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Fungi associated with hibernating bats in New Brunswick caves: the genus *Leuconeurospora*

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Abstract: Two species of *Leuconeurospora*, *L. capsici* and *L. polypaeciloides* (Ascomycota: Pseudeurotiaceae), are reported from the fur and skin of hibernating bats and from other substrata in caves in New Brunswick, Canada. Separate analyses using ITS, *RPB1* and *RPB2* DNA sequence data are in agreement and show these two species and the type species, *L. pulcherrima* form discrete clades within a distinct *Leuconeurospora* clade. The three species are distinguishable morphologically by their anamorphs, having dark conidia in *L. capsici*, hyaline conidia in *L. polypaeciloides* and no conidia in *L. pulcherrima*. Ascomata and ascospores are produced in *L. pulcherrima* and in mated isolates of *L. polypaeciloides*, but have not been observed in *L. capsici*. *Leuconeurospora* species are psychrotolerant, with faster growth and heavier conidial development at 7 C than at 22 C. Of 151 bats sampled from 10 caves, 51 yielded isolates of *L. polypaeciloides* and 15 yielded *L. capsici*. The results were not uniform: neither species was isolated from bats in 3 caves, while 3 caves yielded isolates of both species and 2 yielded *L. polypaeciloides* only. These species were also isolated from cave walls and arthropods in the cave.

Key words: bats, caves, hibernating, phylogeny, mating

INTRODUCTION

Caves have long interested mycologists because of their ecological simplicity. In most fungal habitats plants, living and dead, are major sources of nutrients. In contrast, caves are notable for their paucity of plant materials, requiring resident fungi to utilize other substrates. Vanderwolf et al. (2013b) identified several substrate preference categories among cave fungi in New Brunswick, including keratinophily, coprophily, entomophily and mycoparasitism, as well as the utilization the small amounts of plant material present such as mine timbers and penetrating mycorrhizal roots. These authors also noted that the New Brunswick cave environment favors communities of oligotrophic, psychrotolerant fungi.

The apparent recent introduction of the fungus *Pseudogymnoascus destructans* (Blehert & Gargas) Minnis & D.L. Lindner to North America and the lethal impact of white nose syndrome (WNS) on over-wintering bats has focussed attention on the dearth of information regarding fungi in cave habitats (Vanderwolf et al. 2013a), especially those fungi associated with hibernating bats. In our efforts to address this lack of knowledge in New Brunswick, Canada, we have surveyed hibernating bats and associated cave substrata over several years. Our results yielded a number of the fungi belonging to infrequently reported or undescribed taxa (Vanderwolf et al. 2013b). Among these are two species of the genus *Leuconeurospora* Malloch & Cain (Ascomycota, Pseudeurotiaceae) that have been recovered repeatedly. Similarly, surveys of fungi associated with bats in caves and mines in the United States have identified *Leuconeurospora* species as components of the fungal biota (Minnis and Lindner 2013; Lorch et al. 2013; Zhang et al. 2014). *Leuconeurospora* species have not attracted much attention from mycologists in the past, either because their habitats have not been investigated frequently or because the methods used for their isolation have not been appropriate. Kochkina et al. (2014) recorded *Leuconeurospora* species from Antarctic sediments using conventional microbiological methods and metagenomic analysis.

Members of the genus *Leuconeurospora* are cleistothecial ascomycetes with asci and ascospores occurring in the centrum without obvious orientation. Anamorphs, when present, are represented by dichotomously branched conidiogenous structures bearing chains of conidia on apically elongating annellides. Here we describe the phylogeny, mating systems, cultural features and substrate relationships of *L. capsici* (J.F.H. Beyma) Malloch, Sigler & Hambleton and *L. polypaecioides* Malloch, Sigler & Hambleton (Malloch 2013).

MATERIALS AND METHODS

Isolation and characterization of strains

The 10 collection sites (Table 1) included natural solution caves in limestone and gypsum, and abandoned manganese mines, all populated by over-wintering populations of the bats *Myotis lucifugus*, *M. septentrionalis* and small numbers of *Perimyotis subflavus*. Annual temperature regimes in the dark zones of these sites averaged 6.0 ± 1.3 C and ranged from 2.6 – 14.1 C (Vanderwolf et al. 2012). The sites are characterised by high humidity (RH>85%), and vary in length from 74 m (Harbell's Cave) to 332 m (Berryton Cave).

Following the procedures outlined by Vanderwolf et al. (2013b) hibernating bats were swabbed while roosting. The swab was then immediately streaked across duplicate plates of dextrose-peptone-yeast extract agar (DPYA) (Papavizas and Davey, 1959) and Sabouraud dextrose agar (Hawksworth et al. 1995), both containing 30 m/l each chlortetramycin and streptomycin. DPYA also contained the growth inhibitors oxgall (5 g/l) and sodium propionate (1 g/l). Resulting colonies were subcultured on DPYA without the antimicrobial components. All cultures were incubated at 7 C in the dark.

Fungal samplings were carried out in 2010, before WNS or *Pseudogymnoascus destructans* was found in New Brunswick and in 2012, after WNS had arrived in the province (Table 2). In 2010, 10 *Myotis* spp. were sampled for fungi in each of 8 hibernacula. In 2012, 10 *Myotis* spp. were sampled in each of the 5 hibernacula, and 15 *Perimyotis subflavus* were sampled in 3 hibernacula (Tables 2, 3). In 2013, 6 *Perimyotis subflavus* were sampled in 3 hibernacula (Table 3).

In 2012 and 2013 substrates other than bats were sampled to determine whether species of *Leuconeurospora* occurred in the wider cave environment (Table 4). Two additional localities, Dalling's Cave and Dorchester Mine, were also sampled. The substrates included the walls of the hibernacula and arthropods within hibernacula that might serve as vectors of fungi. The hibernaculum walls were swabbed and streaked in the same way the bats were sampled. Arthropods, whole or in part, were placed directly on the isolation media.

Ten isolates determined as *L. capsici* and 17 isolates determined as *L. polypaeciloides* were accessioned in the University of Alberta Microfungus Collection and Herbarium (UAMH; <https://www.uamh.ca>), Edmonton, Alberta, Canada (Table 5). Cultural features were recorded for selected isolates following growth on DPYA and on malt extract agar (MEA) with peptone (Bills and Foster 2004) for 3 weeks at 7 C and 22 C and compared with the ex-type strains of *Scopulariopsis capsici*, *Torula botryoides* and *Polypaecilum* strains from other sources (Table 5). Measurements of colony diameters represent variation across all the strains. Colony color designations are according to the system of Kornerup and Wanscher (1978). Microscopic structures were

measured in water mounts.

When infertile ascomata were observed in old cultures of several *L. polypaeciloides* strains, mating studies were performed on 10 isolates following the procedures of Gibas et al. (2002). A conidial inoculum of one isolate was streaked across the center of an oatmeal salts agar plate (recipe at <https://www.uamh.ca/~media/uamh/OrderCultures/Documents/Media.pdf>). A second isolate was streaked perpendicularly to the first. Self-self pairings were performed in the same manner. Plates were incubated at 18 C in the dark and examined weekly for 2 months, then monthly. Final results were recorded after 6 months.

Sequencing and phylogenetic analysis

DNA sequences were newly obtained for 31 fungi (Tables 5, 6). Sequences for the ITS (internal transcribed spacer) region of rDNA and two protein coding genes, the largest subunit (*RPB1*) and the second largest subunit (*RPB2*) of RNA polymerase II were determined using genomic DNA extracted from mycelium grown on potato dextrose agar (PDA, BD - Diagnostic Systems, Sparks, Maryland) with an UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Solana Beach, California) or the manufacturer's recommended kit for a Thermo Scientific KingFisher ML magnetic particle processor (VWR, Mississauga, Ontario). PCR was performed using the ITS primer pairs ITS5 / ITS4 (White et al. 1990) and the RNA polymerase II primers *RPB1*-Ac / *RPB1*-CR and *fRPB2*-5F / *fRPB2*-7cR (Liu et al 1999) in 10 µl reactions containing 1µL of DNA, 0.5µL of 2mM dNTPs (Invitrogen Canada Inc. Burlington, Ontario), 0.04 µL of 20µM of each primer, 0.1 µl of 50× Titanium Taq DNA Polymerase and 1µL of 10× Titanium Taq buffer (BD Biosciences, Mississauga, Ontario), and 7.3 µL of S-HPLC H2O, with the following thermal cycling conditions: 95 C for 3 min followed by 40 cycles of 95 C for 1 min, 58 C for 90 sec, 72 C for 2 min, followed by a final extension of 72 C for 8 min. PCR products were direct-sequenced using the BigDye Terminator v. 3.1 Cycle Sequencing Reaction Kit (ABI Prism/ Applied Biosystems, Streetsville, Ontario) and the PCR primers. The reaction mix included 1.75 µl 5× Buffer, 0.5 µl 2.5x BDT sequencing Mix and 1.6 pmol primer. Amplicons were purified by ethanol/sodium acetate precipitation and analyzed by an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Streetsville, Ontario). Sequences were edited using Sequencher version 5 software (Gene Codes Corporation, Ann Arbor, Michigan).

ITS, *RPB1* and *RPB2* data matrices were constructed comprising the new sequences, together with representatives of the closest relatives based on published phylogenies or current BLASTN searches. Compiled sequences were submitted for alignment to the web-based utility MAFFT version 7 (<http://mafft.cbrc.jp/alignment/server/>) using the iterative refinement strategies Q-INS-i (ITS), in which RNA

secondary structure is considered (Kato and Toh 2008), or G-INS-i (*RPB1* and *RPB2*), recommended for <200 sequences with global homology (Kato et al. 2009).

Parsimony analyses were performed using PAUP software (version 4.0b10; Sinauer Associates, Inc., Sunderland Massachusetts [<http://paup.csit.fsu.edu/>]) using the full heuristic search option and 1000 replicates of random sequence addition with gaps treated as missing data. The bootstrap (BS) percentages were determined from 100000 resamplings of the data set and random sequence addition with the number of rearrangements per replicate limited to 1M. Bayesian analysis was performed using MrBayes software version 3.2 (Department of Scientific Computing, Florida State University, Tallahassee, Florida [see <http://mrbayes.csit.fsu.edu/download.php>]). Evolutionary models for each locus were determined using jModelTest 2.1.1 (Darriba et al. 2012) and ranked based on the Bayesian Information Criterion (BIC). All matrices were run using the K2P + gamma evolutionary model with two independent runs using four chains each of 100,000,000 generations, with sampling frequency every 2000 generations. The Bayesian consensus tree was directly generated after 25% burn-in. Clades that were supported by posterior probability (PP) values ≥ 85 and BS values $\geq 75\%$ were recorded.

Raw pairwise distances within the genus *Leuconeurospora* were calculated from the aligned data matrices using the 'ape' package (Paradis et al. 2004) for R (R CORE Team 2014) and distributions of raw pairwise intra- and inter-species distances for each gene and each strain pair were plotted and overlaid using the function ggplot2 (Wickham 2009).

RESULTS

Both *L. capsici* and *L. polypaeciloides* were commonly isolated from skin and fur of hibernating bats and other substrate samples from mines and caves (Tables 2, 3, 4, 5). The genus *Leuconeurospora* was considered by Vanderwolf et al. (2013b) to be among the eight genera of "core fungi" isolated from the skin and fur of bats in pre-WNS New Brunswick. The other genera in this group (*Pseudogymnoascus* [as *Geomyces*], *Penicillium*, *Mortierella*, *Mucor*, *Cephalotrichum*, *Cladosporium*, *Trichosporon*) are all represented by well-known species occurring in a great variety of habitats, underlining the rather special and perhaps more restricted ecology of *Leuconeurospora* species.

Mating

Fertile ascomata were obtained among 7 isolates of *L. polypaeciloides* when mated on oatmeal salts agar. Ascomata were commonly produced deep within the agar and ascospore production required up to 5 months incubation. UAMH 11459 (designated + mating type strain) produced fertile ascomata with UAMH 11251

(designated – mating type strain) and UAMH 11250, 11504 and 11513 (other – mating strains). Two other strains (UAMH 11622 and 11639 designated + mating strains) mated with – mating strains UAMH 11251 and 11250 respectively. Infertile ascomata were commonly produced by UAMH 11513 but no ascospores were observed in self-self crosses.

Cultural features

Asexual reproduction in *L. capsici* and *L. polypaecioides* occurs by means of percurrently proliferating conidiogenous cells marked with closely-spaced annellations (Figs. 1, 2). Fertile hyphae are hyaline, smooth, nearly coenocytic to sparsely septate, 25-125 X 1.7-6.7 μm , dichotomously branched at the apex and terminated by the conidiogenous cells. Conidiogenous cells are nearly cylindrical to slightly swollen below, produced in pairs on the supporting branch and not separated from it by a septum, initially only 5-6 μm in length but gradually elongating to 50 μm or more, 1.7-5.0 μm in diameter. The species differ slightly in the color of the conidia (Figs. 1, 2), in the production of ascomata (Fig. 3) and in their growth responses at different temperatures (Fig. 4). *L. polypaecioides* is heterothallic, producing fertile cleistothecia between compatible mating strains and occasionally producing sterile cleistothecia when unmated.

Colony diameters of *L. capsici* after three weeks were 30-37 mm at 7 C and 13-32 mm at 22 C on DPYA and 28-36 mm at 7 C and 12-26 mm at 22 C on MEA (Fig. 4C, D). Conidial sporulation was heavy at 7 C and 22 C (except for UAMH 11252 which was sterile at 7 C on DPYA. Sporulation was absent at 7 C and slight to absent at 22 C on MEA. Conidia were obovate to obpyriform, truncate at the point of attachment, often notably broadest above the middle and appearing subtruncate at the apex (Fig. 1), Conidia were Grayish green to Dark green (28EF4) in mass and by transmitted light, smooth, 7.4-9.3 X 5.5-9.2 μm ($x = 8.4 \times 6.9$), $D/d = 0.96-1.45$ ($x = 1.23$). Ascomata were not seen.

Colonies of *L. polypaecioides* after three weeks were 30-40 mm at 7 C and 17-40mm on DYPA at 22 C; and 34-40 mm at 7 C and 11-30 mm on MEA at 22 C (Fig. 4). Conidial production was heavy at 7 C and sparse to light at 22 C on DPYA but slight to absent at both temperatures on MEA. Conidia were obovate, truncate at the point of attachment, hyaline in mass and by transmitted light, smooth, 6.3-10.1 X 4.9-6.6 μm ($x = 8.4 \times 5.9$), $D/d = 1.24-1.53$ ($x = 1.42$) (Fig. 2). Cleistothecia were spherical, glabrous or with undifferentiated hyphal attachment, reddish brown by transmitted light and black by reflected light, with a conspicuously cephalothecoid peridium (Fig. 3). The peridium was composed of dehiscent plates of cells 25-50 μm in diameter, with individual cells nearly isometric at the centre of the plates and elongated to 16 μm toward the periphery, 2.5-9.0 μm in diameter. The ascogenous system filled the centrum and was composed of dichotomously branched hyphae

4.0-6.8 μm in diameter, bearing asci at the tips of the branches. Asci were spherical to subspherical, 10.8-16.7 μm in diameter, thin-walled, 8-spored, evanescent, releasing the ascospores into the centrum. Ascospores were ellipsoidal to fusoid, 4.9-6.7 X 3.5-4.1 μm ($x = 5.9 \times 3.8 \mu\text{m}$), $D/d = 1.29-1.73$ ($x = 1.55$), appearing rather pointed at the poles, hyaline, and marked by several low anastomosing ridges up to 1 μm high forming a loose network. Arthroconidia were produced mainly below the surface of the medium and frequently in association with the ascomata (Fig. 3). They were thallic, with schizolytic dehiscence, hyaline, cylindrical, smooth, measuring 6.6-12.7 X 3.6-4.6 μm ($x = 8.3 \times 4.0 \mu\text{m}$), $D/d = 1.81-3.46$ ($x = 2.11$), occasionally produced as subdivisions of the initial conidia and then having an aspect ratio ≤ 1.0 .

Although the growth rates of *L. capsici* and *L. polypaecioides* do not appear to differ greatly between those grown at 7 C and those at 22 C there are quite noticeable differences as seen in Fig. 4. In the four isolates illustrated, colonies grown at 7 C are larger and have more luxuriant aerial growth than those at 22 C. The one exception is *L. capsici* UAMH 11252, in the upper right quadrant, which, although slower, appears to have sporulated more abundantly.

Phylogenetic analysis.—The new DNA sequences determined for this study for each gene region are listed in Tables 5 and 6 with GenBank accession numbers. The isolates sampled included 15 from bats, three morphologically similar isolates, the type species of *Leuconeurospora*, and representatives of other genera hypothesized to be related based on current BLASTN searches and published phylogenies for those taxa. The ITS data matrix comprised 30 taxa and 529 characters of which 102 were parsimony informative. The *RPB1* data matrix comprised 26 taxa and 698 characters of which 169 were parsimony informative. The *RPB2* data matrix comprised 26 taxa and 1078 characters of which 283 were parsimony informative. Maximum parsimony analyses were conducted independently for each alignment, resulting in 2 most parsimonious trees (MPT) of score 184 (ITS), 77 MPT of score 543 (*RPB1*), and 9366 MPT of score 783 (*RPB2*). Bayesian analyses conducted independently resulted in a single consensus tree for each data matrix. One MPT for each gene is shown in Fig. 5A (ITS), Fig. 6A (*RPB1*) and Fig. 6B (*RPB2*).

Phylogenetic analyses of the individual data sets yielded trees of similar topologies in which all the bat isolates, the ex-type strains of *Scopulariopsis capsici* and *Torula botryoides* and *L. pulcherrima* (CBS 343.76), grouped with strong support (PP/BS = 96/100 for ITS, 90/100 for *RPB1* and 100/100 for *RPB2*) as a monophyletic clade distinct from the other taxa (Figs. 5, 6). Within the *Leuconeurospora* clade, two subclades distinct from the one representative of *L. pulcherrima* were strongly supported except in the ITS only analysis. The subclade designated *Leuconeurospora capsici* included the type strains for *Scopulariopsis capsici* and

Torula botryoides (nomen illegitimum) and the ITS sequences were identical for all isolates except for UAMH 11458 which differed from all the others at one ITS2 position (417 in GenBank No. KJ55506, T instead of C). All isolates in the second subclade, designated *L. polypaeciloides*, had identical ITS sequences. Several strains within this subclade formed a *Leuconeurospora* teleomorph when paired *in vitro*. Relative to our 19 GenBank accessions for the genus, the ITS sequences of the two subclades differed at one ITS2 position (394) and both differed at two different positions from *L. pulcherrima* (116 and 452). The percent similarity among the three species for each gene, based on pairwise comparisons of data for one representative sequence from each subclade (UAMH 11251 and 11252) and for *L. pulcherrima* (CBS 343.76), was 99.4–99.8% (ITS), 96.1–97.2% (*RPB1*) and 96.9–97.3% (*RPB2*). The pairwise distances calculated for each gene for all strains in the genus *Leuconeurospora* clearly identifies a distinct gap between the intra- and inter-specific levels for *RPB1* and *RPB2* but not for ITS (Fig. 5B).

DISCUSSION

Recent molecular phylogenetic studies (Minnis and Lindner 2013, Sogonov et al. 2005, Wang et al. 2006) have supported the classification of the “residual” Pseudeurotiaceae (sensu Suh and Blackwell 1999) in the Leotiomycetes with a relatively small number of cleistothecial ascomycete genera as members, including *Leuconeurospora* and the 5 genera represented in the DNA sequence analyses of this study. Most comprise small numbers of species based on Index Fungorum databases (<http://www.indexfungorum.org/names/Names.asp> accessed January 26, 2015): *Connersia* (monotypic genus), *Pleuroascus* (6 species), *Pseudeurotium* (9 species), and the *Pseudogymnoascus/Geomyces* complex (approximately 15 species). The taxonomy database of NCBI currently lists records for many DNA sequences not identified to species under each of these genera (<http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=34379> accessed January 26, 2015), suggesting there is much diversity yet to be understood and evaluated for this lineage. Our multi-gene analyses provided strong support for the reclassification of *Scopulariopsis capsici* in *Leuconeurospora* and for the description of *L. polypaeciloides* as a new species (Malloch 2013). Our data do not support the transfer of *S. capsici* to the genus *Aspergillus* (family Trichocomaceae) as proposed by Samson et al. (2014). Both *L. capsici* and *L. polypaeciloides* have widespread distributions in bat hibernacula in New Brunswick, and it is likely that at least some of the unidentified sequences in NCBI are derived from representatives of these taxa. These species are not readily distinguished by ITS sequences alone since differences between them are few; however, more

confident identifications could be made if in combination with morphological characters. In contrast, based on the sequences generated in this study, data for either of the protein coding genes would be diagnostic.

Leuconeurospora polypaecioides differs notably from the type species *L. pulcherrima* in its conspicuous anamorph. *Leuconeurospora pulcherrima* was first isolated from rabbit and dog dung in Denmark by Emil Chr. Hansen (Hansen, 1876). Georg Winter found additional material on fox dung near Leipzig, Germany (Winter, 1887). It is not clear from either of those publications whether those authors ever saw the other's material. Significantly, Hansen stated that he could find no conidia. A culture from forest soil in Switzerland was deposited at the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands as CBS 343.76 (Table 5). This material, assumed to be conspecific with *L. pulcherrima*, was studied by Suh and Blackwell (1999) who made no mention of conidia. One of us (L.S.) followed the development of this isolate (UAMH 9397 = CBS 343.76) over a period of five to seven months incubation at 9 C and saw no evidence of an anamorph. These cultures produced sparse infertile cleistothecia, suggesting the species might also be heterothallic.

Malloch and Cain (1970) described and illustrated material from Ontario and Alberta, Canada, thought to represent *L. pulcherrima*. Unfortunately they were unable to obtain cultures from either of these collections and did not have the opportunity to observe their development. Because they were unable to comment on conidial development it is unclear now whether they had *L. pulcherrima*, *L. polypaecioides* or some other species. Ascospores of *L. polypaecioides* and *L. pulcherrima* may differ slightly. Winter (1887) gave measurements of 5.0-6.5 X 4.0-5.0 µm for *L. pulcherrima*, differing from the 4.9-6.7 X 3.5-4.1 µm of *L. polypaecioides*, especially in their widths. Malloch and Cain's more variable measurements of 5.5-7.0 X 3.5-5.0 µm in their collections suggest that the ascospores of the two species may not differ greatly.

Isolates of *L. polypaecioides* were obtained from Howe's, Berryton and White Caves and from Markhamville and Glebe Mines, but never from Harbell's and Underground Lake Caves (Tables 2, 3). They were most abundant in Glebe Mine; not only on bats but also on flies, moths and spiders, and on the walls of the mine (Table 4). These additional isolates suggest that *L. polypaecioides* was sporulating heavily in this mine and that its ascospores and/or conidia had become widely distributed. All sites yielding isolates of *L. polypaecioides* showed evidence of vertebrates other than bats, particularly porcupines and raccoons. Porcupines have occupied Markhamville Mine and White Cave to the extent that there are large piles of feces on the floor. Although we have not yet studied the mycota of these piles we suspect that they will support *L. polypaecioides*. Interestingly, the species of *Leuconeurospora* reported by Malloch and Cain (1970) was found abundantly on porcupine dung in an Ontario cave.

The three caves not yielding isolates of *L. polypaecioides* on bats were all quite wet. Harbell's and Kitt's Caves have streams running along their main passages while Underground Lake Cave, as its name implies, is dominated by a relatively large body of water (Table 1). While these streams and ponds seem not to significantly alter the relative humidity of the caves they occupy, they possibly discourage or prevent the entry of most non-flying vertebrates and their associated fungi. Kitt's Cave, however, yielded a single isolate of *L. polypaecioides* from one of its walls (Table 4), suggesting that it may be only a less abundant component of the mycota in this cave. Kitt's Cave does have a beaver den, and McAlpine (1977) reported discovery of beaver scat in this cave.

Leuconeurospora capsici was less abundant on bats than *L. polypaecioides* (Tables 2, 3). However it was isolated several times from *Myotis* spp. in Berryton Cave, the hibernaculum in this study yielding the highest count of bats. It may also be significant that *L. capsici* was also isolated from a bat in this cave that had been infected with *P. destructans* and had died.

Leuconeurospora capsici was long accepted as a member of the genus *Polypaecilum*. It is listed by many authorities under the name *Polypaecilum botryoides* (F.T. Brooks & Handsf.) Vasant Rao & de Hoog, a combination that cannot be maintained because its basionym, *Torula botryoides* F.T. Brooks & Handsf., 1923 is a later homonym of *T. botryoides* Corda, 1829, an unrelated species. Houbraken and Samson (2011) showed the type of *Polypaecilum*, *P. insolitum* G. Sm., to be a member of the Aspergillaceae and therefore only very distantly related to *Leuconeurospora*, a member of the Pseudeurotiaceae (Suh and Blackwell, 1999).

Leuconeurospora capsici is easily distinguished from *L. polypaecioides* by its dark greenish brown conidia with characteristically broad and somewhat flattened apices. It is the only one of the three described species of *Leuconeurospora* not known to produce ascomata.

Recent surveys of bat hibernacula have yielded a diversity of fungi, many of which are psychrotolerant (Minnis and Lindner 2013, Vanderwolf et al. 2013a,b). The two *Leuconeurospora* species reported here grew well at the low temperatures found in the caves (averaging 6.0 ± 1.3 C and ranging from 2.6 – 14.1 C) and in culture at 7 C and 22 C. Our isolates were not tested at lower or higher temperatures because the study was focused more on tolerance to the low temperatures found in the hibernacula. Further evidence of psychrotolerance comes from the isolation of *Leuconeurospora* species from food in cold storage and from Antarctica (Table 5; Kochkina et al. 2014). Interestingly, the isolates of *Leuconeurospora* reported by Lorch et al. (2013) and Minnis and Lorch (2013) appear to represent species different from those reported here. A BLAST search of their RPB2 sequences did not yield matches to either *L. capsici* or *L. polypaecioides* while their ITS sequences appear to show their isolates to be in a separate clade within the *Leuconeurospora* clade. These

results suggest that there may be additional species of this interesting genus of cave-inhabiting fungi.

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Table 1. Sites of caves and mines sampled in New Brunswick, Canada.

Site name (abbreviation)	Nearest town	Geology	Presence of significant water body
Berryton Cave (Be)	Berryton	Limestone	No
Dalling's Cave (Da)	Waterford	Limestone	Yes
Dorchester Mine (Do)	Dorchester	Copper mine	Yes
Glebe Mine (Gl)	Waterford	Manganese mine	No
Harbell's Cave (Ha)	Saint John	Limestone	Yes
Howe's Cave (Ho)	Saint John	Limestone	No
Kitts Cave (Ki)	Hammondvale	Limestone	Yes
Markhamville Mine (Ma)	Markhamville	Manganese mine	No
Underground Lake Cave (Un)	Albert Mines	Gypsum	Yes
White Cave (Wh)	Hillsborough	Gypsum	No

Table 2. *Leuconeurospora* species isolated from surface swabs of 10 bats (*Myotis lucifugus*, *M. septentrionalis*) from eight sites.

	Site ^a								
2010	Be	Gl	Ha	Ho	Ki	Ma	Un	Wh	TOTAL
<i>L. capsici</i>	3 ^b	0	0	0	0	1	0	1	5/80
<i>L. polypaeciloides</i>	3	5	0	2	0	10	0	8	28/80
.....									
2012									
<i>L. capsici</i>	7	ns ^c	0	0	ns	1	ns	1	9/50
<i>L. polypaeciloides</i>	1	ns	0	0	ns	6	ns	3	10/50

^aSite names according to Table 1.
^bNumber of bats yielding isolates.
^cNot sampled due to lack of bats.

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Table 3. *Leuconeurospora* species isolated from surface swabs of *Perimyotis subflavus*.

	Site ^a and number of bats sampled									TOTAL
	Be	Da	Gl	Ha	Ho	Ki	Ma	Un	Wh	
2012	(0 ^b)	(0)	(7)	(1)	(0)	(0)	(7)	(0)	(0)	(15)
<i>L. capsici</i>	nd ^c	nd	0	0	nd	nd	1 ^d	nd	nd	1/15
<i>L. polypaeciloides</i>	nd	nd	6	0	nd	nd	2	nd	nd	8/15
2013	(0)	(1)	(2)	(0)	(0)	(0)	(3)	(0)	(0)	(6)
<i>L. capsici</i>	nd	0	0	nd	nd	nd	0	nd	nd	0/6
<i>L. polypaeciloides</i>	nd	0	2	nd	nd	nd	3	nd	nd	5/6

^aSite names according to Table 1.^bNo *Perimyotis subflavus* present.^cNo data.^dNumber of bats yielding isolates.

Table 4. *Leuconeurospora* species isolated from substrata including cave walls and arthropods.

Site ^a and substrates ^b sampled											
2012	Be	Be	Gl	Gl	Gl	Gl	Do	Do	Do	Do	Total
	(w/12 ^c)	(d/1)	(f/6)	(m/3)	(s/3)	(w/10)	(f/7)	(m/2)	(s/3)	(w/10)	(57)
<i>L. capsici</i>	6 ^d	1	0	0	0	0	0	0	0	0	7/57
<i>L. polypaeciloides</i>	0	0	5	1	3	6	1	0	0	0	16/57
2013	Gl	Gl	Ki	Ma	Ma	Da	Do	Do	Do		Total
	(s/2)	(h/2)	(w/7)	(m/2)	(h/1)	(m/4)	(h/6)	(m/1)	(s/4)		(29)
<i>L. capsici</i>	0	0	0	0	0	1	0	0	0		1/29
<i>L. polypaeciloides</i>	1	2	1	1	1	0	1	0	1		8/29

^aSite names according to Table 1. Note that the sites sampled in 2012 and 2013 differ.

^bSubstrates: d=cadaver of *Myotis lucifugus*, f=fly (*Exechiopsis* sp.), h=harvestman (*Nelima elegans*), m=moth (*Scoliopteryx libatrix*), s=spider (*Meta ovalis*), w=cave wall.

^cNumber of samples taken per substrate.

^dNumber of *Leuconeurospora* isolates obtained from the substrate.

Table 5. *Leuconeurospora* isolates examined in this study.

UAMH No. ^a	Other No.	GenBank No. ^d			Location	Substrate	Isolator(s)	Year	Other data
		ITS	RPB1	RPB2					
<i>Leuconeurospora capsici</i>									
935	CBS 176.44	KJ755509	KJ755457	KJ755483	Netherlands	<i>Capsicum annuum</i> “De Körver”	F.H. van Beyma		Type: <i>Scopulariopsis capsici</i>
11121		KJ755501	KJ755449	KJ755475	Un ^b	skin, bat	KJV & DFM ^c	2009	
11248	NBM F-03666	KJ755502	KJ755450	KJ755476	Wh	fur, female <i>Myotis lucifugus</i>	KJV & DFM	2010	
11249	NBM F-03622	KJ755503	KJ755451	KJ755477	Be	fur, female <i>Myotis lucifugus</i>	KJV & DFM	2010	
11252	CBS 143.23	KJ755504	KJ755452	KJ755478	England	halibut in cold storage	F.T. Brooks & C.G. Hansford	1918	Type: <i>Torula botryoides</i>
11253	CBS 122042	KJ755505	KJ755453	KJ755479	Antarctica	soil, Antarctica	A.M. Stchigel & W.P. MacCormack	1996	<i>Polypaecilum antarcticum</i> (unpubl)
11458	NBM F-03526	KJ755506	KJ755454	KJ755480	Ma	fur, male <i>Myotis lucifugus</i>	KJV & DFM	2010	
11460	NBM F-04960	KJ755507	KJ755455	KJ755481	Be	skin, male <i>Myotis lucifugus</i>	KJV & DFM	2010	
11463	NBM F-04959	KJ755508	KJ755456	KJ755482	Be	fur, male <i>Myotis lucifugus</i>	KJV & DFM	2010	
11721	NBM F-04948	nd ^d	nd	nd	Be	fur, <i>Myotis lucifugus</i>	KJV & DFM	2012	
11722		nd	nd	nd	Be	cave wall	KJV & DFM	2012	
<i>Leuconeurospora polypaeciloides</i>									
11250	NBM F-03557	KJ755510	KJ755458	KJ755484	GI	skin, female <i>Myotis lucifugus</i>	KJV & DFM	2010	– mating strain
11251	NBM F-04962	KC884265	KJ755459	KJ755485	Wh	fur, male <i>Myotis lucifugus</i>	KJV & DFM	2010	– mating type strain
11459		KC884266	KJ755460	KJ755486	Ma	skin, female <i>Myotis lucifugus</i>	KJV & DFM	2010	+ mating type strain
11461		KJ755512	KJ755461	KJ755487	Wh	skin, <i>Pipistrellus subflavus</i>	KJV & DFM	2010	
11504	NBM F-04957	KJ755513	KJ755462	KJ755488	Ma	fur, male <i>Myotis lucifugus</i>	KJV & DFM	2010	– mating strain
11512	NBM F-04953	KJ755514	KJ755463	KJ755489	Ho	fur, male <i>Myotis septentrionalis</i>	KJV & DFM	2010	
11513	NBM F-04954	KJ755515	KJ755464	KJ755490	Ma	fur, male <i>Myotis septentrionalis</i>	KJV & DFM	2010	– mating strain
11622	NBM F-04956	KJ755516	nd	nd	Ma	skin, male <i>Myotis lucifugus</i>	KJV & DFM	2010	+ mating strain

11639	NBM F-04952	KJ755517	nd	nd	Wh	skin, male <i>Myotis lucifugus</i>	KJV & DFM	2010	+ mating strain
11723		nd	nd	nd	Wh	fur, <i>Myotis lucifugus</i>	KJV & DFM	2012	
11724		nd	nd	nd	Wh	skin, <i>Myotis lucifugus</i>	KJV & DFM	2012	
11725		nd	nd	nd	Ma	fur, <i>Perimyotis subflavus</i>	KJV & DFM	2012	
11726		nd	nd	nd	Gl	herald moth, <i>Scoliopteryx libatrix</i>	KJV & DFM	2012	
11727		nd	nd	nd	Do	fly, <i>Exechiopsis sp.</i>	KJV & DFM	2012	
11728		nd	nd	nd	Gl	cave orb weaver, <i>Meta ovalis</i>	KJV & DFM	2012	
11729		nd	nd	nd	Gl	cave wall	KJV & DFM	2012	
11730		nd	nd	nd	Ma	fur, <i>Perimyotis subflavus</i>	KJV & DFM	2012	
<i>Leuconeurospora pulcherrima</i>									
9397	CBS 343.76	KJ755518	KJ75546 5	KJ755491	Switzerland	forest soil	E. Müller		

^aCollection acronyms: CBS – Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; NBM – New Brunswick Museum, Saint John, NB, Canada; UAMH – UAMH Centre for Global Microfungal Biodiversity, University of Toronto, Toronto, ON, Canada [formerly University of Alberta Microfungus Collection and Herbarium].

^bCollection site abbreviations according to Table 1.

^cKJV -- K.J. Vanderwolf; DFM – D.F. McAlpine

^dnd = not sequenced

Table 6. Additional strains used for phylogenetic analysis.

Name	Accession No. (Other No.)	GenBank No. ^b			Provenance (Type Status)
		ITS	RPB1	RPB2	
<i>Connersia rilstonii</i>	CBS 537.74 (DAOM 145387)	KJ755499	KJ755447	KJ755473	Ontario, Canada
<i>Geomyces asperulatus</i>	UAMH 183 (DAOM 231159)	KJ755500	KJ755448	KJ755474	Massachusetts, USA (Type)
<i>Pleuroascus nicholsonii</i>	CBS 345.73	KJ755519	nd	nd	California, USA (Neotype)

<i>Pseudeurotium hygrophilum</i>	UAMH 11527 (CBS 102670)	KJ755520	KJ755466	KJ755492	Karachai-Cherkess Republic, Russia (Type)
<i>Pseudeurotium ovale</i> var. <i>ovale</i>	UAMH 5825	KJ755521	nd	nd	Province De Liège, Belgium
<i>Pseudeurotium zonatum</i>	ATCC 62440 (CBS 480.86)	nd	nd	KJ755493	Newfoundland, Canada
<i>Pseudeurotium zonatum</i>	CBS 329.36	KJ755522	KJ755467	KJ755494	Versailles, France (Type)
<i>Pseudogymnoascus appendiculatus</i>	UAMH 10509	KJ755523	KJ755468	nd	Alberta, Canada (Type)
<i>Pseudogymnoascus roseus</i>	UAMH 1990	KJ755524	KJ755469	KJ755495	Wisconsin, USA
<i>Pseudogymnoascus verrucosus</i>	UAMH 10579	KJ755525	KJ755470	KJ755496	Alberta, Canada (Type)
<i>Pseudogymnoascus</i> sp.	UAMH 875	KJ755526	KJ755471	KJ755497	West Ongul Is., Antarctica (Type: <i>Chrysosporium verrucosum</i>)
<i>Pseudogymnoascus</i> sp.	UAMH 5339 (CBS 388.68)	KJ755527	KJ755472	KJ755498	Netherlands (Type: <i>Chrysosporium merdarium</i> var. <i>roseum</i>)

^aCollection acronyms: ATCC – American Type Culture Collection, Manassas, Virginia; USA; CBS – Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; DAOM – National Mycological Herbarium, Ottawa, Ontario, Canada; UAMH – UAMH Centre for Global Microfungal Biodiversity, University of Toronto, Toronto, ON, Canada.

^bnd = not sequenced

LEGENDS

Fig. 1. *Leuconeurospora capsici* UAMH 11460, conidiogenesis.

Fig. 2. *Leuconeurospora polypaeciloides* Vanderwolf 43SOc2, conidiogenesis.

Fig. 3. *Leuconeurospora polypaeciloides* UAMH 11459x11504, teleomorph and arthroconidia. (A) Cleistothecial peridium. (B) Asci. (C) Ascogenous hyphae. (D) Arthroconidia. (E) Ascospores. A: scale bar = 50 μ m, B-E: scale bar = 10 μ m.

Fig. 4. Colonies of *Leuconeurospora* spp. In 9 cm Petri dishes containing DPYA and MEA after 3 weeks. Top pair of each quadrant on DPYA at 7 C (left) and 22 C (right); bottom pair on MEA at 7 C (left) and 22 C (right). (A) *L. polypaeciloides* UAMH 11251. (B) *L. polypaeciloides* UAMH 11459. (C) *L. capsici* UAMH 11252. (D) *L. capsici* UAMH 11460.

Fig. 5. Phylogenetic relationships of three *Leuconeurospora* species and other members of the Pseudeurotiaceae inferred from parsimony analyses of ITS DNA sequences, and pairwise distances calculated for the genus *Leuconeurospora* for ITS, *RPB1* and *RPB2*. (A) One of 2 equally parsimonious trees inferred from parsimony analyses of ITS DNA sequences. Posterior probability values of $\geq 85\%$ followed by bootstrap values of $\geq 75\%$ and are shown above or beside the branches. T indicates an ex-type culture. For *L. polypaeciloides*, MT (mating type) or M (mating) indicates a positive (+) or negative (–) strain; the holotype was derived from pairing MT strains UAMH 11251 and UAMH 11459. (B) Distribution of intra- and inter-specific pairwise distances for all strains in the genus *Leuconeurospora*, for each gene region analyzed in this study. The average distance is represented by the dotted lines.

Fig. 6. Phylogenetic relationships of three *Leuconeurospora* species and other members of the Pseudeurotiaceae inferred from parsimony analyses of (A) *RPB1* and (B) *RPB2* DNA sequences, showing one of 77 and 9,366 equally parsimonious trees, respectively. Posterior probability values of $\geq 85\%$ followed by bootstrap values of $\geq 75\%$ and are shown above or beside the branches (the values internal to the *L. capsici* clade were omitted due to space limitations). T indicates an ex-type culture. For *L. polypaeciloides*, MT (mating type) or M (mating) indicates a positive (+) or negative (–) strain; the holotype was derived from pairing MT strains UAMH 11251 and UAMH 11459.

182x133mm (300 x 300 DPI)

182x133mm (300 x 300 DPI)

182x236mm (300 x 300 DPI)

182x182mm (300 x 300 DPI)

A Botany

B

A

B