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Paecilomyces clematidis (Eurotiales, Thermoascaceae): a new species from Clematis root

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Abstract

During a survey of endophytic fungi associated with ornamental plants in the Czech Republic, *Paecilomyces*-like strains were isolated from the root of *Clematis*. Analyses based on a combined internal transcribed spacer region (ITS), beta-tubulin (*tub2*) and calmodulin (*CaM*) sequence data matrix were applied to infer the phylogenetic position of these isolates. The novel species is characterized by phialides with a cylindrical basal portion tapering to a thin long neck producing pyriform conidia in chains. The new species is introduced with comprehensive descriptions, illustrations and a phylogenetic tree herein. Two primer pairs targeting the partial *CaM* gene, cm1F/cm1R and cm2F/cm2R, were designed in this study.

Keywords: Calmodulin, Fungi, Phylogeny, Primers, Taxonomy

Introduction

The genus *Paecilomyces* was established by Bainier (1907) and typified by *Paecilomyces* variotii Bainier. Thom (1930) and Samson (1974) noticed a diversity in conidial morphology. The polyphyletic nature of the genus was confirmed by Luangsa-ard *et al.* (2004), who presented a phylogenetic analysis of the 18S rDNA. They demonstrated that *Paecilomyces* is polyphyletic across *Sordariomycetidae* and *Eurotiomycetidae,* which led to its reclassification in *Paecilomyces*. Adding molecular and extrolite data, Samson *et al.* (2009) revised the taxonomy and nomenclature of the accepted taxa and proposed *Paecilomyces* as a monophyletic genus within the order *Eurotiales* connected to its *Byssochlamys* teleomorph. Since this reclassification, only one new species, *P. tabacinus,* was added (Crous *et al.* 2016). Rossman *et al.* (2016) proposed the use of the name *Paecilomyces* over *Byssochlamys,* which was formally introduced by Houbraken *et al.* (2020). Currently, the members of *Paecilomyces* are characterised by producing irregular, branched conidiophores bearing phialides with an inflated base that abruptly narrows to a thin neck and produces olive-brown conidia in chains (Houbraken *et al.* 2020).

Species of *Paecilomyces* have already been reported from various substrates, including human tissues, preserved food (bottled fruit, fruit juice, yogurt, etc.), milk, soil, plants, plant debris; and from various countries all around the world such as Australia, Belgium, Brazil, Canada, China, Denmark, France, Germany, Italy, Japan, Mexico, Netherlands, Switzerland, the United Kingdom, the United States, Uzbekistan, Thailand and Turkey (Samson *et al.* 2009; Brule *et al.* 2020). Several *Paecilomyces* spp. are known for the production of various metabolites that have both harmful (mycotoxins, e.g., byssotoxin A, byssochlamic acid, emodin, mycophenolic acid, patulin, viriditoxin) and beneficial consequences (antitumor metabolites, e.g., byssochlamysol) on human health (Hull 1939; Kramer *et al.* 1976; Rice, 1977; Mori *et al.* 2003; Houbraken *et al.* 2006).

Collection and isolation

During the spring of 2021, roots of 3-year-old *Clematis* L. 'Snow Queen' plants were collected from the ornamental garden of Mendel University in Lednice and immediately transported to the laboratory of Mendeleum – Institute of Genetics, Mendel University, Czech Republic for further processing. The roots were washed with running tap water to remove residual soil, washed again with running sterile distilled water and air-dried on sterile filtration paper. Clean roots were surface sterilized in 1% sodium hypochlorite for one minute and then rinsed three times with sterile distilled water as described in our previous studies (Spetik *et al.* 2019; Spetik *et al.* 2021). The disinfected roots were cut into small segments of 10×2×2 mm and aseptically transferred onto Petri dishes containing potato dextrose agar (PDA, HiMedia, Mumbai, India) supplemented with 0.5 g/l streptomycin sulfate (Sigma–Aldrich, St. Louis, MO, USA). The plates were incubated at 25 °C in the dark for six weeks, and fungal growth was checked every day. Newly developed mycelia were immediately transferred to new PDA plates and purified by hyphal tip isolation (Jensen *et al.* 2013). Two isolates originating from two different plants were maintained as a reference at MEND-F, Fungal Culture Collection of Mendeleum, Mendel University in Brno, The Czech Republic and CBS, Westerdijk Fungal Biodiversity Institute, The Netherlands. The holotype is maintained at the Herbarium (BRNU) Department of Botany and Zoology, Masaryk University, The Czech Republic.

Morphology

Culture characteristics were determined after seven days of cultivation at 25 °C in the dark on PDA, oat meal agar (OA), malt extract agar (MEA) and Czapek yeast autolysate agar (CYA). Colony colours were determined with reference to the colour chart of Rayner (1970). The diameters of the colonies were determined at different temperatures after seven days in the dark. From each culture, the conidiogenous layer and conidia were mounted in 100% lactic acid. A compound Nikon Eclipse Ni-e microscope equipped with NIKON DS-Ri2 camera was used for bright-field digital images of the micromorphological features. For the microscopic measurements, the mean, standard deviation and 95% confidence intervals were calculated from measurements of 30 conidia per isolate. Dimensions are presented as the range of measurements with extreme values in brackets followed by 95% confidence limits and mean ± standard deviation.

DNA extraction and amplification

Genomic DNA was extracted from 7-day-old mycelium grown on PDA at 25 °C in the dark using a NucleoSpin DNA extraction kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. To confirm the identity of the fungal species, multilocus molecular phylogeny based on six genes was used; internal transcribed spacer region (ITS), small ribosomal subunit (SSU), large ribosomal subunit (LSU), beta-tubulin (*tub2*), calmodulin (*CaM*) and RNA polymerase II subunit (*rpb2*). PCR was performed utilizing G2 Flexi DNA polymerase (Promega, Madison, USA) using the primers listed in **Table 1.** For the *CaM* gene, two new primer pairs were designed: cm1F/cm1R and cm2F/cm2R. The PCR amplification conditions were the same for each gene, and only the annealing temperature varied. The reaction mixture was incubated as follows: initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s. The annealing temperatures are shown in **Table 1** for 45 s, extension at 72 °C for 90 s and a final extension step at 72 °C for 7 min. Sequencing was conducted in both directions with the same primer pair used for amplification at the Eurofins Genomics Germany GmbH. Consensus sequences were assembled in BioEdit 7.

TABLE 1. Primers and annealing temperatures used in the PCR amplification step.

Locus	Primer	Primer DNA sequence (5′-3′)	Annealing T [°C]	Reference
ITS	**ITS1**	TCCGTAGGTGAACCTGCGG	55	White *et al.* 1990
	ITS4	TCCTCCGCTTATTGATATGC		
SSU	**NS1**	GTAGTCATATGCTTGTCTC	55	White *et al.* 1990
	NS4	CTTCCGTCAATTCCTTTAAG		
LSU	**LROR**	ACCCGCTGAACTTAAGC	48	Rehner & Samuels 1994
	LR7[b]	TACTACCACCAAGATCT		Vilgalys & Hester 1990
	LR5*[a]	ATCCTGAGGGAAACTTC		Vilgalys & Hester 1990
tub2	**T1**	AACATGCGTGAGATTGTAAGT	58	O'Donnell *et al.* 1997
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC		Glass & Donaldson 1995
CaM	**cm1F**	TTGTCCTCATCCGACTTCT	52	This study
	cm1R	CTTCACGAATCTCCTCCT		
	cm2F	TTTGTCCTCATCCGACTT		
	cm2R	TGCAGCGGAAATAAAACC		
rpb2	**5F2**	GGGGWGAYCAGAAGAAGGC	58	O'Donnell *et al.* 2010
	7cR	CCCATRGCTTGYTTRCCCAT		

Note: ITS, internal transcribed spacer; **SSU**, small ribosomal subunit; **LSU**, large ribosomal subunit; ***tub2,*** beta-tubulin; ***CaM,*** calmodulin; ***rpb2,*** RNA polymerase II subunit; [a]only for sequencing; [b]only for PCR amplification.

TABLE 2. Fungal strains used in phylogenetic analyses.

		GenBank accession numbers					
Species	**Collection no.**	**ITS**	***tub2***	***CaM***	***rpb2***	**Reference**	**Additional genes**
Paecilomyces brunneolus	CBS 370.70[T]	EU037050	EU037068	EU037033	MN969152	Houbraken *et al.* 2020	-
P. clematidis	**CBS 148466[T]**	**MZ923760**	**MZ927740**	**MZ927738**	**OL332317**	**This study**	**LSU: MZ923762; SSU: OL330806**
P. clematidis	**MEND-F-0561**	**MZ923761**	**MZ927741**	**MZ927739**	**OL332317**	**This study**	**LSU: MZ923763; SSU: OL330807**
P. dactylethromorphus	CBS 251.55	FJ389951	FJ390002	FJ389960	-	Houbraken *et al.* 2020	-
P. divaricatus	CBS 284.48[T]	FJ389931	FJ389992	FJ389953	-	Houbraken *et al.* 2020	-
P. formosus	CBS 990.73B[T]	FJ389929	FJ389993	FJ389978	MN969154	Houbraken *et al.* 2020	-
P. fulvus	CBS 132.32[T]	FJ389939	FJ389988	FJ389957	-	Houbraken *et al.* 2020	-
P. fulvus	CBS 146.48	FJ389940	FJ389986	FJ389959	-	Samson *et al.* 2009	-
P. fulvus	CBS 135.62	FJ389943	FJ389989	FJ389976	-	Samson *et al.* 2009	-
P. fulvus	CBS 604.71	FJ389941	FJ389997	FJ389967	-	Samson *et al.* 2009	-
P. laguncullariae	CBS 373.70[T]	FJ389944	FJ389999	FJ389956	JF417414	Houbraken *et al.* 2020	-
P. laguncullariae	CBS 696.95	FJ389945	FJ389996	FJ389956	-	Samson *et al.* 2009	-
P. laguncullariae	CBS 110378	FJ389946	FJ390006	FJ389970	-	Samson *et al.* 2009	-
P. niveus	CBS 100.11[T]	FJ389934	FJ389999	FJ389956	F417414	Houbraken *et al.* 2020	-
P. niveus	CBS 133.37	FJ389935	FJ390000	FJ389958	-	Samson *et al.* 2009	-
P. niveus	CBS 113245	FJ389936	FJ389998	FJ389974	-	Samson *et al.* 2009	-
P. tabacinus	CBS 141098	LT548280	MN969434	LT548288	MN969210	Houbraken *et al.* 2020	LSU: MH878203 (Vu *et al.* 2019)
P. variotii	CBS 338.51	FJ389930	FJ390007	FJ389955	-	Samson *et al.* 2009	-
P. variotii	CBS 102.74[T]	EU037055	EU037073	EU037038	MN969153	Houbraken *et al.* 2020	-
P. zollerniae	CBS 374.70[T]	FJ389933	FJ390008	FJ389966	-	Houbraken *et al.* 2020	SSU: NG_062663 (Luangsa-Ard *et al.* 2004)
Rasamsonia byssochlamydoides	CBS 413.71[T]	JF417476	JF417460	JF417512	-	Houbraken *et al.* 2012	-
Rasamsonia emersonii	CBS 393.64[T]	JF417478	JF417463	JF417510	-	Houbraken *et al.* 2012	-
Thermoascus crustaceus	CBS 181.67[T]	FJ389925	FJ389981	FJ389952	-	Samson *et al.* 2009	-

Note: [T] indicates type strains; **CBS** - CBS culture collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands; **MEND-F** – Fungal collection of Mendeleum, Mendel University in Brno, Czech Republic.

TABLE 3. Morphological characteristics of *Paecilomyces* species, modified from Samson *et al.* (2009).

Species	Conidial length (µm)	Conidial shape (predominant)	Chlamydospores	Colony diam. (mm) on CYA 7 d, 30 °C	Reference
Paecilomyces brunneolus	3.7–5.5 × 1.8–3.4	Ellipsoid to broadly cylindrical with truncate ends	Present, smooth	20–30	Samson *et al.* 2009
P. clematidis	**3.3–3.6 × 2.2–2.5**	**Ellipsoidal with flattened base**	**Present, warted, subglobose to globose, thick walled, dark green, 4.5–6.2 µm**	**>90**	**This study**
P. dactylethromorphus	2.3–7 × 1.7–3.4	Predominantly cylindrical and ellipsoidal without truncate ends	Present, smooth	22–55	Samson *et al.* 2009
P. divaricatus	3.2–4.6 × 1.6–2.5	Ellipsoidal to cylindrical with truncate ends	Absent; in some isolates after prolonged incubation present (40 d)	10–17	Samson *et al.* 2009
P. formosus	3–10 × 1.8–3.5	Ellipsoidal to cylindrical with truncate ends	Present, smooth and often pigmented	18–90	Samson *et al.* 2009
P. fulvus	3.7–7.5 × 1.4–2.5	Cylindrical with truncate ends	Absent; in some isolates after prolonged incubation present (40 d)	50–90	Samson *et al.* 2009
P. laguncullariae	2.7–4.5 × 2.2–3.3	Globose with flattened base	Present, smooth	45–55	Samson *et al.* 2009
P. niveus	3–4.7 × 2.3–4	Globose to ellipsoidal with flattened base	Present, smooth to finely rough	(8–)28–50	Samson *et al.* 2009
P. tabacinus	(2.5–)3–7(–11) × 2.5–7	Ellipsoidal, fusiform, pyriform (tear-shaped), rarely subglobose	Present, thick walled	38-40*	Crous *et al.* 2016
P. variotii	3.3–6.1 × 1.5–4.4	Predominantly ellipsoidal and ellipsoidal with truncate ends	Present, smooth to finely rough	30–45(−55)	Samson *et al.* 2009
P. zollerniae[a]	2.5–4 × 1.5–3	Globose to ellipsoidal, apiculate	Present, warted, globose, brown to dark brown, 5-10.5 µm	30–35	Samson *et al.* 2009; Stolk & Samson 1971

Note: *cultivated at 25 °C; [a] Colonies on MEA reach 80 mm after 14 d at 30 °C (Stock & Samson 1971).

Phylogenetic analysis

Sequences for taxon sampling were obtained from GenBank (**Table 2**). The phylogenetic analysis of the combined ITS, *CaM* and *tub2* sequences comprised 20 ingroup isolates belonging to 11 species of *Paecilomyces* and three outgroup taxa (*Thermoascus crustaceus*, CBS 18167; *Rasamsonia byssochlamydoides,* CBS 41371; *Rasamsonia emersonii*, CBS 39364). Maximum likelihood (ML) and Maximum parsimony (MP) trees were obtained using the software MEGA-X v. 10.2.6 (Kumar *et al.* 2018). The alignment consisted of 1624 characters, of which 83 were excluded, 893 were conserved, 190 were variable and parsimony-uninformative and 458 were parsimony-informative. The heuristic search of the parsimony-informative characters with 1000 bootstrap replicates generated nine equally parsimonious trees through 1226 steps with CI = 0.74, RI = 0.81 and HI = 0.26. One of the nine obtained trees is presented in **Fig. 1**.

The three-gene-based phylogeny (ITS, *tub2* and *CaM*) showed the isolates of the present study in a well-supported sister branch to *P. zollerniae*, which are described here as a taxonomic novelty, *Paecilomyces clematidis sp. nov***.** The novel species described here is highlighted in bold. The alignment and tree files were submitted to Figshare (10.6084/m9.figshare.19929014).

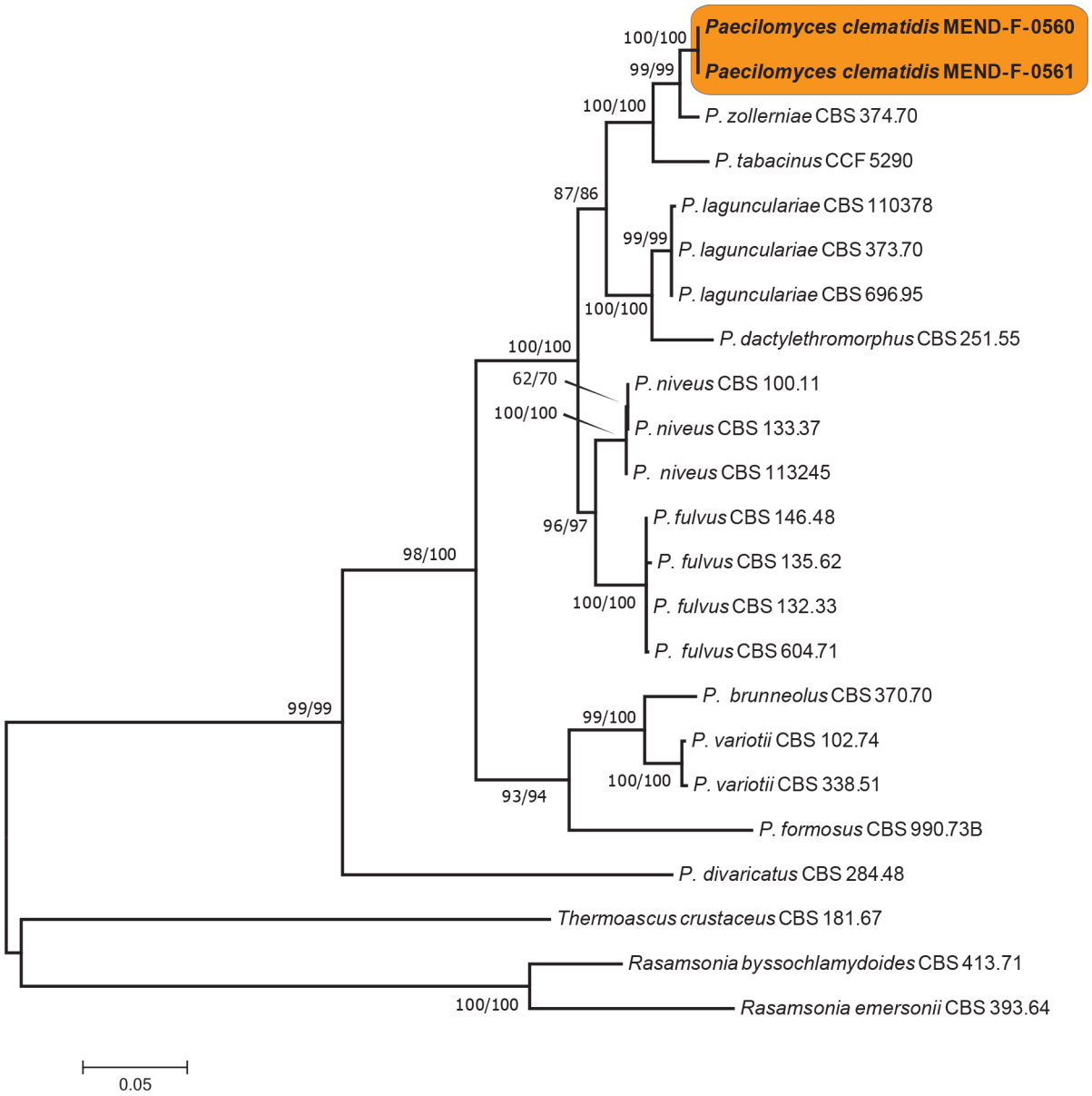

FIGURE 1. Maximum likelihood tree generated from the combined analysis of ITS, *tub2* and *CaM* sequence data. ML/MP bootstrap values are given at the nodes. The tree was rooted to *Thermoascus crustaceus* (CBS 18167), *Rasamsonia byssochlamydoides* (CBS 41371) and *Rasamsonia emersonii* (CBS 39364).

Results

Taxonomy

Paecilomyces clematidis Spetik, Eichmeier, Gramaje & Berraf-Tebbal, *sp. nov*. (**Fig. 2**)
Mycobank number: MB843540

Type: CZECH REPUBLIC, Breclav: Lednice, university garden (48°47'33.3"N, 16°47'55.7"E), isolated from the root of *Clematis* 'Snow Queen' (*Ranunculaceae*), May 2021, M. Spetik, Holotype: BRNU 677844, ex-type living culture CBS 148466 = MEND-F-0560.

Sexual morph: not observed. **Asexual morph:** *Hyphomycetous*. *Conidiophores* hyaline, septate, irregularly branched. *Phialides* with a cylindrical basal portion tapering to a thin long neck, solitary directly on vegetative hyphae or in groups of two–four per metula. *Conidia* (2.5–)3.3–3.6(–4.2) × (1.7–)2.2–2.5(–3.2) µm, mean ± SD 3.4 ± 0.4 × 2.4 ± 0.4 µm hyaline, ellipsoidal with flattened base, pyriform, produced in chains. *Chlamydospores* usually produced

laterally or terminally on short side branches of superficial or immersed hyphae, thick-walled, initially smooth later becoming rough and warted, subglobose to globose, dark green, 4.5–6.2 µm diam. *Culture characteristics*. On PDA after seven days: white to olivaceous, floccose, aerial mycelium, reverse olivaceous, almost no growth at 18 °C; > 90 mm diam at 25°, 30° and 35 °C; 42 mm diam at 40 °C; no growth at 45 °C. On MEA after seven days: white to olivaceous, floccose, aerial mycelium, reverse olivaceous, > 90 mm diam at 25° and 30 °C. On OA after seven days: white to olivaceous, floccose, aerial mycelium, reverse olivaceous, > 90 mm diam at 25° and 30 °C. On CYA after seven days: white to olivaceous, floccose, aerial mycelium, reverse olivaceous, > 90 mm diam at 25° and 30 °C.

Etymology:—refers to the host genus (*Clematis*).

Additional specimens examined: CZECH REPUBLIC, Breclav: Lednice, university garden (48°47'33.4"N, 16°47'55.7"E), isolated from the root of *Clematis* 'Snow Queen' (*Ranunculaceae*), May 2021, M. Spetik, living culture MEND-F-0561.

TABLE 5. Number of single nucleotide polymorphisms of the species most closely related to *P. clematidis* (CBS 148466).

Species	Collection number	Number of single nucleotide polymorphisms				
		ITS	LSU	SSU	*tub2*	*CaM*
P. zollerniae	CBS 374.70	8	-	11	14	1
P. tabacinus	CBS 141098	17	5	-	34	23

FIGURE 2. *Paecilomyces clematidis* (ex-type CBS 148466). **A, F–G.** Chlamydospores. **C.** Conidiophores with phialides. **B, D.** Phialides forming conidia. **E.** Conidia. **H.** Colony on PDA. **I.** Colony on MEA. **J.** Colony on OA. Scale bars: 10 µm.

Discussion

Paecilomyces clematidis shares similar morphological characteristics with *P. zollerniae* and *P. tabacinus* (shape of conidia, **Table 3**). However, *P. clematidis* can be easily distinguished from *P. zollerniae* by its faster growth on CYA and MEA medium. Compared to *P. tabacinus*, *P. clematidis* produces smaller conidia (3.3–3.6 × 2.2–2.5 vs 2.5–11 × 2.5–7) and grows faster on CYA. Despite similar morphologies, all three mentioned species are phylogenetically distinct. The multigene phylogeny (ITS, *tub2* and *CaM*) showed the isolates of the present study were in a well-supported (99/99, ML/MP) sister taxa of *P. zollerniae.* Details of single nucleotide polymorphisms are shown in (**Table 5**), comparing two closest taxa to *P. clematidis*.

During the molecular work, amplification of the partial *CaM* gene using the primer pairs CMD5/CMD6 and CAL-228F/CAL-737R (Carbone & Kohn 1999; Hong *et al.* 2006) was unsuccessful. Consequently, a new primer set was designed and introduced here as cm1F/cm1R and cm2F/cm2R (**Table 1)**.

To the best of our knowledge, this is the first report of *Paecilomyces* spp. from *Clematis* plants in the Czech Republic.

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Author Contributions

A.E. microscopy, primer design. D.G. microscopy. J.B. sampling. K.S. isolation. M.S. drafting the manuscript, molecular and morphological work. A.B.T. and A.E.M. phylogenetic data analysis. All authors reviewed and edited the manuscript. All authors reviewed and approved the final manuscript.

Competing Interests

The authors declare no competing interests.

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