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Notes on *Ceratocystis paradoxa* causing internal post-harvest rot disease on immature coconut in Brazil

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ABSTRACT

Currently there is a large and increasing demand for immature coconuts for fresh consumption of their liquid endosperm (coconut water). The occurrence of post-harvest diseases prevent its sale and change the taste, making it inappropriate for consumption. In 2011, coconut fruits showing internal post-harvest rot symptoms were found in a market in Belo Horizonte and after incubation for 5 days in a humid chamber at room temperature, fungal structures were observed. Fruiting structures show morphology typical of *Ceratocystis paradoxa*. For molecular identification, DNA sequences were generated for the Internal Transcribed Spacer regions 1 and 2 including the 5.8S rRNA gene, part of the β-tubulin and the Transcription Elongation Factor 1-α gene regions. These data were compared with those of other *C. paradoxa* using phylogenetic analysis. Koch's postulates was confirmed by inoculation of 6-mm-diameter PDA plugs with the isolate on fruits of coconut. Morphology of the isolates in culture as well as phylogenetic inference showed that the causal agent of internal post-harvest rot disease on coconut is *C. paradoxa*.
Key words: *Thielaviopsis paradoxa*, Hypocreomycetidae, Microascales, ophiostomatoid fungi, phylogeny, Sordariomycetes.

The coconut palm (*Cocos nucifera* L.) is a tropical crop cultivated in Asia, Oceania, Africa and Latin America continents (Siqueira et al., 2002). In most countries, the copra (dried coconut) and coconut oil are the major derivatives of commercial value in international trade. In Brazil, where the major products are coconut milk, desiccated coconut, coconut oil and coconut water (Mirisola Filho, 2002), there is a large and increasing demand for immature coconuts that are harvested and marketed for consumption of their liquid endosperm (coconut water) (Fontes et al., 2002). Therefore, the commercialization of healthy fruits is important because the post-harvest diseases causes fruit rot that prevent its sale and change the taste, making it inappropriate for consumption (Viana et al., 2002; 2007). In Brazil, coconut fruits have been reported to be susceptible to *Ceratocystis paradoxa* (Dade) C. Moreau, *Cylindrocladium floridanum* Sobers & C.P. Seym. and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. in the field and in post-harvest (Viana et al., 2002; Poltronieri et al., 2003; Halfeld-Vieira & Nechet, 2005; Mariano & Silveira, 2005).

In 2011, coconut fruits showing internal post-harvest rot symptoms were found in a market in the municipality of Belo Horizonte, state of Minas Gerais, Brazil. The fruits had been purchased healthy by the retailer, however, after 7-10 days the coconut water became inadequate for consumption and was associated with an internal rot (Figure 1 A-B). This is a previously unreported of post-harvest symptom on immature coconut. Therefore, the aim of this research was to determine the etiology of this post-harvest disease rot of *C. nucifera*.

Fruits were brought to the Departamento de Fitopatologia at the Universidade Federal de Viçosa and examined under a stereomicroscope Olympus SZX7. Fungal structures were observed only after incubation for 5 days in a humid chamber at room temperature. Some of those structures were scraped, mounted in drops of lactophenol on microscope slides and examined and photographed under a light microscope Olympus BX 51 equipped with a digital camera Olympus e-volt 330. Biometric data were based on 30 measurements of structures. A representative dried specimen was deposited in the local herbarium at the Universidade Federal de Viçosa (VIC 31891).

The fungus was isolated and a single-spore derived culture was obtained and grown on potato-dextrose-agar (PDA) at 25 °C for one week for DNA extraction. A duplicate single-spore derived culture was deposited at Coleção de Culturas de Fungos Fitopatogênicos "Prof. Maria Menezes" (UFRPE) code CMM 1739 and Coleção Micológica de Lavras (UFLA) code CML 2392. Approximately 40 mg of fungal tissue were scraped from the agar surface and placed in a sterile 1.5 mL microcentrifuge tube, which was processed by freezing with liquid nitrogen and grinded into a fine powder using a microcentrifuge tube pestle. The crushing continued with the addition of 100 µL of Nuclei Lysis Solution of

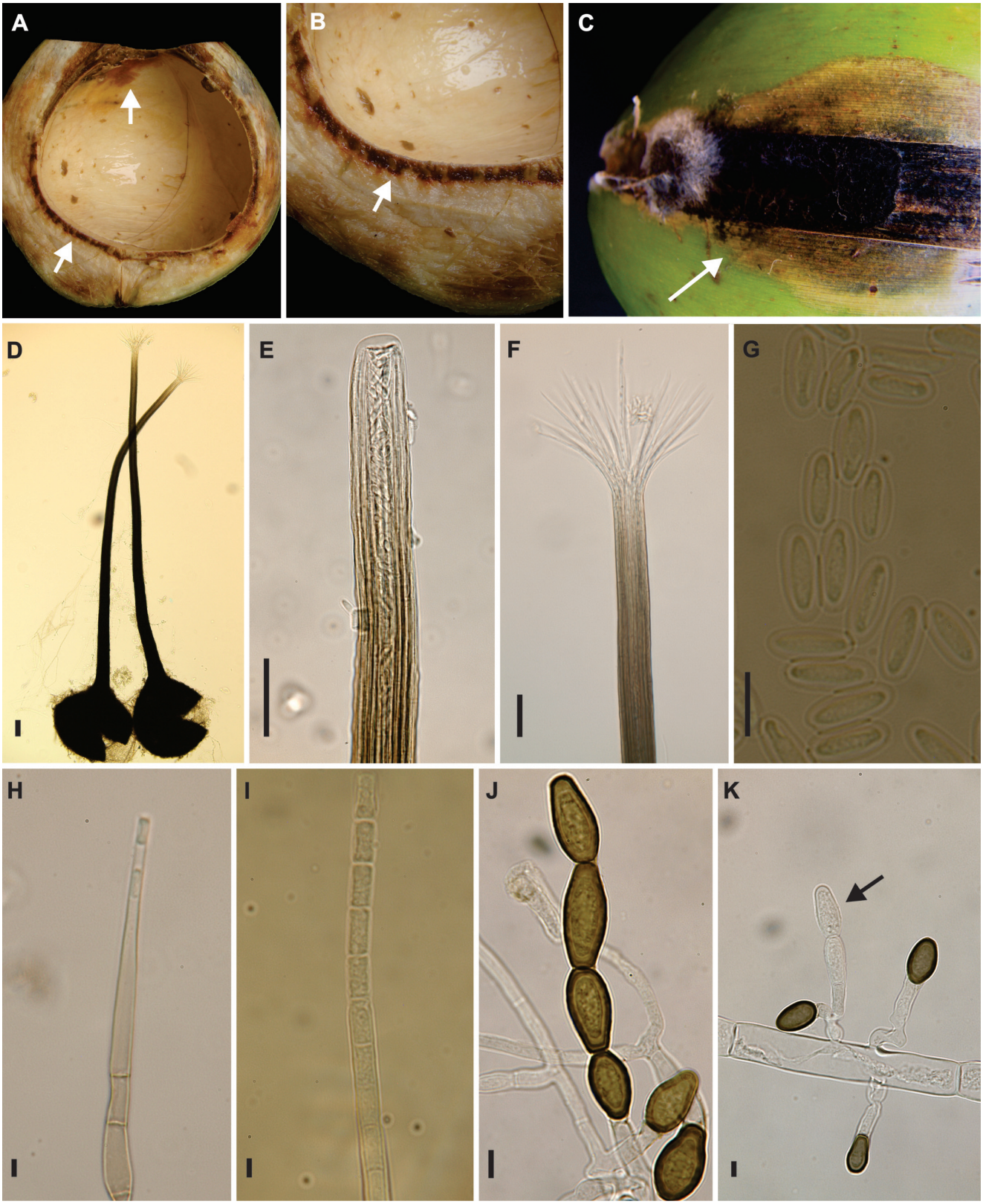

FIGURE 1 - *Ceratocystis paradoxa* strain CMM 1739 ex *Cocos nucifera*. **A.** Darkening of the exocarp, mesocarp and endocarp of coconut fruits from pedicel end and entire endocarp (arrows); **B.** Detail of darkening of the endocarp (arrow); **C.** Pale brown to black lesions around wounds created by removing a rectangular strip of exocarp previously to the inoculation; **D.** Perithecia; **E-F.** Detail of ostiolar hyphae; **G.** Ascospores; **H-I.** Conidiophores producing a succession of conidia through the open end; **J.** Aleurioconidia in chains; **K.** Mature and immature (arrow) aleurioconidia. Bars = 50 µm (D-F), 5 µm (G-K).

the Wizard® Genomic DNA Purification Kit (Promega). Afterwards, an additional 500 µL of the previous solution was added. The extraction continued as described by Pinho et al. (2012).

Polymerase chain reactions (PCR) were set-up using the following ingredients for each 25 µL reaction: 12.5 µL of Dream Taq TM PCR Master Mix 2X (MBI Fermentas), 1 µL of 10 µM of each forward and reverse primer synthesised by Invitrogen, 1 µL of dimethyl sulfoxide (DMSO, Sigma-Aldrich), 5 µL of 100× (10 mg/mL) bovine serum albumin (BSA, Sigma-Aldrich), 2 µL of genomic DNA (25 ng/µl), and nuclease-free water to complete the total volume.

Target regions of the Internal Transcribed Spacer regions 1 and 2 including the 5.8S rRNA gene (ITS), 28S rDNA, β-tubulin and Transcription Elongation Factor 1-α (TEF1-α) were amplified using primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') for ITS (White et al., 1990), LR0R (5'-ACC CGC TGA ACTT AAG C-3') and LR5 (5'-TCC TGA GGG AAA CTT CG-3') for partial 28S rDNA (Vilgalys & Hester, 1990), Bt1a (5'-TTC CCC CGT CTC CAC TTC TTC ATG-3') and Bt1b (5'-GAC GAG ATC GTT CAT GTT GAA CTC-3') for partial β-tubulin (Glass & Donaldson, 1995), EF1F (5'-TGC GGT GGT ATC GAC AAG CGT-3') and EF2R (5'-AGC ATG TTG TCG CCG TTG AAG-3') for partial TEF1-α (Jacobs et al., 2004). The thermal cycle consisted of 95ºC for 5 min, followed by 35 cycles of 94ºC for 1 min (denaturation), 58ºC for 1 min (for β-tubulin and TEF1-α) or 52ºC for 1 min (for ITS and 28S rDNA) (annealing), 72ºC for 2 min (elongation), and 72ºC for 10 min (final extension). PCR products were analyzed by 2% agarose electrophoresis gels stained with GelRed™ (Biotium Inc.) in a 1× TAE buffer (Sambrook et al., 1989) and visualized under UV light to check for amplification size and purity. PCR products were purified using ExoSAP-IT® (USB) following the manufacturer's instructions. Sequencing was performed by Macrogen Inc. (www.macrogen.com). The nucleotide sequences were edited with the DNA Dragon software (Hepperle, 2011). All sequences were checked manually and nucleotides with ambiguous positions were clarified using both primer direction sequences. The sequences obtained in this study were deposited in GenBank (www.ncbi.nlm.nih.gov/genbank) from where sequences of ITS, β-tubulin and TEF1-α of additional species were retrieved for comparison (Table 1).

Consensus sequences were compared against GenBank's database using their Mega BLAST program.

TABLE 1 - Genbank acession numbers of DNA sequences of *Ceratocystis*/*Thielaviopsis* used in the phylogenetic analysis

Species	Isolate	ITS	BT	EF
C. australis	CMW 2333	FJ411325	FJ411351	FJ411299
	CMW 2653	FJ411326	FJ411352	FJ411300
C. coerulescens	CMW 26364	FJ411321	FJ411347	FJ411295
	CMW 26365	FJ411322	FJ411348	FJ411296
	CMW 26366	FJ411320	FJ411346	FJ411294
C. eucalypti	CMW 3254	FJ411327	FJ411353	FJ411301
	CMW 4453	FJ411328	FJ411354	FJ411302
C. fagacearum	CMW 2039	FJ411344	FJ411370	FJ411318
	CMW 2658	FJ411345	FJ411371	FJ411319
C. fimbriata s. str.	CMW 15049	DQ520629	EF070442	EF070394
	CMW 1547	AF264904	EF070443	EF070395
*C. paradoxa***	CMW 8779	FJ411324	FJ411349	FJ411298
	CMW 8790	FJ411323	FJ411350	FJ411297
	CMM 1739	JQ963886	JQ963884	JQ963883
C. radicicola	CMW 1032	FJ411339	FJ411364	FJ411312
	CMW 6728	FJ411340	FJ411365	FJ411313
T. basicola	CMW 25439	FJ411334	FJ411360	FJ411308
	CMW 25440	FJ411335	FJ411361	FJ411309
T. neocaledoniae	CMW 3270	FJ411329	FJ411355	FJ411303
	CMW 26392	FJ411330	FJ411356	FJ411304
T. ovoidea	CMW 22733	FJ411343	FJ411369	FJ411317
T. populi	CMW 26387	FJ411336	FJ411362	FJ411310
	CMW 26388	FJ411337	FJ411363	FJ411311
T. thielavioides	CMW 22736	FJ411342	FJ411367	FJ411315
	CMW 22737	FJ411341	FJ411366	FJ411314

*CMW: Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. CMM: Coleção de Culturas de Fungos Fitopatogênicos "Prof. Maria Menezes" (UFRPE) Brazil. ** CMM 1739 is the isolate obtained in this study. CMW 8779 and 8790 are Indonesian isolates from coconut.

In addition, DNA sequences were selected from van Wyk et al. (2009) to confirm the identity of *Ceratocystis*. As ITS sequences have limited phylogenetic value for *Ceratocystis* species (Harrington et al., 2011), multigene analysis (ITS, β-tubulin and TEF1-α) were used to compare with isolates of coconut from Indonesia. The closest hit sequences were then downloaded in FASTA format and aligned using the multiple sequence alignment program MUSCLE (Edgar, 2004), built in MEGA v. 5 software (Tamura et al., 2011). Alignments were checked and manual adjustments were made when necessary. All the ambiguously aligned regions within dataset were excluded from the analyses. Gaps (insertions/deletions) were treated as missing data. The resulting alignment was deposited into TreeBASE (www.treebase.org) as accession number 12613.

Bayesian inference concatenated (BI) analyses employing a Markov Chain Monte Carlo method were performed with all sequences, first with each gene/locus separately, and then with the concatenated sequences (ITS, β-tubulin and TEF1-α). Before launching the BI, the best nucleotide substitution models was determided for each gene with MrMODELTEST 2.3 (Posada & Buckley, 2004). Once the likelihood scores were calculated, the models were selected according to the Akaike Information Criterion (AIC). The GTR+I+G model of evolution was used for ITS and β-tubulin and SYM+I+G was used for TEF1-α. The phylogenetic analysis of the concatenated alignment was performed on CIPRES webportal (Miller et al., 2010) using MrBayes v.3.1.1 (Ronquist & Huelsenbeck, 2003). In MrBayes, data were partitioned by locus and the parameters of the nucleotide substitution models for each partition were set as described above. Four MCMC chains were run simultaneously, starting from random trees for 10,000,000 generations. Trees were sampled every 1000th generation for a total of 10,000 trees. The first 2,500 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (Rannala & Yang, 1996) were determined from a majority-rule consensus tree generated with the remaining 7,500 trees. Trees were visualized in FigTree (Rambaut, 2009) and exported to graphics programs. The sequences of the species *Ceratocystis fimbriata* Ellis & Halst. strains CMW1547 and CMW15049 were used as outgroup in these analyses.

To check pathogenicity, a 6-mm-diameter plug of the isolate CMM 1739 (single-spore derived culture) was removed from the expanding edge of a 7-day-old culture grown on PDA and placed in contact onto wounds created by removing the calyx or exocarp. Six fruits were inoculated with the isolate and an fungus-free PDA plug was placed onto six fruits. The fruits were maintained in a moist chamber for one week.

The fungus isolated from diseased fruits showed white colonies on PDA, becoming black 1 to 2 days later, with a strong fruity aroma. Perithecia partly or completely immersed, dark brown or black, globose, 775-1803×150-275 µm, with a long neck, black, pale brown towards the tip, tapering, 575-1387×40-77.5 µm, ostiolar hyphae hyaline, erect or moderately divergent. Ascospores ellipsoid, often with unequally curved sides, hyaline, non-septate, smooth, 6-10×2-3 µm. Conidiophores slender, arising laterally from the hyphae, septate, phialidic, hyaline to very pale brown, 103-213×7.5-10 µm, tapering towards the tip and producing a succession of conidia through the open end. Conidia cylindrical to somewhat oval when mature, hyaline to mid-brown, smooth-walled, 6.5-10×3-5 µm. Aleurioconidia terminal, in chains, obovate to oval, thick-walled, brown, 9-24×6-10 µm (Figure 1 D-K).

Amplification of the regions ITS (GenBank Accession No. JQ963886), β-tubulin (GenBank Accession No. JQ963884) and TEF1-α (GenBank Accession No. JQ963883) revealed sequences of ca 490, 540 and 690 bp, respectively. The manually adjusted alignment contained 25 strains and, of all 1322 characters used in the phylogenetic analysis, 289 were parsimony-informative, 307 were variable and 1013 were conserved. Although the 28S rDNA sequences were not used in phylogenetic analyses, they were lodged in GenBank (JQ963885) for future studies and identification purposes.

On inoculated fruits, the symptoms of darkening of the exocarps, mesocarps, and then the endocarps of coconut fruits from pedicel end were observed five days after inoculation and were similar to those expressed in diseased fruits (Figure 1 C). The pathogen was reisolated from the inoculated fruits. All non inoculated fruits remained healthy.

The fungus found causing post-harvest rots on *C. nucifera* in Brazil matched well with the morphological description of *C. paradoxa* (Morgan-Jones, 1967). As no DNA sequences of this species collected in Brazil were available in Genbank to check whether the taxon belongs to *C. paradoxa* (Harrington, 2009), it was decided to identify this isolate based on multilocus phylogenetic tools (Cai et al., 2011a; 2011b; Hyde et al., 2010). Despite the fact that van Wyk et al. (2009) have not provided evidence that their identifications of cultures CMW 8779 and CMW 8790 were based on morphology or on type (ex-type), our study support its identification of *C. paradoxa* (Figure 2).

This species was also reported causing post-harvest diseases in banana, carambola, guava and pineapple (Reyes et al., 1998; Junqueira et al., 2001; Ploetz, 2003). This pathogen causes stem bleeding (Warwick & Passos, 2009; Freire & Martins, 2010) and fruit basal rot of the coconut at field conditions (Rossetti, 1955; Camargo & Gimenes-Fernandes, 1997; Tzeng & Sun, 2009; Tzeng et al., 2010) and external post-harvest disease on coconut (Tzeng & Sun, 2009; Tzeng et al., 2010). Thus, this is the first report of post-harvest internal rot disease on immature coconut caused by *C. paradoxa*. Recently, Lopes et al. (2012) reported the detection of *C. paradoxa* in sap of infected coconut palms. Maybe this is one way used by the fungus to entry inside the fruit with posterior manifestation of post-harvest internal rot, without apparent external symptom.

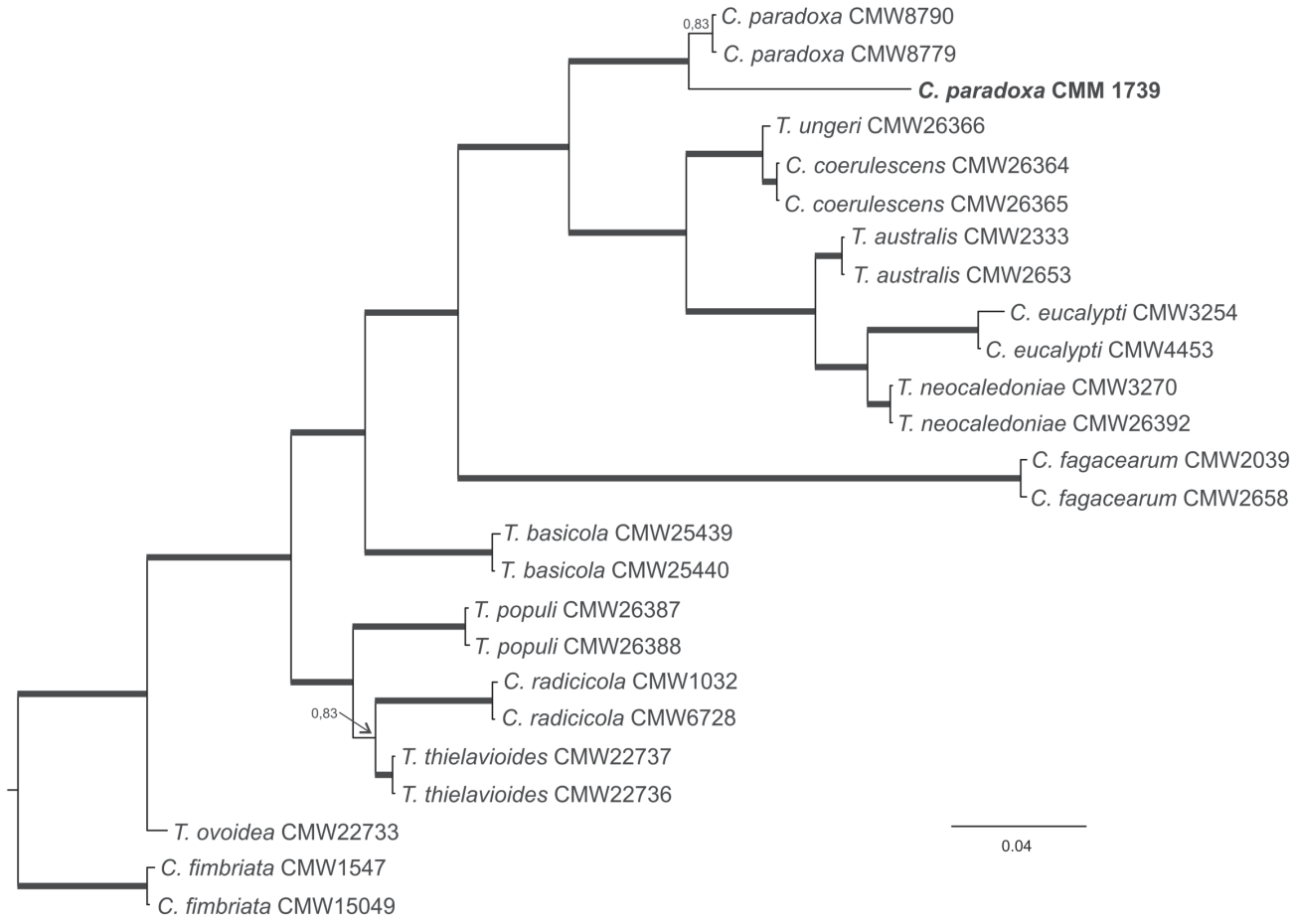

FIGURE 2 - Multilocus phylogenetic tree inferred from Bayesian analysis based on the combined regions of the ITS, β-tubulin and EF-1α. Bayesian posterior probability values are indicated at the nodes. Thickened lines indicate a posterior probability = 1.00. *Ceratocystis fimbriata s. str*. represents the outgroup taxon. The specimen used in this study is highlighted in bold.

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