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Relatedness of *Custingophora olivaceae* to *Gondwanamyces* spp. from *Protea* spp.

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Species of *Gondwanamyces* are unusual amongst the ophiostomatoid fungi in that they are associated with the infructescences of *Protea* in South Africa. These fungi are distinguished from other ophiostomatoid genera by their *Knoxdavesia* anamorphs. *Knoxdavesia* spp. are peripherally similar, but distinct from *Stachybotrys* and *Phialocephala*. They are, however, morphologically identical to *Custingophora*. *Custingophora olivaceae*, was isolated from compost in Germany, a niche very different from that associated with fungi from *Protea* spp. The similarity of *Custingophora* and *Knoxdavesia* is such that they cannot be distinguished morphologically. The notion that *Gondwanamyces* might represent a teleomorph for *Custingophora* is intriguing. To determine whether an anamorph–teleomorph connection exists between *Knoxdavesia* and *Custingophora*, we determined the sequence of approximately 1100 nucleotides from the large subunit (LSU) ribosomal RNA gene. Results suggest that species of *Gondwanamyces* and *C. olivaceae* are phylogenetically related. This, as well as their morphological similarity suggests that *Gondwanamyces* and *C. olivaceae* have a shared ancestry. They are, however, sufficiently different that *Gondwanamyces* is probably not a teleomorph for *Custingophora olivaceae*.

Gondwanamyces proteae (M. J. Wingf., P. S. Van Wyk & Marasas) Marais & M. J. Wingf. is an unusual opthiostomatoid fungus associated with the infructescences of *Protea repens*, in the Cape Province of South Africa (Wingfield, Van Wyk & Marasas, 1988; Marais *et al*., 1996). Recently, other ophiostomatoid fungi have been isolated from *Protea* infructescences. These include *G. capense* (M. J. Wingf. & P. S. Van Wyk) Marais & M. J. Wingf., *Ophiostoma africanum* Marais & M. J. Wingf., *O. protearum* Marais & M. J. Wingf. and *O. splendens* Marais & M. J. Wingf. (Wingfield & Van Wyk, 1993; Marais & Wingfield, 1994; Marais, 1996; Marais *et al*., 1996).

Table 1. List of pyrenomycete taxa and the GenBank loci or source of DNA sequence

	GenBank locus/source
Aphysiostroma stercorarium	U47820
Ceratocystis fimbriata	U47822
C. virescens	U47824
Cercophora septentrionalis	U47823
Chaetomium globosum	U47825
Claviceps paspali	U47826
Cylindrocladium floridanum	U17408
C. scoparium	U17409
Daldinia concentrica	U47828
Diaporthe phaseolorum	U47830
Glomerella cingulata	U48428
Hirsutella thompsonii	U47831
Hypocrea schweinitzii	U47833
Microascus trigonosporus	U47835
Neocosmospora vasinfecta	U47836
Ophiostoma piliferum	U47837
Petriella setifera	U48421
Xylaria curta	U47840
X. hypoxylon	U47841

Species of *Ophiostoma* associated with *Protea* spp. have *Sporothrix* anamorphs, typical of other species in this genus. In contrast, species of *Gondwanamyces* from the same niche have *Knoxdavesia* anamorphs (Wingfield *et al*., 1988; Wingfield & Van Wyk, 1993; Marais & Wingfield, 1994; Marais, 1996; Marais *et al*., 1996). This anamorph genus is thus unusual amongst the ophiostomatoid fungi.

Knoxdavesia is characterized by conidia that arise from phialides with indistinct collarettes (Wingfield *et al*., 1988). The mode of conidial development is not typical of similar fungi but is holoblastic and occurs through an apical wall building process (Minter, Kirk & Sutton, 1982; Mouton *et al*., 1994; Wingfield *et al*., 1988). *Leptographium* is the only anamorph of *Ophiostoma* that resembles this genus (Wingfield *et al*., 1988). *Knoxdavesia* is, however, distinct from *Leptographium* in its absence of metulae and the presence of conidiogenous cells borne directly on the stipe which arise from well developed rhizoids (Wingfield *et al*., 1988).

Wingfield *et al*. (1988) considered *Knoxsdavesia* to be distinct from the morphologically similar *Stachybotrys* and *Phialocephala*. Unlike species of *Gondwanamyces*, *Stachybotrys* spp. typically have dark conidia and one species. *S. albipes* (Berk. & Broom) S. C. Jong & E. E. Davis has a teleomorph in *Melanopsamma* Niesel (Jong & Davis, 1976). Furthermore,

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C. fimbriata  TCAATAAGCG GAGGAAAAGA AACCAACAGG GATTGCCC-T AGTAACGGCG AGTGAAGCGC
O. piliferum  .......... .......... .......... ........-. .......... ..C.....--
G. proteae    .......... .......... .......... ........A. .......... ..C..--...
G. capense    .......... .......... .......... ........A. .......... ..C..--...
C. olivaceae  .......... .......... .......... ........-. .......... ..C..--...

C. fimbriata  GATGGTTTTG GTGAGGTGCC TTCCGAGTTC CCTGGAACGG GACGCCATAG AGGGTGAGAG
O. piliferum  ....C....C .C....C... .......... .......... .......... ..........
G. proteae    ....C..C.. .CA....... .......... .......... .......... ..........
G. capense    ....C..C.. .CA....... .......... .......... .......... ..........
C. olivaceae  ....C..C.. .C........ .......... .......... ......GC.. ..........

C. fimbriata  GGAATGCTGC TCTAAATGGG AGGT-ATATC TCTTCTAAAG CTAAATATAG GCTAGAGACC
O. piliferum  .......... .......... ....C.-... .......... .......--C ..-......-
G. proteae    .......... .......... ....-..... .......... .......--C ..-......-
G. capense    .......... .......... ....-..... .......... .......--C ..-......-
C. olivaceae  .......... .......... ....-..... .......... .......--C ..-......-

C. fimbriata  GCACGTGAAA TTGTTGAAAG GGAAGCGCCT ATGACCAGAC TTGTTTCT-- GGCAGT-TTC
O. piliferum  .......... .......... ........T. G......... ....-G.CC- ...-..-..A
G. proteae    .......... .......... ........T. G......... ....-G.CC- ..-T.AA..A
G. capense    .......... .......... ........T. G......... .-..-G.CC- ..-T.AA..A
C. olivaceae  .......... .......... ........T. G......... ....-G.CC- ..-T.AA..A

C. fimbriata  GGGG-ATGAA AGGCTCTGGG --AACGTAGC TTCTCCCCCT TCTGGGGGGA GTGTTATAGC
O. piliferum  ....-.-... ..A.GG.... --..T..... -.--...--- ..----.... ..........
G. proteae    ....-.-... .AA.GA.... --..T..G.. -....----- ..----.... ..........
G. capense    ....-.-... .AA.GA.... --..T..G.. -....----- ..----.... ..........
C. olivaceae  ....-.-... .AA.GG.... --..T..G.. -..-..---- ..----.... ..........

C. fimbriata  -GTAATGGTC ACAGGACGCC CGTCTTGAAA CACGGACCAA GGAGTTCAAC -CTTATGTGC
O. piliferum  C......... .......... .......... .......... .....-.... -.........
G. proteae    -.-....... .......... .......... .......... .....-.... -.........
G. capense    -.-....... .......... .......... .......... .....-.... -.........
C. olivaceae  -......... .......... .......... .......... .....-.... -.........

C. fimbriata  -CGG-CGCAT CATCGACCGA TTCT-GATGT T-TTCGGATG GATTTGAGTA AGAGCACACA
O. piliferum  -...G..... .T........ .C..-..... .C.-...... .......... ..........
G. proteae    -...G..... .......... ....-..... .C.-...... .......... ..........
G. capense    -...G..... .......... ....-..... .C.-...... .......... ..........
C. olivaceae  A...-..... .......... ....C..... .C.-...... .......... ......T.T.

C. fimbriata  TGGTGGAGGC TCGCCAGCGG TTCTGACGTG CAAATCGATC GTCAAATATG AGCATGGGGG
O. piliferum  .......... ....-..... .......... .......... .......... ..........
G. proteae    .......... ....-..... .......... .......... .......... ..........
G. capense    .......... ....-..... .......... .......... .......... ..........
C. olivaceae  ..-....... ....-..... .......... .......... .......... ..........

C. fimbriata  AGTGTTGAAT TTTCTTCAGT TTT-ATGAGG TAAAGCGAAT GATTAGGGAC TCGGGGGCGC
O. piliferum  .........- ---....... ...T...... .......... -......... ..........
G. proteae    ........-. C-...-.... ...-...... .......... .......... ..........
G. capense    ........-. C-...-.... ...-...... .......... .......... ..........
C. olivaceae  ........-. C-...-.... ...-...... .......... .......... ..........

C. fimbriata  TGAACGTGGG CATTCGAATG TACTCAACAC TAGTGGGCCA TTTTTGGTAA GCAGAACTGG
O. piliferum  .......... .G..A..... ..-....... .......... .......... ..........
G. proteae    .......... .......... .G--...... .......... .......... ..........
G. capense    .......... .......... .G--...... .......... .......... ..........
C. olivaceae  .......... .......... C.A-....T. .......... .......... ..........

AA-CAGCTCA AATTTGAAAT CTGGC-TACA TTCAGTGGT- CCGAGTTGTA ATTTGTAGAG
..-....... .......... .....-.C.- ..TT.G...- .......... ....-C....
..-....... .......... ....--.C-- ...T-G..-C .......... .....C....
..A....... .......... ....--.C-- ...T-G..-C .......... .....C....
..-....... .......... .....C.C.- ..GC.G...- .......... .....C....

CCCCGTACGG TTGGAT-ACC AAACC-TCTG TATAGCTCCT TCAACGAGTC GAGTAGTTTG
.......... .C....TG.. G.G..-.... .......... .......... ..........
.......... .....-C... G.G..G.-.. .G........ .......... ..........
.......... .....-C... G.G..G.-.. .G........ .......... ..........
.......... .....-C... G.G..G.-.. .G........ .-........ ..........

GATAGCGCAC AAGTAGAGTG ATCGAAAGAT GAAAAGCACT TTGAAAAGAG AGTTAAA-CA
.......... .......... .......... .......... .......... G......T-.
.......... ......GA.. .......... .......... .......... .......-..
.......... .......... .......... .......... .......... .......-..
.......... .......... .......... .......... .......... .......-..

GTTAGC-TTC GGGCT-GATT TACTCT-GCC AG--TACAGG CCAGCATCAG TTTGCT-GTC
.C....G... TC...A.-.C G...-.C... G.GC.-.... .......... ..C.-.C...
.C.G.TG... TCA.CA.-.C G...T.-... G.-C.-.... ........G. ....T.AT.-
.C.G.TG... TCA.CA.-.C G...T.-... G.-C.-.... ........G. ....T.AT.-
.C.-..G... TC..CA.-.C G...T.-... G.-C.-.... ........G. ......-...
.C.-..G... TC..CA.-.C G...T.-... G.-C.-.... ........G. ......-...

CCT-C--TG- CATAATACC- TTCGGCAGAC TGAGGACCGC GCTTC-G-CA AGGATGCTGC
.-.G.CG..- --.......C ...TT.G... .....TT... ..A..T.-.. .........G
..-G.CG.-- -........C ..T....... C......... ..A..T.-.. T.........
..-G.CG.-- -........C ..T....... C......... ..A..T.-.. T.........
...-.CG.-- -........- .......... C.......A. A.A..T.-.. T.........

GAGTGTTTGG GTGTAAAACC CC-AGCGCG- TAATGAAAGT GAA-CGTAGG TGAGAGCTT-
A......C.. ....T..... ..T-.....G -......... ...-.....A A........-
A......... ....T..... ..T-.....G -...T..C.. ...-...... .........-
A......... ....T..... ..T-.....G -...T..C.. ...-...... .........-
.......C.. ....T..... ..T-.....G -......C.. ...-...... .......C.C

GGG-TTGGAC CCGAAAGAAG GTGAACTATG CTTGTA-TAG GGTGAAGCCA GAGGAAACTC
...-...... .......... .......... ......-... .......... ..........
...-...... .......... .......... ......-... .......... ..........
...-...... .......... .......... ......-... .......... ..........
...-...... .......... .......... ...C..C... .......... ..........

CGAAAGACTA ATCGAACCTT CTAGTAGCTG GTT-CCAGCC GAAGTTTCCC TCAGGATAGC
.......... .......... .......... ...-...... -..-...... ..........
.......... .......... .......... ...-...... .......... ..........
.......... .......... .......... ...-...... .......... ..........
.......... .......... .......... ...-...... .......... ..........

TATATT--GC CTTCATCCAT TCTCAAACTT TAAATATGTA AGAAGCCCTT GTTACTTAAT
--....TA.. .......... .......... .......... .......... ..........
--....TA.. .......... .......... .......... .......... ........GC
--....TA.. .......... .......... .......... .......... ........GC
--....TA.. .......... .......... .......... .......... ...G......
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Fig. 1. The aligned sequence from the LSU rRNA gene for *Cer. fimbriata*, *O. piliferum*, *G. proteae*, *G. capense* (Viljoen, 1996) and *C. olivaceae*. The sequences are aligned using *Cer. fimbriata* as reference sequence. Homologous bases are indicated by a dot (.), and gaps by a dash (—).

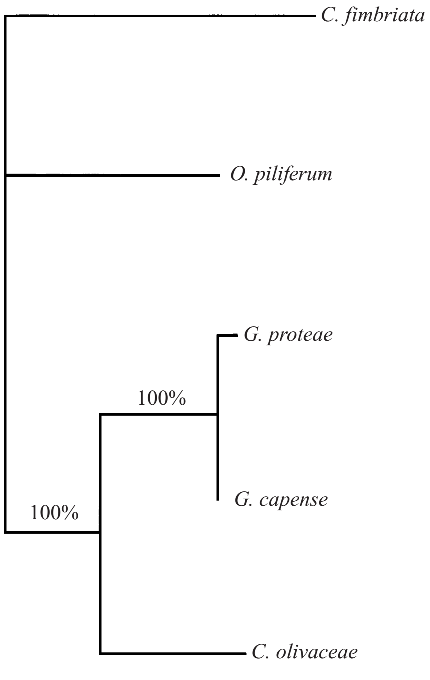

Fig. 2. The most parsimonious phylogenetic tree generated by the branch and bound option of PAUP 3.1.1. The percentages indicated on the tree are frequencies with which branching occurred in 1000 bootstrap replications.

conidiogenous cells in *Phialocephala* are usually subtended by metulae, which are absent in *Knoxsdavesia* (Wingfield *et al.*, 1988).

Custingophora was described to accommodate a single species, *C. olivaceae*, with phialides produced successively on the apex of a conidiophore (Stolk & Hennebert, 1968). The phialospores are produced continuously, collecting, at the apex of the conidial heads in drops of slime (Stolk & Hennebert, 1968). Conidiophores are mononematous, simple or sympodially branched by subapical proliferation, arising from foot cells, which may produce rhizoids (Stolk & Hennebert, 1968).

Species of *Knoxdavesia* have many characters in common with *C. olivaceae*. Colonies of both fungi growing on malt agar have olivaceous to brown pigmentation. Conidiophores develop from foot cells, characterized by well developed rhizoids. Phialides with collarettes develop successively. Phialospores are one celled, hyaline, smooth-walled and cylindrical to allantoid (Stolk & Hennebert, 1968; Wingfield *et al.*, 1988). Furthermore, both these fungi are similar in cell wall composition, have xylose but lack rhamnose, and are sensitive to the antibiotic cycloheximide (Wingfield *et al.*, 1988; Wingfield & Van Wyk, 1993; Marais & Wingfield, 1994; Marais, 1996). These are considered important characters in distinguishing between different ophiostomatoid genera (Smith, Patik & Rosinski, 1967; Jewell, 1974; Harrington, 1981; Upadhyay, 1981; De Hoog & Scheffer, 1984).

Although *Custingophora* and *Knoxdavesia* are virtually identical in morphology, they differ significantly in the niche they occupy. *Custingophora olivaceae* was isolated from compost in Germany and *K. proteae* and *K. capense* occur within the infructescences of various *Protea* spp. in the Western Cape Province of Southern Africa. It is possible that *Gondwanamyces* represents a teleomorph for *C. olivaceae*, or that these fungi share a common ancestor. An alternative is

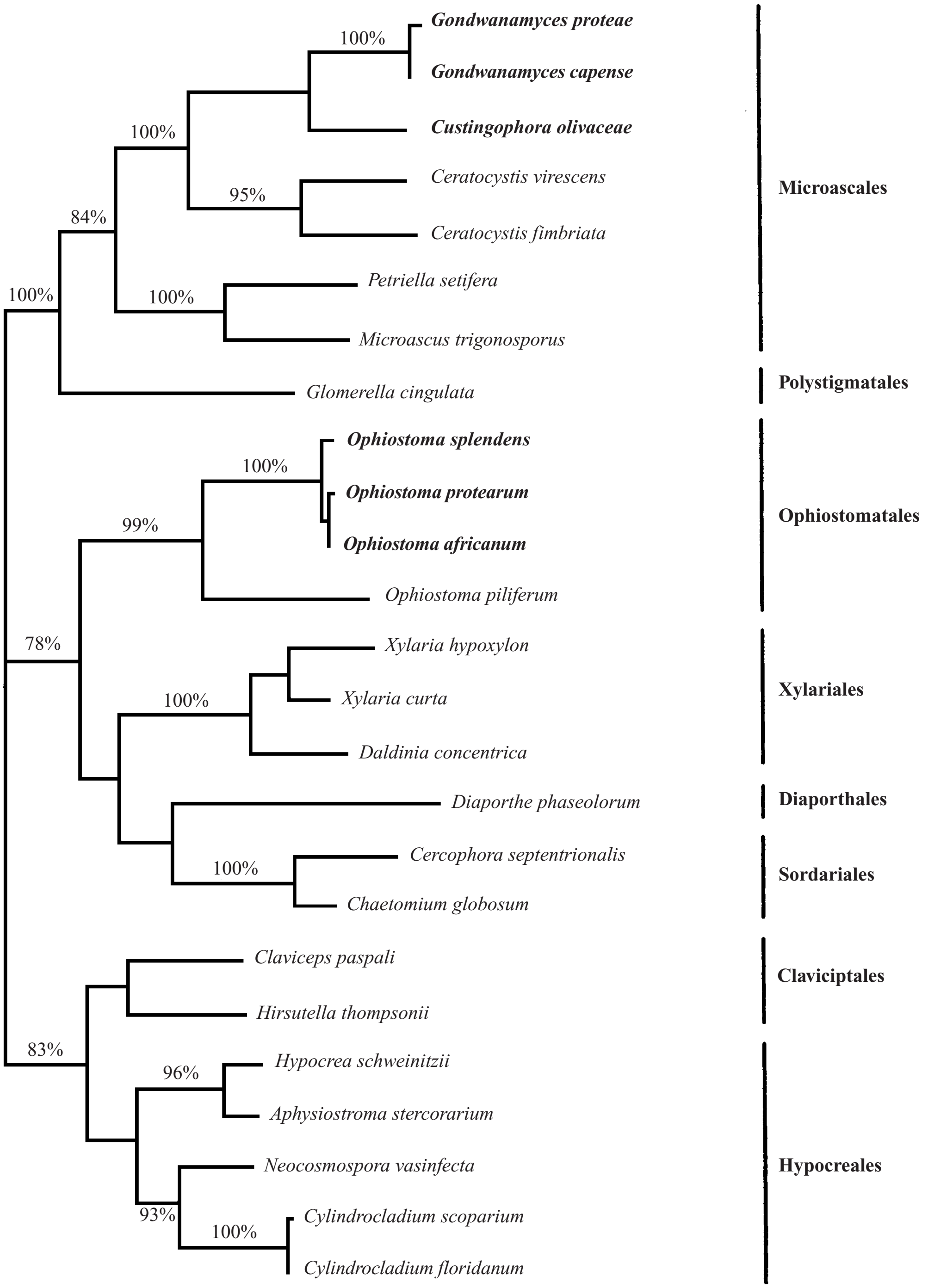

Fig. 3. The most parsimonious phylogenetic tree generated by 10 replicates of an heuristic search option of PAUL 3.1.1. The percentages indicated on the tree are frequencies with which branching occurred in 1000 bootstrap replications.

that they are phylogenetically unrelated but similar as a result of convergent evolution. To test these hypotheses, a PCR generated fragment, using primers for the large subunit (LSU) ribosomal RNA gene, was sequenced and analysed.

MATERIALS AND METHODS

Custingophora olivaceae was grown in Biolab malt extract (20 g l^{-1} at 20 °C. Mycelium was harvested by centrifugation for 10 min at 10 min at 10000 rpm, washed twice with sterile distilled water, lyophilised and stored at −20°. Total nucleic acids were extracted using the method of Viljoen (1996). The Expand High Fidelity PCR System (Boehringer–Mannheim) was used to generate PCR fragments for sequencing. Primer pair LS1 (Gutell & Fox, 1988) and LR6 (Vilgalys & Hester, 1990) were used to amplify a DNA fragment from the LSU rRNA gene in *C. olivaceae*. PCR fragments were treated with Proteinase K and purified using the PCR Wizard Magic Preps (Promega). Forward and reverse sequence reactions were performed using primers LS1, LR3, LR3R, LR5, LR5R and LR6 (Gutell & Fox, 1988; Vilgalys & Hester, 1990). The sequence of *C. olivaceae* was aligned manually to that of *G. proteae*, *G. capense* and a wider range of other pyrenomycetes (Table 1). The sequences were analysed using PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 1993). Bootstrapping (1000 replicates) was performed on sequence data to determine tree confidence intervals.

RESULTS AND DISCUSSION

A sequence of approx. 1100 nucleotides of the LSU rRNA gene from *C. olivaceae* was compared to previously sequenced regions of *G. proteae*, *G. capense*, *Cer. fimbriata* and *O. piliferum* (Fig. 1) (Viljoen, 1996). 137 bases were phylogenetically informative and used to generate a single most parsimonious tree (Fig. 2). The tree consistency index was 0·978 and the homoplasy index was 0·022. In 1000 bootstrap replicates the tree branching had a 100% confidence interval (Fig. 2). The species of *Gondwanamyces proteae* and *G. capense* grouped closely together (Fig. 2). *Custingophora olivaceae* clustered with species of *Gondwanamyces* (Fig. 2). The outgroup species, *Cer. fimbriata* and *O. piliferum* had basal branching points on the tree (Fig. 2).

The sequences of *C. olivaceae* and species of *Gondwanamyces* were compared to other pyrenomycete taxa (Spatafora & Blackwell, 1993). Ten replicates of an heuristic search utilizing the random sequence addition option in PAUP 3.1.1 produced one most parsimonious tree (Fig. 3). Species of *Gondwanamyces* and *C. olivaceae* clustered together within the Microascales with a 100% bootstrap confidence interval (Fig. 3). The tree consistency (CI), homoplasy (HI) and retention (RI) indices were 0·538, 0·462 and 0·705, respectively.

Sequence data from the ribosomal RNA genes have previously been used to demonstrate an anmorph–teleomorph connection between two other ophiostomatoid fungi, *Sporothrix schenkii* and *O. stenoceras* (Berbee & Taylor, 1992). In this study, the grouping of *C. olivaceae* with species of *Gondwanamyces* within the Microascales suggests that these fungi share common ancestry. Unlike the example reported by Berbee & Taylor (1992), *C. olivaceae* does not reside within *Gondwanamyces*. *Custingophora* appears, however, to be allied to *Gondwanamyces*. The sexual state for *C. olivaceae*, if one exists, would therefore, almost certainly be a member of the Microascales.

Custingophora olivaceae forms a monophyletic group together with species of *Gondwanamyces*. It is, therefore, more closely related to *Gondwanamyces* than to other ophiostomatoid genera, *Ceratocystis* (exemplified by *Cer. fimbriata*) and *Ophiostoma* (exemplified by *O. piliferum*). This level of relatedness is reflected by 41 base changes between *C. olivaceae* and *G. proteae*, while there are 81 base changes between *C. olivaceae* and *O. piliferum*. It would, therefore, appear that the similarity in morphology between *Knoxdavesia* and *Custingophora* is a result of shared ancestry between these fungi, and not solely as a result of convergent evolution. We speculate that the divergence between the ancestral state of *Knoxdavesia* and *Custingophora* occurred at the time when the association of *Gondwanamyces* with *Protea* spp. was initiated. In these respective lineages, species of *Gondwanamyces* maintained their teleomorph state, while *Custingophora* has apparently lost the ability to produce a teleomorph.

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