Death of endemic *Virgilia oroboides* trees in South Africa caused by *Diaporthe virgiliae* sp. nov.

N. M. Machingambi[ab], L. L. Dreyer[ab], K. C. Oberlander[cd], J. Roux[b] and F. Roets[bc*]

[a]*Department of Botany and Zoology, Stellenbosch University, Stellenbosch 7600;* [b]*Department of Microbiology and Plant Pathology, DST/NRF Centre of Excellence in Tree Health Biotechnology (CTHB), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Private Bag X20, Hatfield, Pretoria 0028;* [c]*Department of Conservation Ecology and Entomology, Stellenbosch University, Stellenbosch 7600, South Africa; and* [d]*Institute of Botany, Academy of Sciences of the Czech Republic, Průhonice 1, CZ-252 43, Czech Republic*

Numerous dead and dying individuals of the Western Cape endemic tree *Virgilia oroboides* (Fabaceae) were recently observed within a South African national botanical garden. Root-rot fungi and fungi symbiotic with bark beetles (Curculionidae; Scolytinae) from diseased trees were assessed for their respective roles in *V. oroboides* mortality. Disease progression was also monitored over 1 year. Fungi were isolated from surface sterilized bark and root samples from diseased trees and provisionally identified using data from the internal transcribed spacer regions (ITS1, ITS2), including the 5·8S rRNA gene (ITS). Pathogenicity of selected fungi towards *V. oroboides* was tested under field conditions. The pathogenicity of various bark beetle-associated *Geosmithia* (Hypocreales: Hypocreomycetidae) spp. from *V. oroboides* were similarly assessed. The only fungus consistently isolated from lesions on the roots and bark of declining *V. oroboides*, and never from healthy individuals, represented an undescribed *Diaporthe* (Diaporthales, Diaporthaceae) species that was characterized using molecular (using data from the ITS marker and part of the β-tubulin gene, *TUB*), cultural and morphological characters. It is an aggressive pathogen of *V. oroboides*, newly described here as *Diaporthe virgiliae* sp. nov. Trees of all ages are susceptible to this pathogen with subsequent bark beetle attack of mature trees only. All *Geosmithia* spp. from beetles and/or infected trees were nonpathogenic towards *V. oroboides*. *Diaporthe virgiliae* caused a severe decline in the health of the monitored *V. oroboides* population over a period of only 1 year and should be considered as a significant threat to these trees.

Keywords: bark beetle, *Geosmithia*, *Phomopsis*, root pathogen, tree death

Introduction

The Cape Floristic Region (CFR) of South Africa is home to the endemic genus *Virgilia* (Fabaceae). Natural habitats include forest margins where they fulfil an important role in normal ecosystem function (Phillips, 1926). Numerous dead and dying *Virgilia oroboides* trees were recently observed in the Harold Porter National Botanical Garden (HPNBG), Western Cape Province, South Africa (Fig. 1a). Diseased plants exhibited root-rot symptoms that extended to the base of trunks (Fig. 1b,c), and their trunks and branches were heavily colonized by bark beetles (Curculionidae; Scolytinae) (Fig. 1d,e). No serious diseases of *Virgilia* are currently known. Indeed, very few reports of root-associated diseases of endemic CFR plants have been published. One of the better-known examples is phytophthora root disease caused by the non-native stramenopile *Phytophthora cinnamomi*, known to kill various native trees across the CFR (Lübbe & Geldenhuys, 1990). Additional fungal taxa known to cause root diseases in natural CFR vegetation include an undescribed *Pythium* sp. (Jacobsen *et al.*, 2012) and various *Armillaria* spp. (Coetzee *et al.*, 2003).

Botanical gardens are considered sources of pests and pathogens due to outsourcing of planting material from other areas (Von Broembsen, 1989; Coetzee *et al.*, 2003). For example, non-native pathogens such as *Armillaria mellea* and *P. cinnamomi* were accidentally introduced into the Kirstenbosch National Botanical Garden (KNBG, South Africa) from infected plant or soil material (Coetzee *et al.*, 2003) and have killed many native species (Coetzee *et al.*, 2003). There is also a distinct possibility that a recently described fungus (*Immersiporthe knoxdaviesiana*), which causes a severe canker disease of native trees in the HPNBG, could be introduced (Chen *et al.*, 2013). As the HPNBG and the KNBG commonly exchange biological material (J. Forrester, HPNBG, Betty's Bay, South Africa, personal communication), there are concerns that these pathogens may spread between these gardens and into natural vegetation throughout the CFR.

Preliminary isolations from diseased *Virgilia* roots in the HPNBG commonly yielded a fungus that morphologically resembles species of *Diaporthe*. Species in this genus (and their *Phomopsis* asexual states) can be either plant pathogens, pathogens of mammals, nonpathogenic endophytes

*E-mail: fr@sun.ac.za

Figure 1 (a) Dead and diseased (foreground) and healthy (background) *Virgilia oroboides* trees in the Harold Porter National Botanical Garden; (insert to a) close-up of flowers; (b) diseased root lacking young feeder roots; (c) diseased root with outer bark removed, exposing brown necrosis and lesions; (d–f) bark beetle colonization of *Virgilia*; (d) bark beetle holes in outer bark of diseased tree; (e) bark beetle galleries containing eggs, larvae and adults; (f) wood staining by *Geosmithia* associates of bark beetles. (g,h) Field inoculations using *Diaporthe virgiliae* isolates; (g) stem inoculated with *D. virgiliae* mycelia that exudes gum after 6 weeks; (h) lesions resulting from inoculations with *D. virgiliae* after 6 weeks (control on left).

or saprobes (Webber & Gibbs, 1984; Rehner & Uecker, 1994; Garcia-Reyne *et al.*, 2011; Udayanga *et al.*, 2011). Unfortunately, the delineation of *Diaporthe* species is notoriously difficult based on morphological characters alone (Gomes *et al.*, 2013) and it has become a requirement that a combination of morphological and molecular data (including at least two molecular markers) is needed for clear identification of *Diaporthe* spp. (Gomes *et al.*, 2013).

With the recent discovery of a destructive, possibly introduced, pathogen on native trees in the HPNBG (Chen *et al.*, 2013), it is essential to identify the cause of the mortality of *Virgilia* trees in the same garden. In addition to the fungus isolated from *Virgilia* roots, numerous species of *Geosmithia* have previously been isolated from bark beetles and the wood of dying *Virgilia* trees in the HPNBG (Machingambi *et al.*, 2014), but these have not yet been evaluated for their possible role in *Virgilia* tree death in this garden. Specific objectives of this study therefore included: (i) to isolate, identify and determine the pathogenicity of key fungal taxa associated with the roots of diseased *Virgilia* trees; (ii) to test the pathogenicity of various *Geosmithia* taxa associated with *Virgilia*-infesting bark beetles; (iii) to establish which life stages of *Virgilia* are most susceptible to disease; and (iv) to establish whether there was a significant decline in the health of the HPNBG *Virgilia* population over a short time period.

Materials and methods

Sample collection and fungal isolation

Disease surveys of *Virgilia* trees were conducted in the HPNBG (34°20·893′S 18°55·519′E) in Betty's Bay, South Africa during

March 2011–December 2013. The garden houses 10 hectares of cultivated area and 190·5 hectares of pristine natural CFR vegetation (www.sanbi.org). *Virgilia oroboides* was planted in the garden, most probably during the mid-1900s, and has since spread into the surrounding natural vegetation where it has become naturalized. Decline and death of some of these trees were first observed in 2008/2009 and since then the numbers of dead and dying trees have increased considerably.

Dying trees showed clear symptoms of root disease that extended into the base of tree trunks. Consequently, bark (from the base of trees) and root samples were randomly collected from both diseased and healthy individuals. Collected samples were rinsed under flowing tap water and surface sterilized by soaking in 70% ethanol for 5 min and then dried in a laminar flow cabinet. The outer bark was removed from roots with a sterile scalpel to expose any lesions in the inner bark, phloem and/or cambium. Small pieces (*c.* 2 mm²) of tissue from the edges of fresh necrotic lesions were plated onto malt extract agar (MEA, 20 g L^{-1} malt extract and 20 g L^{-1} agar; Biolab) in Petri dishes. A subset of tissue samples was also plated onto PARP (pimaricin + ampicillin + rifampicin + pentachloronitrobenzene) and PARPH (pimaricin + ampicillin + rifampicin + pentachloronitrobenzene + hyrnexazol) media that are selective for *Pythium* and *Phytophthora*, respectively (Jeffers & Martin, 1986). Plates were incubated at room temperature (20–25°C) in the dark and examined daily for fungal growth. Single hyphal tips of developing mycelium were transferred to fresh MEA plates. Pure cultures from isolations were stored at 4°C on MEA until further use.

Isolates of five *Geosmithia* taxa obtained from bark beetles associated with declining *V. oroboides* in a previous study (Machingambi *et al.*, 2014) were acquired from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. These included three isolates each of *Geosmithia flava* (CMW40726, CMW40727, CMW40728), *Geosmithia* sp. 2 (CMW40737, CMW40738, CMW40743), *Geosmithia* sp. 8 (CMW40739, CMW40740, CMW40746), *Geosmithia* sp. 10 (CMW40733, CMW40734, CMW40735) and two isolates of *Geosmithia* sp. A (CMW40741, CMW40742). Nine representative isolates of the newly isolated fungal taxon used in this study were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) and the fungal collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands.

DNA extraction, amplification and sequencing

Fungal mycelia from pure cultures of representative isolates were harvested from MEA plates with a sterile scalpel. Representative isolates of taxa were chosen based on their frequency and consistency of isolation from collected samples. DNA was extracted using a plant PCR kit (Sigma-Aldrich) following the manufacturer's instructions. The nuclear ribosomal internal transcribed spacer region (ITS1, ITS2), including the 5·8S rRNA gene, was amplified using primers ITS1-f (Gardes & Bruns, 1993) and ITS4 (White *et al.*, 1990). As isolates from diseased roots resembled species of *Diaporthe*, part of the β-tubulin gene (*TUB*) was amplified in addition to the ITS region, using primers T1 (O'Donnell & Cigelnik, 1997) and Bt-2b (Glass & Donaldson, 1995).

The 20 μL PCR reaction volumes used consisted of 5 μL ddH_2O, 10 μL REDExtract-N-Amp PCR ready mix (Sigma-Aldrich), 4 μL extracted fungal DNA and 0·5 μL of each 10 mM primer, as per the manufacturer's instructions. A GeneAmp PCR System 2700 thermal cycler (Applied Biosystems) was used to amplify DNA. PCR reaction conditions were: 2 min of initial denaturation at 95°C; 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 55°C, 1 min 30 s elongation at 72°C; and a final elongation step at 72°C for 8 min. PCR products were separated by agarose gel electrophoresis (1·5% agarose gel containing ethidium bromide) and visualized under UV light. Amplified PCR products were purified and sequenced at the Stellenbosch University Central Analytical Facility, Stellenbosch, South Africa.

Phylogenetic analyses

Sequences generated in this study were compared to published sequences in GenBank (http://www.ncbi.nlm.nih.gov/genbank) using the BLAST algorithm (Altschul *et al.*, 1990) and to those in the relevant published literature (Gomes *et al.*, 2013). For the identification of possible *Diaporthe* isolates, the extensive five-marker molecular data set of Gomes *et al.* (2013) was downloaded from www.treebase.org (study S13943) and used as the basis for phylogenetic analyses. Data for taxa chosen for comparisons to the isolates from this study included the type specimens and/or specimens that have been confirmed to be representative of that particular species (as defined in Gomes *et al.*, 2013). No more than two sequences for each representative species were included in the analyses. The newly generated ITS and *TUB* sequences were manually aligned with these published sequences using BioEdit v. 7.0.5 (Hall, 2005). The other three markers were considered missing data for these taxa. Bayesian analyses were conducted to reconstruct molecular phylogenetic trees using MrBayes v. 3.2 (Ronquist *et al.*, 2012) based on a Markov chain Monte Carlo (MCMC) approach. Each marker was allowed its own evolutionary rate and substitution models for each partition were sampled in proportion to their posterior probability using the nst = mixed command. All partitions were assigned gamma plus proportion of invariant sites parameters to estimate among-site rate variation. Chains were run for 2×10^7 generations sampling parameters every 5000 generations. In total, six separate analyses were run to account for convergence on the same posterior and increase estimated sample sizes (ESS) of above 200. Convergence and ESS size were judged using both MrBayes and TRACER v. 1.5.0 (Rambaut & Drummond, 2009). This was also done to circumvent convergence on local maxima in tree space, which was a problem with this data set (see Results). The standard MrBayes burn-in value of 0·25 of total generations (i.e. the first 5×10^7 generations of each run excluded) was used. The resulting phylogenetic consensus tree was printed with FigTree v. 1.3.1 (Rambaut, 2009) and only the relevant portion of the tree that contained sequences from the present study and their closest relatives are presented in the results section. Newly generated sequence data from this study were deposited in GenBank and the alignment and phylogenetic tree were deposited in www.treebase.org (S16794).

Pathogenicity tests

Nine representative isolates of the fungus consistently isolated from lesions on the roots and underneath bark at the base of tree trunks of diseased *V. oroboides* (CMW40748–CMW40756), and three isolates (where available) of each of the five *Geosmithia* taxa isolated in a previous study (Machingambi *et al.*, 2014) were used in pathogenicity tests at the HPNBG. A

7 mm cork borer was used to remove the bark and expose the cambium of *V. oroboides* branches (*c.* 1·5 cm diameter). Similar sized disks from actively growing 2-week-old fungal colonies on MEA were inserted into these wounds with the mycelium facing the xylem. These wounds were covered with masking tape to prevent desiccation and contamination by other organisms. Twenty-five branches were inoculated per tree, with each branch being inoculated with a different fungal strain to control for variation in host resistance. Control branches were inoculated with sterile MEA plugs on these same trees. A total of eight trees were inoculated in this manner.

Inoculations were conducted on plant stems rather than roots due to the unavailability of potted material and because of difficulties in inoculation of roots under field conditions without causing unnecessary damage to the plants, which may influence pathogenicity test results. Containment of infective material in the field after root inoculations (below soil level) would also prove difficult and may have led to the inadvertent spread of the disease to disease-free areas in the HPNBG. In addition, as this is a root disease that extends into the trunk of plants, expected symptoms after stem inoculations could be directly compared to symptoms of infected material from the base of trees (e.g. Maseko *et al.*, 2007).

After 6 weeks, the length of resultant lesions was measured and reisolations of fungi were made to confirm that the inoculated fungi were those responsible for lesion development. For reisolations, branches with wounds were surface sterilized with 70% ethanol and the bark removed. Small pieces of wood (2 mm²) from the lesion front were plated onto MEA in Petri dishes and incubated at room temperature (20–25°C) in the dark. Fungal cultures growing in the plates were identified based on morphological characteristics. All inoculated branches were removed from the trees and destroyed after completion of these experiments to avoid further inoculum build-up in the garden.

To assess pathogenicity, lesion length data from individual fungal isolates were compared to those of the controls using one-way analysis of variance (ANOVA) procedures in STATISTICA v. 11 (Statsoft Corp.) after confirming normality of the data. An LSD post hoc test, as implemented in STATISTICA, was used to test differences between mean group lesion lengths.

Cultural characteristics

Descriptions of cultural characteristics were based on colonies growing on 2% MEA that were incubated at a range of temperatures between 20 and 35°C (at 5°C increments) under a 12 h light/12 h dark cycle. Structures were mounted on microscope slides in clear lactic acid, and studied using a Nikon Eclipse E600 light microscope with differential interference contrast (DIC). Photographs were taken with a Nikon DXM1200 digital camera mounted on the microscope. Measurements (50) of each taxonomically useful structure were made and means (±standard deviation) calculated. Colony colour was described following the colour charts of Rayner (1970).

Disease progression and distribution

Three 50 × 10 m transects were plotted in areas with high numbers of *V. oroboides* trees in the HPNBG and the health status of all individuals encountered therein (including seedlings and saplings) was assessed. Healthy individuals were given a score of 6, those at the leaf yellowing stage a score of 5, those dropping leaves a score of 4, trees with less than 50% shoot die-back a score of 3, trees with more than 50% shoot die-back a score of 2 and dead trees a score of 1. All diseased and dying trees were uprooted, or the roots excavated in the case of large individuals, to determine if the cause of dieback was in fact due to root disease.

The health status of the same individual trees initially assessed in March 2011 was again assessed in June 2012. To determine if population health had stabilized or deteriorated after a year, mean health status of *V. oroboides* individuals from each of the three transects were compared between 2011 and 2012 using the tree health scores in a repeated measures ANOVA in STATISTICA v. 10 (Statsoft Corp.). Significant differences were recorded when $P \leq 0{\cdot}05$.

In order to determine the current extent of this disease, numerous populations of *Virgilia* from across its natural distribution range were assessed for similar disease symptoms. Sites surveyed included Jonkershoek Forestry Reserve, Kirstenbosch National Botanic Garden, Table Mountain Nature Reserve, Silver Mine Nature Reserve and Franschoek Pass for *V. oroboides oroboides*, George for *V. oroboides ferruginea* B–E. van Wyk and George, Knysna, Keurboomstrand and Storms River for *V. divaricata* Adamson.

Effect of tree age on health status

Trunk diameters, used as proxy for plant age, were measured for all individuals encountered within the three transects in HPNBG during 2011. Diameters were taken at breast height (1·37 m above ground level) for mature trees and at 10 cm above soil level for individuals <1 m tall. The effect of tree age on health status was tested for by comparing stem diameters between the different health status categories. The presence or absence of bark beetles on stems and trunks was also recorded. Normality of the stem diameter data was tested using a Shapiro–Wilk test and was subsequently analysed using Kruskal–Wallis ANOVA and Median test procedures in STATISTICA v. 11. Significant differences were reported when $P \leq 0{\cdot}05$.

Results

Sample collection and fungal isolations

Root samples from 23 diseased and 14 healthy *V. oroboides* individuals, of various ages, were collected. Bark beetles were only present in dead and dying older trees that had a minimum stem diameter of 7 cm. Wood around these galleries became stained in a characteristic marbled way, apparently caused by the *Geosmithia* sp. fungal associates of the beetles (Fig. 1e). Samples from diseased roots placed on PARP and PARPH media did not reveal the presence of any *Pythium* or *Phytophthora* spp. However, on MEA medium, a single morphospecies (based on culture characteristics) was consistently isolated from lesions of diseased, but not healthy, roots and bark.

Phylogenetic analyses

The most consistent fungal morphospecies that was isolated from the roots and bark of diseased individuals was confirmed to be a species of *Diaporthe* using ITS sequence data in GenBank (GenBank KC145859.1;

identities = 553/572 (97%), gaps = 7/572 (1%)). All ITS (CMW40748 to CMW40756 GenBank accession numbers KP247566 to KP247574, respectively) and *TUB* (GenBank accession numbers KP247575 to KP247583) sequences of the present isolates were identical. The aligned five-marker data set included 139 taxa and 2444 characters. Bayesian inference of the five-marker data set in MrBayes resulted in analyses converging on two different islands in tree space, with one island 25–30 log likelihood units worse than the other and no movement between islands within each run. Consequently, the three analyses that converged on the island with the higher log likelihood were used as the best estimates of the posterior and the remaining analyses were discarded. However, the position of the relevant *Diaporthe* species was invariant between the two islands (Fig. 2). All three analyses converged on the same posterior (mean ln likelihood as measured in Tracer v. 1.5.0: −43 827·30, standard error of mean: 0·44, 95% highest posterior density interval −43 796·36 to −43 857·47) and gave very similar parameter values even for such a highly partitioned model. Combined parameter ESS values were all >250.

Only the portion of the resulting phylogenetic tree that contained sequences of the taxa isolated in this study and their close relatives is presented in Figure 2. The newly generated sequences resolved in a clade containing *Diaporthe alnea*, *D. eres*, *D. celastrina*, *D. gardeniae*, *D. neilliae*, the *D. nobilis* complex (see Gomes *et al.*, 2013), *D. nomurai* and *D. vaccinii* with strong support. It is sister to a clade containing *D. alnea*, *D. eres*, *D. celastrina*, *D. neilliae*, the *D. nobilis* complex, and *D. vaccinii* with strong support (Fig. 2). Based on these results and morphological comparisons to closely related taxa, isolates of the *Diaporthe* taxon isolated in this study represent an undescribed species that is described here as new.

Diaporthe virgiliae Machingambi N. M, Dreyer L. L & Roets F., sp. nov. (Fig. 3).

MycoBank no. MB811040.

Etymology

Named after *Virgilia*, the first host plant from which this fungus was identified.

Pycnidia black, eustromatic, subglobose to conical, aggregated or scattered. In culture up to 400 μm wide. Pycnidial wall consists of brown, thick-walled cells.

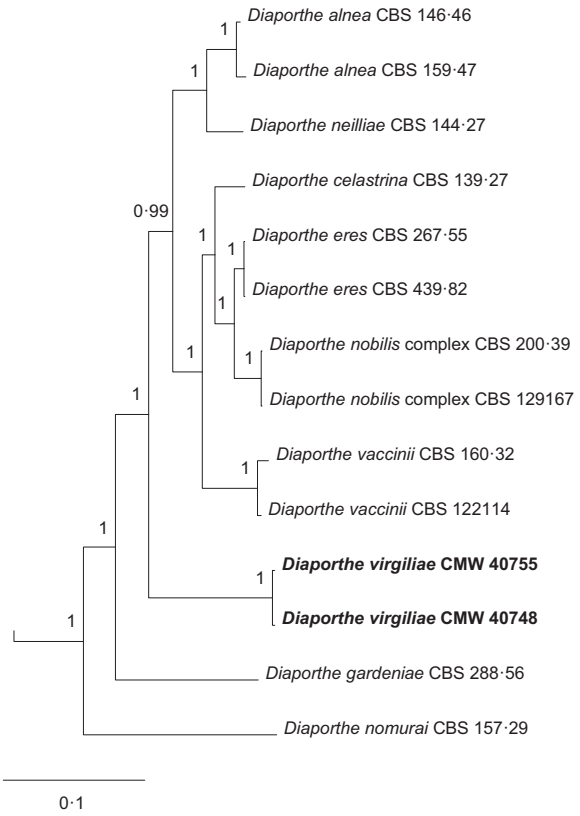

Figure 2 Bayesian strict consensus tree using data available from Gomes *et al.* (2013) and isolates from this study. Bayesian probabilities are shown above branches. South African isolates are in bold.

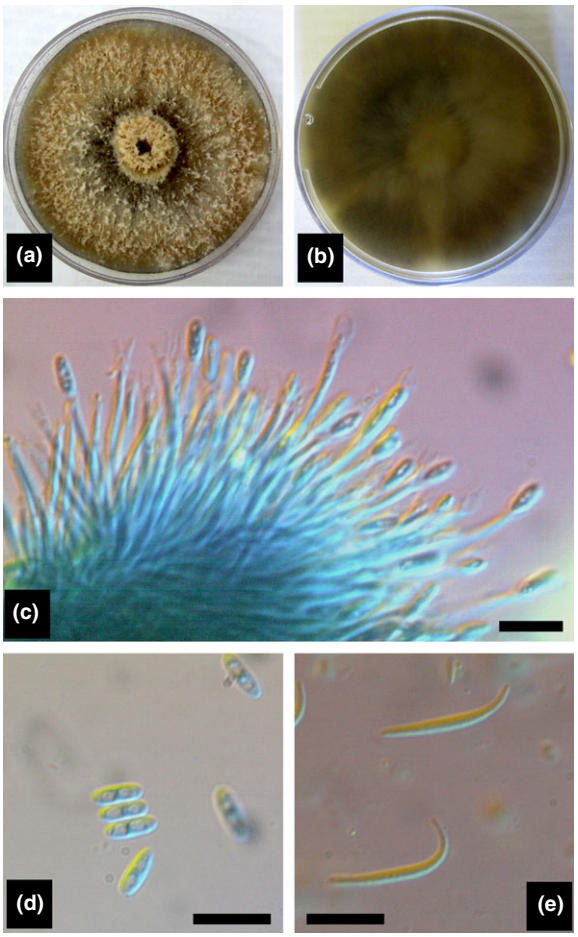

Figure 3 *Diaporthe virgiliae* (a) 2-week old culture on MEA; (b) reverse of 2-week-old culture on MEA; (c) sporulating conidiophores; (d) alpha-conidia; (e) beta-conidia. Scale bars 10 μm.

Conidia exuding from pycnidia in cream to peach-coloured droplets. *Conidiophores* cylindrical, noticeably flexuous, well-developed, simple or branched, 1–2 celled (12·254–)16·579(–21·305) × (0·682–)1·154(–1·468). Conidiogenous cells straight to curved, tapering slightly towards the apex, minute periclinal thickenings and funnel shaped collarettes present. *Alpha-conidia* fusoid-ellipsoidal, apexes bluntly rounded, bases obtuse to subtruncate, biguttulate, (5·19–)6·9(–7·9) × (1·1–)2·3 (–3·5) μm; *beta-conidia* curved and needle like, 17·1–25·4 μm long and 1·0–1·8 μm wide at widest (Fig. 3c–e).

Culture characteristics on MEA
Colonies reaching a diameter of 63 mm after 5 days at an optimum growth temperature of 25°C in the dark. Colonies spreading with sparse, dirty white aerial mycelium with smoke-grey to pale brown surfaces; margins becoming pale brown; reverse smoke-grey at centre becoming darker as it radiates to the edges (Fig. 3a,b).

Teleomorph
Unknown.

Habitat
Causing root disease on *Virgilia oroboides*.

Hosts and distribution
Virgilia oroboides in the Harold Porter National Botanical Garden, Betty's Bay, Western Cape Province, South Africa.

Specimens examined
SOUTH AFRICA. Western Cape Province, the Harold Porter National Botanical Garden (34°20′99″S, 18°55′56″E; 40–45 m a.s.l.), from roots of *Virgilia oroboides*. April 2011, N. Machingambi, HOLOTYPE (National Collection of Fungi, Pretoria, South Africa) PREM 61104, ex-type culture CMW 40755 = CBS 138788; April 2011, N. Machingambi, PARATYPE PREM 61103, living cultures CMW 40748 = CBS 138789.

Pathogenicity tests

Most of the stems inoculated with the *D. virgiliae* isolates from roots and bark produced excessive gum exudation from the wounds (Fig. 1f), a character that could usually be seen at the base of diseased individuals at the HPNBG, while this was absent from stems inoculated with any of the *Geosmithia* isolates and the controls. All *D. virgiliae* isolates caused distinct lesions (Fig. 1g) on stems of *V. oroboides* trees, while control inoculations caused very small lesions. The ANOVA revealed that mean lesion lengths differed significantly between treatments ($F = 18{\cdot}72$, d.f. = 24, $P < 0{\cdot}05$). Lesions caused by all *D. virgiliae* isolates were significantly larger than those of the control (Fig. 4). Isolate CMW40755 caused the most severe lesions, which were significantly longer than those formed by any of the other isolates. None of the *Geosmithia* taxa tested was pathogenic to *Virgilia* trees. All test taxa were successfully reisolated from the lesions after 6 weeks, thus fulfilling Koch's postulates.

Disease progression and distribution

The disease commonly observed in the HPNBG was not found in any of the eight other populations surveyed from across the natural distribution range of *Virgilia*. A total of 91 *V. oroboides* trees were assayed for their health status in the three transects in HPNBG. During the first field visit, 46 of these appeared healthy, 28 were obviously diseased and 17 were dead. A reassessment of the same individuals a year later revealed that only 33 appeared healthy, 14 were diseased and 44 were dead. The overall deterioration in health status of this *Virgilia* population over a year was found to be highly significant ($F = 43{\cdot}5$; d.f. = 90; $P < 0{\cdot}000001$).

Effect of tree age on health status

Trees of all ages and developmental stages were equally susceptible to *D. virgiliae*. Individuals showing initial below-ground signs of disease had only a few fine feeder roots that were dead in individuals at more advanced

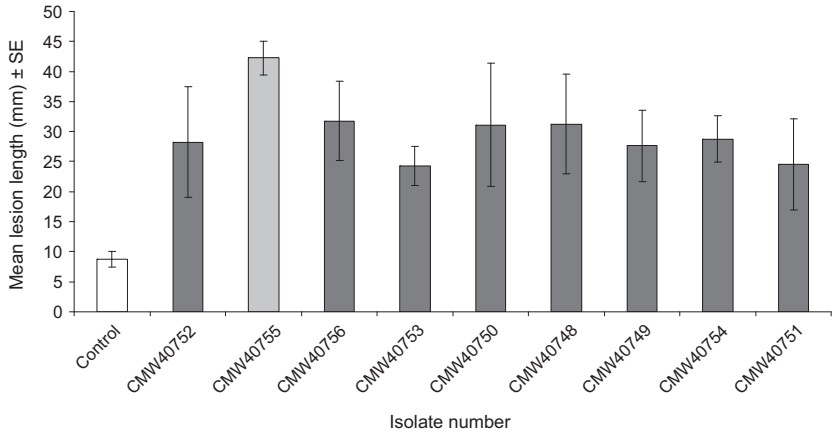

Figure 4 Mean lesion length (mm) caused by isolates of *Diaporthe virgiliae* (CMW40748–CMW40756). Different colours indicate significant differences in mean lesion length.

stages of disease. In wilting specimens, necrotic areas and lesions extended from fine roots into larger roots and into the main tap root. Lesions on dead individuals extended under the bark into the base of the main stem/trunk. This suggests that *D. virgiliae* initially infects the fine, young feeder roots, from where it spreads to larger roots, and ultimately to the root crown and base of stems.

There were no significant differences in size distributions (as surrogate for tree age) between the different disease status categories (Kruskal–Wallis ANOVA $H = 5{\cdot}93$; d.f. = 5; $n = 91$; $P = 0{\cdot}31$).

Discussion

Results from this study indicate that the death of *V. oroboides* in the Harold Porter National Botanical Garden of South Africa is primarily caused by a root-disease fungus, here described as *D. virgiliae*. *Diaporthe virgiliae* was consistently isolated from roots and underneath bark at the base of tree trunks of diseased *V. oroboides* trees and never from healthy specimens, indicating a strong link between the root disease and the fungus. This was supported by pathogenicity tests that showed the formation of very long lesions produced by *D. virgiliae* and with similar wood staining patterns and oozing gum to those observed on diseased plants.

Trees of all ages were susceptible to *D. virgiliae*. Based on the observations of the progression of infection, it seems that *D. virgiliae* may either block water and nutrient conducting cells or kill them. This would lead to subsequent observed symptoms such as leaf yellowing, wilting, leaf drop and shoot dieback over time. With death of the root system, shoot die-back and plant death follow fairly rapidly, as most trees that were diseased in 2011 were dead in 2012.

Species delimitation in *Diaporthe* based on morphological characters is extremely difficult and the description of novel species relies strongly on molecular data (Gomes *et al.*, 2013). In the present analyses *D. virgiliae* resolved as distinct from all currently known taxa based on molecular data (Gomes *et al.*, 2013). It is fairly closely related to the type for the genus, *D. eres*, known from more than 60 hosts (Wehmeyer, 1933). However, no isolates that have been confirmed as species closely related to *D. virgiliae* have been collected from the African continent (Gomes *et al.*, 2013). Many of these species cause disease on their host plants. For example, *D. vaccinii* causes fruit rot, twig blight and leaf spots of blueberries (Alfieri *et al.*, 1984; Farr *et al.*, 2002) and *D. alnea* causes dieback of alder trees (Gomes *et al.*, 2013). However, the only closely related species that the authors are currently aware of that can infect roots of woody host plants is *D. gardenia,* confirmed to infect all parts of their hosts (McKenzie *et al.*, 1940).

Diaporthe species are known to be endophytes, saprobes or pathogens on a wide range of hosts and substrates (Uecker, 1988; Udayanga *et al.*, 2011; Gomes *et al.*, 2013). Many phytopathogenic species cause root disease, rots, spots, cankers, blights and wilts to numerous plants globally (Uecker, 1988; Van Rensburg *et al.*, 2006; Udayanga *et al.*, 2011; Gomes *et al.*, 2013). For example, *D. sclerotioides* is a primary parasite attacking cucumber roots (Ebben & Last, 1973) and *D. pseudophoenicola* causes root disease of date palms (Abbas *et al.*, 1991). To the best of the authors' knowledge, no root disease-causing *Diaporthe* species other than *D. virgiliae* has been described from South Africa. However, numerous species that are associated with aboveground plant diseases have been identified. For example, several *Diaporthe* species have been associated with leaf spot and shoot diseases on grapevines (Pearson & Goheen, 1994; Van Niekerk *et al.*, 2005). A few *Diaporthe* species that are pathogenic to native CFR plants have also been identified. *Diaporthe saccharata*, for example, is responsible for a canker and die-back disease of *Protea repens* (Mostert *et al.*, 2001) while a complex of *Diaporthe* spp. was found to be associated with die-back of rooibos (*Aspalathus linearis*) (Van Rensburg *et al.*, 2006).

This study is the first to record disease of native *V. oroboides* in South Africa. It is currently unknown whether *D. virgiliae* is native to South Africa. However, in assessments of *Virgilia* trees from other areas in the CFR no similar disease symptoms have been observed. As introductions of foreign organisms are common via diseased nursery material or timber (Von Broembsen, 1989; Coetzee *et al.*, 2003) and this disease is currently only known from a botanical garden, it is quite possible that the fungus described here is not native to this area. The high incidence of tree death in this population adds credence to this notion. Being a botanical garden with an active nursery, it is possible that this pathogen could spread to other areas via rooted plants. Therefore, until further studies are conducted, it is recommended that no *Virgilia* plants be translocated from this area.

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