# First report of canker caused by *Chrysoporthe austroafricana* on the plantation-grown eucalypt *Corymbia henryi* in South Africa

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**Abstract**

Species of *Corymbia* are increasingly being tested for plantation establishment in South Africa. During disease surveys in KwaZulu-Natal plantations in 2020, a serious canker disease was found on *Corymbia henryi*. The cankers were seemingly caused by a fungus belonging to the Cryphonectriaceae. The aims of the study were to identify the fungus causing the disease, to test its pathogenicity and to determine the genetic diversity of a population of isolates from infected trees. Phylogenetic analyses using sequence data for the ITS, β-tubulin 1, and β-tubulin 2 gene regions showed that the causal agent of the cankers was *Chrysoporthe austroafricana*. This is the first record of *C. austroafricana* causing cankers on a *Corymbia* species. A pathogenicity trial demonstrated that the fungus could infect *C. henryi* and cause cankers. Fifteen isolates of *C. austroafricana* were subjected to population genetic analyses using microsatellite markers. Eleven multilocus genotypes were detected. Mating type distribution was approximately equal (MAT1-1 : MAT1-2 = 8:7). The results indicate that a wide diversity of genotypes of *C. austroafricana* have undergone a host shift to infect *C. henryi*. This is a similar situation to that observed for *Eucalyptus* in South Africa and suggests that research efforts will be required to reduce the risk of Cryphonectria canker limiting afforestation using *Corymbia* species in the future.

**Keywords**: Cryphonectriaceae, genetic diversity, pathogenicity, pest and disease

## Introduction

Fungi in the Cryphonectriaceae include numerous important tree pathogens that cause stem canker diseases (Gryzenhout et al., 2009). The best-known of these is *Cryphonectria parasitica* (Murrill) M.E. Barr, the causal agent of chestnut blight that led to the near extinction of *Castanea dentata* (Marshall) Borkh. trees in America and Europe (Anagnostakis and Kranz, 1987). In various tropical and sub-tropical areas of the world, *Eucalyptus* species in plantations have been severely affected by Cryphonectria canker caused by various species of the Cryphonectriaceae including those in genera such as *Chrysoporthe, Celoporthe*, and *Microthia* (Gryzenhout *et al.*, 2004; Gryzenhout *et al.*, 2006; Nakabonge *et al.*, 2006a). The most important among these are species of *Chrysoporthe*, of which the best-known pathogen is *Chrysoporthe cubensis* (Bruner) Gryzenh. & M.J. Wingf. (Hodges *et al.*, 1979; Oliveira *et al.*, 2020).

*Chrysoporthe* spp. typically infect wounds on trees, subsequently killing the cambium and giving rise to large cankers, usually on the stems (Gryzenhout *et al.*, 2004). Fruiting bodies of these fungi occur on or in the dead bark covering the cankers, and they typically have yellow to orange coloured necks. There are currently nine known *Chrysoporthe* species (Gryzenhout *et al.*, 2009; Chungu *et al.*, 2010; Oliveira *et al.*, 2020), and all of them have been found on trees or shrubs in the Myrtales, especially the Myrtaceae and Melastomataceae.

Cryphonectria canker on *Eucalyptus* was first reported in South Africa in 1989 (Wingfield *et al.*, 1989). At that time, the causal agent was thought to be the well-known *Cryphonectria cubensis*, which had caused significant losses to *Eucalyptus* in Brazilian plantations (Alfenas *et al.*, 1983). The fungus was later shown to be a distinct species and

was consequently transferred, together with various other species occurring on *Eucalyptus,* to *Chrysoporthe* as *C. austroafricana* Gryzenh. & M.J. Wingf. (Wingfield, 2003; Myburg *et al.*, 2002; Gryzenhout *et al.*, 2004). *Chrysoporthe austroafricana* was shown to be native to Africa and that it had undergone a host jump from *Syzygium* spp. to *Eucalyptus* spp. (Heath *et al.*, 2006; Nakabonge *et al.*, 2006b; Vermeulen *et al.*, 2013). The higher levels of aggressiveness and the lower levels of genetic diversity on *Eucalyptus* spp. than on *Syzygium* spp. added credence to that view (Heath *et al.*, 2006; Vermeulen *et al.*, 2013). Furthermore, the fact that sexual structures were frequently observed on *Syzygium* spp. but rarely on *Eucalyptus* spp., implied that *C. austroafricana* is heterothallic (Gryzenhout *et al.*, 2009). This hypothesis has been proven correct in studies using genome-wide analyses (Kanzi *et al.*, 2019).

During disease surveys in KwaZulu-Natal plantations in 2020, a serious canker disease was found on *Corymbia henryi* (S. T. Blake) K. D. Hill & L. A. S. Johnson. Species of *Corymbia* (previously *Eucalyptus*), including *C. henryi*, are increasingly being tested for plantation establishment in South Africa. The aims of this study were to identify the fungus causing the disease, to test its pathogenicity in order to fulfil Koch's Postulates and to determine the genetic diversity of an available population of isolates.

## Materials and Methods

### *Disease observation and fungal isolates*

Dying *C. henryi* trees were reported in the KwaZulu-Natal Province of South Africa in mid-2020. Affected trees occurred in a single compartment of approximately four-year-

old trees (planted in April 2016) near the town of KwaNgwanase (26°59'23.02"S; 32°45'17.37"E). Inspection of freshly wilted and dead trees was conducted.

Bark samples with fungal fruiting bodies were collected from the bases of 20 *C. henryi* trees. They were kept separately in brown paper bags and transported to the laboratory of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. Direct isolations were made from the fruiting bodies to obtain cultures, whereafter the remaining material was kept in a cool dry environment for further study.

To make pure cultures, a fruiting structure on each bark sample was dissected with a sterile scalpel under a dissecting microscope. The exposed spores and inner structures of the fruiting body were removed and spread over the surface of 2% malt extract agar (MEA: 20 g BioLab malt extract, 20 g Difco Bacto$^{TM}$ agar, 1L ionized water) containing 100 mg streptomycin sulphate (Sigma-Aldrich, Steinheim, Germany). A small block of agar containing a single hyphal tip was transferred with a sterilized needle to fresh MEA. The cultures were deposited for safe keeping in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

***DNA Sequencing and phylogenetic analysis***

Total genomic DNA was extracted from 7-day-old cultures growing on MEA using PrepMan Ultra Sample preparation reagent (Applied Biosystem, Foster City, CA) following the manufacturer's protocols. Polymerase chain reactions (PCRs) were conducted to amplify the internal transcribed spacer (ITS) regions, including the 5.8S gene of the ribosomal RNA and the β-tubulin 1 and β-tubulin 2 regions (BT). The primer

pair ITS1/ITS4 was used for amplification of the ITS region (White *et al.*, 1990) and BT1a/BT1b and BT2a/BT2b to amplify the BT1 and BT2 regions, respectively (Glass and Donaldson, 1995). The PCRs were performed in a final reaction volume of 13.25 µL containing 1 µL template DNA (10 ng/µL), 2.5 µL of 5 × MyTaq™ Reaction Buffer (Bioline, London), 0.3 µL of both forward and reverse primers (10 µM), 0.5 U of MyTaq™ DNA polymerase and 8.65 µL nuclease-free water. The PCR conditions were as follows: initial denaturation at 96 ℃ for 3 min followed by 28 cycles of denaturation at 95 ℃ for 30 sec, annealing at 55 ℃ for 45 sec, and elongation at 72 ℃ for 1 min. A final elongation step at 72 ℃ for 7 min followed. After the PCR products were purified using ExoSAP-IT (Affymetrix), following the manufacturer's protocols, the amplicons were visualized using electrophoresis with GelRed™ (Biotium, CA).

A sequencing reaction was conducted with either the forward or reverse primer using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit v. 3.1 (Applied BioSystems). An ABI PRISM 3100 Autosequencer (Applied BioSystems) was used to generate the sequences. The raw sequences were assembled using MEGA7 (Kumar *et al.*, 2016).

The sequences of twenty-seven isolates representing the currently known *Chrysoporthe* species were obtained from GenBank (http://www.ncbi.nlm.nih.gov) and combined into datasets for analyses with isolates obtained from *C. henryi* (Table 1). The sequences of the ITS and BT gene regions (BT1 and BT2) were analyzed separately as well as in combination and were aligned using MAFFT 7 with the G-INS-I option (http://mafft.cbrc.jp/alignment/server/) (Katoh *et al.*, 2019). Maximum likelihood (ML) analysis was performed using RAxML (Stamatakis, 2014). Confidence levels for nodes were estimated using 1,000 replicate bootstrap analyses. Bayesian inference analyses

**Table 1** − Isolates of *Chrysoporthe* species used in phylogenetic analysis

| Species name | Isolate[a] | Host | Origin | GenBank accession number[d] | | |
|---|---|---|---|---|---|---|
| | | | | ITS | BT1 | BT2 |
| *Chrysoporthe austroafricana* | CMW 2113[b] | *Eucalyptus grandis* | South Africa | AF046892 | AF273067 | AF273462 |
| *C. austroafricana* | CMW 9323 | *Tibouchina granulosa* | South Africa | AF273473 | AF273060 | AF 273455 |
| *C. austroafricana* | CMW 13929 | *Eucalyptus* sp. | Mozambique | DQ246570 | DQ246593 | DQ246570 |
| *C. austroafricana* | CMW 17098 | *Syzygium cordatum* | Malawi | DQ246607 | DQ246561 | DQ246584 |
| *C. austroafricana* | CMW 17101 | *Eucalyptus* sp. | Malawi | DQ246608 | DQ246562 | DQ246585 |
| *C. austroafricana* | CMW 22751 | *S. guineense* | Namibia | GU726948 | GU726960 | GU726960 |
| *C. austroafricana* | CMW 22760 | *S. guineense* | Namibia | GU726949 | GU726961 | GU726961 |
| *C. austroafricana* | CMW 29904 | *S. guineense* | South Africa | GU726950 | GU726962 | GU726962 |
| *C. austroafricana* | CMW 32954 | *S. guineense* | South Africa | GU726951 | GU726963 | GU726963 |
| *C. austroafricana* | CMW 37557 | *S. guineense* | Mozambique | JX842753 | JX842765 | JX842759 |
| *C. austroafricana* | CMW 37563 | *Dissotis* sp. | Mozambique | JX842757 | JX842769 | JX842763 |
| *C. austroafricana* | CMW 56206[c] | *Corymbia henryi* | South Africa | **OP204370** | **OP204385** | **OP204400** |
| *C. austroafricana* | CMW 56209[c] | *C. henryi* | South Africa | **OP204371** | **OP204386** | **OP204401** |
| *C. austroafricana* | CMW 56212[c] | *C. henryi* | South Africa | **OP204372** | **OP204387** | **OP204402** |
| *C. austroafricana* | CMW 56214[c] | *C. henryi* | South Africa | **OP204373** | **OP204388** | **OP204403** |
| *C. austroafricana* | CMW 56217[c] | *C. henryi* | South Africa | **OP204374** | **OP204389** | **OP204404** |
| *C. austroafricana* | CMW 56223 | *C. henryi* | South Africa | **OP204375** | **OP204390** | **OP204405** |
| *C. austroafricana* | CMW 56594 | *C. henryi* | South Africa | **OP204376** | **OP204391** | **OP204406** |
| *C. austroafricana* | CMW 56595 | *C. henryi* | South Africa | **OP204377** | **OP204392** | **OP204407** |
| *C. austroafricana* | CMW 56599 | *C. henryi* | South Africa | **OP204378** | **OP204393** | **OP204408** |

| | | | | | | |
|---|---|---|---|---|---|---|
| *C. austroafricana* | CMW 56601 | *C. henryi* | South Africa | **OP204379** | **OP204394** | **OP204409** |
| *C. austroafricana* | CMW 56606 | *C. henryi* | South Africa | **OP204380** | **OP204395** | **OP204410** |
| *C. austroafricana* | CMW 56609 | *C. henryi* | South Africa | **OP204381** | **OP204396** | **OP204411** |
| *C. austroafricana* | CMW 56617 | *C. henryi* | South Africa | **OP204382** | **OP204397** | **OP204412** |
| *C. austroafricana* | CMW 56620 | *C. henryi* | South Africa | **OP204383** | **OP204398** | **OP204413** |
| *C. austroafricana* | CMW 56621 | *C. henryi* | South Africa | **OP204384** | **OP204399** | **OP204414** |
| *C. cubensis* | CMW 10026 | *Miconia rubiginosa* | Colombia | AY214294 | AY214222 | AY214258 |
| *C. cubensis* | CMW 14394 | *E. grandis* | Cuba | DQ368773 | AH015642 | AH015642 |
| *C. deuterocubensis* | CMW 2631 | *E. marginata* | Australia | GQ290157 | GQ290184 | AF543825 |
| *C. deuterocubensis* | CMW 8650 | *S. aromaticum* | Indonesia | AY084001 | AY084024 | GQ290193 |
| *C. doradensis* | CMW 9124 | *E. deglupta* | Ecuador | DQ224035 | DQ224040 | DQ224041 |
| *C. doradensis* | CMW 11286 | *E. deglupta* | Ecuador | JN942331 | AY214218 | AY214254 |
| *C. hodgesiana* | CMW 10461 | *T. semidecandra* | Colombia | AY692322 | AY692326 | AY692325 |
| *C. hodgesiana* | CMW 10625 | *M. theaezans* | Colombia | AY956970 | AH014900 | AH014900 |
| *C. inopina* | CMW 12727[b] | *T. lepidota* | Colombia | DQ368777 | AH015657 | AH015657 |
| *C. inopina* | CMW 12729 | *T. lepidota* | Colombia | DQ368778 | DQ368808 | DQ368809 |
| *C. puriensis* | CT13[b] | *T. granulosa* | Brazil | MN590029 | MN590041 | MN590041 |
| *C. puriensis* | TGT03 | *T. granulosa* | Brazil | MN590035 | MN590047 | MN590047 |
| *C. syzygiicola* | CMW 29940[b] | *S. guineense* | Zambia | FJ655005 | FJ805230 | FJ805236 |
| *C. syzygiicola* | CMW 29941 | *S. guineense* | Zambia | FJ655006 | FJ805231 | FJ805237 |
| *C. zambiensis* | CMW 29928[b] | *E. grandis* | Zambia | FJ655002 | FJ858709 | FJ805233 |
| *C. zambiensis* | CMW 29930 | *E. grandis* | Zambia | FJ655004 | FJ858711 | FJ805235 |
| *Amphilogia gyrosa* | CMW 10469[b] | *Elaeocarpus dentatus* | New Zealand | AF452111 | AF525707 | AF525714 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *A. gyrosa* | CMW 10470 | *E. dentatus* | New Zealand | AF452112 | AF525708 | AF525715 |

[a]CMW, the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; CT and TGT, the culture collection of the Forest Pathology Laboratory (LPF), Federal University of Lavras, Brazil.

[b]ex-type culture

[c]Isolate used in pathogenicity test

[d]Isolates presented in bold were sequenced in this study

were conducted using MrBayes v.3.2.1 (Ronquist *et al.*, 2012) to estimate the posterior probabilities of tree topologies with Metropolis-coupled Markov Chain Monte Carlo searches. The substitution model was selected for each region with the Akaike information criterion (AIC) in jModelTest 2.1.5 (Posada, 2008). The analysis was performed for 3,000,000 generations with four runs. Trees were sampled every 100 generations, and a 25% burn-in was used to summarize a consensus tree. *Amphilogia gyrosa* (Berk. & Broome) Gryzenh., H.F. Glen & M.J. Wingf. belonging to the Cryphonectriaceae was chosen as the outgroup taxon.

***Genetic diversity and mating type distribution***

The 15 isolates identified as *C. austroafricana* in this study (Table 2) were subjected to microsatellite analysis using 12 primer sets designed by Oliveira *et al.* (2021). Single-plex PCRs were carried out in a total volume of 13.25 µL containing 1 µL of template DNA (10 ng/µL), 0.3 µL of the fluorescent forward primer (10µM), 0.3 µM of the reverse primer (10µM), 2.5 µL of 5 × MyTaq$^{TM}$ Reaction Buffer (Bioline, London, UK), and 0.5 U of MyTaq$^{TM}$ DNA polymerase (Bioline) and 8.65 µL nuclease-free water. PCRs were conducted under the following conditions: initial denaturation at 94 °C for 5 min, followed by 26 cycles of 35 s at 94 °C, 60 to 64 °C depending on the primers for 35 s, 72 °C for 1 min, with a final extension for 7 min at 72 °C.

The PCR products were grouped into panels based on estimated allelic ranges (Table 2), and 1 µL of the diluted product was run in a single lane on an ABI 3500xl sequencer (Thermo Fisher Scientific, MA) using the LIZ500 (Applied Biosystems, Thermo Fisher Scientific) size standard to calibrate fragment sizes. The allele sizes were

**Table 2** − Characteristics of microsatellite markers used for population genetic analysis

| Locus ID | Forward primer sequences | Reverse primer sequences | Motif | Dye | Ta[a] | Panel | Allelic range (bp) | Na[b] | H[c] |
|---|---|---|---|---|---|---|---|---|---|
| CHRY06 | CCCCAAGCTCGCCTACTTC | GACGCTCTCGGGTATTGTCA | AGC | FAM | 60 | 1 | 214−217 | 2 | 0.23 |
| CHRY07 | TTGGTCGGTTCGGTAGAAAG | CATGACGGGAGAGCGATAAG | AGT | VIC | 60 | 2 | 228−255 | 4 | 0.68 |
| CHRY09 | CCTTCTCTTTCCCAATTTTGC | CACCTCGTACAAGGCCTCTC | TGC | NED | 60 | 2 | 210−234 | 3 | 0.5 |
| CHRY10 | TGCGAGTGGGTGAGTGAGTA | ACACGGACGGTCATAGCAC | TGGC | VIC | 60 | 1 | 158 | 1 | 0 |
| CHRY11 | GCTGAAGGCACAGAAAGAGC | AGTCTGCGTTGATCCTTGCT | AGC | NED | 60 | 1 | 156−165 | 2 | 0.48 |
| CHRY13 | GTGTCGTCGACTCAAGCTCA | GACGGAGAGGACACTGAAGC | TGGTC | VIC | 60 | 3 | 212−257 | 4 | 0.44 |
| CHRY15 | AAGGCCAGATTGTCCAAATG | CAGCAACGAACCAAAATCAA | TGA | NED | 60 | 3 | 243 | 1 | 0 |
| CHRY17 | AGATCCAAGTGGGCTTTCCT | CTTAGCTTGACCCAGGGATG | ACC | FAM | 60 | 3 | 149 | 1 | 0 |
| CHRY23 | TGTCCTGCATCTAGGTCGTG | GTCCCAGTCCTCTTGGATCA | AGC | NED | 60 | 2 | 152−158 | 2 | 0.12 |
| CHRY25 | TATCTGCGCAACTACCACCA | GGGAAGTGTGTCTCCTCCTG | ACC | VIC | 60 | 3 | 161 | 1 | 0 |
| CHRY27 | GCCGCCTACAGAGACTATAACG | CGACGAAGACGTAGTTGCAC | AGC | PET | 60 | 3 | 251−272 | 3 | 0.34 |
| CHRY28 | GATATGCCGGTCATGGTGAT | TACTTGAGGGCGAAGGAAAG | TGG | PET | 60 | 1 | 235 | 1 | 0 |

[a]Annealing temperature

[b]Number of alleles

[c]Gene diversity (Nei 1978)

determined using the ABI PRISM GeneMapper 7 software (Applied Biosystems).

The numbers of alleles, gene diversity (*H*) (Nei, 1978) for each microsatellite locus, and the mean gene diversity ($\overline{H}$) were calculated with GenAlEx v.6.5 (Peakall and Smouse, 2012). The data for the polymorphic loci were used to calculate a genotype accumulation curve and draw a minimum spanning network with Bruvo's distance (Bruvo *et al.*, 2004) using R 3.6.1 (R Core Team, 2019) in the Poppr package (Kamvar *et al.*, 2015). A genotype accumulation curve was drawn by means of 1,000 times random sampling for each of the loci.

Mating type distribution in the samples of *C. austroafricana* from *C. henryi* was assayed using PCRs (Table 1). Because the *MAT1-2* idiomorph of *C. austroafricana* includes a truncated *MAT1-1-1* gene and an intact *MAT1-1-2* gene (Kanzi *et al.*, 2019), the *MAT1-1-3* gene for the *MAT1-1* idiomorph and *MAT1-2-1* gene for *MAT 1-2* idiomorph were targeted. The primer sets acdmat113F (5'-TACCATGTCATCCAGCCCCA-3') and acdmat113R (5'-GTACTTTGCTTGGTGTTGAT-3') for the *MAT1-1* idiomorph and acdmat121F (5'-AACCGTCTTCTTGTTGGTC-3') and acdmat121R (5'-GTGGTAGTCTTCTTGGAACG-3') for the *MAT1-2* idiomorph were used (Kanzi *et al.*, 2019). The PCRs were carried out under the same conditions as the PCRs for phylogenetic analyses, except with an annealing temperature of 60 ℃. An exact binomial test was used at P = 0.05 significance level to determine whether the samples deviated from a 1:1 ratio of mating types.

***Pathogenicity tests***

To fulfil Koch's Postulates, a culture of isolate CMW 56217 was grown on 2% MEA for 14 days before being inoculated into the stems of one-year-old, healthy *C. henryi* saplings grown in potting bags. The range of stem diameters of the saplings was between 5 and 10 mm. Twenty *C. henryi* saplings were inoculated by removing the bark from the stems using a cork borer (5 mm diameter). Plugs of the test fungus, made using the same sized cork borer, were removed from the agar and placed into the wounds, with the mycelium facing the cambium, using a sterilized scalpel. For the control inoculations, sterile plugs of 2% MEA were used.

The inoculated wounds were sealed with Parafilm (Bemis Company, Inc, WI) to reduce contamination and to slow the drying out of the agar plugs and wounds. After eight weeks, the trial was terminated, and data were collected by measuring the lengths of bark and cambium lesions on the sapling stems. Stem sections around the point of inoculation containing lesions were sampled and placed in moist chambers to inspect for the presence of the inoculated fungus. The entire inoculation experiment was repeated once.


**Figure 1** − A. *Corymbia henryi* trees infected by *Chrysoporthe austroafricana*. B–F. Symptoms of infected trees. B. Kino exudation. C. Basal swelling and cracking of root collar. D, E. Cracked bark. F. Conidiomata formed on cracked bark. G–K. Close-up of conidiomata on bark with orange to fuscous black necks and orange spore masses at the tips.

## Results

### *Disease observation and fungal isolates*

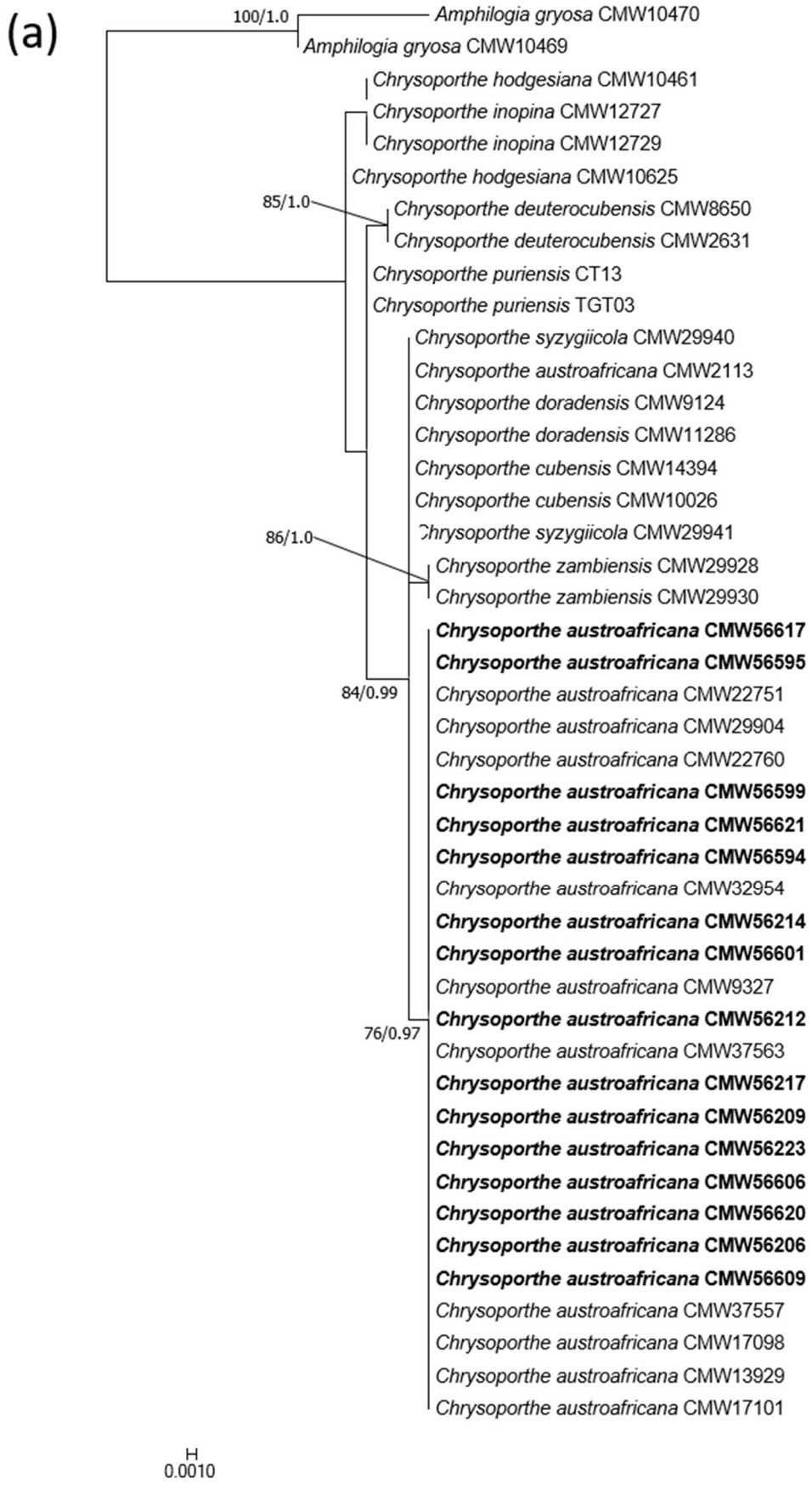
Trees were dying individually and in small patches (Figure 1A). Kino exudation and bark cracking at the bases of the trees were observed (Figure 1B–E). Fungal fruiting bodies with medium to long necks could be seen on the cracked bark (Figure 1F–K), and the root collars of trees were discoloured. In some instances, yellow to orange spore drops were seen exuding from the tips of the fruiting body necks.

One fruiting structure from each of the 20 bark samples was used for isolation. This resulted in 15 pure cultures representing a single fungal morphotype typical of the Cryphonectriaceae with white fluffy aerial mycelium with cinnamon-coloured edges. These isolates were then subjected to phylogenetic and population genetic analyses.

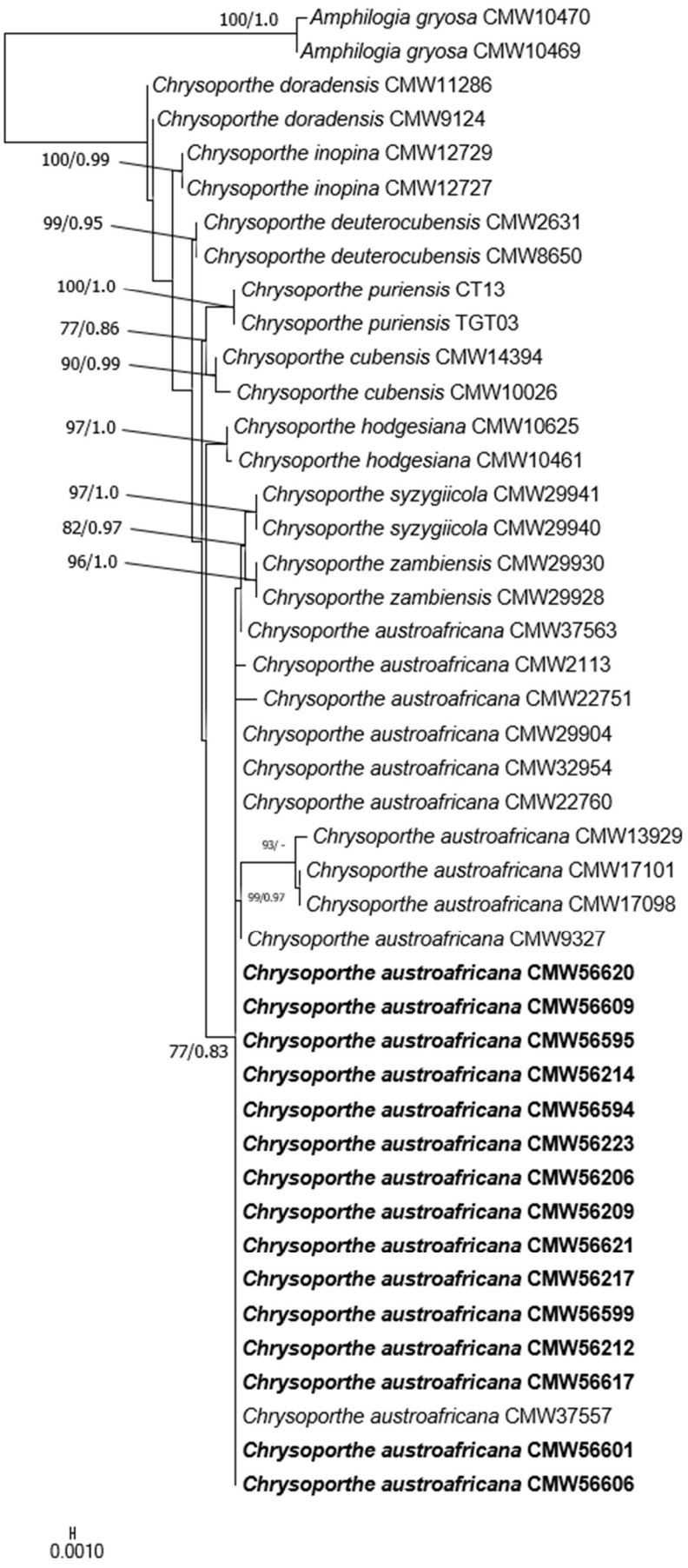
### *DNA sequencing and phylogenetic analysis*

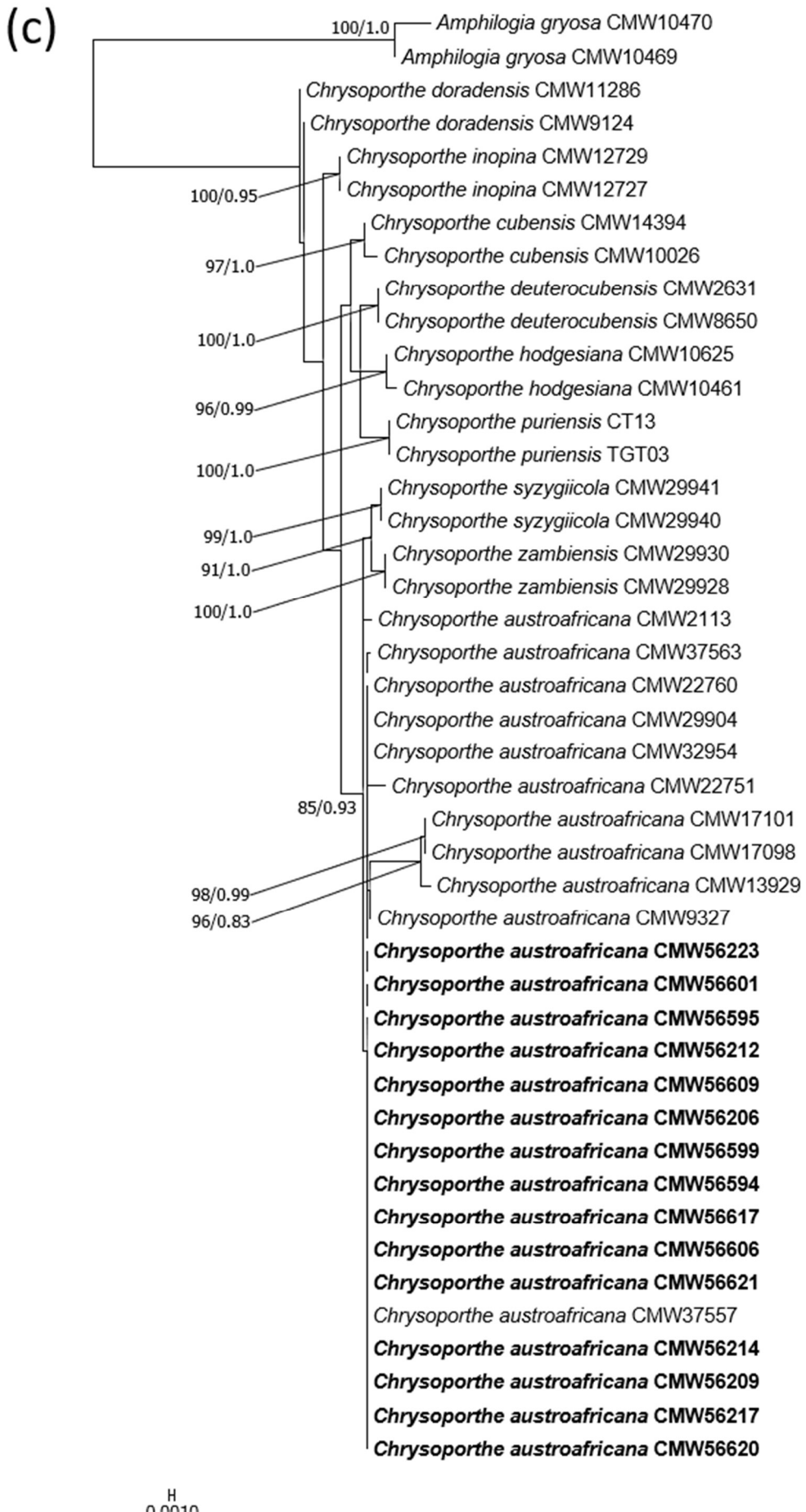
All sequences obtained for the 15 isolates collected in this study were deposited in GenBank (Table 1), and the aligned sequences for the ITS (458 characters), BT (792 characters) and combined (1250 characters) datasets, comprising 44 taxa, were deposited in figshare (doi: 10.6084/m9.figshare.20525349). A summary of important parameters applied in the phylogenetic analysis is presented in Supplementary Table 1.

The ML and BI analyses of the datasets provided trees with consistent topologies. The phylogenetic analyses with ITS, BT and combined datasets showed that the 15 isolates from *C. henryi* resided in the *C. austroafricana* clade, with high bootstrap values of ML and Posterior probabilities (Figure 2).


(a)
100/1.0
Amphilogia gryosa CMW10470
Amphilogia gryosa CMW10469
Chrysoporthe hodgesiana CMW10461
Chrysoporthe inopina CMW12727
Chrysoporthe inopina CMW12729
Chrysoporthe hodgesiana CMW10625
85/1.0
Chrysoporthe deuterocubensis CMW8650
Chrysoporthe deuterocubensis CMW2631
Chrysoporthe puriensis CT13
Chrysoporthe puriensis TGT03
Chrysoporthe syzygiicola CMW29940
Chrysoporthe austroafricana CMW2113
Chrysoporthe doradensis CMW9124
Chrysoporthe doradensis CMW11286
Chrysoporthe cubensis CMW14394
Chrysoporthe cubensis CMW10026
Chrysoporthe syzygiicola CMW29941
86/1.0
Chrysoporthe zambiensis CMW29928
Chrysoporthe zambiensis CMW29930
Chrysoporthe austroafricana CMW56617
Chrysoporthe austroafricana CMW56595
84/0.99
Chrysoporthe austroafricana CMW22751
Chrysoporthe austroafricana CMW29904
Chrysoporthe austroafricana CMW22760
Chrysoporthe austroafricana CMW56599
Chrysoporthe austroafricana CMW56621
Chrysoporthe austroafricana CMW56594
Chrysoporthe austroafricana CMW32954
Chrysoporthe austroafricana CMW56214
Chrysoporthe austroafricana CMW56601
Chrysoporthe austroafricana CMW9327
Chrysoporthe austroafricana CMW56212
76/0.97
Chrysoporthe austroafricana CMW37563
Chrysoporthe austroafricana CMW56217
Chrysoporthe austroafricana CMW56209
Chrysoporthe austroafricana CMW56223
Chrysoporthe austroafricana CMW56606
Chrysoporthe austroafricana CMW56620
Chrysoporthe austroafricana CMW56206
Chrysoporthe austroafricana CMW56609
Chrysoporthe austroafricana CMW37557
Chrysoporthe austroafricana CMW17098
Chrysoporthe austroafricana CMW13929
Chrysoporthe austroafricana CMW17101
H
0.0010

(b)


100/1.0
Amphilogia gryosa CMW10470
Amphilogia gryosa CMW10469
Chrysoporthe doradensis CMW11286
Chrysoporthe doradensis CMW9124
100/0.99
Chrysoporthe inopina CMW12729
Chrysoporthe inopina CMW12727
99/0.95
Chrysoporthe deuterocubensis CMW2631
Chrysoporthe deuterocubensis CMW8650
100/1.0
Chrysoporthe puriensis CT13
Chrysoporthe puriensis TGT03
77/0.86
90/0.99
Chrysoporthe cubensis CMW14394
Chrysoporthe cubensis CMW10026
97/1.0
Chrysoporthe hodgesiana CMW10625
Chrysoporthe hodgesiana CMW10461
97/1.0
Chrysoporthe syzygiicola CMW29941
82/0.97
Chrysoporthe syzygiicola CMW29940
96/1.0
Chrysoporthe zambiensis CMW29930
Chrysoporthe zambiensis CMW29928
Chrysoporthe austroafricana CMW37563
Chrysoporthe austroafricana CMW2113
Chrysoporthe austroafricana CMW22751
Chrysoporthe austroafricana CMW29904
Chrysoporthe austroafricana CMW32954
Chrysoporthe austroafricana CMW22760
93/ -
Chrysoporthe austroafricana CMW13929
Chrysoporthe austroafricana CMW17101
99/0.97
Chrysoporthe austroafricana CMW17098
Chrysoporthe austroafricana CMW9327
Chrysoporthe austroafricana CMW56620
Chrysoporthe austroafricana CMW56609
Chrysoporthe austroafricana CMW56595
77/0.83
Chrysoporthe austroafricana CMW56214
Chrysoporthe austroafricana CMW56594
Chrysoporthe austroafricana CMW56223
Chrysoporthe austroafricana CMW56206
Chrysoporthe austroafricana CMW56209
Chrysoporthe austroafricana CMW56621
Chrysoporthe austroafricana CMW56217
Chrysoporthe austroafricana CMW56599
Chrysoporthe austroafricana CMW56212
Chrysoporthe austroafricana CMW56617
Chrysoporthe austroafricana CMW37557
Chrysoporthe austroafricana CMW56601
Chrysoporthe austroafricana CMW56606
H
0.0010


(c)
100/1.0
Amphilogia gryosa CMW10470
Amphilogia gryosa CMW10469
Chrysoporthe doradensis CMW11286
Chrysoporthe doradensis CMW9124
Chrysoporthe inopina CMW12729
100/0.95
Chrysoporthe inopina CMW12727
Chrysoporthe cubensis CMW14394
97/1.0
Chrysoporthe cubensis CMW10026
Chrysoporthe deuterocubensis CMW2631
100/1.0
Chrysoporthe deuterocubensis CMW8650
Chrysoporthe hodgesiana CMW10625
96/0.99
Chrysoporthe hodgesiana CMW10461
Chrysoporthe puriensis CT13
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Chrysoporthe puriensis TGT03
Chrysoporthe syzygiicola CMW29941
99/1.0
Chrysoporthe syzygiicola CMW29940
91/1.0
Chrysoporthe zambiensis CMW29930
100/1.0
Chrysoporthe zambiensis CMW29928
Chrysoporthe austroafricana CMW2113
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**Figure 2 –** Phylogenetic trees based on ML analysis with *Chrysoporthe* spp. (a) ITS region (b) two regions of BT (BT1 and BT2) (c) the combined dataset of ITS and BT gene regions. The sequences of the isolates in bold were obtained in this study. Bootstrap values >75% for ML and >0.80 for BI PP are shown as ML/BI PP.

### *Population diversity and mating type distribution*

All 12 microsatellite primers were successfully amplified in the 15 isolates of *C. austroafricana* from *C. henryi*. Seven of these 12 loci were polymorphic (Table 2) and used for further statistical analyses, although the genotype accumulation curve did not reach a plateau (Supplementary Figure 1). The gene diversity ($H$) and the number of alleles of each locus are shown in Table 2. All polymorphic loci have a trinucleotide repeat except for CHRY13, which has pentanucleotide repeat. The mean gene diversity ($\overline{H}$) and the mean number of alleles at each of the loci which have trinucleotide repeat was 0.39 ± 0.20 and 2.67 ± 0.82 (mean ± SD), respectively. The 15 isolates included 11 multilocus genotypes (MLG) (Figure 3).

The PCRs successfully amplified either the fragment of the *MAT1-1-3* gene (753 bp) or the *MAT1-2-1* gene (572 bp) in the 15 isolates (Figure 4). The fragment representing the *MAT1-1-3* gene was amplified in eight of the 15 isolates, and the *MAT1-2-1* gene was amplified in the remaining seven isolates. The exact binomial test did not reject the null hypothesis of a *MAT1-1*/*MAT1-2* ratio of 1:1 ($P > 0.05$).

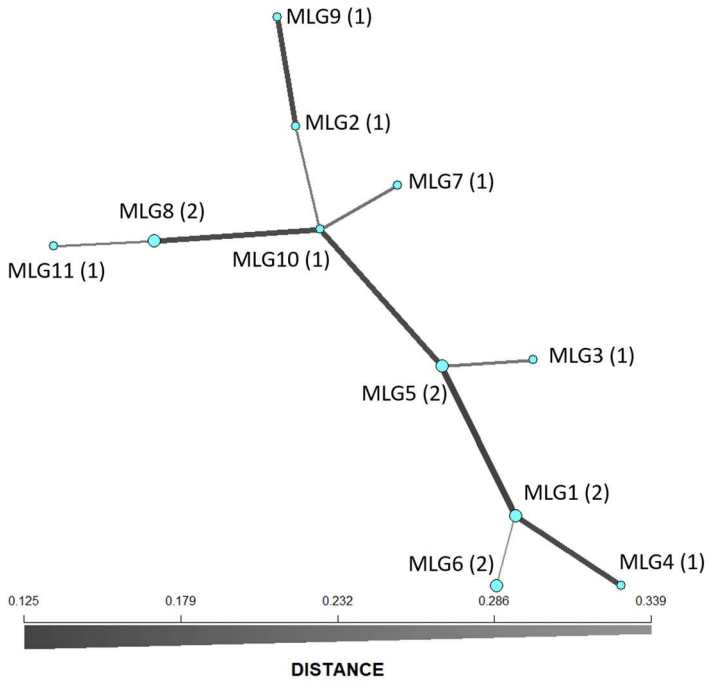


**Figure 3** – Minimum spanning network of multilocus genotypes of *Chrysoporthe austroafricana* isolated from *Corymbia henryi*. Each node indicates a multilocus genotype (MLG). The numbers after MLGs indicate the number of individuals within the multilocus genotype.

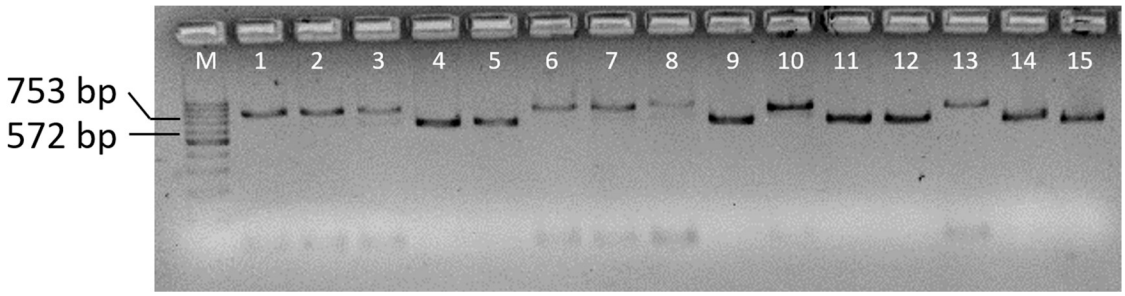


**Figure 4** – The results of the PCR for determination of the mating type of *Chrysoporthe austroafricana*. The upper fragments (753 bp) are amplicons of MAT1-1-3 gene, and the lower fragments (572 bp) are the amplicons of MAT1-2-1 gene. 1: CMW 56206, 2: CMW 56209, 3: CMW 56212, 4: CMW 56214, 5: CMW 56217, 6: CMW 56223, 7: CMW 56594, 8: CMW 56595, 9: CMW 56599, 10: CMW 56601, 11: CMW 56606, 12: CMW 56609, 13: CMW 56617, 14: CMW 56620, 15: CMW 56621, and M: fragment size markers.

***Pathogenicity tests***

After eight weeks, bark and cambial lesions were visible for all *C. henryi* stems inoculated with the selected test isolate. Bark lesion lengths were15.5 ± 4.2 (mean ± SD) mm in the first round of inoculations and 11.5 ± 3.6 mm in the repeat trial. For the control inoculations, most of the wounds had started to heal and were overgrown with callus. The cambial lesion length was 40.1 ± 13.6 mm in the first inoculation trial and 29.7 ± 8.6 mm in the repeat inoculation. Incubation of stem sections, including lesions associated with the *C. austroafricana* inoculations, resulted in yellow to orange mycelium on the lesion surfaces and the formation of fungal fruiting structures typical of *Chrysoporthe*. Examination of the fruiting bodies and spores showed the presence of hyaline, aseptate straight to slightly curved oblong conidia, 3–4 μm in length. No such structures were present for the control inoculations. These results confirmed that the inoculated *C. austroafricana* isolate, originally from dying *C. henryi* trees in the field, can cause disease in *C. henryi*, thus fulfilling Koch's postulates.

**Discussion**

The results of this study showed that the canker disease recently discovered on *C. henryi* is caused by *C. austroafricana*. This identification was based on phylogenetic analyses of DNA sequence data. A small number of isolates of the fungus was shown to include approximately equal numbers of the two MAT loci known for this pathogen. The samples also had a high level of genetic diversity. A pathogenicity test showed that *C. austroafricana* isolated from naturally infected trees is able to infect and cause cankers.

This is the first record of a canker disease caused by *C. austroafricana* on *Corymbia henryi* or any other species of *Corymbia.*

As a result of microsatellite analysis, the 11 MLGs of the 15 isolates were detected. The number of MLGs detected in the study was relatively high compared to those of *C. cubensis* collected from Kenya (two MLGs of the 10 isolates) or Malawi (nine MLGs of the 51 isolates), where *C. cubensis* has been accidentally introduced (Nakabonge *et al*., 2007). Although the accumulation curve using polymorphic primers failed to reach a plateau for the population, the results at least demonstrated that the population has a high gene diversity. Furthermore, a wide diversity of genotypes has undergone a host shift to infect *C. henryi*, which evidently has a high level of susceptibility to the pathogen.

Although the mating type distribution was almost equal in the *C. austroafricana* samples, only conidiomata of the fungus were found on *C. henryi*. This is similar to the situation on *Eucalyptus* in South Africa, where the sexual state of the fungus is seldom found on *Eucalyptus* (Wingfield, 2003). In contrast, these structures are common on branches and stem cankers on native *S. cordatum* Hochst. in the same area (Nakabonge *et al.*, 2006b). Considering the fact that both ascostromata and conidiomata of *C. austroafricana* are commonly observed on *Eucalyptus* and *Syzygium* spp. in Malawi, Mozambique, and Zambia (Nakabonge *et al.*, 2006b), the lack of sexual structures on *Corymbia* and *Eucalyptus* in South Africa is likely associated with a combination of host and environmental factors.

*Chrysoporthe austroafricana* first emerged in South African *Eucalyptus* plantations at the time when vegetative propagation of these trees was first pursued (Wingfield *et al.*, 1989). The disease became less common in subsequent years, largely due to intensive efforts made to select Chrysoporthe-tolerant planting stock for deployment (Van Heerden

*et al.*, 2005). The emergence of a serious new canker disease caused by *C. austroafricana* illustrates the importance of diseases where new species are selected for planting. In this case, *C. henryi* is being considered for larger-scale plantation development, particularly in the sub-tropical areas of South Africa. The results of this study provide evidence that canker caused by *C. austroafricana* may be a limiting factor if susceptible material is used for plantation development.

In comparison to *Eucalyptus* spp., species of *Corymbia* have not been widely deployed for plantation development outside the native range of these trees in Australia. Consequently, relatively little is known regarding the pest and disease threats to these trees, where they are intensively propagated as non-natives. It is likely that as these trees are more extensively planted, new disease problems, such as that observed in this study, will emerge. These could, for example, include those caused by the aggressive shoot and leaf pathogen *Quambalaria pitereka* (J. Walker & Bertus) J.A. Simpson that has damaged *Corymbia* plantations in Australia (Pegg *et al.*, 2009) and the rust fungus *Austropuccinia psidii* (G. Winter) Beenken, which is known to infect various species of *Corymbia* in South America (Rodas *et al.*, 2015; Zauza *et al.*, 2010). Breeding and selection of genotypes of *C. henryi* with tolerance to Cryphonectria canker will need to be implemented to limit the impact of this potentially serious new disease. At the same time, careful monitoring of pest and disease problems will clearly be required, including the application of relevant technologies (Wingfield *et al.*, 2008; Wingfield *et al.*, 2013) as *Corymbia* plantations expand in South Africa and elsewhere in the world.

**Conclusions**

A serious canker disease that has appeared in a South Africa plantation of the eucalypt

*Corymbia henryi* was identified as *C. austroafricana*, a pathogen well-known on *Eucalyptus* in the country. This diagnosis was based on the morphology of the fungus and phylogenetic inference. The isolates from *C. henryi* were shown to be genetically diverse and to have an approximately equal mating type distribution. The results suggest that *C. austroafricana* has undergone a host shift to infect *C. henryi*. As this tree species is considered for plantation development in South Africa and elsewhere, efforts will need to be made to avoid deployment of planting stock that might be susceptible to infection by the pathogen.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

**Data availability statement**

The data underlying this article will be shared upon reasonable request to the corresponding author.

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