MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *ASCOBOLUS* AND *PILOBOLUS* FUNGI IN WILD HERBIVORE DUNG IN NAIROBI NATIONAL PARK

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A Research Thesis Submitted to the Graduate School in Partial Fulfilment for the Requirements of the Award of Master of Science Degree in Biochemistry of Egerton University

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been submitted or presented for examination in any institution

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Recommendation

This thesis has been submitted for examination with our approval as supervisors

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DEDICATION

This thesis is dedicated to my family for their love and support always.

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ABSTRACT

Coprophilous fungi are abundant species found in dung of most wild animals, and an easily available and abundant tool for studying and monitoring ecosystem changes. The focus of this study was to characterize two genera of coprophilous fungi, *Pilobolus* and *Ascobolus.* These fungi are important in decomposing and recycling of nutrients from animal waste. *Ascobolus* fungi are important in genetic studies and have been identified as a source of enzymes and antibiotics. *Pilobolus* fungi play a role in transmission of pulmonary bronchitis because they vector lungworms. Diversity studies of these species give insight on the state of an ecosystem and can be used to predict occurrence of environmental stressors. In this study, wild herbivore dung was collected in Nairobi National Park and incubated for fungal sporulation and afterwards characterized by morphological and molecular methods. The Internal Transcribed Spacers 1 and 2 regions of ribosomal DNA of *Pilobolus* were sequenced. Five species of *Ascobolus* were described using morphological means: *A. immersus, A. amoenus*, *A. bistisii, A. calesco,* and a possible novel *Ascobolus* species with four spores as opposed to the usual eight. Three species of *Pilobolus* were described using morphological means: *P. crystallinus*, *P. heterosporus* and *P. pullus*. Molecular analysis revealed three species of *Pilobolus*: *P. crystallinus*, *P. heterosporus*¸ and *P. pullus*. However, *P. crystallinus* had *P. heterosporus* as the closest match though with low identity. On the other hand, the sequences showed that there was some (89%-99%) similarity between *Pilobolus* collected from this study and those from the United States of America. Consequently, molecular identification of *Pilobolus* offered a confirmation of species identity. In terms of abundance*, Ascobolus immersus* and *P. crystallinus* were the most common species observed. Similarly, waterbuck and zebra dung showed the highest diversity of fungal species while hippopotamus and giraffe had the least number and this could be attributed to the limitation of their feeding areas. The highest observed species richness per dung pile was 5 while the estimated species richness was 15. Therefore, indicating diversity of coprophilous fungi in Nairobi National Park ecosystem is relatively high. The results of this study can be used as a baseline for future monitoring of environmental degradation in the park.

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LIST OF ABBREVIATIONS AND ACRONYMS

BLAST	Basic Local Alignment Search Tool
Bp	Base pairs
dNTPS	Deoxynucleotide Triphosphates
EB	Elution buffer
EDTA	Ethylene Diamine Tetracetic Acid
EF	Elongation Factor
ILRI	International Livestock Research Institute
ITS	Internal Transcribed Spacer
LSU	Large Subunit
PCR	Polymerase Chain Reaction
rDNA	ribosomal Deoxyribonucleic Acid
SSU	Small Subunit
TAE	Tris Acetate EDTA
UV	Ultra Violet
ZR	Zymo Research

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CHAPTER ONE
GENERAL INTRODUCTION

1.1 Background information

The Kenya Wildlife Service is mandated with protecting wildlife (animals and plants) in Kenya and conducting research on their diversity. Diversity research enhances conservation and management of wild animals and plants in Kenya's protected areas such as National Parks, Sanctuaries and Reserves. Wildlife is one of Kenya's top foreign exchange earners in terms of tourism (Ngeta, 2010) and it is necessary to carry out research that will enhance sustainability of wildlife ecosystems. Coprophilous fungi are a group of fungi that grow on dung. They are an important part of the wildlife ecosystem since they help in recycling nutrients in animal dung (Richardson, 2001a). They achieve this through the decomposition of herbivore dung. In addition, they are also important since they positively influence the digestive efficiency of animals besides serving as nutrition for certain arthropods that live in the dung (Wicklow and Angel, 1974b). This group of fungi is also important as indicators of the state of the environment, since their diversity can be used to demonstrate whether there is degradation in an ecosystem (Ebersohn and Eicker, 1992). For these reasons, studying these microorganisms is important for conservation and wildlife protection. These fungi are a potential source of antibiotics and enzymes which is likely to suggest that they may be beneficial for therapeutic uses (Santiago *et al.,* 2011).

Herbivorous animals grazing on vegetation ingest many fungal spores alongside their food (Bell, 1983). These are passed through the gut and excreted with the dung. Under the right environmental conditions, the spores germinate on the dung and begin growing. Once matured, spores are dispersed from the dung and land on new plant material. Some fungi taxa such as *Pilobolus* have developed means of discharging their spores large distances away (Wicklow and Carroll, 1981). This increases the chances of wild herbivores feeding on the spores providing means to continue the life cycle.

Coprophilous fungi diversity and richness is shown to increase with decreasing altitude (Richardson, 2001b). Therefore, the diversity and richness in Nairobi National Park is expected to be much higher than in other parks in low altitude areas. In the current study, the genera *Pilobolus* and *Ascobolus* were chosen for classification by morphological and molecular means into their respective species. This is due to their abundance in nature and the fact that they are among the first genera to be observed after incubation. Previous studies on coprophilous fungi in wild herbivore dung in Kenya revealed that their diversity is high (Mungai *et al.*, 2012).

1.2 Statement of the problem

The Nairobi National Park ecosystem is under considerable stress due to urban development in the surrounding areas and increased human population pressure. The migration corridors such as Kitengela have continued to be closed up due to human settlements. There is also risk of pollution due to the industrialization of areas surrounding the park due to its proximity to the capital city. All these factors are likely to cause a profound impact on the environment. One way to assess the effect of these ecosystem changes for planning intervention is through studying the fungal diversity in the park. Much of the research done on coprophilous fungi in the past has concentrated on domestic animal dung while there are still no clear records on fungal species isolated from wildlife dung. This creates a knowledge gap which needs to be filled in order to obtain a record of this species in the wild. To date there has been no molecular characterization of these species in Kenya to the best of our knowledge. Reliance on morphological characterization of these fungi is not a robust and sufficiently informative tool in species identification due to overlap in certain features among species and even the existence of cryptic species. This view is further supported by the fact that some morphological features are known to change with environmental conditions. Therefore, the same species might have different morphologies depending on the geographical and environmental conditions. When used for identification morphological descriptors can be misleading thus giving inaccurate results. This poses a challenge that can be addressed by using molecular identification as the most reliable in fungal species characterization.

1.3 Objectives

1.3.1 General Objective

To characterize *Ascobolus* and *Pilobolus* fungi from different wild herbivore dung from Nairobi National Park using morphological features and Internal Transcribed Spacer (ITS) molecular markers.

1.3.2 Specific Objectives

1. To identify *Ascobolus* and *Pilobolus* fungi based on their morphological characteristics.
2. To characterise *Pilobolus* fungi using Internal Transcribed Spacer markers.
3. To determine the composition and diversity of *Pilobolus* and *Ascobolus* fungi species among different dung substrates from wild herbivores in Nairobi National Park.

1.4 Hypotheses

1. The morphological characteristics of *Ascobolus* and *Pilobolus* fungi from different wild herbivore dung are not significantly different.
2. The molecular characteristics of *Ascobolus* and *Pilobolus* fungi are not significantly different.
3. *Pilobolus* and *Ascobolus* species composition and diversity do not vary significantly among different dung substrates from wild herbivores in Nairobi National Park.

1.5 Justification of the study

This study aimed to classify coprophilous fungi from the genera *Ascobolus* and *Pilobolus*. *Pilobolus* and *Ascobolus* fungi are the most commonly found in dung, therefore the best candidates for this study. The said fungal genera are important agents in the environment due to their ability to release nutrients from animal waste. They have also been identified as a potential source for enzymes and antibiotics as well as vectors for diseases such as bronchitis. Biodiversity studies are an important part of conservation and help in creating a complete checklist of all the organisms in a given ecosystem. Biodiversity studies on microorganisms such as fungi are also essential. They respond much faster to changes in the environment and can help monitor subtle changes that may not be evident by studying larger organisms. Coprophilous fungal diversity can be used as a tool in monitoring the changes in the ecosystem. Whenever their diversity is high, it infers stability in the environment while low diversity is a sign of stress in the ecosystem.

The use of morphological features has been used and is insufficient in identifying and segregating fungal species. This is mainly because morphological features may sometimes vary even in organisms from the same species. Consequently, the only way to confirm accurate identities of these groups of fungi is through molecular characterization.

The result of this study has helped in creating a checklist for the fungal species in the park. This is a baseline study which will be useful as a reference in future monitoring of the ecosystem. Therefore, allowing for monitoring fungal population trends which will indicate whether there is stress on the ecosystem. Information generated will aid policy makers when making changes to reduce degradation in the environment and thus prevent wildlife decline.

1.6 Scope and limitations

This study assessed the diversity of *Pilobolus* and *Ascobolus* in Nairobi National Park using morphological and molecular descriptors. The samples used were collected from a variety of herbivorous animals in their natural habitat. About 80 samples were obtained randomly from the study area at different times of the day and during different seasons.

The research faced challenges in classifying *Ascobolus* species through molecular methods due to the failure in obtaining pure cultures. This could be attributed to sub-optimal laboratory conditions that are required for growth of these fungi in artificial cultures. For this genus only the morphological descriptors were relied upon during the diversity study. However, this problem was not experienced with *Pilobolus* species which were successfully cultured and genomic DNA extracted for molecular characterization.

CHAPTER TWO
LITERATURE REVIEW

2.1 Overview of Coprophilous fungi

Coprophilous fungi are a group of saprobic fungi that grow on animal dung and become viable once their spores are taken up by animals in their food (Bell, 1983). There are various groups of fungi that fall into this group. These are from coprophilous ascomycetes, basidiomycetes and zygomycetes. However, these do not grow on dung at the same time. They grow in succession which is seen through sequential fruiting of coprophilous fungi on the dung (Richardson, 2002). Therefore, certain groups are observed earlier a few days after incubation while some can grow even three months after incubation.

2.1.1 Composition of coprophilous fungi

Coprophilous fungi composition observed in dung also differs depending on a number of factors. Some of the factors that might influence the mycota in dung include geographical location, insects, competitions and moisture content (Caretta and Piontelli, 1996). This is because each group requires certain conditions for fruiting to occur. In this study, two genera of fungi are of interest, which are *Ascobolus* and *Pilobolus*. These are often found in herbivore dung and depend on the dung for their nutrition. These two genera are common and are observed early within two weeks of incubation.

2.1.2 Life cycle of coprophilous fungi

The life cycle begins when their spores in the dung germinate (Figure 1). They grow to maturity then they disperse onto the surrounding vegetation (Wicklow and Angel, 1974; Bell, 1983). Many herbivores avoid foraging where there are faeces. Therefore, coprophilous fungi have developed means of dispersing their spores far away from the dung in which they are growing. This enables the spores to land on new plant material where they can be ingested by herbivores as they eat (Page, 1962).

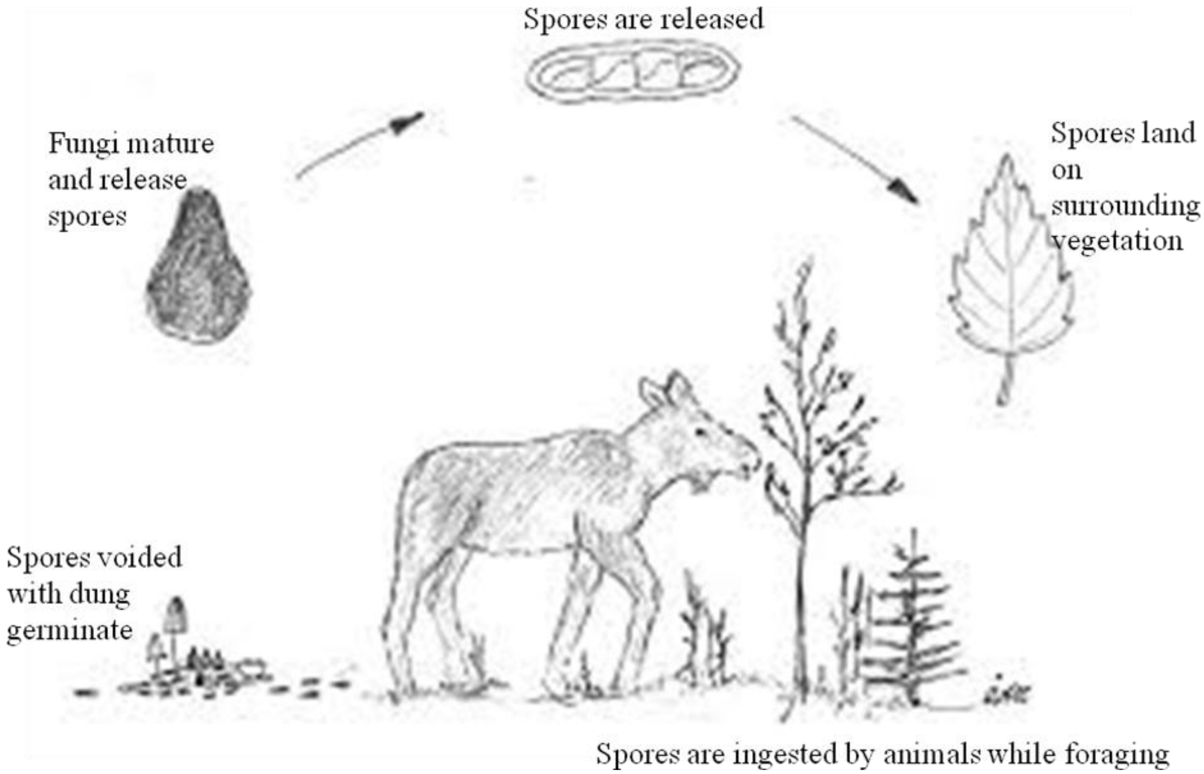

Figure 1: Life cycle of coprophilous fungi (image courtesy of Åsa Nyberg Kruys)

The spores go through the animal's digestive system as the plant material is being digested. Coprophilous fungi spores have thick walls that protect them from digestion as they pass through the digestive system of animals (Wu and Kimbrough, 1992). The spores are then excreted with the faeces from which they grow once they get the right moisture and light conditions.

2.1.3 Biological and economic importance of coprophilous fungi

Coprophilous fungi are quite abundant in the environment and they can be cheaply accessed. They can be exploited for a variety of purposes. They form an important part of the ecosystem and they mostly help in recycling nutrients trapped in animal dung (Richardson, 2008). They are also involved in mineralization of dung (Ebersohn and Eicker, 1992). Bacteria are largely responsible for breakdown of carnivore and omnivore dung. However, herbivore dung is largely dependent on fungi for breakdown. Without coprophilous fungi, there would be piles of herbivore dung in nature because they are able to break down undigested cellulose contained in the dung and release the nutrients back into the environment. In addition, this group of fungi are also important due to their role as biological control agents. They do so by producing biologically active metabolites that potential competitors and predators. This can be exploited for production of bioactive fungal metabolites (Gloer, 1995; Santiago *et al*., 2011).

Coprophilous fungi offer opportunities for discovery of new antibiotics (Bills *et al*., 2013). The fungi have to compete with other microbial communities in the dung for nutrients and thus survival. Therefore, they have become adapted to challenge bacteria, protists and other invertebrates that are also present in the dung. They have high potential of antibiotic discovery through fungal genomics. They have already been identified as a source of antibiotics, for example, antiamoebins that are isolated from *Stilbella* genus (Jaworski and Bruckner, 2000). Coprophilous fungi are also of ecological importance since they can be used as indicator species where they can be used to get information on the ecosystem. Their diversity can be used to monitor changes of the ecosystem. Species diversity help in showing how healthy or stressed an ecological environment is (Ebersohn and Eicker, 1992).

2.2 Genus *Pilobolus*

The genus *Pilobolus* belongs to the class Zygomycetes and the order Mucorales. This group of fungi are identified through their characteristic sporangiophores which have a swollen extension called collumelae and a sporangium that hosts the spores at the top (Kendrick, 2000). *Pilobolus* are among the first fruiting bodies to be observed when dung is incubated (Figure 2). They are normally observed within the first two to three days of incubation at room temperature under natural light (Bell, 1983). Species belonging to the genus *Pilobolus* are the most frequently observed from the Zygomycetes class of fungi (Richardson, 2001a). *Pilobolus* are obligate coprophilous, which means they can only grow on dung material (Krug *et al.*, 2004). They grow while attached to the dung through a swollen trophocyst which is semi immersed in the dung. The trophocysts have been observed to be ovoid to globose in shape and have a rhizoidal extension that is cylindrical. Their sporangiophores are not branched and always straight, phototrophic with orange pigmentation which act as light sensors. The sporangia are black in color while the columellae are smooth and long-elliptical (Viriato, 2008).

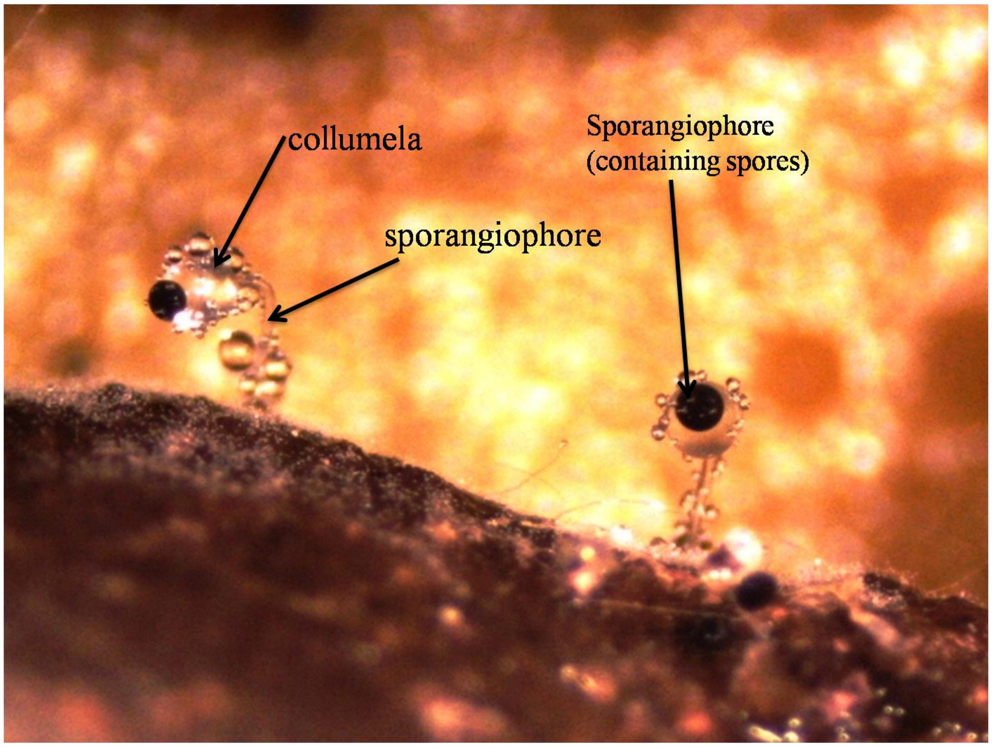

Figure 2: *Pilobolus* (photo by Antoinette Miyunga)

2.3 Genus *Ascobolus*

The genus *Ascobolus* belongs to the class Ascomycetes. Members of this genus are normally identified by certain characteristic features that are also used to distinguish them morphologically (Figure 3). They have apothecia that are either superficial or immersed and can be up to 3cm in diameter. The receptacle can be subglobular, pyriform, villose or downly. The asci can have rounded, dome shaped or truncate apex. They have purple ascospores that change to brown during late maturity. These spores can be arranged in two rows in the asci or irregularly disposed (Brummelen, 1967). They have slender Paraphyses that are found embedded in mucus that can be colorless, yellow or yellowish green. *Ascobolus* species have ascomata that are pale yellow in color and are luteous and superficial (Mungai *et al.*, 2012).

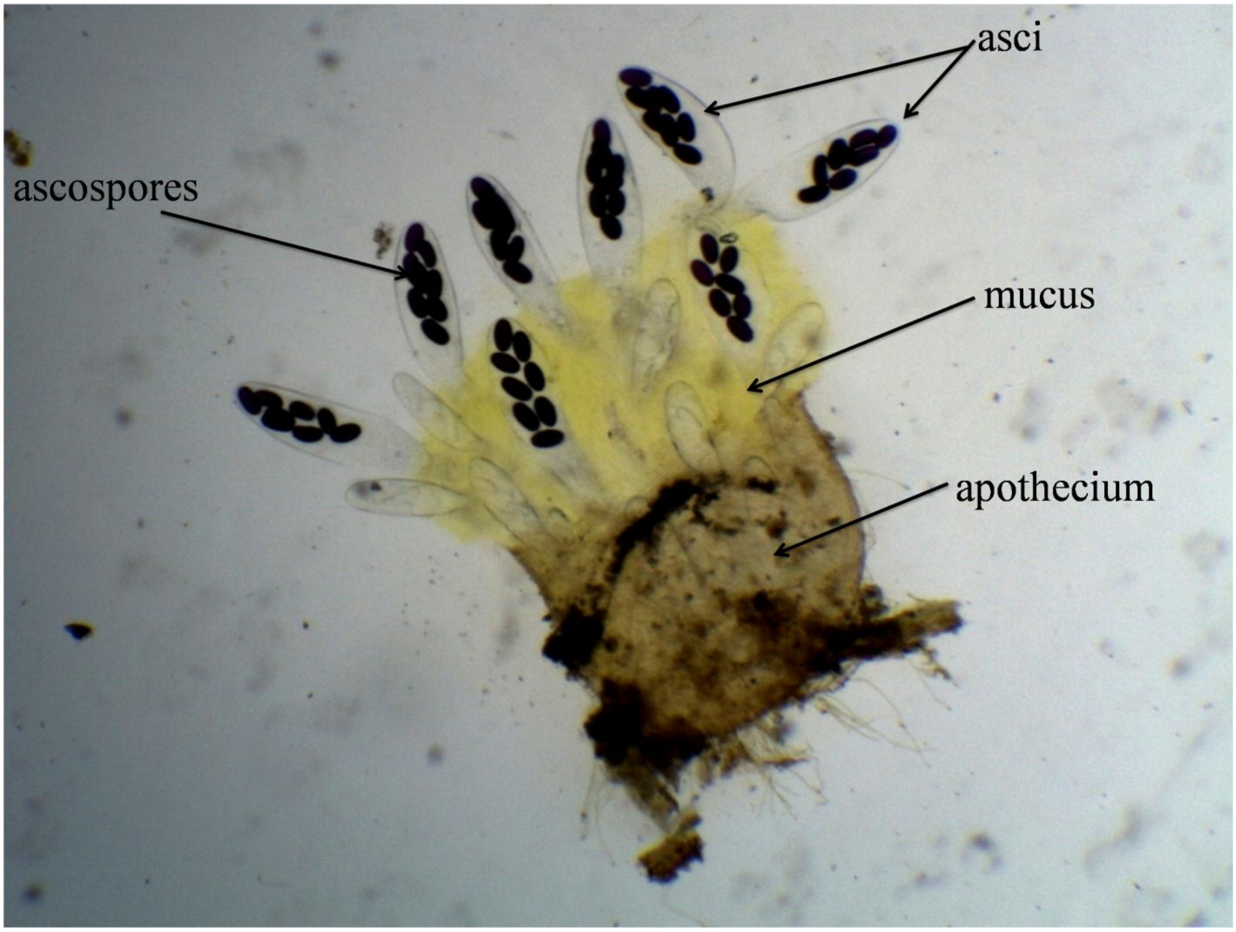

Figure 3: *Ascobolus* (photo by Antoinette Miyunga)

There are about sixty identified species from the genus *Ascobolus* (Ainsworth, 2008). They are phototropic and release their ascospores towards the light which shoot out and end up on surrounding plants where they are eaten again by herbivores. The spores pass through the herbivores digestive system where they end up in waste matter from digestion that is voided as dung where they sporulate to their fruiting bodies and the cycle begins again. The *Ascobolus* fruiting bodies get nutrition from the plant material present in dung (Bell, 1983). Moist chamber incubation is known to yield the highest number of Ascomycetes in the laboratory (Piasai and Manoch, 2009). The incubation period for sporulation of *Ascobolus* in dung is usually 7-20 days (Piasai and Bell, 1983; Mungai *et al.*, 2011). Previous studies on coprophilous fungi on wildlife dung in Kenya revealed that there is abundant *Ascobolus*.

Species of *Ascobolus* fungi identified morphologically from dung in different national parks of Kenya are *Ascobolus amoenus, A. bistisii, A. calesco, A. immersus*, *A. nairobiensis* and *A. tsavoensis* (Mungai *et al*., 2012). Hence, there is need to use molecular techniques to confirm their identities with greater accuracy.

2.4 Economic importance of *Pilobolus* and *Ascobolus*

Pilobolus plays an important role in the spread of parasitic bronchitis in grazing animals. This is a disease that is caused by lungworms (genus *Dictyocaulus*). *Pilobolus kleinii* and *Pilobolus crystallinus* have been identified as notorious vectors for these nematodes (Doncaster, 1981). *Dictyocaulus* spp have been determined to cause diseases of economic importance in domestic animals such as cows and horses. Some animals such as donkeys are not adversely affected by lung worm diseases but can be carriers or the same. Clinical disease in animals develops after initial infection with larvae however the severity of the disease depends on the number of larvae ingested. After the first infection the animals develop an immune response to protect them from future disease. Such animals do not get sick on subsequent exposure to the larvae but act as carriers and cause pasture contamination. However, severe infection develops in unexposed calves (Overview of Lungworm Infection). Lungworm infection and epidemiology has not been extensively studied in wild animals.

The life cycle of lungworms begins when larva are swallowed by animals when they are foraging. The larva travel from the digestive system and into the blood and lymph system. They eventually end up in the lungs and cause infection. When in the lungs where they form cysts and establish an infection. Some of their eggs are swallowed by animals and pass through the gastrointestinal tract and are secreted with faeces. The eggs hatch to produce a larval stage that is infective when swallowed by animals. However, the larvae have to migrate away from the faeces in which they were secreted since animals do not forage near their own faeces. The larvae can move on their own but not for long distances; therefore, they attach to *Pilobolus* sporangia and when the fungus shoots its spores, the larvae are also transported (Figure 4). They land in surrounding which is up to 3 meters away from where the dung is deposited (Eysker, 1991). After infection with lungworms, there is risk of pneumonia and even death resulting from a weakened immune system (Robinson, 1962; Jorgensen *et al*., 1982; Foos, 1997)

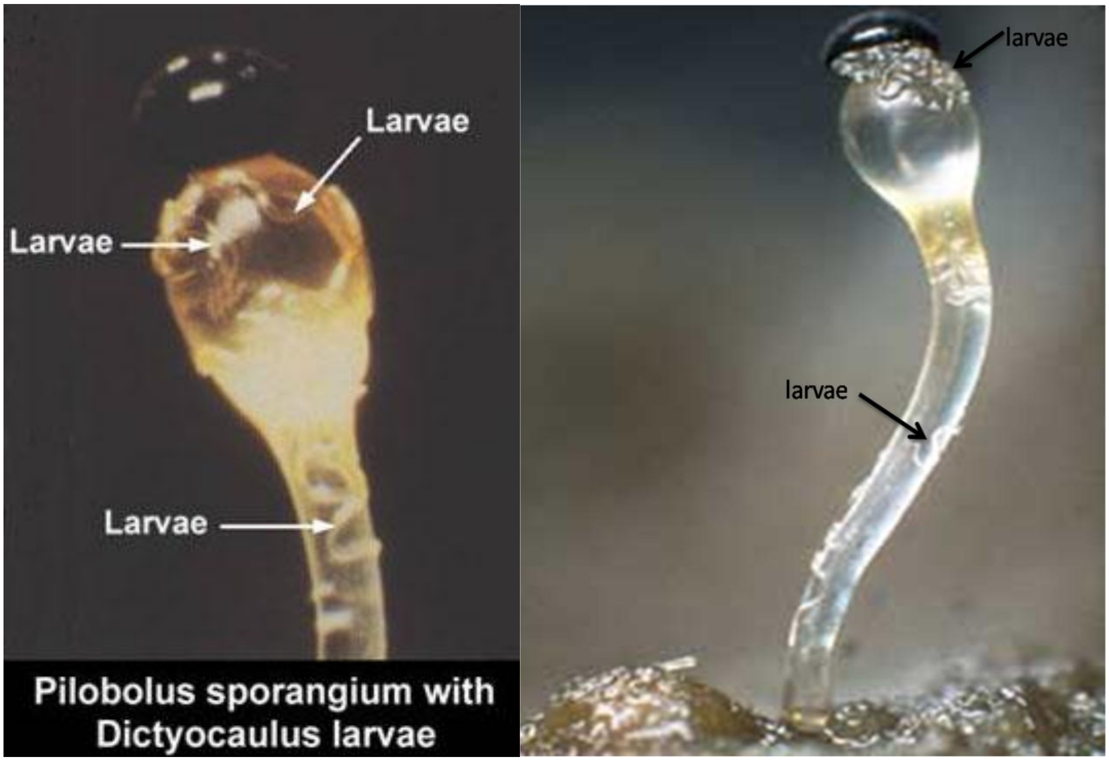

Figure 4: *Pilobolus* and lungworm (Image courtesy of Merial Inc)

Ascobolus fungi are very useful in nature because can digest cellulose which most other organisms are unable to and therefore important in the ecosystem due to their role in recycling nutrients (Kendrick, 2000; Kendrick 2002). Without them, there would be large piles of dung in nature.

2.5 Culture of *Ascobolus* and *Pilobolus* fungi

For molecular analysis, it is essential to get pure cultures of these fungi on artificial media. Some fungi species in these genera are obligate coprophilous and only grow on dung or culture media enriched with dung extract. However, most are facultative coprophilous and can grow on common culture media (Krug *et al*., 2004). *Pilobolus* requires the active growth factors hemin, ferrichrome and coprogen, which are naturally present in dung, in order to grow (Hesseltine *et al*., 1953; Levetin and Caroselli, 1976). Therefore, synthetic media containing these growth factors can facilitate the growth of *Pilobolus* in artificial culture (Levetin and

Caroselli, 1976). In addition, dung agar can also be used to culture these fungi since dung contains all the necessary growth factors (Swartz, 1934).

Numerous apothecia from *Ascobolus* grow best when grown in agar medium containing yeast extract and cellulose. They grow best after prior treatment with Sodium hydroxide solution and incubated at 37°C for twenty four hours. This is done to help soften the hard outer core of the spores and mimic the treatment they go through during the passage in animal digestive system (Yu, 1954). Antibiotics such as terramycin, penicillin and streptomycin are added to such artificial media to prevent invasion/contamination by bacteria. Frutification is poor on agar media generally although better on thin layers (Brummelen, 1967).

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CHAPTER THREE

MORPHOLOGICAL DIVERSITY OF *ASCOBOLUS* AND *PILOBOLUS* FUNGI FROM NAIROBI NATIONAL PARK, KENYA.

3.1 Abstract

The study examined two genera of coprophilous fungi: *Ascobolus* and *Pilobolus* with the aim of species description using their morphological features. Fresh dung samples from wild herbivores were collected in different parts of Nairobi National Park in Kenya and immediately taken to the laboratory for culture by moist chamber method. Isolates studied were obtained from dung of: white rhino, zebra, waterbuck, impala, Cape buffalo, giraffe, Thompson's gazelle, dikdik, hare, grant's hartebeest, hippopotamus and eland. Four species of *Ascobolus* were identified using morphological descriptors namely: *Ascobolus amoenus, A. bistisii, A. calesco* and *A. immersus*. In addition, a possible novel 4-spored *Ascobolus* species was observed differing from the 8-spored species that are described in previous work. Three species of *Pilobolus* were found: *Pilobolus crystallinus var. crystallinus*, *P. heterosporus* and *P. pullus*. The highest abundance was observed for species *Ascobolus immersus* and *Pilobolus crystallinus var. Crystallinus* while the highest diversity was observed in waterbuck dung samples with a total of five different species. This is an indicator that diversity of these genera in the park is relatively high. This shows that Nairobi National Park ecosystem is relatively stable. Future studies can use these findings as a reference to monitor changes in the diversity of these groups of fungi and the park ecosystem in general.

3.2 Introduction

Morphological characterization of fungi is their classification based on differences in their physical characteristics (Bell, 1983). A dichotomous key is used to obtain the specific identity of each fungus by looking at the morphological features. Micro-morphological features of fungi can also be used to classify them phenotypically (Iotti *et al.*, 2005). Classification of *Ascobolus* is based on the type of fruiting body found on the fungi (Bell, 1983; Kendrick, 2002). The ascus can be used to describe a species by looking at their shape, number, size and apex. The ascospores are used to identify species through their shape, whether they occur as single spores or in a cluster and whether they have septa or not.

Prior to dispersal, ascospores are normally found enclosed in the ascus and their arrangement here is used for classification. The average size of the ascospores, pigmentation and whether they are covered by a gelatinous sheath is used in classification. The ascus is also a factor in classification and can be operculate, inoperculate, prototunicate or bitunicate (Doveri, 2004). They are classified through their ascomata which can be apothecial, perithecial, cleistothecial or pseudothecial (Kendrick, 2000). All these features plus the ascospores are used to morphologically characterize Ascomycetes fungus.

The different species from *Pilobolus* are identified by comparing the structures on each fruiting body. The key for identification of *Pilobolus* species involves checking the shape and color of the spores, type of wall on the spores, sizes of spores and Sporangiophore and the form of the columellae (Viriato, 2008).

Previous studies on the genus *Ascobolus* undertaken on wildlife dung in Kenya indicate that there is high species richness (Mungai *et al*., 2012). However, there are no specific studies on *Pilobolus* species in Kenya to the best of our knowledge. *Pilobolus* is especially important due to its role in transmission of bronchitis in animals as a vector for lungworms (Jorgensen *et al*., 1982). Therefore, it is necessary to establish which species are present in the park and their abundance.

The current study focused on morphological diversity of coprophilous *Ascobolus* and *Pilobolus* from wild herbivores with the aim of identifying the different speciespresent in herbivore dung. The findings of of this study provides reference for future biodiversity studies and ecological monitoring of the Nairobi National Park ecosystem.

3.3 Materials and methods

3.3.1 Study area

Wildlife herbivore dung was collected from Nairobi National Park in Kenya. The park is situated approximately 7 km from Nairobi city center with a savannah ecosystem comprising of scattered acacia and open grass plains. The park covers an area of 117km^2 with central coordinates 1°22′24″S, 36°51′32″E. Wild herbivore dung samples were randomly collected from different locations of the park (Figure 5). During collection, all sites were marked using GPS and used to generate a map of sample collection as shown (Figure 6).

Figure 5: Sample collection in the field (Photograph by Asenath Adienge)

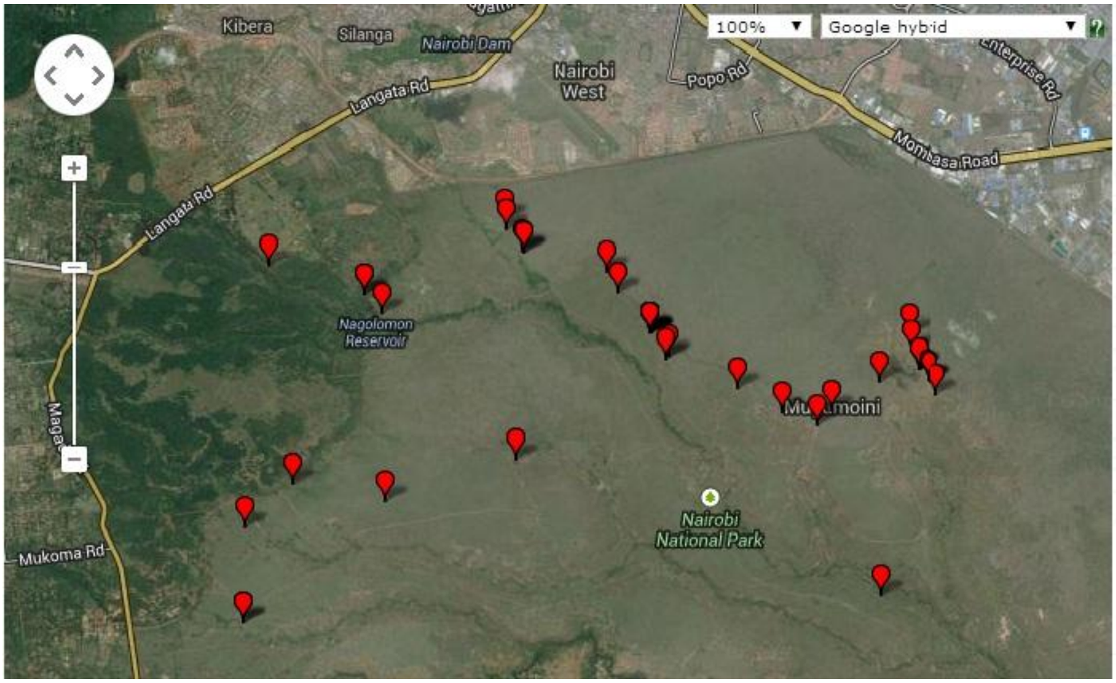

Figure 6: Map of Nairobi National Park showing sample collection points (Google maps)

3.3.2 Sample collection

Sixty four wild herbivore dung piles were collected from Nairobi National Park. These were from the common herbivores found in the park in their natural habitat (Table 1). Fresh dung samples were collected within minutes of being voided (Figure 7). This allowed correct identification of the animal species voiding the dung. It also minimized contamination of the dung by aerial fungal spores. The dung samples were placed in paper envelopes labelled with details of location coordinates, altitude, sample number, date of collection and animal species voiding the dung.

Figure 7: Freshly voided dung from a zebra in Nairobi National Park (photo by Antoinette Miyunga)

3.3.3 Moist Chamber Cultures

After collection, the samples were transported to the KWS laboratory. 10grams of dung from each sample was incubated and the rest stored in a herbarium. The dung incubation was done according to the procedure of Bell (1983). The dung samples were placed in petri dishes (100mm) labelled and lined with a single filter paper of the same size (Figure 8). The dung was moistened with distilled water to encourage fungal sporulation. Afterwards, the samples were incubated at room temperature (18-25°C) in natural light to encourage fungal sporulation. During incubation, samples were monitored daily to check for sporulation and fruiting.

Figure 8: White rhino dung in moist chamber culture (photo by Antoinette Miyunga)

3.3.4 Morphological examination

Once fungal sporulation occurred after about a week the incubated dung was observed under a stereomicroscope and photos taken (Figure 9 and 10). Individual fruiting bodies were collected using a pair of fine forceps and transferred into a drop of sterile water on a glass slide. This was covered using a cover slip and pressed gently to spread out the spores and then examined under a compound microscope under magnification of ×4, ×10, ×40 and ×100 to obtain fine details of the morphological features. Photomicrographs were taken of the fungal images for identification.

Afterwards, permanent slides of each of the fungal species observed were made. This was done by using a drop of a solution of 50% lactic acid and 50% absolute ethanol on a microscope slide. The fruiting body to be preserved was carefully extracted from the dung and placed on the drop. The cover slip was placed gently over the sample while taking care not to form bubbles. This was left to dry for three hours and then the cover slip sealed with clear nail polish.

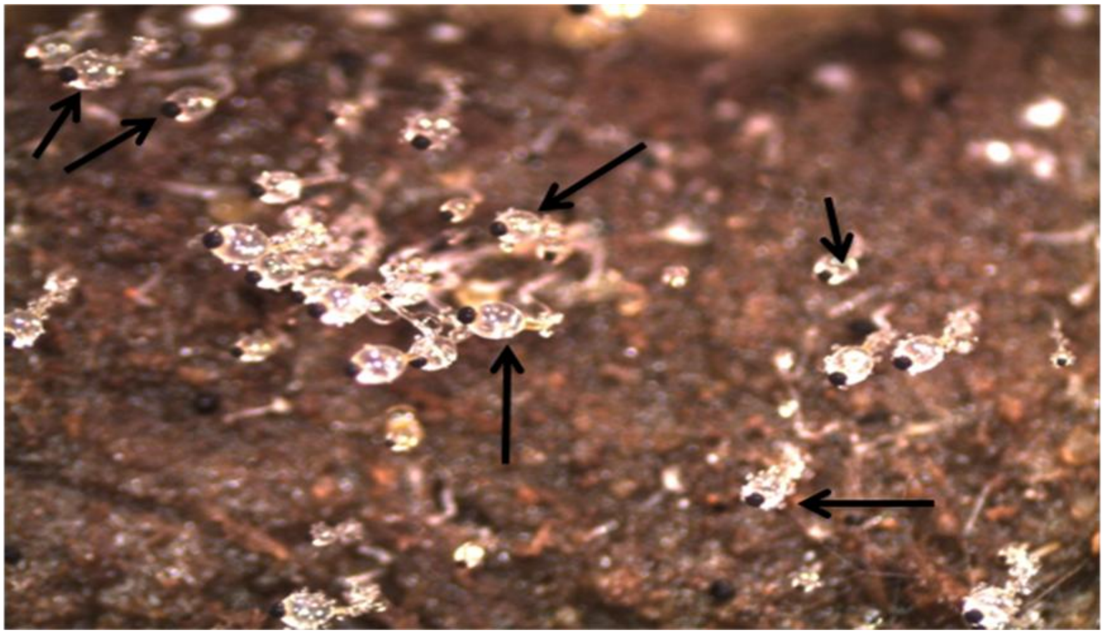

Figure 9: *Pilobolus* fungi (arrows) growing on dung substrate ×4 (photo by Antoinette Miyunga)

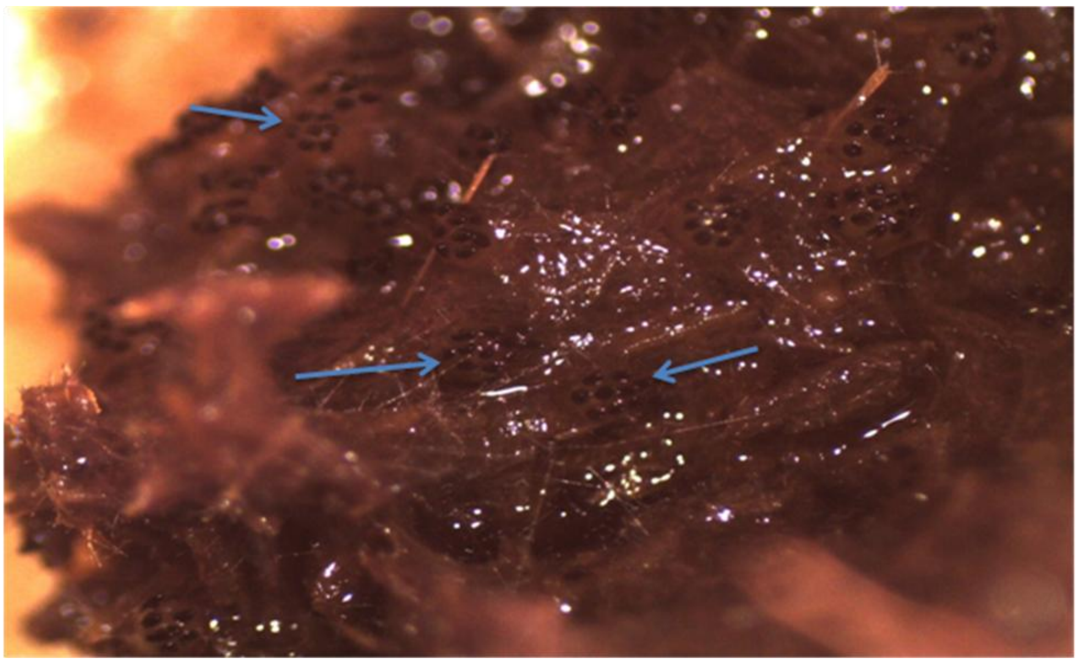

Figure 10: *Ascobolus* fungi (arrows) growing on dung substrate ×4 (photo by Antoinette Miyunga)

3.3.5 Statistical analysis

Different diversity indices were calculated and compared to estimate the diversity of these two genera in the park. These indices offer a quantitative measure to reflect how many different species are present in the dataset, taking into account how evenly these are distributed among the host species.

Shannon-weinner index is commonly used in ecological studies and was chosen as one of the indices for this study. The index was calculated using the following formula, Where pi was the proportion of individuals found in the i^{th} species (Magurran, 2004)

$$H' = -\sum_{I=1}^{R} pi \ln pi$$

Simpson index was calculated using the following formula, where S_{obs} was the number of observations and Ni was the number of individuals in the i^{th} species and Nt was the total individuals in the sample. The index is and the higher the value the greater the diversity (Magurran, 2004)

$$C = \sum_{i}^{Sobs} Pi^2 \qquad Pi^2 = \left\{\frac{\mathrm{Ni}}{\mathrm{Nt}}\right\}$$

Chao's species richness estimation was used to calculate the expected highest species abundance using the following formula, where S_{obs} was the number of species of observed in the sample, a was the number of species represented by a single individual and b is the number of species represented by two individuals (Chao, 1984; Gotelli and Colwell, 2011).

$$S_{chao} = S_{obs} + \left(\frac{a^2}{2b}\right)$$

The curves for the Shannon weinner and Simpson's diversity index were drawn using R package.

3.4 Results

3.4.1 Fungal isolates and Morphology

The following eight species were identified using morphological features:

1. ***Ascobolus amoenus*** (Oudemans, *Hedwigia*21: no. 11, 1882).

Morphological description: Ascomata was apothecioid, solitary or gregarious, semi-immersed, 500–600 µm high, 600–700 µm diam. Receptacle was at first closed then later irregularly opening at the top, cupulate, brown. Disc was flat to convex, greenish yellow, numerous tips of ripe asci protruding and dotting the hymenium. Hypothecium was thin discontinuous and composed of isodiametric and oblong cells 4–6 µm. Medullary excipulum of textura globulosa had thin cells. Ectal excipulum of textura had globulosa-angularis brownish cells 10–20 × 6–10 µm. Paraphyses was filiform, hyaline, tips rarely inflated, embedded in greenish-yellow mucus 2–4 µm broad. Asci measured 180–245 × 50–60 µm, had 8-spores each, narrowly clavate, rounded and curved with walls that characteristically stain blue in Melzer's reagent. Ascospores measured 30–40 × 21–22 µm, were biseriate, ellipsoidal, violet during early growth, and finally brownish (Figure 11)

Material examined: KENYA, Nairobi County, incubated for seven to fourteen days, Nairobi National Park, GPS S1°35'45.11" E36°85'70.55", altitude 1647m, Waterbuck, 23 July 2013, P. Mungai KWSNNP005-2013; Nairobi National Park, Nairobi County, GPS S1°35'91.55" E36°84'48.32", altitude 1649m, Impala, 23 July 2013, P. Mungai KWSNNP007-2013; Nairobi National Park, Nairobi County, GPS S1°35'42.28" E36°85'68.92", altitude 1650m, Hartebeest, 26 August 2013, P. Mungai KWSNNP020-2013; Nairobi National Park, Nairobi County, GPS S1°34'77.08" E36°85'17.54", altitude 1648m, Thompson's gazelle, 26 August 2013, P. Mungai KWSNNP012-2013

Notes: *Ascobolus amoenus* sect *Dasyobolus* was closely similar to *A. elegans* but it could be differentiated by its smaller ascospores. This collection differed from that described by Oudemans (1882) in the size of the asci. The latter has asci with smaller diameter of about 35–40 µm. The ascospores were observed to have double walls.

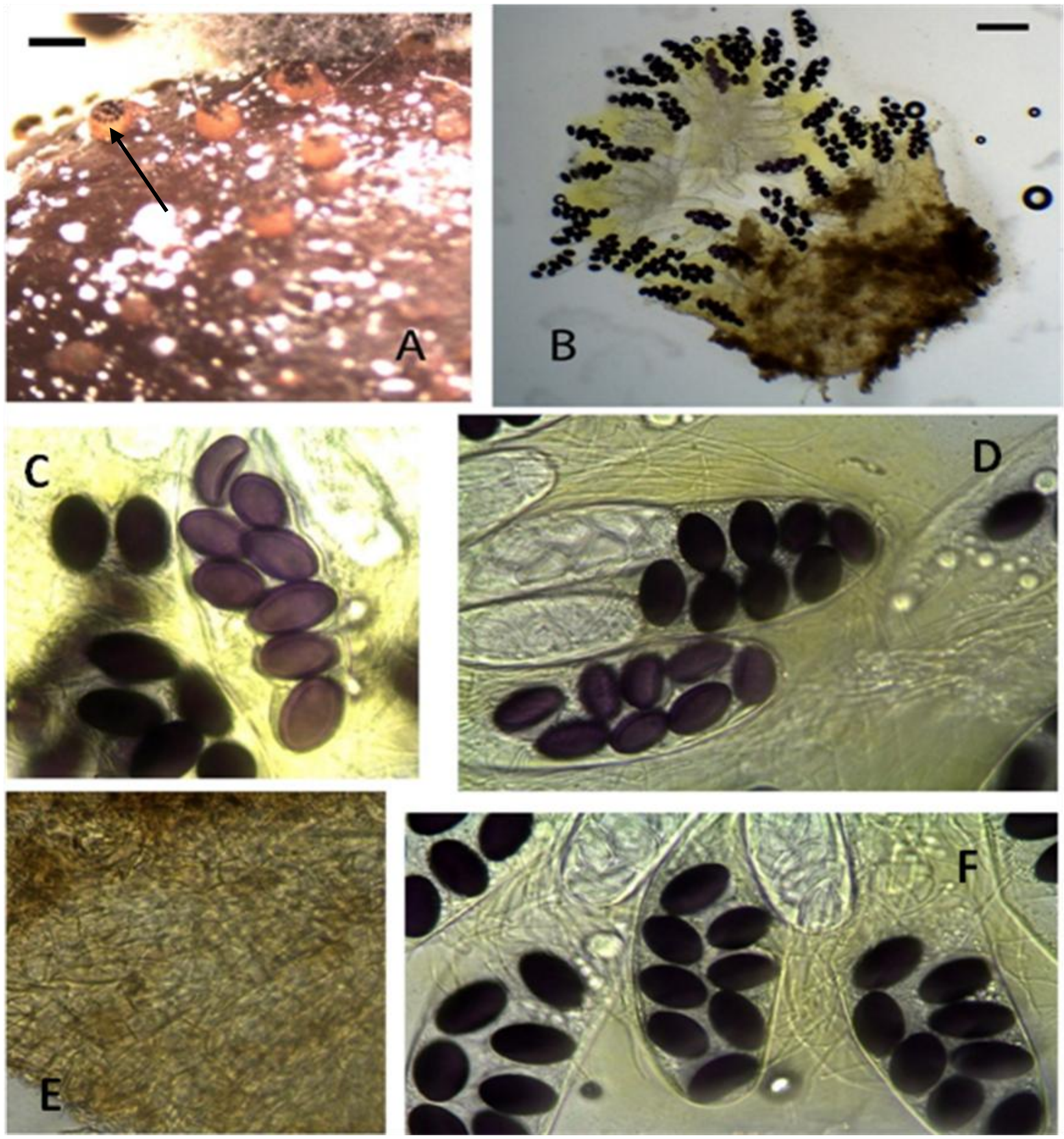

Figure 11: *Ascobolus amoenus* **A** Ascomata on substrate (arrow) **B** Specimen squashed in glycerol **C** 8 spored ascus **D** Asci **E** Ascomatal wall **F** asci and paraphysis **Scale Bar A =** 2000 µm, **B** = 500 µm, **C** = 20 µm, **D** = 50 µm, **E** = 50 µm, **F** = 50 µm (photo by Antoinette Miyunga)

2. ***Ascobolus bistisii*** (Gamundí and Ranalli, *Nova Hedwigia* 10: 347, 1966)

Morphological description: Ascomata were cleistothecioid during early stages, and hymenium exposed later on, gregarious, superficial or semi-immersed, measuring 600–700 μm in height and 400–500 μm diameter. Receptacle was subglobose, brown, dotted with few protruding, finger-like asci, barrel shaped, widest at equatorial part, with a hardly differentiated margin. Disc was convex, light yellow to brown. Hypothecium had very thin of isodiametric cells. Medullary excipulum of textura had angularis cells measuring 5–10 × 6–20 μm. Ectal excipulum of textura had angularis cells measuring 15–20 × 7–8 μm. Paraphyses were cylindric-filiform, with tips not inflated, embedded in clear mucus, numerous septae measuring 3–4 μm. Asci measured 400–500 × 100–105 μm, and were 8-spored, broadly clavate-cylindrical, operculate with dome-shaped apex 30–40 μm wide with wall weakly amyloid. Ascospores measured 53–58 × 30–33 μm and irregularly biseriate, ellipsoidal, rounded at the ends, purple, irregularly distributed at the end of the ascus (Figure 12).

Material examined: KENYA, Nairobi National Park, Nairobi County, Specimens, dung incubate for 7-9 days. GPS S 1°35’43.02” E 36°85’68.79”, altitude 1657m, White Rhino, 23 July 2013, P. Mungai KWSNNP001-2013; Nairobi National Park, Nairobi County, GPS S 1°35’75.45” E 36°84’63.72”, altitude 1649m, Zebra, 23 July 2013, P. Mungai KWSNNP006-2013; Nairobi National Park, Nairobi County, GPS S 1°35’43.02” E 36°85’68.79”, altitude 1657m, Waterbuck, 23 July 2013, P. Mungai KWSNNP003-2013; Nairobi National Park, Nairobi County, GPS S1°34’77.08” E36°85’17.54”, altitude 1648m, Thompson’s gazelle, 26 August 2013, P. Mungai KWSNNP012-2013; Nairobi National Park, Nairobi County GPS S1°85’07.59” E37°02’58.18”, altitude 1658m, Dikdik, 10 January 2014, A. Aluoch KWSNNP024-2014.

Notes- *Ascobolus bistisii* Sect. Dasyobolus (Gamundi and Ranalli, 1966) identified was similar to *Ascobolus immersus* in many ways morphologically. However, this species has regularly ellipsoid spores while those of A. immersus are subcylindrical with rounded ends. This species is quite common in Kenya wildlife dung as observed in this study.

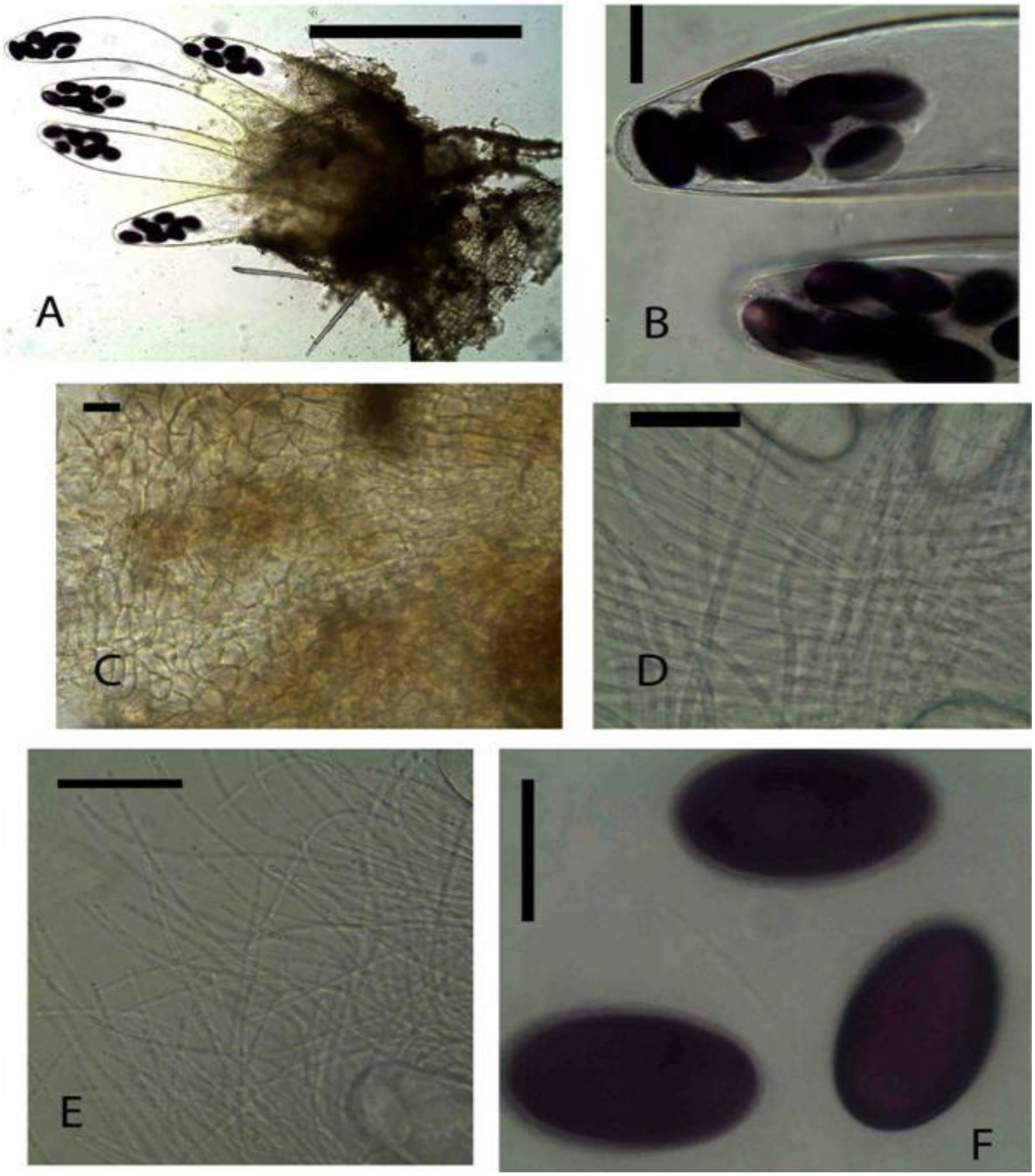

Figure 12: *Ascobolus bistisii* **A** Ascoma squashed in water **B** Ascospores at the tip of Asci **C** Ascomatal wall **D** Paraphysis **E** Paraphysis **F** Ascospores **Scale Bar A =** 200 µm, **B** = 50µm, **C** = 20µm, **D** = 20µm, **E** = 20µm, **F** = 20µm (photo by Antoinette Miyunga)

3. ***Ascobolus calesco*** **(**A.E. Bell and Mahoney, Fungal Planet, no. 11-21: 21: [2], 2007)

Morphological description: Ascomata were apothecioid, scattered or gregarious, semi-immersed and 800 μm high, 700 μm diameter. Receptacle was deep yellow to yellowish-brown, subglobose, barrel shaped with margin not differentiated. Disc was globular flat ripe asci protruding above the hymenium. Hypothecium was not well differentiated from Medullary excipulum. Ectal excipulum of textura had angularis brown cells, 14–21 × 7–11 μm. Paraphyses were filiform, hyaline, simple or sparingly branched at the base, septate, exceeding asci, 2–4 μm broad, tips not swollen and embedded in greenish-yellow mucus. Asci measured 600 × 100 μm, 8-spored, unitunicate. Ascospores measured 48–57 × 27–33 μm, biseriate, single-celled, ellipsoidal, purple, hyaline and had gelatinous sheath on each spore (Figure 13).

Material examined: KENYA, Nairobi National Park, Nairobi County, one specimen, dung incubated for seven to fourteen days, GPS S 1°35’42.28” E 36°85’68.92”, altitude 1650m, Hartebeest, 26th August 2013, P. Mungai KWSNNP020-2013.

Notes: The Kenya *Ascobolus calesco* Sect. *Dasyobolus* described was similar to *A calesco* as identified by Bell and Mahoney (2007).

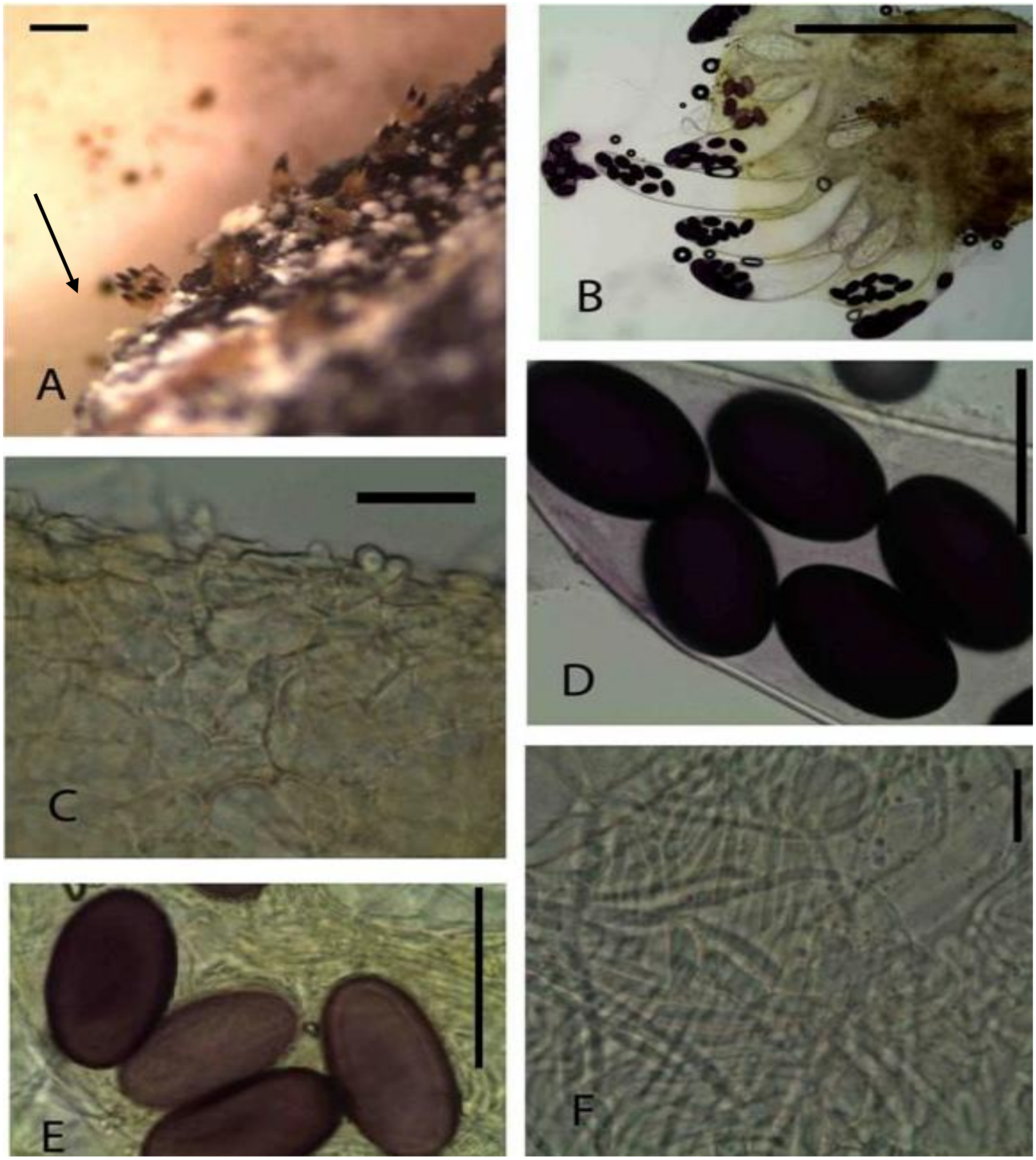

Figure 13: *Ascobolus calesco* **A** Ascomata on substrate **B** Ascoma squashed in lactic acid **C** Ascomatal wall **D** Ascospores **E** Ascospores **F** Paraphyses **Scale bar A =** 1000 µm, **B** = 500 µm, **C** = 20 µm, **D** = 20 µm, **E** = 20 µm, **F** = 20µm (photo by Antoinette Miyunga)

4. *Ascobolus immersus* (Pers., *Neues Mag. Bot.*1: 115,1794)

Morphological description: Ascomata were clestothecoid at first, gregarious or scattered, immersed or superficial, sessile, measuring 700–1000 μm high and 600–800 μm diameter. Receptacle was deep yellow to yellowish-brown or greenish-brown, subglobose, and margin not differentiated. Disc was flat or convex without margin and shiny with a few ripe asci protruding above the hymenium. Hypothecium very thin, of isodiametric cells. Medullary excipulum thin, of textura globulosa or angularis hyaline cells. Ectal excipulum of horizontally elongated textura angularis yellowish-brown thick walled cells, 22–43 × 11–17μm. Paraphyses were filiform, simple or sparingly branched at the base, septate, exceeding asci 2–3.5 μm broad, embedded in greenish-yellow mucus. Asci were broadly clavate, measuring 460–675 × 95–115 μm, 8-spored, unitunicate broadly clavate to sacciform rounded above. Ascospores measured 55–60 × 30–35 μm, biseriate, single-celled, subcylindrical, ends markedly rounded, violet becoming purple at maturity gelatinous sheath on each spore, hyaline, broader on sides and narrow on polar region (Figure 14)

Material examined: KENYA, Nairobi National Park, Nairobi County, seven specimens, dung incubated for 7-15 days, GPS S 1°34’27.03” E 36°82’22.23”, altitude 1657m, Buffalo, 23 July 2013 P. Mungai KWSNNP010-2013; Nairobi National Park, Nairobi County, GPS S 1°35’43.02” E 36°85’68.79”, altitude 1657m, White Rhino, 23 July 2013, P. Mungai, KWSNNP001-2013; Nairobi National Park, Nairobi County, GPS S 1°35’45.11” E 36°85’70.55”, altitude 1647m, Zebra, 23 July 2013, P. Mungai KWSNNP002-2013; Nairobi National Park, Nairobi County, GPS S 1°35’43.02” E 36°85’68.79”, altitude 1657m, Waterbuck, 23 July 2013, P. Mungai KWSNNP003-2013; Nairobi National Park, Nairobi County, GPS S 1°35’42.28” E 36°85’68.92”, altitude 1650m, Hartebeest, 26 August 2013, P. Mungai KWSNNP020-2013; Nairobi National Park, Nairobi County, GPS S1°34’77.08” E36.85’17.54”, altitude 1648m, Thompson’s gazelle, 26 August 2013, P. Mungai KWSNNP012-2013; Nairobi National Park, Nairobi County GPS S1°85’07.59” E37°02’58.18”, altitude 1658m, White Rhino, 10 January 2014, A. Aluoch KWSNNP031-2014; Nairobi National Park, Nairobi County GPS S1°85’07.59” E37°02’58.18”, altitude 1658m, Hare, 10 January 2014, A. Aluoch KWSNNP026-2014; Nairobi National Park, Nairobi County GPS S1°85’07.59” E37°02’58.18”, altitude 1658m, Eland, 10 January 2014, A. Aluoch KWSNNP037-2014; Nairobi National Park, Nairobi County GPS S1°84'64.98" E37°02'65.83", altitude 1619m, Zebra, 14 January 2014, A. Aluoch KWSNNP039-2014.

Notes: *Ascobolus immersus* Sect. Dasyobolus (Persoonia, 1794) was common in dung from wildlife herbivore dung. It was easily distinguished from other members of the *Ascobolus* genus by its characteristically large spores. Each spore was surrounded by a gelatinous sheath. They grew immersed on soft surfaces while growing on the surface of more dense surfaces.

5. *Ascobolus spp*

Morphological description: Ascomata were apothecioid, scattered, immersed, 380 μm high and 540 μm in diameter. Receptacle was deep brown, subglobose, margin not differentiated. Disc flat ripe asci protruding above the hymenium. Hypothecium thin with globose cells. Medullary excipulum of yellowish brown cells of various thickness. Ectal excipulum of textura angularis yellowish-brown cells, 10–25 × 10–12 μm. Paraphyses were filiform, tips not swollen, embedded in greenish-yellow mucus. Asci 365–400 × 60–65 μm, 4-spored sacciform, rounded above. Ascospores measured 35–45 × 20–25 μm, uniseriate, single-celled, subcylindrical, ends markedly rounded, and purple (Figure 15)

Material examined: KENYA, Nairobi National Park, Nairobi County, Hartebeest 26th August 2013 dung incubated for seven days, GPS S 1°35 E 36°86, altitude 1650m, P. Mungai KWSNNP020-2013.

Notes: This species differs from those described earlier due to the fact that they contained four ascospores in each ascus. The ascospores are seen clustered on one end of the ascus.

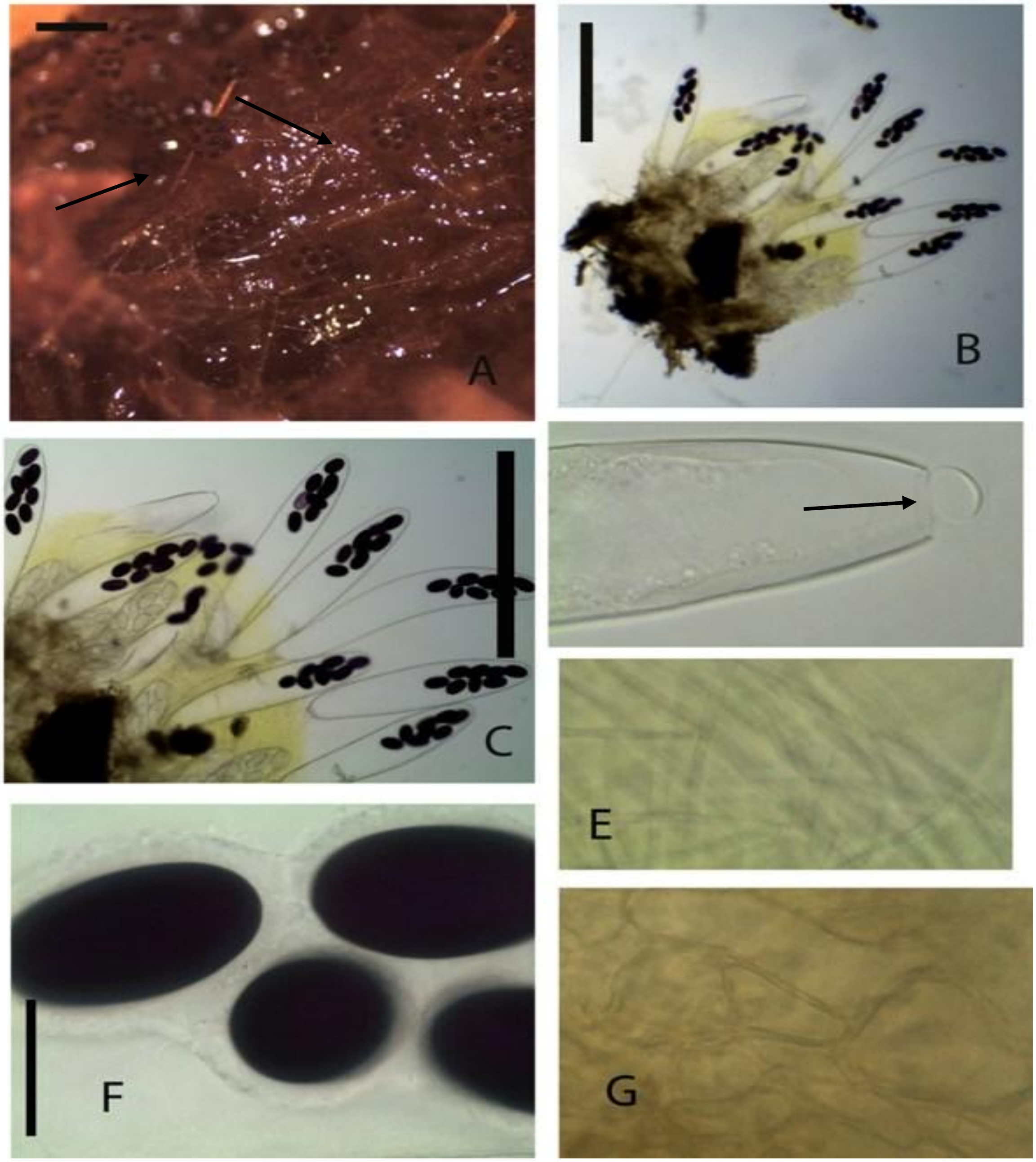

Figure 14: *Ascobolus immersus* A Ascomata on substrate (arrows) B Ascoma squashed in water C 8 spored mature asci D Open operculum (arrow) E Paraphyses F Mature ascopores surrounded by gelatinous sheath G Ascomatal wall. Scale bar A=1000µm, B= 500µm, C=200µm, D=50µm, E=20µm, F=20µm, G=20µm (photo by Antoinette Miyunga)

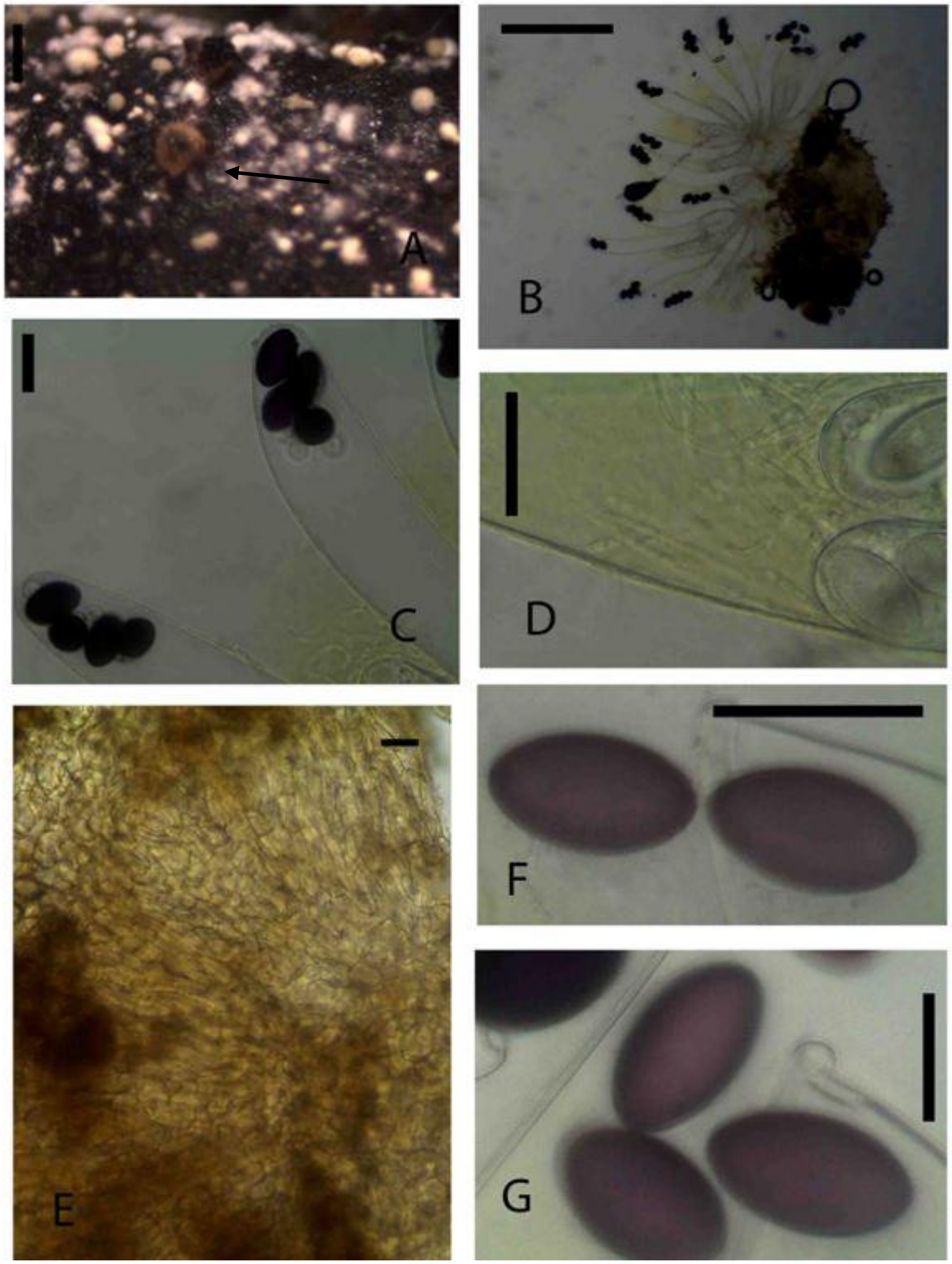

Figure 15: Four spored *Ascobolus* species **A** Ascomata on Substrate **B** Ascoma squashed in water **C** 4 spored asci **D** paraphysis **E** Ascomatal wall **F** Ascospores **G** Ascospores **Scale bar A =** 1000 µm, **B** = 500 µm, **C** = 50 µm, **D** = 20 µm, **E** = 20 µm, **F** = 20µm, **G** = 20µm (Photo by Antoinette Miyunga)

6. *Pilobolus crystallinus* (F. H. Wigg. Tode var. ***crystallinus,*** Schrift. Berl. Gesell. Naturf. Freunde 5: 47, 1784)

Morphological description: Trophocyst was subglobose, 370×360 µm with rhizoidal extension up to 980 µm, with yellowish pigmentation. Sporangiophore was long-cylindrical, unbranched, phototrophic, measuring 4mm×100 µm with black sporangia wall, hemispherical to ovoid 480×250 µm; collumellae smooth walled. Subsporangial vesicle was smooth walled a little orange pigmentation elliptical 700×530 µm; yellow spores, grainy content, smooth wall, ellipsoid 8×5 µm (Figure 16)

Material examined: KENYA, Nairobi National Park, Nairobi County, incubated for four to seven days, GPS S 1°35'45.11" E 36°85'50.89", altitude 1646m, Zebra, 23 July 2013, P. Mungai KWSNNP004-2013; Nairobi National Park, Nairobi County, incubated for three to seven days, GPS S 1°34'50.02" E 36°84'82.34", altitude 1649m, Buffalo, 23 July 2013, P. Mungai KWSNNP009-2013; Nairobi National Park, Nairobi County GPS S1°85'07.59" E37°02'58.18", altitude 1658m, Grant's gazelle, 10 January 2014, A. Aluoch KWSNNP027-2014; Nairobi National Park, Nairobi County GPS S1°84'64.98" E37°02'65.83", altitude 1619m, Zebra, 14 January 2014, A. Aluoch KWSNNP032-2014.

Notes: closely similar to *Pilobolus crystallinus* var. *crystallinus (*F. H. Wigg.) Tode Schrift. Berl. Gesell. Naturf. Freunde 5: 47 (1784). Some specimens had spore sizes differing slightly from those described earlier. *Pilobolus crystallinus* var. *crystallinus* differs from *Pilobolus crystallinus* var. *kleinii* by having pale yellow spores while those of the latter are bright yellow.

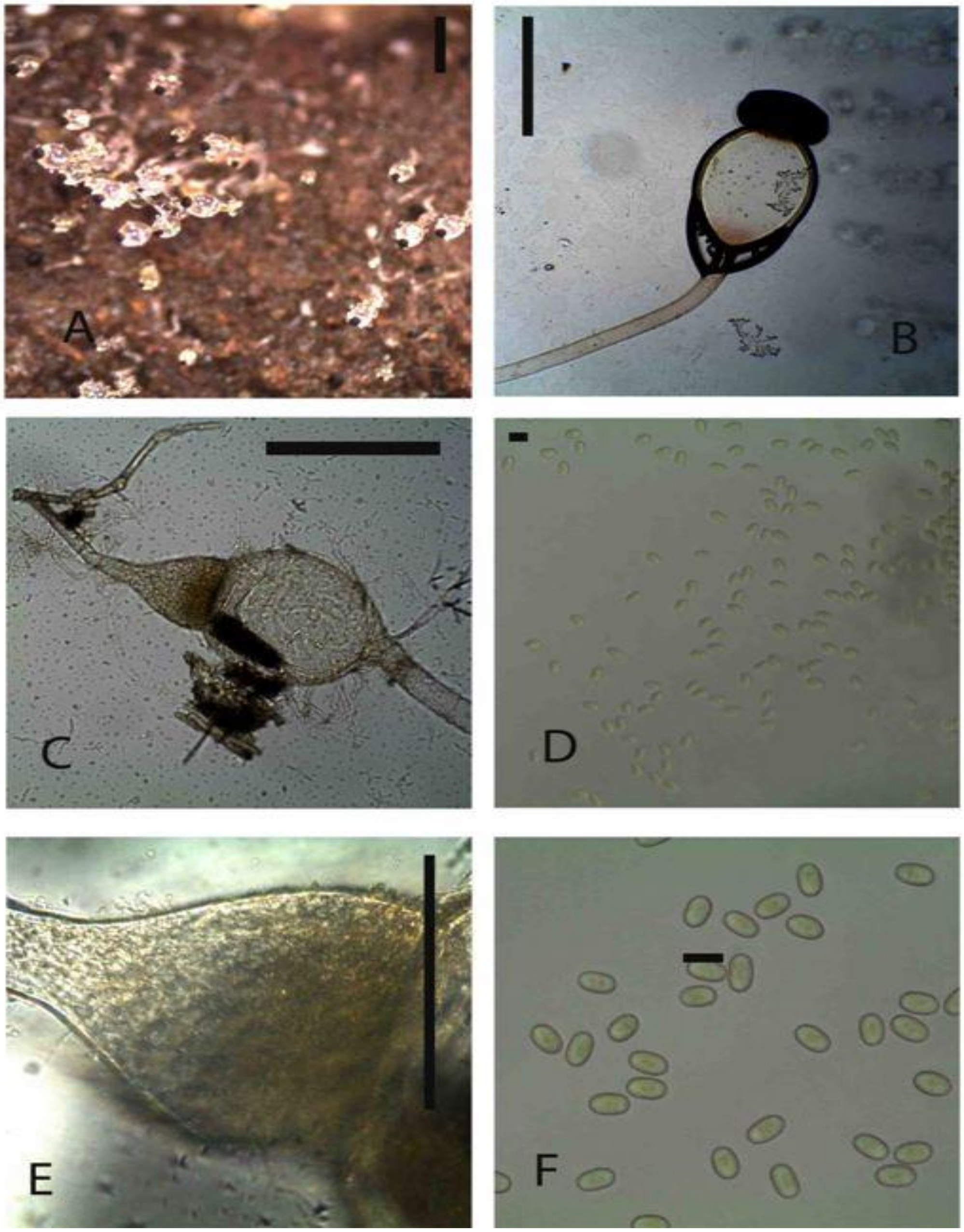

Figure 16: *Pilobolus crystallinus* var. *crystallinus* **A** *Pilobolus* on substrate **B** sporangium and collumellae **C** trophocyst and rhizoidal extension **D** spores **E** Trophocyst **F** spores **Scale Bar A =** 2000 µm, **B =** 500 µm, **C =** 200 µm, **D =** 50 µm, **E =** 50 µm, **F =** 50 µm (Photo by Antoinette Miyunga)

7. *Pilobolus pullus* (Massee, *Bull. Misc. Inf.*, Kew: 160,1901)

Morphological description: Trophocysts were ovoid to globose, hyaline 180 µm diameter, sporangiophore long-cylindrical, 720×90 µm; Black sporangia hemispherical in shape measuring 270×140 µm; Collumellae with smooth walls, subsporangial vesicle with smooth wall hyaline, little pigmentation, ovoid, 370×200 µm: yellow spores with homogenous content, subcylidrical measuing 9×5 µm (Figure 17)

Material examined: KENYA, Nairobi National Park, Nairobi County, incubated for three to seven days, GPS S 1°34'50.02" E 36°84'82.34", altitude 1649m, Buffalo, 23 July 2013, P. Mungai KWSNNP009-2013; Nairobi National Park, Nairobi County, incubated for four to seven days, GPS S 1°35'45.11" E 36°85'50.89", altitude 1646m, Zebra, 23 July 2013, P. Mungai KWSNNP004-2013; Nairobi National Park, Nairobi County GPS S1°85'07.59" E37°02'58.18", altitude 1658m, Giraffe, 10 January 2014, A. Aluoch KWSNNP034-2014

Notes: The isolated material agreed with the description of Massee, Kew Bulletin p.160 (1901) and Naumov (1939). However, the rhizoidal extension for some of the identified species were longer than the 300 µm of the described species.

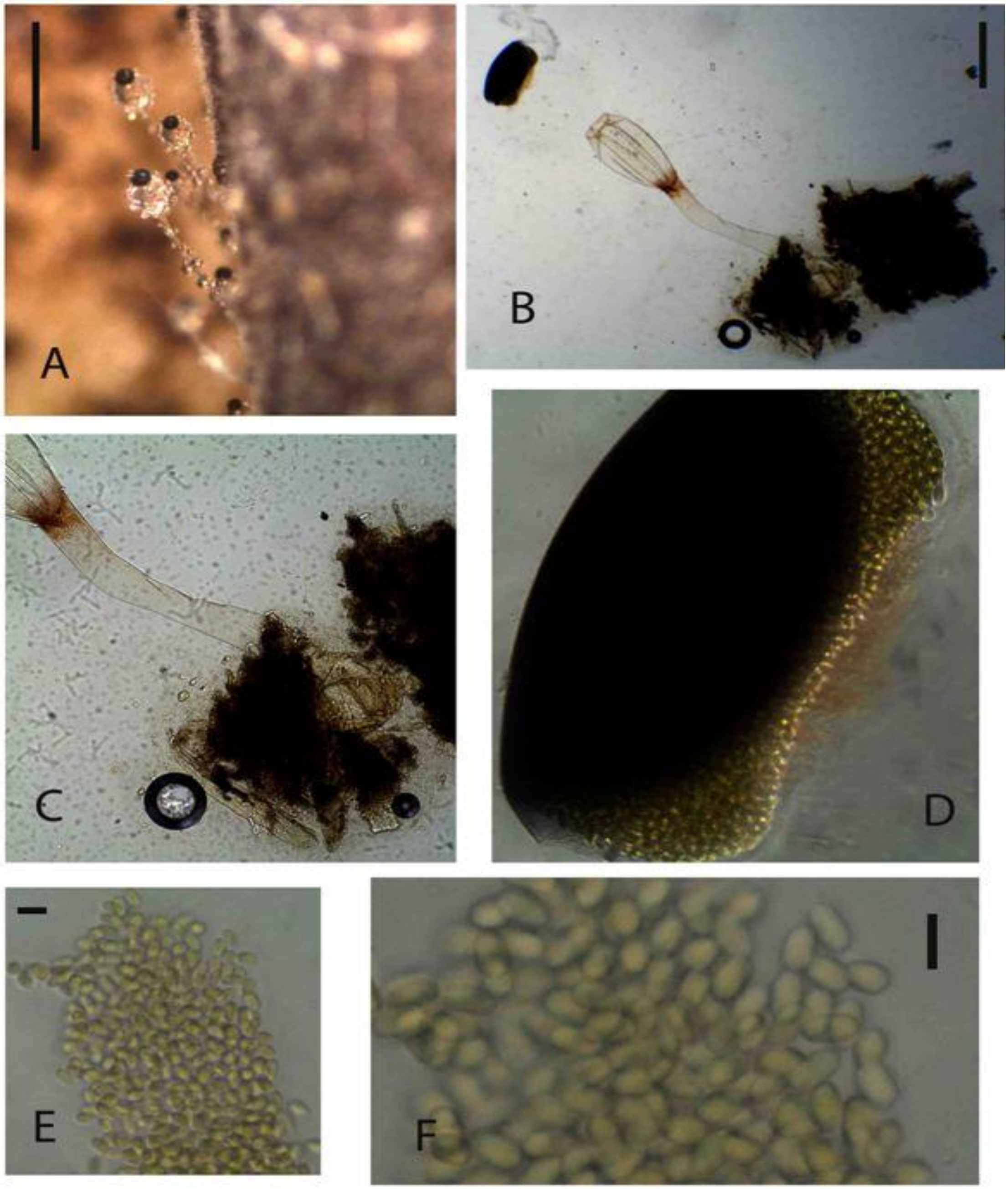

Figure 17: *Pilobolus pullus* **A** *Pilobolus* on substrate **B** *Pilobolus* squashed in glycerol **C** Collumelae and Sporangiophore **D** sporangium **E** spores **F** spores **Scale Bar A =** 2000 µm, **B =** 500 µm, **C =** 200 µm, **D =**50 µm, **E =** 50 µm, **F =** 20 µm (Photo by Antoinette Miyunga)

8. ***Pilobolus heterosporus*** (Palla, *Öst. bot. Z.*50: 349, 1900)

Morphological description: Trophocysts were ovoid to globose, short ellipsoid measuring 200.27×280.46 µm with rhizoidal extensions little pigmentation; sporangiophores, long cylindrical, 1.5mm × 69.23 µm; black sporangia, hemispherical to ovoid 169.7×212.29 µm, resistant wall, conical collumellae, little pigmented subsporangial vesicles ovoid and ellipsoid, 412.31×344.65µm; yellowish spores grainy content, globose, ovoid, cylindrical 5.6-10.08×5.15-6.1 µm (Figure 18)

Material examined: KENYA, Nairobi National Park, Nairobi County, incubated for three to seven days, GPS S1°85’07.59” E37°02’58.18”, altitude 1658m, Dikdik 10 January 2014 A. AluochKWSNNP024-2014.

Notes: This species showed similarities to those described by Naumov (1939) and Viriato (2008). The described species was differentiated from the others due to the different shaped irregular spores with grainy content.

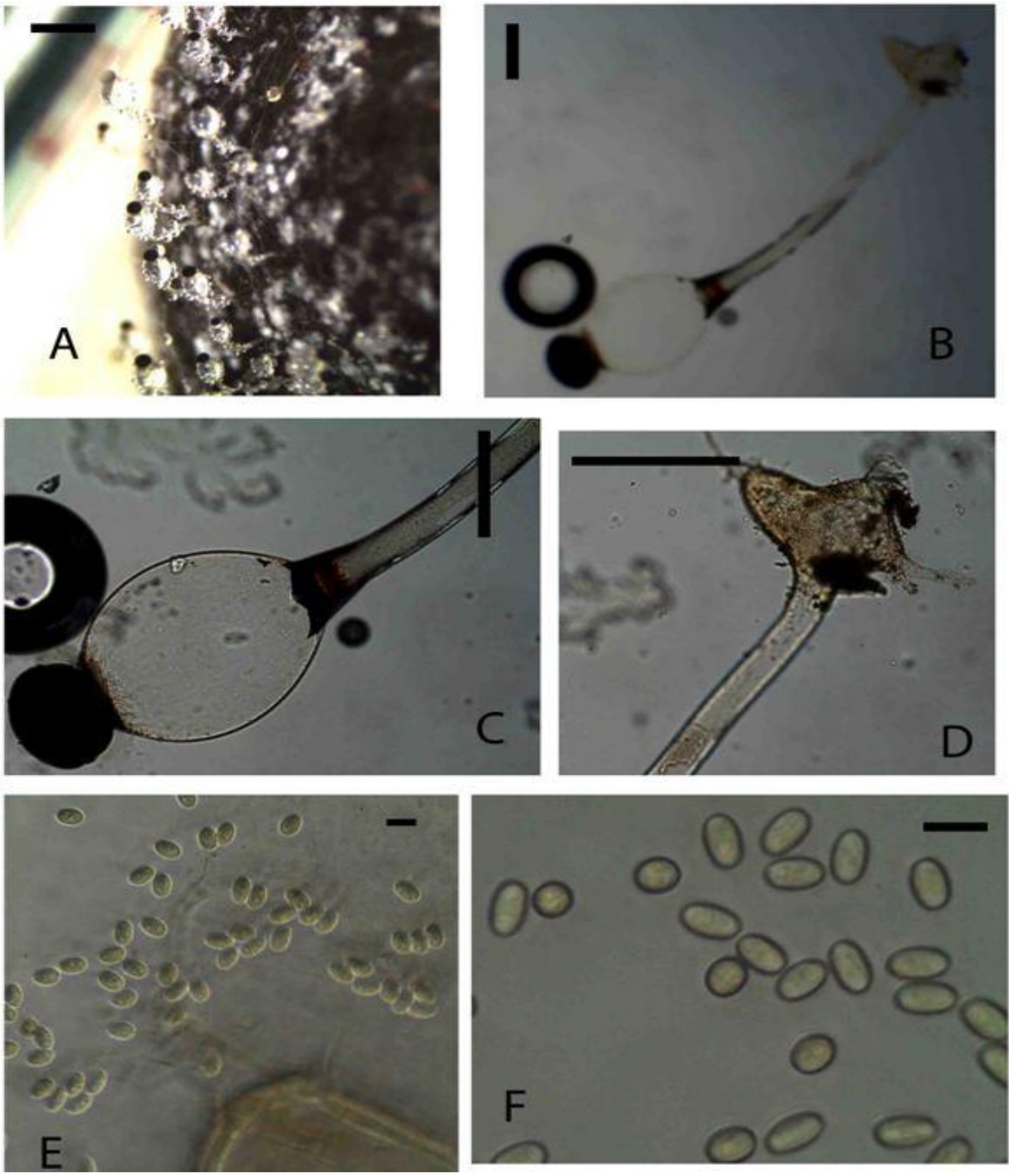

Figure 18: *Pilobolus heterosporus* **A** *Pilobolus* on substrate **B** *Pilobolus* squashed in glycerol **C** Collumelae and Sporangiophore **D** Rhizoidal extension **E** spores **F** spores **Scale Bar** **A** = 2000 µm, **B** = 500 µm, **C** = 200 µm, **D** =50 µm, **E** = 50 µm, **F** = 20 µm (Photo by Antoinette Miyunga)

3.4.2 *Pilobolus* and *Ascobolus* species composition and diversity

Fungi species found in dung from the different animal species were recorded (Appendix IV). There were a total of eight species reported. Three from the genus *Pilobolus* and five from the genus *Ascobolus.* The highest recorded species was *Ascobolus immersus* (28.9%) followed by *Pilobolus crystallinus* (25.2%). These were abundant in a large variety of the animal dung piles sampled. However, since the number of samples collected for each animal species was not equal, the number of coprophilous fungi species found in each dung pile was used to calculate diversity index. There was no growth of any of the two genera of interest in this study in two rhino, two impala, three buffalo, and six giraffe dung piles. This can be attributed to host animals foraging in areas not infected by fungal spores. The highest fungi species diversity of interest in this study was observed in waterbuck dung pile which had 3 *Ascobolus* species and 2 *Pilobolus* species.

Across the 64 dung samples collected, there were 8 different coprophilous fungal species recorded. Since most of them were rare, Chao's formula was used to obtain the estimated richness (Chao, 1984; Gotelli and Colwell, 2011). This gave an estimated richness as 15 different species per dung pile compared to the observed richness of 5 species. R studio was used to draw plots for the different indexes to show diversity and compare Shannon-weinner, Simpson index and diversity numbers for the samples (Figure 3). The Y axis shows the index and X axis shows number of unique species.

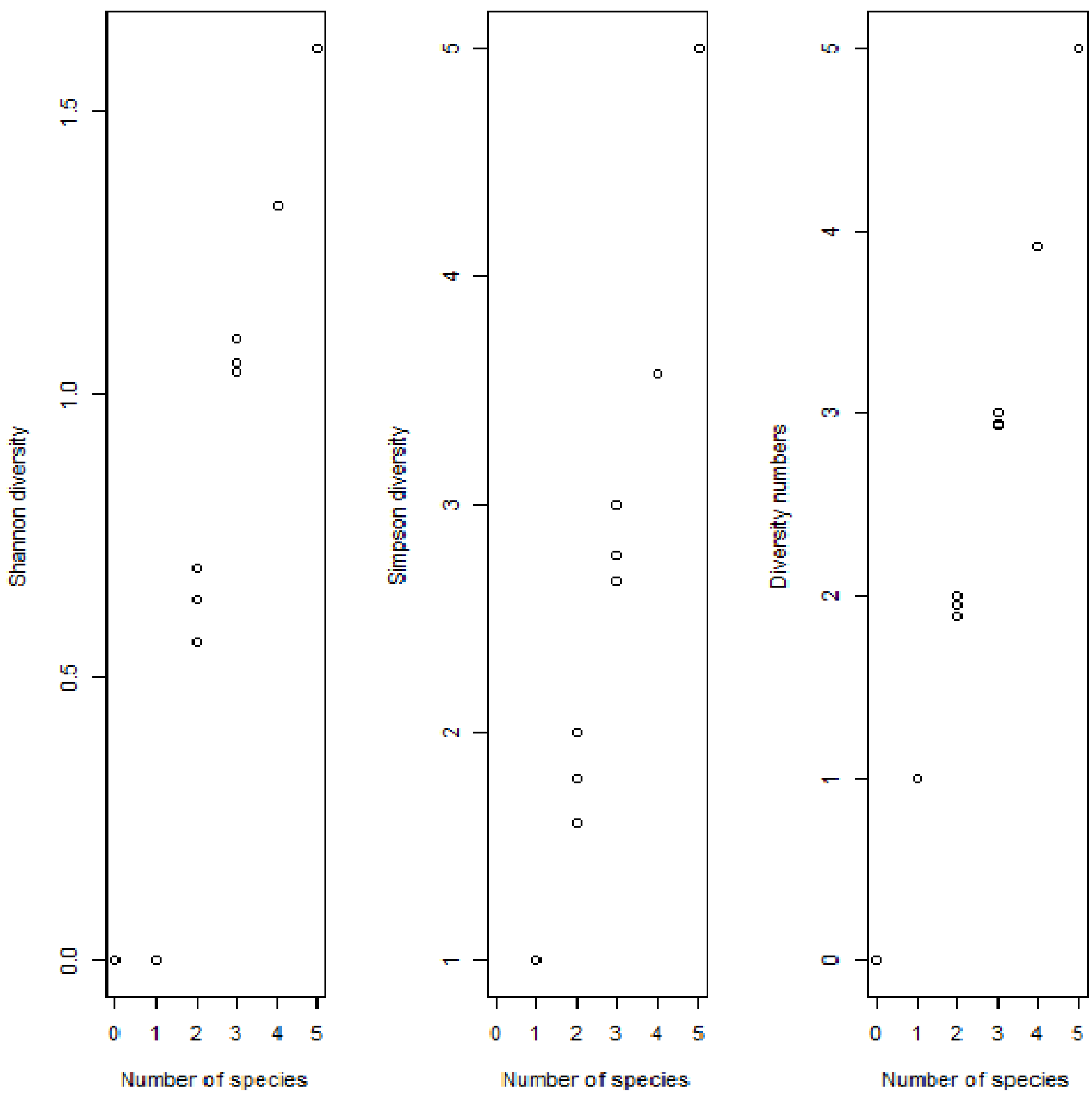

Figure 19: Diversity index plots for the observed species

3.5 Discussion

According to the findings of the current study, five species from the genus *Ascobolus* were identified as: *A. amoenus*, *A. bistisii*¸ *A. calesco*, *A. immersus* and fifth putatively new species (a four-spored asci species) that has never been described to the best of our knowledge. The most abundant species from the animal dung samples was *A. immersus* (28.9%). In addition, there were three *Pilobolus* species identified, that is: *P. crystallinus var. crystallinus*, *P. heterosphorus* and *P. pullus*, *P. crystallinus* (25.2%) was the most abundant species in this genus. Morphological features provided a useful tool in assigning putative species; however, additional information from molecular data is necessary to confirm this.

Fungi from the genus *Pilobolus* were observed to grow and die out within the first week of incubation in a moist chamber. However, for some samples, there was still new growth of *Pilobolus* even after ten days of incubation. This could be attributed to the fact that some of the spores dispersed within the plates were falling on the dung and growing again. This means that *Pilobolus* genus do not require passage in the animal gut or treatment to sporulate.

The observed diversity and expected diversity indicate that there is high species richness of coprophilous fungi in Nairobi National Park. This is likely to indicate that even though the park is located near the capital city, surrounded by upcoming industrial areas as well as increasing human settlements, the park ecosystem itself is relatively well preserved. This is comparable to the work of Tibuhwa *et al*., 2011 who studied macro fungi diversity in Serengeti-Mara ecosystem and reported high diversity in woodland and grassland areas as compared to areas with human settlements. The theory that there is little interference in the ecosystem is also supported by the fact that there was no outright dominant species that are adapted to altered environment conditions since the difference between the abundance of the observed species was not very significant (Odum, 1985; Ebersohn and Eicker, 1992). Future studies on morphological diversity of these coprophilous fungi genera and comparison with the results of this study will aid in monitoring the changes in the park ecosystem.

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CHAPTER FOUR

MOLECULAR CHARACTERIZATION OF *ASCOBOLUS* AND *PILOBOLUS* FUNGAL SPECIES FROM NAIROBI NATIONAL PARK

4.1 Abstract

Pilobolus and *Ascobolus* fungi are abundant in herbivore dung and their species identification has for a long time, relied on morphological data which lacks accuracy due to the overlap of several characters. Misidentification may also be occasioned by different culture conditions. *Ascobolus* fungi have been previously identified from wild herbivore dung from Nairobi National Park using morphological characteristics. Therefore, this study aimed to use molecular tools to classify the species of *Ascobolus* and *Pilobolus* present in Nairobi National Park. In this study we applied molecular identification tools through amplification of sequences that can differentiate these genera down to species level. This method is more reliable than the previously used morphometric analyses and the generated sequences can be used for future study on the genetic composition of these fungi. Dung samples were collected from different wild herbivores in Nairobi National Park. The dung samples were subjected to moist chamber cultures and single sporangium isolates obtained for pure cultures. *Ascobolus* fungi was not isolated in pure cultures due to lack of germination on artificial cultures. On the other hand, *Pilobolus* fungi pure cultures were successful and DNA was extracted from Internal Transcribed Spacer regions amplified and sequenced. Sequence analysis was used to distinguish between species. Three species of *Pilobolus* were identified: *Pilobolus crystallinus*, *Pilobolus heterosporus* and *Pilobolus pullus.*

4.2 Introduction

Pilobolus and *Ascobolus* fungi are globally widespread coprophilous fungi from the class zygomycetes and ascomycetes respectively. Coprophilous fungi form an important part of the wildlife ecosystem since they help in recycling nutrients in animal dung (Richardson, 2001a), and achieve this through the decomposition of herbivore dung. In addition, they are also important since they affect digestive efficiency in animals besides serving as nutrition for certain arthropods that live in the dung (Wicklow and Angel, 1974).

Studies on baseline diversity can be used for future measuring of ecosystem changes (Ebersohn and Eicker, 1992). For these reasons, studying these microorganisms is important for conservation and wildlife protection. Since they help in monitoring subtle changes in the ecosystem by acting as indicators of ecological stress. High diversity is an indication of a thriving ecosystem. However, dominance of a single or few species indicates stress in the ecosystem and thus survival for only species that can adapt to the changing environment.

Much of the research done on *Pilobolus* fungi in the past concentrated on domestic animal dung while there are still no clear records on fungal species isolated from wildlife dung in Kenya. Extensive research on the diversity of *Pilobolus* has been carried out in temperate regions (Foos and Sheehan 2011; Foos *et al*., 2011). There has been no molecular characterization of this genus in Africa before this study and mostly focused on morphological characterization (Caretta *et al*., 1998). Relying on morphological characterization only is not sufficient in species identification due to overlap in certain features among species (Foos and Sheehan, 2011). This is especially true because some of the morphological features are known to change with environmental conditions (Hu *et al*., 1989). Therefore, the same species might have different morphological characters depending on the geographical and environmental conditions. This challenge has necessitated the use of molecular identification as the most reliable in fungal species characterization. This is because ITS region of fungal DNA is relatively stable and can be used in identification with very little margin of error.

Molecular techniques, unlike morphological techniques are particularly useful in the identification of cryptic species (Bidchoka *et al.*, 2001). In addition to complementing morphological identification, molecular techniques may be the only solution for identification and separation of coprophilous species including those whose identity is limited by morphological and culturing techniques (Wu *et al.*, 2003; Iotti *et al.*, 2005; Tarbell, 2008; Herrera *et al.*, 2011). Previous studies on generation of DNA barcodes of fungi suggested that Internal Transcribed Spacer regions of fungal DNA are the best in distinguishing fungi at species level (Schoch *et al*., 2012). They do not have introns which can be variable, have high success rates during PCR amplification and sequencing and can also correctly distinguish between intra and interspecific variation for a broad spectrum of fungi (Schoch *et al*., 2012). In order to identify fungal DNA, fungal specific primers for Internal Transcribed Spacer (ITS) are used (Iotti *et al.*, 2005; Herrera *et al.*, 2011). The ITS region is taxonomically useful and the sequences can be used to identify species (Foos and Sheehan, 2011). The molecular identity of fungi is determined through comparison of the ITS sequences with those in GenBank database of known species (Iotti *et al.,* 2005). The ITS region lies between the 18S SSU (Small Subunit) and the 28S LSU (Large Subunit) genes and contains two noncoding spacer regions the ITS-A and ITS-B (Kendrick, 2000). The ITS region of fungi is about 650bp in size plus the 5.8S gene that separates the noncoding spacer regions (Tarbell, 2008).

The primers specific for ITS regions are ITS1F, ITS1, ITS5 forward and ITS4, ITS4A. ITS4B reverse (Glass and Donaldson, 1995). Sequence analysis of 18S rDNA has revealed distinct divergence between Ascomycetes and Zygomycetes (Wu *et al.,* 2003). The primers specific for this 18S DNA region are NS1 and NS4. B-tubulin sequences are amplified using BT 2a and BT2b primers (Glass and Donaldson, 1995). The nuclear encoded 28S gene can be amplified using a primer combination of LR5 and LR0R (Bresinsky *et al.*, 2008). For this study, ITS4 and ITS5 were used, the oligonucleotide sequences for the primers was 5'TCCTCCGCTTATTGATATGC-3' and 5'-GGAAGTAAAAGTCGTAACAAGG-3' , respectively (Iotti *et al*., 2005).

In order to carry out molecular analysis, it is essential to get pure cultures of these fungi on artificial media. *Pilobolus* requires the active growth factors hemin, ferrichrome and coprogen to grow which are normally found in herbivore dung (Hesseltine *et al*., 1953; Levetin and Caroselli, 1976). Therefore, synthetic media containing these growth factors can facilitate the growth of *Pilobolus* in artificial culture (Levetin and Caroselli, 1976). In addition, dung agar can also be used to culture these fungi since dung contains all the necessary growth factors (Swartz, 1934). *Ascobolus* has been demonstrated to grow well on yeast extract agar with cellulose added (Yu, 1954).

4.3 Materials and Methods

4.3.1 Pure culture isolation of *Ascobolus* on Yeast Extract Agar

Yeast extract agar was prepared according to Yu (1954). 0.4 g of yeast extract, 2.5 grams agar and 0.7 grams of shredded filter paper were added to 100 ml of water. This was autoclaved at 15psi at 120°C for 15 minutes. Chloramphenicol was added to inhibit bacterial growth at 0.05g/L. In a laminar flow hood, to maintain aseptic conditions, 25 ml of the medium was transferred into sterile glass petri dishes with a filter paper on the bottom of each dish. Single fruiting bodies were picked from dung using a sterile needle and crushed in 1% NaOH for 20 minutes. Once the agar solidified, each petri dish was inoculated using a single *Ascobolus* spore. The dishes were placed bottom-up down to prevent the condensation water from falling back on the fungus. The dishes were placed at 37°C for 24hours. Thereafter, they were incubated at 25°C in natural light up to 7 days to allow colony growth as described by Tarbell, 2008 (Figure 20).

4.3.2 Pure culture isolation of *Pilobolus* on Dung Agar

In order to identify fungi with certainty, it is necessary to obtain pure culture with single species. Therefore, dung agar media was prepared according to Swartz, 1934. Three hundred grams of fresh dung was boiled in 1 litre of water until it broke down and there were no clumps. This was sieved using cheese cloth and 15 grams of agar added. The mixture was transferred to 250ml conical flasks which were autoclaved at 15psi and 120°C for 15 minutes. The flasks were slanted and the media allowed to cool to room temperature. Single sporangia were taken from the dung substrate using a sterile needle and transferred to the surface of the medium in the flask. The flasks were sealed using parafilm and placed upright next to a window so that the fungi could get light. Once grown, the spores were shot to the opposite inner surface of the flask (Figure 21). A sterile wire loop was used to collect these spores, which were suspended in 200 µl of distilled water.

4.3.3 Molecular analysis

Extraction of fungal DNA was done using the Zymo Research fungal/bacterial DNA Miniprep™ kit. Spore suspensions were transferred to individual ZR Bashing Bead™ Lysis

Tubes. 750 µl of Lysis solution was added to each tube. These tubes were then secured in a bead beater and processed at 14000 x g rpm for five minutes. The tubes were centrifuged for one minute at 10,000 x g. 400 µl of the supernatant was transferred to a Zymo-Spin^{TM} IV spin filter inside a collection tube and centrifuged for one minute at 7000 x g. To the filtrate in the collection tube, 1200 µl of DNA binding buffer was added. 800 µl of this mixture was transferred to an spin column in a collection tube and centrifuged for one minute at 10,000 x g. The flow through in the collection tube was discarded and the spin column placed back in the collection tube and centrifuged for one more minute at 10,000 x g. 200 µl of DNA pre-wash buffer was added to the spin column in a new collection tube and centrifuged for one minute at 10,000 x g. 500 µl of fungal DNA wash buffer was added to the column and then centrifuged for one minute at 10,000 x g. The spin column was transferred to a clean 1.5ml microcentrifuge tube and a 100 µl of DNA elution buffer added directly into the column matrix. This was centrifuged for thirty seconds at 10,000g to elute the DNA. DNA was quantified on a 1% agarose gel to ascertain success of the extraction process.

The PCR was carried out in a total volume of 25 µl containing 2.5 µl of PCR buffer, 2 µl of dNTPs, 1 µl of forward primer and 1 µl of reverse primer, 0.1 µl of Taq polymerase, 2.2 µl of 25Mm Magnesium Chloride, 15.2 µl of water and 1ul of the sample fungal DNA. This mix was ran in an Applied Biosystems thermocycler under the following cycling regime: 3 min initial denaturation at 95°C, 35 cycles of 30 sec denaturation at 95°C, 30 sec primer annealing at temperatures specific for each of the primers, 1 min extension at 72°C, and a final 10 min extension at 72°C then maintained at 4°C. Universal fungal primers ITS 4 and ITS5 primers were used. The oligonucleotide sequence for the forward primer was 5'TCCTCCGCTTATTGATATGC-3' and reverse 5'-GGAAGTAAAAGTCGTAACAAGG-3'.

Detection of DNA was undertaken using 1% agarose gel electrophoresis to allow for visualization. The PCR products were electrophoresed in an agarose gel stained with Ethidium bromide. The gel was prepared by dissolving one gram of agarose in 100ml of TAE. This was heated to dissolve the agarose and allowed to cool on an electrophoresis tray with combs inserted to form wells. 4ul of the PCR product was mixed with 1µl of loading dye and added into the wells. A 100 bp size marker ladder was added to one of the wells to give indication of the size of amplified product. The gel was electrophoresed at 80 volts for 40 minutes then imaged under UV light to show the bands (UVP transilluminator) (Figure 22).

The PCR products were purified using the QIAquick PCR Purification kit. 100 µl of Buffer PB was added to 20 µl of amplified DNA. The mixture was transferred to a Qiaquick column placed in a 2 ml collection tube. The columns were spun in a centrifuge for 1 minute and the flow through discarded. The columns were returned to the same respective tubes and 750µl of Buffer PE added to the column and centrifuged for 1 minute. The flow through was discarded and the column again returned to the same respective tubes. The column was centrifuged for 1 minute to get rid of any residual wash buffer. Each column was then transferred to clean 1.5 ml microcentrifuge tubes and 30µl of Buffer Elution Buffer (EB) added to the center of the membrane and the columns centrifuged for 1 minute. 20µl of Buffer EB was added to the membrane again and left to stand for 1 minute. This was centrifuged and the columns were discarded. The purified DNA was analyzed on an agarose gel.

The purified PCR DNA products were used for DNA sequencing by direct cycle sequencing. Sequencing reactions was done using ABI PRISM DigDye Terminator v3.1 cycle sequencing kit. Analysis was done on an AB1310 DNA sequencer from Applied Biosystems, CA. The sequencing was done at the International Livestock Research Institute(ILRI) using the 3730 Genetic Analyzer.

4.3.5 Sequence analysis

The sequences obtained were assembled and ends trimmed from the consensus using Geneious software (Kearse *et al*., 2012). DNA Sequence identity was done using the BLAST program from Genbank (Altschul *et al*., 1990). This gave the most probable specific names as described by their genetic sequences. The study sequences were annotated and deposited in Genbank and accession numbers obtained (table 1). To assess the phylogenetic relationship of the sequenced *Pilobolus spp,* blast searches were conducted in Genbank with the recovered haplotypes. All matches with a query cover around over 95% and a fit above 90% were used for phylogenetic analyses. Alignment was done using Muscle software. Geneious software version 8 was also used to draw the phylogenetic tree (Kearse *et al*., 2012). The phylogram was constructed using the nucleotide alignment through neighbour joining method with *Pilobolus longipes* as the outgroup and genetic distance calculated using Tamura-Nei method (Saitou and Nei, 1987; Tamura *et al*., 2013). Resampling tree was created using bootstrap with a support threshold of 50%.

4.4 Results

Sixty five dung piles from 14 different herbivore species found in Nairobi National Park were collected and placed in moist chamber culture. Pure cultures of *Ascobolus* genus did not grow successfully and therefore DNA was not extracted for molecular analysis. Eleven isolates of *Pilobolus* were obtained from 9 host herbivore species. Three different *Pilobolus* species were identified using molecular means namely: *P. crystallinus, P. heterosporus* and *P. pullus.* These were collected from dung of different animals: eland (*Kobus ellipsiprymnus*), impala (*Aepyceros melampus*), dikdik (*Madoqua kirkii*), giraffe (*Giraffa camelopardalis*), hartebeest (*Alcelaphus buselaphus*), thompson's gazelle (*Eudorcas thomsonii*), grant's gazelle (*Nanger granti*) and zebra (*Equus quagga*).

Pilobolus crystallinus (F. H. Wigg.) Tode , *Schr. naturf. Fr. Berlin* 5: 96 (1784)

The ITS sequences from this species varied in length ranging between 676 to 685 base pairs.

Pilobolus pullus Massee, *Bull. Misc. Inf.*, Kew: 160 (1901)

ITS sequences from this species varied in length ranging between 545 to 671 base pairs

Pilobolus heterosporus Palla, *Öst. bot. Z.* 50: 349 (1900)

The ITS sequence was 681 base pairs in length.

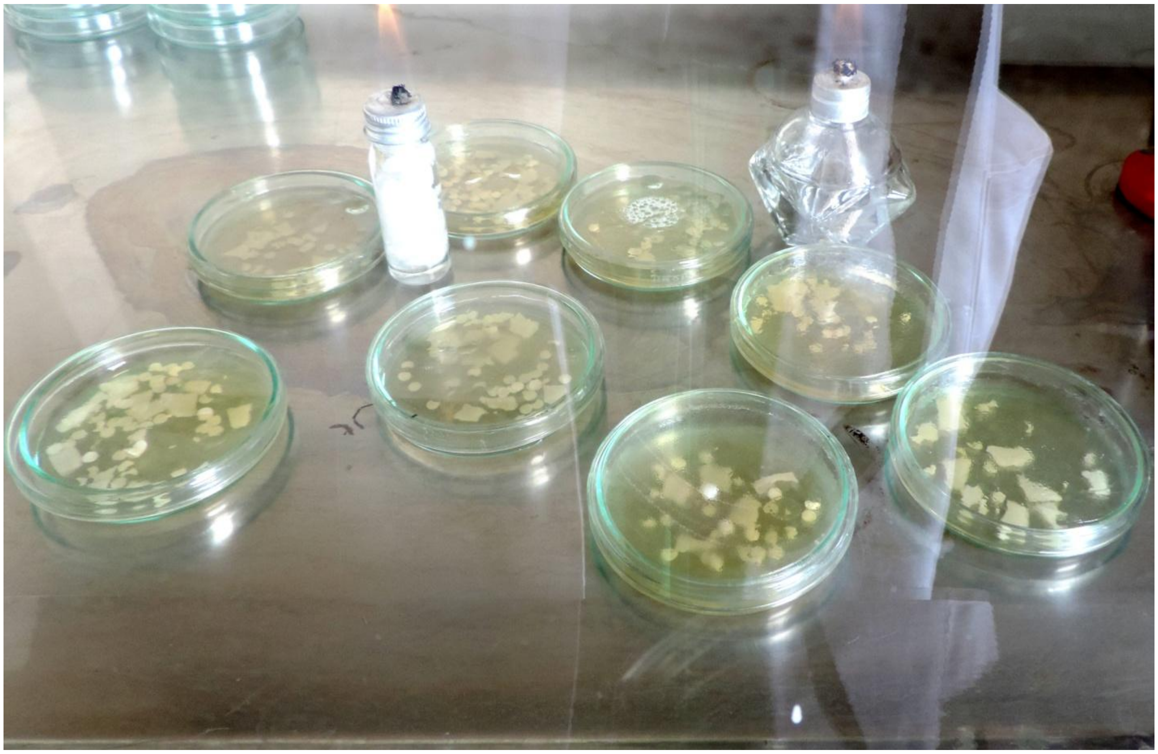

Figure 20: Yeast extract agar cultures in a hood (photo by Antoinette Miyunga)

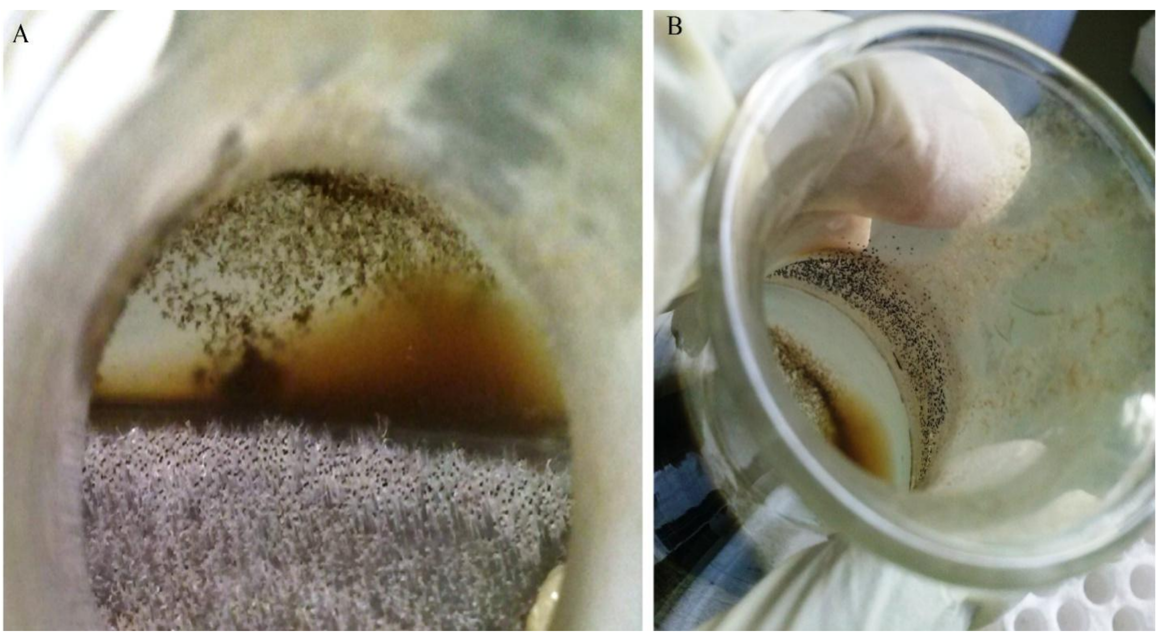

Figure 21: A. *Pilobolus* culture growing on dung agar in a flask B. Sporangia shot on the opposite surface of the flask (photo by Antoinette Miyunga)

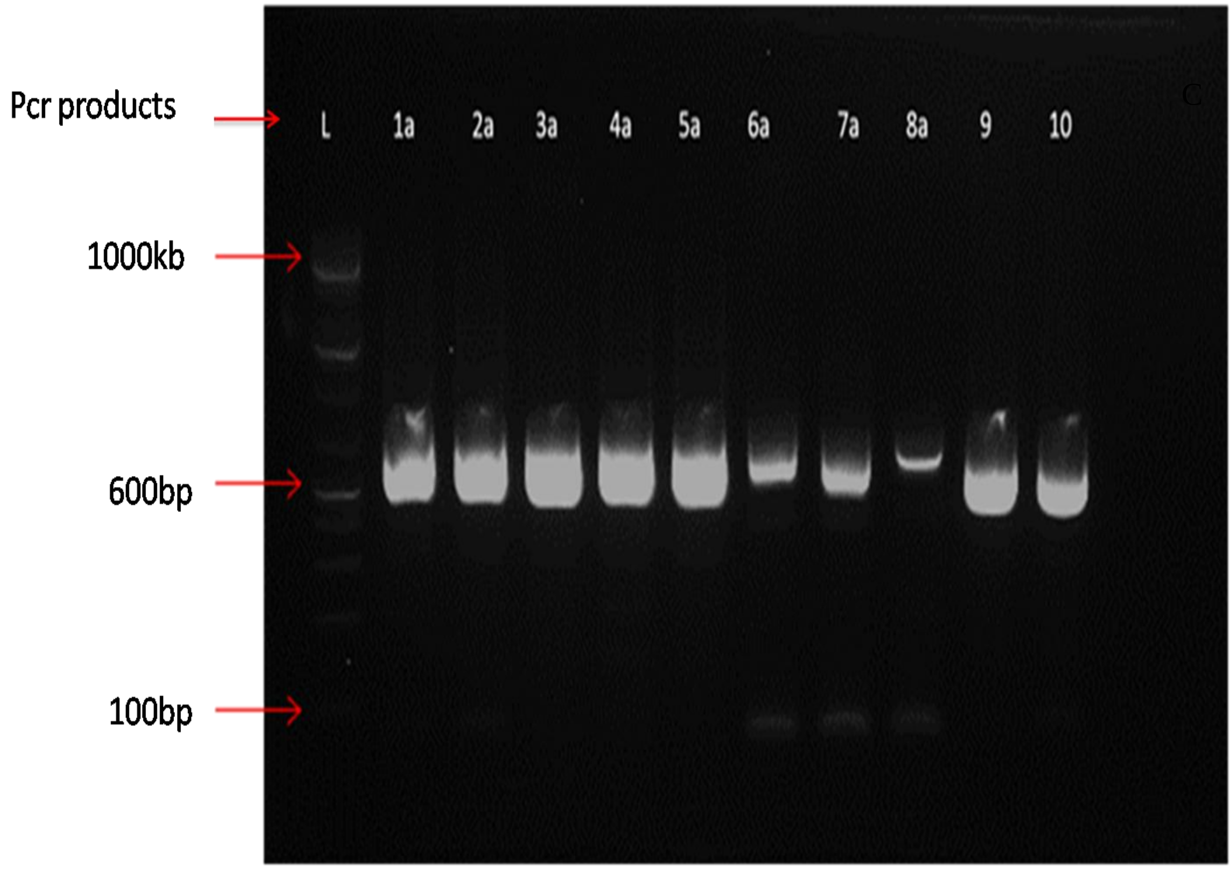

Figure 22: ITS amplified *Pilobolus* DNA. Lane 1-Molecular ladder; Lane 1a -10 Amplified *Pilobolus* ITS sequences

Table 1 shows the accession numbers of the sequences obtained as well as the closest match in gen bank. *Pilobolus* blast searches matched closely *P. heterosporus* and *P. pullus*. *Pilobolus heterosporus* matched to the same species on genbank. However, the matches had low identity of 90%. *Pilobolus pullus* from the study matched to sequences from the same species in genbank some with very high similarity of 99%. *Pilobolus crystallinus* from the study did not match with those of the same species in genbank. The closest match was to *Pilobolus heterosporus* but with low identity.

Figure 23 shows the phylogeny tree from the sequences of the study along with closest matches in genbank. *Pilobolus longipes* was used as an outgroup to root the tree. *Pilobolus pullus* from the study formed a single clade with those of the same species from the US with a high bootstrap support. The same was seen for *Pilobolus heterosporus*; however, *Pilobolus* *crystallinus* clustered separately from the rest of the sequences. This shows how unique they are compared to the rest of the sequences in the study and those available in genbank.

Table 1 *Pilobolus* study samples along with closest matches in Genbank

Sample ID	Species	Accession numbers	Host animal	Closest match in Genbank			
				Identity	Species	Accession number	Host
001	*P. pullus*	KP760859	Buffalo	94%	*P. pullus*	HQ877875	Horse
002	*P. crystallinus*	KP760860	Hartebeest	90%	*P. heterosporus*	HM049604	Elk
003	*P. pullus*	KP760861	Giraffe	99%	*P. pullus*	HQ877875	Horse
004	*P. pullus*	KP760862	Eland	99%	*P. pullus*	HQ877877	Horse
005	*P. pullus*	KP760863	Thompson's gazelle	99%	*P. pullus*	HQ877877	Horse
006	*P. crystallinus*	KP760864	Eland	90%	*P. heterosporus*	HM049604	Elk
007	*P. crystallinus*	KP760865	Impala	91%	*P. heterosporus*	HM049604	Elk
008	*P. crystallinus*	KP760866	Grant's gazelle	90%	*P. heterosporus*	HM049604	Elk
009	*P. heterosporus*	KP760867	Dikdik	90%	*P. heterosporus*	HM049604	Elk
010	*P. crystallinus*	KP760868	Plain's zebra	90%	*P. heterosporus*	HM049604	elk
011	*P. pullus*	KP760869	Buffalo	89%	*P. pullus*	HQ877877	Horse

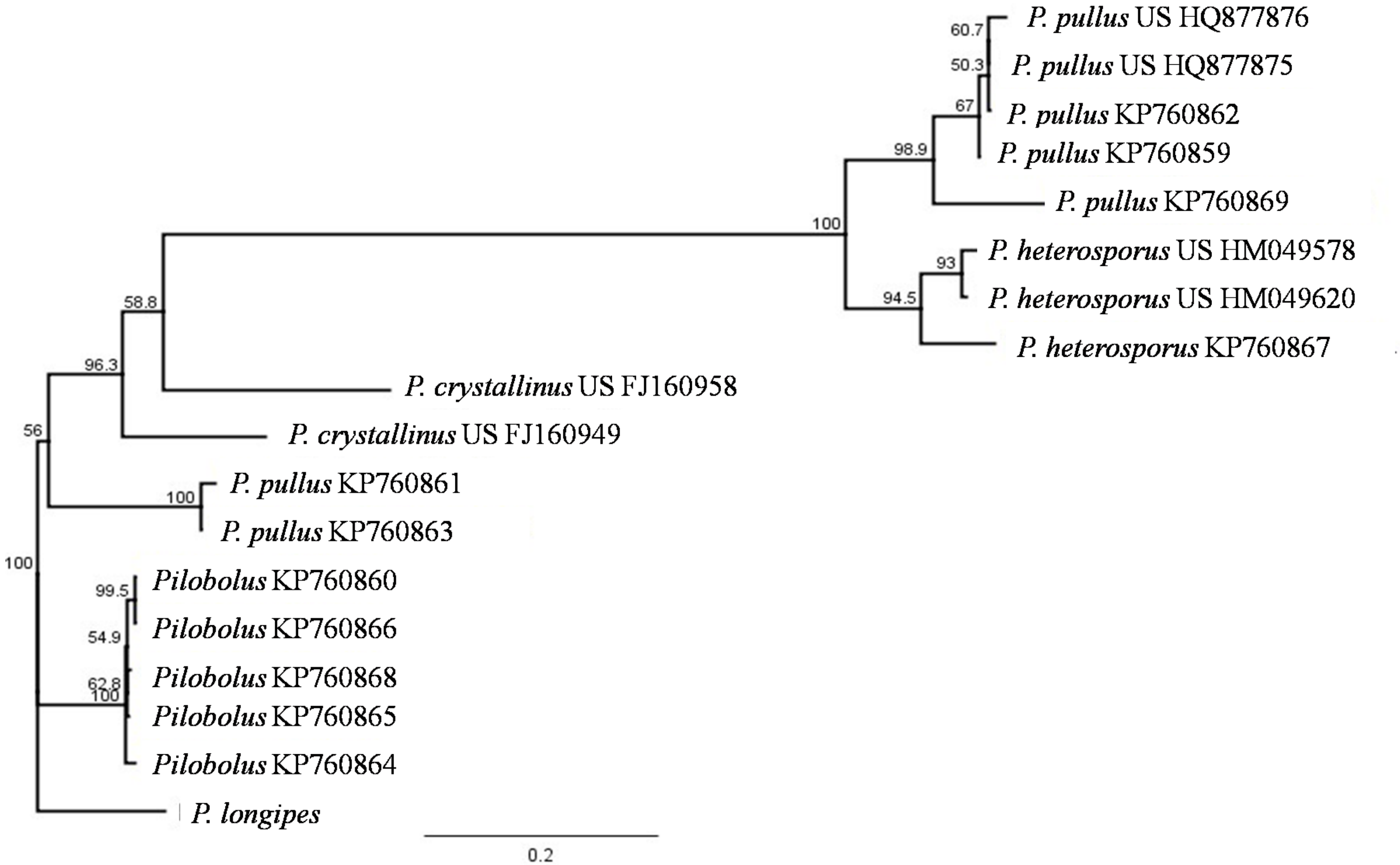

Figure 23: Neighbor joining phylogenetic tree obtained from the study *Pilobolus* sequences and species markers obtained from Gen bank with bootstrap values indicated at the nodes. (Image drawn using Geneious 8.04)

4.5 Discussion

In the past, the taxonomic identification of *Pilobolus* species has mostly relied on morphological characters (Viriato, 2008; Cavalcanti and Trufem, 2008; Hu *et al.,* 1989). However, the classification of such might be obscured by the presence of cryptic species; thus lacking accuracy to asses differences within species and also lacks possible genetic divergence assessment (Pierce and Foos, 2012). This study sought to use molecular identification to asses *Pilobolus* genetic diversity in the dung of several species on large Savannah herbivores from Kenya. For such purpose, universal fungal markers were employed to amplify an ITS region to examine the presence of different *Pilobolus* species (Foos *et al.,* 2011; Foos and Sheehan, 2011). Our results confirm that ITS sequences are an efficient way to distinguish not only at genus level but also at intra generic level (Schoch *et al.,* 2012)

Blast queries of *Pilobolus* from the study were unambiguous since the hits that came up were from the same genus. However, sequences from our study identified as *P. crystallinus* morphologically showed quite high generic variation to other available sequences from the same species. The closest match was to *Pilobolus heterosporus* at relatively low identities between 89-91%. Suggesting the presence a different strain or a previously undescribed species. They formed a monophyletic clade quite distinct from *Pilobolus crystallinus.* Interestingly, they were also quite distinct from *Pilobolus heterosporus* indicating that they were not of the same species despite having *P. heterosporus* as the closest match in genbank. Suggesting that they are not closely related to either. These findings are similar to those of Foos and Sheehan (2011) who reported varying levels of genetic identity between 59.7 to 82% of homologous ITS regions among species of *Pilobolus* in Genbank.

Pilobolus isolate identified morphologically as *P. heterosporus* from Dikdik had matched to the same species in genbank. However, with relatively low identity, 90%. This could indicate a different strain of the same species. The genetic differences could be due to the varying geographic location since it hit to a *Pilobolus* sequence isolated from elk dung in Yellowstone, USA (Foos, 2011). However, on the phylogenetic tree shows that *Pilobolus heterosporus* obtained during the study cluster with those from the US. This indicated close relationship between the species from our study and those obtained from other hosts in a different continent

Pilobolus samples identified morphologically as *P. Pullus* had high similarity to those deposited in genbank (Pierce and Foos, 2012). These had identities of 99% to those suggesting close phylogenetic relationships with those of the same species isolated from another continent with different geographical and environmental conditions. Two isolates also clustered to form a single clade with other sequences from the same species deposited in genbank. However, two *Pilobolus pullus* samples from our study were more divergent than the other three and did not cluster together with those from US. Therefore, we can conclude that this is a new haplotype different from those previously sequenced.

Pilobolus crystallinus which has been identified as the key species in transmission of lungworms, *Dictyocaulus vivparus,* (Foos, 1997) was identified during this study This is an indicator that there could be transmission of lungworms to the animals in the National Park. This suggests that the herbivores in the park are exposed to getting bronchitis while grazing in the park. Therefore, it is essential to do more research to assess the full spectrum of species present in Nairobi National Park and other wildlife protected areas in Kenya to determine whether other *Pilobolus* species identified here are also vectors for the parasites.

Currently, there are less than ten species of *Pilobolus* with sequences deposited in Gen bank out of a possible sixty species described morphologically. To the best of our knowledge, apart from those generated in this study, there are no sequences of these species collected from Africa which could be a different population all together. In addition, very little information exists regarding relationships between these fungi and wildlife hosts (Pierce and Foos, 2012) This is further evidence of the vast lack of knowledge in this field and the need to further understand through consecutive molecular work the biodiversity of the genus and the phylogenetic relationships to those already described.

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CHAPTER FIVE
DISCUSSION CONCLUSION AND RECOMMENDATION

5.1 General Discussion

From the morphological study, five species from the genus *Ascobolus* were identified namely: *A. amoenus, A. bistisii¸ A. calesco, A. immersus* and a four-spored species that has not been described before. The most abundant species from *Ascobolus* genus in the animal dung samples collected were *A immersus* and *A. bistisii.* In addition, there were three *Pilobolus* species identified namely *P. heterosporus, P. pullus* and *P. crystallinus.* These were observed to grow and die out within the first week of incubation in a moist chamber. However, for some samples, there was growth even after ten days of incubation. This could be attributed to the fact that *Pilobolus* does not require passage through animal digestive system to germinate. Therefore, some of the spores being shot in the early days of incubation fell back on the dung and germinated again. Nevertheless, this only went on for as long as there were sufficient nutrients available in the dung.

The genus *Ascobolus* and *Pilobolus* have unique features that differentiate them from other genera. However, during the study, there was overlap in features of some of the specimens making it difficult to classify them into any species. The specimens marked as *Pilobolus* spp and *Ascobolus* spp could not be classified into the known species due to great overlap in features. This points to the need of attaining higher confirmatory levels of identification such as the use of molecular identification.

The molecular characterization revealed three different species with seven different haplotypes from 9 samples. The molecular characterization revealed a possibly new species which was classified as *Pilobolus crystallinus* morphologically but did not match genetically to others of the same species deposited in genbank. This indicates the shortcomings of relying on morphology alone and further supports use of molecular tools to confirm species identification. Nonetheless, it is worth pointing out that more molecular studies need to be carried out for members of this genus to provide adequate references for molecular identification.

5.2 Conclusion

1. Five species of *Ascobolus* were characterized morphologically: *Ascobolus amoenus, Ascobolus bistisii, Ascobolus calesco, Ascobolus immersus* and a fifth putatively new species. Three species of *Pilobolus* were characterized morphologically: *Pilobolus crystallinus, Pilobolus heterosporus* and *Pilobolus pullus*.

2. Three species of *Pilobolus* were characterized using ITS markers: *Pilobolus crystallinus, Pilobolus heterosporus* and *Pilobolus pullus.*

3. The highest observed species richness per dung pile was 5. The estimated species richness diversity was 15 species and this showed that the diversity of *Ascobolus* and *Pilobolus* in Nairobi National Park is high.

5.3 Recommendation

Baseline diversity studies of coprophilous fungi needs to be done in all the National Parks and Reserves. Follow up studies or measures of diversity will help in acting as indicators of ecological stress. More molecular studies should be carried out on these two genera in order to create a library of reference sequences for future molecular characterization. This should be done on the other species as well found in Kenya and Africa as a whole to fill in the existing knowledge gap. Diversity studies on coprophilous fungi should also be extended to other game parks, reserves and wildlife protected areas to determine the presence of these genera.

APPENDIX I: List of Manuscripts

1. Morphological diversity of *Ascobolus* and *Pilobolus* fungi from Wild herbivore dung in Nairobi National Park, Kenya. (Published at Journal of Microbiology Research, September 2015)
2. Molecular identification of *Pilobolus* species from Nairobi National Park. (In press)

APPENDIX II: Publication

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Morphological Diversity of *Ascobolus* and *Pilobolus* Fungi from Wild Herbivore Dung in Nairobi National Park, Kenya

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Abstract The present study examined two genera of coprophilous fungi: *Ascobolus* and *Pilobolus* with the aim of species description using their morphological diversity. Fresh dung samples from wild herbivores were collected in different parts of Nairobi National Park in Kenya and immediately taken to the laboratory for culture by moist chamber method. Isolates studied were obtained from dung of the following animals: white rhino, zebra, waterbuck, impala, Cape buffalo, giraffe, Thomson's gazelle, dikdik, hare, grant's hartebeest, hippopotamus and eland. Five species of *Ascobolus* were studied namely: *Ascobolus amoenus*, *A. bistisii*, *A. calesco* and *A. immersus*. A possible novel 4-spored *Ascobolus* species was observed. Three species of *Pilobolus* were found: *Pilobolus crystallinus* var. *crystallinus*, *P. heterosporus* and *P. pullus*. The most abundant species were: *Ascobolus immersus* and *Pilobolus crystallinus* var. *crystallinus* while the highest diversity was observed in waterbuck dung samples with a total of five different species.

Keywords Ascomycetes, Coprophilous, Wildlife, Zygomycetes

1. Introduction

Coprophilous fungi are saprobic thus an important part of the wildlife ecosystem since they aid in recycling nutrients in animal dung [1]. In addition, they are thought to influence digestive efficiency of animals as well as being part of nutrition for certain arthropods living in herbivore dung [2].

Diversity studies on coprophilous fungi are essential since they give an indication of the state of the environment as they reveal the extent to which environmental stressors contribute to degradation of the environment [3]. High fungi species diversity demonstrates an undisturbed ecosystem that is suitable for the flourishing of wildlife [4]. According to Tibuhwa et al. (2011), macro fungi diversity decreases in areas where agriculture is practiced as opposed to protected wildlife areas with little external environmental stress factors. Species richness is important as an index of the community structure which is useful for conservation [5].

The members of fungi in the genus *Pilobolus* belong to the class Zygomycetes and can be identified through their characteristic sporangiophores that have a swollen extension referred to as collumelae and a sporangium that host the spores at the top [6]. They are observed within two or three days of incubation of dung at room temperature with alternate periods of natural light and darkness [7]. *Pilobolus* are naturally obligate coprophilous species and only grow on dung materials [8]. They are attached to the dung by a swollen trophocyst which is semi immersed in the dung [9]. These trophocysts are normally ovoid to globose while the rhizoidal extensions are long and cylindrical [9]. *Pilobolus* have straight unbranched sporangiophores which grow towards light [9]. The sporangiophores have orange pigments at the base and near the subsporangial vesicles. The sporangia are hemispherical in shape with resistant walls and contain the spores which are spherical or ellipsoid depending on species [9].

The genus *Ascobolus* belongs to the class Ascomycetes which consists of a group of phototropic fungi which release their ascospores towards light. In general, members of this genus take longer than *Pilobolus* to fruit (and is observed after about seven to twenty days following incubation) compared to those of *Pilobolus* [7], [10], [11]. The fruit body of *Ascobolus* has a disk and a receptacle which vary in shape from: perithecoid, pyriform, cylindrical, cupulate, scutullate, discoid and lenticular to pulvinate [12].

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APPENDIX III: Sites of dung samples collected

Date	Sample Number	GPS Latitude	GPS Longitude	GPS Altitude	Dung type (animal species voiding the dung)
23.7.2013	NNP001	S 1.354302	E 36.856879	1657m	White Rhino
	NNP002	S 1.354511	E 36.857055	1647m	Zebra
	NNP003	S 1.354302	E 36.856879	1657m	Water buck
	NNP004	S 1.3511762	E 36.855089	1646m	Zebra
	NNP005	S 1.354511	E 36.857055	1647m	Water buck (unidentified)
	NNP006	S 1.357545	E 36.846372	1645m	Zebra
	NNP007	S 1.359155	E 36.844832	1649m	Impala
	NNP008	S 1.357729	E 36.841099	1648m	White Rhino
	NNP009	S 1.345002	E 36.823411	1649m	Buffalo
	NNP010	S 1.342703	E 36.822223	1657m	Buffalo
7.8.2013	NNP011	S 1.35203	E 36.82693	1643m	Giraffe
26.8.2013	NNP012	S 1.37708	E 36.851754	1648m	Thompson’s Gazelle
	NNP015	S1.349502	E36.85490	1653m	White Rhino
	NNP017	S1.351630	E36.82896	1657m	Buffalo
	NNP020	S1.354228	E36.856892	1650m	Hartebeest
28.8.2013	NNP022	S1.345529	E36.792625	1733m	Giraffe
10.1.2014	NNP023	S1.337257	E36.811041	1671m	Hartebeest

	NNP024	S1.34925	E36.826813	1658m	Dikdik
	NNP025	S1.34925	E36.826813	1658m	Dikdik
	NNP026	S1.34925	E36.826813	1658m	Hare
	NNP027	S1.34925	E36.826813	1658m	Grant's gazelle
	NNP028	S1.34925	E36.826813	1658m	Hartbeest
	NNP029	S1.34925	E36.826813	1658m	Thompson’s Gazelle
	NNP030	S1.34925	E36.826813	1658m	Grant's gazelle
	NNP031	S1.34925	E36.826813	1658m	White Rhino
	NNP032	S1.34925	E36.826813	1658m	Hippopotamus
	NNP033	S1.353055	E36.855815	1643m	Buffalo
	NNP034	S1.353055	E36.855815	1643m	Giraffe
	NNP035	S1.353055	E36.855815	1643m	Impala
	NNP036	S1.353055	E36.855815	1643m	Impala
	NNP037	S1.353055	E36.855815	1643m	Eland
	NNP038	S1.353055	E36.855815	1643m	Water buck
12.1.2014	NNP039	S1.387835	E36.895498	1619m	Zebra
	NNP040	S1.387835	E36.895498	1619m	Zebra
21.1.2014	NNP044	S1.429517	E36.937845		White Rhino
	NNP045	S1.429517	E36.937845		White Rhino
04.4.2014	NNP046				Buffalo

	NNP047				Hartebeest
	NNP048				Hartebeest
	NNP049				Zebra
	NNP050				Hartebeest
	NNP051				Giraffe
	NNP052				Buffalo
	NNP053				Buffalo
20.5.2014	NNP054	S1.365307	E36.7881733	1631m	Giraffe
	NNP055	S1.368350	E34.7289179	1693m	Zebra
	NNP056	S1.368350	E34.7289179	1693m	Impala
	NNP057	S1.368350	E34.7289179	1693m	Impala
	NNP058	S1.368350	E34.7289179	1693m	Impala
	NNP059	S1.380092	E36.7829947	1700m	Eland
	NNP060	S1.384205	E36.791641	1644m	Hartebeest
	NNP061	S1.384205	E36.791641	1644m	Hartebeest
	NNP062	S1.369891	E36.798175	1693m	White Rhino
	NNP063	S1.362626	E36.8122581	1673m	Eland
	NNP064	S1.345271	E36.795970	1711m	Buffalo
30.5.2014	NNP066	S1.340694	E36.813194		Zebra
	NNP067	S1.340694	E36.813194		Zebra

	NNP068	S1.340631	E36.813005		Giraffe
	NNP069	S1.352099	E36.828616		Grant's gazelle
	NNP070	S1.355153	E36.836230		Hartebeest
	NNP071	S1.354389	E36.851619		Impala
	NNP072	S1.355868	E36.857941		Giraffe
	NNP073	S1.347307	E36.797774		Giraffe
	NNP074	S1.342161	E36.785706		Buffalo

APPENDIX IV: Host animals and fungal species described in their dung

Animal	No of dung piles	*P. crystallinus*	*P. pullus*	*P. heterosporus*	*Pilobolus spp*	*A. amoenus*	*A. bistisii*	*A. calesco*	*A. immersus*	*Ascobolus spp*	4spored *Ascobolus*	
White Rhino	7						2		7			3
Zebra	9	4	1				2		8	2		5
Water buck	3		1		3	1	1		2			5
Impala	7	1							1			2
Buffalo	9	1	3						5	1		4
Giraffe	8		2						1			2
Thompson's gazelle	2		2		1	1	1		1			4
Hartebeest	9	1				1			2		2	4
Dikdik	2	1		2	2	3						4
Hare	2					3						1
Grant's gazelle	3	1				1	1					4
Hippopotamus	1					2	1					2
Eland	3	2	2		2	4				1		4
	65	8	5	2	13	17	9	1	27	5	2	

APPENDIX V Study sequences and Genbank accession numbers

1. *Pilobolus pullus* KP760859

TTTCAGATCAAATAATGTGTTTGTCGGCCAGAACTTCCTCATAGCGTCTTACTTGCCA
GCGTAAAATAATTAAACACTTAATATTTATTTTACTCGGCATGAATGACTTGGGTCA
TTCGCCTATAAAGAGATTTAAGCCGCTCAAGTGATTTAACACTTTGTATCGGCCAAG
GATATTAATCATTCCGCCCAGAACTCTGCTAACTAAAGCAAAAGTACGGCGTTGGGG
TTAATATGATACTGAAACAAGTGTACTCTTTGGAATACCAAAGAGTGCAAGATGCGT
TCAAAAACTTGATGATTCACGAGATGCAATTCACAATAGTTATCGCAATTTGCTACG
TTCTTCATCGATGCGAGAGCCTAAAGATCCGTTGTTAAAAGTTGTTTTTAATCATTTT
TTTACGCTGATTGTTTCAATTTATTAACTAAAAAATACTAGGTATAATATTTCGCTAA
CCCGGGGGAGGCCAAAACCTCCCCCAAGCAGCATTTTTTTAGGCCAAATCCCCAAA
CATAAATAGTTCACAAGAGGTAAATTATTTTTTTCTCCTTCCCTCAAAAAAAGAAGA
AAAACGTGGAAAAAATGAACCATTTTAAGACCAAAAGAACCTGATTACCAAATTAA
ATGGAACCCTTCGGCAGGTTCCCTTACGGAAACCTGGTTACGA

2. *Pilobolus crystallinus* KP760860

TTTCCGTAGGTGAACCTGCGGAAGGATCATTAACTATTTTGTTAAAACCATGTTTTTT
TGGCTGGATTAAAAACCTAGTCAATTTTTTACATGTCTTCTTTCGGGGAAGAGAAAA
ATAAATCTACTGTGAAACAATATTTGGGGTTTGGCCATAAAAAAAAAGTTACTTGGG
CGAGCTTTCGAGTTCCCCTGGGTTAACGACATATTATACTATTGATTTTTTAGTTTTA
AAATTGAAAACAATCAGCCTAAAAAACTGATTAAAACAACTTTTAACAACGGATCT
CTAGGCTCTCGCATCGATGAAGAACGTAGCAAATTGCGATAACTATTGTGAATTGCA
TCTCGTGAATCATCAAGTTTTTGAACGCATCTTGCACTCTTTGGTATTCCAAAGAGTA
CACTTGTTTCAGTATCATATTAACCCCCAACGCCGTATGATTTTATTATCATTCTGGG
CGGAATTATGTATCCTTGGCCGAAGAAAGGTATTAAGTTACCTGAGCGGCTTAAATC
TATACTAGGCGAAATGACCCGTTGGTTCATTTTTCTGTTTGTAAAATAAATATTAATG
TGTTTCTTATTTTACGTTTAAGCAGAATTGAATCGTTAAGGGGCATTTCTGGCCAACA
AACACATTATTTTGATCTGAAATCAAGTGGGATTACCCGCTGAA

3. *Pilobolus pullus* KP760861

TAATCATGTTTTTTTGGCCGAGAAATTGGTCATTTTTATCATGTCTTTCCTCTTTTTTT
GAGGGAGGAGAAAAAAATAATTTACTGTGAACTTTTTATGTTTGGGGATTTGGCCTA
AAAAAATGCTGCTTGGGCGAGGTTTTGGCCTCCCCCGGGTTAGCGAAATATTATACC
TAGTATTTTTTAGTTAATAAATTGAAACAATCAGCGTAAAAAACTGATTAAAAACAA
CTTTTAACAACGGATCTCTAGGCTCTCGCATCGATGAAGAACGTAGCAAATTGCGAT
AACTATTGTGAATTGCATCTCGTGAATCATCAAGTTTTTGAACGCATCTTGCACTCTT
TGGTATTCCAAAGAGTACACTTGTTTCAGTATCATATTAACCCCAACGCCGTACTTTT
GCTTTAGTTAGCAGAGTTCTGGGCGGAATGATTAATATCCTTGGCCGATACAAAGTG
TTAAATCACTTGAGCGGCTTAAATCTCTTTCTAGGCGAATGACCCAAGTCATTCATG
CCGAGTAAAATAAATATTAAGTGTTTAATTATTTTACGCTGGCATGTAATGACGCTA
TGAGGAAGTTCTGGCCGACAAACACATTATTTGATC

4. *Pilobolus pullus* KP760862

CGGCCAGAACTTCCTCATAGCGTCATTACATGCCAGCGTAAAATAATTAAACACTTA
ATATTTATTTTACTCGGCATGAATGACTTGGGTCATTCGCCTAGAAAGAGATTTAAG

CCGCTCAAGTGATTTAACACTTTGTATCGGCCAAGGATATTAATCATTCCGCCCAGA
ACTCTGCTAACTAAAGCAAAAGTACGGCGTTGGGGTTAATATGATACTGAAACAAG
TGTACTCTTTGGAATACCAAAGAGTGCAAGATGCGTTCAAAAACTTGATGATTCACG
AGATGCAATTCACAATAGTTATCGCAATTTGCTACGTTCTTCATCGATGCGAGAGCC
TAGAGATCCGTTGTTAAAAGTTGTTTTTAATCAGTTTTTTACGCTGATTGTTTCAATT
TATTAACTAAAAAATACTAGGTATAATATTTCGCTAACCCGGGGGAGGCCAAAACCT
CGCCCAAGCAGCATTTTTTTAGGCCAAATCCCCAAACATAAAAAGTTCACAGTAAAT
TATTTTTTTCCCTCCCTCAAAAAAAGAAGAAA

5. *Pilobolus pullus* KP760863

TAATCATGTTTTTTTGGCCGAGAAATTGGTCATTTTTATCATGTCTTTCCTCTTTTTTT
GAGGGAGAGAAAAAAAATAATTTACTGTGAACTTTTTATGTTTGGGGATTTGGCCTAA
AAAAATGCTGCTTGGGCGAGGTTTTGGCCTCCCCCGGGTTAGCGAAATATTATACCT
AGTATTTTTTAGTTAATAAATTGAAACAATCAGCGTAAAAAACTGATTAAAAACAAC
TTTTAACAACGGATCTCTAGGCTCTCGCATCGATGAAGAACGTAGCAAATTGCGATA
ACTATTGTGAATTGCATCTCGTGAATCATCAAGTTTTTGAACGCATCTTGCACTCTTT
GGTATTCCAAAGAGTACACTTGTTTCAGTATCATATTAACCCCAACGCCGTACTTTTG
CTTTAGTTAGCAGAGTTCTGGGCGGAATGATTAATATCCTTGGCCGATACAAAGTGT
TAAATCACTTGAGCGGCTTAAATCTCTTTCTAGGCGAATGACCCAAGTCATTCATGC
CGAGTAAAATAAATATTAAGTGTTTAATTATTTTACGCTGGCATGTAATGACGCTAT
GAGGAAGTTCTGGCCGACAAACACATTATT

6. *Pilobolus crystallinus* KP760864

TTTCCGTAGGTGAACCTGCGGAAGGATCATTAACTATTTTGTTAAATCCATGTTTTTT
TGGCTGGAGTAAAAACCTAGTCAATTTTTTTACATGTCTTCTTTCGGGGAAGAGAAA
AATAAATCTACTGTGAAACATCATTTGGGGTTTTTGGCCATGAAAAAAAGTTACTTG
GGCGAGCTTTCGAGTTCCCCTGGGTTAACGACATATTATACTATTGATTTTTTAGTTT
TAAAATTGAAAACAATCAGCCTAAAAAACTGATTAAAACAACTTTTAACAACGGAT
CTCTAGGCTCTCGCATCGATGAAGAACGTAGCAAATTGCGATAACTATTGTGAATTG
CATCTCGTGAATCATCAAGTTTTTGAACGCATCTTGCACTCTTTGGTATTCCAAAGAG
TACACTTGTTTCAGTATCATATTAATCCCCAACGCCGTATGATTTTATTATCATTCTG
GGCGGAATTATGTATCCTTGGCCGAAGAAAGGTATTAAGTTACCTGAGCGGCTTAAA
TCTATACTAGGCGAAATGACCCGTTGGTTCATTTTTCTGTTTGTAAAAATAAATATTA
ATGTGTTTCTTATTTTACGTTTAAGCAGAATTGCAATCGTTAAGGGGCATTTCTGGCC
TACAAACACATTATTTTGATCTGAAATCAAGTGGGATTACCCGCTGAACTTAA

7. *Pilobolus crystallinus* KP760865

TTTCCGTAGGTGAACCTGCGGAAGGATCATTAACTATTTTGTTAAAACCATGTTTTTT
TGGCTGGATTAAAAACCTAGTCAATTTTTTACATGTCTTCTTTCGGGGAAGAGAAAA
ATAAATCTACTGTGAAACATTATTTGGGGTTTTTGGCCATGAAAAAAAGTTACTTGG
GCGAGCTTTCGAGTTCCCCTGGGTTAACGACATATTATACTATTGATTTTTTAGTTTT
AAAATTGAAAACAATCAGCCTAAAAAACTGATTAAAACAACTTTTAACAACGGATC
TCTAGGCTCTCGCATCGATGAAGAACGTAGCAAATTGCGATAACTATTGTGAATTGC
ATCTCGTGAATCATCAAGTTTTTGAACGCATCTTGCACTCTTTGGTATTCCAAAGAGT
ACACTTGTTTCAGTATCATATTAATCCCCAACGCCGTATGATTTTATTATCATTCTGG
GCGGAATTATGTATCCTTGGCCGAAGAAAGGTATTAAGTTACCTGAGCGGCTTAAAT
CTATACTAGGCGAAATGACCCGTTGGTTCATTTTTCTGTTTGTAAAATAAATATTAAT
GTGTTTCTTATTTTACGTTTAAGCAGAATTGAATCGTTAAGGGGCATTTCTGGCCAAC
AAACACATTATTTTGATCTGAAATCAAGTGGGATTACCCGCTGAACTTAA

8. *Pilobolus crystallinus* KP760866

TTTCCGTAGGTGAACCTGCGGAAGGATCATTAACTATTTTGTTAAAACCATGTTTTTT
TGGCTGGATTAAAAACCTAGTCAATTTTTTACATGTCTTCTTTCGGGGAAGAGAAAA
ATAAATCTACTGTGAAACAATATTTGGGGTTTGGCCATAAAAAAAAAGTTACTTGGG
CGAGCTTTCGAGTTCCCCTGGGTTAACGACATATTATACTATTGATTTTTTAGTTTTA
AAATTGAAAACAATCAGCCTAAAAAACTGATTAAAACAACTTTTAACAACGGATCT
CTAGGCTCTCGCATCGATGAAGAACGTAGCAAATTGCGATAACTATTGTGAATTGCA
TCTCGTGAATCATCAAGTTTTTGAACGCATCTTGCACTCTTTGGTATTCCAAAGAGTA
CACTTGTTTCAGTATCATATTAACCCCCAACGCCGTATGATTTTATTATCATTCTGGG
CGGAATTATGTATCCTTGGCCGAAGAAAGGTATTAAGTTACCTGAGCGGCTTAAATC
TATACTAGGCGAAATGACCCGTTGGTTCATTTTTCTGTTTGTAAAATAAATATTAATG
TGTTTCTTATTTTACGTTTAAGCAGAATTGAATCGTTAAGGGGCATTTCTGGCCAACA
AACACATTATTTTGATCTGAAATCAAGTGGGATTACCCGCTGAACTTAA

9. *Pilobolus heterosporus* KP760867

TTAAGTTCAGCGGGTAATCCCACTTGATTTCAGATCAAAATAATGTGTTTGTTGGCC
AGAAATGCCCCTTAACGATTCAATTCTGCTTAAACGTAAAATAAGAAACACATTAAT
ATTTATTTTACAAACAGAAAAATGAACCAACGGGTCATTTCGCCTAGTATAGATTTA
AGCCGCTCAGGTAACTTAATACCTTTCTTCGGCCAAGGATACATAATTCCGCCCAGA
ATGATAATAAAATCATACGGCGTTGGGGGTTAATATGATACTGAAACAAGTGTACTC
TTTGGAATACCAAAGAGTGCAAGATGCGTTCAAAAACTTGATGATTCACGAGATGC
AATTCACAATAGTTATCGCAATTTGCTACGTTCTTCATCGATGCGAGAGCCTAGAGA
TCCGTTGTTAAAAGTTGTTTTAATCAGTTTTTTAGGCTGATTGTTTTCAATTTTAAAA
CTAAAAAATCAATAGTATAATATGTCGTTAACCCAGGGGAACTCGAAAGCTCGCCC
AAGTAACTTTTTTTTTATGGCCAAACCCCAAATATTGTTTCACAGTAGATTTATTTTT
CTCTTCCCCGAAAGAAGACATGTAAAAAATTGACTAGGTTTTTAATCCAGCCAAAAA
AACATGGTTTTAACAAAATAGTTAATGATCCTTCCGCAGGTTCACCTACGGAAA

10. *Pilobolus crystallinus* KP760868

TTTCCGTAGGTGAACCTGCGGAAGGATCATTAACTATTTTGTTAAAACCATGTTTTTT
TGGCTGGATTAAAAACCTAGTCAATTTTTTACATGTCTTCTTTCGGGGAAGAGAAAA
ATAAATCTACTGTGAAACATTATTTGGGGTTTTTGGCCATGAAAAAAAGTTACTTGG
GCGAGCTTTCGAGTTCCCCTGGGTTAACGACATATTATACTATTGATTTTTTAGTTTT
AAAATTGAAAACAATCAGCCTAAAAAACTGATTAAAACAACTTTTAACAACGGATC
TCTAGGCTCTCGCATCGATGAAGAACGTAGCAAATTGCGATAACTATTGTGAATTGC
ATCTCGTGAATCATCAAGTTTTTGAACGCATCTTGCACTCTTTGGTATTCCAAAGAGT
ACACTTGTTTCAGTATCATATTAATCCCCAACGCCGTATGATTTTATTATCATTCTGG
GCGGAATTATGTATCCTTGGCCGAAGAAAGGTATTAAGTTACCTGAGCGGCTTAAAT
CTATACTAGGCGAAATGACCCGTTGGTTCATTTTTCTGTTTGTAAAATAAATATTAAT
GTGTTTCTTATTTTACGTTTAAGCAGAATTGAATCGTTAAGGGGCATTTCTGGCCAAC
AAACACATTATTTTGATCTGAAATCAAGTGGGATTACCCGCTGAA

11. *Pilobolus pullus* KP760869

TGATAATGCCTAAAGTTCAGCGGGGTAATCCCACATTGATTTCAGATCAAAAATTAA
TGTGTTTGTCGGCCAGAACTGCCTCTTAGCGACTTTACTATGGCTTCGTAAAATAATT
AAACACTTAATATTATATTTTACTCGGCATGAATGACTTGGGTCATTCGCCTATAAA
GAGATTTAAGCCGCATCCCGTGATTTACAACTTTGTATCGGCCAAGGATATTAATCC
TTCCGCCCAGCCCTAAATCTGCTAACTAAAGCAAAAGTACGGCTTTGGGGTTAATAT
GATACATGAAACAAGTGTACTCTTTGGGAATACCAAGGAAGGCAGATGCGTTTCAA
AACTTGATGATTCACAAGATGCTATTCACCATAGTTATCGCAATTTGCTACGTTCTTC
ATCGATGCGAGAGCCTAAAGATCCGTTGTTAAAAGTTGTTTTTAATCATTTTTTTACC
CGGATTGTTTCAATTTATTAACTAAAAAATACTAAGTATAATATTTCGCTAACCCGG
GGGGGGCCAAAACTTCGCCCAAGCATCTTTTTTTTAGCCCAAATCCCCAATCATAAA
AAGTTCACAATATATTCTCTCCCTCAAAAAAAGAAGAAAGACATGATAAAAATGAT
CCATTTCATCGGCCACAAGGAAACGACTATAA