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Detection of the *Togninia* teleomorph of *Phaeoacremonium aleophilum* in Australia

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Summary. Moist incubation of grapevine wood infected with both *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora* yielded an ascomycete referable to the genus *Togninia* (*Ascomycota, Calosphaeriales*). Single ascospore cultures were morphologically identical to *Pm. aleophilum*. The rDNA ITS sequence of single ascospore isolates was identical to published sequences for the majority of *Pm. aleophilum* isolates. Comparison with the morphology of other wood staining *Togninia* species confirms that the teleomorph of *Pm. aleophilum* is *Togninia minima*.

Key words: grapevine, fungus, Petri disease, wood.

Introduction

Crous *et al*. (1996) described the genus *Phaeoacremonium* with six species. Four of them, *Pm. chlamydosporum*, *Pm. aleophilum*, *Pm. angustius* and *Pm. inflatipes*, were isolated from wood of grapevines (*Vitis* spp.). Subsequently, molecular phylogenetic work of Dupont *et al*., using partial rDNA sequencing, indicated that the genus was heterogeneous and suggested that while *Pm. chlamydosporum* appeared most closely allied to the ascomycete order *Chaetothyriales*, family *Herpotrichiellaceae* (members of which have anamorphs in the similar genus *Phialophora*), the remainder of the genus showed closest affinities to the diaporthalean family *Magnaporthaceae*. Tegli *et al*. (2000a,b) confirmed the degree of divergence between *Pm. chlamydosporum* and *Pm. aleophilum* and agreed that separate genera were needed. Crous and Gams (2000) confirmed the conclusions of Dupont *et al*. (1998) and assigned *Pm. chlamydosporum* to the new genus *Phaeomoniella* Crous & W. Gams. Groenewald *et al*. (2001) published molecular phylogenetic studies confirming the isolated position of *Phaeomoniella chlamydospora*, and demonstrating the close degree of relatedness of remaining species of *Phaeoacremonium*. Tegli *et al*. (2000a) studied the genetic variability of *Pm. aleophilum* and *Pa. chlamydospora* (as *Phaeoacremonium chlamydosporum)* in Italy and found it to be low in both species but higher in *Pm. aleophilum* than in *Pa. chlamydospora*. They suggested that sexual reproduction possibly occurred in both species. Similar conclusions were made by Peros *et al*. (2000) who studied the genetic variability of populations present in French vineyards. Subsequently, Tegli (2001) concluded that sexual reproduction made a major contribution to the genetic structure of populations of *Pm. aleophilum*.

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In 2000, whilst surveying large numbers of grapevine trunks for the presence of these and other fungi, we observed dark, long-necked ascocarps growing amongst extensive colonies of *Pa. chlamydospora* and other fungi after a lengthy period of moist incubation of grapevine trunk pieces. Believing that we may have discovered a teleomorph for *Pa. chlamydospora*, we carried out single-ascospore and single-ascus isolations and consistently recovered colonies identical to typical colonies of *Pm. aleophilum*. This paper describes the morphological and molecular characterisation of the ascomycete and provides proof that it is the teleomorph of *Phaeoacremonium aleophilum*.

Materials and methods

Moist incubation

Pieces of grapevine wood from established vines with vascular streaking were cut transversely and longitudinally to half cylinders up to 10 cm long. They were moist-incubated by placing them on moist paper towelling in suitably sized plastic boxes (take-away food containers) with lids, and incubating at room temperature (18–22°C). The paper towelling was moistened periodically as required. Samples were examined under the dissecting microscope and the fungi present were noted.

Microscopy

When ascocarps were first observed, squash preparations were made to give an indication of the morphology of asci and ascospores. Mounts were made in water, lactic acid and Melzers reagent. Due to the dense melanisation of the ascoma wall, visualisation of the wall tissue required bleaching in 0.5% sodium hypochlorite. Drawings were prepared using a drawing tube.

Culturing

Ascocarps were observed surrounded by conidiophores of *Pa. chlamydospora* and other fungi. Consequently it was acknowledged that attempts to culture from the ascocarps ran the risk of contamination with fungi other than the ascomycete. Because it was difficult to differentiate single ascospores from conidia of *Pa. chlamydospora* at low magnifications, in addition to single-ascospore isolations we attempted single-ascus isolations and single-centrum isolations from isolate VPRI 22559. Single-ascospore isolations were made either by squashing an ascocarp in a drop of sterile water on a microscope slide, or alternatively by taking a mass of ascospores from the tip of the ascocarp neck and suspending the spores in a drop of water. The resulting spore suspensions were streaked with a bacteriological loop onto potato dextrose agar (PDA) in a Petri dish. Single-ascus isolations were made by excising the centrum from an ascocarp with a sterile fragment of razor blade, gently teasing the centrum apart in a drop of sterile water on a microscope slide and streaking out the resulting suspension of asci onto PDA. Single-centrum isolations were made by excising the centrum from an ascocarp and stab-inoculating the centrum onto PDA. Cultures were incubated at 25°C in the light for up to 24 h before examination under the high power (×60) of a dissecting microscope using transmitted-light illumination. In this way, single asci were readily identified and subcultured using a fine needle, while single ascospores could not be differentiated with confidence from conidia. Germinated spores or identifiable asci were subcultured onto fresh plates of PDA, where they were incubated at 25°C until identifiable colonies had grown. Single-centrum cultures were not subcultured until identifiable colonies had formed.

DNA extraction

A single-ascospore isolate (ex VPRI 22559) was grown on PDA for 2 weeks at 22°C. Twelve 1 cm^2 pieces were cut from the growing edge of the colony and transferred into an Erlenmeyer flask containing 100 ml PD broth. The broth culture was incubated for 8 days at room temperature (18–22°C) on a shaker table operating continuously at 150 rpm. Mycelium was harvested by vacuum filtration onto sterile cheesecloth, thoroughly washed with sterile distilled water and then freeze-dried. DNA was extracted from the freeze-dried mycelium according to the protocol described by Lee and Taylor (1990), modified with the addition of 1 mg ml^{-1} RNAase (Progen Ltd., Darra, Australia) to the lysis buffer. The DNA concentration was adjusted to 5 ng μl^{-1}.

rDNA ITS sequencing and phylogenetic analysis

The rDNA ITS region was amplified using PCR primers ITS5 and ITS4 according to White *et al.* (1990), with the exception that the total reaction

volume was 25 µl and using 5 ng of DNA template. The PCR product was visualized on a 1.4% agarose gel containing ethidium bromide.

The PCR product was purified using a Qiagen PCR Product Purification kit (Qiagen, Clifton Hill, Australia). Both strands were sequenced with primers ITS5 and ITS4 using the PE Applied Biosystems Bigdye (Perkin Elmer, Scoresby, Australia) technology according to the manufacturer's instructions. The sequencing products were read on an ABI 373 DNA sequencer. The forward and reverse sequences were compared to determine the complete ITS sequence.

To determine the relationship between our isolate and other fungi including *Pm. aleophilum*, the ITS sequence was compared with relevant sequences in GenBank with a Blast search (Altschul *et al*., 1997). Using ClustalX (Thompson *et al*., 1997), the *Togninia* sp. ITS sequence was aligned with ITS sequences from the ex-type cultures of all described *Phaeoacremonium* species, a selection of additional *Pm. aleophilum* isolates, the published ITS1 sequence of *Togninia novae-zealandiae* (Hausner *et al.,* 1992) and an unusual *Pm. angustius* sequence (GenBank AF295138). The ITS sequence for *Amphisphaeria umbrina* was included as an outgroup (Groenewald *et al*., 2001). A neighbour-joining tree was constructed using MEGA ver 2.1 (Kumar *et al.,* 2001) with 1000 bootstrap replicates. As the *T. novae-zealandiae* sequence covered only the ITS1 region, a distance matrix, using the Kimura 2-parameter method, was calculated using pairwise deletions of gaps and missing data.

Results

Moist incubation

Ascocarps were first noticed on wood of grapevine cv. Riesling from Kyneton, Victoria, Australia, (VPRI 22559) after 157 days moist incubation (specimen collected 18 May 2000, moist incubation prepared 22 May, ascocarps first noticed 16 October). Subsequent samples took 99 days (VPRI 30331), 147 days and 77 days (specimens lost) before ascomata were observed.

Culturing

The predominant fungus isolated by single-ascospore culturing was similar in cultural characteristics to *Pm. aleophilum*, although there was some contamination with *Pa. chlamydospora* and other fungi. In all single-ascus and single-centrum isolations the only fungus isolated was *Pm. aleophilum*-like. These colonies (on PDA) were honey-coloured with greyish aerial mycelium and a yellow pigment diffusing into the agar, typical of *Pm. aleophilum*.

Molecular analysis

The Blast search showed the *Togninia* sp. ITS sequence to be identical or nearly identical (differing at 1 to 3 bases) to all *Pm. aleophilum* sequences in GenBank. The sequence has been deposited in GenBank and given the accession number AY159787. The neighbour-joining tree revealed it to cluster among the *Pm. aleophilum* sequences (Fig. 1). The *T. novae-zealandiae* sequence clustered closest to the sequence of CBS 777.83 (isolated from soil, Argentina), identified as *Pm. angustius* by Crous *et al*. (1996) and Tegli *et al*. (2000a).

Morphological description of the Togninia *teleomorph of* Pm. aleophilum *found in Australia*

Ascomata (Fig. 2 and 3) occurring on bark or exposed wood of *Vitis* spp., single or loosely gregarious amongst a weft of mycelium and conidiophores of the *Phaeoacremonium aleophilum* anamorph (and frequently mixed with other fungi including *Phaeomoniella chlamydospora*). Ascomata black, sphaerical, 150–250 µm diam., with an elongated, tapered neck 150–500 µm long, about 50 µm wide at the base, tapering to 30 µm at the apex; frequently all necks of a group of ascomata curved in the same direction, possibly phototropic (Fig. 2b). Ascocarp wall thick, black, surface layer of textura epidermoidea, the outer cells dark brown, of up to 20 layers of compressed cells, with an inner layer of hyaline cells, 4–6 cells thick, perithecial appendages short, dark hyphae, neck of dark brown textura epidermoidea, verrucose, with a simple, slightly paler apical ostiole composed of slender, paler, incurved hyphae. Centrum consisting of fascicles of paraphyses and ascogenous hyphae which are easily dislodged from the inner hyaline wall layers. Paraphyses hyaline, unbranched, or branched at the base, septate, the septa occurring at intervals of 5–12 µm, cells swollen (monilioid) at the base, 3.5–4.5 µm wide at the base, 2.5–3.5 µm at the apical septum which usually occurs within 6–12 µm of the apex, the apical cell usually ta-

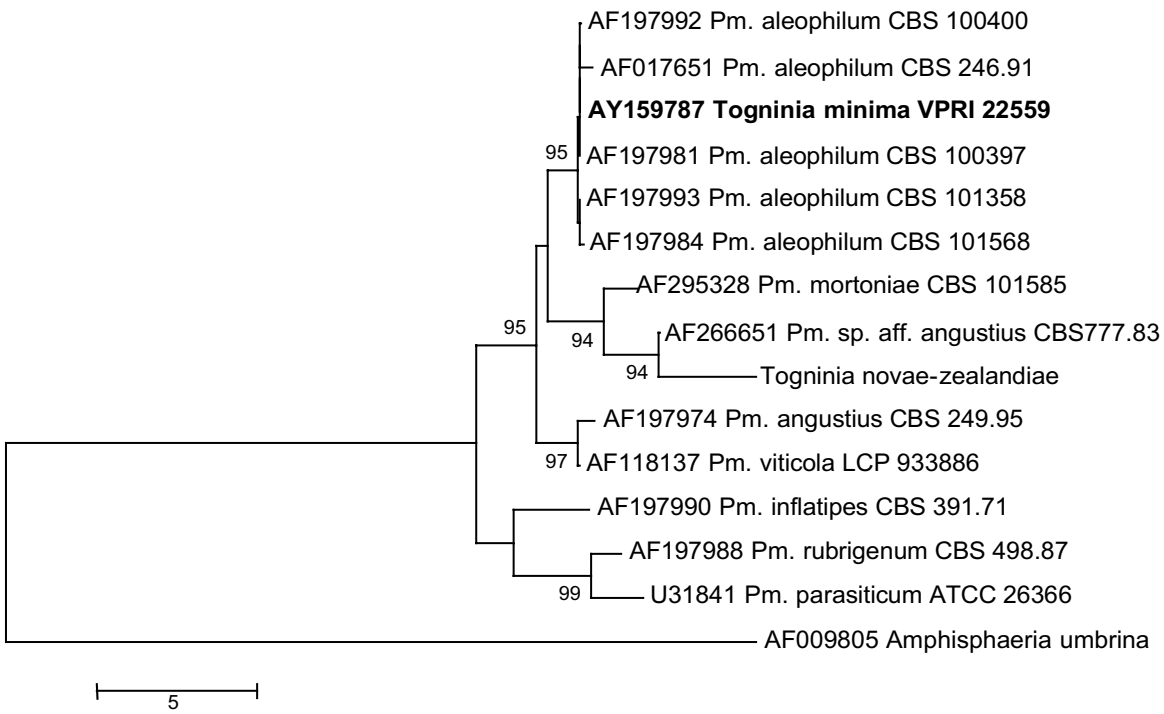

Fig. 1. Phylogenetic tree based on ITS sequences for ex-type cultures of all *Phaeoacremonium* species, four additional isolates of *Pm. aleophilum*, VPRI 22559 (our *Togninia* sp.), *Togninia novae-zealandiae* (from published sequence of Hausner *et al*. 1992), and CBS 777.83 (*P.* sp. aff. *angustius*). Bootstrap numbers lower than 90% are not shown. Scale bar indicates changes per 100 bases. All data except VPRI 22559 and *T. novae-zealandiae* from GenBank.

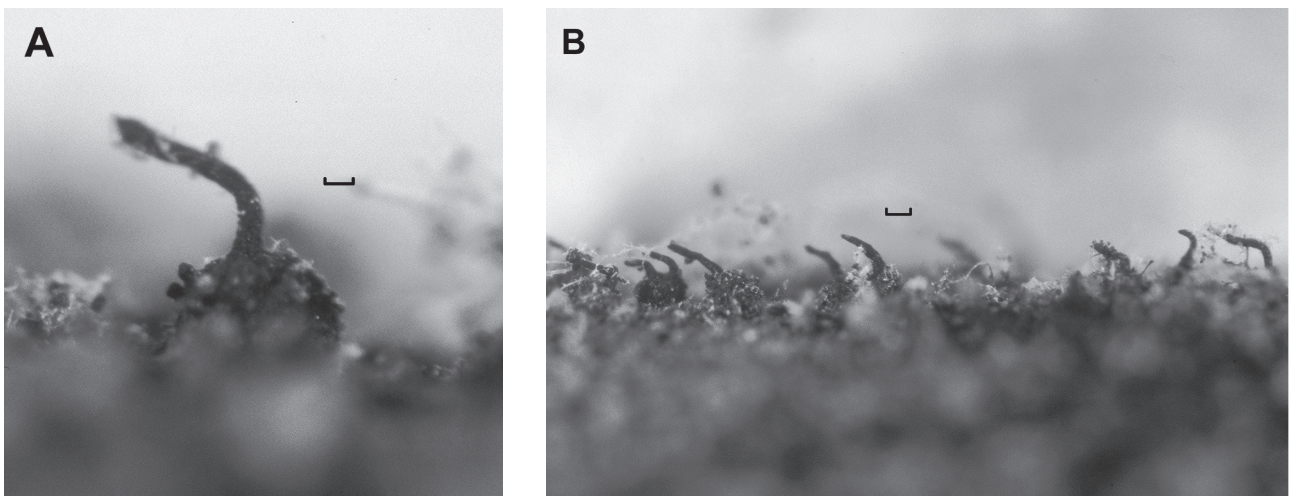

Fig. 2. Ascomata of *Togninia minima* on moist incubated grapevine wood. A. Single ascoma. B. Group of ascomata showing probable phototropic alignment. Scale bars: A = 50 μm; B = 100 μm.

pering abruptly to a point less than 2 μm thick. Asci in fascicles of 5–20 inserted acrogenously on a sympodially proliferating ascogenous hypha arising from the same hyaline pseudoparenchyma cells as the paraphyses. Asci small, sessile on the ascogenous hyphae, unitunicate, oblong to clavate, 14–20×3.5–5.5 μm, with a thick, rounded apical cap 1.5–2.0 μm thick, with a barely visible, inamyloid, cylindrical apical apparatus 1–1.5×1 μm, ascus base truncate, often displaced laterally, basal truncation 1.5–2 μm wide. After secession of the asci, a partial outer ascus wall up to half the length of an ascus remains attached to the ascogenous hypha. Ascospores 8, loosely arranged or biseriate, ellipsoid-oblong to allantoid, 3.5–5.5×1–1.8 μm, each with a single small guttule at each end.

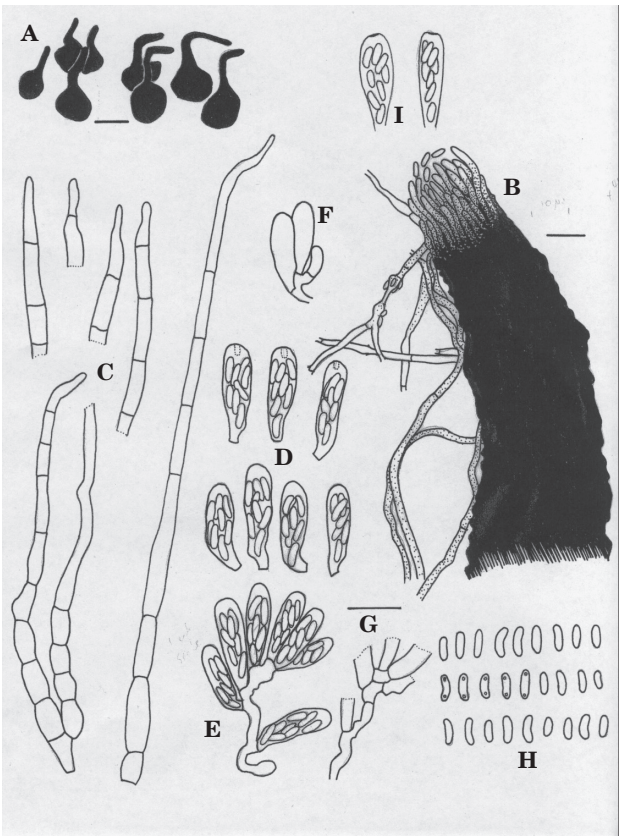

Fig. 3. *Togninia minima* teleomorph A. Ascomata. B. Ascoma neck with ostiolar hyphae, and hyphae and conidiophores of the *Phaeoacremonium* anamorph. C. Paraphyses. D. Asci. E. Ascogenous hypha. F. Immature asci. G. Spent ascogenous hypha, with basal frills. H. Ascospores discharged from ascoma neck. I. Asci discharged from ascoma neck (scale bars 100 and 10 μm).

Anamorph *Phaeoacremonium aleophilum* (Fig. 4). Colonies on MEA growing optimally at 35°C, growth rates variable, 3.5–15.5 mm in 15 days at 35°C. Hyphae forming mid-brown ropes from which arise either single phialides or hyphae which bear various combinations of single phialides or more complex conidiophores. Single hyphae are pale brown, the pigment extending into the conidiophores but becoming progressively more dilute apically, leaving the phialides more or less hyaline. Phialides mono- or less often polyphialidic, slender, tapered, often proliferating percurrently, with a cylindrical or slightly flared collarette. Conidia ellipsoid to allantoid, 2–5×1–1.5 μm.

Found on moist incubated wood of *Vitis vinifera* cv. Riesling, Virgin Hills vineyard, Kyneton, Victoria, Australia, J. Edwards (00-030), 18 May, 2000, VPRI 22559.

Additional specimens examined

Vitis vinifera cv. Mataro, Eagle Spirit Vineyard, Auburn, South Australia, P. Speakman, 14 June 2002, VPRI 30331; cv. Merlot on Kober 5BB, Avoca Vineyard, Wentworth, NSW, D. Dawes, October 2000 (2 specimens, lost); cv. Sultana on Ramsey, Mildura, Victoria, G. Fletcher, November 2000 (specimen lost).

Our specimens of *Togninia* agree well with the description of *T. minima* by Mostert *et al*. (2003) but differ slightly from the description of *T. minima* published by Hausner *et al*. (1992), in which the fungus is described with longer asci and ascospores (asci 20–30 μm long, ascospores 5.0–6.5 μm long, *fide* Hausner *et al*., 1992). However, our material differs more substantially from *T. fraxinopennsylvanica,* which has cylindrical or slightly curved ascospores with truncate ends, and a different anamorph; from *T. novae-zealandiae* which has broader ascospores (2.2–2.6 μm wide); and from *T. inconspicua* O.E. Eriksson & J.Z. Yue which has much longer ascospores (8–10 μm long). Although our isolates have smaller asci and ascospores than *T. minima*, as described by Hausner *et al*. (1992), we concede that this may be caused by natural variation due to different environmental and cultural conditions. We agree therefore with Mostert *et al*. (2003) that *T. minima* is the most appropriate name for the teleomorph of *Pm. aleophilum*.

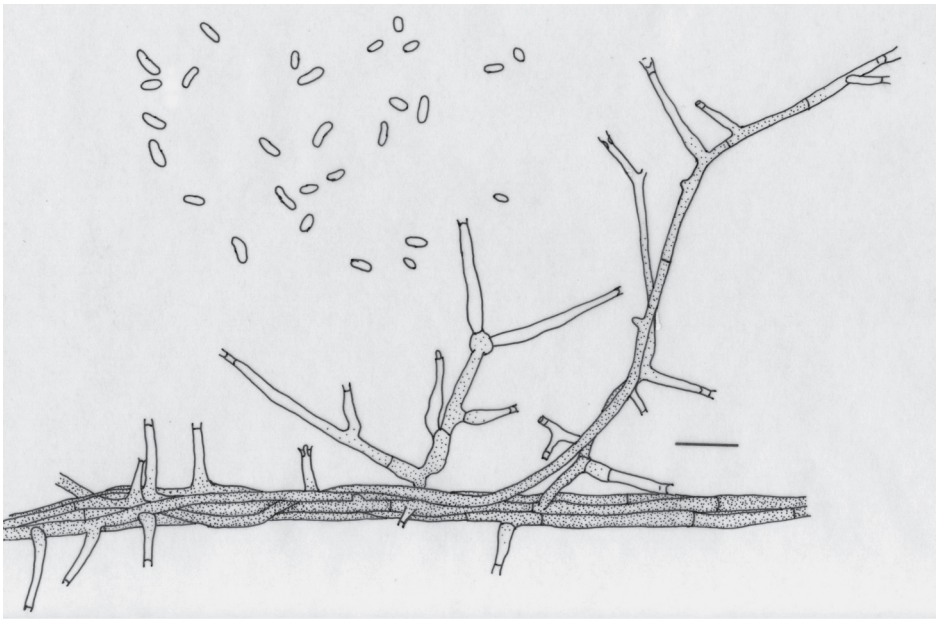

Fig. 4. *Togninia minima*. *Phaeoacremonium aleophilum* anamorph, grown on PDA for 2 weeks at 25°C (scale bar 10 μm).

Discussion

Hausner *et al*. (1992) described in detail the teleomorph of *Togninia minima* and the teleomorphs, anamorphs and cultural characteristics of two additional species of *Togninia* Berl. Anamorphs were said to be intermediate between *Phialophora* Medlar and *Acremonium* Link. Their illustrations of *Togninia* species show remarkable similarities to our ascomycete and their anamorphs appear to be good *Phaeoacremonium* species. Included in the paper were rDNA ITS1 sequences for two species. Although their sequence data is not present in GenBank, a Blast search (Altschul *et al*. 1997) of the published sequence for *T. novae-zealandiae* Hausner *et al*. (1992) confirms that their species is related to species of the genus *Phaeoacremonium*. Based on the descriptions provided by Hausner *et al*. (1992), our ascomycete was identified as a species of *Togninia* and our work confirms the results of Mostert *et al*. (2003) who independently discovered the teleomorph of *Pm. aleophilum* and identified it as *Togninia minima*. Rooney (2002) also found, but did not identify, the teleomorph of *Pm. aleophilum*. The taxonomy and nomenclature of *Togninia* has been comprehensively discussed by Mostert *et al*. (2003).

Eriksson and Yue (1990) figured a cylindrical apical apparatus in the ascus of *T. inconspicua*, which is also visible (but barely) in our material when mounted in water or Melzer's reagent. While ascus dehiscense has not been observed in our material, two observations suggest dehiscence by breakage of the outer wall somewhat above the basal septum. Firstly, remnants of the ascus wall (Fig. 3G) remain on the ascogenous hypha after dehiscence (also illustrated by Hausner *et al*., 1992). Secondly, groups of 8 ascospores can be found occasionally amongst discharged ascospores at the tip of the ascoma neck, surrounded by a thin membrane but lacking the truncate base of intact asci (Fig. 3I).

Hausner *et al*. (1992) were able to produce ascocarps of *Togninia* spp. *in vitro* by pairing strains isolated from dark streaks in wood. Recently, other researchers such as Rooney (2002) and Mostert *et al*. (2003) have also successfully paired isolates of *Phaeoacremonium* species from grapevines and induced ascoma production in culture. Mostert *et al*. (2003) commented that it is not known under which conditions ascocarps are produced in the field. From our moist incubation work we can tentatively predict that ascocarps of *T. minima* develop on the outside or in crevices of infected, proba-

bly dead wood, over a period of between 2–5 months.

Hausner and co-workers also commented that production of perithecia by *T. novae-zealandiae* appeared to be stimulated by other fungi, which it overgrew. This suggested to Hausner *et al*. that the fungus was either mycoparasitic or obtained nutrients from other fungi. This concurs with our observation that perithecia were found mixed with other fungi, in particular *Pa. chlamydospora*, under moist incubation conditions, where an abundance of other fungi was present. There is already some evidence that *Pa. chlamydospora* and *Pm. aleophilum* occur together in a competitive or antagonistic relationship (Sparapano *et al*., 2000).

The discovery of the *Togninia minima* teleomorph of *Phaeoacremonium aleophilum* coupled with Hausner *et al*.'s illustrations and descriptions of *Togninia* anamorphs, and ITS sequence data, leaves little room for doubt that all *Phaeoacremonium* taxa will have teleomorphs in *Togninia* or similar calospherialean genera. The morphology of teleomorphs of *Phaeoacremonium* taxa can be expected to provide additional criteria for identification. The biology of *Togninia* and related genera (wood-inhabiting endophytes associated with vascular streaking) should provide additional clues to the ecology of the *Phaeoacremonium* taxa associated with grapevine.

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