

Article

Keratinophilic and Keratinolytic Fungi in Cave Ecosystems: A Culture-Based Study of Brestovská Cave and Demänovská Ľadová and Slobody Caves (Slovakia)

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Abstract: Despite speleomycological research going back to the 1960s, the biodiversity of many specific groups of micromycetes in underground sites still remains unknown, including keratinolytic and keratinophilic fungi. These fungi are a frequent cause of infections in humans and animals. Since subterranean ecosystems are inhabited by various animals and are a great tourist attraction, the goal of our research was to provide the first report of keratinophilic and keratinolytic fungal species isolated from three caves in Tatra Mts., Slovakia (Brestovská, Demänovská Ľadová and Demänovská Slobody). Speleomycological investigation was carried out inside and outside the explored caves by combining culture-based techniques with genetic and phenotypic identifications. A total of 67 fungal isolates were isolated from 24 samples of soil and sediment using Vanbreuseghem hair bait and identified as 18 different fungal species. The study sites located inside the studied caves displayed much more fungal species (17 species) than outside the underground (3 species), and the highest values of the Shannon diversity index of keratinophilic and keratinolytic fungi were noted for the study sites inside the Demänovská Slobody Cave. Overall, *Arthroderma quadrifidum* was the most common fungal species in all soil and/or sediment samples. To the best of our knowledge, our research has allowed for the first detection of fungal species such as *Arthroderma eboreum*, *Arthroderma insingulare*, *Chrysosporium europae*, *Chrysosporium siglerae*, *Keratinophyton wagneri*, and *Penicillium charlesii* in underground sites. We also showed that the temperature of soil and sediments was negatively correlated with the number of isolated keratinophilic and keratinolytic fungal species in the investigated caves.

Keywords: caves; Slovakia; micromycetes; dermatophytes



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1. Introduction

Underground ecosystems are characterized by unique living conditions [1,2], such as persistent low temperatures over the year in facilities in Central Europe, oscillating from 6 to 10 °C [3–6], high humidity often close to saturation [7], limited presence of UV radiation and even light [8], limited or even no air exchange with the external environment [9], as well as sometimes an increased CO₂ content, especially in the deeper parts of the underground [10,11]. Therefore, cave settlers show peculiar adaptations and cave microbiota occur mainly in the form of spores or other propagation structures [12,13].

Fungi belonging to the phylum Ascomycota dominate in underground sites, where they constitute ca. 69% of all cultured fungi, and some common species found in caves belong to *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, and *Penicillium* genera [12,14,15]. However, such extreme environments promote caves and other underground structures

as a source of unique microbial isolates including extremophiles, due to light and carbon sources scarcity. Recently, we have gained an insight into cold-adapted aeromycota in the Brestovská Cave (Slovakia), providing a first report on the occurrence in underground ecosystems of such fungal species as *Coniothyrium pyrinum*, *Cystobasidium laryngis*, *Leucosporidium drummii*, *Mrakia lollopiis*, *Nakazawaea holstii* [16]. Probably the cause of this state of affairs is global warming, which also reaches the interior of underground facilities, creating more favorable temperature conditions for the development of a wider group of fungi, as well as anthropogenic factors [17,18]. It should be mentioned that the inside temperature of underground ecosystems is correlated with the surface atmospheric temperature [19].

Wigley and Brown [20] proved that external air penetrating caves reaches an almost constant temperature within the entrance sectors as a consequence of two factors: namely, a buffering effect linked to the relative humidity increase, as well as progressive equilibration with the temperature of rocks [20]. Thus, any alternation in the average annual value of the outside temperature proportionally affects mean air temperature inside caves [21]. Consequently, the diversity of microbial communities in underground sites is likely to change with the temperature increase and may lead to the extinction of the cold-adapted in favor of the more mesophilic and competitive species, including potential pathogens [22–24].

It should be mentioned that the biodiversity of mycobiota among caves is not only limited by environmental conditions but also by nutrient availability. Underground ecosystems are seemingly nutrient-poor, which might include organic matter mostly from the outside environment [1,13,25]. However, the presence of various deposits of animal or human origin vastly affects nutrient availability and consequently biodiversity [6,26]. Therefore, Slovak caves have been one of the great examples of fungal biodiversity studies due to their being important underground localities for *Myotis* spp. bats [27] and open tourist attractions [16,28,29].

During most studies of cave mycobiota in Slovak underground environments, the main focus has been on the diversity of microscopic fungi in cave air [16,27,28,30], rock surfaces [26,29] or bat guano [27]. However, from the evaluation of soil and sediment samples taken in and outside of the Harmanecká Cave, we have recently isolated a dermatophyte belonging to the *Microsporium cookei* clade with close affinities to *Paraphyton cookei* [31]. Dermatophytes are representatives of keratinolytic and keratinophilic fungi, which are able to dissolve keratin or keratin-bound substances, respectively, and thus may be infectious towards humans and animals [31–33]. *M. cookei* strains isolated from Harmanecká Cave were capable of dissolving keratin during in vitro tests and proliferating within 37 °C. Both traits suggest their potential as mammalian pathogens [31].

This promising report has directed us to continue our research and thus, the present study provides the first overview of keratinophilic and keratinolytic fungal species isolated from the Brestovská, Demänovská Ľadová and Demänovská Slobody Caves belonging to the Tatra Mountains in north Slovakia. In order to discuss the potential origin of the fungal species within those ecosystems, we determined species diversity in both outdoor and indoor soil and sediment samples.

2. Materials and Methods

2.1. Study Area

All three caves (Brestovská Cave, and Demänovská Ľadová and Slobody Caves) in which speleomycological studies took place are show caves, and the Demänovská Slobody Cave is the most visited underground site in Slovakia [34].

The Brestovská Cave is located near the Zuberec village in the Tatra National Park in Slovakia, in the Western Tatra Mountains [35]. With its length of 1890 m, it is the largest cave in the Orava region and the only one open for tourists (217 m). The exact location of the cave entrance is 867 m a.s.l. The cave itself is part of a large hydrologic system, with unique environmental conditions. The inner temperature of 4–6 °C and the presence of a running river inside the cave enables inhabitation by mammals. Nine bat species have

been described in the Brestovská Cave, and the most common is the greater mouse-eared bat (*Myotis myotis*) [36].

On the other hand, the Demänovská Ľadová and Slobody Caves are both part of the Demänovská Cave system, the longest cave system in Slovakia, located on the northern side of the Low Tatras Mountains, in the national nature reserve Demänovská Valley and in the territory of the Low Tatras National Park [37]. The entrance of the Demänovská Ľadová Cave is located at 840 m a.s.l. [38], whereas the Demänovská Slobody Cave's entrance is at 870 m a.s.l. Both caves differ in their environmental conditions. The Demänovská Ľadová Cave's corridors are 1750 m long (with 650 m available for tourists and a height amplitude of 57 m). The air temperature in the permanent ice zone oscillates around 0 °C, and it rises to 5.7 °C near the cave entrance. The air relative humidity is between 92% and 98%. On the other hand, the Slobody Cave corridors are 8126 m long (with maximal tourist path of 2150 m, and a height amplitude of 86 m). The air temperature fluctuates between 6.1 and 7.6 °C, and the air relative humidity is 94–99% [39,40].

Both caves are important bat-inhabited places in Slovakia. Until now, the presence of 8 bat species has been noted in the Demänovská Ľadová Cave, which seems to be the wintering place of the northern bat (*Eptesicus nilssonii*) and the whiskered/*Brandt's* bat (*Myotis mystacinus/Myotis brandtii*). The Slobody Cave is inhabited by 4 bat species, most frequently by *M. myotis* and the whiskered bat (*Myotis mystacinus* Kuhl) [39,40].

2.2. Temperature Measurement

The soil and sediment temperature was measured nine times at each sampling site, prior to sampling (From I to VIII; Figure 1). For this purpose, a Checktemp® thermometer (HANNA Instruments, range: from −50 °C to +150 °C, accuracy ± 0.2 °C) was used.

2.3. Sample Collection

The sediment and soil samples were taken on 23 August (the Demänovská Ľadová Cave) or 24 August (the Brestovská Cave and the Slobody Cave) 2017 from outdoor and indoor locations of the caves according to Ogórek et al. [31]. Approximately 1000 g of soil/sediment was collected from each tested site using sterile plastic scoops and placed in the sterile bags, followed by the transportation (at 10 ± 2.0 °C) and storage at 7 ± 0.5 °C until mycological testing (up to 7 days).

2.4. Isolation of Fungi from Samples

Fungi were isolated using Vanbreuseghem hair bait [41]. Briefly, a clump of sterile human hair and distilled water were placed on the soil and sediment sample in the Petri dish. Plates were then covered and incubated at 24 ± 1.0 °C for 8–12 weeks, with distilled water supplemented if needed. The mycelium that appeared on the baits were transferred to SGA (Sabouraud's glucose agar: 1000 mL distilled water, 10 g peptone, 40 g glucose, and 15 g agar), supplemented with ampiciline (50 mg·L⁻¹), chloramphenicol (50 mg·L⁻¹), and cycloheximide (100 mg·L⁻¹). The grown colonies of keratinophilic and keratinolytic fungi were subcultured on PDA (potato dextrose agar, Biomaxima, Poland) and incubated in the dark at room temperature (25 ± 0.5 °C) for 4 weeks. Then, fungi were separated by the single spore method and subcultured on PDA slants [31]. The grown cultures were inoculated on PDA plates and such isolates were used for morphological and molecular identification.

2.5. Fungal Identification

Identification was performed by morphological and molecular methods [42–55]. In detail, a molecular analysis was performed, for which DNA was extracted from a 28-day-old culture on PDA using Bead-Beat Micro AX Gravity kit (A&A Biotechnology, Gdańsk, Poland) according to the manufacturer's instructions. Fungal internal transcribed spacer regions (ITS) were amplified using two fungal-specific PCR primers: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [56]. PCR was performed using a T100 Thermal Cycler (Bio-Rad, Berkeley, CA, USA) accord-

ing to Ogórek et al. 2016. The PCR product was purified using Clean-Up Kit (A&A Biotechnology, Gdańsk, Poland), and sequenced at Macrogen Europe.

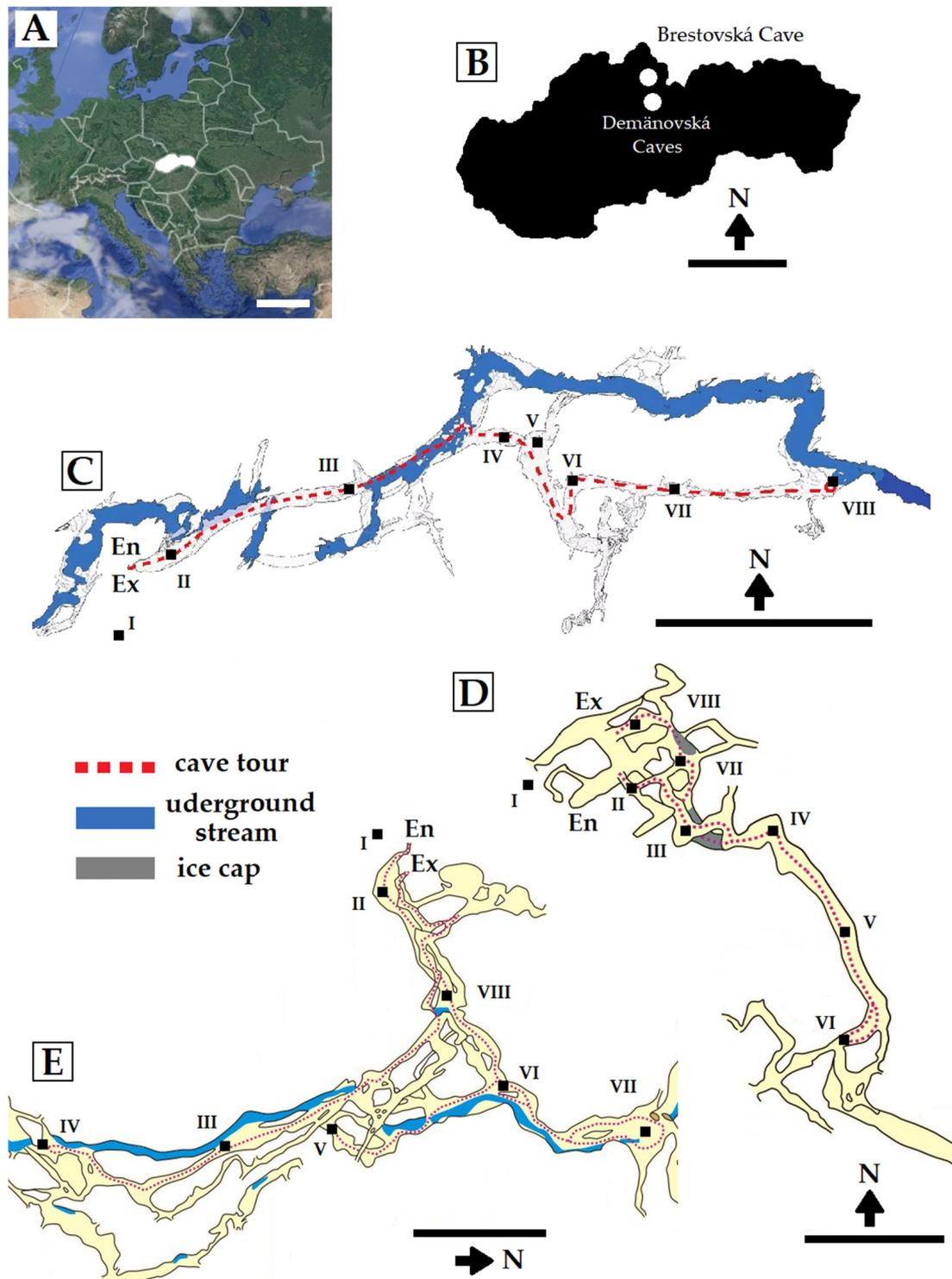


Figure 1. Geographic location of Slovakia (A) and the studied caves (B). Entry (En) and exit (Ex) from the Brestovská Cave (C), the Demänovská Ľadová Cave (D), and the Demänovská Slobody Cave (E). Underground corridors with marked sampling locations on the tourist route: location I outside the caves, and locations II to VIII inside the caves. Scale bars: (A) = 500 km, (B) = 100 km, (C) = 50 m, (D,E) = 100 m.

2.6. Data Analyses

Raw sequences were analyzed with the BioEdit Sequence Alignment Editor, and the obtained PCR products were compared with those deposited in the GenBank of the National Center for Biotechnology Information (NCBI, Bethesda, Rockville, MD, USA) using the BLAST algorithm, and submitted into the mentioned database (Table S1). The isolates were placed in the collection of the Department of Mycology and Genetics, University of Wrocław.

The data obtained on the soil and sediment temperatures were analyzed using one-way analysis of variance (ANOVA) and Tukey's HSD (honest significant difference) test at $\alpha \leq 0.05$ using Statistica 13.0 package (StatSoft Polska Sp. z o.o., Kraków, Poland). The Shannon diversity index (H) was used to determine the diversity of fungal species: $H = -\sum P_i(\ln P_i)$, where P_i stands for the proportion of each species in the sample [57,58]. The Pearson correlation coefficient (r) was used to determine the relationship between the number of keratinophilic and keratinolytic fungal species and the soil/sediment temperatures.

3. Results

Soil and/or sediment temperatures ranged from 10 to 14.7 °C outside the caves (study sites number I) and from 0.8 to 7.4 °C inside them ($p_{\text{study sites I, II}} = 0.000175$ for the Brestovská Cave, $p_{\text{study sites I, VIII}} = 0.000175$ for the Demänovská Ľadová Cave and $p_{\text{study sites I, VI}} = 0.000175$ for the Demänovská Slobody Cave) (Table 1). The lowest temperature of the soil and/or sediment samples was recorded at the study sites VI and VII for the Brestovská Cave ($p_{\text{study sites II, VI}} = 0.002538$), the study site VII for the Demänovská Ľadová Cave ($p_{\text{study sites III, VII}} = 0.000175$), and in the study site VIII for the Demänovská Slobody Cave ($p_{\text{study sites III, VIII}} = 0.030805$) (Table 1).

Table 1. The average of temperature of soil/sediment ($n = 9$) measured in the studied Slovak caves. ¹ See Figure 1 for locations (I—outside underground facilities, II–VIII—inside underground facilities). ² Letters refer to the Tukey's HSD test ($\alpha \leq 0.05$) and different letters indicate significant differences between the temperature of soil and sediment in a given location; they refer to means along the columns.

Study Sites ¹	Temperature of Soil/Sediment (°C)					
	Brestovská Cave		Demänovská Ľadová Cave		Demänovská Slobody Cave	
I	10.0	a ²	10.8	a	14.7	a
II	6.3	b	3.5	c	7.2	c
III	6.2	bc	2.6	d	6.9	d
IV	6.1	bc	3.5	c	6.9	d
V	6.1	bc	2.7	d	7.1	c
VI	6.0	c	2.8	d	7.4	b
VII	6.0	c	0.8	e	7.2	c
VIII	6.2	bc	4.1	b	6.7	e

A total of 67 keratinophilic and keratinolytic fungal isolates were cultured from 24 samples of soil/sediment collected from the 3 studied caves. Isolates were clustered into 32 major groups through macro- and micro- morphological characteristics (Table S1). In turn, the ITS sequence of 32 fungi showed that they belonged to 18 different fungal species (Table 2). PCR products of the nucleotide sequences (ITS rDNA) from 32 tested fungal cultures ranged from 362 to 536 bps. The sequences were submitted to GenBank under the accession numbers from OL362050 to OL362081. Based on a BLAST analysis, the E values were zero, the percentages of the query cover amounted to 100%, and identity ranged from 99.28 to 100% (Table S1).

Table 2. Keratinophilic and keratinolytic fungal species isolated from soil/sediment samples in the studied Slovak caves. ¹ See Figure 1 for locations (I—outside underground facilities, II–VIII—inside underground facilities). ² numbers indicate locations in the particular caves: ³ B—Brestovská Cave, L—Demänovská Ľadová Cave, and S—Demänovská Slobody Cave.

Fungi		Study Sites ¹
Family	Species	
Arthrodermataceae	<i>Arthroderma eboreum</i>	V ² S ³ , VIII S
	<i>Arthroderma insingulare</i>	IV L, VII L, VIII L
	<i>Arthroderma multifidum</i>	VII L, II S, III S, IV S
	<i>Arthroderma quadrifidum</i>	I B, III B, VII B, III L, IV L, V L, VII L, I S, II S, III S, VII S, VIII S
Aspergillaceae	<i>Aspergillus fumigatus</i>	I L
	<i>Penicillium brevicompactum</i>	VII B, IV L, VI S
	<i>Penicillium charlesii</i>	V B
	<i>Penicillium chrysogenum</i>	VII S
	<i>Penicillium griseofulvum</i>	II L
Clavicipitaceae	<i>Metapochonia suchlasporia</i>	II B, III B, I L
Cordycipitaceae	<i>Cordyceps fumosorosea</i>	III B, VIII B
Nectriaceae	<i>Cosmospora viridescens</i>	VII S
Onygenaceae	<i>Aphanoascus keratinophilus</i>	VI S
	<i>Chrysosporium europae</i>	V B
	<i>Chrysosporium merdarium</i>	IV B, VI B, III L, V S
	<i>Chrysosporium siglerae</i>	II L, VII L
	<i>Keratinophyton wagneri</i>	VII S
Pseudeurotiaceae	<i>Pseudogymnoascus pannorum</i>	V B, VII B, III L, IV L, V L, VIII L, III S, V S, VI S

All identified keratinophilic and keratinolytic fungal cultures belonged to Ascomycota, including 7 families (Arthrodermataceae, Aspergillaceae, Clavicipitaceae, Cordycipitaceae, Nectriaceae, Onygenaceae, and Pseudeurotiaceae), and 10 genera (*Aphanoascus*, *Arthroderma*, *Aspergillus*, *Chrysosporium*, *Cordyceps*, *Cosmospora*, *Keratinophyton*, *Metapochonia*, *Penicillium*, and *Pseudogymnoascus*). Most fungal species belonged to the Aspergillaceae family, and *Arthroderma* or *Penicillium* genera (Table 2).

All fungi were detected inside the caves, except *Aspergillus fumigatus*, which was the sole species isolated from the outer study sites of the Demänovská Ľadová Cave (Table 2). Overall, *Arthroderma quadrifidum* was the most common fungus (12 out of 24 study sites) in the soil and/or sediments samples collected from inside and outside locations in all of the three studied caves (Table 2). The propagation structures of this species accounted for 50% of all fungi cultured from the samples outside the underground sites, and 20.8% of all fungi obtained from the samples inside the caves. Moreover, *Pseudogymnoascus pannorum* should also be mentioned, as it accounted for 18.7% of the total from the samples inside the caves (Figure 2).

In the case of the inner underground sites, Demänovská Slobody was the most enriched in fungi (10), whereas in the other two caves the numbers of isolated species were at the same levels (8 in each cave) (Tables 2 and S2). The Shannon diversity index of fungal species was 0.784 for Brestovská Cave, 0.814 for Demänovská Ľadová Cave, and 0.816 for Demänovská Slobody Cave (Table S2). *Arthroderma quadrifidum*, *Chrysosporium europae*, *Ch. merdarium*, *Cordyceps fumosorosea*, *Metapochonia suchlasporia*, *Penicillium brevicompactum*, *P. charlesii*, and *Pseudogymnoascus pannorum* were cultured from the internal samples of the Brestovská Cave, while *A. insingulare*, *A. multifidum*, *A. quadrifidum*, *Ch. merdarium*, *Ch. siglerae*, *P. brevicompactum*, *P. griseofulvum*, and *P. pannorum* were isolated from the interior of the Demänovská Ľadová Cave. On the other hand, soil/sediment samples taken from the inside of Demänovská Slobody Cave contained keratinophilic and kerati-

nolytic species such as: *Aphanoascus keratinophilus*, *A. eboreum*, *A. multifidum*, *A. quadrifidum*, *Ch. merdarium*, *Cosmospora viridescens*, *Keratinophyton wagneri*, *P. brevicompactum*, *P. chrysogenum*, and *P. pannorum* (Figure 3).

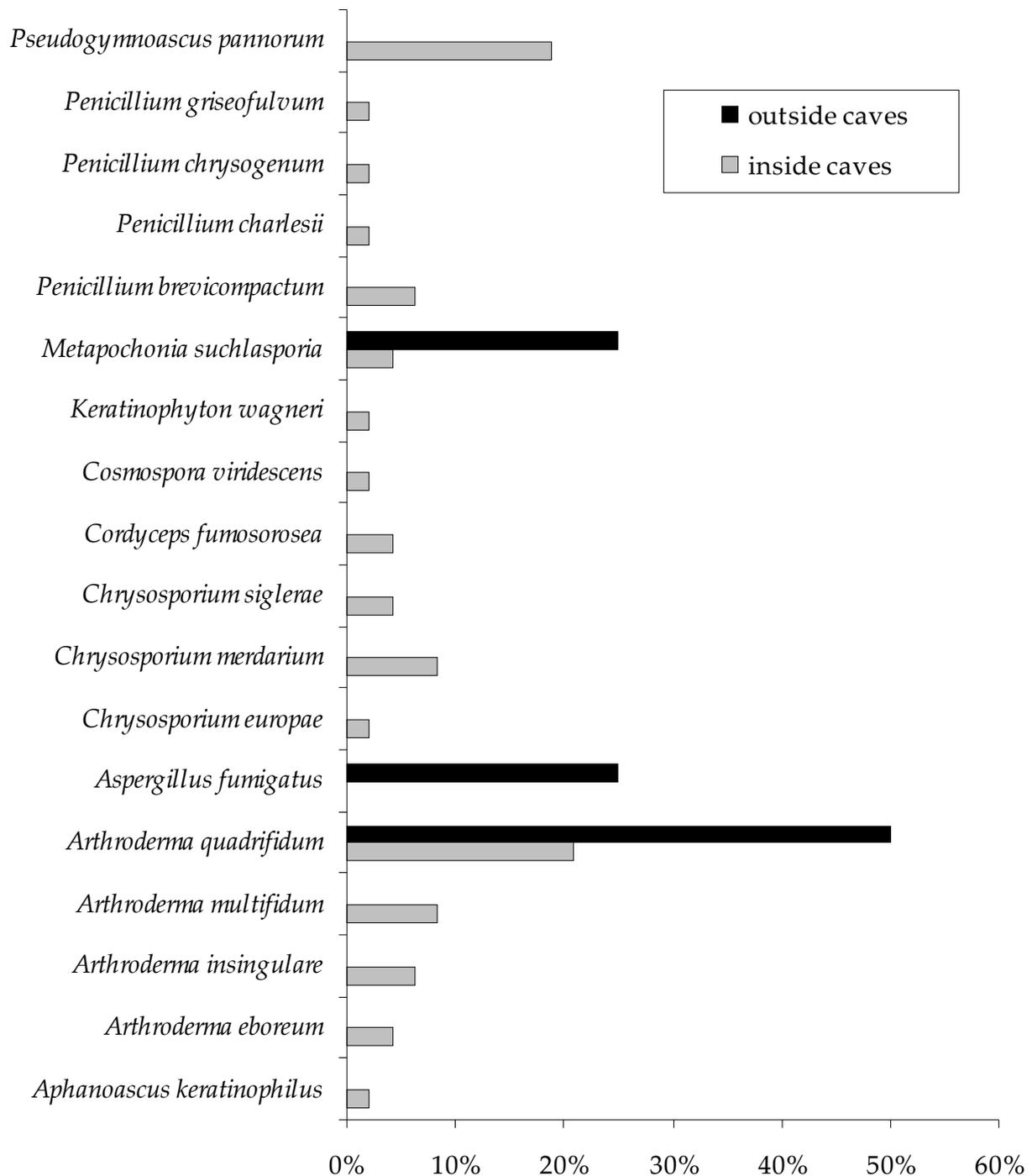


Figure 2. The percentage contribution of each isolate from the soil/sediment samples to the total from all caves.

The most frequently isolated species from the soil/sediment inside the Demänovská Slobody Cave was *A. quadrifidum*, and *A. quadrifidum* and *P. pannorum* in the case of the Demänovská Ľadová Cave, which accounted for 22.2% and 23.5% of all fungi cultured from the samples inside these caves, respectively. In turn, the most often isolated species (15% in

each case) from the interior of the Brestovská Cave were: *A. quadrifidum*, *Ch. merdarium*, *C. fumosorosea*, *M. suchlasporia*, and *P. pannorum* (Figure 3).

Overall, the temperature of soil/sediments correlated negatively with the number of isolated keratinophilic and keratinolytic fungal species in all tested caves ($p < 0.05$; $r = -0.30$) (Figure 4A). The same negative trend was recorded in the case of individual caves: namely, the number of fungal species did not increase with increasing temperature ($p < 0.05$; $r = -0.31$ for the Brestovská Cave, $r = -0.21$ for the Demänovská Ľadová Cave, and $r = -0.54$ for the Demänovská Slobody Cave) (Figure 4B–D).

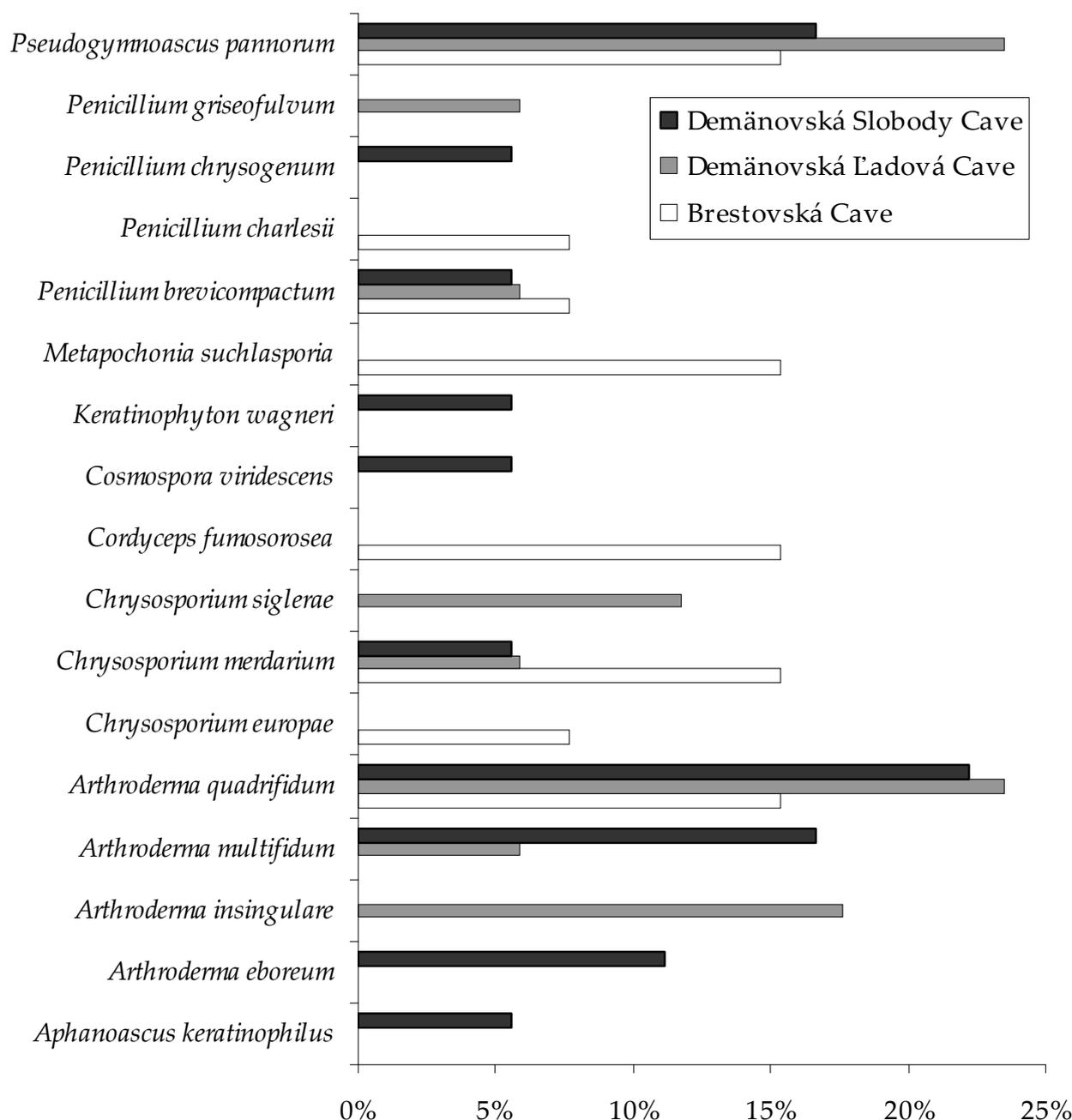


Figure 3. The percentage contribution of each isolate from the soil/sediment samples to the total from particular caves for the interior study sites.

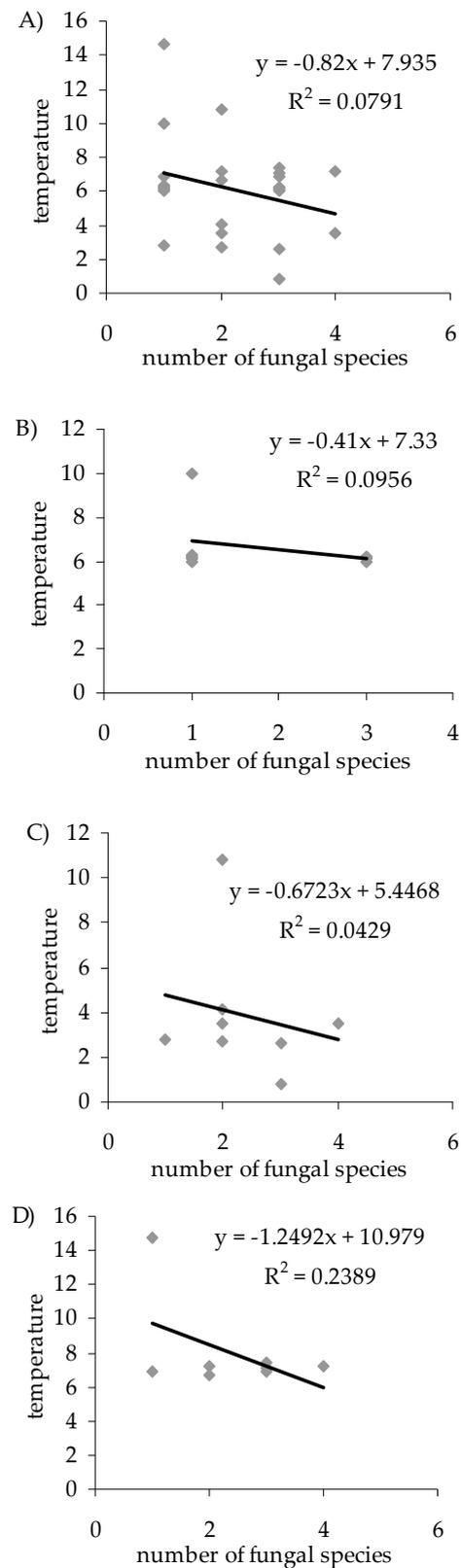


Figure 4. Relationship between the number of keratinophilic and keratinolytic fungal species and the soil/sediment temperatures (°C) in all the caves (A) ($p < 0.05$; $r = -0.30$); the Brestovská Cave (B) ($p < 0.05$; $r = -0.31$); the Demänovská Ľadová Cave (C) ($p < 0.05$; $r = -0.21$); and the Demänovská Slobody Cave (D) ($p < 0.05$; $r = -0.54$).

4. Discussion

Studies on fungal diversity depend on the correct identification of species. Classical identification relies on direct observation of fungi either in their natural condition or after culturing in different growth media [31]. However, currently, mycotaxonomy combines the use of molecular methods along with conventional ones. Such an approach allows discovering new or reinvestigating already known taxa [16,59]. For example, dermatophytes used to be classified as genera *Trichophyton*, *Epidermophyton* and *Microsporum*. However, combinatory molecular analyses (sequencing of ITS rDNA, ribosomal 60S subunit, β -tubulin fragments, and translation elongation factor 3) allowed for the reclassification of dermatophytes into the following genera: *Trichophyton*, *Epidermophyton*, *Nannizia*, *Paraphyton*, *Lophophyton*, *Microsporum*, *Arthroderma*, *Ctenomyces*, and *Guarromyces* [60].

In the present study, we followed this trend by using targeted growth conditions (Vanbreuseghem hair bait) as well as a combination of classical and molecular identification approaches in order to report the occurrence of keratin-dependent fungal species in three studied caves (Brestovská, Demänovská Ľadová and Demänovská Slobody) belonging to the Tatras Mountains in north Slovakia. The interest in soil mycobiota capable of keratin degradation originates in two factors: the biochemical resistance of keratin and the pathogenic potential of keratinolytic saprophytic species [61]. In the present paper, we followed the broad definition of keratin-associated fungi, which divides them into keratinolytic and keratinophilic species. Accordingly, keratinolytic fungi are able to utilize keratin, while keratinophilic fungi utilize non-keratinous components of keratinous substrata or by-products of keratin degradation [62]. In natural environments, the presence of keratin-associated fungi depends on the availability of keratinic substrata of animal or human origin [63]. The constant flow of such organic matter has been associated with the distribution of keratinolytic fungi in environments such as arable soils [64–67], sewage sludge and river bottom sediments [68,69], compost [70], bird feathers [71], the deposits of free-living rodents [72], the nests of water birds [73], and pellets of birds of prey [74].

Keratinolytic fungi are classified as dermatophytes and *Chrysosporium*, or according to Blyskal (2009) into two orders: Onygenales and Eurotiales [75]. Kunert (2000) emphasized that fungi belonging to Onygenales are highly specialized in keratin degradation and include six genera, namely *Arthroderma*, *Aphanoascus*, *Epidermophyton*, *Microsporum*, *Trichophyton*, and *Chrysosporium* [76]. However, the diversity of keratinolytic species is considered to be much broader [67,77] and includes species from the genera *Trichoderma*, *Fusarium*, *Cladosporium*, *Phytophthora* [78], and *Talaromyces* [79], as well as *Aspergillus niger* and *Aspergillus fumigatus* [71]. In fact, the definition can be even wider as some species or genera are newly reported to be keratin-dependent, such as *Pseudogymnoascus destructans* [80].

It should be emphasized that underground objects display harsh living conditions for microorganisms because they are heterotrophic ecosystems (with few exceptions, e.g., the Movile Cave “Peștera Movile” in Romania, a sulfur-based chemolitho-313 autotrophic ecosystem [81]) Despite this fact, in this study we reported the presence of 10 keratin-dependent genera, namely *Aphanoascus*, *Arthroderma*, *Aspergillus*, *Chrysosporium*, *Cordyceps*, *Cosmospora*, *Keratinophyton*, *Metapochonia*, *Penicillium*, and *Pseudogymnoascus* (Table 2). All of these belong to the phylum Ascomycota, which is in agreement with Vanderwolf [12].

Out of the 18 different fungal species identified herein, the following were already reported by Vanderwolf et al. [12] to colonize underground sites: *A. fumigatus*, *A. quadridum*, *A. keratinophilus* (reported as *Chrysosporium keratinophilum*), *Ch. merdarium*, *C. fumosorosea* (reported as *Isaria fumosorosea*), *M. suchlasporia* (reported as *Pochonia suchlasporia*), *P. brevicompactum*, *P. chrysogenum*, *P. griseofulvum*, and *P. pannorum*. Three years later, Vanderwolf et al. [82] reported the presence of *C. viridescens* in two caves in Canada: Grotte de la Baie de la Tour and Grotte du lac Maloin [82]. Held et al. [83] reported *C. viridescens* in an underground iron ore mine in Soudan (Minnesota, USA). Additionally, we came across a record from the early 1960s of the presence of *A. multifidum* (reported as *Chrysosporium* sp.) in a cave in Romania [84]. The literature has also reported on other species of keratinophilic and keratinolytic fungi that were detected in underground ecosystems such as *Arthroderma curreyi*,

Microsporium gypseum, *Trichophyton mentagrophytes*, *T. rubrum*, *T. terrestre*, and *M. cookie*, a clade with close affinities to *P. cookei* [31,85–89]. However, the methodology and results obtained by Lurie and Way [86] were imprecise (lack of adequate controls); the authors themselves admitted that they had no data on the animals being free from *T. mentagrophytes* or if the proper controls were included. In conclusion, to the best of our knowledge, *A. eboreum*, *A. insingulare*, *Ch. europae*, *Ch. siglerae*, *K. wagneri*, and *P. charlesii* have not yet been described in caves nor mines. Thus, we believe that we are the first to report their occurrence in underground sites.

Arthroderma eboreum and *A. insingulare*, together with the most abundant *Arthroderma* in our research, *A. quadrifidum*, are representatives of the former *Trichophyton terrestre* complex. *Arthroderma eboreum* was first reported as *Trichophyton eboreum* in 2005 [90], while its teleomorph *Arthroderma olidum* was described the following year [91]. This species was isolated from the skin of an HIV-positive patient with *tinea pedis*. Despite being cured with the topical combination of cyclopiroxolamine and terbinafine [90], it was later described as fluconazole-resistant [92]. Similarly, *A. insingulare* is reputed to be involved in skin inflammatory disorders (including hyperpigmentation) and was described to be resistant towards griseofulvin [93]. The closely related *A. quadrifidum* is a known example of an opportunistic pathogen, the strains of which might demonstrate resistance towards itraconazole, nystatin, and griseofulvin [94].

Not much is known about *Ch. europae*, *Ch. siglerae* (currently known as *Keratinophyton siglerae* [95] and *K. wagneri*. *Chrysosporium europae* was reported as an isolate from soil and a children's sandpit in Slovakia [96]. This species displays a wide range of thermal tolerance as it was isolated from Antarctic permafrost sediments, but was also reported to survive heating up to 52 °C [97]. In addition, its nutrient requirements must be low as it is one of the most abundant species isolated from nutrient-poor coal ash heaps in Sosnowiec (Poland) [98]. *Chrysosporium siglerae* and *K. wagneri*, based on ITS phylogeny, seem to be closely related as they occupy close (but separate) terminal clusters [95]. Already known distinctive traits include the presence of intercalary conidia in *Ch. siglerae*, the ability of *Ch. siglerae* to grow at 37 °C, and much smaller conidium size in the case of *K. wagneri* [96]. Reporting their presence in underground sites may be of great importance in further understanding the biology of these poorly described fungal species.

On the other hand, *P. charlesii* is a species well-known for its wide metabolic abilities, and is constantly reported as useful in biotechnology. *P. charlesii* was reported to produce tannases—enzymes that catalyze the hydrolysis of ester and depside bonds in hydrolysable tannins, releasing glucose and gallic acid [99]. Additionally, *P. charlesii* is a known producer of alkaline proteases [100] and seven antinematodal and antiparasitic agents, including paraherquamide and its analogs [101]. Isolation and characterization of novel environmental strains of microorganisms already known for their biotransformation abilities may lead to further improving and better understanding biotechnological processes. The potential usefulness of *P. charlesii* strains isolated in our study is yet to be investigated.

It should also be mentioned that the survival and biodiversity of micromycetes in different environments are strictly dependent on microclimatic conditions, with temperature and humidity being the most crucial factors [102]. There is a positive correlation between the abundance and biodiversity of airborne fungi and air temperature in underground ecosystems [16]. However, in our research, we have shown that there is no similar relationship between soil/sediment temperature and the biodiversity of keratinophilic and keratinolytic fungi in the underground sites (Figure 4), as the number of fungal species did not increase with increasing temperature in all studied caves. More importantly, the number of species inside the studied caves was greater than those outside the underground sites. The species biodiversity of keratinophilic and keratinolytic fungi in the underground ecosystems, as in the case of fungi in the air, probably also depends on other factors, such as those of anthropogenic origin (e.g., the number of visitors, the period from which the object was made available to tourists) as well as the abundance of animals living in this type of site [6,103–105]. It can also be assumed that the specific microclimate inside the

caves contributes to the creation of a certain ecological niche in the context of micromycetes where keratinophilic and keratinolytic species have taken over and thus are so abundant inside the underground sites.

5. Conclusions

Our study contributes to gaining new knowledge about the diversity of keratinophilic and keratinolytic fungi inhabiting Slovak caves that are open to tourists in Central Europe. Overall, we isolated 18 fungal species, 17 of which were present inside the cave, but only 3 of which were found outdoors. One of the most commonly isolated species was *A. quadrifidum*, which belongs to the former *T. terrestre* complex. To the best of our knowledge, our research has allowed for the first detection of fungal species in underground ecosystems (*A. eboreum*, *A. insingulare*, *Ch. europae*, *Ch. siglerae*, *K. wagneri*, *P. charlesii*). Not only have we provided new insight into the biology of those species, but we also report that some of them (*A. eboreum*, *A. insingulare*) may present a threat towards immunocompromised patients and animals. Furthermore, we believe that underground sites may be a source of new strains for biotechnological applications, one example being *P. charlesii*. We also showed that the temperature of soil and sediments did not positively correlate with the number of isolated keratinophilic and keratinolytic fungal species in caves. Therefore, the species biodiversity of this group of fungi in underground ecosystems most likely depends on anthropogenic factors as well as the abundance of animals living in this type of site.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app12031455/s1>. Table S1. BLAST analysis of rDNA ITS of keratinophilic and keratinolytic fungi occurring in selected caves in Slovakia. All E values were zero). Table S2. Number of keratinophilic and keratinolytic fungal species isolated from the studied locations of Slovakia caves and the Shannon diversity index for the interior study sites. ¹ See Figure 1 for locations (I—outside underground facilities, II–VIII—inside underground facilities).

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