ITS and β-tubulin phylogeny of *Phaeoacremonium* and *Phaeomoniella* species

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Based on ITS and β-tubulin sequence data of 33 isolates, the newly introduced genus, *Phaeomoniella* was confirmed as being distinct from *Phaeoacremonium* (*Pm.*). Phylogeny inferred from DNA sequences and cultural characteristics also confirmed the species status of *Pm. aleophilum* and *Pm. angustius*, which were recently reduced to synonymy. *Pm. aleophilum* has an optimum growth rate at 30 °C and the ability to grow at 35°, whereas *Pm. angustius* has an optimum growth rate at 25 °C and does not grow at 35°. Furthermore, ITS and β-tubulin sequence data showed *Pm. viticola* to be indistinguishable from *Pm. angustius*, while a new species, *Pm. mortoniae*, could be distinguished from this complex.

INTRODUCTION

Petri grapevine decline, also known as apoplexy, black measles, slow-dieback and black goo decline is a well-known disease of grapevines worldwide (Mugnai, Graniti & Surico 1999). Although the complex aetiology and epidemiology of this disease have not yet been resolved completely, one of the major causal organisms is *Phaeomoniella chlaymydospora*, a fungus that is distributed throughout the world with its host, *Vitis vinifera*. In addition, Petri grapevine decline is a major component of the Esca disease complex of grapevines (primarily *Fomitiporia punctata* and *Stereum hirsutum*), and the Phaeoacremonium disease complex (primarily *Phaeoacremonium* spp.) (Crous & Gams 2000).

As far as could be established, Petri (1912) was the first to report the disease from grapevines with brown and black wood streaks with which he associated species of '*Cephalosporium*' and *Acremonium*. Chiarappa (1959) subsequently isolated and confirmed the pathogenicity of a '*Cephalosporium*' species from grapevine. Hawksworth, Gibson & Gams (1976) subsequently examined this material (IMI 192881, CBS 239.74), and concluded that it represented a new species in this complex, Crous *et al*. (1996) compared morphologically similar isolates originating from human patients and various woody hosts, and established the genus *Phaeoacremonium* for these taxa. The genus contained six species, namely *Pm. aleophilum*, *Pm. angustius*, *Pm. chlamydosporum*, *Pm. parasiticum*, *Pm. inflatipes* and *Pm. rubrigenum*. The '*Cephalosporium*' species isolated by Chiarappa (1959) was found to be representative of *Pm. chlamydosporum* (Crous *et al*. 1996). In a later molecular study, however, Dupont, Laloui & Roquebert (1998) reported that *Pm. chlamydosporum* appeared to be unrelated to the other species of the genus in the *Magnaporthaceae* and closer to *Phialophora sensu stricto* (*Herpotrichiellaceae*). Based on these molecular, as well as additional morphological and pathological differences, a new genus, *Phaeomoniella* (*Pa.*), was introduced and typified by *Pa. chlamydospora* (Crous & Gams 2000).

Other aspects that needed clarification were the pathogenicity of species in this complex (Scheck, Vasquez & Gubler 1998) as well as their delimitation. Dupont *et al*. (1998) synonymized two taxa, and described one species as new (Dupont *et al*. 2000a). *Phaeomoniella chlamydospora* is seen as the dominant organism causing Petri grapevine decline (Crous & Gams 2000). However, as stated previously, other *Phaeoacremonium* spp. are also involved in this disease complex. Accordingly, the aims of this study were to investigate the phylogeny of *Phaeomoniella* and *Phaeoacremonium* species using sequence analysis of two genomic areas and to compare these data to cultural characteristics and morphology.

MATERIALS AND METHODS

Isolates and cultural characteristics

Isolates of *Phaeoacremonium* spp. were either obtained from symptomatic vines or from the Centraalbureau voor Schimmelcultures (CBS). These isolates (Table 1) are maintained in the culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa (STE-U), as well as at CBS.

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Table 1. *Phaeoacremonium* and *Phaeomoniella* isolates studied.

Species	Culture no.	GenBank accession no.		Host and location
		β-tubulin	ITS	
Phaeoacremonium aleophilum	CBS 246.91[a]	AF246811	AF017651	*Vitis vinifera*, Yugoslavia
	CBS 100397	AF246806	AF197981	*V. vinifera*, Italy
	CBS 100399		AF197991	*V. vinifera*, Italy
	CBS 100400	AF246807	AF197992	*V. vinifera*, Italy
	CBS 101358	AF246808	AF197993	*Actinidia chinensis*, Italy
	CBS 100401		AF197982	*V. vinifera*, Italy
	CBS 100402		AF197994	*V. vinifera*, Italy
	CBS 100548		AF197983	*Olea europaea*, Italy
	CBS 101568		AF197984	*V. vinifera*, California
	STE-U 3094 (MT78)	AF246812	AF197996	*V. vinifera*, South Africa
	STE-U 3093 (MT79)	AF246813	AF197985	*V. vinifera*, South Africa
	STE-U 3095 (MT80)		AF197995	*V. vinifera*, South Africa
	CBS 101006			*A. chinensis*, Italy
	CBS 101008			*A. chinensis*, Italy
Pm. angustius	CBS 249.95[a]	AF246814	AF197974	*V. vinifera*, California
	CBS 101739	AF246816	AF197977	*V. vinifera*, France
	CBS 101738	AF192391	AF118137	*V. vinifera*, France
	CBS 101737	AF246817	AF197976	*V. vinifera*, France
	CBS 100947	AF246815	AF197975	*O. europaea*, Italy
Pm. parasiticum	CBS 860.73[a]	AF246803	U31841	Human, California, USA
	CBS 101007	AF246804	AF197980	A. *chinensis, Italy*
	CBS 513.82		U31842	Human, New York, USA
Pm. rubrigenum	CBS 498.94[a]	AF246802	AF197988	Human, USA
	STE-U 3092 (MT11)	AF246800	AF197978	*V. vinifera*, South Africa
	CBS 566.97	AF246801	AF197979	Human, Japan
	CBS 729.97		AF197989	Human, South Carolina, USA
Pm. inflatipes	CBS 391.71[a]	AF246805	AF197990	*Quercus virginiana*, Texas, USA
	CBS 166.75		U31843	*Nectandra* sp., Costa Rica
Pm. mortoniae	CBS 211.97	AF246810	AF295329	*Fraxinus excelsior*, Sweden
	CBS 101585[a]	AF246809	AF295328	*V. vinifera*, California
Phaeomoniella chlamydospora	CBS 229.95[a]	AF253968	AF197973	*V. vinifera*, Italy
	STE-U 3066	AF253969	AF197986	*V. vinifera*, South Africa
	STE-U 3067		AF197987	*V. vinifera*, South Africa

[a] Ex-type culture.

Single-conidial cultures were established for all isolates studied. Isolates were plated onto 2% malt extract agar (MEA; Biolab, Midrand, Johannesburg, South Africa) and incubated at 25 °C under near-ultraviolet light to promote sporulation. Slide preparations were made in lactic acid and 30 examples of each structure measured. The 95% confidence interval was also determined for conidial dimensions; extremes in conidium length and width are given in parentheses. To determine their cardinal temperature requirements for growth, isolates were plated on MEA and incubated for 8 d in the dark at seven different temperatures ranging from 10 to 40° in 5° intervals. Linear mycelial growth was measured by calculating the mean of four perpendicular colony radial measurements of three repeats for every isolate at each temperature studied. The experiment was repeated once. Colony colour (reverse) was determined after 8 d at 25–30° in the dark using the colour designations of Rayner (1970).

DNA isolation and amplification

Single-conidial isolates were grown on MEA plates and incubated at 25° for 2–4 wk. DNA was extracted from fresh mycelium using the Promega Kit for isolation of genomic DNA from plant tissue (Promega Corporation, Madison, WI). The Nuclei lysis solution was substituted with SDS extraction buffer (20% SDS, 2 M Tris–HCl (pH 8), 1 M NaCI, 0.5 M EDTA).

Regions of two genes were amplified. The 5.8S nuclear ribosomal RNA gene and the flanking internal transcribed spacers (ITS1 and ITS2) were amplified using primers ITS1 and ITS4 (White *et al*. 1990). A 600 base pair fragment of the 5′ end of the β-tubulin gene was amplified with primers T1 (O'Donnell & Cigelnik 1997) and Bt2b (Glass *et al*. 1995). PCR reactions (total volume of 25 µl) comprised of 1.5 units Biotaq (Bioline, London), 1 mM deoxynucleoside triphosphates, 4 mM $MgCl_2$, 0.5 µM primer oligonucleotide and approximately 10–30 ng of fungal genomic DNA. Reactions were performed on a Perkin–Elmer (Gene Amp PCR System 2400). PCRs consisted of the following: an initial denaturation for 4 min at 95°, followed by 30 cycles of 60 s at 96°, 30 s at 50° and 90 s at 72°. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). Both strands of the ITS and β-tubulin PCR products were sequenced using the ABI Prism 377 DNA Sequencer (Perkin–Elmer, Norwalk, CN). A Dye Terminator Cycle Sequencing Ready Reaction Kit containing an AmpliTaq DNA Polymerase (Perkin–Elmer)

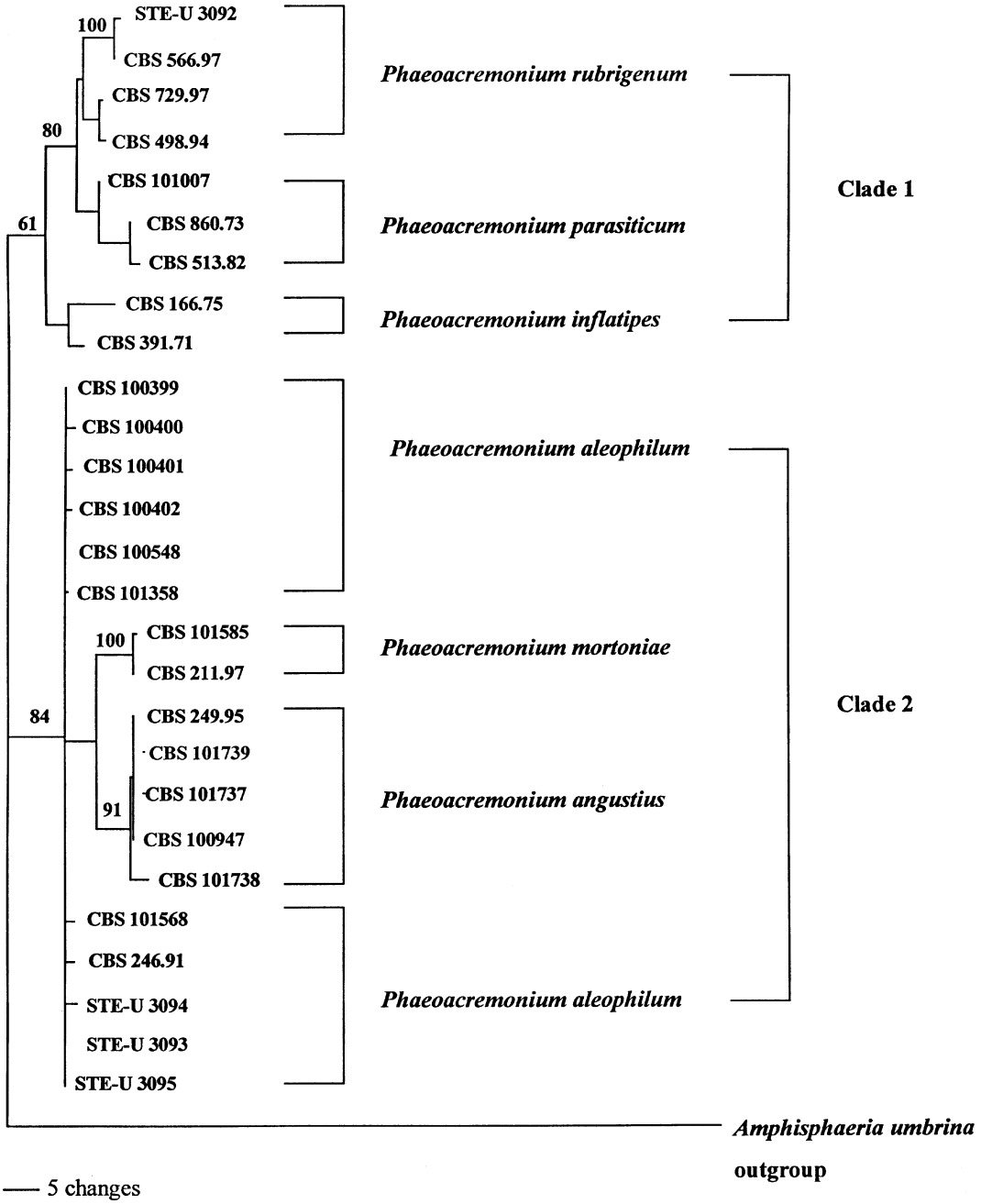


Fig. 1. One of 15 most parsimonious trees generated with PAUP* 4.0b2a from aligned sequences of the 5.8S rRNA gene and flanking ITS1 and ITS2 regions (tree length 396, CI = 0.826, RI = 0.833, RC = 0.688, HI = 0.174). A sequence of *Amphisphaeria umbrina* (AF009805) was used as outgroup.

was used for the sequencing reactions. DNA samples were purified using Centri-Sep Spin columns (Princeton Separations, Adelphia, NJ) and loaded on the sequencing gel. A consensus sequence was created on Sequence Navigator for each isolate.

Phylogenetic analysis

Sequences obtained from this study and GenBank were aligned using the DNA and Protein Sequence Alignment (DAPSA) programme developed by Harley (1998). Alignment gaps were coded as missing data in the analysis. The sequences of *Fusarium* oxysporum were used as outgroup for the joint data of ITS and β-tubulin (GenBank: AF132800 & U34424). *Amphisphaeria umbrina* (GenBank: AF009805) was used as outgroup for the analysis of ITS data. Fifty-four ambiguous characters from site 48 to 101 in the alignment were excluded from the ITS analysis. Phylogenetic analyses were performed with PAUP* version 4.0b2a (Swofford 1999). Maximum parsimony analysis was conducted using branch-and-bound search or heuristic search option using 1000 random addition sequence replicates. Bootstrap support (Felsenstein 1985) for internal branches was evaluated from 1000 heuristic searches and decay indices calculated using AutoDecay (Eriksson 1998) to further test the robustness of branches. Other measures including tree length, consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were also calculated. Congruence between the ITS and β-tubulin sequence data sets

of 18 taxa was measured using the partition homogeneity test in PAUP*.

RESULTS

DNA phylogeny

Maximum parsimony analysis of the ITS1 data set (554 characters, 61 parsimony informative) generated 15 equally most parsimonious trees (MPTs). Maximum parsimony analysis of the combined data sets of ITS and the partial β-tubulin gene sequences (594 characters, 588 parsimony informative) resulted in three MPTs. The result of the partition homogeneity test ($P = 1.00$, where $P \geqslant 0.05$ was significantly incongruent) indicated that the two data sets are congruent. The final phylogenetic trees (Figs 1–2) were compatible with accepted morphological delimitation of taxa in the *Phaeoacremonium*-complex.

In the ITS data set (Fig. 1), clade 1 represents *Pm. rubrigenum*, *Pm. parasiticum* and *Pm. inflatipes*. This clade consists mainly of human isolates. Clade 2 represents isolates of *Pm. aleophilum* and *Pm. angustius*, and with the exception of four isolates, all isolates were obtained from *Vitis vinifera*. This data set alone, however, was insufficient to distinguish between all the morphological species described in *Phaeoacremonium*.

Aligned sequences of primers T1 and Bt2b showed 100% similarity between isolates CBS 101737–101739 (*Pm. viticola*) and CBS 100947 (*Pm. angustius*). The ex-type strain of *Pm. angustius* (CBS 249.95) differed from these isolates with nine informative positions (1.7%). The ITS data showed 100% similarity between CBS 101737, 101739, 100947, and the type of *Pm. angustius*. Five deletions and one transversion (G to C) were observed for CBS 101738 when compared to the ITS sequencing data of other isolates of *Pm. angustius* (Table 2). Interspecies variation for *Pm. aleophilum* and *Pm. angustius* is shown in Table 2. More intraspecific variation was observed among isolates of *Pm. aleophilum* in the β-tubulin data set than in the ITS data set.

The combined data set (Fig. 2) supported the separation of *Pa. chlamydospora* from the *genus Phaeoacremonium*. Furthermore, the separation of *Pm. angustius* from *Pm. aleophilum* was strongly supported by bootstrap (99%) and a decay index (16) (Fig. 2), while isolates CBS 101737, 101738 and 101739 previously identified as *Pm. viticola* (Dupont *et al.* 2000a) were shown to be indistinguishable from *Pm. angustius*. Two similar isolates obtained from *Vitis* and *Fraxinus* (CBS 101585 and

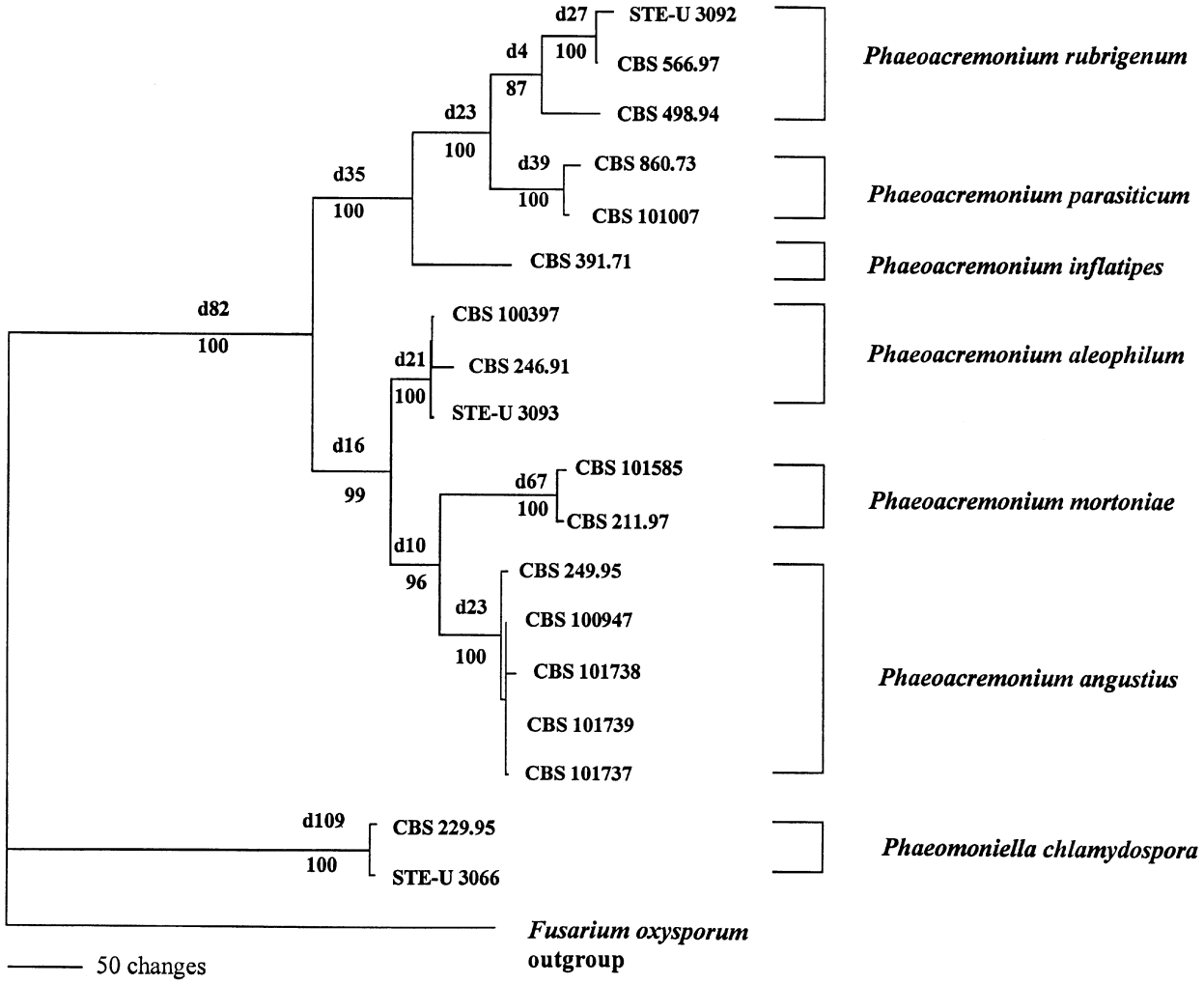


Fig. 2. One of three most parsimonious trees generated with PAUP* 4.0b2a from the combined data set of the aligned sequences of the 5.8S rRNA gene and flanking ITS1 and ITS2 regions and the partial β-tubulin gene (tree length 1571, CI = 0.837, RI = 0.865, RC = 0.724, HI = 0.163). *Fusarium oxysporum* (AF132800 & U34424) was used as outgroup.

Table 2. Intra- and interspecific variation within ITS1-ITS2 and partial β-tubulin gene DNA sequence of selected isolates of *Phaeoacremonium angustius* and *Pm. aleophilum*.

	ITS1-ITS2				β-tubulin			
Species	No. of strains	Length	Differences (bp)	Variation (%)	No. of strains	Length	Differences (bp)	Variation (%)
Intraspecies								
Pm. angustius[a]	5	510	0–6	0.0–1.2	5	529	0–9	0.0–1.7
Pm. aleophilum[a]	14	482–490	0–3	0.0–0.6	6	499–528	0–32	0.0–6.4
Interspecies								
Pm. angustius–*Pm. aleophilum* (CBS 246.91, 249.95)[b]	2	510–514	25	4.9	2	500–502	86	17.1–17.2

[a] Strains listed in Table 1.
[b] Ex-type cultures.

211.97) were found to be distinct from the presently known species based on morphology, cultural characteristics and phylogeny (Fig. 2), and are described as a new species of *Phaeoacremonium*.

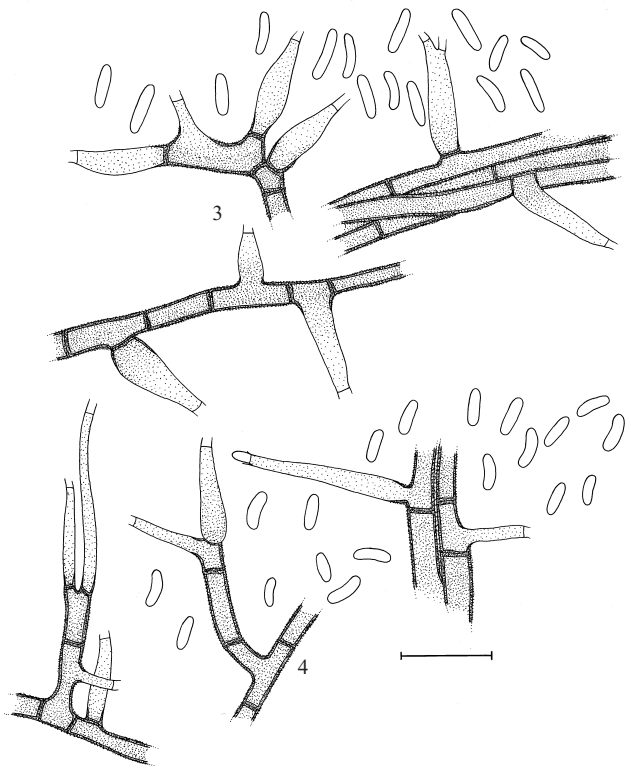
TAXONOMY

Phaeoacremonium mortoniae Crous & W. Gams, **sp. nov.** (Figs 3–4)

Etym.: Named in honour of Lucie Morton, for her contribution towards the understanding of black goo disease of grapevines.

Hyphae ramosae, septatae, singulae vel ad 15 fasciculatae, verruculosae vel tuberculatae, dilute brunneae vel rubro-brunneae, septis obscurioribus divisae, 2–4 µm latae. Conidiophora micronematosa vel macronematosa, ex hyphis aeriis vel submersis oriunda, erecta, simplicia vel ramosa, dilute brunnea, levia vel eximie verruculosa, recta vel flexuosa, 0–3-septata, longitudine variabilia, ad basim 1–3 µm lata, haud constricta ad septa. Cellulae conidiogenae solitariae, terminales vel laterales, monophialidicae, dilute brunneae vel subhyalinae, leves vel minime verruculosae, elongate ampulliformes vel lageniformes vel subcylindricae, vix constrictae ad basim, y 3–20 µm longae, 1–4 µm latae in parte basilari inflata, 1–1.5 µm ad apicem, collari anguste infundibuliformi, 1–2 µm longo et lato praeditae. Conidia in capitulis mucidis cohaerentia, hyalina, dimorphica: seu subcylindrica, recta vel allantoidea, (3–)4–7 × 1–1.5(–2) µm, seu breviora, ellipsoidea, 3–4(–7) × 1.5–2 µm.

Typus: **USA**: *California*: Sonoma County, isolatus e stipite *Vitis viniferae*, 1998, *L. Morton* & *L. van der Water* (PREM 57084 – holotypus; cultura viva CBS 101585).



Figs 3–4. Conidiophores and conidia of *Phaeoacremonium mortoniae* on MEA. **Fig. 3.** CBS 211.97 from *Fraxinus*. **Fig. 4.** CBS 101585 from *Vitis* (ex-type culture).

Mycelium consisting of branched, septate hyphae occurring singly or in strands of up to 15, tuberculate (warts to 0.5 µm) to finely verruculose, pale brown to medium red-brown walls with darker septa, becoming lighter towards the conidiogenous region, 2–4 µm wide. *Chlamydospore*-like structures not observed. *Conidiophores* micronematous to macronematous, arising from aerial or submerged hyphae, erect, simple, cylindrical, pale brown, smooth to finely verruculose, straight to flexuous, 0–3-septate, variable in length, 3–80 µm tall, 1–3 µm wide at base, generally not constricted at septa. *Conidiogenous cells* solitary, terminal or lateral, mostly monophialidic, pale brown to subhyaline, smooth to finely verruculose, elongate-ampulliform to lageniform or subcylindrical, not to prominently constricted at base, 3–20 µm long, 1–4 µm wide at the swollen part, 1.0–1.5 µm wide at the apex, with a terminal, narrowly funnel-shaped collarette, 1–2 µm long and wide. *Conidia* becoming aggregated in slimy heads at apices of conidiogenous cells, hyaline, dimorphic, partly subcylindrical, straight to allantoid, (3–)4–7 × 1–1.5(–2) µm, and partly shorter and ellipsoidal, 3–4(–7) × 1.5–2 µm.

Cultural characteristics. Colonies on MEA (reverse) fuscous black (7′′′′k), or alternating rings of fuscous black and greyish sepia (15′′′′i), outer ring of growth greyish sepia to honey (21′′b); surface pale mouse grey to mouse grey (15′′′′′d — 15′′′′′i), uniform in colour, or with a slightly lighter outer ring of smoke grey (21′′′′f) mycelium, with or without a brown pigment that can diffuse up to 3 cm from the colony into the agar; colony margins smooth, surface forming

radiating ridges in the agar with sparse aerial hyphae that tend to form hyphal strands, giving colonies a slightly woolly appearance in the middle; colonies reaching a radial growth of 10–11 mm at 30° in the dark after 8 d. Cardinal temperatures for growth: min above 10°, opt 30°, max below 35°.

Hosts: *Fraxinus excelsior*, *Vitis vinifera*.

Distribution: Sweden, USA (California).

Additional culture examined: **Sweden**: stem wound in *Fraxinus excelsior*, under stripped bark, Dec. 1996, *J. Stenlid* (CBS 211.97).

Phaeoacremonium mortoniae can be distinguished from other species in the genus by its unique cultural characteristics. Of the species that have an optimal growth rate at 30° (*Pm. rubigenum* and *Pm. inflatipes*; Crous *et al*. 1996), it is distinguished by having darker, fuscous black to greyish sepia colonies (reverse), as well as a diffuse brown pigment that can also form in the agar. These cultural differences are also supported by its distinct phylogeny based on ITS and β-tubulin sequence data. Based on morphology alone, however, it would be difficult to distinguish these species, which once again stresses the importance of integrating cultural characteristics and molecular data for the identification of these *taxa*.

Although regarded as similar (Dupont *et al*. 1998, 2000a, b), isolates of *Pm. aleophilum* and *Pm. angustius* could be distinguished in the present study based on DNA phylogeny (Fig. 2) and cultural characteristics. All 12 isolates of *Pm. aleophilum* tested obtained optimum growth on MEA at 30° after 8 d, and were able to grow at 35°. In contrast, however, isolates of *Pm. angustius* and *Pm. viticola* (CBS 101737, 101738 and 101739) obtained optimum growth at 25°, and were unable to grow at 35°. A diffuse yellow pigment was observed in the agar for some isolates of *Pm. aleophilum* when incubated as described above. A similar yellow diffused pigment was also observed for isolates of *Pm. angustius*, while others became red-purple in reverse, similar to that observed for isolates of *Pm. viticola*. Isolates of *Pm. mortoniae* produced a more brownish diffused pigment. These pigments were more pronounced in older cultures, but generally disappeared with successive subculturing.

DISCUSSION

The molecular data obtained in this study confirm those of previous studies (Dupont *et al*. 1998, Groenewald, Bellstedt & Crous 2000), showing that *Pm. chlamydosporum* was more closely related to *Phialophora s. str*. (*Herpotrichiellaceae*), than other species of *Phaeoacremonium* (*Magnaporthaceae*). These data, as well as the morphological differences discussed by Crous & Gams (2000), therefore support the new genus *Phaeomoniella* as being distinct from *Phaeoacremonium*. Dupont *et al*. (1998, 2000a, b) found insufficient evidence to distinguish *Pm. aleophilum* and *Pm. angustius*. Using sequence data (ITS and β-tubulin) of the type strains, as well as RFLP patterns of additional strains, they concluded that these two species were conspecific, with the ITS1 rDNA region showing only two nucleotide differences between these species, and the 5′ end of the β-tubulin gene showing no differences (Dupont *et al*. 2000a). Furthermore, they also found growth patterns to be similar, though the incubation period was significantly longer than that proposed in previous studies (Crous *et al*. 1996, Crous & Gams 2000). In the present study we were able to distinguish these two species by their ability to grow on MEA at 35° (*Pm. aleophilum*) or below (*Pm. angustius*) after an incubation period of 8 d in the dark. Furthermore, although similar, we observed more genotypic divergence between these two species than that initially reported by Dupont *et al*. (1998, 2000a, 2000b). Sequences of the partial β-tubulin gene of the ex-type strains of *Pm. aleophilum* (CBS 246.91) and *Pm. angustius* (CBS 249.95) showed distances of 15.5% from each other and for the rDNA data set differences of 4% were observed. Three isolates representing *Pm. viticola* (Dupont *et al*. 2000a) (CBS 101739, 101737, 101738) were also found to be the same as *Pm. angustius* (CBS 249.95), with a 100% sequence similarity in the ITS sequence. Phylogenetic analysis of the combined data set supported the separation of *Pm. angustius* from *Pm. aleophilum* with a strong bootstrap value and decay index. A practical and easy method for distinguishing these species seems to remain their cardinal temperatures for growth. *Pm. aleophilum* has an optimum growth rate at 30°, and can grow at 35°, whereas *Pm. angustius* has an optimum growth at 25° and does not grow at 35°. Although morphologically similar, the separation of *Pm. angustius* and *Pm. aleophilum* is therefore supported based on the DNA phylogeny and cultural growth characteristics.

This study has further shown that *Pm. aleophilum* is a prominent organism in grapevine decline with a wide geographic distribution, and that isolates thought to be *Pm. inflatipes* were in fact *Pm. aleophilum*. In fact, no records of *Pm. inflatipes* could be confirmed from grapevine, and its occurrence in this host remains to be proven.

In conclusion, the *Phaeoacremonium* complex in *Vitis vinifera* is extremely difficult to identify based on morphological characteristics alone. It is therefore not surprising that in this study we found many *Phaeoacremonium* isolates to be misidentified. More isolates need to be included in future studies to resolve questions concerning the species that occur in grapevines and which of these play a role in diseases of this host, other plants, and in human disorders. Molecular techniques will play an increasingly important role in identification of relevant isolates. Here diagnostic testing with specific primers (Groenewald *et al*. 2000, Tegli, Betelli & Surico 2000) will also be important.

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