FUSE

Fungal Systematics and Evolution

VOLUME 5
JUNE 2020
PAGES 1–15

doi.org/10.3114/fuse.2020.05.01

Penicillium diversity in Canadian bat caves, including a new species, *P. speluncae*

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Key words:
Thysanophora sect. *Fasciculata*
Genealogical Concordance Phylogenetic Species Recognition (GCPSR) concept
new taxon
Pseudogymnoascus destructans (Pd)
secondary metabolites

Abstract: *Penicillium* species were commonly isolated during a fungal survey of bat hibernacula in New Brunswick and Quebec, Canada. Strains were isolated from arthropods, bats, rodents (*i.e.* the deer mouse *Peromyscus maniculatus*), their dung, and cave walls. Hundreds of fungal strains were recovered, of which *Penicillium* represented a major component of the community. *Penicillium* strains were grouped by colony characters on Blakeslee's malt extract agar. DNA sequencing of the secondary identification marker, beta-tubulin, was done for representative strains from each group. In some cases, ITS and calmodulin were sequenced to confirm identifications. In total, 13 species were identified, while eight strains consistently resolved into a unique clade with *P. discolor*, *P. echinulatum* and *P. solitum* as its closest relatives. *Penicillium speluncae* is described using macro- and micromorphological characters, multigene phylogenies (including ITS, beta-tubulin, calmodulin and RNA polymerase II second largest subunit) and extrolite profiles. Major extrolites produced by the new species include cyclopenins, viridicatins, chaetoglobosins, and a microheterogenous series of cyclic and linear tetrapeptides.

Effectively published online: 9 August 2019.

INTRODUCTION

The study of fungi associated with bats and their habitats has become important after the spread of White-nose Syndrome (WNS) caused by *Pseudogymnoascus destructans* (Pd), resulted in an ongoing rapid decline of bat populations in North America. Much effort has focused on populations of Pd from positive caves. White-nose Syndrome is named for characteristic white growth caused by *P. destructans,* which was previously known as *Geomyces destructans* (Gargas *et al.* 2009, Minnis & Lindner 2013). Characterization of fungal populations and identification of other fungal species may reveal possible antagonists to Pd (Micalizzi *et al.* 2017).

White-nose Syndrome was first reported in New York in 2006 (Blehert *et al.* 2009), while the first report from Canada was from Ontario in 2010. In both cases, it led to mass mortality of the hibernating bat populations (McAlpine *et al.* 2012). The disease only occurs while bats hibernate. *Pseudogymnoascus destructans* cannot grow at temperatures above ± 20 °C (Gargas *et al.* 2009), and it is thought that the cool caves and mines inhabited by bats during hibernation serve as environmental reservoirs of Pd (Lorch *et al.* 2013, Reynolds *et al.* 2015). The presence of Pd in bat populations was confirmed in many countries in Europe and Asia but no significant mortality was observed, despite the fact that some European bats have been found with clinical WNS (Wibbelt *et al.* 2010, Puechmaille *et al.* 2011). Why bats remain healthy in these areas is unclear.

The study of fungal diversity is important to determine the true impact of a potential invasive species such as Pd on fungal community structure among bats and hibernacula (Johnson *et al.* 2013). Understudied environments such as caves are rich sources of undescribed microbial species. Many new fungi have recently been described from underground environments as more studies are conducted, although it is still unknown whether obligate troglobiotic fungi exist (Zhang *et al.* 2017). Previous studies commonly reported the isolation of *Cladosporium*, *Fusarium*, *Mortierella*, and *Penicillium* species from bat wings, caves and mines (Johnson *et al.* 2013, Vanderwolf *et al.* 2013a, b). *Penicillium* is one of the most common genera isolated from caves on multiple substrates, particularly sediment and air, although no new *Penicillium* species have been described from caves apart from *P. cavernicola*, which has also been found outside of caves on dairy products (Frisvad & Samson 2004, Vanderwolf *et al.* 2013a, b), and *P. gravinicasei* recently described from a cave in Italy from ripening Apulian cave cheeses (Anelli *et al.* 2018). Vanderwolf *et al.* (2016) studied the fungi associated with over-wintering arthropods in Pd positive hibernacula in Canada. They isolated 87 fungal taxa from four arthropod genera. In the current study, we report *Penicillium* isolated from these arthropods, but also include strains isolated from various other substrates associated with caves and/or bats. The aims of this study were (1) to determine the *Penicillium* species diversity in bat caves and hibernacula in New Brunswick and Quebec, and (2) formally describe the new species that was isolated during the survey.

MATERIALS AND METHODS

Strains, sampling and isolations

Strains were isolated from arthropods, bats, rodents, rodent dung, and walls of bat hibernacula in New Brunswick (Berryton Cave, Dallings Cave, Dorchester Mine, Glebe Mine, Markhamville Mine, White Cave) and Quebec (Grotte à la Patate), Canada (Vanderwolf *et al.* 2013b, 2016, 2017). Fungi were also isolated from a dead big brown bat that was found in a parking garage in Fredericton, New Brunswick. Isolation media included dextrose-peptone yeast extract agar (DPYA), sabouraud agar (SD) or malt extract agar (MEA), with plates incubated at 7 °C. Representative strains for each species found were submitted to the Canadian Collection of Fungal Cultures (DAOMC) and the holotype specimen of the new species deposited in the Canadian National Mycological Herbarium (DAOM). Strains isolated during this study are summarized in Table 1.

DNA extraction, sequencing and phylogenetic analysis

Strains were grown on Blakeslee's (1915) malt extract agar (MEAbl) for 7 d and DNA extracted using the Ultraclean™ Microbial DNA isolation Kit (MoBio Laboratories Inc., Solana Beach, USA). DNA was amplified with a PCR master mix consisting of 0.5 µL dNTPs (2 µM), 0.04 µL for each primer (20 µM), 1 µL 10× Titanium Taq buffer (Clontech, California, USA), 0.1 µL 50× Titanium Taq enzyme (Clontech, California, USA), 0.5 µL template DNA and 7.82 µL sterile purified water. ITS barcodes (Schoch *et al.* 2012), partial beta-tubulin (*BenA*), partial calmodulin (*CaM*) and RNA polymerase II second largest subunit (*RPB2*) genes were amplified using PCR conditions and primers suggested by Visagie *et al.* (2014b). PCR products were verified by agarose gel electrophoresis and subsequently sequenced with the BigDye Terminator Cycle Premix Kit (Applied Biosystems, Waltham, USA). Contigs were assembled and edited in Geneious v. 8.1.5 (BioMatters Ltd., Auckland, New Zealand). Newly generated sequences were submitted to GenBank and accession numbers provided in Table 1. Gene sequences of the new species were compared to a reference sequence dataset built around the ex-type sequences published in Visagie *et al.* (2014b), also including reference sequences from (Samson *et al.* 2004, Houbraken *et al.* 2011, 2012, 2014, 2016, Frisvad *et al.* 2013a, b, Visagie *et al.* 2014a) where needed (Suppl. Table S1). Additional unpublished sequences related to the new species were included and originate from various past projects. Sequences were aligned in MAFFT v. 7.407 (Katoh & Standley 2013), with the G-INS-i option and manually trimmed and adjusted in Geneious where needed. Datasets were subsequently analysed using Maximum Likelihood (ML) and Bayesian tree inference (BI). For concatenated phylogenies, each gene was treated as a separate partition. ML trees were calculated in IQtree v. 1.6.8 (Nguyen *et al.* 2015) with the most suitable model for each gene and/or partition calculated using Modelfinder (Kalyaanamoorthy *et al.* 2017) and bootstrapping done using UFBoot (Minh *et al.* 2013), both integrated into IQtree. Bayesian inference trees were calculated in MrBayes v. 3.2.6 (Ronquist *et al.* 2012) with the most suitable model selected by ParitionFinder v. 2.1.1 (Lanfear *et al.* 2017) using the corrected Akaike information criterion (Akaike 1974). Alignments and command blocks used for analyses were uploaded to TreeBASE (https://treebase.org) with accession 23575. Trees were visualized in Figtree v. 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree) and visually edited in Affinity Designer v. 1.7.1 [Serif (Europe) Ltd, Nottingham, UK].

Morphology

Morphological characters were captured using standardized protocols proposed by Visagie *et al.* (2014b). Colony characters were captured on Czapek yeast autolysate agar (CYA), MEAbl, yeast extract sucrose agar (YES), oatmeal agar (OA) and creatine sucrose agar (CREA). Strains were inoculated in a three-point pattern on these media in 90 mm Petri dishes. Plates were incubated for 7 d at 25 °C in darkness in perforated plastic bags. Colour names and codes used in descriptions are from Kornerup & Wanscher (1967). Microscopic observations were made using an Olympus SZX12 dissecting microscope and Olympus BX50 compound microscope equipped with Infinity3 and InfinityX cameras driven by Infinity Analyze v. 6.5.1 software (Lumenera Corp., Ottawa, Canada). Colonies were captured with a Sony NEX-5N camera. Plates were prepared in Affinity Photo v. 1.6.6 [Serif (Europe) Ltd, Nottingham, UK]. For aesthetic purposes, micrographs were adjusted using the "inpainting brush tool" without altering areas of scientific significance. Line drawings were prepared in Affinity Photo v. 1.7.1 [Serif (Europe) Ltd, Nottingham, UK] running on an iPad Pro with an Apple Pencil.

Extrolites

For extrolite analyses, all strains were grown in 9 cm polystyrene Petri dishes on CYA (Pitt 1980) and YES (Frisvad 1981, Filtenborg *et al.* 1990) incubated at 25 °C for 14 d. Six agar plugs from each fungal isolate were excised with a sterilized 7 mm cork-borer and transferred to a 13 mL polypropylene tube. Two mL of ethyl acetate was then added and vortexed for 30 s, followed by sonication at 30 °C for 30 min and vortexed again for 30 s. The supernatants were transferred into new polypropylene tubes and dried on a centrifugal vacuum concentrator at 35 °C. Extracts were then reconstituted in 1 mL of methanol:water (8:2) and filtered into 2 mL amber glass HPLC vials using a 0.45 µm PVDF syringe filter. Extracts were immediately stored at -20 °C until analysis by liquid chromatography mass spectrometry (LC-MS). Extracts were analyzed in both positive and negative polarities using a Q-Exactive Orbitrap coupled to an Agilent 1290 HPLC. The chemical formula of observed extrolites were determined with Xcalibur® software using accurate mass measurements (< 3.0 ppm) and manually verified by isotopic pattern. The chemical formulae were then searched against microbial extrolite databases [AntiBase2013 (Wiley-VCH, Weinheim, Germany)] and KNApSAcK (Afendi *et al.* 2012) and putative matches were scrutinized by comparing their MS/MS fragmentation with those published in the literature or predicted by CFM-ID (Allen *et al.* 2014).

RESULTS

Sampling, isolations & identifications

During the survey, 70 *Penicillium* strains were isolated from six different caves in New Brunswick and one in Quebec, Canada. Eight strains of the new *Penicillium* species were isolated from walls of the Glebe Mine and White Cave in New Brunswick and Grotte à la Patate in Quebec, and three strains were

Table 1. Species isolated from Canadian bat caves.

Species	Section	Strain	Date collected	Isolation medium	Province	Location	Cave name	Substrate	ITS	*BenA*	*CaM*	*RPB2*
P. bialowiezense	*Brevicompacta*	KAS 7465, W7430A	31-Mar-2015	MEA	New Brunswick	Dorchester	Dorchester Mine	Cave wall	n/a	MG490896	n/a	n/a
		DAOMC 252097, KAS 7466, W72102	07-Jul-2014	DPYA	Quebec	Anticosti Island	Grotte à la Patate	Cave wall	n/a	MG490897	n/a	n/a
		DAOMC 252098, KAS 7476, W29304	30-Apr-2015	MEA	New Brunswick	Moncton	Berryton Cave	Cave wall	n/a	MG490903	n/a	n/a
		KAS 7480, W24103	30-Apr-2015	DPYA	New Brunswick	Moncton	Berryton Cave	Cave wall	n/a	MG490906	n/a	n/a
		KAS 7511, S11101	18-Mar-2013	DPYA	New Brunswick	Dorchester	Dorchester Mine	Spider (*Meta ovalis*)	n/a	MG490924	n/a	n/a
		KAS 7517, M50103	11-Apr-2014	DPYA	New Brunswick	Sussex	Glebe Mine	Gnat (*Exechiopsis* sp)	n/a	MG490929	n/a	n/a
		KAS 7522, H27101	11-Apr-2014	DPYA	New Brunswick	Sussex	Glebe Mine	Harvestman (*Nelima elegans*)	n/a	MG490933	n/a	n/a
		KAS 7523, H26208	11-Apr-2014	SD	New Brunswick	Sussex	Glebe Mine	Harvestman (*Nelima elegans*)	n/a	MG490934	n/a	n/a
		DAOMC 252099, KAS 7525, H09108	18-Mar-2013	DPYA	New Brunswick	Dorchester	Dorchester Mine	Harvestman (*Nelima elegans*)	n/a	MG490936	n/a	n/a
		KAS 7542, 742102	16-Apr-2014	DPYA	New Brunswick	Fredericton	Fredericton parking garage	Bat (*Eptesicus fuscus*)	n/a	MG490949	n/a	n/a
P. brevistipitatum	*Robsamsonia*	DAOMC 252100, KAS 7514, P06101	14-Mar-2014	DPYA	New Brunswick	Dorchester	Dorchester Mine	Rodent (*Peromyscus maniculatus*)	MG490876	MG490926	MG490966	n/a
		DAOMC 252101, KAS 7520, M26108	16-Apr-2013	DPYA	New Brunswick	Sussex	Dallings Cave	Moth (*Scoliopteryx libatrix*)	MG490878	MG490932	MG490968	n/a
		DAOMC 252102, KAS 7531, D3303	25-Mar-2014	DPYA	New Brunswick	Dorchester	Dorchester Mine	Rodent dung (*Peromyscus maniculatus*)	MG490879	MG490938	MG490969	n/a
		DAOMC 252103, KAS 7534, D2203	21-Mar-2014	DPYA	New Brunswick	Dorchester	Dorchester Mine	Rodent dung (*Peromyscus maniculatus*)	MG490881	MG490941	MG490971	n/a
		DAOMC 252104, KAS 7538, D1007A	21-Mar-2014	DPYA	New Brunswick	Dorchester	Dorchester Mine	Rodent dung (*Peromyscus maniculatus*)	MG490882	MG490945	MG490972	n/a
		DAOMC 252105, KAS 7539, D1007	21-Mar-2014	DPYA	New Brunswick	Dorchester	Dorchester Mine	Rodent dung (*Peromyscus maniculatus*)	MG490883	MG490946	MG490973	n/a
P. chrysogenum	*Chrysogena*	DAOMC 252106, KAS 7505, W05100	21-Apr-2015	DPYA	New Brunswick	Hillsborough	White Cave	Cave wall	n/a	MG490919	n/a	n/a
		DAOMC 252107, KAS 7540, 742110	16-Apr-2014	DPYA	New Brunswick	Fredericton	Fredericton parking garage	Bat (*Eptesicus fuscus*)	n/a	MG490947	n/a	n/a
P. concentricum	*Robsamsonia*	KAS 7459, W98105	16-Apr-2015	DPYA	New Brunswick	Sussex	Glebe Mine	Cave wall	n/a	MG490890	n/a	n/a
		DAOMC 252108, KAS 7467, W72101	07-Jul-2014	DPYA	Quebec	Anticosti Island	Grotte à la Patate	Cave wall	n/a	MG490898	n/a	n/a
		KAS 7470, W59104	07-Jul-2014	DPYA	Quebec	Anticosti Island	Grotte à la Patate	Cave wall	n/a	MG490900	n/a	n/a
		KAS 7471, W59104	07-Jul-2014	DPYA	Quebec	Anticosti Island	Grotte à la Patate	Cave wall	n/a	MG490901	n/a	n/a

Table 1. (Continued).

Species	Section	Strain	Date collected	Isolation medium	Province	Location	Cave name	Substrate	ITS	*BenA*	*CaM*	*RPB2*
		KAS 7478, W29203	30-Apr-2015	SD	New Brunswick	Moncton	Berryton Cave	Cave wall	n/a	MG490904	n/a	n/a
		DAOMC 252109, KAS 7479, W24103A	30-Apr-2015	DPYA	New Brunswick	Moncton	Berryton Cave	Cave wall	n/a	MG490905	n/a	n/a
		DAOMC 252110, KAS 7483, W22102A	30-Apr-2015	DPYA	New Brunswick	Moncton	Berryton Cave	Cave wall	n/a	MG490908	n/a	n/a
		KAS 7486, W20408	30-Apr-2015	DPYA	New Brunswick	Moncton	Berryton Cave	Cave wall	n/a	MG490910	n/a	n/a
		KAS 7513, P06102	14-Mar-2014	DPYA	New Brunswick	Dorchester	Dorchester Mine	Rodent (*Peromyscus maniculatus*)	n/a	MG490925	n/a	n/a
		KAS 7515, P05201	14-Mar-2014	SD	New Brunswick	Dorchester	Dorchester Mine	Rodent (*Peromyscus maniculatus*)	n/a	MG490927	n/a	n/a
		KAS 7532, D3301	25-Mar-2014	DPYA	New Brunswick	Dorchester	Dorchester Mine	Rodent dung (*Peromyscus maniculatus*)	n/a	MG490939	n/a	n/a
		KAS 7535, D2111	21-Mar-2014	DPYA	New Brunswick	Dorchester	Dorchester Mine	Rodent dung (*Peromyscus maniculatus*)	n/a	MG490942	n/a	n/a
		KAS 7536, D1204	21-Mar-2014	DPYA	New Brunswick	Dorchester	Dorchester Mine	Rodent dung (*Peromyscus maniculatus*)	n/a	MG490943	n/a	n/a
		KAS 7537, D1106	21-Mar-2014	DPYA	New Brunswick	Dorchester	Dorchester Mine	Rodent dung (*Peromyscus maniculatus*)	n/a	MG490944	n/a	n/a
P. consobrinum	*Exilicaulis*	DAOMC 252111, KAS 7464, W76401	31-Mar-2015	DPYA	New Brunswick	Dorchester	Dorchester Mine	Cave wall	MG490873	MG490895	MG490963	n/a
		DAOMC 252112, KAS 7491, W19102	30-Apr-2015	DPYA	New Brunswick	Moncton	Berryton Cave	Cave wall	MG490874	MG490913	MG490964	n/a
P. corylophilum	*Exilicaulis*	DAOMC 252113, KAS 7481, W24100	30-Apr-2015	DPYA	New Brunswick	Moncton	Berryton Cave	Cave wall	n/a	MG490907	n/a	n/a
		DAOMC 252114, KAS 7484, W22102	30-Apr-2015	DPYA	New Brunswick	Moncton	Berryton Cave	Cave wall	n/a	MG490909	n/a	n/a
		KAS 7489, W20103	30-Apr-2015	DPYA	New Brunswick	Moncton	Berryton Cave	Cave wall	n/a	MG490912	n/a	n/a
		KAS 7493, W16200B	30-Apr-2015	SD	New Brunswick	Moncton	Berryton Cave	Cave wall	n/a	MG490914	n/a	n/a
P. expansum	*Penicillium*	DAOMC 252115, KAS 7501, W07104	21-Apr-2015	DPYA	New Brunswick	Hillsborough	White Cave	Cave wall	n/a	MG490917	n/a	n/a
		KAS 7502, W05406	21-Apr-2015	DPYA	New Brunswick	Hillsborough	White Cave	Cave wall	n/a	MG490918	n/a	n/a
		KAS 7506, W04407	21-Apr-2015	DPYA	New Brunswick	Hillsborough	White Cave	Cave wall	n/a	MG490920	n/a	n/a
		KAS 7510, W00200	21-Apr-2015	SD	New Brunswick	Hillsborough	White Cave	Cave wall	n/a	MG490923	n/a	n/a
		DAOMC 252116, KAS 7519, M26109	16-Apr-2013	DPYA	New Brunswick	Sussex	Dallings Cave	Moth (*Scoliopteryx libatrix*)	n/a	MG490931	n/a	n/a

Table 1. (Continued).

Species	Section	Strain	Date collected	Isolation medium	Province	Location	Cave name	Substrate	ITS	*BenA*	*CaM*	*RPB2*
		KAS 7529, H06108	18-Mar-2013	DPYA	New Brunswick	Dorchester	Dorchester Mine	Harvestman (*Nelima elegans*)	n/a	MG490937	n/a	n/a
		KAS 7545, 702115	04-Apr-2013	DPYA	New Brunswick	Markhamville	Markhamville Mine	Bat (*Perimyotis subflavus*)	n/a	MG490951	n/a	n/a
		KAS 7546, 701115	04-Apr-2013	DPYA	New Brunswick	Markhamville	Markhamville Mine	Bat (*Perimyotis subflavus*)	n/a	MG490952	n/a	n/a
		KAS 7547, 701106	04-Apr-2013	DPYA	New Brunswick	Markhamville	Markhamville Mine	Bat (*Perimyotis subflavus*)	n/a	MG490953	n/a	n/a
P. glabrum	*Aspergilloides*	DAOMC 252117, KAS 7475, W54101	07-Jul-2014	DPYA	Quebec	Anticosti Island	Grotte à la Patate	Cave wall	n/a	MG490902	n/a	n/a
		DAOMC 252118, KAS 7494, W16200A	30-Apr-2015	SD	New Brunswick	Moncton	Berryton Cave	Cave wall	n/a	MG490915	n/a	n/a
		KAS 7524, H11101	18-Mar-2013	DPYA	New Brunswick	Dorchester	Dorchester Mine	Harvestman (*Nelima elegans*)	n/a	MG490935	n/a	n/a
P. glaucoalbidum	*Thysanophora*	DAOMC 252119, KAS 7460, W88411	31-Mar-2015	DPYA	New Brunswick	Dorchester	Dorchester Mine	Cave wall	MG490870	MG490891	MG490960	n/a
		DAOMC 252120, KAS 7461, W88405	31-Mar-2015	DPYA	New Brunswick	Dorchester	Dorchester Mine	Cave wall	MG490871	MG490892	MG490961	n/a
		KAS 7462, W88405	31-Mar-2015	DPYA	New Brunswick	Dorchester	Dorchester Mine	Cave wall	MG490872	MG490893	MG490962	n/a
		DAOMC 252122, KAS 7508, W04210	16-Apr-2015	SD	New Brunswick	Sussex	Glebe Mine	Cave wall	MG490875	MG490921	MG490965	n/a
P. rubens	*Chrysogena*	DAOMC 252123, KAS 7488, W20104	30-Apr-2015	DPYA	New Brunswick	Moncton	Berryton Cave	Cave wall	n/a	MG490911	n/a	n/a
		KAS 7495, W16200	30-Apr-2015	SD	New Brunswick	Moncton	Berryton Cave	Cave wall	n/a	MG490916	n/a	n/a
		KAS 7509, W02400	16-Apr-2015	DPYA	New Brunswick	Sussex	Glebe Mine	Cave wall	n/a	MG490922	n/a	n/a
		DAOMC 252124, KAS 7543, 741102	16-Apr-2014	DPYA	New Brunswick	Fredericton	Fredericton parking garage	Bat (*Eptesicus fuscus*)	n/a	MG490950	n/a	n/a
P. spathulatum	*Brevicompacta*	KAS 7468, W71101	07-Jul-2014	DPYA	Quebec	Anticosti Island	Grotte à la Patate	Cave wall	n/a	MG490899	n/a	n/a
		DAOMC 252125, KAS 7541, 742105	16-Apr-2014	DPYA	New Brunswick	Fredericton	Fredericton parking garage	Bat (*Eptesicus fuscus*)	n/a	MG490948	n/a	n/a
P. speluncae	*Fasciculata*	DAOMC 251696, KAS 7473, W54119	07-Jul-2014	DPYA	Quebec	Anticosti Island	Grotte à la Patate	Cave wall	MG490864	MG490884	MG490954	MN170736
		DAOMC 251697, KAS 7474, W54102	07-Jul-2014	DPYA	Quebec	Anticosti Island	Grotte à la Patate	Cave wall	MG490865	MG490885	MG490955	MN170737
		DAOMC 251698, KAS 7500, W07302	21-Apr-2015	MEA	New Brunswick	Hillsborough	White Cave	Cave wall	MG490866	MG490886	MG490956	MN170738
		DAOMC 251699, KAS 7503, W05404	21-Apr-2015	DPYA	New Brunswick	Hillsborough	White Cave	Cave wall	MG490867	MG490887	MG490957	MN170739
		DAOMC 251700, KAS 7504, W05202	16-Apr-2015	SD	New Brunswick	Sussex	Glebe Mine	Cave wall	MG490868	MG490888	MG490958	MN170740

Table 1. (Continued).

Species	Section	Strain	Date collected	Isolation medium	Province	Location	Cave name	Substrate	ITS	*BenA*	*CaM*	*RPB2*
		DAOMC 251701[T], KAS 7512, P06201	14-Mar-2014	SD	New Brunswick	Dorchester	Dorchester Mine	Rodent (*Peromyscus maniculatus*)	MG490869	MG490889	MG490959	MN170741
		DAOMC 252126, KAS 7516, P01202	12-Mar-2014	SD	New Brunswick	Dorchester	Dorchester Mine	Rodent (*Peromyscus maniculatus*)	MG490877	MG490928	MG490967	MN170742
		DAOMC 252127, KAS 7533, D3108	25-Mar-2014	DPYA	New Brunswick	Dorchester	Dorchester Mine	Rodent dung (*Peromyscus maniculatus*)	MG490880	MG490940	MG490970	MN170743
P. westlingii	*Citrina*	DAOMC 252128, KAS 7463, W77200	31-Mar-2015	SD	New Brunswick	Dorchester	Dorchester Mine	Cave wall	n/a	MG490894	n/a	n/a
		DAOMC 252129, KAS 7518, M34108	14-Mar-2014	DPYA	New Brunswick	Dorchester	Dorchester Mine	Moth (*Scoliopteryx libatrix*)	n/a	MG490930	n/a	n/a

DAOMC: Culture collection of the National Mycological Collections, Agriculture & Agri-Food Canada, Ottawa, Canada; KAS: Internal working culture collection at DAOMC; Remaining acronyms represent internal isolate numbers from the personal collection of Karen Vanderwolf and Dave Malloch.

isolated from a deer mouse (*Peromyscus maniculatus*) and its dung from the Dorchester Mine in New Brunswick (Table 1). Based on the *BenA* phylogeny (Fig. 1), and in some cases additional ITS and *CaM* BLAST searches, the remaining strains were identified as *Penicillium bialowiezense* (n = 10), *P. brevistipitatum* (n = 6), *P. chrysogenum* (n = 2), *P. concentricum* (n = 14), *P. consobrinum* (n = 2), *P. corylophilum* (n = 4), *P. expansum* (n = 9), *P. glabrum* (n = 3), *P. glaucoalbidum* (n = 4), *P. rubens* (n = 4), *P. spathulatum* (n = 2), and *P. westlingii* (n = 2).

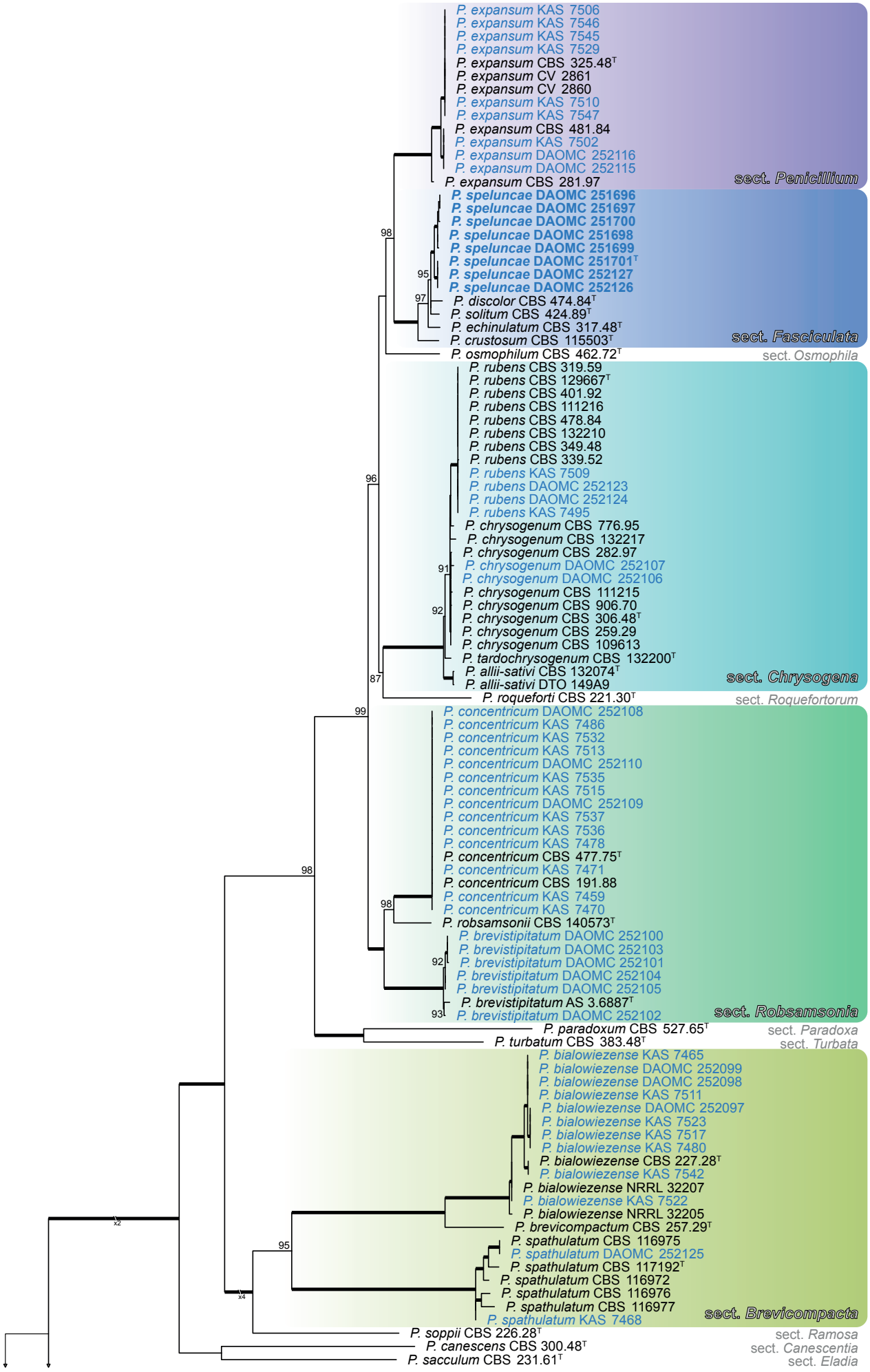
Phylogeny

A multigene phylogeny was used to show identities of strains isolated during this study (Fig. 1). The alignment contained 175 taxa and was 2 375 bp long (*BenA* 1–453; *CaM* 454–1019; *RPB2* 1020–1823; ITS 1824–2375). The most appropriate substitution model for each partition was: *BenA* TIM2e+I+G4; *CaM* TNe+I+G4; *RPB2* TNe+R3; ITS TIM2+F+I+G4. Generally, *BenA* sequences from strains isolated during this study matched well with reference sequences in terms of resolving in a particular clade. However, many of the newly generated sequences represented minor deviations from previously known sequences. In some cases, variation was such that calmodulin was sequenced to make a final identification of a species (e.g. *P. consobrinum*, *P. brevistipitatum*). One clade was found to represent a new species in section *Fasciculata*.

To demonstrate the genealogical concordance of the new species in relation to its close relatives, phylogenies of all known species from section *Fasciculata* were calculated based on *BenA, CaM* and *RPB2* (Fig. 2). To demonstrate the overall phylogenetic relationship, a concatenated dataset, based on ITS, *BenA*, *CaM* and *RPB2* was calculated. Alignment metadata is summarised in Suppl. Table S2.

As expected, ITS (not shown) lacked sufficient variation to distinguish among species. For example, *P. speluncae* shared similar ITS sequences with *P. cavernicola*, *P. echinulatum*, *P. discolor* and *P. solitum*, noting that strains DAOMC 251696 and DAOMC 251697 formed a distinct clade because of an A-T transversion. *BenA, CaM* and *RPB2* distinguished among close relatives much better. The exception was the clade associated with cheese; *P. biforme, P. camemberti* (1 bp difference)*, P. caseifulvum, P. commune* and *P. palitans* had identical *CaM* sequences, while *P. camemberti* had 1 bp difference from these species. *RPB2* sequences for *P. caseifulvum* and *P. commune* were identical (albeit with limited sampling). *BenA* was not helpful to distinguish between *P. camemberti* and *P. commune*, supporting the hypothesis that the former is a domesticated form of the latter (Pitt *et al.* 1986, Polonelli *et al.* 1987). Much sequence variation was observed within the clade containing *P. speluncae*, *P. discolor*, *P. echinulatum* and *P. solitum*. This resulted in all phylogenies having poor backbone support in both ML and BI, mainly because of the strains identified as *P. speluncae*. Nonetheless, all phylogenies resulted in three distinct clades corresponding with *P. discolor*, *P. solitum* and *P. echinulatum*. CBS 271.97 and CBS 278.97 previously considered typical of *P. discolor* (Frisvad & Samson 2004, Samson *et al.* 2004) were phylogenetically resolved distinct from the ex-type CBS 474.84[T] within the broad concept applied to *P. speluncae*.

Fig. 1. ML tree based on ITS, *BenA, CaM* & *RPB2* showing identities and diversity of *Penicillium* associated with bats or bat caves. Bootstrap values ≥ 80% are shown above branches while thickened branches indicate 100 % support. Sequences obtained from ex-type cultures are indicated by [T]. Sequences obtained from strains during this study are indicated by blue text, while the new species, *P. speluncae*, is in bold blue text. The tree was rooted to *Talaromyces pinophilus*.



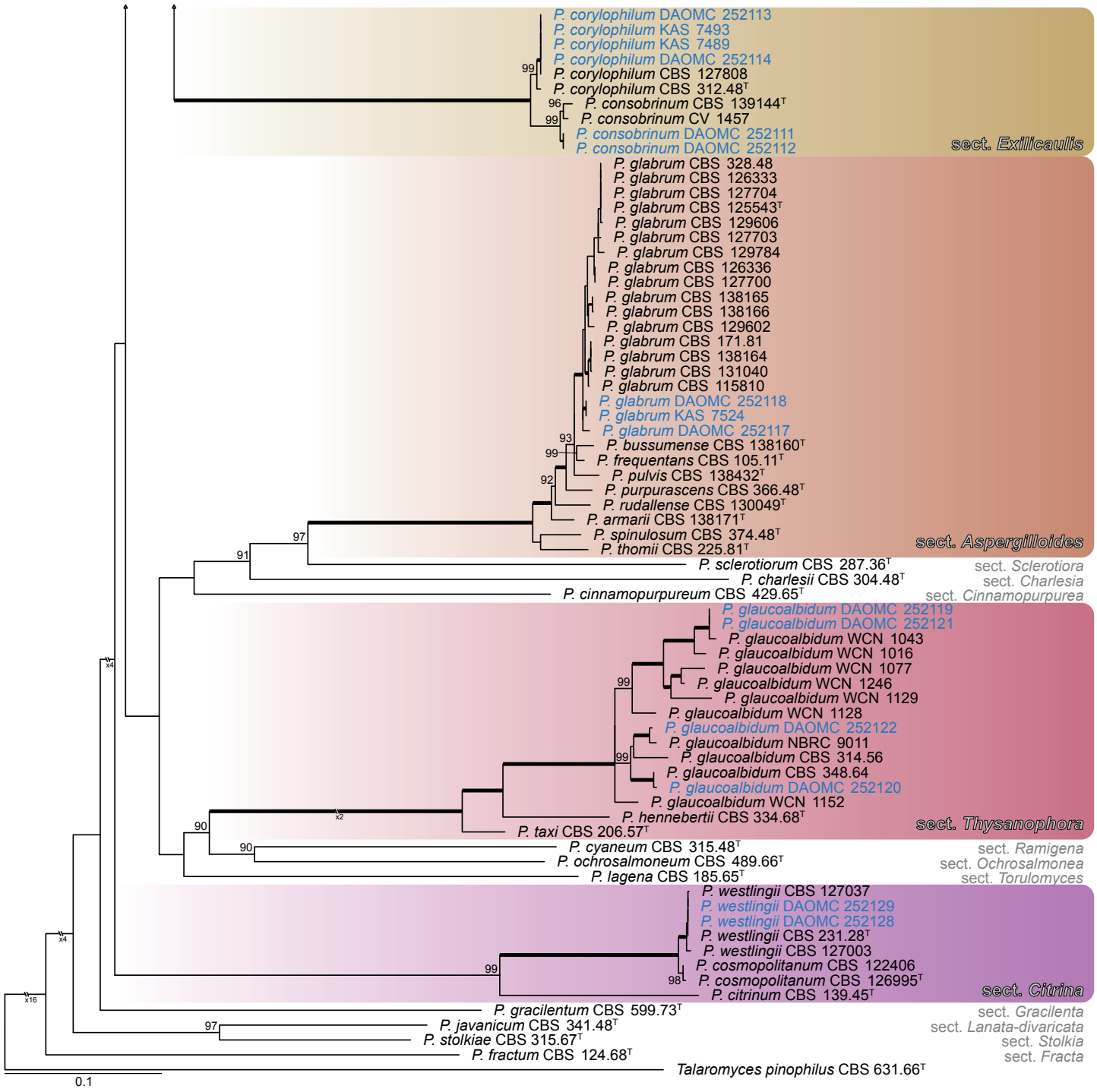


Fig. 1. (Continued).

Extrolites

As analysed by LC-MS, there were five classes of compounds produced by *P. speluncae* under the reported growth conditions: cyclopenins, viridicatins, chaetoglobosins, cyclic dipeptides, and tetrapeptides. Cyclopenins and viridicatins are derived from a shared biosynthetic pathway (Simonetti *et al.* 2016) and are among the most widely distributed extrolites across species in *Penicillium* subgenus *Penicillium* (Frisvad *et al.* 2004). Chaetoglobosins are a large class of metabolites biosynthesised by a polyketide derived macrocycle fused to a modified tryptophan amino acid and are produced by *Chaetomium globosum* as well as *P. discolor* (Frisvad *et al.* 1997), *P. expansum* (Frisvad & Filtenborg 1989) and *P. marinum* (Frisvad *et al.* 2004). One of the major chaetoglobosins produced by these isolates is a newly described natural product, tetrahydrochaetoglobosin

Fig. 2. ML trees of *Penicillium* section *Fasciculata*, based on concatenated, *BenA*, *CaM* and *RPB2* alignments, showing the relationship of *P. speluncae* within the section. PP and BS values ≥ 0.95/80 are shown above thickened branches (* = 1.00/100; - = <0.95/80). Sequences obtained from ex-type cultures are indicated by T. Strains of the new species characterised based on morphology and extrolites is indicated by bold blue text. Trees were rooted to *Penicillium robsamsonia*.

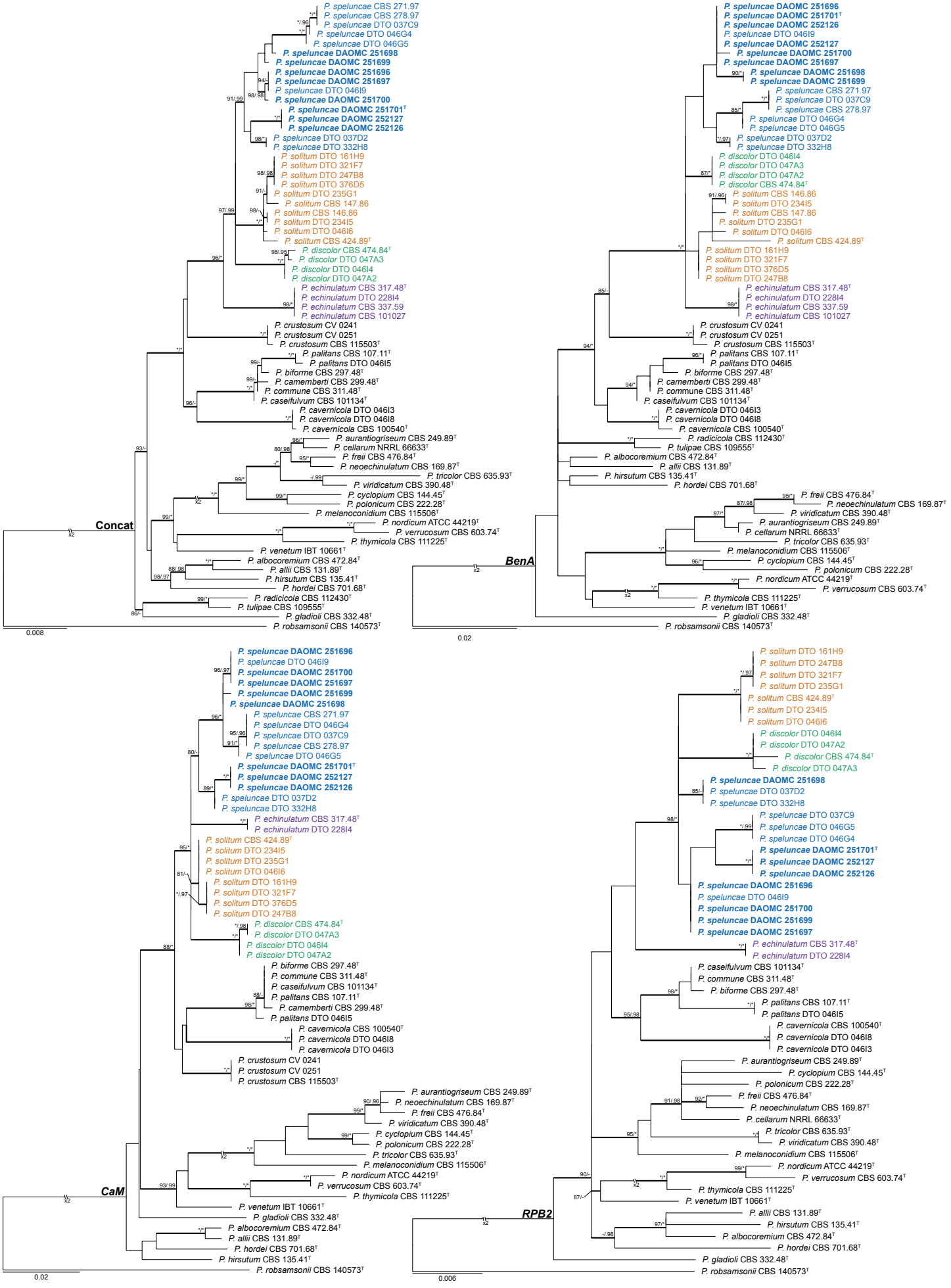


Table 2. Extrolites produced by *Penicillium speluncae.*

Extrolite name	Formula	m/z [M+H]$^+$	RT	% strains producing
cyclopenin	$C_{17}H_{14}N_2O_3$	295.1076	3.03	86 %
cyclopenol	$C_{17}H_{14}N_2O_4$	311.1025	2.69	86 %
cyclopeptine	$C_{17}H_{16}N_2O_2$	281.1285	3.11	100 %
dehydrocyclopeptine	$C_{17}H_{14}N_2O_2$	279.1130	3.17	86 %
viridicatin	$C_{15}H_{11}NO_2$	238.0865	3.36	100 %
viridicatol	$C_{15}H_{11}NO_3$	254.0812	2.95	100 %
chaetoglobosin F	$C_{32}H_{38}N_2O_5$	531.2852	3.54	100 %
tetrahydrochaetoglobosin A	$C_{32}H_{40}N_2O_5$	533.3009	3.27	100 %
chaetoglobosin A	$C_{32}H_{38}N_2O_5$	531.2852	3.63	100 %
chaetoglobosin C	$C_{32}H_{36}N_2O_5$	529.2698	3.78	57 %
prochaetoglobosin I	$C_{32}H_{38}N_2O_2$	483.3005	4.41	100 %
cyclo(VP)	$C_{10}H_{16}N_2O_2$	197.1286	2.32	100 %
cyclo(LP)	$C_{11}H_{18}N_2O_2$	211.1441	2.50	100 %
cyclo(IP)	$C_{11}H_{18}N_2O_2$	211.1443	2.55	100 %
cyclo(FP)	$C_{14}H_{16}N_2O_2$	245.1285	2.62	100 %
fungisporin	$C_{28}H_{36}N_4O_4$	493.2809	3.77	100 %
cyclo(Phe-Val-Phe-Val)	$C_{28}H_{36}N_4O_4$	493.2809	3.55	100 %
Val-Phe-Val-Phe	$C_{28}H_{38}N_4O_5$	511.2919	2.87	100 %
cyclo(Phe-Phe-Val-Ile)	$C_{29}H_{38}N_4O_4$	507.2360	4.00	100 %
Phe-Val-Ile-Phe	$C_{29}H_4N_4O_5$	525.3074	2.96	100 %
cyclo(Phe-Tyr-Val-Val)	$C_{28}H_{36}N_4O_5$	509.2761	3.42	100 %
Phe-Val-Val-Tyr	$C_{28}H_{38}N_4O_6$	527.2866	2.68	100 %
Phe-Ile-Val-Tyr	$C_{29}H_{40}N_4O_6$	541.3022	2.74	100 %
cyclo(Phe-Trp-Val-Val)	$C_{30}H_{37}N_5O_4$	532.2914	3.68	100 %
Phe-Val-Val-Trp	$C_{30}H_{39}N_5O_5$	550.3024	2.89	100 %
cyclo(Tyr-Trp-Val-Val)	$C_{30}H_{37}N_5O_5$	548.2866	3.39	100 %
Tyr-Val-Val-Trp	$C_{30}H_{39}N_5O_6$	566.2973	2.68	100 %
cyclo(Trp-Trp-Val-Val)	$C_{32}H_{38}N_6O_4$	571.3025	3.62	100 %

(Walsh *et al.* 2018). In addition to these extrolites, a series of cyclic and linear tetrapeptides, composed of combinations of valine, phenylalanine, leucine/isoleucine, tyrosine and tryptophan were consistently detected across all tested strains (Table 2). These peptides could be putatively characterized by *de novo* sequencing and are likely similar to the series of linear and cyclic tetra peptides previously identified in cultures of *P. chrysogenum*, including fungisporin; cyclo(D-Phe-L-Phe-D-Val-L-Val) (Ali *et al.* 2014).

Taxonomy

Penicillium speluncae Visagie & Yilmaz, ***sp. nov.*** MycoBank MB828614. Figs 3, 4.

Etymology: Latin, *speluncae,* meaning from a cave.

ITS barcode: MG490869. Alternative identification markers: *BenA* = MG490889, *CaM* = MG490959, *RPB2* = MN170741.

Colony diam, 7 d (at 25 °C; in mm): CYA 30–35; CYA 15 °C (12–) 17–22(–25); CYA 30 °C 12–21(–26); CYA 37°C no growth; CYAS 29–32(–37); MEAbl 25–30; YES 45–47; OA 25–27; CREA 25–26.

Colony characters: CYA 25 °C, 7 d: Colonies moderately deep, sulcate; margins low, narrow to wide, entire; mycelia white; texture velutinous to fasciculate; sporulation moderately dense, conidia *en masse* greyish green (25E7), dull green (26D3–4); soluble pigments absent; exudates absent; reverse greyish yellow (4B6), greyish orange (5B4), yellowish white (4A2). MEA 25 °C, 7 d: Colonies low, plain; margins low, narrow, entire; mycelia white; texture velutinous to fasciculate; sporulation moderately dense, conidia *en masse* greyish green (25E5–26E5); soluble pigments forming a yellow halo surrounding colony; exudates absent; reverse greyish yellow (4B6), light yellow (3A5), greyish green (29C6). YES 25 °C, 7 d: Colonies low, sulcate; margins low, wide, entire; mycelia white; texture velutinous to fasciculate; sporulation moderately dense, conidia *en masse* greyish green (25C5–D5), dull green (26D3); soluble pigments absent; exudates absent; reverse orange yellow to orange (4A7–6A7). OA 25°C, 7 days: Colonies moderately deep, plain; margins low, narrow, entire; mycelia white; texture fasciculate; sporulation dense, conidia *en masse* greyish to dark green (25E7–F7); soluble pigments forming a yellowish halo surrounding colony; exudates absent. CREA 25 °C, 7 d: Growth strong, acid produced, colony reverse orange.

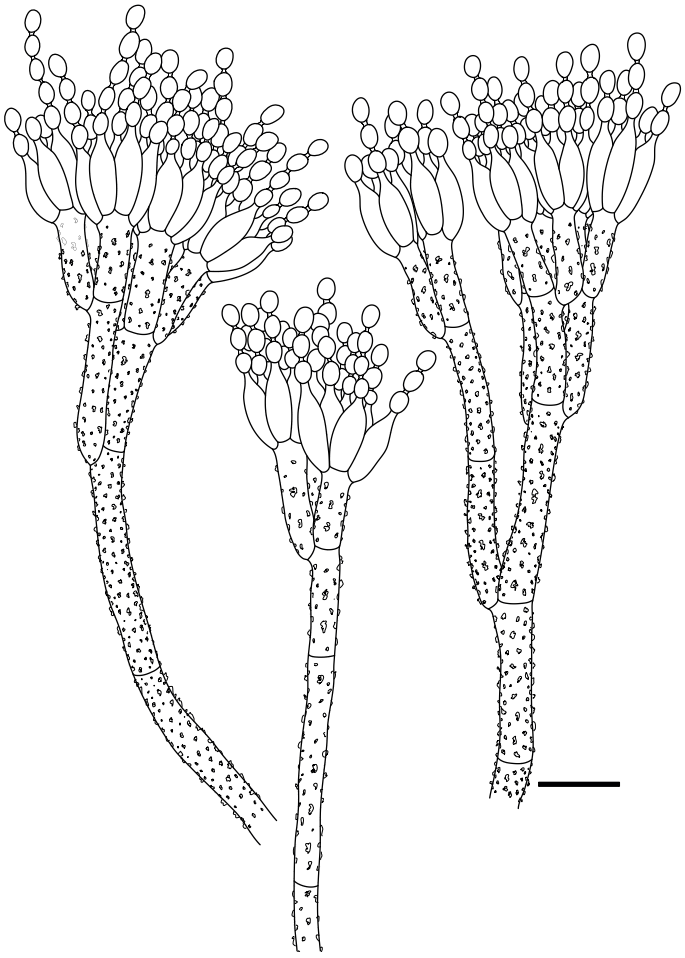


Fig. 3. Line drawing of *Penicillium speluncae.* Scale bar = 10 µm.

Micromorphology: Conidiophores terverticillate, minor proportion bi- and quarterverticillate; stipes rough, 180–600 × 3.5–4.5 µm; branches 15–29 µm; metulae (2–)3–4, 10–16 × 3–4.5 µm; phialides ampulliform, 4–6 per metula, 8.5–11 × 3–4 µm (9.9±0.7 × 3.3±0.2); average length metula/phialide 1.3; conidia smooth, broadly ellipsoidal, 3–4 × 2.5–3.5 µm (3.6±0.2 × 3±0.2), average width/length = 0.82, n = 72.

Extrolites: cyclopenins, viridicatins, chaetoglobosins, fungisporin, cyclic and linear tetrapeptides (See Table 2).

Typus: **Canada**, New Brunswick, Dorchester, Dorchester mine, from a swab of deer mouse fur (live *Peromyscus maniculatus*), 14 Mar. 2014, *K. Vanderwolf* (**holotype,** DAOM 745788 (dried culture); ex-type strain DAOMC 251701 = KAS 7512 = P06201).

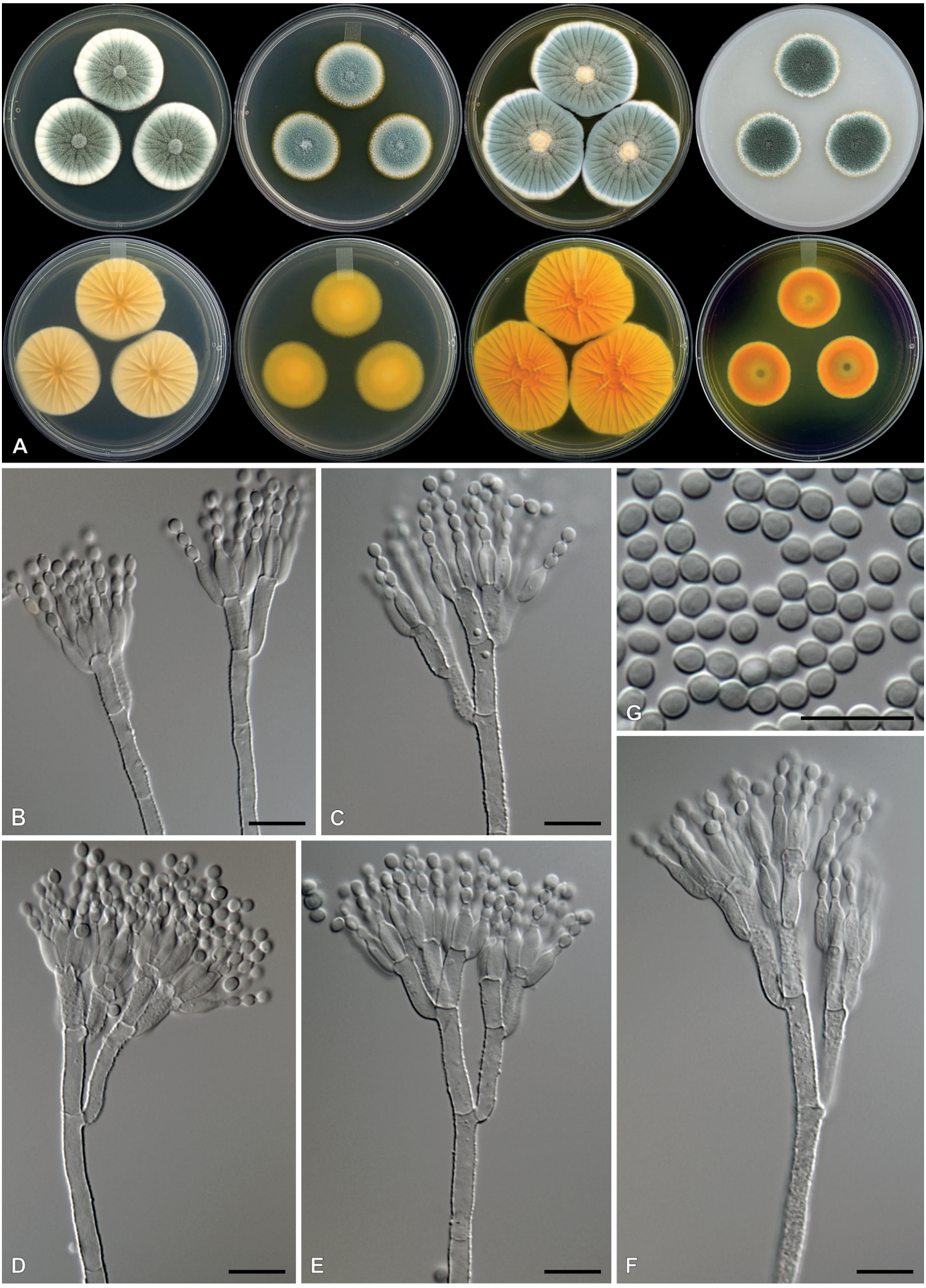
Notes: *Penicillium speluncae* is resolved in a clade with *P. discolor, P. echinulatum* and *P. solitum* (Fig. 2). Of these, *P. speluncae* showed relatively good growth on CYA at 30 °C, compared to poor growth observed for the others. Both *P. discolor* and *P. echinulatum* produce roughened globose to subglobose conidia, in contrast to the new species' smooth, broadly ellipsoidal conidia. *Penicillium solitum* is morphologically most similar to the new species. Both species have smooth conidia and produce a striking yellow orange reverse on YES. However, *P. speluncae* produces broadly ellipsoidal conidia (globose to subglobose in *P. solitum*), grows faster on YES compared to *P. solitum* (45–47 mm *vs* 25–39 mm) and has the ability to grow on CYA at 30 °C. *Penicillium solitum* has several synonyms examined before (Frisvad & Samson 2004), and showed no growth on CYA at 30 °C. Of the extrolites produced in this clade, chaetoglobosins are produced by only *P. speluncae* and *P. discolor*, territrems only by *P. echinulatum*, compactin only by *P. solitum,* while penitrem and roquefortine are produced by *P. crustosum* and other distantly related Penicillia. *Penicillium speluncae* produces cytoglobosin and prochaetoglobosin, which are absent in *P. discolor*, while palitantin was not detected for the new species (comparisons summarised in Table 3; data from Frisvad *et al.* 2004).

Additional materials examined: **Canada**, New Brunswick, Dorchester, Dorchester copper mine, from rodent fur (*Peromyscus maniculatus*), 12 Mar. 2014, *K. Vanderwolf* (culture DAOMC 252126 = KAS 7516 = P01202); Dorchester mine, from rodent dung (*Peromyscus maniculatus*), 25 Mar. 2014, *K. Vanderwolf* (culture DAOMC 252127 = KAS 7533 = D3108); Hillsborough, White Cave (gypsum), from cave wall, 21 Apr. 2015, *K. Vanderwolf* (cultures DAOMC 251698 = KAS 7500 = W07302, DAOMC 251699 = KAS 7503 = W05404); Sussex, Glebe mine (limestone), from cave wall, 16 Apr. 2015, *K. Vanderwolf* (culture DAOMC 251700 = KAS 7504 = W05202); Quebec, Anticosti Island, Grotte à la Patate (limestone), from cave wall, *K. Vanderwolf* (cultures DAOMC 251696 = KAS 7473 = W54119, DAOMC 251697 = KAS 7474 = W54102).

DISCUSSION

This study focused on *Penicillium* species isolated from six Pd-positive bat hibernacula in New Brunswick, Canada and one Pd-negative bat hibernaculum in Quebec, Canada. The isolates were collected from arthropods, bats, rodents and their dung (*i.e*. the deer mouse *Peromyscus maniculatus*), cave walls, and one dead bat found in a parking garage. During the survey, hundreds of fungal strains were obtained and *Penicillium* represented one of the most frequently isolated genera, probably because of the ability of these species to grow at low temperatures (Frisvad & Samson 2004, Vanderwolf *et al.* 2016). Previous studies had similar results, but the diversity of *Penicillium* in caves is even greater than previously reported and several of these species have never been reported from caves or mines, including *P. bialowiezense, P. brevistipitatum, P. consobrinum, P. rubens, P. spathulatum* and *P. westlingii* (Nováková 2009, 2018, Vanderwolf *et al.* 2013b, 2016, Anelli *et al.* 2018). Other species identified were *P. chrysogenum, P. concentricum, P. corylophilum, P. expansum, P. glabrum* and *P. glaucoalbidum*. One species could not be identified based on DNA reference sequences and further study showed it to represent a new species, described above as *P. speluncae,* classified in section *Fasciculata*.

Phylogenetic analyses of sect. *Fasciculata* revealed a large degree of genetic variation within *P. speluncae*. Single gene trees based on *BenA, CaM* and *RPB2* resulted in inconsistent groupings and poor backbone support for this clade meaning that genealogical concordance could not be applied to delimit segregate species. The basal branch encompassing this clade was relatively well supported in the concatenated tree. DAOMC strains were characterized based on morphology and extrolite data, with very few differences noted. For example, colony growth rates varied on CYA at 30 °C and CYAS, but a similar variation was previously observed in *P. solitum* (Frisvad & Samson 2004). Extrolite data also distinguish among this group of species. Chaetoglobosins are produced only by *P. speluncae* and *P. discolor*, while the former produces cytoglobosin and prochaetoglobosin,



which are absent in *P. discolor*. *Chaetomium globosum* is the best-known producer of chaetoglobosins, including the major chaetoglobosins A, C, and F, also shown here to be produced by *P. speluncae*. A distinguishing feature between chaetoglobosin production by *C. globosum* and *P. speluncae* is a newly described natural product, tetrahydrochaetoglobosin A (Walsh *et al.* 2019), which was not observed in *C. globosum*. Our data hint that *P. speluncae* may be a species complex with so far cryptic species that may be resolved with additional data. Considering the available data, we conservatively propose the name *P. speluncae* for this clade. In principle, an analogous situation occurred with *P. glabrum* (sect *Aspergilloides*). This complex was studied several times morphologically but a satisfactory conclusion was never found (Pitt *et al.* 1990). Houbraken *et al.* (2014) provided an extensive phylogenetic analysis and distinguished between *P. glabrum* and *P. frequentans* using a concatenated phylogeny of *BenA*, *CaM* and *RPB2*, even though these species had poor backbone support in the single gene trees for *BenA* and *CaM* and could not be distinguished.

Even though we adopt a consilient species concept for *Penicillium*, there is often bias towards DNA sequences for making a species identification or deciding whether strains are new or not. This situation is a direct consequence of the accepted species list and associated ex-type reference sequences published by Visagie *et al.* (2014b) and resulted in a generally aggressive approach to describing new species or reinstating old names. Many of the resulting taxa are often based on a single strain. This in turn complicates sequence-based identifications because the reference data do not encapsulate infraspecies variation. We thus encourage a more holistic approach to introducing new species, noting that singleton species will always be a part of our science. An example is *P. brevistipitatum*, which before this study was known only from ex-type sequences. *BenA* sequences obtained from our strains differed at several nucleotide positions and only after *CaM* was sequenced could we identify strains as this species. The additional reference sequences generated here will thus aid future identifications of *P. brevistipitatum*. Several new genotypes were also discovered for *P. bialowiezense, P. consobrinum, P. glabrum, P. glaucoalbidum* and *P. spathulatum* (Fig. 1). Several strains were identified as *P. glaucoalbidum* (≡ *Thysanophora glaucoalbida*). Although this species is often encountered as an endophyte of conifer needles, the name is not currently accepted because no type material is available (Visagie *et al.* 2014b). Lectotypification is complicated by the large degree of variation observed in available sequences (Iwamoto *et al.* 2005); this will be the focus of a future study.

Table 3. Distinguishing features of species closely related to *Penicillium speluncae*.

	Conidia	CYA texture	YES soluble pigment	Yes reverse	Chaetoglobosins	Compactin	Penitrem	Roquefortine	Cytoglobosin	Prochaetoglobosin	Palitantin
P. speluncae	Smooth, broadly ellipsoidal	Velutinous to fasciculate	None	Orange yellow to orange	+	-	-	-	+	+	-
P. crustosum	Smooth, globose to subglobose	Velutinous to weakly fasciculate, becoming crustose	Pale brown or none	Strongly yellow	-	-	+	+	-	-	-
P. discolor	Rough, globose to subglobose	Velutinous to fasciculate	Brilliant red diffusible colour on YES	Orange turning into deep red with age	+	-	-	-	-	-	+
P. echinulatum	Rough, globose to subglobose	Velutinous to weakly fasciculate	None	yellow	-	-	-	-	-	-	+
P. solitum	Smooth to finely rough, globose to subglobose	Velutinous	None	yellow to orange	-	+	-	-	-	-	+

Fig. 4. *Penicillium speluncae*. **A.** Colonies, from left to right, top row: CYA, MEA, YES, OA; bottom row: reverse on CYA, MEA, YES, CREA. **B–F.** Conidiophores. **G.** Conidia. Scale bars = 10 µm.

ACKNOWLEDGEMENTS

We acknowledge DNA sequencing support from Lisa James of the Molecular Technologies Laboratory of ORDC, AAFC, Ottawa, and research support from AAFC project J-1848. We also acknowledge Martin Meijer for sequencing support provided at the Westerdijk Fungal Biodiversity Institute. This manuscript was written while the first author worked at ORDC, and was subsequently first submitted while he worked at the Agricultural Research Council, South Africa.

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Table S1. Strains used for phylogenetic analyses.

Table S2. Metadata related to the phylogenetic analysis of sect. *Fasciculata*.