Multiple Didymella teleomorphs are linked to the Phoma clematidina morphotype

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Key words
Ascochyta vitisae 
ß-tubulin 
Clematis 
Didymella clematidis 
Didymella vitamina 
DNA phylogeny 
ITS 
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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 petichial-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 ß-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. vitamina 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 clematid-is-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 colonies were subcultured onto fresh media. Strain CBS 123707 was isolated from leaves of C. vitalba plants at Gampelsteg, Swiss Valley, Switzerland (Table 1). Strain CBS 123705 was isolated from leaves of C. ligusticifolia at Toppenish, Washington State, USA (Table 1). Isolates were stored on V8 agar slants at 3 °C. For the phylogenetic study of the two Phoma strains isolated (CBS 123705, CBS 123707), all P. clematidina strains which were available from the CBS and PD collection, one Didymella vitamina strain and six Phoma reference strains were included.
<table>
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<th>Species</th>
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<th>Origin</th>
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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 phylogenic analyses, parts of the ITS1, ITS2 and 5.8S rRNA gene (ITS), the β-tubulin gene (TUB) and 28S rRNA gene (LSU) were analysed. The primers V9C (de Hoog & Gerrits van den Ende 1998) and ITS4 (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 Btb4Rd (5’-CCR GAY TGY TCR 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 (15 s at 94 °C), annealing and elongation (45 s at 52 °C for the ITS region and 48 °C for the TUB region) and a final elongation step of 7 min at 72 °C. For the ITS region the 35 cycles consisted of 30 s at 94 °C, 30 s at 48 °C and 60 s at 72 °C, for the TUB region 30 s at 94 °C, 30 s at 52 °C and 30 s at 72 °C.

PCR amplicons were visualized 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 (Swoford 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 Neighbour-Joining analysis with the uncorrected “p” distances and 1000 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., 1985).
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](image-url) 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.
Growth rate 50–65 mm diam after 7 d, with entire margin. Aerial mycelium present in irregular zones, felt 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 felt, 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
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. 

**Asci** 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). **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 (–) 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 fleshy, white to rosy-buff, near colony margin iron-grey. Colonies iron-grey to olivaceous. Reverse similar.

**Specimens examined.** **AUSTRIA,** Vienna, Gaisberg, on stem of Clematis vitalba, April 1939, F. Petrak, holotype 2644. — **FRANCE,** Var, Jouques, on leaves of Clematis vitalba, Dec. 1964, E. Müller, CBS H-11972, culture ETH2672 = CBS 454.64. — **SWITZERLAND,** Gampel-Steg, on leaves of Clematis vitalba, 10 Oct. 1991, A.G. Spiers, epitype designated here PDD69378, culture ex-epitype ICMP 13663 isolate 9 = PD 97/13460-2 = CBS 123707.

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.


**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 up 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...
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, outer cells isodiametric to oblong. Conidigenous 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, asceptate conidia occur, c. 6–8 × 2–3 µm. Conidial matrix saffron to salmon. Chlamydospores absent.


Notes — Didymella clematidia 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.

DISCUSSION

The present study is the first to assess the diversity of P. clematidina by means of DNA sequence comparisons. The reconstructed phylogeny (Fig. 1) indicates that multiple taxa are present within the morphological variation understood to represent P. clematidina. Three distinct and well-supported groups were identified which are elevated to species level. A first clade (clade A) includes the representative culture of P. clematidina CBS 108.79 (Boerema & Dorenbosch 1979). This species is characterised by chlamydospore production and a wide ostiolar opening. Thus far, no teleomorph connection has been established. The observed difference in susceptibility to Clematis wilt caused by P. clematidina between cultivated and wild Clematis spp. (van de Graaf et al. 2001) may be explained by the existence of three genetically distinct fungal species. Strains that now belong to the newly defined P. clematidina have been isolated from Clematis hybrids, whereas D. vitalbina is recorded exclusively from C. vitalba. Another feature that suggested a high level of variability within P. clematidina is their resistance against benzimidazole fungicides. Van Kuik & Brachter (1997) and van de Graaf et al. (2003) have reported on two groups of P. clematidina within their collections that clearly responded differently to these fungicides. Van de Graaf et al. (2003) could link these groups to slight differences in morphological appearance in culture and to differences in pathogenicity. Although resistance studies could not be conducted within this study, it is worthwhile to conduct further research on the phytopathological features of all taxonomic groups observed.

The previous misidentification of C. clematis-rectae PD 99/2069 as P. clematidina and the introduction of the Ascochyta anamorph of D. clematidis, previously reported as the P. clematidina biocontrol agent, illustrates the difficulties within the Phoma generic complex. Coniothyrium is characterised by holoblastic, annellidic conidigenous cells and brown conidia (Crous et al. 2007, Damm et al. 2008). In contrast, Phoma spp. produce enteroblastic, phialidic conidigenous cells with hyaline conidia (Sutton 1980). Differences in conidogenesis between these genera are best observed by means of electron microscopy. The difference in conidial pigmentation is also sometimes hard to observe as the conidia of several Phoma spp. have been reported to darken with time (Boerema et al. 2004), whereas (young) conidia of some Coniothyrium species may appear almost hyaline (Taylor & Crous 2001, Verkley et al. 2004). Moreover, both C. clematis-rectae and P. clematidina can be simultaneously isolated from infected material. The main difference between Ascochyta and Phoma are the annellidic conidiogenesis and distoseptation of Ascochyta (Boerema & Bollen 1975). When septa occur in Phoma, they are secondary. In Ascochyta spp. the septation is an essential part of the conidial maturation, which explains why mature conidia are nearly always septiculate, both in vivo and in vitro.

As reported by de Gruyter et al. (2009), the distinction among the different coelomycete genera based on morphological features is not always supported by molecular studies. Some species of the anamorph genera such as Coniothyrium, Ascochyta, Ampelomyces and Microsphaeropsis cluster with Phoma species in the Didymellaceae. This is also seen in our study where strains of C. clematis-rectae and the Ascochyta anamorph of D. clematidis cluster amidst Phoma isolates within the Didymellaceae. It is therefore recommended to improve the current classification of the anamorphic Pleosporales by further evaluating the Phoma, Ascochyta and Coniothyrium complexes and strive to establish monophyletic groups.

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