., Wingfield, M.J., Wingfield, B.D. and Kang, J.-C. (1999). *Uwebraunia* and *Dissoconium*, two morphologically similar anamorph genera with distinct teleomorph affinity. Sydowia 52: 155-166.

Crous, P.W., Aptroot, A., Kang, J.-C., Braun, U. and Wingfield, M.J. (2000). The genus *Mycosphaerella* and its anamorphs. Studies in Mycology 45: 107-121.

Crous, P.W., Hong, L., Wingfield, B.D. and Wingfield, M.J. (2001a). ITS rDNA phylogeny of selected *Mycosphaerella* spp. and their anamorphs occurring on Myrtaceae. Mycological Research 105: 425-431.

Crous, P.W. Kang, J.-C. and Braun, U. (2001b). A phylogenetic redefinition of anamorph genera in Mycosphaerella based on ITS rDNA sequence and morphology. Mycologia 93: 1081-1101.

Crous, P.W., Groenewald, J.Z., Mansilla, J.P., Hunter, G.C. and Wingfield, M.J. (2004). Phylogenetic reassessment of *Mycosphaerella* spp. and their anamorphs occurring on *Eucalyptus*. Studies in Mycology 50: 195-214.

Gams, W., Hoekstra, E.S. and Aptroot, A. (eds) (1998). CBS *Course of mycology*. *4th ed*. Centraalbureau voor Schimmelcultures, Baarn, the Netherlands.

Kirschner, R., Piepenbring, M. and Chen, C.J. (2004). Some cercosporoid hyphomycetes from Taiwan, including new species of *Stenella* and new reports of *Distocercospora pachyderma* and *Phacellium paspali*. Fungal Diversity 17: 57-68.

Lee, S.B. and Taylor, J.W. (1990). Isolation of DNA from fungal mycelia and single spores. In: *PCR Protocols: a guide to methods and applications* (eds. M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White). Academic Press, San Diego, USA: 282-287.

Mondragon-Jacobo, C. and Perez-Gonzalez, S. (1996). Native cultivars of cactus pear in Mexico. In: *Progress in new crops*. (ed. J. Janick). ASHS Press, Arlington, VA.: 446-450.

Rayner, A.W. (1970). A Mycological Colour Chart. Commonwealth Mycological Institute, Kew.

Schubert, K. and Braun, U. (2005). Taxonomic revision of the genus *Cladosporium s.l.* 4. Species reallocated to *Asperisporium*, *Dischloridium*, *Fusicladium*, *Passalora*, *Pseudo-asperisporium* and *Stenella*. Fungal Diversity 20: 187-208.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: a guide to methods and applications* (eds. M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White). Academic Press, San Diego, USA: 315-322.

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