Life glowing in the darkness: fungal richness in Gruta da Viola cave, Minas Gerais

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Abstract

Caves are environments capable of forming a unique ecosystem that shelters a great diversity of organisms, including fungi. The fungal richness in cave is still little studied. This study aimed to identify the richness of culturable fungi in the Gruta da Viola marble cave in the Southern Espinhaço Mountain in Brazil. Samples of airborne particles, animal dung, organic litter, and sediment were collected. Total genomic DNA was extracted from the isolates and the ITS, LSU, *TUB*, *CAL*, and *RPB2* regions were amplified and sequenced for fungal identification. A total of 398 isolates were obtained, of which 127 have been identified at the genus level. *Ascomycota* was the dominant phylum, with *Eurotiales* and *Hypocreales* as the most frequently sampled orders. Notably, *Aspergillus* and *Penicillium* were the most abundant genera, while *Amphichorda* was prominent in animal dung. Several genera, including *Humicola*, *Staphylotrichum*, and *Trichocladium*, are reported in Brazilian caves for the first time. These findings underscore the potential of Brazilian caves as reservoirs of fungal diversity and highlight the need for further taxonomic studies to discover novel taxa in this unique environment.

Resumo

As cavernas são ambientes capazes de formar um ecossistema único que abriga uma grande diversidade de organismos, incluindo fungos. A riqueza fúngica em cavernas ainda é pouco estudada. Este estudo teve como objetivo identificar a riqueza de fungos cultiváveis na caverna de mármore Gruta da Viola, localizada na porção sul da Serra do Espinhaço, no Brasil. Amostras de partículas aéreas, fezes de animais, material orgânico em decomposição e sedimento foram coletadas. O DNA genômico total foi extraído dos isolados, e as regiões ITS, LSU, *TUB*, *CAL* e *RPB2* foram amplificadas e sequenciadas para a identificação fúngica. Foram obtidos 398 isolados, dos quais 127 foram identificados ao nível de gênero. *Ascomycota* foi o filo dominante, com *Eurotiales* e *Hypocreales* sendo as ordens mais frequentemente amostradas. Notadamente, *Aspergillus* e *Penicillium* foram os gêneros mais abundantes, enquanto *Amphichorda* foi predominante nas fezes de animais. Vários gêneros, incluindo *Humicola*, *Staphylotrichum* e *Trichocladium*, são relatados pela primeira vez em cavernas brasileiras. Esses achados destacam o potencial das cavernas brasileiras como reservatórios de diversidade fúngica e ressaltam a necessidade de estudos taxonômicos adicionais para descobrir novos táxons nesse ambiente.

1. Introduction

Cave ecosystems harbour a great diversity of microorganisms, including fungi (BARTON & JURADO, 2007; CUNHA et al. 2020). Although most fungi that inhabit the cave environment have evolved outside, they are well-adapted to live inside this ecosystem (ZHANG et al., 2018). The limited amounts of available nutrients and organic matter in a cave can be a challenge for the survival of several organisms. However, fungi have adapted to live in different cave substrates, such as animal dung and organic litter (ZHANG et al., 2018; ZHANG et al., 2021; ALVES et al., 2022; POLI et al., 2024). Nearly 2,000 fungal have already been documented in caves worldwide (ZHANG et al., 2021; VANDERWOLF et al. 2013). However, little is known about cave mycobiota in Brazilian caves.

Brazilian territory has more than 23,000 caves registered all over its different biomes, with nearly half of these recorded caves occurring in Minas Gerais state (ICMBio/CECAV, 2022). The fungal diversity in Brazilian caves has only recently been explored by researchers from different regions and biomes (PRAZERES et al., 2025). These advances have led to new discoveries regarding the mycobiota inhabiting Brazilian caves. Studies conducted in caves from the Southern Espinhaço Mountain in Brazil, located in Minas Gerais state, have discovered new fungal species in quartzite and limestone caves (CONDÉ et al. 2023, DUTRA et al., 2023; LEÃO et al., 2024).

In the view of new discoveries and the lack studies in marble caves of Minas Gerais, this study aimed to identify the richness of culturable fungi in the Gruta da Viola marble cave in the Southern Espinhaço Mountain in Brazil.

2. Material and Methods

2.1. Study area and fungal sampling

Gruta da Viola is a marble cave located in Santana do Riacho municipality, Minas Gerais state. This is the largest cave in the geosite, located in a protected area known as Morro da Pedreira (SOUZA et al., 2019).

Sampling of airborne fungi and sediment were performed in three points along the cave. Organic litter (leaf litter and animal remains), and animal dung were collected when present along the cave. The Koch sedimentation method (KUZMINA et al., 2012) was adapted to obtain airborne fungi using a one-meter-high support to elevate Petri plates. At each point, six plates containing culture media Malt Extract Agar added with chloramphenicol (2 mg/L) (MEA+), Dichloran-Rose Bengal-Chloramphenicol Agar (DRBC), and National Botanical Research Institute's Phosphate growth medium (NBRIP) were exposed to the cave environment for 15 minutes. Sediment samples were collected at a depth of 1–5 cm after removing 1 cm of the surface. Leaf litter samples were stored in paper bags (LEÃO-FERREIRA et al., 2013) and animal dung samples were stored in falcon tubes. All samples were stored under refrigeration and transported to the Laboratório de Micologia e Etiologia de Doenças Fúngicas da Universidade Federal de Viçosa.

2.2. Fungal isolation

For the isolation of fungi on animal dung and sediment samples, the serial dilution method was used until reaching a concentration of 10^{-5} (ZHANG et al., 2017). One hundred microliters of each concentration were spread on the surface of the MEA+, DRBC and NBRIP culture media in duplicates. The dilution plates and the plates exposed to cave air were incubated at 25 °C for 30 days, during which fungal was observed daily, and colonies were periodically transferred to potato dextrose agar (PDA) plates.

Leaf litter samples were processed using the washing technique in running water, followed by storage in a humid chamber at room temperature (CASTAÑEDA-RUIZ et al., 2005). Samples were periodically observed under a stereoscopic microscope for 30 days, and reproductive fungal structures were transferred to PDA plates.

Pure cultures were obtained using the hyphal tip method (TUITE, 1969) and then preserved in 2 mL microtubes containing a 10% glycerol solution at –20 °C. All isolates are stored in the cave fungi collection of the Laboratório de Micologia e Etiologia de Doenças Fúngicas da Universidade Federal de Viçosa.

2.3. DNA extraction, amplification and sequencing

Fungal isolates were cultivated in PDA for 7 days for total genomic DNA extraction. Fungal mycelia were collected, and the commercial Wizard Genomic DNA Purification kit (Promega®) was used.

Informative regions of the fungal genome, such as the internal transcribed spacer regions 1 and 2, together with the 5.8S subunit (ITS), the 28S rDNA nuclear region (LSU), partial beta-tubulin gene region (*TUB*), calmodulin gene region (*CAL*), and RNA polymerase II subunit gene region (*RPB2*) were amplified by PCR. The PCR results were visualized by agarose gel electrophoresis, and purification and sequencing were performed at Macrogen Inc., South Korea (http://www.macrogen.com).

2.4. Fungal identification

Isolates were identified by comparing their DNA sequences with sequences deposited in GenBank database using the megablast tool within the BLAST platform (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Searches in the BLAST searches were limited to curated sequences from type material deposited in GenBank. Isolates with similarity greater than 95% with type sequences were considered within the related genus and isolates below were assigned only into family.

2.5. Data analyses

Graphs representing fungal richness in Gruta da Viola cave were constructed using GraphPad Prism v.8. A Venn diagram was constructed using the online tool Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/index.html).

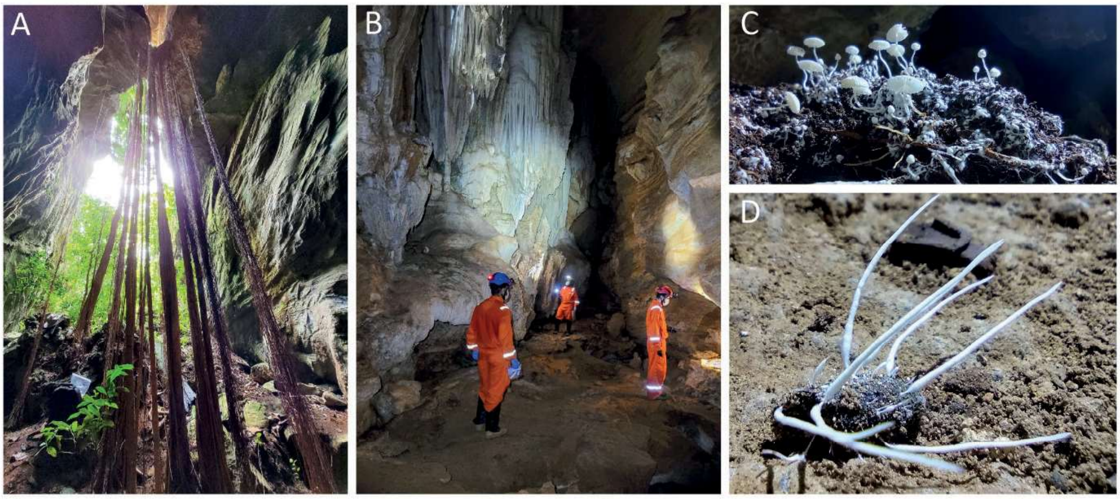

Figure 1: *Gruta da Viola cave. A: Entrance of the cave. B: Interior of the cave. C: Mushrooms growing on organic litter. D: Amphichorda sp. growing on animal dung.*

3. Results

A total of 398 fungal isolates were obtained. Among these, 127 strains were identified by molecular analysis of DNA sequences, being 61 from sediment, 37 from animal dung, 24 from air particles and five from organic litter. These isolates represent three phyla, eight classes, 15 orders, 30 families, and 45 fungal genera (Table 1).

Ascomycota was the most represented phylum and the only found in all the substrates sampled (117 isolates and 37 genera). Among the *Ascomycota*, 11 fungal orders were identified, with *Eurotiales* and *Hypocreales* being the most frequently sampled (54 and 33 isolates, respectively). In *Mucoromycota* five isolates were obtained, most of them isolated from animal dung (three isolates), with only one isolate from organic litter and sediment each. *Mucorales* was the only order found in *Mucoromycota*, represented by three families and four genera. Four isolates and genera from *Basidiomycota* were obtained, with three orders represented in the samples: *Agaricales, Exobasidiales*, and *Polyporales*.

Most of the fungal isolates identified in the Gruta da Viola cave were obtained from sediment and soil samples (61 isolates and 26 genera), followed by animal dung (37 isolates and 14 genera), air particles (24 isolates and 18 genera), and organic litter (five isolates and four genera) (Fig. 2A). Although animal dung presented more strains than air particles, it showed greater genus diversity than animal dung.

Among the fungal isolates identified, 14 genera were found exclusively in sediment: *Absidia, Apinisia, Cephalotrichum, Clonostachys, Collariella, Coprinellus, Dactylonectria, Microascus, Pithoascus, Simplicillium, Sodiomyces, Stachybotrys, Staphylotrichum*, and *Trichocladium*. Nine fungal genera were found only in air particles: *Crinipellis, Daldinia, Diaporthe, Malbranchea, Memnoniella, Mollisia, Paraphaeosphaeria, Pseudophlebia, Xepicula*. Six were isolated only in animal dung: *Chrysosporium, Lichtheimia, Mucor, Nigrograna, Preussia*, and *Yunnania*; and the genus Meira was exclusive from organic litter.

The analysed substrates did not share any common genera across all samples. *Aspergillus, Penicillium* and *Talaromyces* were found in air, animal dung, and sediment (Fig. 2B). Furthermore, *Fusarium* was also found in three substrates (animal dung, organic litter, and sediment). Air particles and sediment shared four common genera: *Cladosporium, Gliomastix, Metarhizium*, and *Xylaria*. Organic litter and air are the only substrates that did not share any common genera (Fig. 2B).

The genus *Aspergillus* was the most abundant (29 isolates), followed by *Penicillium* (20 isolates), *Amphichorda* (eight isolates), and *Metarhizium* (five isolates) (Fig. 2C). Many of the genus identified had only one isolate identified, whereas three isolates could not be identified at genus level.

Eurotiales was the most abundant order in the animal dung, air particles and sediment samples (Fig. 2D). However, *Hypocreales* was the only order present in all substrates sampled, and the most abundant on organic litter. Moreover, other orders were found in three substates: *Mucorales* (animal dung, organic litter and sediment), as well as *Onygenales* and *Pleosporales* (air, animal dung, and sediment) (Fig. 2D). On the other hand, the orders *Diaporthales, Helotiales*, and *Polyporales* were represented only in air samples.

Table 1: *List of identified genera and number of isolates obtained from each substrate in Gruta da Viola cave.*

	Cave substrates			
Genus	**Airborne particles**	**Animal dung**	**Organic litter**	**Sediment**
Absidia		1		1
Amphichorda	1	7		
Apinisia				1
Aspergillus	1	12		16
Bulbithecium			2	2
Cephalotrichum				1
Chrysosporium		1		
Cladosporium	1			1
Clonostachys				1
Collariella				1
Coprinellus				1
Crinipellis	1			
Cunninghamella		1	1	
Dactylonectria				1
Daldinia	1			
Diaporthe	1			
Fusarium		2	1	1
Gliomastix	2			1
Humicola		1		1
Lecanicillium		1		1
Lichtheimia		1		
Malbranchea	1			
Meira			1	
Memnoniella	1			
Metarhizium	1			5
Microascus				3
Mollisia	1			
Mucor		1		
Nigrograna		1		
Paraphaeosphaeria	1			
Penicillium	6	5		9
Pithoascus				1
Preussia		1		
Pseudophlebia	1			
Simplicillium				1
Sodiomyces				1
Striatibotrys				1
Staphylotrichum				2
Talaromyces	1	1		2
Trichocladium				1
Xepicula	1			
Xylaria	2			2
Yunnania		2		

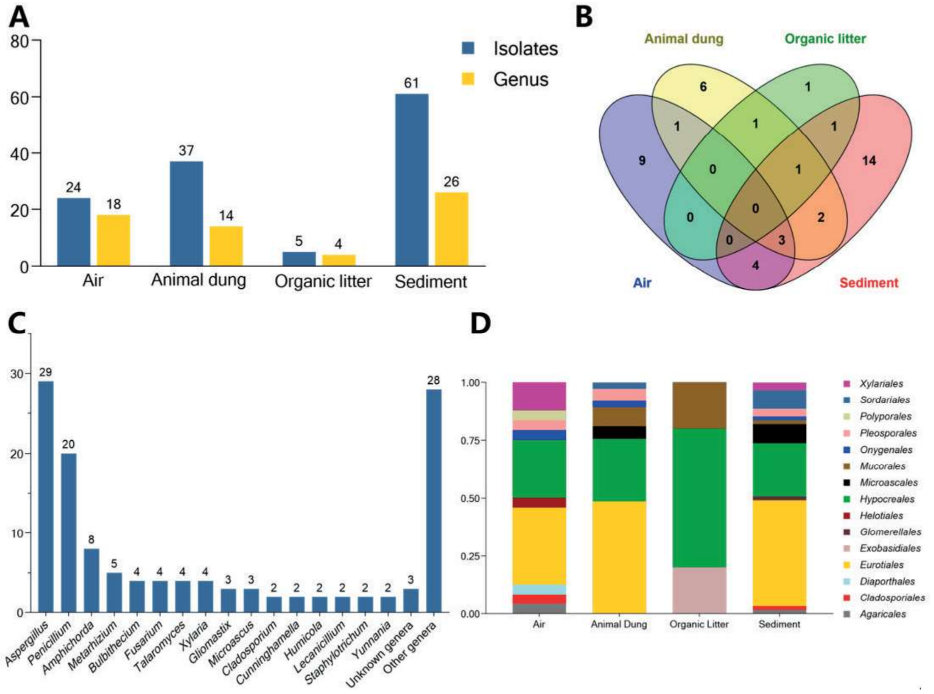

***Figure 2:** Statistics of fungal richness from Gruta da Viola cave. A: Number of isolates and fungal genera obtained from different substrates. B: Venn diagram of fungal genera obtained in four substrates. C: Most common genera found in Gruta da Viola cave. D: Abundance of fungal orders found in each substrate.*

4. Discussion

Fungi belonging to the phylum *Ascomycota* are often more abundant in caves worldwide (BIAGIOLI et al., 2023). The plasticity of ascomycetous species allows them to grow successfully in different environments, including those with poor nutrient availabilities, such as caves (ZHANG et al., 2021; POLI et al., 2024). Ascomycota represents the majority of the isolates obtained in this study, with 117 isolates and 37 of 45 of the fungal genera identified.

The order *Eurotiales* comprises fungi with different lifestyles capable of colonize several substrates due to its low specificity (LOCKHART et al. 2023). Member of this order, such as *Aspergillus, Penicillum* and *Talaromyces*, have been frequently found in caves worldwide. New species from these three genera were recently described in Brazilian caves uncovering the diversity of these fungi in a little studied environment (ALVES et al., 2022; NÓBREGA et al., 2024; LIMA et al., 2024). In the present study, *Aspergillus* and *Penicillium* were the most abundant genera, being isolated from air, animal dung, and sediment, similar to those of previous studies.

Cladosporium is one of the most common genus found in caves worldwide (BIAGIOLI et al., 2023). Brazilian caves harbour a great diversity of *Cladosporium* species (PEREIRA et al., 2022). *Cladosporium diamantinense* and *C. speluncae* were described in caves from Southern Espinhaço Mountain, in Minas Gerais (DUTRA et al., 2023). Nevertheless, only two *Cladosporium* isolates, from air and sediment, were obtained in Gruta da Viola cave. An unexpected result considering the previous studies in caves worldwide and near to the one sampled.

The genus *Amphichorda* was one of the most abundant in this study, with seven isolates from bat dung and one from air particles. Species of *Amphichorda* found in caves in China were also isolated from animal dung (ZHANG et al. 2017; ZHANG et al. 2021). This genus was previously identified in Minas Gerais caves, where the new species *A. monjolensis* was described in Gruta Velha Nova cave, associated with an insect (LEÃO et al., 2024). Therefore, our results expand the substrates that *Amphichorda* can be found in Brazilian caves.

Fungi belonging to *Chaetomiaceae* family have been identified in different substrates from Brazilian caves, such as airborne particles, bat guano and leaf litter (ALVES et al., 2022; CONDÉ et al., 2023). In this study six *Chaetomiaceae* isolates were obtained from animal dung and sediment, representing four genera. Condé et al. (2023) reported the species *Collariella bostrychodes* in Velha Nova cave on leaf litter, and here this genus is reported occurring on cave sediment. Additionally, this is the first report of *Humicola*, *Staphylotrichum* and *Trichocladium* in Brazilian caves.

Microorganisms inhabiting the cave environment are frequently associated with human pathogens, such as *Aspergillus fumigatus* and *Histoplasma capsulatum* (VANDERWOLF et al. 2013; SILVA et al., 2014). However, many other fungal genera and species can be found in caves, including new taxa that helps to fill the knowledge gap in current estimates of fungal species on Earth. A further taxonomic investigation, combining molecular phylogenetic analyses and morphological examination of fungal structures, will enable the identification of potentially novel genera and species within the Gruta da Viola cave.

5. Conclusion

In this study the richness of culturable fungi from Gruta da viola was explored. A total of 168 fungal isolates were obtained in Gruta da Viola cave, however, 127 could be identified by molecular analysis. The isolates that could not be identified at genus level will be studied and published in future research. Furthermore, 45 fungal genera belonging to three phyla were obtained in four substrates sampled.

No studies aiming to identify fungi from Gruta da Viola cave were reported until date. To the best of our knowledge, this is report of culturable fungi found in this cave. Expanding the knowledge about fungi that occurring in Brazilian caves from Minas Gerais.

The isolates obtained here will continue to be analysed for the correct identification at species level. Therefore, new fungal taxa can be identified and described among the isolates from this study.

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