Investigations into *Encephalartos* insect pests and diseases in South Africa identifies *Phytophthora cinnamomi* as a pathogen of the Modjadji cycad

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South Africa holds the greatest diversity of *Encephalartos* species globally. I In recent years several reports have been received of *Encephalartos* species in the country dying of unknown causes. The aim of this study was to investigate the presence of, and identify the causal agents of diseases of *Encephalartos* species in the Gauteng and Limpopo Provinces of South Africa. Symptomatic plant material and insects were collected from diseased plants in private gardens, commercial nurseries and conservation areas in these regions. Insects collected were identified based on morphology, and microbial isolates based on morphology and DNA sequence data. Insect species identified infesting cultivated cycads included the beetle *Amorphocerus talpa*, and the scale insects, *Aonidiella aurantii*, *Aspidiotus capensis*, *Chrysomphalus aonidum*, *Lindingaspis rossi*, *Pseudaulacaspis cockerelli*, *Pseudaulacaspis pentagona* and *Pseudococcus longispinus*. Fungal species isolated from diseased plants included species of *Diaporthe*, *Epicoccum, Fusarium, Lasiodiplodia, Neofusicoccum, Peyronellaea, Phoma, Pseudocercospora* and

Toxicocladosporium. The plant pathogen *Phytophthora cinnamomi* was identified from *E. transvenosus* plants in the Modjadji Nature Reserve. Artificial inoculation studies fulfilled Koch's postulates, strongly suggesting that *P. cinnamomi* is responsible for the deaths of these plants under field conditions.

Keywords: Cycadales, *Cycas*, *Encephalartos*

Introduction

Cycads (Cycadales) are a group of ancient gymnosperms, with a lineage dating back to more than 300 million years ago (MYA), with extant cycads evolving ~12 MYA (Nagalingum *et al*., 2011). Since their first discovery in the 18th century, cycads have become highly desired as ornamentals worldwide (Donaldson, 2003; Cousins *et al*., 2012). They are thought to represent the oldest pot-plants in the world (Crous *et al*., 2008) and have a long history in gardens in several parts of Asia. Over the past centuries a large number of cycad species have been removed from the wild for private collections and landscaping purposes (Cousins *et al*., 2012).

The removal of cycads from their natural habitats for plant collection purposes is a major reason for the recent decline of cycad populations globally (Donaldson, 2003). In South Africa, wild populations of the African cycad genus *Encephalartos*, are targeted by collectors to supply the increasing demand for mature plants (Department of Environmental Affairs, 2013). As a result, a large number of South Africa's *Encephalartos* species are facing extinction in the wild (Donaldson, 2003; Department of Environmental Affairs, 2013). While the removal of plants is considered the main threat to wild cycad populations in South Africa, other factors such as

diseases and insect pests could also be affecting cycad populations. This aspect, however, has received no attention and deserves investigation.

Although resilient to most environmental stresses, cycads, like most cultivated plants, are susceptible to a number of insect pests, most of which are specific to cycads. The best known of these is the cycad aulacaspis scale (CAS), *Aulacaspis yasumatsui* Takagi, which, due to increased cycad trading, has spread globally and become a significant threat to both cultivated and wild cycad populations (Song *et al*., 2012). It has reduced the natural populations of two *Cycas* species, *Cycas micronesica* Hill in Guam and *Cycas taitungensis* Shen, Hill & Tsou, in Taiwan, to the point of extinction (IUCN/SSC, 2005) and is now referred to by the IUCN as the "single most important threat to natural cycad populations" (IUCN/SSC, 2005). In 2015 *A. yasumatsui* was reported in South Africa, infesting cultivated *Cycas* and *Encephalartos* plants (Nesamari *et al*., 2015).

Literature on diseases of cycads caused by micro-organisms is very limited, especially on *Encephalartos* species. Microbial genera that have been identified, to date, from diseased *Encephalartos* species include species of *Catenulostroma* (Crous *et al*., 2008), *Cladophialophora* (Crous *et al*., 2008), *Exophiala* (Crous, 2010), *Leptothyrium* (Doidge, 1950), *Pestalotia* (Nag Raj, 1993), *Phaeomoniella* (Crous *et al*., 2008), *Phoma* (Castellani & Ciferri, 1950), *Phyllosticta* (Nag Raj, 1993), *Phytophthora* (Grobbelaar, 2004), *Saccharata* (Crous *et al*., 2008), *Sporidesmium* (Crous *et al*., 2008) and *Teratosphaeria* (Crous *et al*., 2008). Of these, only *Phytophthora* has been associated with a serious disease of cycads (Ann *et al*., 2004; Grobbelaar, 2004; McDougall, 2005; Ho *et al*., 2010), while most were associated with leaf spots and diebacks on cultivated plants.

In recent years several reports have been received from private collectors and conservation agencies of *Encephalartos* species in South Africa dying of unknown causes. In one instance it was reported that approximately 1000 plants had died within a single year (S. Rodgers, pers. Comm). The lack of recent, scientific data regarding the presence and identities of pests and pathogens of cycad species, particularly species of *Encephalartos* in South Africa, hampers conservation efforts to protect these threatened plants. The aim of this study was, therefore, to investigate possible biotic causes of cycad deaths in the Gauteng and Limpopo Provinces of South Africa and to identify the casual agents of these deaths. We also provide a list of insect pests and pathogens found during the surveys, as a foundation for further studies. Use was made both of morphology and DNA sequence data to identify specimens obtained.

Materials and methods

Collection of samples

Three nurseries and two botanical gardens in the Tshwane Metropolitan area (Gauteng Province) of South Africa were surveyed at regular intervals over a period of one year (2013-2014) for the presence of diseases and pests of *Encephalartos* species. Where reports of diseased and dying plants were received from other nurseries and private gardens in the area samples were also obtained..

Three areas in the Limpopo Province, where *E. transvenosus* occurs naturally, and from where disease reports were received, were surveyed for the presence of insect pests and diseases of these plants. These areas were the Modjadji Nature Reserve, the Soutpansberg area and the Wolkberg Wilderness area. Each area was visited multiple times over a period of one year (2013-2014),

with the exception of the Wolkberg Wilderness area that was visited only once. Plants of different age classes were examined for symptoms of disease and multiple line transects were walked in each area to cover the entire area in which the plants occurred. Symptomatic plant material and soil samples were collected from diseased/dying *E. transvenosus* plants when encountered.

Symptomatic plant material was surface sterilized using 70% ethanol and rinsed with sterile water. Small pieces of material, from the leading edges of lesions, were plated onto 2% malt extract agar (MEA) (Biolab, Midrand, South Africa) for fungal isolation and NARPH selective medium (Shearer & Dillon, 1995) to obtain possible *Phytophthora* species. For soil samples, an oomycete baiting method was used in which a suspension, one part soil and one part sterile distilled water, was made in a large container and left at room temperature for three days, to induce zoospore production. After three days the suspension was agitated by hand to induce release of zoospores, and leaves from a susceptible avocado cultivar (R0.12) were placed on the surface of the suspension. The leaves were left in the suspension at room temperature for two days whereafter all the leaves were rinsed with sterile distilled water, blotted dry and plated onto NARPH medium. Pure cultures of microbial isolates were obtained by sub-culturing onto 2% MEA.

Insect specimens were collected into vials and/or ziplock bags and were transferred to 90% ethanol and sent to the Agricultural Research Council (ARC) in Pretoria, South Africa, for identification. Reference voucher specimens have been deposited in the South African National Collection of Insects, at the Agricultural Research Council in Pretoria (SANC).

Identification of microbial isolates

Cultures of all obtained microbial isolates were grouped based on their geographical origin, host plants and plant parts from which they were obtained. They were then further grouped based on colony colour, morphology and spore structures, when present. Two to three representatives from each group were selected for identification using DNA sequence data. For fungi, isolates from the same plant, identified as the same fungal species, were grouped together and one representative of each group was selected for identification using multi-gene sequence data. Representative microbial isolates have been deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

For DNA extraction mycelium was scraped from the surfaces of one to two week old cultures, freeze-dried and ground to a fine powder using a Retsch cell disrupter (Retsch Gmbh, Germany). Genomic DNA was extracted from the ground mycelia using the CTAB (cetyl trimethyl ammonium bromide) protocol described by Möller *et al*. (1992). DNA concentrations were measured using a Thermo Scientific NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and adjusted to a working concentration of approximately 75ηg μL^{-1}. PCR amplifications of sections of the internally transcribed spacer (ITS) regions (including the 5.8S gene) were performed for initial identification of all isolates. For more accurate species level identification additional genome regions were included for analyses. These were sections of the actin (ACT), β-tubulin (BT) and translation elongation factor 1-alpha (TEF-1α) regions. Primer combinations utilized for the four gene regions were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') for the ITS (White *et al*., 1990), ACT-512 (5'-ATGTGCAAGGCCGGTTTCGC-3') and ACT-783 (5'-TACGAGTCCTTCTGGCCCAT-3') for the ACT (Carbone & Kohn, 1999), βt2a (5'-

GGTAACCAAATCGGTGCTGCTTTC-3') and βt2b (5'-AACCTCAGTGTAGTGACCCTTGGC-3') for BT (Glass & Donaldson 1995) and EF1F (5'-TGCGGTGGTATCGACAAGCGT-3') and EF2R (5'-AGCATGTTGTCGCCGTTGAAG-3') for TEF-1α (Jacobs *et al*., 2004).

PCR reagents and protocols provided by the KAPATaq PCR Kit (Kapa Biosystems, Cape Town, South Africa) were used and PCR reactions were run on a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, U.S.A.). The thermal cycling conditions for the ITS and BT regions included an initial denaturation step at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for the ITS and 56 °C for the BT regions for 30 s and an extension at 72 °C for 60 s, and a final extension step at 72 °C for 7 min. The ACT thermal cycle comprised of an initial denaturation step at 95 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 45s, annealing at 52 °C for 30s and extension at 72 °C for 90s, and a final extension step at 72 °C for 6 min. For TEF-1α, an initial denaturation at 94 °C for 3 min followed by 30 cycles of 30 s at 94 °C, 45 s at 54 °C and 2 min at 72 °C, and a final extension step at 72 °C for 7 min. Successful PCR amplification was confirmed by staining 2 µL of PCR products with 2 µL GelRedTM (Biotium Incorporation, USA) nucleic acid dye and performing electrophoresis on 2% agarose gels. A DNA molecular weight marker (100 bp ladder) (Fermentas O'Gene RulerTM) was included for determination of band lengths and gels were visualized under UV light. PCR products were purified by gel filtration using 6% Sephadex G-50 (50-150 µm bead size) (Sigma, Steinheim, Germany).

Sequencing reactions were performed at a final volume of 12 µl with the same primers as used for the PCR amplification reactions. Sequencing reaction mixtures contained 2.5 µl sequencing buffer, 4 µl of PCR grade water, 0.5 µl BigDye terminator cycle sequencing kit (Applied

Biosystems, Foster City, CA, U.S.A.), 1 µl of the selected primers (10 mM) and 4 µl purified PCR product. Thermal cycling conditions comprised 25 cycles of denaturation at 96 °C for 10 s, annealing at 52 °C for ITS and ACT, 54°C for TEF-1α, and 56 °C for BT for 5 s and elongation at 60 °C for 4 min. Sequencing PCR products were purified through filtration using Sephadex G-50 and concentrated in an Eppendorf 5301 vacuum concentrator, at 60 °C. Sequencing was performed on an ABI PRISM™ 3100 DNA Analyzer (Applied BioSystems, Foster City, CA, U.S.A.).

DNA sequences were edited using MEGA v5.2 and BioEdit v7.2.5 and aligned with closely related species obtained from GenBank (http://www.ncbi.nlm.nih.gov/) using the online interface of MAFFT v7 (http://mafft.cbrc.jp/alignment/software/). Alignments were checked manually and the appropriate substitution models for the sequence data sets were determined using the Akaike Information Criterion (AIC) in jModelTest v2.1.5 (http://darwin.uvigo.es). Models obtained were used to conduct maximum likelihood (ML) analyses in PAUP v4.0b10 (Swofford, 2000) using random sequence addition and tree bisection-recognition (TBR) branch swapping. DNA sequences of isolates belonging to the same family or sub-family as the ingroups were used as outgroups. A bootstrap method with 100 replications was used to estimate the confidence levels of the ML phylogenies.

Determination of oomycete mating type

The mating types of two oomycete isolates (CMW44108, CMW44109) were determined by pairing with isolates of known mating types, A1 and A2, on carrot agar plates (Linde *et al*., 1997). Plates were incubated at room temperature in the dark and examined for the presence of oogonia after four weeks.

Pathogenicity trials

Trial 1: Because of restrictions on working with *Encephalartos* species, only a limited number of plants could be used for artificial inoculation studies. Initially only 20 *E. transvenosus* plants, 2-5 years old, could be obtained and it was decided to only use two *Phytophthora* isolates in pathogenicity trials. These two isolates (CMW44108, CMW44109) were grown for two weeks on 2% MEA and the fully grown cultures were transferred separately into 2L sterile 2% malt extract broth. Each day, for two weeks, the liquid cultures were agitated by hand to prevent clumps from forming. The liquid cultures were used to inoculate *E. transvenosus* seedlings, which were acclimatized in a phytotron for four weeks at ~22℃. Seedlings were inoculated in February 2015 by submerging their roots in the 2L broth cultures for one hour. Eight seedlings were inoculated for each of the two putative pathogen isolates and four seedlings were inoculated with sterile broth as negative controls. The seedlings were thereafter transferred into sterile soil mixed with 100ml liquid culture of each respective isolate. No microbial cultures were added to the soil used for the negative controls. The seedlings were then grown for five to eight weeks in a phytotron at ~22℃ and watered twice every week. Seedlings were checked twice a week for symptoms of leaf wilt and stem rot. When symptoms were observed, the symptomatic seedlings, as well as two controls, were uprooted, rinsed with distilled water and cut open. Symptomatic tissue from the tap roots and stems was rinsed with 70% ethanol and sterile distilled water, and then plated onto NARPH selective medium. The same was done with tissue from the control seedlings. Disease severity in the roots of the seedlings was rated on a scale of 0 to 3, with 0 as no symptoms, 1 as mild (less than 50% of roots discoloured), 2 as intermediate (50-75% of roots discoloured) and 3 as severe (75-100% of roots discoloured and/or plants dead).

Trial 2: Forty-five *E. transvenosus* seedlings of a more similar age, two to three years old, were obtained in 2016 for pathogenicity trials to fullfill Koch's postulates with the putative pathogens of *E. transvenosus* obtained from the Modjadj Nature Reserve. Only putative pathogens obtained from symptomatic stems and roots of dying *E. transvenosus* trees were selected for artificial inoculation studies. Isolates were selected to represent those species most likely to be involved in disease. Inoculum was prepared for eight isolates, CMW44108 (*Phytophthora*), CMW44109 (*Phytophthora*), CMW44302 (*Diaporthe*), CMW44309 (*Fusarium*), CMW44310 (*Fusarium*), CMW44311 (*Fusarium*), CMW44315 (*Fusarium*), and CMW44319 (*Lasiodiplodia*), by growing them in 700ml of 2% malt extract broth for one week in a shaking incubator at 25℃. Forty-five *E. transvenosus* seedlings, ranging in age from two to three years old, were planted in groups of five in nine plastic crates (120L volume), filled with river sand. Each seedling was planted together with a wooden rod (~0.5mm diam) of 20cm length, with the roots of seedlings close to the rods. An extra 8 rods were then stuck into the soil of each crate, close to the seedlings, to ensure an even distribution of rods in each crate. During the inoculation process the wooden rods were carefully removed from the soil and the holes filled with 50ml of the inoculum suspension. Eight of the crates were inoculated with an individual isolate each, while in the 9th crate sterile broth was used as a negative control. The inoculated seedlings were watered once a week and monitored twice a week for symptoms of leaf wilt. Inoculated seedlings were kept in an open area under a roof and at ambient environmental conditions. Inoculations were done on the 25th February 2016.

The experiment was terminated after 7 weeks. Seedlings were evaluated for symptoms of leaf wilt/death and root death. To evaluate root infections, seedlings were uprooted, rinsed with distilled water and the roots and stems inspected for symptoms of necrosis. Where encountered,

symptomatic tissue was rinsed with 70% ethanol (for 30 seconds) and sterile distilled water, and plated onto NARPH selective medium for oomycete isolates and 2% MEA for fungal isolates. This was done to determine if the observed symptoms were caused by the inoculated fungi, and thus to fulfill Koch's postulates. Disease severity in the roots of the seedlings was rated on a scale of 0 to 3, as explained for trial one.

Results

Collection of samples

A total of 112 diseased plants were sampled from the three nurseries and two botanical gardens monitored over a one year period in the Gauteng Province. A further four commercial nurseries were visited once each and insect samples collected from a total of 11 plants. Additionally, samples were obtained from four plants in a commercial nursery and one plant in a private garden, in the Limpopo Province. Symptoms observed on nursery and garden plants included leaf chlorosis and necrosis, cone discolouration and death as well as leaf spots, leaf scorch and dieback (Figure 1a, b, c, d; Figure 2b, e, l).

A total of 38 diseased plants were sampled in the Modjadji Nature Reserve and the Wolkberg wilderness area. These included 28 small plants of approximately 10-30cm in trunk height and 10 plants in excess of 1m in trunk height. The small plants showed signs of leaf wilt (Figure 1b) and when cut open it was observed that their apical meristems were rotting (Figure 1c, e), necrotic lesions were forming in the core of the stems (Figure 1d) and the roots were dead. Only five of these plants, sampled in the Modjadji Nature Reserve, showed early symptoms of disease, where the apical meristems were just starting to rot, while in the other plants sampled, the apical

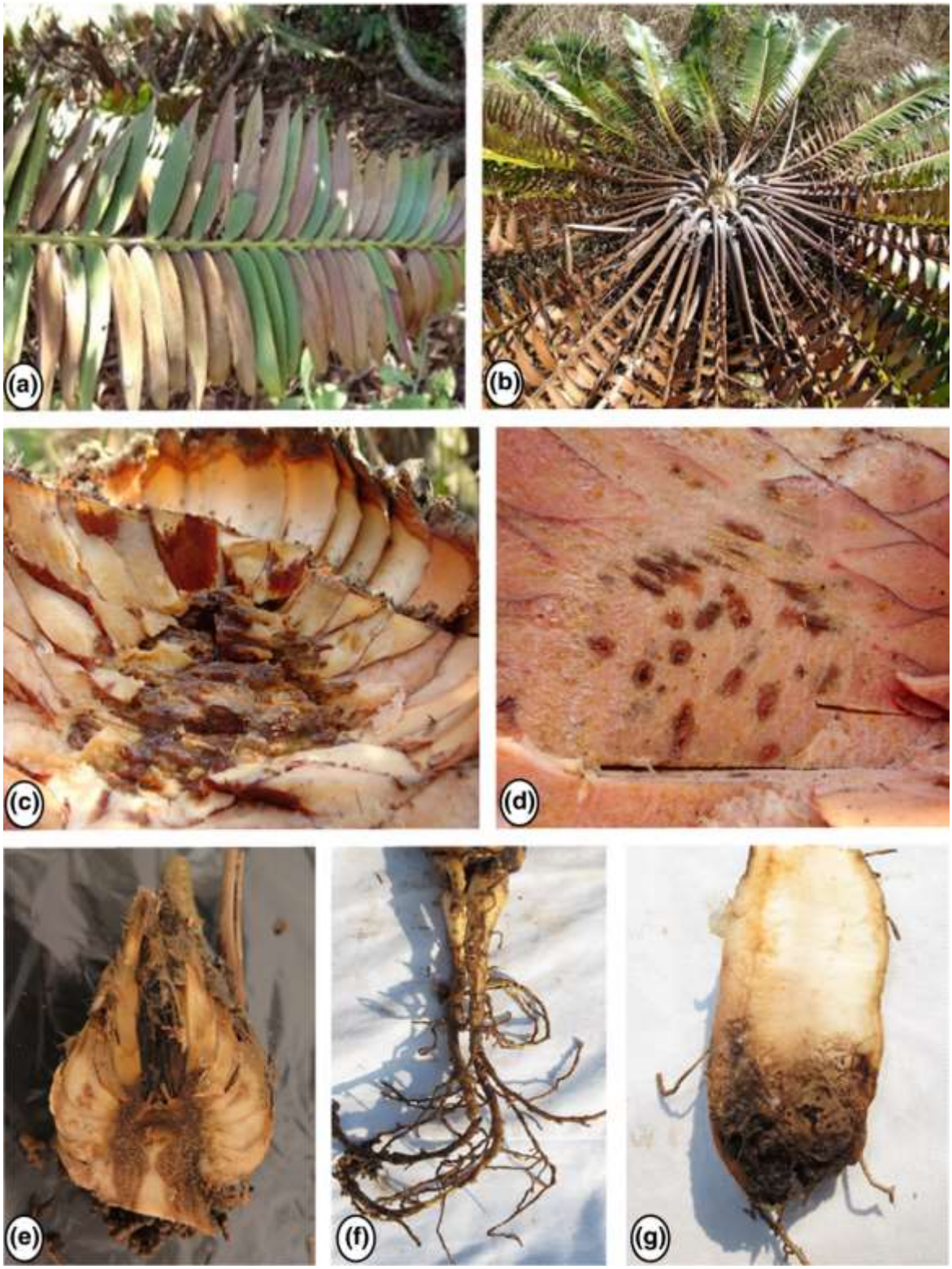

Figure 1: Disease symptoms observed on *Encephalartos tranvenosus* in the Gauteng and Limpopo Provinces. (a) Leaf blotch and death, (b) leaf collapse on a dying plant, (c) apical meristem rot of a dying plant, (d) lesions in the inner stem of a dying plant, (e) apical meristem and core rot of an inoculated seedling, (f) seedling inoculated with isolate CMW44109 showing symptoms of root death and (g) rot of the stem base.

Figure 2: Insect species and symptoms identified on diseased cycad plants in the Gauteng Province. (a) *Amorphocerus talpa*, (b) cone discolouration and death on *E. transvenosus* caused by *A. talpa*, (c) leaf chlorosis on *C. thouarsii* caused by *Aonidiella aurantii*, (d)) *Aspidiotus capensis*, (e) leaf chlorosis and necrosis on *E. transvenosus* caused by *A. capensis*, (f) leaf chlorosis on *C. revoluta* caused by *Chrysomphalus aonidum*, (g) *Lindingaspis rossi* infesting an *E. nubimontanus* leaflet, (h) *Pseudaulacaspis cockerelli*, (i) *P. cockerelli* infesting a *C. revoluta* stem, (j) leaflet chlorosis on *C. thouarsii* caused by *Peudaulacaspis pentagona*, (k) *Pseudococcus longispinus* and (l) leaf chlorosis on *C. revoluta* caused by *P. longispinus*.

meristems and most of the inner stems were already dead upon examination and often infested with insects. No insects or tunnels were, however, observed on the five small plants showing early symptoms of disease. Although there were reports of large numbers of plants dying in the Soutpansberg area, only two diseased plants, an adult of approximately 2m in height and its sucker, were found. However, these plants were already dead upon examination and infested with stem boring insects.

Identification of insect specimens

Nine insect species were identified from specimens collected in this study (Table 1). Among these, a beetle species, *Amorphocerus talpa* Schoenherr (Figure 2a), was observed infesting female cycad cones in the Pretoria National Botanical Gardens. Infestation by this beetle species caused cone discolouration and death (Figure 2b). Several hard scale insects (Hemiptera, Diaspididae) and a soft scale insect (Hemiptera, Coccoidea) were encountered infesting the leaflets and stems of *Cycas* and *Encephalartos* plants in gardens and nurseries in the Gauteng and Limpopo Provinces. These included the hard scale insects *Aonidiella aurantii* Maskell (Figure 2c), *Aspidiotus capensis* Newstead (Figure 2d), *Chrysomphalus aonidum* Linnaeus (Figure 2f), *Lindingaspis rossi* Maskell (Figure 2g), *Pseudaulacaspis cockerelli* Cooley (Figure 2h), *P. pentagona* Tozzetti (Figure 2j), and the soft scale insect *Pseudococcus longispinus* Tozzetti (Figure 2k). The scale insects *A. aurantii*, *C. aonidum*, *P. cockerelli* and *P. pentagona*, were observed infesting only *Cycas* species, while *A. capensis*, *L. rossi* and *P. longispinus* were observed on both *Cycas* and *Encephalartos* species. Among all the scales collected, *A. capensis* was the most common, occurring in all nurseries surveyed in the Gauteng Province and on a wide range of *Encephalartos* species. Cycad species infested with scale insects showed symptoms of chlorosis and leaf death (Figure 2e, l). The non-native scale insect, *Aulacaspis yasumatsui* (CAS)

Table 1: Insect species and associated symptoms identified from diseased cycad species in nurseries and gardens in the Gauteng and Limpopo Provinces of South Africa.

Species	Host	Region	Symptoms	ARC accession nr.
Nurseries				
Aonidiella aurantii	*Cycas revoluta*	Pretoria	Leave chlorosis	HC 7234
	C. thouarsii	"	"	
Aspidiotus capensis	*Encephalartos dyerianus*	Pretoria	Leave chlorosis	HC7214
	E. friderici-guilielmi	"	"	
	E. lehmanni	"	"	
	E. manikensis	"	"	
	E. trispinosus	"	"	
	E. transvenosus	"	"	
Aulacaspis yasumatsui	*C. revoluta*	Pretoria	Leaf chlorosis	HC7227
	"	Tzaneen	"	
Chrysomphalus aonidum	*C. revoluta*	Tzaneen	Leaf chlorosis	HC 7235
Lindingaspis rossi	*E. nubimontanus*	Pretoria	Leaf Infestation	HC7215
Pseudaulacaspis cockerelli	*C. revoluta*	Pretoria	Leaf infestation	HC 7233
	"	Tzaneen	"	
Pseudaulacaspis pentagona	*C. thouarsii*	Pretoria	Leaf chlorosis	HC 7231
Pseudococcus longispinus	*C. revoluta*	Tzaneen	Leaf infestation	HC 7236

	E. transvenosus	Pretoria	‘‘	
Gardens				
Amorphocerus talpa	*Encephalartos laevifolius*	Pretoria	Cone discolouration, death	
	E. longifolius	‘‘	‘‘	
	E. tranvenosus	‘‘	‘‘	
Aspidiotus capensis	*E. altensteinii*	Pretoria	Leave chlorosis, death	HC 7214
	E. arenarius	‘‘	‘‘	
	E. ferox	‘‘	‘‘	
	E. lehmanni	‘‘	‘‘	
	E. longifolius	‘‘	‘‘	
	E. natalensis	‘‘	‘‘	
	E. princeps	‘‘	‘‘	
	E. senticosus	‘‘	‘‘	
Lindingaspis rossi	*C. revoluta*	Pretoria	Leaf Infestation	HC7215

was found affecting non-native *Cycas* species in nurseries in Pretoria and Tzaneen (Table 1). No insect pests were observed on field plants, although feeding damage to the leaves of *E. transvenosus* was observed in the Wolkberg Wilderness area.

Identification of microbial isolates

A total of 118 fungal and three oomycete isolates were obtained from diseased *Encephalartos* species in this study. Based on sequence results, isolates were identified as species of *Diaporthe* (Diaporthales, Diaporthaceae), *Epicoccum* (Pleosporales, Pleosporaceae), *Fusarium* (Hypocreales, Nectriaceae), *Neofusicoccum* (Botryosphaeriales, Botryosphaeriaceae), *Lasiodiplodia* (Botryosphaeriales, Botryosphaeriaceae), *Phoma* (Pleosporales, Pleosporaceae), *Pseudocercospora* (Capnodiales, Mycosphaerellaceae) and *Toxicocladosporium* (Capnodiales, Cladosporiaceae). A ninth morphological group comprised of three isolates of *Phytophthora* sp. based on ITS sequence data. For fungi, representatives of each species, identified based on ITS sequence data, was selected for identification using multi-gene sequence data.

Diaporthe isolates

Three *Diaporthe* isolates (Table 2), were identified based on ITS sequence data. Two representative isolates (CMW44302, CMW44303) were selected for multi-gene sequence analysis. The two *Diaporthe* isolates were identical in their ITS and BT gene sequences, but differed in their TEF-1α gene sequences. Based on sequence data they represent a novel species (Suppl Figure 1), most closely related to *Diaporthe cf. nobilis* Saccardo & Spegazzini in the ITS, separate from other *Diaporthe* species in the BT and TEF-1α, and closely related to *D. nomurai* Hara in the combined analyses.

Table 2: Microbial genera and associated symptoms associated with diseased cycad species in the Gauteng and Limpopo Provinces of South Africa.

Identity	**Region**	**Host**	**Symptoms**	**Number of plants**	**CMW numbers**
Nurseries and gardens				34 total	
Epicoccum sorghi	Pretoria Nursery	*E. hildebrandtii*	Leaf spot, death	3	44305
	"	*E. whitelockii*	"	2	44306
Epicoccum sp. nov. 1	Pretoria Nursery	*E. hildebrandtii*	Leaf spot	1	44307
Epicoccum sp. nov. 2	Pretoria Nursery	*E. ferox*	Leaf spot	1	44335
	Pretoria Nursery	*E. middelburgensis*	Leaf die-back	3	44304
Epicoccum sp. nov. 3	Pretoria Garden	*E. transvenosus*	Leaf spot	2	44332
Fusarium oxysporum species complex (Clade 3)	Pretoria Nursery	*E. princeps*	Dying leaf bract	1	44316
Fusarium solani species complex (FSSC 5)	Pretoria Nursery	*E. paucidentatus*	Stem rot	1	44317
Neofusicoccum sp. nov.	Pretoria Garden	*E. transvenosus*	Leaf death	1	44320
Peyronellaea coffeae-arabicae	Pretoria Garden	*E. ferox*	Leaf spot	2	44322
	"	*E. natalensis*	"	3	44323
	"	*E. paucidentatus*	"	1	44325
	"	*Cycas thoursii*	"	4	44324

Peyronellaea sp. nov. 1	Pretoria Nursery	*E. lebomboensis*	Leaf spot	1	44328
Peyronellaea sp. nov. 2	Pretoria Garden	*E. paucidentatus*	Leaf spot	1	44326
Peyronellaea sp. nov. 3	Pretoria Garden	*Cycas thoursii*	Leaf spot	1	44329
Peyronellaea sp. nov. 4	Pretoria Garden	*E. altensteinii*	Leaf spot	1	44330
Phoma sp. nov. 1	Pretoria Garden	*Cycas thoursii*	Leaf spot	1	44331
Pseudocercospora sp. nov. 1	Pretoria Nursery	*E. ferox*	Leaf die-back	2	44333
Pseudocercospora sp. nov. 2	Pretoria Nursery	*E. vilosus*	Leaf die-back	1	44983
Taxicladosporium sp. nov.	Pretoria Nursery	*E. arenarius*	Leaf die-back	1	44334
Field				18 total	
Diaporthe sp. nov.	Modjadji Nature Reserve	*E. transvenosus*	Cortex, lesions	1	44302
	Wolkberg Wilderness	*E. transvenosus*	Vascular lesions	1	44303
Fusarium oxysporum species complex	Modjadji Nature Reserve	*E. transvenosus*	Leaf bract lesions	2	44311
(Clade 3)					
Fusarium solani species complex	Modjadji Nature Reserve	*E. transvenosus*	Stem lesions	4	44308, 44312, 44315
	Soutpansberg	"	"	2	44313, 44314
Fusarium sp. nov. 1 (FFSC)	Modjadji Nature Reserve	*E. transvenosus*	Leaf bract lesions	1	44309
Fusarium sp. nov. 2 (FFSC)	Modjadji Nature Reserve	*E. transvenosus*	Leaf bract lesions	1	44310

Lasiodiplodia sp. nov. 1	Modjadji Nature Reserve	*E. transvenosus*	Leaf bracts	1	44319
Lasiodiplodia sp. nov. 2	Wolkberg Wilderness area	*E. transvenosus*	Vascular tissue	1	44318
Neofusicoccum parvum	Wolkberg Wilderness area	*E. transvenosus*	Leaf die-back	1	44321
Peyronellaea sp. nov. 5	Wolkberg Wilderness area	*E. transvenosus*	Leaf bract lesions	1	44327
Phytophthora cinnamomi	Modjadji Nature Reserve	*E. transvenosus*	Rotting apical meristem	2	44108, 44109, 44110

Epicoccum and Phoma isolates

A total of 32 *Phoma* and two *Epicoccum* isolates were identified (Table 2). Sixteen representative isolates, which included 14 *Phoma* isolates and two *Epicoccum* isolates were selected for multi-gene sequence analyses. Phylogenetic analyses of the ITS and BT gene regions grouped the isolates in three *Phoma* sections, namely *Peyronellaea*, *Phoma* and *Sclerophomella* (Suppl Figure 2). Isolates CMW44335 and CMW44304 represented a single novel *Epicoccum* species closely related to *Epicoccum nigrum* Link in the ITS, BT and combined analyses. CMW44331 represents a novel *Phoma* species, separate from all other species in the ITS, BT and combined analyses. Isolates CMW44322, CMW44323, CMW44324 and CMW44325 grouped as *Peyronellaea coffeae-arabicae* Aveskamp, Gruyter & Verkley in the ITS and combined analyses and as closely related to *P. coffeae-arabicae* in the BT analysis. Isolates CMW44326, CMW44327, CMW44328, CMW44329, CMW44330 represent five novel *Peyronellaea* species. CMW44332 represents a novel *Epicoccum* species, grouping as *P. coffeae-arabicae* in the ITS, as *Epicoccum sorghi* in the BT and close to, but separate from *E. sorghi* in the combined analyses. Isolates CMW44305 and CMW44306 grouped as *E. sorghi* in the ITS and combined analyses and as closely related to *E. sorghi* in the BT analysis. CMW44307 represents a novel *Epicoccum* species closely related to *E. sorghi* in the ITS, BT and combined analyses.

Fusarium isolates

A total of 36 *Fusarium* isolates were identified (Table 2). ITS sequence data identified them as belonging to three species complexes, the *Fusarium solani* (FSSC), *Fusarium oxysporum* (FOSC) and *Fusarium fujikuroi* (FFSC) species complexes. Ten representative isolates were selected for multi-gene sequence analysis.

Six of the 10 representative isolates grouped in the FSSC. The ITS, TEF-1α and combined analyses grouped five of the isolates in one major clade close to, but separate, from the *F. petroliphilum* Chen & Fu clade, and one isolate close to members of the FSSC 5 clade (Suppl Figure 3). No BT sequences could be found on GenBank for isolates used to construct the ITS and TEF-1α trees.

Of the four other representative *Fusarium* isolates, two isolates grouped in the FOSC and two in the FFSC. Analysis of the TEF-1α region grouped CMW44311 and CMW44316 in clade 3 of the FOSC (Suppl Figure 4) and CMW44309 and CMW44310 in two clades, close to, but separate from *F. phyllophilum* Nirenberg in the FFSC (Suppl Figure 5). No ITS and BT sequences could be found on GenBank for isolates used to construct the TEF-1α trees.

Botryosphaeriaceae isolates

The ITS, BT, TEF-1α and combined analyses grouped the two *Lasiodiplodia* isolates in two clades closely related to *L. mahajangana* Begoude, Roux & Slippers (Suppl Figure 6). Analyses of these three gene regions also grouped the *Neofusicoccum* isolates as *N. parvum* Pennycook & Samuels (CMW44321) and as a novel *Neofusicoccum* species (CMW4420) (Suppl Figure 7).

Pseudocercospora isolates

Two *Pseudocercospora* isolates were identified (Table 2). ITS analysis grouped CMW44333 as *P. acericola* Woronichin and CMW44983 closest to CMW4433. ACT analysis grouped the isolates in one clade closely related to *P. lythracearum* Heald & Wolf and *P. crispans* Hunter & Crous. The TEF-1α grouped them in two clades closest to each other, but separate from other *Pseudocercospora* species and the combined analysis in two clades closely related to *P. rubi*

Sacc. These results suggested that CMW44333 and CMW44983 represent two novel, closely related, *Pseudocercospora* species (Suppl Figure 8).

Toxicocladosporium isolate

A single *Toxicocladosporium* isolate was identified from a single plant with leaf dieback symptoms (Table 2). Analysis of the ITS region grouped the isolate as *T. veloxum* Crous & Wingfield, closely related to *T. irritans* Crous & Braun (Suppl Figure 9). No BT and TEF-1α sequences could be obtained on GenBank for species in this genus.

Phytophthora isolates

Phylogenetic analysis of the ITS region of the oomycete isolates grouped them with *P. cinnamomi* (Suppl Figure 10). Two of these isolates were further characterized using mating studies to determine their mating types. Both isolates formed oogonia when grown with an isolate of the A1 mating type, identifying both as belonging to the A2 mating type. *Phytophthora cinnamomi* was obtained from two dying plants in the Modjadji Nature Reserve (CMW44108, CMW44109) (Table 2), as well as from soil adjacent to one of these plants (CMW44110).

Pathogenicity trials

Trial 1: Five weeks post inoculation symptoms of leaf wilt were observed in most of the seedlings inoculated with one of the *P. cinnamomi* isolates (CMW44109). These symptoms were not seen in seedlings inoculated with isolate CMW44108, or in the controls. When the diseased seedlings were cut open symptoms of necrosis could be seen in the tap roots and stems (Figure 1e) of all the inoculated seedlings, while the control seedlings showed no symptoms of necrosis. Re-isolations from lesions resulted in *P. cinnamomi. Phytophthora cinnamomi* was not isolated from any of the controls.

Eight weeks post inoculation seedlings inoculated with isolate CMW44108 still showed no symptoms of leaf wilt. Severe leaf wilt was, however, observed on one of the two control seedlings. Re-isolations from lesions produced *P. cinnamomi* from the diseased control plant. *Phytophthora cinnamomi* was, however, not isolated from any of the seedlings inoculated with CMW44108 or the remaining control.

Trial 2: Seven weeks post inoculation symptoms of leaf wilt were observed in some of the seedlings inoculated with the *P. cinnamomi* isolates (CMW44108 and CMW44109). These symptoms were not seen in seedlings inoculated with fungal isolates or in the controls. When the seedlings were examined, symptoms of severe necrosis could be seen in the roots of seedlings inoculated with isolate CMW44109 (Figure 1f), while those inoculated with isolate CMW44108 showed less severe symptoms, with tissue necrosis restricted to root tips. In some plants inoculated with *P. cinnamomi* necrosis was also observed in the stem base (Figure 1g). No symptoms of root necrosis were observed in any of the seedlings inoculated with the fungal isolates or in the controls. It was, however, observed that two seedlings inoculated with the *Fusarium* isolate CMW44309 had a mild discolouration on the tips of their collaroid roots. Re-isolations from disease roots inoculated with *P. cinnamomi* isolates resulted in the growth of this pathogen. None of the other inoculated microbes were re-isolated and none were obtained from the controls.

Discussion

This study aimed to provide information on the possible causes of disease and death of *Encephalartos* species in South Africa. Focus was placed on the Gauteng and Limpopo

Provinces, based on reports received from conservation agencies and cycad growers. No serious diseases were encountered in the Gauteng Province, however, a number of insect species were encountered feeding off the leaves and cones of cycads and causing leaf chlorosis, necrosis and cone death. A disease of significant concern was found affecting *E. transvenosus* plants in the Modjadji Nature Reserve in the Limpopo Province. Although reports of plant death were also received from two other areas in the province (Wolkberg Wilderness, Soutpansberg), only two plants were observed showing symptoms of disease in these areas. Several novel fungal species were identified in the study. Among these were a single *Neofusicoccum* species, two *Pseudocercopsora* species, a *Toxicocladosporium* species, and several species in the *Peyronellaea*, *Phoma* and *Sclerophomella* sections, associated with leaf dieback. A novel *Diaporthe* sp. and two *Lasiodiplodia* species, associated with stem rot were also obtained. These fungi, however, need to be characterized further.

Only one serious disease, resulting in the death of *E. transvenosus*, was encountered in this study, namely in the Modjadji Nature Reserve in the Limpopo Province. *Phytophthora cinnamomi* was isolated from fresh symptoms and this represents the first official report of the pathogen on native cycad species in South Africa. *Phytophthora cinnamomi* is a soilborne pathogen with a host range of over 3000 plant species which include economically important crop plants, forest trees, ornamentals and plants in native ecosystems (Hardman, 2005). It is having lasting detrimental impacts on native plant diversity, threatening ecosystem functioning in Australia, Europe and the USA (Brasier, 1996; Shearer *et al*., 2007). Previous reports of *P. cinnamomi* causing diseases on cycad species include species of *Cycas* in New Zealand (Ho *et al*., 2010), *Macrozamia* in Australia (McDougall, 2005) and *Zamia* in Taiwan (Ann *et al*., 2004). Although there are previous reports of *Phytophthora* species causing disease on cultivated cycads in South Africa

(Grobbelaar, 2004), none of these reports identified the species involved. Pathogenicity trials conducted under greenhouse conditions fulfilled Koch's postulates, validating *P. cinnamomi* as a pathogen of *E. transvenosus* plants and, therefore, a potential causal agent of the observed deaths in the Modjadji Nature Reserve.

Phytophthora cinnamomi has been known in South Africa for nearly a century and has become established widely in the country (Linde *et al*., 1997). The centre of origin of the pathogen is hypothesized to be South East Asia, but it has been spread globally. It has two mating types, designated A1 and A2, both of which have been reported from South Africa (Linde *et al.*, 1997). Isolates obtained from diseased *E. transvenosus* plants in the Modjadji Nature Reserve were of the A2 mating type. In South Africa, the A2 mating type is often associated with agricultural crops such as avocado, as well as native and cultivated forests, while the A1 mating type has been reported only from native ecosystems in the Western Cape Province of the country (Linde *et al*., 1997). The pathogen has been known as a major pathogen of avocado and other tree crops in agricultural areas near the Modjadji Nature Reserve for several decades (Darvas & Bezuidenhout, 1987) and could easily have spread to *E. transvenosus* plants from those areas.

A beetle pest, *Amorphocerus talpa*, was encountered feeding on the cones of several *Encephalartos* species in the Pretoria National Botanical Gardens. *Amorphocerus talpa* is endemic to Africa and well known to attack exclusively *Encephalartos* species (Grobbelaar, 2004). The larvae of this beetle feed off the sporophylls and axis of cones, causing the cones to darken and die. To date, reports of *A. talpa* in South Africa have mainly been from the Eastern Cape Province, where the beetles have been observed feeding off the male and female cones of *Encephalartos* plants (Downie *et al*., 2008). In the current study, plants observed infested with *A. talpa* showed severe cone necrosis.

Leaf-feeding scale insects were common in all areas surveyed in the Gauteng Province. The scale insect *Aspidiotus capensis*, was observed infesting several *Encephalartos* and *Cycas* species, and was common in all gardens and nurseries surveyed in this region. *Aspidiotus capensis* has previously been reported from *Encephalartos* species in Port Elizabeth, South Africa (Annecke & Mynhardt, 1970)Several other scale insects were identified infesting cultivated cycads in the Gauteng and Limpopo Provinces. These include well known fruit pests such as *Aonidiella aurantii*, *Chrysomphalus aonidum*, *Lindingaspis rossi*, *Pseudaulacaspis cockerelli*, *P. pentagona* and *Pseudococcus longispinus*. *Aonidiella aurantii*, *Chrysomphalus aonidum*, *Pseudaulacaspis cockerelli* and *P. pentagona* are native to Asia, while *Lindingaspis rossi* and *Pseudococcus longispinus* are native to Australia (ScaleNet database, www.sel.barc.usda.gov). Due to movement of infected plant material, these scale insects have become widely dispersed and are now invasive pests in many countries in Africa, Asia, Europe, the Western Hemisphere and Oceania (ScaleNet database, www.sel.barc.usda.gov). Although these insects have been reported from cycads, as well as other plant families, they are not common on cycad species in South Africa (ScaleNet database, www.sel.barc.usda.gov; IM Millar, ARC, personal communication, 2014) and in this study were mostly encountered on non-native *Cycas* species, often associated with leaf chlorosis.

Several fungal species were obtained from cycad species in this study. However, none of those tested in the inoculation studies produced disease on *E. transvenosus*, making it unlikely that they are the primary cause of the observed disease on these plants. *Neofusicoccum parvum* is a member of the Botryosphaeriaceae and a pathogen of a wide range of hosts, including many economically important plants (Slippers & Wingfield, 2007). Species in this family have been previously identified on cycads associated with leaf and shoot dieback diseases (Weber, 1944;

Chakraborty *et al*., 2011). In the current study, *N. parvum* was only found on dead plant material and was not associated with freshly developing lesions. The majority of fungal species identified in the study were those of *Fusarium* and *Phoma. Fusarium* isolates identified included members of the *F. fujikuroi*, *F. oxysporum* and *F. solani* species complexes. Members of these three complexes include both soilborne plant pathogens and saprophytes (Leslie & Summerell, 2006;). Previous reports of *Fusarium* species on cycads include reports of leaf blight diseases (Weber, 1944) and root death (Polizzi, 1995). *Phoma* species were common on cultivated plants and often associated with leaf spots and diebacks. *Phoma* species have been previously reported on *Encephalartos* species associated with similar leaf diseases as observed in the current study (Doidge, 1950; Crous *et al.*, 2008).

Although many cultivated cycads were encountered in the Gauteng Province showing symptoms of leaf diseases, to date, none of the symptoms observed have resulted in any plant death, suggesting that, currently, these symptoms are not of major concern. Further surveys are, however, needed to determine the extent and severity of *P. cinnamomi* infection on *Encephalartos* species in South Africa. Areas near the Modjadji Nature Reserve include avocado plantations, and other host plants of *P. cinnamomi*, on which the pathogen has been causing disease for many years (Darvas & Bezuidenhout, 1987). Such areas could be a source of inoculum for this disease, therefore measures may need to be implemented to restrict pathogen movement between these areas. Continued surveys in the Wolkberg Wilderness area and the Soutpansberg area will be necessary to resolve the cause/s of plant death in those areas.

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References

Ann PJ, Tsai JN, Wang IT, 2004. *Phytophthora cinnamomi* on *Zamia furfuracea* in Taiwan. *Plant Pathology Bulletin* **13**, 233-236.

Annecke DP, Mynhardt MJ, 1970. On some species of *Habrolepsis* Foerster and *Adelencyrtus* Ashmead (Hymenoptera: Encyrtidae) in Southern Africa and Mauritius. *Entomophaga* **15**, 127-148.

Brasier CM, 1996. *Phytophthora cinnamomi* and oak decline in southern Europe. Environmental constraints including climate change. *Annals of Forest Science* **53**, 347-358.

Carbone I, Kohn LM, 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* **91**, 553-556.

Castellani E, Ciferri R, 1950. Mycoflora of Eritrea, Somaliland, and Ethiopia. Atti dell'Istituto Botanico e Laboratorio Crittogamico, University of Pavia.

Chakraborty MR, Ojha S, Chatterjee NC, 2011. Die-back disease of *Cycas* - a new record from Burdwan, West Bengal. *Journal of Mycopathological Research* **49**, 195-96.

Cousins SR, Williams VL, Witkowski ETF, 2012. Uncovering the cycad taxa (*Encephalartos* species) traded for traditional medicine in Johannesburg and Durban, South Africa. *South African Journal of Botany* **78**, 129-138.

Crous PW, Wood AR, Okada G, Groenewald JZ, 2008. Foliicolous microfungi occurring on *Encephalartos*. *Persoonia* **21**, 135-146.

Crous PW, 2010. *Exophiala encephalarti*. Fungal Planet 58, *Persoonia* **25**, 136-137.

Darvas JM, Bezuidenhout JJ, 1987. Control of *Phytophthora* root rot of avocados by trunk injection. *Proceedings of the First World Avocado Congress, South African Avocado Growers' Association Yearbook* **10**, 91-93.

Department of Environmental Affairs, Republic of South Africa, 2013. National management strategy and action plan for cycads. Pretoria, South Africa.

Doidge EM, 1950. The South African fungi and lichens to the end of 1945. *Bothalia* **5**, 1-1094.

Donaldson JS, 2003. Cycads: status survey and conservation action plan. Gland, Switzerland and Cambridge, UK: IUCN/SSC Cycad Specialist Group.

Downie DA, Donaldson JS, Oberprieler RG, 2008. Molecular systematics and evolution in an African cycad-weevil interaction: Amorphocerini (Coleoptera: Curculionidae: Molytinae) weevils on *Encephalartos*. *Molecular Phylogenetics and Evolution* **47**, 102-116.

Glass NL, Donaldson GC, 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Applied and Environmental Microbiology* **61**, 1323-1330.

Grobbelaar N, 2004. Cycads, with special reference to the southern African species. Published by author. Pretoria, South Africa.

Hardman AR, 2005. Pathogen profile, *Phytophthora cinnamomi. Molecular Plant Pathology* **6** (6), 589-604.

Ho W, Thangavel R, Romberg M, Griffin R, Murugan S, Alexander B, 2010. *Phytophthora* records from passive surveillance programme in New Zealand. 5th IUFRO Phytophthoras in Forests and Natural Ecosystems. Auckland and Rotorua, New Zealand.

IUCN/SSC Cycad Specialist Group, 2005. Report and recommendations on cycad aulacaspis scale, *Aulacaspis yasumatsui* Takagi (Hemiptera: Diaspididae). United States Department of Agriculture (USDA).

Jacobs K, Bergdahl DR, Wingfield MJ, Halik S, Seifert KA, Bright DE, Wingfield BD, 2004. *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. *Mycological Research* **108**, 411-418.

Leslie JF, Summerell BA, 2006. The *Fusarium* Laboratory Manual. Iowa, USA: Blackwell Publishing.

Linde C, Drenth A, Kemp GHJ, Wingfield MJ, von Broembsen SL, 1997. Population Structure of *Phytophthora cinnamomi* in South Africa. *Phytopathology* **87**, 822-827.

McDougall KL, 2005. The responses of native Australian plant species to *Phytophthora cinnamomi*. Appendix 4. O'Gara E, Howard K, Wilson B, Hardy GEStJ, eds. Management of *Phytophthora cinnamomi* for biodiversity conservation in Australia: Part **2**, 1-52.

Möller EM, Bahnweg G, Sandermann H, Geiger HH, 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies and infected plant tissue. *Nucleic Acids Research* **20**(22), 6115-6116.

Nagalingum NS, Marshall CR, Quental TB, Rai, HS, Little DP, Mathews S, 2011. Recent synchronous radiation of a living fossil. *Science* **334**, 796-799.

Nag Raj TR, 1993. Coelomycetous anamorphs with appendage-bearing conidia. Waterloo, Ontario: Mycologue Publications, 101.

Nesamari R, Millar IM, Coutinho TA, Roux J, 2015 South African cycads at risk: *Aulacaspis yasumatsui* (Hemiptera: Coccoidea: Diaspididae) in South Africa. *African Entomology* **23**, 198-206.

Polizzi G, 1995. The diseases of *Cycas revoluta*. *Informatore Fitopatologico* **45**(12), 27-33.

Shearer BL, Dillon M, 1995. Susceptibility of plant species in *Eucalyptus marginata* forest to infection by *Phytophthora cinnamomi*. *Australian Journal of Botany* **43**, 113-134.

Shearer BL, Crane EM, Barrett S, Cochrane A, 2007. *Phytophthora cinnamomi* invasion, a major threatening process to conservation of flora diversity in the South-west Botanical province of Western Australia. *Australian Journal of Botany* 55, 225-238.

Slippers B, Wingfield MJ, 2007. Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact. *Fungal Biology Reviews* **21**, 90-106.

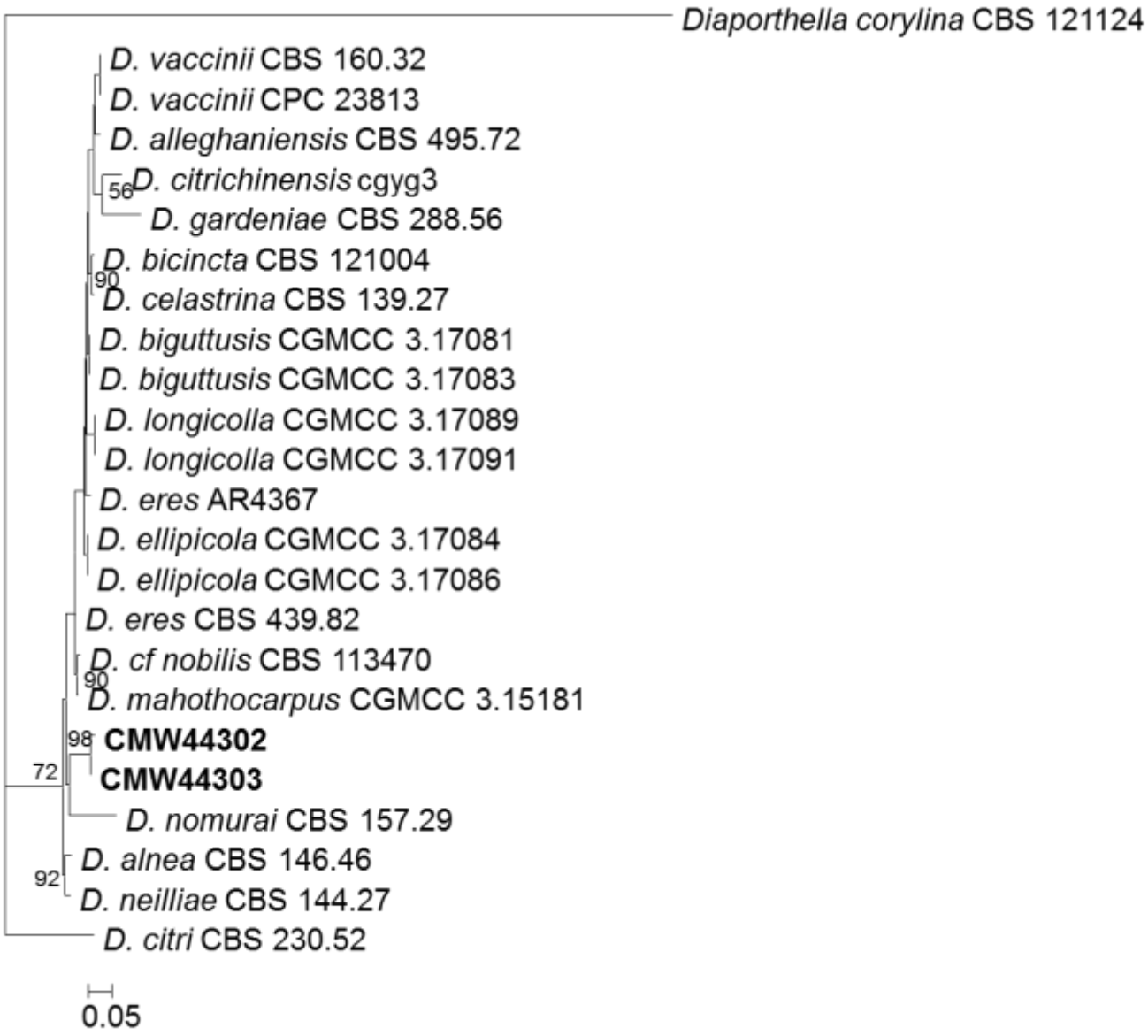
Song SY, Tan CW, Hwang SY, 2012. Host range of *Cybocephalus flavocapitis* and *Cybocephalus nipponicus*, two potential biological control agents for the cycad aulacaspis scale, *Aulacaspis yasumatsui. Journal of Asia-Pacific Entomology* **15**, 595-599.

Swofford DL, 2000. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). 4th version. Sinauer Associates, Sunderland, Massachusetts, U.S.A.

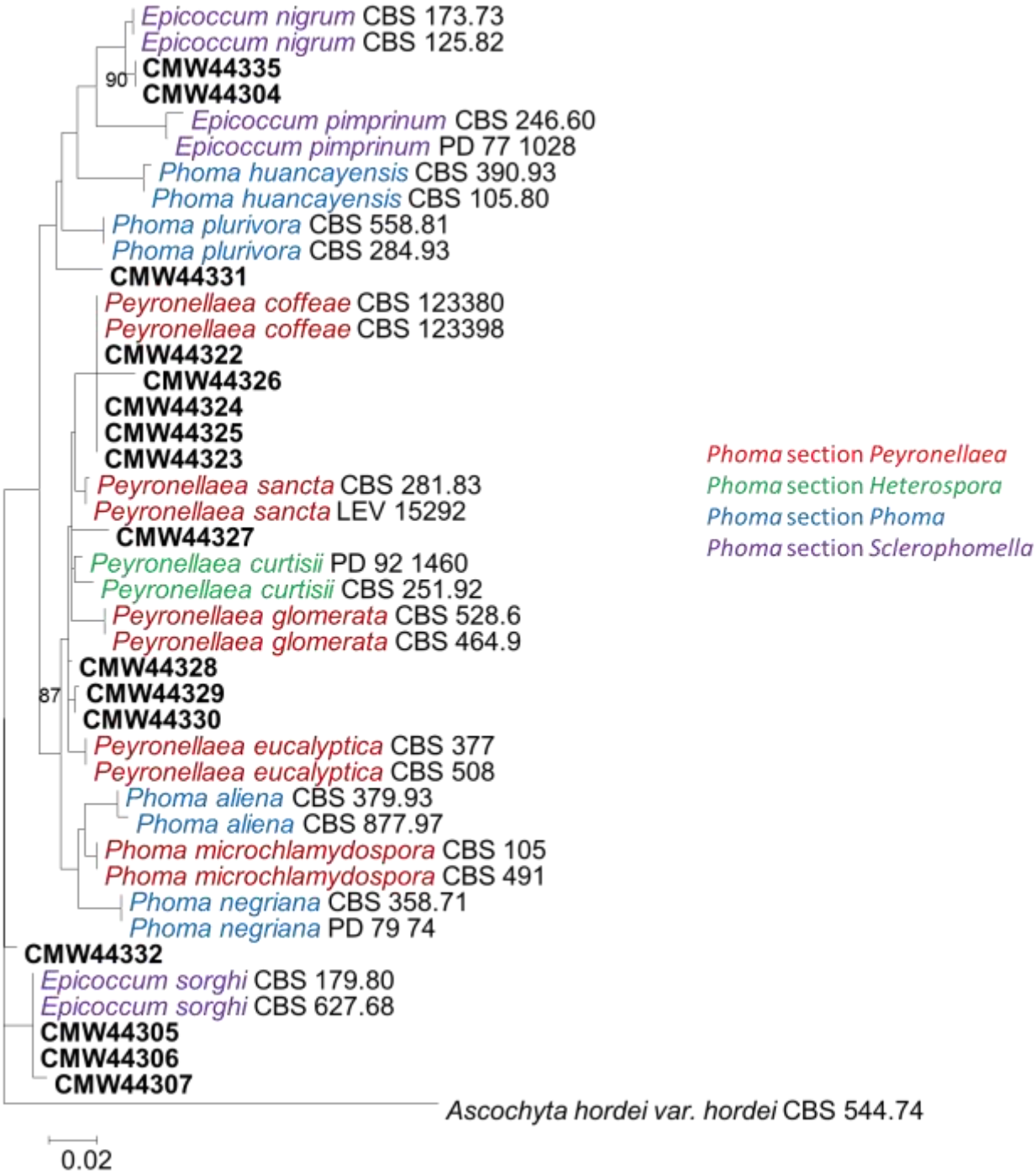
Weber GF, 1944. The blight disease of *Cycas revoluta*. *Florida Academy of Sciences* **7**, 129-132.

White TJ, Bruns T, Lee SJWT, Taylor JW, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications* **18**, 315-322.

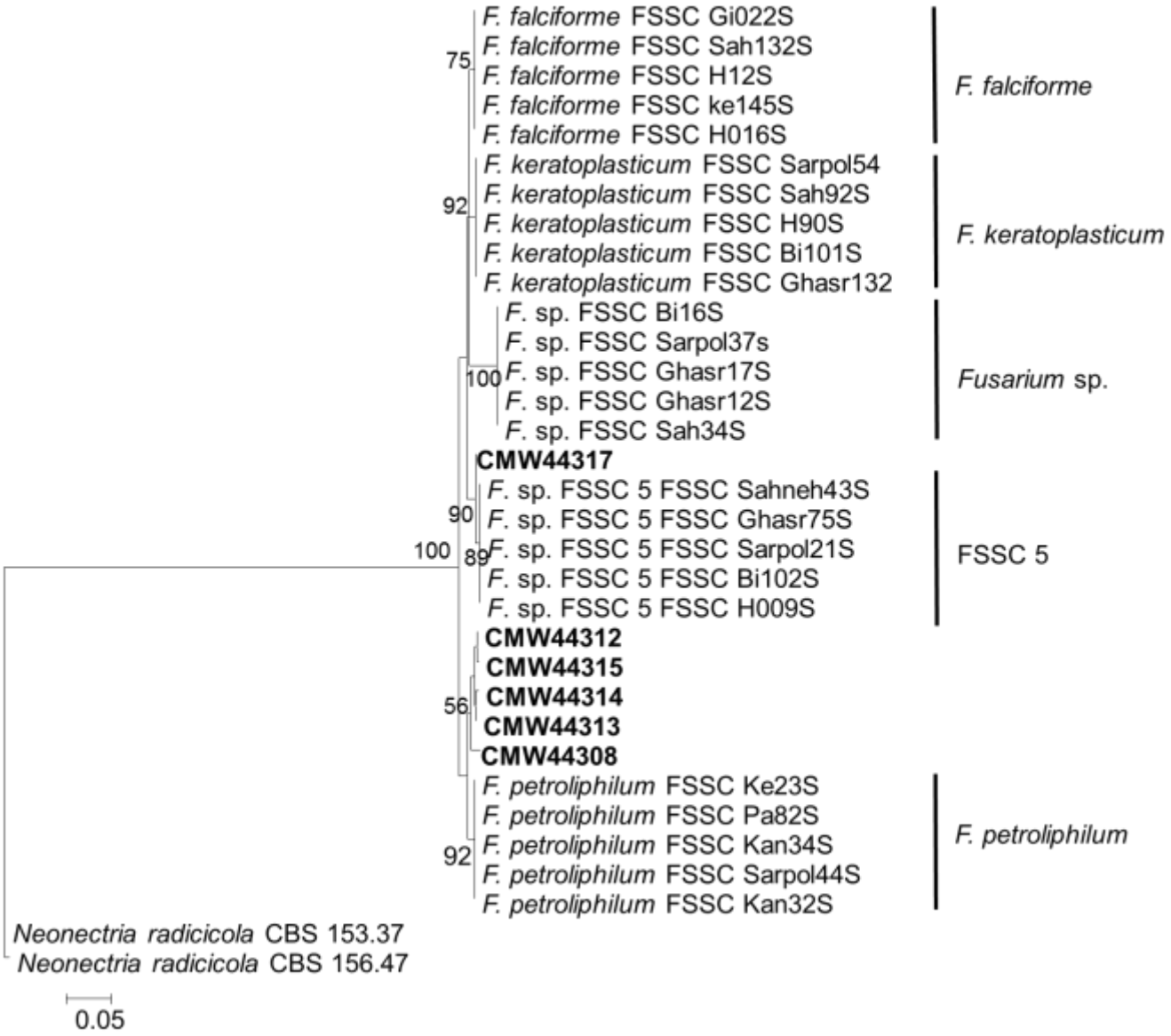
Supplementary material



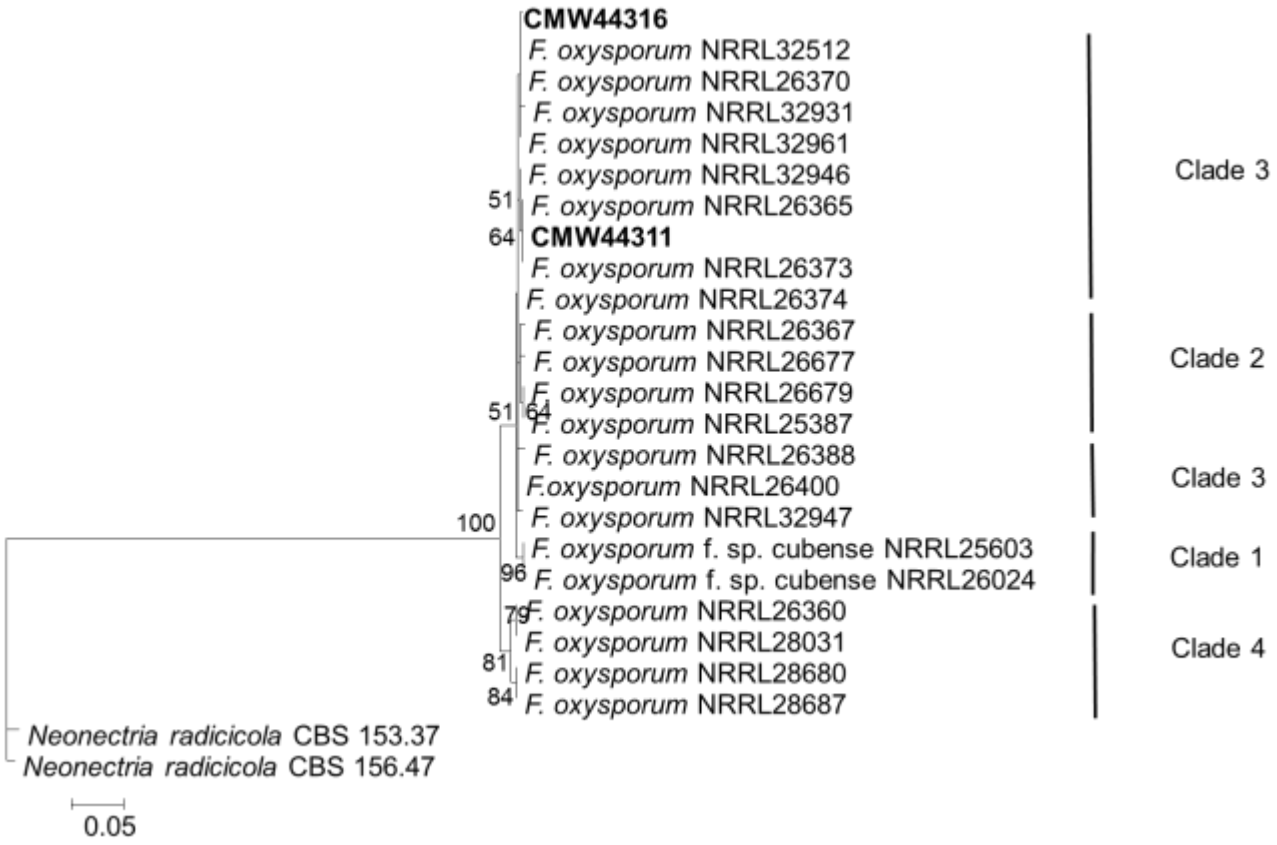
Supplementary Figure 1: Maximum likelihood (ML) phylogenetic tree based on combined ITS, BT and TEF-1α genomic DNA sequences showing identities of *Diaporthe* isolates. Numbers next to branches indicate ML bootstrap values.



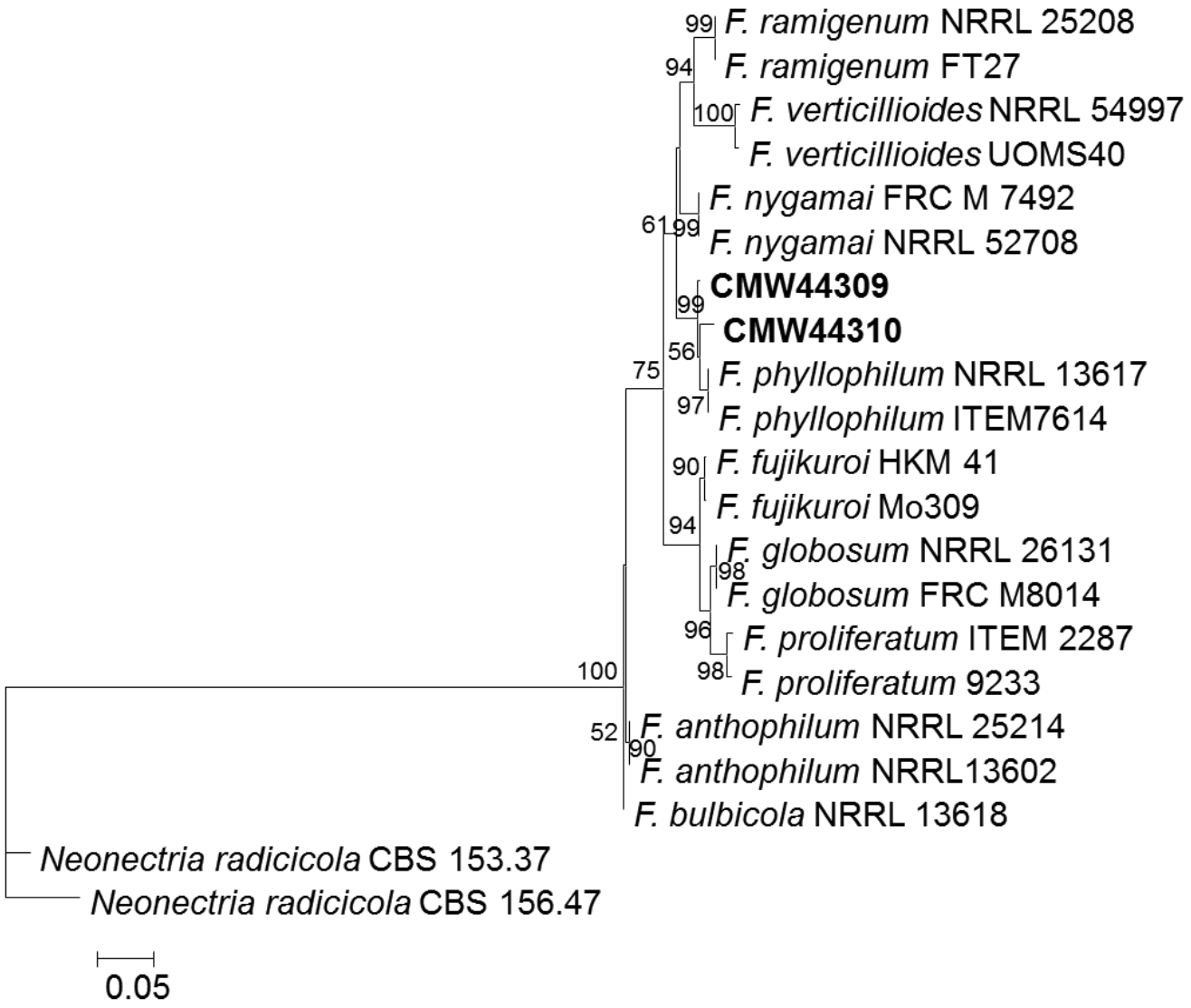
Supplementary Figure 2: Maximum likelihood (ML) phylogenetic tree based on combined ITS and BT genomic DNA sequences showing identities of *Epicoccum* and *Phoma* isolates. Numbers next to branches indicate ML bootstrap values.



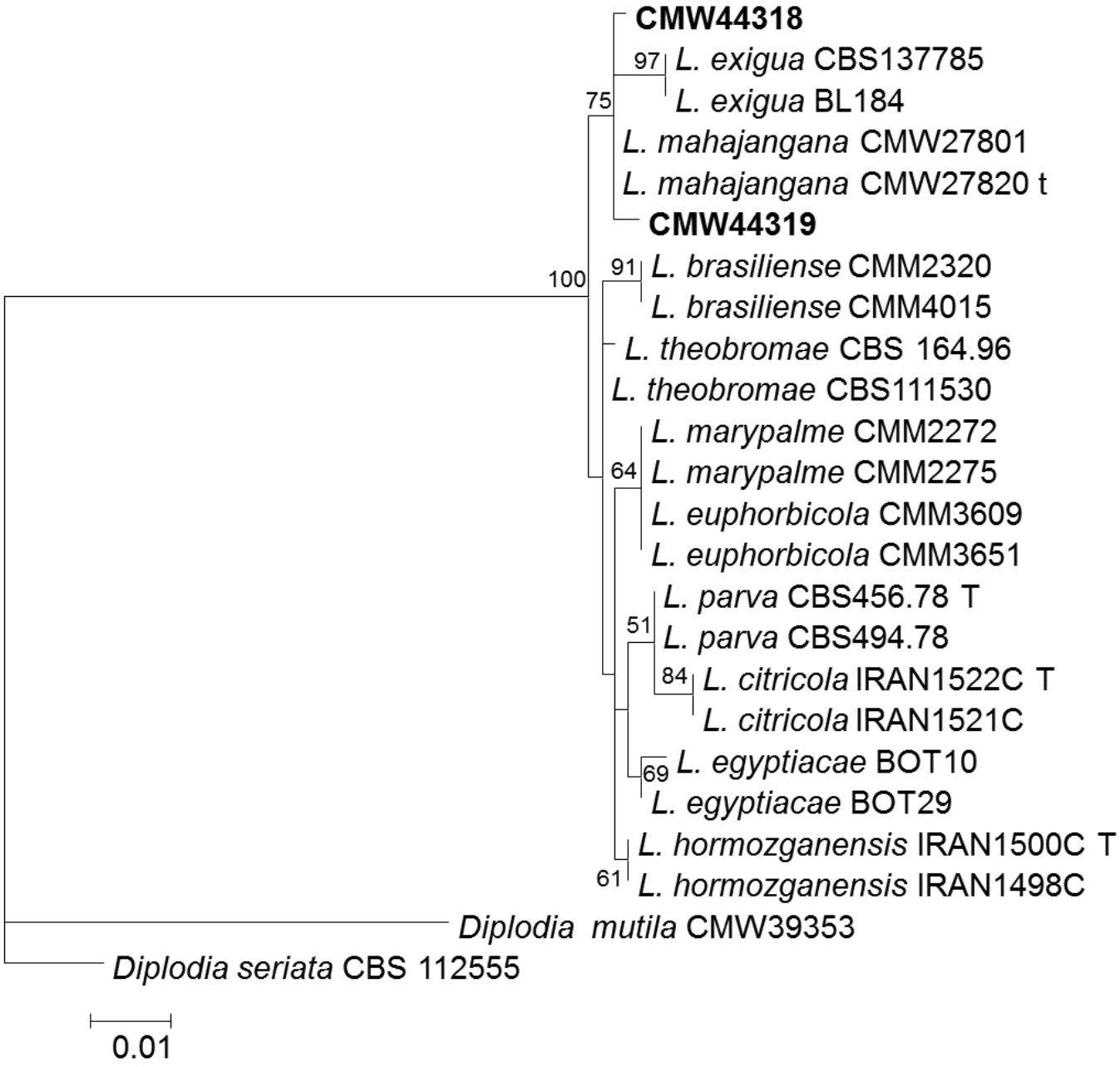
Supplementary Figure 3: Maximum likelihood (ML) phylogenetic tree based on combined ITS and TEF-1α genomic DNA sequences showing identities of *Fusarium* isolates in the *Fusarium solani* species complex. Numbers next to branches indicate ML bootstrap values.



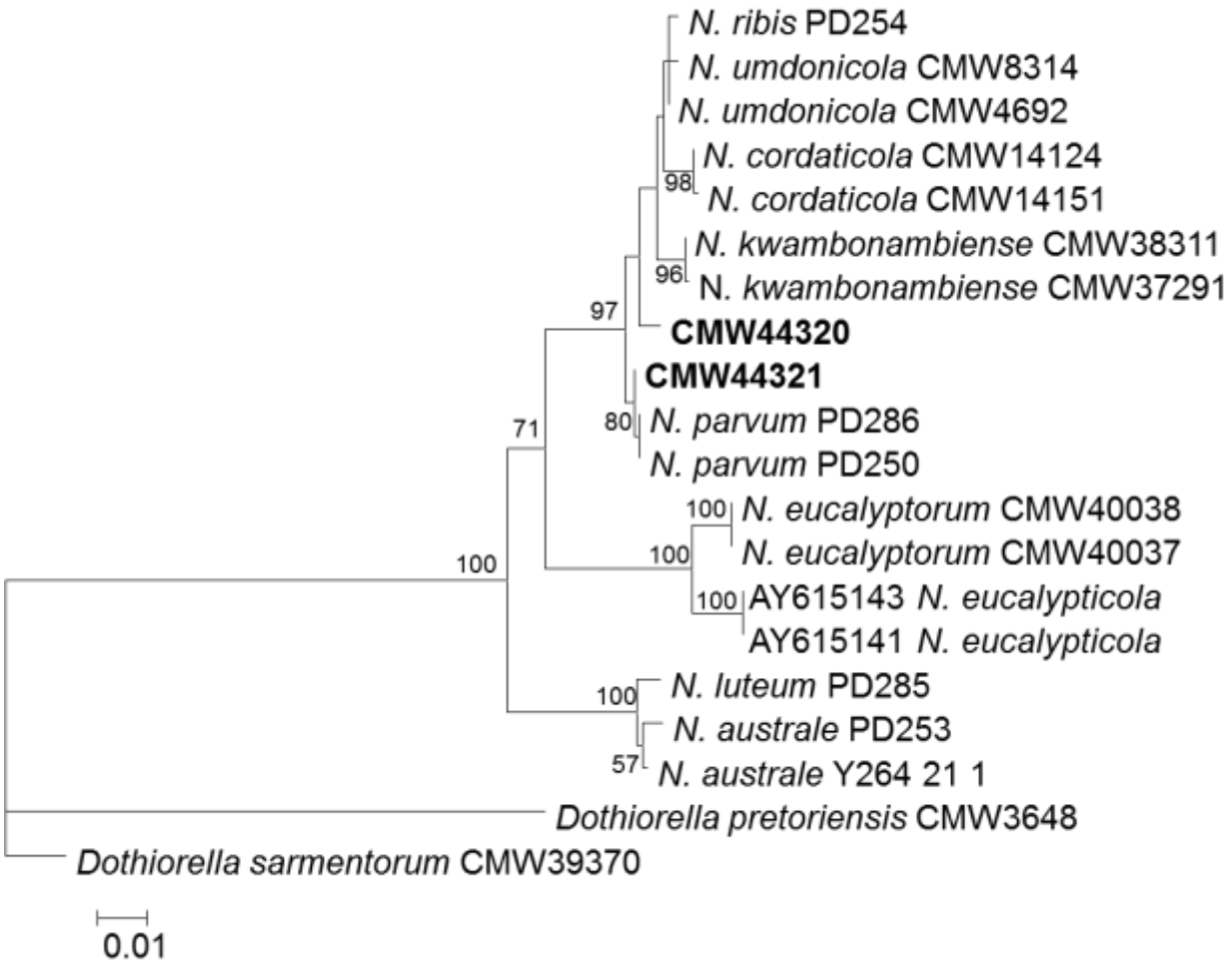
Supplementary Figure 4: Maximum likelihood (ML) phylogenetic tree based on TEF-1α genomic DNA sequences showing identities of *Fusarium* isolates in the *Fusarium oxysporum* species complex. Numbers next to branches indicate ML bootstrap values.



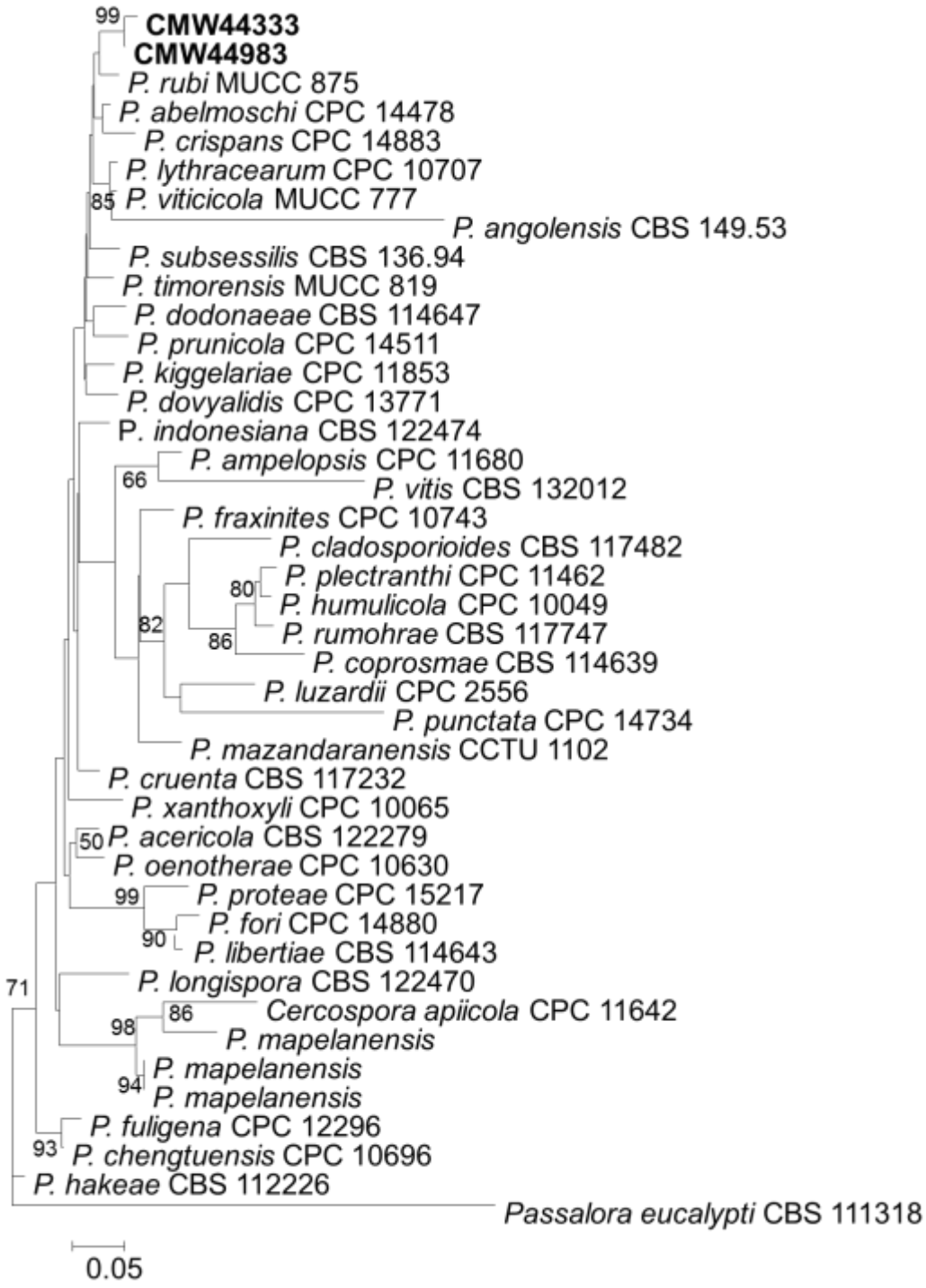
Supplementary Figure 5: Maximum likelihood (ML) phylogenetic tree based on TEF-1α genomic DNA sequences showing identities of *Fusarium* isolates in the *Fusarium fujikuroi* species complex. Numbers next to branches indicate ML bootstrap values.



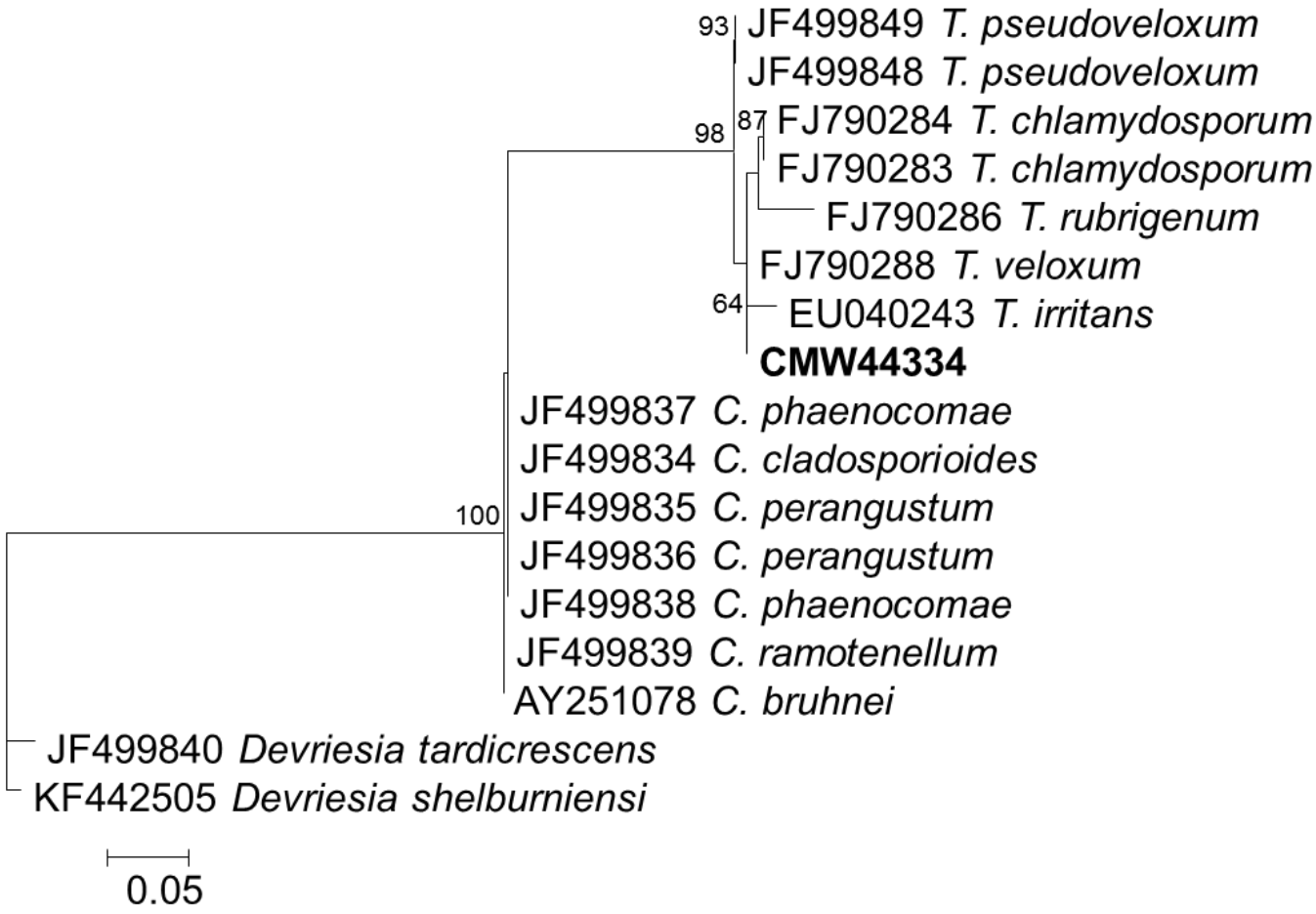
Supplementary Figure 6: Maximum likelihood (ML) phylogenetic tree based on combined ITS and TEF-1α genomic DNA sequences showing identities of *Lasiodiplodia* isolates. Numbers next to branches indicate ML bootstrap values.



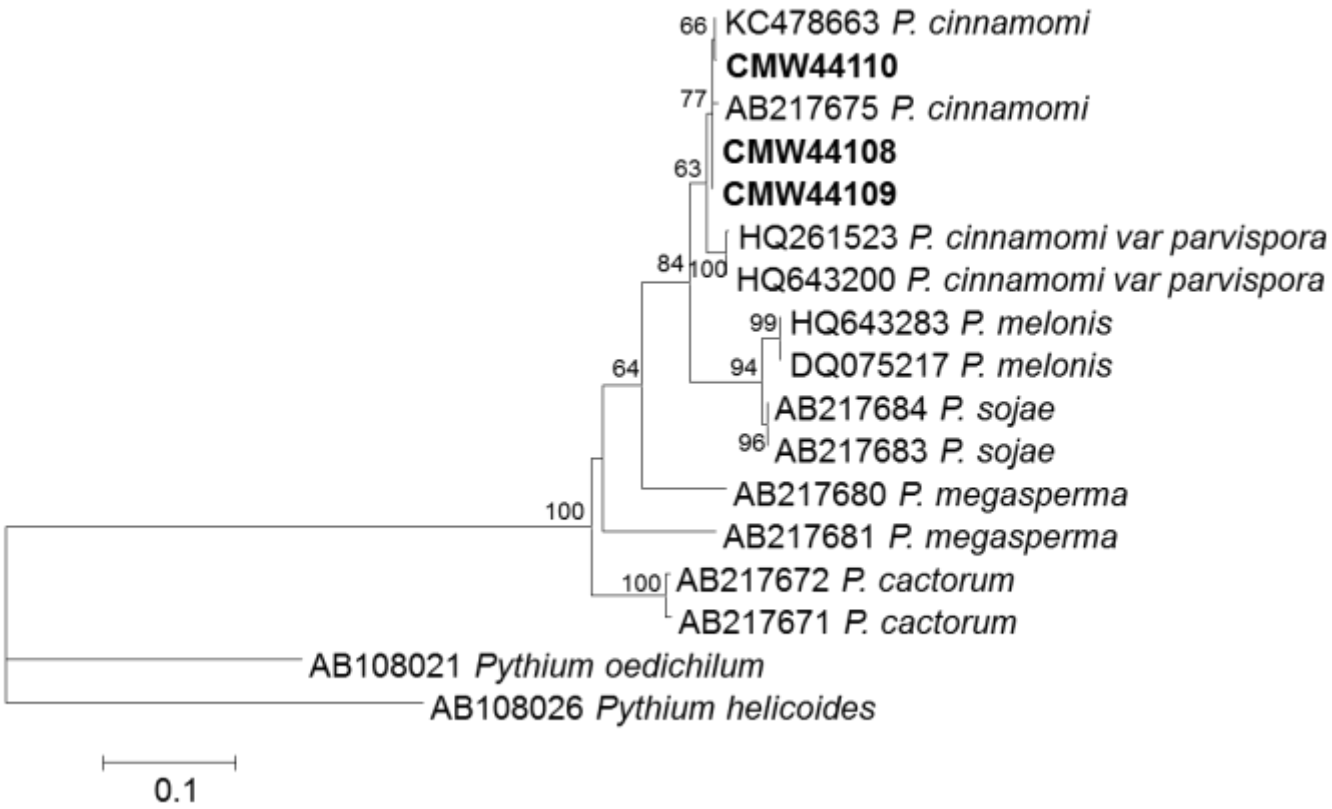
Supplementary Figure 7: Maximum likelihood (ML) phylogenetic tree based on combined ITS, BT and TEF-1α genomic DNA sequences showing identities of *Neofusicoccum* isolates. Numbers next to branches indicate ML bootstrap values.



Supplementary Figure 8: Maximum likelihood (ML) phylogenetic tree based on combined ACT, ITS and TEF-1α genomic DNA sequences showing identities of *Pseudocercospora* isolates. Numbers next to branches indicate ML bootstrap values.



Supplementary Figure 9: Maximum likelihood (ML) phylogenetic tree based on ITS genomic DNA sequences showing identity of the *Toxicocladoporium* isolate from *Encephalartos arenarius*. Numbers next to branches indicate ML bootstrap values.



Supplementary Figure 10: Maximum likelihood (ML) phylogenetic tree based on ITS genomic DNA sequences showing identities of *Phytophthora* isolates. Numbers next to branches indicate ML bootstrap values.