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Cymadothea trifolii, an obligate biotrophic leaf parasite of *Trifolium*, belongs to *Mycosphaerellaceae* as shown by nuclear ribosomal DNA analyses

U.K. Simon[1], J.Z. Groenewald[2], P.W. Crous[2]

Key words

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Capnodiales
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Mycosphaerella kilianii
Mycosphaerellaceae
sooty/black blotch of clover
SSU

Abstract The ascomycete *Cymadothea trifolii*, a member of the *Dothideomycetes*, is unique among obligate biotrophic fungi in its capability to only partially degrade the host cell wall and in forming an astonishingly intricate interaction apparatus (IA) in its own hyphae, while the attacked host plant cell is triggered to produce a membranous bubble opposite the IA. However, no sequence data are currently available for this species. Based on molecular phylogenetic results obtained from complete SSU and partial LSU data, we show that the genus *Cymadothea* belongs to the *Mycosphaerellaceae* (*Capnodiales*, *Dothideomycetes*). This is the first report of sequences obtained for an obligate biotrophic member of *Mycosphaerellaceae*.

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INTRODUCTION

The obligate biotrophic ascomycete *Cymadothea trifolii* (*Dothideomycetes*, *Ascomycota*) is the causal agent of sooty/black blotch of clover. Although the fungus is not regarded as a serious agricultural pathogen, it has a significant impact on clover plantations used for animal nutrition, and is often found at natural locations. In one study it was observed that sooty blotch was "the most frequently recorded disease" at sampling sites in England and Wales, with the number of leaves damaged ranging from 4–21 % (Lewis & Thomas 1991). *Cymadothea trifolii* is widespread on *Trifolium* species (*Fabaceae*), but has also been reported on alfalfa (*Medicago sativa*) (Puschner 2005). The fungus is considered a likely cause of edema, erythema, vesiculation and necrosis of the light pigment areas of horses grazing infected clover (Puschner 2005). It is characterised by small black pustules, the stromata, on the lower side of clover leaflets. The asexual state (*Polythrincium trifolii*) can easily be identified by thick-walled, melanised, sympodially growing conidiophores with a spiral appearance. In its sexual state it produces pseudothecial ascomata and spermatogonia. Infection of the host occurs via stomata (Roderick 1993, Simon et al. 2005b). Inside the leaf the fungus proliferates intercellularly, but forms an intricate interaction apparatus (IA) to obtain nutrients from its host (Simon et al. 2004). During the interaction with the attacked host cell the wall of the latter is partially degraded. Pectins are dissolved while cellulose and xyloglucans remain intact (Simon et al. 2005a). This structure is thus far unique among ascomycetes.

Although the morphology of *C. trifolii* has been accurately documented (Wolf 1935), no molecular evidence is currently available to clarify its taxonomy. Due to the unique interaction this obligate pathogen has with its host, the aim of the present study was to obtain DNA sequence data to resolve its phylogenetic position.

[1] Karl-Franzens-University Graz, Institute for Plant Sciences, Department of Plant Physiology, Schubertstraße 51, 8010 Graz, Austria; corresponding author e-mail: uwe.simon@uni-graz.at.
[2] CBS Fungal Biodiversity Centre, P.O. Box 85167, 3508 AD, Utrecht, The Netherlands.

MATERIALS AND METHODS

Sampling

Infected leaves of *Trifolium repens* were collected at the edge of an alfalfa (*Medicago sativa*) field near Hohenentringen (Tübingen, Baden-Württemberg, Germany) on 31 July 2007 (Herbarium CBS H-20110). Furthermore, 53 species from the CBS culture collection were included to supplement sequences obtained from GenBank due to the paucity of complete small subunit (SSU) data of related fungal nuclear ribosomal DNA in GenBank (Table 1).

DNA extraction and amplification

Approximately 12 conidial stromata of *C. trifolii* were dissected from one spot of an infected leaf with a sterilised razor blade, and washed in 20 µL of AE-buffer (Qiagen, Hilden, Germany). Each stroma was examined with a light microscope to check for possible contaminations with other fungi. Apparently uncontaminated stromata were collected in a fresh drop of AE-buffer, gently washed, and re-examined before placing them onto another 20 µL drop of AE-buffer in an Eppendorf tube (1.5 mL). The procedure was repeated from another spot on the same leaf. This method was chosen because earlier attempts to isolate DNA of this fungus had always resulted in contaminations with other species of fungi (not shown). Secondly, it allowed us to exclude plant material.

To break up the thick melanised cell walls of conidiophores and conidia, the cups containing fungal material were placed in liquid nitrogen for 5 min and heated immediately afterwards for 5 min at 96 °C in a heating block (Dri-block DB-2A, Techne, Cambridge, UK). This step was repeated twice. Because little DNA was present in the samples, the whole genome was amplified

Table 1 Species names, culture collection and GenBank accession numbers of fungal strains used in this study. The GenBank accession number is for the concatenated 18S rDNA, ITS1, 5.8S rDNA, ITS2 and partial 28S rDNA sequence, unless otherwise indicated.

Species	Strain no.[1]	GenBank no.
Ascochyta fabae	CBS 114.36	EU167566
Ascochyta pisi var. *pisi*	CBS 108.26	EU167557
Ascochyta vicia-pannonicae	CBS 254.92	EU167559
Ascochyta viciae-villosae	CBS 255.92	EU167560
Asteroma alneum	CBS 109840	EU167609
Bagnisiella examinans	CBS 551.66	EU167562
Cercospora beticola	CBS 116456	AY840527
Cladosporium sp. 1	CBS 280.49	EU167574
Cladosporium sp. 2	CBS 282.49	EU167586
Cladosporium sp. 3	CBS 266.53	EU167592
Cymadothea trifolii	Herbarium CBS H-20110	EU167612 (SSU)
	Herbarium CBS H-20110	EU167613 (SSU)
	Herbarium CBS H-20110	EU167610 (LSU)
	Herbarium CBS H-20110	EU167611 (LSU)
Davidiella macrospora	CBS 138.40	EU167591
Davidiella tassiana	CBS 723.79	EU167558
Didymella bryoniae	CBS 233.52	EU167573
Didymella exitialis	CBS 446.82	EU167564
Didymella phacae	CBS 184.55	EU167570
Didymella rabiei	CBS 237.37	EU167600
Dothidea berberidis	CBS 186.58	EU167601
Dothidea muelleri	CBS 191.58	EU167593
Guignardia vaccinii	CBS 114751	EU167584
Kabatiella caulivora	CBS 242.64	EU167576
Kabatiella microsticta	CBS 342.66	EU167608
Mycosphaerella aleuritidis	CBS 282.62	EU167594
Mycosphaerella arbuticola	CBS 355.86	EU167571
Mycosphaerella berberidis	CBS 324.52	EU167603
Mycosphaerella brassicicola	CBS 174.88	EU167607
Mycosphaerella coacervata	CBS 113391	EU167596
Mycosphaerella crystallina	CBS 681.95	EU167579
Mycosphaerella flageoletiana	CBS 114302	EU167597
Mycosphaerella fragariae	CBS 719.84	EU167605
Mycosphaerella gregaria	CBS 110501	EU167580
Mycosphaerella handelii	CBS 113302	EU167581
Mycosphaerella harthensis	CBS 325.52	EU167602
Mycosphaerella laricina	CBS 326.52	EU167595
Mycosphaerella linorum	CBS 261.39	EU167590
Mycosphaerella microsora	CBS 100352	EU167599
Mycosphaerella milleri	CBS 541.63	EU167577
Mycosphaerella punctata	CBS 113315	EU167582
Mycosphaerella populicola	CBS 100042	EU167578
Mycosphaerella pseudoellipsoidea	CBS 114709	EU167585
Mycosphaerella punctiformis	CBS 113265	EU167569
Mycosphaerella pyri	CBS 100.86	EU167606
Mycosphaerella grossulariae	CBS 235.37	EU167588
Mycosphaerella rosigena	CBS 330.51	EU167587
Mycosphaerella rubi	CBS 238.37	EU167589
Mycosphaerella stromatosa	CBS 101953	EU167598
Phaeosphaeria rousseliana	CBS 580.86	EU167604
Phoma exigua var. *exigua*	CBS 118.94	EU167567
Phoma medicaginis var. *medicaginis*	CBS 533.66	EU167575
Phoma pinodella	CBS 110.32	EU167565
Phoma sojicola	CBS 567.97	EU167568
Pleiochaeta ghindensis	CBS 552.92	EU167561
Pleiochaeta setosa	CBS 496.63	EU167563
Pseudocercospora vitis	CPC 11595	DQ073923
Ramichloridium cerophilum	CBS 103.59	EU041798
Schizothyrium pomi	CBS 486.50	EF134948
	CBS 406.61	EF134949
Teratosphaeria fibrillosa	CPC 1876	EU019282
Teratosphaeria microspora	CBS 101951	EU167572
Teratosphaeria molleriana	CBS 118359	EU167583

[1] CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC: Culture collection of Pedro Crous, housed at CBS.

using the GenomiPhi kit (GE Healthcare, Munich, Germany) according to the manufacturer's protocol: 1 µL of each sample was placed into 9 µL of sample buffer and placed in a heating block (Techne) at 96 °C for 3 min, cooled down on ice and mixed with 10 µL of a prepared solution consisting of 9 µL reaction buffer and 1 µL enzyme mix. The resulting 20 µL solution was incubated for 24 h at 30.5 °C in a thermal cycler (model 2720, Applied Biosystems, Foster City, CA, USA) equipped with a heated lid. Afterwards, the samples were heated up to 96 °C for 10 min in a heating block (Techne) and subsequently cooled on ice to stop polymerase activity of the kit. DNA was extracted with the DNAeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions.

Additionally, DNA was isolated from 52 fungi of the class *Dothideomycetes* and one fungus of the class *Sordariomycetes* from the CBS culture collection (Table 1) using a CTAB-based method modified from Möller et al. (1992) as described in Gams et al. (2007).

Polymerase chain reactions (PCRs) were performed in a total volume of 50 µL containing 5 µL 10 × PCR-buffer (Life Technologies, Eggenstein, Germany), 34.1 µL H_2O, 2 µL $MgCl_2$ (50mM, Life Technologies), 2 µL dNTPs (5 mM, Life Technologies), 1 µL forward and 1 µL reverse primers (25 pmol/µL each), 0.2 µL Bovine Serum Albumin (1 %, BSA, Sigma-Aldrich, Munich, Germany), 0.2 µL *Taq* polymerase (Life Technologies) and 5 µL DNA extract diluted 1 : 10. The following primers were used for amplification: SSU: a) forward: NS17, NS19, NS21, NS23, b) reverse: NS18, NS20, NS22, NS24 (Gargas & Taylor 1992); LSU: a) forward: LR0R (Rehner & Samuels 1994), b) reverse: LR5 (Vilgalys & Hester 1990). PCR was carried out on a 2720 Thermal Cycler (Applied Biosystems) equipped with a heated lid. Initial denaturation and enzyme activation took place at 94 °C for 5 min and was followed by amplification for 35 cycles. The parameters were as follows: 30 s at 94 °C, 90 s at either 50, 55, 60 or 65 °C (depending on primers), 4 min at 72 °C, plus a final 7 min extension at 72 °C with subsequent cooling down to 4 °C.

Sequencing

For cycle sequencing the same primers were applied as for PCR using the ABI PRISM BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol, but with a reaction volume of 10 µL, and the enzyme diluted 1 : 6 with the supplied dilution buffer. Electrophoresis and data sampling were performed on an ABI 3100 Genetic Analyser (Applied Biosystems). Sequences were manually edited with SEQUENCHER™ v. 4.1.2 (Gene Codes Corporation, Ann Arbor, Mi, USA).

Phylogenetic analyses

DNA sequences were assembled, added to the outgroup and complemented with further GenBank sequences using Sequence Alignment Editor v. 2.0a11 (Rambaut 2002). Manual adjustments for improvement were made by eye where necessary. Any large insertions were excluded. Phylogenetic analyses of sequence data were done with PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2003) and consisted of neighbour-joining analysis with the uncorrected ("p"), the Kimura 2-parameter and the HKY85 substitution model. Alignment gaps were treated as missing data and all characters were unordered and of equal weight. Any ties were broken randomly when encountered. For parsimony analysis, alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed using the heuristic search option with 100 random taxa additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1 000 bootstrap replications (Hillis & Bull 1993). Tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency index (RC) were calculated and the resulting trees were printed with TreeView v. 1.6.6 (Page 1996).

Bayesian analysis was conducted on the same aligned dataset after MrModeltest v. 2.2 (Nylander 2004) was used to determine the best nucleotide substitution model for each data partition (18S rDNA and 28S rDNA). Phylogenetic analyses were per-

Fig. 1 One of 141 equally most parsimonious trees obtained from the combined SSU and LSU sequence alignment. The scale bar shows 10 changes and bootstrap support values from 1 000 replicates are shown at the nodes. The tree was rooted to *Asteroma alneum*. The relevant order and class names are given on the right with family designations included for *Capnodiales*. D = *Dothideomycetes*.

formed with MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003) applying a general time-reversible (GTR) substitution model with gamma (G) and proportion of invariable site (I) parameters to accommodate variable rates across sites and with inverse gamma rates and dirichlet base frequencies. The Markov Chain Monte Carlo (MCMC) analysis of four chains started with a heating parameter of 0.1 from a random tree topology and lasted 1 031 000 generations. Trees were saved each 100 generations, resulting in 10 311 saved trees. Burn-in was set at 2 500 generations after which the likelihood values were stationary, leaving 7 811 trees from which the 50 % majority rule consensus trees and posterior probabilities were calculated. All trees were rooted with *Asteroma alneum* (*Sordariomycetes*) as outgroup taxon.

RESULTS

Complete SSU and partial LSU sequences were obtained for *Cymadothea trifolii* using the GenomiPhi Kit (GE Healthcares). Despite repeated attempts and specific primer design we were

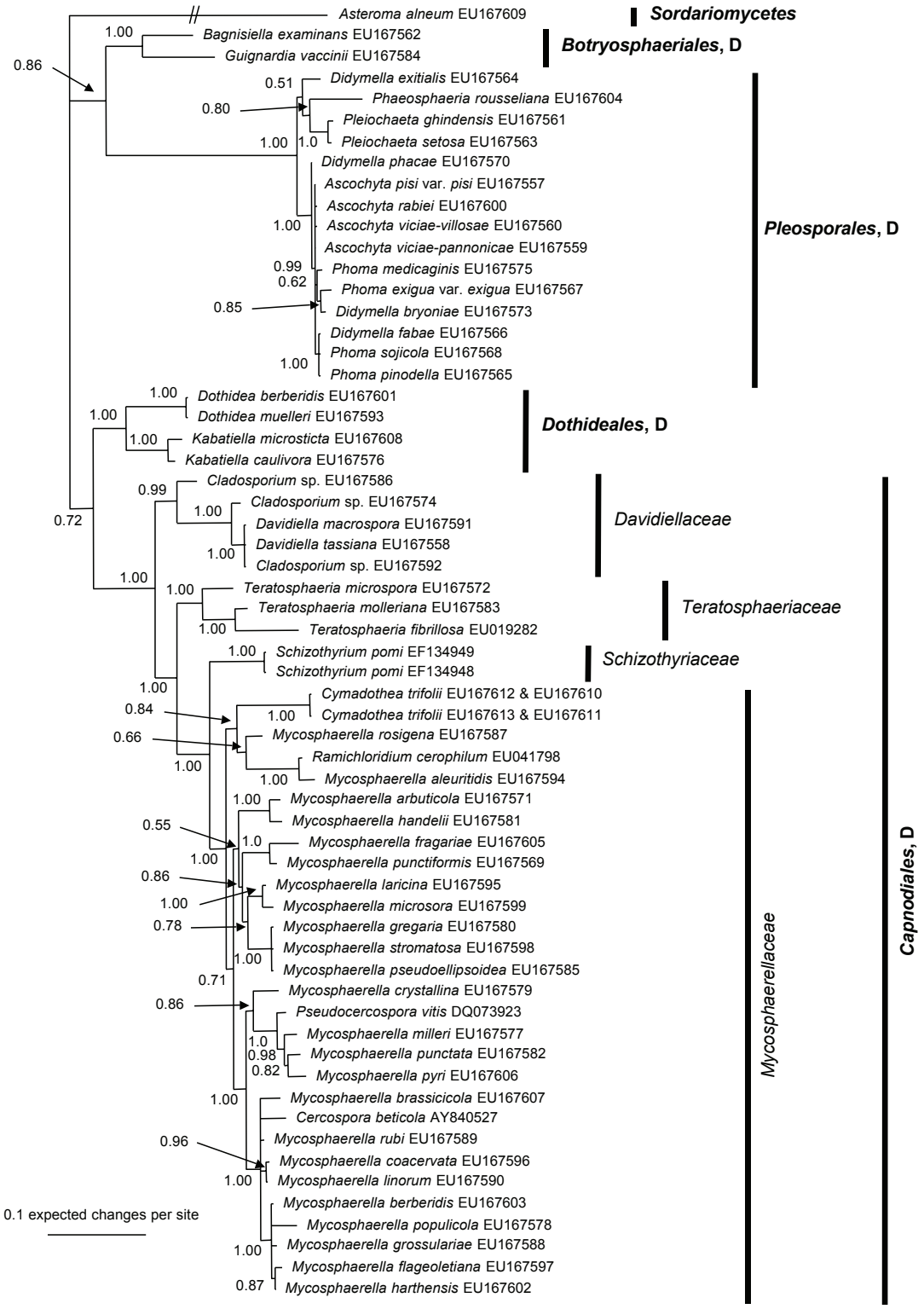

Fig. 2 The 50 % majority rule tree of 7 811 trees obtained from a Bayesian analysis of the combined SSU and LSU sequence alignment. Bayesian posterior probabilities are given at the nodes and the scale bar shows 0.1 expected changes per site. The tree was rooted to *Asteroma alneum*. D = *Dothideomycetes*.

unable to obtain ITS sequences for this species. Sequence comparisons of the two isolations showed that both isolates were 100 % identical. Additionally, we were able to generate sequences from another 19 species thus far absent from GenBank (checked both by CoreNucleotide search of names and by using BLAST searches against the NCBI database, (http://www.ncbi.nlm.nih.gov/)). GenBank accession numbers are provided in Table 1.

The manually adjusted alignment contains 60 taxa plus the outgroup sequence. 2 419 characters including alignment gaps (available in TreeBASE) were used for phylogenetic analyses. Of these, 371 were parsimony-informative, 154 were variable and parsimony-uninformative, and 1 894 were constant. Neighbour-joining analyses using the three substitution models on the sequence data yielded trees with similar overall topology and bootstrap support values. The parsimony analysis yielded 141 equally most parsimonious trees (TL = 1 438 steps; CI = 0.524; RI = 0.855; RC = 0.448), the first of which is shown in Fig. 1. These trees differed mainly with regard to the order of taxa in the *Pleosporales* and in the *Mycosphaerellaceae* (see thickened consensus lines in Fig. 1). Also, the *Schizothyriaceae* was placed within the *Mycosphaerellaceae* in some of these

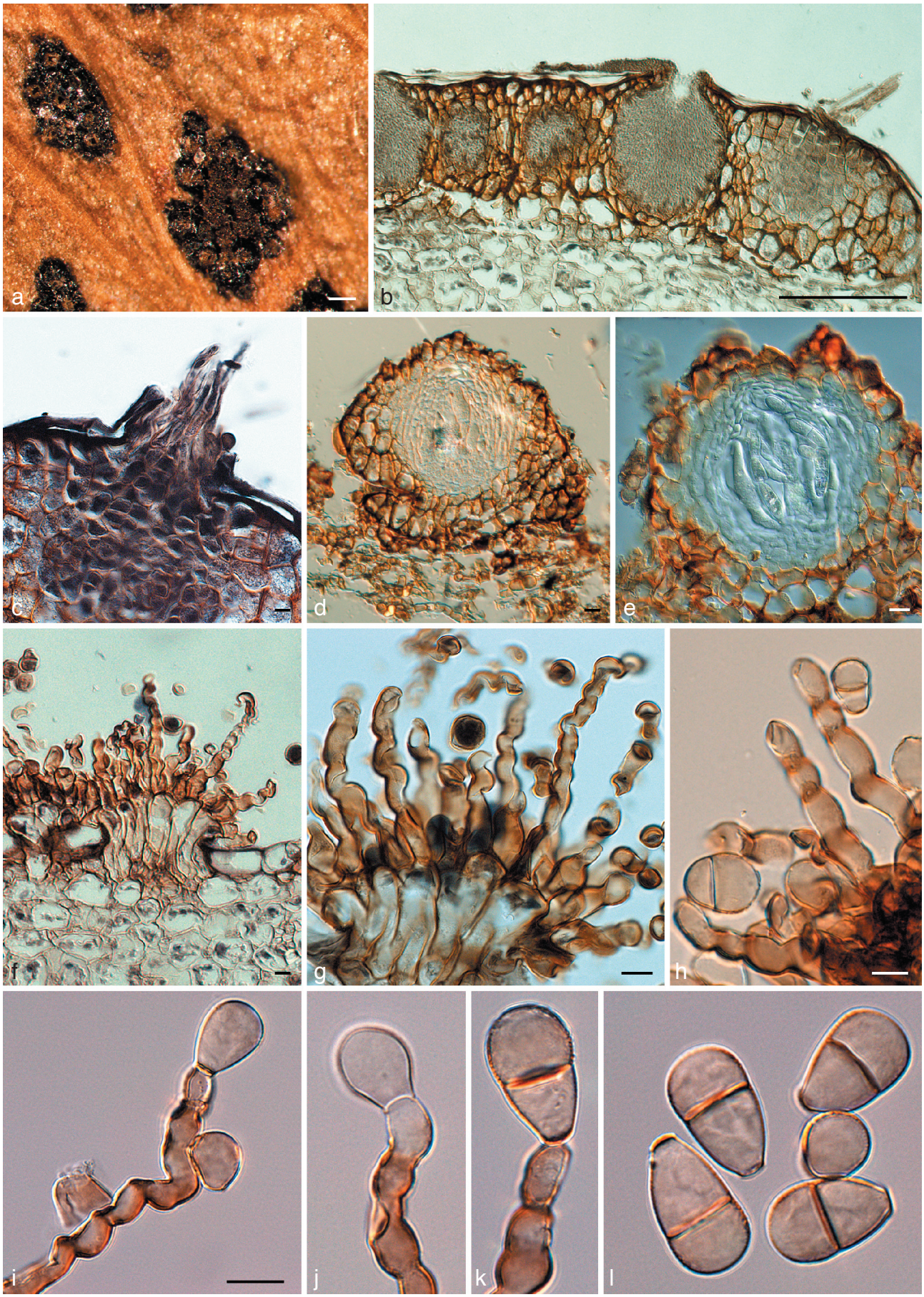

Fig. 3 *Cymadothea trifolii* and its anamorph *Polythrincium trifolii*. a. Ascomata and spermatogonia on the leaf surface; b. vertical section through spermatogonia; c. trichogynes arising from developing ascoma; d, e. vertical section through ascomata; f–h. fasciculate conidiophores arising from leaf tissue. Basal part consisting of tightly aggregated subcylindrical cells that give rise to one or more curved conidiogenous cells with flattened, darkened scars along the length of one side of each conidiogenous cell; i–k. conidiogenous cells with developing conidia; l. mature (0–)1-septate conidia. — Scale bars = 10 µm, except a = 150 µm, b = 120 µm.

trees. MrModeltest identified the same substitution parameters for both data partitions. The Bayesian analysis resulted in a consensus tree (Fig. 2) with the same topology and clades as that obtained from the parsimony analysis.

Both analyses show that *C. trifolii* unequivocally belongs to *Mycosphaerellaceae* s.str. (*Dothideomycetes*, *Capnodiales*) with *M. aleuritidis*, *M. rosigena* and *Ramichloridium cerophilum* as nearest relatives. However, the Bayesian analyses supported this cluster with a posterior probability value of 0.84 (Fig. 2), whereas the parsimony analysis failed to provide bootstrap or consensus support for the association (Fig. 1). The *Mycosphaerellaceae* as family was highly supported only in the Bayesian analysis while many subgroupings received only little support or appeared paraphyletic in both analyses. In the parsimony analysis, the clustering of *Mycosphaerellaceae* and *Schizothyriaceae* were well supported but not the *Mycosphaerellaceae* in itself.

DISCUSSION

Our analyses based upon whole nuclear ribosomal SSU and partial LSU (D1-D3) sequence data show that *Cymadothea trifolii* belongs to the *Mycosphaerellaceae* s.str. However, the position of this clade within the *Mycosphaerellaceae* remains uncertain due to the lack of high MP bootstrap support values or Bayesian posterior probabilities. Furthermore, recent studies have led to the conclusion that the genus *Mycosphaerella* is polyphyletic (Crous et al. 2007a), and that this morphology type occurs in several families within the *Capnodiales*, including *Mycosphaerellaceae*, *Schizothyriaceae* (Batzer et al. 2008) and *Davidiellaceae* (Crous et al. 2007b, Schubert et al. 2007). Within the *Mycosphaerellaceae*, however, several genera other than *Mycosphaerella* can be distinguished. Although these are chiefly recognised based on their anamorphs, the fact that these anamorph genera are also paraphyletic within the order is cause for more confusion (Arzanlou et al. 2007, 2008, Cheewangkoon et al. 2008, Crous et al. 2007a, 2008a, b).

On a general scale, the phylogenetic placement of the included orders is congruent with the multi-gene phylogeny for *Dothideomycetes* published recently (Schoch et al. 2006). The relatively comprehensive representation of *Pleosporales* is due to the fact that some of our preliminary analyses had pointed to the genus *Didymella* as the group to which *C. trifolii* might belong. Later we discovered that these earlier findings were due to contaminations (see below). We were also able to contribute sequences for 19 species hitherto unrepresented in GenBank, including *M. aleuritidis* and *M. rosigena*, which have turned out to be most closely related to *C. trifolii*. Morphologically, *C. trifolii* is a typical member of the *Mycosphaerellaceae* (Fig. 3), having spermatogonia, and hyaline, 1-septate ascospores in 8-spored, bitunicate asci, formed in fascicles in pseudothecial ascomata. Its anamorph, which is placed in the monotypic genus *Polythrincium*, is passalora-like (Crous & Braun 2003), but should be retained as separate due to the unique morphology of its conidiophores and arrangement of its conidial scars. Subsequently, based on its phylogeny and unique anamorph, the genus *Cymadothea* should be regarded as a distinct genus within the *Mycosphaerellaceae*. Apparently, the closest relatives of *Cymadothea* have yet to be found. Since this species is an obligate biotroph, other members of its group may turn out to be well-known biotrophic *Mycosphaerellaceae* that could thus far not be grown on agar media. In our analyses, only the Bayesian analysis provided strong support for the *Mycosphaerellaceae* with little resolution within the family. Using only LSU data, Crous et al. (2007a) obtained bootstrap support of 76 % and a posterior probability value of 0.83 for parsimony and Bayesian analyses respectively for the *Mycosphaerellaceae*. The lack of support for this family obtained during this study could be due to the high homoplasy because of the taxon sampling and/or selected gene regions adversely influencing the phylogenetic signal. Using only LSU and representatives of the *Mycosphaerellaceae* and *Teratosphaeriaceae*, Cheewangkoon et al. (2008) obtained a bootstrap support value of 88 % for the *Mycosphaerellaceae*.

To our knowledge, this is the first report of sequence data for a truly obligate biotrophic member of this economically important family, which contains thousands of serious plant pathogens, including some of which the genomes are, or soon will be, available, such as *M. graminicola* (septoria leaf and glume blotch of wheat) and *M. fijiensis* (black leaf streak of banana). During this study it was extremely difficult to obtain uncontaminated DNA of *C. trifolii* – a problem well known to all researchers working with obligate biotrophs. While the SSU sequences were relatively easy to obtain, more than 50 previous attempts to generate clear LSU sequences with a variety of primer combinations tested were unsuccessful. Even cloning produced no obvious results because contaminations were so abundant. Only after extremely careful removal, washing and light microscopic examination of conidiostromata and then applying the GenomiPhi kit (GE Healthcares), which allows amplification of DNA from very small samples (see Tan & Murray 2006), we succeeded in obtaining clear sequences. Thus, we strongly recommend using this kit in combination with the cleaning procedure described in this manuscript when there is little DNA present, or when there is a high risk of contamination.

Cymadothea trifolii has a hitherto unique mode of nutrient acquisition via an extremely complex IA as documented in previous work (Simon et al. 2004, 2005b). Applying immunocytochemical methods it was found that *C. trifolii* differentially dissolves the host cell wall at the contact area: skeletal elements (cellulose and xyloglucans) are left intact, while the pectin matrix gets degraded (Simon et al. 2005b). Thereby the pathogen presumably increases the host cell wall pore size without totally disrupting the integrity of the attacked cell. Both the cellular interaction and the highly localised differential host wall degradation make *C. trifolii* at present unique among fungi.

Such an intricate cellular interaction is unlikely to have evolved without intermediate forms. Accordingly, there should be fungi producing structures resembling those of *C. trifolii* as shown by Bauer et al. (1997) for a somewhat similar kind of interaction in the *Exobasidiales* (*Basidiomycota*). However, these species have yet to be discovered. Only by widening the sampling, especially of biotrophic species, will we be able to tell whether or not this mode of interaction is mirrored in phylogenetic relationships and evolutionary trends.

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