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Fungal Diversity in Korean Caves and Cave-Inhabiting Bats with Attention to *Pseudogymnoascus* Species

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Abstract: *Pseudogymnoascus* is a psychrophilic fungus, which is a genus widely distributed in cold regions around the world. Recently, the presence of *Pseudogymnoascus destructans* (*Pd*), the causative agent of white-nose syndrome (WNS) belonging to *Pseudogymnoascus*, has been reported in neighboring countries of Korea. However, no investigation on *Pd* has been reported in Korea. In this study, cave-inhabiting bats and their habitats were investigated in terms of the diversity of cave fungi, and we tried to confirm the presence of *Pd*. Three caves suspected of hosting *Pd* were selected, and 83 environmental and 53 bat samples were collected. A total of 154 fungal strains belonging to 31 different genera were isolated, and 20 of 154 were confirmed to belong to *Pseudogymnoascus*. *Pd*-diagnostic PCR was performed to check whether *Pd* was present in the isolated *Pseudogymnoascus*, and seven positives were confirmed. However, phylogenetic analyses revealed that no isolates belonged or were closely related to the clade with *Pd*. Although samples were collected from limited areas, undescribed *Pseudogymnoascus* species were isolated, and it was confirmed that Korean isolates were distributed in various clades. In conclusion, it is hypothesized that Korean *Pseudogymnoascus* presents high diversity.

Keywords: fungal diversity in caves; *Pseudogymnoascus*; multi-locus phylogenetic analyses; fungal pathogens; white-nose syndrome

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1. Introduction

Fungi are organotrophic microorganisms and the second-largest eukaryote with an estimated 11.7 million to 13.2 million species [1]. Fungi are most commonly associated with terrestrial ecosystems, but they are present in almost every environment on Earth, from deep-sea deposits to animal skin [2–6]. Among fungal habitats, caves are generally considered an extreme environment for life because of the lack of organic carbon inflow through photosynthesis [7,8]. In these stringent environments, fungal diversity is generally considered to be low. However, several papers underline that caves harbor unexpectedly high diversity [9,10]. Previous studies have suggested that some of the fungal communities in caves were introduced from the outside [11–14]. They have been reported to be affected by external factors, such as airflow, water movement, and visitors [11,12,15,16]. Among these, cave-inhabiting bats are estimated to be one of the most important factors in the inflow of fungal spores [17,18].

Pseudogymnoascus is one of the fungal genera most closely related to bats [19,20]. In addition, the genus *Pseudogymnoascus* is a keratolytic and psychrophilic fungus that has a wide geographic distribution in cold regions worldwide [21–23]. The genus *Pseudogymnoascus* was established by Raillo to encompass the species *Pseudogymnoascus roseus* and *Pseudogymnoascus vinaceus*; decades later, Samson integrated *P. vinaceus* into *P. roseus* [24,25]. Attention to cave fungi has soared since 2009 when the pathogen *Geomyces destructans*, responsible for the fatal white-nose syndrome (WNS) disease, was identified [19,20,26].

Minnis and Lindner reorganized *Geomyces* and allied species after a multi-locus analysis. *Geomyces destructans* has been included in the *Pseudogymnoascus* genus [26], and many other species have been described in recent years [22,26–28].

Traditional classifications in taxonomy and systematics were mainly based on morphological approaches [29]. However, there may be limitations in systematically classifying fungal species because the morphological characteristics are not observed to be as diverse as numerous fungal species [1,30]. To redeem these limitations, molecular methods using a DNA barcode have emerged [31–33]. However, each gene marker used has different advantages and limitations [34–37]. Hence, multi-locus sequence typing, which uses multiple gene markers to redeem for the limitations, is used in fungal phylogeny [38,39].

Research on fungi associated with caves and bats in Korea is insufficient. In addition, the presence of *Pd*, the causative agent of WNS, has been reported in countries around Korea recently [40,41]. However, no studies on *Pseudogymnoascus*, including *Pd*, have been conducted. Therefore, the investigation of fungi present in bats and bat caves in Korea and the diversity of *Pseudogymnoascus* species is required. In this study, samples collected from bats and bat caves were used to cultivate fungi and to conduct *Pd*-specific diagnostic PCR and phylogenetic analyses to assess the diversity of Korean *Pseudogymnoascus* and the presence of *Pd*.

2. Materials and Methods

2.1. Sample Collection and Laboratory Processing

In 2018, we were provided information about a bat showing WNS-like symptoms in Geum cave by a bat ecologist (Dr. Chung CU, personal communication). Based on this information, we investigated the presence of *Pd* infection in bat caves and the surrounding environment. Environmental and bat samples were collected from three karst caves (Geum: 36°59′ N, 128°21′ E; Eun: 37°00′ N, 128°21′ E; and Handemy: 36°59′ N, 128°26′ E) in Danyang, Republic of Korea, during September 2019 to April 2020 (Figure 1). The temperature of the caves was 6–15 °C and the humidity was 50–83%. The bat guano and cave wall samples were collected using sterilized cotton swabs. Bats were captured using a bat mist net within the caves and were immediately released after oral and skin swab collection using sterilized cotton swabs. All bats in the caves were examined for the presence of *Pd* using ultraviolet (UV) light at a wavelength of 365 nm (Analytik Jena, Jena, Germany) [42,43]. All bats showed negative results for the UV light test. Skin swabs were stroked approximately 10 times, focusing on the bat's wing membrane, ears, nose, and side of the mouth. Furthermore, oral swabs were obtained from the tongue and palate mainly. Bat guano (1 g) was suspended in 10 mL phosphate-buffered saline (PBS), containing 100 μg/mL of gentamycin and chloramphenicol. The swab samples were suspended in 1 mL of the mixture of antibiotics in PBS. A total of 83 environmental samples (Geum: 52; Eun: 14; Handmy: 17) and 53 bat samples (Geum: 15; Eun: 20; Handmy: 18) were collected. All samples were transported to the laboratory in a cooler and stored at 4 °C in the fridge until processed (three days).

2.2. Culture and Isolation of Fungi

To isolate fungi, 83 environmental samples (44 cave wall swabs and 39 bat guano) and 53 bat samples (19 oral swabs and 34 skin swabs) were cultured on Sabouraud dextrose agar (Becton Dickinson Co., Sparks, NV, USA), containing 100 μg/mL gentamycin and chloramphenicol (MBcell, Seoul, Republic of Korea). The supernatants of environmental samples, skin and oral swabs were collected through centrifugation at 3000 rpm for 1 min at 4 °C and then diluted to 1×10^5 fold using PBS. Diluted sample supernatants (100 μL) were inoculated onto the media, and PBS was used as a negative control. The inoculated media were divided into samples at room temperature (20 °C) and at low temperature (6 °C) groups, and dark incubation was performed at room temperature for two weeks and at low temperature for 40 days. Media were checked daily. To obtain fungal cultures, the

single-spore isolation method was used [44]. Single fungal cells were washed once with distilled water and harvested in 2 mL of PBS.

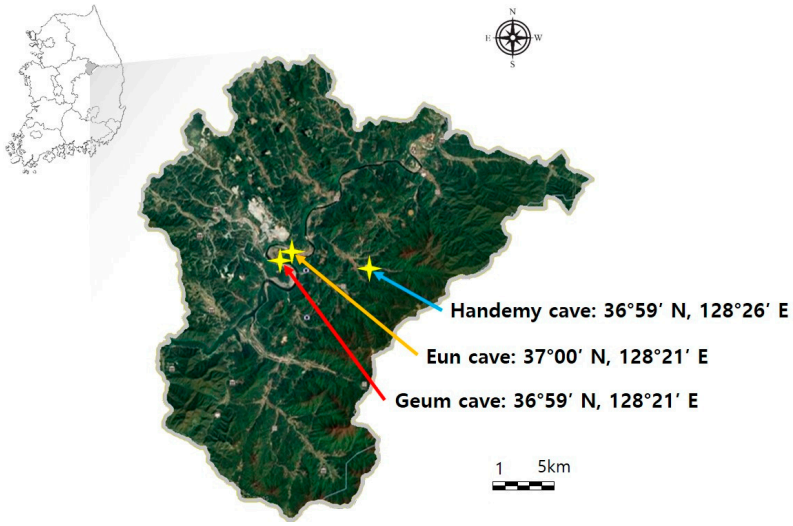


Figure 1. Map of the sample collection sites for this study. The picture is a magnification of the gray area of the South Korean map, and the location of the three caves within Danyang is marked with yellow crosses. The environmental samples, bat skin swabs, and oral swabs samples were collected from each site.

2.3. Genomic DNA Extraction, PCR Amplification, and Sequencing

Genomic DNA from pure fungal colonies was extracted using the i-genomic BYF DNA Extraction Mini Kit (iNtron Biotechnology, Seongnam, Republic of Korea) according to the manufacturer's instructions. The isolates were first identified at genus level by internal transcribed spacer (ITS) sequencing and BLASTn comparison. The strains belonging to the *Pseudogymnoascus* genus were furtherly processed for the 28S large subunit rRNA gene (LSU), translation elongation factor 1 alpha (TEF1-α), minichromosomal maintenance protein 7 (MCM7), and RNA polymerase II second-largest subunit (RPB2) [22,26–28]. In addition, amplification was performed using nu-SSI(1506)-184-5′-Gd and nu5.8S-144-3′-Gd primer pair for *Pd* diagnosis [45]. The primer sequences used to amplify the markers are listed in Table S2. This amplification was performed using Maxime™ PCR Premix i-StarTaq (iNtron Biotechnology) according to the manufacturer's instructions. The PCR mixture (20 µL) contained 50 ng of DNA template and 10 pmol of each primer. Sequencing was performed by barcode-tagged sequencing (BTSeqTM; CELEMICS, Seoul, Republic of Korea). All sequences generated in this study were deposited in the GenBank database, and their accession numbers are listed in Table S3.

2.4. Strain Identification and Phylogenetic Analysis

The sequences generated in this study were combined with the ones downloaded from GenBank to generate a sequence dataset (Table S3). Available reference sequences were retrieved from the NBCI database, and fungal sequences were aligned with the reference sequences using MAFFT v7.3113. Both sequence editing and concatenation were performed using MEGA 7.0.26 [46]. Minnis and Lindner observed that LSU and TEF1 introns have limited phylogenetic value because they are present and scattered among unrelated *Pseudogymnoascus* members [26]. Therefore, homologous gaps corresponding to LSU and TEF1 introns were excluded. In addition, the non-overlapping ends of the sequences in each alignment were trimmed. Phylogenetic analysis was conducted using maximum likelihood (ML) and Bayesian inference (BI) methods. ML analyses were performed using IQ-TREE v 1.6.8 [47]. The best-fit nucleotide substitution model for each locus was estimated using IQ-TREE's Model Finder function [48] following the Bayesian information criterion (BIC). Bootstrap analyses were performed using ultrafast bootstrap approximation with

1000 replicates [49]. BI analyses were performed using MrBayes v.3.2.6 [50]. The analyses included two independent runs of five million generations with four chains each. The substitution model was set to K2 + I + G, and the first 25% of the samples and trees were discarded as burn-ins. The remaining trees were used to construct a 50% majority rule consensus tree.

3. Results

3.1. Fungus Isolation

Fungal colonies were cultivated from 31 of 83 environmental samples, 17 of 19 oral swab samples, and 13 of 34 skin swab samples. A total of 154 fungi were isolated, including 100 from the environment, 22 from oral swabs, and 32 from skin swabs.

3.2. Identification of Isolated Fungi

In the identification of isolated fungi using the ITS region molecular marker, the occurrence of isolated fungi from environmental samples by family level was as shown in Table 1. The occurrence of isolated fungi from bat samples by family level was as shown in Table 2. The occurrence frequencies of total isolated fungi by phylum level were as follows: *Ascomycota* (83.8%); *Basidiomycota* (14.3%); and *Mucoromycota* (1.9%). The most abundantly isolated family was *Saccharomycetaceae* (32.5%), followed by *Trichocomaceae* (22.1%), *Pseudeurotiaceae* (15.6%), and *Cladosporiaceae* (2.6%). The highest-frequency genera were *Debaryomyces* (40 strains), *Penicillium* (33 strains), and *Pseudogymnoascus* (20 strains) (Table S1).

Table 1. Fungal diversity frequencies by phylum and family level of isolated from cave environmental samples at each cave: (a) Geum; (b) Eun; (c) Handemy. *Trichocomaceae* was most abundantly isolated.

Phylum	Family	No. Isolates	Isolates %
	(a)		
Ascomycota	*Trichocomaceae*	17	27.4
	Pseudeurotiaceae	16	25.8
	Saccharomycetaceae	2	3.2
	Clavicipitaceae	2	3.2
	Hypocreaceae	1	1.6
	Nectriaceae	1	1.6
	Cladosporiaceae	1	1.6
	Myxotrichaceae	1	1.6
	Plectosphaerellaceae	1	1.6
	Sordariomycetes	1	1.6
Basidiomycota	*Mrakiaceae*	8	12.9
	Trichosporonaceae	7	11.3
	Tremellaceae	1	1.6
Mucoromycota	*Mucoraceae*	3	4.8
	(b)		
Basidiomycota	*Trichosporonaceae*	3	42.9
Ascomycota	*Trichocomaceae*	2	28.6
	Chaetomiaceae	1	14.3
	Torulaceae	1	14.3
	(c)		
Ascomycota	*Trichocomaceae*	10	32.3
	Saccharomycetaceae	9	29.0
	Pseudeurotiaceae	3	9.7
	Cladosporiaceae	2	6.5
	Didymellaceae	1	3.2
	Nectriaceae	1	3.2
	Incertae sedis	1	3.2
	Microascaceae	1	3.2
Basidiomycota	*Mrakiaceae*	2	6.5
	Trichosporonaceae	1	3.2

Table 2. Fungal diversity frequencies by phylum and family level of isolated from bat samples (oral and skin swab) at each cave: (a) Geum; (b) Eun; (c) Handemy. *Saccharomycetaceae* was the most frequently isolated.

Phylum	Family	No. Isolates	Isolates %
	(a)		
Ascomycota	*Saccharomycetaceae*	14	72.2
	Cladosporiaceae	1	1.9
	(b)		
Ascomycota	*Saccharomycetaceae*	4	36.4
	Pseudeurotiaceae	4	36.4
	Trichocomaceae	2	18.2
	Pleosporaceae	1	9.1
	(c)		
Ascomycota	*Saccharomycetaceae*	21	75.0
	Dothioraceae	3	10.7
	Trichocomaceae	3	10.7
	Pseudeurotiaceae	1	3.6

3.3. *Pd*-Specific Diagnosis

A total of *Pseudogymnoascus* samples were confirmed from 16 environmental samples, 1 oral sample, and 3 skin samples (Figure 2 and Table S1). *Pd*-diagnostic PCR results revealed that 7 out of 20 isolates tested positive for a 650 bp size (skin swab: 3 isolates; environment: 4 isolates) (Figure 3).

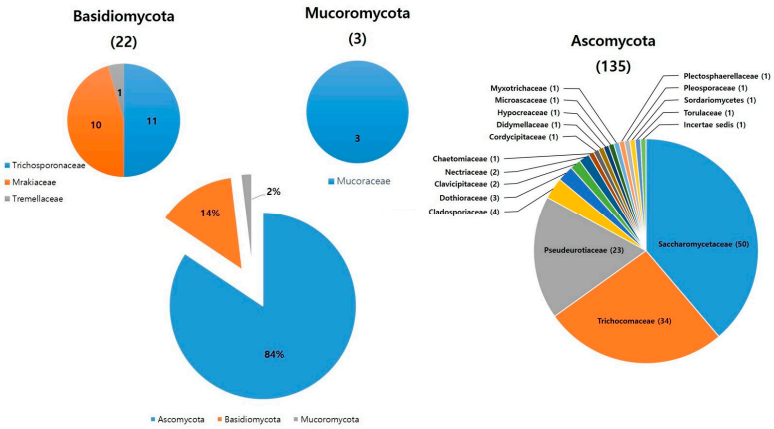
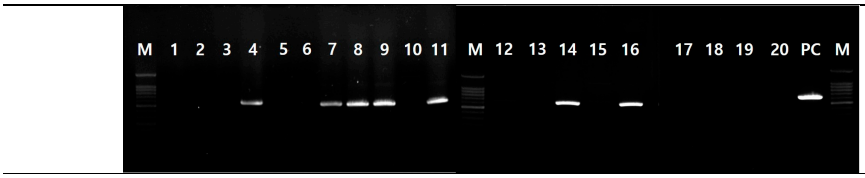


Figure 2. Fungal diversity frequencies by phylum and family. One hundred and fifty-four fungal colonies were isolated. *Ascomycota* was the most dominant (84%).



No.	Strain	No.	Strain	No.	Strain
1	19BE01LM3	8	20BSS17OM1	15	20BE20LM3
2	19BE02LM3	9	20BSS17OM2	16	20BE25LM2
3	19BE05LM1	10	20BE01LM1	17	20BE27LM1
4	19BG05LM3	11	20BE05LM1	18	20BE35LM1
5	19BG18LM2	12	20BE06LM1	19	20BE36LM4
6	19BOS05LM2	13	20BE09LM2	20	20BE38LM1
7	19BSS04LM4	14	20BE10LM1	PC	20631-21 (ex-type)

Figure 3. Confirmation of *Pseudogymnoascus* species by *Pd*-specific diagnostic primers. All the isolated *Pseudogymnoascus* species were verified using a *Pd*-specific diagnose primer. Of the 20 fungi, excluding positive control, 7 tests were positive [45].

3.4. Phylogenetic Analysis of Pseudogymnoascus

After excluding non-overlapping ends and LSU and TEF1 introns, the concatenated alignment contained 3199 nucleotides (ITS, 486; LSU, 934; MCM7, 503; RPB2, 526; and TEF1, 750). The BI and ML trees showed identical topologies. Therefore, only the BI tree is shown (Figure 4).

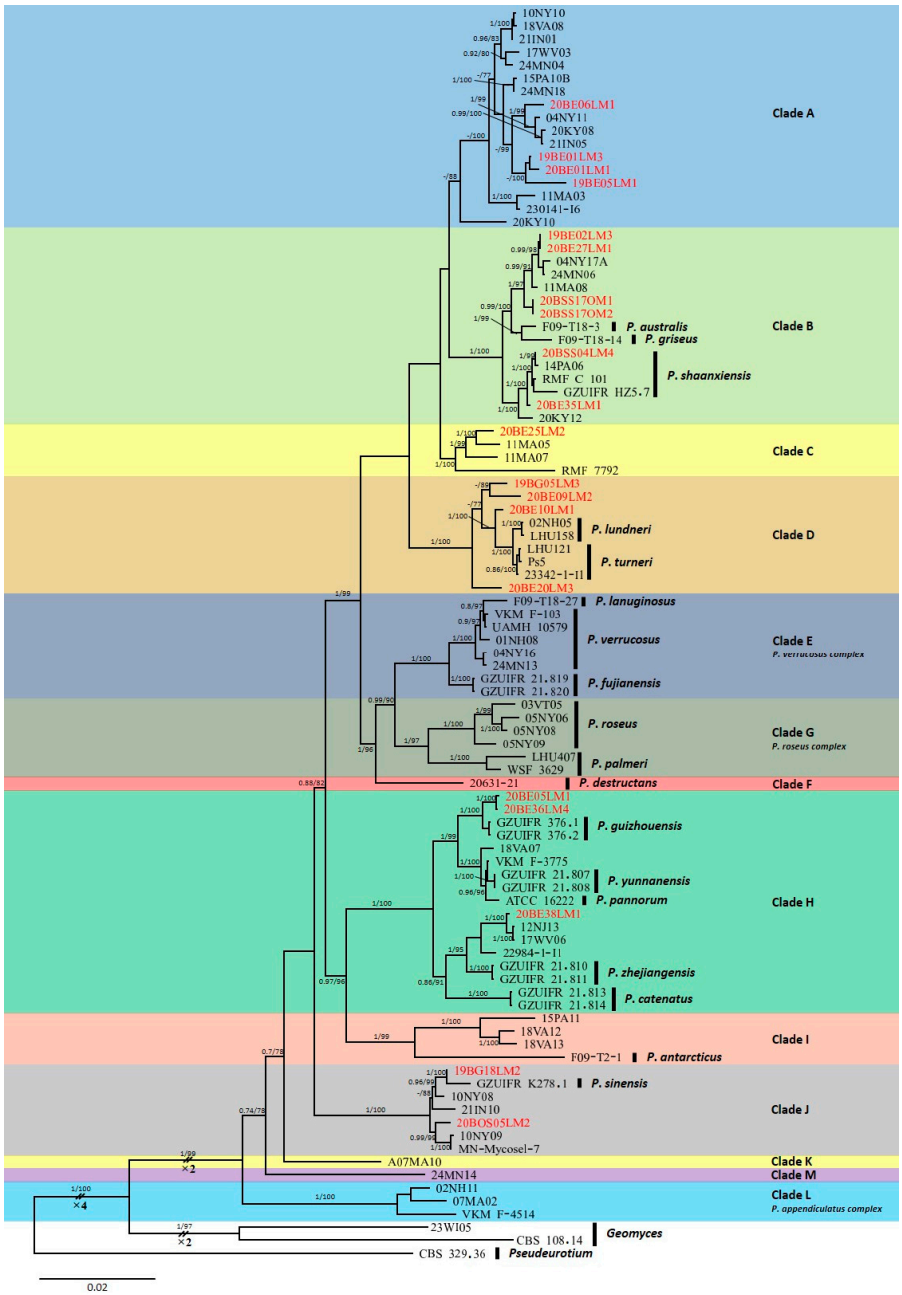


Figure 4. Bayesian inference phylogenetic tree of *Pseudogymnoascus* generated from the concatenated dataset of five loci (ITS, LSU, TEF1, RPB2, and MCM7) [22,26–28]. Bayesian posterior probabilities (BPPs) and significant ML bootstrap (BS) values are indicated with branches. Only clades that received 0.70 BPP and 70% BS simultaneously were considered to be strongly supported and are presented at the branches. Clades are identified using clade nomenclature (A to M), formally defined by Minnis and Lindner [26]. The scale bar indicates 0.02 nucleotide changes per site. Isolated strains in this study are highlighted in bold and red.

The clades achieved by the BI method were named A to M, as defined by Minis and Lindner [26]. The *Pseudogymnoascus* isolates fell in clades A (4), B (5), C (1), D (4), H (3), and J (2) (Figure 4). None of the *Pseudogymnoascus* isolates fell into *Pd* as clade F.

4. Discussion

A total of 154 fungi were isolated from the bat and habitat environment samples. The most dominant family of isolated fungi from environmental samples was *Trichocomaceae*, which includes *Penicillium* and *Aspergillus* (Tables 1 and S1). These two genera have been reported as the most common fungi in the cave environment, along with *Cladosporidium* [51]. The most abundant family of isolated fungi from bat samples was Saccharomycesaceae, which includes Debaryomyces and Candida (Tables 2 and S1). To begin with, there was a report that Candida and Debaryomyces were identified in bat feces [52,53]. In addition, Debaryomyces is known as a common yeast found in bat skin [54,55]. In conclusion, it is assumed that these fungi continue to circulate in bat-dwelling environments and bats. Except for these two families, the notable one was the family *Pseudeurotiaceae* (24), which was abundantly isolated in both environmental and bat samples (Tables 1 and 2). This family contains *Pseudogymnoascus* (20) and *Leuconeurospora* (4) (Figure 2 and Table S1). The genus that constituted the most significant proportion of *Pseudeurotiaceae* was *Pseudogymnoascus*, which is abundantly present as a keratolytic fungus in cave environments and as a saprophyte in cold soils and tree roots [4,21,23]. In addition, the high abundance of *Pseudogymnoascus* is assumed to be caused by the fact that the cave environment is suitable for its growth. The optimal temperature for *Pseudogymnoascus* growth is 15 °C [22], and the average temperature in the cave from which the samples were collected was 11 °C.

A *Pd*-diagnostic test using *Pd*-specific primers showed that 7 out of 20 *Pseudogymnoascus* species were positive. However, the phylogenetic tree revealed they were not located within clade F where *Pd* belongs. Lorch reported a 100% *Pd*-specific diagnostic PCR primer specificity [28]. However, in this study, a 35% false positive rate was observed (Figures 3 and 4). The *Pd*-diagnostic primer described by Lorch contains a portion of the intron of SSU and the ITS1 5.8 s region [28]. SSU is used to analyze high taxonomic levels (family, order, class, and phyla) due to low variation between taxa [56]. In addition, ITS is a region used to analyze low taxonomic levels due to relatively high variations between taxa groups, but it does not work well in some fungal genera, and it was reported that the intergenomic ITS variation does not occurs largely in 3–5% of Ascomycota and Basidiomycota [37,56]. Considering this information, false positive results were presumed to be because *Pd* and the isolates were of the same genus. Even when isolates were not *Pd* (Figure 4), because this region is analogous to *Pd*, it is assumed that the diagnostic PCR result was positive (Figure 3). Further studies, such as improving the false positive rate of diagnostic primers for fungi in the same genus or developing new diagnostic methods using other target regions, are required.

Isolates 19BE01LM3, 20BE01LM1, and 19BE05LM1 fell in clade A. 19BG05LM3, 20BE09LM2, 20BE10LM1, and 20BE20LM3 were located in clade D. However, they were in independent branches of previously known species (Figure 4) [22,26–28]. These isolates are new undescribed species, which require future morphological analyses.

Pseudogymnoascus isolates known to date are predominantly from the United States and China, except for some from Antarctica (Table S3). Most studies have been conducted in North America and China, and recent studies in Antarctica have been reported [22,26–28]. *Pseudogymnoascus* species have been isolated several times throughout the United States and constitute an entire clade, most of which have been isolated from bat caves [22,26]. Furthermore, the *Pseudogymnoascus* species reported from urban soil in various regions of China were located in clades B, E, H, and J [27,28]. In this study, *Pseudogymnoascus* species were isolated from samples collected from bat caves in limited areas, but isolates were located in various clades (A, B, C, D, H, and J). Some of those isolated are new undescribed species. Samples collected from more regions in the future may contribute more to the study of *Pseudogymnoascus*.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/d15020198/s1: Table S1: Identification of isolated fungi and percentage at phylum, family, and genus level; Table S2: Primer information and PCR protocols used in this study; and Table S3: GenBank accession numbers of the sequences used in this study [57–65].

Author Contributions: J.-K.O. is the senior author of this study and, as such, has full access to all data in the study and takes responsibility for the integrity of the data and the accuracy of the data analyses. Y.-S.K., S.-Y.L. and J.-K.O. conceived and designed the study. J.-S.P. and Y.-J.K. collected samples from bats and caves. C.-U.C. captured the bats and supplied the bat information. All authors contributed to the materials and data collection. Y.-S.K. performed the experiments and analyzed the data. Y.-S.K., S.-Y.L. and J.-K.O. drafted the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The authors confirm that the study was performed in compliance with the ethical policies of the journal as specified in the author guidelines. This study was approved by Jenobuk National University Laboratory Animal Research Center (JBNU2021-067).

Informed Consent Statement: Not applicable.

Data Availability Statement: Isolated fungal sequences were submitted to the NCBI GenBank database under accession numbers.

Conflicts of Interest: The authors declare no conflict of interest.

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