IDENTIFICATION OF FUNGI FROM SOIL AND SEDIMENT IN JEFRIZ CAVE; THE FIRST SURVEY IN A CAVE FROM IRAN

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Abstract

The aim of this study was to characterize the mycobiota in soil and sediment samples of Jefriz cave in Kerman, Iran. During 2018–2019, the culturable mycobiota from several sites within the Jefriz cave, resulted in 82 fungal isolates. Morphological characteristics of the isolates, as well as molecular sequence data, were used for species identifications. The fungi were identified as species of Fusarium, Fusicolla, Geomyces (Pseudogymnoascus), Humicola, Chalastospora, Penicillium, Aspergillus, Epicicium, Podospora and Mucor. The most prevalent was Aspergillus spelunceus, followed by Geomyces pannorum and Humicola grisea. The majority of these species have been reported as cave residents in previous studies of cave environments. Our data showed that the fungal community composition varied between the samples from the entrance and less visited sites deeper in the cave. This study is the first cave mycological investigation in Iran, and one of the identified species is reported for the first time from a cave.

INTRODUCTION

Caves are considered as extreme environments characterized by darkness, low temperatures, high humidity, and limited organic matter (Barton and Northup, 2007). Microbial communities in caves are uniquely adapted to oligotrophic conditions (Jurado et al., 2009). Fungi are an important group of cave microflora that play critical roles as decomposers, mutualists, and pathogens (Shapiro and Pringle, 2010) and they interact with cave fauna. Some fungal species are involved in processes of disintegration and formation of cave speleothems by providing nucleation sites for precipitation of calcium carbonate (Barton and Northup, 2007), while some other species play roles in biodeterioration of cave structures via physical penetration, metabolite secretion, and pigmentation (Cañveras et al., 2001; Wang et al., 2011). These facts make the caves microorganisms interesting to microbiologists.

A number of studies have explored the fungal community occurring in caves soil and sediments and have proved that caves harbor a high diversity of fungi (Vanderwolf et al., 2013). In July 2001, an outbreak of Fusarium solani in the prehistoric painted Lascaux Cave in France was discovered and biocide treatments were applied (Dupont et al., 2007; Bastian et al., 2009b). Pseudogymnoascus destructans, the causal agent of white-nose syndrome emerged in North America and spread in 2006, drawing more attention to fungal cave communities (Meteyer et al., 2009). Vanderwolf et al. (2013) published a list of species and genera of fungi, slime molds, and yeasts reported from caves worldwide. The fungal community composition is affected by factors such as sampling site, isolation method, time of year (Wang et al., 2011), human activity, and presence of nutrient sources such as bat guano and droppings (Nováková, 2009; Shapiro and Pringle, 2010).

No data have been reported on metabolic properties and the mechanisms of adaptation of the fungal flora in caves in Iran. Jefriz cave, located in Baft, Kerman province, Iran (Fig. 1), is an attractive limestone cave with unique speleothems. This cave is important since there are reports of bat species residing in the cave (personal communication with local people). In this study, we aimed to investigate fungal flora of Jefriz cave based on culture-dependent methods to determine the diversity of fungi and thereby enhance our understanding of the evolution of adaptations to the extreme ecosystems in caves.

MATERIALS AND METHODS

Sampling Site

Jefriz is a limestone cave located 3 km east of Jefriz village, Baft, Kerman province, Iran (Fig. 1). This cave has a vertical, eight-meter deep entrance located on a hillside. A gate has been installed over the entrance (Fig. 2). Exploration into the cave is by rappelling through a shaft that requires the use of specialized equipment such as a rope or cable ladder. The cave length is about 332 m (personal communications from the explorers). The cave is linear with few side passages, but there are two main tunnels. After entry, there is a big chamber with passageways covered with stalactites and stalagmites. The main passage in some parts is only accessible through narrow tunnels that requires the explorer to crawl to pass. The cave floor is covered with wet debris and tiny ponds are present. Jefriz cave is not scientifically

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explored and a map has not been published. A gate at the entrance (Figure 2b) of the cave was installed by local people to prevent entering of unprofessional cavers, to preserve the cave from damages produced by some careless visitors, and to protect the untrained visitors because the exploration needs training and special equipment.

Sample Collection and Isolation of Culturable Fungi

Soil and sediment samples were collected using a sterilized spatula from 0-4 cm of the cave floor, placed in sterile bags, transferred to the laboratory, and stored at 4 °C. Four visits were carried out for sampling and four samples were taken during each visit. Sample collections took place in all four seasons, on January 11, 2018, and May 14, 2019, August 19, 2019, and October 22, 2019. For each trip, one sample was collected from sites close to the entrance corridor in the first large chamber, and three samples were collected randomly throughout the cave from deeper sites. Isolations were carried out by direct plating and plate dilution methods (Garrett, 1981). Soil samples (1 g) were suspended in nine mL sterile distilled water, vortexed vigorously for 1 min and allowed to stand for 3 min. Two hundred μL of 10⁻² to 10⁻⁴-fold diluted suspensions was inoculated onto potato dextrose agar (PDA; Merck, Germany) and dichloran rose bengal chloramphenicol (DRBC) agar (peptone 5 g, KH2 PO4 1 g, MgSO4 0.5 g, glucose 10 g, dichloran solution (0.2% (w/v) in ethanol, Rose Bengal 0.025 g, chloramphenicol 0.1 g, agar 15 g, and distilled water to 1 L; King et al., 1979). All ingredients of culture media were obtained from Merck, Germany. Chloramphenicol (Sigma–Aldrich, Germany) was added to eliminate bacteria. Cultures were incubated at 25 °C for four weeks in darkness. Plates were examined for fungal growth every three days. Colonies that appeared on cultures were transferred to new plates for further examination. All the isolates were deposited in the KGUT Fungal Culture Collection at Kerman Graduate University of Advanced Technology, Kerman, Iran.

Morphological Studies

The morphological identification of fungal isolates was based on the macroscopic and microscopic characteristics of the isolates, such as colony morphology, conidia, and conidiophores according to reliable identification guides (Domsch et al., 1980; Barnett and Hunter, 1998; Hanlin et al., 1998; Leslie and Summerell, 2006; Seifert et al., 2011). All cultures were done in triplicate. Media used were Malt Extract Agar (MEA: malt 20 g/L, glucose 20 g/L, peptone 1 g/L, agar 16 g/L), Czapek Yeast autolysate Agar (CYA: sucrose 30 g/L, powdered yeast extract 5 g/L, K2HP04 1 g/L, NaN03 2 g/L, KCl 0.5 g/L, MgSO4·7H2O 0.5 g/L, FeSO4·7H2O 0.01 g/L, ZnSO4·7H2O 0.01 g/L, CuSO4·5H2O 0.005 g/L, agar 20 g/L) and Czapek–Dox agar (CZ: sucrose 30 g/L, K2HP04 1 g/L, NaN03 2 g/L, KCl 0.5 g/L, MgSO4·7H2O 0.5 g/L, FeSO4·7H2O 0.01 g/L, ZnSO4·7H2O 0.01 g/L, CuSO4·5H2O 0.005 g/L, agar 20 g/L) (Hubka et al., 2016) to observe colony characteristics. All ingredients of culture media were obtained from Merck, Germany. Lactophenol, lactophenol cotton blue, and 50% lactic acid (Merck, Germany) solutions were used to prepare microscope slides. The measurements and microphotographs of fungal features were taken using Dino-eye microscope camera USB lens (The Microscope Store, LLC., USA) from agar plates and slides. Colony diameter was assessed on plates after 7–28 days at 25 °C. The mean diameter was obtained from three replicates.
Molecular Examination Of Isolates

Genomic DNA of fungal isolates was extracted using a CTAB extraction procedure (Doyle and Doyle, 1987; Zhang et al., 2010) from mycelia grown on PDA for 7–15 days at 25 °C. The DNA concentrations were estimated by a NanoDrop spectrophotometer (NanoDrop Technologies, USA). ITS-rDNA regions were amplified using two primers, ITS1 and ITS4 (White et al. 1990). A ~700 bp portion of the TEF1-ɑ gene (Translation Elongation Factor 1ɑ) was amplified from Fusarium isolates using the primers, EF1 (5’-ATG GG TAA GGA RGA CAA GAC-3’) and EF2 (5’-GGA RGT ACC AGT SAT CAT G-3’) (O’Donnell et al., 1998; Geiser et al., 2004; Edel-Hermann et al., 2012). Primers were synthesized by Macrogen, Inc. (South Korea). Twenty-five μL polymerase chain reaction (PCR) contained 1X reaction buffer, 2.5 mM dNTPs, 0.4 mM of each primer, 20 ng of DNA, and 1 unit of Taq polymerase (Ampliqon, Denmark). A Biometra T Advanced Thermal Cycler (Biometra, Göttingen, Germany) were used to perform PCRs. The cycling conditions for ITS1 and ITS 4 consisted of 95 °C for 5 min, followed by 30 cycles of 95 °C for 45 s, 60 °C for 30 s, and 72 °C for 1 min, and then 5 min at 72 °C. The annealing temperature of 54 °C was used for TEF1-ɑ. The sequencing was carried out by Macrogen (Macrogen Inc., South Korea). The sequences were edited by Geneious (Biomatters Inc., USA) when needed and compared to the sequences in the NCBI (GenBank) database using BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1990) to find the most likely taxonomic designation of each isolate. All nucleotide sequences obtained in this study were deposited in GenBank and accession numbers were obtained as MN643060 to MN643071.

RESULTS

Fungal Isolates

In our current investigation, 16 samples of soil and sediments from Jefriz cave were collected in 4 visits and a total of 82 fungal isolates were isolated. The morphological and molecular investigations led to the identification of Fusarium solani, Fusicolla septimanifiniscientiae, Pseudogymnoascus (Geomyces) pannorum, Humicola grisea, Chalastospora gossypii (formerly Alternaria malorum), Penicillium expansum, P. brevicompactum, Aspergillus speluncus, Epicicicum nigrum, Podospora sp., Ochroconis sp. and Mucor spp. (Figs. 3 and 4). Fifteen isolates were mycelia sterilia and were not identified to species level. The most prevalent genera were Aspergillus speluncus (21%), Pseudogymnoascus pannorum (13%) and Humicola grisea (10%) (Table 1). Species diversity in this cave were not attempted to estimate due to the method of isolation that was limited to culture-dependent methods. These methods give an estimate of the fungi present but is biased toward isolation of spore-forming species. Fungal species in cave air were not attempt ed because the air, especially in the entrance corridor, is in contact with outside air resulting in biased isolation toward common airborne fungi from outside of the cave.

Sampling was carried out in four visits, one in each season of the year. In each visit, samples were collected from the sites close to the entrance corridor and from sites located deep in the cave. Comparisons showed that the composition of fungal species was different among sampling sites. Penicillium expansum and Aspergillus speluncus were the most abundant species detected from samples close to the entrance of the cave in the first large room while the most abundant species in remote and out of reach parts were Pseudogymnoascus pannorum and Humicola grisea. In addition, there was no observed difference in the composition of fungal species isolated in different seasons of the year.
DISCUSSION

Our goal was to provide preliminary data on fungal communities of caves to study biological interactions in subterranean and extreme environments. The present study is the first one studying the mycobiota of a cave in Iran. We were able to successfully identify eleven fungal genera from soil and sediment samples of Jefriz cave on the basis of macroscopic, microscopic, and molecular analyses.

Literature suggests that most of these species are commonly associated with cave habitats around the world (Cunningham et al., 1995; Dupont et al., 2007; Jurado et al., 2009; Bastian et al., 2010; Docampo et al., 2010; Wang et al., 2011; Jacobs et al., 2017). Some of the isolated species such as *Fusarium* spp. are common soil inhabitants (Leslie and Summerell, 2006) and it is possible that they have been carried in by the water streams that run from outside through the walls of Jefriz cave. A similar scenario was suggested for *F. solani* entering Lascaux Cave by Dupont et al. (2007). Human visitors, small animals, and arthropods are other vectors that may have contributed to the fungal flora composition of Jefriz cave. The relationship between fungi and arthropods in caves is reported in several studies. Greif and Currah (2007) and Dromph (2003) isolated several fungal species from collembolans. *Isaria farinosa* (syn: *Paecilomyces farinosus*) is reported as the one of frequent parasites occurring on insects in underground environments in the Czech Republic by Kubátová and Dvořák (2005), and also as a parasite of *Stenophylax* (syn: Micropterna *fissus*) in Spanish caves by Jurado et al. (2008). Arthropod roles as host and vectors of fungal species (Bastian et al., 2009a) in Jefriz cave remain to be studied in further research by entomologists.

Water films resulting from condensation cover Jefriz walls and speleothems. These wet surfaces are a suitable niche for fungal growth (Barton and Jurado, 2007). *Lecanicillium psalliotae, L. aranearum, Engyodontium album* and *Torrubiella* spp. in biofilm samples of Roman catacombs are reported (Saarela et al., 2004; Jurado et al., 2008). Another factor influencing the cave mycobiota is availability of potential food sources. Droppings and guano especially of bats may serve as possible source of nutrients and energy input for many fungal species as well (Zhang et al., 2017).

The predominant species in Jefriz cave were *Aspergillus spelunceus* (21%), *Pseudogymnoascus pannorum* (13%), and *Humicola grisea* (10%). *Aspergillus spelunceus* (sect. *Nidulantes*) have been frequently isolated from caves (Vanderwolf et al., 2013; Hubka et al., 2016). Species related to *A. nidulans* may have medical importance (Hubka et al., 2016); the prevalence of this species should receive more attention from cavers. *Aspergillus* spp. are known to be allergenic, and causal agents of aspergillosis and mycotoxin producers (IARC 2002; Perrone et al., 2007). Mycotoxins are associated with immune deficiency and cancer (Shephard, 2008). *Pseudogymnoascus pannorum* was one of the dominant species in Jefriz cave. This species expresses keratinolytic enzymes and can cause skin infections and nail geomycosis (Gianni et al., 2003; Zelenková; 2006; Reynolds and Barton, 2014) and may be dangerous to humans. *Mucor* spp. (2) tolerate low temperatures and higher salinity than seawater (Poole and Price, 1971; Leushkin et al., 2015). Characteristics such as tolerating low temperatures justifies its high prevalence in extreme environments such as caves. This species is reported from caves, arctic, and low temperature soils worldwide (Out et al., 2016). It should be noted that its close relative, *P. destructans*, is one of the commonly reported fungi from caves all over the world.

### Table 1. Fungal species isolated from Jefriz cave. The relative frequency is calculated by dividing the frequency of each species by the total number of all isolates.

| Species                          | Relative frequency | Isolate used for sequencing | Accession numbers obtained from GenBank |
|----------------------------------|-------------------|----------------------------|----------------------------------------|
| *Pseudogymnoascus pannorum*      | 13                | J2                         | MN643060                               |
| *Humicola grisea*                | 10                | J4                         | MN643061                               |
| *Chalastospora gossypii*         | 5                 | J10                        | MN643062                               |
| *Penicillium brevicaeptum*       | 5                 | J11                        | MN643063                               |
| *Penicillium expansum*           | 7                 | J17                        | MN643064                               |
| *Aspergillus spelunceus*         | 21                | J14                        | MN643065                               |
| *Epicoccum nigrum*               | 4                 | J20                        | MN643066                               |
| *Podospora* sp.                  | 2                 | J12                        | MN643067                               |
| *Ochroconis* sp.                 | 1                 | J21                        | MN643068                               |
| *Fusicolla septimanifiniscientiae* | 2          | J0                         | MN643069                               |
| *Fusicolla septimanifiniscientiae* | ...          | ...                        | ...                                    |
| *Fusarium solani*                | 2                 | J22                        | MN643071                               |
| *Mucor* spp.                     | 7                 | ...                        | ...                                    |
| *Mycelia sterilia*               | 20                | ...                        | ...                                    |
(Martinkova et al., 2010; Turner et al., 2011) causing white nose syndrome in bats (Shelley et al., 2013). Another commonly-occurring species isolated from the cave samples was *Humicola grisea*. This species has high potential in starch saccharification (Maheshwari et al. 2000), which makes it a promising species in the process of producing bioethanol by microbial fermentation (Pervez et al., 2014). Several studies have reported bio-deterioration of structures in caves associated with fungal species extracellular metabolites. Gargani (1968) studied the microbial floras of damaged wall paintings in Florence after a flood and associated the damage with the growth of microorganisms. The deteriorations are the result of extracellular metabolites secreted while hyphae are penetrating inside the wall painting and cave speleothems (Strzcelczyk, 1981; Garg et al., 1995).

*Fusicolla septimanifinscientiae* is reported here from a cave for the first time. *Fusarium solani* was one of the especially interesting microorganisms that was identified. An aggressive isolate of this species was discovered in 2001 threatening the Paleolithic paintings of Lascaux cave, France, and resulted in multiple applications of biocides (Dupont et al., 2007). Arthropods have symbiotic relationships with *Fusarium* spp. (Morales-Ramos et al., 2000; Sharma and Marques, 2018) and are suggested as possible vectors of some species (Jurado et al., 2008). The pathogenicity of *Fusarium verticillioides* on grasshoppers (Pelizza et al., 2011) and *F. keratoplasticum* and *F. proliferatum* on *Tribolium* species (Chehri, 2017) have been reported.

*Penicillium expansum*, followed by *Aspergillus spelunceus*, prevailed in samples collected near the cave entrance, while *Humicola grisea* was the most abundant species in the samples collected in remote parts of the cave. Air exchange with the outside environment and the activities of cave explorers are possibly impacting on the cave airborne fungal community at the entrance. The number of visitors is not officially reported, however, the number is recently reduced because of the door implemented by locals. The impact of human activity on fungal diversity in caves is documented by Shapiro and Pringle, (2010). Their investigation showed complex associations between levels of human disturbance and the diversity of fungal species in four caves in Kentucky and Tennessee. They showed that the fungal diversity was higher in sites with moderate levels of disturbance than highly visited sites.

**CONCLUSIONS**

This survey was conducted to determine the major components of the mycoflora present in Jefriz cave to understand the complex biological interactions in subterranean habitats. In conclusion, 12 fungal species associated with Jefriz cave soil and sediments were identified in this study. Our data showed that the biological and climatic condition of this cave is selective. The fungal species were composed of both cosmopolitan species and common cave residents. Fungal species that can be harmful to the human health were found in Jefriz cave that evidenced the need to further investigate the hygienic safety of this place for cavers, although no illnesses have been reported. Work on unculturable species is needed to provide a thorough perspective of the fungal community in this cave. Furthermore, studies on metabolic activity and bioactive compounds of fungi, such as *Humicola grisea*, help our understanding of the biological interactions in a subterranean environment. We suggest studying the possible agents contributing to the dispersion of spores (*i.e.* arthropod vectors) in order to better take preventive actions, if needed, about the preservation of the cave and the safety of the explorers.

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