Multiple Didymella teleomorphs are linked to the Phoma clematidina morphotype

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Key words
Ascochyta vitatae
β-tubulin
Clematis
Didymella clematidis
Didymella vitatae
DNA phylogeny
ITS
LSU
taxonomy

Abstract The fungal pathogen Phoma clematidina is used as a biological agent to control the invasive plant species Clematis vitalba in New Zealand. Research conducted on P. clematidina as a potential biocontrol agent against C. vitalba, led to the discovery of two perithecial-forming strains. To assess the diversity of P. clematidina and to clarify the teleomorph-anamorph relationship, phylogenetic analyses of 18 P. clematidina strains, reference strains representing the Phoma sections in the Didymellaceae and strains of related species associated with Clematis were conducted. Partial sequences of the ITS1, ITS2 and 5.8S rRNA gene, the β-tubulin gene and 28S rRNA gene were used to clarify intra- and inter-species relationships. These analyses revealed that P. clematidina resolves into three well-supported clades which appear to be linked to differences in host specificity. Based on these findings, Didymella clematidis is newly described and the descriptions of P. clematidina and D. vitatae are amended.

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INTRODUCTION

The genus Clematis (Ranunculaceae) accommodates (semi-) woody, climbing plants and shrubs. Species of Clematis occur throughout the temperate regions of the northern and southern hemispheres and can also be found in the tropics and mountainous regions. Clematis contains more than 400 species, and more than 600 varieties are grown commercially. In the 19th century the cultivation of Clematis became popular but soon after the start of its large scale cultivation, a widespread destructive disease which caused high yield losses emerged in Europe and America (van de Graaf et al. 2001). This disease was referred to as Clematis wilt, exhibiting symptoms of stem rot and wilting of above-ground plant parts (Gloyer 1915). Ascochyta clematidina and Coniothyrium clematidis-rectae were identified as the causal organisms of Clematis wilt (Gloyer 1915, Blok 1965).

On the basis of new circumscriptions of Phoma and Ascochyta (Boerema & Bollen 1975), A. clematidina was transferred to Phoma as P. clematidina (Boerema & Dorenbosch 1979). Phoma clematidina is presently regarded as a widespread pathogen of Clematis spp. Incidentally, P. clematidina has also been isolated from plants other than Clematis, including a cultivated Selaginella sp. (Boerema & Dorenbosch 1979). Gloyer (1915) inoculated a series of plant species such as bean, pea, muskmelon, pumpkin, eggplant and elm with P. clematidina to assess its host range and found no development of disease symptoms. However, in the necrotic tissue at the point of inoculation developing pycnidia could be observed, indicating that P. clematidina may survive as a saprobe on different plant hosts.

Clematis vitalba (old man’s beard) is a vine that is native to Europe but has become widespread primarily due to its introduction as an ornamental. As an invasive plant species, C. vitalba is a threat to native trees and shrubs, as it reduces light levels and smothers crowns of trees with its prolific foliage (Gourlay et al. 2000). In New Zealand, C. vitalba is regarded as a serious pest, and much research has been undertaken in order to save the native forest remnants from disappearing due to smothering caused by C. vitalba (Hume et al. 1995, Ogle et al. 2000, Hill et al. 2001, 2004, Paynter et al. 2006). After extensive laboratory tests, a virulent strain of P. clematidina, which was originally isolated from an American C. ligusticifolia, was introduced to New Zealand in 1996 as a biological control agent of C. vitalba (Gourlay et al. 2000). Remarkably, a teleomorph was observed to develop on agar slants in vitro after storage for approximately 2 yr. A similar finding was observed in a strain isolated from C. vitalba from Switzerland.

In the present study the sexual strains of P. clematidina are phylogenetically and morphologically compared to reference strains housed in the culture collections of the Centraalbureau voor Schimmelcultures (CBS) and the Dutch Plant Protection Service (PD). The aims of this study were to assess the variation within this species, and to clarify the morphology of its potential sexual state.

MATERIALS AND METHODS

Fungal isolation and DNA extraction

Small fragments (< 1.0 mm2) of necrotic leaf tissue were removed with a dissecting needle and plated onto filtered V8-juice agar (V8) (Gams et al. 2007), and incubated at 20 °C under a 12 h near-ultraviolet / 12 h dark photo period. After 7 d, the agar slants were amended.

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### Table 1: Isolates included in the phylogenetic analyses.

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession no.</th>
<th>Host</th>
<th>Origin</th>
<th>GenBank no.</th>
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<td><strong>Phoma exigua var. exigua</strong></td>
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<td><strong>Phoma herbarum</strong></td>
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<td>Rosa multiflora</td>
<td>Netherlands</td>
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<tr>
<td><strong>Phoma zeae-maydis</strong></td>
<td>CBS 588.69</td>
<td>Zea mays</td>
<td>USA</td>
<td>FJ427086 FJ427190 EU754192</td>
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</table>


DNA extraction from all isolates was performed using the UltraClean Microbial DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA), according to the manufacturer’s instructions. All DNA extracts were diluted 10× in milliQ water and stored at 4 °C before their use as PCR templates.

### DNA amplification and phylogenetic analyses

For phylogenetic analyses, parts of the ITS1, ITS2 and 5.8S rRNA gene (ITS), the 9-tubulin gene (TUB) and 28S rRNA gene (LSU) were analysed. The primers V9G (de Hoog & Gerrits van den Ende 1998) and IITS4 (White et al. 1990) were used for the amplification of the ITS region, primers Btub2Fd (5’-GTB CAC CTY CAR ACC GGY CAR TG-3’) and Btub4Rd (5’-CCR GAY TGR CCR AAR ACR AAG TGG TC-3’) for the TUB region (J.Z. Groenewald, CBS) and primers LR0R (Rehner & Samuels 1994) and LR7 (Vilgalys & Hester 1990) for the LSU region.

The LSU PCR was performed as described by de Gruyter et al. (2009). The ITS and TUB PCR mixtures both contained 0.5 units of Taq polymerase E (Genaxxon Bioscience, Biberach, Germany), 0.2 µM of each primer and 1× PCR buffer E incomplete (Genaxxon Bioscience). The remaining PCR mixture consisted of 0.5 µL diluted genomic DNA, 0.04 mM dNTPs and 1 mM MgCl₂ for the ITS region and 1.0 µL diluted genomic DNA, 0.02 mM dNTPs and 2 mM MgCl₂ for the TUB region. The amplification reactions were performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, California, USA) and had a total volume of 12.5 µL. Conditions for PCR amplification were comparable for both regions and consisted of an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of denaturation (94 °C for 30 s), annealing and elongation (52 °C and 72 °C for 30 s), and 35 cycles of denaturation (94 °C for 5 min), followed by 30 cycles of annealing and elongation (52 °C and 72 °C for 30 s) for the ITS region and 35 cycles of annealing and elongation (48 °C and 72 °C for 60 s) for the TUB region.

PCR amplicons were visualised by electrophoresis and sequenced as described by de Gruyter et al. (2009). DNA sequences obtained from forward and reverse primers were used to obtain consensus sequences using Bionumerics v. 4.60 (Applied Maths, St-Martens-Latem, Belgium) and phylogenetic analyses of the sequence data were conducted in PAUP v. 4.0b10 (Swofford 2003). To test whether the three different loci could be used in combined analyses, a partition homogeneity test was executed (Farris et al. 1995). Phylogenetic analyses consisted of Neighbor-Joining analysis with the uncorrected “p”, Jukes-Cantor and Kimura 2-parameter substitution models, and a parsimony analysis using the heuristic search option with 100 random taxa additions. Alignment gaps were set as fifth state, and tree bisection and reconstruction (TBR) was used as the branch-swapping algorithm. The robustness of the most parsimonious tree was evaluated by 1 000 bootstrap replicates (Hillis & Bull 1993). The resulting trees were printed with TreeView v. 1.6.6 (Page 1996) and are deposited in TreeBASE (www.treebase.org).

### Morphology

Cultural characteristics of the strains (Table 1) were studied on oatmeal agar (OA) and malt extract agar (MEA) (Gams et al. (Table 1)). The *Phoma* reference strains represent the type species of the five *Phoma* sections recently classified in the Didymellaceae (de Gruyter et al. 2009), including the type species of the genus *Phoma*, *P. herbarum* (CBS 615.75). *Phoma clematidina* has been placed in Phoma sect. Heterospora (Boerema et al. 1997), however, the type species of this section, *Phoma heteromorphospora* proved not to be related to the Didymellaceae (de Gruyter et al. 2009). Therefore *Phoma glaucii* (CBS 114.96) was used as the reference strain for this section. Two *C. clematidis-rectae* strains were also included, as they are closely related to *Phoma* and have also been found associated with wilting symptoms of *Clematis* (Table 1).
analyses. The combined alignment consisted of 2 150 bp (ITS 490 bp, TUB 333 bp, LSU 1327 bp), of which 1 978 characters were constant, 60 were parsimony uninformative and 112 were parsimony informative. The Neighbour-Joining trees obtained with the three different substitution models, and the single most parsimonious tree exhibited identical topology. The most parsimonious tree is presented in Fig. 1 (TL = 314 steps, CI = 0.697, RI = 0.888, RC = 0.619). This phylogenetic tree supports division of the P. clematidina strains into three distinct and well-supported groups (Fig. 1). A first group (clade A) contains the representative culture of P. clematidina CBS 108.79 (Boerema & Dorenbosch 1979) and strains isolated from symptomatic Clematis species and hybrids. A second group (clade B) comprises strains isolated from C. vitalba, including the freshly isolated strain CBS 123707, producing perithecia in pure culture, and the D. vitalbina strain CBS 454.64. Strain CBS 123705 is closely related to strains in clade B but forms a distinct clade (C) on its own. Strain PD 99/2069 clusters with the two Coniothyrium clematidis-rectae strains (100 % bootstrap support) among the other clades. The morphological characters of this strain proved to be similar to those of both C. clematidis-rectae strains and therefore strain 99/2069 requires renaming.

RESULTS

Phylogenetic analyses of ITS, TUB and LSU

The partition homogeneity test indicated that the DNA sequence data from the three loci were combinable (P = 0.737). Concatenated sequences were thus used in all phylogenetic analyses. The combined alignment consisted of 2 150 bp (ITS 490 bp, TUB 333 bp, LSU 1327 bp), of which 1 978 characters were constant, 60 were parsimony uninformative and 112 were parsimony informative. The Neighbour-Joining trees obtained with the three different substitution models, and the single most parsimonious tree exhibited identical topology. The most parsimonious tree is presented in Fig. 1 (TL = 314 steps, CI = 0.697, RI = 0.888, RC = 0.619). This phylogenetic tree supports division of the P. clematidina strains into three distinct and well-supported groups (Fig. 1). A first group (clade A) contains the representative culture of P. clematidina CBS 108.79 (Boerema & Dorenbosch 1979) and strains isolated from symptomatic Clematis species and hybrids. A second group (clade B) comprises strains isolated from C. vitalba, including the freshly isolated strain CBS 123707, producing perithecia in pure culture, and the D. vitalbina strain CBS 454.64. Strain CBS 123705 is closely related to strains in clade B but forms a distinct clade (C) on its own. Strain PD 99/2069 clusters with the two Coniothyrium clematidis-rectae strains (100 % bootstrap support) among the other clades. The morphological characters of this strain proved to be similar to those of both C. clematidis-rectae strains and therefore strain 99/2069 requires renaming.

Fig. 1 Parsimony tree obtained from a heuristic search with 100 random taxon additions of the combined ITS, BT and LSU sequences alignment. Scale bar indicates 1 change and bootstrap support values from 1 000 replicates are shown in percentages at the nodes.
Taxonomy

Clade A


≡ Phyllosticta clematidina Ellis & Dearn., Canad. Rec. Sci 5: 268. 1893; not Phyllosticta clematis Brunaud, see above.


Description in vitro (amended from Boerema 1993). *Pycnidia* subglobose, mostly solitary on the agar surface, 110–120 µm diam, or larger, up to 350 µm diam, glabrous or with some hyphal outgrows around the ostioli. *Ostioli* 1(–3), papillate, relatively wide, up to 50 µm diam. *Pycnidial wall* 1–4 cells thick, pseudoparenchymatous, composed of isodiametric, somewhat elongated cells, dark pigmented around the ostioli. *Conidiogenous cells* phialidic, hyaline, simple, smooth, flask-shaped, 6–7.5 × 5.5–7 µm. *Conidia* ellipsoidal, occasionally slightly allantoid, thin-walled, smooth, hyaline, mostly aseptate, (3.5–)4–6.5(–9) × 2–3(–3.5) µm, occasionally larger and 1-septate, 9–13 × 3–4 µm, usually guttulate. *Conidial matrix* honey to salmon. *Chlamydospores* usually scanty, un- or multi-cellular, where unicellular usually intercalary in short strains, guttulate, thick-walled, green-brown, 8–10 µm diam, where multicellular irregular dicyto/phragmosporous, often somewhat botryoid and in combination with unicellular chlamydospores, tan to dark brown, 3–50 × 12–25 µm.

Cultural characteristics — Colonies on OA: growth rate 50–65 mm diam after 7 d, with entire margin. Aerial mycelium present in irregular zones, felty or scarcely floccose, white to olivaceous-grey. Colonies olivaceous to iron-grey. Reverse similar. A rosy-buff discoloration of the agar medium often occurs due to the presence of anthraquinone needle-shaped crystals which persist after application of NaOH. Colonies on MEA: growth rate variable, 30–55 mm diam after 7 d, with entire margin. Aerial mycelium felty, white to pale olivaceous-grey, or absent near centre. Colonies rosy-buff to rosy-vinaceous. Reverse similar.


Notes — The holotype has apparently been lost, and is not in LE or LEP. The isotype is selected here, with similar host, location and collector. The specimen and associated strain designated here as epitype represent the modified taxonomy of this species.

Clade B

**Didymella vitalbina** Petr., Ann. Mycol. 38: 348. 1940 — Fig. 2


Description in vitro. *Perithecia* superficial, solitary or clustered, globose/subglobose to pyriform, (75–)200–300 µm, with prominent, ostiolate, elongated neck, 30–60 µm. *Perithecial wall* black, *textura globulosa*, 6.5–10 µm, ectal excipulum 3–4 layers of elongated cells (c. 8 × 3 µm), medullary excipulum

![Fig 2 Didymella vitalbina (CBS 123707)](image-url)
a. Colony on OA after 14 d; b. colony on MEA after 14 d; c. longitudinal section through a perithecium; d. ascus; e. ascospores; f. pycnidial wall with conidiogenous cells; g. pycnidium; h. longitudinal section through a pycnidium; i. conidia. — Scale bars: c, h = 50 µm; d, f = 5 µm; e, i = 10 µm; g = 100 µm.
8–10 layers of globular cells (5.5 × 5 µm), integrated with 6–8 basal layers of smaller globular cells (5 × 3 µm). Ascospore mass white. Ascii bitunicate, 8-spored uniseriate, cylindrical with club-shaped base, 50–80 × 6.5–9.5 µm, paraphyses septate, but not obvious. Ascospores hyaline, septate, ovate to obpyriform, smooth, 9–15 × 3–5.5 µm (av. 11.2 × 4.1 µm). Pycnidia solitary or confluent, highly variable in shape and size, (sub)globose, to elongated or flask-shaped, gibbrous, dark brown, superficial on the agar (105–)135–290(–330) × 95–210(–250) µm. Ostioli (1–)2–3(–5), prominent, 11–22 µm diam on an elongated neck. Pycnidial wall pseudoparenchymatous, thin, 5.5–9.5 µm, consisting of up to only 2 cell layers, outer cells isodiametric to oblong. Conidiogenous cells phialidic, hyaline, simple, smooth, variable in shape and size, 6.5–8.5 × 7–11 µm. Conidia ellipsoidal, hyaline, smooth, mainly aseptate, (5.5–)6.5–10(–11) × 2–4 µm, or 1-septate up to 18 × 4 µm, usually guttulate. Conidial matrix honey to rosy-buff/salmon.

Chlamydospores absent.

Cultural characteristics — Colonies on OA: growth rate 50–60 mm diam after 7 d, with entire, smooth, sharp margins. Aerial mycelium absent or with some floccose tufts, white to (pale) olivaceous-grey. Colonies olivaceous to iron-grey. Reverse similar. Colonies on MEA: growth rate 45–55 mm diam after 7 d, with entire margin or undulate, smooth. Aerial mycelium feltly, white to rosy-buff, near colony margin iron-grey. Colonies iron-grey to olivaceous. Reverse similar.


Notes — The first observations of the teleomorph in vitro were made on V8 subcultures obtained from V8 slants stored at 3 °C for 2 yr. It is not likely that the teleomorph will be observed in vitro after routine cultivation. The anamorph of Didymella vitalbina would be more appropriately accommodated in Phoma than in Ascochyta. However, the priority of the teleomorph name makes a new combination in the anamorph superfluous.

Clade C

Didymella clematidis Woudenberg, Spiers & Gruyter, sp. nov. — MycoBank MB513003; Fig. 3

Anamorph. Ascochyta sp.

Asci bitunicati, octospori, cylindracei, 65–125 × 10–20 µm. Paraphyses septatae, inaequales, 2 µm latae. Ascosporeae hyalinae, septatae, ovatae usque ad obpyriformes, laeves, 15–22 × 4.5–8 µm (av. 19 × 5.7 µm).

Etymology. Named after its host, Clematis.

Description in vitro. Perithecia superficial, solitary or clustered, globose/subglobose to pyriform, (130–)250–370 µm, with prominent elongated neck, up to 75 µm, with central ostiole. Perithecial wall black, textura globulosa 6–10 µm, ectal excipulum to several layers of elongated cells (8 × 5 µm), medullary excipulum 4–5 layers of globular cells (5.5 × 8 µm), integrated with 2–3 layers of smaller globular cells (5 × 5.5 µm). Ascospore

Fig. 3 Didymella clematidis (CBS 123705). a. Colony on OA after 14 d; b. colony on MEA after 14 d; c. perithecium with asc; d. e. asc with ascospores; f. pycnidia; g. longitudinal section through a pycnidium; h. pycnidial wall with conidiogenous cells; i. conidia. — Scale bars: c, f = 100 µm; d, e, h, i = 10 µm; g = 50 µm.
mass white. Asci bitunicate, 8-spored uniseriate/biseriate, cylindrical with club-shaped base, 65–125 × 10–20 µm, paraphyses septate, inconspicuous, 2 µm wide. Ascospores hyaline, septate, ovate to obpyriform, smooth, 15–22 × 4.5–8 µm (av. 19 × 5.7 µm). Pycnidia mostly solitary but also confluent, globose to subglobose or irregular, glabrous, sienna to brown, superficial on the agar but also immersed or in aerial mycelium, (100–)130–360–(560) × 110–340–(475) µm. Ostioli 1–2 or up to 5, 20–50 µm diam, initially non-papillate but forming an elongated neck in a later stage. Pycnidial wall pseudoparenchymatous, thin, 8–12 µm, consisting of up to 5 cell layers, 5.5–8.5 µm.

**DISCUSSION**

Up to 5, 20–50 µm diam, initially non-papillate but forming an elongated neck in a later stage. Pycnidial wall pseudoparenchymatous, thin, 8–12 µm, consisting of up to 5 cell layers, outer cells isodiametric to oblong. Conidiogenous cells phialidic, hyaline, simple, smooth, globose or flask-shaped, c. 7–9.5 × 5.5–8.5 µm. Conidia elongate, sometimes slightly allantoid, constricted in the middle, hyaline, smooth, mostly uniseptate, (14.5–)16–23–(30) × 4–7–(7.5) µm, with numerous guttules. Only incidentally smaller, aseptate conidia occur, c. 6–8 × 2–3 µm. Conidial matrix saffron to salmon. Chlamydospores absent.


Notes — *Didymella clematidina* produces large 2-celled conidia both in vitro and in vivo. Strain CBS 123705 produced both the teleomorph and anamorph state in pure culture. The species is highly virulent on *C. vitalba*.

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REFERENCES


