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Characterization of the fungal flora present in bat guano: survey for species of clinical interest as a contribution for a public health study

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Trabalho efectuado sob a orientação de:
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"A natureza reservou para si tanta liberdade
que nunca a poderemos conhecer completamente
com o nosso saber e a nossa ciência"

Johann Wolfgang von Goethe

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Caracterização da flora fúngica presente em excrementos de morcego: pesquisa de espécies de interesse clínico como contributo para um estudo de saúde pública

RESUMO

Neste trabalho foi realizada uma prospecção da flora fúngica presente em guano de morcego, em algumas cavernas de Portugal continental. O objectivo global do trabalho foi conhecer a diversidade de fungos existentes nas diferentes cavernas e detectar a presença de fungos potencialmente patogénicos para os seres humanos.

Um total de dezassete caves foram estudadas e várias amostras de guano foram colhidas em cada uma delas. Estas amostras foram processadas e inoculadas em três meios de cultura distintos e a diferentes temperaturas. Os fungos isolados foram identificados de acordo com as características macro e micromorfológicas, nomeadamente o aspecto das colónias e a forma e estrutura das conídias. Nos casos em que a identificação não foi possível tendo em conta apenas as características fenotípicas foi efectuada a sequenciação da região ITS dos genes de DNA ribossomal 28S. Finalmente, todos os resultados foram analisados estatisticamente, fazendo os cálculos dos seguintes índices: Shannon, que permite calcular a abundância de espécies de fungos em cada cave; Evenness, cujo valor demonstra a uniformidade da população e Sorenson, que permite uma comparação entre duas caves diferentes.

Um total de 165 isolados fúngicos foram obtidos, dos quais 62 foram identificados ao nível da espécie, 88 ao nível do género e 15 ficaram por identificar. 137 espécies cresceram nas placas inoculadas a 25°*C*, 20 espécies cresceram a 15°*C* e 8 cresceram a 4°*C*. O filo que prevaleceu foi o Ascomicota, com um total de 91 espécies, seguindo-se o filo Basidiomicota, com 45 espécies isoladas e, finalmente. o filo Zigomicota, com apenas 14 espécies. Entre todas as caves, o género que dominou foi *Trichosporon*, com um total de 43 espécies isoladas e nas três temperaturas de incubação, seguindo-se o género *Penicillium*. Cova da Moura, foi a cave onde foi obtida uma maior média de fungos e valores mais altos para os índices de Shannon e Evenness.

Em Portugal só existe publicado um estudo semelhante e apenas uma espécie, *Mucor hiemalis*, é comum em ambos os estudos, sendo todos os outros novos registos nas cavernas Portuguesas. Uma das espécies encontradas, *Sporothrix shenkii,* constitui o primeiro registo de um isolado ambiental deste fungo no nosso país.

Alguns dos fungos detectados durante esta pesquisa são patogénicos oportunistas e, desta forma, os investigadores devem tomar precauções extra aquando do seu manuseamento e os espeleologistas ou mesmo turistas visitando caves, devem estar cientes dos riscos que poderão correr.

Characterization of the fungal flora present in bat guano: survey for species of clinical interest as a contribution for a public health study

ABSTRACT

The purpose of this project was to perform a survey of a few caves in Portugal mainland in order to assess the fungal diversity present in bat guano and detect fungi that may be potential human pathogens.

A total of seventeen caves were surveyed during this work, and several guano samples were collected from each. These samples were processed and inoculated in three different media at three different temperatures. All the fungi isolated were identified according to their morphological features, namely the colony morphology and shape of reproductive structures. When identification was not possible based only on phenotypic characteristics, sequencing of the ITS regions of 28S rDNA was performed. Finally all data were statistically treated, calculating the Shannon index, which discriminate the abundance of the species present among the caves; Evenness index, which demonstrate the uniformity of the species present and Sorenson index, that permit a comparison between two caves.

A total of 165 isolates were obtained, from which 62 were identified to the specie level, 88 to the genus level and 15 remained unidentified. Regarding the temperatures of incubation, 137 isolates were able to grow on plates inoculated at 25*ºC*, 20 isolates grew on plates inoculated at 15*ºC,* and only 8 isolates grew at 4*ºC*. The phylum that prevailed was Ascomycota, with a total of 91 species, followed by the Basidiomycota, with 45 species and finally the Zygomycota with only 14 species present. The most preeminent genus among all caves was *Trichosporon,* with a total of 43 species isolated and in the three temperatures of incubation, followed by *Penicillium*. "Cova da Moura", was the cave with higher fungal species average and higher values of Shannon and Evenness indexes.

In Portugal there is only one known similar survey and only *Mucor hiemalis* was found in both studies, which makes all the other fungi found new records in Portuguese caves. One of the species found, *Sporothrix shenkii,* represents the first record of an environmental isolate of this fungus in our country.

Some fungi isolated during this survey were opportunistic pathogens to humans, so the investigators should take extra precaution when handling them and speleologists or even people visiting caves should be aware of the risks.

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
CFU	Colony Forming Units
CMA	Corn Meal Agar
DNA	Deoxyribonucleic acid
e-value	Expect value
HIV	Human Immunodeficiency Virus infection
ITS	Internal Transcribed Spacer
NCBI	National Centre for Biotechnology Information
PBS	Phosphate-Saline Buffer
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
rDNA	ribosomal DNA
rRNA	ribosomal RNA
SDS	Sodium Dodecyl Sulfate
TAE	Tris-acetate-EDTA
WNS	White Nose Syndrome
YGC	Yeast extract Glucose Chloramphenicol

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CHAPTER I

INTRODUCTION

1.1. CAVES

Beneath Earth surface various kinds of cavities, both natural and artificial, can be found. In these subterranean environments are included caves, catacombs, tombs, hypogean chapels, among others. Humankind has been using caves for millennia: early humans used them as places of shelter, in many societies they served as places of worship or resting places for the dead, in many cultures caves played a prominent role in myths and legends. Finally, they are also of great interest to scientists and explorers. The scientific study of subterranean habitats is known as Speleology and incorporates knowledge of chemistry, biology, geology, microbiology, and topography, among others.

Caves are covered and protected from abrupt climatic changes by a rock mantle, which creates an environment peculiar and stable. Usually these closed environments are characterized by lack of natural light, reduced variability in the number of specific abiotic conditions like moisture, temperature, as well as by isolation and restriction in space. Additionally they are nutrient-limited, and have extensive areas of mineral surfaces. Together these conditions provide a quasi-extreme environment for microorganisms, however as normally nutrients are sequestered from the outside environment it results in novel and diverse microbial population (Saiz-Jimenez, 2012; Sugita et al., 2005).

Cave fauna is a mixture of forms with different origins characterized by convergent features, such as adaptation to life in permanent darkness and high humidity. In the cave ecosystem, fungi are an important component of the chain food that prevails. A variety of fungal spores and mycelium might enter into a cave by means of natural agents, such as flood and air or/and even through organic substances like plant and animal remains. Once inside the cave fungi begin to grow on suitable substrates like remnants of insects and bat guano (Saiz-Jimenez, 2012; Sugita et al., 2005). Fungi affect the inhabitants dynamics of cave biota and play a role in the decomposition of organic matter, making it accessible to other members of the cave community, like cavernicole insects, which diet include fungal spores and mycelia (Koilraj et al., 1999). Inside a cave and when organic matter is available fungi are by far the most disturbing microorganisms. What makes this true is their ability of spore production, dissemination and colonization capability (Nieves-Rivera, 2003). Another contribution to this disturbance is the human-induced changes, especially inside the

touristic caves. This was observed in Castañar de Ibor Cave (Spain) when, in 2008, the cave walls and sediments showed colonization by long and white fungal mycelia. Latter it was proven that this fungal outbreak was initiated by *Mucor circinelloides* and *Fusarium solani* (Figure 1), two species with explosive rate of reproduction, due to a detritus input, the vomit of a visitor (Jurado et al., 2010b). Fernandez-Cortes and collaborators (2011) also proved that the majority of airborne fungi are originated from external environment penetrating through the cave entrance.

In this study, caves play a special role. Some are roosting habitat of bats, making these places the ideal ones to obtain bat guano samples.

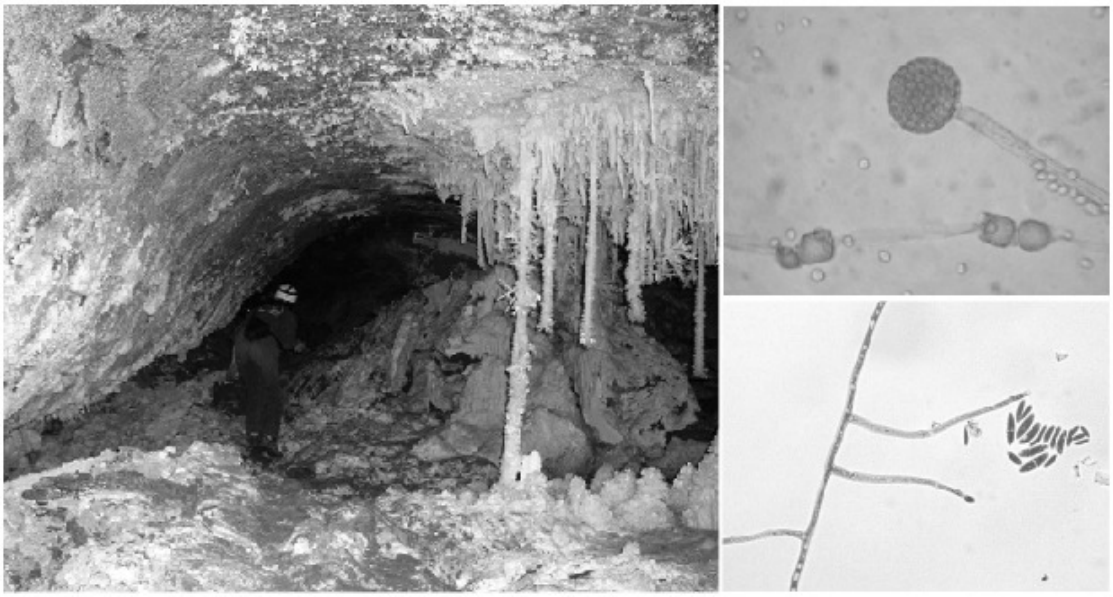

Figure 1: On the left, white mycelia growth on the cave walls; top right, *Mucor circinelloides* and on the bottom right *Fusarium solani* (adapted from Jurado et al., 2010b).

1.2. BATS AND GUANO

Bats are social species that coexist in aggregates of significant numbers (up to several millions) and they can be found all over the world in tropical and temperate environments. They are an ecological diverse mammalian taxon with 1232 species currently described. In Portugal inhabit 27 species of bats that are grouped into four families: *Rhinolophidae*, *Vespertilionidae*, *Miniopteridae* and *Molossidae*. About half of these species are cave dwelling and the ones that we can find in this habitat usually

belong to the *Rhinolophidae, Vespertilionidae* and *Miniopteridae* families. Additionally, bats inhabiting caves are essentially insectivorous (Cabral et al., 2006).

Human economics and the natural world are realities that can benefit with bat communities because they play an integral part of the ecosystem. On one hand insectivorous bats are super efficient insect eaters, and each one is able to consume up to 1,000 per hour. So, they are considered to be important agents that keep the level of growing insect populations under check. On the other hand, frugivorous bats, that feed on fruit and nectar, help to regenerate forests by seed dispersing, and along with these feeding habit the process of pollinate plants also occurs. Additionally, their guano is a source of agricultural fertilizer (Jurado et al., 2010b). Thus, bats provide a harm free and priceless alternative to pesticides and fertilizers.

However, bats can also represent a health concern to human and animal heath. These concerns include rabies-like lyssaviruses, classical rabies and histoplasmosis. Vampire or sanguivorous bats, which feed on blood, are the vectors of paralytic rabies and are potentially more serious threat to livestock than to humans. The other health concern, histoplasmosis is directly related with bat guano because it provides optimal growth conditions for the pathogenic fungus *Histoplasma capsulatum* especially in humid conditions (Fleming et al., 2009).

It is also important to mention that bats can also be targets of diseases. The most recent one discussed and studied all over the world is an emerging fungal disease called White Nose Syndrome (WNS). WNS is associated with a cutaneous infection caused by a keratinophylic and psychrophilic species named *Geomyces destructans*. This fungi has the ability to grow on exposed tissues of hibernating bats, such as on hair follicles and sebaceous glands, causing the presence of delicate, exuberant, white filaments or powdery growth that obscure the muzzle, ears and wing membranes (Figure 2). Curiously, throughout histological examination of infected bats, Meteyer and co-workers, 2009, demonstrated that *G. destructans* doesn't lead to inflammation or immune response in the tissues (Mickleburgh et al., 2002). The first evidence of WNS appeared on February 2006 in a photograph taken at Howes Cave, 52 km west of Albany, New York (Gargas et al., 2009). Latter it was witnessed that WNS was causing unparalleled mortality among hibernating bats in North America and has rapidly spread occurring throughout the north-eastern and mid-Atlantic regions, in Ontario and Québec provinces in Canada (Blehert et al., 2009). In Europe, it was only in 2008-2009 that WNS was identified and curiously monitoring hasn´t documented

high mortality events. Over the last years several studies have shown that *G. destructans* is present in seven European countries: France, Germany, Switzerland, Czech Republic, Slovakia, Hungary and more recently in Italy (Blehert et al., 2009; Foley et al., 2011; Frick et al., 2010; Meteyer et al., 2009). Nevertheless, the distribution of *G. destructans* in Europe still remains poorly known since geographical coverage of these studies is limited.

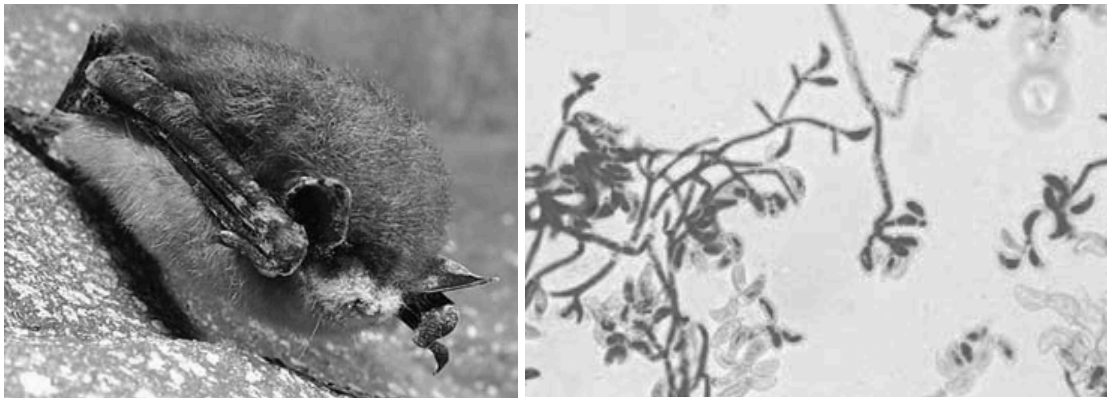

Figure 2: On the left, little brown bat exhibiting white fungal growth on its muzzle, ears and wings. On the right, microscopic morphology of *Geomyces spp.* stained with lactophenol cotton blue (adapted from Blehert et al., 2009).

Guano is the scientific term for the accumulation of faeces of particular animals in time and space. Specifically in caves, guano can come from three different animals: birds, bats and crickets. Bat guano piles are the most usual source of organic matter in caves, and it forms the basis of a food web comprising bacteria, fungi, protozoans, nematodes and arthropods, being essential for sustaining the health of the cave ecosystem. There are many cave organisms, such as fish, salamanders and arthropods, which depend on bat guano as food resource (Blehert et al., 2009). The input of organic nutrients from bat guano provides a source of carbon, nitrogen, water and also important minerals, like potassium, sodium, phosphorous, calcium, iron and magnesium, which often are nutrients lacking in many ecosystems. The inorganic nutrients and amount of energy provided from bat guano depends on the feeding habits, on how well bats are able to assimilate ingested food and on the energetic content of the food. Based on this, there are three main types of guano produced: frugivorous, that contains small-undigested seed; sanguivorous, that have a pasty consistency and a reddish colour when fresh, becoming black and powdery when

older; and insectivorous, with chitinous pieces of insects and other arthropods (Emerson & Roark, 2007).

1.3. CHARACTERISTICS OF FUNGI

Fungi comprise one of the major eukaryotic lineages, exceeding in number plants and animals. They are a unique group of organisms, distinctive from all others in their behaviour and cellular organization. This group includes yeast, moulds, and mushrooms.

The total number of described fungi until today is about 99 000 species and there is no doubt that basidiomycetes and ascomycetes represent the vast majority of fungal diversity (Blackwell, 2011). However, with the current standard laboratory methods it is still difficult to grow and isolate all fungi in the laboratory, but there is molecular data supporting an estimative existence of 1,5 to 5,1 million species all over the world (Hawksworth, 2001; Blackwell, 2011).

All fungi have to acquire organic nutrients as energy sources and for cellular synthesis, but there is a broad distinction on how these nutrients are obtained. Fungi can grow as parasites or symbionts on another organism, and as saprophytes on non-living materials. Saprotrophic fungi are important for the environment because they have major roles in recycling the nutrients in natural and agricultural environments. This is accomplished throughout the decomposition of organic material, which is performed from the simplest one, such as methane, to the most complex polymers, like lignin. However, some saprotrophic fungi can cause a serious threat to humanity since they can be significant spoilage agents, causing timber decay in buildings, and also by growing on stored food products, producing mycotoxins. Fungi can also parasite prokaryotes, plants and animals in order to obtain nutrients from the living tissues of these hosts. This intimate association can cause the killing of the host tissues through the production of toxins, or the parasite can live in complete harmony with the host, without killing its cells. Within this mutualistic symbiosis between fungi and host, there are some hosts that can beneficiate from the association, because it enables them the exploitation of novel habitats and resources. Finally, there is the pathogenic group of fungi, which are parasites that cause disease in bacteria, plants, animals, including humans, and even in other fungi. Regarding humans, relatively few fungi infect

humans or other warm-blooded animals. Nevertheless, as most of these fungi are opportunistic parasites, they may be a real threat to immunocompromised individuals. The economic impact of this group of fungi is colossal for the civilization. On one hand, they are beneficial for the pharmaceutical companies by the production of antibiotics and for the agricultural business by the production of pesticides, but on the other hand, they can cause several mycosis, even deadly for certain individuals, and can also be extremely detrimental through the devastation impact in plant diseases (Hawksworth, 2001; O'Brien et al., 2005).

1.3.1 FUNGAL TAXONOMY AND FEATURES

The separation of fungi from other organisms heralded the Five Kingdom approach, which was proposed by the American biologist Robert Whittaker in 1969. In his publication Whittaker proposed Five Kingdoms: Monera, Protista, Plantae, Fungi and Animalae. The kingdom Fungi was proposed based on their unique structures and in their nutrition: unlike animals, fungi don't ingest their diet; unlike plants, they don't obtain their own nutrients by photosynthesis; fungi rather secrete digestive enzymes around their nutriment and finally absorb it into their cells (Deacon, 2009). However, the organisms considered as fungi are polyphyletic, very complex and diverse, which latter was confirmed by ultra-structural, biochemical and molecular biological studies.

The "true fungi", often termed Mycota or Eumytoa, comprise a range of characteristics that separate them from other organisms. They are eukaryotic organisms with distinctive cell wall components, which include chitin and glucans, polymers of glucose with β-1.3 and β-1.6 linkages. Typically they are haploid but many yeasts are diploid. Regarding their reproduction, it can be by both sexual and asexual means, and they normally produce spores. They grow as apical filaments, termed hyphae, by repeated cell divisions in a chain of cells, which consequently branch, giving rise to a network called mycelium. There are also some fungi that can grow as single-celled yeasts through budding or fission. Furthermore, some of them can switch from a yeast phase to a hyphal phase, and vice-versa, in response to environmental changes, and they are called dimorphic. Finally, fungi are heterotrophs, meaning that they require organic compounds as energy sources and carbon skeletons for cellular synthesis (Guarro et al., 1999).

The kingdom Fungi is organized into phyla, classes and then into orders. The five phyla accepted in this kingdom are Ascomycota, Basidiomycota, Zygomycota, Chytridiomycota and Glomeromycota. The former Deuteromycota group is no longer accepted as a taxonomic category, and presently they are addressed as mitosporic fungi that have lost a sexual phase or are anamorphs of Ascomycota and some Basidiomycota (Deacon, 2009).

The phylum Ascomycota is the larger one, containing approximately 75% of all fungi described. It is also the most diverse and ubiquitous, occurring in numerous ecological niches and all aquatic and terrestrial ecosystems. The species from this phylum are characterized and distinct from the species of other phyla by the presence of the ascus. The ascus is a cell where two compatible nuclei of distinctive mating types come together and fuse, creating a diploid nucleus, which undergo meiosis to produce haploid sexual spores, known as ascospores. This group includes ascomycetous yeasts, many economically important pathogens of plants, livestock, domesticated animals and even humans. Additionally, in a different context various ascomycetes form mycorrhizal associations with forest trees (Deacon, 2009).

The phylum Basidiomycota contains about 35% of all fungi described. Despite the majority of examples for this group being the mushrooms and toadstools, there is a vast diversity of species, including many important plant and serious human pathogens and basidiomycetous yeasts. There is only one feature that characterizes this group, the presence of the basidium, a structure where meiosis occurs and leads to the production of sexual spores called basidiospores (Deacon, 2009; Guarro et al., 1999).

The phylum Zygomycota comprises a group of lower fungi that have some distinctive features: the hyphae generally are nonseptate, forming a coenocytic mycelium and the isogamic sex organs, termed gametangia, after fusion produce a single, thick-walled, dark, sexual spore, called zygospore. Within this group several common members, such as the Mucorales, can cause serious threatening infection in humans (Deacon, 2009; Guarro et al., 1999).

The phylum Chytridiomycota is a group unique in respect to one characteristic: they are the only true fungi capable to produce motile, flagellate zoospores (Deacon, 2009; Guarro et al., 1999).

Finally, the phylum Glomeromycota is a recent one that comprehends arbuscular mycorrhizal fungi. They can be found growing in the majority of plant roots, with which they are obligate symbionts, without killing the root cells. The

reproduction is through large, multinucleated spores and additionally form coenocytic mycelia (Deacon, 2009)

1.3.2 FUNGAL DIVERSITY IN BAT GUANO

Fungi, such as other microorganisms, inhabit all niches of the biosphere. Antiquity, diversity, longevity and ubiquitous distribution are characteristics that make these organisms remarkable. Naturally, they occur in all environments including soils, water and also in caves (Deacon, 2009), where they take part in the cave communities as saprotrophs or pathogens. As mentioned before one of the most important substrates for fungi inside a cave is bat guano. One of the first evidences supporting this matter emerged in 1965 when Edwin S. Kajihiro observed the existence of dermatophytes in fresh bat guano. Along this study, *Microsporum gypseum* was the species with higher incidence, followed by the species *Trichophyton mentagrophytes*, *T. rubrum* and *T. terrestre*. Among the human pathogenic fungi he was able to isolate also *Candida sp.*, *Cladosporium sp.*, *Coccidioides immitis*, *Cryptococcus neoformans*, *H. capsulatum*, and *Sporotrichum sp.* (Koilraj et al., 1999). Over the time, some publications about surveys on fungal diversity in bat guano emerged on several caves around the world (Table 1), but the information on this matter is still poorly known. Nevertheless, in these studies several species have been described: in 2003, Nieves-Rivera performed a mycological survey of Río Camuy Caves Park, in Puerto Rico sampling among other substrates, bat guano and recorded 33 fungi species; in 2005, Sugita and co-workers surveyed for yeast in caves, collecting bat guano samples from 20 caves in Japan and documented 18 species, from which seven were potentially novel ones. In 2006, Ulloa and co-workers performed another survey for mycological diversity in guano samples from Mexico and recorded 19 fungi species. In Portugal, there is only one published paper about the mycological flora and it was performed in “Fojo dos Morcegos” cave, in “Serra da Arrábida”. It was executed in 1994 and the authors only found 3 species of fungi on guano samples (Almeida et al., 1994) (Table 1).

Table 1: Some fungi species isolated from bat guano samples.

Kingdom Ascomycota			
Species	References	Species	References
Acremonium berkeleyanum	1	*Geomyces pannorum*	1
Acremonium murorum	1	*Geotrichum candidum*	1,6
Acremonium sp.	3	*Hanseniaspora sp.*	3,4
Alternaria alternata	1	*Histoplasma capsulatum*	3,8
Aphanoascus fulvescens	3	*Malbranchea sp.*	1
Arthrinium arundinis	1	*Microsporum gypseum*	8
Aspergillus flavus	2,5,6	*Oidiodendron griseum*	1
Aspergillus fumigatus	1,2,5,6	*Paecilomyces sp.*	2,5
Aspergillus niger var. niger	2,5,6	*Penicillium chrysogenum*	1,2
Aspergillus terreus	3,6	*Penicillium citrinum*	1
Aspergillus versicolor	1,3,6	*Penicillium roqueforti*	1,2,5
Aureobasidium pullulans	7	*Saccharomyces cerevisiae*	4
Botryotrichum piluliferum	1	*Scopulariopsis croci*	1
Candida lusitaniae	4	*Sepedonium sp.*	2,5,6
Candida palmioleophila	4	*Sporothrix sp.*	8
Chrysosporium sp.	1,3,6	*Talaromyces flavus*	1
Cladosporium cladosporoides	2,5	*Tolypocladium niveum*	1
Coccidioides immitis	8	*Trichoderma viridae*	2,5,6
Eurotium amstelodam	1	*Trichophyton mentagrophytes*	8
Fusarium solani	1,5	*Verticillium sp.*	1
Kingdom Basidiomycota			
Species	References	Species	References
Cryptococcus neoformas	8	*Trichosporon laibachii*	4
Cryptococcus podzolicus	4	*Trichosporon porosum*	4
Kingdom Zygomycota			
Species	References	Species	References
Absidia corymbifera	6	*Mucor circinelloide*	1,7
Absidia glauca	1	*Mucor dimorphosporus*	1
Circinella umbellata	2	*Mucor hiemalis*	1,7
Cunninghamella echinata	6	*Rhizopus stolonife*	1,6
Mortierella sp.	1	*Syncephalis sp.*	6

1_(Nováková, 2009); 2_(Nieves-Rivera, Santos-Flores, Dugan, & Miller, 2009); 3_(Ulloa et al., 2006), 4_(Sugita et al., 2005); 5_(Nieves-Rivera, 2003); 6_(Koilraj et al., 1999); 7_(Almeida et al., 1994); 8_(Kajihiro, 1965).

Most caves are healthy places and furthermost cavers are healthy people. However, during the 20th century it became evident that some cave explorers became sick, due to particular caves and their inhabitants. One inhabitant that is particularly common in bat environment and causes disease is *H. capsulatum*. Nowadays it is known that *H. capsulatum* is endemic in the Mississipi valley (USA), most of Latin America, Africa and Southeast Asia (Eisenberg & Goldman, 1991). In Europe however, only a few indigenous cases have been described (Antinori et al., 2006; Calza et al., 2003; Jariwalla et al., 1977; Mahvi et al., 2004) demonstrating that this continent

is not endemic. Nevertheless it is suggested that the environmental conditions inside European caves are capable of supporting the growth of this fungi.

H. capsulatum naturally lives in soil containing bird and bat excrements, which are a nitrogen-rich organic matter where the conditions accelerate sporulation under optimal temperature and humidity conditions (Blache et all, 2011; Muñoz et al., 2010). It is typically a dimorphic fungus: in nature and at environment temperatures below 25ºC exists in a mycelial phase, however in mammalian host and at the temperature of 37ºC it is transformed into yeast. The mycelia phase is characterized by a white or brown, hair-like mycelium and the yeast phase produce a creamy white and textured colony. Regarding mycelia form morphology, the organism characteristically produces two types of conidia: macroconidia and microconidia (Figure 3). While macroconidia have 8 to 15 μm in diameter and on the surface we can observe numerous distinctive projections, microconidia have 2 to 4 μm, tiny pear-shaped smooth structures which are likely the main infectious form (Kauffman, 2007). This fungi is the etiological agent of systemic histoplasmosis. It is considered a disease that especially affects guano collectors, miners, geologists, anthropologists, speleologists and biologists (Muñoz et al., 2010)

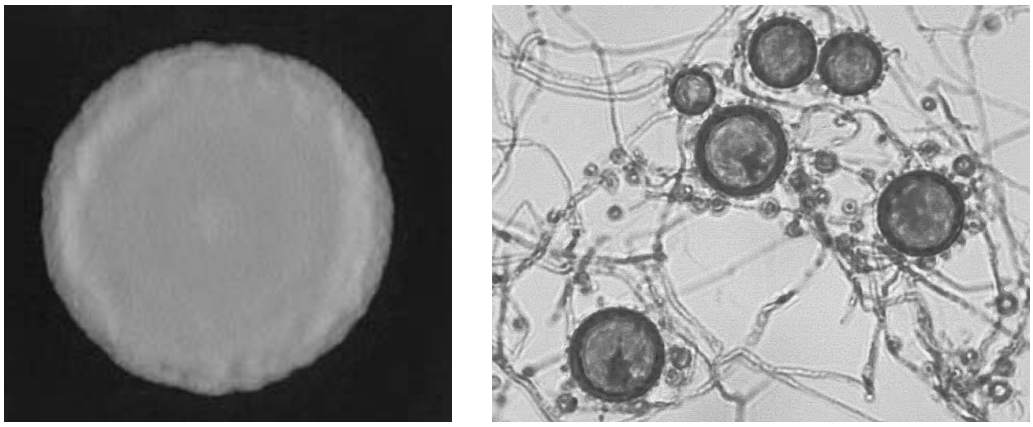

Figure 3: Culture (on the left) and microscopic morphology (on the right) of *H. capsulatum*, where is possible to observe large, round, single celled macroconidia formed on short and hyaline conidiophores (adapted from Ellis et al., 2007).

In addition to histoplasmosis, there are other cave fungi that can cause several pulmonary diseases, which include cryptococcosis, coccidioidomycosis, aspergillosis, blastomycosis, among others. This group of diseases is a particular problem to individuals with impairments of the immune system, such as persons with human immunodeficiency virus infection (HIV).

While guanophilic fungi are important to microbiologists and their roles in cave communities are important to speleologists, this kind of research however, can have other applications. For microbial ecologists, for example, the identification of fungi is crucial to the protection of the cultural heritage, like Palaeolithic paintings, Roman catacombs and ancient mural paintings, from fungi colonization. Nowadays this is of the utmost importance and is vital to find out the origin and causes of these colonisations, in order to prevent them and protect the historical patrimony (Jurado et al., 2010b; Jurado et al., 2008).

1.3.3 MORPHOLOGICAL IDENTIFICATION

In an era of microbial strain identification by molecular techniques, some will ask why there is still a need for morphological tests, especially considering that identification by molecular procedures is faster and more accurate. The fact is that for species identification, many laboratories sequence one or two diagnostic genes, others may sort isolates using morphology and then sequence representative strains. However, some laboratories have no sequencing facilities, so identification relies on standard morphologic criteria.

Historically, classification of organisms is based on observable characteristics, therefore is based on what we designate a phenotypic approach. Briefly, the most common procedure used worldwide is, in a first step, the growth of isolates in appropriate culture media, secondly observe specific cell structures enhanced by a stain under a light microscope, and third the identification by using current literature and determination keys.

Yeast can be defined as fungi whose asexual growth predominantly results from budding or fission and have sexual states that are not enclosed in fruiting bodies. Their cells can be globose, subglobose, ellipsoid, ovoid, obovoid, cylindrical, botuliform, elongate, apiculate, ogival, lunate or triangular, which may reflect the mode of reproduction. Some examples include the lemon-shape cells of the yeast *Hanseniaspora* and the bottle-shape cells of *Malassezia*. Additionally, when observing how the ballisto and blastoconidia are formed (conidiogenesis) it can provide information that aids the identification of a particular genera or species. Regarding the

macro-morphological characteristics it relies on some detail observable on the agar growing colonies, such as texture, colour, surface and margin (Kurtzman et al., 2011).

Concerning filamentous fungi, spores and spore bearing structures are one of the most important characteristics for fungi identification. A huge variety of spores are produced: ascospores, basidiospores, conida, zygospores, sporangiospores, chamydospores, among others, depending on the phyla. Other feature that can be additionally used for fungi identification is the presence or absence of hyphae septa. For example, usually the Zygomycota phylum can be characterized for the absence of hyphae septa. Agar growing colonies are also important in the fungi identification. Culture characteristics such as colour, smell, texture, pigments produced should also be appointed and considered.

Despite all utilities, the phenotypic approach has been criticized over its limitation or disadvantages. This approach has a lack of standardized and stable terminology, is highly subjective and some phenotypic characteristics are considered to be unstable and dependent on environmental conditions. But most importantly, there is a severe limitation in this approach, which is the lack of applicability in fungi that don't grow in culture, and this is the principal reason why many fungi remain unclassified (Guarro et al., 1999).

1.3.4 MOLECULAR IDENTIFICATION

A high rate of molecular-based approaches for microbial communities have been developed to overcome some limitations related to traditional culture-based techniques. Many of these approaches rely on a primary polymerase chain reaction (PCR) step in order to amplify genes of interest.

Microbiologists have been using DNA and RNA sequences for the classification and identification of fungi over the years. There are several highly conserved genes and gene families within the fungal genome that have been used to classify microorganisms based on their phylogeny. The most commonly used are the ribosomal DNA (rDNA) gene complex, because it can be found in all microorganisms and rDNA is a non-protein coding, multiple copy gene, whose repeated copies in tandem are homogenised by gradual evolution (Guarro et al., 1999; Kirk et al., 2004; Manter & Vivanco, 2007). Additionally, due to the intensive use of these sequences for

fungal molecular studies over time (Bruns et al., 1991), nowadays various public databases are available where the rDNA gene complex sequences from diverse fungal taxa are deposited (Vilgalys, 2003).

The rDNA gene complex is a section of the genome that has become a critical marker for species identification in eukaryotes. It is constituted for three genes that code for ribosomal RNA (rRNA) – 18S, 5.8S and 28S – and have a fairly conserved nucleotide sequence among fungi. This complex also includes variable DNA sequences of the intervening internal transcribed spacer (ITS) regions called ITS1 and ITS2. Since these sequences are not transcribed, they do not have any functional purpose, in fact the function of these regions isn´t completely known. What is effectively known is that the ITS are spacers that separate rDNA genes of the functional DNA sequences, and are targets of mutation with a higher frequency than the rDNA genes. Therefore, due to these mutations, sequence variation among species occurs and this sequence heterogeneity within this region is like a signature for molecular assays in separating genera and when possible species. Summarizing, the sequence homology within the rDNA genes and the differences accumulated within the ITS regions are the genetic basis for the organization of fungi into taxonomic groups (Iwen et al., 2002).

Several steps have to be accomplished in order to apply molecular identification techniques. Typically, in a first step DNA extraction of the query species is performed, followed by an amplification of the rDNA loci by PCR. Then, the PCR products are purified and sequenced. Finally, the query sequences are compared to store ones in order to locate regions of similarity between them. These comparisons are carried out in public databases of nucleotide sequences such as GenBank, a database that was built and is distributed by the National Centre for Biotechnology Information (NCBI), which uses sequence-similarity searching thought the Basic Local Alignment Search Tool (BLAST). These searches are the basic type of analysis most frequent performed on GenBank database, which is the sequence repository most world widely used in the field (Benson, 2006).

Together nucleic acid extraction and PCR amplification have provided novel insights into the genetic-functional diversity in fungi communities. Over the years a variety of primer sets have been designed and the same was observed specifically for the 18S and ITS regions of fungi. As a result of relatively short period of evolution in the kingdom fungi, a lack of variation within the eukaryotic ribosomal small subunit,

18S rRNA, of closely related species is observed. Consequently, taxonomic identification based on these sequences is problematic, with identification usually limited to genus or family level. The ITS region however, benefit from a faster rate of evolution, resulting in more sequence variation within closely related species. Hence, ITS sequences normally provide greater taxonomic resolution than sequences of coding regions (Anderson & Cairney, 2004). The PCR primers design for amplification of fungal ITS region are the ITS1F (Gardes & Bruns, 1993) and ITS4 (White et al, 1990), which amplify from the ITS1 to the ITS2 region and apparently they have specificity for ascomycetous, basidiomycetous and zygomycetous fungi (Anderson & Cairney, 2004; Gardes & Bruns, 1993; Lord et al., 2002).

Among all the ribosomal regions, the ITS region has the highest probability of successful identification for the wide range of fungi, containing the most clear define barcode gap between inter and intraspecific variation. Actually the ITS region has already been proposed as the primary fungal barcode marker by the Consortium for the Barcode of Life (Conrad et al., 2012).

1.4. AIMS

Europe is known among speleologists as exceptional for its abundance and variety of subterranean environments. Many of them can be visited and are important centres of mass tourism attraction. With today´s leisure tourism, the frequency of visiting caves increased, consequently it became necessary to know about potentially pathogenic microorganisms present in caves, and inform the public about it. Unfortunately, information on this subject is scarce, and visitors remain unaware of the dangers surrounding them (Jurado et al., 2010a). In Portugal this kind of information is practically absent and to our knowledge, there is a single study published about a survey of the mycological flora in "Fojo dos Morcegos" cave (Almeida et al., 1994). Additionally, fungal infections nowadays are a serious problem in terms of public health. Both population aging and progress in invasive techniques for diagnosis led to increasing numbers of immunocompromised patients subject to risk factors predisposing to the development of severe infections. This is why the determination of the extent of potential dangers inside a cave is of great importance.

The main aim of this project is to determine the fungal diversity present in bat guano from caves in Portugal mainland and compare the diversity found among the caves sampled.

Upon the general purpose, our objective was also to search for fungal species of clinical interest, namely *H. capsulatum*, in order to evaluate the potential risks for public health. Finally, another objective was to look for the presence of *Geomyces destructans*, a fungi causing mass mortality among American bats.

CHAPTER II

MATERIALS AND METHODS

2.1 CAVES SELECTION

A survey of the fungal diversity in bat guano samples at 17 bat roosts distributed in various regions of Portugal mainland was conducted (Figure 4). These roosts were chosen because they were places targeted for monitoring bat colonies by different speleologists (Table 2). The requisites needed for the collection of bat guano samples were that the species of bats had to be known and the guano had to be present in great amounts.

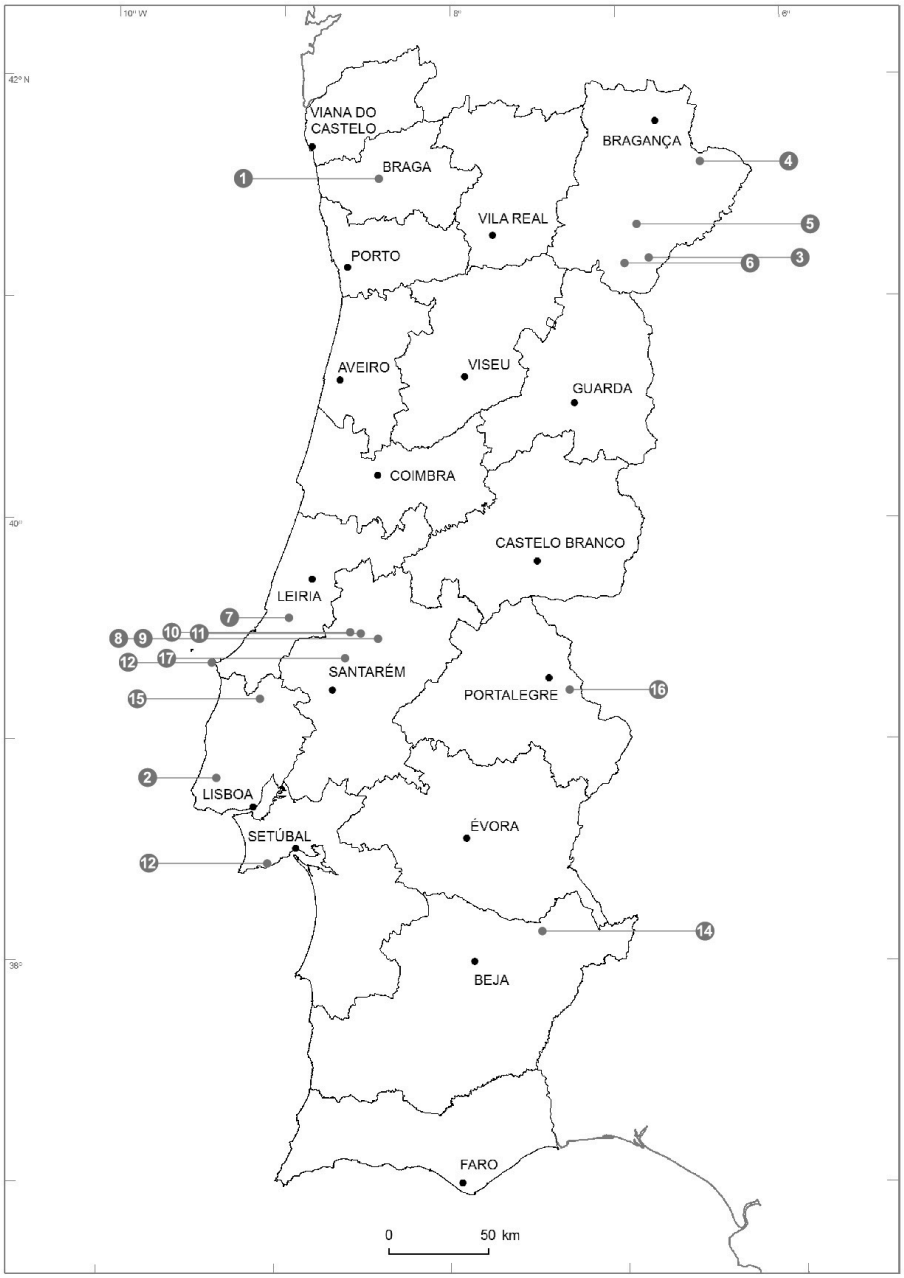

Figure 4: Location of the caves where the guano sampling occurred. Information relative to each number is found in Table 2.

2.2 GUANO SAMPLING

This research was conducted from January 2012 to May 2012 in 43 guano piles of insectivorous bats located in the caves already mentioned. Guano piles varied greatly in size, thickness and shape (Figure 5).

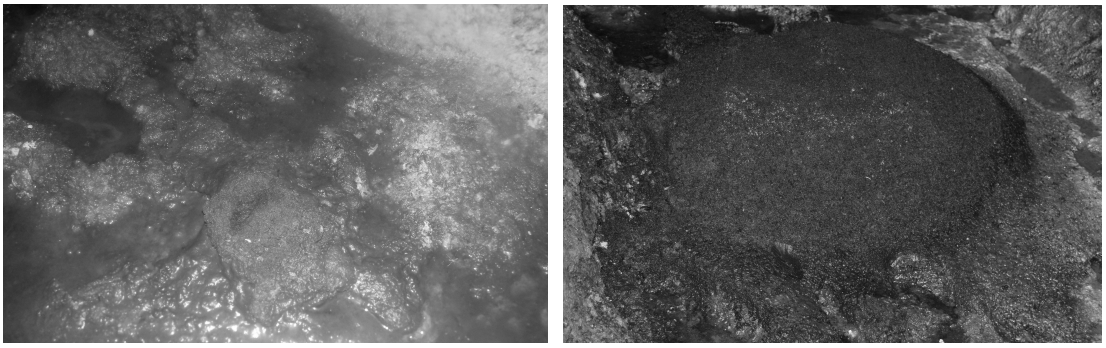

Figure 5: Guano piles. On the left guano from "Mina de Felgar", largely distributed all over the cave with a muddy and damp aspect. On the right guano from "Sendim da Serra", concentrated in one site of the cave and with a dried aspect (photos courtesy of Vírginia Duro).

Once inside a cave different spots containing large amounts of guano were selected to collect the samples, assuring that there was a good coverage of the entire cave. All samples were collected right bellow the surface of the guano pile. Using a sterile spatula, the surface of the pile was removed sideways and the guano right bellow was collected into a 50 ml sterilized falcon tube. Afterwards, this falcon was sealed with parafilm and labelled with a sample number and the cave name. Finally, the spatula was cleaned with ethanol 70% and paper. This process was repeated the necessary number of times. All collected samples were stored in a refrigerator at 4℃ until their arrival at the laboratory, for a period no longer than 10 days to avoid the germination of fungi spores stimulated by humidity, and thus prevent any change in the microflora.

Cave related data, like environmental temperature and relative humidity were measured whenever possible, and a questionnaire was filled by the person who collected the sample (Appendix I). Data related to each cave is presented in Table 2.

Table 2: List and characteristics of bat guano collection caves and bat genera/species inhabiting those caves.

Number*	Cave	Location	Sampling day	Bat Genera/Species in the cave	Number of samples	Temperature	Humidity	Author(s) of sampling
1	Tibães	Braga	20/1/2012	*Rhinolophus*	1	(-)	(-)	Vírginia Duro, Frederico Hintze and Filipa Vale
2	Regaleira	Sintra	28/1/2012	*Rhinolophus hipposideros*	1	20.8℃	67%	Filioa Vale
3	Carviçais	Carviçais, Torre de Moncorvo	26/1/2012	*Rhinolophus ferrumequinum* *Miniopeterus schreibersii*	1	8.9℃	82%	Vírginia Duro, Frederico Hintze and Francisco Amorim
4	Campo de Víboras	Vimioso	24/1/2012	*Rhinolophus euryale* *Rhinolophus mehelyi*	1	15.7℃	77.9%	Vírginia Duro, Frederico Hintze and Francisco Amorim
5	Sendim da Serra	Alfândega da Fé	24/1/2012	*Rhinolophus*	1	10.2℃	77.9%	Vírginia Duro, Frederico Hintze and Francisco Amorim
6	Mina Felgar	Felgar, Torre de Moncorvo	25/1/2012	*Rhinolophus*	1	14.6℃	86.6%	Vírginia Duro, Frederico Hintze and Fran isco Amorim
7	Louro	Alcobaça	4/2/2012	(+)	3	14.5℃	85.5%	Sr. Gabriel Mendes
8	Almonda	Torres Novas	4/2/2012	*Myotis myotis*	3	(-)	(-)	Sr. Gabriel Mendes
9	Malhada	Bairro, Torres Novas	4/2/2012	*Rhinolophus euryale* *Rhinolophus mehelyi*	3	(-)	(-)	Sr. Gabriel Mendes
10	Picos	Telhados Grandes	4/2/2012	*Rhinolophus ferrumequinum*	1	(-)	(-)	Sr. Gabriel Mendes
11	Lapa da Ovelha	Minde	5/2/2012	*Miniopterus schreibersii*	3	11℃	67%	Sr. Gabriel Mendes
12	Zambujal	Sesimbra	12/2/2012	*Miniopterus schreibersii*	3	10,7℃	46%	Bárbara Monteiro
13	Sem nome	Peniche	12/2/2012	(+)	3	(-)	(-)	Bárbara Monteiro
14	Preguiça	Moura	16/2/2012	*Rhinolophus ferrumequinum* *Rhinolophus hipposideros* *Rhinolophus euryale* *Rhinolophus mehelyi* *Myotis myotis* *Myotis blythii* *Myotis escalerai* *Myotis daubentonii* *Miniopterus schreibersii* *Plecotus austriacus* *Eptesicus serotinus*	6	f. 1_19.9℃ f. 2_21.9℃ f. 3_23℃ f. 4_21.2℃ f. 5_20.5℃ f. 6_15.3℃	f. 1_43% f. 2_76% f. 3_69% f. 4_69% f. 5_72% f. 6_75%	Dra. Luísa Rodrigues

Table 2 (cont.)

Number*	Cave	Location	Sampling day	Bat Genera/Species in the cave	Number of samples	Temperature	Humidity	Author(s) of sampling
15	Gralhas	Cadaval	17/2/2012	*Rhinolophus ferrumequinum* *Rhinolophus euryale* *Rhinolophus mehelyi* *Myotis myotis* *Myotis blythii* *Myotis escalerai* *Myotis daubentonii* *Myotis bechsteinii* *Miniopterus schreibersii*	5	f. 1_10.1ºC f. 2_10.2ºC f. 3_10.8ºC f. 4_12.3ºC f. 5_12.5ºC	f. 1_70% f. 2_72% f. 3_68% f. 4_69% f. 5_71%	Dra. Luísa Rodrigues
16	Cova da Moura	Marvão	24/2/2012	*Rhinolophus ferrumequinum* *Rhinolophus euryale* *Rhinolophus mehelyi* *Myotis myotis* *Myotis blythii* *Myotis escalerai* *Myotis daubentonii* *Myotis bechsteinii* *Myotis emarginatus* *Miniopterus schreibersii*	6	f. 1_12.4ºC f. 2_12.3ºC f. 3_11.3ºC f. 4_11.4ºC f. 5_14.5ºC f. 6_10.4ºC	f. 1_45% f. 2_48% f. 3_49% f. 4_47% f. 5_68% f. 6_51%	Dra. Luísa Rodrigues
17	Gruta do Alviela	Alcanena	3/5/2012	*Miniopterus schreibersii*	1	16ºC	87%	Sr. Gabriel Mendes
Total					43			

* The location of each cave is shown on figure X; (-) weren´t able to mesure; (+) waiting for the identification; (f.) falcon.

2.3 SAMPLE INOCULATION AND FUNGAL ISOLATION

As soon as the samples arrived to the laboratory, 1g of each guano sample was mixed with 5 ml phosphate-saline buffer (PBS) (Appendix II) and the solution was homogenised for at lest 3 minutes in a vortex, to ensure that the microorganisms were released in the supernatant. The supernatant was centrifuged at 300x *g* at 4℃, for 10 minutes to precipitate all the residues and separate them from the microorganisms. Subsequently, the supernatant was transferred to a new sterilized falcon. When there were different samples from the same cave, the supernatants were mixed together in a single falcon. Finally, 0.1 ml supernatant was inoculated on triplicate on different agar media supplemented with 0,005% chloramphenicol (Taylor et al., 2005). The entire procedure was carried under aseptic conditions.

Potato dextrose agar (PDA), yeast extract glucose chloramphenicol (YGC) and corn meal agar (CMA) were the media chosen for fungal growth. After the inoculation, plates of PDA and YGC were stored at 25℃ for 3 weeks and plates of CMA were stored at 15 and 4℃ for 4 to 5 weeks, a longer period because the growth of fungi was much slower (Gargas et al., 2009).

A few days later, several colonies with different appearance began to arise. All the colonies that appeared to differ from each other where isolated into new media, under aseptic conditions in a flow chamber with horizontal air flux, and kept at the correspondent temperature. After this first isolation, only 3 more occurred in each inoculated set of samples, one in the first week, one in the second and one in the third week after inoculation.

The isolation plates were regularly checked during incubation to make sure that there were no contaminations. Whenever some contaminations were observed the isolation was repeated until the target species were separated from it

2.4 SOLATES STORAGE

All isolates were stored in order to preserve the different strains for undefined period of time and to prevent any change in their viability, morphology and composition. The preservation method was different for yeasts and filamentous fungi

and all the processes involved were performed in a flow chamber with horizontal air flux, to ensure aseptic conditions and protection against any pathogenic strain.

Regarding yeasts, all strains where grown in Petri dishes, filled with YGC medium for 2-3 days and at the corresponding growth temperatures. Simultaneously, the cryotubes for the preservation where prepared: in each 1.5 ml of glycerol 30%, was added in sterile conditions. Then, through the aid of a toothpick with flattened tip, a small amount of the colony was collected and immersed in the solution, scraping it against the walls of the cryotube. Finally, all the cryotubes where stored in a deep freezer at a -80℃.

Concerning filamentous fungi, the efficiency of the preservation depends on the type and degree of sporulation: spore-forming strains can usually be frozen successfully, however the same type of preservation for non-sporulating strains is far less successful. For this reason, and to avoid any loss of samples, all filamentous fungi were preserved in PDA medium and stored at 4℃. For this purpose, all strains were grown in Petri dishes, filled with PDA medium, for at least 15 days and at the corresponding growth temperature. At the same time, the cryotubes for the preservation where prepared: in each 1 ml of PDA medium was added, they were autoclaved and, immediately after exiting the autoclave, they were inclined to allow the solidification of the medium in the form of a ramp. After, a small amount of the colony was inoculated in the cryotube and grown for at least 15 days and at the corresponding grow temperature. Finally, all the cultures in the cryotubes were stored in a refrigerator at 4℃.

2.5 DIRECT IDENTIFICATION

The isolates were identified at the genus level, and when possible at the species level according to their morphological characteristics.

Along the incubation time, pictures were taken of the plates, using ChemiDoc, to register the fungal diversity on each Petri dish. Using a stereo microscope, every different colony was morphologically described. Finally it was also performed the CFU (colony forming units) count.

Micro-morphological characteristics were also recorded. For this purpose, isolated filamentous samples were prepared with adhesive tape mounted on a glass

slide, using the mounting method previously described (Harris, 2000). Regarding yeasts samples, the preparations were done simply using a toothpick. A stain solution was used – Cotton Blue 0.05% – to enhance the reproductive structures and yeast cells. Once these preparations were ready they were sealed with varnish, observed under a Laborlux S microscope (Wild Leitz) with a built-in camera and various pictures were taken.

2.6 MOLECULAR IDENTIFICATION

In order to complement direct identification a molecular approach was carried out. DNA extraction was performed and it was distinctly held in filamentous fungi and yeast cells.

2.6.1 DNA EXTRACTION FROM FILAMENTOUS FUNGI SAMPLES

Initially, each isolate was grown on Petri dishes filled with PDA medium, for at least 15 days, at the corresponding incubation temperature. After this time or when the cultures were mature, a 2 ml eppendorf was filled up to approximately 500 µL with the sample biomass. Then, the eppendorf was submerged in liquid nitrogen and the biomass was well macerated with the aid of a pestle. After, 3 x 200 µL of TES buffer were added (Appendix II), always macerating after each addition. The eppendorf was then placed on boiling water (95-100*ºC*) for 5 minutes, vortexed and left to cold down to room temperature. Later, two centrifugations were performed: the first at 3000 rpm, at 4*ºC*, for 10 minutes and the second at 13000 rpm, at 4*ºC*, for 3 minutes, to improve the efficiency of the DNA extraction. Afterwards, the same volume of chloroform: isoamylalcohol (24:1) was added, the mixture vortexed and centrifuged at 14000 rpm, at 4*ºC*, 10 minutes. This centrifugation resulted in a gradient: the first layer is where the DNA is located, the second layer is where the proteins are found and the third layer is where we can find other contaminates, such as RNases. So, the first layers was carefully removed, transferred to a new eppendorf and the same process was performed twice. Next, 1/10 of the total volume of sodium acetate 3M was added and 2.5X of the final volume of ethanol absolute was added.

This mixture was homogenised by inverting 50 to 60 times and centrifuged at 14000 rpm, at 4℃, for 30 minutes. With this centrifugation the DNA was precipitated and the supernatant was discarded. The pellet was washed with 5 ml of ethanol 70%, and then centrifuged at 14000 rpm, at 4℃, for 10 minutes. Once again, the supernatant was discarded and, when the pellet was dry, it was suspended in 50 µL of ultra pure H_2O and was placed in a refrigerator at 4℃, overnight. In the next day, the eppendorf was placed in a ThermoBlock™ at 65℃, for at least 45 minutes, in order to dissolve the pellet. Finally, the DNA was quantified in a NanoDrop Spectrophotometer and stored in a freezer at -20℃ until their utilization.

2.6.2 DNA EXTRACTION FROM YEAST SAMPLES

Each isolated sample was grown on Petri dishes filled with YGC medium, for at least 3 days at the corresponding incubation temperature. With the aid of a blue tip an amount of colony was withdrawn to a 1,5 ml eppendorf. Then the eppendorfs were placed in the microwave for 3 minutes to promote cell lysis. Once outside the microwave the eppendorfs were immediately placed on ice. The lysed cells were suspended on 100 µL of solution A (Appendix II) and then 5 µL of Sodium Dodecyl Sulfate (SDS) 10% were added. The mixture was incubated at 65℃, for 5 minutes in a ThermoBlock™. After incubation, the eppendorfs were placed on ice, 80 µL of potassium acetate 5M was added and the mixture was homogenised on a vortex. Later, the mixture was centrifuged at the maximum velocity at 4℃, 30 minutes and the supernatant was transferred to a new eppendorf, where 200 µL of isopropanol were added. Finally, the eppendorfs where stored in a refrigerator at 4℃, overnight.

In the next day, the eppendorfs where placed at room temperature for 5 minutes, followed by a centrifugation at the maximum velocity at 4℃, for 15 minutes. The supernatant was rejected and the pellet was washed twice with 500 µL of etanol 70% and once with 500 µL of ethanol 96%. In between the washes the eppendorfs were centrifuged at the maximum velocity at 4℃, for 5 minutes. Finally, after the pellet dry, it was dissolved in 50 µL of TE buffer (Appendix II). After their quantification in a NanoDrop Spectrophotometer, the DNA's were stored at -20℃ until their utilization.

2.6.3 PCR AMPLIFICATION AND SEQUENCING

After the DNA extraction, a PCR was performed (Table 3) in a iCycler Thermocycler, amplifying the ITS region and using the primer forward ITS1 (Gardes & Bruns, 1993) and reverse ITS4 (White et al., 1990).

Table 3: Reaction components and conditions of the PCR.

PCR Mix	**Volume (µl)**
Taq (10x) with $NH_4(SO_4)_2$	5
MgCl2 (25 mM)	4
ITS1 (10 mM) (5'-TCCGTAGGTGAACCTGCGG-3')	4
ITS4 (10 mM) (5'-TCCTCCGCTTATTGATATGC-3')	4
dNTPs (10 mM)	1
Taq polimerase (5 U/ µL)	0.6
Ultra pure H_2O	29.4
DNA	2
Total	50
PCR Conditions	
Initial denaturation	96℃ for 4 minutes
40 Cycles of denaturation	96℃ for 1 minute
Annealing	55℃ for 5 seconds
Extension	72℃ for 1 minute
Final extension	72℃ for 10 minutes

DNA amplification was confirmed by electrophoresis: PCR products were separated by agarose gel 1.2%, diluted in Tris-Acetate-EDTA (TAE) buffer 0.5 M (Appendix II). This electrophoresis ran at 70 mV and 100 mA, during 30-45 minutes. Then the gel was stained with GelRed™ (VWR) for at least 30 minutes in agitation and photographed under UV-light on a transilluminator. The PCR products were purified using GenElute™ PCR Clean-Up Kit (Sigma-Aldrich) and sent to DNA sequencing in STAB VIDA laboratory (http://www.stabvida.com/). Finally, the forward and reverse sequences obtained were assembled through the CodonCode Aligner program, and the contigs were submitted to a BLAST search on NCBI´s database GenBank

2.7 DATA ANALYSIS

Once the fungi identification was achieved, a data analysis was carried out (according to Figueira & Barata, 2007). For this purpose the following values were

determined: (1) Total number of incidences of each fungi per temperature and cave; (2) Number of species per cave; (3) Number of plates that yielded fungi; (4) Average number of species per cave = Total number of species divided by the total number of samples inoculated; (5) Number of unique species per cave; (6) Shannon index: $H' = -\sum_{i=1}^{S} pi.Ln\,(pi)$, where *pi* is the amount of individuals of the species *i* that contributes to total diversity; (7) Evennes index: $E = H'/H'max$, where $H'\,\max = Ln(S)$ is the maximum value of diversity of species that are present, and S the total number of species at the respective cave; and (8) Sorenson index: $Cs = 2j/(a+b)$, where *j* is the number of species common in the caves analysed, *a* is the number of species in cave A and *b* is the number of species in cave B.

Shannon index is one of the most popular indexes in community ecology and it allows the discrimination of the abundance of the species present among the caves. Its value can vary between 0 and 4,5: *H'* = 0 only if the sample includes only a single species; commonly is 1.5 <*H'*< 3.5 and is rarely grater than 4,5, when all species are equally abundant. Evenness can vary between 0 and 1 and closer to 1 means a more even population (Zak & Willig, 2004). Sorenson-index is used for comparison of two different caves and can vary from 0 to 1: 1 complete similarity and 0 complete dissimilarity (Abdel-Wahab & El- Sharouny, 2002).

CHAPTER III

RESULTS AND DISCUSSION

3.1 GENERAL RESULTS AND ORGANIZATION

For the isolation of fungi from the samples of bat guano different growth media were used: PDA specifically for the growth of filamentous fungi and YGC, mainly to favour the growth of yeast. However, it was verified that both yeast and filamentous fungi were able to grow either on PDA and YGC, with no significant differences in the number of isolates recovered. Finally, latter on emerged the issue to survey for *Geomyces destructans* and for this purpose CMA medium was chosen.

A total of 165 fungal isolates were obtained, from which 62 were identified to the species level, 88 to the genus level, and 15 remain to identify. As expected, the majority of the isolates, specifically 137, grew on the plates inoculated at 25*ºC*, followed by 20 isolates that grew on the plates inoculated at 15*ºC,* and only 8 isolates grew on the samples inoculated at 4*ºC* (Figure 6).

It is also important to mention that samples from the caves 1 to 6 where only incubated at 25*ºC*, because at the time the issue about the possible presence of *G. destructans* had not emerged yet. From cave 7 to 16 all inoculated samples were incubated at 25*ºC*, 15*ºC* and 4*ºC*. At these lower temperatures other fungi that were not *G. destructans* developed and they were also isolated and accounted for the project results. From cave 17 the inoculated samples were only incubated at 25*ºC* and 15*ºC* (Figure 6). The incubation at 4*ºC* was not performed, in part, due to lack of time and also because the optimal temperature for *G. destructans* growth was already proven to be 15*ºC*.

The fungi isolated in each cave are listed separately from table 4 to table 20. Some of these fungi were only identified according to their morphological characteristics. Therefore the majority of the taxa could only be identified to the genus level. Additionally, as this kind of identification is extremely difficult and under inexperienced eyes is even harder, there is some margin of error and probably some taxa may be misidentified. The characteristics through which the morphologic identification was achieved are also listed in the tables and are marked with a $^{(*)}$ right before the description. Others were identified according to the sequencing of ITS regions of rDNA genes and the two parameters throughout this molecular identification was achieved are also listed, in the same tables, and are marked with a $^{(+)}$. These two parameters provide an indication whether the alignment performed was "good" and they are: the e-value, that represent the number of hits and its value

decrease exponentially as the score of the match increases, meaning that the ideal value for this parameter is zero; and the pairwise % identity, which represent the pairwise residues that are identical in the alignment, meaning that the ideal value for this parameters is equal to 100. Additionally, there is the information on the CFU count whose values vary from uncountable (in the tables "In") to 43 colonies. In the tables 4 to 20 there are also some unidentified taxa and this occurred for the following main reasons: (*i*) the cultures from where the slide and coverslip preparation where performed weren't old enough and they didn't present the characteristic reproductive structures necessary for their morphological identification, (*ii*) the DNA extraction for these taxa didn´t work, it was needed more time for protocol optimization (*iii*) they might correspond to unknown species.

The data analysis values are shown in table 21, where the fungi isolated are alphabetically organized by phylum and cave, and where some data, like the Shannon and Evenness indexes, are displayed. Additionally, in table 22, the values of Sorenson index comparing the caves among each other, are presented. Also, from Figure 7 to 19, there are some photographs of identified and unidentified fungi during this master project.

The analysed caves will be addressed for their number code (Table 2): 1 - "Tibães", 2 - "Regaleira", 3 - "Carviçais", 4 - "Campo de Víboras", 5 - "Sendim da Serra", 6 - "Mina Felgar", 7 - "Louro", 8 - "Almonda", 9 - "Malhada", 10 - "Picos", 11 - "Lapa da Ovelha", 12 - "Zambujal", 13 - "Sem Nome", 14 - "Preguiça", 15 - "Gralhas", 16 - "Cova da Moura" and 17 - "Gruta do Alviela".

3.2 ANALISYS OF FUNGAL DIVERSITY IN EACH CAVE

In "Tibães", a total of four fungi were isolated. From these, only one was identified to the species level as *Trichosporon chiropterorum* and this identification was achieved by comparing the colony characteristics and the yeast cells with others, which were identified by sequencing of the ITS region of rDNA 28S. Two isolates were identified to the genus level, according to their reproductive structures, respectively as *Penicillium sp.* and *Verticillium sp.* (Figure 7); and one species remained to identify (Table 4). This isolate 1.2 was not identified because it was not

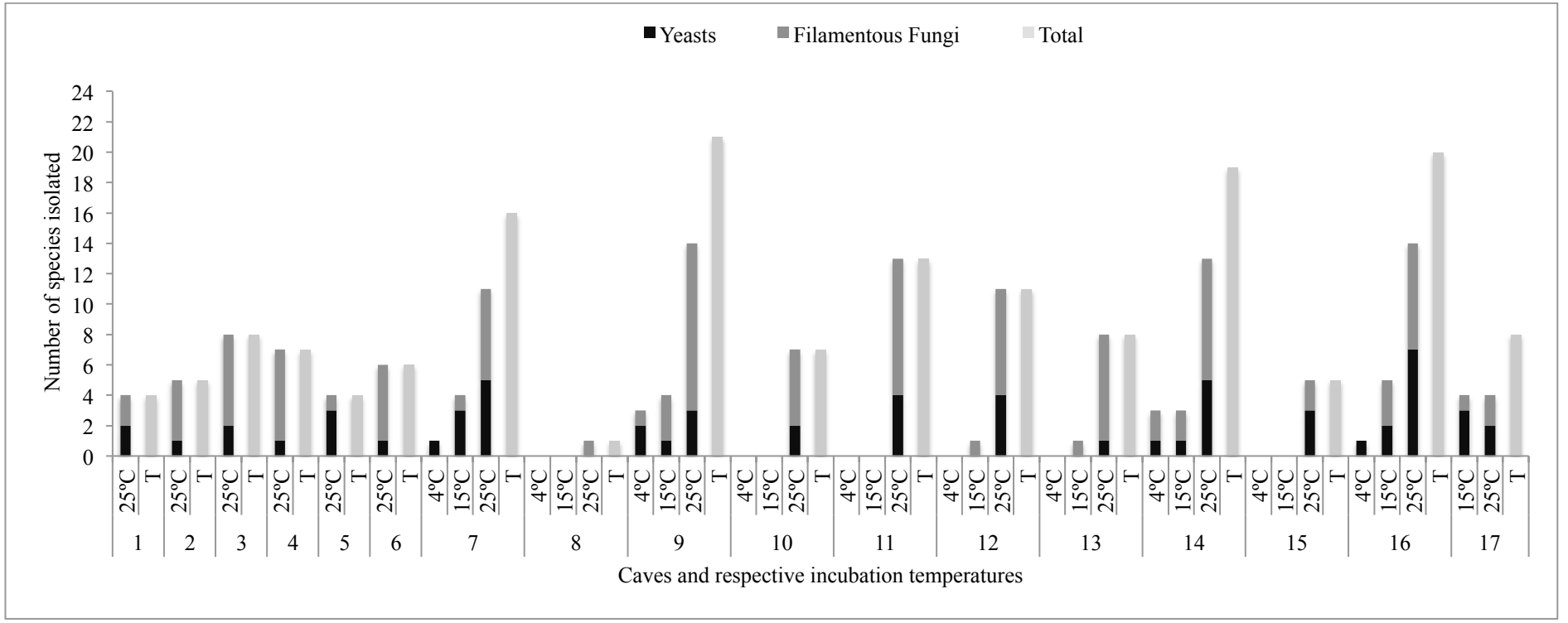

Figure 6: Number of filamentous fungi and yeast species isolated from each cave at the different temperatures of incubation.

possible just through the morphological characteristics exhibited and the sequencing results were not conclusive.

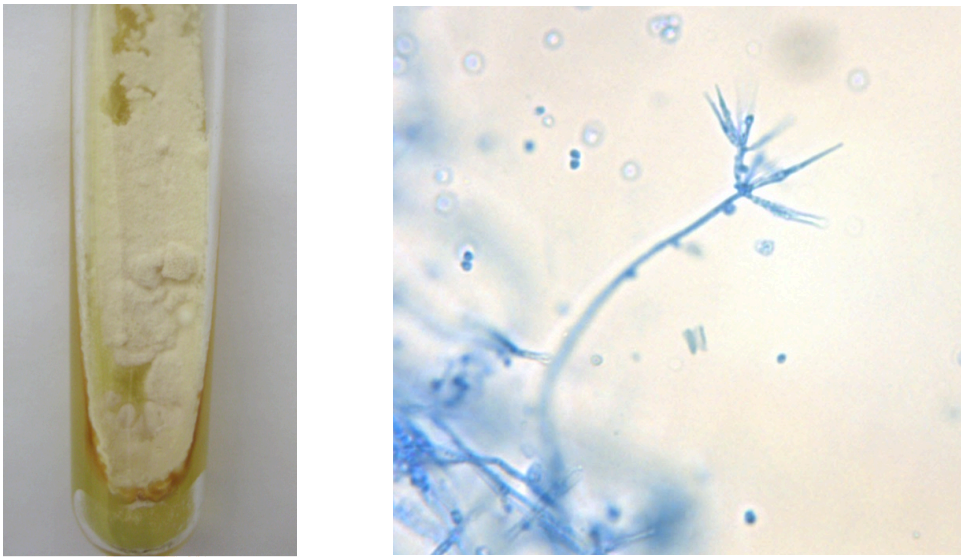

Figure 7: Fungi isolated from cave 1, with the code 1.A, identified as *Verticillium sp.* On the left, culture on PDA medium and, on the right, conidiophore and divergent phialides observed under an optical microscope at x400 magnification, both after three weeks of incubation.

Table 4: Fungi species isolated from the sample collected at the cave "Tibães", incubation temperature and CFU count. The parameters used for identification are also shown.

Code	Species	Temp.	CFU	(+) e-value (*)Colony Characteristic	(+) % Pairwise identity (*) Micro Characteristics
1.1	*Trichosporon chiropterorum*	25ºC	12	-	-
1.2	Unidentified	25ºC	17	-	-
1.A	*Verticillium sp.*	25ºC	1	(*) Suede-like, white colony and brown reverse	(*) Conidiophores well differentiated, erect and verticillately branched. Phialides divergent.
1.B	*Penicillium sp.*	25ºC	1	(*) Velutinous, dark-green colony and colourless reverse	(*) Conidiophores branched (biverticillated). Phialides in a ampulliform shape

From "Regaleira", a total of five fungi were isolated. Two of them were identified to the species level as *Mucor fragilis* and *Zygosaccharomyces florentinus*, both displaying the ideal values for e-value, which was equal to zero, and for pairwise % identity, that was equal to one hundred. The remaining three were identified to the genus level, according to the morphology of their reproductive structures, respectively as *Mucor sp.* (Figure 8), *Penicillium sp.* and *Rhizomucor sp.* (Table 5).

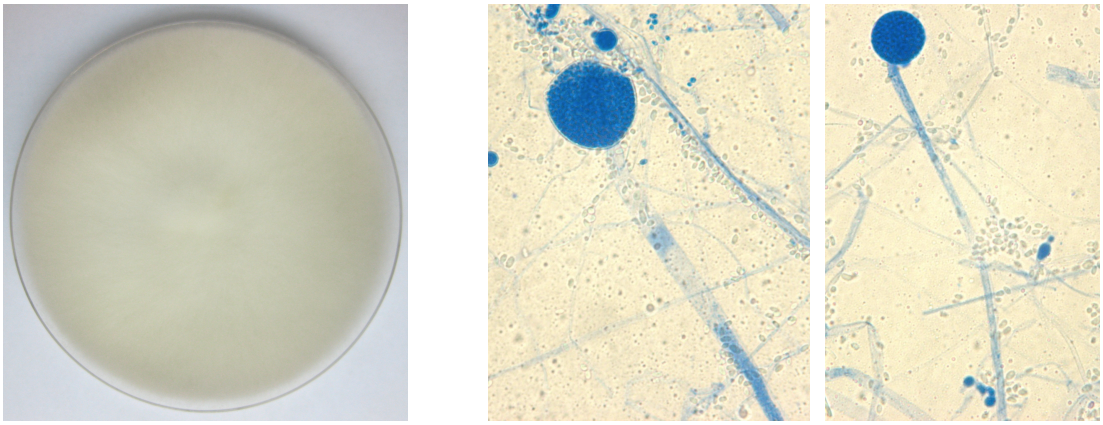

Figure 8: Fungi isolated from cave 2, with the code 2.B, identified as *Mucor sp.* On the left, culture on PDA medium and, on the right, sporangiophores observed under an optical microscope at x400 magnification, both after two weeks of incubation.

Table 5: Fungi species isolated from the sample collected at the cave "Regaleira" incubation temperature and CFU count. The parameters used for identification are also shown.

Code	**Species**	**Temp.**	**CFU**	**(+) e-value**	**(+) % Pairwise identity**
				(*)Colony Characteristic	**(*) Micro Characteristics**
2.1	*Zygosaccharomyces florentinus*	25ºC	8	(+) 0.0	(+) 100
2.A	*Mucor fragilis*	25ºC	42.3	(+) 0.0	(+) 100
2.B	*Mucor sp.*	25ºC	In	(*) Hairy, white colony and colourless reverse	(*) Sporangiophores without basal rhizoids, unbranched and multispore sporangia. Presence of chlamydospores along the hyphae
2.C	*Penicillium sp.*	25ºC	In	(*) Powdery, green colony and brown reverse	(*) Conidiophores branched (biverticillated). Phialides in a acerose shape
2.D	*Rhizomucor sp.*	25ºC	In	(*) Hairy and ochre colony and buff-ochre reverse	(*) Sporangiophores branched ending in a sporangium. Chlamydospores abundant

From "Carviçais", a total of eight fungi were isolated. Half of them were identified to the species level as *Trichosporon cavernicola*, *T. chiropterorum*, *Tolypocladium cylindrosporum* and *Verticillium leptobactrum*. This identification was quite accurate with the e-values equal to zero and pairwise % identity equal to 99. The only one that left doubts was *Verticillium leptobactrum*, but when observing and comparing its reproductive structures with confirmed ones, it showed a great similarity. From the other four, three were identified to the genus level, according to

the morphology of their reproductive structures, as *Paecilomyces sp.* (apparently two different species) and *Verticillium sp.* One isolate remained to identify (Table 6), because the cultures were not old enough and when observing under a microscope they didn't present the characteristic reproductive structures necessary for their morphological identification and after the DNA extraction for these taxa didn´t work.

Table 6: Fungi species isolated from the samples collected at the cave "Carviçais", incubation temperature and CFU count. The parameters used for identification are also shown.

Code	Species	Temp.	CFU	(+) e-value	(+) % Pairwise identity
				(*)Colony Characteristic	(*) Micro Characteristics
3.1	*Trichosporon chiropterorum*	25ºC	1	(+) 0.0	(+) 99
3.2	*Trichosporon cavernicola*	25ºC	3	(+) 0.0	(+) 99
3.A	*Verticillium sp.*	25ºC	1	(*) Woolly, white colony and colourless reverse. Production of orange pigments	(*) Conidiophores well differentiated, erect and verticillately branched. Phialides divergent.
3.B	Unidentified	25ºC	1	-	-
3.C	*Verticillium leptobactrum*	25ºC	1	(+) $4e^{-174}$	(+) 99
3.D	*Paecilomyces sp.*	25ºC	14.3	(*) Suede-like, grey-green colony and brown revers	(*) Septate hyphae from where the conidiophores branch vertically. Phialides are swollen at their base and ovoid conidia, forming long chains
3.E	*Tolypocladium cylindrosporum*	25ºC	In	(+) 0.0	(+) 100
3.F	*Paecilomyces sp.*	25ºC	1	(*) Velutinous, grey-green colony and brown reverse	(*) Septate hyphae from here the conidiophores branch vertically. Phialides are swollen at their base and ovoid conidia, forming long chains

From "Campo de Víboras", a total of seven isolates were obtained. From these, four remain to identify, two were identified as *Paecilomyces sp.* and *Trichoderma sp.,* according to the morphology of their reproductive structures, and one was molecularly identified as *Trichosporon otae*, displaying a e-value of zero and pairwise % identity equal to 99 (Table 7). The reasons why the isolates 4.A, 4.B and 4.C were not identified were the follow: the cultures were too young and when observing under a microscope they didn't present reproductive structures necessary for their morphological identification and after the extraction, the DNA was not pure.

Regarding the isolate 4.D it was also very young and the DNA extraction was not successful.

Table 7: Fungi species isolated from the samples collected at the cave "Campo de Víboras", incubation temperature and CFU count. The parameters used for identification are also shown.

Code	Species	Temp.	CFU	(+) e-value	(+) % Pairwise identity
				(*)Colony Characteristic	(*) Micro Characteristics
4.1	*Trichosporon otae*	25ºC	1.6	(+) 0.0	(+) 99
4.A	Unidentified	25ºC	1	-	-
4.B	Unidentified	25ºC	1	-	-
4.C	Unidentified	25ºC	1	-	-
4.D	Unidentified	25ºC	1	-	-
4.E	*Paecilomyces sp.*	25ºC	23	(*) Suede-like, white colony and light brown reverse	(*) Septate hyphae from where the conidiophores branch vertically. Phialides are swollen at their base and ovoid conidia, forming long chain
4,F	*Trichoderma sp.*	25ºC	20.5	(*) Suede-like, white colony and brown reverse	(*) Septate hyphae from where the conidiophores branch vertically. Phialides are swollen at their tips and round conidia

In "Sendim da Serra", a total of four fungi were recovered from the plates inoculated. One isolate was identified to the genus level as *Paecilomyces sp*, according to the morphology of its reproductive structures. The remaining ones were analysed through ITS region sequencing, however the results obtained do not allow species identification since the sequence homologies found were lower than 98%. Hence, these identifications remained at the genus level, as *Trichosporon sp.* (apparently two different species) (Figure 9) and *Rhodotorula sp.* (Table 8).

Table 8: Fungi species isolated from the samples collected at the cave "Sendim da Serra", incubation temperature and CFU count. The parameters used for identification are also shown.

Code	Species	Temp.	CFU	(+) e-value	(+) % Pairwise identity
				(*)Colony Characteristic	(*) Micro Characteristics
5.1	*Trichosporon sp.*	25ºC	4	(+) 0.0	(+) 94
5.2	*Rhodotorula sp*	25ºC	1	(+) $2e^{-158}$	(+) 97
5.3	*Trichosporon sp.*	25ºC	6	(+) 0.0	(+) 95
5.A	*Paecilomyces sp.*	25ºC	6.5	(*) Velutinous, white colony and light brown reverse	(*) Septate hyphae from where the conidiophores branch vertically. Phialides are swollen at their base and ovoid conidia, forming long chains

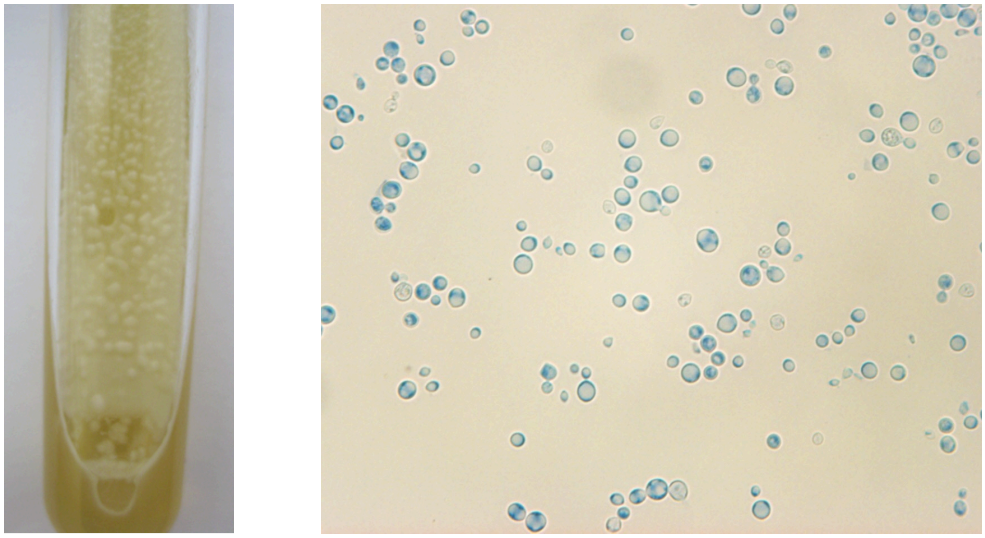

Figure 9: Yeast isolated from cave 5, with the code 5.1, as *Trichosporon sp.* On the left, culture on YGC medium and, on the right, the cells, observed under an optical microscope at x400 magnification, both after one week of incubation.

In "Mina Felgar" a total of six fungi were recovered. One taxa was identified to the species level according to the sequencing results, as *Rhodotorula bloemfonteinensis*, displaying a e-value equal to $1e^{-175}$ and Pairwise % identity equal to 98. Cell and colony morphology were concordant with the sequencing results. From the remaining ones, they where identified according to their reproductive structures: one to the species level, as *Penicillium citrinum*, and the rest to the genus level, as *Aspergillus sp.*, *Paecilomyces sp.* (Figure 10), *Penicillium sp.,* and *Trichoderma sp.* (Table 9).

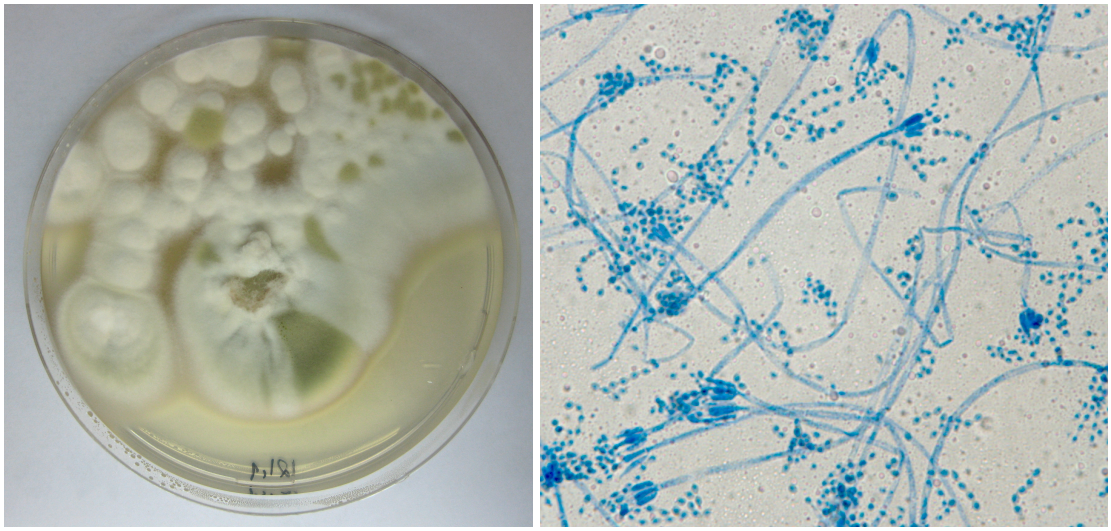

Figure 10: Fungi isolated from cave 6, with the code 6.D, identified as *Paecilomyces sp*. On the left, culture on PDA medium and, on the right, conidiophores, divergent phialides and ovoid conidia in chains, observed under an optical microscope at x400 magnification, both after two weeks of incubation.

Table 9: Fungi species isolated from the samples collected at the cave "Mina Felgar", incubation temperature and CFU count. The parameters used for identification are also shown-

Code	Species	Temp.	CFU	(+) e-value (*)Colony Characteristic	(+) % Pairwise identity (*) Micro Characteristics
6.1	*Rhodotorula bloemfonteinensis*	25ºC	6	(+) $1e^{-175}$	(+) 98
6.A	*Penicillium sp.*	25ºC	1	(*) Velutinous, green yellow colony and colourless reverse	(*) Conidiophores branched (monoverticillated). Phialides in a shape of ampulliform. Ovoid conidia forming chains
6.B	*Penicillium citrinum*	25ºC	4.3	(*) Velutinous, greyish colony and colourless reverse	(*) Conidiophores branched (biverticillated). Phialides in flask-shaped. Spherical conidia.
6.C	*Aspergillus sp.*	25ºC	2	(*) Grey colonies with black reverse. Production of orange pigments	(*) Conidiophores erects, unbranched and without septum at the base. Conidia are grouped in chains forming columns
6.D	*Paecilomyces sp.*	25ºC	3.5	(*) Velutinous, white-light green colony and light brown reverse	(*) Septate hyphae from where the conidiophores branch vertically. Phialides are swollen at their base and ovoid conidia, forming long chains
6.E	*Trichoderma sp.*	25ºC	2.3	(*) Suede-like, white colony and brown reverse	(*) Septate hyphae from where the conidiophores branch vertically. Phialides are swollen at their tips and round conidia

In "Louro", a total of sixteen fungal isolates were obtained. Eight of them were identified by sequencing the ITS region of 28S rDNA genes as *Oidiodendron sp.*, *Pochonia chlamydosporia*, *Trichosporon jirovecii*, *T. moniliiforme* (occurring twice), *T. mycotoxinivorans* and *T. shinodae* (occurring twice). However, as the results of the molecular identification are only considered when there is at least 98% sequence homology, three of the isolates were only identified to the genus level as *Chrysosporium sp*. (apparently occurring twice) (Figure 11) and *Trichosporon sp.* Additionally, one of the isolates was identified as *Aspergillus sp*, according to the morphology of its reproductive structures, and three remained to identify. The isolates 7.3 and 7.5 were not identified because by observation of the morphological characteristics exhibited we couldn't reach it and the DNA extraction required optimization. Regarding the isolate 7.E there was no match with known fungi and the DNA sequencing results were not conclusive, they need to be repeated. The genera most abundant in these cave was *Trichosporon* with a total of seven species present in

the three temperatures of incubation. Some of the isolates were able to grow both at 25ºC and 15ºC, one was able to grow at 4ºC, and *T. mycotoxinivorans* was only able to grow at 15ºC, meaning that these species are competent to grow at lower temperatures and can be called psychrophilic fungi (Table 10).

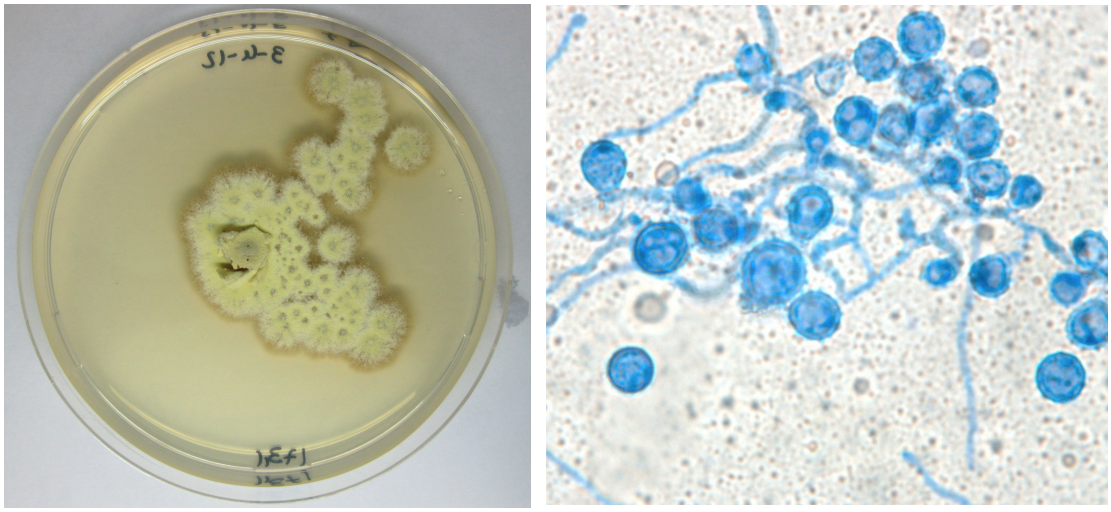

Figure 11: Fungi isolated from cave 7, with the code 7.D, identified as *Chrysosporium sp.* On the left, culture on PDA medium and, on the right macroconidia, observed under an optical microscope at x1000 magnification, both after two weeks of incubation.

Table 10: Fungi species isolated from the samples collected at the cave "Louro", incubation temperature and CFU count. The parameters used for identification are also shown-

Code	Species	Temp.	CFU	(+) e-value (*)Colony Characteristic	(+) % Pairwise identity (*) Micro Characteristics
7.1	*Trichosporon shinodae*	25ºC	2.5	(+) 0.0	(+) 99
7.2	*Trichosporon moniliiforme*	25ºC	1	(+) 0.0	(+) 99
7.3	Unidentified	25ºC	1	-	-
7.4	*Trichosporon jirovecii*	25ºC	1	(+) 0.0	(+) 98
7.5	Unidentified	25ºC	8.5	-	-
7.A	*Acremonium strictum*	25ºC	1.8	(*) Velutinous, with colony and orange reverse. Production of orange pigment	(*) Conidiophores simple and sometimes branched. Oval conidia aggregated at the apex of each phialide.
7.B	*Oidiodendron sp.*	25ºC	1.6	(+) $5e^{-101}$	(+) 98
7.C	*Aspergillus sp.*	25ºC	5.75	(*) Black colonies with black reverse. Production of brownish pigments	(*) Conidiophores erects, unbranched and without septum at the base. Conidia are agrouped in chains forming columns
7.D	*Chrysosporium sp.*	25ºC	1	(+) $2e^{-149}$	(+) 85

Table 10 (Cont.)

Code	Species	Temp.	CFU	(+) e-value	(+) % Pairwise identity
				(*)Colony Characteristic	(*) Micro Characteristics
7.E	Unidentified	25ºC	1	-	-
7.F	*Pochonia chlamydosporia*	25ºC	1	(+) 0.0	(+) 98
7.7	*Trichosporon mycotoxinivorans*	15ºC	1.6	(+) 0.0	(+) 98
7.8	*Trichosporon moniliiforme*	15ºC	3	(+) 0.0	(+) 99
7.9	*Trichosporon shinodae*	15ºC	4	(+) 0.0	(+) 99
7.G	*Chrysosporium sp.*	15ºC	3	(+) $5e^{-145}$	(+) 84
7.6	*Trichosporon sp.*	4ºC	3	(+) 0.0	(+) 92

From "Almonda", only one fungal isolate was recovered, the lowest value registered among all the caves surveyed. To understand the low diversity inside this cave several parameters have to be taken into account such as: (*i*) the kind of cave formation, if by physical process, if by mechanical process or both, (*ii*) the local of sampling according to cave zonation, depending on the zone of the light distribution, temperature fluctuation vary greatly and it has a massive influence in cave organisms and (*iii)* which vertebrates and invertebrates reside inside the cave. These parameters were not recorded during this project, but now looking at the final results we realized that they were needed, because this data was important to have a better understanding of the differences found among the caves. The only species isolated from this sample was molecularly identified as *Sporothrix schenckii* and it was exclusive to this cave. It displayed an e-value of zero and Pairwise % identity equal to 100 (Table 11). However, further studies need to be performed to confirm this identity since species from the *Sporothrix* species complex are difficult to separate and it is accepted that the sequences of calmodulin genes are the most adequate to separate species from this complex (Marimon et al., 2007).

Table 11: Fungi species isolated from the samples collected at the cave "Almonda", incubation temperature and CFU count. The parameters used for identification are also shown.

Code	Species	Temp.	CFU	(+) e-value	(+) % Pairwise identity
				(*)Colony Characteristic	(*) Micro Characteristics
8.A	*Sporothrix schenckii*	25ºC	1	(+) 0.0	(+) 100

In "Malhada" a total of twenty-one fungal isolates were obtained. Seven of them were identified by sequencing the ITS region of 28D rDNA as *Geomyces*

pannorum (Figure 12), *Trichosporon cavernicola*, *T. chiropterorum* (with tree occurrences), *T. moniliiforme* and *T. porosum*; and one to the genus level as *Lecanicillium sp.*, displaying e-values of zero and Pairwise *%* identities higher then 98%. Additionally, one isolate was identified as *Rhizomucor variabilis* according to the morphology of its reproductive structures, and eleven to the genus level as, *Beauveria sp.*, *Fusarium sp.*, *Microsporum sp.*, *Mucor sp.*, *Paecilomyces sp.* (apparently two different species), *Penicillium sp.* (apparently four different species) and *Verticillium sp.* One isolate remained to identify (Figure 13), because through the morphological characteristics exhibited we couldn't reach it and after there was not enough time to receive the DNA sequencing results (Table 12).

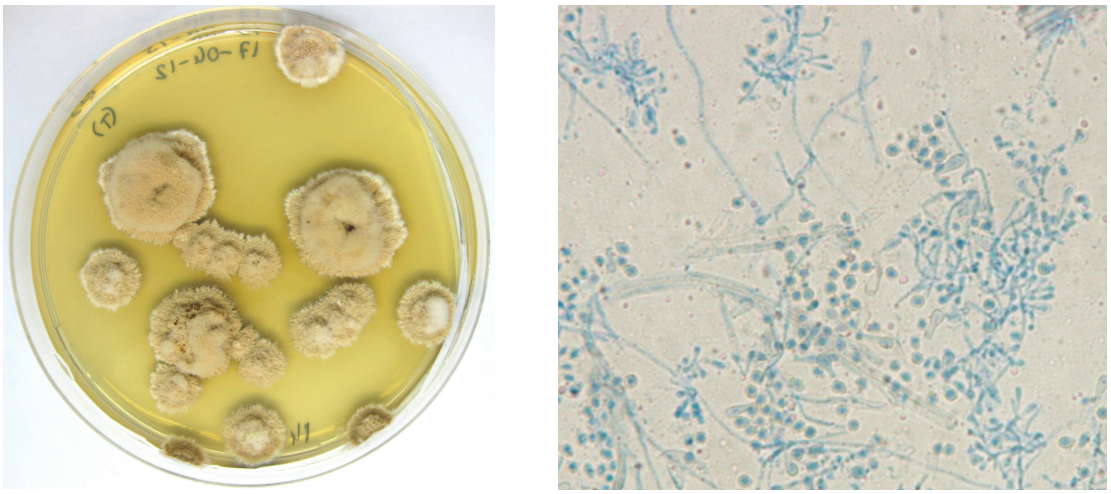

Figure 12: Fungi isolated from cave 9, with the code 9.F, identified as *Geomyces pannorum*. On the left, culture on PDA medium and, on the right, conidiophores and conidia, observed under an optical microscope at x400 magnification, both after three weeks of incubation.

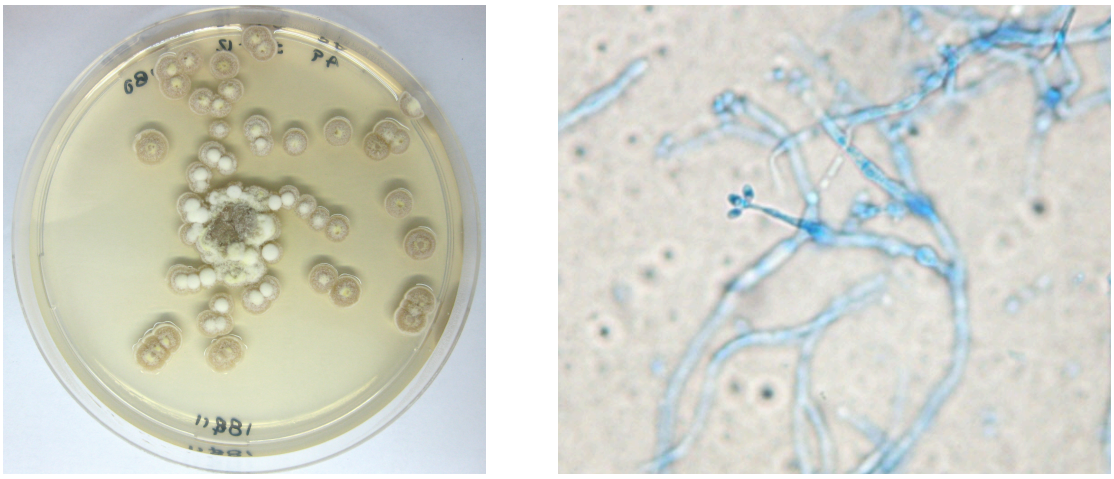

Figure 13: Fungi isolated from cave 9, with the code 9.K, which was one that remained to identify. On the left, culture on PDA medium and, on the right reproductive structure under an optical microscope at x400 magnification, both after three weeks of incubation.

The genera more abundant in this cave was again *Trichosporon,* as already observed in cave 7, with a total of six species present at the three temperatures of incubation. *T. chiropterorum* was the only species able to grow in the three incubation temperatures. There were also some other species able to grow at the lower temperatures (Table 12).

Table 12: Fungi species isolated from the samples collected at the cave "Malhada", incubation temperature and CFU count. The parameters used for identification are also shown.

Code	Species	Temp.	CFU	(+) e-value (*)Colony Characteristic	(+) % Pairwise identity (*) Micro Characteristics
9.1	*Trichosporon chiropterorum*	25ºC	5.57	(+) 0.0	(+) 99
9.2	*Trichosporon moniliiforme*	25ºC	14.5	(+) 0.0	(+) 98
9.3	*Trichosporon cavernicola*	25ºC	3	(+) 0.0	(+) 99
9.A	*Beauveria sp.*	25ºC	14	(*) Woolly, white colony and colourless reverse	(*) Conidiogenous cells growing in aggregation in dense clusters along the hypha. Conidia are single celled oval
9.B	*Penicillium sp.*	25ºC	2	(*) Velutinous, green colony and brown reverse	(*) Conidiophores branched (triverticillated). Phialides in a shape of ampulliform. Ovoid conidia forming chains
9.C	*Rhizomucor variabilis*	25ºC	1.2	(*) Hairy and ochre in the middle; and yellow in the borders colony and yellow reverse	(*) Sporangiophores branched ending in a sporangium. Chlamydospores abundant
9.D	*Penicillium sp.*	25ºC	1.6	(*) Powdery, green colony and brown reverse	(*) Conidiophores branched (triverticillated). Phialides in a acerose shape
9.E	*Paecilomyces sp.*	25ºC	5	(*) Velutinous, vinaceous colony and brown reverse	(*) Septate hyphae from where the conidiophores branch vertically. Phialides are swollen at their base and ovoid conidia, forming long chains
9.F	*Geomyces pannorum*	25ºC	56	(+) 0.0	(+) 99
9.G	*Fusarium sp.*	25ºC	17.6	(*) Floccose white colony, with pink shades and reverse colourless	(*) Conidiogenous cells formed on aerial hyphae. Microconidia in ovoid shape present
9.H	*Penicillium sp.*	25º C	1	(*) Velutinous, light green colony and brown reverse	(*) Conidiophores branched (triverticillated). Phialides in a shape of ampulliform. Round conidia forming chains.

Table 12 (cont.)

Code	Species	Temp.	CFU	(+) e-value (*) Colony Characteristic	(+) % Pairwise identity (*) Micro Characteristics
9.I	*Microsporum sp.*	25ºC	4	(*) Cottony white colony, with production of salmon pigments and reverse cream-coloured	(*) Macroconida present, with fusiform shape and rounded apex
9.J	*Paecilomyces sp.*	25ºC	13	(*) Velutinous, winaceous colony and brown reverse	(*) Septate hyphae from where the conidiophores branch vertically. Phialides in fusiform shape and round conidia
9.K	Unidentified	25ºC	1	-	-
9.L	*Verticillium sp.*	25ºC	3	(*) Woolly, cream-coloured colony and colourless reverse. Production of orange pigments	(*) Conidiophores well differentiated and erect, verticillately branched. Phialides divergent.
9.6	*Trichosporon chiropterorum*	15ºC	7	(+) 0.0	(+) 99
9.N	*Penicillium sp.*	15ºC	In	(*) Velutinous, green colony with the edges white and brown reverse	(*) Conidiophores branched (biverticillated). Phialides in a acerose shape
9.O	*Lecanicillium sp.*	15ºC	In	(+) 0.0	(+) 98
9.4	*Trichosporon porosum*	4ºC	6.6	(+) 0.0	(+) 100
9.5	*Trichosporon chiropterorum*	4ºC	14	(+) 0.0	(+) 99
9.M	*Mucor sp.*	4ºC	2	(*) Hairy, greyish-brown colony and colourless reverse	(*) Sporangiophores without basal rhizoids, irregularly branched and multispore sporangia. Presence of clamydospores along the hyphae

From "Picos" a total of seven fungi were isolated from all the plates incubated. Two of them were identified by sequencing the ITS region of 28S rDNA to the species level as *Trichosporon dulcitum* (Figure 14) and *T. multisporum*, displaying e-values of zero and Pairwise *%* identities equal to 100. The remaining ones were identified to the genus level according to the morphology of their reproductive structures, as *Basidiobolus sp.*, *Geomyces sp.*, *Mucor sp.*, *Scopulariopsis sp.* and *Verticillium sp.* (Table 13).

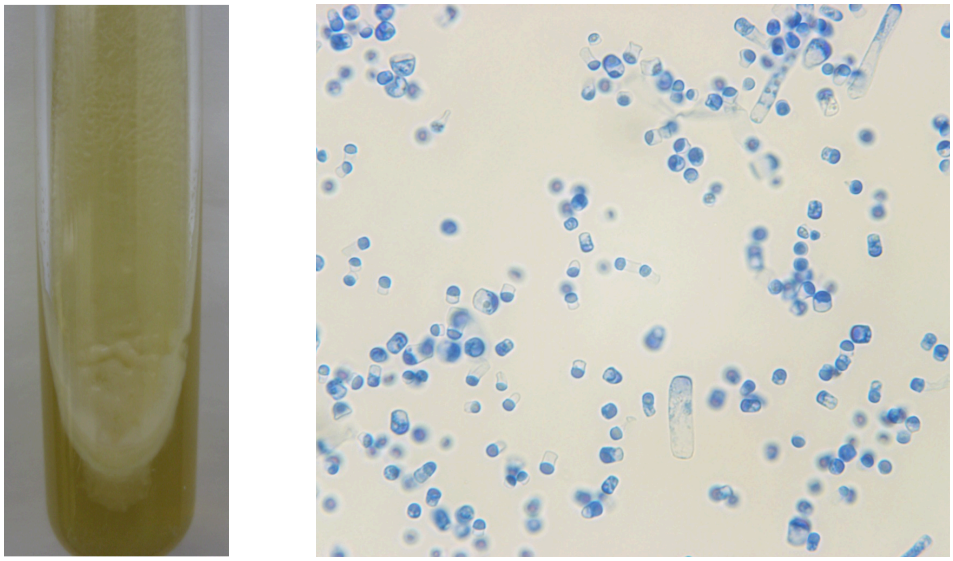

Figure 14: Yeast isolated from cave 10, with the code 10.2, identified as *Trichosporon dulcitum*. On the left, culture on YGC medium and, on the right, the cells, observed under an optical microscope at x400 magnification, both after one week of incubation.

Table 13: Fungi species isolated from the samples collected at the cave "Picos", incubation temperature and CFU count. The parameters used for identification are also shown.

Code	Species	Temp.	CFU	(+) e-value (*)Colony Characteristic	(+) % Pairwise identity (*) Micro Characteristics
10.1	*Trichosporon multisporum*	25ºC	3.8	(+) 0.0	(+) 100
10.2	*Trichosporon dulcitum*	25ºC	1	(+) 0.0	(+) 100
10.A	*Mucor sp.*	25ºC	1	(*) Hairy, yellowish-brown colony and colourless reverse	(*) Sporangiophores without basal rhizoids, irregularly branched and multispore sporangia. Absence of clamydospores along the hyphae
10.B	*Verticillium sp.*	25ºC	5	(*) Woolly, white colony with yellow shades and colourless reverse. Production of yellow pigments	(*) Conidiophores well differentiated and erect, verticillately branched. Phialides divergent.
10.C	*Scopulariopsis sp*	25ºC	1	(*) Velvety white colony and cream-coloured reverse	(*) Conidiophores erect, short and unbranched. Conidiogeneous cells ampulliform.
10.D	*Geomyces sp.*	25ºC	3.6	(*) Floccose light orange colony and cream-coloured reverse	(*) Conidiophores erect, bearing short chain of arthroconidia. Conidia terminal.
10.E	*Basidiobolus sp.*	25ºC	6	(*) Flat creamy-grey colony becoming radially folded and covered by a fine, woolly, white surface mycelium	(*) Big hyphae forming numerous round zygospores. The sporophore has a distinct swollen area right below the conidium

From “Lapa da Ovelha” a total of thirteen species were isolated. Five were identified to the species level as *Kluyveromyces dobzhanskii*, *Verticillium leptobactrum*, *Trichosporon akiyoshidainum*, *T. moniliiforme* and *T. multisporum*. These identifications had the e-values equal to zero and pairwise % identity higher then 99. One identification was not accurate since the sequence homology was much lower than 98%, and as such the result was not considered. Hence this identification remained at the genus level as *Chrysosporium sp*., but further analysis is needed to confirm this identification. From the remaining ones, one was not identified and the others were identified according to the morphology of their reproductive structures, one to the species level as *Geotrichum candidum* (Figure 15), and the rest to the genus level as *Acremonium sp.*, *Exophiala sp.* (Figure 16), *Penicillium sp.* (apparently two different species) and *Mucor sp.* (Table 14).

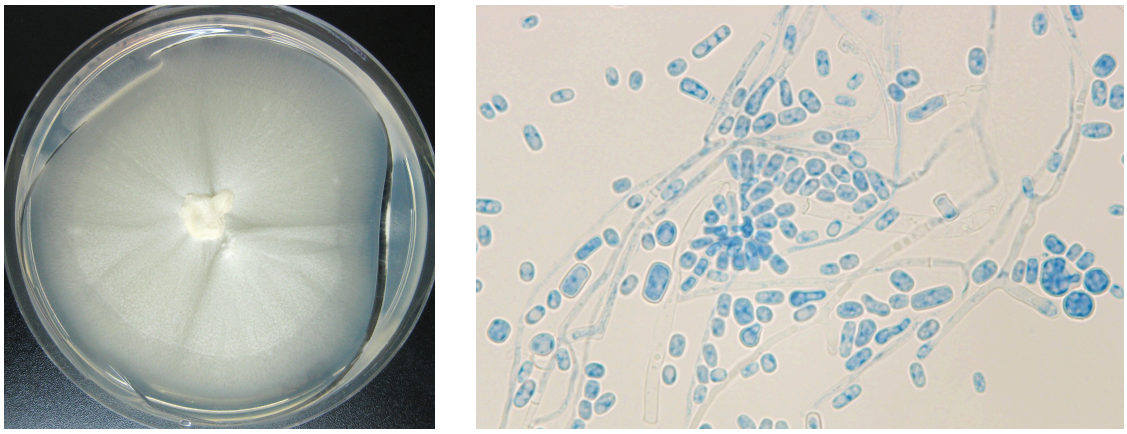

Figure 15: Yeast isolated from cave (11), with the code 11.H, identified as *Geotrichum candidum*. On the left, culture on PDA medium and, on the right, cylindrical and barrel shaped cells, observed under an optical microscope at x400 magnification, both after one week of incubation

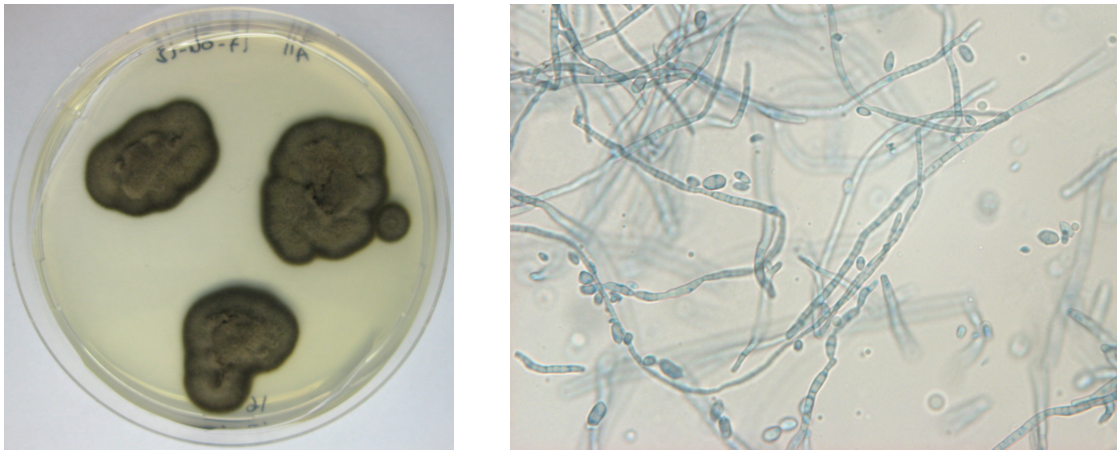

Figure 16: Fungi isolated from cave 11, with the code 11.D, identified as *Exophiala sp.* On the left, culture on PDA medium and, on the right, conidiogenous cells and conidia, observed under an optical microscope at x400 magnification, both after two weeks of incubation.

Table 14: Fungi species isolated from the samples collected at the cave "Lapa da Ovelha", incubation temperature and CFU count. The parameters used for identification are also shown.

Code	Species	Temp.	CFU	(+) e-value (*)Colony Characteristic	(+) % Pairwise identity (*) Micro Characteristics
11.1	*Kluyveromyces dobzhanskii*	25ºC	1	(+) 0.0	(+) 99
11.2	*Trichosporon moniliiforme*	25ºC	12	(+) 0.0	(+) 100
11.3	*Trichosporon akiyoshidainum*	25ºC	4.3	(+) 0.0	(+) 100
11.4	*Trichosporon multisporum*	25ºC	1.5	(+) 0.0	(+) 100
11.A	*Penicillium sp.*	25ºC	2.3	(*) Powdery, green colony and brown reverse	(*) Conidiophores branched (triverticillated). Phialides in a shape of ampulliform. Round conidia forming chains.
11.B	*Mucor sp.*	25ºC	1	(*) Hairy, yellow colony and colourless reverse	(*) Sporangiophores without basal rhizoids, irregularly branched and multispore sporangia. Absence of clamydospores along the hyphae
11.C	*Acremonium sp.*	25ºC	2.5	(*) Velutinous, withe colony and brown reverse.	(*) Phialides hyaline and erect. Oval conidia grouped at the top of the phialides.
11.D	*Exophiala sp.*	25ºC	1	(*) Smooth black colony and black reverse	(*) Conidiogenous cells flask-shaped and conidia formed in slimy heads
11.E	*Penicillium sp.*	25ºC	61.6	Powdery, green colony and brown reverse	(*) Conidiophores branched (biverticillated). Phialides in a shape of ampulliform. Round conidia forming chains.
11.F	Unidentified	25ºC	1	-	-
11.G	*Verticillium leptobactrum*	25ºC	1	(+) 0.0	(+) 99
11.H	*Geotrichum candidum*	25ºC	In	(*) Flat cream-coloured colony, with reverse colourless	(*) Cylindrical and barrel shaped cells present
11.I	*Chrysosporium sp.*	25ºC	2	(+) $5e^{-150}$	(+) 84

In "Zambujal" a total of twelve fungi were isolated. Four were identified by sequencing the ITS region of 28S rDNA as *Trichosporon cavernicola*, *T. chiropterorum*, *T. moniliiforme* and *T. otae*, displaying e-values of zero and Pairwise *%* identities higher than 99. The remaining ones were identified to the genus level as *Acremonium sp.*, *Mucor sp.* (apparently three different species), *Penicillium sp.* (apparently two different species) and *Scedosporium sp.*, according to the morphology of their reproductive structures. One isolate remains to identify (Figure 17), because

the morphological characteristics exhibited were not conclusive and the sequencing results need to be repeated. All of the isolated fungi from this cave grew on the plates inoculated at 25ºC, except one isolate of the genus *Acremonium*, that was only able to grow on the plates incubated at 15ºC (Table 15).

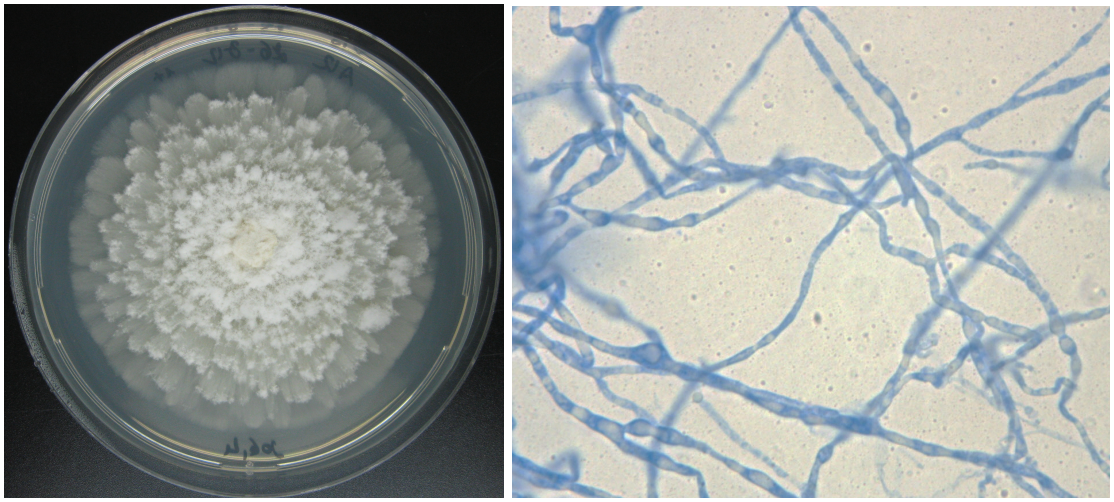

Figure 17: Fungi isolated from cave 12, with the code 12.D, which was one that remained to identify. On the left, culture on PDA medium and, on the right reproductive structure under an optical microscope at x400 magnification, both after three weeks of incubation.

Table 15: Fungi species isolated from the samples collected at the cave "Zambujal", incubation temperature and CFU count. The parameters used for identification are also shown.

Code	Species	Temp.	CFU	(+) e-value (*) Colony Characteristic	(+) % Pairwise identity (*) Micro Characteristics
12.1	*Trichosporon chiropterorum*	25ºC	1.8	(+) 0.0	(+) 99
12.2	*Trichosporon cavernicola*	25ºC	2	(+) 0.0	(+) 99
12.3	*Trichosporon otae*	25ºC	1	(+) 0.0	(+) 99
12.4	*Trichosporon moniliiforme*	25ºC	1	(+) 0.0	(+) 100
12.A	*Scedosporium sp.*	25ºC	12.5	(*) Hairy white-greyish colony with yellow reverse	(*) Conidia flask-shaped and conidiogenous cells formed percurrently
12.B	*Mucor sp.*	25ºC	1	(*) Hairy, white colony and colourless reverse	(*) Sporangiophores without basal rhizoids, irregularly branched and multispore sporangia. Absence of clamydospores along the hyphae
12.C	*Penicillium sp.*	25ºC	2.25	(*) Powdery, green colony and brown reverse. Production of yellow pigments	(*) Conidiophores unbranched. Phialides in a shape of ampulliform. Round conidia forming chains.

Table 15 (Cont.)

Code	Species	Temp.	CFU	(+) e-value (*) Colony Characteristic	(+) % Pairwise identity (*) Micro Characteristics
12.D	Unidentified	25ºC	28.2	-	-
12.E	*Mucor sp.*	25ºC	1	(*) Hairy, ochre colony and colourless reverse	(*) Sporangiophores without basal rhizoids, irregularly branched and multispore sporangia. Presence of clamydospores along the hyphae
12.F	*Mucor sp.*	25ºC	1	(*) Hairy, yellow colony and colourless reverse	(*) Sporangiophores without basal rhizoids, irregularly branched and multispore sporangia. Absence of clamydospores along the hyphae
12.G	*Penicillium sp.*	25ºC	2	(*) Powdery, green colony and brown reverse. Production of brown pigments	(*) Conidiophores unbranched. Phialides in a shape of ampulliform. Round conidia forming chains.
12.H	*Acremonium sp.*	15ºC	In	(*) Velutinous, withe colony and orange reverse.	(*) Phialides hyaline and erect. Oval conidia grouped at the top of the phialides.

In "Sem Nome" a total of nine isolates were recovered. Three of them were identified by sequencing the ITS region of 28S rDNA as *Humicola sp., Trichosporon mucoides* and *Verticillium leptobactrum*. The identification of this last species raised some doubts, but when observing and comparing it with other isolates of the same species, it showed a great similarity. There was also one isolate whose identification was not accurate, since the sequence homology with the ones in the database was much lower than 98%. Hence this identification was only considered at the genus level as *Malbranchea sp.* The remaining ones were identified as *Acremonium sp.*, *Paecilomyces sp.*, *Penicillium sp.*, *Scopulariopsis sp.* and *Basidiobolus sp*, according to the morphology of their reproductive structures (Table 16).

Table 16: Fungi species isolated from the samples collected at the cave "Sem Nome", incubation temperature and CFU count. The parameters used for identification are also shown.

Code	Species	Temp.	CFU	(+) e-value (*) Colony Characteristic	(+) % Pairwise identity (*) Micro Characteristics
13.1	*Trichosporon mucoides*	25ºC	1	(+) 0.0	(+) 100

Table 16 (Cont.)

Code	Species	Temp.	CFU	(+) e-value (*) Colony Characteristic	(+) % Pairwise identity (*) Micro Characteristics
13.A	*Scopulariopsis sp.*	25ºC	5	(*) Velvety purple colony and brown reverse	(*) Conidiophores erect, short and branched. Conidiogeneous cells ampulliform. Conida arranged in chains
13.B	*Verticillium leptobactrum*	25ºC	4	(+) $2e^{-166}$	(+) 98
13.C	*Malbranchea sp.*	25ºC	1	(+) 0.0	(+) 91
13.D	*Paecilomyces sp.*	25ºC	2.4	(*) Velutinous, winaceous colony and brown reverse. Production of fluorescent yellow pigments	(*) Septate hyphae from where the conidiophores branch vertically. Phialides are swollen at their base and ovoid conidia, forming long chains
13.E	*Penicillium sp.*	25ºC	1	(*) Powdery, green colony and colourless reverse	(*) Conidiophores unbranched. Phialides in a shape of ampulliform. Round conidia forming chains
13.F	*Basidiobolus sp.*	25ºC	14	(*) Flat creamy-grey colony becoming radially folded and covered by a fine, woolly, white surface mycelium	(*) Big hyphae forming numerous round zygospores. The sporophore has a distinct swollen area right below the conidium
13.G	*Humicola sp.*	25ºC	9	(+) 0.0	(+) 99
13.H	*Acremonium sp.*	15ºC	3	(*) Velutinous, withe colony and orange reverse.	(*) Phialides hyaline and erect. Oval conidia grouped at the top of the phialides.

From "Preguiça" a total of nineteen fungi were isolated from the plates incubated. Six isolates were identified as *Mucor hiemalis*, *Penicillium griseofulvum*, *Trichosporon chiropterorum* (which occurred tree times) and *T. moniliiforme*. These identifications were the ideal ones, with the e-values equal to zero and pairwise % identity higher then 99. Also there were two identifications not accurate, since the sequence homology was much lower than 98% and as such the result can not be considered. Hence this identification remained at the genus level as *Blastobotrys sp.* (Figure 18) and *Candida sp.* The remaining ones were identified according to the morphology of their reproductive structures as *Acremonium sp.* (apparently two different species), *Mucor sp.*, *Paecilomyces sp.*, *Penicillium sp.* (apparently four different species), *Sporothrix sp.* and *Trichosporon laibachii*, remaining one to identify, because through the morphological characteristics exhibited we couldn't

reach it and after there the PCR amplification the DNA had contaminants present. Two genera stood out in these cave, *Penicillium* and *Trichosporon*, each one with a total of five isolates. From the isolates of to the genus *Penicillium* two grew at 25*ºC*, two were able to grow at 15*ºC*, and one at 4*ºC*. From the species belonging to the genus *Trichosporon* only *T. chiropterorum* was able to grow at the three temperatures of incubation. The isolates of *Mucor hiemalis* were also able to grow at 4*ºC* (Table17).

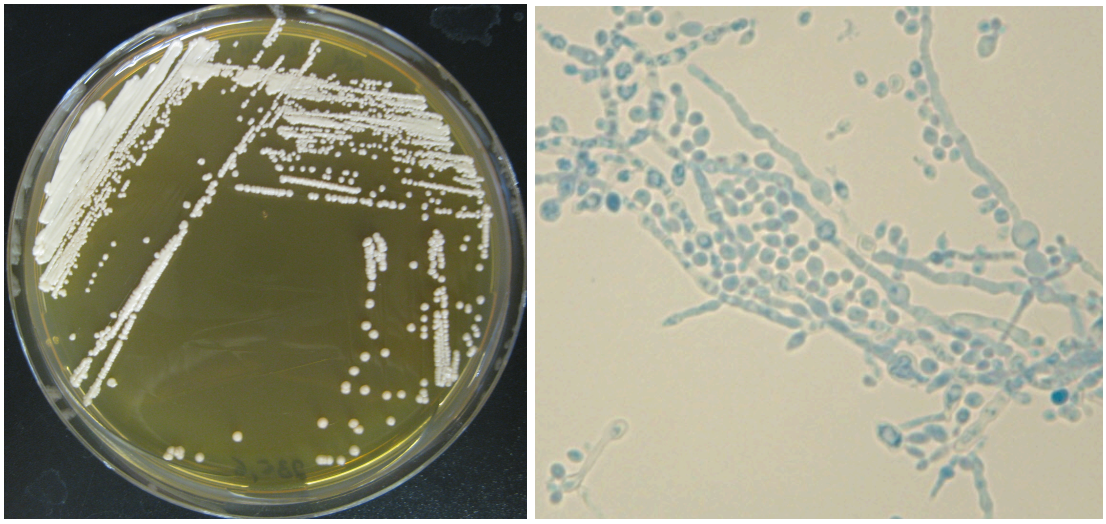

Figure 18: Yeast isolated from cave 14, with the code 14.1, identified as *Blastobotrys sp*. On the left, culture on YGC medium and, on the right, the cells, observed under an optical microscope at x400 magnification, both after one week of incubation.

Table 17: Fungi species isolated from the samples collected at the cave "Preguiça", incubation temperature and CFU count. The parameters used for identification are also shown.

Code	Species	Temp.	CFU	(+) e-value (*)Colony Characteristic	(+) % Pairwise identity (*) Micro Characteristics
14.1	*Blastobotrys sp.*	25ºC	13.2	(+) 0.0	(+) 92
14.2	*Trichosporon chiropterorum*	25ºC	3	(+) 0.0	(+) 99
14.3	*Candida sp.*	25ºC	16	(+) 0.0	(+) 92
14.4	*Trichosporon moniliiforme*	25ºC	13	(+) 0.0	(+) 100
14.5	*Trichosporon laibachii*	25ºC	2	(*) Farinose white colony	(*) Septate hyphae and presence of arthroconida
14.A	*Mucor sp.*	25ºC	1.3	(*) Hairy, ochre colony and colourless reverse	(*) Sporangiophores without basal rhizoids, irregularly branched and multispore sporangia.
14.B	*Penicillium sp.*	25ºC	25	(*) Powdery, green colony and colourless reverse.	(*) Conidiophores unbranched. Phialides in a shape of ampulliform.
14.C	Unidentified	25ºC	96.8	-	-

Table 17 (Cont.)

Code	Species	Temp.	CFU	(+) e-value	(+) % Pairwise identity
				(*)Colony Characteristic	(*) Micro Characteristics
14.D	*Penicillium sp.*	25ºC	22.5	(*) Velutinous, green colony and brown reverse	(*) Conidiophores branched (biverticillated). Phialides in a acerose shape
14.E	*Acremonium sp.*	25ºC	In	(*) Velutinous, withe-yellowish colony and brown reverse. Production of brown pigments	(*) Phialides hyaline and erect. Oval conidia grouped at the top of the phialides.
14.F	*Paecilomyces sp.*	25ºC	In	(*) Velutinous, white green colony and light brown reverse	(*) Septate hyphae from where the conidiophores branch vertically. Phialides are swollen at their base and ovoid conidia, forming long chains
14.G	*Sporothrix sp.*	25ºC	In	(*) Wrinkled white colony and reserve colourless	Conidia in short chains, arising along the hyphae
14.H	*Acremonium sp.*	25ºC	In	(*) Velutinous, yellow colony and brown reverse. Production of brown pigments	(*) Phialides hyaline and erect. Oval conidia grouped at the top of the phialides.
14.7	*Trichosporon chiropterorum*	15ºC	6.3	(+) 0.0	(+) 99
14.K	*Penicillium sp.*	15ºC	93.5	(*) Powdery, green colony and colourless reverse.	(*) Conidiophores branched (biverticillated). Phialides in a shape of ampulliform.
14.L	*Penicillium sp.*	15ºC	7	(*) Velutinous, green colony with the edges white and brown reverse	(*) Conidiophores branched (biverticillated). Phialides in a acerose shape
14.6	*Trichosporon chiropterorum*	4ºC	4	(+) 0.0	(+) 99
14.I	*Mucor hiemalis*	4ºC	3	(+) 0.0	(+) 99
14.J	*Penicillium griseofulvum*	4ºC	59.5	(+) 0.0	(+) 99

In “Gralhas” a total of five isolates were recovered. Two were identified to the species level by sequencing the ITS region of 28S rDNA as *Trichosporon middelhovenii* and *T. moniliiforme*, displaying the e-values equal to zero and pairwise % identity higher then 99. One of the identifications was not accurate, since the sequence homology was much lower than 98% and as such the result cannot be considered. Hence this identification remained at the genus level as *Sporothrix sp*. From the remaining ones, one was identified according to the morphology of its reproductive structures as *Penicillium sp.* and one remained unidentified (Table 18), because through the morphological characteristics exhibited we couldn’t reach it and DNA extraction procedure needs to be improved.

Table 18: Fungi species isolated from the samples collected at the cave "Gralhas", incubation temperature and CFU count. The parameters used for identification are also shown.

Code	Species	Temp.	CFU	(+) e-value (*)Colony Characteristic	(+) % Pairwise identity (*) Micro Characteristics
15.1	*Trichosporon moniliiforme*	25ºC	4.75	(+) 0.0	(+) 99
15.3	*Sporothrix sp.*	25ºC	7	(+) 0.0	(+) 90
15.5	*Trichosporon middelhovenii*	25ºC	2	(+) 0.0	(+) 99
15.A	*Penicillium sp.*	25ºC	1	(*) Velutinous, green colony and green reverse	(*) Conidiophores branched (biverticillated). Phialides in a acerose shape
15.B	Unidentified	25ºC	1	-	-

In "Cova da Moura" a total of twenty fungi were isolated. Eight isolates were identified as *Candida palmioleophila* (which occurred twice), *Candida psychrophila*, *Geomyces sp., Kluyveromyces dobzhanskii*, *K. lactis*, *Trichosporon chiropterorum* (which occurred twice) (Figure 19) and *T otae*. The e-values displayed were equal to zero and pairwise % identities higher then 99. There were also two cases with sequence homologies lower than 98% and that need to be further studied. Hence this identification remained at the genus level as *Torrubiella sp.* and *Trichosporon sp.* The remaining ones were identified according to the morphology of their reproductive structures to the genus level, as *Acremonium sp.*, *Beauveria sp.*, *Penicillium sp.* (apparently three different isolates), *Scopulariopsis sp.* (apparently two different isolates) and *Trichosporon sp.* (Table19).

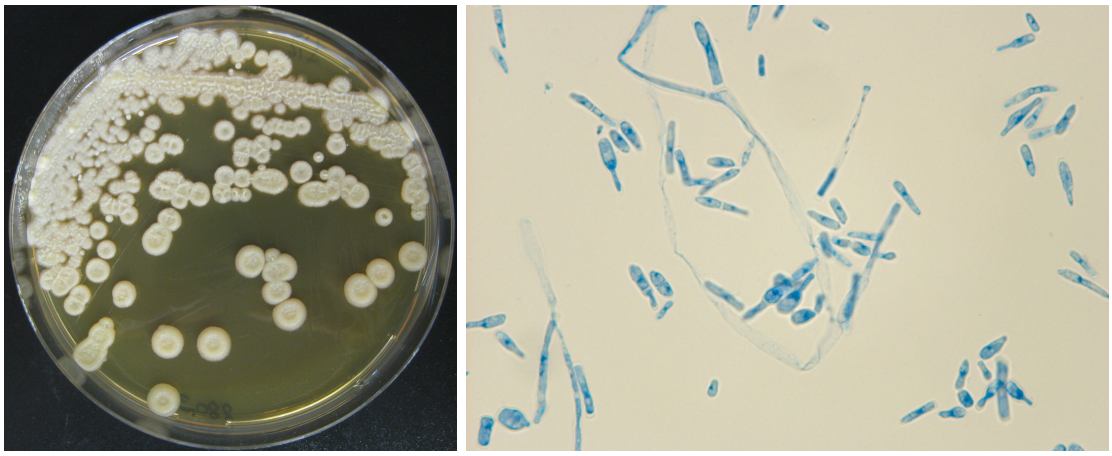

Figure 19: Yeast isolated from cave 16, with the code 16.5, morphologically identified as *Trichosporon chiropterorum*. On the left, culture on YGC medium and, on the right, the cells, observed under an optical microscope at x400 magnification, both after one week of incubation.

The genus *Trichosporon* stood out in this cave with a total of five species, as already observed in the caves 7 and 9. From the species belonging to this genus the only one that was able to grow at the lower temperatures was *T. chiropterorum*, which grew well at the three temperatures of incubation. Additionally, the isolates of *Candida palmioleophila* were also able to grow at 25ºC and 4ºC (Table19).

Table 19: Fungi species isolated from the samples collected at the cave "Cova da Moura", incubation temperature and CFU count. The parameters used for identification are also shown.

Code	Species	Temp.	CFU	(+) e-value (*)Colony Characteristic	(+) % Pairwise identity (*) Micro Characteristics
16.1	*Trichosporon sp.*	25ºC	1	-	-
16.2	*Kluyveromyces lactis*	25ºC	38	(+) 0.0	(+) 100
16.3	*Kluyveromyces dobzhanskii*	25ºC	18	(+) 0.0	(+) 100
16.4	*Trichosporon sp.*	25ºC	40.3	(+) 0.0	(+) 94
16.5	*Trichosporon chiropterorum*	25ºC	7	(+) 0.0	(+) 99
16.6	*Candida palmioleophila*	25ºC	6.16	(+) 0.0	(+) 100
16.7	*Candida psychrophila*	25ºC	2	(+) 0.0	(+) 99
16.8	*Trichosporon otae*	25ºC	7.5	(+) 0.0	(+) 99
16.A	*Beauveria sp.*	25ºC	10.6	(*) Woolly, white colony and pink reverse.	(*) Conidiogenous cells growing in aggregation in dense clusters along the hypha. Conidia are single celled oval
16.B	*Torrubiella sp.*	25ºC	3	(+) 0.0	(+) 97
16.C	*Acremonium sp.*	25ºC	2	(*) Woolly white colony and pink reverse.	(*) Phialides hyaline and erect. Oval conidia grouped at the top of the phialides.
16.D	*Scopulariopsis sp.*	25ºC	4	(*) Velvety grey colony and cream-coloured reverse	(*) Conidiophores erect, short and branched. Conidiogeneous cells ampulliform. Conida arranged in chains
16.E	*Penicillium sp.*	25ºC	7	(*) Powdery, green colony and colourless reverse. Production of yellow pigments	(*) Conidiophores branched (biverticillated). Phialides in a shape of ampulliform. Round conidia sometimes forming short chains
16.F	Unidentified	25ºC	9	-	-
16.G	*Scopulariopsis sp.*	25ºC	In	(*) Velvety white and yellow colony and cream-coloured reverse	(*) Conidiophores erect, short and branched. Conidiogeneous cells ampulliform. Round conidia arranged in chains

Table 19 (cont.)

Code	Species	Temp.	CFU	(+) e-value (*)Colony Characteristic	(+) % Pairwise identity (*) Micro Characteristics
16.10	*Trichosporon chiropterorum*	15ºC	4.6	-	-
16.H	*Penicillium sp.*	15ºC	9.5	(*) Powdery, green colony and colourless reverse.	(*) Conidiophores branched (triverticillated). Phialides in a shape of ampulliform. Round conidia sometimes forming short chains
16.H	*Penicillium sp.*	15ºC	9.5	(*) Powdery, green colony and colourless reverse.	(*) Conidiophores branched (triverticillated). Phialides in a shape of ampulliform. Round conidia sometimes forming short chains
16.I	*Geomyces sp.*	15ºC	1	(+) 0.0	(+) 99
16.J	*Penicillium sp.*	15ºC	1	(*) Velutinous green colony and green reverse	(*) Conidiophores branched (triverticillated). Phialides in a acerose shape
16.9	*Candida palmioleophila*	4ºC	3	(+) 0.0	(+) 100

In "Gruta do Alviela" a total of eight fungi were isolated. Two isolates were identified to the species level as *Rhodotorula mucilaginosa* and *Trichosporon porosum*, both identifications displaying the perfect values for the e-value and pairwise *%* identity. The remaining ones were identified according to the morphology of their reproductive structures, one to the species level as *Trichophyton concentricum,* and five to the genus level as *Candida sp.*, *Geotrichum sp.*, *Penicillium sp.* (apparently 2 different ones) and *Pichia sp.* Isolates from *Pichia sp.*, *Penicillium sp.*, *R. mucilaginosa* and *T. porosum* were able to grow at 15*ºC* (Table 20).

Table 20: Fungi species isolated from the samples collected at the cave "Gruta do Alviela", incubation temperature and CFU count. The parameters used for identification are also shown.

Code	Species	Temp.	CFU	(+) e-value (*)Colony Characteristic	(+) % Pairwise identity (*) Micro Characteristics
17.1	*Geotrichum sp.*	25ºC	1	(*) Flat cream-colored colony, with reverse colourless	(*) Cylindrical and barrel shaped cells present
17.2	*Candida sp.*	25ºC	1	(*) Smooth cream-colored colony	(*) Small oval cells
17.A	*Trichophyton concentricum*	25ºC	14	(*) Wrinkled pink colony and brown reserve	(*) Septate branched hyphae. Round and smooth microconidia

Table 20 (Cont.)

Code	Species	Temp.	CFU	(+) e-value (*)Colony Characteristic	(+) % Pairwise identity (*) Micro Characteristics
17.B	*Penicillium sp.*	25ºC	2	(*) Powdery, green colony and brown reverse.	(*) Conidiophores branched (biverticillated). Phialides in a shape of ampulliform. Round conidia sometimes forming short chains
17.3	*Pichia sp.*	15ºC	1	(*) Smooth and bright cream-colored colony	Presence of budding yeasts, pseudohyphae and ascorpores
17.4	*Rhodotorula mucilaginosa*	15ºC	25	(+) 0.0	(+) 100
17.5	*Trichosporon porosum*	15ºC	17	(+) 0.0	(+) 100
17.C	*Penicillium sp.*	15ºC	2	(*) Velutinous, green colony and green reverse	(*) Conidiophores branched (biverticillated). Phialides in a elongate shape

3.3 COMPARISON AMONG CAVES

From the four fungi isolated in "Tibães" three were apparently different from all the others, making them exclusive to these cave, namely *Penicillium sp., Verticillium sp.* and the unidentified one (Table 21). According to Sorenson index, there are five caves that could be considered similar to this one. The most similar was cave 3, followed by the caves 12, 9, 14 and 16 (Table 22). They all share isolates of the species *T. chiropterorum*. Shannon index was equal to 1.39 and the Evenness index was equal to 1, the maximum value for this index, meaning that the fungi population present in this cave is evenly abundant (Table 21).

The five fungi isolated in "Regaleira" are all different from the isolates of the remaining caves (Table 21). Hence they are exclusive to this one, resulting in Sorenson indexes always equal to zero (Table 22). Shannon index was equal to 1.61 and Evenness was equal to 1, the maximum value for this index, meaning that the fungi population present in this cave is also evenly abundant (Table 21).

From the eight species isolated in "Carviçais", apparently five isolates were registered only in this cave, namely *Paecilomyces sp.* (both isolates)*, Tolypocladium cylindrosporum, Verticillium sp.* and the unidentified one (Table 21). Concerning Sorenson index, there are seven caves that are similar to this one. The most similar cave was cave 12, with which it shares the species *T. cavernicola* and *T. chiropterorum*, followed by cave 1, sharing *T. chiropterorum*, and cave 9, with which

it shares the species *T. cavernicola* and *T. chiropterorum*. Next, in order there are caves 13 and 11, sharing the species *Verticillium leptobactrum*, and caves 14 and 16, with which it shares *T. chiropterorum* (Table 22). Shannon index was equal to 1.91 and the Evenness index was 0.92 (Table 21).

From the eight fungi species isolated from "Campo de Víboras", apparently six isolates were exclusive to it, namely *Paecilomyces sp., Trichoderma sp.,* and the four unidentified ones (Table 21). Shannon index was equal to 1.15, the Evenness index was equal to 0.59, the lowest value observed (Table 21). Regarding Sorenson index, there are two similar caves, 12 and 16, with which it shares the species *Trichosporon otae* (Table 22).

The four fungi isolated from "Sendim da Serra" all differ from the isolates of the remaining caves (Table 21). Hence they are exclusive to this cave, resulting in a Sorenson index equal to zero (Table 22). Shannon index was equal to 1.04 and Evenness was equal to 0.75 (Table 21).

In "Mina Felgar" from the six fungi isolated five were apparently exclusive to it, namely *Paecilomyces sp.*, *Penicillium citrinum*, *Penicillium sp.*, *Rhodotorula bloemfonteinensis* and *Trichoderma sp.* (Table 21). Shannon index was equal to 1.79 and the Evenness index was equal to 1, the maximum value for this index, meaning that the fungi population present in this cave is evenly abundant (Table 21). Regarding Sorenson index, there is only one cave, cave 7, that shows some similarity, sharing *Aspergillus sp.* (Table 22).

From the sixteen species isolated in "Louro", ten were apparently exclusive to it, namely *Acremonium strictumi, Oidiodendron sp.*, *Pochonia chlamydosporia*, *T. jirovecii*, *T. mycotoxinivorans*, *T. shinodae, Trichosporon sp.* and the three unidentified ones (Table 21). Shannon index was equal to 2.31, one of the highest values for this index, meaning that the species population are equally abundant; and the Evenness index was equal to 0.83, a high value for this index, meaning that the fungi population present in this cave is highly even (Table 21). Concerning Sorenson index, there were six caves that were similar to this one. The most similar was cave 11, followed by the caves 15, 6, 12, 9 and 14. With cave 11 two isolates are shared, *Chrysosporium sp.* and *T. moniliiforme* and with cave 6 *Aspergillus sp.* With the reaming ones it shared the species *T. moniliiforme* (Table 22).

In "Almonda" Shannon index was equal to zero, as expected, because there is only one species, and in these cases the Evenness index can not be calculated (Table

21). Finally, as the single species existent was exclusive to this cave, no other caves were similar to this one (Table 22).

From the twenty one species isolated in "Malhada", apparently fourteen of them were exclusive to it, namely *Beauveria sp.*, *Fusarium sp.*, *Geomyces pannorum*, *Lecanicillium sp.*, *Microsporum sp.*, *Mucor sp.*, two *Paecilomyces sp.*, four *Penicillium sp.*, *Rhizomucor variabilis*, *Verticillium sp.* and the unidentified taxa (Table 21). Shannon index was equal to 2.56, one of the highest values for this index, meaning that the species population are equally abundant; and the Evenness index was equal to 0.84, a high value for this index, meaning that the fungi population present in this cave is highly even (Table 21). Concerning Sorenson index, there were nine caves that were similar to this one. The most similar was cave 12, followed by caves 15, 3, 1, 14, 7, 17, 11 and 16 (Table 22). With cave 12 it shared *T. cavernicola*, *T. chiropterorum* and *T. moniliiforme*; with cave 3 the species *T. cavernicola* and *T. chiropterorum*; with cave 14 *T. chiropterorum* and *T. moniliiforme*; with caves 7, 11 and 15 the species *T. moniliiforme*; with caves 1 and 16 *T. chiropterorum*; and finally with cave 17 the species *T. porosum* (Table 21).

From the seven fungi isolated in "Picos" six were exclusive to it, namely *Trichosporon multisporum*, *Mucor sp.*, *Verticillium sp., Scopulariopsis sp*, *Geomyces sp.* and *Basidiobolus sp.* Shannon index was equal to 1.95, and the Evenness index was equal to 1, the maximum value for this index, meaning that the fungi population present in this cave is evenly abundant (Table 21). Regarding Sorenson index, there were two caves similar to these one, cave 13, sharing *Basidiobolus sp.* and cave 11, with which it shared *T. multisporum* (Table 22).

Among the thirteen fungi isolated from "Lapa da Ovelha", apparently eight isolates were exclusive to it, namely *Acremonium sp.*, *Exophiala sp.*, *Geotrichum candidum*, *Mucor sp.*, two *Penicillium sp. Trichosporon akiyoshidainum*, and the unidentified one (Table 21). Shannon index was equal to 2.46 and the Evenness index was equal to 1. This was one of the caves displaying the highest values for these indexes, meaning that the fungi population present in this cave is very diverse and evenly abundant (Table 21). Concerning Sorenson index, there were nine caves that can be considered similar to this one. The most similar was cave 7, followed by caves 15, 3, 10, 13, 12, 9, 14 and 16 (Table 22). With cave 7 it shares two isolates, *Chrysosporium sp.* and *T. moniliiforme*; with caves 3 and 13 the species *Verticillium*

leptobactrum; with caves 9, 12, 14 and 15 it shares *T. moniliiforme*; with cave 10 *T. multisporum* is shared and with cave 16 *Kluyveromyces dobzhanskii*.

From the twelve fungi isolated in "Zambujal", eight isolates were exclusive to this cave, namely *Acremonium sp.*, three *Mucor sp.*, two *Penicillium sp.*, *Scedosporium sp.* and the unidentified one (Table 22). The Shannon index was equal to 2.09, and the Evenness index was equal to 0.84, a high value for this index, meaning that the fungi population present in this cave is highly even (Table 21). Concerning Sorenson index, there were nine caves similar to this one. The most similar was cave 3, sharing two species, *T. cavernicola* and *T. chiropterorum*, followed by cave 9, sharing *T. cavernícola*, *T. chiropterorum* and *T. moniliiforme*. With cave 14 it shares *T. chiropterorum* and *T. moniliiforme.* With cave 1 it shares *T. chiropterorum;* with cave 16 it shares two species, *T. chiropterorum* and *T. otae*; with cave 4 only *T. otae*. Finally, with the caves 15, 11 and 7 it shares *T. moniliiforme* (Table 22).

From the nine species isolated in "Sem Nome", apparently seven were exclusive to it, namely *Acremonium sp.*, *Basidiobolus sp.*, *Humicola sp.*, *Malbranchea sp.*, *Paecilomyces sp.*, *Penicillium sp.*, *Scopulariopsis sp.* and *T. mucoides* (Table 22). Just like in cave 12, all of the isolated fungi from this cave were able to grow on the plates inoculated at 25ºC, except one isolate of the genus *Acremonium*, that only grew at 15ºC. Shannon index was equal to 2.2, and the Evenness index was equal to 1, meaning that the fungi population present in this cave is very diverse and evenly abundant (Table 21). Regarding Sorenson index, there are three caves similar to this one. The most similar is cave 10, with which it shares *Basidiobolus sp.*, followed by caves 3 and 11 sharing the species *Verticillium leptobactrum*.

In "Preguiça" practically all fungi that were isolated were exclusive to this cave, except *T. chiropterorum* and *T. moniliiforme*. The Shannon index was equal to 2.4, and the Evenness index was equal to 0.82, being one of the caves with the highest values, meaning that the fungi population present in this cave is very diverse and evenly abundant (Table 21). Concerning Sorenson index, there are eight caves that share species to this one. With caves 12 and 9 it shares the species *T. chiropterorum* and *T. moniliiforme*. With caves 1, 3 and 16 it shares *T. chiropterorum*. Finally, with caves 7, 11 and 15, it shares the species *T. moniliiforme* (Table 22).

From the cave "Gralhas" three of the species isolated were exclusive to it, namely *Penicillium sp.*, *Sporothrix sp.* and the unidentified one. The Shannon index was equal to 1.61, and the Evenness index was equal to 1, the maximum value for this

index, meaning that the fungi population present in this cave is evenly abundant (Table 21). Regarding Sorenson index, there are five caves sharing *T. moniliiforme* with this one namely cave 9, followed by the caves 7, 12, 11 and 14.

In "Cova da Moura" the majority of the isolated fungi were exclusive to this cave, except for four species, namely *Kluyveromyces dobzhanskii*, *Trichosporon chiropterorum*, *T. otae* and *Trichosporon sp.* (which occurred twice) (Table 21). The Shannon index was equal to 2.62 and the Evenness index was equal to 0.88, which where overall the highest values among the caves, meaning that the fungi population present in this cave is the most diverse and evenly abundant (Table 21). Concerning Sorenson index, there are seven caves sharing species with this one. With cave 12, it shares the species *T. chiropterorum* and *T. otae*; with cave 11, it shares *Kluyveromyces dobzhanskii*; finally with the caves 1, 3, 9 and 14, it shares *T. chiropterorum*.

In "Gruta do Alviela" all the fungi isolated were exclusive to this cave, except for *Trichosporon porosum.* Regarding the data analysis, the Shannon index was equal to 1.92, and the Evenness index was equal to 0.92, a high value for this index, meaning that the fungi population present in this cave is evenly abundant (Table 22). Considering Sorenson index there is only one cave, cave 9, that showed some similarity, sharing the species *T. porosum* (Table 22).

The most abundant genus among all caves was *Trichosporon*, with a total of 43 species isolated and in the three temperatures of incubation. This result is similar to the results obtained in a study performed in Japan, where it was suggested that *Trichosporon spp.* were the most frequent yeasts in bat guano (Sugita et al., 2005). In this study 20 bat-inhabited caves were guano sampled and 75% of the fungi found were *Trichosporon* species, from which nine were potentially novel species. In addition to the *Trichosporon spp.*, others like *Candida palmioleophila*, *Zygosaccharomyces florentinus* where also isolated, species that were also found in caves 16 and 2, respectively. The next most frequent genus was *Penicillium*, with 23 occurrences also at the three temperatures of incubation. Again there is a similar study, performed in 2009 in Slovakia where, from a total of 195 taxa isolated, the genus *Penicillium* was one of the most frequent in guano samples and the authors considerer that it may represent a typical one on bat guano (Nováková, 2009). Regarding the occurrences in terms of phyla, the prevalent phylum was Ascomycota, with a total of

91 species, followed by the Basidiomycota, with 45 species and finally the Zygomycota with only 14 species present (Table 21).

Concerning the data analysis values there is a huge diversity among the results. In general caves 7, 9, 13, 14 and 16 displayed high values, meaning that the fungi obtained from these caves are more diverse and evenly abundant. For these caves the Shannon index values were in fact the highest, however, regarding Evenness index it was equally high in practically all the caves, except for caves 4 and 8 (Table 21). The Sorenson index was very low between every cave, meaning that the fungi isolated in these caves were not very similar, nevertheless the highest values were observed between 3-12 and 9-12, followed by 1-3 and 7-11 (Table 22).

In Portugal there is only one known survey similar to this and it was performed in the cave "Fojo dos Morcegos" (Almeida et al., 1994). In this survey the authors collected samples of clay and bat guano and some fungal species were isolated from both subtracts. For comparison with our results only the fungi isolated from bat guano are important and they were *Aureobasidium pullulans*, *Mucor hiemalis* and *Mucor circinelloides*, as already mentioned before. Only *Mucor hiemalis* was found in the present study, making all the other fungi found new records in Portuguese caves.

Some of the isolated taxa during this work are recognized as opportunistic pathogens of man. Some *Acremonium spp.*, as the isolated *A. strictum* can cause mycetoma, onychomycosis and hyalohyphomycosis. Also, *Candida spp.*, *Exophiala spp.*, *Fusarium spp.* and *Sporothrix schenckii* are well documented human pathogens. *Geomyces pannorum* occasionally can initiate superficial infection of skin and nails in humans. *Geotrichum candidum* is the causative agent of geotrichosis. The genus *Microsporum*, *Trichophyton* and *Verticillium* comprise a number of important species that are causative agents of human dermatophytoses. *Paecilomyces spp.* and *Scopulariopsis spp.* are common environmental moulds but may also be associated with human infection. The infections caused by the genus *Scedosporium spp.* are mainly mycetomas, including infections of the eyes, ears and more commonly the lungs (de Hoog *et al.,* 2001 and Ellis et al., 2007). Others, like *Tolypocladium cylindrosporum* have been studied as a biological control agent against insects (Scorsetti et al., 2012).

Although many fungi are capable of causing disease among humankind, others are indeed very useful to us. These last ones are of great value for industries, from

which beer, cheese, bread are manufactured, and for biotechnology, with inumerous investigations been performed every day and everywhere.

Caves are amazing places, with many secrets hidden and still largely unexplored. Hence there is a high probability that these places can harbour many new and unknown fungi species, which can be extremely deadly to us or quite important to the new developments in our way of life

Table 21: Total number of isolated fungi in each cave and data analyses, specifically Shannon and Evenness indexes of each cave.

Specie	Total	CAVES																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
ASCOMYCOTA																		
Acremonium sp.	6											1	1	1	2		1	
Acremonium strictum	1							1										
Aspergillus sp.	2						1	1										
Beauveria sp.	2									1							1	
Blastobotrys terrestris	1														1			
Candida palmioleophila	2																2	
Candida psychrophila	1																1	
Candida sp.	2														1			1
Chrysosporium sp.	3							2				1						
Exophiala sp.	1											1						
Fusarium sp.	1									1								
Geomyces pannorum	1									1								
Geomyces sp.	2										1						1	
Geotrichum candidum	1											1						
Geotrichum sp.	1																	1
Humicola sp.	1													1				
Kluyveromyces dobzhanskii	2											1					1	
Kluyveromyces lactis	1																1	
Lecanicillium sp.	1									1								
Malbranchea sp.	1													1				
Microsporum sp.	1									1								
Oidiodendron sp.	1							1										

Table 21 (cont.)

Specie	Total	CAVES																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Penicillium citrinum	1						1											
Penicillium griseofulvum	1														1			
Penicillium sp.	22	1	1				1			4		2	2	1	4	1	3	2
Pichia sp.	1																	1
Pochonia chlamydosporia	1							1										
Scedosporium sp.	1												1					
Scopulariopsis sp.	4										1			1			2	
Sporothrix schenckii	1								1									
Sporothrix sp.	2														1	1		
Tolypocladium cylindrosporum	1			1														
Torrubiella sp.	1																1	
Trichoderma sp.	2				1		1											
Trichophyton concentricum	1																	1
Verticillium leptobactrum	3			1								1		1				
Verticillium sp.	4	1		1						1	1							
Zygosaccharomyces florentinus	1		1															
BASIDIOMYCOTA																		
Trichosporon akiyoshidainum	1											1						
Trichosporon cavernicola	3			1						1			1					
Trichosporon chiropterorum	11	1		1						3			1		3		2	
Trichosporon dulcitum	1										1							

Table 21 (cont.)

Specie	Total	CAVES																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Trichosporon jirovecii	1							1										
Trichosporon middelhovenii	1															1		
Trichosporon moniliiforme	7							2		1		1	1		1	1		
Trichosporon mucoides	1													1				
Trichosporon multisporum	2										1	1						
Trichosporon mycotoxinivorans	1							1										
Trichosporon otae	3				1								1				1	
Trichosporon porosum	2									1								1
Trichosporon shinodae	2							2										
Trichosporon sp.	5					2		1									2	
Rhodotorula bloemfonteinensis	1						1											
Rhodotorula mucilaginosa	1																	1
Rhodotorula sp	1					1												
ZYGOMYCOTA																		
Basidiobolus sp.	2										1			1				
Mucor fragilis	1		1															
Mucor hiemalis	1														1			
Mucor sp.	8		1							1	1	1	3		1			
Rhizomucor variabilis	1									1								
Rhizomucor sp.	1		1															
UNIDENTIFIED	15	1		1	4			3		1		1	1		1	1	1	

Table 21 (cont.)

		CAVES																
Specie	**Total**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Total	165	4	5	8	7	4	6	16	1	21	7	13	12	9	19	5	20	8
Total number of species	154	4	5	8	7	4	6	11	1	19	7	13	12	9	17	5	18	8
Number of unique species	116	3	5	5	6	3	5	8	1	14	5	8	8	7	15	3	14	7
Inoculated samples	202	4	6	6	6	6	6	16	16	16	16	16	16	16	16	16	15	9
Samples that yield fungi	157	4	6	6	6	3	6	15	1	16	12	11	13	14	16	6	15	7
Average number of fungi per sample		1	0.83	1.3	1.6	0.67	1	1	0.06	1.31	0.44	0.81	0.75	0.56	1.19	0.31	1.33	0.89
Shannon index		1.39	1.61	1.91	1.15	1.04	1.79	2.31	0	2.56	1.95	2.46	2.09	2.2	2.4	1.61	2.62	1.92
Evenness index		1	1	0.92	0.59	0.75	1	0.83	-	0.84	1	0.96	0.84	1	0.82	1	0.88	0.92

Table 22: Values of Sorenson index. Information on each number is found in Table 2.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
1	-	0	0.17	0	0	0	0	0	0.11	0	0	0.13	0	0.1	0	0.1	0
2	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0.17	0	-	0	0	0	0	0	0.15	0	0.1	0.2	0.12	0.08	0	0.08	0
4	0	0	0	-	0	0	0	0	0	0	0	0.11	0	0	0	0.08	0
5	0	0	0	0	-	0	0	0	0	0	0	0	0	0	0		0
6	0	0	0	0	0	-	0.12	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0.12	-	0	0.07	0	0.17	0.09	0	0.07	0.13	0	0
8	0	0	0	0	0	0	0	-	0	0	0	0	0	0	0	0	0
9	0.11	0	0.15	0	0	0	0.07	0	-	0	0.06	0.19	0	0.11	0.17	0.05	0.07
10	0	0	0	0	0	0	0	0	0	-	0.1	0	0.13	0	0	0	0
11	0	0	0.1	0	0	0	0.17	0	0.06	0.1	-	0.08	0.09	0.07	0.11	0.06	0
12	0.13	0	0.2	0.11	0	0	0.09	0	0.19	0	0.08	-	0	0.14	0.12	0.13	0
13	0	0	0.12	0	0	0	0	0	0	0.13	0.09	0	-	0	0	0	0
14	0.1	0	0.08	0	0	0	0.07	0	0.11	0	0.07	0.14	0	-	0.05	0.09	0
15	0	0	0	0	0	0	0.13	0	0.17	0	0.11	0.12	0	0.09	-	0	0
16	0.1	0	0.08	0.08	0	0	0	0	0.05	0	0.06	0.13	0	0.08	0	-	0
17	0	0	0	0	0	0	0	0	0.07	0	0	0	0	0	0	0	-

CHAPTER IV

CONCLUSIONS AND FUTURE PRESPECTIVES

In conclusion, these results provided new and basic information on fungal species present in Portuguese caves and potential sources of infection for speleologists or people visiting caves, especially the immunocompromised ones.

The only survey, like this one, performed in Portugal was in "Fojo dos Morcegos" by Almeida and co-workers, 1994, and they only isolated three fungal species from bat guano. Therefore this work represents a major contribute to the knowledge of fungal diversity inside Portuguese caves. Furthermore, in this study there is the first record of an environmental *Sporothrix* species in Portugal.

Cave 16, "Cova da Moura" was the one with higher fungal species average and higher values of Shannon and Evenness indexes (Table 21). Therefore, this cave was the one whose fungal population was more diverse and evenly abundant. Cave 12, "Zambujal", was the more similar one with cave 16, sharing two species, *T. chiropterorum* and *T. otae*. Nevertheless, on the surveyed caves the majority of the taxa were only identified to the genus level and this identification was achieved mainly by analysis of the morphological characteristics. Thus, besides the reported taxa there are probably more different species and eventually novel ones. As mentioned before, the results provided by this work are preliminary, since the available time did not allow a more complete survey.

Geomyces destructans wasn´t found in this survey, but Portuguese bats with WNS have already been reported (personal communication). To assess this subject a more complete survey has to be accomplished, including also the use of other culture media and making the sample collections at a specific time – during the bat hibernation season.

Some fungi isolated during this survey were opportunistic pathogens to humans, so the investigators should take extra precaution when handling them and speleologists or even people visiting caves should be aware of the risks. Assous and collaborators, 2009, even stated that "*public education concerning the risks involved in entering caves, combined with prevention measures in countries in which ticks lives primarily in caves and similar sites, is effective in reducing the number of cases. Persons entering caves must wear appropriate shoes and clothes covering all the body. It is recommended to spray shoes and the trouser bottoms with a repellant. Avoidance of long stays in caves reduces the opportunities for ticks to identify the host. Sleeping in caves or at nearby sites must be avoided...If prolonged work is*

necessary, the work should be stopped for several days for respraying" (Assous & Wilamowski, 2009).

Finally, a total of twenty-eight psychrophilic fungi where isolated during this work, from which some were also able to grow at 25*ºC*. For this to happen structural and physiological features undergo changes. One example is the production of different enzymes, secondary metabolites or changes in the cell wall and cellular membrane. One interesting approach for these fungi is to study what kind of enzymes they produce and if they can be applied to biotechnology research. This may be very useful for adding in laundry detergents making them more efficient in washing in low temperatures. This way energy can be saved and the planet spared.

This was a project and a subject that I enjoyed to work on and I really hope to be able to continue and improve my knowledge in this matter.

CHAPTER IV

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APPENDIX

APPENDIX I

SAMPLING QUESTIONNAIRE

Dados da Colheita

Cidade:_____

Abrigo:_____Temperatura:_____

Humidade:_____

Espécies identificadas:_____

_____

Número de amostras:_____

Data da colheita_____

Dados da Colheita

Cidade:_____

Abrigo:_____Temperatura:_____

Humidade:_____

Espécies identificadas:_____

_____

Número de amostras:_____

Data da colheita_____

Dados da Colheita

Cidade:_____

Abrigo:_____Temperatura:_____

Humidade:_____

Espécies identificadas:_____

_____

Número de amostras:_____

Data da colheita_____

APPENDIX II

SOLUTIONS

⇒ **PBS**

Reagents:

- Sodium chloride
- Potassium chloride
- Sodium phosphate
- Potassium phosphate

⇒ **TAE buffer**

Reagents:

- Tris base in water
- Glacial acetic acid
- 0.5 M EDTA (pH 8.0) solution

⇒ **TES buffer**

Reagents:

- 0.05 M EDTA
- 20% Sucrose
- 1 M Tris base or 0.01 M HCl (pH 8.0)

⇒ **Solution A**

Reagents:

- 0.05 M Tris-HCl (pH 7.4)
- 0,2 M EDTA

⇒ **TE buffer**

Reagents

- 10 mM Tris-HCl (pH 7.4)
- 1 mM EDTA