# *Caliciopsis pleomorpha sp. nov.* (*Ascomycota: Coryneliales*) causing a severe canker disease of *Eucalyptus cladocalyx* and other eucalypt species in Australia

I.G. Pascoe[1*], P.A. McGee (Maher)[2], I.W. Smith[3], S.-Q. Dinh[4], J. Edwards[4]

[1]30 Beach Road, Rhyll, Victoria 3923, Australia
[2]RMIT University, GPO Box 2476, Melbourne VIC 3001, Australia
[3]Bushbury Forest Pathology Service, 31 Old Monbulk Road, Belgrave, Victoria 3160, Australia
[4]Agriculture Victoria Research, AgriBio, Centre for AgriBioscience, 5 Ring Road, La Trobe University, Bundoora, Victoria 3083, Australia

*Corresponding author: pascoeig@bigpond.net.au

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one new taxon
pathogenicity
stem canker
taxonomy

**Abstract:** *Caliciopsis pleomorpha sp. nov.* is described from a severe stem canker disease of cultivated *Eucalyptus cladocalyx* 'Nana' (dwarf sugar gum) in Australia. The fungus is a pleomorphic ascomycete (*Coryneliales*), with pycnidial (pleurophoma-like) and hyphomycetous (phaeoacremonium-like) morphs, and differs in these respects and in ITS sequences from other *Caliciopsis* spp. The fungus was also found associated with cankers on other *Eucalyptus* species growing in native habitats, and was successfully inoculated under glasshouse conditions into a wide range of *Eucalyptus* species on which it caused cankers of varying severity.

## INTRODUCTION

In 1979, a stem canker disease was observed on *Eucalyptus cladocalyx*, at Frankston, Victoria, Australia. Cankers were first seen on *E. cladocalyx* 'Nana', a dwarf selection of *E. cladocalyx* that is widely grown as a shelter-belt and street tree. Subsequently the disease was found to be extremely damaging on *E. cladocalyx* 'Nana', in urban amenity trees and rural shelter-belts in many localities, in some cases virtually destroying shelter-belt plantings on farms.

A fungus consistently found sporulating on the cankers was identified as a species of *Caliciopsis* (*Ascomycota, Coryneliales*). Comparison of the fungus with other species of the genus *Caliciopsis* showed the fungus to be a new species. Its morphology and taxonomy, the disease symptoms caused and experiments on pathogenicity, host range and control are described in detail in the MSc thesis of Maher (1993).

The present paper describes the symptoms of the disease (Fig 1), the morphology of the various morphs of the fungus (Figs 2–7) both on host tissue and in culture, investigates the phylogeny of the fungus using ITS sequencing (Fig. 8), formally describes the fungus as a new species, *Caliciopsis pleomorpha*, and documents its pathogenicity to *E. cladocalyx 'Nana'* and 14 other taxa of *Eucalyptus* (Fig. 9) while listing 9 Eucalyptus taxa and 2 Corymbia taxa on which the fungus has been recorded from field collections in 4 Australian states.

## MATERIALS AND METHODS

### Microscopic observations

Ascomata and pycnidia were rehydrated with a drop of water for ease of manipulation, and observed by hand sectioning or crushing. Specimens were mounted in water, a solution of 50 % lactic acid/50 % glycerol or a lactic acid/glycerol solution with acid fuchsin added, and examined under the compound microscope. Slides were sealed with nail varnish for long-term storage and for observations using oil immersion magnification. Drawings were prepared using a drawing tube. Observations and descriptions are a compilation of data from all the *Eucalyptus* canker specimens listed in "Specimens Examined" below.

### Growth in culture

Standard mycological media used are as in Crous *et al.* (2009). All isolations from the host were derived from single ascospores or single conidia and grown initially on potato dextrose agar (PDA). Experiments were conducted to determine the best media for vegetative growth and sporulation. Isolates were grown on PDA, malt extract agar (MEA), distilled water agar (WA), corn meal agar (CMA), oatmeal agar (OMA) Czapek agar (CZA) and synthetic nutrient-poor agar (SNA) (Nirenberg 1976, Crous *et al*. 2009) ). In an attempt to improve *in vitro* production of ascomata and conidiomata, additional media were trialed which consisted of WA with added plant material, including gamma-irradiated carnation leaves (CLA), autoclaved millet seeds (MSA) and autoclaved or gamma-irradiated *Eucalyptus* twigs (*Eucalyptus* twig agar - ETA). The different plant materials were added to freshly poured, still molten WA.

**Fig. 1.** Symptoms of Caliciopsis canker. **A.** Severely diseased young shelter-belt trees at Flinders, Victoria. **B.** Severe cankers on twigs (1–2 cm diam) of *E. cladocalyx* 'Nana'. **C.** Developing lesions on vigorous main stem (3–4 cm diam) of *E. cladocalyx* 'Nana', centred on *Aulographina eucalypti* lesions. **D.** Lesion on *E. cladocalyx* 'Nana' 3 mo after agar plug inoculation. **E.** Uninoculated lesion 3 mo after wounding.

Single ascospore isolations from VPRI 16689 were plated onto PDA to assess the growth of the fungus at a range of temperatures and with or without light. PDA plates were inoculated with 5 mm diam colonised agar plugs (2 per plate) taken from 4-wk-old PDA cultures. To determine growth rate and optimal temperature for growth, three plates per temperature were grown at 5, 10, 15, 20, 25, 30 and 35 °C. In a second experiment to further refine the optimum temperature cultures were grown at 23, 25, 28, 30, 32 and 35 °C. Growth of the colonies in each experiment was assessed by measuring the diameter of each colony in two directions, perpendicular to each other. The diameters were then averaged and the results, including descriptions of visual appearance of cultures, recorded.

## Molecular analysis

DNA from mycelium of pure cultures VPRI 17721 and VPRI 15646, was extracted with Bioline Plant DNA Kits following the manufacturer's instructions. The ribosomal DNA internal transcribed spacer region was amplified and sequenced using primers ITS1F and ITS4 (White *et al*. 1990). Attempts to obtain LSU sequences were unsuccessful. The PCR products were purified and sequenced using Sanger 454 by Macrogen (Korea). The sequence data were visually corrected and aligned (ClustalW), and the Neighbour-Joining tree was built using Geneious v. 7.

## Pathogenicity testing

Pathogenicity tests were conducted initially with *E. cladocalyx* 'Nana'. Later, additional pathogenicity tests were conducted with 15 commercially important taxa of *Eucalyptus* including *E. cladocalyx* 'Nana'. Inoculum for experiments was produced by inoculating PDA plates with 5 mm diam agar mycelial plugs colonised by the isolates VPRI 16689 (*E. cladocalyx* 'Nana' experiment) and VPRI 16907 (multi-species experiment). The inoculum plates were incubated for 4 wk at 25 °C, prior to experimentation.

**Pathogenicity to *E. cladocalyx* 'Nana'** was investigated by inoculation of four randomly selected branches (each with one of four treatments) on each of 10 two-yr-old *E. cladocalyx* 'Nana' saplings growing in pots in a glasshouse. Inoculum of isolate VPRI 16689 was applied as the following treatments: 1) Agar plug inoculations consisting of either 5 mm diam colonised PDA plugs or 5 mm diam sterile PDA plugs respectively, inserted under a 5–6 × 2–3 mm flap cut into the periderm with a sterile scalpel blade; or 2) spore suspension inoculations consisting of either a suspension of conidia (10$^6$ conidia per ml derived from pycnidia produced on sterile eucalypt twigs on water agar) or sterile water respectively, inserted into the periderm with a sterile syringe and hypodermic needle. The resulting wounds produced by the spore injections were approximately the same size as the plug inoculations. The plug inoculations were placed between the second and third node and the suspensions were injected at the third node. Inoculation sites were wrapped in Parafilm© for two weeks to protect the site from desiccation. The experiment was performed in a temperature-controlled glasshouse and the saplings were watered by hand. Lesion extension was measured fortnightly over a period of three months. Lesions which formed were assessed for the presence of fruiting bodies and reisolations of the fungus were attempted. To isolate the fungus, tissue from the lesion margin was removed, surface-sterilised

with sodium hypochlorite and placed onto distilled water agar and incubated at 25 °C, for 1–3 wk.

**Pathogenicity to some commercially important taxa of *Eucalyptus*** was tested with the following 15 taxa: *E. botryoides*, *E. cladocalyx* (tall form), *E. cladocalyx* 'Nana', *E. delegatensis*, *E. fastigata*, *E. globulus*, *E. grandis*, *E. marginata*, *E. nitens*, *E. obliqua*, *E. radiata*, *E. regnans*, *E. saligna*, *E. sieberi* and *E. viminalis*. The experiment was performed in temperature-controlled glasshouses with an automatic watering system using overhead sprinklers.

Five randomly chosen saplings of each species were inoculated with colonised 5 mm PDA discs, while five saplings of each species randomly chosen as controls were inoculated with sterile 5 mm PDA discs. Prior to inoculation the surface of the chosen stem was sterilised by swabbing with 70 % ethanol. Inoculum and control discs were inserted under a 5–6 × 2–3 mm flap cut into the periderm with a sterile scalpel blade at a point 150 mm above soil level. The inoculation sites were wrapped in Parafilm© for 2 wk for protection against desiccation of the sapwood. Assessments of lesion development were made at 1, 2.5, 4, 5, 6 and 7 mo after inoculation. Lesions were assessed for length and width and presence or absence of pycnidia and ascomata and rated according to their general appearance.

## RESULTS

### Symptoms of disease

On severely affected trees of *E. cladocalyx* 'Nana', cankers can be found on branches, twigs and the main trunk and the tree gradually defoliates over a number of years and dies back as the cankers coalesce (Fig. 1A). Severe cankers are mostly seen on smaller twigs where they destroy the bark and leave an elongated dark brown, ragged canker (Fig. 1B) exposing the sapwood and quickly girdling the twig. Callus production appears to be suppressed. Milder cankers are seen on larger, vigorously growing branches (> 3 cm diam), and these develop into lesions up to 5 cm diam (Fig. 1C) while leaving the bark relatively intact, producing a raised, darkened, cracked area that rarely exposes the sapwood. These large cankers on branches (Fig. 1C) have been seen centred on old lesions typical of those caused by *Aulographina eucalypti,* confirmed by the presence of *A. eucalypti* ascomata. Later, as the bark dies, a more open canker develops. Observations suggest that infection may occur through wounds including leaf abscission scars, insect feeding or oviposition damage and fungal lesions.

### Microscopic examination and growth in culture

Microscopic examination of naturally occurring cankers revealed globose to depressed-globose black pycnidia and elongate, ventricose black ascomata (Fig. 2), often deeply embedded in cracks, but sometimes freely produced over the exposed surfaces of the affected bark. Isolation of the fungus from well-established cankers by placing surface-sterilised symptomatic wood or bark directly onto agar, was seldom successful as other faster growing fungi were frequently isolated. These included *Botryosphaeria* spp., *Endothiella* spp. and *Cytospora eucalypticola*, all of which are known pathogens of *Eucalyptus*. Consequently, to achieve an accurate diagnosis of cankers, single spore isolation of

**Fig. 2.** Ascomata of *C. pleomorpha* on stem of *E. cladocalyx* 'Nana'.
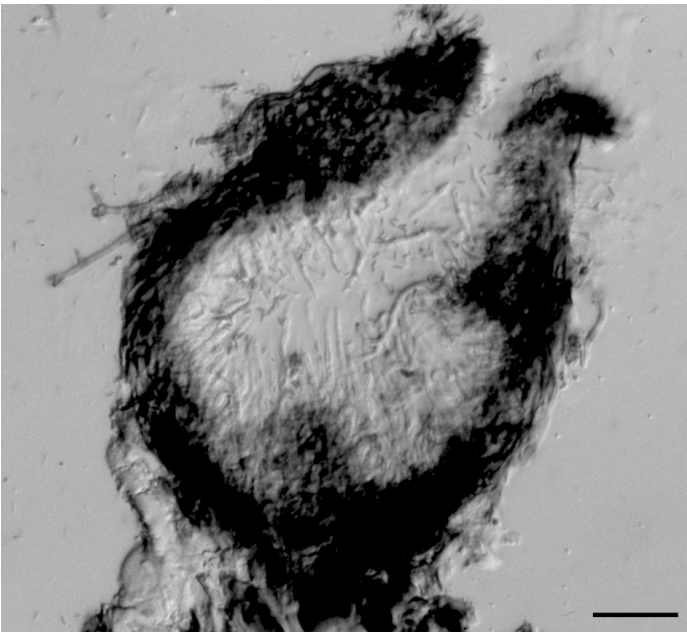


**Fig. 3.** Pycnidium of *C. pleomorpha* on stem of *E. cladocalyx* 'Nana'.

either conidia or ascospores was essential and yielded identical cultures of a phaeoacremonium-like hyphomycete, which on further incubation on a range of appropriate media produced both pycnidia and ascomata identical to those seen on the host.

The ascomata on host tissue (Fig. 2) have swollen median to submedian ascigerous locules surmounted by an elongated neck from which ascospores are discharged and accumulate as a dry, reddish-brown mass (mazaedium). Pycnidia (Fig. 3) are found on the canker surface where they are usually produced before the ascomata. Pycnidia may exude an inconspicuous cirrus of colourless conidia.

In culture (Fig. 4) the fungus grew at all temperatures between 5 °C and 30 °C and is able to survive but not grow at 35 °C (removal from 35 °C and incubation at 25 °C caused growth to resume). Optimal growth on PDA of around 3 cm in 14 d occurs at 25–28 °C. The fungus grew more quickly on WA (Fig. 4A), PDA (Fig 4B, and OMA and than it did on MEA, CMA and SNA and growth on CZA was very poor. Light did not significantly affect growth rate, but did enhance the sporulation of the hyphomycete morph compared with incubation in darkness.

On some media the fungus produces a hyphomycete morph (Fig. 4F) that resembles *Phialophora* or *Phaeoacremonium*.

Sporulation of the hyphomycete morph occurred abundantly on PDA, OMA and WA and poorly on CMA. Sporulation did not occur on any of the other media used, including MEA. Funk (1963) and Ray (1936) did not report a hyphomycete morph using only MEA and CMA as culture media. The hyphomycete morph was rarely visible on field-collected specimens although it was sometimes induced by moist-incubating the cankers for a few days. It was occasionally seen on lesions developing on inoculated eucalypt stems in the glasshouse during pathogenicity trials.

The fungus also grew and sporulated well on WA with added plant material, including gamma-irradiated carnation leaves (CLA), millet seeds (MSA) and autoclaved *Eucalyptus* twigs (ETA). Of these, ETA was most successful in stimulating production of fertile ascomata (Fig. 4C), fertile pycnidia (Fig. 4D, E), and the hyphomycete morph (Fig 4C, D, F) as well as structures that resembled abortive ascomata (Fig. 4D) (which we referred to as "feathery digitate structures") with multi-branched tips and liberal hyphal growth and hyphomycete sporulation from their apices. These structures appeared to be similar to the structures that Funk (1963) found in *C. pinea* cultures and which he described as grotesque looking ascocarps. We speculate that these "feathery digitate structures" might be aberrant, culture-induced aborted ascomata which grow on to produce the hyphomycete morph. The structures were not seen *in vivo* and did not develop into functional ascomata *in vitro*.

## Molecular analysis

ITS sequences obtained for the two isolates VPRI 15646 (GenBank MG641785) and VPRI 17721 (GenBank MG641784) were over 99 % similar to one another and were compared with existing *Coryneliales* sequences on GenBank. The two VPRI isolates were most similar to *Caliciopsis calicioides* (GenBank JX968549) with which they shared 74.88 % and 75.29 % similarity in the ITS sequence, respectively. The second closest species were *C. valentina* and *C. eucalypti* with which our isolates shared approximately 73–74 % similarity in the ITS sequence. A phylogram of known *Caliciopsis* and *Corynelia* species ITS sequence data, including that of the two VPRI isolates, is presented as Fig. 8, and demonstrates that our fungus is well separated from other *Caliciopsis* taxa.

## Taxonomy

The *Eucalyptus* fungus was compared with specimens of morphologically similar species of *Caliciopsis*. Specimens examined included a number of non-type specimens of *C. pinea* and the holotypes of *C. myrticola, C. rapaneae, C. veillonii, C. xanthostemonis,* and *C. podocarpi*. Of the taxa examined, *C. xanthostemonis* (known only from the holotype) most closely resembles the *Eucalyptus* fungus, from which it is almost indistinguishable on the basis of ascoma and ascospore morphology. The small number of ascomata of *C. xanthostemonis* present on the holotype generally appear shorter and narrower than those of the *Eucalyptus* fungus although there is considerable overlap. Ascomata of the *Eucalyptus* fungus range in mean lengths from 292 µm (VPRI 16907) to 695 µm (15646) and in mean locule widths from 70 µm (16821) to 119 µm (15646) while the overall mean for all eight collections measured was length 463 µm, width at locule 89 µm. The range for the holotype of *C. xanthostemonis* was length 280–390 µm, width at locule 28–56 µm). So, while some individual specimens of the *Eucalyptus* fungus have mean
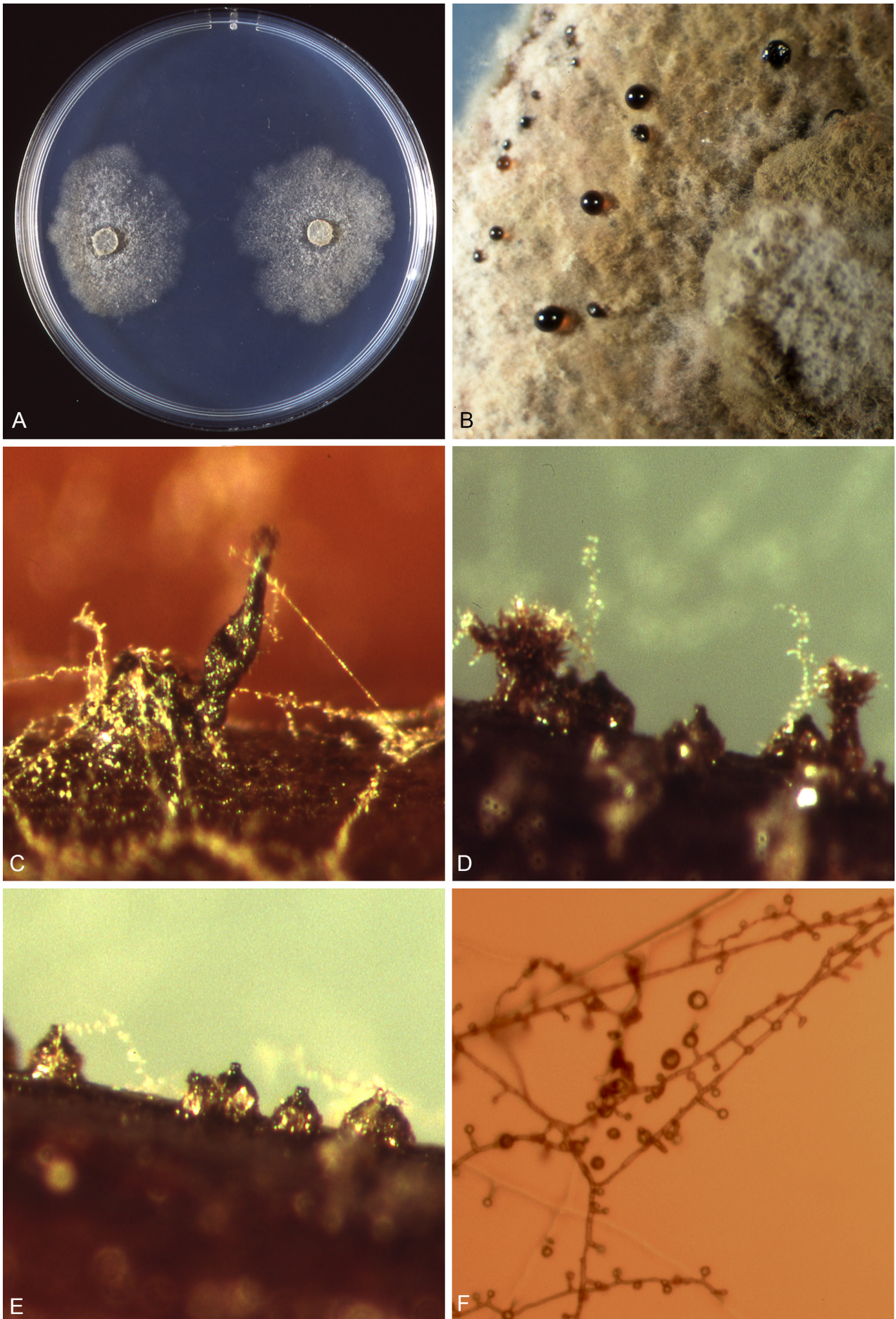
**Fig. 4.** *Caliciopsis pleomorpha* in culture. **A.** Culture on WA at 2 wk. **B.** PDA culture showing reddish exudate and felty texture. **C.** Ascoma and hyphomycete morph on ETA. **D.** Feathery digitate structures, pycnidia and hyphomycete morph on ETA. **E.** Pycnidia on ETA. **F.** Hyphomycete morph on WA.

lengths that fall within the range for *C. xanthostemonis* the majority are considerably longer and almost all are considerably wider. Unfortunately, there are so few ascomata on the holotype that the full variability of *C. xanthostemonis* is not known and only additional collections of *C. xanthostemonis* will provide sufficient data to separate the two species on the basis of ascomatal dimensions. The ascospores of the two species were virtually identical, globose to subellipsoid, brown and smooth-walled (*C. xanthostemonis* 3.1–4.7 μm, *Eucalyptus* fungus 3.3–3.7 μm diameter). Huguenin (1969) did not observe pycnidia, stating "les spermogonies n'ont pas été observées". In this study, however, pycnidia similar to those of *C. pleomorpha* were observed on the holotype of *C. xanthostemonis*, although they were effete and no internal structures or conidia could be found in the single pycnidium sectioned. Since there was no evidence of a hyphomycete state on the type, it is therefore not possible to describe properly any asexual morphs for *C. xanthostemonis*. In addition, *C. xanthostemonis* is apparently a saprophyte and is known only from leaf necrosis associated with the margins of insect damage, while the *Eucalyptus* fungus has not been found to infect leaf tissue and is a virulent pathogen of stems.

*Caliciopsis xanthostemonis* therefore differs from the *Eucalyptus* fungus only in minor details of ascoma dimensions and habitat. Moreover, its occurrence on *Xanthostemon*, a genus of the *Myrtaceae*, does raise the suspicion that it may be phylogenetically close to the *Eucalyptus* fungus. Several taxa of *Xanthostemon* occur in northern Australia and therefore share habitat with *Eucalyptus* so the possibility of movement between hosts exists. Unfortunately, molecular comparison of the two species is not an option unless fresh material can be obtained, as the holotype of *C. xanthostemonis* has fewer than 20 ascomata. The characters of pycnidium centrum morphology, conidium shape and size, hyphomycete conidiogenesis and conidia and cultural characters are also not available for *C. xanthostemonis*. Funk (1963) found it difficult to differentiate *C. orientalis* from *C. pinea* based on morphology alone, since there was considerable overlap in the dimensions of ascomata, ascospores and pycnidia, but concluded that the considerable differences in cultural behaviour as well as pathogenicity to two different genera respectively, justified recognition of two different species. The lack of knowledge of pycnidial and hyphomycetous morphs as well as a lack of knowledge of behaviour in culture for *C. xanthostemonis*, the apparent difference in nutrition (pathogenic vs. saprophytic) of the *Eucalyptus* fungus and *C. xanthostemonis* and finally the lack of DNA sequence data for *C. xanthostemonis* are the principal factors which prevent us from treating the two taxa as conspecific.

The eucalypt canker fungus was also compared with *C. eucalypti* Crous, from leaves of *Eucalyptus marginata*. The description and illustrations of *C. eucalypti* include only the pycnidial morph and its conidia, which are similar to those of our fungus. However, Crous provided sequence data for *C. eucalypti* and our sequence data show that that the fungi are not conspecific.

Consequently we choose to erect a new species for the eucalypt canker fungus, as *C. pleomorpha sp. nov.*

**Caliciopsis pleomorpha** Patricia McGee & Pascoe, **sp. nov.** MycoBank MB823818. Figs 2–7.

*Etymology*: *pleo Gr:* - many or multi; *morpha Gr*: - forms or shapes.

*Sporocarps* (ascomata and pycnidia) seated on rough-edged, longitudinally splitting stem cankers (Fig. 1B, C). *Ascomata* (Figs 2, 5) separate or loosely grouped, not arising from a visible stroma, dark brown to black, ventricose, 250–700 (rarely up to 900) μm high, 65–130 μm diam, straight or curved, elongate with a submedian to suprabasal swollen ascigerous locule. Ascoma wall of *textura porrecta* to *textura intricata*, heavily gelatinised, apex of immature ascomata with a conspicuous cap of clear mucilage, asci (Fig. 5B) 10–13 × 7–8 μm, containing eight ascospores, elongating at maturity and extending up the ascoma neck to the apex before deliquescing to release ascospores at or below the ostiole; discharged ascospores accumulating in a dry reddish brown mass (mazaedium) at the ostiole, ascospores (Fig. 5C) golden brown, thick-walled, smooth, depressed globose to subellipsoid, 3.3–3.7 μm diam, with a thick (0.3 μm) wall. Pycnidial and hyphomycetous morphs produced, the hyphomycete morph usually occurring only in culture, but occasionally sparse on canker surfaces close to sporocarps. *Pycnidial conidiomata* (Figs 3, 6A) solitary, sub-caespitose or adjoined to ascomata, dark brown to black, globose or depressed globose, 80–150 μm diam, with a prominent papillate ostiole, wall of *textura angulata* to *textura intricata*, centrum containing short ampulliform conidiophores and elongated, septate acropleurogenous conidiophores (Fig. 6B) and heavily gelatinised paraphysis-like structures (immature conidiophores?) (Fig. 6C). *Conidiophores* hyaline, arising from the inner cells of the pycnidial wall, occurring either as simple ampulliform single-celled conidiogenous cells 6–12 × 3–4 μm or more frequently elongate and acropleurogenous conidiophores, 15–45 μm long comprising up to six or more cells, 6–10 × 2–4 μm, producing conidogenous loci as short lateral projections arising directly from beneath each septum; *conidiogenous cells* phialidic with an inconspicuous collarette; conidia hyaline, asymmetrical, oblong to allantoid, aseptate, smooth, 3–5 × 1–2 μm, with 1–2 guttules. *Growth in culture* occurring readily on a range of media, cardinal temperatures 5 °C and 35 °C, optimum 25 °C, cultures on potato dextrose agar (PDA) dark olivaceous, felty, with a pale, wet margin, aerial mycelium dense, matted, with false heads of conidia scattered on short lateral phialides or irregularly developed conidiophores, mycelium frequently developing droplets of dark reddish exudate (Fig. 4B); hyphae *in vitro* sub-hyaline to mid-brown, branched, thick-walled, rough, 4–9 μm wide. *Conidiophores* of hyphomycete morph (Figs 4F, 7A) phaeoacremonium-like, in culture and occasionally seen on canker tissue, simple or branched, sub-hyaline to pale brown, smooth or rough, conidiogenous cells lageniform, the collarettes usually inconspicuous and parallel-sided but occasionally flared (phialophora-like), mono- or polyphialidic, discrete, smooth or roughened with bubble-like adhesions, 5–22 × 2–5 μm, hyphomycetous conidia (Fig. 7B) aseptate, ellipsoid-ovoid, smooth, 2.8–4.5 × 1–2.5 μm, containing 1–2 guttules.

*Holotype*: **Australia**, Victoria, Flinders, Orcadia Park, on *Eucalyptus cladocalyx* 'Nana', Nov. 1991, *L. Wesley* (VPRI 17721).

*Additional specimens examined*: **Australia**, Victoria, Flinders, Orcadia Park, on *E. cladocalyx 'Nana'*, 11 Dec 1991, *P. Maher*, VPRI 17851; 11 Dec 1991, *P. Maher, I.G. Pascoe*, VPRI 32273, VPRI 32274; 28 Mar 1992, *P. Maher*, VPRI 17852; 10 Apr. 1992, *P. Maher*, 17860; 04 Aug 1992, *P. Maher*, VPRI 18063, VPRI 18064; 08 Sep 1992, *P. Maher*, VPRI 18235, VPRI 18236; *E. botryoides*, 10 Apr. 1992, *P. Maher*, VPRI 17861; *Corymbia ficifolia*, 9 Jul. 1992, *P. Maher*, VPRI 32281; Kew, Forestry
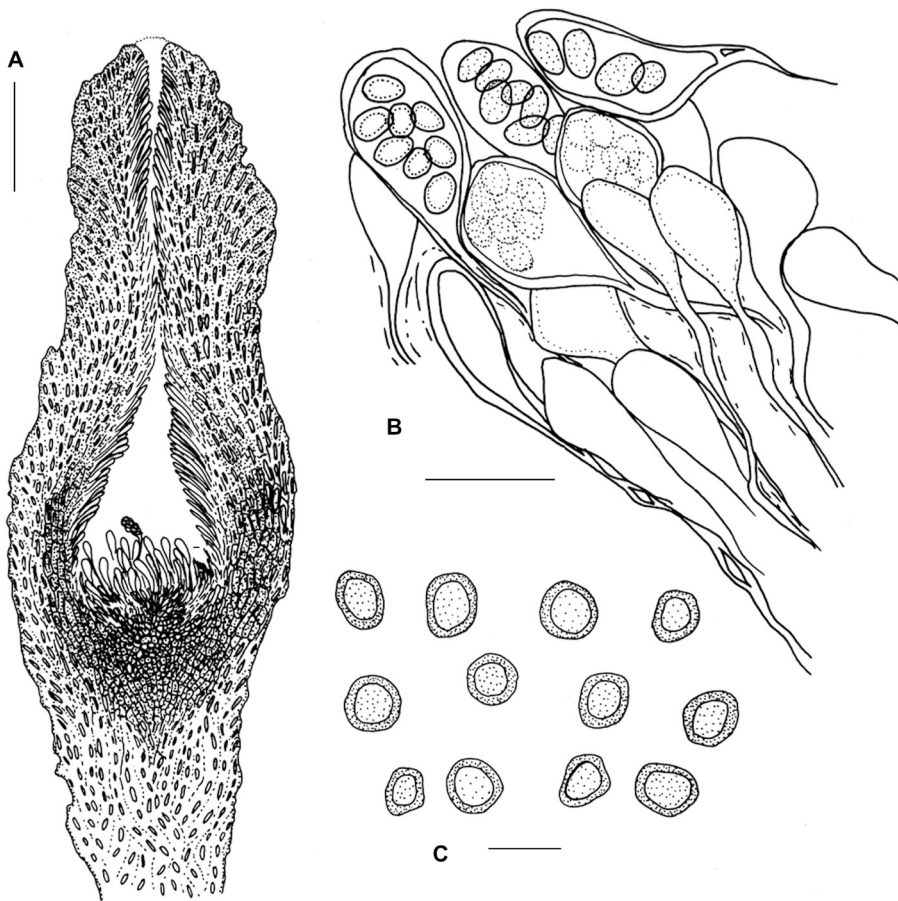
**Fig. 5. A.** Young ascoma of *C. pleomorpha* from host tissue showing *textura porrecta* tissue type. **B.** Developing asci. **C.** Ascospores. Scale bars: A = 40 µm, B = 10 µm, C = 5 µm.
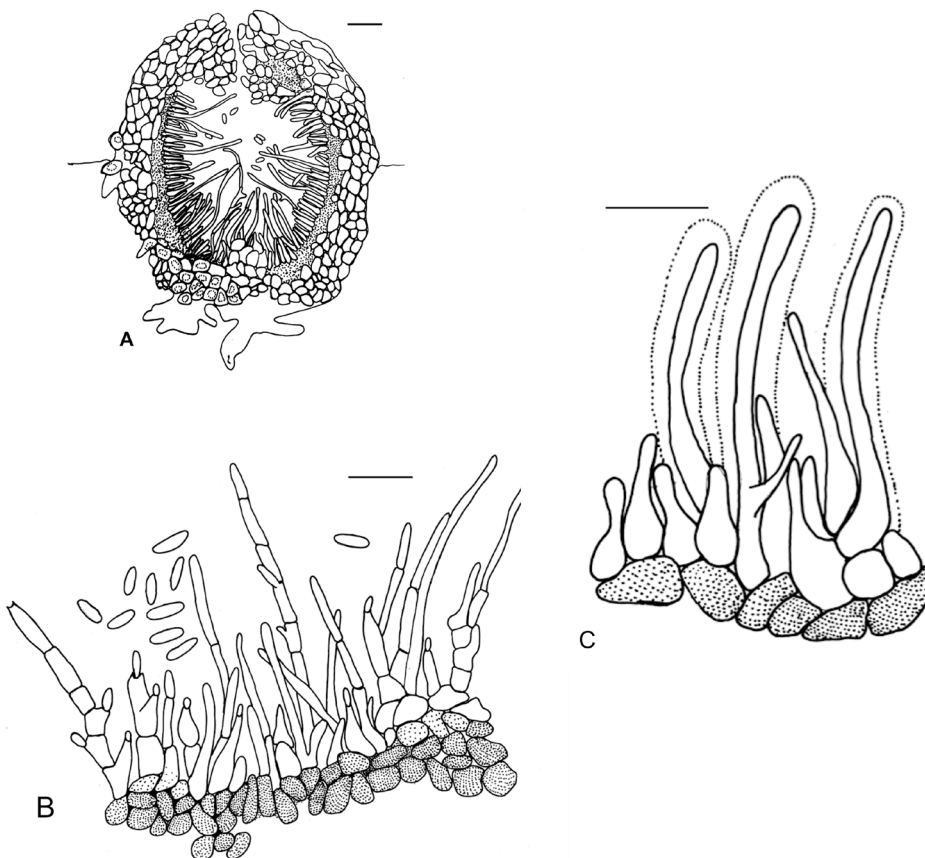


**Fig. 6. A.** Pycnidium from host tissue. **B.** Conidiophores and conidia from pycnidia. **C.** Developing conidiophores showing simple phialides and gelatinised immature acropleurogenous conidiophores. Scale bars = 10 µm.

glasshouse, *Eucalyptus botryoides*, 2 Nov. 1993, *P. Maher*, VPRI 19598, VPRI 19599, VPRI 19600; *E. fastigata*, 02 Nov 1993, *P. Maher*, VPRI 19602; *E. globulus*, 02 Nov 1993, *P. Maher*, VPRI 19603; *E. grandis*, 02 Nov 1993, *P. Maher*, VPRI 19604, VPRI 19606; *E. radiata*, 02 Nov 1993,

*P. Maher*, VPRI 19608; *E. regnans*, 02 Nov 1993, *P. Maher*, VPRI 19609; *E. saligna*, 02 Nov 1993, *P. Maher*, VPRI 19610; *E. sieberi*, 02 Nov 1993, *P. Maher*, VPRI 19611; *E. obliqua*, 02 Nov 1993, *P. Maher*, VPRI 19607; *E. viminalis*, 02 Nov 1993, *P. Maher*, VPRI 19612; *E. delegatensis*, 02
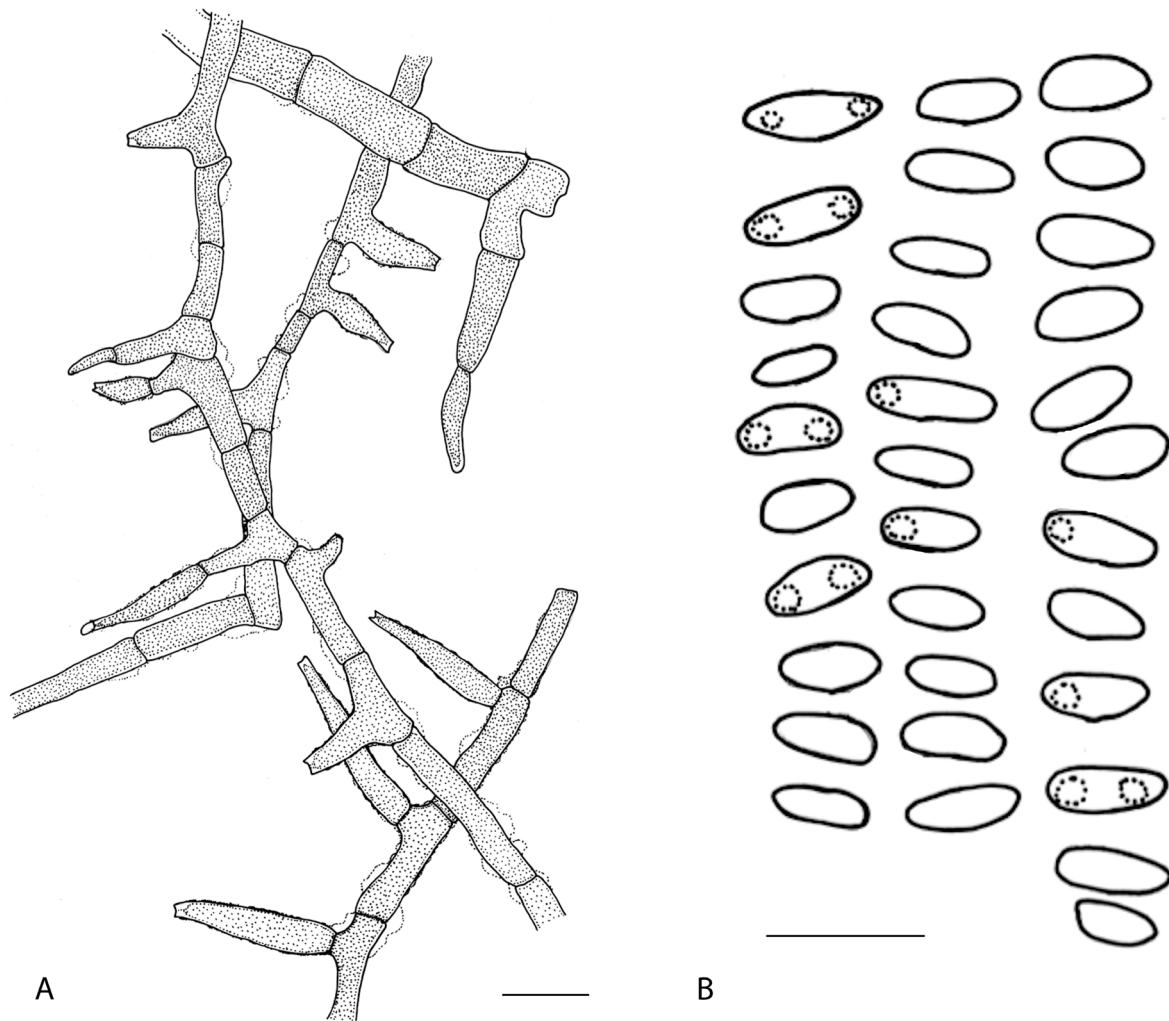
**Fig. 7. A.** Conidiophores of the hyphomycete morph of *C. pleomorpha* on WA. **B.** Conidia of the hyphomycete morph. Scale bars = 10 μm.

Nov 1993, *P. Maher*, VPRI 19601; Traralgon, *E. cladocalyx 'Nana',* 23 Mar 1990, *R. Cantrill*, VPRI 16665; 25 Apr 1990, *P. McGee*, VPRI 16689; 01 Jun 1990, *P. McGee & I.G. Pascoe*, VPRI 16754, VPRI 16757, VPRI 16753, VPRI 16755; 23 Mar 1991, *P. McGee*, VPRI 32290; 05 Apr 1991, *P. McGee*, VPRI 17235, VPRI 17236; *E. spathulata*, 05 Apr 1991, *P. McGee*, VPRI 17237; Narre Warren North, *E. cladocalyx 'Nana'*, 30 Nov 1992, *D. Phillips*, VPRI 18526; Burnley, glasshouse, *E. cladocalyx 'Nana',* 1991, *P. Maher*, VPRI 17709; 20 Jan 1991, *P. McGee*, VPRI 17366; 15 Sep 1991, *P. Maher*, VPRI 17708; 27 Jul 1990, *I.G. Pascoe*, VPRI 16834; Morwell, *E. cladocalyx 'Nana',* 01 Jun 1990, *P. McGee & I.G. Pascoe*, VPRI 16756; Woodford, *E. cladocalyx,* 10 May 1980, *M. Hall*, VPRI 11001; Frankston, *E. cladocalyx,* Dec 1979, *L. Smith*, IMI 260278, DAR 35408, VPRI 10928; Frankston, George Pentland Botanical Gardens, *E. cladocalyx,* 22 Jul 1984, *I.G. Pascoe*, VPRI 12381; *E. spathulata,* 22 Jul 1984, *I.G. Pascoe*, VPRI 12382; Warrnambool, *E. cladocalyx,* 10 May 1980, *M Hall*, VPRI 11002; Nar Nar Goon, *E. cladocalyx 'Nana',* 23 Aug 1990, *P. McGee*, Barker P, VPRI 16907; Locality unknown, *E. cladocalyx,* 22 Mar 2002, *I.W. Smith*, VPRI 24952; Springvale, *E. cladocalyx,* 25 Sep 1990, *P. McGee*, VPRI 16981; Linton, *E. cladocalyx,* 25km W of Ballarat, 27 Nov 1987, *G Marks*, VPRI 15646; Glen Aire, *E. cypellocarpa*, 08 Jul 1990, *I.G. Pascoe*, VPRI 16821; Main Ridge, Mornington-Flinders Rd, near Roberts Rd, *E. dives*, 09 Jul 1992, *P. Maher*, VPRI 17985; Beenak, Hansens Creek Rd., *E. obliqua,* 20 May 1987, *I.G. Pascoe*, VPRI 15393; NSW, Dyraaba, Dyraaba Station, on *Corymbia variegata*, 7 Jun. 2005, *A.J. Carnegie*, VPRI 40612; Dorrigo Plateau, *E. nitens*, Nov 2006, *I.W. Smith*, VPRI 40644, VPRI 40645; Locality Unknown, "Stop 3", *E. dunnii*, 02 Nov 2006, *I.W. Smith*, VPRI 40646; Queensland, Davies Creek, on *E. camaldulensis*, Aug. 2005,

*A.J. Carnegie*, VPRI 40613; South Australia, Port Lincoln, Kirten Point Caravan Park, *E. cladocalyx,* Feb. 1993, *P. McGee*, VPRI 32277; Tod River Reservoir, Koppio Hills, *E. cladocalyx,* Feb. 1993, *P. McGee*, VPRI 32279, VPRI 32278; Koppio, *E. cladocalyx,* Feb. 1993, *P. McGee*, VPRI 32280.

*Type specimens of other taxa*: *Caliciopsis myrticola*, on *Myrtus emarginata*, Faux Bon Secours, New Caledonia, 1 Jun. 1967, B. Huguenin, NC 67083 (PC); *Caliciopsis podocarpi*, on *Podocarpus minor*, Plaine des Lacs, lieu dit "Le Goulet", New Caledonia, 18 Aug. 1966, B. Huguenin, NC 66058 (PC); *Caliciopsis rapaneae*, on *Rapanea lanceolata* Mez*,* Mont Mou, New Caledonia, 27 Dec. 1966, J-M Veillon, NC 67032 (PC); *Caliciopsis veillonii*, unknown host, Montagne des Sources, New Caledonia, 27 Apr. 1967, J.-M Veillon, NC 67050 (PC); *Caliciopsis xanthostemonis*, on *Xantostemonis baudounii*, Néhoué, New Caledonia, 20 Aug. 1967, Mc Kee, NC 67082 (PC).

## Pathogenicity experiments

### *Eucalyptus cladocalyx* 'Nana'
Pathogenicity of *C. pleomorpha* to *E. cladocalyx* 'Nana' was demonstrated by artificial inoculation, resulting in development of typical lesions, sporulation of the fungus on infected tissues and re-isolation of the fungus into axenic culture. While canker symptoms (Fig. 1D) were generally less severe than those seen under field conditions, the general appearance of the cankers was similar to naturally occurring lesions. Lesions examined 3

**Table 1.** Final observations of mean lesion length and width (in mm) and the presence of fruiting bodies 3 months after inoculation of *E. cladocalyx* 'Nana' with *C. pleomorpha*.

| Treatments | Mean Length | Mean Width | Lesions containing ascocarps | Lesions containing pycnidia |
|---|---|---|---|---|
| 1) plug - inoculated | 9.76 ± 1.25 | 5.45 ± 0.55 | 0 | 7 |
| 2) plug - control | 6.56 ± 0.84 | 3.40 ± 0.53 | 0 | 2 |
| 3) suspension - inoculated | 8.50 ± 2.27 | 4.81 ± 0.75 | 3 | 7 |
| 4) suspension - control | 7.45 ± 2.70 | 4.00 ± 0.52 | 0 | 3 |

mo after mycelial plug inoculation had a mean lesion length 44 % greater than the initial inoculation wound, while control lesions had a mean lesion length 16 % longer than the initial wounds. Sporulating pycnidia appeared within 1–2 mo after inoculation on all inoculated treatments, and sporulating ascomata appeared within 3–4 mo on 3 of the syringe inoculated lesions. After 3 mo pycnidia were found in 2 of 10 control replicates of the plug inoculated treatment, and in 3 of 10 controls of the syringe inoculated treatment, indicating that some controls had become infected during the experiment, perhaps due to dispersal of propagules by overhead sprinklers, and at 7 mo these control replicates had developed cankers comparable to the inoculated treatments. Table 1 shows 3-mo data for lesion size and occurrence of fruiting bodies. Isolation from lesion margins onto WA resulted in growth typical of *C. pleomorpha*, and sporulation of the hyphomycete morph.

*Other eucalypt species*

Inoculations of other *Eucalyptus* species were successful in causing canker development on all species tested, although there was substantial variation in severity. Within 1 mo there were visible differences between control and inoculated seedlings, with many inoculated wounds becoming darkened with a concave surface and necrotic margins spreading into surrounding tissue. Necrotic tissue was frequently swollen and distorted, and in some inoculated saplings the tissue surrounding the lesion developed red pigments. In some lesions, kino exudate appeared. By contrast control wounds were dry and pale and showed no indication of spreading within 1 mo. At 2.5 mo there was still no change in the appearance of wounds in uninoculated controls, while symptoms continued to progress in inoculated treatments. However at 4 mo, many of the controls had become infected and began to show similar symptoms to those seen in inoculated lesions. At six mo, two of the inoculated *E. marginata* saplings and two of the inoculated *E. cladocalyx* 'Nana' saplings died. The stems broke at the lesion site on one inoculated sapling of *E. obliqua* and one inoculated sapling of *E. saligna*. The remaining plants were generally healthy despite the lesions. New growth was commonly initiated below the wound site after six months infection. The symptoms varied greatly between the different species ranging from elongate, distorted lesions in susceptible species such as *E. radiata,* and *E. globulus* to relatively short, broadly swollen lesions in species such as *E. obliqua*. In the relatively resistant species *E. botryoides* and *E. saligna* there was little lesion extension and little swelling. Distortion of the stems was common in many species due to the spread of the lesions and associated swelling.

At 5 mo (Fig. 8), data for lesion length shows the seven most severely affected species (lesion length 24–34 mm) to be *E. cladocalyx*, *E. cladocalyx* 'Nana', *E. globulus*, *E. nitens*, *E. radiata, E. regnans* and *E. sieberi*. The least affected species (lesion length 12–15 mm) were *E. botryoides*, and *E. saligna*

while other taxa *E. delegatensis*, *E. fastigata*, *E. grandis*, *E. marginata*, *E. obliqua* and *E. viminalis* had lesion lengths of 17–20.5 mm and may be regarded as moderately susceptible. At 7 mo the seven most severely affected taxa in terms of lesion length were *E. cladocalyx*, *E. cladocalyx* 'Nana', *E. globulus*, *E. nitens*, *E. radiata*, *E. regnans* and *E. sieberi* and the least affected were again *E. botryoides* and *E. saligna*. Width of lesions gave different results with *E. cladocalyx* and *E. cladocalyx* 'Nana' having low values and *E. delegatensis*, *E. fastigata*, *E. globulus*, *E. grandis*, *E. nitens* and *E. viminalis* having the highest values at four mo. Since *E. cladocalyx* and *E. cladocalyx* 'Nana' were both qualitatively and quantitatively the most severely affected taxa in the trial, it is clear that width of lesions is not a good
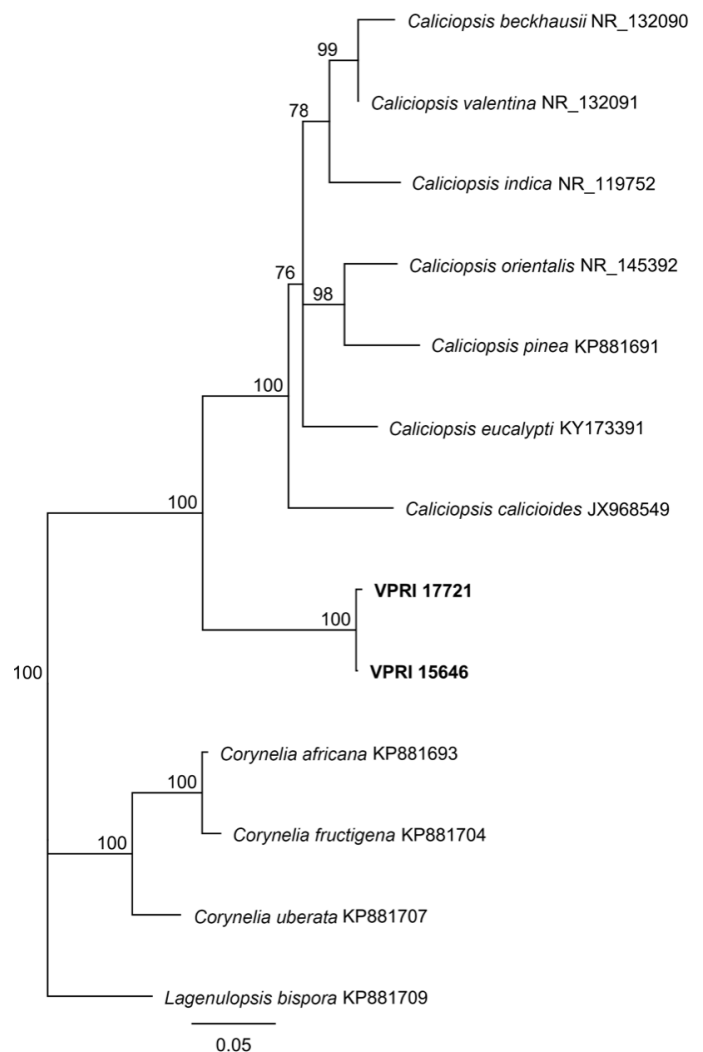


**Fig. 8.** Phylogenetic placement of VPRI isolates of *Caliciopsis pleomorpha* obtained using ITS sequence data, aligned with ITS sequences from available GenBank reference strains of species of *Caliciopsis* and other *Coryneliaceae*. The tree was rooted to *Lagenulopsis bispora*.
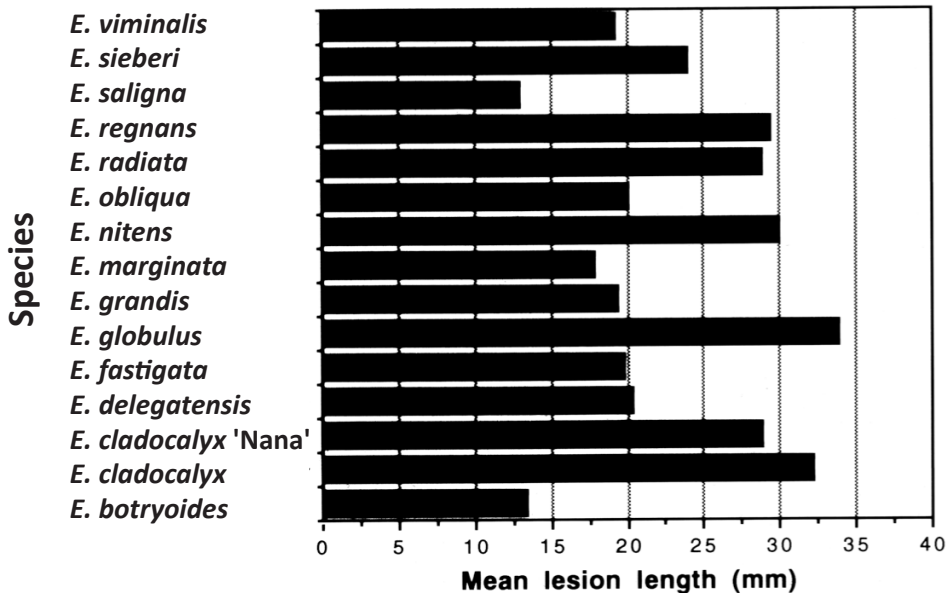
**Fig. 9.** Mean lesion length (mm) in 15 eucalypt taxa five mo after inoculation with *C. pleomorpha*.

indicator of susceptibility. Indeed, much longer-term trials on field-infected trees are required before a good indication of overall susceptibility of different species can be arrived at.

## DISCUSSION

Stem canker diseases are not uncommon on the genus *Eucalyptus* in Australia and there have been several comprehensive studies of the fungi that cause eucalypt cankers. Davison & Tay (1983) described cankers from *Eucalyptus marginata* (Jarrah) in Western Australia, and Old *et al*. (1986) summarised the fungi associated with eucalypt cankers in south-eastern Australia. Old *et al*. (1990) studied the effect of drought and defoliation on the susceptibility of eucalypts to cankers caused by *Endothia gyrosa* and *Botryosphaeria ribis*, and Wilkes (1987) looked at the interactions between the various fungi associated with injured sapwood in eucalypts. The canker diseases of *Eucalyptus* are also comprehensively summarised by Keane *et al.* (2000). None of these publications mentions any species of *Caliciopsis* associated with eucalypt cankers.

The genus *Caliciopsis* was erected by Peck (1880), based on *C. pinea*, a pathogen of *Pinus*, and was first monographed by Fitzpatrick (1942) who accepted 10 species. The genus now includes 35 species (Index Fungorum - http://www.indexfungorum.org/names/Names.asp). *Caliciopsis* is characterised by elongate, often ventricose ascomata with a swollen ascigerous locule, which is elevated above the substrate and usually surmounted by an elongate cylindrical neck. The ascoma tissue is gelatinised. Asci have multiple wall layers (Johnston & Minter 1989), the outer wall breaking early to allow the internal ascus stipe to elongate, extending the ascus into the neck of the ascoma where ascospores are released by deliquescence of the ascus wall. Ascospores initially accumulate in a gelatinous mass at the ostiole, later drying to a powdery mass. Benny *et al.* (1985) compared *Caliciopsis* with related genera of the Coryneliales and concluded that the genus was unique in the presence of gelatinised *textura intricata* in the central tissues of the ascoma, and in having the centrum elevated above the substratum on a stalk. Huguenin (1969) described a number of new species of *Caliciopsis* and other *Coryneliales* from New Caledonia. More recently, Garrido-Benavent & Perez-Ortega (2015) described a new species *C. valentina*, recombined *Coniocybe beckhausii* into *Caliciopsis*, compared the ITS and LSU sequences of those species with other available *Caliciopsis* sequence data and published a worldwide key to the genus. Prior to the present paper, only *C. xanthostemonis*, *C. myrticola* and *C. eucalypti* have been recorded on *Myrtaceae*, and only *C. eucalypti* has been described from *Eucalyptus*. No doubt many other species, both phytopathogenic and saprotrophic, exist in nature and remain to be discovered.

McCormack (1936) attempted to germinate conidia of *C. pinea* from pycnidia and since they did not germinate, identified them as spermatia. Funk (1963), on the other hand, had no difficulty in germinating conidia on agar and growing cultures which were identical to single ascospore cultures, although he continued to regard the conidia as spermatia. He was able to produce pycnidia of *C. pseudotsugae* on twig cultures and of *C. pinea* and *C. orientalis* on agar. In *C. pleomorpha*, pycnidial conidia germinate readily and grow in culture to produce the hyphomycetous morph and, under appropriate conditions (*Eucalyptus* twig agar), pycnidia and ascomata. Pratibha *et al*. (2010) did not observe pycnidia in *C. indica* but did succeed in isolating the fungus onto MEA from single ascospores. However they did not observe a hyphomycetous morph.

The present paper demonstrates that in *C. pleomorpha* the pycnidial morph is capable of propagule germination and growth in culture (and is therefore conidial and not spermatial), that a hyphomycetous morph exists that can be demonstrated on appropriate media, and that pycnidia and ascomata form readily on media containing sterilized host tissue. The key to all of these discoveries seems to be the use of media other than MEA, which was the only medium used by Funk (1963), McCormack (1936) and Pratibha *et al*. (2010), while Ray (1936) used only CMA. While *C. pleomorpha* grows well on MEA, it does not produce the hyphomycetous morph and it grows poorly and sporulates sparsely on CMA. On other media such as PDA, WA and OMA, *C. pleomorpha* produces only the hyphomycetous morph and it is necessary to use *Eucalyptus* twig agar to induce development of pycnidia and ascomata. Deliberate spermatisation was not necessary, but the fungus frequently produced aborted ascomata which might indicate that some form of fertilisation is required.

Previously, *C. pinea* was the only species to have produced ascomata in culture (Ray 1936, Funk 1963). Ray grew *C. pinea* on CMA and obtained spermogonia, but was only able to induce formation of ascomata with the addition of blocks of white pine (*Pinus strobus*) to the CMA, and by the use of many combinations of single spore isolates, only two of which resulted in production of ascospores. Funk (1963) was able to obtain ascomata on sterilized twigs, noting that this occurred in single ascospore cultures and inferring that spermatization was not necessary and that the ascospores appeared to be homothallic. It is clear from the papers of Funk, McCormack and Ray that they looked carefully enough at their cultures (having observed such structures as hyphal anastomoses and dendroid hyphal tips) to have detected a hyphomycete state if it had occurred. It would be interesting to grow *C. pinea, C. pseudotsugae* and *C. orientalis* on PDA, WA, and OMA to see if a hyphomycete morph is able to be produced by those species under appropriate conditions.

Susceptibility to *C. pleomorpha* seems to be related to the ability to form callus in infected lesions. Moderately resistant species (such as *E. viminalis*) produced large amounts of callus and succeeded in healing the lesion, while highly susceptible species such as *E. cladocalyx* typically failed to produce any callus so that symptoms continued to progress into a perennially developing canker. *Eucalyptus botryoides* and *E. saligna* simply failed to develop significant cankers without production of large amounts of callus. It is probable that the parameters measured during the glasshouse trial are not predictive of susceptibility to *Caliciopsis* cankers and their consequences including death of twigs and branches. On *E. cladocalyx* 'Nana' in the field, well developed cankers are open with the wood exposed, with very little callus and ragged, torn edges. On the other hand, natural field infections on moderately susceptible species such as *E. obliqua* have large amounts of callus development and tend to close over but the stem is swollen to about twice its normal diameter and may be very susceptible to breaking.

Since the original discovery of *C. pleomorpha*, the disease has been detected in many naturally infected forest trees (I.W. Smith, J. Simpson & A. Carnegie, pers. com.), and found to be particularly damaging in plantation forestry. In NSW forests it has been recorded on *E. grandis, E. globulus, Corymbia variegata, C. maculata, E. nitens* and *E. grandis* × *E. camaldulensis* clones (Carnegie 2007 a, b). Cankers associated with *C. pleomorpha* were mostly found on stressed or damaged trees in NSW, such as those severely defoliated by insects (chrysomelid leaf beetles) or fungi (*Teratosphaeria* spp.) and on most hosts cankers were small, perennial and eventually occluded (Carnegie 2007a). However, in 2004 the fungus caused significant damage to an *E. nitens* plantation in the Dorrigo Plateau in NSW, killing at least 50 % of trees (Carnegie 2007b). Carnegie (2007a) also reported Caliciopsis canker from *E. camaldulensis* in northern Queensland.

In 2008 a canker disease was detected in plantations of *E. grandis* in northern NSW and suspected, on the basis of its distinctive symptoms, of being caused by the exotic *Teratosphaeria zuluensis* and brought to the attention of the senior author by quarantine authorities. Microscopic examination and culturing by the senior author and colleagues showed that the black conidiomata contained hyaline (rather than brown) conidia and further examination and comparison of ITS sequences showed that the fungus was in fact *C. pleomorpha*. No ascomata were detected. In this case the symptoms consisted of deep but restricted cankers on the main trunk, quite distinct from typical lesions on *E. cladocalyx* 'Nana'.

*Caliciopsis pleomorpha* is most commonly a severe pathogen of *E. cladocalyx*. However, there are enough records of the fungus attacking other *Eucalyptus* species within plantations and natural populations to indicate that the pathogen has the potential to be an occasional but significant threat to natural and commercial *Eucalyptus* forests. Further studies on environmental and genetic factors affecting the susceptibility of different species are needed to elucidate this threat.

## ACKNOWLEDGEMENTS

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