# Novel ophiostomatalean fungi from galleries of *Cyrtogenius africus* (Scolytinae) infesting dying *Euphorbia ingens*

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## Abstract

*Euphorbia ingens* trees have been dying in large numbers in the Limpopo Province of South Africa for approximately 15 years. The ambrosia beetle *Cyrtogenius africus* is often found infesting diseased and dying trees. The aim of this study was to identify the ophiostomatoid fungi occurring in the galleries of *C. africus*. Logs infested with this beetle were collected from the KwaZulu-Natal, Limpopo, Mpumalanga, and North West Provinces of South Africa. Fungi belonging to the Ophiostomatales were identified based on morphology and comparison of sequence data for the β-tubulin, ITS1-5.8S-ITS2 and LSU gene regions. A novel species of *Ophiostoma* and a novel genus in the Ophiostomatales were identified. Inoculation studies with these fungi produced lesions in the branches of healthy *E. ingens* trees.

**Keywords:** *Ophiostoma*, Ophiostomataceae, ophiostomatalean fungi, Ophiostomatales, Scolytinae

## Acknowledgements

We thank the Department of Science and Technology (DST), National Research Foundation (NRF) and DST/NRF Centre of Excellence in Tree Health Biotechnology (CTHB), for financial support; Dr. Hugh Glen for assistance with Latin names and Dr. Roger Beaver for assistance with beetle identification. Mr. Gert van der Merwe, Mr. Thomas Ndala, Mr. Henk Fourie and Buyskop Game Farm are thanked for permission to use their properties for field studies.

## 1. Introduction

The ophiostomatoid fungi were originally classified, based on morphology, as a group of fungi with similar sexual structures that had evolved in close association with insects (Wingfield et al. 1993, De Beer et al. 2013). These structures include flask-shaped ascomata with long necks raised above the substrate and exuding sticky spore drops (containing ascospores) that aid in dispersal via insects (Dowding 1984, Malloch and Blackwell 1993). Three ascomycete genera, *Ceratocystis*, *Ceratocystiopsis*, and *Ophiostoma*, were originally included in the group referred to as ophiostomatoid fungi (Wingfield et al. 1993). Phylogenetic inference based on DNA-sequence analyses later revealed that the ophiostomatoid fungi represent a polyphyletic assemblage, comprising two distinct orders, the Ophiostomatales and the Microascales (Hausner et al. 1993, Spatafora and Blackwell 1994, Zipfel et al. 2006). The Ophiostomatales contains only one family (Ophiostomataceae) with six genera, *Ceratocystiopsis*, *Fragosphaeria*, *Leptographium sensu lato*, *Ophiostoma sensu lato*, *Raffaelea sensu stricto* and *Graphilbum* (De Beer et al. 2013), while the Microascales is comprised of five families, two of which, the Ceratocystidaceae and Graphiaceae, accommodate the ophiostomatoid genera (Réblová et al. 2011, De Beer et al. 2013, De Beer et al. 2014).

Ophiostomatalean fungi have various ecological associations with ambrosia and bark beetles (Paine et al. 1997, Harrington 2005). While bark beetles feed in the phloem and sometimes have dependent associations on the fungi they vector, ambrosia beetles bore into the xylem of host trees where they are completely dependent upon their fungal associates for food (Beaver 1989, Hulcr and Dunn 2011, Six 2012). Ambrosia beetles are known to infest dead or severely stressed trees (Batra 1967, Wood 1982). However, studies have shown that increasing numbers of these beetles, together with their fungal partners, can infest and kill healthy trees leading to substantial tree mortality (Hulcr and Dunn 2011, Ploetz et al. 2013, Ranger et al. 2015). For example, in Japan increasing levels of oak die-back caused by *Platypus quercivorus* Murayama and its fungal symbiont *Raffaelea quercivora* Kubono & Shin. Ito, have been reported (Kamata et al. 2002, Kubono and Ito 2002). Likewise, in the United States of America (USA) laurel wilt disease is caused by *Raffaelea lauricola* T.C. Harr., Fraedrich & Aghayeva that is vectored by the invasive ambrosia beetle *Xyloborus glabratus* Eichhoff (Fraedrich et al. 2008, Harrington et al. 2008).

*Euphorbia ingens* E. Meyer: Boissier trees, native in the savanna landscape of South Africa, have been dying in large numbers for several years (Roux et al. 2008, 2009, Van der Linde et al. 2011a,b). The first reports of die-offs were from the Limpopo Province of South Africa (RSA) and were associated with various biotic factors (Malan 2006, Roux et al. 2008, 2009). Pilot studies showed the presence of several ophiostomatalean fungi in the tunnels of beetles infesting dying *E. ingens* (Roux et al. 2008, 2009). Two weevils [*Cossonus* sp. Claireville and *Stenoscelis* sp. Wollaston (Cossoninae)] as well as the ambrosia beetle *Cyrtogenius africus* Wood (Scolytinae) were identified from diseased and dying *E. ingens* trees (Van der Linde et al. 2011a,b). Van der Linde et al. (2011b), however, identified only a single species of ophiostomatalean fungi, *Knoxdaviesia serotectus* (J.A. van der Linde & Jol. Roux) Z.W. de Beer & M.J. Wingf. (Ceratocystidaceae), from the secondary phloem of dying trees associated with a *Cossonus* species.

The aim of this study was to identify ophiostomatalean fungi collected in the tunnels of *C. africus* on *E. ingens* in all provinces of South Africa where the tree occurs. Ophiostomatalean fungi were identified using sequence data of multiple gene regions and their potential role in tree die-offs was considered using artificial inoculation studies to assess pathogenicity to the host tree.

## 2. Materials and Methods

### Collection of samples and isolations

Sections of *E. ingens* stems were cut from trees showing signs of beetle infestation at six sites in South Africa over a period of three years (2012-2014), from early autumn (March) to early spring (August) during each year. One log was sampled from each of 10 trees at each site. The sites were located in KwaZulu-Natal (Eshowe - March 2013, Coordinates: 28°48'42.64"S 31°30'30.10"E), Limpopo (Bela-Bela - August 2012/July 2013, 24°51'48.30"S 28°20'5.90"E; Last Post - June 2014, 23°17'21.39"S 29°55'27.93"E), Mpumalanga (Lydenburg - July 2012, 24°55'53.87"S 30°19'7.09"E) and North West Provinces (Brits - July 2013, 25°42'59.27"S 27°42'9.24"E; Enzelsberg - July 2013, 25°22'58.05"S 26°16'4.21"E).

Logs were carefully dissected in the laboratory to expose galleries of *C. africus*. Isolations were made directly from fungal structures, typical of ophiostomatalean fungi, in the galleries of *C. africus* as well as from stained tissue surrounding the galleries. Spore drops from ascomata were placed, using a sterilised needle, on 2 % MEA (MEA; 15 g agar and 20 g malt extract $l^{-1}$) containing 0.4 g streptomycin sulphate $l^{-1}$. MEA plates were incubated at 25 °C for up to five days and single hyphal tips from germinating spores transferred to fresh MEA plates. Isolates were deposited in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, and representative isolates were deposited in the Centraalbureau voor Schimmelcultures (CBS),

Utrecht, The Netherlands. Herbarium specimens representing novel species were deposited with the National Fungal Collection (PREM), Pretoria, South Africa.

## Fungal Morphology and Growth

Fruiting structures of the ophiostomatalean fungi, obtained from both plant material and MEA plates, were examined by mounting structures in distilled water on glass microscope slides and examining these using a Zeiss microscope. Images of relevant, representative structures were captured with an Axiocam digital camera (Axiovision 3.1). Informative characteristics for the ophiostomatalean fungi were measured (50 measurements each) and presented as (min–) avg. ± std. dev. (–max) for the length and width of the structures (l/w).

Optimal temperatures for growth of the fungi were determined by placing 5 mm agar discs, obtained from five-day-old cultures, with the mycelium facing down, at the centers of 90 mm MEA plates. Five replicates were used for each isolate at each temperature. Plates were incubated at temperatures ranging from 10 °C to 35 °C at 5 °C intervals. Incubation was conducted in the dark for 10 days with measurements taken every 24 hours. Two measurements of the total diameter were made perpendicular to one another. Averages of the diameters were calculated and one-way analysis of variance (ANOVA) was conducted ($P < 0.05$ as significant, JMP 12.0.1 SAS Institute 2015) to determine at which temperature each of the species grew best. Data were tested for normality (Shapiro-Wilk's W, Shapiro and Wilk 1965) with non-normal data transformed (ln+1; to account for zeros in the data) and analysed using a Kruskal-Wallis one way ANOVA.

## DNA extraction, PCR, Sequencing and Phylogenetic analyses

DNA was extracted from the mycelium of five-day-old isolates (with ophiostomatalean culture morphology) using PrepMan® Ultra (Applied Biosystems, Foster City, USA). DNA concentrations were determined using a Nanodrop ND-1000 Spectrophotometer (Thermo-Fisher Scientific, Wilmington, USA). The polymerase chain reaction (PCR), using an Applied

Biosystems Veriti thermocycler, was used to amplify the β-tubulin (BT) gene region using the primers T10 (O'Donnell and Cigelnik 1997) or Bt2A together with Bt2B (Glass and Donaldson 1995), the internal transcribed spacer (ITS) regions 1 and 2 including the 5.8S gene using the primers ITS1 and ITS4 (White et al. 1990), and the ribosomal large subunit (LSU) gene region using the primers LR5 and LROR (Vilgalys and Hester 1990). PCRs were conducted using the protocol described by Van der Linde et al. (2011b). PCR products were confirmed using an agarose gel (2 %; Whitehead Scientific, Cape Town, South Africa) loaded with GelRed (Anatech, USA), visualised under UV illumination. A 100 bp DNA molecular marker (O'RangeRuler™ 100 bp DNA ladder, Fermentas Life Sciences, Vilnius, Lithuania) was used to estimate the sizes of the PCR products. Amplification products were purified using DNA Clean & Concentrator™-5 (ZYMO Research, Irvine, USA).

Purified PCR products were sequenced using an ABI 3700 DNA analyser (Applied Biosystems) following the instructions provided by the manufacturer. Mega 5.0 (Tamura et al. 2007) was used to construct contigs based on forward and reverse sequences. Sequences were submitted to searches in the BLASTn database to establish closest matches. Sequences of the most closely related fungi for all three gene regions were downloaded from Genbank and included in phylogenetic analyses. Sequences obtained from this study and Genbank were aligned using MAFFT 5.851 (Katoh et al. 2002).

Aligned sequences were analyzed with Maximum Parsimony (heuristic searches, with random stepwise addition and tree bisection as branch swapping algorithms) in PAUP* 4.0b10 (Swofford 2002). Bootstrap analyses, with 1000 replicates (Felsenstein 1985), were determined for all datasets. Posterior probabilities were determined using Bayesian inference (MrBayes 3.1.2, Huelsenbeck and Ronquist 2001) using the Monte Carlo Markov chain (MCMC) method (parameters set at four chains producing 5,000,000 generations, recording trees every 100 generations). The appropriate nucleotide substitution model was determined using jModelTest

0.1.1 (Posada 2008) with burn-in values determined using graphical analysis (Tracer 1.5) at the point where values converged.

## Pathogenicity study

Three isolates of each of the fungi identified in this study were selected for pathogenicity trials conducted on *E. ingens* trees growing under field conditions at Bela-Bela (24°51'48.30"S 28°20'5.90"E) in the Limpopo Province. Sterilized wooden tooth picks were autoclaved in malt extract broth and placed onto the surfaces of 2 % MEA plates. These plates were left for three days to ensure sterility before inoculation with the test fungi (Van der Linde et al. 2011b). Five plates were prepared for each of the isolates as well as the control and grown for five days prior to inoculation. The control consisted of sterile toothpicks on the surface of MEA that had not been inoculated with fungi. Toothpicks for each isolate (seven in total), including the control, were inserted into five separate branches on seven trees. Toothpicks were inserted to a depth of 3 mm in secondary tier healthy succulent branches. Branches ranged between 25 and 30 cm in circumference.

Six weeks after inoculation, all inoculated branches were removed for evaluation. External lesions extending from the entry point of the toothpicks were measured (parallel with branch length). The depth and width of internal rotting was also measured after cutting open the branches at the point of inoculation. Isolations were made from any visible lesions to confirm fungal identity and to comply with Koch's postulates. The entire experiment was repeated once. Data from both experiments were analysed separately and then combined for each isolate to determine variation in external lesion length and internal rotting (depth and width). The mean of the external lesion length and area of internal rotting (depth and width) was compared among all the isolates tested using one-way analysis of variance (ANOVA) with $P < 0.05$ set as significant (JMP 12.0.1 SAS Institute 2015). Normality of data was tested with Shapiro-Wilk's W with non-normal data transformed (ln+1; to account for zeros in the data) and analysed with a Kruskal-Wallis one way ANOVA. No variance was found with the control (replicates showed

zero lesion length) and consequently, each isolate was compared against zero using independent, one sided t-tests, Bonferroni-corrected for multiple comparisons ($\alpha = 0.05$, JMP 12.0.1).

# 3. Results

## Fungi isolated

*Cyrtogenius africus* was obtained from logs at all sites. Fifty-five isolates resembling species of ophiostomatalean fungi were obtained from beetle galleries and surrounding stained tissues from 40 of the 60 logs. Of the 55 isolates obtained in this study two different species were identified. Thirty-nine of the isolates belonged to one species, with 13 directly isolated from sporocarps in *C. africus* tunnels and 26 obtained from fungal stain surrounding the tunnels. Of the 39 isolates four were obtained from Bela-Bela (three trees), four from Brits (four trees), three from Eshowe (three trees), six from Enzlesberg (six trees), 15 from Last Post (10 trees) and seven from Lydenburg (seven trees). Sixteen of the 55 isolates were identified as another species with six obtained directly from sporocarps in *C. africus* tunnels and 10 obtained from fungal stain surrounding the tunnels. All sixteen isolates of the second species were obtained from Bela-Bela (7 trees), with none from the other sites investigated. The two fungal species identified were not found in the same *C. africus* tunnels in Bela-Bela.

## Fungal Morphology and Growth

Sexual and asexual structures were observed in the galleries of *C. africus*, with the two states not observed to occur within the same tunnels. The one state was characterized by ascomata with spore droplets and the other state had hyaline sporothrix-like conidiophores in the tunnels. All cultures obtained were white with two distinct culture morphologies; one with radiate-to chrysanthemum-like (in reference to the flower morphology of this plant genus, usually used to describe *Phytophthora* and *Pythium* culture morphology, Mrázková et al. 2011)

**Table 1** Locality and Genbank accession numbers of representative isolates sequenced and used in phylogenetic analyses

| Species | CMW no.[a] | CBS no.[b] | PREM no.[c] | Locality | ITS | LSU | β-tubulin |
|---|---|---|---|---|---|---|---|
| *Ophiostoma thermarum* | 38929[d] | 140081 | 61239 | Bela-Bela | KR051114 | KR051126 | KR51102 |
| *O. thermarum* | 38930[d,e] | 139747 | 61238 | Bela-Bela | KR051115 | KR051127 | KR51103 |
| *O. thermarum* | 38931 | – | – | Bela-Bela | KR051116 | KR051128 | KR51104 |
| *O. thermarum* | 38932 | – | – | Bela-Bela | KR051117 | KR051129 | KR51105 |
| *O. thermarum* | 38940[d] | 140082 | 61240 | Bela-Bela | KR051118 | KR051130 | KR51106 |
| *Aureovirgo volantis* | 41238[d,e] | 139648 | 61235 | Last Post | KR051119 | KR051131 | KR51107 |
| *A. volantis* | 41250[d] | 139649 | 61236 | Last Post | KR051120 | KR051132 | KR51108 |
| *A. volantis* | 42282 | – | – | Eshowe | KR051123 | KR051133 | KR51109 |
| *A. volantis* | 42285 | – | – | Lydenburg | KR051121 | KR051134 | KR51110 |
| *A. volantis* | 42287 | – | – | Bela-Bela | KR051124 | KR051135 | KR51111 |
| *A. volantis* | 42290 | – | – | Enzelsberg | KR051122 | KR051136 | KR51112 |
| *A. volantis* | 42292[d] | 139645 | 61237 | Brits | KR051125 | KR051137 | KR51113 |

[a] CMW, Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), South Africa

[b] CBS, Centraalbureau voor Schimmelcultures, Netherlands

[c] PREM, South African National Collection of Fungi, South Africa

[d] Isolates used in pathogenicity and growth study

[e] Type strains

aerial mycelial growth, with the other culture morphology type having no aerial mycelia and an inconspicuous, near translucent, shiny appearance.

The six isolates (three each of the two species, Table 1) selected for further study had significant differences in growth rates at the different temperatures. The optimal growth rate was 30 °C for species 1 (F statistic: 327.906, df = 5, $P < 0.001$) and species 2 (F statistic: 1394.727, df = 5, $P < 0.001$). The temperature range supporting growth was similar for both species.

## DNA sequence analyses

Analyses of the ITS data (TL = 717, CI = 0.517, RI = 0.856, 45.7 % of characters parsimony informative, TreeBase: TB17782) (Fig. 1) revealed that the isolates obtained from *E. ingens* in the present study formed two groups, representing taxa distinct from all known species in the Ophiostomatales. The first taxon was most closely related to *Ophiostoma bragantinum*, and grouped peripheral to the *S. schenckii–O. stenoceras* complex within *Ophiostoma sensu lato*. The second taxon grouped with a lineage closest to, but clearly distinct from the *Sporothrix lignivora* complex and the genus *Graphilbum*. The two taxa exhibited similar relationships with other ophiostomatalean lineages in the LSU tree (TL = 283, CI = 0.525, RI = 0.832, 24.8 % of characters parsimony informative, TreeBase: TB17783) (Fig. 2). The ITS and LSU datasets were supported by high bootstrap values as well as posterior probabilities obtained from Bayesian analysis (ITS model: GTR+G, LSU model: TIM3+I+G, burn-in values for both datasets were 3000). BT sequences are not available for all taxa included in the ITS and LSU trees and so a similar tree comparison could not be made for these gene sequences. However, based on BLAST searches (data not shown) the sequences for both taxa were distinct from all available BT sequences for the Ophiostomatales.

**Fig. 1.** The most parsimonious tree obtained from maximum parsimony analyses of ITS1-5.8S-ITS2 sequence data. Nodes in bold indicate MCMC posterior probabilities values ≥ 0.95 and values at the nodes are mean parsimony bootstrap values. T indicates ex-type strains

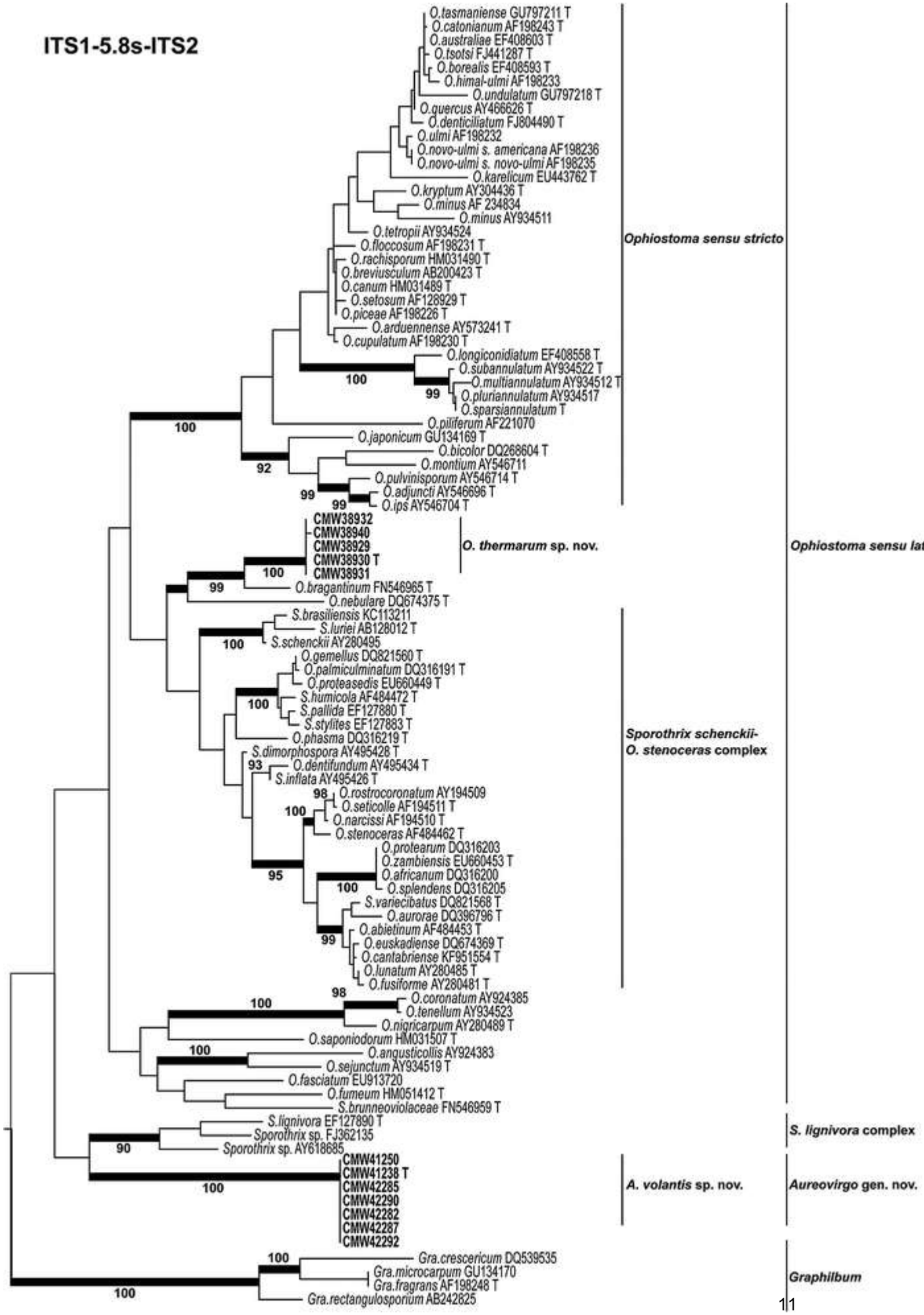
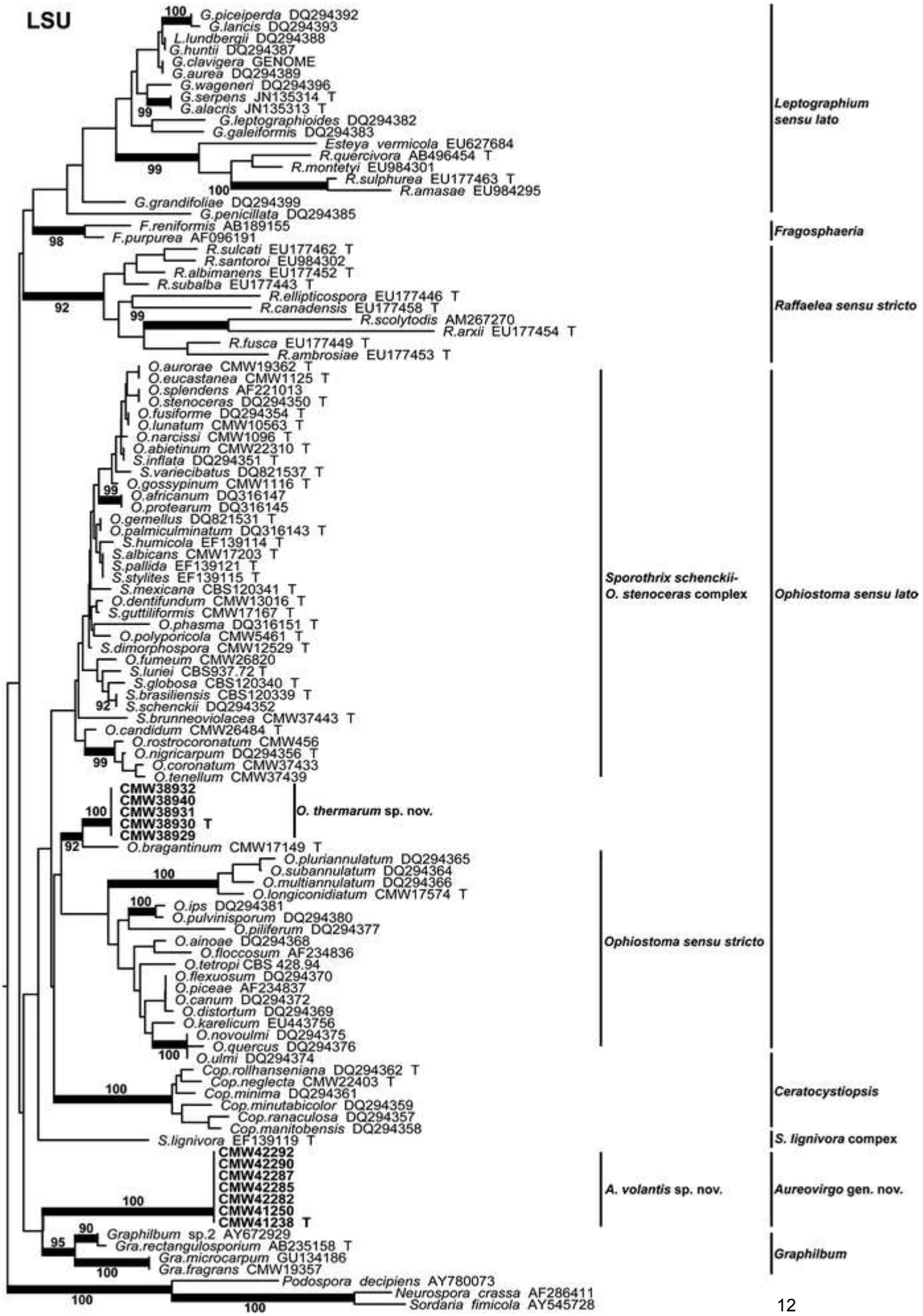


**Fig. 2.** The most parsimonious tree obtained from maximum parsimony analyses of LSU sequence data. Nodes in bold indicate MCMC posterior probabilities values ≥ 0.95 and values at the nodes are mean parsimony bootstrap values. T indicates ex-type strains



## Taxonomy

Analyses of DNA sequences of the ITS, LSU and BT gene regions of isolates obtained in this study confirmed that these isolates represented two novel taxa distinct from previously described species. One of these clearly represents a new genus in the Ophiostomatales, while the other fungus is described as a new species of *Ophiostoma sensu lato*.

***Aureovirgo*** J.A. van der Linde, Z.W. de Beer & Jol. Roux gen. nov.

Mycobank MB813870

**Etymology:** Genus name refers to the golden appearance of the immature ascomata and the pure white color of the cultures ("Aureovirgo" refers to a golden maiden with an unstated overtone of virginal whiteness).

Type species: ***Aureovirgo volantis*** J.A. van der Linde, Z.W. de Beer & Jol. Roux

Ascomatal bases honey colored (19") when immature to fuscous (13""k) when mature, necks dark, ostiolar hyphae hyaline, ascospores allantoid with ellipsoidal sheaths. Leptographium-like asexual state: Conidiophores mononematous, hyaline, stipe cylindrical and simple. Conidia oblong-elliptical and oval ovate.

***Aureovirgo volantis*** J.A. van der Linde, Z.W. de Beer & Jol. Roux sp. nov. (Fig. 3)

Mycobank MB813872

**Etymology**: the species name is derived from the Latin word "volanti" for flying. This describes the distribution of the fungus with the insect *C. africus*.

Mycelium on MEA produces pure white radiate to chrysanthemum aerial growth. Sexual state found only in insect galleries, not observed in culture. *Ascomatal bases* honey coloured (19") when immature, to fuscous (13""k) when mature (77.5–) 78.7 – 94.9 (–97.3) x (90.61–) 92.1 – 106.9 (–110.6) µm (86.8 x 99.5 µm, l/w 0.9), *necks* dark brown (164.5–) 202.2

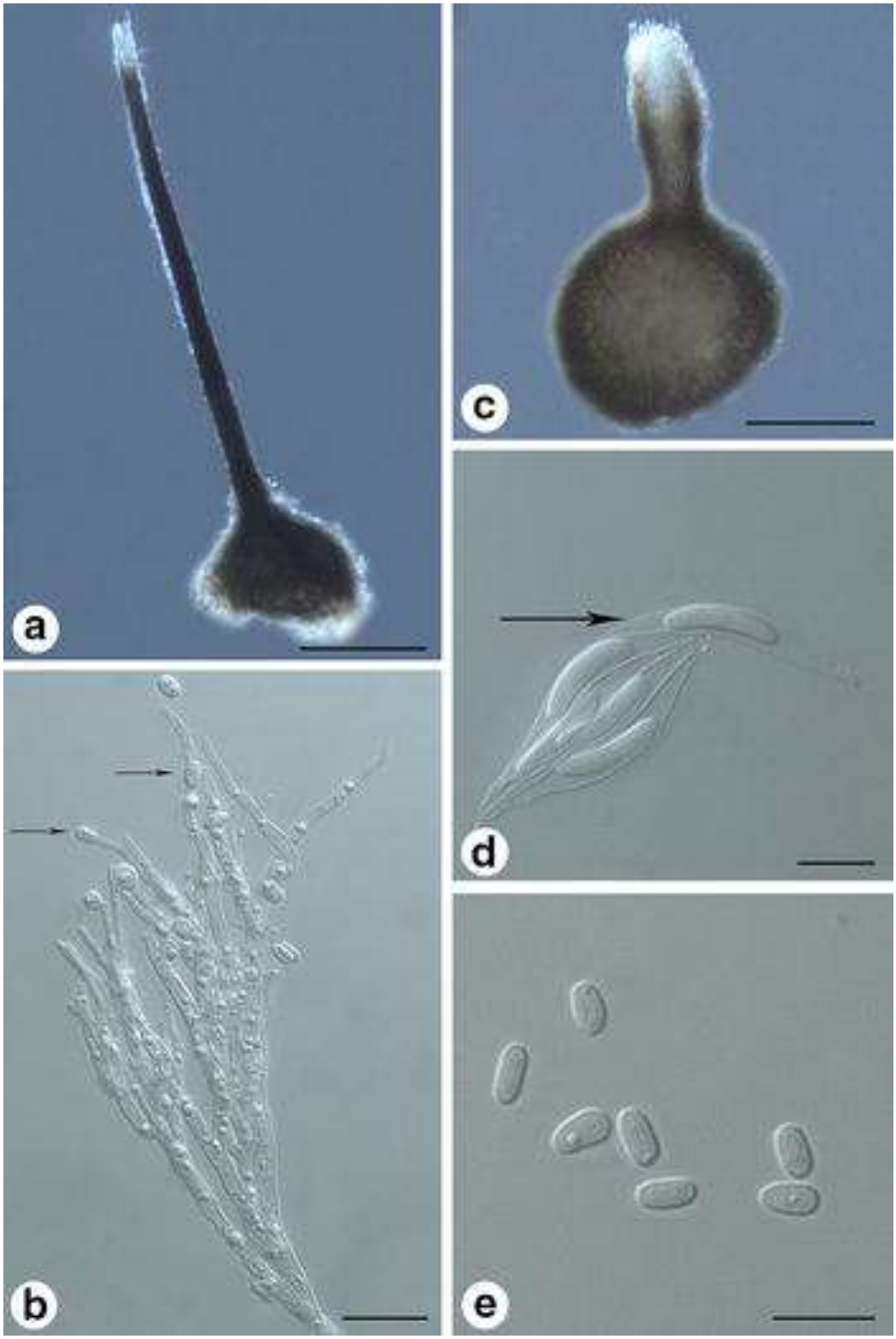



**Fig. 3.** *Aureovirgo volantis* (CMW41238). **a** Mature ascocarp with ostiolar hyphae. **b** Leptographium-like conidiophore with conidiogenous cells (*arrows* indicate conidiogenous cells). **c** Immature ascocarp with typical honey coloured base. **d** Allantoid ascospores with ellipsoidal sheath (*arrows* indicate ellipsoidal sheath). **e** Oblong-elliptical and oval ovate conidia. *Scale bars* **a**, **c** = 100 µm, **b** = 10 µm, **d**, **e** = 5 µm

– 345.2 (–359.3) x (16.0–) 15.9 – 21.3 (–23.9) µm (273.7 x 18.6 µm, l/w 14.7), *ostiolar hyphae* hyaline (33.6–) 34.1 – 67.1 (–72.9) x (3.6–) 3.7 – 4.5 (–4.6) µm (50.6 x 4.1 µm, l/w 12.3), *ascospores* allantoid with ellipsoidal sheaths (6.9–) 7.2 – 8.0 (–8.3) x (1.5–) 1.7 – 2.1 (–2.2) µm (7.6 x 1.9 µm, l/w 4.0). *Asexual state* leptographium-like. *Conidiophores* mononematous, hyaline (22.4–) 25.2 – 46.4 (–52.0) x (1.0–) 0.9 – 1.7 (–1.9) µm (35.8 x 1.3 µm, l/w 27.5), *conidia* oblong-elliptical and oval ovate (3.5–) 3.7 – 4.8 (–5.9) x (1.6–) 1.9 – 2.9 (–3.8) µm (4.3 x 2.4 µm, l/w 1.8). Optimum temperature for growth temperature 30 °C, growing at 9.7 mm/day, with minimum growth at 15 °C and maximum growth at 35 °C.

HOLOTYPE. SOUTH AFRICA, LIMPOPO PROVINCE: Last Post, isolated directly from *Cyrtogenius africus* galleries on diseased *Euphorbia ingens* trees, June 2014, Van der Linde JA, holotype PREM 61236 dry culture on MEA, ex-holotype culture CMW41238 = CBS139648; paratype PREM 61236, ex-paratype culture CMW41250 = CBS139649. Ascomata not observed in culture.

*Additional specimens examined*: Limpopo Province (Bela-Bela; July 2013, Van der Linde JA; CMW42287), KwaZulu-Natal (Eshowe; March 2013, Van der Linde JA; CMW42282), Mpumalanga (Lydenburg; July 2012, Van der Linde JA; CMW42285), North West (Brits; July 2013, Van der Linde JA; CMW42292 = CBS139645 and Enzelsberg; July 2013 Van der Linde JA; CMW42290) isolated from *C. africus* galleries on diseased *E. ingens* trees.

***Ophiostoma thermarum*** J.A. van der Linde, Z.W. de Beer & Jol. Roux sp. nov. (Fig. 4)

Mycobank MB813873

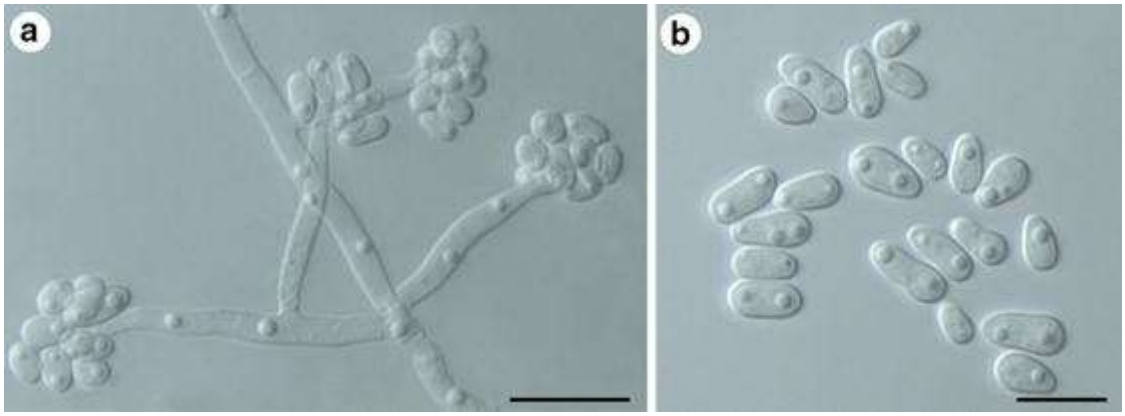



**Fig. 4.** *Ophiostoma thermarum* (CMW38930). **a** Sporothrix-like condiophore with conidiogenous cells. **b** Oblong-elliptical and obovate conidia. *Scale bars* **a** = 10 µm, **b** = 5 µm

**Etymology**: The species name refers to the locality from where this species was collected, Bela-Bela, which is surrounded by geothermal springs ("thermarum" meaning place of warm baths).

Growth on MEA barely visible, shiny white yeast-like growth with no aerial mycelium. Sexual state not observed on plant material or culture. *Asexual state* sporothrix-like. *Conidiophores* (56.0–) 62.3 – 101.3 (–107.4) x (1.00–) 1.1 – 2.1 (–2.4) µm (81.8 x 1.6 µm, l/w 51.1), *conidia* oblong-elliptical and obovate (4.1–) 4.4 – 5.6 (–6.5) x (1.4–) 2.0 – 2.8 (–3.3) µm (5.0 x 2.4 µm, l/w 2.1). Optimum temperature for growth 30 °C, growing at 9.4 mm/day, with minimum growth at 15 °C and maximum growth at 35 °C.

HOLOTYPE. SOUTH AFRICA, LIMPOPO PROVINCE: Bela-Bela, isolated from *Cyrtogenius africus* galleries on diseased *Euphorbia ingens* trees, August 2012, Van der Linde JA, holotype PREM 61238 dry culture on MEA, ex-holotype culture CMW38930 = CBS139747; paratype PREM 61240, ex-paratype culture CMW38940 = CBS140082.

*Additional specimens examined*: Bela-Bela, isolated from *C. africus* galleries on diseased *E. ingens* trees, August 2012, Van der Linde JA; CMW38929 = CBS140081; CMW38932; CMW38931.

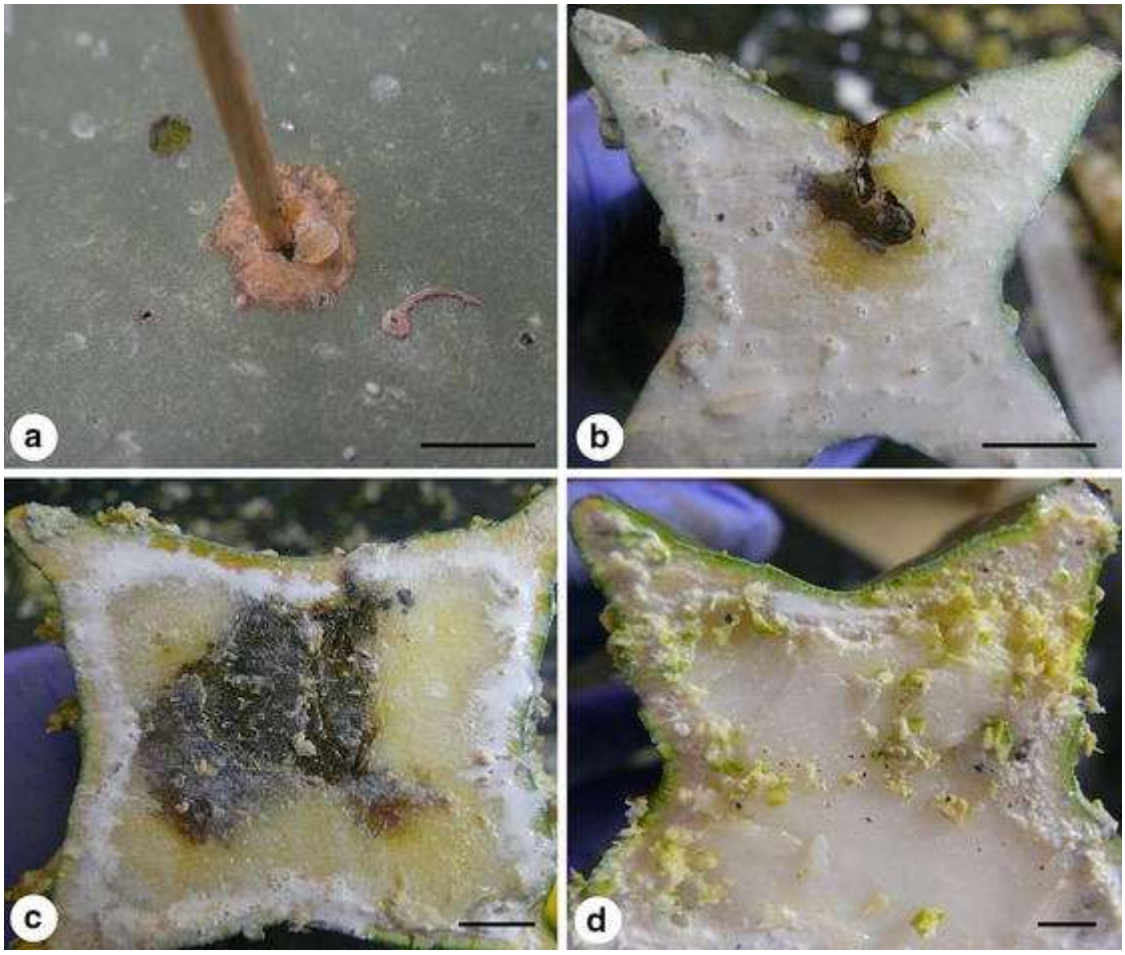



**Fig. 5.** Sections through *E. ingens* branches inoculated with *A. volantis*, *O. thermarum* and control inoculation. **a** Lesion produced by *A. volantis* (CMW42292) on the exterior of the succulent branches. **b** Internal rotting of succulent tissue produced by *O. thermarum* (CMW 38929). **c** Extensive rotting found in some cases, in this case produced by *A. volantis* (CMW41250). **d** Control inoculation showing no infection. *Scale bars* **a**–**d** = 10 mm

## Pathogenicity

Isolates of *O. thermarum* (CMW38940, CMW38930, CMW38929) and *A. volantis* (CMW41238, CMW41250, CMW42292) produced internal rotting and external lesions at the points of inoculation on healthy *E. ingens* trees. The external lesions at the points of inoculation surrounding the toothpicks were brown in color with dark brown rotted areas leading into the internal succulent tissue (Fig. 5a, b, c). Control inoculations produced no external lesions or internal rotting of the succulent branches (Fig. 5d). Both experiments produced similar results

with significant differences in the length of the external tissue lesions, between the isolates extending from the points of inoculation (F test statistic = 13.250, df = 5, P < 0.001). There was no significant difference among depths (F test statistic = 1.6907, df = 5, P = 0.1673) and widths, between the isolates, (F test statistic = 1.0913, df = 5, P = 0.3854) of the internal lesions (Fig. 6). Both species produced lesions that were significantly larger (Values from the Bonferroni procedure were all significant with P < 0.05) than those of the controls. The two fungi used in the inoculations were consistently re-isolated and identified based on morphology, while no growth was produced from isolations from the control inoculations.

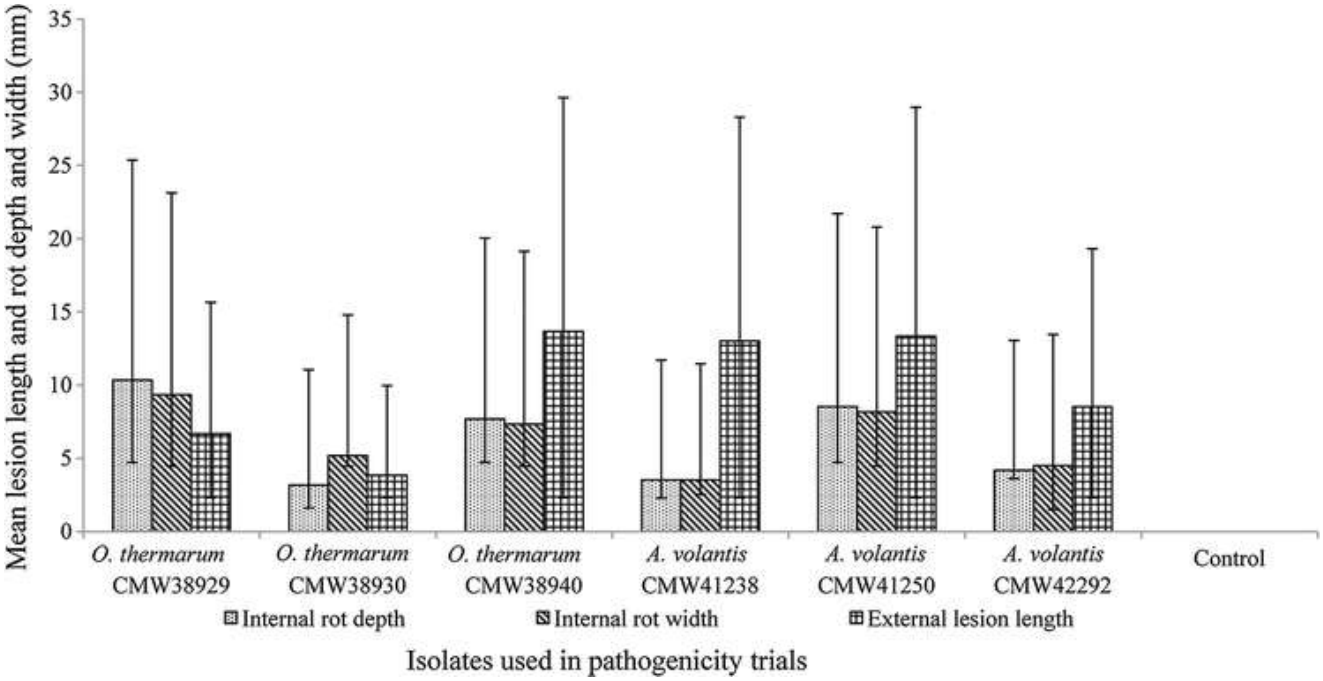



**Fig. 6**. Mean external lesion and internal rotting depth and width measurements obtained in inoculations of *E. ingens* branches with *O. thermarum* (CMW38929, CMW38930, CMW38940) and *A. volantis* (CMW41238, CMW41250, CMW42292). Error bars indicate 95 % confidence limits for each isolate

## 4. Discussion

Fungi in the Ophiostomatales were identified from dying *E. ingens* trees at all sites investigated in this study. Previously, fungi in the Microascales were identified from dying *E.*

*ingens* trees infested by *Cossonus* species in the Limpopo Province (Roux et al. 2009, Van der Linde et al. 2011b), but these fungi were not found here. This is the first report of fungi in the Ophiostomatales from dying *E. ingens* trees in South Africa. A species in a novel genus and a new *Ophiostoma* species were identified and described from the fungi isolated from the galleries of the ambrosia beetle *C. africus* infesting dying *E. ingens* trees.

The newly described genus, *Aureovirgo*, can be distinguished from other genera in the Ophiostomatales based on DNA sequence data and distinct morphological features. *Aureovirgo* is phylogenetically most closely related to *Graphilbum* H.P. Upadhyay & W.B. Kendr. and species in the *Sporothrix lignivora* complex. It can, however, be distinguished from those genera based on its conidiophore morphology, as well as its conidia and ascospores. *Aureovirgo* has a leptographium-like asexual state as opposed to the sporothrix-like state in *S. lignivora* and pesotum-and hyalorhinocladiella-like states in *Graphilbum* (De Beer and Wingfield 2013). The ascospores are the most distinctive feature of *Aureovirgo. Graphilbum* species have rod-shaped ascospores with ossiform sheaths (De Beer and Wingfield 2013), while those of *Aureovirgo* are uniquely allantoid with ellipsoidal sheaths. A single species of *Aureovirgo* was identified in this study and described as *A. volantis* sp. nov.

A new species in the genus *Ophiostoma* Syd. & P. Syd was identified and described as *Ophiostoma thermarum* sp. nov. *Ophiostoma thermarum* grouped peripheral to the *Sporothrix schenckii–Ophiostoma stenoceras* complex (De Beer et al. 2003), a group of fungi known to be associated with soil, hardwoods, *Protea* infructescences and mites (De Beer et al. 2003; Roets et al. 2008). Phylogenetic analyses revealed *O. thermarum* to be closely related to *O. bragantinum* Pfenning & Oberw., described in Brazil from soil samples (Pfenning and Oberwinkler 1993). *Ophiostoma bragantinum* has shorter conidiophores (20 to 40 µm) and different conidial morphology (guttuliform or fusiform) compared to *O. thermarum* (Pfenning and Oberwinkler 1993).

Previous studies on dying *Euphorbia* trees in South Africa identified two ophiostomatalean fungi from these trees, namely *Knoxdaviesia serotectus* (J.A. van der Linde, Jol. Roux) Z.W. de Beer & M.J. Wingf. (on *E. ingens*) and *K. ubusi* (J.A. van der Linde, Jol. Roux) Z.W. de Beer & M.J. Wingf. (on *E. tetragona* Haw.) (Van der Linde et al. 2011b). These species reside in the Microascales and were associated with rotting branches and a weevil in the genus *Cossonus*. Neither of these species were obtained from *C. africus* tunnels in diseased *E. ingens* trees in the current study. The weevil *Cossonus* infests only succulent parts of diseased *E. ingens* branches which is very different from *C. africus*, an ambrosia beetle that infests the woody xylem of diseased trees. The absence of *Knoxdaviesia* spp. in this study is thus not surprising.

*Aureovirgo volantis* and *O. thermarum* both produced external lesions and internal rotting in the inoculated *E. ingens* branches in the pathogenicity tests. Previously Van der Linde et al. (2011b) inoculated healthy *E. ingens* branches with two *Knoxdaviesia* species, which produced results similar to those found in the present study. Relatively small lesions and areas of internal rot were found associated with fungal inoculations in both studies although the results were variable (possibly due to genetic variation of *E. ingens* in their natural environment). This is in contrast to inoculations with *Lasiodiplodia* spp., also isolated from diseased *E. ingens* and capable of producing larger lesions (Van der Linde et al. 2011a). The results of the present study failed to show that either of the two ophiostomatalean fungi are primary pathogens. However, in the case of mass infestations, they could possibly contribute to tree death.

This study expands the base of knowledge regarding the diversity of ophiostomatalean fungi that occur in diseased *E. ingens* trees. It is now known that that this group of fungi occurs on diseased *E. ingens* trees across South Africa (where *E. ingens* populations occur). It was surprising that only two fungal species were obtained from the tunnels of *C. africus*. Interestingly, *A. volantis* and *O. thermarum* were never isolated from the same tunnel. Many

ambrosia beetles carry more than one fungal partner and these fungi are often isolated together (Carrillo et al. 2014, Kostovcik et al. 2015). The association of ophiostomatalean fungi with *C. africus* and *E. ingens* die-offs deserves further study including additional collections from trees and beetles at different times of the year. Mass inoculations may also be sued to simulate the effect of fungi on trees after of mass attacks of host beetles.

## 5. References

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