CHARACTERISATION AND DEVELOPMENT OF PROPAGATION SPAWNS FOR SELECTED WILD EDIBLE MUSHROOMS FROM ABERDARE NATIONAL FOREST, KENYA

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A thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Science (Biotechnology) in the School of Pure and Applied Sciences of Kenyatta University

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DECLARATION

I, Crispus Mbaluto, declare that this thesis is my original work and has not been presented for the award of a degree in any other university or for any other award.

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We confirm that the work reported in this thesis was carried out by the candidate under our supervision and has been submitted with our approval as University supervisors.

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DEDICATION

This thesis is hereby dedicated to the family of Mr and Mrs Bernard M. Mukeku to whom I am privileged to belong, for their encouragement with love and respect. They imbibed in me the discipline of hard work and self-esteem.

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I set my unfeigned and humble thanksgiving before **ALMIGHTY** God who favoured and reinforced me with the fortitude and capability to aptly complete my research work. A research project such as this is the product of many minds and many voices. It would not have been possible were it not for the immense contributions of the following people and institutions to which I am very grateful and hereby acknowledge. With profound gratitude and deep sense of devotion, I acknowledge Dr. Steven Runo of the Department of Biochemistry and Biotechnology Kenyatta University, Dr. Wanjiru Wanyoike of the Department of Botany Jomo Kenyatta University of Agriculture & Technology (JKUAT) and Dr. Ing. Calvin Onyango of the Food Division Kenya Industrial Research & Development Institute (KIRDI). It was their confidence in my capabilities and appreciation of my work that encouraged me to keep on fighting against all odds till the success was ensured. Most of us have true stalwarts in our lives, and I acknowledge Daniel Ojwang of KIRDI, who supported, listened and often worked on these ideas with me without reservation. Many others in the forest contributed to this research, they are a generous and hardy group who make research in the forest a worthwhile endeavour. Allow me to mention a few: Mr. Mervyn Mwangangi and Mr. David M Musee (Forest Rangers), Mzee Geoffrey Mashauri and Catherine Waruguru Gichoi, Aberdare National forest residents; without forgetting the driver Judith Ngina who carefully drove us in the entire field work journey and back to our work stations. I thank my lovely parents Mr and Mrs. Bernard Mukeku who taught me the meaning of patience and I saw hope in their eyes. Special thanks are due to my siblings for their prayers, encouragement and endless support. Generally, there were many more people who were an important part of this research. Thanks for your intellectual support and insight. Last but by no means least, I have the good fortune to social or special close friend who remained involved in this process, even when they had no idea where I was, or whether I would ever finish; I am highly indebted to Ketrina Mpetaphiri.

ABSTRACT

Characterization and identification of mushrooms at the species level is an important first step in systematic exploitation in specific applications. Mushrooms industry is growing rapidly in Kenya, importation of cultures and propagation spawns is growing too. The imported strains are susceptible to pest and diseases, and are low yielding. This study aimed at characterising selected wild edible mushrooms using morphological and molecular characteristics and developing of propagation spawns of the selected Kenyan wild edible species of mushrooms. Mushrooms samples were collected with assistance of a taxonomist and local forest dwellers. Structured questionnaires were used for ethno-mycological study. Dominant phenotypic features of the fruiting bodies and spores were observed for morphological analysis. The Ribosomal DNA Internal Transcribed Spacer and Large Sub-Unit regions (rDNA-ITS and nLSU) were amplified and amplicons sequenced for molecular identification. Phylogenetic trees constructed using Neighbour-joining method in MEGA5. Pure cultures were grown in 3 different media potato dextrose agar (PDA), yeast extra agar (YEA) and malt extra agar (MEA) for 10 days for culture morphology studies. The spawning experiment was arranged in completely randomized design with three replicates raised on optimal condition studied from cultures. Ethno-mycology findings at $p \leq 0.05$ showed no significant difference among respondents. Collected accessions had numerous striking phenetic features. Based on the basidiomata and spores morphology data, the accessions were classified as members of genus *Macrolepiota*. The ITS sequences revealed identity (homology percentage from GenBank data base) of *Macrolepiota dolichaula* [KAB03, RMK04 and RMK08, % identity 100 (AF482839.1)], [MAT06, MAT08 and ZAI02, % identity 100 (AF382839.1)], [KAB07, % identity 100 (HM125516.1)]. The nLSU sequences revealed identity (homology percentage from GenBank data base) of *Macrolepiota procera* [KAB01, KAB03 KAB07 and ZUT06, % identity 100 (JN940269.1)], *Macrolepiota dolichaula* [MAT06, RMK04, RMK08 and ZAI02, % identity100 (AF482883.1)], [MAT08 % identity 100 (DQ411537.1)] and *Galerina sp.* For ZUT016 both ITS and nLSU sequences [% identity 99 (HQ60475.1)]. Nine out of 10 mushrooms could be identified up-to species level. Cultural studies revealed MEA at 25°C and pH 7 was most optimal for the mushrooms samples. Spawn production showed that 100 % sorghum grains can be successfully colonized by mycelia to produce high quality spawn. The ethno-mycology findings in this study envisage the purposeful strengthening of wild edible mushrooms exploitation. Characterization provides additional information enriching GenBank database. Successful development of propagation spawns suggest samples can be cultivated. Diversity studies and cultivation trials are recommended.

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LIST OF ABBREVIATIONS

AFLP .Amplified Fragment Length Polymorphism

CRD Completely Randomised Design

DNA Deoxyribonucleic Acid

ECM Ectomycorrhizae

FAO Food and Agriculture Organisation

GPS Geographical Positioning System

HCL Hydrochloric Acid

ITS Internal Transcribed Space

JKUAT Jomo Kenyatta University of Agriculture and Technology

KFS Kenya Forest Service

KU Kenyatta University

NaOH Sodium Hydroxide

PCR Polymerase Chain Reaction

RAPD Random Amplified Polymorphic DNA

RFLP Random Fragmented Length Polymorphism

RNA Ribonucleic Acid

SSR Short Sequence Repeats

YEA Yeast Extra Agar

CHAPTER ONE

INTRODUCTION

1.1 Background to the study

Mushrooms are fungal organisms with distinctive fruiting body, which can be hypogenous or epigeous, large enough to be seen with the naked eye and to be picked by hand (Chang and Miles, 1992; Hall *et al.,* 2003; Oyetayo, 2011). They do not contain chlorophyll and are therefore eukaryotic heterotrophs which obtain food from decaying organic matter (Dada *et al.,* 2008; Kumar and Sharma, 2011).

Millions of people in many developing countries depend on wild resources to meet their food needs especially in periods of food crisis. According to Central Bureau of Statistics (2001), in Kenya, as in all developing countries, the rapid growth in population is a great threat to natural resources. In addition, poor economy, unemployment and unfavourable climatic changes contribute to the high prevalence of extinction of natural habitat of the wild species of the edible higher fungi (Lillian *et al.,* 2007).

Growth of mushroom industry requires new strains with better characteristics. Farmers require mushroom varieties with fast maturity period, increased resistance to both pests and diseases and high yield.

Kenya's rich mushroom biodiversity has great potential to provide new mushroom strains with desirable characteristics for commercial cultivation. The exact characterization and identification at the species level is thus an important step in systematically utilizing the full potential of fungi in specific applications (Lieckfeldt *et al.*, 2001). Many *mushrooms* species have been identified and characterized in the past using morphological features. Studies on *Pleurotus and Auricularia auricula* (L. ex Hook.) Underw species in Kenya have been based on morphological characteristic.

However, morphological features in Basidiomycetes fungi are influenced by environmental factors and often fail to detect variations among species and strains that are closely related. Elsewhere, Iotti *et al*. (2005) used morphological characterization of ectomycorrhizal mycelia using basidiomycetous species to determine their ecological importance.

In developed world, grouping of mushroom species was based on the region or locality of origin; for example, North American mushrooms, mushrooms of the Western Hemisphere, and mushrooms of South Africa. Certain mushrooms are easy to identify, many are not. In fact, there are a great number of look-alikes among the mushroom strains.

To avoid any unpleasant experiences, especially when identifying mushrooms for the purpose of determining edibility, characterisation of these wild mushrooms requires a basic knowledge of the structure of fungi and of the way in which they live (Shu-Ting, 2008).

Molecular approaches are required to determine genotypic identity, analyse population structure and evolutionary within and among species thus justifying the morphological observations (Samiya *et al.,* 2011). They are more stable, reproducible and are not affected by environmental factors hence provide more information on genetic characteristics of any species. Identification and characterization of local mushrooms strains using molecular tools is necessary in selecting new strains for commercial cultivation. Morphological characters alone used in the past are often inadequate for exact strain identification and in resolving the systematics and evolutionary relationships within Basidiomycetes fungi. The aim of this study was to identify, characterize wild edible mushrooms from Aberdare National forest in Kenya and subsequently develop propagation spawns for selected as first step towards their domestication.

1.2 Problem statement and justification

In recent times, edible mushrooms have assumed greater importance in the diets of both rural and urban dwellers (Khan *et al.,* 2011). In Kenya, unlike previously when consumption was confined to rural communities, urban dwellers are increasing consuming mushrooms (Wambua, 2004). Increase in demand for edible mushrooms has resulted in setting up of several mushroom units in different parts of the country. Mushrooms cultivation has not been given due importance and the sector is underdeveloped with only two exotic species grown for the hotel industry (Gateri *et al.,* 2004; Odendo *et al.,* 2009; Onyango *et al.,* 2011a; Onyango *et al.,* 2011b). Currently the mushroom production stands at slightly over 500 tons per annum with the production of *Pleurotus* species being the second most produced after *Agaricus* (Concern/GTZ/MOA., 2005). The total annual mushroom production in Kenya is low and hardly enough to meet the local demand. Rural communities therefore, rely on the collection and consumption of wild edible species during the rainy seasons.

Unfortunately, the seasonality of wild edible mushrooms makes them unreliable source of nutrition. Similarly, lack of clear-cut identification and limited information on their genetic diversity limit their exploitation for commercial production and breeding purposes.

Increased productivity of mushroom industry in Kenya requires new mushroom species with improved characteristics such as high yields and increased resistance to pests and diseases. Characterization and identification of wild species is likely to provide strains with desired characteristics. Accurate taxonomic identification and phylogenetic classification is therefore necessary for selecting strains with potential for propagation and breeding purposes. Molecular markers including rapid amplified polymorphic DNA (RAPD) markers, amplified fragment length polymorphic markers (AFLP), restriction fragment length polymorphic (RFLP) markers and microsatellite have all been employed to discriminate different kinds of organisms including mushrooms.

Similarly, the internal transcribed spacer (ITS) and nuclear Large subunit (nLSU) regions of the ribosomal DNA (rDNA) have also been widely used for the phylogenetic identification of mushrooms at both the species and genus level (Sanchez-Ballesteros *et al.*, 2000). Different regions of rDNA also evolve at variable rates and this makes them suitable for investigating fungal relationships at different taxonomic levels. Grain formulations have been successfully utilized in spawns development for exotic strains and therefore recommended for this study.

1.3 Hypothesis

Wild edible mushrooms propagation spawns can be developed for domestication.

1.4 Research questions

a) Are ethno-mycological applications still dominant among local communities?

b) What are the phenotypic and genotypic characters which are important at the deeper levels of the classification?

c) Can pure cultures be developed from wild edible mushrooms strains from Aberdare National Forest in Kenya and propagation spawns developed?

1.5 Research objectives

1.5.1 General objective

To identify, characterize and develop propagation spawns for selected wild edible mushrooms from Aberdare National forest Reserve in Kenya.

1.5.2 Specific objectives

a) To establish ethno-mycology and identify selected wild edible mushrooms using phenotypic features.

b) To identify the characterized selected wild edible mushrooms using molecular techniques.

c) To develop grain propagation spawns from pure cultures of the identified wild edible mushrooms species.

1.6 Significance of the study

Characterisation will contribute to addition of indigenous edible mushrooms knowledge in Kenya, also provide information on the genetic characteristics which is important in selecting suitable strains for breeding purposes. Spawn propagation techniques will provide data on propagation feasibility.

CHAPTER TWO

LITERATURE REVIEW

2.1 Ecology and life cycle of mushrooms

Fungi are regarded as the second largest group of organisms in the biosphere after insects. Known fungal species constitute only about 5 % of their species in the world. Thus, the large majority of fungi are still unknown. Out of about 70,000 described species of fungi, it has been suggested that around 14,000-15,000 species produce fruiting bodies of sufficient size and suitable structure to be considered as macrofungi (mushrooms). Of these, about 5,000 species have varying degrees of edibility, and more than 2,000 species from 31 genera are regarded as prime edible mushrooms. Only 100-200 of them are experimentally grown, 50~100 economically cultivated, around 30-60 commercially cultivated, and only about 6-10 to have reached an industrial scale of production in many countries. Furthermore, about 1,800 have medicinal value. The number of poisonous mushrooms is relatively small (approximately 10%), and of these about 30 species are considered to be lethal (Miles and Chang, 1997; Chang and Mshigeni, 2004).

Mushrooms can be classified into three groups, namely the saprophytes, the parasites and the symbiotic (which include mycorrhizal) species. There are only a few parasitic mushrooms. Most of the cultivated gourmet mushrooms are saprophytic fungi. Some of the edible mushrooms are mycorrhizal species, e.g. Perigold black truffle, *Tuber melanosporum*, and matsutake mushroom, *Tricholoma matsutake*. It is difficult to bring these highly celebrated wild mushrooms species into cultivation because they are mycorrhizal (Miles and Chang, 1997; Chang and Mshigeni, 2004).

These species have a symbiotic relationship with some vegetation, particularly trees, that is there is a relationship of mutual need (Asian and Pacific Centre for Agricultural Engineering and Machinery: APCAEM, 2007). Saprophytes obtain nutrients from dead organic materials; parasites derive food substances from living plants and animals and causing harm to the hosts; and mycorrhiza live in a close physiological association with host plants and animals thereby forming a special partnership where each partner enjoys some vital benefits from the other. However, some mushrooms do not fall neatly within these man-made categories and can share two of these categories. For example, some *Ganoderma spp.* including *Ganoderma lucidum* are common saprophytes, however, they can be pathogenic too; *Tricholoma matsutake* while initially appearing to

be mycorrhizal on young roots, soon become pathogenic and finally exhibits some saprobic ability (APCAEM, 2007).

In nature, typically spores sprout from the gills, the thin tissue found on the underside of the mushroom cap as the cap fully expands due to maturity. Borne by wind, some kinds of spores are capable of travelling great distances from the fruit-body to start their own fungus colonies. The spores are produced in large numbers to guarantee the spread of the fungus in the environment (Halpern, 2006). If conditions are favourable (optimum temperature and moisture), the spores will germinate to form a mass of mycelium. This is the start of the vegetative phase of the mushroom (Figure 2.1). Given an unrestricted amount of nutrients and favourable growing conditions, it is capable of unlimited growth.

The mycelium developing from the germinating spore is the so-called primary mycelium and is usually uni-nucleate and haploid. This stage is short-lived because mycelia from different spores tend to ramify and fuse to form the secondary mycelium with two compatible nuclei, which continues to grow vegetatively and is able to form fruiting bodies (Stuntz *et al.,* 1978; Halpern, 2006).

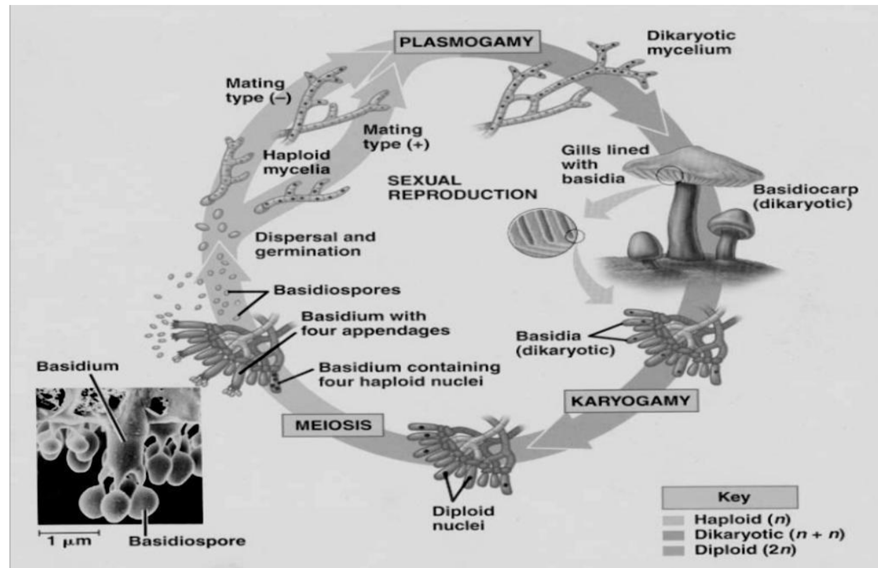

Figure 2.1 Life cycle of mushroom forming basidiomycetes (Carlson, 2008)

Due to lack of chlorophyll, mushrooms can feed on almost any organic substrate such as soil, wood rot, or food left for too long. However, how fast and large the mycelium grows depends on environmental factors such as soil temperature and the accessibility of food (Halpern, 2006).

2.2 Mushroom production

Mushroom cultivation is a worldwide practice. Global mushrooms production since 20th Century has showed a phenomenal growth pattern in tonnage. This is attributed to rapid - growth within the industrial activity (Chang and Miles, 1991).

The global economic value, although difficult to evaluate has been estimated to be more than 9.8 billion USD per annum (Chang, 1996).

This estimation included several species of eleven main genera namely *Agaricus*, *Lentinula*, *Volvariella*, *Pleurotus*, *Auricularia*, *Flammulina*, *Tremella*, *Hypsizygus*, *Pholiota*, *Grifola*, and *Hericium* (Chang, 1999b; Martínez-Carrera, 2000). Strong consumer demands due to their unique culinary, medicinal properties (Onyango *et al.,* 2011b) and threats of depletion of mushrooms are factors that have stimulated increased worldwide production in the past few decades.

Overall, world production of mushrooms is increasingly being dominated by species that are both edible and have medicinal properties. Only two of the major medicinal mushrooms, viz. *Ganoderma lucidum* and *Trametes (Coriolus) spp*. are distinctly inedible (National Research Centre for Mushroom: NRCM, 2003). Whereas, Asia contributed 63.6% of the total world mushroom tonnage, Europe, 14.3% and North America, 7.0%, both Africa and Latin America's shares were less than 1% (Chang, 1996). This is largely due to lack of know-how, lack of understanding that mushroom can play vital roles towards enhancing human health when used as dietary food supplements, lack of reliable sources of good quality mushroom spawn for supporting the efforts of local mushroom growers, lack of

venture capital to support mushroom farming entrepreneurs, and absence of systematic government support towards promoting mushroom farming as a valuable non-traditional new food and cash crop (comparable to coffee, tea, cotton, tobacco, etc.) (APCAEM, 2007; Sanjeev and Yash, 2011).

2.3 Characterisation of mushrooms

2.3.1 Classical approaches

The term mushroom is not a taxonomic division (Oyetayo, 2011), while modern identification of mushrooms is quickly becoming molecular the traditional taxonomy methods for identification are still used by most combined with microscopic examinations.

These classical approaches included; ecological growth requirements such as wood, soil, moisture, and morphological features of mushrooms such as colour, shape and size which gave rise to the designation of their respective names. Current phenotypic approaches still include the classical approaches with emphasis on micro-morphological features such as basidiospores or ascospores-shape, size and staining colour reaction (Tibuhwa, 2011). Srivastava and Soreng (2012) in an effort to domesticate wild edible mushrooms used phenotypic methods mostly used by the folk communities, to collect and macro-morphologically characterise them.

2.3.2 Modern approaches

One of approaches that are commonly used in studies of genetic diversity within and among populations or groups of individuals, and is applied with all types of markers and organisms, is based on comparisons of individual genotypes within and between populations (Dalirsefat *et al.,* 2009). In such cases, a genetic similarity (or dissimilarity) matrix constructed from all potential pair wise combinations of individuals are used to characterize population structure based on relative affinities of each individual to all other individuals tested. This approach requires suitable methods for evaluating similarity between individuals, and it is particularly useful in the case of possible linkages between different loci.

The choice of an appropriate coefficient of similarity is a very important and decisive point to evaluate clustering, true genetic similarity between individuals, analyzing diversity within populations and studying relationship between populations, because different similarity coefficients may yield conflicting results (Dalirsefat *et al.,* 2009). Molecular markers are commonly used to characterize genetic diversity within or between populations or groups of individuals because they typically detect high levels of Polymorphism (Dalirsefat *et al*., 2009; Haq, 2009).

Furthermore Random amplified polymorphic DNA (RAPDs) and Amplified fragment length polymorphism (AFLPs) are efficient in allowing multiple loci to be analysed for each individual in a single gel run.

In analysing banding patterns of molecular markers, the data typically are coded as (0,1)-vectors, 1 (One) indicating the presence and 0 (Zero) indicating the absence of a band at a specific position in the gel. With diploid organisms and codominant markers, such as Allozymes, RFLPs or SSRs, the banding patterns may be translated to homozygous or heterozygous genotypes at each locus and the allelic structure derived is utilized for comparison between individuals (Maguire *et al.,* 2002; Dalirsefat *et al.,* 2009). More often, however, the binary patterns obtained are used directly in comparisons of similarity of individuals.

Globally, the molecular revolution in fungal taxonomy commenced in the early 1990s, with analyses of PCR-amplified ribosomal RNA genes (Hibbett *et al*., 2007). Today, fungal molecular systematics is a mature discipline in which multi-locus datasets, extensive taxon sampling and rigorous analytical approaches are standard as reported by researchers in the mycological field: However, the combination of morphological studies and molecular phylogenetic analysis provides a good tool to understand the

systematics and species boundaries. However, phylogenies based on the coding genes of the ribosomal DNA strongly support several taxonomic groups within any genus (Menolli *et al*., 2010).

Atri *et al.* (2012a) studied taxonomy of *Coremiopleurotus* (*Pleurotus cystidiosus* O.K. Miller) using macro-micro morphological feature, cultural characteristic and molecular features using Internal Transcribed spacer (ITS) - sequencing and chemical analysis to ascertain nutraceutical attributes.

Menolli *et al*. (2010) used both morphological features of basidiomata and LSU rDNA gene to identify commercial isolates of *Pleurotus spp*. Khan *et al.* (2011) used both morphological and random amplified polymorphic DNA (RAPD) markers to characterise seven *Pleurotus* spp mushrooms and showed different pattern of genetic diversity among different *Pleurotus* species.

Kenya, has well documented vascular plant species and forest communities (Peltorinne, 2004). Mushroom forming fungi are poorly collected, sparingly studied and relatively underutilized (Onyango *et al.,* 2011).

Some studies on morphological characters and spawn production procedures of three Kenyan native strains of wood ear mushroom [*Auricularia auricula* (L. ex Hook.) Underw by Onyango *et al*. (2011) showed that, utilization of mushrooms collected from the wild required adequate description of useful phenetic features and domestication protocols.

Otieno (1968) used morphological features to further knowledge of termite fungi in East Africa. New species of *Termitomyces* were documented as well as some species which had been previously reported from the Congo (Heim, 1942; 1958).

2.4 Edible mushroom propagation technology

Intentional cultivation of edible mushrooms probably predate recorded history which is an indeed ancient one (Singh and Mishra, 2012). Empirical mushrooms cultivation methods were independently developed in China about 1,000~1100 AD for *Auricularia* spp. *Lentinula edodes* (Berk.) Pegler. Cultivation in France was before 1900 about 350 years ago for *Agaricus bisporus* (Lange) Imbach. During the last 50 years, these methods have been significantly improved and modern technologies permit the cultivation of about 20 species at different levels around the world (Chang and Miles, 1989).

Mushrooms can be cultivated through a variety of methods. Some methods are extremely simple and demand little or no technical expertise. On the other hand, cultivations which require aspects of sterile handling technology are much more technically demanding (Chang and Miles, 1989). Mushroom cultivation involves several different operations each of which must be performed properly if the enterprise is to be successful (Table 2.1).

Table 2. 1: Operations involved in mushroom cultivation

No	Operations involved
1.	Selection of mushroom spores or strains
2.	Raising and maintenance of mushroom mycelial cultures
3.	Development of spawn/Inoculum
4.	Preparation of growing (substrate) medium
5.	Spawn inoculation and colonisation of substrate
6.	Crop management for mushroom production
7.	Post-harvest handling

(Source: Chang and Miles, 1989)

While step (1), (2) and (3) are more or less common and similar for most of the mushrooms, it is the substrate preparation, crop raising, and post-harvest technology which vary with the type of mushroom (Marcel, 2004; Mshandete and Cuff, 2008;). However, propagation technologies depend on the existing natural conditions, the applied solutions vary widely, starting with the traditional empiric ones and reaching up to the computer-assisted ones (Zăgrean, 2011).

2.4.1 Strain selection and maintenance

This is the first stage in mushroom cultivation (Stamets, 1993). Pure cultures are prepared either from single or multispore or tissue from a mushroom of a high yielding and vigorous strain; the former is suitable for obtaining fruiting cultures of homothallic species such as *Agaricus bisporus* but is not a suitable technique for heterothallic species (Marcel, 2004).

Culturing tissues can be derived from stipe or (cap) pileus of the mushrooms following surface sterilization. The tissue is transferred onto sterile growth medium; both homothallic and heterothallic species can be used to raise fruiting cultures *http://www.ia-micron.com/chapt4.pdf.* For single or multispore culture, a healthy and mature mushroom is first washed in sterile water, surface-sterilized with alcohol, and is placed on a spiral wire loop kept in sterile petriplate covered with a beaker (Marcel, 2004). This process is not feasible in most laboratories.

Pure cultures have been traditionally maintained by periodic sub-culturing and/or cold storing between 2-5°C which is costly and time consuming (Smith and Onions, 1983). However, modern technologies for maintenance of vigour and genetic characteristics especially productivity and quality are advisable (Chang and Miles, 1989).

A strain maintenance method depends on factors such as requirements, resources, cost (Smith and Onions, 1983). It is advisable that each mushroom strain should be maintained by at least two different methods. Liquid nitrogen and mineral oil preservation have been found highly suitable and are popular for preservation of mushroom cultures (Smith and Onions, 1983; Jong, 1989; Smith and Kolkowski, 1996; Singh and Upadhyay, 2002).

2.4.2 Spawn production

The term "spawn" is used for vegetative growth of mushroom mycelium on a suitable medium, to be used as inoculum or "seed" for the substrate in mushroom cultivation. Spawn production is a fermentation process in which the mushroom mycelium will be increased by growing through a solid organic matrix under controlled environmental conditions. In almost all cases the organic matrix will be sterilised grain, such as sorghum, millet, rye or wheat (Sinden, 1932; 1937; Marcel, 2004). The purpose of the grain spawn is to boost the mycelium to a state of vigour such that it will rapidly colonise the selected bulk growing substrate.

The grain is an important nutrient support as well as a vehicle for the eventual even distribution into the growing medium of the mushroom inoculant.

Each individual grain becomes coated with the mycelium and in fact becomes a mycelial capsule. All operations from pure culture isolation through spawn preparation must be conducted under sterile techniques and performed as rapidly as possible to lessen the possibility for contamination to occur. An extensive technology has been developed throughout the world to ensure the production of high quality mushroom spawn (Sinden, 1932; 1937; Marcel, 2004).

2.4.3 Mushroom production techniques

The historical development of mushrooms cultivation techniques started with log cultivation in early 1950s and changed over to bottle cultivation in early 1960s and in polypropylene bags and stainless steel trays in 1970s. In Kenya at present, most production of king oyster, white button and porta bella are on sawdust, wheat straws, sugar bagasse or corn cob substrates contained in polypropylene bags (Sinden, 1932; 1937; Marcel, 2004).

2.4.3.1 Log culture

This method is applicable to mushroom species which grow as saprophytes. Logs are cut from fast growing deciduous species (which have thick outer bark) and inoculated with spores or with mycelial plugs inserted into drilled holes and then stacked in piles with mild to heavy

soaking. Mycelial growth through the log occurs over several months and the logs then placed in an upright position partly embedded in the soil.

The advantage of log culture is that it is a simple and natural method but with the disadvantages that the process is labour-intensive and slow in comparison to growing mushrooms in sterilised sawdust mixtures. Log cultivation is not technically demanding and is relatively easy to carry out but is seasonal and cannot meet demands for high productivity. Mushroom production primarily occurs in the cooler spring and autumn months. Since this is an open, non-sterile procedure contamination with other wood rotting mushroom species can occur (Kwon, 2004).

2.4.3.2 Bottle cultivation

A bottle cultivation system favours the small particle-sized growth media types such as sawdust, spent grains and grain hulls. Prepared and moisture-conditioned substrate mixture is loaded into the bottle feeder. Through the feeder, bottles are filled with the preset quantity of mixture. Once bottles are filled, compactors press the mixture in the bottle down to the pre-set height and hole-makers go through the compacted mixture. Proper compaction gives the substrate high density, which means more nutrients will be available to mycelia and thus produce a higher yield.

Vertical holes in the bottle permit even distribution of mushroom spawn to the bottom, which allows for fast, even colonization. Fast depletion of nutrients in the substrate, in turn, leads to early fruiting (Kwon, 2004).

The system, however, might be impractical for growers who use pasteurized bulk substrates or composted substrates that are not appropriate for bottling. In addition, the initial set-up cost of the system may be too high for many small-scale growers to adopt. Still, some growers may be able to develop some viable ideas from this up-to-date growing method (Kwon, 2004).

2.4.3.3 Enriched sawdust culture

This is an intensive and regulated cultivation technique developed in several Asian laboratories over the last 2 - 3 decades. In this innovative approach various hardwood sawdust or wood chips supplemented with nitrogen-rich additives such as rice bran and other cereal brans are mixed together and then compacted into special autoclavable polypropylene bags of various dimensions (Stamets, 1993, Yamanake, 1997; Stamets, 2000; Kwon, 2004). The bags are then autoclaved to ensure complete internal sterility, allowed to cool to 20°C and then aseptically inoculated with the desired amount of spawn.

This stage demands complete sterile handling techniques and any relaxation of standards allows microbial contamination with concomitant financial losses. The inoculated bags are moved to growing rooms with computer controlled environments giving accurate humidity and temperature conditions. Following inoculation, the bags are stacked on trays or suspended from wires for several weeks during which time the mycelium grows through the sawdust mix secreting enzymes which degrade the complex macromolecules of the substrate lignin, cellulose, hemicellulose the breakdown products being absorbed by the advancing mycelium. When the mycelium has reached maturity, the bag is given a cold temperature shock for 12 - 24 h, restacked and the bag opened and within a few days the mushrooms develop. Overall, this new method greatly shortens the production time and gives much higher yields. Using natural log cultivation the time from spawning to harvesting of mushrooms can be between 8 months to one year with complete exhaustion of the log up to 3 years. In contrast, with the synthetic log, mushrooms can be harvested about 80 days after spawning and completion of economic production from then takes less than 6 - 8 weeks.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

The study was carried out in Ragati, Kabage, Zaina, Zuti and Mathioya reserves within Aberdare National Forest between 7th and 9th August 2012 (Figure 3.1). Aberdare National Forest lies within the Aberdare ranges which are isolated volcanic range forming the eastern wall of the rift valley, and running roughly 100 km North-South between Nairobi and Thomsons falls. They are situated to the west of Mount Kenya, in Nyeri County. The ranges cover 766 - 767 km^2, altitude 1829 - 4001 m above sea level. The topography is quite diverse with deep ravines that cut through the forested Eastern and Western slopes. They have red volcanic soils rich in organic matter. The park is surrounded by a predominantly indigenous forest with climatic conditions of mist and rain occurring throughout much of the year. Precipitation varies from around 1000 mm yearly on the north western slopes to as much as 3000 mm in the south east (Kenya Forest Services: KFS, 2012).

3.2 Ethnomycological study

Semi-structured questionnaires were used to collect data from informants within Aberdare National Forest Reserve community (Figure 3.1) between 7th and 9th August, 2012.

Information sought was on historical background, traditional usage, edibility status, folk taxonomy, methods of preservation and commercial importance of mushrooms as described in structured questionnaire (Appendix 1).

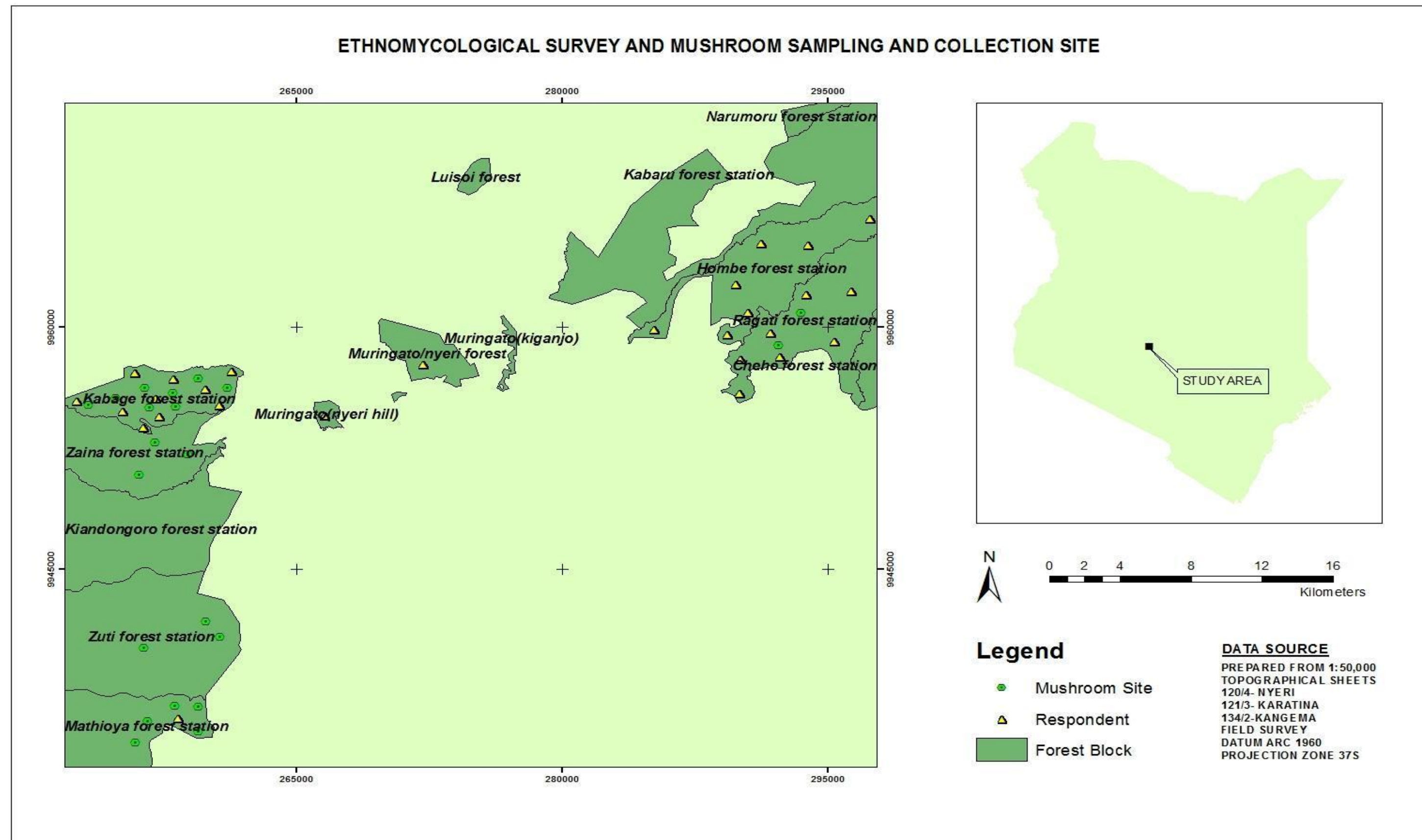

Figure 3. 1 Ethno-mycological survey and mushrooms sampling and collection sites

3.3 Sampling and Collection

Mushrooms samples were expediently collected in company of local mushrooms collectors. Ethno-mycological survey was done concurrently with collection of mushrooms in a completely randomly design from each habitat. Each collection points were recorded using a Garmin *e*-trex-20-GPS (Garmin, Kansas, USA) global positioning system. Each observed mushroom was photographed *in-situ* using DSC~W550 Cyber Shot Sony 14.1 Mega Pixel (Sony Corporation, Tokyo, JAPAN) prior to picking from its substrate. Picking was done with the aid of scalpel and in special cases a knife was used to uproot the long rooted mushroom.

The collected mushrooms were packed into digestible paper bag, which were labelled with collection/accession number, location co-ordinates, collection date and name of collector. Appendix 2 shows detailed collection protocol which was filled on spot for field identification features as outlined by Mueller *et al*. (2004). Sporocarps exhibiting a range of developmental stages were examined for macro-morphological characters. Tissues for cultures were taken and spore prints set up. Some specimens were oven dried at 50°C for 8 hours for microscopy work while others were packed in paper bags for further characterization in the laboratory (Lorge *et al*., 2004).

Three representative mushrooms accessions were selected for both characterization and propagation studies as outlined in Appendix 2, 3 and 4.

3.4 Macro and micro morphological characterization

3.4.1 Macro-morphological studies

Mushrooms exhibiting a range of developmental stages were examined for macro-morphological characters including; cap, stipe, ring, hymenia and spore print colour determined by eye observation; additional surface features such as scales, a X3 hand lens was used for exhaustive observation.

The following features were observed for the cap; size (Diameter in cm); shape (apex, margin); cap flesh (cross sectional view); colour of cap; texture of surface and features; whether surface was dry or wet; latex presence and finally colour of latex if present. For stipe the following features were examined; size (Length cm) from smallest to largest in-case of a collection; shape (cross sectional and longitudinal view); shape of base; attachment to cap; texture of stipe and distribution of other surface features; stipe colour; stipe consistency; base attachment to substrate; stipe flesh; stipe annulus/Ring (presence/absence, shape, size, consistency, colour, position, attachment).

The structure, colour, margin colour; gills attachment to stipe; spacing; thickness (side view); margin of edges and lamellulae presence (short gills which extend only part of the way from the cap margin to the stipe) were observed for hymenia (Leonard, 2010; Lorge *et al*., 2004).

3.4.2 Spore prints set up

For mushroom specimens which had a distinct cap and stem, the cap was removed and placed with fertile-side down on Petri-plate and allowed spores to drop overnight. A commercial colour chart was used to determine observed colours. The caps were then covered with a bowl to prevent air currents and left over night (12 hours). The prints were necessary to determine overall spore colour and also source of mature spores for microscopic examination and measurements.

3.4.3 Micro-morphological studies

3.4.3.1 Spore microscopy

A clean, dry surgical blade was used to scrape lightly the spore print and tapped off the spores onto a clean stained slide. Three stains; Congo red, Melzers reagent (White *et al.,* 1993) and cotton blue in lactic acid were used for observation under microscope.

A cover slip was gently placed on top. Observations were made at x20, x40 and x100 magnification of bright field Multipurpose Biological microscope (XSZ-21 Series-Ningbo Shengheng Optics and Electronics Co., Ltd: Gao Qiao Town: China). Measurements were made by using graduated ocular lens.

Calibration was achieved by counting the number of eyepiece divisions that correspond to usually 10 µm divisions on a stage micrometre which was placed in the position normally occupied by a slide. 1µm = 0.000001 m (m x 10^{-6}). Sizes were measured to the nearest 0.5µm. Measurements of spores in focus (length and width) was done on at least 30 mature spores and recorded the dimensions of the smallest and largest spores found. In cases of aberrant spores, their dimensions were excluded from counting. Finally the dimensions of the spores were expressed as Mean±SD for two ranges of possibilities in µm (most narrow to the broadest spore) and (shortest to the longest spore observed). These phenetic and microscopic features were used in taxonomy guided by a taxonomy key (Appendix 5).

3.5 Development of tissue propagation cultures

Experiments were done under a laminar flow where aseptic conditions were maintained by thoroughly cleaning the hood using cotton swabs soaked in 70 % ethanol after which the fan and UV light were switched on for 30 minutes to sterilize the working chamber. Culture media was prepared according to the manufacturer's instructions; 39, 50 and 23 g of PDA, MEA and YEA, respectively, was added into 1 litre beaker containing distilled water and boiled. This was put into autoclavable bottles and then sterilized at 121°C for 15 minutes for PDA and YEA while MEA was sterilised at 115°C for 10 minutes. The media was aseptically poured into sterile disposable Petri plates (85 x 15 mm) and quickly covered using Petri plate lids and allowed to cool and solidify. Fresh healthy representative sample of clade's 1, 2 and 3 were selected for tissues sectioning for cultures. Small tissue sections (about 2×2 mm^2) were cut using sterile surgical blades from inner surfaces of the cap (oftenly between the stipe and cap joint) cleaned with 70% ethanol. Cut fragments were placed in the middle surface of the media, then covered with a plate lid and tightly sealed with a parafilm. Inoculated dishes were labelled with same accession numbers as the sample. Cultured plates were incubated in dark sterile cabinets at 25°C to enable mycelia establishment. Sub culturing was done onto a fresh media until pure cultures were obtained.

3.6 Molecular characterisation

3.6.1 Genomic DNA isolation from mushrooms fruiting bodies

Total genomic DNA was extracted from eleven dried mushrooms fruiting bodies using modified *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB) method of DNA extraction (Borges *et al.,* 2009, Hariprakash *et al.,* 2010; Nasim *et al.,* 2010). The fruiting bodies were immersed in liquid nitrogen and crushed using mortar and pestle. Approximately, ~1-2 g of powder was put into 1.5 ml eppendorf tubes and re-suspended in 65°C pre-warmed 400 µL of extraction buffer (100 mM Tris-HCl (pH 7.5), 2% (wt/vol) cTAB, 50 mM EDTA, 5M NaCl, 14M (vol/vol) β-mercaptoethanol and 2% (w/v) PVP) and incubated for 1 h at 65°C in water bath with continuous shaking and mixing by inverting at 15 min interval. Tubes were removed and allowed to cool for 10 min. To this preparation, 500 ml of chloroform : isoamyl alcohol (24:1 Vol / Vol) was added. Two phases were observed upon mixing by gently inverting the tubes 10 times. The resultant emulsion was disrupted by centrifugation at 3500 rpm for 10 min at room temperature (MSE minor centrifuge, England, London). The upper aqueous phase was transferred into freshly labelled new 1.5 mL tubes and then 50 µL of NaOAc (5.2) followed by 400 µL ice cold 100% isopropanol was added and left to precipitate for overnight at -20°C. The precipitates were centrifuged at 3500 rpm for 10 min at room temperature and supernatant discarded.

Pellets were then air-dried by inverting the tubes for 5 min and washed twice with equal volumes (400 µL) of 70 % ethanol. The DNA pellets were completely dried by inverting the tubes and re-suspended in 100 µL of double distilled water and left to dissolve overnight. Two microliters of DNAse free RNAse A [10mg/ml] were added to each tube and incubated for 30 min at 37°C in water bath. The resulting purified DNA was stored at -20°C for further analyses.

3.6.2 DNA standardization

3.6.2.1 Determination of DNA purity [Quantity and Quality]

DNA concentration and purity was determined by loading 1 µL of DNA samples onto a NanoDrop-2000 spectrophotometer at wave length 260 and 280 nm. The DNA concentration was calculated using the formula below;

(DNA) = Optical density x dilution factor x constant (50 µg/ml).

Purity was determined using the 260/280 and 260/230 nm ratio. DNA samples were diluted to a working concentration of 50 ng / μL^{-1} and stored at 4ºC, as per the laboratory procedure.

3.6.2.2 Visualisation of genomic DNA bands

Agarose gel (0.8%) was prepared by melting 0.8 g of agarose in 100 ml of 0.5X TBE (0.89 M Tris base, 0.89M Boric acid, 20 mM EDTA pH 8.0) in a microwave until completely dissolved. GelRed™ (visualising dye 2.5 µL) was added and content allowed to cool while stirring (Borges *et al.,* 2009; Nasim *et al.,* 2010). The gel was cast in a tray with fixed comb and allowed to set for 20 min at room temperature on a flat surface. Three micro litres of genomic DNA were mixed with 3 µL 2X loading dye (Orange dye) and loaded into the wells. This was run alongside 6 µL lambda (λ) DNA for 60 min at 70 V. The resulting DNA bands on the gel were exposed to UV light (UVP-Entela, U.S.A) and photographed using a Sony WD 550 Cyber Shot Camera (Sony Corporation, Tokyo, Japan).

3.6.3 Genomic DNA amplification with ITS and nLSU primers

Genomic DNA isolated from mycelia was diluted 1:100 using sterile milli-Q water. The ITS and nLSU regions of rDNA were amplified using forward and reverses primers. ITS5 (5' GGAAGTAAAAGTCGTAACAAGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') were used to amplify the ITS region while LROR (5'-ACCCGCTGAACTTAAGC-3') and LR16 (5'-TTCCACCCAAACACTCG-3') amplified nLSU regions.

A master mix was prepared using 0.8 μM of each Forward and Reverse primers, 16.4 μL sterile Milli-Q water and 2 μL DNA (50 ng/μL). All added to Bioneer-PCR premix (Accupower premix Cat No. K2012). The mixture was amplified in Gene Amp PCR system 9700 thermocycler. A control with no DNA was done in every series of amplification to test for the presence of contamination of reagents and or reaction buffers. Thirty cycles of PCR were performed in a thermocycler fitted with a heated lid using modified temperature profiles (Muruke *et al.,* 2002). Thermocycling scheme is outlined in Appendix 6.

3.6.4 Agarose gel analysis of PCR amplicons

The size of amplified DNA was examined by gel electrophoresis. Several modifications were made on Muruke *et al.* (2002) protocol. Agarose gel 1.8 % was prepared in 0.5 x TBE buffer. GelRed™ was used as visualising dye. Loading was done with 3 μL PCR products mixed with 3 μL loading dye (2 x) in 0.5 x TBE buffer. A size marker lane containing 6 μL of 1 kb+ (plus) DNA ladder was included in every gel. The gel was run for about 60 min at 100 V. Bands pattern were visualised on a UV trans-illuminator and photographed.

3.6.5 Sequencing of amplicons

Prior to sequencing, the PCR products (amplicons) were cleaned by precipitating. The amplicons were transferred into 0.5 ml eppendorf tubes, $1/10^{th}$ volume of 3 M NaOAc (5.2) and 2.5 volumes of 100 % (absolute) ethanol was added. This was thoroughly mixed by vortexing gently. Centrifuging at 14,000 RPM for 20 min at 4 °C followed. The supernatant was carefully discarded and pellets air dried by inverting on tissue paper for 1 min. This was followed by addition of 300 µL 70 % ethanol and gentle vortexing for homogenity. Centrifugation at 14,000 rpm for 15 min at 4 °C. The supernatant was carefully discarded and pellets air dried by inverting on tissue paper for 2 min. The pellets were suspended in 20 µL of nuclease free water. The contents were gently vortexed 10 times with even interval, followed by brief (30 s) centrifugation, incubation at 65 °C for 5 min, vortex at low speed 10 times with even interval, centrifuge briefly and placed samples on ice. The concentration of purified PCR products was determined using Nano drop 2000c spectrophotometer. Concentrations above 25 ng / µL were considered enough for sequencing using Big dye Terminator method with ABI 3730 *xL* Genetic Analyzer (Applied Biosystem).

3.6.6 Agarose gel analysis of purified PCR amplicons

A total of 3 µL of purified/cleaned PCR products mixed with 3 µL of 2 x loading dye were used in gel analysis. Agarose gel of 1.8 % was prepared with 100 ml 0.5 x TBE buffer and GelRed™ was used as visualising dye. This was run at 100V for 60 min against 6 µL of 1 kb+ (plus) DNA ladder. The image was photographed for determination of size and integrity of the amplicons.

3.7 Screening for optimal growth conditions for spawn development

Modified methods of Imatiaj *et al.* (2008) and Jayasinghe *et al.* (2008) were used; three different culture media Potato Dextrose Agar, Malt Extract Agar and Yeast Extract Agar (PDA, MEA and YEA) were prepared to investigate the mycelial growth of the strains. Media was prepared by dissolving 39, 23, 50 g of PDA, YEA and MEA respectively in 1 litre distilled water, dissolving and sterilizing at 121°C for 15 min for PDA and YEA and at 115°C for 10 min for MEA. Optimum *p*H for mycelial growth was obtained by evaluating media *p*H levels to 5, 6, 7 and 8 for all medias', subject to control (manufacturers *p*H ±0.2) with the addition of 1 N NaOH or HCl. *p*H was adjusted using Milwaukee (SM101) *p*H meter with accuracy of ±0.02.

The *p*H adjusted molten media was aseptically poured into sterile Petri plates and allowed to solidify. Nine (9) mm diameter plug of an inoculum was taken from 10 days old culture grown on PDA and placed at centre of each plate of the 3 different culture media. Culture morphology was made on density, texture, colour, presence of aerial hyphae and type of colony growth including pattern and presence of exudates. Growth rate was determined at 25 °C, 30 °C, and 35 °C in dark conditions. This experiment was done in triplicate.

3.8 Development of grain propagation spawns

3.8.1 Spawn production using optimal conditions of *p*H, media and temperature

The modified method of Onyango *et al.* (2011a) was used for spawn development. Sorghum and millet formulations, supplemented with wheat bran and protein additive branded Koppa-kula at varying combinations were tested to determine their suitability for spawn production. Mycelia obtained from 10 days old cultures incubated at optimal conditions were used to develop grain spawns. The grains were boiled in fresh clean water (1:1.5 w/v) for 30 min to soften before being utilized for spawn production. A total of nine different treatments (150 g each at grains ratio; 1, 1:1, 4:1 and 2:2:1 were used) see Appendix 7. The weighed grains, wheat bran and koppa kula were then thoroughly mixed by hand.

Each grain formulation (150 g) was mixed with calcium carbonate powder at a ratio of 3 g / kg to regulate *p*H. To produce grain spawn of 50 % moisture level, water was added at a rate of 25 ml per 150 g formulation. Grain combinations were put in 500 ml heat resistant glass bottles and autoclaved (Wisconsin Aluminum Foundry Co., Inc; Manitowoc) for 15 min at 121°C. Bottles were allowed to cool in a sterile lamina flow hood after shaking them to loosen and evenly distribute the grains. Ten 9 mm agar pieces were carefully transferred to upper surfaces of prepared grains for each grain combination. Inoculated grain bottles were tightly secured using moist cotton wool and covered with sterile aluminium foil and bottle lids. They were incubated in dark at optimal temperature until they were fully colonized. Spawn production experiments were laid out in a completely randomized design (CRD) and replicated three times.

3.9 Data analysis

The data obtained from ethno-mycological study and morphological characterisation was scored and analysed using statistical software (SPSS-V16) for similarity and dissimilarity and dendrogram constructed, while all the numerical data of both tissue and spore cultures plus compatibility determinations of the cultures, in terms of growth rate (mm / 24 h) was expressed in Mean ± SD and analysed using students *t*-test for significance difference between the means of individual *p*H, values at $p \leq 0.05$.

The partial ITS and nLSU [rDNA] sequences were assembled for each sample using the forward and reverse primers. The sequences were aligned using the ClustalW2 multiple alignment program *http://www.ebi.ac.uk/Tools/clustalw2*. Sequences were submitted to National Centre for Biotechnology Information (NCBI); BOLD and UNITE databases for identification of the strains.

CHAPTER FOUR

RESULTS

4.1 Ethno-botanical cultural background

The plant species and vegetation in the areas sampled were identified through the botanical inventory carried out by Kenya Forest Service team. The area was dominated by closed canopy highland forest with low alpha-diversity (low diversity within community). Beta-diversity (species diversity generated through habitat diversity) was relatively low with many additional communities being anthopogenic, such as forest degraded to scrub through farming. Wetlands/swampy area had been reclaimed into grassland. The forest indigenous vegetation included; *Vitex kiniensis, Albizia gumifera, Croton megalocapus, Olea europaea, Polycias kikuyuensis, Cordia africana, Prunus africana, Cassiporee molosonia and Juniperus procera* while the plantation forest had the C*oppressus lustanica* and *Croton macrostachyus.* The indigenous area was most rich in both vegetation and mushrooms species such as *Auricularia* spp*, Pleurotus* spp*, Agaricus* spp*, Ganoderma* spp among other unknown species. Forest reserves on waterlogged soils had different vegetation, less species-rich, mostly shrubs than on well drained soils. A distinctive riparian community is found along river banks. All species-rich forest reserves had both old growth and mature second growth.

4.2 Ethno-mycological survey

Twenty seven respondents were interviewed for ethno-mycological study (Tables 4.1 – 4.5). Traditional knowledge (Table 4.1), the local inhabitants have accumulated a large quantity of traditional knowledge and experience in utilization of the wild edible mushroom resources. They are well familiar about the morphological features, habitats, qualities of various edible mushrooms. It was observed that male respondents were more knowledgeable than females. The respondents also reported that the ecological growth requirements such as wood, soil, moisture, and morphological features of mushrooms such as colour, shape and size gave rise to the designation of their respective names.

Table 4. 1: Indigenous knowledge findings for respondents around Aberdare National forest Reserve

	Indigenous knowledge			
Respondent age	**Good**	**Poor**	**Row total**	**P≤0.05**
<20	2(10%)	0	2	*P*=0.51
21-40	12(63%)	7(87.5)	19	
>41	5(26%)	1(12.5%)	6	
Column total	19	8	27	
Gender				
Male	14(63.6%)	4(80%)	18	*P*=0.05
Female	8(36.4%)	1(20%)	9	
Column total	22	5	27	

The results are expressed as percentages at P≤0.05 for 27 respondents

Collection season (Table 4.2), according to the respondents the frequency and diversity of fructification of mushrooms depended upon various factors such as temperature, percentage of rainfall, humidity among other factors. It was reported to occur mostly during rainy seasons.

Table 4. 2: Collection seasons findings for respondents around Aberdare National forest Reserve

Collection season					
Respondent age	**Rain season**	**All year**	**Don't know**	**Row total**	**P≤0.05**
<20	2 (8.7%)	0	0	2	*P*=0.16
21-40	17(73.9%)	0	2(100%)	19	
>41	4 (17.4%)	2(100%)	0	6	
Column total	23	2	2	27	
Gender					
Male	12(54.5%)	2(50%)	1(100%)	15	*P*=1
Female	10(45.5%)	2(50%)	0	12	
Column total	22	4	1	27	

The results are expressed as percentages at P≤0.05 for 27 respondents

Consumption (Table 4.3), the use of mushrooms as accessible folk food predates all other usage underpinning their economic importance to humans. This was reported by all respondents. Also great inclusion of wild edible mushrooms as part of their daily meals was supported by males more than the female respondents.

Table 4. 3: Consumption findings for respondents around Aberdare National forest Reserve

	Consumption			
Respondent age	**Edible**	**Non-edible**	**Row total**	**P≤0.05**
<20	2(9.5%)	0	2	P=0.99
21-40	14(66.7%)	5(83.3%)	19	
>41	5(23.8%)	1(16.7%)	6	
Column total	21	6	27	
Gender				
Male	13(61.9%)	2(33.3%)	15	P=0.18
Female	8(38.1%)	4(66.7%)	12	
Column total	21	6	27	

The results are expressed as percentages at P≤0.05 for 27 respondents

Preservation methods (Table 4.4), the most reliable method portrayed by respondents was sun-drying. The local people hardly preserved mushrooms for culinary uses.

Table 4. 4: Preservation methods findings for respondents around Aberdare National forest Reserve

	Preservation methods			
Respondent age	**Sun-dried**	**Not dried**	**Row total**	**P≤0.05**
<20	0	2(8.7%)	2	P=0.45
21-40	2(50%)	17(73.9%)	19	
>41	2(50%)	4(17.4%)	6	
Column total	4	23	27	
Gender				
Male	5(71.4%)	10(50%)	15	P=0.41
Female	2(28.6%)	10(50%)	12	
Column total	7	20	27	

The results are expressed as percentages at P≤0.05 for 27 respondents

Market/selling (Table 4.5), surplus dried and fresh mushrooms were found to be sold irrespective of the variety. However, this was not supported by many respondents. According to locals the rainy season is the favourable time for the collection of wild edible mushroom. Although some mushrooms were appearing all seasons.

Table 4. 5: Market findings for respondents around Aberdare National forest Reserve

	Market			
Respondent age	**Sold**	**Not sold**	**Row total**	**P≤0.05**
<20	0	2(8.3%)	2	P=0.99
21-40	2(66.7%)	17(70.8%)	19	
>41	1(33.3%)	5(20.8%)	6	
Column total	3	24	27	
Gender				
Male	2(50%)	13(56.5%)	15	P=0.4
Female	2(50%)	10(44.5%)	12	
Column total	4	23	27	

The results are expressed as percentages at P≤0.05 for 27 respondents

4.3 Mushroom habitat characteristics

The mushrooms samples collected were distinctively grouped (named clades) into three based on their habitat and morphological characteristics (Plate 4.1; Figure 4.1; Table 4.6). Fruit bodies of Clade1 were found growing in gregarious manner. The surrounding vegetation was *Vitex kiniensis* commonly known as Meru Oak. Clade 2 accessions were found growing in Caespitose and gregarious manner, in mixed forest (plantation, grassland and indigenous) blocks.

The vegetation included *Copressus lustanica* of the family *Copressuceae* for plantation and *Vitex kiniensis, Croton macrostachyus, Albizia gummifera, Olea europaea, Polycia kikuyuensis, Cordia africana, Prunus africana, Cassipuree molosonia, Juniperus procera and Croton megalocapus*. Accession in Clade 3 were growing singly (solitary) on soil. The surrounding vegetation was plantation of *Copressus lustanica.*

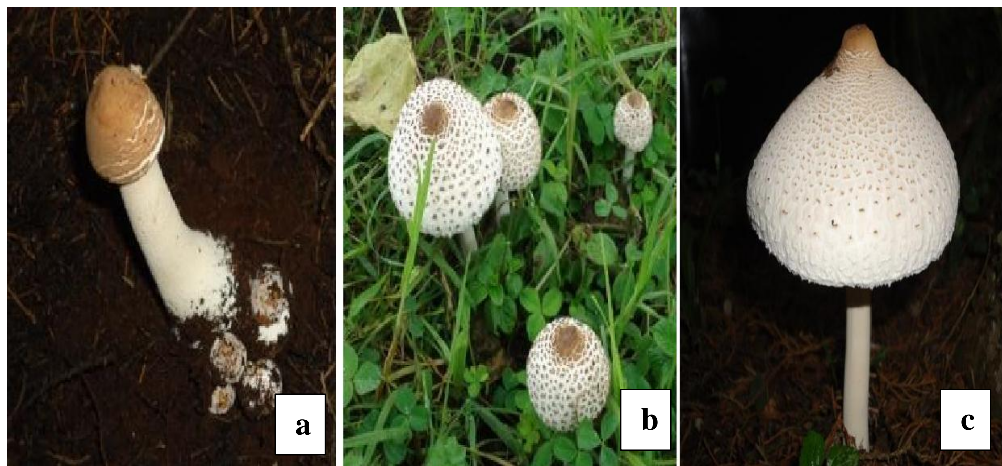

Plate 4. 1: Panels A, B and C represents mushrooms samples natural habitat at Aberdare National forest Reserve. A- Caespitose B- Gregarious and C- Solitary growth

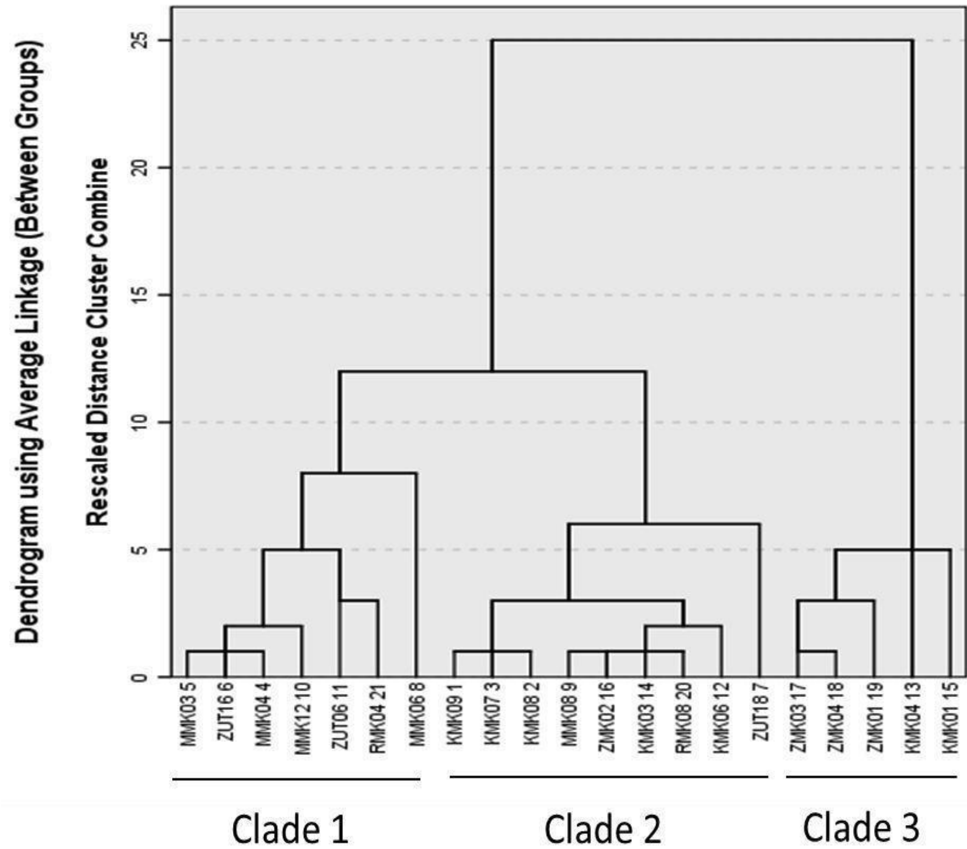

Figure 4. 1 Cluster dendogram illustrating morphological diversity between mushroom accessions collected from Aberdare National forest Reserve. The scale shows dissimilarity distance at 95% confidence limits and dissimilarity index of 0.38

Table 4. 6: Natural habitation characteristics of the edible mushroom accessions collected in Aberdare National forest Reserve

	Characteristics			
Accession	**Growth**	**Substrate**	**Type of forest**	**Vegetation**
Clade 1	Gregarious	Soil	Indigenous	*Vitex kiniensis*
Clade 2	Caespitose/ Gregarious	Soil	Mixed	*Croton megalocapus/Vitex Kiniensis*
Clade 3	Solitary	Soil	Plantation	*Copressus lustanica*

4.4 Characterisation of mushrooms fruiting bodies

4.4.1 Morphological characteristics of mushroom accessions

The cap of mature fruit-body when fresh ranged 100 - 215 mm in diameter while the young fruit bodies were 25 mm. The young caps were closed and possed parabolic to oval shape. The middle aged fruiting bodies caps were convex with appressed apex and the mature caps were applanate (plano-spread) with a small, rounded umbo dark brown to brown in colour while aging. The surfaces were covered with clearly visible brownish tile like arranged scales. The surfaces at the middle (apex) were smooth. The edges of caps were slightly tomentous to entire for accessions in clade 1; clade 2 and 3. The young fruit bodies had in-rolled margins. Clade 1 had thin fruit body flesh while clade 2 and clade 3 had thick flesh. Two main basidiocarps colours were observed, these included whitish (accessions in clade 2 and 3) and brownish (accessions in clade 1). However, some mushrooms accessions bore lighter or darker shades of these colours and were grouped together with the predominant colour (Table 4.7).

Table 4. 7: Morphological characteristics of the cap of the edible mushrooms accessions collected from Aberdare National forest Reserve

Accession No.	Clade1	Clade2	Clade3
Shape	Convex	Convex-plano	Convex-plano
Colour	Brownish	Whitish grey	Whitish grey
Surface features	Squamulose	Squamulose	Squamulose
Apex	Appressed	Umbonate	Umbonate
Margin	Entire	Entire	Entire
Diameter	1 Cm	11.5 Cm	10 Cm
Flesh	Thin	Thick	Thick

The stipes were slim, cylindrical, hollow inside, bulbous at the base except for clade 1 accessions which was un-swollen, 25-270 mm high and 10 mm wide. The base had maximum diameter of up to 40 mm. Colour was whitish (Table 4.8), with small brownish scales characterised by zigzag pattern (This occurred on some accessions) especially mature accessions. They were centrally attached to the cap, fibrous with simple base attachment to the host substrate.

Table 4. 8: Morphological characteristics of the stipe of the edible mushrooms accessions collected from Aberdare National forest Reserve

Accession No	Clade1	Clade2	Clade3
Attachment to cap	Central	Central	Central
Colour	White	White	White
Length	30 mm	125 mm	150 mm
Longitudinal Shape	Cylindrical	Cylindrical	Cylindrical
Base shape	Un-swollen	Bulbous	Bulbous
Surface features	Smooth	Smooth	Smooth
Consistency	Fibrous	Fibrous	Fibrous
Base attachment	Simple	Simple	Simple
Flesh	Hollow	Hollow	Hollow

The remnants of complete velum made on the stipe a double ring, which was relatively stable, and was observable on mature accessions only. The rings were positioned towards the stipe top and were movable along the stipe. Accession in clade1 lacked a ring. The flesh was white when fresh and cream when dry. Ring size ranged between 1-20 mm with rubbery texture. It was membranous for clade 2 accessions and upturned for accession in clade 3 (Table 4.9). Ring was only observable on all accessions with convex to plano basidiocarps.

Table 4. 9: Morphological characteristics of the ring of the edible mushrooms accessions collected from Aberdare National forest Reserve

Accession No.	Clade1	Clade2	Clade3
Present/absent	Absent	Present	Present
Shape	-	Membranous	Up turned
Size	-	2 mm	15 mm
Consistency	-	Rubbery	Rubbery
Colour	-	White	White
Position on stipe	-	Top	Top
Attachment to stipe	-	Movable	Movable

The hymenophore was composed of the lamellae and lamellulae in intercalcated version except for all young accessions which had their cap closed. The lamellae were crowded, bulging out and free except for accessions in clade 1 which had adnexed lamellae. Both lamellae and their margins were white except for clade 1 which bore brown shades. Accessions in clade 3 had thin gills while clade 1 and 2 had broad lamellae, all of which were entire (Table 4.10).

Table 4. 10Table 4.10: Morphological characteristics of the Hymenia of the mushrooms accessions collected from Aberdare National Forest Reserve

Accession No.	Clade1	Clade2	Clade3
Structure	Gills	Gills	Gills
Attachment to stipe	Adnexed	Free	Free
Gills colour	Brown	Whitish	Cream
Margin texture	Entire	Entire	Entire
Margin colour	Brown	Whitish	Cream
Lamellulae	Intercalcated	Intercalcated	Intercalcated
Thickness	Broad	Broad	Thin
Spacing	Spaced	Crowded	Crowded

The spores were white, elliptical and asymmetrical in colour, shape and symmetry, respectively. Under magnification X400 the lengths ranged from 11.30±0.72, 11.70±0.58 to 13.15±0.67 for Clade 1, 2 and 3 respectively (Plate 4.2; Table 4.11). The widths ranged from 7.67±0.50, 8.35±0.66 to 8.80±0.51 for Clade 2, 1 and 3 respectively. Stain reaction observed were cynophillic, congophilous for Cotton Blue and Congo Red. It was Inamyloid for accessions in Clade 1 and 2 in Melzers reagent and Dextrinoid for Clade 3.

Table 4. 11: Spore morphology for mushroom accessions collected from Aberdare National forest Reserve as observed at (X400)

Accession No	**Clade1**	**Clade2**	**Clade3**
Spore characteristics			
Colour (fresh)	White	White	White
Shape	Ellipsoid	Ellipsoid	Ellipsoid
Symmetry	Asymmetric	Asymmetric	Asymmetric
Size at (X400)			
Length (µm)	11.30±0.72	11.70±0.58	13.15±0.67
Width (µm)	8.35±0.66	7.67±0.50	8.80±0.51
Stain reaction			
Cotton blue	Cyanophillic	Cyanophillic	Cyanophillic
Congo red	Congophilous	Congophilous	Congophilous
Melzers reagent	Inamyloid	Inamyloid	Dextrinoid

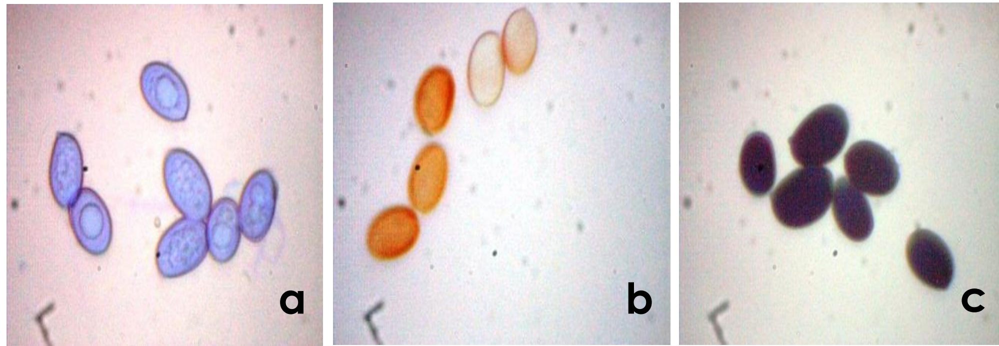

Plate 4. 2: Spore stains reaction; Left to Right; A-Cotton Blue, B-Congo Red and C-Melzers Reagent for accessions in all clades

4.4.2 Molecular characterization

Eleven samples were selected for DNA extraction. These samples were selected from the three clades and coded based on area collected. The total genomic DNA yielded highest in accession RMK08 1755.4 ng/µL, followed by KAB03 with 1462.2 ng/µL while the lowest yielding was MAT08 with 241.6 ng/µL. The quality of DNA as shown in Appendix 8 was indicated by spectrophotometric absorbance at 260 and 280 nm. Values ranging 1.8-2.0 for 260/280 ratio were considered pure DNA. Total genomic DNA was amplified using Ribosomal DNA primers ITS and nLSU. As shown in Appendix 10, purified PCR amplicon yielded products ranging between 33.3 – 110.5 ng/µL for the ITS while nLSU yielded 21 – 110.5 ng/µL. The purity of amplicons as determined by spectrophotometric at 260 / 280 nm was 1.75 - 1.87 nm and 260 / 230 nm was 0.9 – 2.22 for ITS primers. nLSU at 260 / 280 nm was 1.74 – 1.86 and 260 / 230 was 0.94 – 2.22 nm.

Purified PCR products as run on 1.8 % agarose electrophoretic gel showed distinct bands of 700 bp for all accessions (Plate 4.3).

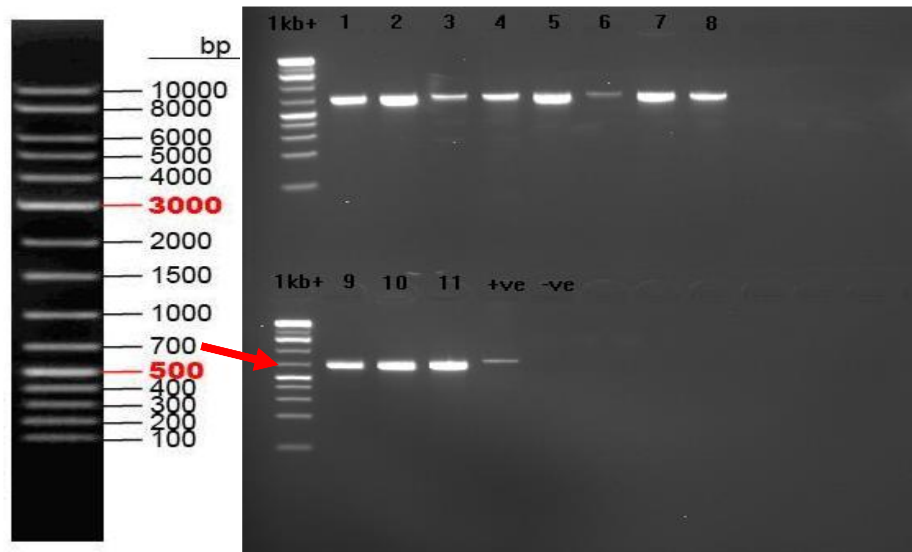

Plate 4. 3: Purified PCR product gel of the mushroom samples (1kb+ lane DNA ladder) Legend: lane 1 – 11 represents, RMK08, RMK04, ZUT06, ZUT16, KAB03, KAB01, KAB07, MAT04, MAT06, MAT08 and ZAI02, respectively

The partial nucleotide sequences were deposited GenBank using Basic Local Alignment Search Tool (BLAST) search program in National Centre for Biotechnology Information (NCBI) site. The BLAST results are shown in Table 4.12. Based on percentage identity, mushrooms were identified as follows using ITS; ZUT06 and KAB01 didn't have identity match from GenBank, MAT06, MAT08, ZAI02, RMK08, RMK04 and KAB03 – (*Macrolepiota dolichaula, %* identity 100 (AF482839.1)), KAB7- *[Macrolepiota dolichaula, %* identity 100 (HM125516.1)] while ZUT16-

[Galerina sp, % identity 99, (HQ604745.1)). Using nLSU; ZUT06, KAB03, KAB07 and KAB01-(*Macrolepiota procera, %* identity 100 (JN940269.1)); RMK08, RMK04, ZAI02 and MAT06 – (*Macrolepiota dolichaula, %* identity 100 (AF482883.1)), MAT08- (*Macrolepiota dolichaula, %* identity 100 (DQ411537.1))*;* while ZUT16 - (*Galerina sp, %* identity (HQ60475.1)). Out of the 10 mushroom samples, 9 belong to *Macrolepiota dolichaula* while 1 belonged to *Galerina sp.* However, it could not be identified upto species level from available GenBank sequences.

Phylogenetic trees (Figure 4.2a and b) constructed revealed close relationship between ZAI02, RMK08, RMK04, MAT06, KAB03, KAB07 and MAT08 with *Macrolepiota dolichaula* (HM125522.1; HM125516.1 and AF482839.1) for the ITS region, while ZUT06, KAB07, KAB01 and KAB03 *Macrolepiota procera* (JN940269.1) with RMK04, MAT06, RMK08 and ZAI02, and MAT08 *Macrolepiota dolichaula* (AF482883.1 and DQ411537.1 respectively) for nLSU region analysed. However, sample ZUT16 *Galerina sp* (HQ604745.1) showed a distant relationship with other samples in both cases.

Table 4. 12: Evolutionary analysis of both ITS and nLSU sequences BLAST results

Samples	Blast Seq length (bp)		Query coverage	% identity (Accessions)		Identified names of samples	
	ITS	***n*LSU**		**ITS**	***n*LSU**	**ITS**	***n*LSU**
RMK08	659	581	100%	AF482839.1	AF482883.1	*Macrolepiota dolichaula*	*Macrolepiota dolichaula*
RMK04	668	596	100%	AF482839.1	AF482883.1	*Macrolepiota dolichaula*	*Macrolepiota dolichaula*
ZUT06	692	535	100%	-	JN940269.1	-	*Macrolepiota procera*
ZUT16	610	591	99%	HQ604745.1	HQ60475.1	*Galerina sp*	*Galerina sp*
KAB03	658	596	100%	AF482839.1	JN940269.1	*Macrolepiota dolichaula*	*Macrolepiota procera*
KAB01	-	426	100%	-	JN940269.1	-	*Macrolepiota procera*
KAB07	647	601	100%	HM125516.1	JN940276.1	*Macrolepiota dolichaula*	*Macrolepiota Procera*
MAT06	666	591	100%	AF482839.1	AF482883.1	*Macrolepiota dolichaula*	*Macrolepiota dolichaula*
MAT08	673	582	100%	AF482839.1	DQ411537.1	*Macrolepiota dolichaula*	*Macrolepiota dolichaula*
ZAI02	668	531	100%	AF482839.1	AF482883.1	*Macrolepiota dolichaula*	*Macrolepiota dolichaula*

Evolutionary analyses were conducted using molecular evolutionary genetics analysis 5 (MEGA 5).
Legend: - Sequences could not be assembled

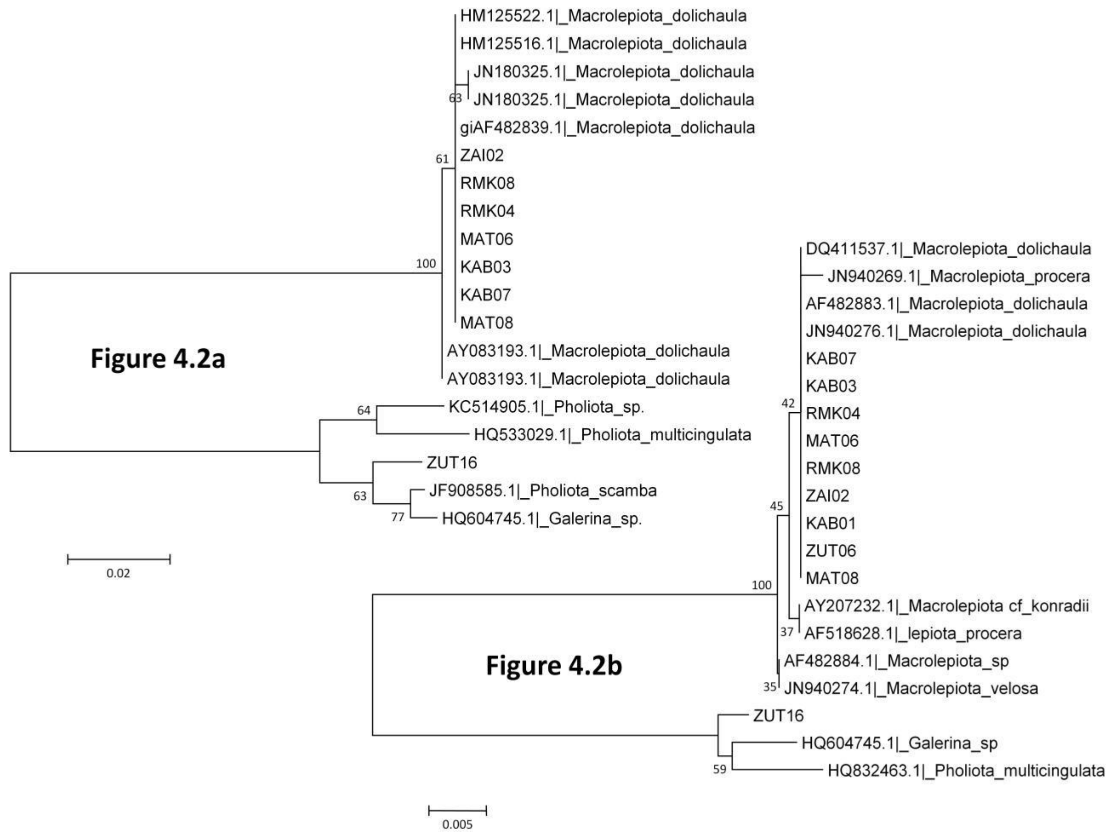

Figure 4. 2 Phylogenetic trees showing relationships among mushroom samples using ITS (a) and nLSU (b) regions, respectively

4.5 Characterisations of tissue culture

4.5.1 Morphology of pure cultures

There was no mycelia development when accessions in Clade 1 were cultured in MEA, PDA and YEA and incubated at 35°C (Table 4.13; Plate 4.4). Mycelia developed at the other incubation conditions (25°C and 30°C in MEA, PDA and YEA). In these cases, density was compact at 30°C for MEA and PDA, somehow compact at 25°C for PDA and MEA. It was somehow thin when grown on YEA at 25°C. The texture was cottony in all media at 25°C and 30°C. The growth pattern was regular-concentric or irregular concentric. Zonation was circular whereas colour was white; while hyphae were regular for all media at 25°C and 30°C and abundant in PDA at 30°C.

Table 4. 13: Mycelia characteristics of clade 1 accessions

Temperature	Density	Texture	Growth and pattern	Zonation	Colour	Hyphae
MEA						
25°C	SC	Cottony	Regular-concentric	Circular	White	Regular
30°C	C	Cottony	Regular-concentric	Circular	White	Regular
35°C	-	-	-	-	-	-
PDA						
25°C	SC	Cottony	Regular-concentric	Circular	White	Regular
30°C	C	Cottony	Irregular-concentric	Circular	White	Abundant
35°C	-	-	-	-	-	-
YEA						
25°C	ST	Cottony	Regular-concentric	Circular	White	Regular
30°C	SC	Cottony	Irregular-concentric	Circular	White	Regular
35°C	-	-	-	-	-	-

C - Compact; SC- Somewhat compact; ST-Somewhat thin. Regular (short hyphae); Abundant (Plenty long hyphae); Scarce (few hyphae); Circular (concentric rings); Lateral (flat) and Dash (-) no growth observed

There was mycelia development when accessions in Clade 2 were cultured and incubated at 25°C, 30°C and 35°C in MEA, PDA or YEA (Table 4.14; Plate 4.4 and 4.5). Density was compact at all incubation temperature on MEA and PDA; but on YEA it was ST, SC and C at 25, 30 and 35°C, respectively. The texture was velvety or cottony. Growth and its pattern was regular-plane or regular-concentric whereas zonation was lateral or circular. Colour was white while hyphaes were abundant and regular for MEA and YEA at all temperature while both abundant and scarce for PDA at 25°C, 30°C and 35°C, respectively.

Table 4. 14: Mycelia characteristics of clade 2 accessions

Temperature	Density	Texture	Growth and pattern	Zonation	Colour	Hyphae
MEA						
25°C	C	Velvety	Regular-plane	Lateral	White	Abundant
30°C	C	Velvety	Regular-plane	Lateral	White	Abundant
35°C	C	Velvety	Regular-plane	Lateral	White	Abundant
PDA						
25°C	C	Velvety	Regular-plane	Lateral	White	Abundant
30°C	C	Cottony	Regular-plane	Lateral	White	Abundant
35°C	C	Cottony	Regular-plane	Lateral	White	Scarce
YEA						
25°C	ST	Cottony	Regular-concentric	Circular	White	Regular
30°C	SC	Cottony	Regular-plane	Lateral	White	Regular
35°C	C	Cottony	Regular-concentric	Circular	White	Regular

C - Compact; SC- Somewhat compact; ST-Somewhat thin. Regular (short hyphae); Abundant (Plenty long hyphae); Scarce (few hyphae); Circular (concentric rings); Lateral (flat) and Dash (-) no growth observed.

There was mycelia development when accessions of Clade 3 were cultured and incubated at 25 °C, 30 °C and 35 °C in MEA, PDA and YEA (Table 4.15; Plate 4.4 and 4.5). Mycelial density was compact, somewhat compact or somewhat thin whereas texture was velvety or cottony. The growth and it pattern was regular-plane or regular-concentric or irregular-concentric. Zonation was lateral or circular; colour was white while hyphaes were abundant and regular for MEA and YEA at all temperature while both they were abundant and scarce for PDA at 25 °C, 30 °C and 35 °C, respectively.

Table 4. 15: Mycelia characteristics of clade 3 accessions

Temperature	Density	Texture	Growth and pattern	Zonation	Colour	Hyphae
MEA						
25°C	C	Velvety	Regular-plane	Lateral	White	Abundant
30°C	C	Velvety	Regular-concentric	Circular	White	Abundant
35°C	C	Velvety	Regular-plane	Lateral	White	Abundant
PDA						
25°C	C	Velvety	Regular-plane	Lateral	White	Abundant
30°C	C	Cottony	Irregular-concentric	Circular	White	Abundant
35°C	C	Cottony	Regular-plane	Lateral	White	Scarce
YEA						
25°C	ST	Cottony	Regular-concentric	Circular	White	Regular
30°C	SC	Cottony	Regular-plane	Lateral	White	Regular
35°C	C	Cottony	Regular-concentric	Circular	White	Regular

C - Compact; SC- Somewhat compact; ST-Somewhat thin. Regular (short hyphae); Abundant (Plenty long hyphae); Scarce (few hyphae); Circular (circles); Lateral (flat) and Dash (-) no growth observed

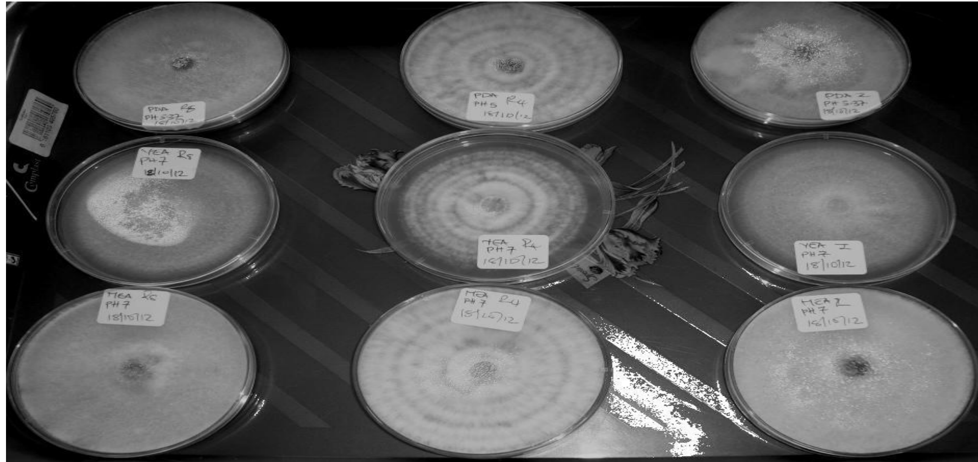

Plate 4. 4: Mycelial growth on different media 10 days after inoculation. (Top to bottom per column is PDA, YEA and MEA. Left to Right per column Clade-1, 2, 3 accessions).

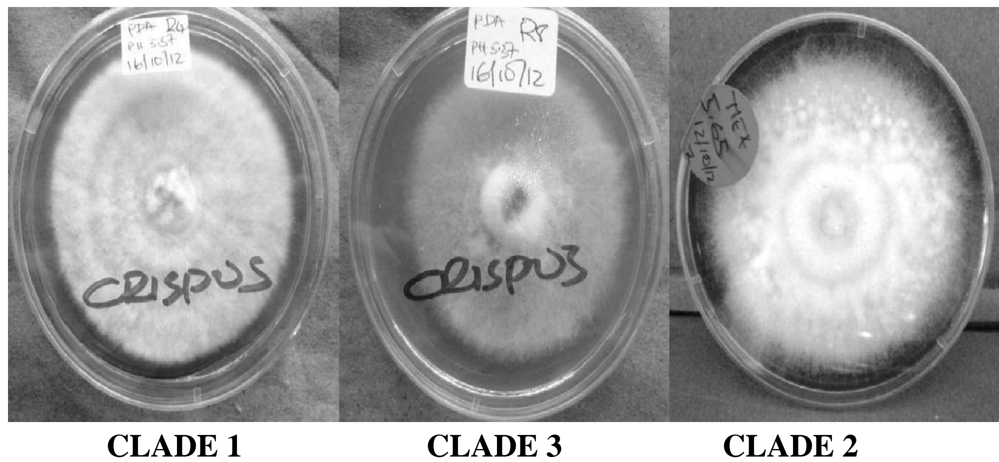

Plate 4. 5: Pure cultures of mushroom accessions 10 days after inoculation

4.6 Optimization of mycelial growth conditions

There was no growth observed at temperature 35 °C in all media and *p*H. As shown in Table 4.16; at temperature 25 °C *p*H 6 all media had no significant difference in growth PDA had highest growth ($75.67±2.42^{AB}$) at *p*H control, *p*H 5 YEA showed the highest growth ($83.33±2.42^{C}$), *p*H 7 PDA had highest growth [$74.33±1.63^{AB}$] while *p*H 8 MEA had the highest growth ($71.67±1.63^{B}$). At temperature 30 °C PDA had the highest growth ($41.33±3.56^{B}$), at *p*H 5, 6, 7 and 8 PDA had the highest growth ($38.67±1.21^{AB}$; $42.17±2.64^{A}$; $45.50±4.23^{AB}$; $47.67±1.86^{B}$) respectively. There was no significant difference on MEA and PDA at temperature 25 °C on all *p*H levels, at 30 °C *p*H 7 and 8 showed significantly high growth. YEA at temperature 25 °C on *p*H 5 and 6 showed significant difference while at 30 °C *p*H 6 and 7 had significant mycelial growth.

Table 4. 16: Effects of different media, temperature and *p*H on mycelial growth of clade 1 accessions

Accession	Temp	*p*H Range				
		Control	**5.0**	**6.0**	**7.0**	**8.0**
MEA	25°C	74.67 ± 3.78^{A}	75.83 ± 3.97^{AB}	75.67 ± 3.08	74.17 ± 2.48^{A}	71.67 ± 1.63^{B}
	30°C	33.67 ± 4.27	36.17 ± 1.94^{A}	36.17 ± 1.94	43.50 ± 2.07^{Aa}	40.67 ± 1.21^{Ab}
	35°C	9.0 ± 0	9.0 ± 0	9.0 ± 0	9.0 ± 0	9.0 ± 0
PDA	25°C	75.67 ± 2.42^{AB}	73.0 ± 2.37^{A}	75.83 ± 1.72	74.33 ± 1.63^{AB}	74.33 ± 1.21^{BC}
	30°C	41.33 ± 3.56^{Ba}	38.67 ± 1.21^{AB}	42.17 ± 2.64^{Aab}	45.50 ± 4.23^{ABac}	47.67 ± 1.86^{Bad}
	35°C	9.0 ± 0	9.0 ± 0	9.0 ± 0	9.0 ± 0	9.0 ± 0
YEA	25°C	64.17 ± 1.94	83.33 ± 2.42^{Ca}	74.50 ± 2.67^{b}	64.0 ± 1.79	52.50 ± 2.07
	30°C	27.17 ± 4.07	31.67 ± 1.37	34.83 ± 1.17^{ac}	34.67 ± 2.25^{b}	31.17 ± 3.25
	35°C	9.0 ± 0	9.0 ± 0	9.0 ± 0	9.0 ± 0	9.0 ± 0

Results are expressed as Mean±SD in mm/24h for six determinations per Media, *p*H and temperature. *p*H and temperature means per media followed by different lower-case letters along the rows are statistically different and media and temperature means per *p*H followed by different upper-case letters down columns are statistically different by Tukey's and LSD test at $p\leq0.05$.

Clade 2 accessions their mycelial growth at temperature 25 °C, as shown in Table 4.17 had *p*H control MEA with the highest growth of ($77.67\pm.3.20^{AB}$), *p*H 5 YEA had the highest growth of (82.33 ± 1.63^{C}), *p*H 6, 7 and 8 MEA had the highest growth of (81.0 ± 1.26^{C}; 81.0 ± 1.26^{A}; 84.50 ± 1.22^{BC}) respectively. At temperature 30 °C *p*H control had highest growth (76.0 ± 2.83^{A}), *p*H 5 YEA had the highest growth of (76.50 ± 4.04^{A}) while *p*H 6, 7 and 8 PDA showed the highest mycelial growth of (78.67 ± 3.98^{A}; 79.0 ± 3.58^{AB}; 83.17 ± 1.94^{AB}) respectively. At temperature 35 °C YEA at all *p*H levels showed the highest mycelial growth rates. Different *p*H level showed significance in mycelial growth for MEA at temperature 25 °C *p*H 6, 7 and 8 had significantly different growths of (81.0 ± 1.26^{Cad}; 81.0 ± 1.26^{Abe}; 84.50 ± 1.22^{BCcfgh}), at 30 °C *p*H 7 and 8 were significant different and only *p*H 7 (37.17 ± 2.40^{abc}) was significant different at temperature 35 °C. On PDA *p*H 8 showed significantly different growths at all temperatures, while for YEA, there was no significant difference at temperature 25 °C. However, *p*H 5 had significant different growth at temperature 30°C and 35 °C (76.50 ± 4.04^{Aa}; 45.17 ± 1.33^{Aa}) respectively.

Table 4. 17: Effects of different media, temperature and *p*H on mycelial growth of clade 2 accessions

Accession	Temp	*p*H Range				
		Control	**5.0**	**6.0**	**7.0**	**8.0**
	25°C	77.67±.3.20^{AB}	76.17±1.47^{AB}	81.0±1.26^{Cad}	81.0±1.26^{Abe}	84.50±1.22^{BCcfgh}
MEA	30°C	73.17±2.56	71.83±1.94	70.50±3.94	75.17±1.94^{Aad}	82.17±1.47^{Abcef}
	35°C	31.17±1.72^{A}	33.17±1.17	32.0±2.10	37.17±2.40^{abc}	27.17±0.75
	25°C	75.0±2.10^{A}	75.0±1.41^{A}	74.33±2.42^{A}	76.33±2.50	81.83±2.40^{Babcd}
PDA	30°C	76.0±2.83^{A}	72.17±3.19	78.67±3.98^{Aad}	79.0±3.58^{ABbe}	83.17±1.94^{ABcf}
	35°C	33.17±1.47^{AB}	33.50±1.38	36.17±1.72^{Aad}	41.50±1.87^{Abeg}	38.50±1.05^{ABcf}
	25°C	74.33±5.50	82.33±1.63^{C}	79.83±6.43^{AB}	76.33±3.61	74.33±5.82
YEA	30°C	72.33±5.47	76.50±4.04^{Aa}	73.50±4.09	69.67±2.58	72.0±3.41
	35°C	41.0±1.79^{C}	45.17±1.33^{Aa}	40.33±1.21^{B}	42.0±2.37^{AB}	38.17±1.17^{A}

Results are expressed as Mean±SD in mm/24h for six determinations per Media, *p*H and temperature. *p*H and temperature means per media followed by different lower-case letters along the rows are statistically different and media and temperature means per *p*H followed by different upper-case letters down columns are statistically different by Tukey's and LSD test at $p \leq 0.05$.

As shown in Table 4.18, accession RMK-08 mycelial growth at temperature 25 °C at *p*H control PDA showed the highest growth (76.0 ± 1.55^{AB}) while MEA had the highest mycelial growth for *p*H 5, 6, 7 and 8 of (75.17 ± 3.97^{AB}; 76.67 ± 3.78^{A}; 83.17 ± 1.94^{C}; 85.0 ± 0^{BC}) respectively. At temperature 30 °C all *p*H control values were statistically similar while *p*H 5, 6, 7 and 8 PDA had the highest growth of (75.0 ± 2.68^{A}; 79.0 ± 3.63^{AC}; 79.83 ± 3.37^{AB}; 83.83 ± 1.60^{AB}) respectively. At temperature 35 °C YEA showed the highest mycelial growth at all *p*H levels. Accession RMK-08 on MEA showed significant growth at temperature 25 °C, 30 °C on *p*H 7 and 8 (83.17 ± 1.94^{Cace}; 85.0 ± 0^{BCbdf} and 75.67 ± 2.50^{Abeg}, 83.17 ± 1.94^{Acfhi}) respectively and *p*H 7 at 35 °C had (39.83 ± 1.47^{abc}). PDA had most significant growths at *p*H 8 on all temperatures (82.50 ± 1.76^{Bacde}; 83.83 ± 1.60^{ABabc}; 42.33 ± 2.50^{Bceg}) for 25, 30 and 35 °C respectively, while YEA *p*H 8, 5 and 6 for temperature 25, 30 and 35 °C had the significant growths of (73.50 ± 3.27^{d}; 79.17 ± 2.14^{Ba}; 41.0 ± 1.10^{Bb}).

Table 4. 18: Effects of different media, temperature and *p*H on mycelial growth of clade 3 accessions

Accession	Temp	*p*H Range				
		Control	**5.0**	**6.0**	**7.0**	**8.0**
MEA	25°C	75.33±3.93^{A}	75.17±3.97^{AB}	76.67±3.78^{A}	83.17±1.94^{Cace}	85.0±0^{BCbdf}
	30°C	71.17±2.23	67.0±2.37	74.33±3.27^{Aad}	75.67±2.50^{Abeg}	83.17±1.94^{Acfhi}
	35°C	34.33±2.16^{A}	35.50±1.05	34.33±1.86	39.83±1.47^{abc}	29.50±1.05
PDA	25°C	76.0±1.55^{AB}	73.17±1.47^{A}	74.83±3.87	77.67±1.86^{Bb}	82.50±1.76^{Bacde}
	30°C	77.50±3.51	75.0±2.68^{A}	79.0±3.63^{AC}	79.83±3.37^{AB}	83.83±1.60^{ABabc}
	35°C	32.0±1.55	33.67±3.08	36.17±2.23^{A}	41.50±2.07^{ABbdf}	42.33±2.50^{Bceg}
YEA	25°C	65.50±3.51	84.17±1.60^{Ca}	75.17±3.06^{b}	72.67±1.75^{c}	73.50±3.27^{d}
	30°C	74.33±1.75	79.17±2.14^{Ba}	77.33±1.21^{AB}	69.83±2.99	72.67±1.37
	35°C	39.33±1.21^{B}	42.17±2.17^{Aa}	41.0±1.10^{Bb}	40.17±1.47^{A}	40.0±1.10^{A}

Results are expressed as Mean±SD in mm/24h for six determinations per Media, *p*H and temperature. *p*H and temperature means per media followed by different lower-case letters along the rows are statistically different and media and temperature means per *p*H followed by different upper-case letters down columns are statistically different by Tukey's and LSD test at $p \leq 0.05$.

4.7 Development of grain propagation spawns

Mycelia obtained from tissue culture were used to develop grain spawns. Three mushrooms accessions were selected for spawn development based on pure culture morphology (Table 4.19 and Plate 4.6). However, RMK04, ZMK02 and RMK08 gave the best pure cultures. Sorghum 100 % gave significantly ($p \leq 0.05$) the fastest rate of colonization of 8 -10 days for the three accessions. This was followed by 80 % sorghum with 20 % wheat bran which took 9 – 11 days. Other formulations followed as listed; 40 % sorghum, 40% millet and 20% wheat-bran, 50 % millet with 50 % sorghum; 100 % millet, 80% millet and 20% wheat bran, 80% sorghum with 20% kopa-kula, 40 % sorghum 40% millet and 20% kopa-kula. The slowest growth ($p \leq 0.05$) of colonization was witnessed in 80 % sorghum and 20 % kopa-kula which took up-to 25 days for mycelia colonisation.

Table 4. 19: Time taken (days) for mycelial establishment on grain spawn formulation

No.	Grain formulation	Accession RMK 04	ZMK 02	RMK 08
1.	100% Sorghum	10.50±0.71[b]	9.0±0.00[a]	8.50±0.71[a]
2.	100% Millet	13.50±0.71[de]	9.50±0.71[a]	11.0±0.0[abc]
3.	50% Millet+50% Sorghum	13.0±1.41[cd]	10.0±0.00[a]	10.0±0.0[ab]
4.	80% Millet+20% KopaKula	0.0±0.0[a]	24.50±0.71[d]	25.0±1.41[g]
5.	80% Millet+20% Wheat bran	15.0±0.0[e]	14.50±0.71[b]	14.50±0.71[cde]
6.	80% Sorghum+20% KopaKula	0.0±0.0[a]	21.50±0.71[c]	21.50±2.12f
7.	80% Sorghum+20% Wheatbran	11.50±0.71[bc]	10.0±1.41[a]	9.0±1.41[a]
8.	40% Sorghum+40% Millet+20% KopaKula	0.0±0.0[a]	24.0±1.41[d]	23.0±141[fg]
9.	40% Sorghum+40% Millet+20% Wheatbran	12.50±0.71[bcd]	9.0±1.41[a]	13.0±0.0[bcd]
CV [%]		8.37	5.9	6.21
LSD ≤ 0.05		2.0	2.0	3.0

Results are expressed as Mean±SD in days for three determinations per accession at optimal mycelial establishment conditions.

CLADE 1

CLADE 2

CLADE 3

Plate 4. 6: Completely colonized mushrooms spawns after 10; 9 and 8 days for clades 1, 2 and 3, respectively

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Ethno-mycology

Ethno-mycology is study of use of mushrooms by human (Kik *et al.,* 2013). The study findings, showed good flow of Indigenous knowledge on mushrooms among the respondents. A report on traditional forest related knowledge by Chang and Lee (2004) and Kumar and Sharma (2011) emphasises its importance in; sustainable forest management, understanding ecology and ecosystems. It provides invaluable information on the diversity of organisms within the ecosystem which is supported by the current study. Mushrooms collection mostly occurred during long and short rains onset (March-June and October-December). This agreed with report by Sibounnavong *et al.* (2008) and Kik *et al.* (2013) that there is an optimum period or mushroom season when they abundantly appear. Peak mushrooms and macrofungi season for each region differ due to differences in ecological climate such as rainfall, humidity and temperature.

Factors that encouraged mushrooms collections were gathering of fallen tree logs for use as firewood, herding of animals, open and unrestricted access to the forest and grasslands. This agreed with findings of Hyde (2003); Chang and Lee (2004) and Kumar and Sharma (2011).

Fischer exact test, based on the interview with the locals indicated that the knowledge of the mushrooms domain with respect to mushroom ecology and seasonal availability was consistent across the Aberdare community regardless of gender, age and lineage as there was no significant difference at $p \leq 0.05$. Most mushrooms were consumed when fresh. A few were sundried for preservation purposes. In addition, mushrooms were reported to be delicacies among the ethnic groups. Similar observations have been reported by other researchers including Apetorgbor *et al.* (2006); Baysal *et al*. (2007); Giri and Rana (2008); Synytsya *et al*. (2008); Kumar and Sharma (2011) and Okoro and Achuba (2012). The use of mushrooms as an item of commerce was not common among the respondents. A few respondents reported to have traded on wild edible mushrooms. However, in other developing countries literature documents trade of wild edible mushrooms. Also cultivation for consumption and the surplus is sold to earn income Apetorgbor *et al.* (2006) and Onuoha *et al.* (2009).

5.2 characteristics and importance of mushrooms habitats

Plant species and vegetation was sampled and found to be dominated by closed canopy highland forest with low alpha diversity although some areas had been degraded through human activities like farming. Wild edible mushrooms form mycorrhizal associations with the vegetation (Boa, 2004).

African mycorrhizal fungi are specialised to co-occur with indigenous tree species only, and are thus endemic to tropical Africa (Alexander and Hogberg, 1986). Ninety nine percent of the tree species are mycorrhyza dependent. Without the mycorrhizas the trees would grow poorly and the ecological integrity of forests around the world would be threatened. Therefore, being able to infect their roots with macrofungi / mushrooms will improve tree fitness. The biology and ecology of wild edible fungi are therefore important, as is a fundamental knowledge of which species grow with particular trees (Boa, 2004). Some of the vegetation reported to have mycorrhizal association include Causaurina, Cupressus, Pinus, Picea, Abies and Larix species (Appendix 9).

5.3 Characterisation of accession collected

Morphological and molecular characteristic were used in this study to identify wild mushrooms. Morphological characteristics have been shown to distinguish different genera of fungi (Iotti *et al*., 2005). The mushroom isolates characterised had numerous striking features, both macroscopically and microscopically. Phenotypic data of the mushrooms and spores classified the accessions as members of genus *Macrolepiota* as also compared to the genera keys used in the study (Appendix 6).

Ge *et al.* (2010) indicated that the genus *Macrolepiota* belongs to the Family-*Agaricaceae*, Order- *Agaricales*, Phylla-*Basidiomycota* as established by Singer (1948). Macroscopically, basidiomata of species in this genus are typically big, fleshy, and often with squamules on the pileus; lamellae are white to cream; a prominent annulus is usually present which is often movable (Velliga, 2003). Spore phenology and microscopy results revealed basidiospores to be white in colour when fresh to cream when dry, Ellipsoid shape and asymmetric. They were relatively big, cyanophilic in cotton blue, congophilous in congo red and inamyloid in melzers reagent to dextrinoid for clade3. These observations were similar to Ge *et al.* (2010) and Sysouphanthong *et al.* (2011) study findings.

Precise morphological identifications are essential to ensure the correct assignment of molecular approach to macrofungi identification (Barseghyan *et al.,* 2012). Molecular characterization involves use of genetic markers for identification. The ITS and nLSU partial nucleotide sequences of mushrooms samples were searched against available sequences on GenBank data for molecular identification (Das *et al.,* 2013). Internal Transcribed Spacer (ITS) sequences for accessions; RMK08, RMK04, KAB03, MAT06, MAT08 and ZAI02 identified as *Macrolepiota dolichaula* (trichodermal pileus covering; Whitish spore print, long stipe with annulus) matched morphologically (trichodermal pileus covering;

Whitish spore print, long stipe with annulus) with reported mushrooms *Macrolepiota dolichaula* by Vellinga *et al.* (2003) from New South Wales in Australia. Accession KAB07 was identified to be *Macrolepiota dolichaula* from available sequences of GenBank database (100% Homology) this was also reported by Ge *et al.* (2010) from China. Sample ZUT16 belonged to different genera *Galerina sp.* and matched with (99% homology) with sample reported by Berbee *et al.* (2010). This was also noted from the phylogenetic tree. The large sub-unit (nLSU) fragment sequences for accession; RMK08, RMK04, MAT06 and ZAI02 identified as *Macrolepiota dolichaula* matched with *(R. P. J. de Kok 901 (CANB)]* samples from Canberra Black mountain in Australia.

KAB07, KAB01, KAB03 and ZUT06 identified as *Macrolepiota procera* matched GeneBank databases sequences (100% Homology) with samples reported by Ge (2011) in Germany. Accession MAT08 was identified as *Macrolepiota dolichaula* also reported by Ge *et al.* (2006).

From molecular point of view on the phylogenetic tree, *Macrolepiota dolichaula* and *Macrolepiota procera* mushrooms had distinct relationship with same species from GenBank though collected from different regions.

The present study however, suggests that, GenBank data base for mushrooms is not sufficiently rich in Africa and or Kenya local strain sequences. In this study it was possible to resolve and match the morphological identity to molecular taxonomy. Clearly, there is need for additional full length molecular data from additional samples from different geographical origin to have enough data for taxonomic work.

5.4 Tissue culture morphology studies

5.4.1 Effect of temperature on mycelial growth

The optimum temperature for the mycelial growth and density of tested three representative mushroom accessions was 25 °C. As the temperature increased growth was suppressed with lowest mycelial growths and morphological characteristics being recorded at 35 °C. In case of RMK04, mycelial growth was completely suppressed at 35 °C on all media and *p*H levels. Mycelium growth of RMK-04 may not have occured at 35 °C due to denaturation of important enzymes which catalyse fungal metabolic processes (Kibar and Peksen, 2011). Reports by Shim *et al.* (2005); Imtiaj *et al*. (2008); Jayasinghe *et al.* (2008) and Lai *et al*. (2011) state that the favorable mycelial growth of *Schizophyllum commune*, *Lignosus rhinoceros, Macrolepiota procera* and *Ganoderma lucidum* were at 30 °C.

Shim *et al*. (2003) reported that the mycelial growth of *Paecilomyces fumosoroseus* had been expedited gradually in proportion to the rise of temperature and was most suitable at 25 °C. Likewise, Hur *et al.* (2008) worked on *Phellinus* spp and reported that 25 °C was most favourable for mycelial development. Even though mycelial growth of the *Macrolepiota* accessions was favorable at 25 – 30 °C and had been expedited in proportion to the rise of temperature, the mycelial growth appeared to be suppressed at the temperature above 30 °C. These may be attributed to enzyme inactivation at higher temperatures. The study results were found similar to findings of Sung *et al.* (1999) and Shim *et al.* (2005) but incompatible to Shim *et al.* (2003).

5.4.2 Effect of *p*H on mycelial growth

The growth of fungi is promoted by temperature and *p*H of the media (Kibar and Peksen, 2011). The *p*H of the medium is an important factor for mycelial growth and thus different types of macrofungi prefer different *p*H conditions. Mycorrhizal fungi favour acidic conditions while ectomycorrhizal members of *Agaricales* favour neutral to near neutral *p*H (Kibar and Peksen, 2011). Favorable mycelial growth of the three studied accessions was obtained in a *p*H range of 7-8. However, these accessions showed good mycelial growth and density in *p*H 5, 6 and control. Results indicated that *Macrolepiota* species can grow at broad *p*H levels.

This result also implies that different *Macrolepiota* species prefer different *p*H values tending towards nueutral *p*H (7). The results agreed with Shim *et al.* (2005) study on *Macrolepiota procera* who obtained good mycelial growth at *p*H 7. Jayasinghe *et al*. (2008) also reported that *Ganoderma lucidum* strain grew over a wide range of *p*H but optimum growth was obtained at *p*H towards neutrality. Shim *et al.* (2003) also reported *Paecilomyces fumosoroseus* showed favorable growth at *p*H 7 though these mushrooms favourably accommodated *p*H's ranging from 6 - 9. Imtiaj *et al.* (2008) and Lai *et al.* (2011) worked on *Lignosus rhinoceros* and *Schizophyllum commune* and reported that *p*H 6 and 7 and *p*H 5 were favourable respectively. They suggested that mushrooms may have a broad pH range for their favorable mycelial growth, which was observed in this study.

5.4.3 Screening of favourable culture media

Culture media are important as they supply required nutrients for mycelia growth (Kibar and Peksen, 2011). Therefore, the effect of culture media on the mycelial growth varies according to mushroom species. Three different culture media were used to screen the optimal mycelial growth of the three different species of *Macrolepiota* genera. Mycelium growth was completed within 8 and 10 days for PDA, MEA or YEA.

All culture media showed comparatively good mycelial growth for mycelial growth of *Macrolepiota* genera. As Kibar and Peksen (2011) reported, PDA promotes growth of mushroom mycelia. This agreed partially with ZMK-02 and RMK-08 accession results.

The study results showed that, MEA was the most favourable for mycelial establishment though PDA had equally good mycelial growth. Cultural condition of *Macrolepiota procera* study by Shim *et al.* (2005) showed good mycelia growth on PDA medium. Likewise, Hur *et al.* (2008) studied the effects of different growth culture medias' on *Phellinus spp* and recorded that PDA promoted most mycelial phenotype.

Even though PDA was favourable to *Macrolepiota procera*, this media was unfavourable to *Paecilomyces fumosoroseus* as reported by Shim *et al.* (2003). Accession RMK-04 results agreed with Lai *et al*. (2011) study on the effect of seven culture medias' on *Lignous rhinocerus* and reported that optimum mycelial growth and density was observed in glucose-peptone and yeast extract peptone dextrose (YEPD), followed by mushroom complete medium (MCM) media. Lastly, Ukoima *et al.* (2009) stated that supernatant culture media stimulated higher mycelia growth than synthetic agar culture media such as PDA and YEA after studying mycelia of *Volvariella volvacea, Pleurotus tuber-regium* and *Pleurotus sajor-caju* on

different culture media. This contradictory result indicates that taxonomically distinct fungal groups have different nutritional requirement. Even though the mycelial growth in three different media showed wide range of variations, the mycelial densities were compact in PDA and MEA culture media tested.

5.5 Spawn development

Rapid mycelia growth on spawn grain observed in this study may be attributed to a greater food reservoir in sorghum grains, whereas the millet grains provided a greater number of inoculation points. According to Onyango *et al.* (2011a) high rates of colonization may be attributed to mycelia getting the most suitable ratio of mixture with a high reservoir of energy and all the nutritional ingredients such as carbon, nitrogen, lipids and minerals (Beyer and Wilkinson, 2002; Choi, 2004). Vigorous substrate colonization by the mycelium during spawn run is desirable because it reduces mushroom cropping time and may allow mycelium to outgrow competitors in the substrate (Ekpo, 2009; Onyango *et al.,* 2011a). Beyer and Wilkinson (2002); Choi (2004) and Onyango *et al.* (2011a) reported high influence of nutritional additives supplements on mycelial establishment which disagrees with value observed for non supplemented sorghum grain which established mycelium the fastest. However, some of the supplemented formulation also showed rapid mycelia growth.

Bran supplementation provides a protein rich medium which can increase rate of mycelia growth two-fold (Onyango *et al*., 2011a).

Conversely, kopa-kula supplemented grains gave significantly the slowest ($p \leq 0.05$) growth rates as observed in 80% sorghum + 20% kopa-kula, 40% sorghum + 40% millet and 20% kopa-kula and 80% millet + 20% kopa-kula lasting 21.5, 24, 25 days for ZMK02 and RMK08. It was therefore clear that supplementation with wheat bran increased the speed of mycelia growth while kopa-kula greatly decreased the growth rate. Additionally, compactness or poor aeration of the grains may have resulted in inefficient utilization of nutrients thereby slowing mycelia growth rate in kopa-kula supplemented grains. Comparison between the supplements showed that wheat bran was better than kopa-kula regarding their influence on mycelia growth. All the grains supplemented with wheat bran took a shorter time to fully colonize compared to those combined with kopa-kula. Although the brans were not evaluated for nutritive composition, it is highly probable that the wheat bran used in this study had a higher protein content increasing the nitrogen level in the grains.

Uhart *et al*. (2008) analyzed the nutritional values of different supplements and concluded that wheat bran contained better quality nutrients that increase the rate of mycelia growth.

Oei (2003) and Onyango *et al.* (2011a) attributed enhanced performance of mycelia on availability of several amino acids and protease as well as transaminase enzyme activities on wheat bran. Another factor that may have influenced the rate of mycelia growth is quality of the inoculants used and aeration of the grains. Stamets (2000) recommended dense and thick mycelia to be used for inoculation of grains for spawn formation.

5.6 Conclusions

1. Mushrooms have been a common source of food, medicine and income to the Aberdare community. Ethno-mycological results showed clear understanding of importance and traditional practises within the generations. Hence, ethno-mycological documentation of such mushroom activity is very important, which is unfortunately not available as of yet. In view of the increasing commercialization of the wild edible mushrooms, more studies on the ethno-mycology of mushrooms in the State are called for. These mushrooms are abundant in Aberdare National forest Reserve; the present findings can serve to stimulate further investigations on ethno-mycology and to gather different views of the local populace about the value of mushrooms.

2. Morphological features discriminated mushrooms into three clades and identified them as members of genus *Macrolepiota*. Our results also indicated that although it is relatively easy to isolate wild mushrooms strains using phenotypic features, it is necessary to use molecular techniques to avoid misidentification. Molecular characterization, confirmed two clades to be members of genus *Macrolepiota and and one as genus Galerina. Macroleptia* species was further classified into *Macrolepiota procera,* and *Macrolepiota*

dolichaula. These results enrich and provide additional information to mushroom biodiversity and GenBank data base resource aiding to molecular phylogenetic analysis in Kenya. Further, identification knowledge may also be significant for human benefits.

3. Mushrooms industry is rapidly growing and farmers demand strains with high agro-economic traits. Development of propagation spawns is the first step in efforts to test starch digestibility and compost utilizations by mushrooms among other conditions in this study spawn preparation results showed good utilization of local grains; sorghum and millet. With or without supplementation recommendation for their application in spawn production can be drawn. Apparently, these locally available supplements are rich in nutrients which can be utilized by mushroom mycelium to aid in spawn production. This study documents for the first time a new *Basidiomycota* in Kenya with probable high agronomic potential.

5.7 Recommendations

From this study the following recommendations are made

1. Ethno-mycology worldwide is broadening in scope and design that now addresses the knowledge base across gender, age groups and occupational categories. The results of this study underscore the need to conduct a nationwide survey on the indigenous uses of mushrooms to safe guard the knowledge handed down from generations. The study also recommends regular surveys over an extended period in order to assess the patterns of abundance of mushrooms in different seasons.

2. In this study ITS and nLSU primers were used for identification. However, development of SSR markers for the genotyping the various species of *Macrolepiota* genus is highly recommended.

3. There is need to explore other cheaply available options such as ground maize cobs for spawn development. This would reduce dependency on sorghum and millet which are food material. Finally it would lower the cost incurred in acquiring the grain and hence increase food security.

4. There is need to undertake compost preparation using supplementation material like wheat bran, rice bran or pigeon manure (instead of chicken manure) in order to establish any difference in productivity and quality of mushrooms obtained. This understanding novel technical knowledge that could be deployed for the successful cultivation of hitherto uncultivated edible and other valuable mush-rooms underpins the need for further research.

5. *Macrolepiota* species are well known for several applications including culinary use, and ability to produce interesting enzymes with potential uses in biotechnological processes like bioremediation, biodegradation, bio-pulping and detoxification of recalcitrant substances since they have some bioactive compounds. This study thus, recommends a thorough investigation on the possible bioactive compound found in this new described *Macrolepiota* species for biotechnological applications.

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APPENDICES

Appendix 1: Structured Interview Questionnaire

QUESTIONNAIRE FOR BASELINE SURVEY ON THE DISTRIBUTION AND UTILIZATION OF WILD EDIBLE MUSHROOM SPECIES IN EASTERN AFRICA UTAFITI JUU YA UFAHAMU NA MATUMIZI YA UYOGA ASILIA KATIKA ENEO LA AFRICA MASHARIKI

1.0. SURVEY QUALITY CONTROL/ TAARIFA ZA MTAFITI NA MTAFITIWA

1. Date of interview (dd/mm/yyyy) / Tarehe (siku/mwezi.mwaka)…………………………………........
2. Researcher name………………………………………………………… ……….........................
3. Name of interviewee (optional)/ Jina la mhojiwa……………………………….........................
4. Interviewee's family profile / Habari za familia ya mhojiwa………………………………………….
5. Number of adults in the household/ Namba ya watu wazima………………………………………
6. Number of children under 14 years / Namba ya watoto chini ya miaka 14……..........................
7. Contacts (optional) / Anuani ya mtahiniwa ………………………………….........................

2.0. SITE IDENTIFICATION/ ENEO HUSIKA

1. Region/Mkoa..
2. District /Wilaya…………………………………………………….……........ ....
3. Division/ Jimbo/Kata……………………………………………………… ……..…...............................
4. Village/ Kijiji…….……………………………………………………..…… …………………………………
5. Nearby forest/ Msitu wa asili ulipo karibu…… GPS readings from house/Eneo la kijiografia; Altitude/Mwuinuko......../Latitude/Latitudo…....Longitude/Longitudo..

3.0. DEMOGRAPHIC DATA OF INTERVIEWEE/ TAARIFA ZA MHOJIWA

1. Sex / Jinsia ya mhojiwa
    a. Male/Mme [ ]; b. Female/Mke [ ]
2. Age /Umri wa mhojiwa
    a. Under 20 years/Chini ya miaka 20 [ ]; b. 21-40 years/ Kati ya miaka 21-40 [ ] ; c. 41 years –and above/ Miaka 41 na zaidi[ ]
3. Education level / Kiwango cha elimu:
    a. Not attended school/ Hajasoma kabisa [ ]
    b. Primary level education/ Elimu ya msingi [ ]
    c. Secondary / high school education/ Elimu ya sekondari [ ]
    d. College level and above / Elimu ya juu:cheti/diploma/digrii [ ]
    e. Level of education vs eating habits
4. Main occupation / Kazi :
    a. Farmer /Mkulima[ ];
    b. Employed /Ameajiriwa [ ];
    c. Other/Nyinginezo [ ] (Please specify/Eleza tafadhali).

4. KNOWLEDGE OF WILD EDIBLE MUSHROOMS UFAHAMU JUU YA UYOGA

1. Do you know what mushrooms are?Je unaufahamu uyoga?
    a. Yes/Ndiyo [ ]; b.No /Hapana [ ]
2. Have you ever used mushrooms? Je, ulikwishawahi kutumia uyoga?
    a. Yes/Ndiyo [ ]; b. No/Hapana / [ ]
3. For what purpose do you use mushrooms? Huwa unautumia uyoga kwa madhumuni gani?
    a. Food/ Chakula [ ]; b. Medicinal purpose /matumizi ya dawa [ ]; c. For sale/Kuuza []; d. Others/Sababu nyinginezo [ ]. Please explain/Elezea tafadhali……………….
4. If you eat mushrooms, how often? Kama unakula uyoga, ni mara ngapi?
    a. Very Often /Mara nyingi[ ]; b. Sometimes/ Mara mojamoja [ ] c. Rarely/Mara chache[ ]; d. On special occasions/Siku maalumu. Please explain/Tafadhali eleza
5. If you eat mushrooms, where do you obtain them? Kama unakula uyoga, huwa unaupata wapi?
    a. Wild/ Porini[ ]
    b. Cultivated/ Uliyolimwa [ ]

6. If you use mushrooms in your diet, how are they cooked? Kama unakula uyoga, huwa unauandaa vipi? ………………………………………………………………………….

7. If you use mushrooms for medicinal purpose, explain. Kama huwa unautumia uyoga kwa dawa tafadhali elezea ……………………………………………………………

8. If you sell mushrooms, where do you sell them? Kama huwa unauza uyoga, ni wapi unauuza?

   a. Open air local markets/ Soko mjinga [ ]
   b. Local supermarkets / Maduka ya supamaketi [ ]
   c. Local restaurants/hotels/ Hoteli zilizopo jirani [ ]
   d. Other [ ] Please specify/ Sehemu nyinginezo, tafadhali eleza…

8. If you sell mushrooms, what portions or weights do you use (kg, pack, basket etc) and at what price? Kama huwa unauza uyoga, ni vipimo gani unavitumia? (kilo, fungu, ndoo n.k) na kwa bei gani?...

9. Which species/varieties do you sell most? Huwa unauza uyoga wa aina gani?

11. In which form do you mostly sell them? Unauza uyoga wako ukiwa katika hali gani ?

   a. Fresh/ Ukiwa mbichi [ ]; b. Dried/ Umekaushwa kwa jua [ ]; c. Salted/ Umehifadhiwa kwa kuwekewa chumvi [ ]; d. Smoked/ umeukaushwa kwa moshi [ ]

12. Where do you collect wild mushrooms? Unapata wapi uyoga pori unaoliwa?

   b. From the forests/ Toka msituni[ ]; b. From culitvated fields/ Toka mashambani [ ]; c. From ant-hills/ toka kwenye vichuguu [ ]; c. Other/Kwingineko [ ] Please Specify /Tafadhali elezea ………………………………………………….

13. Which periods of the year do you collect mushrooms? Kipindi gani cha mwaka huwa unakusanya uyoga?…………………divide year in quarter Jan-April/May-Aug/Sept-Dec .

14. How do you differentiate between edible and poisonous mushrooms? Unatofautishaje kati ya uyoga unao liwa na ule wenye sumu?.............………morphology colour/ growth habitat/

15. Have you heard of any incidence of reported food poisoning due to mushroom consumption? Ulikwishawahi kusikia habari za watu walidhurika kwa kula uyoga wenye sumu?

a. Yes/ Ndiyo [ ]; b. No/ Hapana[ ]

16. If yes state, the number of deaths heard/ Kama ulikwishawahi, wangapi walifariki?……..

17. How do you keep/store mushrooms after collection from the field? Ni vipi huwa unahifadhi uyoga wako baada ya kuuvuna porini?...

18. Have you ever grown mushrooms? Ulikwishawahi kulima uyoga?

Yes /Ndiyo[ ] Proceed to question No. 19/ Endelea na swali namba 19/;

b. No/ Hapana [ ] Proceed to question No. 20/Endelea na swali namba 20.

19. If you grow mushrooms, what type? Kama unalima uyoga, ni wa aina gani?..................

20. If you do not cultivate mushrooms, why? Kama hulimi uyoga, ni kwa nini?....lack of knowledge/ lack of resource/ cost of acquring skill/ market of products..

21. Given an opportunity, would you be interested to learn how to cultivate mushrooms? Kama ukipewa nafasi, je utapenda kulima uyoga? a. Yes/Ndiyo [ ]; b. No/ Hapana [ ];

22. What socio-cultural beliefs about mushrooms in your community do you know? Kuna imani gani juu ya uyoga katika jamii yenu?……………………………………………………………………...

23. Do you have any question or comment regarding mushrooms in general?
Je unalo swali au maoni yoyote kuhusiana na uyoga?..

Appendix 2: Samples collection form (phenetic features to be recorded from fresh material).

No.	Item	Remarks
1.	Cap shape: (Applanate(flat)=01; Convex(rounded)=02; Conical(cone like)=03; Campanulate(bell shaped)=04; Infundibuliform(centrally depressed)=05; Auriform=06; discoid=07; ear shaped=08; Cup shaped=09.)	
2.	Cap colour (colour chart)	
3.	Cap surface texture: (Glabrous(smooth)=01; Pubescent(finely hairy)=02;Velutinous (like velvet)=03; Villose (coarsely hairy)=04; Fibrillose(radiating fibres)=05; Squamulose(with scales)=06; Areolate(breaking into patches)=07.)	
4.	Cap apex (Top): (Umbonate(Central raised bump)=01; Umbilicate(Central indent)=02; Papillate(Pimple in indent)=03; Other(specify e.g. flat)=04.)	
5.	Cap margin (Entire(smooth)=01; striate(lines at edge)=02; Tuberculate(furrowed)=03; Plicate(pleated)=04; Rimose(splitting)=05; Inrolled)=06.)	
6.	Cap diameter (Take diameter from the widest to the least wide)	
7.	Cap flesh (cross sectional view) (Thin =01; Thick=02)	
8.	Cap (Chemical colour test 15%KOH;30%NH_3;10%$FeSO_4$)	
9.	Stipe (Present =01; Absent/Reduced =02)	
10.	Stipe attachment to cap (Central=01; Dorsal=02; Eccentric=03; Lateral=04; Sessile=05.)	
11.	Stipe colour(use colour chart)	
12.	Stipe size- long >5cm=(01); Average >1<5cm=(02); Short<1cm=(03); Absent/Reduced=(04)	
13.	Stipe shape longitudinal view (Cylindrical (Equal)=01; Clavate=02; Tapering to apex=03; Tapering to base=04; Bulbous=05; Ventricose (swollen)=06.)	
14.	Stipe shape Cross sectional view (Circular (terete)=01; Compressed=02.)	

15.	Stipe base shape (Bulbous=01; Angular bulbous=02; Tapering=03; Radicating=04; Unswollen=05; Clapsing-marginate volva=06; Powdery friable volva=07; Saccate volva=08.)	
16.	Stipe surface features texture (Glabrous(smooth)=01; Pubescent(finely hairy)=02; Velutinate(Velvety)=03; Reticulate(with network)=04; Fibrillose(Vertical fibres)=05; Squamulose(scales)=06; Punctate(small dots)=07.)	
17.	Stipe consistency (Fragile=01; Robust=02; Cartilaginous=03; Fibrous=04; Woody=05; Corky=06; Chalky=07; Tough outer layer=08; Rind outer layer=09; Rubbery=10; Brittled=11).	
18.	Stipe base attachment (Simple=01; Caespitose(Attached at base=02); Rhizoids(With basal roots)=03; (Insititious(Direct into wood)=04; Disc(With basal disk)=05; Rhizomorph(with bulb)=06; Volva (Sac)=07; Mycellial pad (With bunch of hair at base)=08.)	
19.	Stipe flesh (Solid=01; Stuffed=02; Chambered (Lacunose)=03; Hollow=04.)	
20.	Stipe (Chemical colour test 15%KOH;30%NH_3;10%$FeSO_4$)	
21.	Annulus -Ring on stipe (Absent (No ring)=01; Present=02.)	
22.	Ring shape- Membranous (Hanging down)=01; Up turned(Edge up)=02; Sheathing (Sock line)=04; Cortinate(Cobweb like)=05.)	
23.	Ring Size (mm)	
24.	Ring consistency (Fragile=01; Robust=02; Cartilaginous=03; Fibrous=04; Woody=05; Corky=06; Chalky=07; Tough outer layer=08; Rind outer layer=09; Rubbery=10; Brittled=11; Leathery=12)	
25.	Ring Colour(Use colour chart)	
26.	Ring Position (Bottom=01; Middle=02; Top=03.)	
27.	Ring attachement (Fixed=01; Can it move=02.)	
28.	Spore print colour (use colour chart)	

29.	Hymenium structure (Gills=01; Pore=02; Teeth=03; Reticulate=04; Non gilled(Jelly)=05)	
30.	Hymenial (gills/lamellulae/tubes/teeth) attachment to stipe (Decurrent (extending down the stem/running down stem)=01; Adnate (Right angled/broadly attached)=02; Adnexed (acute angled/partially attached)=03; Free (not reaching stipe/not attached)=04; Sinuate (notched near the attachment to stipe)=05; Receding (attached at first becoming free later)=06; Emarginated(very acute)=07; Arcuate(arched)=08); Ridges =(09).	
31.	Hymenial colour (use colour chat)	
32.	Hymenial (gill/pore/teeth/ridges) margin (Smooth (entire)=01; Variously uneven as seen through a hand lens, e.g. Crenate=02; Serrate=03;Eroded(Ragged)=04; Wavy=05; The edge may differ in colour to the face may be glistening=06; Fimbriate (minutely fringed, often due to presence of cystidia-then referred to as cystidiate)=07).	
33.	Hymenial margin colour (use colour chart)	
34.	Hymenial lamellulae arrangement (Regular/unbranched (All gills reach stipe)=01; Intercalcated (with some short gills either in one or two series)=02; Furcate (Splitting)=03; Anastomising-Costate (Cross gills present)=04; Pores (sponge like)=05; Hydnoid (with teeth)=06); Reticulate(ridges)=07).	
35.	Lamellulae (Present (agaric)=01; Absent(not agaric)=02).	
36.	Gills thickness in side view (Thin=01; Broad=02; Ventricose(swollen in the middle)=03).	
37.	Gills spacing (Crowded=01; Well-spaced apart (distant)=02; Arbitrarily somewhat in between (close or sub distant)=03).	
38.	Hymenium (Chemical colour test 15%KOH;30%NH_3;10%$FeSO_4$)	

Appendix 3: Microscopic Examination

Item No	Item	Remarks
1.	Spore shape (Ellipsoid(sides are curved and ends rounded)=01; Ovoid(egg-shaped, one end larger than the other)=02; Globose (spherical)=03; Oblong/cylindrical(ends round, sides parallel)=04; Fusiform(tapering of both ends)=05; Citriniform (lemon-shaped. ends of the spores with a beak-like Projection)=06; Amygdaliform(almond-shaped)=07; Phaseoliform(bean-shaped)=08; Angular(various angular spore shapes)=09.	
2.	Spore size/length/width (……………………… µm)	
3.	Spore reaction in melzer's reagent (Amyloid=01; Dextrinoid=02; Inamyloid=03)	
4.	Spore reaction in Congo red – spore walls & other structures (Positive Reaction)=01; (Hyaline)=02)	
5.	Spore reaction in 1% Cotton Blue in Lactic acid – spore walls & other structures (Positive Cyanophilic Reaction)=01; (Hyaline)=02)	
6.	Spore wall ornamentation(Oil emulsion objective)	
7.	Basidia location: (Pleurocystidia(at tip)=01; Cheilocystidia(on stem)=02)	
8.	Basidia size	
9.	Basidia shape	
10.	Colour of Basidia	
11.	Number of sterigma(spore bearing projection)	
12.	Cystidia location: (Pleurocystidia (on the gill faces)=01; Cheilocystidia(on the gill edges)=02; Pileocystidia(on the cap stem)=03; Caulocystidia (on the stem)=04).	
13.	Cystidia size	
14.	Cystidia shape	
15.	Colour of cystidia	
16.	Abudance of cystidia	
17.	Structure of trama (Gill);(Regular (parallel)=01; Irregular(interwoven)=02; Bilateral(divergent)=03; Inverse (convergent)=04).	
18.	Pellis (skin/cuticle of fungal organ): Pileipellis-skin on cap (pileus); Stipitipellis-skin on stem (stipe).	
19.	Trama: Cap; Stipe & Lamellae trama	

Appendix 4: Structured Laboratory Fungus Inventory Recording Sheet

FUNGUS RECORDING SHEET	
Locality	Date
Habitat species Associated species	Substrate
Cap Details Size in diameter…………….. (mm) Texture…………………………..…. Surface……………………………… Latex (presence/absence):………………; latex colour:….………. Peeling nil/quarter/half/all Cap margin……..…,… Shape………..……… Shape at top…..……. Veil…………….……... Colour…………………	
Flesh Smell………….. Taste………….. Colour………… Colour (chemical) change	Chemicals 1. 15% KOH 2. 10% $FeSO_4$ 3. 30% NH_3
Gills Pore/teeth/gills…..…… Colour………………..…. Gill margin…………..…. Gill thickness ………………..(side view) Gill spacing………………….. Attachment to stipe…………	
Stipe Colour…………………………Height…………….. (mm) Width ……………………(mm) Texture………………………… Shape…………….(longitudinal)……………..(cross sectional) Base shape…………………… Stipe consistency……………. Ring …………...(present/absent)………….consistency Volva……………………..….Attachment to	

<table>
<tr><td colspan="3">cap……….......
Base attachment………….......</td><td></td></tr>
<tr><td colspan="3"><u>Spore</u>
Size………………...(µm)
Shape……………………
Colour……………………
Type (Chemical test).……</td><td><u>Chemicals</u>
1. Melzer's reagent.
2. 1% Cotton Blue in Lactic acid
3. Congo Red</td></tr>
<tr><td colspan="3">Cystidia</td><td></td></tr>
<tr><td colspan="3">Basidia</td><td></td></tr>
<tr><td colspan="3">Notes</td><td></td></tr>
<tr><td>Photo Ref No.</td><td></td><td></td><td></td></tr>
</table>

Appendix 5: phenetic features taxonomy keys

Key to the recognized species of *Macrolepiota* were adopted from (Ge et al. 2010) who worked on sample from china.

1 Basidiomata with a volva at the base of the stipe…………………………………………………….*M. velosa*.

1* Basidiomata without a volva at the base of the stipe.

2 Pileus surface with brown plate-like squamules; annulus complex; clamp connections common at the base of the basidia.

3 Stipe surface with conspicuous fine brown squamules on whitish background; pileus squamules made up of yellowish-brown walled long hyphal segments, mainly 25–90×7–11 (14) µm…………………………………………..……………………………

……………………………….*M. procera*.

3* Stipe surface with fine brown squamules on whitish background; pileus squamules made up of yellowish-brown walled short hyphal segments, mainly 15–25×7–11 µm………………………….….*M. detersa*.

2* Pileus surface with pale ochraceous to brown fine squamules; annulus simple, or only slightly thicker near the edge; clamp connections absent or present.

4 Stipe surface with brown squamules; usually without clamps at the base of

basidia………………………………………………………………………

…………………...……….*M. mastoidea.*

4* Stipe surface smooth; usually with clamps at the base of basidia.

5 Stipe base sometimes becomes orange when cut, pileus squamules composed of more frequently branched hyphae, cheilocystidia mainly clavate to broadly clavate.……………………..…*M. dolichaula.*

5* Stipe base not changing colour when cut, pileus squamules composed of seldom branched hyphae, cheilocystidia mainly obtusely fusiform to clavate……………………………………………*M. orientiexcoriata.*

Appendix 6: PCR profile

No.	Stages	Activity	Temp (°C)	Duration
1.	Stage 1	Initial denaturation	95	5 min
2.	Step 1	Denaturation	95	30 s
3.	*Step 2* *stage 2* [30 cycles]	Primer annealing	60	30 s
4.	*Step 3*	Primer extension	72	1 min
5.	Stage 3	Final extension	72	10 min

Reaction stopped by chilling to 4°C
Store at -20°C ∞

Appendix 7: Grain and supplement formulations for spawning

S/N	Grain and supplement formulation
1.	1 Sorghum
2.	4 Sorghum + 1 Wheat bran
3.	4 Sorghum + 1 Koppa kula
4.	1 Millet + 1 Sorghum
5.	2 Sorghum + 2 Millet + 1 Wheat bran
6.	2 Sorghum + 2 Millet + 1Koppa kula
7.	1 Millet
8.	4 Millet + 1 Wheat bran
9.	4 Millet + 1 Koppa kula

Appendix 8: Vegetation family and species with Mycorrhiza association with mushrooms

No	Family	Species
1.	*Casaurinaceae*	*Causaurina*
2.	*Cupressaceae*	*Cupressus*
3.	*Pinaceae*	*Pinus – Pines;* *Picea – Spruces;* *Abies –Firs;* *Larix - Larches*

Appendix 9: Total genomic DNA concentration in ng/µL

No.	Sample ID	Nucleic Acid Conc	Unit	A260	A280	260/280	260/230
1	RMK08	1755.4	ng/µl	35.1	17.9	1.96	0.64
2	RMK04	969.3	ng/µl	19.4	9.88	1.96	1.7
3	ZUT06	988.2	ng/µl	19.8	10.4	1.9	1.45
4	ZUT16	282.7	ng/µl	5.65	3.07	1.84	0.93
5	KAB03	1462.2	ng/µl	29.2	14.8	1.97	1.76
6	KAB01	777.5	ng/µl	15.6	7.99	1.95	1.58
7	KAB07	467.5	ng/µl	9.35	4.83	1.94	1.33
8	MAT04	726.5	ng/µl	14.5	7.71	1.88	1.34
9	MAT06	684.6	ng/µl	13.7	7.48	1.83	1.02
10	MAT08	241.6	ng/µl	4.83	2.54	1.9	1.11
11	ZAI02	393	ng/µl	7.86	4.16	1.89	1.22

Appendix 10: Purified PCR products concentration

No.	Sample ID	Nucleic Acid Conc	Unit	A260	A280	260/280	260/230
ITS PCR FRAGMENTS							
1	RMK08	44	ng/μl	0.88	0.474	1.86	2.22
2	RMK04	71.2	ng/μl	1.425	0.763	1.87	2.15
3	ZUT16	79.4	ng/μl	1.587	0.878	1.81	1.24
4	KAB03	65.6	ng/μl	1.312	0.703	1.87	2.04
5	KAB01	33.3	ng/μl	0.667	0.382	1.75	0.90
6	KAB07	76.4	ng/μl	1.528	0.825	1.85	1.95
7	MAT06	84	ng/μl	1.68	0.916	1.83	1.65
8	MAT08	91.9	ng/μl	1.839	1.008	1.82	1.80
9	ZAI02	110.5	ng/μl	2.209	1.199	1.84	1.97
10	+VE CONTROL	36.8	ng/μl	0.736	0.403	1.83	1.06
LSU PCR FRAGMENTS							
1.	RMK08	55.5	ng/μl	1.111	0.609	1.82	1.68
2.	RMK04	83.6	ng/μl	1.672	0.925	1.81	1.60
3.	ZUT16	55.2	ng/μl	1.103	0.613	1.80	1.58
4.	KAB03	110.5	ng/μl	2.209	1.23	1.80	1.55
5.	KAB01	16.4	ng/μl	0.328	0.181	1.81	1.48
6.	KAB07	88.1	ng/μl	1.762	0.991	1.78	1.50
7.	MAT06	95.4	ng/μl	1.908	1.059	1.80	1.63
8.	MAT08	95.1	ng/μl	1.903	1.049	1.81	2.07
9.	ZAI02	116.4	ng/μl	2.328	1.288	1.81	1.68
10.	+VE CONTROL	21.8	ng/μl	0.435	0.249	1.74	0.94