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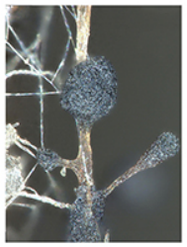

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Remarkable fungal biodiversity on northern Belgium bats and hibernacula

Pierre Becker[a], Claudia van den Eynde[b], Frederik Baert[a], Elizabet D'hooge[a], Robby De Pauw[c,d], Anne-Cécile Normand[e], Renaud Piarroux[e], and Dirk Stubbe[a]

[a]BCCM/IHEM Fungi Collection, Mycology and Aerobiology, Sciensano, Brussels 1050, Belgium; [b]Department of Enzootic, Vector-Borne and Bee Diseases, Sciensano, Brussels 1180, Belgium; [c]Department of Epidemiology and Public Health, Sciensano, Brussels 1070, Belgium; [d]Department of Rehabilitation Sciences, Ghent University, Ghent 9000, Belgium; [e]INSERM, Institut Pierre-Louis d'Epidémiologie et de Santé Publique, Assistance Publique-Hôpitaux de Paris (AP-HP), Groupe Hospitalier Pitié-Salpêtrière, Service de Parasitologie-Mycologie, Sorbonne Université, Paris 75013, France

ABSTRACT

Bats can be affected by fungal pathogens such as *Pseudogymnoascus destructans*, the causative agent of the white-nose syndrome. Their body surface can also be colonized by fungal commensals or carry transient fungal species and participate in their dispersal. In this study, 114 bat specimens belonging to seven species were sampled from various locations in northern Belgium. Culture-based methods revealed an important mycological diversity, with a total of 209 different taxa out of the 418 isolates. Overall, a mean of 3.7 taxa per bat was recorded, but significant differences were observed between sampling sites and seasons. The mycobiomes were dominated by cosmopolitan and plant-associated species, in particular from the genera *Cladosporium, Penicillium*, and *Aspergillus*. Other species known to be related to bats or their environment, such as *Apiotrichum otae*, were also retrieved. Sampling of hibernacula indicated that diverse fungal species can inhabit these sites, including a yet undescribed *Pseudogymnoascus* species, distinct from *Ps. destructans*, namely, *Ps. cavicola*.

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Mycobiome; *Pseudogymnoascus cavicola*; *Pseudogymnoascus destructans*; white-nose syndrome; 1 new taxon

INTRODUCTION

The emergence and spread of the white-nose syndrome (WNS) epizootics in various North American bat species has revealed the knowledge gap on the mycobiota occurring on bats and their hibernacula. WNS is caused by the psychrophilic fungus *Pseudogymnoascus destructans* and resulted in the death of millions of bats since it was first reported in 2006 in the state of New York (Blehert et al. 2009; Drees et al. 2017; Frick et al. 2010). The disease causes lesions to the wing membranes and alters the torpor patterns during hibernation, resulting in premature depletion of fat reserves and starvation (Janicki et al. 2015; Pikula et al. 2017). Genomic and typing analyses of *Ps. destructans* isolates showed that it was probably introduced in the United States and Canada from a European strain (Drees et al. 2017; Leopardi et al. 2015). The presence of this fungal pathogen is indeed reported throughout Europe but is not associated with mass mortality in Palearctic bat populations (Martinkova et al. 2010; Puechmaille et al. 2011; Wibbelt et al. 2010; Zukal et al. 2016, 2014). Contrary to the American hosts, it is thus believed that the long established coexistence between European bats and *Ps. destructans* promoted the tolerance toward the fungus (Zukal et al. 2016).

However, variation in host susceptibility to WNS has been documented in North American bats, with some species being resistant to infection by *Ps. destructans*. This protection could potentially result from the higher abundance, in the skin mycobiome, of commensal yeast species that would inhibit the growth of *Ps. destructans* (Njus 2014; Vanderwolf et al. 2021a, 2021b). This antagonist, strain-dependent, effect was notably shown in vitro in the presence of *Cutaneotrichosporon moniliiforme, Aureobasidium pullulans*, and *Holtermanniella takashimae* strains (Vanderwolf et al. 2021a, 2021b). In addition to commensals, many fungal species found on bats are considered to be transient, since they substantially overlap with the fungal taxa present in the environment (AngeStark et al. 2019). Geographic location is consequently an important factor that determines diversity and composition of skin fungal assemblages (Vanderwolf et al. 2021a).

Hibernacula are considered to be the main environmental reservoir of *Ps. destructans* from which bats become infected annually during the cold season

CONTACT Pierre Becker pierre.becker@sciensano.be
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(Fischer et al. 2022). The fungus is indeed psychrophilic and does not survive the elevated body temperature of active animals, allowing the bats to clear the infection during summer. Bats also have an influence in the fungal diversity found in hibernacula by introducing fungal propagules and producing organic waste such as guano (Borda et al. 2014; Kokurewicz et al. 2016; Ogorek et al. 2020; Vanderwolf et al. 2013).

In Belgium, the presence of *Ps. destructans* is only reported in the southeastern end of the country (Leopardi et al. 2015). Compared with this latter area, the northern part of Belgium (i.e., Flanders) is characterized by a softer climate, low-lying plains, important urbanization, and large agricultural lands. One of the goals of the present study was therefore to assess the presence of *Ps. destructans* in Flemish hibernacula. Although it was not isolated during the sampling campaign, a new psychrophilic *Pseudogymnoascus* species was found and described as *Ps. cavicola*, sp. nov. The study also aimed at describing the skin mycobiome of Flemish active bats during summer and autumn and to analyze possible differences in fungal diversity and composition among bat species, locations, and seasons.

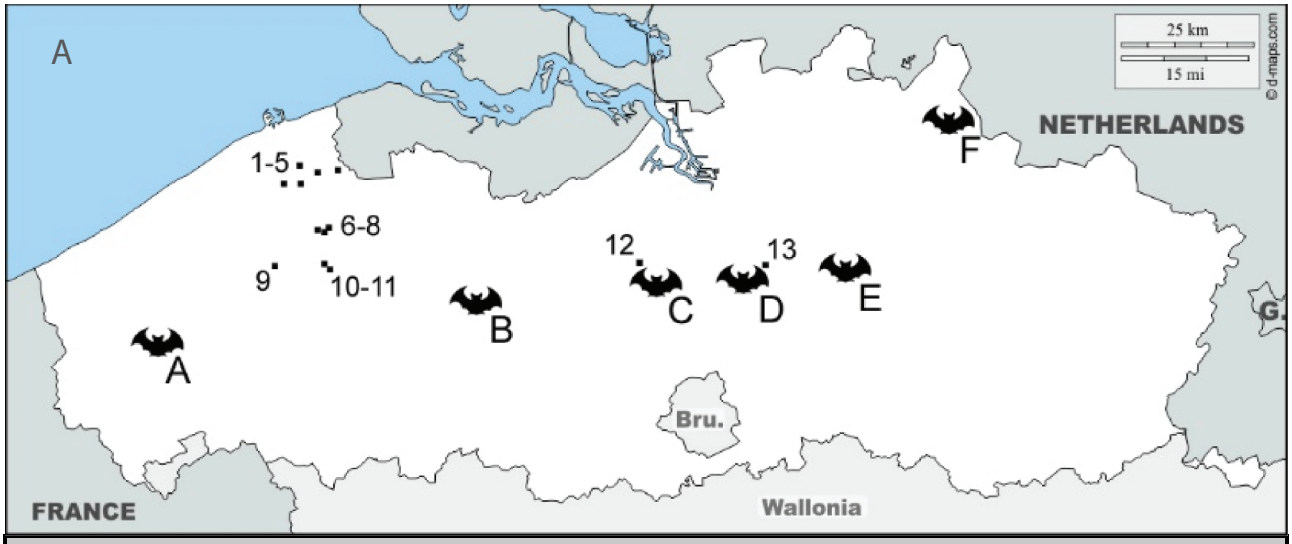
MATERIALS AND METHODS

Ethics.—Permission for the capture and handling of bats was granted from the ethical commission of Sciensano and authorized by the "Agentschap voor Natuur en Bos" (Governmental Flemish Agency for Nature and Forest) and "Natuurpunt" (Flemish Nature Conservation Organization). The permit (reference number ANB/BL/FF-V18-00095) was, however, limited, and the sampling on bats was not allowed during hibernation. Only the hibernation sites (i.e., the hibernacula) could be sampled during the winter season. All samples were taken in collaboration with the Bats Working Group of Natuurpunt.

Sampling.—From mid-July to mid-October 2018, 114 bat specimens belonging to seven different species (*Eptesicus serotinus, Myotis dasyscneme, M. daubentonii, M. emarginatus, M. mystacinus, M. natteri, Plecotus auritus*) were sampled from six localities across Flanders (FIG. 1). The sampling sites consisted of buildings (forts, cellars, farms) surrounded by meadows, small wooded areas, and urban zones. Bats were captured with mist nets at sunset and then placed in cotton holding bags in order to reduce stress. Medical gloves were worn during the handling. Both wings (on the inner part of the plagiopatagium and the dactylopatagium), nose, and ears were sampled using a single swab (ESwab, Copan, Brescia, Italy) per bat. Swabs were held in collection tubes containing 1 mL of Liquid Amies (Copan, Brescia, Italy). The latter allowed the moistening of the swab before sampling and was used as transport and preservation medium in which the swabs were maintained for a maximum of 5 days at 4 C prior to plating. Additionally, hibernacula were sampled in January and February 2019 from 13 locations (FIG. 1). At each location, one sample of wall surface (bricks) was swabbed below the hibernating bats and one sample of ground sediment was taken, both as close as possible to the bats without disturbing their hibernation and as far as possible from the cave entrance. Caves were inhabited mostly by *Myotis daubentonii, M. mystacinus, M. natteri, Plecotus auritus, Pipistrellus pipistrellus*, and *Pi. nathusii*. Sediments were made up of sand and/or gravel, and a few grams were collected in sterile plastic pots using sterile plastic spoons.

Isolation.—Swabs from the bats were first vortexed in their liquid, and 100 µL of the latter were then plated in the laboratory on nonselective culture media, including malt extract agar supplemented with 0.05% (*m*/*v*) chloramphenicol, at 25 C, as well as diluted Sabouraud supplemented with 0.05% (*m*/*v*) chloramphenicol, at 15 and 25 C. These media were prepared in house (see the recipes in SUPPLEMENTARY MATERIAL 1). For the hibernacula, the detection of *Ps. destructans* was targeted by using more selective conditions, namely, cultures on dextrose-peptone-yeast extract agar (DPYA) at 10 C, as suggested by Vanderwolf et al. (2016a). Swabs from the hibernacula were processed in the same way as for the swabs from the bats. For the sediment samples, 1 g was first suspended in 9 mL of a 0.85% sodium chloride solution under stirring for 1 h. After settling of the sediment, 200 µL of the supernatant was then plated on DPYA. All cultures were examined regularly for 3 weeks for fungal growth, and distinct morphotypes were isolated on diluted Sabouraud or malt extract agar.

Identification.—Isolates were first identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) using the standardized procedure described previously (Becker et al. 2014; Normand et al. 2013). For each isolate, mass spectra were acquired in quadruplicates with a microflex LT MALDI-TOF MS instrument (Bruker Daltonics, Bremen, Germany) using the default settings. Their identification was performed with the software Biotyper 4.1 (Bruker Daltonics) using the MSI 2.0 database. The latter is shared online through a free Web application (https://msi.happydev.fr) (Imbert et al. 2020). A cutoff score of 1.70 was applied for species-



B

Sampling sites			Bats species						
Ref	Locality	GPS coordinates	Eptesicus serotinus (n=1)	Myotis dasyscneme (n=1)	Myotis daubentonii (n=66)	Myotis emarginatus (n=13)	Myotis mystanicus (n=4)	Myotis natteri (n=3)	Plecotus auritus (n=26)
A.	Diksmuide	N50.9831, E2.8682		1	11				
B.	Merelbeke	N51.0203, E3.7472	1						
C.	Puurs	N51.0675, E4.2852			5	2	4		3
D.	Duffel	N51.0752, E4.4918			50	3		2	13
E.	Herentals	N51.1403, E4.8249				7			
F.	Arendonk	N51.3480, E5.1228				1		1	10

C

Ref	Locality	GPS coordinates	Ref	Locality	GPS coordinates	Ref	Locality	GPS coordinates
1.	Brugge	N51.2082, E3.2708	6.	Beernem	N51.1133, E3.3208	10.	Wingene	N51.0763, E3.3393
2.	Brugge	N51.2091, E3.2886	7.	Beernem	N51.1145, E3.2927	11.	Wingene	N51.0866, E3.3079
3.	Damme	N51.2325, E3.3264	8.	Beernem	N51.1176, E3.3283	12.	Bornem	N51.0830, E4.2383
4.	Damme	N51.2425, E3.3923	9.	Oostkamp	N51.0847, E3.2311	13.	Lier	N51.0833, E4.5665
5.	Damme	N51.2490, E3.2835						

Figure 1. Geographic localization of the sampling sites. (a). Map of Flanders (Belgium) showing the sampling sites of the bat individuals (bat icons A to F) and of the hibernacula (squares 1 to 13). (b). Localization of the bat sampling sites with the repartition of the bat specimens according to sampling site and bat species. (c). Localization of the hibernaculum sampling sites.

level identification. Identification was regarded as valid (i) if it was above the cutoff and (ii) if the identification was identical in at least three out of the four replicates.

Isolates for which MALDI-TOF MS–based identification was nonconclusive (generally because the species was not represented in the database) were processed for DNA sequencing identification. Genomic DNA was extracted, after a prior treatment including freeze-drying and bead-beating, using the Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany) following the manufacturer's instructions. Species-level identification required the sequencing of one or several phylogenetic markers. The loci that were used include the internal transcribed spacer (ITS) of the rRNA gene, the beta-tubulin (*TUB*), the calmodulin (*CAM*), the translation elongation factor 1-alpha (*TEF1*), the actin (*ACT*), the 28S large subunit ribosomal RNA (28S), the 18S small subunit ribosomal RNA (18S), the RNA polymerase II largest subunit (*RPB1*), the RNA polymerase II second largest subunit (*RPB2*), and the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Primers (Berbee et al. 1999; Carbone and Kohn 1999; Glass and Donaldson 1995; Liu et al. 1999; Manitchotpisit et al. 2009; Matheny et al. 2002; Miller and Huhndorf 2005; O'Donnell and

Cigelnik 1997; O'Donnell et al. 1998, 2000; Peterson 2008; Quaedvlieg et al. 2011; Rehner and Buckley 2005; Stiller and Hall 1997; Sung et al. 2007; Vilgalys 2018; Voigt and Wöstemeyer 2000; White et al. 1990) and polymerase chain reaction (PCR) conditions used to amplify these genes are provided in SUPPLEMENTARY TABLE 1. PCRs were performed using 1 µL of DNA, 0.25 µM of each primer, 0.2 µM of dNTP mix, 5 µL of 10× PCR buffer, 1 U of Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), and distilled water to a final volume of 50 µL. Amplicons were purified with the ExoSAP-IT Express PCR Product Cleanup kit (Applied Biosystems, Waltham, Massachusetts) and sequenced in a 3130xl genetic analyzer (Applied Biosystems) using the same primers and the BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were identified by comparison against the GenBank database using the BLAST tool (Altschul et al. 1990) and the MycoBank database (https://www.mycobank.org/page/Pairwise_alignment). Species-level identification was achieved if sequences showed at least 98.5% similarity to reference strains and if identity was consistent among loci. Higher taxonomic levels (species complex, genus, family, or order) were assigned to the strains that did not meet these criteria. Sequences obtained in the present study were deposited in the European Nucleotide Archive database (SUPPLEMENTARY TABLE 2). Noteworthy, after DNA identification, strains belonging to species that were not represented in the MALDI-TOF MS database were added to the latter to allow MALDI-TOF MS identification of conspecific isolates during subsequent samplings.

Phylogenetic analyses.—A multilocus phylogenetic analysis involving *Pseudogymnoascus* strains was performed with *TEF1* and ITS sequences obtained in the present study and from GenBank (SUPPLEMENTARY TABLE 3). Sequences from isolates included in the phylogenetic evaluation by Minnis and Lindner (2013) were chosen, supplemented with sequences of the type strains of *Pseudogymnoascus* species described since then by Crous et al. (2019), namely, *Ps. lindneri* and *Ps. turneri*; by Crous et al. (2020), namely, *Ps. palmeri*; and by Zhang et al. (2020), namely, *Ps. guizhouensis, Ps. shaanxiensis*, and *Ps. sinensis*. *TEF1* and ITS loci were selected because their sequences were available for all the isolates and strains considered. The multiple-sequence alignment (SUPPLEMENTARY MATERIAL 2) was constructed with MAFFT 7.394 using the FFT-NS-i iterative refinement method. The scoring matrix for nucleotide sequences was set to 1PAM/κ = 2. The alignment was then manually assessed and checked for inconsistencies. Based on this data set, a maximum likelihood (ML) phylogeny was constructed using IQ-TREE 1.6.12 (Trifinopoulos et al. 2016). Branch support was calculated using 1000 bootstrap replicates. The data set was subdivided into four gene partitions: ITS1+ITS2, 5.8S+28S, *TEF1* introns, and *TEF1* exons. ModelFinder was used to determine the best-fit model for each partition (Kalyaanamoorthy et al. 2017). Subsequently, a Bayesian analysis was conducted using MrBayes 3.2.7. (Ronquist et al. 2012). Two parallel runs with four chains ran for 107 generations, sampling frequency was set to 1000, and the first 25% of trees were discarded as burn-in. Single-gene phylogenies and a narrower analysis involving strains around *Ps. cavicola* were conducted using the same approach.

Statistical analyses.—Sample coverage (C) was used as an estimator of the completeness of the samples. It was estimated according to Good (1953): $C = 1 - (f1/N)$, where f1 is the number of singletons (i.e., taxa represented each by one isolate) and N is the total number of isolates (Chao and Jost 2012). Only one taxon was counted if several isolates from the same bat specimen were identified as the same species. Statistical analyses were performed in R using the EMMEANS (https://cran.r-project.org/web/packages/emmeans/index.html) and MASS (https://cran.r-project.org/web/packages/MASS/index.html) packages. To evaluate the hypothesis that the number of fungal species per bat is related to the location, bat species, and/or season, a negative binomial regression count model was constructed with the number of fungal species per bat as outcome and the bat species (6 factors), location (5 factors), and season (2 factors) as explanatory factors. For the seasons, samples obtained in July and August were regarded as summer whereas those obtained in September and October were regarded as autumn. The *E. serotinus* bat specimen obtained in Merelbeke was excluded from the analysis. Afterward, pairwise comparisons of the number of fungal species were carried out among bat species, locations, and seasons of sampling. To assess diversity, the Shannon diversity index (SI) of fungal species on each bat was calculated using the diversity function in the VEGAN package (https://cran.rproject.org/web/packages/vegan/vegan.pdf). Afterward, a Gaussian model was constructed with SI as the outcome variable and the bat species (6 factors), location (5 factors), and season (2 factors) as explanatory factors. To assess whether fungal assemblage composition varied among bat species, sites, and seasons, a nonparametric permutational multivariate analysis of variance (PERMANOVA) was applied on the SI. Samples that had no composition were excluded for the analysis. PERMANOVA was run for 1000 iterations, and R^2 values were reported from the

model where the variable entered the model last. All analyses were performed at the 5% significance level.

RESULTS

A total of 493 isolates were obtained from the 114 sampled bats. Some fungal species were retrieved multiple times on the same bat specimen, whereas fungal cultures were negative for 6 bats, resulting in 418 unique isolates representing 209 different species out of 108 bats (SUPPLEMENTARY TABLE 2). Incubation at 15 C resulted in the isolation of species that were also able to grow at 25 C and thus failed to detect psychrophilic taxa. Overall, the sample coverage was estimated at 0.65, meaning that the isolated species represent about two thirds of the fungi present in the communities. An average of 3.7 ± 2.6 (SD) fungal taxa were recorded per bat (range: 0–12). The statistical analyses (SUPPLEMENTARY TABLE 4) showed that this parameter did not vary significantly between bat species ($P \geq 0.05$). However, significant differences were observed according to the location. In particular, a higher number of fungal taxa were obtained on bats from Puurs and Diksmuide, as compared with the other sampling sites, 5.9 ± 3.3 (SD) ($P = 0.02$) and 5.9 ± 2.5 (SD) ($P < 0.001$) taxa, respectively. The number of fungal taxa per bat was also significantly higher ($P = 0.017$) in the autumn (5.3 ± 2.6 [SD]) than in the summer (2.8 ± 2.0 [SD]). Similar results were obtained using the SI (SUPPLEMENTARY TABLE 4), with no significant difference between bat species, and a higher diversity in the autumn ($P = 0.008$). Regarding locations, bats from Diksmuide appeared to have a significantly higher diversity of fungal species, as compared with the other sites ($P = 0.002$).

TABLE 1 provides the list of fungal taxa that were identified and their frequency. This diversity was dominated by *Ascomycota* species and comprised mainly cosmopolitan and plant-associated fungi. Isolates belonging to *Cladosporium* (n = 80), *Penicillium* (n = 58), *Aspergillus* (n = 39), *Microascus* (n = 19), and *Alternaria* (n = 16) accounted for more than half of the total isolates. Within these fungal genera, a high degree of species diversity was also observed, with 70 different taxa. Interestingly, some species that are more rarely isolated, such as *Absidia stercoraria, Blastobotrys arbuscula, Microascus hollandicus*, and *Microascus trautmannii*, were detected during the study. *B. arbuscula* in particular was isolated from 10 bats belonging to four different species and sampled in four different locations. Furthermore, a dermatophyte

Table 1. Identification and distribution of fungal taxa isolated from the body surface of 7 species of bats in 6 sampling sites in Flanders, Belgium.

	No. of bats for each bat species*							
Identification	Es	Mds	Mdb	Me	Mm	Mn	Pa	No. of sampling sites
Ascomycota								
Aaosphaeria arxii							1	1
Acaulium caviariforme			1					1
Acaulium sp.			1					1
Acremonium charticola				1				1
Acremonium rutilum			1					1
Acremonium sclerotigenum			1					1
Acremonium sp. 1			3	1		1	2	2
Acremonium sp. 2				1				1
Acrodontium crateriforme							1	1
Akanthomyces sp.							1	1
Alternaria alternata			2				3	4
Alternaria arborescens		1	2					3
Alternaria kordkuyana						1		1
Alternaria section *Infectoriae*		1	2	2	1			4
Alternaria sp.						1		1
Amphichorda cavernicola			1					1
Apiospora rasikravindrae			1					1
Apodus oryzae			1					1
Arachniotus aurantiacus				1			1	2
Arthrinium arundinis							1	1
Arthroderma onychocola				1				1
Ascobolus sp.			1					1
Aspergillus alliaceus					1			1
Aspergillus candidus			2		1	1		2
Aspergillus dobrogensis			2	2	1	1	1	2
Aspergillus fumigatus			1					1
Aspergillus jensenii		1	10	2	1		1	3

(*Continued*)

Table 1. (Continued).

Identification	No. of bats for each bat species*							No. of sampling sites
	Es	Mds	Mdb	Me	Mm	Mn	Pa	
Aspergillus montevidensis			1					1
Aspergillus pragensis			2				1	2
Aspergillus pseudoglaucus				1			1	2
Aspergillus puulaauensis						1		1
Aspergillus section *Candidi*			2				1	1
Aspergillus subalbidus					1			1
Aureobasidium leucospermi			1					1
Aureobasidium pullulans			2			1	8	3
Auxarthron sp.			1					1
Beauveria bassiana							1	1
Beauveria pseudobassiana				1				1
Blastobotrys arbuscula			5	2		1	2	4
Botrytis cinerea			7				2	2
Candida sp.			1					1
Catenulostroma sp.							1	1
Cephalotheca sp. 1			1			1		1
Cephalotheca sp. 2			1					1
Cephalotheca sp. 3			1					1
Cephalotheca sp. 4			1					1
Cephalotrichiella penicillata			3					1
Chaetomium angustispirale			1		1		1	1
Chaetomium cochliodes							1	1
Chaetomium sp.			1					1
Chlamydosporiella sp.				1				1
Cladosporium aggregatocicatricatum			1					1
Cladosporium allicinum		1	9	3	2		7	5
Cladosporium cladosporioides			3					2
Cladosporium cladosporioides complex	1		1			1	2	4
Cladosporium delicatulum	1		2	1	2	1	5	5
Cladosporium europaeum		1					3	3
Cladosporium halotolerans			1					1
Cladosporium herbarum complex							1	1
Cladosporium inversicolor			1		1			2
Cladosporium langeronii			1				1	2
Cladosporium neolangeronii			1					1
Cladosporium phyllophilum			1				2	3
Cladosporium pseudocladosporioides			1		2		3	4
Cladosporium psychrotolerans			1					1
Cladosporium ramotenellum		1	4			2	2	4
Cladosporium sinuosum							2	2
Cladosporium sp.							1	1
Cladosporium sphaerospermum			1					1
Cladosporium uwebraunianum							1	1
Cladosporium westerdijkiae				1			1	1
Clonostachys solani			2					2
Cryptendoxyla sp.				1				1
Debaryomyces fabryi			3					1
Debaryomyces hansenii	1							1
Debaryomyces sp. 1			1					1
Debaryomyces sp. 2			1	1				2
Debaryomyces sp. 3	1							1
Debaryomyces sp. 4			1					1
Diaporthe columnaris							1	1
Entoleuca mammata							1	1
Epicoccum nigrum			3		2	2	1	4
Fusarium cerealis			1					1
Fusarium solani species complex			1					1
Fusarium sp.			1					1
Fusarium witzenhausenense			1					1
Gamsia columbina							1	1
Gremmenia infestans							2	1
Gymnoascus reessii				1				1
Hanseniaspora uvarum							1	1
Helotiales sp.							1	1
Hendersonula pinicola							1	1
Hypoxylon fragiforme			1					1
Hypoxylon rubiginosum			1					1
Kernia hippocrepida			1					1
Lachnum sp.							1	1
Lasionectria sp.				1				1

(*Continued*)

Table 1. (Continued).

Identification	No. of bats for each bat species*							No. of sampling sites
	Es	Mds	Mdb	Me	Mm	Mn	Pa	
Lasiosphaeriaceae sp.			1					1
Lecanicillium aphanocladii							1	1
Metschnikowia sp.							1	1
Microascaceae sp.			1					1
Microascus atrogriseus			1	1				2
Microascus hollandicus			1			1		2
Microascus paisii			2	1		1		4
Microascus pseudolongirostris			1					1
Microascus sp.			4					2
Microascus trautmannii			5	1				3
Microdochium neoqueenslandicum							1	1
Mollisia sp.							1	1
Myriodontium sp.			1	1				2
Myrmecridium schulzeri			1					1
Myxotrichum sp.			1					1
Neoroussoella sp.					1			1
Niesslia tenuis			1					1
Oidiodendron griseum			1					1
Oidiodendron sp.			1					1
Onygenaceae sp. 1			1					1
Onygenaceae sp. 2			1					1
Paraphaeosphaeria neglecta							1	1
Paraphoma fimeti			2					1
Penicillium allii-sativi					1			1
Penicillium bialowiezense		1	3			1	2	3
Penicillium brevicompactum				1				1
Penicillium buchwaldii							1	1
Penicillium chrysogenum							1	1
Penicillium citreonigrum			3					1
Penicillium corvianum			1					1
Penicillium corylophilum			1				1	2
Penicillium crustosum			1					1
Penicillium flavigenum					1			1
Penicillium glandicola			1					1
Penicillium kongii			2	1			1	3
Penicillium lilacinoechinulatum			4					1
Penicillium mexicanum				1	1			1
Penicillium minnesotense			1					1
Penicillium murcianum			1				1	2
Penicillium phoeniceum			1					1
Penicillium polonicum					1		1	2
Penicillium roseopurpureum			2				1	3
Penicillium rubens			6				3	3
Penicillium sanguifluum					1			1
Penicillium scabrosum			2					2
Penicillium section *Citrina*			1					1
Penicillium section *Exilicaulis*				1			1	2
Penicillium section *Fasciculata*					1			1
Penicillium section *Ramosa*			1					1
Penicillium section *Torulomyces*			1					1
Penicillium vulpinum							1	1
Periconia cookei			2					2
Phaeosphaeria sp.							1	1
Phaeosphaeriaceae sp. 1			1					1
Phaeosphaeriaceae sp. 2			1					1
Phialemonium inflatum			3					2
Phialocephala sp.							1	1
Phialophora dancoi							1	1
Phialophora intermedia							1	1
Phomatosporales sp.			1					1
Pithoascus sp.				1				1
Plectosphaerellaceae sp.			1					1
Pseudopithomyces chartarum			2		1			2
Purpureocillium lilacinum			1					1
Rhexocercosporidium sp.			1					1
Sarocladium sp.							1	1
Scopulariopsis brevicaulis			1					1
Scopulariopsis candida			1	1				2
Scopulariopsis soppii			2	1				3
Setoseptoria scirpi			1					1

(*Continued*)

Table 1. (Continued).

Identification	No. of bats for each bat species*							No. of sampling sites
	Es	Mds	Mdb	Me	Mm	Mn	Pa	
Sporendonema casei			1					1
Stachybotrys chlorohalonata			1					1
Talaromyces section *Trachyspermi*			1					1
Thyronectria sinopica							1	1
Trichoderma atrobrunneum			1					1
Trichoderma citrinum							1	1
Volutella ciliata			1					1
Wickerhamomyces anomalus			1					1
Xenoacremonium sp.			1					1
Basidiomycota								
Apiotrichum dulcitum					1			1
Apiotrichum otae			1					1
Coprinaceae sp.						1		1
Coprinellus disseminatus			1					1
Coprinellus radians							1	1
Cystobasidium laryngis					1			1
Cystobasidium slooffiae				1				1
Filobasidium magnum				1				1
Filobasidium oeirense			1				1	2
Fomes fomentarius			2					1
Ganoderma resinaceum			2					2
Holtermanniella sp.							1	1
Hormographiella aspergillata				1				1
Hormographiella verticillata					1			1
Hypholoma fasciculare					1			1
Mycosarcoma maydis			2				1	3
Papiliotrema sp.			1					1
Phlebia subochracea			1					1
Psathyrella candolleana							1	1
Solicoccozyma terrea				1				1
Sporobolomyces roseus							1	1
Vishniacozyma carnescens			1				1	2
Vishniacozyma sp.			1					1
Mucoromyceta								
Absidia cylindrospora			1					1
Absidia pseudocylindrospora			1					1
Absidia stercoraria			1					1
Actinomucor elegans			1					1
Mortierella alpina			1					1
Mucor circinelloides				1				1
Mucor flavus				1				1
Mucor fragilis			1					1
Mucor janssenii							1	1
Mucor ucrainicus							1	1

*Es = *Eptesicus serotinus*; Mds = *Myotis dasycneme*; Mdb = *M. daubentonii*; Me = *M. emarginatus*; Mm = *M. mystanicus*; Mn = *M. natteri*; Pa = *Plectotus auritus*.

fungus, *Arthroderma onychocola*, was isolated from a single bat specimen but without apparent skin lesions on the body. Fungal species known to be associated with bats or their environment were also identified. This includes an *Apiotrichum otae* strain and an *Acaulium caviariforme* (formerly *Microascus caviariformis*) strain, both obtained from *Myotis daubentonii* specimens. Finally, numerous yeast species were isolated, including representatives of *Apiotrichum, Candida, Cystobasidium, Debaryomyces, Filobasidium, Hanseniaspora, Holtermanniella, Metschnikowia, Solicoccozyma, Sporobolomyces, Vishniacozyma*, and *Wickerhamomyces*. Some yeast taxa were shared between bat species and locations. Skin fungal assemblage composition was not associated with bat species (R^2 = 0.066, P = 0.256), sampling site (R^2 = 0.055, P = 0.197), and season of collection (R^2 = 0.037, P = 0.057).

The presence of *Ps. destructans* was not detected during the study, including in the hibernacula. In the latter, sample coverage was, however, low (C = 0.05). Compared with the bats, less isolates (n = 22) were obtained in the hibernacula due to the more selective culture conditions (SUPPLEMENTARY TABLE 2). These isolates displayed an important diversity, with 21 species representing 15 genera (TABLE 2). One of these isolate was identified as a yet undescribed *Pseudogymnoascus* species, distinct from *Ps. destructans*. We therefore propose the description of a new *Pseudogymnoascus* species, namely, *Ps. cavicola*.

A selection of the strains isolated from the bats and the hibernacula were deposited in the BCCM/IHEM collection (SUPPLEMENTARY TABLE 2).

Table 2. Identification of fungal taxa isolated from hibernacula in 13 different locations in Flanders, Belgium.

Identification	Sampling site*
Ascomycota	
Beauveria caledonica	2
Cephalotrichum nanum	3
Cephalotrichum purpureofuscum	5
Enterocarpus grenotii	3
Gamsia columbina	5
Gamsia sp.	13
Gliomastix murorum	13
Metapochonia suchlasporia	12
Myxotrichum deflexum	11
Parengyodontium album	1
Penicillium ibericum	4
Penicillium janczewskii	4, 5
Penicillium section *Fasciculata*	1
Penicillium vulpinum	2
Pseudogymnoascus cavicola, sp. nov.	10
Spiromastix sp.	9
Tolypocladium cylindrosporum	7
Trichoderma viridarium	7
Mucoromyceta	
Absidia cylindrospora	6
Mucor flavus	8
Mucor hiemalis	3

*See FIG. 1 for the references and the localization of the hibernacula.

TAXONOMY

Pseudogymnoascus cavicola F. Baert, E. D'Hooge & P. Becker, sp. nov. FIG. 2
MycoBank MB840116

Typification: BELGIUM. WEST FLANDERS: Wingene, from soil sediment of a potato cellar inhabited by hibernating bats, 12 Jan 2019, C. van den Eynde (**holotype** IHEM 28275). GenBank: ITS = OU989484; 28S = OU641157; *TEF1* = OU641535.

Etymology: *cavicola* (Latin), referring to the environment (cellar) from which it was isolated.

Specimen examined: IHEM 28275 (type strain); no other material available for examination.

Description: Colonies growing restrictedly on potato dextrose agar (PDA) at 25 C (15 mm diam after 7 days), raised, dense, velutinous, becoming radially sulcate with time, margin regular. Aerial mycelium sparse, scattered, floccose, and whitish. Colony color white at the edge of the colony, changing to rosy buff and vinaceous buff in the center, becoming buff and honey with age. Exudate droplets hyaline at first, becoming very abundant and brown vinaceous to very dark vinaceous with age (FIG. 2). Reverse buff, honey, and umber, becoming sepia, brown vinaceous, and very dark vinaceous with age. Pigment diffuses in the agar, umber-colored. Colonies growing restrictedly on Sabouraud at 25 C (13 mm diam after 7 days), raised, dense, floccose, becoming radially sulcate with time, margin regular. Colony color white with a pink hue at first, becoming rosy buff, buff, and honey with age. Exudate droplets hyaline at first, becoming very abundant and brown vinaceous to very dark vinaceous with age. Reverse ochraceous near the margin to fulvous and umber in the center, becoming sepia, brown vinaceous, and very dark vinaceous with age. Pigment produced after 10 days, umber-colored. Hyphae hyaline, smooth-walled, 0.9–2.0 µm diam. Conidiophores hyaline, branched at

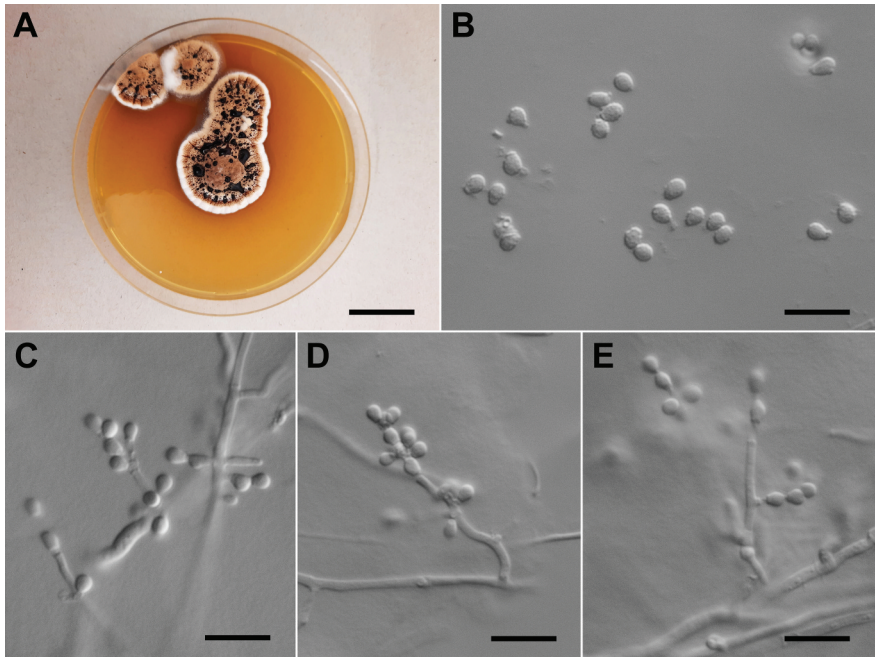


Figure 2. *Pseudogymnoacus cavicola*. (a). Front colony on potato dextrose agar after 28 days at 25 C. (b–e). Conidiophores and conidia. Bars: A = 2 cm; B–E = 10 µm.

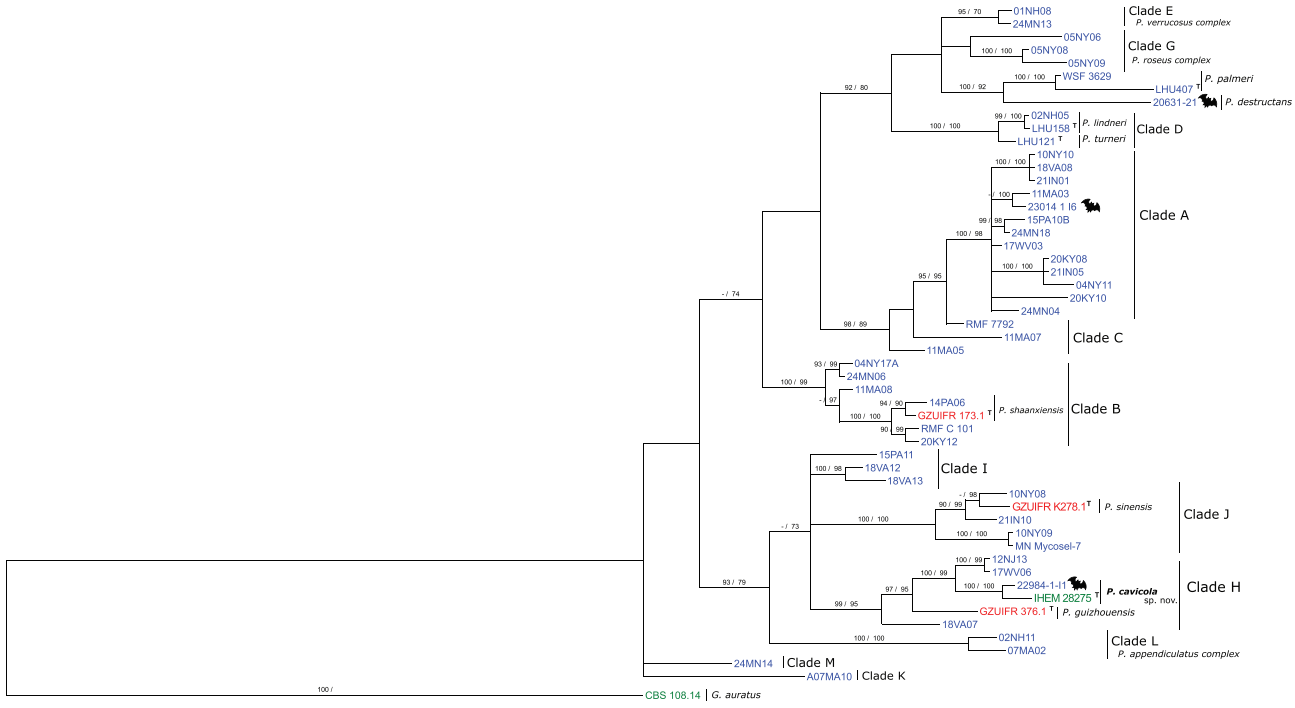


Figure 3. Multilocus phylogenetic tree of the *Pseudogymnoascus* genus based on ITS and *TEF1* gene regions, with type strain of *Geomyces auratus* (CBS 108.14) used as outgroup. Bayesian posterior probabilities (as percentage) and maximum likelihood bootstrap values are shown at the nodes, respectively. Bootstrap values lower than 70% and posterior probabilities lower than 90% are not shown. Clades A to M named following Minnis and Lindner (2013). Strains isolated from bats are tagged with bat icons. The color indicates the geographic origin of the strains: blue for the USA, red for China, and green for Belgium.

acute angles near the apex and sometimes verticillate. Conidia formed terminally and laterally, on side branches, on short pedicels, or directly on the hyphae. Mature conidia smooth-walled to roughened, yellow-green, obovoid, subglobose, pyriform, or clavate (2.5–)3.0–5(–6) × 2–3.5 (–4) µm (avg. ± SD = 3.8 ± 0.67 × 2.7 ± 0.40 µm, n = 50), with wide basal scars (FIG. 2). Intercalary conidia often present, formed in the fertile hyphae or in the conidiophore, where they appear solitary or in series of two, preceding the terminal conidia, rough-walled, yellow-green, barrel-shaped, cylindrical, or irregularly shaped, 3.5–5.5(–6.5) × (1.5–)2–3(–3.5) µm (avg. ± SD = 4.4 ± 0.65 × 2.5 ± 0.39 µm, n = 50), with abscission scars. Sexual morph not observed. Growth in a temperature range between 10 and 25 C. No growth at 30 C and above. No other culture was available for examination; the morphological description is based solely on the observation of the holotype.

Notes: Minnis and Lindner (2013) investigated the genetic diversity among *Pseudogymnoascus* isolates obtained mainly from bat hibernacula soils across northeastern United States, revealing a total of 13 clades (named A to M), most of them including yet undescribed species. Strain IHEM 28275 is part of clade H and is closely related to isolate 22 984-1-I1 isolated from a *Perimyotis subflavus* bat in Tennessee, USA. Their ITS and 28S sequences are identical, and their *TEF1* sequences share 99.6% similarity (FIG. 3, SUPPLEMENTARY FIGS. 1–3). Phylogenetically, *Ps. cavicola* is close to *Ps. guizhouensis*, described by Zhang et al. (2020), but the lower genetic similarity of ITS (99.0%) and *TEF1* (98.3%) markers suggests that they are separate species. Moreover, the morphology of *Ps. cavicola* differs from that of *Ps. guizhouensis* by the slightly smaller conidia (4.0 × 3.0 µm vs. 3.8 × 2.7 µm) and slightly longer and more slender intercalary conidia (4 × 3 µm vs. 4.4 × 2.5 µm). *Ps. cavicola* also differs from *Ps. guizhouensis* by the shape of its intercalary conidia (barrel-shaped, cylindrical, or irregularly shaped vs. barrel-shaped or cuneiform). Macromorphologically, *Ps. cavicola* can be distinguished from *Ps. guizhouensis* by the production of dark vinaceous exudate droplets, the vinaceous color of the reverse of the colony, and the diffusion of an amber-colored pigment in the agar.

DISCUSSION

The fungal diversity found on Flemish bats was high, indicating that bats are important carriers of fungal spores and yeast cells and participate in the dispersion of numerous fungal species. The fungal diversity varied according to the location. This suggests that the fungal

richness found on bats is reflective of the diversity of their environment. Interestingly, the sampling sites were geographically similar and were all situated in rather rural surroundings with agricultural parcels, wooded areas, and small urbanized zones. There were thus no particular differences in the landscape that could explain the higher diversity in Diksmuide and Puurs. However, these two sampling sites were both located in small nature reserves. The limitation of human activities in these reserves and their biological interest could account for the observed differences. Regarding fungal diversity among bat species, no significant difference was detected. This result differs from the study of Vanderwolf et al. (2021a) where the skin fungal diversity varied with both site and bat species. Other studies performed on bacterial communities associated with bats skin also showed different diversities among bat species (Li et al. 2022b, 2022a). The absence of difference in our study could indicate that diversity among bat species is not always the case, although it can be due to the variable size of the samples. The seasonal change of the fungal diversity observed in the present study was previously found on Mexican free-tailed bats from Colorado, with an increased diversity during summer (Fenster et al. 2019). In our case, a higher diversity is measured in the autumn, which could be related to seasonal variations in the fungal diversity of the environment (Rudolph et al. 2018). This could also result from bat behavior, with activities such as swarming, mating, or building-up of fat reserves occurring during that period.

No significant difference in the fungal skin assemblage composition was detected among bat species, locations, or seasons of collection, in contrast to previous similar studies performed across the United States (Vanderwolf et al. 2021a, 2021b). The reduced geographic area and the similarity of the surroundings could explain the greater homogeneity found in northern Belgium samples. In the latter, the most represented fungal genera were *Aspergillus, Penicillium*, and *Cladosporium*. These groups are commonly observed within bat microbiota, worldwide. In Australian bent-winged bats, a total of 115 fungal species were recognized, including representatives of these groups (Holz et al. 2018). *Penicillium* and *Cladosporium* were also among the most commonly isolated taxa in Canadian bats, the same observation being made on U.S. bats from various hibernacula in Illinois (Johnson et al. 2013; Vanderwolf et al. 2015, 2016b). In Brazil, fungal diversity on Molossidae bats was dominated by *Aspergillus, Penicillium, Chrysonilia*, and *Cladosporium* (Furtado et al. 2020; Shapiro et al. 2015). In Italy, *Aspergillus* and *Cladosporium* species were the only molds isolated from three bat individuals (Voyron et al. 2011).

Less common species were also detected, which illustrates how sampling campaigns can extend our knowledge on the fungal ecology, the geographic distribution of the species, and the substrates on which they can be found. *Absidia stercoraria*, for instance, was previously isolated from rat dung in Korea (Li et al. 2016), whereas *Microascus hollandicus* and *Microascus trautmannii* are two recently described species isolated respectively from an indoor horse arena in the Netherlands and an oriented strand board in Germany (Woudenberg et al. 2017). *Arthroderma onychocola* is presumed to be a geophilic dermatophyte and was only known from a few clinical cases of human onychomycosis (Hubka et al. 2014, 2015). *Blastobotrys arbuscula*, isolated in multiple bats and locations in our study, was originally described from a strain isolated from indoor air in Finland (Kurtzman et al. 2011).

Fungal species known to be associated with bats were isolated as well, including *Apiotrichum otae*, which was described from strains isolated from bat guano in Japan (Takashima et al. 2020). It is also the case of *Acaulium caviariforme*, which is known to occupy caves and was obtained from bats in Canada (Sandoval-Denis et al. 2016; Vanderwolf et al. 2015, 2016b). Regarding the various yeast species isolated in the present study, their diversity is particularly interesting. Yeast assemblages on bat skin are indeed believed to represent primarily commensal rather than transient members of fungal communities, compared with filamentous fungi. They are thus more likely to be permanent residents of the skin (Vanderwolf et al. 2021b). It was, however, not possible to assign a potential antagonist effect against *Ps. destructans* of one of these yeast commensals, since all the sampled bat species were previously reported to be susceptible to WNS (Puechmaille et al. 2011; Wibbelt et al. 2010; Zukal et al. 2016, 2014).

A high diversity of fungal species were retrieved in the hibernacula despite the selective culture conditions targeting *Ps. destructans*. Representatives of fungal genera commonly found in caves were isolated, including *Absidia, Beauveria, Mucor, Penicillium*, and *Trichoderma* species (Vanderwolf et al. 2013). Most of these species grow relatively fast. During isolation, plates were indeed incubated for 3 weeks, which is enough to detect *Ps. destructans* (Gargas et al. 2009) but may be insufficient to accommodate for the slow growth of some species at 10 C. A longer incubation could therefore have resulted in an even higher diversity.

The presence of *Ps. destructans* in Flanders was not detected during the sampling campaign but cannot be ruled out given the low sample coverage in the hibernacula. Additional sampling is thus required to confirm its absence. Another *Pseudogymnoascus* species, *Ps.*

cavicola, sp. nov., was, however, isolated in one of the sampled hibernacula. The isolation of non-*destructans Pseudogymnoascus* species in caves was observed in previous studies. Mycological analyses of American hibernacula indeed showed that they can be inhabited by various nonpathogenic *Pseudogymnoascus* species (Lorch et al. 2013; Minnis and Lindner 2013).

CONCLUSION

Investigation of underexplored ecological niches is essential for increasing our knowledge on fungal biodiversity but also for revealing new species of fungi. In this study, sampling of a hundred bat specimens resulted in nearly 25% (50 out of 209) of taxa that were limited to the genus or higher taxonomic level using multilocus DNA sequencing. These taxa could potentially correspond to new species, and more work is required for their description.

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ORCID

Pierre Becker http://orcid.org/0000-0001-6158-102X

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