**Research Papers**

**Neocosmospora** spp. associated with dry root rot of citrus in South Africa

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**Summary.** Citrus is one of the most important fruit crops cultivated in South Africa. Internationally, citrus dry root rot is a common disease in major citrus production areas. Several abiotic and biotic factors are involved in disease development, in which *Neocosmospora* species are important biotic agents. The diversity of *Neocosmospora* species associated with dry root rot symptoms of *Citrus* trees cultivated in South Africa was evaluated using morphological and molecular analyses. Multi-locus analysis was conducted, based on fragments of seven loci including: ATP citrate lyase (*acl1*), calmodulin (*cal*), internal transcribed spacer region of the rRNA (ITS), large subunit of the rRNA (LSU), RNA polymerase largest subunit (*rpb1*), RNA polymerase second largest subunit (*rpb2*), and translation elongation factor 1-alpha (*tef1*). A total of 62 strains representing 11 *Neocosmospora* species were isolated from crowns, trunks and roots of citrus trees affected by dry root rot, as well as from soils sampled in affected citrus orchards. The most commonly isolated taxa were *N. citricola*, *N. ferruginea* and *N. solani*, while rarely encountered taxa included *N. brevis*, *N. crassa*, *N. hypothemenni* and *N. noneumartii*. Furthermore, four *Neocosmospora* species are also newly described, namely *N. addoensis*, *N. citricola*, *N. gamtoosensis* and *N. lerouxii*.

**Keywords.** Citrus decline, morphology, multigene phylogeny, systematics.

**INTRODUCTION**

Citrus is one of the most important world fruit crops, and South Africa is among the largest producers and exporters of citrus fruit (FAOSTAT, 2019). Citrus dry root rot (DRR) is a common problem among citrus growers, reported in major production areas such as Australia (Broadbent, 2000), Florida, California and Texas in the United States of America (Graham et
al., 1985), Italy (Polizzi et al., 1992), Oman (Nemec et al., 1980; Bender, 1985), Pakistan (Kore and Mane, 1992; Conzulex et al., 1997; Verma et al., 1999; Rehman et al., 2012), Turkey (Kurt et al., 2020), Tunisia, Greece and Egypt (El-Mohamedy, 1998; Yaseen and D’Onghia, 2012).

While the aetiology of DRR is multifactorial and not completely understood, it is usually attributed to Neocosmospora (Fusarium) solani sensu lato. However, several species of Neocosmospora, but also Fusarium, are commonly found in orchard soils and citrus plants. These two closely related fusarioid genera encompass important plant pathogens, and are associated with major diseases of citrus (Menge, 1988; Derrick and Timmer, 2000; Sandoval-Denis et al., 2018), including DRR, root rot, feeder root rot, wilt, twig dieback and citrus decline (Menge, 1988; Spina et al., 2008). Fusarium equiseti was recovered from citrus roots in Florida (Smith et al., 1988), while F. proliferatum, F. sambucinum and Neocosmospora solani were found in Greece (Malikoutsaki-Mathioiud et al., 1987). Fusarium oxysporum f. sp. citri was reported as responsible for the wilt of citrus in Tunisia (Hannachi et al., 2014). Fusarium oxysporum and strains first assigned to “F. ensiforme” and later reidentified as Neocosmospora brevis were also reported from DRR in Italy (Sandoval-Denis et al., 2018; 2019), while a number of Neocosmospora species have been reported in association with DRR of citrus in Europe (Sandoval-Denis et al., 2018).

Neocosmospora (Hypocreales, Nectriaceae), comprises species with varied ecologies, including saprobes, endophytes, and plant and animal pathogens. Pathogenic species of Neocosmospora are known to affect more than 100 plant host families and diverse animal species, including humans (Sandoval-Denis et al., 2019). Although Neocosmospora (1899) is an old and well-established name, recent phylogenetic, morphological and ecological data (Lombard et al., 2015) provided additional support for this genus as one of several distinct fusarioid genera in the Nectriaceae. Follow-up revisions have corrected the taxonomy of most Neocosmospora species known to date, including the main pathogenic clades (Sandoval-Denis and Crous, 2018; Sandoval-Denis et al., 2019).

Previous studies have demonstrated how DRR, caused by the association between stressed plants and Neocosmospora species, can generate sudden decline of plants weakened by abiotic and biotic factors, such as root injuries, Phytophthora root rot, graft incompatibility, poor drainage, poor soil aeration, excess fertilizer, or soil pH (Menge, 1988; Polizzi et al., 1992). Chlorosis, poor vigour, wilt, leaf abscission and degeneration are visible in affected plants for several years before they suddenly die. Examination of scaffold roots, crowns and basal trunks usually shows wood staining (Timmer et al., 1979; Timmer 1982). Rot of the fibrous roots is also visible and associated with canopy size reductions, defoliation, dieback and sloughing of root cortices (Nemec and Baker, 1992). This disease has been managed by planting resistant rootstocks. However, during the last decade, trifoliolate orange (Poncirus trifoliata) rootstocks, which are very susceptible to DRR, have been widely used, due to their resistance to virus and soilborne pathogens (i.e.: Citrus Tristeza Virus) (Fang et al., 1998).

Since 2013, sudden, devastating decline and death of citrus trees has been reported in the Gamtoos and Sundays River Valleys production areas in the Eastern Cape province of South Africa. This decline is typically observed on 4- to 10-year-old trees with the trifoliolate rootstocks Carrizo citrange and Swingle citrumelo. As scions, these declining trees are of various citrus types, including lemons, oranges and mandarins. To date, little is known about DRR-like diseases in citrus orchards in South Africa. Given the importance of citrus production, and specifically in the two areas of South Africa, as well as the relevant economic impact of DRR in other countries, further research was needed to increase understanding of the aetiology of this disease.

Morphological, cultural and molecular characteristics of the fungal species associated with symptomatic trees were investigated in this study by employing large-scale sampling to isolate the pathogens involved, and to identify their strains according to modern taxonomic concepts via morphological characterization and multilocus DNA sequence data. In 2018 several surveys were conducted in citrus orchards with the aims to: (1) conduct extensive surveys to sample symptomatic plant material; (2) cultivate as many of the associated fungi as possible; (3) conduct DNA multi-locus sequence analyses combined with morphological characterization of isolates obtained; and (4) compare the obtained results with known wood decay fungi previously associated with trees displaying characteristic DDR symptoms.

**MATERIALS AND METHODS**

**Sampling, fungal collection and isolation**

The Patensie (Gamtoos River Valley) and Kirkwood (Sundays River Valley) areas were surveyed during the second half of 2018. During these visits, the external and internal symptoms of diseased trees were examined. Scaffold roots, crown and trunk portions taken from between soil level and scion unions, were collected in
both the survey areas. Samples were each transversally cut into 3-cm-thick discs, which allowed observation of internal wood decay symptoms.

Wood fragments (3 × 3 mm) were cut from necrotic and healthy tissues and also from the margins between them. Each fragment was then surface sterilised by soaking in 70% ethanol for 5 s, 4% sodium hypochlorite for 90 s, sterile water for 60 s and then dried on sterile filter paper. Fragments were placed on potato dextrose agar (PDA) amended with 100 μg mL⁻¹ streptomycin (PDA-S), and were then incubated at 25°C. Characteristic Neocosmospora colonies were collected from these plates by hyphal tipping onto clean PDA-S plates. The isolates used in this study are maintained in the culture collection of the Department of Plant Pathology, University of Stellenbosch, Stellenbosch, South Africa, and at the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, The Netherlands (Table 1).

**Morphological studies of isolates**

Morphological studies were carried out as indicated elsewhere (Leslie and Summerell, 2006; Sandoval-Denis and Crous, 2018; Sandoval-Denis et al., 2019). Macroscopic characteristics and fungal colony appearance of each isolate was determined after culturing on oatmeal agar (OA), potato dextrose agar (PDA) and synthetic nutrient-poor agar (SNA; Nirenberg, 1976), and incubation for 7–14 d at 24°C in darkness under a 12 h/12 h light/dark cycle using cool fluorescent light. Colour nomenclature follows that of Rayner (1970). Fungal micromorphology was studied using 7–14-d-old cultures on carnation leaf agar (CLA; Fisher et al., 1982) and SNA, incubated at 24°C in a 12 h/12 h near UV light/dark cycle. Photomicrographs were captured using a Nikon Eclipse 80i microscope with Differential Interference Contrast (DIC) optics and a Nikon AZ100 dissection microscope, both equipped with a Nikon DS-Ri2 high definition colour digital camera. Measurements were recorded using Nikon NIS-elements D software v. 4.50, from at least 50 randomly selected elements for each structure.

**Molecular studies of isolates**

Total genomic DNA was extracted from isolates grown on malt extract agar (MEA; Crous et al., 2019), incubated for 7 d at room temperature (approx. 24°C). Mycelium was scraped from the colony surfaces with the aid of sterile scalpels, and DNA was isolated using the Wizard® Genomic DNA purification Kit (Promega Corporation) following the manufacturer’s protocol.

Seven gene fragments were PCR amplified using the following primer combinations with protocols described elsewhere: acl1-230up and acl1-1220low for the larger subunit of the ATP citrate lyase (acl1; Gräfenhan et al., 2011), CAL-228F and CAL2Rd for calmodulin (cal; Carbono and Kohn, 1999; Quaedvlieg et al., 2014), ITS4 and ITS5 for the internal transcribed spacer region of the rRNA (ITS; White et al., 1990), LR0R and LR5 for a partial fragment of the large subunit of the rRNA (LSU; Vilgalys and Hester, 1990; Vilgalys and Sun, 1994), Fa and G2R for the RNA polymerase largest subunit (rpb1; O’Donnell et al., 2010), 5f2 and 7cr plus 7cf and 11ar for two non-contiguous fragments of the RNA polymerase second largest subunit (rpb2; Liu et al., 1999; Sung et al. 2007), and EF-1 and EF-2 for the translation elongation factor 1-alpha gene (tefl; O’Donnell et al., 2008). Sequencing was carried out in both directions on an ABI Prism 3730XL DNA Analyzer (Applied Biosystems) using the same primer pairs used for amplification, plus the internal sequencing primers F6, F8 and R8 for rpb1 (O’Donnell et al., 2010). Consensus sequences were assembled using Seqman Pro v. 10.0.1 (DNASTAR).

Sequence alignments were constructed and analysed individually for each gene partition, including DNA sequences representing the phylogenetic diversity of Neocosmospora selected according to recently published phylogenies (Guarnaccia et al., 2019; Sandoval-Denis et al., 2019). Alignments were achieved using MAFFT (Katoh et al., 2019) as implemented on the European Bioinformatics Institute (EMBL-EBI) portal (www.ebi.ac.uk), and were visually inspected and then manually corrected if needed using MEGA v. 6 (Tamura et al., 2013).

Phylogenetic analyses were based on two independent algorithms: Maximum-Likelihood, using Random Accelerated (sic) Maximum Likelihood (RAxML) v. 8.2.10 (Stamatakis, 2014) and Bayesian inference (BI) under MrBayes v. 3.2.6 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The analyses were carried out using the CIPRES Science Gateway portal (www.phylo.org: Miller et al., 2012). Single-gene phylogenies were compared visually to check for topological conflict between significantly supported clades, and then as combined multilocus phylogenies (Mason-Gamer and Kellogg, 1996; Wiens 1998). A first analysis based on combined rpb2 and tefl sequence data was directed to identify Neocosmospora spp. from isolates obtained from symptomatic citrus trees. A second analysis including the combined seven gene dataset was directed to clarify the phylogeny of South African citrus Neocosmospora isolates with uncertain phylogenetic position or deter-
Table 1. Collection data and GenBank accession numbers of isolates included in this study.

| Species                  | Strain number | Country     | Host                  | GenBank sequence accession number |
|--------------------------|---------------|-------------|-----------------------|----------------------------------|
|                          |               |             |                       | **Species Strain number** | **Country** | **Host** | | **GenBank sequence accession number** |
|                          |               |             |                       | **ad** | **cal** | **ITS** | **LSU** | **rpb1** | **rpb2** | **tefl** |
| *Geejayessia atrofusca*  | NRRL 22316    | USA         | Staphylea trifolia   | -     | -      | AF178423 | AF178392 | JX171496 | EU329502 | AF178361 |
| *Geejayessia cicatricum* | CBS 125552    | Slovenia     | Dead twig            | HQ728171 | HQ728145 | MH875038 | HQ728153 | HM626644 |
| *Neocosmospora addoensis*| CBS 146508 = VG268 = CPC 37126 | South Africa | *Citrus sinensis* - crown | MW218003 | MW218050 | MW173040 | MW173031 | MW218096 | MW446573 | MW248739 |
|                          | CBS 146509 = VG279 = CPC 37127 | South Africa | *Citrus sinensis* - crown | MW218004 | MW218051 | MW173041 | MW173032 | MW218097 | MW446574 | MW248740 |
|                          | CBS 146510T = VG281 = CPC 37128 | South Africa | *Citrus sinensis* - crown | MW218005 | MW218052 | MW173042 | MW173033 | MW218098 | MW446575 | MW248741 |
| *Neocosmospora ampla*    | CBS 202.32T   | German East Africa | Coffea sp.          | -     | -      | LR583701 | LR583909 | -      | LR583815 | LR583594 |
| *Neocosmospora bataticola* | CBS 144397    | USA         | Ipomoea batatas      | MW218006 | MW218053 | AF178407 | AF178376 | MW218099 | EU329509 | AF178343 |
|                          | CBS 144398T   | USA         | Ipomoea batatas      | MW218007 | MW218054 | AF178408 | AF178377 | MW218100 | FJ240381 | AF178344 |
| *Neocosmospora borneensis* | CBS 145462T | Indonesia  | Bark or recently dead tree | -     | -      | AF178415 | AF178384 | -      | EU329515 | AF178352 |
| *Neocosmospora bostryoides* | CBS 144.25NT | Honduras  | Soil                  | MW218008 | MW218055 | LR583704 | LR583912 | MW218101 | LR583818 | LR583597 |
|                          | CBS 392.66 | Unknown Bertholletia excelsa | MW218009 | MW218056 | LR583705 | LR583913 | MW218102 | LR583819 | LR583598 |
| *Neocosmospora brevicona* | CBS 204.31T | Indonesia | Gladiolus sp.         | MW218010 | MW218057 | LR583707 | LR583915 | MW218103 | LR583821 | LR583600 |
| *Neocosmospora brevis*   | CBS 130326 | USA         | Human eye             | -     | -      | DQ094351 | DQ236393 | -      | EF470136 | DQ248669 |
| VG150                    | South Africa | *Citrus sinensis* - crown | -     | -      | MW173043 | -      | -      | MW446576 | MW248742 |
| VG152                    | South Africa | *Citrus sinensis* - crown | -     | -      | MW173044 | -      | -      | MW446577 | MW248743 |
| VG157                    | South Africa | *Citrus sinensis* - crown | -     | -      | MW173045 | -      | -      | MW446578 | MW248744 |
| *Neocosmospora catenata* | CBS 143228 | USA         | Stegostoma fasciatum  | MW218011 | MW218058 | KC808255 | KC808255 | MW218104 | KC808354 | KC808213 |
| CBS 143229T              | USA         | Stegostoma fasciatum  | MW218012 | MW218059 | KC808256 | KC808256 | MW218105 | KC808355 | KC808214 |
| *Neocosmospora citricola* | CBS 146511 = VG202 = CPC 37129 | South Africa | *Citrus sinensis* - crown | MW218013 | MW218060 | MW173046 | MW173034 | MW218106 | MW446579 | MW248745 |
| CBS 146512 = VG307 = CPC 37130 | South Africa | *Citrus sinensis* - crown | MW218014 | MW218061 | MW173047 | MW173035 | MW218107 | MW446580 | MW248746 |
| CBS 146513T = VG343 = CPC 37131 | South Africa | *Citrus sinensis* - crown | MW218015 | MW218062 | MW173048 | MW173036 | MW218108 | MW446581 | MW248747 |
| VG17                     | South Africa | *Citrus sinensis* - crown | -     | -      | MW173049 | -      | -      | MW446582 | MW248748 |
| VG30                     | South Africa | *Citrus sinensis* - crown | -     | -      | MW173050 | -      | -      | MW446583 | MW248749 |
| VG139                    | South Africa | *Citrus sinensis* - crown | -     | -      | MW173051 | -      | -      | MW446584 | MW248750 |
| VG140                    | South Africa | *Citrus sinensis* - crown | -     | -      | MW173052 | -      | -      | MW446585 | MW248751 |
| VG183                    | South Africa | *Citrus sinensis* - crown | -     | -      | MW173053 | -      | -      | MW446586 | MW248752 |
| VG197                    | South Africa | *Citrus sinensis* - root scaffold | -     | -      | MW173054 | -      | -      | MW446587 | MW248753 |
| VG203                    | South Africa | *Citrus sinensis* - crown | -     | -      | MW173055 | -      | -      | MW446588 | MW248754 |
Table 1. (Continued).

| Species                     | Strain number¹ | Country          | Host             | GenBank sequence accession number² |
|-----------------------------|----------------|------------------|------------------|------------------------------------|
| Neocosmospora crassa        | CBS 144386T    | France           | Unknown          | MW218016 MW218063                  |
| Neocosmospora cucurbitae    | CBS 410.62     | Netherlands      | Cucurbita viticifolia | - - MW173061 MW173037 MW173060 MW173037 |
| Neocosmospora cyanescens    | CBS 518.82T    | Netherlands      | Human foot       | MW218017 MW218064                  |
| Neocosmospora diminuta      | CBS 144390T    | Unknown          | Coelocaryon preussi | - - MW173061 MW173037 MW173060 MW173037 |
| Neocosmospora elegans       | CBS 144395     | Japan            | Xanthoxylum piperitum | - - MW173061 MW173037 MW173060 MW173037 |
| Neocosmospora falciformis   | CBS 475.67T    | Puerto Rico      | Human mycetoma   | MW218021 MW218068                  |
| Neocosmospora ferruginea    | CBS 109028T    | Switzerland      | Human subcutaneous nodule | - - MW173061 MW173037 MW173060 MW173037 |

(Continued)
| Species                     | Strain number$^1$ | Country     | Host                         | GenBank sequence accession number$^2$ | acl | cal | ITS | LSU | rpb1 | rpb2 | tef1 |
|----------------------------|-------------------|-------------|------------------------------|----------------------------------------|-----|-----|-----|-----|------|------|------|
|                            |                   |             |                              |                                        |     |     |     |     |      |      |      |
| Neocosmospora gamsii        | CBS 143207$^T$    | USA         | bronchoalveolar lavage fluid |                                        |     |     |     |     |      |      |      |
|                            | CBS 143211        | USA         | Humidifier coolant           |                                        |     |     |     |     |      |      |      |
| Neocosmospora gamtoosensis  | CBS 146502$^T$ = VG16 = CPC 37120 | South Africa | Citrus sinensis - crown | MW218023 MW218070 MW173063 MW173038 MW218116 MW446611 MW248762 |     |     |     |     |      |      |      |
| Neocosmospora haematococca | CBS 119600$^T$    | Sri Lanka   | Dying tree                  |                                        |     |     |     |     |      |      |      |
| Neocosmospora hypothenemi   | CBS 145464$^T$    | Benin       | Hypothenemus hampei         | MW218024 - LR583715 LR583923 MW218117 JF741716 JF740850 |     |     |     |     |      |      |      |
|                            | CBS 145466        | Uganda      | Hypothenemus hampei         | MW218025 MW218071 - - MW173066 - - MW446614 MW248765 |     |     |     |     |      |      |      |
| VG11                       | South Africa      | Citrus sinensis - crown | - - | MW173064 - - | MW446612 MW248763 | |     |     |     |      |      |      |
| VG14                       | South Africa      | Citrus sinensis - crown | - - | MW173065 - - | MW446613 MW248764 | |     |     |     |      |      |      |
| VG49                       | South Africa      | Citrus sinensis - root scaffold | - - | MW173066 - - | MW446614 MW248765 | |     |     |     |      |      |      |
| VG189                      | South Africa      | Citrus sinensis - crown | - - | MW173067 - - | MW446615 MW248766 | |     |     |     |      |      |      |
| VG328                      | South Africa      | Citrus sinensis - crown | - - | MW173068 - - | MW446616 MW248767 | |     |     |     |      |      |      |
| Neocosmospora ipomoeae     | CBS 353.87        | Netherlands | Gerbera sp.                 | MW218026 MW218072 LR583717 LR583925 MW218119 LR583831 DQ247639 |     |     |     |     |      |      |      |
| CBS 833.97                 | Netherlands       | Rosa sp.    | MW218027 MW218073 LR583719 LR583927 MW218120 LR583833 LR583611 | |     |     |     |     |      |      |      |
| Neocosmospora keratoplastica| CBS 490.63$^T$   | Japan       | Human                       | MW218028 MW218074 LR583721 LR583929 MW218121 LT906562 LT906670 |     |     |     |     |      |      |      |
| CBS 144389                 | Belgium           | Greenhouse humic soil | MW218029 MW218075 LR583722 LR583930 MW218122 LR583836 LR583613 | |     |     |     |     |      |      |      |
| Neocosmospora lerouxiix     | CBS 146514$^T$ = VG48 = CPC 37132 | South Africa | Citrus sinensis - root scaffold | MW218030 MW218076 MW173069 MW173039 MW218123 MW446617 MW248768 | |     |     |     |     |      |      |      |
| Neocosmospora lichenicola  | CBS 509.63        | Brazil      | Air                          | - - LR583728 LR583936 - - LR583843 LR583618 | |     |     |     |     |      |      |      |
| CBS 623.92$^T$             | Germany           | Human necrotic wound         | - - LR583730 LR583938 - - LR583845 LR583620 | |     |     |     |     |      |      |      |
| Neocosmospora liriodendri  | CBS 117481$^T$    | USA         | Liriodendron tulipifera      | MW218031 MW218077 AF178404 AF178373 MW218124 EU329506 AF178340 |     |     |     |     |      |      |      |
| Neocosmospora longisima     | CBS 126407$^T$    | New Zealand | Tree bark                    | - - LR583731 LR583939 - - LR583846 LR583621 | |     |     |     |     |      |      |      |
| Neocosmospora macrospera    | CBS 142424$^T$    | Italy       | Citrus sinensis              | MW218032 MW218078 LT746266 LT746281 MW218125 LT746331 LT746218 | |     |     |     |     |      |      |      |
| CPC 28193                  | Italy             | Citrus sinensis              | MW218033 MW218079 LT746268 LT746283 MW218126 LT746333 LT746220 | |     |     |     |     |      |      |      |

(Continued)
| Species                        | Strain number | Country   | Host                        | ad    | cal    | ITS     | LSU     | rpc1   | rpc2   | tefl   |
|-------------------------------|---------------|-----------|-----------------------------|-------|--------|---------|---------|--------|--------|--------|
| Neocosmospora martii          | CBS 115659TT   | Germany   | Solanum tuberosum           | -     | -      | JX435206| JX435206| JX435256| JX435156|
| Neocosmospora metavorans      | CBS 135789TT   | Greece    | Human pleural effusion      | MW218034 | MW218080 | LR583738| LR583946| MW218127| LR583849| LR583627|
| Neocosmospora mori            | CBS 143219     | Spain     | Human foot                  | MW218035 | MW218081 | LR583744| LR583948| MW218128| LR583851| LR583629|
| Neocosmospora mori            | CBS 145467TT   | Japan     | Morus alba                  | -     | -      | DQ943055| DQ236347| -       | -      | EU329499| AF178358|
| Neocosmospora noneumartii     | CBS 115658TT   | Israel    | Solanum tuberosum           | MW218036 | MW218082 | LR583745| LR583949| MW218129| LR583630|
| Neocosmospora oblonga         | CBS 130325T    | USA       | Human eye                   | -     | -      | LR583746| LR583950| LR583853| LR583631|
| Neocosmospora paracemartii    | CBS 123669TT   | Argentina | Solanum tuberosum           | -     | -      | LR583747| LR583951| LR583855| DQ247549|
| Neocosmospora parcemososa     | CBS 115695TT   | South Africa | Soil                      | MW218037 | MW218083 | JX435199| JX435199| JX435249| JX435149|
| Neocosmospora persoae         | CBS 144142T    | Italy     | Persea americana            | MW218038 | MW218084 | LT991940| LT991947| MW218130| LT991909| LT991902|
| Neocosmospora petrophila      | CBS 203.32     | South Africa | Pelargonium sp.            | MW218039 | MW218085 | DQ943202| DQ236362| MW218131| LR583857| DQ246835|
| Neocosmospora piperis         | CBS 145470TT   | Brazil    | Piper nigrum                | -     | -      | AF178422| AF178539| -       | -      | AF178360|
| Neocosmospora pisi            | CBS 123669TT   | USA       | Progeny of parentals from Pisum sativum and soil | -     | -      | LR583755| LR583957| LR583862| LR583636|
| Neocosmospora protoensiformis | CBS 145471T    | Venezuela | Dicot tree                  | -     | -      | AF178399| AF178368| LR583646| KY556454|
| Neocosmospora pseudoradicicola| CBS 145472TT   | Papua New Guinea | Diseased cocoa pods         | MW218041 | MW218087 | JF740899| JF740899| MW218133| JF741084| JF740757|
| Neocosmospora quercicola      | CBS 141.90T    | Italy     | Quercus cerris              | -     | -      | LR583760| LR583964| LR583869| DQ247634|
| Neocosmospora regularis       | CBS 230.34T    | Netherlands | Pisum sativum              | -     | -      | LR583763| LR583967| LR583873| LR583643|
| Neocosmospora solivicola      | CBS 123846T    | USA       | Liriodendron tulipifera     | -     | -      | LR583766| LR583971| LR583876| LR583646|
| Neocosmospora solani          | CBS 140079TT   | Slovenia  | Solanum tuberosum           | MW218042 | MW218088 | KT313633| KT313633| KT313623| KT313611|
| VG36                          | South Africa   | Citrus sinensis - root scaffold | -     | -      | MW173072| -       | -       | MW446621| MW248771|
| VG38                          | South Africa   | Citrus sinensis - root scaffold | -     | -      | MW173073| -       | -       | MW446622| MW248772|

(Continued)
| Species                      | Strain number | Country          | Host                          | GenBank sequence accession number | acl  | cal  | ITS   | LSU   | rpb1  | rpb2  | tef1  |
|------------------------------|---------------|------------------|-------------------------------|-----------------------------------|------|------|-------|-------|-------|-------|-------|
| Neocosmospora sp. (FSSC12)   | CBS 143212    | USA              | Turtle egg                    | MW218043 MW218089                 | -    | -    | MW173074 | -    | MW446623 MW248773 |
| Neocosmospora spathulata      | CBS 143226    | USA              | Kemps Ridley turtle           | MW218044 MW218090                 | -    | -    | MW173075 | -    | MW446624 MW248774 |
| Neocosmospora stercicola     | CBS 142481\(T\) | USA              | Human synovial fluid          | MW218045 MW218091                 | -    | -    | MW173076 | -    | MW446625 MW248775 |
| Neocosmospora suttoniana     | CBS 143214\(T\) | USA              | Human wound                   | MW218046 MW218092                 | -    | -    | MW173077 | -    | MW446626 MW248776 |
| Neocosmospora tonkinensis     | CBS 115.40\(T\) | Vietnam          | Musa sapientium               | MW218048 MW218094                 | -    | -    | MW173078 | -    | MW446627 MW248777 |
| Neocosmospora vasinfecta     | CBS 446.93    | Japan            | Soil                          | MW218049 MW218095                 | -    | -    | MW173079 | -    | MW446628 MW248778 |
| CBS 533.65                   | USA           | Unknown          | Solanum lycopersicum          | MW218049 MW218095                 | -    | -    | MW173080 | -    | MW446629 MW248779 |

1 CBS: Westerdijk Fungal Biodiversity Institute (WI), Utrecht, The Netherlands; CPC: Collection of P.W. Crous, held at WI; NRRL: Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, USDA, Peoria, IL, USA; VG: Working collection of J. Van Niekerk held at Department of Plant Pathology, University of Stellenbosch, South Africa; ET: Ex-epitype, IT: Ex-isotype, NT: Ex-lectotype.

2 acl: ATP citrate lyase largest subunit; cal: calmodulin; ITS: internal transcribed spacer region of the rDNA; LSU: large subunit of the rDNA; rpb1: RNA polymerase largest subunit; rpb2: RNA polymerase second largest subunit; tef1: translation elongation factor 1-alpha. Sequences generated in the present study are shown in **bold font**.
mined as putative novel species in the previous analyses. For RAxML analyses, the default parameters were selected and clade stability was determined by bootstrap (BS) analysis using 1000 repetitions. Bayesian analyses consisted of two parallel runs of 5 M generations, with the stop-rule on, set to 0.01. The sampling frequency was set to 1000 generations, and consensus trees and posterior probability values (PP) were calculated after discarding the first 25% of sampled trees as the burn-in fraction. The best evolutionary model for each gene partition was determined using MrModelTest v. 2.3 (Nylander, 2004).

RESULTS

Sampling, fungal collection and isolation

In the Patensie and Kirkwood areas, diseased trees initially showed yellowing, wilting leaves and dieback of branch tips. Symptoms subsequently progressed with defoliation and sudden decline before the plants died. Inspection of affected trees showed cracks or blisters on the trunks above the crowns with, rarely, gum exudates (Figure 1). If each trunk was transversely cut, brown to black discolouration and necrosis of the vascular tissue became visible with different extensions (Figure 2). Similar discolouration and stains were visible into the scaffold roots. Symptoms were observed in orchards older than 8 years. Incidence of symptomatic plants was in some cases up to 50% of affected trees in orchards.

A total of 62 monosporic isolates resembling those of Neocosmospora were collected from the sampled citrus trees. Among them, 33 isolates were obtained from the Kirkwood area and 29 from Patensie. Thirty-eight were isolated from trunk portions, 22 from scaffold roots, and two from soil surrounding infected roots. Among the isolates collected from trunks, 17 were from necrotic tissue, two from healthy tissue and 14 from the margins between necrotic and healthy tissues.

Phylogenetic studies and identification of the pathogens

A first analysis, based on combined rpb2 and tef1 loci, was conducted to identify the Neocosmospora isolates obtained from symptomatic citrus trees. The dataset contained 129 isolates, representing 62 South African isolates, as well as 67 ex-type or reference strains representing 46 taxa in Neocosmospora, and two outgroup taxa (Geeyajessia atrofuscus NRRL 22316 and G. cicatriscum CBS 125552). The alignment included 2290 positions (1614 rpb2, 676 tef1) of which 748 were variable (480 rpb2, 268 tef1), and 562 positions were phylogenetically informative sites (379 rpb2, 183 tef1). For both gene partitions, a GTR + I + G model was selected and incorporated in the analyses. The BI lasted for 1,855,000 generations, and the consensus tree and PP were calculated from 1392 trees after discarding 494 trees as burn-in fraction. Phylogenetic trees inferred using ML and BI analyses resulted in very similar topologies, and therefore only the ML tree is presented in Figure 3a. The South African isolates were distributed among 11 distinct phylogenetic lineages, of which seven corresponded to known Neocosmospora species, which were, in order of frequency of isolation: N. ferruginea and N. solani (15 isolates each), N. hypothemieni (five isolates), N. brevis (three isolates), N. noneumartii (two isolates), and N. crassa and N. falciformis (one isolate each). The remaining 20 South African isolates grouped within four undescribed phylogenetic lineages, among which 15 isolates clustered in a well-supported clade (“Neocosmospora sp. 1", BS = 93/PP = 0.96), sister to N. bataticola; three isolates (VG268, 279 and 281) clustered in a fully-supported clade (“Neocosmospora sp. 2", BS = 100/PP = 100), sister to N. metavaranis; while two isolates (singletons VG16 and VG48) were resolved as single lineages (respectively, “Neocosmospora sp. 3" and “Neocosmospora sp. 4"), however, with low statistical support values compared with those in the rpb2 and tef1 analyses.

To further assess the phylogenetic position of the putative novel phylogenetic clades, a second, more robust multi-locus phylogenetic analysis was performed using seven loci (acl, cal, ITS, LSU, rpb1, rpb2 and tef1) and selected strains representing closely related species, as determined in the previous phylogenetic assessment of the genus Neocosmospora. The combined dataset included 5904 positions (616 acl, 573 cal, 467 ITS, 480 LSU, 1489 rpb1, 1613 rpb2 and 666 tef1) from 47 strains, representing a subset of 28 phylogenetic clades of Neocosmospora, plus two outgroup taxa. From the total sites included, 1405 were variable (188 acl, 103 cal, 100 ITS, 34 LSU, 372 rpb1, 390 rpb2 and 218 tef1), and 856 were phylogenetically informative (81 acl, 82 cal, 70 ITS, 22 LSU, 196 rpb1, 266 rpb2 and 139 tef1). Optimal model selection for each gene partition was determined as follows: GTR + G for tef1, GTR + I + G for LSU and ITS; K80 + G for acl, K80 + I + G for cal, and SYM + I + G for rpb1 and rpb2. The BI lasted for 1,520,000 generations, and PP were calculated from 1141 trees after discarding 380 trees as the burn-in fraction. The BI analysis (shown in Figure 3b) confirmed the topology obtained by ML.

The analyses confirmed the results obtained in the two-locus phylogeny, and the four novel lineages were resolved with high BS and PP support. Neocosmospora sp. 2 and representative isolates of clade Neocosmospora spp. associated with dry root rot of citrus in South Africa
Figure 1. Dry root rot symptoms of citrus observed in South Africa. Tree decline progression: initial leaves wilting (A), yellowing, loss of leaves, and dieback of branch tips (B) and plant death (C). External cracks or blisters on the trunk portion above the crown (D) and internal dry rot (E) of the same plant. Gum exudate at the crown level (F). Brown to black discolouration and necrosis of the vascular tissue visible in longitudinal and transverse sections (G).
Neocosmospora spp. associated with dry root rot of citrus in South Africa

sp. 1 were both resolved as fully supported clades (BS = 100/PP = 100), while the lone lineages Neocosmospora sp. 3 and Neocosmospora sp. 4 were confidently resolved as well-supported branches (respectively, BS = 96/PP = 0.97 and BS = 86/PP = 0.96). These four phylogenetic lineages are therefore here proposed as the novel species Neocosmospora addoensis, N. citricola, N. gamtoosensis and N. lerouxii.

Taxonomy

Neocosmospora addoensis Sand.-Den. & Guarnaccia, sp. nov. – MycoBank MB837939; Figure 4.

Etymology. Named after the geographical area Addo, South Africa where first collected.

Ty pus. South Africa, Eastern Cape, Kirkwood, from Citrus sinensis crown, May 2018, V. Guarnaccia (holo-type CBS H-24565 designated here, culture ex-type CBS 146510 = CPC 37128 = VG281).

Conidiophores borne on aerial mycelium, 53.5–425 μm long, unbranched or less commonly laterally branched, bearing terminal single phialides, proliferating percurrently; aerial phialides monophialidic, subulate to subcylindrical, smooth- and thin-walled, 34–64.5 × 2–4 μm, with short and flared apical collarettes and inconspicuous periclinal thickening; aerial conidia arranged in false heads on phialide tips, hyaline, broadly ellipsoidal to clavate and slightly asymmetrical, smooth- and thin-walled, aseptate, (5.5–)7–10(–14.5) × (2–)3–4 μm (av. 8.5 × 3 μm). Sporodochia pale luteous to pale peach coloured, formed abundantly on carnation leaves. Sporodochial conidiophores unbranched or laterally and irregularly branched bearing apical groups of 2–3 monophialides; sporodochial phialides subulate to subcylindrical, 12.5–25 × 2–4.5 μm, smooth and thin-walled, commonly proliferating sympodially, collarettes and periclinal thickening absent or inconspicuous. Sporodochial conidia falcate, slightly curved dorsoventrally to almost straight, broadest near the half portion or the upper third, tapering towards both ends, with blunt and slightly curved apical cells and blunt, sometimes inconspicuous foot-like basal cells, (1–)2–5-septate, predominantly 4-septate, hyaline, smooth- and thick-walled; one-septate conidia: (18.5–)19–24(–25) × 3–4.5 μm (av. 21.5 × 4 μm); two-septate conidia: (24–)26–30 × 3.5–5 μm (av. 27.5 × 4.5 μm); three-septate conidia: (27–)33–43(–45) × (3–)4–5.5(–6) μm (av. 38 × 5 μm); four-septate conidia: (39–)42–47.5(–51.5) × 4.5–6 μm (av. 49 × 5.5 μm); five-septate conidia: (37.5–)42.5–51 × 5–6 μm (av. 47 × 5.5 μm). Chlamydospores subspherical to spherical, hyaline to pale yellow, smooth-walled or slightly roughened, thick-walled, 4–10 μm, single or in chains, terminal or intercalary on hyphae and conidia.

Figure 2. Small (A), medium (B) or large (C) extensions of internal discoulouration in transverse sections through citrus tree trunks.
Figure 3. Maximum-likelihood (ML) phylograms obtained from combined \(rpb2\) and \(tef1\) (A) and \(acl\), \(cal\) ITS, \(LSU\), \(rpb1\), \(rpb2\) and \(tef1\) (B) sequences, of 62 isolates of \textit{Neocosmospora} spp. from South African \textit{Citrus} (shown in red), and representative and ex-type isolates of \textit{Neocosmospora}. Names of new species described here are shown in bold font. Numbers on the nodes are ML bootstrap values greater than 70% followed by Bayesian posterior probability values greater than 0.95. Branch lengths are proportional to distance. Ex-type, ex-epitype and ex-neotype strains are indicated, respectively, with T, ET and NT. The trees are rooted to \textit{Geejeyesia atrofusca} (NRRL 22316 and \textit{G. cicatricum} (CBS 125552).
Figure 4. *Neocosmospora addoensis* (ex-type culture CBS 146510). (a and b) sporodochia formed on the surface of carnation leaves; (c to f) sporodochial conidiophores and phialides; (g to i) aerial conidiophores; (j and k) aerial conidia; (l) sporodochial conidia. Scale bars: a and b = 100 μm; c to l = 10 μm.
**Culture characteristics.** Colonies on PDA reaching 79 mm diam. at 24°C after 7 d (growth rate: 4.1–5.6 mm d⁻¹). Colony surface white to primrose, becoming scarlet to bay, flat with abundant dense aerial mycelium, cottony to woolly; colony reverse pale luteous to sulphur yellow, a vivid scarlet to rust pigment can be formed. On SNA, white to pale buff, membranous to woolly with scant aerial mycelium, becoming powdery; colony reverse white to pale buff. On OA, pale luteous to pale rosy buff, flat, membranous to cottony; colony reverse pale luteous to pale rosy buff.

**Additional materials examined.** South Africa, Eastern Cape, Patensie, from *Citrus sinensis* crown, May 2018, V. Guarnaccia (CBS 146508 = CPC 37126 = VG268, CBS 146509 = CPC 37127 = VG279).

**Notes.** Both phylogenetic analyses resolved *Neocosmospora addoensis* as the closest genetic relative to *N. metavorans* (96 to 98% sequence similarity among individual gene datasets). *Neocosmospora metavorans* is a frequent opportunistic pathogen of animals, including humans (Sandoval-Denis et al., 2019). Nevertheless, in addition to its genetic exclusivity, these two species are morphologically quite distinct, particularly in the size and septation of the aerial conidia (aseptate, up to 14.5 μm in *N. addoensis* and multisepate, up to 25 μm in *N. metavorans*), while sporodochial conidia of *N. addoensis* are more slender (up to 6 μm wide) than those of *N. metavorans* (up to 7.5 μm wide).

*Neocosmospora addoensis* is characterized by its small and slender macroconidia, which are much smaller than the average macroconidial type in *Neocosmospora*. Based on its macroconidial size, this species is close to *N. brevis* and *N. pseudoradicicola*; however, these two species are well-delimited phylogenetically, clustering in far separate lineages of the genus (96% sequence similarity with *N. brevis* and 97% with *N. pseudoradicicola*). Morphologically, *N. addoensis* differs from *N. pseudoradicicola* by its macroconidial shape and curvature, with more rounded apical cells, rather inconspicuous foot cells and less pronounced dorsoventral curvature; and from *N. brevis* by the absence of aerial macroconidia and slightly more elongated and hooked macroconidial apical cells.

**Neocosmospora citricola** Guarnaccia & Sand.-Den., sp. nov. – MycoBank MB837940; Figure 5.

**Etymology.** In reference to occurrence of this fungus on *Citrus* plants.

**Typus.** South Africa, Eastern Cape, Patensie, from *Citrus sinensis* crown, May 2018, V. Guarnaccia (holotype CBS H-24566 designated here, culture ex-type CBS 146513 = CPC 37131 = VG343).

**Conidiophores** borne on aerial mycelium, 66.5–198.5 μm long, unbranched or irregularly laterally branched, bearing terminal single phialides; **aerial phialides** monopodialic, subulate to subcylindrical, smooth- and thick-walled, 39.5–73.5 × 2–4.5 μm, each showing a discrete flared collarette and inconspicuous to evident periclinal thickening; **aerial conidia** arranged in false heads on phialide tips, hyaline, broadly ellipsoid to obovoidal, rarely clavate, smooth- and thin-walled, 0–1-septate, (6–)9–17–(24.5) × 3–5–(6.5) μm (av. 13 × 4.5 μm). **Sporodochia** pale luteous to pale orange, formed abundantly on carnation leaves and on the agar surface. **Sporodochial conidiophores** laterally and irregularly branched, bearing single terminal monopodialic or terminal groups or up to three monopodialic; **sporodochial phialides** subulate to subcylindrical, 11–27.5 × 3–5.5 μm, smooth and thin-walled, with inconspicuous or absent apical collarettes and periclinal thickening. **Sporodochial conidia** falcate, curved dorsoventrally to almost straight, each with broadening in the upper third, tapering towards both ends, with a blunt to papillate and slightly curved apical cell and a blunt, foot-like basal cell, (2–)3–5–(6)–septate, predominantly five-septate, hyaline, robust, smooth- and thick-walled; two-septate conidia, 44 × 5.7 μm; three-septate conidia: 33.5–49.5 (–58) × 4.5–6 μm (av. 43 × 5.5 μm); four-septate conidia: (46.5–)47.5–56–(59.5) × 5–6.5 μm (av. 52 × 6 μm); five-septate conidia: (49.5–)53–60.5–(65) × (4.5–)5.5–6.5–(7) μm (av. 57 × 6 μm); six-septate conidia: 60 × 6 μm. **Chlamydospores** subspherical to spherical, hyaline to pale golden brown, smooth to slightly roughened and thick-walled, 5–10 μm, single or in chains, terminal or intercalary on hyphae and conidia.

**Culture characteristics:** Colonies on PDA reaching 69 mm diam. at 24°C after 7 d (growth rate: 3.2–4.9 mm d⁻¹). Colony surfaces straw, buff to pale luteous, with pale luteous to orange centres and abundant aerial mycelium, flat, felty, woolly to cottony with abundant concentric rings of aerial mycelium, colony reverse pale luteous to orange. On SNA, white and translucent, flat, woolly, becoming slightly pulverulent with sporulation, colony reverse white. On OA, saffron to peach, flat, membranous to cottony, colony reverse intense peach to flesh.

**Additional materials examined.** South Africa, Eastern Cape, Patensie, from *Citrus sinensis* crown, May 2018, V. Guarnaccia (CBS 146511 = CPC 37129 = VG302, CBS 146512 = CPC 37130 = VG307).

**Notes.** *Neocosmospora citricola* resolved as a highly supported monophyletic clade, basal to a fully supported lineage containing *N. bataticola* and *N. elegans*, which clearly differentiated genetically (96 to 98% sequence similarity to *N. citricola* in the single gene datasets).
Figure 5. *Neocosmospora citricola* (ex-type culture CBS 146513). (a and b) sporodochia formed on the surface of carnation leaves; (c to f) sporodochial conidiophores and phialides; (g and h) aerial conidiophores; (I and j) aerial conidia; (k) sporodochial conidia. Scale bars: a and b = 100 μm; c to k = 10 μm.
Although genetically distant, Neocosmospora citricola is morphologically similar to N. nirenbergiana, N. piperis and N. protoensiformis (92% sequence similarity with N. nirenbergiana and 96% with N. piperis and N. protoensiformis; data not shown), the four species producing very similar macroconidia in shape and overall size. Nevertheless, N. citricola differs from N. nirenbergiana and N. piperis by the absence of aerial macroconidia. Conversely, N. nirenbergiana and N. piperis do not produce aerial conidia, and the aerial conidiophores of N. citricola are much more robust than those of N. nirenbergiana and N. piperis. Neocosmospora protoensiformis also lacks aerial macroconidia; however, in addition to forming smaller microconidia (up to 15 μm long, average size 7.6 × 3.6 μm in N. protoensiformis vs up to 24 μm long, average size 13 × 4.5 μm in N. citricola), and shorter sporodochial phialides (up to 19.5 μm long in N. protoensiformis vs up to 27.5 μm long in N. citricola), macroconidia of N. protoensiformis differ from those of N. citricola by usually being more tapered at both ends.

**Neocosmospora gamtoosensis** Sand.-Den. & Guar- naccia, sp. nov. – Mycobank MB837941; Figure 6.

*Etymology.* Named after the valley where this fungus was collected, Gamtoos River Valley, South Africa.

*Type.* South Africa, Eastern Cape, Patensie, from Citrus sinensis (holo-crown, May 2018, V. Guarnaccia was collected, Gamtoos River Valley, South Africa.

Conidiophores borne on aerial mycelium, 96.5–291 μm long, unbranched or irregularly laterally branched, bearing terminal single phialides; aerial phialides monophialidic, subulate to subcilindrical, smooth- and thin-walled, 17.5–61 × 2–3.5 μm, collarettes and pericinial thickening evident; aerial conidia arranged in false heads on phialide tips, hyaline, broadly ellipsoid, ovoid to short clavate, smooth- and thin-walled, aszate, (4.5–)5.5–9(–11.5) × 2–3.5(–6) μm (av. 7 × 3 μm). Sporodochia citrine to honey, formed abundantly on carnation leaves. Sporodochial conidiophores commonly unbranched and densely packed, bearing terminal, single monopialides or groups of 2–3 phialides; sporodochial phialides lageniform to ampulliform, 7.5–17 × 3–5 μm, smooth and thin-walled, each with an often conspicuous pericinal thickening and a reduced, flared collarette. Sporodochial conidia falcate, slightly curved dorsoventrally to almost straight on their ventral faces, broadening in the upper third, tapering towards both ends, with blunt and hooked apical cells and blunt to slightly pointed and extended foot-like basal cells, (4–)5–6–7-septate, predominantly five-septate, hyaline, smooth- and thick-walled; four-septate conidia: (37–)40–55(–56.5) × 4.5–5.5 μm (av. 48.5 × 5 μm); five-septate conidia: (46.5–)51.5–60(–62) × 4.5–5.5 μm (av. 56 × 5 μm); six-septate conidia: 55.5–64(–65) × 4.5–5.5 μm (av. 60 × 5 μm); seven-septate conidia: 60.5 × 5 μm. Chlamydospores subspherical, hyaline to pale yellow, inconspicuously roughened, thick-walled, 5–12 μm diam., single or forming chains or clusters, terminal or intercalary on hyphae.

**Culture characteristics:** Colonies on PDA reaching 60 mm diam. at 24°C after 7 d (growth rate: 3.8–4.3 mm d⁻¹). Colony surfaces pale luteous, amber to pure yellow, flat with abundant dense aerial mycelium in radial patches, cottony to woolly, colony reverse pale luteous to vivid pure yellow. On SNA, colonies white to pale buff, translucent, flat, woolly with scant aerial mycelium, becoming slightly powdery; reverse white to pale buff. On OA, the colonies are pale luteous, pale buff to primrose, flat, membranous to cottony, and colony reverse pale luteous to pale rosy buff.

*Notes.* In the combined rpfb2 and tef1 analysis, Neocosmospora gamtoosensis formed an unsupported lone lineage, basal to a larger lineage containing N. hypothenemi, N. perseae and N. pseudoradicicola. The combined seven-loci analysis resolved N. gamtoosensis within the larger lineage, with high statistical support for all the earlier listed species. Base pair similarities between the novel species and its closest relatives ranged from 98% in the combined dataset to between 96 and 99% in the individual gene datasets.

Neocosmospora gamtoosensis is morphologically reminiscent of N. hypothenemi, both species having predominantly five-septate macroconidia of very similar size and shape; however, N. gamtoosensis has conspicuously flared collarettes on its aerial phialides, also producing shorter (length up to 11.5 μm, average = 7 μm in N. gamtoosensis vs up to 13.5 μm, average = 8.2 μm in N. hypothenemi), aszate aerial conidia, and honey coloured sporodochia (yellow-green in N. hypothenemi), and lacking reddish pigments on PDA. Neocosmospora noneumartii, another genetically distant (97% sequence similarity in the combined analysis), but morphologically similar species, differs from N. gamtoosensis by forming dimorphic conidia from aerial phialides and longer sporodochial conidia (five-septate sporodochial conidia of average length 56 μm vs 63 μm in N. noneumartii). Neocosmospora gamtoosensis is also morphologically very similar to N. lerouxii. However, N. gamtoosensis has shorter (five-septate sporodochial conidia average length 63 μm in N. lerouxii) and more curved sporodochial conidia.

**Neocosmospora lerouxii** Guarnaccia & Sand.-Den., sp. nov. – Mycobank MB837942; Figure 7.
Figure 6. *Neocosmospora gamtoosensis* (ex-type culture CBS 146502). (a to c) Sporodochia formed on the surface of carnation leaves; (d and e) sporodochial conidiophores and phialides; (f to h) aerial conidiophores; (i and j) aerial conidia; (k) sporodochial conidia. Scale bars: a and b = 100 μm; c to k = 10 μm.
Figure 7. Neocosmospora lerouxii (ex-type culture CBS 146514). (a and b) sporodochia formed on the surface of carnation leaves; (c) sporodochial conidiophores and phialides; (d to g) aerial conidiophores and phialides; (h and i) aerial conidia; (j) sporodochial conidia. Scale bars: a and b = 100 μm; d and e = 50 μm; f to j = 10 μm.
**Etymology.** In memory of Dr Hennie Le Roux (10 Jul 1967 – 4 Oct. 2016), who made major contributions to the South African and international citrus industries.

**Typus.** South Africa, Eastern Cape, Patensie, from *Citrus sinensis* root scaffold, May 2018, V. Guarnaccia (holotype CBS H-24567 designated here, culture ex-type CBS 146514 = CPC 37132 = VGE8).

Conidiophores borne on aerial mycelium, 139.5–295 \( \mu \text{m} \) long, simple or most commonly abundantly and irregularly branched, proliferating percurrently, bearing terminal single phialides; **aerial phialides** monophialidic, subulate to subcylindrical, smooth- and thin-walled, 37–61.5 \( \times \) 2–4 \( \mu \text{m} \), with pericllinal thickening and collarettes abundant; **aerial conidia** arranged in false heads on phialide tips, hyaline, ovate, broadly ellipsoidal to subspherical, hyaline to pale yellow-brown, smooth- and thick-walled; two-septate conidia, 29 \( \times \) 4 \( \mu \text{m} \); three-septate conidia: 40 \( \times \) 5 \( \mu \text{m} \); four-septate conidia: (44–)45–49(–50.5) \( \times \) (4–)4.5–5 \( \mu \text{m} \); five-septate conidia: (46.5–)56.5–67(–73.5) \( \times \) 4.5–5(–6.5) \( \mu \text{m} \); six-septate conidia: 60–74 \( \times \) 4.5–5.5 \( \mu \text{m} \) (av. 67 \( \times \) 5 \( \mu \text{m} \)). **Chlamydoospores** subspherical to spherical, hyaline to pale yellow-brown, smooth- and thick-walled, 4–8 \( \mu \text{m} \) diam., single or in chains, terminal or intercalary on hyphae.

Culture characteristics. Colonies on PDA reaching 61 mm diam. at 24°C after 7 d (growth rate: 3.5–4.3 mm d\(^{-1}\)). Surfaces buff, pale luteous to pale flesh, with abundant and dense whitish aerial mycelium, flat to slightly raised, feltly to cottony. Colony reverse pale luteous, quickly becoming amber to sulphur yellow, with or without pale apricot patches. On SNA, colonies white and translucent, flat, feltly, with white reverse sides. On OA, colonies white, saffron to buff, flat, membranous to cottony, with reverse sides buff to pale luteous with pale salmon patches.

Notes. The combined rpb2 plus tef1 analysis showed this taxon to form a well-supported (BS = 74, PP = 0.96) lone lineage, basal to a larger, unsupported lineage containing *N. catenata*, *N. cyanescens*, *N. ferruginea*, *N. macrospora*, and *N. spatulata*, and the undescribed phylogenetic species FSSC 12. The analysis of the combined seven-gene dataset confirmed the previous results, with all the species described here resolved as highly- to fully-supported monophyletic clades. Genetic similarity between *N. lerouxii* and its closest phylogenetic relatives also support phylogenetic exclusivity of *N. lerouxii* (98% sequence similarity with all the above taxa in the combined alignment, and 97 to 99% similarity for the individual gene datasets).

Morphologically, *Neocosmospora lerouxii* most closely resembles the three distantly related species *N. gamtoosensis*, *N. hypothenemi* and *N. noneumartii* (respectively, 97, 98 and 97% sequence similarity, in the seven-loci combined dataset). While the three species were clustered in well-separated lineages in all analyses, morphologically they share very similar characteristics. Although *N. lerouxii* has similar macroconidial shape to *N. gamtoosensis* and *N. hypothenemi*, the macroconidia of *N. lerouxii* are longer and straighter than in the other two species (five-septate macroconidia average length 62 \( \mu \text{m} \) vs 56 \( \mu \text{m} \) in *N. gamtoosensis* and 59 \( \mu \text{m} \) in *N. hypothenemi*). Macroconidia of *N. lerouxii* also have thinner walls in comparison to those of *N. noneumartii*. In addition, has a slower growth rate in culture than *N. noneumartii*, (3.5–4.3 mm d\(^{-1}\) for *N. lerouxii* vs 4.7–8 mm d\(^{-1}\) in *N. noneumartii*).

**DISCUSSION**

Since 2013, severe sudden decline and death of citrus plants has been observed in citrus production areas of the Eastern Cape province of South Africa. Several species of *Colletotrichum*, *Diaportheae* and *Botryosphaeriaceae* have been reported as causing wood decay of citrus plants internationally (Guarnaccia and Crous, 2018; Mayorquin et al., 2019; Berraf-Tebbal et al., 2020; Esparham et al., 2020; Bezerra et al., 2021). Considering the very large economic losses to the South African citrus industry due to the observed sudden decline of trees, and because no surveys and isolations had been previously conducted for this disease and associated pathogens in the Eastern Cape citrus production area, a large-scale survey of affected citrus plants was required. The present study provides the first preliminary survey and sampling of citrus trees affected by dry root rot, and characterization of *Neocosmospora* diversity related to the observed disease in two important citrus production areas of South Africa.
Neocosmospora species are well-established in geographical areas with Mediterranean, sub-tropical or tropical climates, where these fungi are associated with diseases of important agricultural crops (Sandoval-Denis et al., 2018; Guarnaccia et al., 2018; Guarnaccia et alii, 2019).

Fusarium oxysporum, F. proliferatum and N. solani s. tr. were previously considered as pathogens associated with dry root rot of citrus plants. (Menge, 1988; Adesemoye et al., 2011). Specifically F. oxysporum and N. solani were previously reported from South Africa. Diversity of Fusarium (three species) and Neocosmospora (five species) was revealed associated with dry root rot in restricted areas of three European countries by Sandoval-Denis et al. (2018). However, that study considered it likely that many other Neocosmospora spp. would also be isolated if a wider sampling area was surveyed.

In the present study, several citrus orchards in two major citrus production area of South Africa were investigated. A total of 62 Neocosmospora strains were collected from symptomatic tree trunks, roots and soil surrounding the roots. Phylogenetic analyses as well as morphological characters, revealed ten Neocosmospora species associated with infections on Citrus in South Africa, plus one species (N. falciformis) from soil from affected citrus orchards. The analyses included several of the closest related taxa to each of the Neocosmospora species recovered, based on BLAST searches of NCBI’s GenBank nucleotide database. The final phylogenetic tree revealed four previously undescribed species (N. addoensis, N. citricola, N. gamtoosensis, and N. lerouxii) and six known species (N. brevis, N. crassa, N. ferruginea, N. hypothenemi, N. noneumartii, and N. solani) all of which were always associated with abovementioned symptomatic material.

Neocosmospora citricola, N. ferruginea and N. solani were the predominant species, largely found associated with the affected tissues of symptomatic plants cultivated in all the investigated orchards. Although follow-up studies will conduct pathogenicity trials to confirm these observations, it is assumed that these species represent the major biotic factors causing DRR of citrus in South Africa as they were consistently associated with the symptoms described from the diseased trees. These results also partially confirm what was recently demonstrated after surveys conducted in Mediterranean countries, where N. ferruginea (formerly FSSC28) and N. solani were isolated from typical DRR of citrus (Sandoval-Denis et al., 2018). Neocosmospora citricola was not found before the present study, and considering the broad distribution on affected plants, this fungus is likely to be important in DRR. Neocosmospora addoensis was isolated with low frequency, from one orchard and from necrotic trunk tissue. The other novel species described in this study, N. gamtoosensis and N. lerouxii, were found only sporadically, and are thus not considered as important pathogens. However, their description provides new insights into the taxonomy of Neocosmospora. Neocosmospora brevis, N. crassa, N. hypothenemi and N. noneumartii were also isolated sporadically, and future studies will investigate their roles in DRR. The complexity of pathogens associated with artificially reproducing DRR of citrus is well-known (Graham et al., 1985), but needs to be confirmed in further field trials. Furthermore, additional surveys in South Africa and other citrus-producing areas, and pathogenicity trials of Neocosmospora spp. in association with abiotic factors, should also be conducted.

The present study has provided the first overview of Neocosmospora diversity associated with DRR of citrus trees in South Africa, and has given useful information about taxonomic characterization within Neocosmospora. All the Neocosmospora species were isolated from crowns, trunks, roots and soil from the affected citrus orchards. Infected propagation material and soil can spread the pathogens nationally and internationally as the fungi can survive as chlamydospores in the soil and systemic infections in plant material. Further studies are required to resolve the host range and pathogenicity of all the species recovered. These fungi can survive as endophytes or as latent infections within citrus plants, so healthy propagation material should be used by growers. Favourable climatic conditions and, especially, plant stress factors could also play major roles in disease development. Further research on the epidemiology of DRR of citrus should be conducted to develop specific knowledge as the basis for effective disease prevention and management.

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