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RESEARCH ARTICLE

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Multiple *Didymella* teleomorphs are linked to the *Phoma clematidina* morphotype

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Key words

Ascochyta vitalbae
ß-tubulin
Clematis
Didymella clematidis
Didymella vitalbina
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 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. vitalbina* 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 mm^2) 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 vitalbina* strain and six *Phoma* reference strains were included

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Table 1 Isolates included in the phylogenetic analyses.

Species	Accession no.[1]	Host	Origin	GenBank no.		
				ITS	TUB	LSU
Coniothyrium clematidis-rectae	CBS 507.63, PD 07/03486747	*Clematis* sp.	Netherlands	FJ515606	FJ515624	FJ515647
	PD 95/1958	*Clematis* sp.	Netherlands	FJ515607	FJ515625	FJ515648
Didymella vitalbina	CBS 454.64	*Clematis vitalba*	France	FJ515605	FJ515623	FJ515646
Phoma clematidina	CBS 201.49	*Clematis* sp.	Netherlands	FJ426991	FJ427102	FJ515628
	CBS 195.64	*Clematis jackmannii*	Netherlands	FJ426990	FJ427101	FJ515629
	CBS 102.66	*Clematis* sp.	England	FJ426988	FJ427099	FJ515630
	CBS 520.66, PD 64/657	*Selaginella* sp.	Netherlands	FJ426992	FJ427103	FJ515631
	CBS 108.79, PD 78/522	*Clematis* sp.	Netherlands	FJ426989	FJ427100	FJ515632
	CBS 911.87	*Clematis vitalba*	Germany	FJ515592	FJ515610	FJ515633
	CBS 123705, PD 97/13460.1, ICMP 13664	*Clematis ligusticifolia*	USA	FJ515593	FJ515611	FJ515634
	CBS 123706, PD 08/04373904.5	*Clematis vitalba*	Netherlands	FJ515594	FJ515612	FJ515635
	CBS 123707, PD 97/13460.2, ICMP 13663	*Clematis vitalba*	Switzerland	FJ515595	FJ515613	FJ515636
	PD 75/294	*Clematis* sp.	Unknown	FJ515596	FJ515614	FJ515637
	PD 80/683	*Clematis* sp.	Netherlands	FJ515597	FJ515615	FJ515638
	PD 91/1865	*Clematis* sp.	Netherlands	FJ515598	FJ515616	FJ515639
	PD 95/895	*Clematis* sp.	Netherlands	FJ515599	FJ515617	FJ515640
	PD 97/12061	*Clematis* cv. Purple spider	Netherlands	FJ515600	FJ515618	FJ515641
	PD 97/12062	*Clematis* cv. New Dawn	Netherlands	FJ515601	FJ515619	FJ515642
	PD 99/2069	*Clematis* sp.	England	FJ515602	FJ515620	FJ515643
	PD 08/04373904.2B	*Clematis vitalba*	Netherlands	FJ515603	FJ515621	FJ515644
	PD 08/04417700.3	*Clematis vitalba*	Netherlands	FJ515604	FJ515622	FJ515645
Phoma complanata	CBS 268.92, PD 75/3	*Angelica sylvestris*	Netherlands	FJ515608	FJ515626	EU754180
Phoma exigua var. *exigua*	CBS 431.74, PD 74/2447	*Solanum tuberosum*	Netherlands	FJ427001	FJ427112	EU754183
Phoma glaucii	CBS 114.96, PD 94/888	*Chelidonium majus*	Netherlands	FJ515609	FJ515627	FJ515649
Phoma glomerata	CBS 528.66, PD 63/590	*Chrysanthemum* sp.	Netherlands	FJ427013	FJ427124	EU754184
Phoma herbarum	CBS 615.75, PD 73/665, ATCC 2499, IMI 199779	*Rosa multiflora*	Netherlands	FJ427022	FJ427133	EU754186
Phoma zeae-maydis	CBS 588.69	*Zea mays*	USA	FJ427086	FJ427190	EU754192

[1] ATCC: American Type Culture Collection, Virginia, USA; CBS: CBS Fungal Biodiversity Centre, Utrecht, The Netherlands; ICMP: International Collection of Micro-organisms from Plants, Auckland, New Zealand; IMI: International Mycological Institute, CABI-Bioscience, Egham, United Kingdom; PD: Dutch Plant Protection Service, Wageningen, The Netherlands.

(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).

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 ß-tubulin gene (TUB) and 28S rRNA gene (LSU) were analysed. The primers V9G (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 Btub4Rd (5'-CCR GAY TGR CCR AAR ACR AAG TTG 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 $MgCl_2$ for the ITS region and 1.0 µL diluted genomic DNA, 0.02 mM dNTPs and 2 mM $MgCl_2$ 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, annealing and elongation 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 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-Marthens-Lathem, 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 Neighbour-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.

2007) as described by Boerema et al. (2004). The growth rates on both plates were examined after 7 and 14 d of incubation. Colony colours were determined after 2 wk using the colour charts of Rayner (1970).

Sexual structures were studied on V8 whereas other morphological features were described from OA as soon as sporulation occurred. Fruiting bodies were mounted in water and examined with the aid of a Nikon 80i light microscope. Pycnidial wall structure and the shape of the conidiogenous cells were studied using microtome sections of 9 µm thickness that were prepared with a Leica CM3050 freezing microtome and mounted in lactic acid. For electron microscopy, small segments (5 × 5 mm) of agar with pycnidia/perithecia were fixed in 3 % glutaraldehyde and 2 % formaldehyde in 0.1 M phosphate buffer (Karnovsky 1965) and prepared for scanning and transmission electron microscopy as previously described (Spiers & Hopcroft 1992).

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.

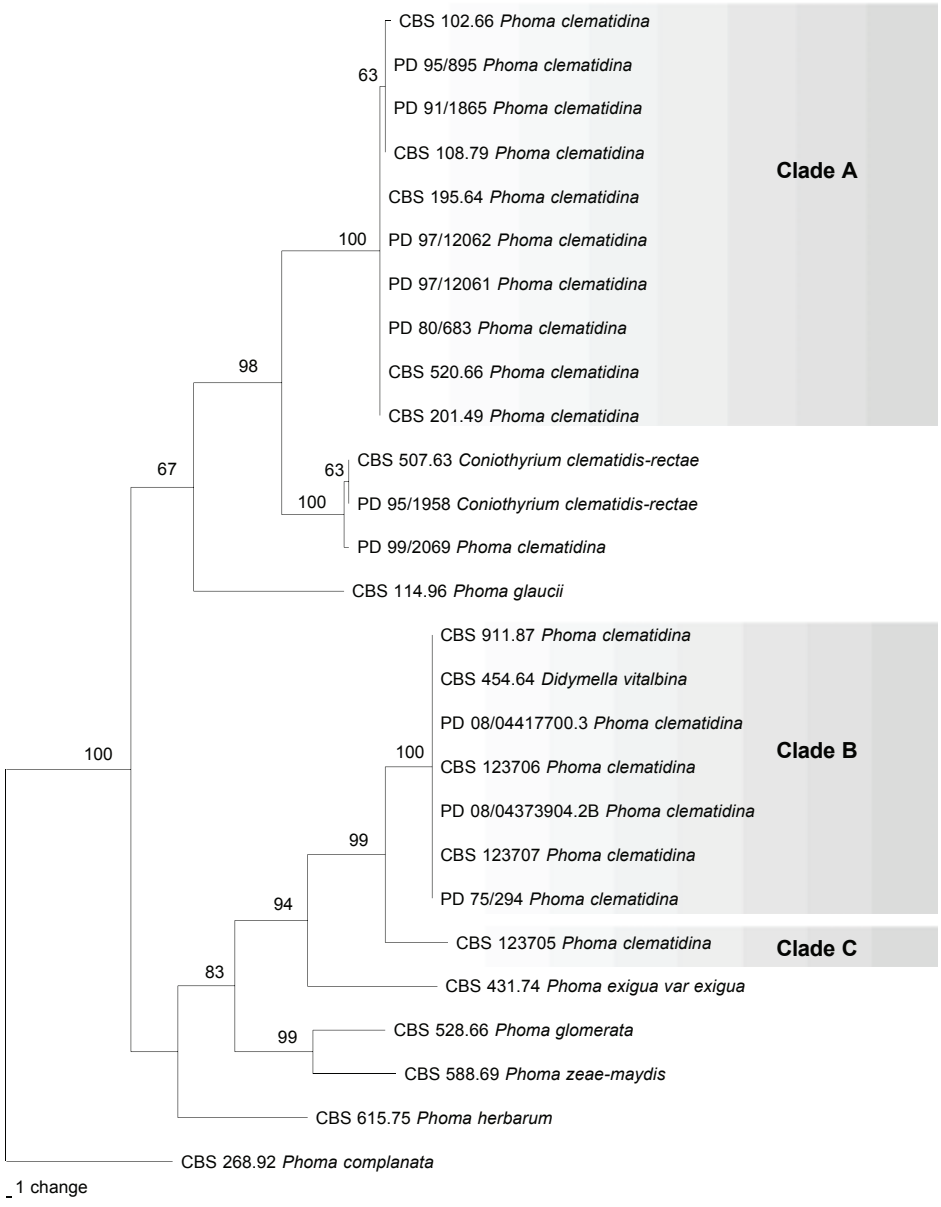

Fig. 1 Parsimony tree obtained from a heuristic search with 100 random taxon additions of the combined ITS, BT and LSU sequences alignment. Scale bar indicates 1 change and bootstrap support values from 1 000 replicates are shown in percentages at the nodes.

Taxonomy

Clade A

Phoma clematidina (Thüm.) Boerema, Versl. Meded. Plziektenk. Dienst Wageningen 153 (Jaarb. 1978): 17. 1979

Basionym. Ascochyta clematidina Thüm., Bull Soc. Imp. Naturalistes Moscou 55: 98. 1880.

= *Phyllosticta clematis* Brunaud, Ann. Soc. Sci. Nat. Charente-Infér. 26: 9. 1889.

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

= *Ascochyta indusiata* Bres., Hedwigia 35: 199. 1896.

= *Ascochyta davidiana* Kabát & Bubák, Oesterr. Bot. Z. 54: 25. 1904.

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–)4–8.5(–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, uni- or multicellular, where unicellular usually intercalary in short strains, guttulate, thick-walled, green-brown, 8–10 µm diam, where multicellular irregular dictyo/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.

Specimens examined. Russia, Minussinsk, on leaves of *Clematis glaucae*, *N. Martianoff,* isotype LE 40082. – The Netherlands, Spaubeek, on the stem of *Clematis* sp., July 1978, *G.H. Boerema*, epitype designated here CBS H-16193, culture ex-epitype CBS 108.79 = PD 78/522.

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

Anamorph. '*Ascochyta*' *vitalbae* Briard & Har. apud Briard, Rev. Mycol. (Toulouse) 13: 17. 1891.

≡ *Diplodina vitalbae* (Briard & Har.) Allesch., Rabenh. Krypt.-Fl., ed. 2. Pilze 6 (Lief. 69): 683. 1900 (vol. dated 1901).

= *Diplodina clematidina* Fautrey & Roum. apud Roum., Rev. Mycol. (Toulouse) 14: 105. 1892.

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

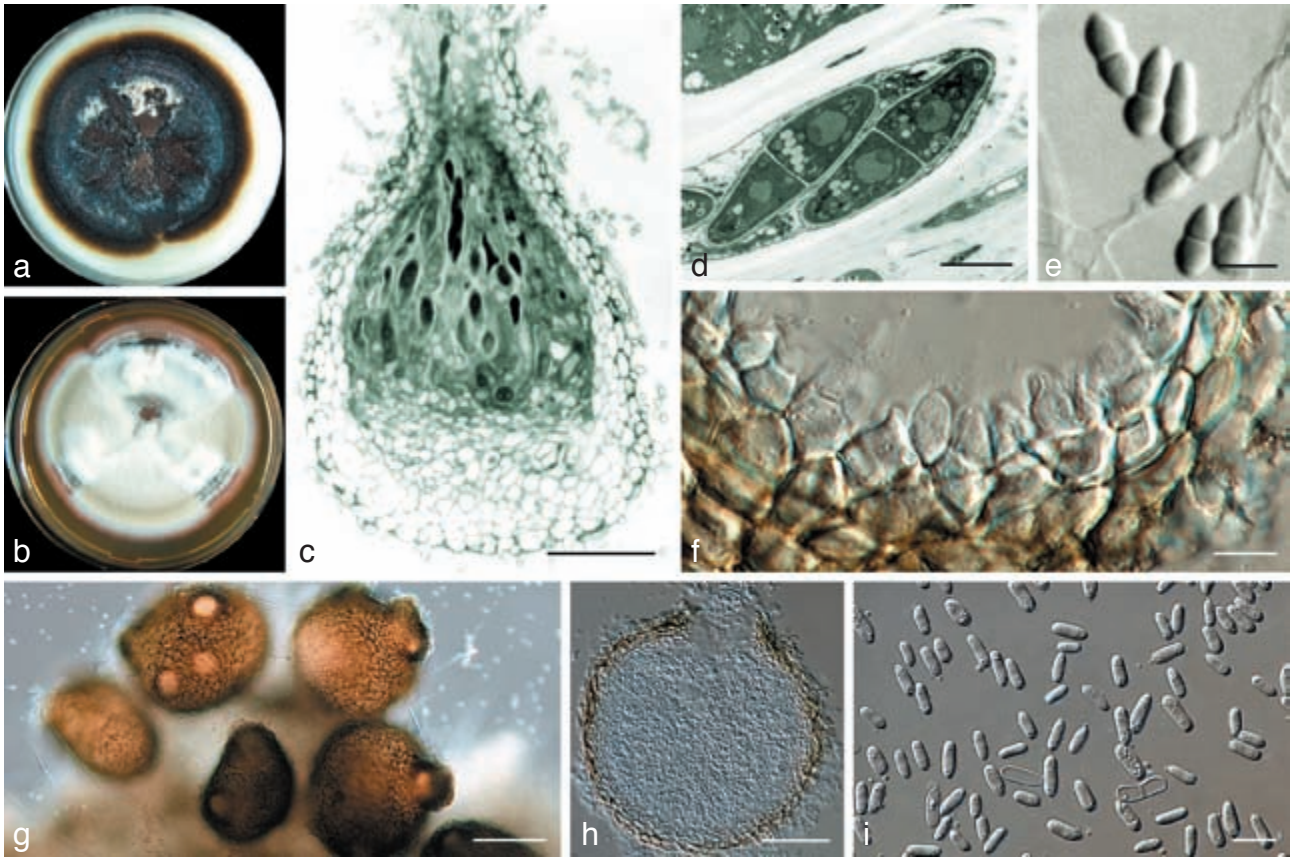

Fig. 2 *Didymella vitalbina* (CBS 123707) a. Colony on OA after 14 d; b. colony on MEA after 14 d; c. longitudinal section through a perithecium; d. ascus; e. ascospores; f. pycnidial wall with conidiogenous cells; g. pycnidia; h. longitudinal section through a pycnidium; i. conidia. — Scale bars: c, 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. *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). *Pycnidia* solitary or confluent, highly variable in shape and size, (sub)globose, to elongated or flask-shaped, glabrous, 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 felty, 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.

Asci bitunicati, octospori, cylindracei, 65–125 × 10–20 µm. Paraphyses septatae, inconspicuae, 2 µm latae. Ascosporae 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 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*

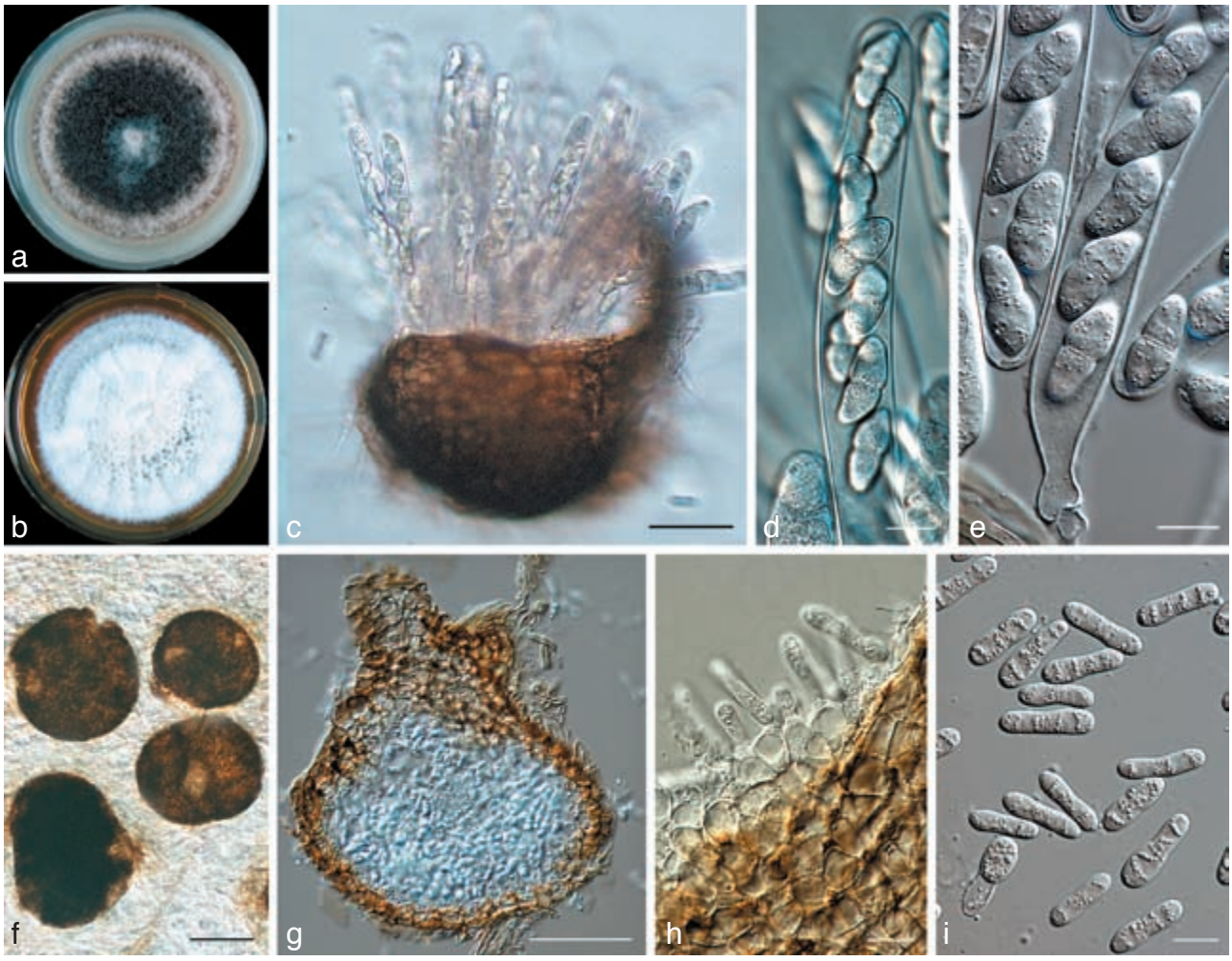

Fig. 3 *Didymella clematidis* (CBS 123705). a. Colony on OA after 14 d; b. colony on MEA after 14 d; c. perithecium with asci; d, e. asci with ascospores; f. pycnidia; g. longitudinal section through a pycnidium; h. pycnidial wall with conidiogenous cells; i. conidia. — Scale bars: c, f = 100 µm; d, 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, 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.

Cultural characteristics — Colonies on OA: growth rate 55–65 mm diam after 7 d, with entire, smooth, sharp margins. Aerial mycelium woolly to floccose, pale olivaceous-grey. Colonies olivaceous-grey. Reverse iron-grey to (pale) olivaceous-grey. Colonies on MEA: growth rate 55–65 mm diam after 7 d, with entire, smooth, sharp margins. Aerial mycelium woolly to floccose, white. Colonies olivaceous-grey. Reverse iron-grey, with greyish sepia tinges or striated olivaceous-grey zones.

Specimens examined. USA, Washington State, Toppenish, on leaves of *Clematis ligusticifolia*, 7 Sept. 1991, *A.G. Spiers*, holotype PDD69379, culture ex-holotype ICMP 13664 isolate 30/32 = PD 97/13460-1 = CBS 123705.

Notes — *Didymella clematidis* 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 with this species. In contrast, *Phoma* strains from clade B, which were identified as anamorphs of *D. vitalbina*, were lacking chlamydospore production but regularly formed an elongated neck with ostiole. A third novel taxon (clade C), of which we currently have only one single isolate, is described here as *D. clematidis*. Due to the presence of only two-celled conidia both in vitro and in vivo, the anamorph stage of *D. clematidis* is classified in the genus *Ascochyta.* This species has been applied in New Zealand as a biological control agent of the environmental weed old man's beard (Gourlay et al. 2000). Its phytopathological value as a highly aggressive strain may be not only due to it being a genetically different entity, but also to its distinct geographical origin. *Clematis vitalba* originates from Europe and therefore may not have been adapted to this *D. clematidis*, which is found in the USA. Further research on a larger set of strains originating from various geographical origins is required to obtain more detailed information about the genetic diversity of this species. This may also provide a better assessment of the variation within clade A. The isolates in this clade have been obtained from various *Clematis* species and hybrids, however, almost exclusively originated from the Netherlands.

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. clematidis-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 conidiogenous cells and brown conidia (Crous et al. 2007, Damm et al. 2008). In contrast, *Phoma* spp. produce enteroblastic, phialidic conidiogenous cells with hyaline conidia (Sutton 1980). Differences in conidiogenesis 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. clematidis-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 septate, 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. clematidis-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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