

Uncovering the Mycobiota of the Teto de Seixos Cave

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Abstract

Caves are unique environments with a great diversity that is still unknown, especially when it comes to tropical caves. In Brazil, recent studies have shown a great potential for discovering new cave species. Thus, the objective of this study was to identify the cultivable filamentous fungi of the Teto de Seixos cave, located in the Serra do Espinhaço Meridional of Minas Gerais. Samples of airborne particles, sediments, organic litter and animal dung were collected. Total genomic DNA was extracted from the isolates and the ITS, LSU, TUB and CAL regions were amplified and sequenced for fungal identification. A total of 480 isolates were obtained, of which 164 isolates have been identified at the genus level. Of these, 28 isolates were obtained from airborne particle samples, 28 from animal dung, 44 from litter samples and 68 from sediments. The 164 isolates were grouped into 45 genera, 36 families, 20 orders, 7 classes, and 3 phyla. *Aspergillus*, *Penicillium* and *Cladosporium* were the genera with the highest number of isolates found. Preliminary results indicate that there are potential new fungal taxa in the cave.

Resumo

As cavernas são ambientes únicos com uma grande diversidade ainda desconhecida, principalmente quando se refere a cavernas tropicais. No Brasil, estudos recentes demonstram um grande potencial de descobertas de novas espécies de fungos cavernícolas. Assim, o objetivo desse trabalho foi investigar os fungos filamentosos cultiváveis da caverna Teto de Seixos, situada na Serra do Espinhaço Meridional de Minas Gerais. Amostras de partículas suspensas no ar, de sedimento, serapilheira e fezes de animais foram coletadas. Para identificação a nível de gênero, foi extraído o DNA genômico total dos isolados e as regiões ITS, LSU, TUB e CAL foram amplificadas e sequenciadas. Ao todo, 480 isolados foram obtidos, dos quais 164 isolados foram identificados a nível de gênero, sendo 28 isolados provenientes de amostras de partículas suspensas no ar, 26 de fezes de animais, 44 de amostras de serapilheira e 66 de sedimento. Os 164 isolados foram agrupados em 45 gêneros, 36 famílias, 20 ordens, 7 classes e 3 filos. Os gêneros *Aspergillus*, *Penicillium* e *Cladosporium* foram os gêneros com maiores números de isolados encontrados. Os resultados preliminares indicam que existem potenciais novos táxons fúngicos na caverna.

1. Introduction

Caves are unique ecosystems with spatial delimitation, partial or total absence of direct sunlight, little variation in temperature and limited amount of available nutrients (ALVES et al., 2022; POLI et al., 2024; ZHANG et al., 2021). These characteristics classify the caves as extreme environments for life development, but various studies demonstrate the great occurrence of fungal in caves, including the discovery of new taxa (ALVES et al., 2022; CONDÉ et al., 2023; DUTRA et al., 2023; LEÃO et al., 2024; POLI et al., 2024; ZHANG et al., 2021).

In Brazil, more than 23 thousand caves are registered in the Centro Nacional de Pesquisa e Conservação de Cavernas – ICMBIO/CECAV (MINISTÉRIO DO MEIO AMBIENTE, 2022), and nearly 50% of these are in the state of Minas Gerais. The mycobiota of caves in Minas Gerais are little studied, recently some studies showed a great potential for fungal

discoveries. Forty-seven species distributed in 15 genera, mainly in the genera *Aspergillus* and *Penicillium*, were isolated from bat guano and air samples from the dolomitic cave Lapa Nova (TAYLOR et al., 2013). In 2014, TAYLOR et al., (2014) identified 32 fungal species in sediment samples collected in the RM3 cave located in the Iron Quadrangle. Recent studies in caves in the Serra do Espinhaço Meridional (SEM) resulted in the description of one new fungal genus and seven new species of filamentous fungi (CONDÉ et al., 2023; DUTRA et al., 2023; LEÃO et al., 2024). However, there are still many to discover on the mycobiota of caves in Minas Gerais. Thus, these study aimed was to report the cultivable filamentous fungi present in samples collected in the Teto de Seixos marble cave, located in the SEM of Minas Gerais.

2. Material and methods

2.1. Study area and collections

Teto de Seixos cave is constituted of marble rocks which is located in Santana do Riacho, in the part of the SEM known as Serra do Cipó. This cave is located within a conservation unit named APA Morro da

Pedreira. The cave is approximately 500 meters long and has three entrances (SOUZA et al., 2011; 2019).

Samples of airborne particles, sediments, organic litter, and animal dung were collected at three different points in the cave. The collection of airborne particles was performed using the Koch sedimentation method (KUZMINA et al., 2012), using a one-meter-high support to

elevate the Petri plates. At each point, two 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 layer. When present, organic litter samples were collected and stored in paper bags (LEÃO-FERREIRA et al., 2013), while animal dung samples were collected and 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 animal dung and sediment samples, the serial dilution method was used until reaching a dilution factor of 10⁻⁵ (ZHANG et al., 2017). One hundred microliters of each dilution were spread on the surface of the MEA+, DRBC and NBRIP culture media in duplicates. All plates were incubated at 25 °C for 30 days, the fungal growth was observed daily and the new colonies were periodically transferred to plates containing potato dextrose agar (PDA).

The organic litter samples were washed in running tap water, followed by storage in a humid chamber (CASTAÑEDA-RUIZ et al., 2005). The samples were periodically observed under a stereoscopic microscope for 30 days, and reproductive fungal structures were transferred to PDA plates, and incubated at 25 °C.

Pure cultures were obtained using the hyphal tip method (TUIE,

1969) and then stored 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

Total genomic DNA was extracted by collecting mycelia grown in PDA medium for 7 days, using the commercial Wizard Genomic DNA Purification kit (Promega®) and the protocol described by PINHO et al., (2013). The DNA amplification of the internal transcribed spacer regions 1 and 2, together with the 5.8S subunit (ITS), 28S rDNA nuclear region (LSU), partial beta-tubulin gene region (TUB), and calmodulin gene region (CAL) were used for fungal identification. The PCR results were visualized by 0.8% agarose gel electrophoresis. The PCR products were purified and sequencing at Macrogen Inc., South Korea (<http://www.macrogen.com>).

2.4. Fungal identification

Isolates were identified by comparing sequences in the NCBI database using the megablast tool within the BLAST platform (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). BLAST searches were limited to sequences from type material to enhance accuracy. Isolates with sequence identity greater than 95% were considered within the related genus. Those sequences that were below 95% were assigned only to family.

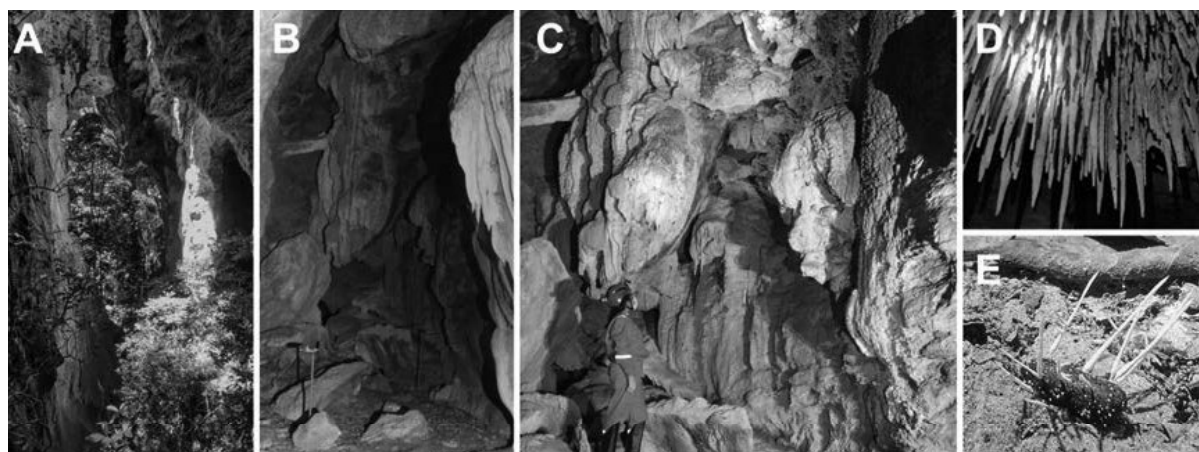


Figure 1: Teto de Seixos cave. A: Entrance to the cave; B: Airborne particles sample collection; C–D: Speleothems; E: Animal dung colonized by *Amphichorda* sp. and other fungi.

3. Results

A total of 480 fungal isolates were obtained from Teto de Seixos cave, of which 164 were identified at the genus level. Of these, 28 isolates were recovered from airborne particle samples, 26 from animal dung, 44 from organic litter, and 66 from sediment (Fig. 2A). The 164 isolates were grouped into 45 genera, 36 families, 20 orders, and seven classes distributed in three phyla. The genera identified in this study are listed in Table 1.

The phylum *Ascomycota* presented the largest number of representatives (157 isolates and 42 genera), followed by *Mucoromycota* (5 isolates and 2 genera), and *Basidiomycota* (2 isolates and one genus) (Fig. 2B). The most representative ascomycetous genera were *Aspergillus*, *Penicillium*, *Cladosporium*, *Amphichorda*, and *Wardomyces*, with 35, 18, 10, 7, and 6 isolates, respectively (Fig. 2C). *Mucoromycota* was the second most abundant phylum, with two isolates of *Absidia*, two of *Mucor*, and one

unidentified isolate from the family *Lichtheimiaceae*. In *Basidiomycota*, only two isolates were found, one belonged to the genus *Minimedusa* and one unidentified genus within the family *Polyporaceae*.

Fourteen genera were found in airborne particle samples (Fig. 2A), with *Cladosporium* (six isolates), *Aspergillus* (3), *Mycosphaerella* (2), and *Microascus* (2) being the most found. Eight genera (*Amycosphaerella*, *Annulohypoxylon*, *Botryotinia*, *Cercospora*, *Epicoccum*, *Exutisphaerella*, *Mycosphaerella*, and *Neokalmusia*) were found exclusively from this sample (Fig. 2D). Eleven genera were identified in animal dung (Fig. 2A), of which *Aspergillus* (8) and *Amphichorda* (5) were the most abundant. Four genera (*Furcaterigmium*, *Gibellulopsis*, *Marquandomyces*, and *Nigrograna*) were found only in animal dung samples (Fig. 2D).

In organic litter, the isolates were distributed in 21 genera (Fig. 2A),

among which *Aspergillus* (10) was the most abundant. Nine genera were exclusively found in this substrate (Fig. 2D), namely, *Alternaria*, *Botryosphaeria*, *Lecanicillium*, *Minimedusa*, *Orbilia*, *Scolecobasidium*, *Striatibotrys*, and *Xepicula*. Sediment samples presented the highest number of identified genera (23), of which we observed a predominance of isolates within the order *Eurotiales*, accounting for almost 50% of the identified isolates (32 of the 66). *Aspergillus* (14) and *Penicillium* (13) were the most abundant, whereas nine genera were exclusive to

this substrate (*Collariella*, *Humicola*, *Malbranchea*, *Metarhizium*, *Mucor*, *Paracamarosporium*, *Staphylotrichum*, *Talaromyces*, and *Trichocladium*) (Fig. 2D). Only *Aspergillus* and *Penicillium* were found in all analyzed substrates (Figure 2D).

In total, 22 isolates could not be identified in any of the known genera (Fig. 2C). These isolates were included in 14 families, 11 orders and three phyla.

Table 1: List of identified genera and number of isolates obtained from each substrate in Teto de Seixos cave.

Genus	Cave substrates			
	Airborne particles	Animal dung	Organic litter	Sediment
<i>Absidia</i>		1		1
<i>Alternaria</i>			1	
<i>Amphichorda</i>		5	1	1
<i>Amycosphaerella</i>	1			
<i>Annulohypoxylon</i>	1			
<i>Aspergillus</i>	3	8	10	14
<i>Botryosphaeria</i>			1	
<i>Botryotinia</i>	1			
<i>Cercospora</i>	1			
<i>Cladosporium</i>	6		3	1
<i>Clonostachys</i>			2	1
<i>Collariella</i>				1
<i>Epicoccum</i>	1			
<i>Exutisphaerella</i>	1			
<i>Furcasterigmium</i>		1		
<i>Fusarium</i>		1	1	1
<i>Gibellulopsis</i>		1		
<i>Gliomastix</i>			1	1
<i>Humicola</i>				1
<i>Lecanicillium</i>			1	
<i>Malbranchea</i>				3
<i>Marquandomyces</i>		1		
<i>Metarhizium</i>				1
<i>Microascus</i>	2			3
<i>Minimedusa</i>			1	
<i>Monocillium</i>			1	1
<i>Mucor</i>				2
<i>Mycosphaerella</i>	1			
<i>Neokalmusia</i>	1			
<i>Nigrograna</i>		1		
<i>Orbilia</i>			1	
<i>Paracamarosporium</i>				1
<i>Penicillium</i>	1	1	3	13
<i>Purpureocillium</i>			1	2
<i>Scolecobasidium</i>			1	
<i>Simplicillium</i>	1		1	
<i>Sirastachys</i>		1	2	
<i>Staphylotrichum</i>				1
<i>Striatibotrys</i>			1	
<i>Talaromyces</i>				4
<i>Trichocladium</i>				1
<i>Wardomyopsis</i>		1		5
<i>Xepicula</i>			2	
<i>Xylaria</i>	1			2

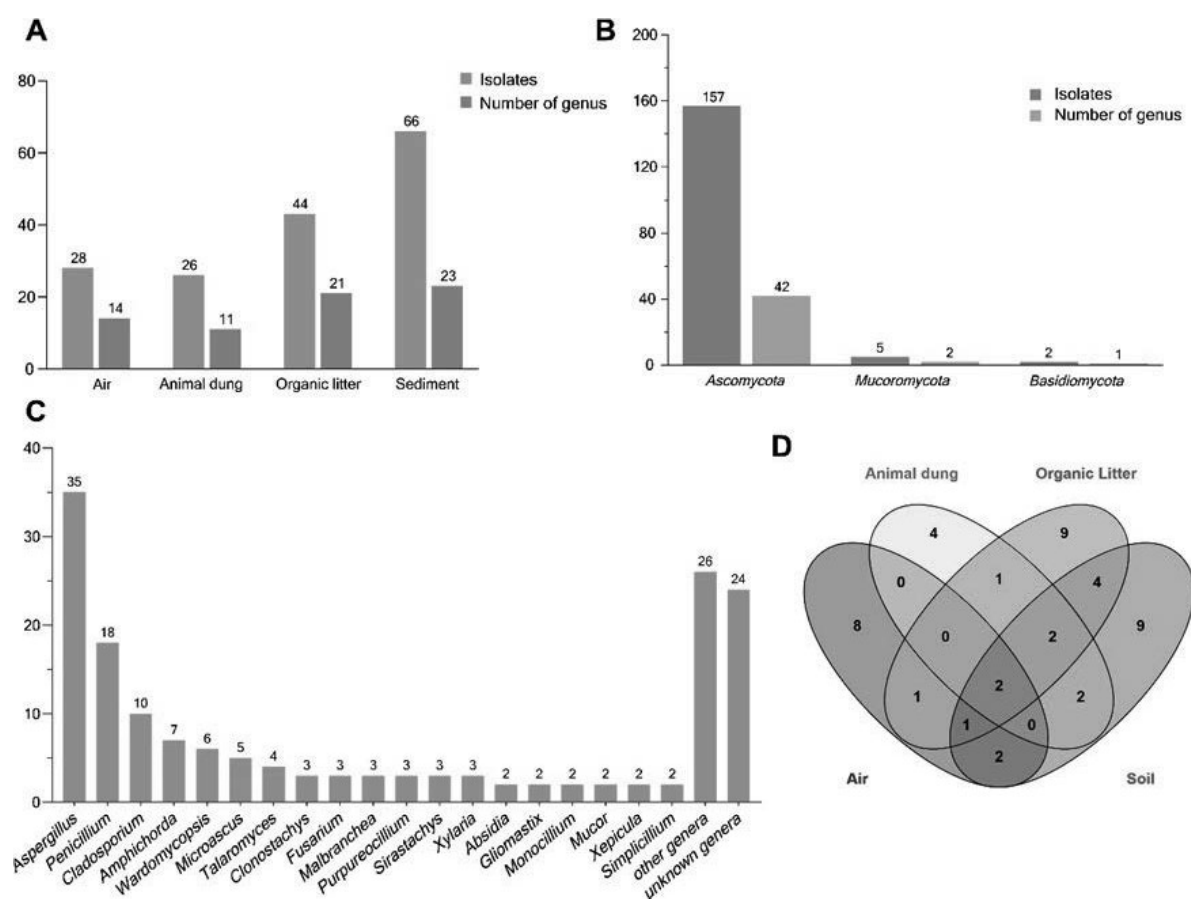


Figure 2: Statistics of fungi isolated from Teto de Seixos cave. A: number of isolates and fungal genera obtained from different substrates. B: number of isolates and genus found in each fungal phylum. C: most common genera found in this study. D: Venn diagram of fungal genera found in this study.

4. Discussion

Airborne particles are one of the main sources of fungal dispersal into the cave environment, and the genera *Aspergillus*, *Penicillium*, and *Cladosporium* are among the genera with the highest number of reports in cave studies (CONDÉ et al., 2023; CUNHA et al. 2020; PEREIRA et al., 2022; ZHANG et al., 2021). In this study, *Aspergillus* and *Cladosporium* isolates were identified in air particle samples in large quantities; however, only one isolate of *Penicillium* was identified.

Samples of animal dung in caves, especially bat dung, are often associated with pathogenic fungi such as *Histoplasma capsulatum* (CUNHA et al., 2020; SILVA et al., 2013). Conversely, it is also a substrate that can be a source for new fungal discoveries (CONDÉ et al., 2023; ZHANG et al., 2017; 2021). CONDÉ et al. (2023) described the genus *Parahumicola*, and species *P. guana*, from bat dung in the quartzite cave Gruta Monte Cristo in the SEM. In the animal dung samples collected from Teto de Seixos cave, we identified isolates belonging to the genus *Amphichorda*. This genus has some species described as coprophilous (ZHANG et al., 2017; 2021) and has potential for biotechnological use (BELOUSOVA et al., 2023; JIANG et al., 2021; 2023; XU et al., 2018; YUAN et al., 2022). In Brazil, one species of this genus, *Amphichorda monjolensis*, has been described in the carbonate cave Gruta Velha Nova in Monjolos, Minas Gerais (LEÃO et al., 2024).

Approximately 45% of the fungal isolates obtained in the present study were retrieved from the sediment samples. Sediment samples are generally among the richest in organic matter content when compared to other samples studied in caves, which provides greater fungal growth and consequently greater numbers of isolates (ALVES et al., 2022; CUNHA et al. 2020; ZHANG et al., 2021).

The genera *Aspergillus*, *Penicillium* and *Cladosporium* have been widely reported in caves (CONDÉ et al., 2022; CUNHA et al., 2020; DUTRA et al., 2024; POLI et al., 2024; VANDERWOLF et al., 2013; ZHANG et al., 2017; 2021). In this study, these genera were the most abundant, representing more than 40% of the isolates, and were found in all the samples analyzed. These genera present several characteristics that allow for great adaptation to different substrates, including spore dispersal over long distances, low substrate selectivity, and the ability to colonize animals and plants (POLI et al., 2024; VANDERWOLF et al., 2013; ZHANG et al., 2021).

Twenty-two isolates could not be assigned to any known genera. Further analysis based on a taxonomic polyphasic approach, using molecular phylogeny and morphological characterization of reproductive structures, will allow the identification of possible new genera and species from Teto de Seixos cave.

5. Conclusion

Forty-five genera were identified in the Teto do Seixos cave, demonstrating that caves harbor a great fungal richness, with potential for identifying new taxa. Until this work, Teto de Seixos cave was still

unknown from a mycological point of view, which demonstrates a potential for new fungal discoveries and reinforces the importance of studying the mycology of caves.

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