Prosopidicola mexicana gen. et. sp. nov., causing a new pod disease of *Prosopis* species

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Abstract: Species of *Prosopis* introduced into South Africa from the Americas for fuel wood, shade and fodder, have become naturalized and widespread in the dry northwestern areas of this country. Invasive *Prosopis* species have been the target of a biological control programme in South Africa since 1985. During a survey for potential fungal biological control agents in Mexico and Texas in 2001, a pod disease was recorded on *Prosopis glandulosa* in both countries. The disease is characterized by black/grey pycnidia, flattening of the pods, and seed decay. Morphological investigations of the causal organism showed it to be a *Coniothyrium*-like coelomycete. However, based on conidiogenous cell morphology and proliferation, we concluded that the organism is not congeneric with *Coniothyrium s. str*. Phylogenetic analysis of the SSU gene placed this fungus in the *Diaporthales*. Parsimony analysis of the ITS region (ITS-1, 5.8S, ITS-2) revealed it to group closely to *Cryphonectria* and *Endothia*. Consequently, a new genus, *Prosopidicola*, with type species *Prosopidicola mexicana*, is proposed.

Taxonomic novelties: *Prosopidicola* Crous & C.L. Lennox gen. nov., *Prosopidicola mexicana* Crous & C.L. Lennox sp. nov.
Key words: *Coniothyrium*, invasive weed, pod rot, *Prosopidicola, Prosopis*, systematics.

INTRODUCTION

Members of the genus *Prosopis* L. are thorny, leguminous shrubs or trees. During the late 1800s a number of *Prosopis* species were introduced into South Africa from North and South America for fodder (pods), shade and firewood (Harding 1978, Poynton 1987). Harding (1978) recognized the presence of six taxa in southern Africa, four of which have become naturalized and widespread in the dry northwestern areas of South Africa. In this country *Prosopis glandulosa* Torr. var. *torreyana* (L.D. Benson) E. Murray (honey mesquite) hybridises with *P. velutina* Wooton (velvet mesquite) and *P. chilensis* Stuntz (mesquite), making identification difficult (Henderson 2001). These *Prosopis* species and their hybrids have invaded riverbeds, riverbanks and drainage lines in the arid northwest (Henderson 2001). In some areas the invasion has been so extensive that impenetrable stands have formed and the land has been largely lost for agricultural purposes. Mechanical and chemical control is both difficult, due to the tree's thorny multi-stemmed nature, and expensive, in an area of low land value. Moran *et al.* (1993) stated that long-term, economically viable management of *Prosopis* in South Africa will probably only be achieved through biological control. As the plant still has some useful attributes (fodder, firewood, charcoal and wood for flooring), biological control has, until recently, been restricted to the use of seed-feeding beetles that reduce the reproductive capacity of the plant without

diminishing its usefulness (Zimmerman 1991, Moran *et al.* 1993, Impson *et al.* 1999). The extent of the area invaded by *Prosopis*, and recent estimates of water usage by this plant (Le Maitre *et al.* 1996), have lent government support for research on the use of fungi as agents for its biological control. Both classical and mycoherbicide approaches are being investigated by researchers in the Weed Pathology Unit of ARC-PPRI, Stellenbosch, South Africa.

In September and October 2001, an extensive survey and collections were made of pathogens occurring on *P. glandulosa* var. *torreyana* and *P. velutina* in Mexico and Texas. All necessary export and import permits had been obtained prior to the start of the survey and collection trip. Of particular interest was a pod disease collected in Mexico and Texas. *Prosopis glandulosa* pods with similar symptoms had previously been collected by S. Neser in 1989 near La Joya, Texas (Fig. 1). The fungal causal organism had been isolated and lodged (Culture number C158) in the culture collection of the Weeds Pathology Unit, ARC-PPRI, Stellenbosch, South Africa.

The objectives of this paper are to report on a new pod disease of *Prosopis*, and to discuss the taxonomy and identity of this pod disease in the light of the morphological and molecular characteristics of the pathogen.

MATERIALS AND METHODS

Isolates

During a survey and collection of pathogens occurring on *P. glandulosa* and *P. velutina* species in Mexico and Texas in 2001, a severe pod disease was noted on these trees growing north of Chihuahua (Mexico), and near the towns of Zapata, La Joya and Raymondville (Texas). Symptoms of this pod disease, caused by an as yet unidentified coelomycete, were characterised by black/grey pycnidia, flattening of the pods and seed decay. Symptomatic pods were collected, labelled and placed in brown paper bags. The pods collected each day were examined using a Zeiss field microscope for signs of the causal organism. Symptomatic material was wrapped in tissue paper, placed in brown paper bags and stored in a cooler box. Collected pods were sent to South Africa via a courier service. On arrival in South Africa, the material was taken directly into the plant pathogen quarantine facility at ARC-PPRI Weeds Pathology Unit in Stellenbosch. Once in quarantine, the diseased pods were re-examined for signs of the causal organism, and isolations made from symptomatic tissue. Symptomatic tissue was surface-sterilized in 70 % ethanol for 30 s, 1 % sodium hypochlorite for 2 min, and 70 % ethanol for 30 s. Sections of tissue (5 mm^2) were transferred to Petri dishes containing potato dextrose agar (PDA) (39 g Difco PDA, 1 L distilled H$_2$O) and incubated at 25 $^{\circ}$C under continuous cool day-light-type white fluorescent light. Single-conidial cultures were subsequently established for all pod disease isolates. Axenic cultures were maintained on PDA slants at 5 $^{\circ}$C in the dark in the fungal culture collection of the ARC-PPRI Weeds Pathology Unit, Stellenbosch, South Africa. Reference strains and herbarium specimens have been deposited at the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, The Netherlands.

DNA isolation and amplification

The isolation protocol of Crous *et al.* (2000) was used to isolate genomic DNA from fungal mycelia grown on MEA plates. The primers ITS1 and ITS4 (White *et al.* 1990) were used to amplify part of the nuclear rRNA operon spanning the 3' end of the 18S (small subunit) rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS (ITS2) region and the 5' end of the 28S (large subunit) of the rRNA gene. Part of the 18S rRNA gene was amplified using primers NS1 and NS4 (White *et al.* 1990). The reaction mixture contained 5 µL of diluted sample, 1 × PCR buffer (Bioline), 8 mM MgCl$_2$, 500 µM of each of the dNTPs, 2.5 U Taq polymerase (Bioline) and 10 pmoles of each primer and made up to a total volume of 25 µL with sterile water. The cycling conditions comprised denaturing at 96 $^{\circ}$C for 5 min, followed by 30 cycles of denaturation at 96 $^{\circ}$C (30 s), annealing 55 $^{\circ}$C (30 s) and elongation at 72 $^{\circ}$C (90 s). A final elongation step at 72 $^{\circ}$C for 7 min was included. PCR products were separated by electrophoresis at 75 V for 1 h in a 0.8 % (w/v) agarose gel in 0.5 × TAE buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualised under UV light using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, U.K.) following ethidium bromide staining.

Fig. 1. Disease symptoms associated with *Prosopidicola mexicana* on pods of *Prosopis glandulosa.*

Table 1. *Coniothyrium* and *Coniothyrium*-like isolates included in this study for sequence analysis and/or morphological comparison.

Species	Accession no.[1]	Substrate	Country	Collector	GenBank accession	
					ITS	SSU
"Coniothyrium" leuco-spermi	CBS 114035 = STE-U 2852	*Protea repens*	South Africa	J.E. Taylor	AY720707	AY720711
Coniothyrium palmarum	CBS 400.71	*Chamaerops humilis*	Italy	W. Gams	AY720708	AY720712
"Coniothyrium ovatum"	STE-U 18	*Eucalyptus cladocalyx*	South Africa	P.W. Crous		AY720713
"Coniothyrium ovatum"	CBS 111149 = STE-U 23	*E. cladocalyx*	South Africa	P.W. Crous		AY720714
"Coniothyrium ovatum"	CBS 110906 = STE-U 40	*E. cladocalyx*	South Africa	P.W. Crous		AY720715
"Coniothyrium ovatum"	CBS 113621 = STE-U 42	*E. cladocalyx*	South Africa	P.W. Crous		AY720716
Prosopidicola mexicana	CBS 113529	*Prosopis glandulosa*	U.S.A.	C. Lennox	AY720709	AY720717
Prosopidicola mexicana	CBS 113530	*P. glandulosa*	U.S.A.	S. Neser	AY720710	

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The amplification products were purified by using a GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech Europe GmbH, Germany) according to the manufacturer's instructions. The cycle sequencing reaction with 20 to 40 ng of purified PCR products and 10 pmoles of primer in a total volume of 10 μL was carried out with an ABI PRISM BigDye Terminator v. 3.0 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA, U.S.A.) containing AmpliTaq DNA Polymerase following the instructions of the manufacturer. The resulting fragments were analysed on an ABI Prism 3100 DNA Sequencer (Perkin-Elmer, Norwalk, Connecticut, U.S.A.).

Phylogenetic analysis
The nucleotide sequences of the 18S rDNA gene generated in this study were added to the outgroups, *Candida fukuyamaensis* AB013566 and a *Debaryomyces* sp., AB022440 and other sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/) using Sequence Alignment Editor v. 2.0b11 (Rambaut 2002). For the ITS alignment, *Coniothyrium palmarum* and two *Phaeosphaeria* species were used as outgroups. Adjustments for improvement were made by eye where necessary. Phylogenetic analyses with both parsimony and neighbour-joining (using uncorrected "p", Jukes-Cantor and Kimura-2-parameter models) were done using PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2000). Any ties that were encountered were broken randomly. For parsimony analysis, alignment gaps were treated as both a new character and as missing, and all characters were taken as unordered and of equal weight. The robustness of the trees was evaluated by 1000 bootstrap replications (Hillis & Bull 1993). Other measures calculated included tree length, consistency index, retention index and rescaled consistency index

(CI, RI and RC). Resulting trees were printed with TreeView v. 1.6.6 (Page 1996).

Morphology
Isolates were plated onto PDA and incubated at 25 °C under near-UV light to promote sporulation. Slide preparations were made in lactic acid and 30 examples of each structure measured (× 1000) using a Zeiss Axioskop light microscope. The 95 % confidence intervals were also determined for conidial dimensions, with the extremes in conidium length and width given in parentheses. Colony colour (reverse) was determined on PDA after 8 d at 25–30 °C in the dark using the colour designations of Rayner (1970). For additional clarification of morphological characteristics, fungal pycnidia were prepared for scanning electron microscopy according to the methodology employed by Lennox & Rijkenberg (1989). This material was examined using a Leo S440 scanning electron microscope.

RESULTS

Phylogenetic analysis
Approximately 1 kb was determined for the SSU rRNA gene and the alignment contained 1046 characters and 41 taxa including the two outgroup taxa. Approximately 550 to 600 bases were determined for each isolate, of which approximately 520 to 580 bases per sequence (spanning ITS1, 5.8S rRNA gene, ITS2 and the first part of the small subunit gene) were added to the alignment. The manually adjusted alignments of the nucleotide sequences contained 590 characters including alignment gaps (data not shown) and 24 taxa including the three outgroup taxa. Sequences were deposited in GenBank (Table 1), and the alignment in TreeBASE (S1150).

The phylogenetic analysis of the SSU sequence data resulted in the neighbour-joining tree shown in Fig. 2. The topology of the tree stayed the same, irrespective of which substitution model was used. Four well-supported clades were obtained, each containing taxa originally identified as *Coniothyrium* or *Coniothyrium*-like. The first clade (100 % bootstrap support) places the type strain of *Prosopidicola mexicana* in the *Diaporthales*. The *Mycosphaerellaceae* form the second main clade and contain a well-supported group (100 % bootstrap support) of four *Coniothyrium ovatum* H.J. Swart isolates. The third clade (99 % bootstrap support) contains representatives of the black yeasts and also contains the isolate *Coniothyrium leucospermi* Crous & Denman which groups with 92 % bootstrap support with *Dothidea insculpta* Wallr. and *Dothidea ribesia* (Pers.) Fr. The last clade (100 % bootstrap support) contains *Coniothyrium palmarum* Corda and belongs to the *Pleosporales*.

To study the phylogenetic placement of *Prosopidicola mexicana*, the ITS gene was also sequenced.

Setting the gapmode to a new state yielded two equally parsimonious trees (one of which is shown in Fig. 3) with the same topology but a slight difference in branch length, while scoring gaps as missing characters increased the number of equally parsimonious trees to 22. The strict consensus tree of the 22 equally parsimonious trees had the same topology as the bootstrap consensus tree obtained when scoring gaps as fifth characters. The results of the neighbour-joining analyses of the ITS data were the same for the three substitution models tested (data not shown). However, when compared to the parsimonious trees, *Leucostoma persoonii* (Nitschke) Höhn. was found to group with sequences of *Diaporthe* Nitschke species, apart from *Prosopidicola mexicana* and *Cryphonectria* (Sacc.) Sacc. & D. Sacc. A BLASTn search of the ITS sequences of the two strains of *Prosopidicola mexicana* did not return any hits with high similarity, as is evident from the ITS phylogram. The two *Prosopidicola mexicana* isolates grouped with a clade containing sequences of *Cryphonectria* and *Endothia* (93 % bootstrap support).
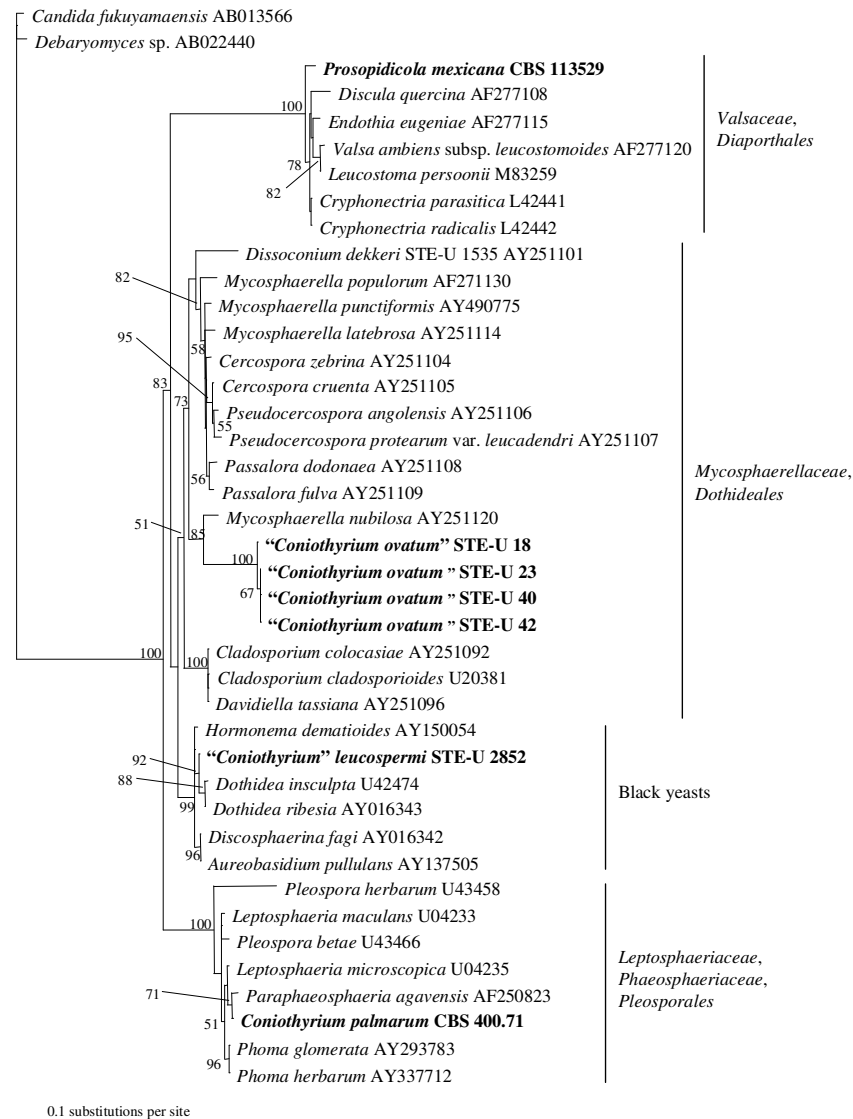
Fig. 2. Neighbour-joining tree obtained from a search of the SSU data set using the Kimura 2-parameter substitution model. The tree was rooted to *Candida fukuyamaensis* and a *Debaryomyces* species. Bootstrap support values from 1000 replicates are shown at the nodes. The bar indicates 0.1 substitutions per site and all new sequences are shown in bold type.
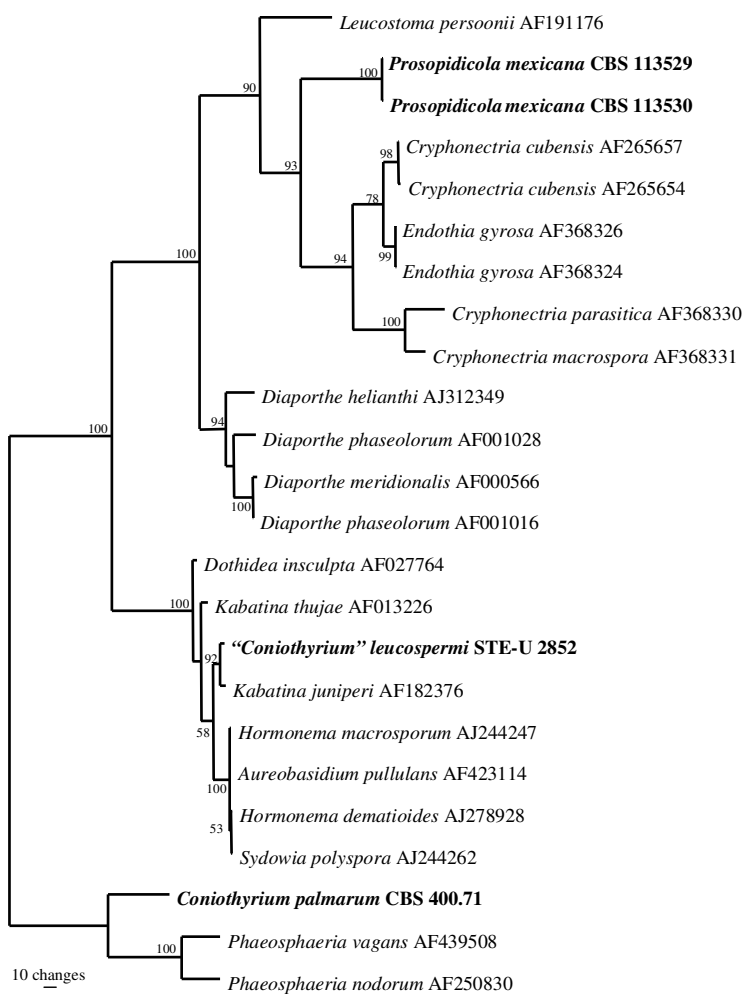
Fig. 3. One of two most parsimonious trees obtained from a heuristic search of the ITS data set with 100 random taxon additions (TL = 940 steps, CI = 0.751, RI = 0.880, RC = 0.661). The trees were rooted to *Coniothyrium palmarum* and two *Phaeosphaeria* species. Bootstrap support values from 1000 replicates are shown at the nodes. The bar indicates 10 changes and all new sequences are shown in bold type.

Morphology

The *Prosopis* pathogen was initially identified as a member of *Coniothyrium*. This complex is characterized by having thin-walled, unilocular pycnidia, with hyaline, percurrently proliferating conidiogenous cells that give rise to brown, thick-walled, 0–1-euseptate, ellipsoidal to subcylindrical conidia. The *Prosopis* pathogen is clearly not congeneric with *Coniothyrium s. str.* in that it has branched conidiophores which have green-brown conidiogenous cells that proliferate percurrently, and in some cases also sympodially. The conidiogenous cells are also unique in that they have an irregular, wart-like, green-brown apical region. From these differences it is clear that a new genus has to be proposed to accommodate this fungus.

Taxonomy

Prosopidicola Crous & C.L. Lennox, **gen. nov.** MycoBank MB500048.

Etymology: *Prosopidicola* (L. genitive of *Prosopis*) indicating the species association with the genus *Prosopis* L.

Genus anamorphum pycnidiale, *Coniothyrio* simile. Conidiophora ramosa, cellulae conidiogenae percurrenter, nonnumquam sympodialiter proliferentes, viridi-brunneae, in summo verrucosae.

Typus: *Prosopidicola mexicana* Crous & C.L. Lennox, sp. nov.

Genus asexual, conidiomata pycnidial, resembling those of *Coniothyrium*. Conidiophores brown; conidiogenous cells proliferating percurrently, rarely sympodially, green-grown, roughened at apex.

Prosopidicola mexicana Crous & C.L. Lennox, **sp. nov.** MycoBank MB500049. Figs 4–12.

Legumina hospitis putrescens, laesiones irregulares, ad 7 × 2–3 mm, pallide brunneae, margine elevata, rubro-brunnea vel fusca. Mycelium internum, ex hyphis ramosis, septatis, levibus, brunneis, 2–3.5 μm latis compositum. Conidiomata amphigena, sparsa, nigra, subepidermalia, deinde

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erumpentia, globosa vel subglobosa, uniloculariа, pycnidialia, nonnumquam acervulos fingentia, ad 250 µm diam, paries ad 15 µm crassus, e 3–4 stratis cellularum angularium rubro-brunnearum compositus. Conidiophora totam superficiem internam obtegentia, subcylindrica, ramosa, 0–3-septata, recta vel irregulariter curvata, dilute brunnea vel deorsum viridi-brunnea, deinde etiam viridi-brunnea in parte distali, 5–50 × 3–4 µm. Cellulae conidiogenae pallide viridi-brunneae, primum leves, deinde fuscius viridi-brunneae et verrucosae, subcylindricae vel lageniformes; primum sicut phialides sporulantes, apice angusto (1.5–2 µm) periclinaliter inspissato, sed etiam percurrenter proliferentes, collaria extensa, 3–4 µm lata relinquentes; raro duo loci conidiogeni iuxta formati; cellulae 5–16 × 3–4 µm. Conidia in massa nigra exsudata, late ellipsoidea, brunnea, recta vel modice curvata, sursum rotundata, basi subtruncata, hilo inconspicuo praedita, levia, tenuitunicata, unicellularia, (8–)10–13(–20) × (3.5–)4.5–5.5(–6) µm.

Typus: Herb. CBS 7948, in legumine *Prosopidis glandulosae*, U.S.A., Texas, La Joya, 24 Sept. 2001, C.L. Lennox C 564 (cultura viva CBS 113529).

Lesions associated with pod rot symptoms, irregular, covering the pod, 2–3 mm wide and up to 7 mm long, extending across the width of the pod, pale brown with a raised, red-brown to dark brown margin. *Mycelium* internal, consisting of branched, septate, smooth, medium brown hyphae, 2–3.5 µm wide. *Conidiomata* amphigenous, scattered, black, subepidermal, becoming erumpent, globose to subglobose, unilocular, pycnidial, in some cases appearing acervular, up to 250 µm diam; wall up to 15 µm thick, consisting of 3–4 layers of medium red-brown textura angularis. *Conidiophores* lining the inner layer of the wall, covering the whole cavity, subcylindrical, branched, 0–3-septate, straight to irregularly curved, pale brown to green-brown below, becoming medium green-brown in the upper conidiogenous region, 5–50 × 3–4 µm.
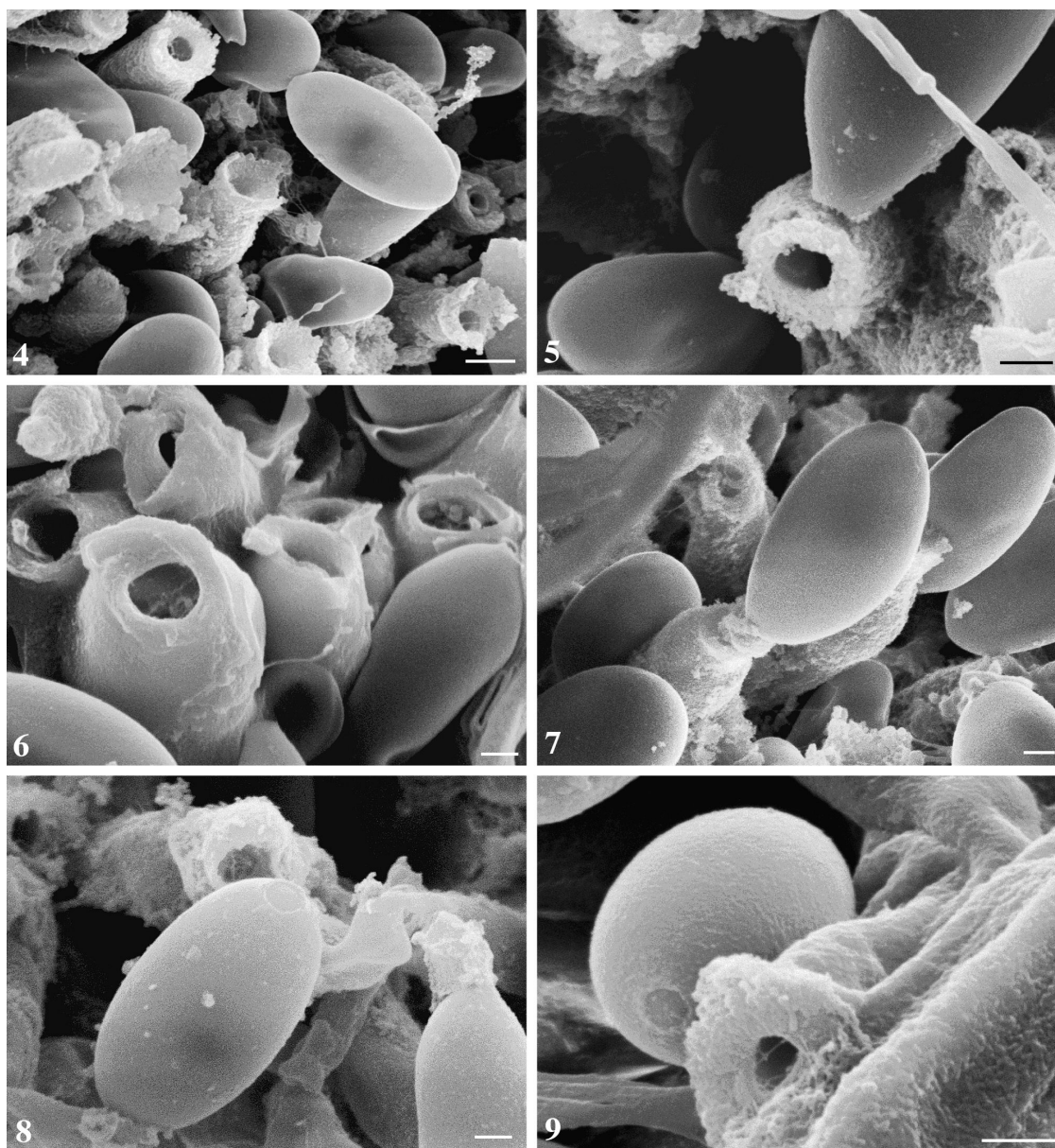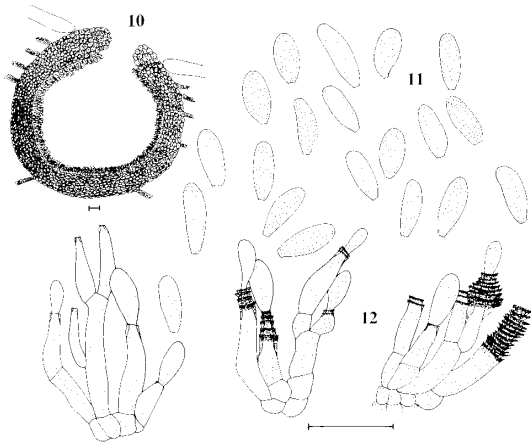

Figs 4–9. Conidiogenous cells and conidia of *Prosopidicola mexicana*, showing percurrent proliferation, the verrucose apical region of conidiogenous cells, and the basal scar on conidia. Scale bars = 1 µm.

Conidiogenous cells green-brown and smooth when young, becoming medium to dark green-brown and warty with maturity, subcylindrical to lageniform; proliferation appearing phialidic when young, with prominent periclinal thickening and a narrow apex (1.5–2 µm), but proliferating percurrently with age, apex obtaining flared collarettes, 3–4 µm wide; rarely with two loci per conidiogenous cell, proliferations aggregating and becoming dark green-brown, providing a warty, irregular appearance with age, with prominent periclinal thickening visible at the apical part of the conidiogenous cell, 5–16 × 3–4 µm. *Conidia* solitary, exuding in black masses, broadly ellipsoidal, medium brown, straight to slightly curved, rounded at the apex, tapering to a subtruncate base, with an inconspicuous dehiscence scar, smooth, thin-walled, aseptate, (8–)10–13(–20) × (3.5–)4.5–5.5(–6) µm (av. 11 × 5 µm).

Cultural characteristics: Colonies on PDA (reverse) smoke grey (21''''d) to greyish sepia (15'''ï); colony margin fimbriate; colonies reaching a mean radial growth of 46–55 mm at 30 °C in the dark after 8 d. Cardinal temperatures for growth: minimum above 10 °C, optimum 30 °C, maximum below 40 °C.

Substrate and distribution: Pathogenic to pods of *Prosopis glandulosa*; Mexico, U.S.A. (TX).

Figs 10–12. *Prosopidicola mexicana.* 10. Vertical section through a pycnidium. 11. Conidia. 12. Range of variation observed among young (left) to mature (right) conidiophores and conidiogenous cells. Scale bars = 10 µm.

Specimens examined: **Mexico**, Chihuahua, on pods of *Prosopis glandulosa*, 8 Sept. 2001, C.L. Lennox C 559, Herbarium specimen WH 65 (6). **U.S.A.**, Texas, La Joya, on pods of *Prosopis glandulosa*, 24 Sept. 2001, C.L. Lennox, **holotype** Herb. CBS 7948, culture ex-type CBS 113529; Texas, Zapata, on pods of *Prosopis glandulosa*, 24 Sept. 2001, C.L. Lennox 72c; Texas, Raymondville, on pods of *Prosopis glandulosa*, 25 Sept. 2001, C.L. Lennox C 570, C 571, C 572, C 573, Herbarium specimen WH 65 (7); Texas, La Joya, on pods of *Prosopis glandulosa*, 24 Aug. 1989, S. Neser, C 158, culture CBS 113530.

DISCUSSION

Coelomycetes having pycnidia with brown, 0–1-septate conidia that are formed on conidiophores that have been reduced to percurrently proliferating conidiogenous cells, usually suggest that the fungus is representative of *Coniothyrium s.l.* However, the phylogeny derived in Fig. 2 clearly illustrates that this morphology type has evolved more than once, and can even be found in different orders.

The type species of *Coniothyrium* is *C. palmarum*, which is allied to the *Leptosphaeriaceae/Phaeosphaeriaceae/Pleosporales* subclade (Fig. 2), and clusters with species having *Paraphaeosphaeria*-like teleomorphs. The genus *Paraphaeosphaeria*, however, is typified by *P. michotii* (Westend.) O.E. Erikss., and clusters quite apart from this clade, and appears to be conspecific to *C. minitans* W.A. Campb. The latter fungus is an important biocontrol organism, which also appears to be incorrectly classified in *Coniothyrium* and is transferred to *Paraconiothyrium* by Verkley *et al*. (this volume).

Other than these two subclades, additional subclades within the *Pleosporales* can also be found that have *Coniothyrium*-like anamorphs, suggesting that more anamorph and possibly teleomorph genera need to be distinguished here (Câmara *et al.* 2001, 2002, 2003).

The yeast-like growth and peculiar nature of *C. leucospermi*, a minor foliicolous pathogen of *Proteaceae*, has been discussed in detail by Taylor & Crous (2001). It is not surprising, therefore, to see this fungus cluster with other members of the black yeasts (Fig. 2). Although it is clearly not congeneric with *Coniothyrium s. str.*, its correct taxonomic position remains unresolved.

Although Corlett (1991) lists *Coniothyrium* as an anamorph of *Mycosphaerella*, this has never been proven in culture, and hence Crous *et al*. (2000) argued that these links were probably incorrect. Much to our astonishment, however, we found that certain species that resemble *Coniothyrium* in general morphology, notably the *Eucalyptus* leaf pathogen *C. ovatum* H.J. Swart (Crous 1998), and the canker pathogen *C. zuluense* M.J. Wingf., Crous & T.A. Cout. (Wingfield *et al.* 1997), belong in *Mycosphaerella*, and would need to be accommodated in a genus other than *Coniothyrium*.

The clustering of *Prosopidicola* in a clade separate from *Coniothyrium s. str.* is not surprising, given its rather unusual conidiogenous cell morphology, and mode of conidium proliferation. The fact that it clusters in the *Diaporthales* with genera such as *Diaporthe* (*Phomopsis*), *Cryphonectria* and *Endothia* (*Endothiella*) is, however, totally unexpected. From these results it is clear that the *Coniothyrium*-like morphology has evolved more than once within different fungal orders, and that plant pathologists will

be faced with increasing difficulties related to the identification of these organisms, as the standard circumscription of *Coniothyrium* (Sutton 1980), can no longer be applied with certainty. Furthermore, the value of conidiogenesis within the *Coniothyrium/Microsphaeropsis* complex, as well as conidiomatal structure in *Coniothyrium/Cyclothyrium*, will also require further clarification.

For the long-term management of *Prosopis* as an alien invasive weed in South Africa, a decision was taken at a national workshop to fully explore those biological control agents that target flowers, pods and seeds. Based on this, and preliminary tests, the potential of *Prosopidicola mexicana* as a biological agent to control invasive *Prosopis* species is being further investigated.

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