A novel species of *Microsphaeropsis* causing cankers on *Rafnia amplexicaulis* in South Africa

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Abstract: Cankers leading to branch, stem and plant death were observed on the South African endemic *Rafnia amplexicaulis* (*Fabaceae*) in the Cederberg Wilderness Area, South Africa, during September 2021. Conidiomatal pycnidia were found developing on the cankers, and isolations consistently yielded a *Microsphaeropsis* species. Phylogenetic analysis based on partial nucleotide sequences of the internal transcribed spacers (ITS), the nuclear large subunit (LSU) and RNA polymerase II second largest subunit (*RPB2*) regions showed that the fungus represented an undescribed species. Based on the multigene phylogeny and morphological characteristics, we describe the species here as *M. rafniae sp. nov*. Pathogenicity tests and the fulfilment of Koch's postulates confirmed that *M. rafniae sp. nov*. is the cause of the cankers of *R. amplexicaulis*. Presently, this disease is known from a single location in South Africa, and further surveys are required to determine its distribution and relative importance.

INTRODUCTION

Rafnia amplexicaulis (*Fabaceae*) is a perennial, woody shrub endemic to the Northern and Western Cape Provinces of South Africa. As a resprouter, coppice shoots are produced from an underground lignotuber following fire, resulting in plants being multi-stemmed at ground level (Campbell & van Wyk 2001). The leaves and roots of *R. amplexicaulis* have been utilised as traditional remedies by the local Cape indigenous communities (Kinfe *et al.* 2015).

During a field visit to the Cederberg mountains in September 2021, yellowing and dying *R. amplexicaulis* shrubs were observed in a single location within the Cederberg Wilderness Area, Western Cape Province, South Africa. Closer inspection revealed girdling cankers present on the symptomatic stems. Fungal structures (pycnidia) characteristic of a *Microsphaeropsis* species were visible on the surface of the cankers.

Microsphaeropsis (*Didymellaceae*) was introduced by Von Höhnel (1917) to accommodate pycnidial fungi with small dark aseptate conidia produced from phialides. The genus has a cosmopolitan distribution, with species commonly described as plant endophytes or saprophytes. The genus also contains a number of plant pathogens described from necrotic spots and/ or lesions on leaves and twigs (Swart *et al.* 1998, Hou *et al.* 2020).

The aim of this study was to describe the disease occurring on *R. amplexicaulis* and identify its causal agent.

METHODS AND METHODS

Disease description and isolations

The diseased *R. amplexicaulis* plants were restricted to an area of approximately 1 000 m^2 on a south-east facing slope within the Cederberg Wilderness Area, Western Cape Province, South Africa (-32.412743, 19.174894). Shrubs were visibly yellowing, and on closer examination cankers were commonly found on symptomatic stems and branches. In instances where cankers were girdling, these led to stem and branch death (Fig. 1). Removal of the outer bark showed distinct necrosis of the cambium at the leading edges of the lesions. Sections of symptomatic stems were removed from plants, placed in brown paper bags and transported to the laboratory for further examination.

Conidiomatal pycnidia that oozed conidial masses typical of *Microsphaeropsis* species were observed on the surfaces of the cankers. Conidia were lifted from the pycnidia using a sterile hypodermic needle and transferred to the surface of 2 % malt

Fig. 1. *Rafnia amplexicaulis* in the field. **A.** Population of *Rafnia amplexicaulis* in the Cedarberg Wilderness Area, Western Cape Province, South Africa. **B.** Symptomatic stems.

extract agar (MEA: 20 g Biolab malt extract, 20 g Difco agar, 1 L deionised water) amended with 1 % streptomycin sulphate (Sigma-Aldrich). Cultures were purified by transferring single hyphal tips to fresh MEA plates and maintained at 25 °C.

The resulting cultures were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Lunnon Road, Pretoria, South Africa. The holotype and ex-holotype were deposited in the H.G.W.J. Schweickerdt Herbarium (PRU) and the culture collection of Innovation Africa (CMW-IA), respectively, at the University of Pretoria, Lunnon Road, Pretoria, South Africa.

Morphology

The top part of pycnidial structures were cut with a scalpel. The exposed insides of the structures were moistened with a piece of agar, and conidiogenous cells and conidia were extracted and mounted on slides in water. For measurements, the water was replaced with 85 % lactic acid. Images were captured using Nikon microscopes (Eclipse Ni and SMZ18, Japan) mounted with a DS-Ri2 camera. The image program NIS-Elements BR was used for measurements and taking photos. Bark samples containing pycnidia were cut into small pieces. The pieces were boiled for a few seconds to soften the structures and mounted on a disc with freezing medium. Vertical sections were prepared in 10–12 µm thickness using a cryomicrotome (Leica CM1520, Germany). The sections were mounted in 85 % lactic acid for observation. Fifty conidia were measured and presented as min–max (average ± standard deviation), whereas less than ten structures were measured for conidiogenous cells and conidiomata and presented as min–max, due to the shortage of samples.

Isolate CMW 57792 was used for the growth study and colony morphology. Culture characteristics and growth rates were determined on MEA, potato dextrose agar (PDA; BD Difco) and oatmeal agar (OA; liquid extract of 30 g of oats cooked in 800 mL water for an hour used to make 1 L, 20 g Difco agar). Colours were described using the colour chart of Rayner (1970).

Cultures were grown at seven temperatures, ranging from 5 to 35 °C in 5 °C intervals. At each temperature, five replicates of the isolate were incubated in the dark. Diameters perpendicular to each other were measured after 10 d, when the colony margins reached the edges of the Petri-dishes at optimum temperature. After measuring the diameters, the plates were returned to the incubators for an additional few weeks to observe possible changes with age. An NaOH spot test was performed on a culture grown on OA (Boerema *et al*. 2004).

DNA isolation, PCR amplification and sequencing

DNA was extracted from 7-d-old isolates grown on 2 % MEA at 25 °C using Prepman® Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocols. The nuclear internal transcribed spacer regions 1 and 2 (ITS), including the 5.8S rRNA gene region, were amplified using primers ITS1F and ITS4 (White *et al*. 1990, Gardes & Bruns 1993); part of the nuclear large subunit (LSU) of ribosomal RNA gene with primers LR0R and LR5 (Vilgalys & Hester 1990, Rehner & Samuels 1994) and a fragment of the DNA-directed RNA polymerase II second largest subunit gene (*RPB2*) with primer pair RPB2-5F2 and fRBP2-7cR (Liu *et al*. 1999; Sung *et al*. 2007). PCR amplifications were prepared following the protocols described by Pham *et al*. (2019). For ITS and LSU regions, the thermal cycling included an initial denaturation at 95 °C for 5 min followed by 10 primary amplification cycles of 30 s at 95 °C, 30 s at 56 °C, and 60 s at 72 °C, then 30 additional cycles of the same reaction sequence, with a 5 s increase in the annealing step per cycle, and the reactions were completed with a final extension at 72 °C for 10 min. The amplification for *RPB2* was performed following the method of Liu *et al*. (2020). Amplified fragments of all loci were purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Amplicons were sequenced in both directions using an ABI PRISM™ 3100 DNA sequencer (Applied Biosystems, USA) at the Sequencing Facility

Table 1. Collection details and GenBank accessions of isolates included in the phylogenetic analyses.

Species	Isolates	Substrate	Locality	GenBank accessions			References
				RPB2	LSU	ITS	
Microsphaeropsis fusca	CBS 116669	*Sarothamnus scoparius*	The Netherlands	MT018219	EU754170	MN973572	Hou *et al.* (2020)
	CBS 116670^T	*Sarothamnus scoparius*	The Netherlands	MT018220	MN943779	MN973573	Hou *et al.* (2020)
	CBS 139603; MFLUCC 14-0507	*Sarothamnus scoparius*	Germany	MT018218	MN943778	MN973571	Hou *et al.* (2020)
Microsphaeropsis olivacea	CBS 233.77	*Pinus laricio*	France	MT018217	GU237988	GU237803	Hou *et al.* (2020)
	CBS 320.76	*Cronartium ribicola*	France	MT018214	MN943775	MN973568	Hou *et al.* (2020)
	CBS 617.83	*Pinus sylvestris*	Switzerland	MT018213	MN943774	MN973567	Hou *et al.* (2020)
	CBS 608.72	*Tremella foliacea*	The Netherlands	MT018216	MN943777	MN973570	Hou *et al.* (2020)
	CBS 336.78	*Picea abies*	Germany	MT018215	MN943776	MN973569	Hou *et al.* (2020)
Microsphaeropsis proteae	CBS 113685; UPSC 1926	*Dryas octopetala*	Sweden	MT018262	MN943800	MN973593	Hou *et al.* (2020)
	CBS 111303^T; CPC 1423	*Protea nitida*	N/A	MT018221	JN712561	JN712495	Hou *et al.* (2020)
	CBS 111319; CPC 1425	*Protea nitida*	N/A	MT018223	JN712563	JN712497	Hou *et al.* (2020)
	CBS 111320; CPC 1424	*Protea nitida*	N/A	MT018222	JN712562	JN712496	Hou *et al.* (2020)
Microsphaeropsis rafniae	**CMW 57792^T**	*Rafnia amplexicaulis*	South Africa	**OR211858**	**OR209716**	**OR209698**	This study
	CMW 57793	*Rafnia amplexicaulis*	South Africa	**OR211859**	**OR209717**	**OR209699**	This study
	CMW 57794	*Rafnia amplexicaulis*	South Africa	**OR211861**	**OR209718**	**OR209700**	This study
	CMW 57795	*Rafnia amplexicaulis*	South Africa	**OR211862**	**OR209719**	**OR209701**	This study
Microsphaeropsis taxicola	CBS 469.80	*Rhus typhina*	The Netherlands	MT018210	MN943772	MN973565	Hou *et al.* (2020)
	CBS 442.83	*Taxus baccata*	The Netherlands	MT018211	EU754171	GU237865	Hou *et al.* (2020)
	CBS 427.92	*Opuntia cladodes*	The Netherlands	MT018212	MN943773	MN973566	Hou *et al.* (2020)
Microsphaeropsis viridis	CBS 763.73	*Populus tremula*	France	MT018205	MN943768	MN973561	Hou *et al.* (2020)
	CBS 762.73	*Pseudotsuga menziesii*	France	MT018206	MN943769	MN973562	Hou *et al.* (2020)
	CBS 354.69	*Berberis* sp.	The Netherlands	MT018207	MN943770	MN973563	Hou *et al.* (2020)
	CBS 432.71^T	*Sarothamnus* sp.	The Netherlands	MT018209	GU237987	GU237863	Hou *et al.* (2020)
	CBS 639.80	*Abies alba*	Germany	MT018208	MN943771	MN973564	Hou *et al.* (2020)
Neomicrosphaeropsis alhagi-pseudalhagi	MFLUCC 17-0825^T	*Alhagi maurorum*	Uzbekistan	MH069682	MH069670	MH069664	Hou *et al.* (2020)
Neomicrosphaeropsis elaeagni	MFLUCC 17-0740^T	*Elaeagnus angustifolia*	Russia	MH069684	MH069672	MH069666	Hou *et al.* (2020)
Neomicrosphaeropsis italica	MFLUCC 15-0484	*Tamarix* sp.	Italy	KU695539	KU729853	KU900319	Hou *et al.* (2020)
	MFLUCC 16-0284	*Tamarix* sp.	Italy	KU714604	KU900296	KU900321	Hou *et al.* (2020)
Nothomicrosphaeropsis welwitschiae	CBS 146829^T; CPC 38879	*Welwitschia mirabilis*	Namibia	MW890067	MW883826	MW883434	Crous *et al.* (2021)
Paramicrosphaeropsis ellipsoidea	CBS 197.97	*Quercus ilex*	Spain	MT018224	MN943780	MN973574	Hou *et al.* (2020)
	CBS 194.97^T	*Quercus ilex*	Spain	MT018225	MN943781	MN973575	Hou *et al.* (2020)
Calophoma parvula	CBS 620.68	*Aegopodium podagraria*	The Netherlands	MT018234	MN943783	MN973577	Hou *et al.* (2020)

CBS = culture collection of Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; CMW = culture collection of the FABI, University of Pretoria, Pretoria, South Africa; CMW-IA = culture collection of Innovation Africa, University of Pretoria, Pretoria, South Africa; CPC = culture collection of Pedro Crous, housed at Westerdijk Fungal Biodiversity Institute; MFLUCC = Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; UPSC: Uppsala University Culture Collection, Sweden.

ITS = internal transcribed spacer regions 1 & 2 including the 5.8S region of the nrRNA; LSU = 28S large subunit of the nrRNA; RPB2 = DNA-directed RNA polymerase II second largest subunit gene.

^T Denotes ex-type strain.

Isolates and sequences obtained in this study are indicated in **bold**.

of the Faculty of Natural and Agricultural Sciences, University of Pretoria, South Africa. Geneious Prime v. 2022.1.1 was used for assembling and editing raw sequences (https://www.geneious.com). All sequences generated in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov) (Table 1).

Phylogenetic analyses

Reference sequences for species closely related to those emerging from this study were downloaded from the GenBank nucleotide database (Table 1). All sequences were aligned using MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server/) (Katoh & Standley 2013), then confirmed manually in MEGA v. 7 (Kumar *et al*. 2016) where necessary. Maximum likelihood (ML) and Bayesian inference (BI) analyses were performed on the combined dataset of three regions. The most appropriate model was obtained using the software jModeltest v. 1.2.5 (Posada 2008). For ML, analyses were conducted using RAxML v. 8.2.4 on the CIPRES Science Gateway v. 3.3 (Stamatakis 2014) with default GTR substitution matrix and 1 000 rapid bootstraps. For BI, analyses were performed using MrBayes v. 3.2.6 (Ronquist *et al.* 2012) on the CIPRES Science Gateway v. 3.3. Four Markov chain Monte Carlo (MCMC) chains were run from a random starting tree for five million generations and trees were sampled every 100th generation. The first 25 % of trees sampled were eliminated as burn-in and the remaining trees were used to determine the posterior probabilities. *Calophoma parvula* (CBS 620.68) was used as the outgroup taxon. Resulting trees were viewed using MEGA v. 7 (Kumar *et al*. 2016) and FigTree v. 1.4.3 (Rambaut 2010).

Pathogenicity tests

The pathogenicity of the isolated *Microsphaeropsis* species towards *R. amplexicaulis* was determined in a natural population of the host plant in the Cedarberg Wilderness Area, Western Cape Province, South Africa (-32.42956; 19.15981). Inoculations were initiated during spring (October) of 2021 using two isolates (CMW 57792 and CMW 57793).

Thirty plants were randomly chosen for inoculation, distributed in an area of approximately 1 ha. A 7-mm-diam cork borer was used to remove the bark and expose the cambium on a single branch (2–3 cm diam) per plant. Similar sized discs, taken from the actively growing margins of 2-wk-old cultures on PDA, were inserted into these wounds with the mycelial growth facing the xylem (n = 10 branches per isolate). Each branch received only one inoculation. An additional 10 branches were inoculated with an agar-only control. Wounds were covered with Parafilm™ (Amcor, Zürich, Switzerland) to prevent desiccation and contamination by other organisms.

After 7 wk, inoculated branches were harvested by removal at the base of the main stem, and transferred in brown paper bags to the laboratory for assessment. Leaves were removed from the branches, and the length of the lesions (mm) was determined by removing the bark around each inoculation point with a sterile scalpel and measuring the length of the longest distance of the stained portion of the vascular tissues using digital calipers.

Isolations were made from stained portions of vascular tissues of all inoculated branches including control treatments, to determine whether *Microsphaeropsis* was the causal agent for lesion development. Branch sections containing lesions were surface sterilised with 70 % ethanol for 1 min, after which approximately 5 mm³ sections from the leading edges of lesions were plated onto ½ PDA. These were incubated in the dark at 25 °C for approximately 7–10 d and resulting fungal cultures were morphologically evaluated to confirm identity.

Lesion length data were normally distributed after implementing a Shapiro-Wilks test (W = 0.931, p = 0.053) using R Software v. 3.6.3 (https://www.rstudio.com). The influence of treatment (different isolates and controls) on lesion length data was thereafter tested using a linear model (*lm*) using base R. Significant main effects were separated using a conservative Tukey post-hoc test in the multcomp package in R (Hothorn *et al*. 2008). A probability level of 5 % was considered significant.

Permitting

Permission to collect samples was provided by the Western Cape Nature Conservation Board. Collections were made under permit number CN44-87-16977.

RESULTS

Disease description and pathogen identification

A *Microsphaeropsis* species was consistently found sporulating on cankered *R. amplexicaulis* stems. Four isolates (CMW 57792, CMW 57793, CMW 57794 and CMW 57795), originating from separate shrubs, were purified and used for further morphological study and molecular identification. Two isolates (CMW 57792 and CMW 57793) were used in the pathogenicity trials.

Phylogenetic analyses

Amplicons of approximately 520 bp were generated for the ITS region, 900 bp for the LSU, and 880 bp for the *RPB2.* The concatenated aligned dataset consisted of 31 ingroup taxa and 1 835 characters, including alignment gaps. Based on the results of jModeltest, a TrNef+G model was selected for ITS, the TPM1uf for LSU and the TIM3+I for *RPB2,* and these models were applied to individual loci in the concatenated dataset for the BI analyses. ML and BI analyses resulted in phylogenetic trees with concordant topologies and showed similar phylogenetic relationships between taxa. The ML tree with bootstrap support values, and the posterior probabilities obtained from BI, is presented in Fig. 2. The four isolates considered in this study were identical and clustered in a well-supported clade (ML/BI = 100/1.00), clearly distinct from the most closely related species, *Microsphaeropsis proteae,* and thus represent a novel taxon.

Taxonomy

Microsphaeropsis rafniae M.J. Wingf., N.Q. Pham & Marinc., ***sp. nov***. MycoBank MB 849562. Fig. 3.

Etymology: Name refers to *Rafnia*, the host genus on which it occurs.

Diagnosis: Similar to *M. proteae* (conidia 5–8 × 3.5–4 µm *in vivo*) but differs in its smaller conidial dimensions (3–5 × 2–3 µm *in vivo*).
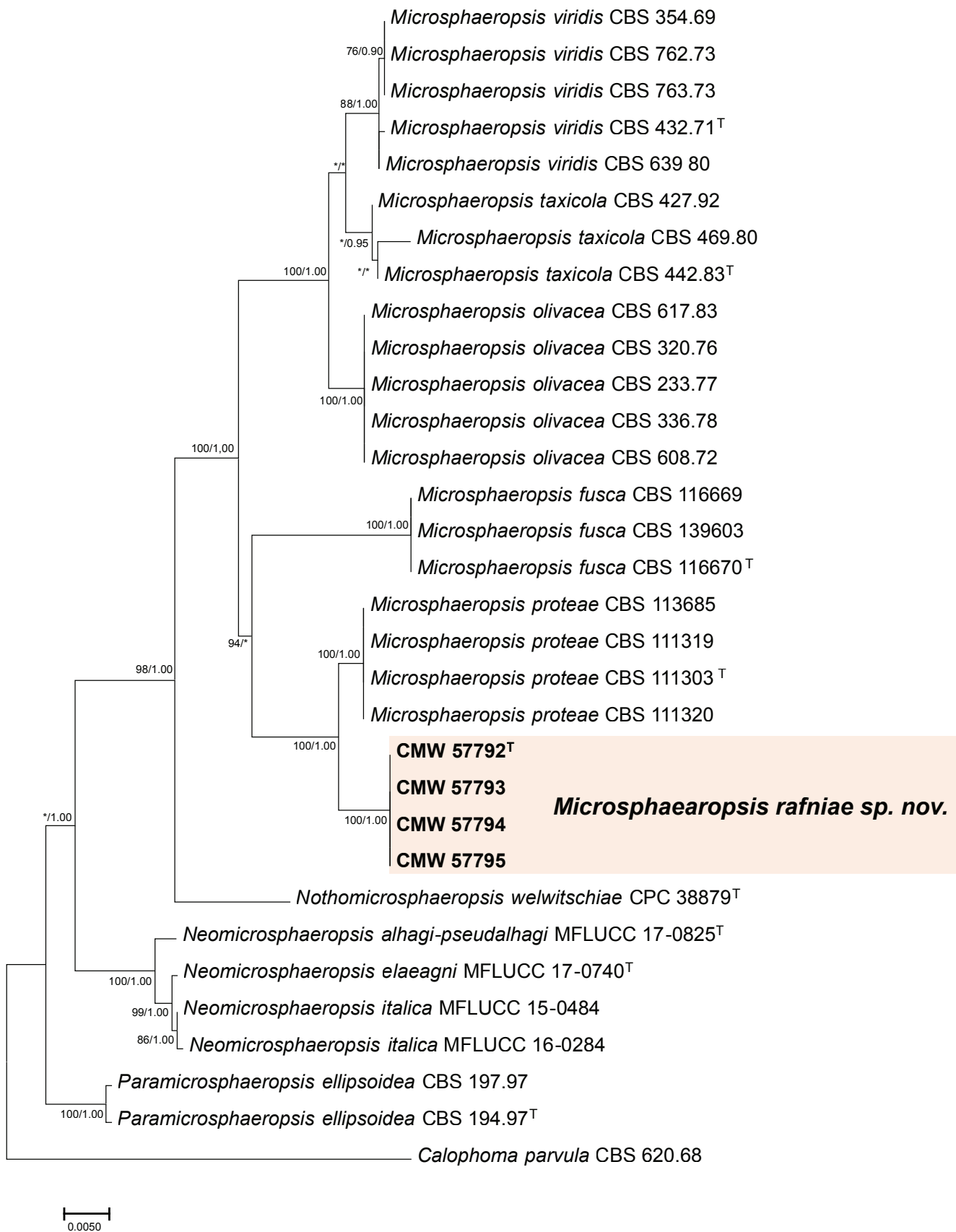
ITS+LSU+*RPB2*

Fig. 2. Phylogenetic tree based on a Maximum Likelihood (ML) analysis of a combined DNA data set of ITS, LSU and *RPB2* sequences representing *Microsphaeropsis* spp. and closely related groups in *Didymellaceae*. Isolates sequenced in this study are presented in bold face. Bootstrap values ≥ 70 % for ML analyses and posterior probabilities values ≥ 0.90 obtained from Bayesian inference (BI) are indicated at the nodes as ML/BI. Isolates representing ex-type cultures are marked with a "T". *Calophoma parvula* (CBS 620.68) represents the outgroup taxon.
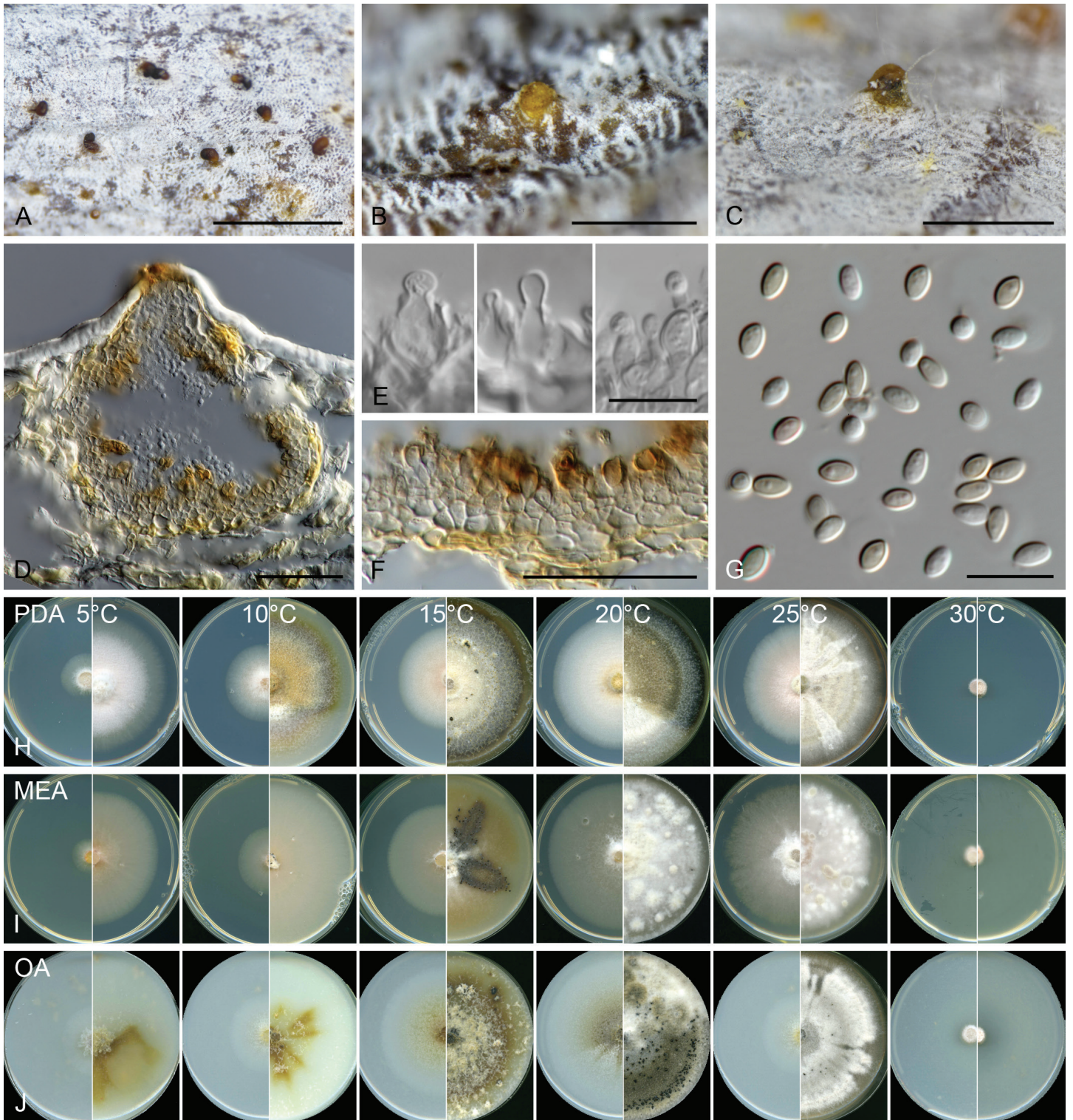
Fig. 3. Micrographs and culture characteristics of *Microsphaeropsis rafniae sp. nov.* (holotype: PRU(M) 4551, ex-holotype CMW-IA 52, CMW 57792). **A.** Conidiomata immersed in the substrate. **B, C.** Conidioma with protruding ostiole and conidial mass on the tip. **D.** Vertical section of conidioma in the substrate. **E.** Conidiogenous cells showing periclinal thickening (left) or percurrent proliferation (middle). **F.** Conidiomatal wall. **G.** Conidia. **H–J.** Colonies of the ex-holotype grown at six temperatures after 10 d (left) /30 d (right) in the dark on PDA (H), MEA (I) and OA (J).

Typus: **South Africa**, Western Cape Province, West Coast District, Cederberg Mountains, *Rafnia amplexicaulis*, 4 Sep. 2021, *M.J. Wingfield* (**holotype** PRU(M) 4552; ex-holotype culture CMW-IA 52, CMW 57792), [GenBank: OR209698 (ITS), OR209716 (LSU), OR211858 (*RPB2*)].

Description: *Sexual morph* not observed. *Conidiomata* in substrate pycnidial, solitary, scattered, immersed becoming erumpent, subglobose, 159 × 149 µm; ostiole inconspicuous;

pycnidial wall pseudoparenchymatous, consisting of 3–4 layers of compressed cells, outer layers pale brown. *Conidiophores* reduced to conidiogenous cells, formed along pycinidial cavity. *Conidiogenous cells* phialidic, hyaline, ampulliform to lageniform, 4–8 × 3–4 µm, showing periclinal thickening or percurrent proliferation. *Conidia* oozing out in yellow mass, becoming brown droplets at tip of ostiole, ellipsoidal, ovoid, rarely pyriform, hyaline, thin-walled at beginning, becoming sub-

hyaline to pale brown, thick-walled with age, smooth, aseptate, 3–5 × 2–3 (4.2 ± 0.35 × 2.4 ± 0.14) μm *in vivo*, 3–6 × 2–3 (4.2 ± 0.45 × 2.4 ± 0.27) μm *in vitro*, guttulate.

Culture characters: Colonies on PDA in the dark for 10 d, optimum growth temperature at 20 °C reaching 68.6 mm, followed by 25 °C (61.3 mm), 15 °C (52.1 mm), 5 °C (17.1 mm), 30 °C (10.5 mm) and 35 °C (no growth); on MEA optimum growth temperature at 20 °C reaching 71.5 mm, followed by 25 °C (65.3 mm), 15 °C (52.6 mm), 5 °C (18.6 mm), 30 °C (12 mm) and 35 °C (no growth). Colonies on PDA, MEA and OA showing circular growth with smooth margins, with superficial, flat and medium dense mycelia, fertile in 30 d, morphology homogeneous in 10 d, becoming diverse with age, having streaks or patches of white aerial mycelia. Colony colours on PDA and MEA in 10 d above peach (7d) to sienna (13i), fading towards edges, in 30 d sienna, umber (13m) to olivaceous grey (21''''''i) at higher temperatures, covered with white aerial mycelia partly or in patches. Colony colours on OA in 10 d colourless with tint of citrin (21k) near centre, in 30 d greenish olivaceous (23'''i) streaks to olivaceous gray with white aerial mycelial patches or streaks and black fruiting structure.

Distribution: South Africa, Western Cape Province, West Coast District, Cederberg Wilderness Area.

Additional specimens examined: **South Africa**, Western Cape Province, West Coast District, Cederberg Wilderness Area, -32.412743, 19.174894, on stems and branches of *Rafnia amplexicaulis*, 4 Sep. 2021, *M.J. Wingfield,* PRU(M) 4552, culture CMW-IA 53, CMW 57793 [GenBank: OR209699 (ITS), OR209717 (LSU), OR211859 (*RPB2*)]; PRU(M) 4553, culture CMW-IA 54, CMW 57794 [GenBank: OR209700 (ITS), OR209718 (LSU), OR211861 (*RPB2*)]; PRU(M) 4554, culture CMW 57795 [GenBank: OR209701 (ITS), OR209719 (LSU), OR211862 (*RPB2*)].

Notes: *Microsphaeropsis rafniae* is phylogenetically close to *M. proteae* and *M. fusca*. However, it can be distinguished from those species by its smaller conidia (3–5 × 2–3 μm *in vivo*); *M. fusca* (5–10.5 × 3.5–6.5 μm *in vitro*) (Hou *et al.* 2020) and *M. proteae* (5–8 × 3.5–4 μm *in vivo*) (Swart *et al.* 1998, Crous *et al.* 2011). *Microsphaeropsis fusca* was originally isolated from twig lesions of *Sarothamnus scoparius* (*Fabaceae*) in the Netherlands, and *M. proteae* from leaves of *Protea nitida* (*Proteaceae*) in Hermanus, Western Cape Province, South Africa (Swart *et al.* 1998, Hou *et al.* 2020). The NaOH spot test was positive on OA, turning from pale luteous to sienna colour, whereas *M. fusca* was reported as negative (Hou *et al.* 2020). There is no record of a spot test for *M. proteae*. The species showed strong growth at lower temperatures (5–25 °C). Cultures at 35 °C failed to grow when plates were returned to an optimum temperature (20 °C) and incubated for another 10 d, indicating this temperature results in death.

Pathogenicity tests: After 7 wk of incubation, lesions were evident around the inoculation points under the bark of the hosts. These consisted of dark brown to reddish-brown vascular staining in the form of streaks. Controls showed similar staining, but this did not extend far from the inoculation points. *Microsphaeropsis rafniae* was consistently isolated from the stained areas of the treatments but was never recovered from the controls. Treatment had a significant effect on lesion length (F = 35.83, DF = 2, 27, p < 0.001). Post-hoc analyses revealed that lesions caused by the two isolates were significantly longer than those caused by the controls, but lesion length did not differ between the two test isolates (Fig. 4).

DISCUSSION

A previously unreported canker disease was observed on stems and branches of *R. amplexicaulis* in the Cederberg Wilderness Area, Western Cape, South Africa. *Microsphaeropsis* isolates were recovered from these cankers and identified based on multi-locus (ITS, LSU and *RPB2*) phylogenetic analyses and
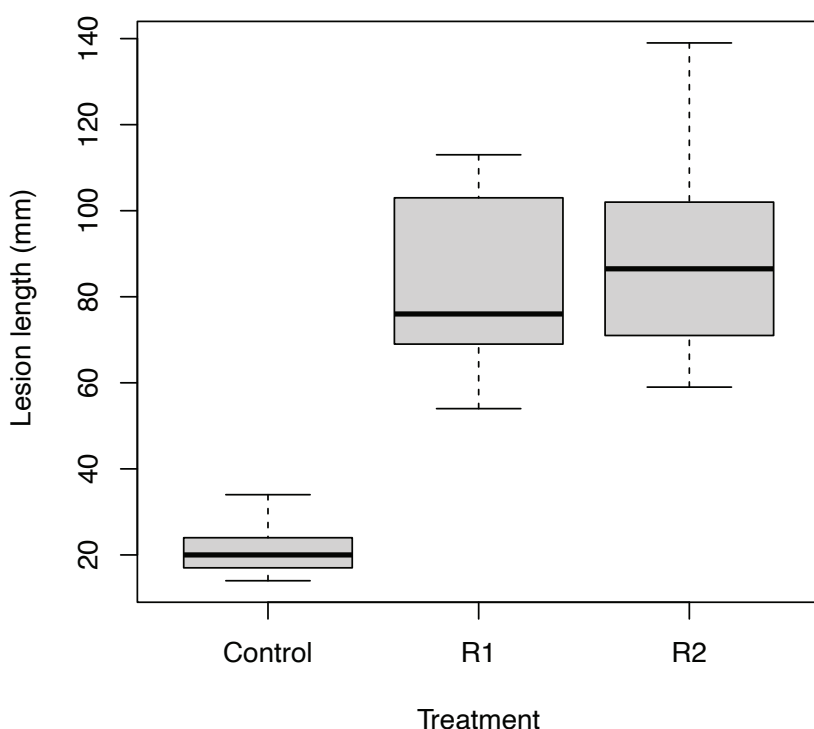

Fig. 4. Lesion length of two isolates of *Microsphaeropsis rafniae sp. nov.* (R1: CMW 57792, R2: CMW 57793) inoculated into *Rafnia amplexicaulis* branches after 7 w of incubation. Boxes indicating a 25–75 % data range, whiskers indicating a 1.5 × interquartile range.

morphological characters. These analyses revealed the isolates represented a novel species, described here as *M. rafniae*. Pathogenicity tests confirmed that *M. rafniae* is a canker pathogen of *R. amplexicaulis*.

The genus *Microsphaeropsis* was originally placed in *Montagnulaceae*, a family established to accommodate species with pigmented, phoma-like conidia. De Gruyter *et al.* (2009) proposed the family *Didymellaceae* to accommodate species in *Phoma* s. *l.* and related genera, including *Microsphaeropsis*. A new family, *Microsphaeropsidaceae*, was introduced to accommodate the genus by Chen *et al.* (2015). However, Hou *et al.* (2020) found that *Microsphaeropsis* spp. clearly reside in the *Didymellaceae* and reduced *Microsphaeropsidaceae* to synonymy, with *Microsphaeropsis* again placed in *Didymellaceae*.

Microsphaeropsis spp. have been reported from a wide variety of plant hosts, including as pathogens causing twig lesions (Hou *et al.* 2020) and leaf spots (Swart *et al.* 1998). This study characterised a novel pathogenic *Microsphaeropsis* species, *M. rafniae,* causing cankers on *R. amplexicaulis*, an endemic South African shrub. *Microsphaeropsis rafniae* is phylogenetically closest to *M. proteae*, also described from an endemic South African plant, *Protea nitida* (Swart *et al.* 1998). The two species are, however, phylogenetically distinct, with all isolates of *M. proteae* clustered in a well-supported clade. While morphologically similar, the two species can be distinguished based on their conidial size. Currently, neither the distribution range nor epidemiology of *M. rafniae* are known, with further studies required to better understand the role of this fungus as a disease-causing agent of *R. amplexicaulis.*

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Conflict of interest: The authors declare that there is no conflict of interest.

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