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

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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.

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), resulting 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. graminicasei 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, Dellings 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 (DYPa), saubouraud 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 (CoM) and RNA polymerase II second 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 PartitionFinder v. 2.1.1 (Lanfear et al. 2017) using the corrected Akaikie information criterion (Akaikie 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), MEAbI, 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. Colony 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 “impainting 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.

Extrtolites

For extrtolite 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 extrtolites were determined with Xcalibur® software using accurate mass measurements (< 0.3 ppm) and manually verified by isotopic pattern. The chemical formulae were then searched against microbial extrtolite 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 | Dorchester     | Dorchester Mine   | Spider (Meta ovalis) | n/a       | MG490906 | n/a   | n/a   |
|                       |               | KAS 7511, S11101 | 18-Mar-2013 | DPYA             | New Brunswick | Sussex         | Grotte à la Patate | Gnat (Exechiopsis sp) | n/a       | MG490924 | n/a   | n/a   |
|                       |               | KAS 7517, S50103 | 11-Apr-2014 | DPYA             | New Brunswick | Sussex         | Grotte à la Patate | Harvestman (Nelima elegans) | n/a       | MG490929 | n/a   | n/a   |
|                       |               | KAS 7522, H27101 | 11-Apr-2014 | DPYA             | New Brunswick | Sussex         | Grotte à la Patate | Harvestman (Nelima elegans) | n/a       | MG490933 | n/a   | n/a   |
|                       |               | KAS 7523, H26208 | 11-Apr-2014 | SD               | New Brunswick | Sussex         | Grotte à la Patate | Harvestman (Nelima elegans) | n/a       | MG490934 | n/a   | n/a   |
|                       |               | DAOMC 252099, KAS 7525, H90108 | 18-Mar-2013 | DPYA             | New Brunswick | Dorchester     | Dorchester Mine   | Rodent (Peromyscus maniculatus) | MG490876 | MG490926 | MG490966 | 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 dung (Peromyscus maniculatus) | MG490887 | MG490926 | MG490966 | n/a   |
|                       |               | DAOMC 252101, KAS 7520, M26108 | 16-Apr-2013 | DPYA             | New Brunswick | Sussex         | Dallings Cave     | Moth (Scoliopteryx bilatic) | 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   | DAOMC 252108, KAS 7459, W98105 | 16-Apr-2015 | DPYA             | New Brunswick | Sussex         | Grotte à la Patate | 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   |
| Species          | Section   | Strain                        | Date collected | Province          | Location     | Cave name            | Substrate         | ITS       | BenA  | CaM  | RPB2 |
|------------------|-----------|-------------------------------|----------------|-------------------|--------------|----------------------|-------------------|-----------|-------|------|------|
| P. consobrinum   | Exilicaulis | KAS 7478, W29203             | 30-Apr-2015    | SD                | New Brunswick | Moncton              | Cave wall         | n/a       | MG490904 | n/a  | n/a   |
|                  |           | DAOMC 252109, KAS 7479, W24103A | 30-Apr-2015    | DPYA              | New Brunswick | Moncton              | Cave wall         | n/a       | MG490905 | n/a  | n/a   |
|                  |           | DAOMC 2521110, KAS 7483, W22102A | 30-Apr-2015    | DPYA              | New Brunswick | Moncton              | Cave wall         | n/a       | MG490908 | n/a  | n/a   |
|                  |           | KAS 7486, W20048              | 30-Apr-2015    | DPYA              | New Brunswick | Moncton              | Cave wall         | n/a       | MG490910 | n/a  | n/a   |
|                  |           | KAS 7513, P06102              | 14-Mar-2014    | DPYA              | New Brunswick | Dorchester           | Rodent (Peromyscus maniculatus) | n/a       | MG490925 | n/a  | n/a   |
|                  |           | KAS 7515, P05201              | 14-Mar-2014    | DPYA              | New Brunswick | Dorchester           | Rodent (Peromyscus maniculatus) | n/a       | MG490927 | n/a  | n/a   |
|                  |           | KAS 7532, D3301              | 25-Mar-2014    | DPYA              | New Brunswick | Dorchester           | Rodent dung (Peromyscus maniculatus) | n/a       | MG490939 | n/a  | n/a   |
| P. corylophilum  | Exilicaulis | KAS 7535, D2111              | 21-Mar-2014    | DPYA              | New Brunswick | Dorchester           | Rodent dung (Peromyscus maniculatus) | n/a       | MG490942 | n/a  | n/a   |
|                  |           | KAS 7536, D1204              | 21-Mar-2014    | DPYA              | New Brunswick | Dorchester           | Rodent dung (Peromyscus maniculatus) | n/a       | MG490943 | n/a  | n/a   |
|                  |           | KAS 7537, D1106              | 21-Mar-2014    | DPYA              | New Brunswick | Dorchester           | Rodent dung (Peromyscus maniculatus) | n/a       | MG490944 | n/a  | n/a   |
| P. expansum      | Penicillium | DAOMC 252111, KAS 7464, W76401 | 31-Mar-2015    | DPYA              | New Brunswick | Dorchester           | Cave wall         | MG490873 | MG490895 | MG490963 | n/a |
|                  |           | DAOMC 252112, KAS 7491, W19102 | 30-Apr-2015    | DPYA              | New Brunswick | Moncton              | Cave wall         | MG490874 | MG490913 | MG490964 | n/a |
|                  |           | DAOMC 252113, KAS 7481, W24100 | 30-Apr-2015    | DPYA              | New Brunswick | Moncton              | Cave wall         | MG490907 | n/a    | n/a   | n/a   |
|                  |           | DAOMC 252114, KAS 7484, W22102 | 30-Apr-2015    | DPYA              | New Brunswick | Moncton              | Cave wall         | MG490909 | n/a    | n/a   | n/a   |
|                  |           | KAS 7489, W20103             | 30-Apr-2015    | DPYA              | New Brunswick | Moncton              | Cave wall         | MG490912 | n/a    | n/a   | n/a   |
|                  |           | KAS 7493, W16200B            | 30-Apr-2015    | SD                | New Brunswick | Moncton              | Cave wall         | MG490914 | n/a    | n/a   | n/a   |
|                  |           | DAOMC 252115, KAS 7501, W07104 | 21-Apr-2015    | DPYA              | New Brunswick | Hillsborough         | Cave wall         | MG490917 | n/a    | n/a   | n/a   |
|                  |           | KAS 7502, W05406             | 21-Apr-2015    | DPYA              | New Brunswick | Hillsborough         | Cave wall         | n/a       | MG490918 | n/a  | n/a   |
|                  |           | KAS 7506, W04407             | 21-Apr-2015    | DPYA              | New Brunswick | Hillsborough         | Cave wall         | n/a       | MG490920 | n/a  | n/a   |
|                  |           | KAS 7510, W02007             | 21-Apr-2015    | DPYA              | New Brunswick | Hillsborough         | Cave wall         | n/a       | MG490923 | n/a  | n/a   |
|                  |           | DAOMC 252116, KAS 7519, M26109 | 16-Apr-2013    | DPYA              | New Brunswick | Sussex               | Dallings Cave     | MG9031    | n/a    | n/a   | n/a   |
|                  |           |                                |                |                   |              |                      |                  |           |        |      |      |
Table 1. (Continued).

| Species         | Section     | Strain                     | Date collected | Isolation medium | Province | Location          | Cave name                | Substrate             | ITS                  | BenA     | CaM     | RPB2 |
|-----------------|-------------|----------------------------|----------------|------------------|----------|-------------------|--------------------------|-----------------------|----------------------|----------|---------|-------|
| *Penicillium*   |             | 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   |
|                 |             | KAS 7524, H11101           | 18-Mar-2013    | DPYA             | New Brunswick | Dorchester        | Dorchester Mine          | Harvestman (Nelima elegans) | n/a                  | MG490935 | n/a     | n/a   |
| *P. glaucolbidum* | Thysanophora | DAOMC 252119, KAS 7460, W88411 | 31-Mar-2015 | DPYA             | New Brunswick | Dorchester        | Dorchester Mine          | Cave wall              | MG490870 | MG490891 | MG490960 |
|                 |             | KAS 7462, W88405           | 31-Mar-2015    | DPYA             | New Brunswick | Dorchester        | Dorchester Mine          | Cave wall              | MG490871 | MG490892 | MG490961 |
|                 |             | KAS 7425, W16200A          | 16-Apr-2015    | SD               | New Brunswick | Sussex            | Glebe Mine                | Cave wall              | MG490872 | MG490893 | MG490962 |
| *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   |
| *P. spathulatum* | Brevicompacta | DAOMC 251696, KAS 7473, W54119 | 07-Jul-2014 | DPYA             | Quebec       | Anticosti Island  | Grotte à la Patate       | Cave wall              | MG490864 | MG490884 | MG490954 |
|                 |             | KAS 7509, W02040           | 16-Apr-2015    | DPYA             | New Brunswick | Sussex            | Glebe Mine                | Cave wall              | n/a                  | MG490916 | n/a     | n/a   |
|                 |             | KAS 7425, W16200A          | 16-Apr-2015    | SD               | New Brunswick | Sussex            | Fredericton parking garage | Cave wall              | n/a                  | MG490922 | 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              | MG490865 | MG490885 | MG490955 |
|                 |             | DAOMC 251697, KAS 7474, W54102 | 07-Jul-2014 | DPYA             | Quebec       | Anticosti Island  | Grotte à la Patate       | Cave wall              | MG490866 | MG490886 | MG490956 |
|                 |             | DAOMC 251698, KAS 7500, W07302 | 21-Apr-2015 | MEA              | New Brunswick | Hillsborough      | White Cave                | Cave wall              | MG490867 | MG490887 | MG490957 |
|                 |             | DAOMC 251699, KAS 7503, W05404 | 21-Apr-2015 | DPYA             | New Brunswick | Hillsborough      | White Cave                | Cave wall              | MG490868 | MG490888 | MG490958 |
|                 |             | DAOMC 251700, KAS 7504, W05202 | 16-Apr-2015 | SD               | New Brunswick | Sussex            | Glebe Mine                | Cave wall              | MG490868 | MG490888 | MG490958 |

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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. glaucoaibidum* (n = 4), *P. rubens* (n = 4), *P. pithathulum* (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 2375 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* TN+R3; ITS TIM2e+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\(^2\) within the broad concept applied to *P. speluncae*.

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**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 \(^\dagger\). 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*. 

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**Table 1.** (Continued).
Penicillium diversity in bat caves

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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 *Penicillum* (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. marinium* (Frisvad *et al.* 2004). One of the major chaetoglobosins produced by these isolates is a newly described natural product, tetrahydrochaetoglobosin (Frisvad & Filtenborg 1989). One of the major chaetoglobosins produced by these isolates is a newly described natural product, tetrahydrochaetoglobosin.

*Fig. 1. (Continued).*

*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 †. Strains of the new species characterised based on morphology and extrolites is indicated by bold blue text. Trees were rooted to Penicillium robosamsonia.*
(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 

denovo

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, RP2B = 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.

| Extrolite name          | Formula   | m/z [M+H]+    | RT | % strains producing |
|-------------------------|-----------|---------------|----|---------------------|
| cyclopenin              | C₂₈H₂₈N₁₀O₅ | 295.1076      | 3.03 | 86 %                |
| cyclopenol              | C₂₈H₂₈N₁₀O₅ | 311.1025      | 2.69 | 86 %                |
| cyclopeptine            | C₂₈H₂₈N₁₀O₅ | 281.1285      | 3.11 | 100 %               |
| dehydrocyclopeptine     | C₂₈H₂₈N₁₀O₅ | 279.1130      | 3.17 | 86 %                |
| viridicasin             | C₂₈H₂₈N₁₀O₅ | 238.0865      | 3.36 | 100 %               |
| viridicatox             | C₂₈H₂₈N₁₀O₅ | 254.0812      | 2.95 | 100 %               |
| chaetoglobosin F        | C₂₈H₂₈N₁₀O₅ | 531.2852      | 3.54 | 100 %               |
| tetrahydrochaetoglobosin A | C₂₈H₂₈N₁₀O₅ | 533.3009      | 3.27 | 100 %               |
| chaetoglobosin A        | C₂₈H₂₈N₁₀O₅ | 531.2852      | 3.63 | 100 %               |
| chaetoglobosin C        | C₂₈H₂₈N₁₀O₅ | 529.2698      | 3.78 | 57 %                |
| prochaetoglobosin I     | C₂₈H₂₈N₁₀O₅ | 483.3005      | 4.41 | 100 %               |
| cyclo(VP)               | C₂₈H₂₈N₁₀O₅ | 197.1286      | 2.32 | 100 %               |
| cyclo(LP)               | C₂₈H₂₈N₁₀O₅ | 211.1441      | 2.50 | 100 %               |
| cyclo(IP)               | C₂₈H₂₈N₁₀O₅ | 211.1443      | 2.55 | 100 %               |
| cyclo(FP)               | C₂₈H₂₈N₁₀O₅ | 245.1285      | 2.62 | 100 %               |
| fungisporin             | C₂₈H₂₈N₁₀O₅ | 493.2809      | 3.77 | 100 %               |
| cyclo(Phe-Val-Phe-Val)  | C₂₈H₂₈N₁₀O₅ | 493.2809      | 3.55 | 100 %               |
| Val-Phe-Val-Phe         | C₂₈H₂₈N₁₀O₅ | 511.2919      | 2.87 | 100 %               |
| cyclo(Phe-Phe-Val-Ile)  | C₂₈H₂₈N₁₀O₅ | 507.2360      | 4.00 | 100 %               |
| Phe-Val-Ile-Phe         | C₂₈H₂₈N₁₀O₅ | 525.3074      | 2.96 | 100 %               |
| cyclo(Phe-Tyr-Val-Val)  | C₂₈H₂₈N₁₀O₅ | 509.2761      | 3.42 | 100 %               |
| Phe-Val-Tyr             | C₂₈H₂₈N₁₀O₅ | 527.2866      | 2.68 | 100 %               |
| Phe-Ile-Val-Tyr         | C₂₈H₂₈N₁₀O₅ | 541.3022      | 2.74 | 100 %               |
| cyclo(Phe-Trp-Val-Val)  | C₂₈H₂₈N₁₀O₅ | 532.2914      | 3.68 | 100 %               |
| Phe-Val-Trp             | C₂₈H₂₈N₁₀O₅ | 550.3024      | 2.89 | 100 %               |
| cyclo(Tyr-Trp-Val-Val)  | C₂₈H₂₈N₁₀O₅ | 548.2866      | 3.39 | 100 %               |
| Tyr-Val-Trp             | C₂₈H₂₈N₁₀O₅ | 566.2973      | 2.68 | 100 %               |
| cyclo(Trp-Trp-Val-Val)  | C₂₈H₂₈N₁₀O₅ | 571.3025      | 3.62 | 100 %               |

Colonie 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 (48B), 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 (48B), 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.
Penicillium diversity in bat caves

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Penicillium speluncae

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 (9.6±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 length to width = 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. 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.

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**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.

| Table 3. Distinguishing features of species closely related to *Penicillium speluncae*. |
| Conidia | CVA texture | YES soluble pigment | CVA reverse | YES reverse |
| --- | --- | --- | --- | --- |
| *P. speluncae* | Smooth, broadly ellipsoidal | Velutinous to fasciculate | Brilliant red diffusible colour on YES | Orange yellow to orange |
| *P. chatrasum* | Smooth, globose to subglobose | Velutinous to weakly fasciculate, becoming crustose | Orange turning into deep red with age | Strongly yellow |
| *P. frequentans* | Rough, globose to subglobose | Velutinous to weakly fasciculate | None | Yellow |
| *P. echinulatum* | Smooth, globose to subglobose | Strongly yellow | Orange turning into deep red with age | Strongly yellow |
| *P. solitum* | Smooth to finely rough, globose | Brilliance red diffusible colour on YES | None | Yellow to orange |

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Table S1. Strains used for phylogenetic analyses.

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