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# *Togninia* (Calosphaeriales) is confirmed as teleomorph of *Phaeoacremonium* by means of morphology, sexual compatibility and DNA phylogeny

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***Abstract:*** Petri disease, or black goo, is a serious disease of vines in most areas where grapevines are cultivated. The predominant associated fungus is *Phaeomoniella chlamydospora* (Chaetothyriales). Several species of *Phaeoacremonium* (*Pm.*) also are associated, of which *Pm. aleophilum* is the most common. Although no teleomorph is known for *Phaeoacremonium*, the genus *Togninia* previously has been linked to phaeoacremonium-like anamorphs. To investigate the possible anamorph-teleomorph connection of *Phaeoacremonium* to *Togninia*, anamorphs of *Togninia minima*, *T. fraxinopennsylvanica* and *T. novae-zealandiae* morphologically were compared with *Pm. aleophilum* and some representative cultures were mated in all combinations. Although no interspecies mating proved fertile, matings between isolates of *Pm. aleophilum* produced a *Togninia* teleomorph within 3–4 weeks. Certain field isolates of *Pm. aleophilum* commonly produced the teleomorph, demonstrating that both mating types can occur in the same vine and thus also explaining the genetic diversity observed for this fungus in some vineyards. To elucidate the phylogenetic relationships among these taxa, isolates were subjected to sequence analysis of the nuclear ribosomal internal transcribed spacers (ITS1, ITS2) and the 5.8S rRNA gene, as well as portions of the translation elongation factor 1 alpha (EF-1α) gene. The generic placement of teleomorphs within *Togninia* (Calosphaeriales) further was confirmed via phylogenetic analyses of 18S small subunit (SSU) DNA. From these sequences, morphological and mating data, we conclude that *T. minima* is the teleomorph of *Pm. aleophilum*, and that it has a biallelic heterothallic mating system. An epitype and mating type tester strains also are designated for *T. minima*.

***Key words:*** Calosphaeriales, EF-1α, ITS, 18S SSU DNA, sexual compatibility, systematics

## INTRODUCTION

Petri disease is a well-known disease of grapevines worldwide (Mugnai et al 1999). Affected grapevines exhibit a slow dieback as well as stunted growth. The predominant associated fungus is *Phaeomoniella chlamydospora* W. Gams, Crous, M.J. Wingf. & L. Mugnai (Chaetothyriales, Herpotrichiellaceae). Whether this fungus is the sole or only a contributing causal agent of the disease is uncertain. Several species of *Phaeoacremonium* (*Pm.*) (Diaporthales, Magnaporthaceae) also commonly grow from vines affected by Petri disease, including *Pm. aleophilum* W. Gams, Crous, M.J. Wingf. & L. Mugnai, *Pm. angustius* W. Gams, Crous & M.J. Wingf., *Pm. inflatipes* W. Gams, Crous & M.J. Wingf., *Pm. mortoniae* Crous & W. Gams, *Pm. parasiticum* (Ajello, Georg & C.J.K. Wang) W. Gams, Crous & M.J. Wingf., *Pm. rubrigenum* W. Gams, Crous & M.J. Wingf., and *Pm. viticola* Dupont. Of the *Phaeoacremonium* species associated with Petri disease, *Pm. aleophilum* consistently has been the most frequently isolated (Scheck et al 1998, Ari 2000, Gatica et al 2001). In various studies, *Pm. aleophilum* has been found to be associated with brown-streaking symptoms in Petri-diseased grapevines (Mugnai et al 1999, Ari 2000) and sectorial brown necrosis (Gatica et al 2001).

The genetic variation within populations of *Phaeomoniella chlamydospora* and *Pm. aleophilum* has been studied by various workers (Péros et al 2000, Tegli et al 2000a, b). Using RAPDs (Random Amplified Polymorphic DNA) and RAMS (Random Amplified Micro- or Mini-Satellites), Tegli et al (2000b) showed that considerable variation existed among isolates of *Pm. aleophilum* collected from the same field, sug-

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gesting that sexual reproduction might occur (Tegli 2000). Considerable genetic variation suggestive of ongoing recombination also was found in Universally Primed-PCR studies done with *Pm. aleophilum* isolates from Australia (Cottral et al 2001). Rooney et al (2002) subsequently reported inducing teleomorphs for *Pm. inflatipes* and *Pm. aleophilum* under laboratory conditions.

In a study of the genus *Togninia* Berl. (Calosphaeriales), Hausner et al (1992) treated *Togninia minima* (Tul. & C. Tul.) Berl. (lectotype of *Togninia*) and at the same time they described two new species, *T. fraxinopennsylvanica* (Hinds) Hausner, Eyjólfsdóttir & J. Reid and *T. novae-zealandiae* Hausner, Eyjólfsdóttir & J. Reid. Although no cultures of *T. minima* were available, the two newly described species were cultured and were shown to produce anamorphs that were intermediate between *Acremonium* Link : Fr. and *Phialophora* Medlar. Several anamorph genera in recent years have been described with this approximate appearance (Gams 2000). Of these, the genus *Phaeoacremonium* W. Gams et al closely fits the description of the *Togninia* anamorphs illustrated by Hausner et al (1992).

The first aim of the current study was to investigate the possible link between *Phaeoacremonium* and *Togninia*. *Phaeoacremonium aleophilum*, a species similar in appearance to the anamorphs illustrated by Hausner et al (1992), was chosen for morphological comparison with the anamorphs of *T. minima*, *T. fraxinopennsylvanica* and *T. novae-zealandiae*. A further aim was to determine if the formation of a teleomorph could be elicited in *Pm. aleophilum*. This was done by mating a selection of vine isolates in culture. A final aim was to elucidate the genetic diversity within and among these species and to clarify the higher-order phylogenetic placement of *Togninia*. To address this, a phylogenetic analysis was conducted using sequences of the nuclear ribosomal DNA region encompassing the internal transcribed spacers (ITS1, 5.8S and ITS2), as well as translation elongation factor 1 alpha (EF-1α), and 18S ribosomal small subunit (SSU).

## MATERIALS AND METHODS

*Morphology.*—Field isolations were made from rooted nursery plants and older, diseased grapevines, from which single-conidial isolates were obtained (TABLE I). Anamorph morphology was studied on 2% malt-extract agar (MEA; Biolab, Midrand, South Africa), while perithecia were induced on twice-autoclaved pieces of grapevine cane placed on 2% water agar (Biolab) (GWA). Cultures were incubated at 22 C under a 12 h fluorescent white light/dark regime. For microscopy, material was mounted in lactic acid. Thirty measurements were taken of each type of morphological structure, and averages and 95% confidence intervals were determined for spore dimensions. Measurements are given with minimum and maximum ranges in parentheses.

Vertical sections (10 μm) of fruiting bodies were cut with a Leica CM1100 freezing microtome. Colony colors were determined according to Rayner (1970). Cultures are maintained in the collection of the Department of Plant Pathology at the University of Stellenbosch, and representative strains have been deposited at the Centraalbureau voor Schimmelcultures (CBS, Utrecht, the Netherlands). Reference strains of *T. fraxinopennsylvanica* (CBS 110212, ex-type), *T. novae-zealandiae* (UAMH 9589, UAMH 9590, ex-type) and *T. minima* (CBS 213.31, ex-type of *Longoa paniculata* Curzi) also were studied.

*Matings.*—Twenty-one *Pm. aleophilum* isolates were grown on MEA plates for 2 wk, using 10 plates per isolate. Conidia were dislodged from the agar surface by means of a glass rod, and suspensions were prepared in 5 mL sterile distilled water. Two aliquots of 100 μL each, representing two different isolates, were pipetted onto the canes of GWA plates. Isolates were mated in all possible combinations. Controls consisted of a 200 μL aliquot of one isolate only. Plates were incubated at 22 C under continuous white light. Successful crosses were noted 3–4 wk after mating. For a mating to be considered successful, perithecia had to produce large quantities of ascospores that germinated readily in culture. One such mating was chosen (LM 54 × LM 463), and 20 single ascospore isolates obtained (LM 227–LM 240, LM 243–LM 247, LM 249). Further crosses were made with these ascospore isolates using the procedure described above. Two strains found to be of opposite mating type arbitrarily were designated as MAT1-1 (LM 463) and MAT1-2 (LM 54). Inter-species matings were done to investigate the biological species boundaries of *Pm. aleophilum*, *T. minima*, *T. novae-zealandiae* and *T. fraxinopennsylvanica*.

*DNA isolation and amplification.*—Twenty-seven *Pm. aleophilum* isolates were selected for sequence comparisons (TABLE I). Sequences of the ITS, EF-1α and SSU of *T. fraxinopennsylvanica* (CBS 110212), *T. novae-zealandiae* (UAMH 9589 and UAMH 9590), *T. minima* (CBS 213.31) and an unknown *Phaeoacremonium* sp. (STE-U 3394) also were included. Genomic DNA was extracted using the isolation protocol of Lee and Taylor (1990). In studies intended to determine the degree of genetic diversity within *Pm. aleophilum*, the 5.8S nuclear ribosomal RNA gene and the flanking internal transcribed spacers (ITS1 and ITS2) were amplified with primers ITS1 and ITS4 (White et al 1990) and translation elongation factor 1 alpha (EF-1α) was amplified with primers EF1-728F and EF1-986R (Carbone and Kohn 1999). These PCR amplification cycles run on a GeneAmp PCR System 2700 (Perkin-Elmer, Norwalk, Connecticut) were for both regions: 96 C for 5 min, followed by 36 cycles of (1) denaturation (94 C for 30 s), (2) annealing (50 C for 30 s) and (3) elongation (72 C for 90 s), and a final 7 min extension step at 72 C.

In studies intended to determine the higher order phylogeny of *Togninia*, primers NS1 and NS4 (White et al 1990) were used to amplify the 5′ end of the 18S ribosomal

TABLE I. *Phaeoacremonium* and *Togninia* isolates studied

| Species | Culture No. | GenBank No. ITS | GenBank No. EF | GenBank No. SSU | Host and location |
|---|---|---|---|---|---|
| *Togninia minima* (Anamorph: *Pm. aleophilum*) | LM44[c] | | | | *Vitis vinifera*, Paarl, South Africa |
| | LM45[c] | | | | *Vitis vinifera*, Paarl, South Africa |
| | LM46[b] | | | | *Vitis vinifera*, Paarl, South Africa |
| | LM47[c] | | | | *Vitis vinifera*, Paarl, South Africa |
| | LM48[c] | | | | *Vitis vinifera*, Paarl, South Africa |
| | LM49 = CBS 110701[b] | | | | *Vitis vinifera*, Paarl, South Africa |
| | LM50 = CBS 110831[c] | | | | *Vitis vinifera*, Paarl, South Africa |
| | LM51[b] | | | | *Vitis vinifera*, Paarl, South Africa |
| | LM53 = CBS 110702[c] | | | | *Vitis vinifera*, Paarl, South Africa |
| | LM54 = CBS 110703[a,c] | | | | *Vitis vinifera*, Paarl, South Africa |
| | LM56 = CBS 110705[b] | | | | *Vitis vinifera*, Paarl, South Africa |
| | LM58[b] | | | | *Vitis vinifera*, Paarl, South Africa |
| | LM61[c] | | | | *Vitis vinifera*, Paarl, South Africa |
| | LM65 = CBS 110711[b] | | | | *Vitis vinifera*, Paarl, South Africa |
| | LM76 = CBS 110827[b] | | | | *Vitis vinifera*, Paarl, South Africa |
| | LM77[c] | | | | *Vitis vinifera*, Paarl, South Africa |
| | LM78[b] | | | | *Vitis vinifera*, Paarl, South Africa |
| | LM467[c] | | | | *Vitis vinifera*, Stellenbosch, South Africa |
| | LM468 = CBS 110753[b] | | | | *Vitis vinifera*, Stellenbosch, South Africa |
| | LM458 = CBS 111014[c] | | | | *Vitis vinifera*, Wellington, South Africa |
| | LM463 = CBS 111015[a,b] | AY179930 | AY179896 | | *Vitis vinifera*, Wellington, South Africa |
| | LM4 = CBS 110707 | AY179935 | AY179901 | | *Vitis vinifera*, Robertson, South Africa |
| | LM5 = CBS 110830 | AY179929 | AY179895 | | *Vitis vinifera*, Robertson, South Africa |
| | LM12 = CBS 110828 | AY179932 | AY179898 | | *Vitis vinifera*, Paarl, South Africa |
| | LM23 = CBS 110835 | AY179933 | AY179899 | | *Vitis vinifera*, Paarl, South Africa |
| | LM34 = CBS 110708 | AY179928 | AY179894 | | *Vitis vinifera*, Paarl, South Africa |
| | LM74 = CBS 110709 | AY179937 | AY179903 | | *Vitis vinifera*, Paarl, South Africa |
| | LM119 = CBS 110834 | AY179936 | AY179902 | | *Vitis vinifera*, Paarl, South Africa |
| | LM466 | AY179925 | AY179891 | | *Vitis vinifera*, Wellington, South Africa |
| | LM440 | AY179927 | AY179893 | | *Vitis vinifera*, Wellington, South Africa |
| | CBS 246.91[d] | AY179934 | AY179900 | | *Vitis vinifera*, Yugoslavia |
| | CBS 100402 | AY179938 | AY179904 | | *Vitis vinifera*, Italy |
| | CBS 101006 | AY179939 | AY179905 | | *Actinidia chinensis*, Italy |
| | CBS 101357 | AY179942 | AY179908 | | *Actinidia chinensis*, Italy |
| | CBS 100400 | AY179941 | AY179907 | | *Vitis vinifera*, Italy |
| | CBS 100399 | AY179940 | AY179906 | | *Vitis vinifera*, Italy |
| | CBS 100398 | AY179931 | AY179897 | | *Vitis vinifera*, Italy |
| | LM24 = CBS 110833 | AY179923 | AY179889 | | *Vitis vinifera*, Paarl, South Africa |
| | LM44 | AY179950 | AY179916 | | *Vitis vinifera*, Paarl, South Africa |
| | LM52 = CBS 110832 | AY179951 | AY179917 | | *Vitis vinifera*, Paarl, South Africa |

TABLE I. Continued

| Species | Culture No. | GenBank No. ITS | EF | SSU | Host and location |
|---|---|---|---|---|---|
| | LM75 | AY179952 | AY179919 | AY179956 | *Vitis vinifera*, Paarl, South Africa |
| | LM83 = CBS 110829 | AY179953 | AY179921 | | *Vitis vinifera*, Paarl, South Africa |
| | LM113 | AY179954 | AY179920 | | *Vitis vinifera*, Paarl, South Africa |
| | LM115 = CBS 111016 | AY179955 | AY179918 | | *Vitis vinifera*, Paarl, South Africa |
| | LM441 | AY179924 | AY179890 | | *Vitis vinifera*, Wellington, South Africa |
| | LM443 | AY179926 | AY179892 | | *Vitis vinifera*, Wellington, South Africa |
| | LM460 | AY179922 | AY179888 | | *Vitis vinifera*, Wellington, South Africa |
| | CBS 213.31 | AY179943 | AY179909 | AY179957 | Unknown, Italy |
| *Togninia novae-zealandiae* | UAMH 9589 | AY179944 | AY179910 | | *Cupressus macrocarpa*, New Zealand |
| | UAMH 9590 | AYA79445 | AY179911 | AY179958 | *Pinus radiata*, New Zealand |
| *Togninia fraxinopennsylvanica* | CBS 110212 | AY179947 | AY179913 | AY179959 | *Fraxinus pennsylvanica*, USA |
| *Phaeacremonium* sp. | STE-U 3394 | AY179946 | AY179912 | | *Desmoschoenus spiralis*, New Zealand |

[a] Designated mating type tester strains.
[b] Mat1-1 mating type.
[c] Mat1-2 mating type.
[d] Ex-type culture of *Pm. aleophilum*.

DNA (SSU) gene for a subset of four isolates representing the different *Togninia* species and *Pm. aleophilum*. The cycling conditions consisted of an initial denaturation step of 94 C for 7 min, followed by 36 cycles of (1) denaturation (95 C for 45 s), (2) annealing (55 C for 60 s) and (3) elongation (72 C for 120 s), and finally a 2 min extension step at 72 C.

PCR products were analyzed by electrophoresis at 85 V for 30 min in a 0.8% (w/v) agarose gel in 0.5 × TAE buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M ethylene diamine tetraacetic acid [EDTA], pH 7.85) and visualized under UV light with a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, United Kingdom) following ethidium bromide staining.

PCR products were purified according to the manufacturer's instructions using a commercial kit (Nucleospin Extract 2 in 1 Purification Kit, Machery-Nagel GmbH & Co., Germany). Sequencing reactions were carried out with ABI PRISM Big Dye Terminator version 3.0 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, California), according to the manufacturer's recommendations, and were analyzed on an ABI Prism 3100 DNA Sequencer (Perkin-Elmer, Norwalk, Connecticut). Sequences were deposited at GenBank (TABLE I), and the alignment was deposited in TreeBase (ITS and EF-1α: SN1269–3614; SSU: SN1269–3617).

*Phylogenetic analysis.*—Raw sequence data were analyzed using EditView 1.0.1 (http://www.appliedbiosystems.com), and sequences were manually aligned by inserting gaps. Phylogenetic analyses were conducted using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2000). Gaps were treated as a fifth character, and all characters were unordered and of equal weight. *Phialophora richardsiae* (Nannf.) Conant (CBS 270.33, GenBank ITS = AY179948, EF-1α = AY179914) and *Cercospora apii* Fresen. (CBS 119.25, GenBank ITS = AY179949, EF-1α = AY179915) were used as outgroups for both the EF-1α and ITS analyses. Six *Pm. aleophilum* isolates (LM 44, LM 52, LM 75, LM 83, LM 113, and LM 115) were excluded from the combined analyses. Their respective EF and ITS sequences were 100% similar to the sequences of LM 24, LM 441, LM 466, LM 443, LM 440, LM 463, LM 460, LM 34 and LM 5. Maximum-parsimony analysis was performed using the heuristic search option with a 1000 random-taxon additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Bootstrap support for the ITS and EF-1α analysis for internal branches was evaluated from 1000 heuristic search replicates and 1000 random taxon additions. Tree length, consistency index (CI), retention index (RI) and the rescaled consistency index (RC) values also were calculated. A partition homogeneity test in PAUP (Swofford 2000) was conducted to test the congruence between the ITS and EF-1α sequence datasets. Small subunit sequences were added to an alignment obtained from TreeBase (M911). Sequences representative of the different orders within the class Sordariomycetes, as well as the order Chaetothyriales (Chaetothyriomycetes), were retrieved from GenBank and added to the alignment. Neighbor-joining analyses of the SSU alignment (using uncorrected "p",

Kimura-2-parameter and Jukes-Cantor substitution models) were done with PAUP version 4.0b10 (Swofford 2000). *Rhodosporidium toruloides* Banno and *Athelia bombacina* Pers. were used as outgroups for the neighbor-joining analysis.

## RESULTS

*Morphology.*—In culture, *T. minima* strain CBS 213.31 produced a *Phaeoacremonium* anamorph similar to *Pm. aleophilum*, but distinct from the *Phaeoacremonium* anamorphs associated with *T. fraxinopennsylvanica* and *T. novae-zealandiae*. A detailed description of *T. minima*, based on the teleomorphs formed by isolates of *Pm. aleophilum* (TABLE I), as well as the material designated by Hausner et al (1992), is provided below:

*Togninia minima* (Tul. & C. Tul.) Berl., Icon. Fung. 3: 11. 1900. FIGS. 1–24
≡ *Calosphaeria minima* Tul. & C. Tul., Sel. Fung. Carpol. 2: 112, plate XIII:23–24. 1863.
≡ *Calosphaeria* (*Erostella*) *minima* (Tul. & C. Tul.) Sacc., Syll. Fung. 1: 101. 1882.
≡ *Erostella minima* (Tul. & C. Tul.) Traverso, Fl. Ital. Crypt. 1: 156. (1905) 1906.
= *Calosphaeria alnicola* Ellis & Everh., Proc. Acad. Nat. Sci. Phila. 221. (1890) 1891.
≡ *Togninia alnicola* (Ellis & Everh.) Berl., Icon. Fung. 3: 10. 1900.
= *Longoa paniculata* Curzi, Atti Ist. Bot. R. Univ. Pavia, Ser 3, 3: 204. 1927.

*Anamorph. Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & Mugnai, Mycologia 88: 791. 1996.

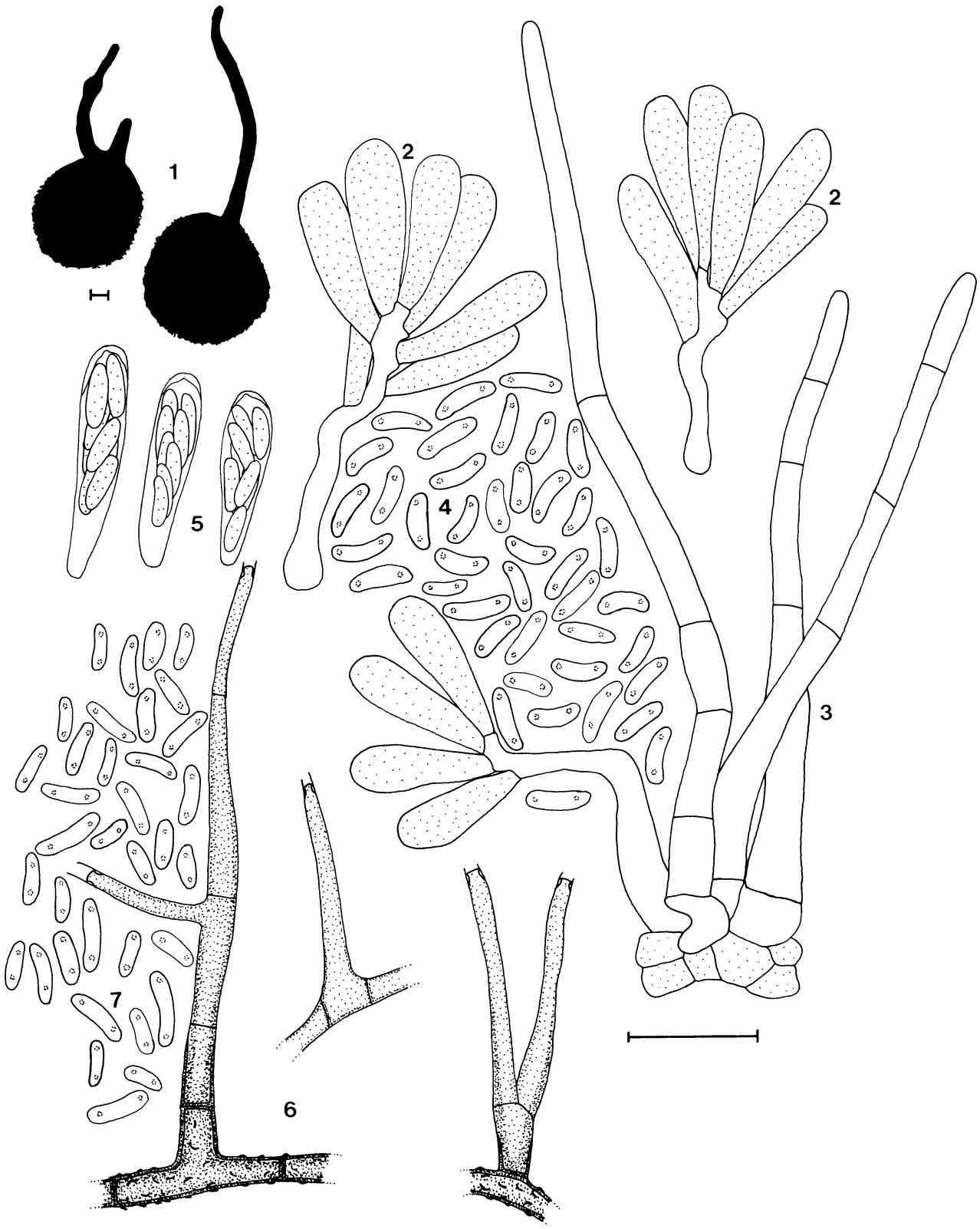
*Mycelium* consisting of branched, septate hyphae; hyphae occurring singly or in strands of up to 10, tuberculate (with warts to 1 μm) to verruculose, pale brown, becoming paler toward the conidiogenous region, 1.5–3 μm wide. *Chlamydospores* absent. *Perithecia* heterothallic, mostly aggregated, not valsoid, sometimes solitary, mostly subepidermal also on the surface of the epidermis; perithecia subglobose, sometimes obpyriform, with a long cylindrical neck, (160–)250–285(–420) μm diam and basal part (200–)285–325(–400) μm tall. Wall consisting of two regions of *textura angularis*: outer region dark brown, cells smaller and more rounded than inner layer, approx. 8–10 cells thick (individual cells not visible further outward), 20–40 μm thick; inner region hyaline (centrum) to pale brown, 5–7 cells and 12–28 μm thick; surface covered with brown, septate hyphal appendages that become hyaline towards their tips (more abundant on older perithecia). *Perithecial necks* black, 1–3(–6) per perithecium, curved, verrucose, with apex often proliferating secondarily upon aging and then appearing nodulose; nodules (–120 μm wide) also appearing lower down on the neck; necks 800–1800 (av. 1055) μm long, 35–130 (av. 69) μm wide at the base, and 20–60 (av. 41) μm wide at the apex, neck sometimes dividing into two near the apex. Multinecked perithecia often with a thin wall dividing the perithecial chamber. *Paraphyses* hyaline, septate, cylindrical, narrowing towards the tip, 45–125 (av. 83) μm long, 2–4 μm wide at the base and 1.5–2 μm at the apex, persistent. *Asci* arising in acropetal succession from sympodially proliferating ascogenous hyphae that appear spicate when mature, hyaline, clavate, with bluntly rounded apices and with sides straight or tapering towards the truncate or bluntly obtuse bases (17–)19–20(–27) × 4–5 μm; *apical complex* 0.5–1 μm thick, of indistinct structure, with a nonamyloid apical ring (negative in Melzer's reagent). *Ascogenous hyphae* hyaline, branched, smooth-walled, 2–3 μm wide (inflated bases −5 μm wide). *Ascospores* 1-celled, hyaline, oblong-ellipsoidal to allantoid with rounded ends, sometimes containing small guttules at the ends, (4–)4.5–5(–6.5) × 1–2 μm (av. 5 × 1.5 μm).

*Conidiophores* mostly micronematous, arising from aerial or submerged hyphae, erect, simple, frequently reduced to conidiogenous cells, rarely 1–2-septate, subcylindrical, pale brown, paler towards the tip, smooth to verruculose, straight to gently curved, 4–40 μm tall, 2–3 μm wide. *Conidiogenous cells* terminal or lateral, mostly monophialidic, subcylindrical to narrowly ellipsoidal, smooth to verruculose, subhyaline, 3–21 μm long, 1–2.5 μm wide at the base, 1–1.5 μm wide at the apex, with a terminal, inconspicuous, almost convergent, 0.5–1 μm long, 1 μm wide collarette. *Conidia* aggregating in slimy heads, hyaline, oblong-ellipsoidal to allantoid, when larger oblong to reniform, becoming 2-guttulate with age, (2.5–)3–4.5(–7) × 1.5–2.5(–3) μm (av. 4 × 2 μm).

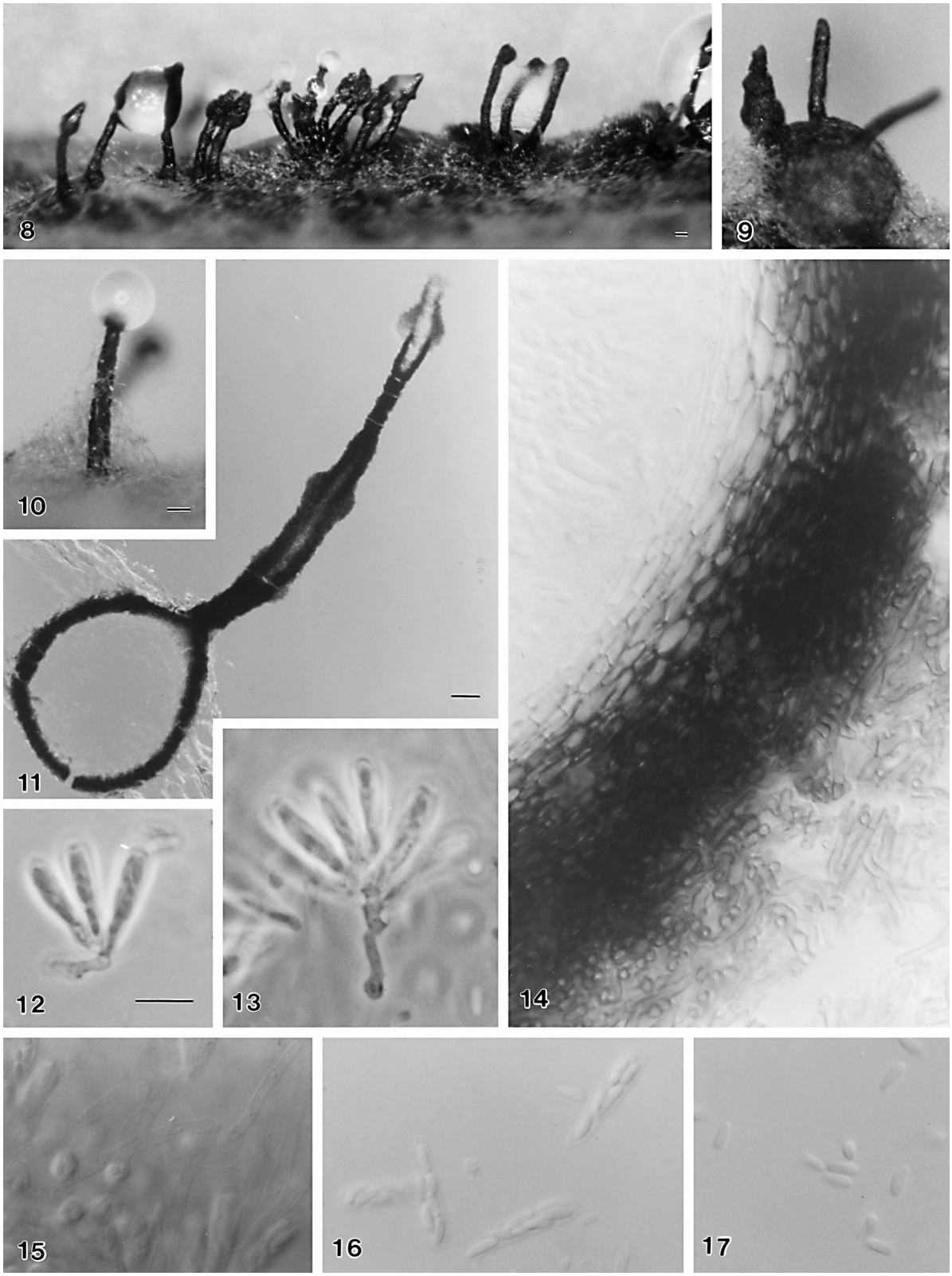
*Substrates and geographical distribution.* In vascular bundles of vines and twigs of woody plants: *Vitis vinifera* (Argentina, Australia, Chile, France, Italy, New Zealand, South Africa, Spain, Turkey, U.S.A. and Yugoslavia), *Actinidia sinensis* (Italy), bark of a tropical rain forest tree (Papua New Guinea), *Olea europaea* (Italy) (Crous et al 1996, Larignon et al 1997, Ari 2000, Crous and Gams 2000, Armengol et al 2001, Groenewald et al 2001).

*Cultural characteristics.* See Crous et al (1996).

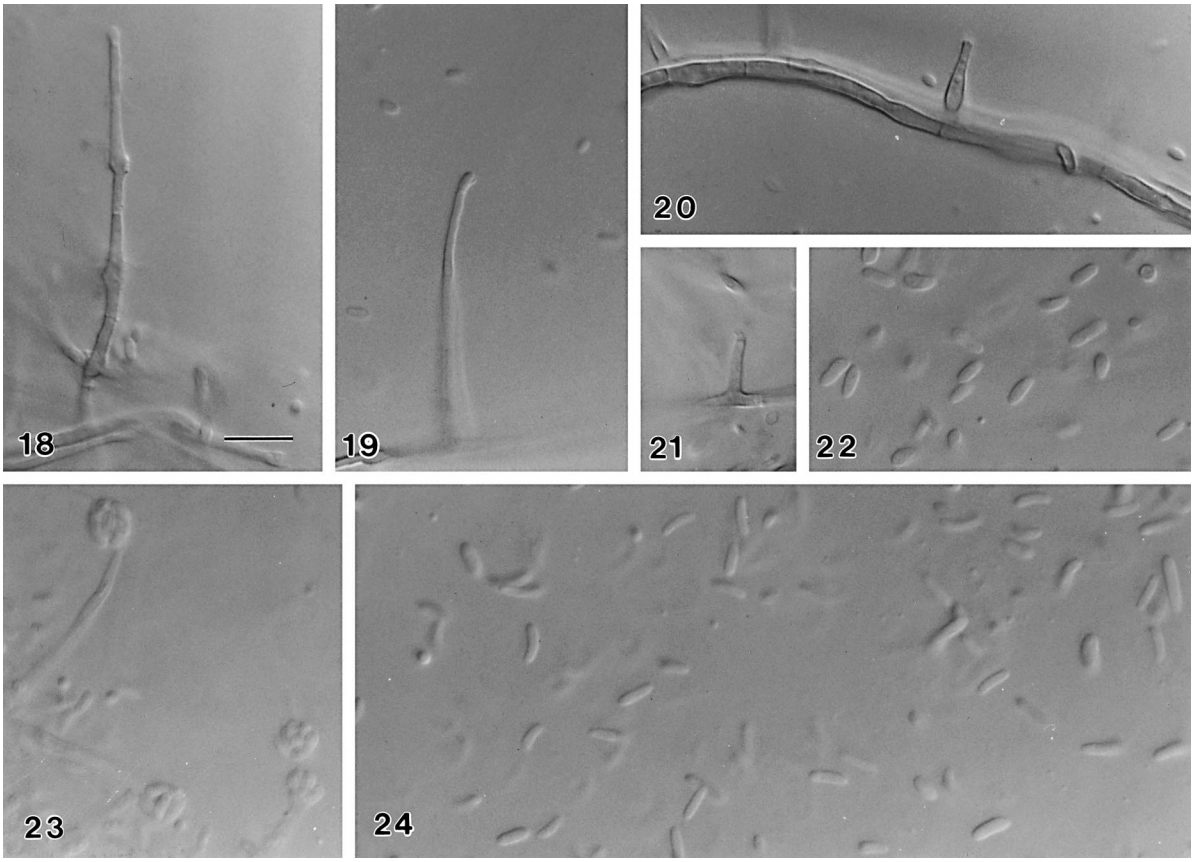
*Type specimens.* YUGOSLAVIA. On roots and stems of *Vitis vinifera*, 1990, *M. Muntañola-Cvetković* (CBS 246.91 dried specimen and ex-type culture of *Pm. aleophilum*). SOUTH AFRICA. WESTERN CAPE PROVINCE: Wellington and Paarl, respectively, stems of *Vitis vinifera*, 2001, *L. Mostert*, LM 463 (MAT1-1 = CBS 111015) × LM 54 (MAT1-2 = CBS 110703),




FIGS. 1–7. *Togninia minima* and its anamorph *Phaeoacremonium aleophilum*. 1. Perithecia. 2. Asci with spicate arrangement on ascogenous hyphae. 3. Paraphyses. 4. Ascospores. 5. Asci. 6. Conidiophores and conidiogenous cells. 7. Conidia. Bars = 10 μm.




FIGS. 8–17. *Togninia minima*. 8. Perithecia on *Vitis* cane. 9. Perithecium with three necks. 10. Perithecium with globoid spore mass. 11. Vertical section through a perithecium. 12, 13. Asci with spicate arrangement on ascogenous hyphae. 14. Vertical section through a perithecium wall. 15. Asci and paraphyses. 16. Asci. 17. Ascospores. Bars = 40 μm in FIGS. 8–11, 10 μm in FIGS. 12–17.




FIGS. 18–24. *Phaeoacremonium aleophilum*, anamorph of *Togninia minima*. 18–21. Conidiophores and conidiogenous cells. 22. Conidia after 3 mo. 23. Conidia aggregated in globoid masses. 23. Conidia after 7 d. Bar = 10 μm.

[**epitype** of *T. minima*, designated here] dried specimen, herb. CBS 6580.

*Notes.* No information previously has been available regarding the anamorph of *T. minima*. The only culture currently available that previously has been identified as *T. minima* is CBS 213.31. It differs from freshly isolated strains of *Pm. aleophilum* in several morphological characters. *Phaeoacremonium aleophilum* isolates in general have buff (19″d) to honey (21″b) colonies, while colonies of CBS 213.31 are white to buff (19″d) with woolly mycelial tufts. The difference might be due to cultural degeneration. Conidiogenous cells (1.5 μm at the base) and conidia (1 μm wide) of CBS 213.31 were slightly narrower than those of *Pm. aleophilum* (2.5 and 2 μm, respectively).

*Phaeoacremonium aleophilum* differs from anamorphs of *T. fraxinopennsylvanica* and *T. novae-zealandiae*. Conidia of *T. fraxinopennsylvanica* and *T. novae-zealandiae* were up to 9 and 10.5 μm long, respectively, significantly longer than those of *Pm. aleophilum*, which did not exceed 7 μm. Also, the collarettes of *T. fraxinopennsylvanica* and *T. novae-zealandiae* (1.0–1.8 μm) were longer than those of *Pm. aleophilum* (0.5–1 μm).

*Matings.*—Perithecia produced by crossing *Pm. aleophilum* isolates were contrasted with those described for *T. minima*, *T. fraxinopennsylvanica* and *T. novae-zealandiae* (TABLE II). No interspecies mating was observed, nor were any matings obtained with CBS 213.31. The teleomorph of *Pm. aleophilum* was identical, however, to that described by Hausner et al (1992) for *T. minima*. Furthermore, ascospores of *T. minima* are longer and perithecia are wider and have longer necks than those of *T. fraxinopennsylvanica* and *T. novae-zealandiae* (TABLE II).

Of the 21 isolates of *Pm. aleophilum* that were mated, 10 belonged to one mating type and 11 to the other (FIG. 25). The bi-allelic heterothallic mating system suggested by these results was verified with crosses among the F1 ascospore progeny. Of 20 single-spore isolates used from one perithecium, 10 grouped in one mating type and 10 in the other, thus agreeing with a 1:1 Mendelian segregation of mating type (FIG. 26).

TABLE II. Perithecial morphology of the various *Togninia* species studied

| | Ascospore shape and dimensions (µm) | Ascal dimensions (µm) | Perithecial dimensions (µm) | Neck length (µm) | Reference |
|---|---|---|---|---|---|
| *T. minima* | Allantoid 5.0–6.5 × 1–2 | 20–30 × 4.5–6 | 225–490(–525) diam | | Tulasne and Tulasne (1863) |
| *T. minima* | 3–5.5 × 1–2 | 15–21(–25) × 3.5–5(–6) | 220–490 diam | | Berlese (1900) |
| *T. minima* | Oblong-ellipsoidal to allantoid (4–)4.7–5(–6.5) × 1–2 | (17–)19–20(–27) × 4–5 | (160–)250–285(–420) diam (200–)285–325(–400) tall | 800–1800 | Present study |
| *T. fraxinopennsylvanica* | Cylindrical to slightly curved 4–5.2 × 1.6–1.8(–2) | 16.5–21 × 4–4.8 | 195–290 diam | 400–900 | Hausner et al (1992) |
| *T. novae-zealandiae* | Ellipsoidal to oblong-ellipsoidal, slightly rounded at the ends 3.8–5.6 × (1.8–)2.2–2.6 | 15.5–24 × 4.7–5.5(–6.5) | 175–265 diam | 450–1300 | Hausner et al (1992) |

*Phylogenetic analysis.*—The combined alignment (ITS and EF-1α) had a total length of 863 characters, of which 301 were constant, 329 were parsimony uninformative and 233 were parsimony informative. The result of the partition homogeneity test showed that the ITS and EF-1α datasets were congruent ($P$ = 0.72) and therefore could be combined. Parsimony analysis of the combined datasets, using a heuristic search with 1000 random taxa additions, resulted in 10 most-parsimonious trees (Tree length = 901 steps, CI = 0.937, RI = 0.903, RC = 0.846 and HI = 0.063). *Togninia minima* (CBS 213.31) grouped in the clade with all *Pm. aleophilum* sequences, as well as the extype strain of *Pm. aleophilum*. The analysis clearly delimits the three *Togninia* species with good bootstrap support (FIG. 27). Some variation was observed within the *Pm. aleophilum* clade, as two isolates (CBS 100400, 101357) formed a subclade with 100% bootstrap support within the *Pm. aleophilum* clade. These two isolates differed from the rest of the *Pm. aleophilum* isolates at 11 (for EF-1α) and two (for ITS) nucleotide positions. Another group within the *Pm. aleophilum* clade also received significant support (87%). These sequences, however, differed from the other isolates in the *Pm. aleophilum* clade at only two nucleotide positions in the EF-1α dataset. A total of 21 characters proved variable in the combined dataset (2.4%). The EF-1α area was found to be more variable (15 nucleotides, 4.6%) than the ITS area (6 nucleotides, 1.1%).

In the phylogram of the SSU sequence data (FIG. 28), *Pm. aleophilum*, *Togninia minima* (CBS 213.31), *T. fraxinopennsylvanica* and *T. novae-zealandiae* grouped together, forming the Calosphaeriales clade (Sordariomycetes) (100% bootstrap). *Magnaporthe grisea* (T.T. Hebert) M.E. Barr, as well as a teleomorph of *Harpophora* W. Gams (*Gaeumannomyces graminis* (Sacc.) Arx & D.L. Olivier, Magnaporthaceae, incertae sedis), formed a well-supported subclade (100% bootstrap) within the class Sordariomycetes. *Phialophora verrucosa* Medlar, anamorphic *Capronia semiimersa* (Cand. & Sulmont) Unter. & F.A. Naveau clustered with *Capronia* spp. (Chaetothyriales) with 100% bootstrap support.

## DISCUSSION

*Togninia* is accepted as being typified by *T. minima* (Clements and Shear 1931). This viewpoint has been followed by Hausner et al (1992), as explained in Holm (1992), and also is accepted by Barr (M. E. Barr pers comm, 20 Feb 2002), contrasting with her earlier view (Barr et al 1993) that *Togninia* was a synonym of *Pleurostoma* Tul. & C. Tul. A more complicated issue addressed by Hausner et al (1992) was

| Isolate | LM44 | LM45 | LM46 | LM47 | LM48 | LM49 | LM50 | LM51 | LM53 | LM54 | LM56 | LM58 | LM61 | LM65 | LM76 | LM77 | LM78 | LM458 | LM463 | LM467 | LM468 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LM44 | - | | | | | | | | | | | | | | | | | | | | |
| LM45 | - | - | | | | | | | | | | | | | | | | | | | |
| LM46 | + | + | - | | | | | | | | | | | | | | | | | | |
| LM47 | - | - | + | - | | | | | | | | | | | | | | | | | |
| LM48 | - | - | + | - | - | | | | | | | | | | | | | | | | |
| LM49 | + | + | - | + | + | - | | | | | | | | | | | | | | | |
| LM50 | - | - | + | - | - | + | - | | | | | | | | | | | | | | |
| LM51 | + | + | - | + | + | - | + | - | | | | | | | | | | | | | |
| LM53 | - | - | + | - | - | + | - | + | - | | | | | | | | | | | | |
| LM54 | - | - | + | - | - | + | - | + | - | - | | | | | | | | | | | |
| LM56 | + | + | - | + | + | - | + | - | - | + | - | | | | | | | | | | |
| LM58 | + | + | - | + | + | - | + | - | + | + | - | - | | | | | | | | | |
| LM61 | - | - | + | - | - | + | - | + | - | - | + | + | - | | | | | | | | |
| LM65 | + | - | - | + | + | - | + | - | + | + | - | - | + | - | | | | | | | |
| LM76 | + | + | - | + | + | - | + | - | + | + | - | - | + | - | - | | | | | | |
| LM77 | - | - | - | - | - | + | - | + | - | - | + | + | - | + | + | - | | | | | |
| LM78 | + | - | - | + | + | - | + | - | + | + | - | - | + | - | - | + | - | | | | |
| LM458 | - | - | + | - | - | + | - | + | - | - | + | + | - | + | + | - | + | - | | | |
| LM463 | + | + | - | + | + | - | - | - | - | + | - | - | + | - | - | + | - | - | - | | |
| LM467 | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | + | - | - | - | |
| LM468 | - | + | - | + | + | - | + | - | + | + | - | - | + | - | - | + | - | + | - | - | - |

FIG. 25. Schematic representation of a mating study with single conidial isolates of *Phaeoacremonium aleophilum*. A (−) indicates that no perithecia formed, while (+) indicates formation of perithecia that exuded copious amounts of fertile ascospores.

whether the genus name *Togninia* had precedence over *Erostella*. *Erostella* first was described as subgenus of *Calosphaeria* Tul. & C. Tul., and subsequently was elevated to generic level as *Erostella* (Sacc.) Trav. (1905) and again as *Erostella* (Sacc.) Sacc. (1906). Both *Erostella* and *Togninia* have *Calosphaeria minima* Tul. & C. Tul. as lectotype. The key to resolving this mystery lies in the precise interpretation of the Latin description of *Togninia* provided by Berlese (1900), who placed 12 species and one variety in this genus. The decision of Clements and Shear (1931) to designate *T. minima* as lectotype of *Togninia* is not obviously supported by Berlese's text. Berlese (1900, p. 20), stated "*C.*[*alosphaeria*] *herbicola* E.[llis] et E.[verhart] id. *C. ambigua* Berl.[ese] est novi generis *Togninia* typus." Two interpretations of this text are possible. The first is that Berlese actually typified *Togninia* by *T. ambigua* Berl.; in this case the lectotypification of Clements and Shear (1931) should be rejected and *Erostella* can be resurrected for the taxa treated by Hausner et al (1992), with *Calosphaeria minima* as lectotype. The second interpretation,

| Isolates | LM227 | LM228 | LM229 | LM230 | LM231 | LM232 | LM233 | LM234 | LM235 | LM236 | LM237 | LM238 | LM239 | LM240 | LM243 | LM244 | LM245 | LM246 | LM247 | LM249 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LM227 | - | | | | | | | | | | | | | | | | | | | |
| LM228 | + | - | | | | | | | | | | | | | | | | | | |
| LM229 | - | + | - | | | | | | | | | | | | | | | | | |
| LM230 | - | + | - | - | | | | | | | | | | | | | | | | |
| LM231 | + | - | + | + | - | | | | | | | | | | | | | | | |
| LM232 | + | - | + | + | - | - | | | | | | | | | | | | | | |
| LM233 | + | - | + | - | - | - | - | | | | | | | | | | | | | |
| LM234 | - | + | - | - | + | + | + | - | | | | | | | | | | | | |
| LM235 | + | - | + | - | - | - | - | - | - | | | | | | | | | | | |
| LM236 | + | - | + | + | - | - | - | - | - | - | | | | | | | | | | |
| LM237 | - | + | - | - | + | + | + | - | - | + | - | | | | | | | | | |
| LM238 | + | - | + | + | - | - | - | + | - | - | - | - | | | | | | | | |
| LM239 | - | - | - | + | - | - | - | - | - | - | + | - | - | | | | | | | |
| LM240 | + | - | + | - | - | - | - | + | - | - | - | - | - | - | | | | | | |
| LM243 | + | - | + | + | - | - | - | - | - | - | + | - | - | - | - | | | | | |
| LM244 | - | + | - | - | + | + | + | - | + | + | - | + | + | + | - | - | | | | |
| LM245 | - | + | - | - | + | + | + | - | + | + | - | + | + | + | + | - | - | | | |
| LM246 | - | + | - | - | - | + | + | - | + | + | - | + | - | + | + | - | - | - | | |
| LM247 | - | + | - | - | + | + | + | - | + | + | - | + | + | + | + | - | - | - | - | |
| LM249 | - | + | - | - | + | + | + | - | + | + | - | + | - | + | + | - | - | - | - | - |

FIG. 26. Schematic representation of crosses from single ascospores of *Togninia minima* obtained from a fertile mating (LM 54 × LM 463). A (−) indicates that no perithecia formed, while (+) indicates formation of perithecia that exuded copious amounts of fertile ascospores.

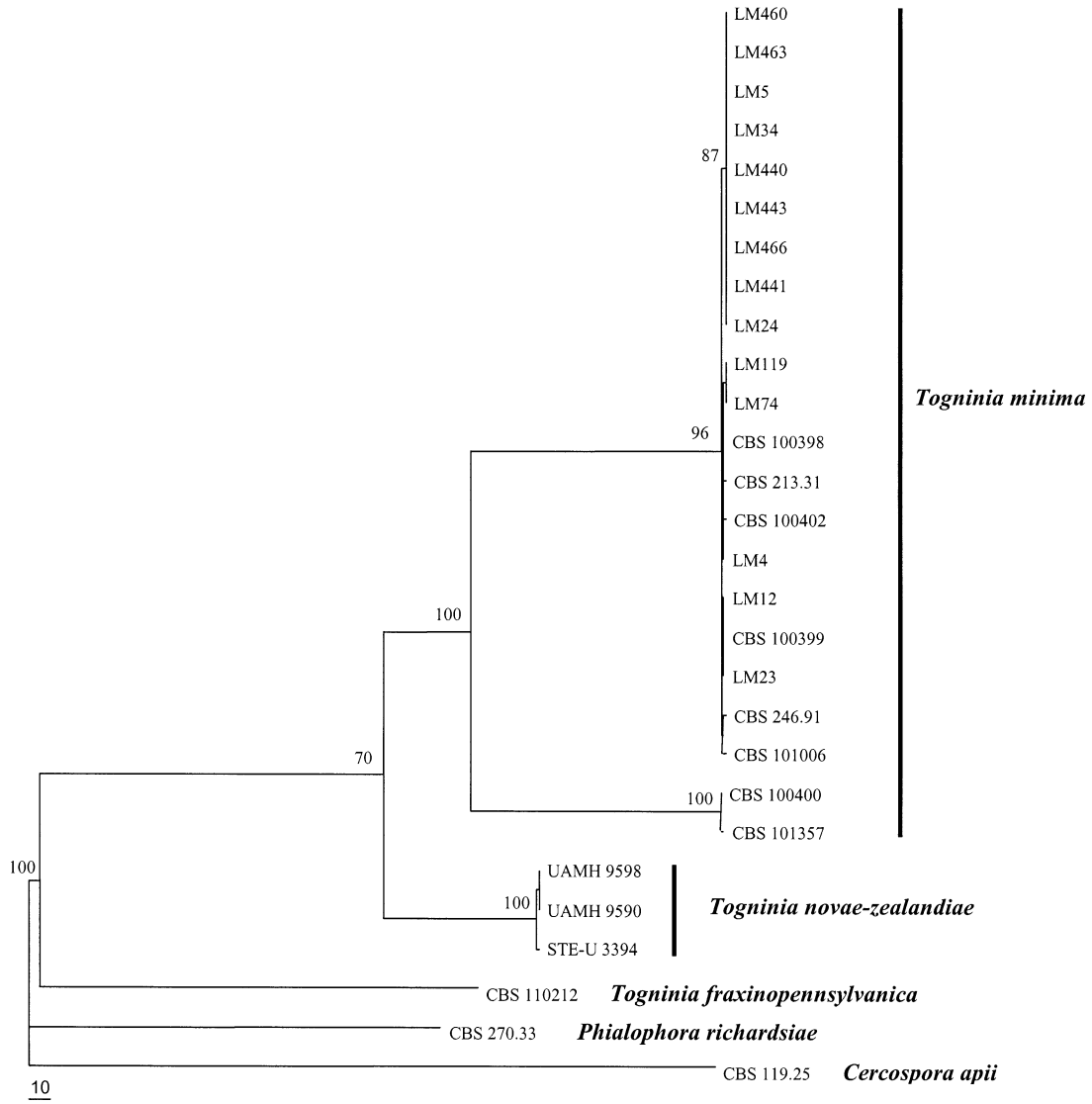



FIG. 27. One of 10 most-parsimonious trees obtained from heuristic searches of a combined alignment of the 5.8S rRNA gene and flanking ITS1 and ITS2 regions and the translation elongation factor 1-alpha gene (length = 901 steps, CI = 0.937, RI = 0.903, RC = 0.846 and HI = 0.063). Bootstrap support values (1000 repl.) above 65% are shown at the nodes. *Phialophora richardsiae* and *Cercospora apii* were used as outgroups.

which was explained by Holm (1992) and subsequently followed by Hausner et al (1992), is that Berlese did not consider *C. herbicola* to be synonymous with *C. ambigua*. Under the genus *Togninia*, Berlese (1900, p. 9) listed only *T. ambigua*, while he transferred *C. herbicola* to *Jattaea* on p 8 but *C. minima* to *Togninia* on p 11. The Latin ''id.'', ''idem'', means ''the same as'', but it also has been translated as ''ditto'', which is more appropriate here, as Berlese (1900) clearly did not treat *C. herbicola* as synonymous with *C. ambigua* but actually placed them in different genera. The species in this *Calosphaeria* complex are thus ''novi generis *Togninia* typus'', meaning that they are of the *Togninia*-type (Holm 1992), and consequently the lectotypification of *Togninia* with *T. minima*, as proposed by Clements and Shear (1931), can be accepted.

Because no authentic material of *T. minima* exists, Hausner et al (1992) designated the original illustration (Tulasne and Tulasne 1863) as iconotype. *Togninia* thus is conceived as a genus that has solitary to clustered, globose perithecia with papillate to beak-like apices that can be smooth or ornamented. Asci are unitunicate with truncate bases and thickened apices, appearing in a spicate arrangement on ascogenous hyphae. Paraphyses are present, hyaline, septate, and ascospores are hyaline, aseptate, allantoid to ellipsoidal. Anamorphs are acremonium- to phi-

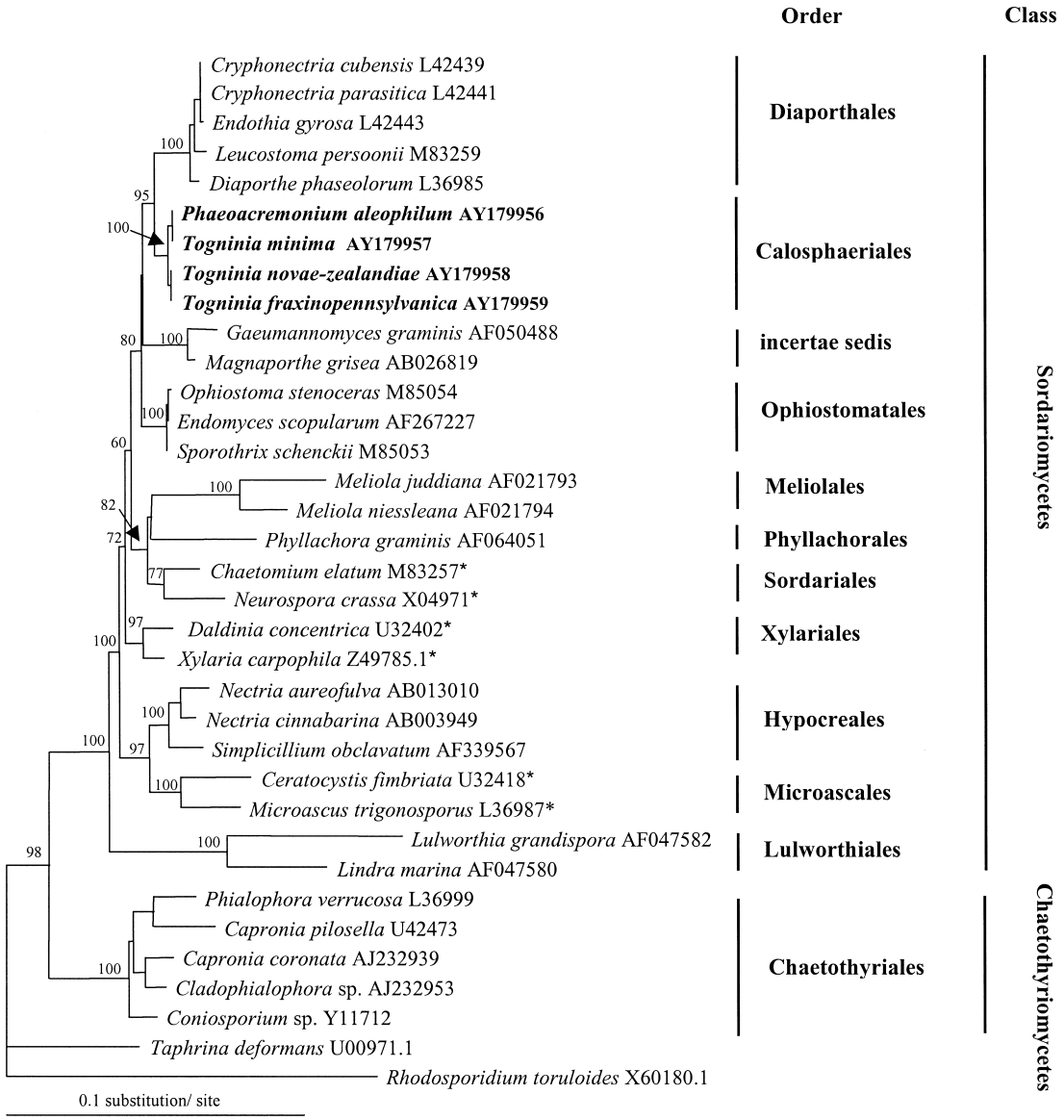



FIG. 28. Neighbor-joining phylogenetic tree obtained from partial 18S rDNA sequences. Bootstrap support values (1000 repl.) are shown above the nodes. *Taphrina deformans* and *Rhodosporidium toruloides* were used as outgroups. Sequences marked with an asterisk were taken from TreeBase (M911).

alophora-like (Hausner et al 1992). Hausner et al (1992) based their redescription of *T. minima* in part on two specimens, one collected from *Alnus* in the United States (N.A.F. 2514) and another from *Prunus pennsylvanica* collected in Canada (SSMF 725–7179). No cultures, however, were available for study. Clements and Shear (1931) treated *Longoa* Curzi as synonym of *Calosphaeria*, a genus that is heterogeneous (M. E. Barr pers comm). In her revision of the Calosphaeriales, Barr (1985) did not treat *Longoa*, but based on its morphology Eriksson and Hawksworth (1986) considered the type species, *Longoa paniculata* (CBS 213.31, ex-type), to be a synonym of *T. minima*. This synonymy is supported by the original description and molecular data obtained in this study.

A comparison of our fungus with the original descriptions of *Togninia* provided by Tulasne and Tulasne (1863) and Berlese (1900), as well as with the redescription of Hausner et al (1992), shows that our fungus clearly belongs in *Togninia*. Furthermore, its generic relationship with *T. fraxinopennsylvanica* and *T. novae-zealandiae* is confirmed via the tight cluster seen in 18S SSU sequence data (FIG. 28).

Before any teleomorph was known for *Phaeoacremonium*, the genus was considered to have affinities for the Magnaporthaceae (Dupont et al 1998). Members of this family generally are characterized by hav-

ing long, hairy necks and septate ascospores (Kirk et al 2001). The family is considered to be close to the Diaporthales (Winka and Eriksson 2000). The *Togninia* teleomorphs of *Phaeoacremonium* differ strongly from those of other Magnaporthaceous fungi. In addition, our 18S rDNA sequence analysis shows that the *Togninia* and *Phaeoacremonium* species investigated here form a distinct clade apart from the Diaporthales (FIG. 28), supporting their placement in the order Calosphaeriales for reasons outlined by Barr (1985). Phylogenetic analyses of DNA sequence data have shown that *T. minima* forms a separate cluster with *T. fraxinopennsylvanica* and *T. novae-zealandiae*, as well as other species presently known in *Phaeoacremonium* (Groenewald et al 2001). *Togninia* teleomorphs also have been induced for several other *Phaeoacremonium* spp., which await further description once their mating strategies have been resolved.

Results from mating studies clearly have shown that *T. minima* has a biallelic heterothallic mating system. Such systems in fungi have been reviewed by Glass and Nelson (1994). Field isolates obtained from individual diseased vines could be induced to form the teleomorph in vitro on cane sections, indicating that compatible mating types co-occur in nature on the same vine. The conditions necessary for perithecial formation in the field remain unknown. The extent to which teleomorphs occur in the field also is unknown. The discovery of the teleomorph is important for the design and deployment of disease-control strategies, as well as for the overall understanding of the epidemiology of Petri disease.

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