# IDENTIFICATION OF MUSHROOMS FROM PINE PLANTATIONS WITHIN THE TSITSIKAMMA REGION, SOUTH AFRICA

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Submitted in fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

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# DECLARATION

I, Maryke Herselman, hereby declare that the work in this thesis entitled “Identification of mushroom species from pine plantations in the Tsitsikamma region, South Africa” that I herewith submit at the University of the Free State, is my independent work and that I have not previously submitted it for other qualification at another institution of higher education.

November 2022

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# ABSTRACT

Mushrooms have been exploited for ages by mankind for their astoundingly wide application as a sustainable dietary supplement that also carries economical, ecological value and medicinal qualities. Although some mushrooms are considered edible and flavoursome others are deadly. Mushrooms also play ecologically vital roles in nature as decomposers, pathogens and symbionts of plants, animals and humans. Mushrooms have in recent times been heavily explored for new-age biotechnological and medical innovations, but without knowledge of species present in a country, regulation is difficult. In South Africa, knowledge about the biodiversity of macro fungi seems to be lacking. To expand this biodiversity knowledge, this study focused on the coastal Tsitsikamma region in the Eastern Cape province, which represents the largest native forest area of South Africa. However, these forests are interspersed with commercial tree plantations, agriculture and urban development. Specifically, this study focused on mushrooms occurring in plantation areas, to initiate a knowledge base of macro fungi associated with these alien plants, before future studies can determine which are more likely native mushrooms, and if mushrooms from these alien plants can also be found in native vegetation. Therefore, the first aim of the study was to collect and document mushroom diversity and morphology from plantations, and to highlight distinguishable and identifiable characteristics. Morphological studies were aided in the second aim of using rDNA nuclear Internal Transcribed Spacer (ITS) DNA sequence comparisons to confirm specimen identities. A total of 13 species were collected and identified from various plantations in the region. These included species of *Amanita*, *Russula* and *Lactarius*, as well as *Panaeolus, Chlorophyllum*, *Clitopilus, Imleria* and *Gymnopilus*. One specimen identified to be a *Chlorophyllum* species could not be identified to species level, and may possibly represent a novel species. The study yielded three first reports for South Africa, namely *L. quieticolor*, *P. antillarum* and *A. morissi*, with the latter species having vulnerable red list status and is only known from North America. It was also found that the South African described *R. capensis* could possibly be conspecific to *R. caerulea*, which occurs widely in the Northern Hemisphere. A large number of species found were also ectomycorrhizal, having a symbiotic relationship with plant roots, which were pines in this study. The use of DNA sequence comparisons in this study revealed novel associations and reports, in some cases different from the better known morphologically identified species previously known from the region. This study thus shows that careful surveys should be done in future, using both morphological and DNA sequence based identification.



**KEYWORDS:** *Amanita,* Biodiversity, *Chlorophyllum*, *Clitopilus, Gymnopilus, Imleria*, Internal Transcribed Spacer, *Lactarius*, *Panaeolus*, *Pinus, Russula*

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# LIST OF ACRONYMS

| | |
|---|---|
| **%** | percentage |
| **µL** | microliter |
| **µM** | micromolar |
| **$^{o}$C** | degree Celsius |
| **18S** | small subunit ribosomal RNA |
| **28S** | large subunit ribosomal RNA |
| **5-HT** | 5-hydroxy-tryptamine |
| **5-HT2A** | 5-hydroxy-tryptamine 2A receptor |
| **AM** | arbuscular mycorrhiza |
| **BLAST** | Basic Local Alignment Search Tool |
| **bp** | base pair |
| **BS** | bootstrap support |
| **CFK** | Cape Floristic Kingdom |
| **CFR** | Cape Floristic Region |
| **Chl** | trichloromethane |
| **cm³** | cubic centimetre |
| **CNS** | central nervous system |
| **DAFFT** | Department of Agriculture Forestry and Fisheries |
| **$dH_2O$** | distilled water |
| **DNA** | deoxyribonucleic acid |
| **EAA** | essential amino acids |
| **ECM** | ectomycorrhizal |
| **EDTA** | ethylenediaminetetraacetic acid |
| **GRNP** | Garden Route National Park |
| **h** | hour |
| **ha** | hectare |
| **HCL** | hydrochloric acid |
| **ITS** | internal transcriber spacer |
| **ITS1** | first internal transcribed spacer |
| **ITS4** | forth internal transcribed spacer |
| **IUCN** | internal union of conservation of nature |
| **kg** | kilogram |
| **km** | kilometre |
| **LSD** | lysergic acid diethylamide |
| **LSU** | large subunit |
| **m** | meter |
| **m.a.s.l** | meters above sea level |
| **Ma** | milliampere |
| **MAFFT** | Multiple Alignment using Fast Fourier Transform |
| **MEGA** | Molecular Evolutionary Genetics Analysis |
| **mg** | milligram |
| **min** | minute |
| **ml** | millilitre |
| **ML** | maximum likelihood |
| **mm** | millimetre |
| **mM** | millimolar |
| **MM** | megamolar |
| **MTO** | Mountain to Ocean Group |
| **NCBI** | National Center for Biotechnology Information |
| **ng** | nanogram |
| **nLSU** | nuclear large ribosomal subunit gene |
| **nm** | nanometer |
| **OCD** | obsessive compulsive disorder |
| **PCR** | polymerase chain reaction |
| **pH** | potential of hydrogen/power of hydrogen |
| **rDNA** | ribosomal DNA |
| **RNA** | ribonucleic acid |
| **RPB1** | RNA (ribonucleic acid) Polymerase I |
| **RPB2** | RNA (ribonucleic acid) Polymerase II |
| **rpm** | revolutions per minute |
| **rRNA** | ribosomal RNA |
| **s** | second |
| **SDS** | sodium dodecyl sulfate |
| **SSU** | small subunit |
| **TAE** | Tris-Acetate EDTA |
| ***Tef*-1α** | translocation elongation factor 1-α |
| **Tris** | tris (hydroxymethyl) aminomethane |
| **tRNA** | transfer RNA |
| **UFS** | University of the Free State |
| **UK** | United Kingdom |
| **USA** | United States of America |
| **V** | voltage |
| **W** | watt |
| **WNP** | Wilderness National Park |

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# CHAPTER 1 – INTRODUCTION

## 1.1 Introduction to macro fungi

The fungal realm boasts with an enormous level of diversity. However, there are several opinions regarding the total estimated number of species, resulting in a projected range of 3.8 million (Hawksworth & Lücking, 2017) to 13.2 million (Wu et al., 2019) species that are encountered in all habitational environments world-wide (Claridge et al., 2000). Mushrooms form intricate interactions with an array of organisms including plants (Bonfante & Genre, 2010; de Mattos-Shipley et al., 2016), animals such as insects (Crous et al., 2006; Fisher et al., 2020; Hawksworth & Lücking, 2017), as well as humans (Boukes et al., 2017; Fisher et al., 2020; Schoch et al., 2012). These relationships are either considered mutually beneficial to extremely detrimental, and carry out vital roles in any structured ecosystem, with particular functions being determined by the relationship type with a particular host or substrate (Fisher et al., 2020; Rasalanavho et al., 2020). For example, certain fungi form special symbiotic relationships with plant roots and are called mycorrhiza (Bonfante & Genre, 2010; Itoo et al., 2016). These mycorrhizal relationships are mutually beneficial and both parties interact in definite ways regarding each other's overall health. Due to this broad range of functionalities, this boldly diverse kingdom is represented by numerous eccentric shapes, characters and sizes (Rubina et al., 2017), such as the well-recognized vibrant red bulbous bell button silhouettes of *Amanita muscaria*, to that of the single-celled yeast. However, several species with enormous probable impact on all aspects of human life remain undiscovered and undescribed, therefore remaining under-utilized and undervalued (Hrudayanath & Sameer, 2014; Lindequist et al., 2005).

Some of the earliest representations of relations between the Fungal Kingdom and mankind dates back to prehistoric times. For instance, mushrooms were found among the Iceman Ötzi's belongings, and well documented cave paintings are located in the heart of the Sahara Desert that portrays mushrooms used by humans (Debnath et al., 2019; El Enshasy et al., 2013; Gründemann et al., 2020; Lindequist et al., 2005; Molitoris, 1994; Rasalanavho et al., 2020; Samorini, 2001; Yuan et al., 2016). Knowledge on mushrooms that impact humans sociologically is called ethnomycology and represents the traditional uses of any fungus for

purposes by local peoples through beliefs carried down through generations (Gupta et al., 2019). Today, mushrooms are still used traditionally and industrially.

## 1.2 South African Mycology

In South Africa, a known census for all fungal species is lacking. An under-estimated number of 171 500 fungal species is believed to be present in the country (Crous et al., 2006). Despite the high number of fungal species predicted for South Africa and the wide application to modern day life, little effort is being articulated towards fully understanding the unknown and overall fungal diversity exclusive to South Africa. Recently, the first ever checklist illustrating the level of diversity of macro fungi, such as mushrooms, found in the country was presented (Kinge et al., 2020), concluding that the directory will serve as a foundation for future additions and refinement.

### 1.2.1 Indigenous vs non-native areas in South Africa

One of the most significant and well known indigenous vegetation types in South Africa include that of the fynbos biome. The Cape Floristic Region (CFR) or Cape Floral Kingdom (CFK) includes fynbos vegetation and is localized in the Western Cape and Eastern Cape provinces, South Africa. It has extensively been investigated, more than any other part of the sub-Saharan territories of Africa (Crous et al., 2006). The CFK is one of only six floral kingdoms in the world (Crous et al., 2006; Rutherford et al., 2006), demonstrating high levels of diversity. An estimated total number of 8650 vascular plant species have been documented, although it is being considered the smallest regarding land area (Goldblatt, 1997). Of the species recorded an estimated 65% are believed to be endemic to the country. This significant indigenous vegetation is dominantly threatened by natural occurring fires induced by lighting (Kraaij, Cowling, & Van Wilgen, 2013; Myers, Mittermeier,Mittermeier, 2000) and the ever growing agricultural sector and rapidly development of urbanized areas (Crous et al., 2006; Leis, 2022; Newbound et al., 2010). The native vegetation also faces tribulation competing with fire-sensitive plantations of invasive alien trees species for natural resources including, land space, water and soil nutrients (Kraaij et al., 2011; Pauw, 2009).

The Tsitsikamma indigenous forest is an iconic tourist district that forms part of the Garden Route National Park (GRNP) in South Africa. Different from the CFK it consists of *Podocarpus* species (Yellowwood), *Ocotea bullata* (Black Stinkwood) and *Olea capensis* (Black Ironwood) (Baard & Kraaij, 2019; Bellingan, 2010; Ella, 2005; Hawley & Dames, 2004; Tchoumi et al., 2020) .The GRNP stretches across the Western and Eastern Cape. Located between the Bloukrans river (provincial border) and Storms river, the region includes the southern foothills of the Tsitsikamma mountain range. This area receives a mean annual rainfall of 800–1100 mm that gradually increases as one passes from west to east, and south inward to the northern situated mountains ranges (Kraaij, Cowling, & van Wilgen, 2013). The GRNP is fragmented into at least 30 protected areas, of which the Tsitsikamma forms part. The region is characterized by cattle farms, settlements, and dense indigenous forests (Pauw, 2009). The Tsitsikamma plateau also has large areas devoted to plantations of pine trees, which are harvested for timber and paper production (Kraaij et al., 2011; Tchoumi et al., 2020). Plantation and farmlands are further dividing smaller recognized sites (Oudebosch, Witelsbos, Kou-Kamma, Storms River). The abundance of fauna and flora presented by the dynamic landscapes, along with an ideal climate and rainfall, create ideal tropical warm climates (Goldblatt, 1997; Rocha et al., 2019).

The introduction of non-native vegetation types including *Quercus*, *Eucalyptus* and *Pinus* species were imported by the first Europeans to colonize the southern Cape region, now known as Cape Town (Western Cape province) in 1652 (Ella, 2005; Fitzgerald, 2018; Rutherford et al., 2006; Showers, 2010). These settlers brought various propagation materials for the establishment of important domestic cultivated plantations and other crop trees, originating from the Northern Hemisphere (Fitzgerald, 2018; Showers, 2010). These cultivated areas were established among the natural occurring fynbos vegetation. Alongside these introduced vegetation types various fungal species were also introduced into the country, since fungi tend to form significant relationships with their hosts. Through the increase in demand of timber and the economic value associated with these cultivated plantations, the forestry industry has developed extensively (Flemming & Keith Martin, 2018; Geldenhuys, 1997; Showers, 2010). Plantations of various *Pinus* species, including *Pinus patula, Pinus elliottii, Pinus radiata* and *Pinus taeda* according to the DAFFT Timber Report (2010/2011), and *Eucalyptus* trees have been established across the country, including the

Eastern Cape, Western Cape, KwaZulu-Natal, Limpopo and Mpumalanga provinces (Geldenhuys, 1997; Hawley et al., 2008; Hugo et al., 2012).

The vast majority of macrofungi with names and listed in field guides in South Africa most likely do not represent native fungi (Goldman & Gryzenhout, 2019; Gryzenhout, 2021; Kinge et al., 2020). This is because these are mushrooms that occur in other areas of the world and that could thus be identified from field guides from abroad, and that most likely have been introduced into South Africa. Although a small number of native species has been described (Goldman & Gryzenhout, 2019), most of the truly native mushrooms cannot be identified because they are still undescribed. Very few DNA sequence based studies also exist for South African macro fungi. Research is many focused on plant pathogenic genera such as *Ganoderma* (Coetzee et al., 2015; Tchoumi et al., 2018) and *Armillari*a (Coetzee et al., 2002). This means that even the presumed non-native mushrooms, such as *Amanita* and *Russula* species, in urban areas and plantations where their plant hosts grow, have not yet been properly characterized based on more than morphology.

## 1.3 Problem statement

Some biodiversity studies have been done on fungi in the Tsitsikamma forests and surrounding regions and other cultivated plantations within the country. However, these only focussed on species that were plant pathogens causing wood rot, and the mycorrhizal status of indigenous vegetation as well as non-native plants (Dames et al., 1999; De Koker et al., 2000; Hawley et al., 2008; Hawley & Dames, 2004; Musvuugwa, 2014; Tchoumi et al., 2020). As a result, the remaining biodiversity is still unstudied. This includes the diversity of mushrooms found in cultivated pine plantations from the Tsitsikamma region that have not been documented yet, especially based on the most up to date DNA sequence based phylogenies. In fact, very few of these mushrooms have been sequenced throughout South Africa, despite their prominence in the environment. The first ever state owned plantations in the Tsitsikamma area were established in 1883 near the town of Knysna (Van Der Zel & Brink, 1980). These plantations focussed mainly on *Pinus* species rather than that of *Eucalyptus*. Due to the developing forestry sector in 1891, forest gaps created by the harvesting of indigenous trees were filled with more than 100,000 alien trees (*Pinus,*

*Eucalyptus* and *Quercus* spp.) (Baard & Kraaij, 2014). Knowing that some fungal species behave in a host specific manner (Chen et al., 2018), it is expected to find a copious amount of fungi preferring these coniferous and hardwood trees. Having their identities verified with up to date DNA sequence data, will be important for conservation efforts in the area protecting the indigenous vegetation, and management of the pine plantation areas, since most of these mushrooms are mycorrhizal.

## 1.4 Aims and objectives

The aim of this study was to identify macro fungi from the Tsitsikamma region from non-native pine plantations by morphological observations and DNA sequencing. The area is considered to be a significant ecological niche with a diverse array of plant growth and landscapes (Baard & Kraaij, 2014; Kraaij, Cowling, & Van Wilgen, 2013). The generated data will be used to investigate the identities, phylogenetic relationships and fungal diversity of the gathered specimens.

Fruiting bodies of macro fungi collected from various locations in the Tsitsikamma area, including Mountain to Ocean (MTO) owned properties, will be identified as follows:

- Observations of macromorphology and reviewing relevant literature to identify specimens.
- DNA sequence comparisons based on the Internal Transcribed Spacer (ITS) of the ribosomal region to confirm morphological identifications.

Successfully investigating the biodiversity of mushrooms observed in the Tsitsikamma area will bring forth information regarding the numerous species of macro fungi localized to this remote area (Gründemann et al., 2020). Moreover, molecular results will verify the phylogenetic relationships aiding correct identification, or it could be possible that errors in the concepts of what we think certain species should be or look like, could be discovered. The species identified will aid in reporting fungal diversity from this area in future and will greatly aid conservation and future comparisons between plantations and natural areas.

# CHAPTER 2 – LITERATURE REVIEW

## 2.1 Introduction

Although the relationship between humans and mushrooms dates back to prehistoric times and the fungal realm boast with an enormous level of diversity (Claridge et al., 2000; He et al., 2022; Li et al., 2014), the overarching question regarding the true amount of species within the fungal kingdom remains unanswered ever since the establishment of the mycological field (He et al., 2022; Zhang et al., 2021). Due to the complex nature regarding the classification of organisms it was believed for long that all fungi were part of the Plantae Kingdom. However, fungi have been proven to be closer related to the Animal Kingdom and later through the way of molecular analysis in the 1990s fungal associations were placed within their own kingdom (Whittaker, 1969).

## 2.2 Diversity

The Fungal Kingdom consists of members that presents in various shapes, sizes and forms and includes smuts, rust, mushrooms, mildews, yeasts, molds and toadstools. The kingdom is divided into phyla that includes Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota (He et al., 2022; Musvuugwa, 2014). The term “mushroom” is generally associated with species of macro fungi that are classified as ‘higher fungi’ or that of fungi that produce fruiting bodies (Hrudayanath & Sameer, 2014; Martinez-Medina et al., 2021; Wasser, 2011). Mushroom fruiting bodies can either be epigeous growing above ground or hypogenous found underground (Anderson & Lake, 2013; Claridge et al., 2000; Hrudayanath & Sameer, 2014; Lindequist et al., 2005; Pala et al., 2012). Fungal species producing macroscopic fruiting bodies are mainly represented by the taxonomic phyla Basidiomycota and Ascomycota (Babasaheb, Parkhe & Palghadmal, 2019; He et al., 2022; Jayasiri et al., 2015; Maharachchikumbura et al., 2021). The Basidiomycota, alone is estimated to include between 35,000 to 50,300 species (de Mattos-Shipley et al., 2016; He et al., 2022; Thu et al., 2020).

The vagueness regarding the total true amount of fungal species is believed to be due to a lack of correct identification and further documentation of findings (Mueller & Schmit, 2007; Schoch et al., 2014). A working hypothesis is that the diversity is estimated to be represented by 14 million species (Bhunjun et al., 2022; Fisher et al., 2020; He et al., 2022; Mueller & Schmit, 2007) found world-wide. The ambiguity regarding the level of diversity leave several species with enormous probable impact on all aspects of human life remain undiscovered and undescribed, therefore remaining under-utilized and valued (Hrudayanath & Sameer, 2014; Lindequist et al., 2005).

## 2.3 Habitat

Mushroom are found in a variety of habitats all across the world. Ideal and favourable conditions include environmental factors such as soil health and composition, climate, humidity and rainfall. Thus most macrofungal growth is visible within montane moist evergreen forests regions with warmer tropical climates and high humidity levels (Kengni Ayissi & Mossebo, 2014; Panda et al., 2021; Rumainul et al., 2015). Fruiting bodies grow in almost all soil types but can also be found, thrive on living plant species and organic decaying wood-substrates such as logs, stumps, branches and forest litter (Alsohaili, 2018; He et al., 2022; Reynolds et al., 2018). Numerous fungal species are also observed flourishing in heavily composted grass fields and herbivorous animal manure. These dung associated mushrooms are collectively known as coprophilous mushrooms (Ediriweera, 2015; Wang & Tzean, 2015). Although the fertiliser substrate does not support a long life cycle for these fruiting bodies it demonstrates the adaptability of several fungal species to able to survive within harsh and intolerable conditions (Manimohan et al., 2007; Mumpuni et al., 2020). Other punitive conditions include desert sand and mountainous sandstone areas with dry and hot climates (Kaul, 2009; Pauline et al., 2021).

## 2.4 Significant ecology relationships

Encountered in all habitational environments world-wide (Claridge et al., 2000), mushrooms form intricate interactions with an array of other organisms, including plants, animals and humans. Being considered as either mutually beneficial or extremely detrimental, they carry out vital roles in any structured ecosystem, with particular functions being influenced and determined by the relationship type with said host or substrate (Fisher et al., 2020; Rasalanavho et al., 2020). The major ecological roles are discussed below and include saprophytes, parasites and symbionts.

### 2.4.1 Saprophytes

Saprophytic mushrooms feed on dead or decaying plant matter as well as the remains of animals (Ascough et al., 2010; Grangeia et al., 2011; Kinge et al., 2020; Reynolds et al., 2018). These mushrooms are considered as important decomposers within nature and have the unique ability to breakdown, for example, cellulose material (Adenipekun & Lawal , 2012; de Mattos-Shipley et al., 2016). The capability of recycling organic material is essential to the overarching health of the surrounding ecosystem (Bhunjun et al., 2022; He et al., 2022; Leonardi et al., 2021; Xu, 2016). The processing of dead leaves, logs and plant roots results in the production of beneficial organic material, highly concentrated with significant minerals and nutrients (Ghadmal, 2019). These are reabsorbed by the intimate environment and is utilised by plants to promote and sustain overall health (Kinge et al., 2020). Although, saprophytic mushrooms are significant regarding the overall health of environments they also pose a threat to harvested sub-tropical fruit, causing fruit rot that can immensely impact the agricultural and export sectors (Crous et al., 2006).

### 2.4.2 Mycorrhiza

Mutual beneficial symbiotic relationships form between mushrooms and living hosts (Chen et al., 2018; Itoo et al., 2016). Many of these significant interactions form amongst the mycorrhizal mushrooms and the roots of the living plant. Mycorrhizal mushrooms are divided

into two main groups, namely Arbuscular (AM) and Ectomycorrhizal (ECM) mushrooms. While ECM mushrooms form a protective mantle net surrounding plant roots, without the hyphae entering hosts cells, hyphae of AM mushrooms penetrates the cortex of plant roots, (Chen et al., 2018; Hawley & Dames, 2004). Both of these subdivided groups provide advantages to host substrates by obtaining carbohydrates from the host to develop an extensive mycelium network in the surround substrate (Gąsecka et al., 2017; Hawley & Dames, 2004). The spreading mycelium mat in return aids in the absorption of essential minerals and water from surrounding environment that encourages and promotes plant growth and health (Gąsecka et al., 2017; Rocha et al., 2019; Wyatt et al., 2014). The underlying mycelium forming around the plant roots forms a protective layer against various plant pathogens (Chen et al., 2018; de Mattos-Shipley et al., 2016; Kloepper, 2019).

### 2.4.3 Parasites

Some relationships of mushrooms can be detrimental. In the case of plants (Fisher et al., 2020), the parasitic nature of various fungal species can cause enormous damage to the wellbeing of the host plant resulting in a decreased growth rate and fruiting yields, and possible death. Some infections manifest as wilt, scabs, rust and rotted tissue (Pujari et al., 2015) and thus over all affect the functioning of the host and agriculture sector (Tchoumi et al., 2018). Although parasitic mushrooms can be beneficial towards providing space and nutrition for growing seedlings it can also lead to the infestation of agricultural crops, that can lead to significant economic losses and great food shortages.

Entomo-pathogenic mushrooms such as species within the *Cordyceps* genus are known to grow on the larvae of insects' (Kiho & Ukai, 1995; Massee, 1895). After fungal infection that leads to the death of these insect larvae the fungal organism replaces the bodily tissue of the dead remaining larvae with fungal pro-life structures and start to grow from within these insect corpses (Vega et al., 2009).

Zoo-pathogenic mushrooms are parasitic functioning mushrooms that are associated with animals (Powers & Howard, 2021). These fungal members can cause various diseases such as ringworm and tinea versicolor in their animal host (Bonifaz et al., 2010). Favus is a chronic

skin condition cause by the dermatophytic fungus *Trichophyton megninii* and is mainly associated with poultry (Arné & Lee, 2019; Powers & Howard, 2021). One of the most common parasitic mushrooms associated with humans are species within the *Candida* genus (Arné & Lee, 2019). These species are known to cause mycosis, the fungal infection of the skin that effects the mucous membrane, nails and other human body parts (Alanio et al., 2017). Also members of the *Blastomyces* and *Sporotrichum* are known to attack the subcutaneous tissue, bones and internal organs of their animal and human host. Some mushrooms have been found that feed on other mushrooms, thus mushrooms that also parasitize other fungal species . Members of the genus *Trichoderma* are known to produce a powerful enzyme able to break down the cell walls of other fungal species (Adnan et al., 2019). These species bind themselves to the growing hyphae of other fungal species, and a specialised appressorium protrude through and injects toxic enzymes into the cells of the host. This allows the parasitic fungus to thrive within the host.

### 2.4.4 Insect and animal associations

Some mushrooms have even evolved to the extent as to form specialized associations with specific hosts. One of the best known example, an intricate relationship is observed between termites and members of the *Termitomyces* genus. *Termitomyces* mushroom species are completely dependent on termites and their nest. The mushroom feeds on the organic matter brought back by the insects from their feeding on trees. (Adejumo et al., 2015; Sitotaw et al., 2020). These wood-destroying termites deposit faecal pellets containing partially digested wood debris underneath the mycelial network, which then extracts nutrients they require to grow and further form small nodules of hyphae. These nodules in return serve as additional food source for the termite colony (Adejumo et al., 2015).

## 2.5 Applications

### 2.5.1 Ethnomycology

Some of the earliest representations of relations between the Fungal Kingdom and mankind dates back to prehistoric times. For instance, mushrooms were found among the Iceman Ötzi's belongings, and well documented cave paintings located in the heart of the Sahara Desert portrays mushrooms used by humans (Debnath et al., 2019; El Enshasy et al., 2013; Gründemann et al., 2020; Lindequist et al., 2005; Molitoris, 1994; Rasalanavho et al., 2020; Samorini, 2001; Yuan et al., 2016). Today, mushrooms are still used traditionally and.

Knowledge on mushrooms that impact humans sociologically is called ethnomycology (Debnath et al., 2019; Osarenkhoe et al., 2014; Sitotaw et al., 2020). This is represented by the traditional use of any fungus for various purposes by local people and beliefs carried down through generations (Gupta et al., 2019). Thus, ethnomycology refers to the investigation surrounding man's long standing history of selecting and using mushrooms (Sitotaw et al., 2020). It is based on the merging of biological focused concepts and other scientific disciplines including that of ethnobiology, anthropology and ethnobotany, creating an integrated concept of cultural uses. This was established by the need of food improvement and further expanding in the exploration of other medicinal and cultural aspects. The field prioritises the diversity of species that are considered to be useful to that of the species that are considered to be inedible and/or poisonous. The knowledge and information is normally carried across generations and expressed during cultural, historical and religious events by indigenous folklore (Osarenkhoe et al., 2014). Parts of these events included the actual collection of the natural substance and handling of it during these actions. This also included the correct morphological identifications of the specimens and the documentation of all relevant information. Problematic uses identified regarding the field of ethnomycology is the lack of coherence of species documentation and utilization of these significant fungal species.
Ethnomycological practices are deeply imbedded in the beliefs and myths carried out by the cultures of indigenous communities all across the world. Fungal identification based on ethonomycological beliefs can be difficult in that different tribes of indigenous people from various localities may refer to the same species of mushrooms by different names. The Semai

people from Penisular Malaysia refers to the sclerotia mushroom belonging to the *Lignosus* genus as betes kismas where other tribes know it as susu rimau (Lau et al., 2015).

### 2.5.2 Edibility and nutrition

The utilization of edible fruiting bodies applies to many fields. These include gourmet mushrooms that are a culinary food enjoyed due to their umami taste and earthy aroma (Buruleanu et al., 2018; Muszyńska et al., 2020; Sharifi-Rad et al., 2020; Wang et al., 2014). Others form a staple food source in many cultures (Anderson & Lake, 2013; Ndifon, 2022) . Edible mushrooms range in shapes and sizes and in a recent study it was found that about 3283 species of higher macro fungi were considered to be edible (Zhang et al., 2021). Notable edible species include boletes (*Boletus edulis*), hypogeous truffles and cup-shaped morrels (El Enshasy et al., 2013; Sande et al., 2019; Trappe et al., 2008). Due to the significant chemical composition of edible fungi, mushrooms are considered to be very valuable in the healthy human diet. They contain a range of nutritional components that are often not even all included in plant and animal derived foods. Below follows a discussion of the most important components.

#### *2.5.2.1 Carbohydrates*

Edible mushrooms are considered to be rich in high energy providing carbohydrates but are low in calorie count (Gupta et al., 2019; Hrudayanath & Sameer, 2014). These carbohydrates measure an estimated 50-65% of dried sample weight, varying between fruiting body structures (Muszyńska et al., 2020; Rasalanavho et al., 2020). Often some of the polysaccharides have useful medicinal properties (Daba & Ezeronye, 2003; Jong & Birmingham, 1992).

#### *2.5.2.2 Fat*

The over-all amount of lipids within fruiting bodies are considered to be very low (Sande et al., 2019). Rasalanavho et al. (2020) documented an average of 0.8-5.3% lipids found within dried wild edible fruiting bodies, namely *B. edulis, Boletus mirabilis* and *Lactarius deliciosus*. The dominant fat type found within edible mushrooms is unsaturated fatty acids. Wild growing edible mushroom being a good source of this essential fatty acid, namely omega-6,

is comparable to many edible vegetables (Sande et al., 2019; Wang et al., 2014), thus being considered a healthy alternative to unhealthy fat sources and reducing overall fat intake.

#### *2.5.2.3 Protein*

Proteins are considered to be the main flavour contributing compound to edible mushroom species (Gupta et al., 2019; Zhang et al., 2021). The distribution and amount of protein not only depends on the species and size of the fruiting body but environmental factors also influence composition (Zhang et al., 2021). Universal proteins measure 12.3 mg/g of a dried mushroom sample. Fungal proteins are considered to be of higher quality compared to plant proteins and are even viewed as analogous to eggs, milk and meat (Adejumo et al., 2015; Hrudayanath & Sameer, 2014; Wang et al., 2014).

Alongside these valuable proteins mushrooms also contains all essential amino acids except for tryptophan (Urtzman, 2005; Wang et al., 2014). Essential amino acids (EAA) have to be obtained from food because they cannot be produced by the body naturally (Ogbe et al., 2009; Urtzman, 2005). Thus, edible mushrooms can be considered a noble substitute for animal products high in protein (Sharifi-Rad et al., 2020).

#### *2.5.2.4 Vitamins*

Vitamins found in mushrooms include riboflavin, tocopherol and Vitamin D. These concentrations vary between specimens and species. Preserving and cooking processes have proven to decrease the viability and potential of the vitamins in the mushroom (Wang et al., 2014). Mushrooms are also renowned source of Vitamin B, D and K (Anderson & Lake, 2013; Hrudayanath & Sameer, 2014).The precursor to Vitamin D, ergosterol (pro-vitamin D) is abundant in various bolete species namely *Imleria badia*, *B. edulis* and *Boletus reticulatus*. Ergosterol is converted to Vitamin D by ultraviolet light exospore (Adejumo et al., 2015) and functions as an anti-inflammatory that is capable of cytotoxicity (Panda & Tayung, 2016) and shows anti-cancer activity against various damaging and dangerous enemy cells and cancer cell lines (Cao et al., 2012; Muszyńska et al., 2020).

### 2.5.2.5 Minerals

Mushrooms contain many essential minerals namely iron, phosphor, copper and potassium (de Mattos-Shipley et al., 2016; Sharifi-Rad et al., 2020; Urtzman, 2005). Iron and copper are fundamental components in the production of red blood cells and the transportation of oxygen within the body (Alaimo et al., 2018). Potassium is significant in controlling blood pressure, while phosphor in combination with calcium is needed for the formation of structures such as teeth and bone (Urtzman, 2005). Another mineral abundant in mushrooms is selenium, a powerful antioxidant that protects the cells from damage (Panda & Tayung, 2016). This essential mineral is rarely found in vegetables compared to that present in mushrooms, which is found to be a very rich sources of this mineral (Alaimo et al., 2018).

### 2.5.2.6 Toxicology

Although, many species are considered to be edible some can cause various levels of intoxication and poisonings (Stebelska, 2013; Stöver et al., 2019). This occurrence is known as mycetism or mycetismus. A large number of fungal species are recognised for their unique mycotoxin toxicology profiles. Mycotoxins have been divided into categories, including amatoxins mainly associated with *Amanita*, *Lepiota* and *Galerina* (Hallen et al., 2002; Li et al., 2014). Gyromitrin, found in *Gyromitra esculenta, Gyromitra gigas* and *Gyromitra fastigiata*, is an oxidizale substrate, thus making it an unstable chemical (Jo et al., 2014). Muscarine and ibotenic acid (Poliwoda et al., 2014; Stebelska, 2013) reported form *Amanita muscaria*, psilocybin (Stebelska, 2013) produced by *Psilocybe cubensis* and coprine found in *Coprinopsis atramentaria* (Ndifon, 2022) are hallucinogenic compounds.

Amantoxins are considered to be thermostable, thus the application of heat, e.g. during cooking, does not affect the toxicology level. The manifestation and the extent of symptoms experienced are dependent on the amount and way of exposure either after the ingestion or inhalation of vapour of toxic mushrooms. The intoxication by these mycotoxic compounds presents by a wide range of symptoms experienced, varying from acute gastric intestinal distress such as nausea, vomiting and possibly diarrhoea. Some can affect the central nervous system (CNS) by delaying motor functions effecting an individual's sight and speech and some

present by altering psychological functioning causing delirium, depression and states of agitation. Severe poisoning by certain mycotoxins can proceed with cytotoxic-hepatotoxic action and can lead to fatalities (Kowalczyk et al., 2015).

### 2.5.3 Economic value

Mushrooms can be a functional food, exploited for nutritional features, their chemical composition and diversity in cooking applications (Sharifi-Rad et al., 2020). Fungi such as mushrooms are thus considered as a valuable economic trade entity. The consumption of some edible wild mushrooms as a promotional food group is important for countries with nutrition deficient diets (Rasalanavho et al., 2020). Mushrooms play a significant role in traditional markets, being sold as food as well as a form of traditional medicine in many African countries (Makhado et al., 2009; Tibuhwa, 2018). Mushrooms observed at these markets are often wild growing (Khaund & Joshi, 2014; Loyd et al., 2018).

Gourmet mushrooms command a high commercial price (Anderson & Lake, 2013). Species within the *Morchella* genus are considered to be a delicacy due to its pungent, nutty and slightly earthy taste (Pildain et al., 2014; Sambyal et al., 2014). The exterior surface of the mushrooms resembles that of honeycomb and provides an enjoyable texture similar to that of meat, thus making it applicable in various cooking applications (Turkoglu et al., 2006). Morrels are not commercially grown and are therefore rarely sold (García-Pascual et al., 2006; Sambyal et al., 2014). These fungi occur naturally and are thus considered a valuable and rare find.

### 2.5.4 Pharmacology

Higher classed fungi are sources rich in biological compounds (Hleba et al., 2016). Some selected fungal species are idolised for their psychoactive and hallucinogenic properties, thus being widely applied in spiritual worshipping by traditional folk (Okhuoya et al., 2010). Mushrooms have for long also been appraised for probable medicinal significance and were, and still are, used in old traditional folklore medicine as a treatment for a number of physical and psychological human ailments (Cao et al., 2012; Daba & Ezeronye, 2003; Hrudayanath &

Sameer, 2014; Khatua et al., 2017; Pieroni et al., 2005; Sharifi-Rad et al., 2020). Traditional treatment of a various number of ailments are performed in many underdeveloped countries (Rasalanavho et al., 2020). In South Africa 70% of indigenous people still rely on the admission of natural medical significant organisms after consultation of traditional healers (Andrade-Cetto et al., 2016).

The bioactive secondary metabolites found within some fungal species have a wide application to the developmental field of mycopharmacology by presenting with numerous advantages to human health (Money, 2016; Thu et al., 2020). These properties include immune enhancement, regulation and maintaining homeostasis, regulating of biological activities as well as the ability to prevent and possibly aid in the treatment of a variety of life threatening disorders and diseases such as cancer the most common death causing disease investigated in humans (Hereher et al., 2018), ischemic strokes and cardio-vascular ailments. The medicinal properties presented by various fungal species include the ability to act as an anti-inflammatory, immunomodulatory, anti-carcinogenic, antiviral, anti-bacterial, antidiabetic, anti-oxidative agent (Cheung et al., 2003).

In recent times mushrooms have been investigated for alternative treatment options for cancerous tumours and as an aid in healing diabetic induced wounds (Pringle et al., 2021). Selected macro fungi are beneficially used to aid in the treatment of multiple ailments. Many human disorders are promoted through oxidative damage caused by the imbalance of free floating radicals in the body (Cheung et al., 2003). Stressors effect the oxidation level of sugars, proteins and lipids. High concentrations of anti-oxidants such as phenolic, organic compounds and alkaloids found in abundance within various fungal species can neutralize stress when disparity occurs. Unregulated oxidative stress can lead to cardio vascular complications, neuro degenerative disorders, various cancers and diabetes (Fadeyi et al., 2019). The nutritional composition of many species of edible mushrooms are investigated and found that essential fatty acids found in numerous species have the potential to reduce blood cholesterol levels and regulate cell physiology (Sande et al., 2019).

More research describes the distribution of toxic chemicals in inedible and edible macro fungi (Rasalanavho et al., 2019; Stewart et al., 1999), also recognise species associated with significant bioactive-chemicals that prompt antibacterial, anticancer and anti-inflammatory responses. Such research aids in the expansion of natural resources in conjunction with drug

development possesses, a great potential for the health sector (Andrade-Cetto et al., 2016; Boukes et al., 2017; Daba & Ezeronye, 2003; Nkadimeng, et al., 2020).

#### *2.5.4.1 Psychology and mind altering properties*

Certain mushrooms have been extensively investigated for the potential to treat many psychological conditions such as depression, anxiety and obsessive-compulsive behaviour (Nkadimeng & Steinmann, et al., 2020; Stebelska, 2013; Wasser, 2015). The ability of some mushrooms to cause hallucinogenic symptoms are mainly caused by the compounds psilocybin and psilocin (the active form of psilocybin) that is found in species of the genera such as *Psilocybe*, *Panaeolus* and *Gymnopilus* (Jo et al., 2014). The utilization of these magic mushrooms often calls for the ingestion of fruiting bodies or the consumption of fungal extracts considered similar to teas, as well as the inhalation of smoke produced by the burning of selected fungal matter (Okhuoya et al., 2010). Investigated cave paintings located in the Sahara Desert depicts the interaction of indigenous people with fungal shaped objects that suggested that the art illustrates the usage of mushrooms during traditional religious activities since the moving human and fruiting body structures were connected with dotted lines, thus demonstrating the probable believed mind-altering properties possessed by these mushrooms (Samorini, 2001).

Psychoactive properties have also been identified in *A*. *muscaria* and *Amanita pantherina*, members of the *Amanita* genus. *Amanita muscaria* and *A. pantherina* contain trace amounts of muscimol and ibotenic acid, respectfully (Guzmán et al., 1997). These are considered toxic substances when ingested that lead to mentioned intoxication symptoms, and the use of less detrimental fruiting bodies are regularly preferred (de Mattos-Shipley et al., 2016).

Chemically psilocybin and psilocin are very similar to lysergic acid, also known as LSD. LSD is a class A drug that was discovered and synthesized by Hofmann in 1938. The lab altered synthesised derivative, namely N,N-diethyllysergamide is considered to be one of the most compelling illegal psychoactive pharmaceutical to date. The drug when used produces hallucinations, visions and experiences similar to that of an individual suffering from a mental illness like schizophrenia (Molitoris, 1994). This comparison lead to the possession and usage

of magic mushrooms or mushrooms containing psilocybin to be considered illegal and against the law in most parts of the world (de Mattos-Shipley et al., 2016).

The ability of these recognised neurotrophic mushrooms to act upon the human body and mind has brought on countless opportunities regarding other aspects of human health (Guzmán et al., 1997; Money, 2016). The neuro-modulation of psilocybin (prodrug) is instigated mainly by the ingestion of hallucinogenic mushrooms containing psilocybin (Reynolds et al., 2018). Under acidic conditions psilocybin is metabolised and converted to active psilocin by a dephosphorylating reaction, from where it is absorbed in the gastro-intestinal track and can cross the brain barrier (de Mattos-Shipley et al., 2016; Stebelska, 2013). Psilocin effects the serotonergic system, causing hallucinogenic effects (Bacqué-cazenave et al., 2020; Varley et al., 2020) and acting as a psycho-active agonist of the neurological system of the subtype serotonin 5-HT2A receptor. The serotonin 5-HT is the major modulator of motor behaviours and cognitive functioning (Jo et al., 2014; Reynolds et al., 2018). Due to this ability, a large number of research is being conducted showing that psilocin can be used to treat depression, anxiety and obsessive compulsive disorder (OCD) (Isbell, 1959; Nkadimeng, et al., 2020). The compound is especially appealing because it is non-addictive and intoxication by psilocybin rarely leads to fatalities because the considered lethal dosage of magic mushrooms in humans is 17 kg/70 kg, an amount considered to be very low (Nkadimeng & Steinmann, et al., 2020).

### 2.5.5 Bioremediation and industrial agriculture

Bioremediation refers to the cost effective and ecological advantageous degradation of toxic environmental chemicals by the utilisation of microorganisms (Adenipekun & Lawal, 2012; Ascough et al., 2010). Due to the ability to breakdown and decompose organic matter numerous fungal species are exploited for these unique capabilities during acts of bioremediation. White rot mushrooms are capable to decompose lignin and shows ability to transform heavy metals in contaminated soil through unique enzymatic activities (Babasaheb, Parkhe & Palghadmal, 2019; De Koker et al., 2000). They are used to decontaminate oil-polluted land in bioremediation activities (Adenipekun & Lawal, 2012). This aids in forming the needed basis for sustainable agricultural and forestry through the recycling of organic

matter that can be used as a growing substrate and that can be returned to the ground as natural fertilizers (Gupta et al., 2019; Odelade & Babalola, 2019).

In the agricultural industry within rural communities' natural remedies and traditional healing is not only restricted to be used for human medical care but is also sometimes considered and applied in the treatment of diseases of animals such as livestock (Kaul, 2009; Mumpuni et al., 2020). The practise is considered to be underutilized regarding the lack of research focusing on this topic, thus it is essential that the use of medical mushrooms is explored. This could be especially useful for under-developed or developing countries. For example in South Africa, specifically the Eastern Cape province, it has been found that 75% of small scale famers in rural areas are still relying on natural herbal remedies to treat their livestock, but they are still lacking the knowledge regarding accurate dosages and the most appropriate herb or organism to use for a variety of diseases observed in these animals (Masika & Afolayan, 2002). Several advantages of utilizing mushrooms as a source of biological active compounds over that found in plants are that fruiting bodies are produced within a reduced amount of time and mycelium can be manipulated in various ways to produce the specific desired concentration of these wanted active compounds (Pringle et al., 2021).

## 2.6 Identification

Due to the broad range of presence and interactional functioning, the boldly diverse Fungal Kingdom is represented by numerous eccentric shapes, shades and sizes (Rubina et al., 2017), such as the well-recognised vibrant red bulbous bell button silhouettes of *A. muscaria*, to that of the single-celled yeast. These macro fungi within the Basidiomycota taxa are mainly identified by the observation of morphological features. These features include all physical characteristics that can be observed from the investigated specimen (Adejumo et al., 2015; Hawley & Dames, 2004), the substrate or host of the specimen and environment surrounding the sample (Badotti et al., 2017; Itoo et al., 2016), and all significant notable features such as smell (Anderson & Lake, 2013) or discoloration of the example due to tissue damage (Reynolds et al., 2018). Discoloration can be caused by bruising or handling (Zai Wei Ge et al., 2018; Itoo et al., 2016; Kaur et al., 2014) or can be in the form of bleeding (Leonardi et al., 2021; McKenzie et al., 2002). Microscopic investigation of characteristics such as spore,

basidium and cystidial morphology, and tissue arrangements are needed for some species identifications and descriptions (Adejumo et al., 2015; Alsohaili, 2018).

Relying only on this way of species identification has been proven to present difficulties. Some morphological characteristics are considered to be extremely subjective to observational bias of the collector (Jayasiri et al., 2015; Schoch et al., 2014). Furthermore, confusion of true species identification are due to a high morphological plasticity observed between closely related species within the same genus (Alanio et al., 2017; Khaund & Joshi, 2014; Menolli et al., 2010; Silva-Filho et al., 2020). Other methods of identification, such as the use of DNA sequence comparisons, are thus also used to compliment morphological studies and to address taxonomical problems (Alsohaili, 2018; Itoo et al., 2016).

### 2.6.1 Morphological

Identification of macro fungi have for long been only based on morphological observed features. This is known as the phenotypic classification concept (Jayasiri et al., 2015). Key macro morphological characteristics considered when identifying macro fungi based on observations only include the shape, size, colour, cap, stalk and gills of the fruiting body, as well strong associations observed in some species with the habitat or host substrate that the fruiting body is growing on or from, is considered during identification (Itoo et al., 2016). For example, *Lactarius* and *Russula* species are believed to be pioneered by alienated pine host associations (Kaul, 2009). Vegetative mycelia observation is yet another way of using physical attributes to correctly identify different mushroom species, but can be difficult due to the complex nature of branched hyphae structures that can differ within minute measurements in width, length and thickness (Wasser, 2014). Other microscopic features include spore, basidium and cystidial morphologies.

Morphological based identification techniques are exploited by foragers, scientists and mycologists. It is considered to an extent to be a swift, reliable, quick and informal way of fungal identification. However, due to species plasticity it is also only accurate to some degree (Menolli et al., 2010) because some closely related species within the same genus may present features that can overlap or be inter changeable (Tchoumi et al., 2020). This means

of identification is also considered to be very subjective to the observer, some expertise is needed in various cases and it may not be able to indicate the taxonomic relationships between species (Jayasiri et al., 2015). For instance, grouping specimens become more difficult as some species present differently through their life cycle and can look different from a developing fruiting body to that of a mature older specimen of the same species. The environment can also play a role in the alteration of appearance in some fungal species. The surrounding flora for example, have the ability to influences macro fungi production by pH, carbon and nitrogen regulation (Debnath et al., 2020). Species that present with warts or scales can be washed away or off due to heavy rainfall, or specimen colours can vary due to varying surround climate (Goldman & Gryzenhout, 2019).

Classification of mushrooms based on physical characters alone can result in the wrong identification that can lead to detrimental effects. Numerous accounts of fungal poisoning are documented each year across the world (Hallen et al., 2002). Poisonings are largely due to the inability to distinguish between edible and inedible specimens that leads to wrongful identification of the specimen and the lack of sufficient data regarding poisonous mushroom profiles (Jo et al., 2014; Kowalczyk et al., 2015). The overall inability to distinguish numerous groups of macro fungi from each other either as edible, inedible, deadly poisonous, possible suspects or unknown toxicology results in the under estimation of the level of fungal diversity (Schoch et al., 2014) and the erroneous identification of many species (Menolli et al., 2010).

### 2.6.2 Phylogenetic

Utilizing DNA based technologies, scientist have been able to design ways of more accurate fungal identification on a molecular level. This method of identification heavily relies on DNA sequencing and DNA barcoding (Badotti et al., 2017; Khaund & Joshi, 2014; Xu, 2016). The term DNA barcoding refers to a relatively short gene sequence present in the genome that is considered unique enough for species identification. The universal gene region used for the molecular identification of species members of the Fungal Kingdom is known as the fungal barcode (Itoo et al., 2016; Schoch et al., 2012).

Many markers in the fungal genome have been investigated for their usefulness, including the locus positions ITS (internal transcriber spacer), LSU (large subunit) and SSU (small

subunit) of the ribosomal operon, and protein-coding gene regions such as the translation elongation factor 1α and the largest subunit of RNA polymerase 2 (RPB1) (Schoch et al., 2012; Xu, 2016). The rRNA cistron is transcribed as a single unit by RNA polymerase I and further slitting after transcription, resulting in the removal of two internal transcribed spacer regions. The spacer pair that includes the 5.8S gene is collectively refered to as the ITS region, with the remaining 18S nuclear ribosomal gene forming the smaller subunit rRNA known as the SSU and the 28S nuclear ribosomal gene forming the larger subunit (LSU) (Lee et al., 2000; Maharachchikumbura et al., 2021; Menolli et al., 2010). The overriding direction steers towards using the 18S, 5.8S and 28S ITS region of nuclear ribosomal genes, which has been sequenced for most fungi (Schoch et al., 2012). Of these, the ITS region is found to be more effective to discriminate taxonomically between species than the more conserved LSU and SSU regions that are used for higher level classifications, and these regions have a high PCR (polymerase-chain-reaction) amplification success rate (Purty & Chatterjee, 2016).

Using DNA techniques to identify fungal species is considered to beneficial to decrease the worldwide knowledge gap related to fungal diversity and to provide more evidence regarding relationships between taxa and evolutionary trends (Jayasiri et al., 2015). It can be more accurate in the identification of macro fungi to species level compared to only relying on morphological identification. It can also distinguish between specimens that present with high levels of morphological plasticity and in cases when morphological identification is not always possible (Alsohaili, 2018), such as when the life stage of development of the fungus is too young or for a mature older specimen, and specimens that are incomplete. Even the mycelium from which the fruiting body grows can be used or even cultivated from spore specimens and grown for DNA application (Liu et al., 2022; Menolli et al., 2010).

The fungal DNA barcoding initiative provide a potent and rapid approach to identify cryptic species, investigate phylogenetic relationships between specimens and offers a reliable way of documenting the true diversity of the Fungal Kingdom (Xu, 2016). This approach focusses on the principle that a unique short 500-800bp sequence code produced by an applicable primer set can be compared with other barcodes and used to identify a species (Purty & Chatterjee, 2016; Tchoumi et al., 2020). The universally recognised code serve as an worldwide understood language, compared to multiple names documented for the same species around the globe (Menolli et al., 2010).

Although, the means of fungal identification through DNA derived techniques are very advantages, more expenses and external resources are required compared than that of only relying on ways of morphological identification (Adenipekun & Lawal, 2012; Avin et al., 2013). However, morphology has formed the base for taxonomic classification of species and phylogenetic analysis has aided the effort to correctly identify unknown specimens or where morphological identification was challenged (Jayasiri et al., 2015). This fact can be counteracted by the complementing morphology based approaches with molecular identification techniques. Using both identification techniques can aid in the accurate discovery of new species (Maharachchikumbura et al., 2021; Song & Cui, 2017; Wisitrassameewong et al., 2020) and help to taxonomically classify species correctly (Trappe et al., 2008). Thus, consistent a combination of using morphological feature characterisation alongside phylogenetic investigation is recommended for the classification and correct identification of fungal species (Alsohaili, 2018; Badotti et al., 2017; Itoo et al., 2016; Khatua et al., 2017; Kiran et al., 2021; Li et al., 2019; Menolli et al., 2010).

This way of sufficient fungal species identification thus aids in resolving problematic disadvantages presented by morphological species plasticity (Menolli et al., 2010). Molecular identification does not come without challenges. The physical cellular structure of mushrooms is incomparable to that of other organisms, presenting hardy construction that makes the breakdown of fungal tissue and DNA exposure difficult. For example, the arrangement of internal chitin sleeves considered to provide cell walls with an level of firmness and to structural protection caused fungal cells to be somewhat resistant to processes of lysis (Kumar & Mugunthan, 2018). The lysis of cells is considered the most important step in any fungal focused method of DNA extraction because it results in the exposure of the internal cellular content. Many ways of lysis have been investigated, such as considering mechanical techniques by exposing samples to liquid nitrogen or dried ice in efforts to improve fine gridding by mortar and pestle sets (Griffin et al., 2002), as well as chemical breakdown by means of utilizing digestive enzymes and other chemicals such as benzyl chloride for cellular lysis (Aamir et al., 2015; Shaolan et al., 2002). Alongside numerous investigated extraction protocols and methods a variety of extraction kits are commercially available specifically designed for the purpose of fungal genomic DNA extraction (Kumar & Mugunthan, 2018; Loyd et al., 2018). These set out kits provide researchers with step by step

699 guidance following a standardized protocol and usually includes all that is needed to
700 successfully complete the extraction process.

701

## 702 2.7 Fungal biodiversity of South Africa
703

704 The first ever checklist documenting the macrofungal diversity within the country by Kinge et
705 al., (2020) reviewed relevant literature and documentations of fungal species observed within
706 the country based on previous studies and records of the National Fungarium of South Africa.
707 For example, Reid et al. (1991) documented 13 *Amanita* species from South Africa, resulting
708 in the discovery of new taxa. Members of the genus include the well-known poisonous *A.*
709 *muscaria*, *A. pantherina* as well as the edible *A*. *pantherina* look-a-like species *Amanita*
710 *excelsa* and *Amanita rubescens* (Kinge et al., 2020), Amanita *phalloides* and sub-species *A.*
711 *phalloides* var. *alba* and *A. phalloides* var*. umbrina*, *Amanita foetidissima*, *Amanita pleropus*
712 and *Amanita reidii*, with some species considered to be native to the country (Hallen et al.,
713 2002; Zhang et al., 2015). The research was based on morphological characterisation and
714 relevant available literature.

715 Other well studied genera include the medically important *Ganoderma* genus renowned for
716 its healing properties (Cao et al., 2012; Loyd et al., 2018; Tchoumi et al., 2018), and *Armillaria*,
717 known as the 'honey fungus' because of its vibrant golden yellow colour (de Mattos-Shipley
718 et al., 2016; Molitoris, 1994; Wang et al., 2014). *Armillaria* species are responsible for causing
719 major health concerns in economically important host trees, including *Eucalyptus* and *Pinus*
720 plantations (Tchoumi et al., 2018; Wartchow & Cortez, 2016). New species in *Ganoderma*,
721 such as the destructive species Ganoderma *austroafricanum* (Coetzee et al., 2015), were also
722 discovered.

723 *Ganoderma* species are represented by fruiting bodies classified morphologically as brackets
724 with an inherit parasitic and saprophytic nature causing numerous diseases in associated
725 hosts (Paterson, 2006). The *Ganoderma lucidium* complex, commonly known as the Reishi or
726 Lingzhi mushroom, belongs to the Ganodermataceae and is identified by macroscopic
727 features such as its distinctive kidney-shaped, brown fruiting body, that is woody, cork-like
728 textured, with the typical reddish brown shiny laccate colour with and cream-white outer

margin (Money, 2016; Pauline et al., 2021; Rumainul et al., 2015). The bracket mushrooms are generally located growing on broad-leaved tree trunks, cut-down stumps and emerging tree roots (Pringle, 2017). This bracket fungus is considered to be inedible due to its fibrous texture and hard woody exterior but its medical potential can be explored by oral administration through making traditional tea and soup extracts with other herbs as a 'health tonic' (Bulam et al., 2019). The fungal body in some circumstances can be burned and some is inhaled for medical purposes (Ekandjo & Chimwamurombe, 2012). In modern times the medical benefits presented by the *Ganoderma* species are divided into three main types that includes the fruiting body, fruiting body producing mycelia and fungal spores that is dried and ground down to powder form. From these parts of the macro fungi many drugs and dietary nutraceuticals are derived, as well as supplemental beverages, other oral liquids and even chewable tablets (Bulam et al., 2019). The genus was also investigated in neighbouring country Namibia were it was found that species members were easily found in abundance on dead or dying trees, stumps or plant roots.

Some studies have been done on macro fungi in the Tsitsikamma region (Coetzee et al, Tchoumi et al.). Research conducted by Tchoumi et al. (2020) investigated the wood rotting mushrooms associated with the indigenous forest in the region and revealed novel species and relationships between mushrooms and their hosts, revealing that some species are not limited to a single host. For instance, the occurrence of *Fomitiporia capensis*, was previously limited to South African vineyards (*Vitis vinifera*) but then recorded on *Quercus* and *Psidium*, indicating that these mushrooms have to ability to occupy a wide range of hosts (Tchoumi et al., 2020). Furthermore, a total of seven new *Ganoderma* species have been identified from this area by Tchoumi et al. (2020), with four associated with trees showing wood-rot symptoms. Three species of *Ganoderma* described by Tchoumi et al. (2018, 2019) totalled up the amount of *Ganoderma* species observed in the country to 13 (Kinge et al., 2020), as well as four more that are native to the country. The prevalence of the these parasitic macro fungi in the region supports the interpretation that some of these fungal species are responsible for causing symptoms of wood rot in this native forest (Tchoumi et al., 2018) and that they pose a threat to the agriculture and forestry sectors in the whole of the GRNP.

However, studies from Tsitsikamma usually focussed on species that were plant pathogens and causing tree rot, resulting that the remaining fungal biodiversity is still unstudied. This

760 includes the diversity of mushrooms found in cultivated pine plantations that have not been
761 documented yet.

# CHAPTER 3 – MATERIALS AND METHODS

## 3.1 Sampling location

The Garden Route National Park (GRNP) is located in the Western Cape and Eastern Cape province of South Africa (Fig. 3.1). Spanning over 145, 000 ha, the entire forest complex is the largest and one of the richest biodiversity regions in the country (Baard & Kraaij, 2014). It includes not only terrestrial vegetation but steep rock-faced shore lines (Flemming & Keith Martin, 2018; Hugo et al., 2012; Parker-Nance et al., 2019). The area lies within the fynbos biome, which is considered to be the smallest but most biologically diverse plant kingdom in the world (Bellingan, 2010; Rutherford et al., 2006). The GRNP was established in 2005 by various land groups and is managed for the conservation of water, biodiversity and indigenous forests by Cape Pine (formerly Mountain to Ocean Forestry) and the Garden Route National Park for the Tsitsikamma region.

The Garden Route is classified by a humid to sub humid climate and receives rain throughout the year, with a mean annual of 800 mm to 1100 mm peaking in winter months (June-August) (Milne & Haynes, 2004). The measured rain-fall increases in a north-easterly direction as an increased altitude is recognised. The climate in the region is considered to be stable showing no significant seasonal temperature differences (Kraaij, Cowling, & Van Wilgen, 2013). Winters are mild with temperatures ($18^{o}C$ – $21^{o}C$), but occasional frost occurring on the highest mountain peaks can be observed during this time. Mild warm summers ($22^{o}C$ – $25^{o}C$) are reinforced with the warm Agulhus ocean current that creates warmer south easterly winds during the this time (Baard & Kraaij, 2014; Flemming & Keith Martin, 2018). Altitudinal range for the region is between 0 – 1675 m.a.s.l due to the extreme topography of the areas, rugged mountain range and coastal beaches (Baard & Kraaij, 2014).

Approximately 60 500 ha of fragmented indigenous forests are dispersed across the Garden Route National Park. The range extends for about 225 km from the Outeniqua, located east of the Touw river and Tsitsikamma mountain ranges, to the southern coastal plateau of the Tsitsikamma (Ella, 2005; Kraaij, Cowling, & van Wilgen, 2013). The whole of the region is separated into smaller portions by roads, various land uses such as farm lands and towns

(Milne & Haynes, 2004; Tchoumi et al., 2020). Vegetation includes indigenous forests, fynbos shrub lands, agricultural fields and plantations of alien tree species (Baard & Kraaij, 2014; de Beer et al., 2014; Milne & Haynes, 2004). The business of manufacturing usable biomaterial from trees is the primary contribution to the increased number of alien plants in the region and a large percentage of land use is plantation based. Although the Western Cape plantations represents only 6.3% of South Africa plantation, the region hosts more than 15% of sawn timber production plants in the country and represent 15.2% of all wood rounding factories in South Africa (de Beer et al., 2014). In particular, *Pinus* and *Eucalyptus* species consist of the majority of alien plants, cultivated for timber production.

The first cultivated state owned plantations in the GRNP was established in 1883 close to the coastal town Knysna in the Western Cape to provide an alternative source of timer than that of indigenous tree species, and focussed more on the cultivation of *Pinus* (Hugo et al., 2012; Rocha et al., 2019). The plantation areas became more extended since 1891 as land gaps were created by the harvesting of indigenous forest. The development and expansion of the private and state owned plantations in the coastal region grew steadily as the plantation sector developed, to a current estimated extent of 70,000 ha cultivated plantations between the Garden Route's bordering towns George in the western area and Kareedouw in the east (Avis, 1995; Baard & Kraaij, 2014; Rutherford et al., 2006).

The area is subdivided into two main sections (Fig. 3.1), due to the aggressive fragmentation of the native areas of the protected park by the evolving plantation sectors. The first area is the Wilderness National Park (WNP) situated in the Western Cape portion of the GRNP. The area is located around and between the larger towns of George, Sedgefield and Knysna (Baard & Kraaij, 2019; de Beer et al., 2014; Hawley & Dames, 2004). The other portion is the acclaimed Tsitsikamma plateau (Hugo et al., 2012) localised to the Eastern Cape province that stretches between 30 km east from Plettenberg Bay and 30 km west form Humansdorp for approximately 80 km of coastline (Baard & Kraaij, 2014; Hugo et al., 2012; Pauw, 2009). Considering being recognised as one of the world's biodiversity "hotspots" the overall management and conservation efforts regarding forest biomes should be seen priority on a global scale (Crous et al., 2006; Hawley & Dames, 2004).

## 3.2 Sample collection

Samples were collected from various substrates including damp soil, under shaded trees, dead tree stumps and decaying roots of commercial pine (*Pinus*) plantations. Samples were collected from these natural habitats from March 2021 to March 2022 from various locations within the Garden Route National Park area, Tsitsikamma South Africa (Fig. 3.1). Samples were collected from numerous commercial plantations, grass lands and disjointed vegetation within the region.

Samples of various different species of macro fungi, depending on availability, was photographed and collected following the methodology of Gryzenhout (2012), Goldman & Gryzenhout (2019) and Gryzenhout (2021). Photographs were taken from various viewpoints (dorsal, ventral, lateral) of each specimen. Features captured to facilitate correct identification included top (cap), under (gill when present) and lateral side views (shape illustration), representing all parts (cap, stipe, basal parts and gills) of each of the observed specimens and any other interesting features such as discolouration. Collection bags were marked with a collection number, probable fungal species name and geographic location corresponding with different collection sites. Any physical changes of importance and useful in species identification was documented for each specimen during the collection and eventual drying process.

For sampling the entire fruiting body of the mushrooms was handpicked, dug out or cut off from their natural environment or host substrates, further cleaned of residual debris, soil and sand with a soft bristled brush and stored in individually labelled paper bags. Samples were dried at 65$^{o}$C for 8 – 10h or until dry to the touch over a heater. The dried specimens were stored in new individually labelled paper bags until genomic DNA extraction was performed at the University of the Free State (UFS). Collection tools were sterilised with 70% ethanol in between each specimen to prevent cross contamination between samples. For publication the specimens will be deposited in the National Fungarium of South Africa (Agricultural Research Council, Pretoria, South Africa).

## 3.3 Morphological Identification

Samples of various different species of macro fungi, depending on availability, was photographed and collected following the methodology of Gryzenhout (2012), Goldman & Gryzenhout (2019) and Gryzenhout (2021) (Table 3.1). Morphological identification of collected samples was based on the South African fungal field guide of Gryzenhout (2012), Goldman and Gryzenhout (2019) and Gryzenhout (2021) (Table 1). An infield feature capturing table (Table 3.2) was developed for this thesis that allows for the rapid documentation of physical characteristics and aids in morphological identification. Key macroscopic morphological characteristics of each specimen were considered, as well as on which substrate and habitat they were collected from. Macroscopic features included the shape and location of the hymenium that determines the form of the fungus, the shape, texture and colour of the fungal cap, gill colour, shape and spacing or pores, stipe attachment type, shape, size, colour and texture were documented, as well as the absence or presents of a ring, a veil or a volva. Any other observed morphological changes such as staining, discoloration after bruising or emitted fluid or smell was noted while the identification was conducted.

Samples of various different species of macro fungi, depending on availability, was photographed and collected following the methodology of Gryzenhout (2012), Goldman & Gryzenhout (2019) and Gryzenhout (2021) (Table 3.1). To aid in documenting these physical characteristics a fungal illustration for the infield table was created to simplify the documentation of specimen characteristics that include all the features mentioned above (Fig 3.2). The complete illustration was divided into three sections. The top section included the cap and gills, illustrating the shape of the cap, cap margins, colour, additional observations such as warts or striations, as well as presence of gills, attachment type and colour of the lamellae. The middle section deals with the stipe, including its shape, colour and accompanying structures such as a volva or ring. The bottom section of the fungal illustration documents the habitat and substrate that the fruiting body was found in or on.

In addition to Gryzenhout (2012), Goldman & Gryzenhout (2020) and Gryzenhout (2021) relevant morphological characteristics found in literature including Branch (2015) was applied to compile an overall morphological characteristic table for each sampled species. The overall

886 aim of the table was to be as user friendly as possible including key observational
887 characteristics to aid identification, to document each aspect of morphology as un-objectively
888 as possible but that can still be applied to aid in the identification of various fungal forms
889 (Nuytinck & Verbeken, 2007). The table was supplemented by photographs and based on DNA
890 sequence based identifications.

## 3.4 Phylogenetic Identification

### 3.4.1 DNA Extraction

Subsections 1cm³ of the dried mushroom samples was cut out to be further pulverized in a 2ml Eppendorf using a small pestle inside the tube. Approximately 40 mg of the pulverized material was used for genomic DNA extraction. The genomic DNA was extracted using the method mentioned in Alvin et al. (2012) with minor modifications. The DNA isolation called for the addition of approximately 900 µL extraction buffer (100 mM Tris–HCL pH 8.0, 10 mM EDTA, 2% SDS) to the isolated 40mg dried pulverised fungal material. Each sample was incubated for 30 min at 65 $^{o}$C. A modification include that during the incubation time each sample was vortexed every 5 min to ensure optimal lysis of the fungal cells. Following the incubation, Eppendorf tubes were centrifuged at 13,000 rpm, 4$^{o}$C for 5 min. The aqueous phase was pipetted into new Eppendorf tubes and was extracted twice with 600 µL Chl:1AA: isoamyl alcohol (24:1). DNA was precipitated with cold (100%) ethanol, and pelleted by centrifuging at 16,000 rpm for a modified total amount of 30 min at 4$^{o}$C. Finally, the pellet was re-suspended in 20 µL nuclease free water for this protocol and stored at -20$^{o}$C prior to use.

The genomic DNA was visualised using a 2% agarose gel (Cleaver Scientific Ltd, UK) with Gel-Red™ Nucleic Acid Gel Stain (ThermoFisher Scientific, USA). In a geldoc (Vacutec, Roosevelt Park, South Africa). Standard electrophoresis conditions (100V, 400Ma, 45 min) were used in a BioRad Power Pack 300V, 400Ma, 75W device. Further quantification of the genomic DNA was done utilizing a NanoDrop® Spectrophotometer ND-1000 (ThermoFisher Scientific). DNA purity was evaluated by 260/280nm absorbance values and multiples of DNA concentrations (ng/ µL) were averaged and documented for each sample.

### 3.4.2 PCR Amplification

The ITS rRNA gene region was amplified using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3')(White et al., 1990). Every 25 µL PCR reaction contained genomic DNA template, 1.25 µL of each primer, 12.5 µL One Taq® 2X MM w/standard buffer (New England BioLabs, inqaba Biotechnical Industries

(Pty) Ltd, Pretoria, South Africa) and nucleus free water. PCR cycling conditions was as follows: 94°C for 2min 30s; 40 cycles of 94°C for 30s, 54°C for 30s, 72°C for 40s, followed by a final extension at 72°C for 10 min (Song & Cui, 2017) and was conducted using a BioRad T100 Thermal Cycler (BIORAD, Johannesburg, South Africa) .Amplification products (5µL) were ran through 1.5% agarose gels with Gel-Red™ Nucleic Acid Gel Stain and results were viewed on a geldoc (Vacutec, Roosevelt Park, South Africa).

Amplified products (5µL) was treated with ExoSAP-IT™ PCR Product Cleanup Reagent. PCR amplification (2µL) was added to Big Dye Terminator, One Tag® 2X MM w/standard buffer (New England BioLabs, inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa), Big Dye buffer (Big Dye Terminator v1.1, v3.1, 5X Sequencing Buffer, Applied Biosystems, ThermoFisher), 5µM primer, and $dH_2O$ solution in preparation for sequencing. PCR amplification with conditions as follows: 96°C for 1 min; 35 cycles of 96°C for 3 min, 52°C for 30s, 60°C for 4 min, followed by a final extension at 60°C for 3 min and was conducted using a BioRad T100 Thermal Cycler (BIORAD, Johannesburg, South Africa). The PCR amplification products was analysed by automated Multicapillary Electrophoresis on an ABI Prism 3730 Genetic Analyzer in Department of Genetics.

### 3.4.3 Phylogenetic Analysis

ITS gene region searches were done on sequence deposits in Genbank (Alsohaili, 2018; Badotti et al., 2017; Itoo et al., 2016) and compiled into aligned FASTA format contigs with Geneious V.11, for preliminary identification. Sequence searches excluded uncultured/environmental sample sequences alongside limited and un-limited sequences from type material. Generated sequences where selected based on quality controlling parameters. This included percentage identity (%), the expected value or E-value, the sequence query coverage percentage (%) and the length of the sequences measured in base pairs (bp). Sequences were also carefully chosen based on current names and applied to a single type (homotypic names) to reduce confusion of further tree analysis (Jayasiri et al., 2015; Schoch et al., 2014).

954 Multiple sequences were assembled into datasets (Table 3.3) and aligned using the MAFFT
955 (Multiple Alignment using Faster Fourier Transform) server set to align without eliminating
956 gappy regions (https://mafft.cbrc.jp/alignment/software/). Phylogenetic relationships
957 between taxa were visualised by creating phylogenetic trees based on best fitted models
958 (Table 3) and analysed and visualized by using Maximum Likelihood (ML) in MEGA (Molecular
959 Evolutionary Genetics Analysis) V.7 (https://www.megasoftware.net/), with 500 bootstrap
960 value (BS). Separate datasets and analyses were done per specimen unless specimens were
961 from the same species or genus.

962 **Table 3. 1** All samples identified within this study.

963

| SCIENTIFIC NAME | DISTRIBUTION WITHIN SOUTH AFRICA | COMMON NAMES | ECOLOGY | HABITAT | SIMILAR MORPHOLOGICAL SPECIES | EDIBILITY | MORPHOLOGICAL GROUP | LOCATION |
|---|---|---|---|---|---|---|---|---|
| *Amanita morrisii* | Found within Eastern Cape, possibly more widespread . | | Mycorrhizal | Under Coniferous and Broadleaved trees (fruiting bodies single or grouped). | *Amanita pantherina* | | Gilled | Koomansbos |
| *Amanita muscaria* | Widespread across the country. | Fly agaric; Fly Amanita | Mycorrhizal | Under forests oak and pine tree species (fruiting bodies single or grouped). | *Amanita caeasarea* | Poisonous | Gilled | Plaatbos |
| *A. pantherina* | Widespread across the country. | Panther Cap; Panther Amanita | Mycorrhizal | Under Coniferous and Broadleaved trees (fruiting bodies single or grouped). | *Amanita excesla; Amanita rubescens* | Deadly Poisonous | Gilled | Koomansbos |
| *A. rubescens* | Widespread across the country. | Blusher; Blushing Amanita; False Pantherina | Mycorrhizal | Under Coniferous and Broadleaved trees (fruiting bodies single, grouped or scattered). | *Amanita excesla; A. pantherina* | Edible | Gilled | Plaatbos |
| *Chlorophyllum sp.* | Found within Eastern Cape, possibly more widespread. | | | Under Coniferous and Broadleaved trees (fruiting bodies single or grouped). | Other *Chlorophyllum* species. | | Gilled | Lottering |
| *Clitopilus prunulus* | | Sweetbread Fungus; The Miller | Saprophytic | Under Coniferous and Broadleaved trees (fruiting bodies single or grouped). | | Edible | Gilled | Plaatbos |
| *Gymnopilus junonius* | Widespread across the country. | Laughing Jack; Giant Gymnopilus | Saprophytic | On unhealthy or dying Coniferous and Broadleaved trees (fruiting bodies single, grouped and or clustered). | Other *Gymnopilus* species*; Omphalotus plearius; Lactarius delisciosus* | Inedible | Gilled | Koomansbos |
| *Imleria badia* | Widespread across the country. | Bay Bolete; Bay-capped Bolete | Mycorrhizal | Under Coniferous and Broadleaved trees (fruiting bodies single or grouped). | Other *Boletus* species; *Boletus edulis; Boletus reticulatus* | Edible | Boletes | Plaatbos |
| *Lactarius quieticolor* | Found within the Eastern Cape, possible more widespread. | | Mycorrhizal | Under Coniferous and Broadleaved trees (fruiting bodies single or grouped). | *L. delisciosus* | Edible | Gilled | Kleinbos |
| *Panaeolus antillarum* | Found within Eastern Cape, possible more widespread. | | | In dung mounds (Coprophilous Mycota). | *Panaeolus subbalteatus; Panaeolus foenisecii* | | Gilled | Koomansbos |
| *Russula caerulea* | Found within the Eastern Cape, possible more widespread. | Humpback brittlegill Russula | Mycorrhizal | Under Coniferous trees, especially pine species (fruiting bodies single or grouped). | *Russula capensis* | Edible | Gilled | Lottering |

| SCIENTIFIC NAME | DISTRIBUTION WITHIN SOUTH AFRICA | COMMON NAMES | ECOLOGY | HABITAT | SIMILAR MORPHOLOGICAL SPECIES | EDIBILITY | MORPHOLOGICAL GROUP | LOCATION |
|---|---|---|---|---|---|---|---|---|
| *R. capensis* | Found within the Eastern Cape, possible more widespread. | Cape Russula | Mycorrhizal | Under Coniferous trees, especially pine species (fruiting bodies single or grouped). | *R. caerulea* | Suspect | Gilled | Plaatbos |
| *Russula sardonia* | Found within Gauteng, Western Cape and Eastern Cape, possibly more widespread. | Primrose Brittlegill; Purple-Stemmed Russula | Mycorrhizal | Under Coniferous and Broadleaved trees (fruiting bodies single or grouped). | *Russula xerampelina* | Poisonous | Gilled | Kleinbos |

964

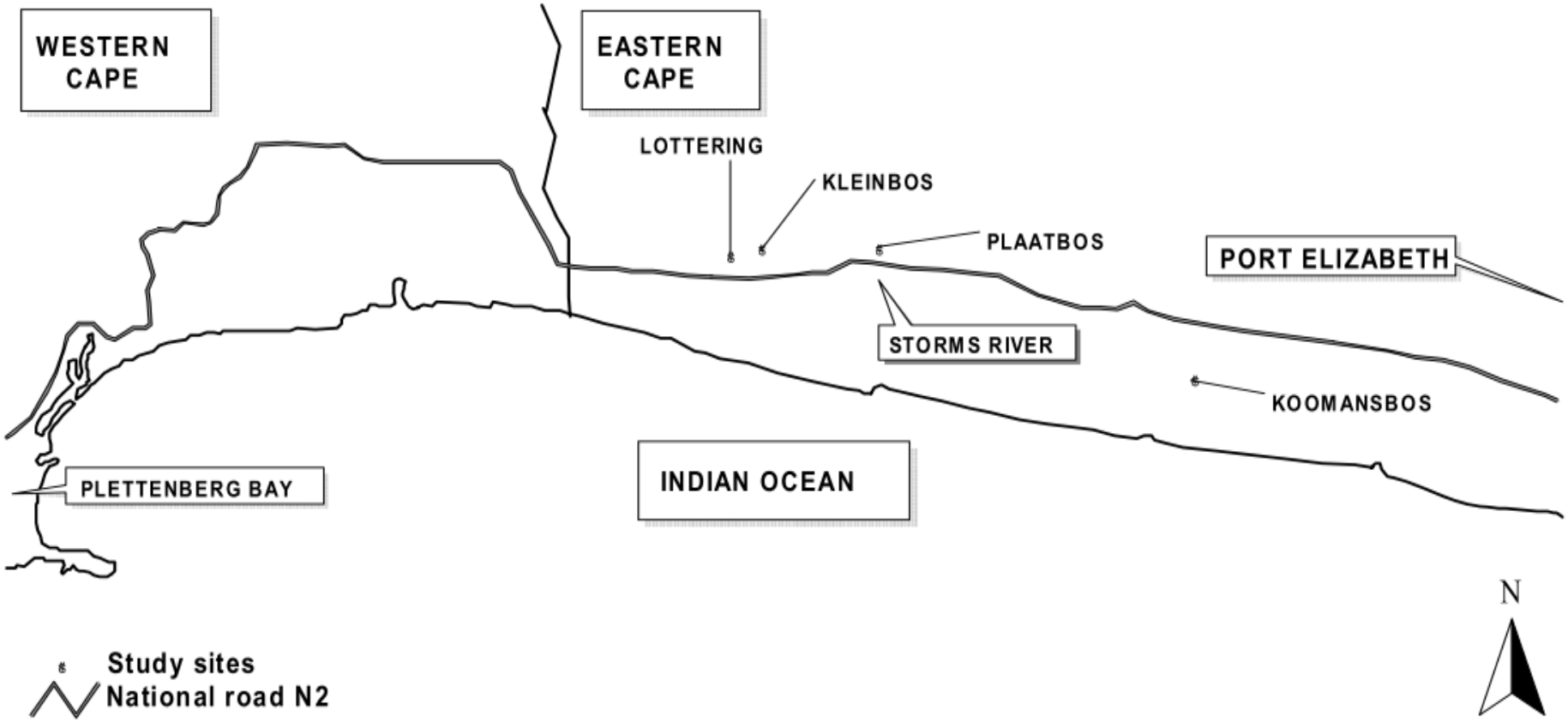
**Table 3. 2** Infield morphology documentation table.

| | | |
|---|---|---|
| Specimen Number: | | |
| Species Identification: | | |
| Section 1: Cap and Hymenium | Cap: Structure supported on the stipe or stalk. | Colour: |
| | | Shape: (Bell-Shaped/Broadly Convex/Broadly Umbonate – Wavy /Conical/Convex/Deeply Depressed/Depressed - Central part of the Cap sunken /Egg-Shaped/Funnel Shaped/Globose – spherical or globe-shaped /Hemispherical/Plane/Flat/Umbonate) |
| | | Surface Texture: (Dry/Hairy/Scaly/Smooth/Sticky) |
| | | Structures: (Scales/Warts – remnants of the universal veil) |
| | Hymenium (Gills/Tubes): The layer of fertile cells that produce the spores. | Colour: |
| | | Margins: The connective area of the cap and gills |
| | | Surface: (Appendiculate/Crenate – edged with rounded teeth/Crisped – finely wavy/Entire – smooth and more even /Lobate – lobate /Undulate – Wavy) |
| | | Sectional: (Acute/Decurved/Incurved – curved or rolled inward toward the stipe (margin) /Inrolled – rolled up on the side next to the stipe, when young (margin) /Obtuse/Plane – flat /Upturned) |
| | | Type Attachment: (Free/Adnexed/Adnate/Notched/Notched Decurrant Tooth/Seceding/Decurrent – Down Stipe/Depressed) |
| | | Length: (Close – spaced close together, between crowded and distant/Crowded – arranged extremely close together – full appearance/Distant – spaced far appart/Fanned/Full and Intermediated/Forked) |
| Section 2: Stipe and Structures | Stipe: Stem or stalk. | Colour: |
| | | Shape: (Bulbous base/Club-shaped/Cylindrical/Rooting base/Tapering Down/Tapering towards base/Tapering Upward) |
| | | Type: (Central – stipe attached at the centre of the cap /Excentric – stipe not centrally attached to the cap /Lateral – at the side) |
| | Structures | Ring: A band of tissue encircling the stem |
| | | (Absent/Present) |
| | | Position on the stipe: (Top/Middle/Bottom) |
| | | Volva: Cup – like structure remains of the universal veil around the base of the stipe |
| | | (Absent/Present) |
| Section 3: Habitat/Substarte and Additional Information | Habitat/Substarte: The surface or material, such as soil or bark, to which the fungus is attached or on which it grows. | In soil/On tree/Other: |
| | | Soil: (Sandy/Muddy/Manure/Grass/Forest) |
| | | Tree: (Dead/Fallen/Decaying/Alive) |
| | | Surrounding Environment: (Forest/Pasture/Grass/Other) |
| | Bruising/Bleeding/Staining | Colour: |
| | | Action: (Touching/Cutting/Damaged) |
| | Odour/Smell | Similar: |
| Similar species: | | |

968 **Table 3. 3** Technical information regarding sequencing within this study.

969

| **Taxon** | ***Amanita morrisii*** | ***Amanita muscaria*** | ***Amanita pantherina*** | ***Amanita rubescens*** | ***Chlorophylulm sp.*** | ***Clitopilus prunulus*** | ***Gymnopilus junonius*** | ***Imleria badia*** | ***Lactarius quieticolor*** | ***Panaeolus antillarium*** | ***Russula caerulea*** | ***Russula capensis*** | ***Russula sardonia*** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Fruiting body** | Gilled | Gilled | Gilled | Gilled | Gilled | Gilled | Gilled | Boletes | Gilled | Gilled | Gilled | Gilled | Gilled |
| **No. Characters** | 680 | 550 | 520 | 703 | 625 | 604 | 700 | 634 | 698 | 670 | 626 | 689 | 619 |
| **No. Taxa** | 12 | 7 | 9 | 8 | 7 | 6 | 7 | 6 | 6 | 6 | 8 | 6 | 8 |
| **No. Sequences** | 27 | 19 | 17 | 18 | 17 | 16 | 17 | 17 | 19 | 19 | 18 | 16 | 18 |
| **No. Sites** | 870 | 760 | 756 | 845 | 779 | 705 | 859 | 820 | 924 | 744 | 831 | 772 | 760 |
| **No. Conserved** | 725 | 680 | 637 | 735 | 631 | 641 | 759 | 505 | 818 | 586 | 619 | 588 | 537 |
| **No. Variable Sites** | 128 | 57 | 99 | 109 | 142 | 61 | 92 | 314 | 106 | 150 | 186 | 150 | 195 |
| **Parsim-Info** | 108 | 43 | 57 | 95 | 121 | 54 | 69 | 298 | 98 | 132 | 180 | 128 | 119 |
| **Singleton** | 20 | 14 | 42 | 12 | 21 | 7 | 23 | 16 | 8 | 18 | 6 | 22 | 75 |
| **Evolutionary Model** | Tamura 3-parameter + Gamma distribution | Tamura 3-parameter + Gamma distribution | Tamura 3-parameter + Gamma distribution | Tamura 3-parameter + Gamma distribution | Kimura 2-parameter | Tamura 3-parameter + Gamma distribution | Tamura 3-parameter + Gamma distribution | Tamura 3-parameter + Gamma distribution | Kimura 2-parameter | Tamura 3-parameter + Gamma distribution | Kimura 2-parameter | Kimura 2-parameter | Kimura 2-parameter |
| **Phylogeny** | Fig. 4.2 | Fig. 4.3 | Fig. 4.4 | Fig. 4.5 | Fig. 4.6 | Fig 4.8 | Fig 4.10 | Fig 4.12 | Fig 4.14 | Fig 4.16 | Fig 4.19 | Fig 4.20 | Fig 4.21 |




970 **Figure 3. 1** Sampling locations namely, Lottering, Kleinbos Plaatbos and Koomansbos plantations in the Garden Route National Park (Ella, 2005).

971

972

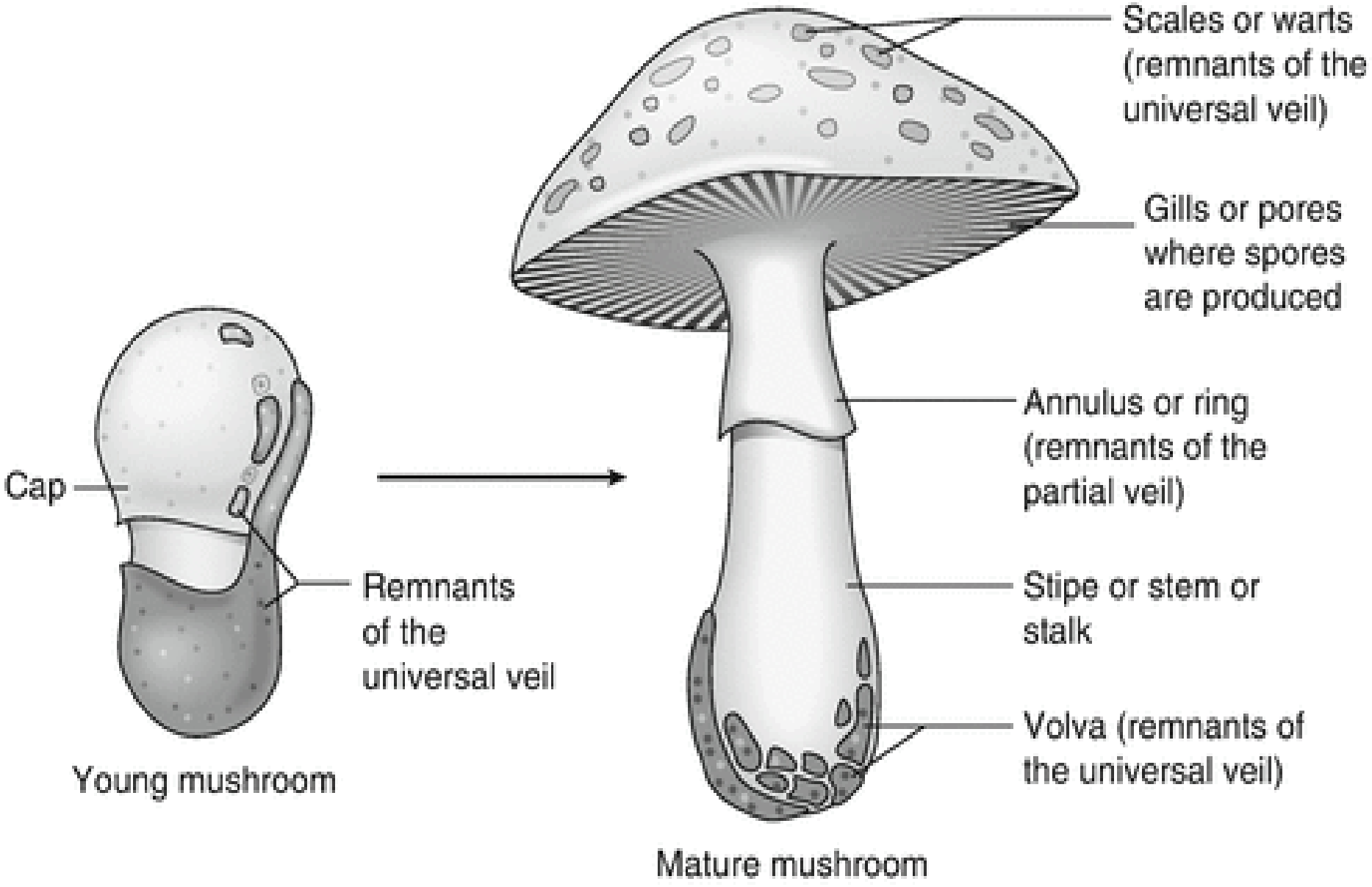



**Figure 3. 2** Universal mushroom illustration by The Mushroom Diary (https://rrcultivation.com/blogs/mn/mushroom-anatomy-caps-stems).

# CHAPTER 4 – RESULTS AND DISCUSSION

## 4.1 Sampling Collection

There were 13 species collected (Table 3.1) in this study from *Pinus* (pine) plantations in the Tsitsikamma. These species represented 8 genera including *Amanita, Chlorophyllym, Clitopilus, Gymnopilus, Imleria, Lactarius, Panaeolus* and *Russula*, their identities were confirmed by morphological comparison to field guides. Specimens taxonomic ranks area captured in (Table 4.1). Of these samples various species were confirmed from previous literature, such as *Amanita muscaria*, *Amanita rubescens*, *Amanita pantherina*, *Russula capensis*, *Russula sardonia* and *Russula caerulea* (Tonjock et al. 2020; Goldman & Gryzenhout, 2019; Gryzenhout, 2021). Others were first reports of species within South Africa, such as *Amanita morrisii*, *Lactarius quieticolor* and *Panaeolus antillarum* (Halama, 2014; Silva-Filho et al., 2020; Tulloss, 2016). These latter species have previously been reported in countries around the world, for example *L. quieticolor* has been identified in Europe, South Central Chile, Brazil and India (Almonacid-Muñoz et al., 2022; Leonardi et al., 2021; Nuytinck & Verbeken, 2007; Silva-Filho et al., 2020) whereas *A. morrisii* has only ever been identified in USA (United States of America) (Simmons et al., 2002; Thongbai et al., 2016; Tulloss, 2016). Whereas all these species are considered not to be native, due to their Northern Hemisphere origins but *R. capensis* could be native to South Africa. The size of the fruiting body was estimated for species following guidelines and research by Gryzenhout (2012), Goldman & Gryzenhout (2019) and Gryzenhout (2021).

994

995 Table 4. 1:Taxonomic ranking of specimens found within this study.

| Sample | Morphological Identification | Phylum | Class | Order | Family | Genus | Species | Field Guides |
|---|---|---|---|---|---|---|---|---|
| D5 | *Amanita morrisii* | Basidiomycota | Agaricomycetes | Agaricales | Amanitaceae | *Amanita* | *morrisii* | No |
| D18 | *Amanita muscaria* | Basidiomycota | Agaricomycetes | Agaricales | Amanitaceae | *Amanita* | *muscaria* | Yes |
| D7 | *Amanita pantherina* | Basidiomycota | Agaricomycetes | Agaricales | Amanitaceae | *Amanita* | *pantherina* | Yes |
| D32 | *Amanita rubescens* | Basidiomycota | Agaricomycetes | Agaricales | Amanitaceae | *Amanita* | *rubescens* | Yes |
| D2 | *Chlorophylulm sp.* | Basidiomycota | Agaricomycetes | Agaricales | Agaricaceae | *Chlorophyllum* | *N/A* | N/A |
| D19 | *Clitopilus prunulus* | Basidiomycota | Agaricomycetes | Agaricales | Entolomataceae | *Clitopilus* | *prunulus* | Yes |
| D61 | *Gymnopilus junonius* | Basidiomycota | Agaricomycetes | Agaricales | Stophariaceae | *Gymnopilus* | *junonius* | Yes |
| D8 | *Imleria badia* | Basidiomycota | Agaricomycetes | Boletales | Boletaceae | *Imleria* | *badia* | Yes |
| D10 | *Lactarius quieticolor* | Basidiomycota | Agaricomycetes | Russulales | Russulaceae | *Lactarius* | *quieticolor* | No |
| D17 | *Panaeolus antillarium* | Basidiomycota | Agaricomycetes | Agaricales | Incertae sedis | *Panaeolus* | *antillarium* | No |
| D6 | *Russula caerulea* | Basidiomycota | Agaricomycetes | Russulales | Russulaceae | *Russula* | *caerulea* | Yes |
| D14 | *Russula capensis* | Basidiomycota | Agaricomycetes | Russulales | Russulaceae | *Russula* | *capensis* | Yes |
| D66 | *Russula sardonia* | Basidiomycota | Agaricomycetes | Russulales | Russulaceae | *Russula* | *sardonia* | Yes |

996

## 4.2 Morphological and DNA sequence based identification

### 4.2.1 *Amanita*

The sequencing result of various *Amanita* species, morphologically presented in (Fig. 4.1) were presented in separate *Amanita* subset phylogenies to enhance the resolution of the clades in the individual trees, and included *A. morrisii* (subset 1) (Fig. 4.2), *A. muscaria* (subset 2) (Fig. 4.3), *A. pantherina* (subset 3) (Fig. 4.4) and *A. rubescens* (subset 4) (Fig 4.5). The results from the DNA sequence comparison confirmed the findings of the macroscopic characteristics.

***Amanita morrisii***

The *A. morrisii* (subset 1) phylogenetic tree (Fig 4.2) consisted of 27 sequences including the unknown specimen (Sample D5). That was collected under pine trees in the Koomansbos plantation. The data set included various *Amanita* species, including *A. rubescens*, *Amanita flavoconia*, *Amanita detersa* and *Amanita augusta.* These sequences were mostly form the Northern Hemisphere including countries such as China, India, Pakistan and the United States of America (USA). The sample investigated in this study grouped within a clade of *A. morrisii* sequences originating from the USA with a supportive bootstrap value of 98%.

Morphologically the sample is medium to large in size (Fig. 4.1 A-E). The cap colour ranged from dark brown to dark grey, fading with age. The glistening moist cap is broadly convex with flatten umbo, depressed in the centre in mature specimens. The cap is covered in white to pale grey warts. Gills are white, close or intermediate, either free, sometimes adnate or notched with decurrent tooth attachment. Margins are non-striated, with smooth entire surface view and plane flat sectional view. The stipe is central, white to pale grey, cylindrical in shape, tapering upwards. Ring is present on the top of the stipe. The conical volva is completely absent or present as irregular greyish-brown of white patches. The sample was found in moist forest soil under coniferous trees.

The morphological characteristics corresponded with previous documentation by Perk. (1910). Similar species within South Africa include *A. pantherina*, *Amanita excelsa* and *A. rubescens*. Distinctive features are considered the glistening appearance of the greyish cap, the central depression of the cap and the absence on the volva, compared to these other mentioned species (http://www.Amanitaceae.org/?Amanita%20morrisii). This is thus, considered to be the first report of *A. morrisii* in South Africa. *Amanita morrisii* was first reported and described by Peck (1910). The species belongs to the section *Validae* (Tulloss, 2016). According to http://www.Amanitaceae.org/ the

species is considered vulnerable according to the IUCN red list category and criteria. Specimens have only ever been identified within the United States of America. The documentation of the species from other localities indicates the possible further distribution of specimens than previously thought. The vulnerability status of the species and the current decreasing population trend, highlights the importance of further investigating the fungal diversity, documenting true species identification and updating of existing fungal registries within South Africa.

***Amanita muscaria***

The *A. muscaria* (subset 2) phylogenetic tree (Fig 4.3) showing results of A. muscaria and related species, consisted of 19 sequences including the unknown Sample D18. The sample was collected under pine trees in the Plaatbos plantation. The data included various *Amanita* species, namely *A. muscaria*, *Amanita ibotengutake*, *Amanita flavopantherina* and *Amanita griseopantherina*. The sequences were mostly form the Northern Hemisphere including countries such as Europe, China and the United State of America (USA). The sample investigated in this study grouped within a clade of *A. muscaria* sequences originating from with a supportive bootstrap value of 99%.

Morphologically the sample is one of the most distinctive fungal species (Fig 4.1 F-H), known around the world. The cap colour is a recognised vibrant red, with a white dotted appearances (Poliwoda et al., 2014). The cap is hemispherical that flattens with age. Loose pyramidal warts are present in irregular patterns, on the cap and margins, that gives a scaly appearance. Gills are pure white, crowded or intermediate freely attached. Margins are striated, with adhering veil remnants. The stipe is central, pure white, cylindrical in shape, with bulbous base. Membrameous ring is present near the apex of the stipe. The conical volva is absent. Flesh is soft and white. The sample was found in moist forest soil under coniferous trees. The morphological characteristics corresponded with species documentation in relevant field guides by Goldman & Gryzenhout (2019); Gryzenhout (2021).

The fungal genus *Amanita* is quite diverse and contains many different species. The genus contains members that are considered to be edible and poisonous and is widely distributed worldwide (Hallen et al., 2002; Itoo et al., 2016; Rasalanavho et al., 2019). The 'Fly Agaricus' (Obermaier & Müller, 2020) originated from the Siberian-Beringian region (Reid & Eicker, 1991). The mushroom is considered to be neurotropic and have hallucinogenic properties due to it containing toxins such as ibotenic acid and muscimol (Poliwoda et al., 2014; Stebelska, 2013). The ingestion of these toxins effect the central nervous system (CNS) and cause CNS excitation. Ibotanic and muscimol intoxication thus, can lead to delirium, states of agitation and cause various behavioural changes (Jo et al., 2014; Stebelska, 2013).

Due to these exhibited properties it was and still is considered an important and sacred fungus in the Siberian region of Russia, by the Chukchee and Koryak people of the area (Guzmán et al., 1997), being utilised for various religious and cultural rituals (Garibay-Orijel et al., 2007; Lau et al., 2015; Stebelska, 2013). Species in the genus usually displays mycorrhizal relationships with host substrates and surrounding environment. *A. muscaria* is known to form strong association with coniferous trees including various *Pinus* spp. as well as broadleaved trees such as *Eucalyptus* species (Fitzgerald, 2018; Hawley et al., 2008; Itoo et al., 2016). Therefore, it is believed that most members of the genus have been introduced to the country from Europe and Australia (de Ronde et al., 1990; Guzmán et al., 1997). The introduction of exotic tree species to the country was to support the growing and developing timber industry (De Koker et al., 2000; Tchoumi et al., 2020).

### *Amanita pantherina*

The *A. pantherina* (subset 3) phylogenetic tree (Fig 4.4) consisted of 17 sequences including the unknown specimen (Sample D7). The specimen was collected under pine trees in the Koomansbos plantation. The data set included various *Amanita* species including *A. pantherina*, *Amanita pseudopantherina, A. griseopantherina* and *A. flavopantherina, Amanita aprica* and *A. ibotengutake.* These sequences were mostly form the Northern Hemisphere including countries such as China and the USA as well as from Northern Europe including Russia and Czech Republic. The sample investigated in this study Sample D7 grouped within a clade of *A. pantherina* sequences originating from Northern Europe with a supporting bootstrap value of 88%. A closely related sequences included is that of *A. pseudopantherina* and *A. griseopantherina* originating from China.

Morphologically the sample is medium in size (Fig 4.1 I-J). The pale greyish-brown cap is dotted with white pyramidal warts, hemispherical to flat with even and smooth margins. Gills are white, thin, crowded and free. The stipe is thick and central, smooth that widens towards the base. The mebraneous ring is tattered and white in color. The volva encloses around the bulbous base, forming white rings and ridges on the stipe. The sample is similar in appearance to *A. excelsa* and *A. rubescens.* The morphology of the specimen corresponds with the previous documentation of the species within the country by Goldman & Gryzenhout (2019);Gryzenhout (2021); Reid & Eicker (1991).

The species is considered to be deadly poisonous. Intoxication by the species leads to symptoms of nausea, vomiting and if untreated unconsciousness 1-3 hours after consumption (Guzmán et al., 1997; P. Li et al., 2014). The poisoning caused by the species is due to the toxic compound ibotenic acid of which the species is high in concentration (Poliwoda et al., 2014; Stebelska, 2013). The species is also considered to be a neurotrophic fungus due to the presents of ibotenic acid (Guzmán et al., 1997).

### *Amanita rubescens*

The *A. rubescens* (subset 4) phylogenetic tree (Fig 4.5) consisted of 18 sequences including the unknown specimen (Sample D32). The specimen was collected under pine trees in the Plaatbos plantation. The data included various *Amanita* species namely *Amanita orsonii, Amanita flavorubens, A. detersa* and *A. rubescens*. The sequences were mostly from the Northern Hemisphere including countries such as China and within Europe. The sample investigated in this study grouped within a clade of *A. rubescens* sequences originating from Europe with a supporting bootstrap value of 95%.

Morphologically the sample is medium in size (Fig 4.1 K-Q). The cap colour ranged from reddish to blushing-brown. The cap is hemispherical that flattens with age. Loose warts are present in irregular

patterns, gives a scaly appearance. Gills are pure white, crowded or intermediate freely attached. Margins are faintly striated, with even and smooth surface view and plane flat sectional view. The stipe is central, white to reddish brown flushes, cylindrical in shape, with bulbous base. Rings is present near the apex of the stipe. The conical volva is present as concentric warty circle rings. Flesh stains red when damaged. This specimen was found in moist forest soil under coniferous trees. The morphological characteristics corresponded with species documentation in relevant field guides by Goldman & Gryzenhout (2019); Gryzenhout (2021).

*Amanita rubescens* originates from Europe and was introduced to the country via the establishment of cultivated plantations (Hallen et al., 2002; Reid & Eicker, 1991). The specimen is considered to be edible when cooked, but poisonous when consumed raw (Reid & Eicker, 1991). It occurs widely throughout South Africa stated by Goldman & Gryzenhout (2019); Gryzenhout (2021).

***South African Diversity of Amanita species***

Overall, morphologically the species of the genus *Amanita* can be widely recognized by pale gills carrying white spores, located under the margins of the fruiting body cap. While the gills are mainly free from the stem, the remnants of a universal protective veil, ruptured during maturation of the organisms is observed as a volva around the stipe or as wart-like structures on the cap of the mushroom (Hallen et al., 2002; Itoo et al., 2016; Poliwoda et al., 2014; P. Zhang et al., 2015). Species members can either be considered edible or poisonous and have well established mycorrhizal relationship with host as mentioned.

The members of the genus *Amanita* are of the most well recognised mushrooms across the world (Thongbai et al., 2016; Wasser, 2011). Due to do that some members of the genus can easily be recognised by their 'universal mushroom shape' and dotted appearance (Samorini, 2001; Simmons et al., 2002). *Amanita* is widespread and comprises of more than 600 species that, although are found all over the world (De Koker et al., 2000; Itoo et al., 2016; Y. S. Liu et al., 2022; Pala et al., 2012; Poliwoda et al., 2014; P. Zhang et al., 2015), approximately, only more than half have been documented through publications. The others are only recorded by regional names or other codes of possible identification and some still remain invalidly or misidentified (Thongbai et al., 2016).

In South Africa a total of 17 species have been reported by (Kinge et al., 2020) based on relevant literature (Hallen et al., 2002; Reid & Eicker, 1991), namely *Amanita aureofloccosa*, *Amanita capensis*, *A. excelsa*, *A. flavoconia*, *Amanita foetidissima, A. muscaria, A. pantherina, A. phalloides, Amanita*

*pleropus, Amanita praeclara, Amanita roseolescens, A. rubescens, Amanita singer, Amanita solitaria, Amanita strobiliformis* and *Amanita veldiei.* Some of these species including *A. muscaria, A. pantherina and A. phalloides* are most likely introduced with their alien host (Hallen et al., 2002; Wartchow & Cortez, 2016; Wood, 2017). Native species within the genus include *A. foetidissima*, *A. roseolescens*, *A. veldiei* and *A. praeclara* (Reid & Eicker, 1991). Species that have been reported to be indigenous to other countries in Africa include *Amanita zambiana* from Zambia (Pegler & Piearce, 1980; Ndifon, 2022).

### 4.2.2 *Chlorophyllum*

The *Chlorophyllum* phylogenetic tree (Fig 4.6) consisted of 17 sequences including the unknown specimen (Sample D2). The set included various *Chlorophyllum* species, including *Chlorophyllum palaeotropicum, Chlorophyllum lusitanicum, Chlorophyllum molybdites, Chlorophyllum globosum* and *Chlorophyllum pseudoglobosum*. It also included sequences representing the species *Secotium queinzii.* These sequences were mostly from South Africa and the Northern Hemisphere including counties such as Spain, USA and India. The sample investigated (Sample D2) grouped separately from any other species of *Chlorophyllum* species currently sequenced. The most closely related branch of sequences represented *C. pseudoglobosum* originating from India.

Morphologically (Fig 4.7) the sample is large in size. The white hemispherical cap is subglobose to convex with a central umbo. The cap is covered in tough brown scales. The margin is even to striated. The gills are freely attached, crowded and full to intermediated in length. The stipe is thick, tapering upwards, white darkening to brown below the conspicuous ring. The morphological characteristics of the sample corresponds with previous documentation of the species within the genus e.g. *C. molybdites* (Goldman & Gryzenhout 2019; Gryzenhout 2021; Ge et al., 2018).

Conventionally, this genus was considered to be monotypic and only contains green-spored species, such as *C. molybdites*. Overall the species within *Chlorophyllum* is characterised by a hemispherical, white convex cap, covered in brown scales, with a dark brown low umbo. Gills are free, closely crowed and greenish to grey in colour (Ge & Yang, 2006; Ge et al., 2018).

Historically three *Chlorophyllum* species have been documented within the country including *C. molybdites*, *C. palaeotropicum* and *Chlorophyllum africanum* (Kinge et al., 2020). *Chlorophyllum rhacodes* was also identified by Van der Westhuizen and Eicker (1994). Recently *C. palaeotropicum* and *C. africanum* was described within the country by multiple gene phylogeny (Ge et al., 2018). The description of these new *Chlorophyllum* species from South Africa indicates that the species diversity

of this genus in South Africa are still unexplored. The fact that the specimen collected in this study thus grouped on its own, indicates that it could most likely be another new species. However, it could also represent an already named *Chlorophyllum* species from another country that simply has not been sequenced yet. Future morphological comparisons will aid to resolve this question.

A sequence labelled as *S. queinzii* grouped in the *Chlorophyllum* tree. *S. queinzii* rather is a species known from South Africa (Singer, 1960). It was first described based on morphological features by Kunze in 1840. Further, molecular documentation of the species was done in 1963 on samples collected from the Cape region. The genus *Secotium* has for long been synonymous with species within the *Chlorophyllum* genus (Loizides et al., 2020). Thus, the phylogenetic analysis from this study indicates that this species could possibly represent a *Chlorophyllum* species, which should be investigated in more detail in future.

### 4.2.3 *Clitopilus*

The *Clitopilus* phylogenetic tree (Fig. 4.8) consisted of 16 sequences including the unknown specimen (Sample D19). The specimen was collected under pine trees in the Plaatbos plantation area. The data set included *Clitopilus prunulus, Clitopilus brunneiceps, Clitopilus amygdaliformis, Clitopilus abprunulus*, *Clitopilus fusiformis* and *Clitopilus yannanensis.* These sequences were mostly form the Northern Hemisphere. The sample (Sample D19) investigated in this study grouped within a clade representing *C. prunulus,* including sequences originating from Europe with a supporting bootstrap value of 89%. Other closely related species are that of *C. yannanensis* and *C. brunneiceps,* originating from China.

Morphologically (Fig. 4.9) the sample is medium in size. The white to grey-brown cap is convex with a velvety surface. Margins are curved inwards or slightly undulate. Gills equally distributed in length are white to light pink and decurrent. The stipe is central concolorous. Flesh is white to soft pink. The morphology of the sample corresponds to documentation of the species by Jian et al. (2020).

The genus is considered small with a total of 30 members that have been documented (Jian et al., 2020; Noordeloos & Gates, 2012). Pleuromutilin a compound associated with the genus, was first discovered by Kavanagh et al. (1951). This secondary metabolite binds to the bacterial ribosomal subunit and hinders the correct positioning of the tRNAs for the transfer of necessary peptides for protein synthesis, thus functioning as antibiotics. Chemical derivatives from the compound Pleuromutilim, namely Tiamulin and Valnemulin, has been used to treat immunocompromised patients, and Retapapmulin is the first antibiotic of this class to be developed for use in human therapeutics (Hartley et al., 2009). These by-products have also been used within veterinary practises (Hartley et al., 2009; Molitoris, 1994).

The discovery of these compounds proposes that the genus should be studied further in future for pharmaceutical development. However, despite the medicinal potential of the genus, the poor taxamonic classification within the genus often leads to the wrongful identification of species and leads to the fact that its medical potential is overlooked and underutilised (Hartley et al., 2009). Species members that are known to produce the secondary metabolite namely Pleuromutilin include *Clitopilus hobsonii, Clitopilus passeckerianus, Clitopilus scyphoides, Clitopilus pinsitus and C. prunulus* (Hartley et al., 2009).

*Clitopilus prunulus* is mainly characterised by its white to pinkish flesh and soft pink to brownish pink spore print (Noordeloos & Gates, 2012). The fungus commonly known as the sweetbread fungus (Grangeia et al., 2011), is considered to be a saprophytic mushroom, thus functioning as natural

decomposers and recyclers of organic material in the ecosystem (Gryzenhout et al., 2020; Vizzini et al., 2011) and further play an important role in the health of the surrounding environment. These symbiotic mushrooms are found growing within pastures, on forest leaf litter and dead tree branches and logs or within or under coniferous and broad-leaved forests (Alaimo et al., 2018).

In South Africa, *C. prunulus* is the only species member from the genus *Clitopilus* that has been reported within the country (Kinge et al., 2020). Although the species members from the genus present with a distinguishable pinkish colored flesh, morphological differentiation between species within the genus is considered to be difficult due to the lack of documentation of all species within the genus (Noordeloos & Gates, 2012).

### 4.2.4 *Gymnopilus*

The *Gymnopilus* phylogenetic tree (Fig. 4.10) consisted of 17 sequences including the unknown sample (Sample D61). The specimen was collected under pine trees in the Koomansbos plantation. The data set included various *Gymnopilus* species, including *Gymnopilus junonius, Gymnopilus dunensis, Gymnopilus voitkii, Gymnopilus speciosissimus, Gymnopilus ochraceus, Gymnopilus ventricosus and Gymnopilus sordidostipes*. These sequences were mostly from the Northern Hemisphere including countries such as India, Pakistan and the USA, as well as from France and Canada. The sample investigated in this study grouped within a clade of *G. junonius* sequences originating from New Zealand, Iran and France with a supportive bootstrap value of 99%.

Morphologically the sample (Fig. 4.11) is large in size. The cap colour ranged from warm yellow to bright dark orange. The smooth fibrilous cap is convex with a slight protruding umbo, with even to undulate margins. Gills are thin yellow to rust brown, adnexed, full and crowded and intermediate in length. The yellow-orange central stipe is thick and wide at the base. The membraneous ring near the apex of the stipe is concolorous to the fruiting body. The specimen has a mild, to slightly pleasant odour. Morphologically the sample's characteristics corresponded with previous documentation of the species by Goldman & Gryzenhout (2019); Gryzenhout (2021).

Almost 200 species have been recognised for the *Gymnopilus* genus, that was further subdivided by Kühner (1980) and Singer (1986) into subdivisions, namely *Annulati* members with a prominent partial veil and *Gymnopilus* members without a partial veil (Holec, 2005; Marchant et al., 2004). Members of the genus is often recognised by their medium to large golden-bright orange to rusty-brown fruiting bodies. The fruiting body of the macro fungi is often found solitary or clustered (Ragupathi et al.,

2018), typically growing on various stages of wood, from living trees or decaying branches and logs (Holec, 2005; Guzman, 2009).

The species *G. junonius* is commonly known as the Laughing Gym or the Big Laughter mushroom, due to the uncontrollable laughter expressed after consumption of this fungus (S. Lee et al., 2020; Ragupathi et al., 2018). *Gymnopilus junonius* was previously known as *Gymnopilus spectabilis*, where *G. junonius* was represented by a slenderer and smaller specimen, morphologically similar to that of *G. spectabilis* which was considered the more robust and larger specimen between the two species (S. Lee et al., 2020). Although, these two species are somewhat synonymous to each other, Holec (2005) stated that if it is proven that these two species are in fact represented by only one it should be refered to as *G. junonius*.

Overall *Gymnopilus* species are widely recognised as inedible poisonous hallucinogenic mushrooms (Cho et al., 2021; S. Lee et al., 2020). A total of 14 species within the genus of neurotropic fungi contain the psychedelic compound psilocybin (Guzmán et al., 1997). A psilocybin containing species within the genus includes *G. junonius*, hence the species is considered a medicinally valued fungal species and serves as a great source of research regarding the investigation of hallucinogens (Cho et al., 2021). In South Africa only four species of *Gymnopilus* have been documented namely *Gymnopilus hybridus*, *G. junonius*, *Gymnopilus penetrans* and *Gymnopilus sapineus* (Kinge et al., 2020). *G. junonius* is morphologically very similar to the other species recognised within the country. Further the species is widespread within the country. It also occurs in the UK, Europe, USA, Japan, Australia, New Zealand and Russia (Gryzenhout, 2021;Holec, 2005; Lee et al., 2020; Ragupathi et al., 2018).

### 4.2.5 *Imleria*

The *Imleria* phylogenetic tree (Fig. 4.12) consisted of 17 sequences including the unknown specimen (Sample D8). The specimen was collected under pine trees in the Plaatbos plantation area. The data set included various *Imleria* species namely, *Imleria badia, Imleria parva* and *Imleria pallida*. The sequences were mostly from the Northern Hemisphere including countries such as China, Germany and India. Sample D8 grouped as a haplotype within a clade of *I. badia* sequences originating from Germany with a supporting bootstrap value of 100%. A closely related branch is that of *I. parva* originating from China.

Morphologically the sample (Fig. 4.13) is medium in size. The smooth cap is brown to dark ochre-brown in colour, convex with even margins. Pores rather than gills have a spongy texture and are white to olivaceous, small and slightly depressed around the stipe. The streaked paler concolorous stipe is

thick and cylindrical. Ring and volva are absent. Flesh is firm and fibrous with a mild mushroomy odour and bruise blue when damaged. The morphological characteristics corresponded with species documentation in relevant field guides by Goldman & Gryzenhout, (2019); Gryzenhout, (2021).

*Imleria badia* is also known as the bay bolete (Muszyńska et al., 2020) and is heavily enjoyed for its texture and earthy flavour (Jaworska et al., 2015). This bolete is frequently harvested for culinary applications, and is a valuable food source because it contains proteins, sugars and carbohydrates while being low in calories. The species normally displays mycorrhizal relationships with coniferous tree species and is often found on tree trunks within mixed forests (Duñabeitia et al., 1996; Gąsecka et al., 2017). *Imleria badia* is also valuable to the surrounding environment due to its ability to absorb heavy metals form the nearby surrounding habitat, therefore acting as an important bio-accumulator that reduces pollution by hazardous chemicals (Gąsecka et al., 2017; Malinowska et al., 2004), thus playing a major role in the overall health of the ecosystem. The species is also regarded as one of the most valuable medicinal wild growing edible mushrooms (Muszyńska et al., 2020). It demonstrates anti-oxidative potential via free radical scavenging and has been investigated as an alternative treatment option for various ailments including diabetic wound healing (Pringle et al., 2021).

In South Africa, *I. badia* is morphologically similar to other boletes such as *Boletus edulis* and *Boletus reticulatus*. However, the blueing of the pores when damaged is distinctive and is due to the oxidation of boletol. This aids in the morphological identification of the only species member within this genus known within the country (Kinge et al., 2020). The species occurs widespread across the country and has also been documented in the UK, Europe, Canada, USA, Mexico and Russia (Goldman & Gryzenhout, 2019).

### 4.2.6 *Lactarius*

#### *L. quieticolor*

The *Lactarius* phylogenetic tree (Fig 4.14) consisted of 19 sequences including the unknown specimen (Sample D10). The specimen was collected under pine trees in the Kleinbos plantation. The data set included various *Lactarius* species such as *L. quieticolor, Lactarius hatsudake* and subspecies *Lactarius deliciosus* var. *olivaceosordidus*. These sequences were mostly from the Northern Hemisphere. The sample investigated in this study Sample D10 grouped in a clade of *L. quieticolor* sequences originating from Czech Republic, France, Sweden, Poland and MT007126, which is a specimen from Brazil with a supporting bootstrap value of 100%. A closely related haplotype includes *L. hatsudake* sequences originating from China with a supporting bootstrap value of 86%.

Morphologically the sample is orange to dark warm red in colour (Fig. 4.15 A-C). The cap is funnel shaped with a central sunken depressed. The surface is smooth and sticky when wet. Gills are orange to warm red in colour, with smooth, undulate wavy margins, that becomes upturned. Further decurrent attached and crowded with intermediate lengths. The stipe is con-coloured with the cap, cylindrical shaped and central. Ring and volva is absent. The sample was found in soil under pine trees and covered in pine needles. Sample bled saffron orange coloured milk when cut or when flesh was damaged. The morphological characteristics corresponded with previous documentation for *L. quieticolor* (Das, 2015; Nuytinck & Verbeken, 2005, 2007; Silva-Filho et al., 2020). This study reported the first occurrence of this species in the Tsitsikamma, and also for South Africa.

*Lactarius delisciosus*

A sample of *L. delisciosus* was also found besides *L. quieticolor.* Sequences of this specimen was unavailable but was included to document the similarity in morphological appearance to that of *L. quieticolor*. The *Lactarius* genus is known as the 'milk cap fungi' due to exuding a milky-like fluid that varies in colour depending on the species member when the fruiting body is bruised, broken or crushed in any way (Nuytinck & Verbeken, 2005, 2007). This physical characteristic is often used to taxonomically distinguish members of the genus from each other. It is a large genus of ectomycorrhizal mushrooms that contains about 400 species (Vieira et al., 2014). Research regarding multigene analysis has shown that the genus *Lactarius* is not monophyletic and consists of two clades (Leonardi et al., 2021). Although, the genus has been investigated for numerous functional properties research has found that correct species descriptions are still lacking. Mostly only diversity of the genus is documented by listing observed species alongside poor morphological descriptions. Due to the morphological placidity within the genus found among species this has led to various wrongful miss-identification (X.-H. Wang, 2007).

Morphologically the sample was orange in colour (Fig. 4.15 D-F). The funnel shaped cap is slightly dressed in the centre. The sunken centre is emphasised by tan to greenish concentric zones on the cap. The overall surface of the fruiting body is smooth but sticky when wet. The decurrent crowded gills are orange with a greenish tinge, intermediate in length, thick and forked. The concolorous stipe is thick, marked with small orange depressions, smooth and central. Ring and volva structures are absent. Flesh exudes orange coloured milk when damaged or bruised. Specimens was found growing under pine trees in sandy alkaline soil, covered in pine needles. The morphology from the table corresponds with earlier documentation of the species within the country (Goldman & Gryzenhout, 2019).

*Lactarius* is also known to form mycorrhizal relationships with coniferous trees like pine. These highly host specific relationships are so significant that it can be utilized in the morphological characterisation of species within the genus (Nuytinck & Verbeken, 2007). Although, not all of the genus members' edibility status is known, some of the members of the genus, such as *L. delisciosus* also known as ' the pine ring' or saffron milk-cap (Leonardi et al., 2021), *Lactarius sanguifluus*, *Lactarius vinisus* and *L. quieticolor* are widely enjoyed edible mushrooms that is renowned for their excellent taste and meaty texture as well as wide cooking applications (Silva-Filho et al., 2020), making it favourable as a commercially sold export (Nuytinck & Verbeken, 2007).

*Lactarius delisciosus* is considered an edible fungus, that has an excellent flavour and is valued in various cooking applications (Nuytinck & Verbeken, 2007). The species is also known as the 'pine ring' due to the significant relationship with host genera of *Pinus* species. Naturally the species is distributed within Europe and Asia. Compared to *L. delisciosus* the morphological similar species *L. quieticolor* is also considered to be edible and is enjoyed for their excellent flavour. This species also forms significant relationships with *Pinus* host species, but is limited by distribution throughout Europe (Nuytinck & Verbeken, 2005). *Lactarius quieticolor* is found to be growing in more acidic soil compared to *L. delisciosus* that prefers more neutral calcareous soils. Thus, the presences of a species can be limited to the environmental factors and this can possibly be considered as a macromorphological characteristic to identify between the two species.

***South African Diversity of Lactarius species***

Only two species of *Lactarius* is documented in South Africa namely, *L. delisciosus* and *Lactarius hepaticus* (Goldman & Gryzenhout, 2019; Kinge et al., 2020). This study thus represents a first report of *L. quieticolor* for South Africa. The species *L. quieticolor* is considered very similar in appearance to *L. delisciosus* but can possibly be distinguished by the surrounding environment associated with the sample as well, as that the latex fluid excided after the flesh of species within the genus has been damaged, that can vary in color between *L. delisciosus* and *L. quieticolor*. The color of the fluid latex observed in samples of *L. delisciosus* is more bright orange compared to the more red-dark orange seen in *L. quieticolor* samples. Based on the phylogenetic evidence it is likely that *L. quieticolor* is present in the Tsitsikamma and it was probably misidentified as *L. delisciosus* in the past.

### 4.2.7 *Panaeolus*

The *Panaeolus* phylogenetic tree (Fig. 4.16) consisted of 20 sequences including the unknown sample (Sample D17) that was collected under pine trees in the Koomansbos plantation. The data set included various *Panaeolus* species and some species of *Deconica*, namely *Panaeolus fimicola*, *Panaeolus semiovatus*, *P. antillarum*, *Panaeolus foenisecii*, *Panaeolus axfordii* and *Deconica chionophila*. These sequences were mainly from the Northern Hemisphere including countries such as China, France and the USA. The sample investigated in this study grouped within a clade of *P. antillarum* sequences form the Egypt, Thailand and the Dominican Republic with a supporting bootstrap value of 99%.

Morphologically the sample (Fig. 4.17) is small in size. The dry pale to light grey-brown buff cap is conical convex and smooth. The margin is regular and non-striated. The moderate broad gills are adnate to adnexed, unequal in lengths, greyish-black in colour and crowded. The subbulbous based stipe is long and slender, smooth and powdery, white to light brown in colour and cylindrical in shape. The ring and volva are absent. It was found on a dung heap. Morphology of the sample corresponded with previous descriptions of the species (Desjardin & Perry, 2017; Halama et al., 2014; Kaur et al., 2014).

*Panaeolus antillarium* was first reported and described from the U.S Virgin Islands in the late 1820s (Halama et al., 2014). Since the first report of the species it has been documented in Africa, Australia, China, Europe, India, Taiwan, Poland, Philippines and Thailand (Bustillos, 2014; Desjardin & Perry, 2017; Halama et al., 2014; Manimohan et al., 2007). The species is universally characterised as a small mushroom with convex buff to light brown cap, with regular margins. Gills are adnate and unequal in length, crowded and greyish-black in colour. The stipe is long, slender and cylindrical (Desjardin & Perry, 2017; Halama et al., 2014). The fungus is considered a pantropical-sub temperate species. Due to the high morphological variability between specimen confusion often arises when identifying the species.

*Panaeolus* contains species that are known as coprophilous macro fungi, thus growing within dung or substrate that contains dung remnants (Kaur et al., 2014). Mushrooms are rarely seen growing in or on dung, because this ephemeral substratum cannot support a long life cycle and the larger sizes of various fruiting body producing macro fungi (Manimohan et al., 2007). These mushrooms often demonstrate facultative behaviour by being able to grow on dung from a wide range of herbivores, including cattle, horses and wild life (Halama, 2014). *Panaeolus* are also considered to be neurotrophic mushrooms with hallucinogenic properties when consumed. This is because some species members within the genus contains psilocybin that cause hallucinogenic effects (Bustillos, 2014).

In South Africa a total of 11 *Panaeolus* species have been documented by Kinge et al. (2020). These include *Panaeolus caliginosus*, *Panaeolus campanulatus*, *P. fimicola*, *Panaeolus fimicoloides*, *Panaeolus papilionaceus*, *Panaeolus retirugus*, *P. semiovatus*, *Panaeolus semiovatus* f. exannulatus, *Panaeolus solidipes*, *Panaeolus sphinctrinus* and *Panaeolus subbalteatus*. These species are difficult to identify and distinguish because they vary from very small to almost medium in size, are similar in colour and significant overlap occurs in substrate.

### 4.2.8 *Russula*

The sequencing result of *Russula* species (Fig. 4.18) were presented in separate *Russula* subset phylogenies to enhance the resolution of the clades in the individual trees, and these included *R. caerulea* (subset 1) (Fig 4.19), *R. capensis* (subset 2) (Fig 4.20), and *R. sardonia* (subset 3) (Fig 4.21). The results from the DNA sequence comparison, confirmed the findings of the macroscopic characteristics.

#### ***Russula caerulea***

The *R. caerulea* (subset 1) phylogenetic tree (Fig. 4.19) consisted of 18 sequences including the unknown specimen (Sample D6). The specimen was collected under pine trees in the Lottering plantation. The data set included various *Russula* species including *Russula clavatohyphata*, *Russula minor, R. caerulea* and *Russula purpureomaculata.* These sequences were mainly form the Northern Hemisphere including six countries such as China and within Northern Europe. The sample noted within this study Sample D6 grouped within a clade of *R. caerulea* sequences originating from Europe with a confirming bootstrap value of 100%. A closely related branch is that of *R. clavatohyphata* originating from India.

Morphologically the sample (Fig. 4.18 A-C) is a medium sized mushroom. The cap is a dark deep cool-toned red to purple colour. Convex in shape with prominent umbo. The cap is sticky covered in pine needles with no scales of warts present. The gills are a pale cream colour that slightly protrudes up along the cap margin, although the surface it is smooth and even. Aged specimens become upturned compared to the decurved cap margins of growing specimens. The brittle gills are adnexed attached, crowded and full in length. The stipe is firm, white and central, tapering upwards towards the apex. Ring and volva are absent. The morphological characteristics corresponded with previous documentation of the species by Gryzenhout (2010).

***Russula capensis***

The *R. capensis* (subset 2) phylogenetic tree (Fig 4.20) consisted of 17 sequences including the unknown specimen (Sample D14). The specimen was collected under pine trees in the Plaatbos plantation. The data set included various *Russula* species including *R. caerulea, Russula ayubiana, Russula laeta, Russula velenivskyi, Russula gnathangensis* and *Russula tengii*. These sequences were mostly from the Northern Hemisphere including countries such as India, Pakistan, China and within Northern Europe. The sample investigated in this study Sample D14 grouped within a clade of R. caerulea, with a supporting bootstrap value of 100%, but as a haplotype. There are no other sequences are available for *R. capensis*, thus with supporting morphology this is the first reference sequences for the species.

Morphologically the sample is medium in size (Fig. 4.18 D-F). The cap is purple to dark cool-toned red, convex with a central depression. Margin is smooth and even. Gills are pale white to cream, adnexed, slightly protruding beyond the margin and crowded. The stipe is solid, central, tapering towards the apex. Ring and volva is absent. The morphology corresponds with pervious documentation of the species by Goldman & Gryzenhout, (2019); Gryzenhout, (2021).

*Russula capensis* was first documented and describe by Pearson (1950) from the Western Cape, South Africa. It is believed that the species is endemic to the country, due to that it is not known form other locations, although it shares similar characteristics to various other *Russula* species. Additional sequencing information regarding *R. capensis* is lacking, moreover results suggest that it could be conspecific to *R. caerulea*, a much older species from Europe. Other gene regions have been investigated for the correct molecular identification of *Russula* species, including the large subunit of the nuclear ribosomal (nLSU), translation elongation factor 1-α (*Tef*-1α), largest subunit (RPB1) and second largest (RPB2)(Li et al., 2019). Therefore, future studies should include sequencing of these genes to confirm if *R. capensis* is a separate species or conspecific to other *Russula* such as *R. caerulea* species within the country.

The *R. sardonia* (subset 3) phylogenetic tree comprised of 18 sequences including the unknown specimen (Sample D66). The specimen was obtained from under pine trees in the Kleinbos plantation. The data set included various *Russula* species including *Russula sanguinea, R. sardonia*, *Russula indohimalayana* and *Russula ryukokuensis.* These sequences were mostly from the Northern Hemisphere including countries such as India, Japan and within Northern Europe. The sample

investigated in this study Sample D66 grouped within a clade of *R. sardonia* sequences originating from across Europe and one KY693646 from Spain, with a supporting bootstrap value of 100%.

Morphologically the sample's cap is a purplish blush to grey-ruby color and convex, with a slight central depression (Fig. 4.18 G-I). Margins are wavy, with a smooth surface, dry to sticky texture. Gills are adnexed to slightly decurrent, crowded and white to pale lemon cream in color. Stipe is central, solid, widening at apex, white, flushed pale rose pink. Ring and volva are absent and overall has a faint, pungent odor. The morphological characteristics correspond with previous documentation of the species by Goldman & Gryzenhout (2019); Gryzenhout (2021).

### *Russula sardonia*

*Russula sardonia* was first documented in 1838. It is also commonly known as the purple-stemmed russula, due to its purple and lilac tinged stipe. It occurs in the UK, Europe, Canada, Mexico and Russia. The species is mycorrhizal with coniferous tree species. The species is considered to be poisonous and can induce diarrhea, vomiting and abdominal cramps if consumed (Gryzenhout, 2019).

The *Russula* genus is the largest in the Russulacea family. Members of the genus is spread worldwide and are considered to be highly diverse, forming significant mycorrhizal relationships with host plants, conifer and broadleaved trees (Kiran et al., 2021; Li et al., 2019; Pala et al., 2012; Wisitrassameewong et al., 2022). Over 2500 names and 750 species have been document since the genus was recognised in 1971 (Wisitrassameewong et al., 2020). Universally specimens of the genus are morphologically characterised by a velvety pileus, a fleshy pink, red or purple stipe and a light white creamy spore print (Wisitrassameewong et al., 2022).

### *South African Diversity of Russula species*

In South Africa in total only seven *Russula* species are recognised, namely *Russula agaricina, R. caerulea, R. capensis, Russula fallax, R. sardonia, Russula sororia* and *Russula xerampelina*. Overall *Russula* species found in South Africa have a pink, vibrant red or dark purple colour. The cap is convex with central depression observed. Gills vary in colour from creamy white to dark brown or black as the specimen ages. Spore prints can be either a cream colour or deep ochre (Li et al., 2019; Pala et al., 2012; Panda et al., 2021; Song et al., 2022). Distinguishing between members of the gDenus, species features are used such as the cap colour, with *R. capensis* being more purple compared to the pinkish

red presented by *R. sardonia* (Goldman & Gryzenhout, 2019). The stipe colour is pure white in *R. capensis* and slightly flushed in *R. sardonia* and *R. xerampelia*. Odour is also used to correctly identify *R. xerampelia* form that of *R. sardonia,* since *R. xerampleia* specimens present with similar features to those of *R. sardonia* but have a distinctive fishy seafood smell (Goldman & Gryzenhout, 2019); Gryzenhout, (2021).

The Tsitsikamma is one of the richest biodiverse areas for mushrooms in South Africa. Yet very little research has been done regarding macro fungi from this area. This is especially concerning regarding large regions of plantations and cultivated agricultural land is in such close proximity to native vegetative growth. This study is largely focussed on macro fungi from pine plantations, most likely worldwide known non-native species due to their strong and significant host associations. Yet even for these, supposedly well-known fungi, results yielded surprises, e.g. two first reports of which one fungus is considered vulnerable and only known from the USA. This also indicated that a South African described species could be wrongly described. However, other results by far confirmed identities and often represent first DNA results for South Africa for these species. Results from this study thus serves as a useful foundation to start characterizing mushroom diversity in South Africa.


**Figure 4. 1** Various *Amanita* species from the Tsitsikamma region.

A-E: *Amanita morrisii* A: Cap with central depression.; B: Ring on stipe.; C: Mature and growing fruiting bodies.; D: Warts and bulbous stipe.; E: Immature fruiting body. F-H: *Amanita muscaria* F: Scarlet cap with fleshy central stipe.; G: Cap, covered with white warts.; H: Gills white and intermediate in length. I-J: *Amanita pantherina* I: Side view of the fruiting body.; J: Cap, covered in white pyramidal warts. K-Q *Amanita rubescens* K: Side view of the fruiting body.; L: Blushing stipe.; M: Side view of the fruiting body.; N: Top view of the cap, warts.; O: Ring on stipe .; Q: Bulbous stipe.

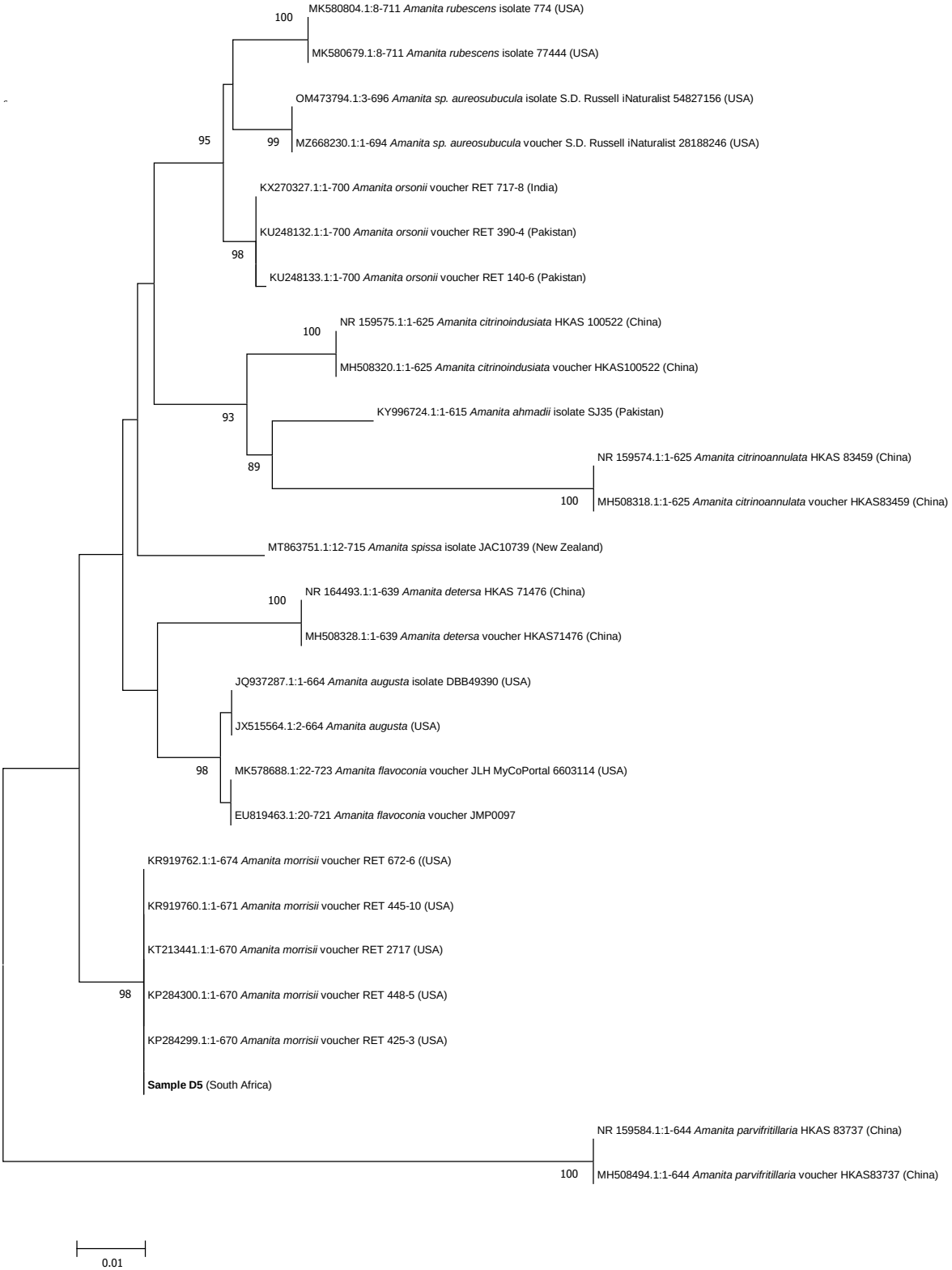



**Figure 4. 2** *Amanita morrisii* phylogram.

Unrooted phylogram of 27 internal transcriber region (ITS) sequences from 12 *Amanita* species based on Maximum Likelihood. The sequence labeled as Sample D5 in bold was collected in this study. Values observed on the left of each group of sequences represent bootstrap support percentages (≥80 is shown).

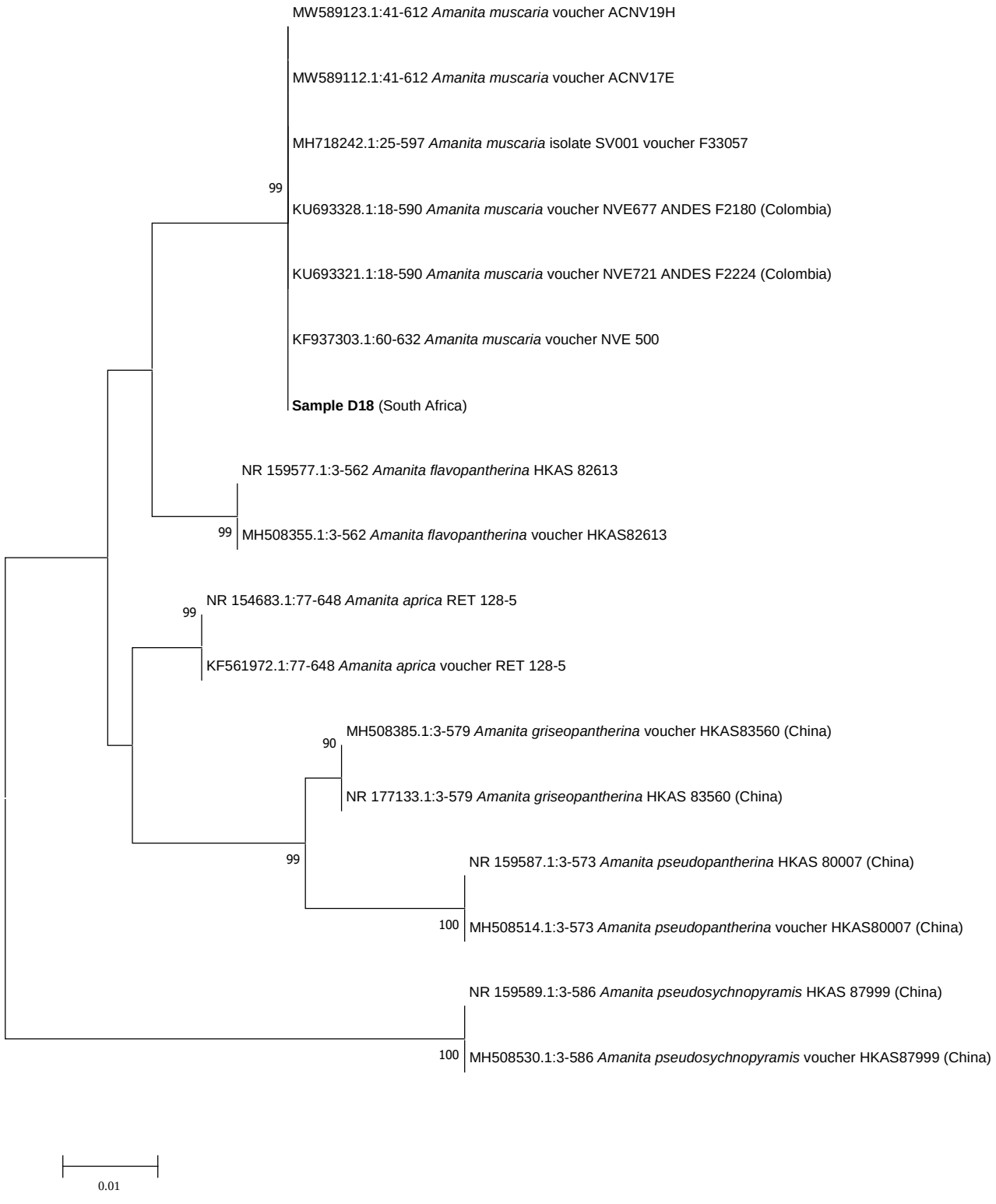



**Figure 4. 3** *Amanita muscaria* phylogram.

Unrooted phylogram of 17 internal transcriber region (ITS) sequences from 6 *Amanita* species is based on Maximum Likelihood. The sequence characterized as D18 in bold was collected in this investigation. Values noted on the left of each group of sequences represent bootstrap support percentages (≥80 is shown).

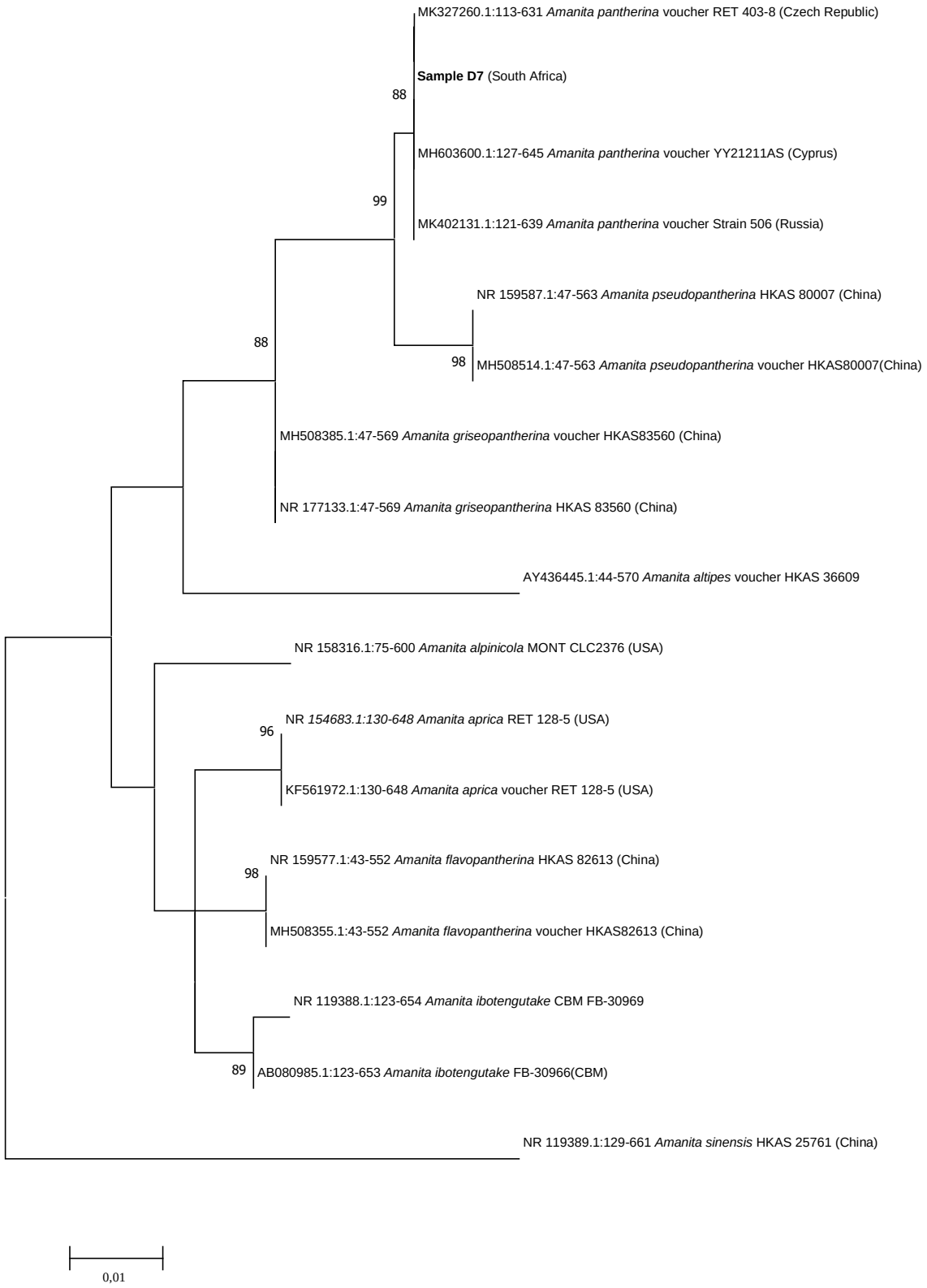



**Figure 4. 4** *Amanita pantherina* phylogram.

Unrooted phylogram of 17 internal transcriber spacer region (ITS) sequences from 9 *Amanita* species is based on Maximum Likelihood. The sequence labelled as Sample D7 in bold was collected in this study. Values observed on the left of each group of sequences represents bootstrap support percentages (≥80 values are shown).

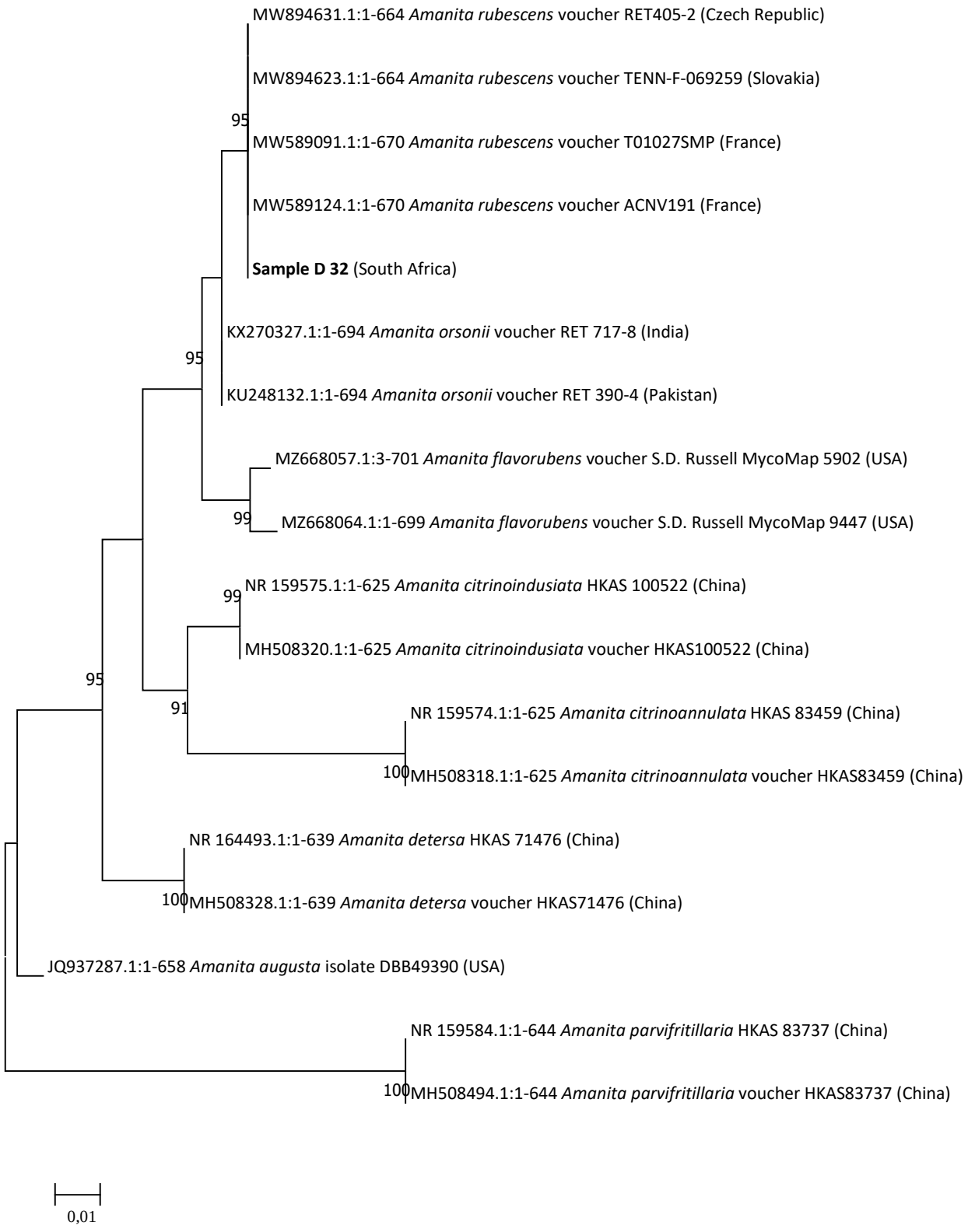



**Figure 4. 5** *Amanita rubescens* phylogram.

Unrooted phylogram of 18 internal transcriber region (ITS) sequences from 8 *Amanita* species is based on Maximum Likelihood. The sequence characterized as D32 in bold was gathered in this study. study Values seen on the left of each group of sequences represent bootstrap conformation percentages (≥80 is shown). The box indicates the clade which the collected sample is in.

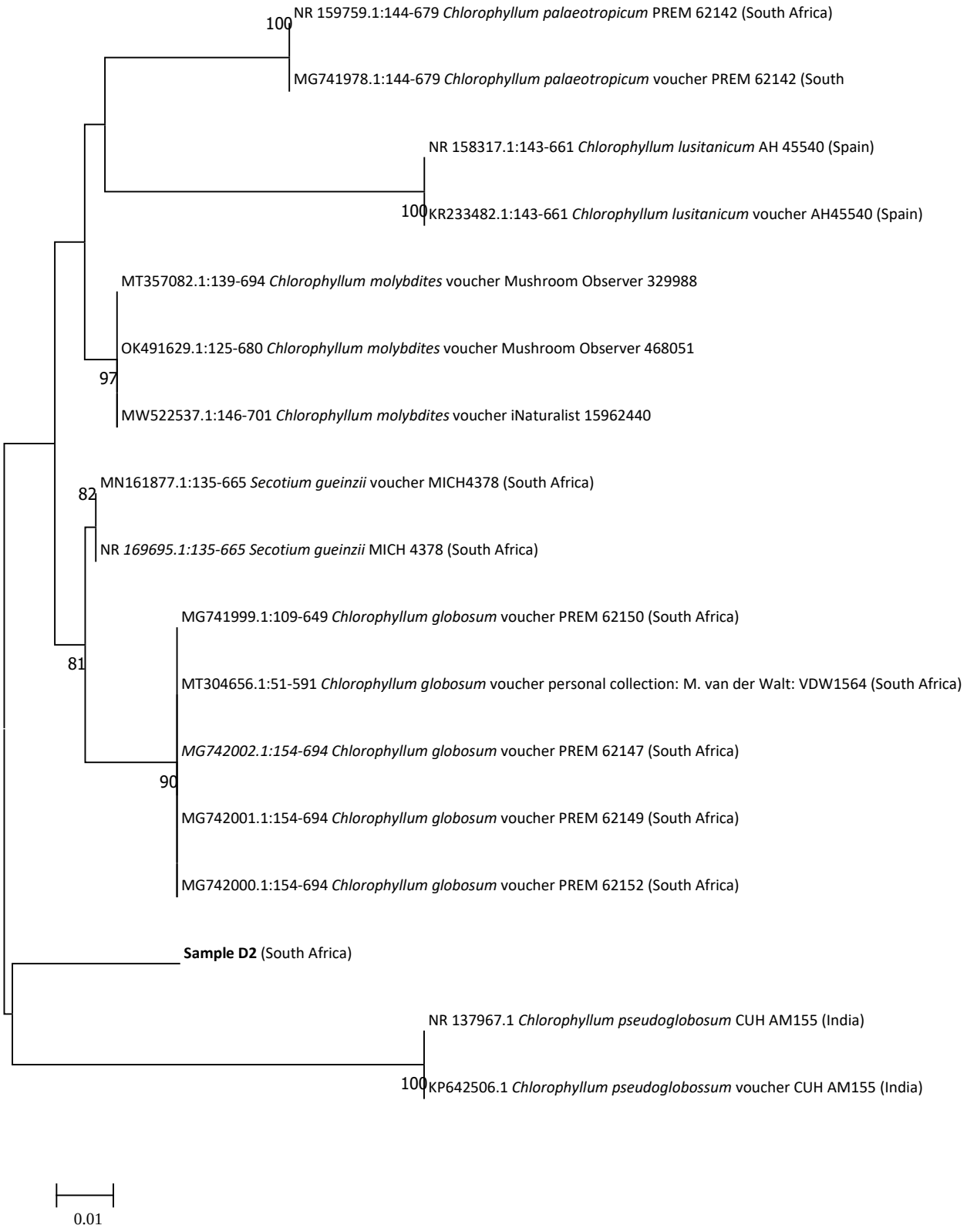



**Figure 4. 6** *Chlorophyllum* sp. phylogram.

Unrooted phylogram of 17 internal transcriber spacer region (ITS) sequences from 5 *Chlorophyllum* and 1 *Secotium* species based on Maximum Likelihood. The sequence labelled as Sample D2 in bold was investigated in this study. Values observed on the left of each group of sequences represent bootstrap supporting percentages (≥80 values are shown).

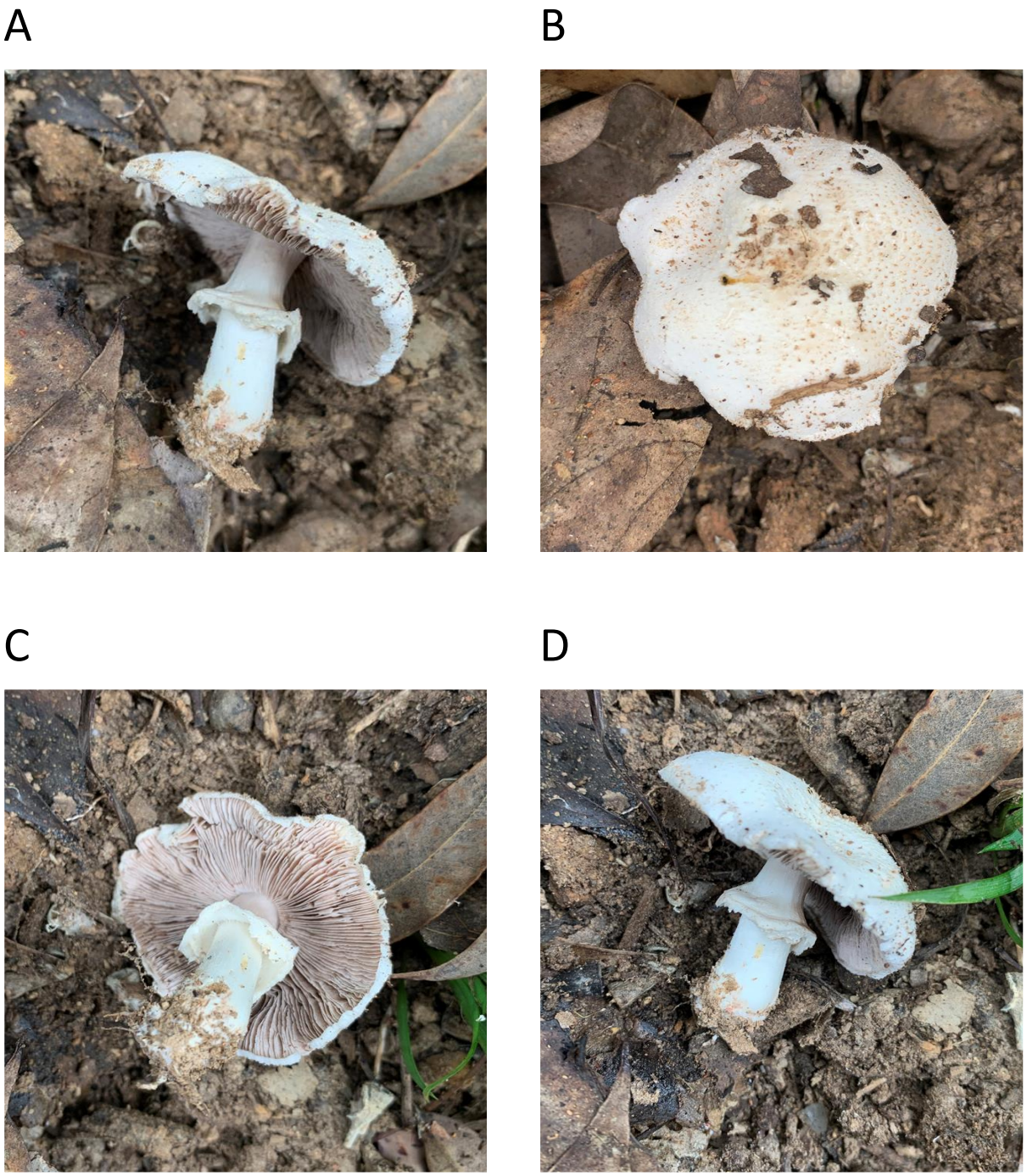



**Figure 4. 7** *Chlorophyllum* sp. from the Tsitsikamma region.

1539 A: Side view of the fruiting body.; B: Top view of the cap.; C: Gills and stipe.; D: Ring and basal bulb.

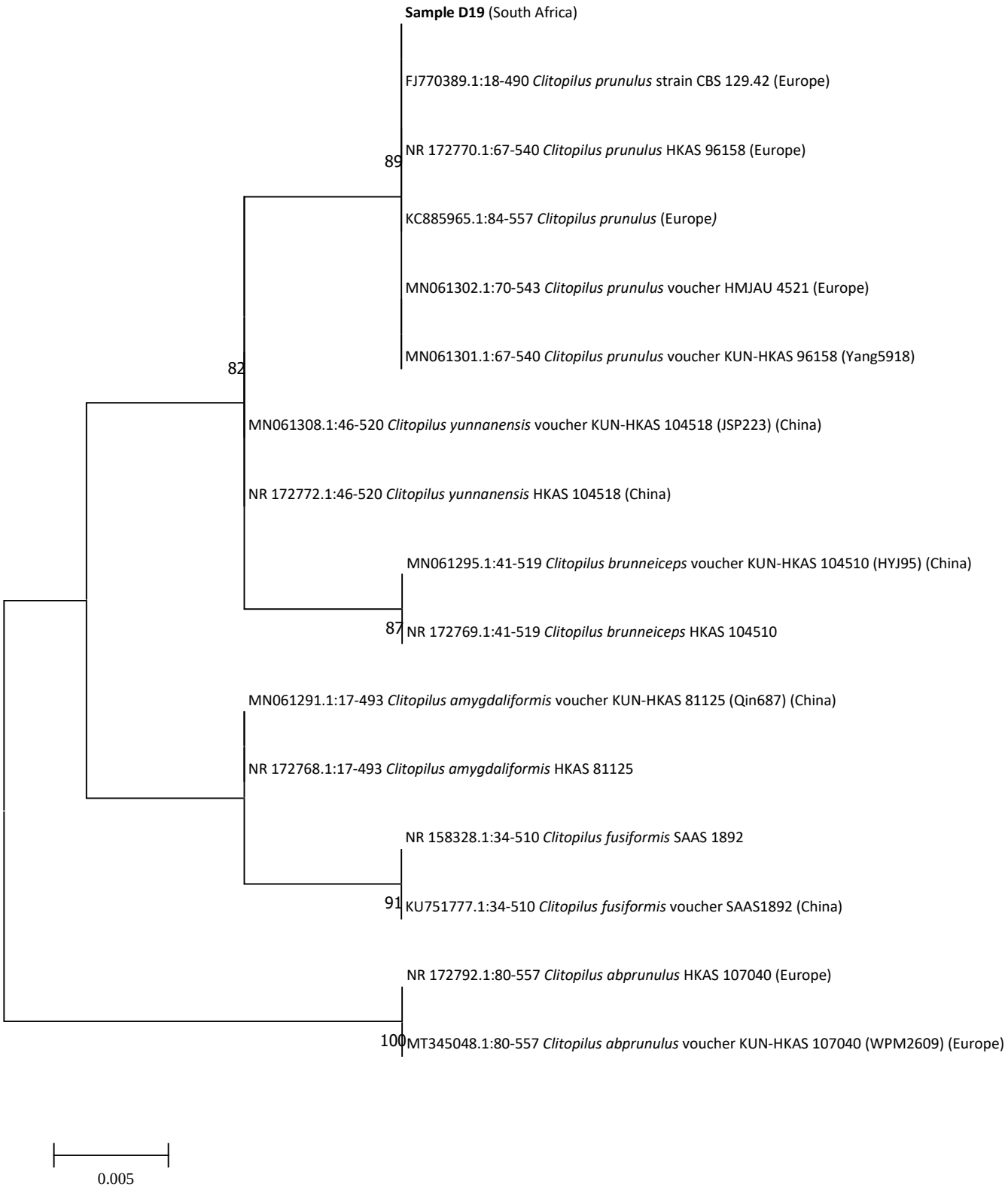



**Figure 4. 8** *Clitopilus prunulus* phylogram.

Unrooted phylogram of 17 internal transcriber spacer region (ITS) sequences from 5 *Chlorophyllum* and 1 *Secotium* species based on Maximum Likelihood. The sequence labelled as Sample D2 in bold was investigated in this study. Values observed on the left of each group of sequences represent bootstrap supporting percentages (≥80 values are shown).

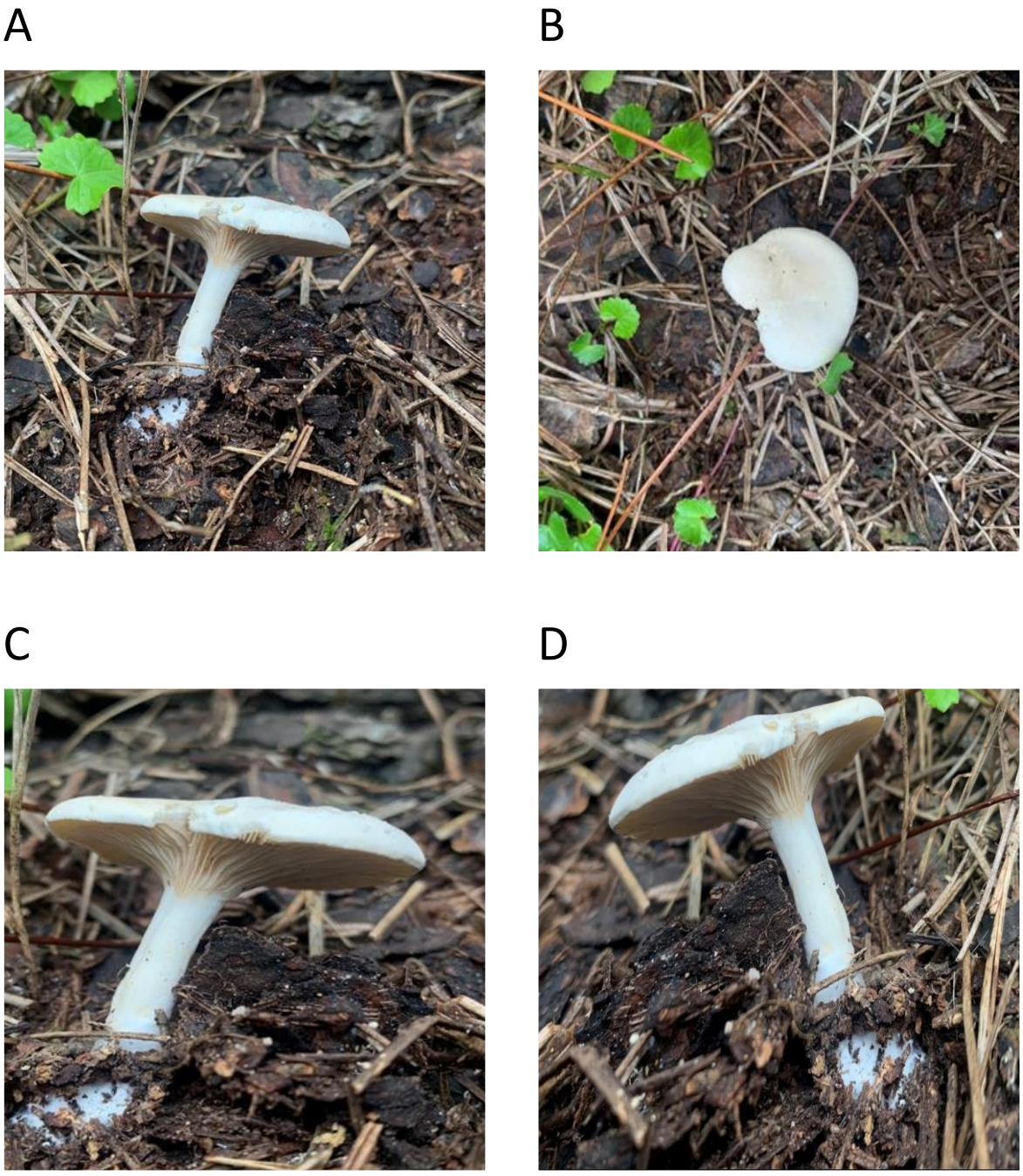



**Figure 4. 9** *Clitopilus prunulus* from the Tsitsikamma region.

A: Side view of the fruiting body, broadly convex shape of the cap.; B: Top view of the fruiting body.; C: Decurrent gills attached to the stipe.; D: Absent ring and cylindrical stipe.

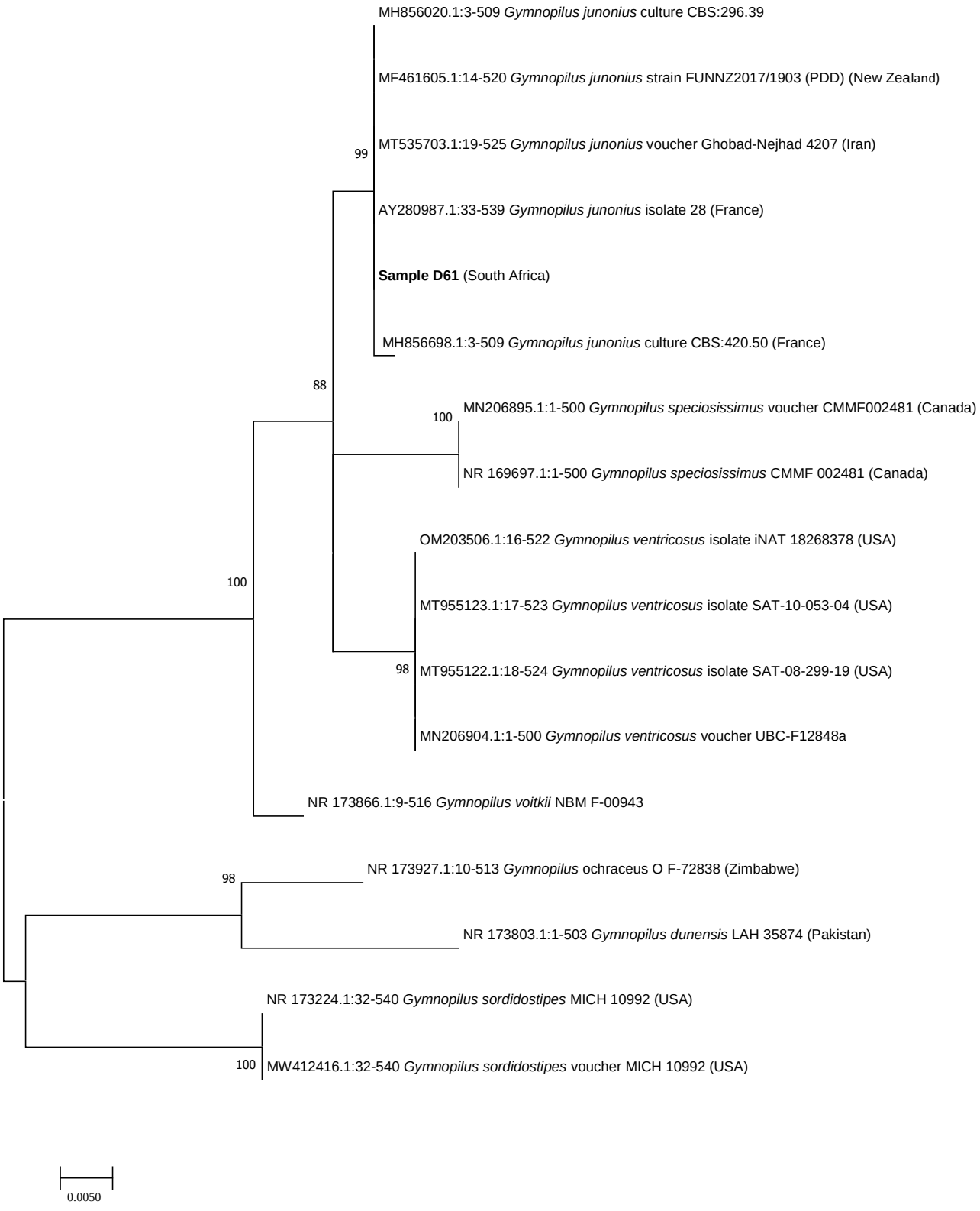



**Figure 4. 10** *Gymnopilus junonius* phylogram.

Unrooted phylogram of 17 internal transcriber spacer region (ITS) sequences from 6 *Gymnopilus* species is based on Maximum Likelihood. The sequence labelled as Sample D61 in bold was investigated in this study. Values observed on the left of each group of sequences represent bootstrap supporting percentages (≥80 values are shown).

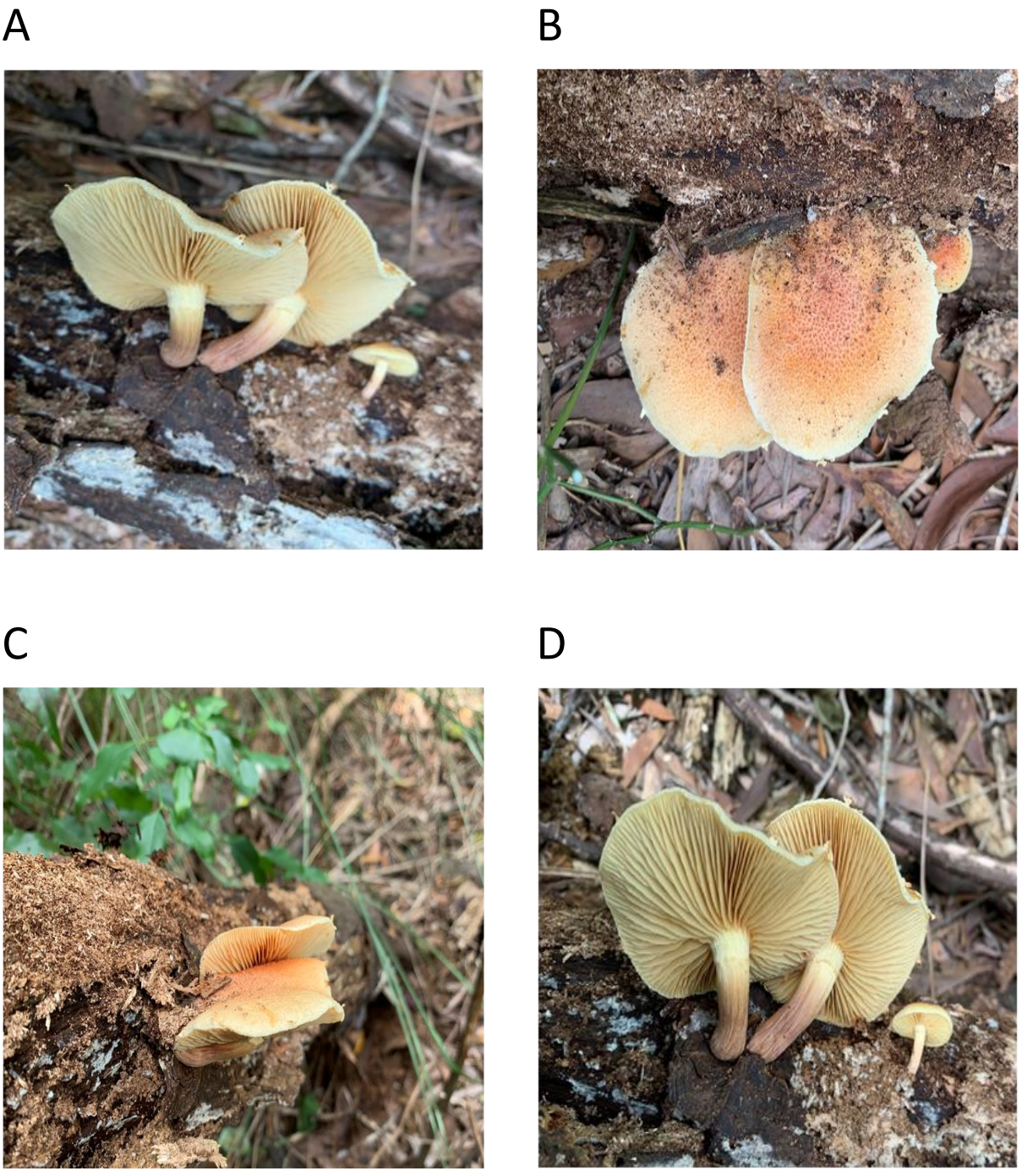



**Figure 4. 11** *Gymnopilus junonius* from the Tsitsikamma region.

A: Side view of the fruiting bodies, colour variation of the stipe .; B: Top view of the caps, circular shape of the fruiting body.; C: Fruiting bodies clustered in overlapping clumps .; D: Gills, crowded and adnexed attached to the stipe.

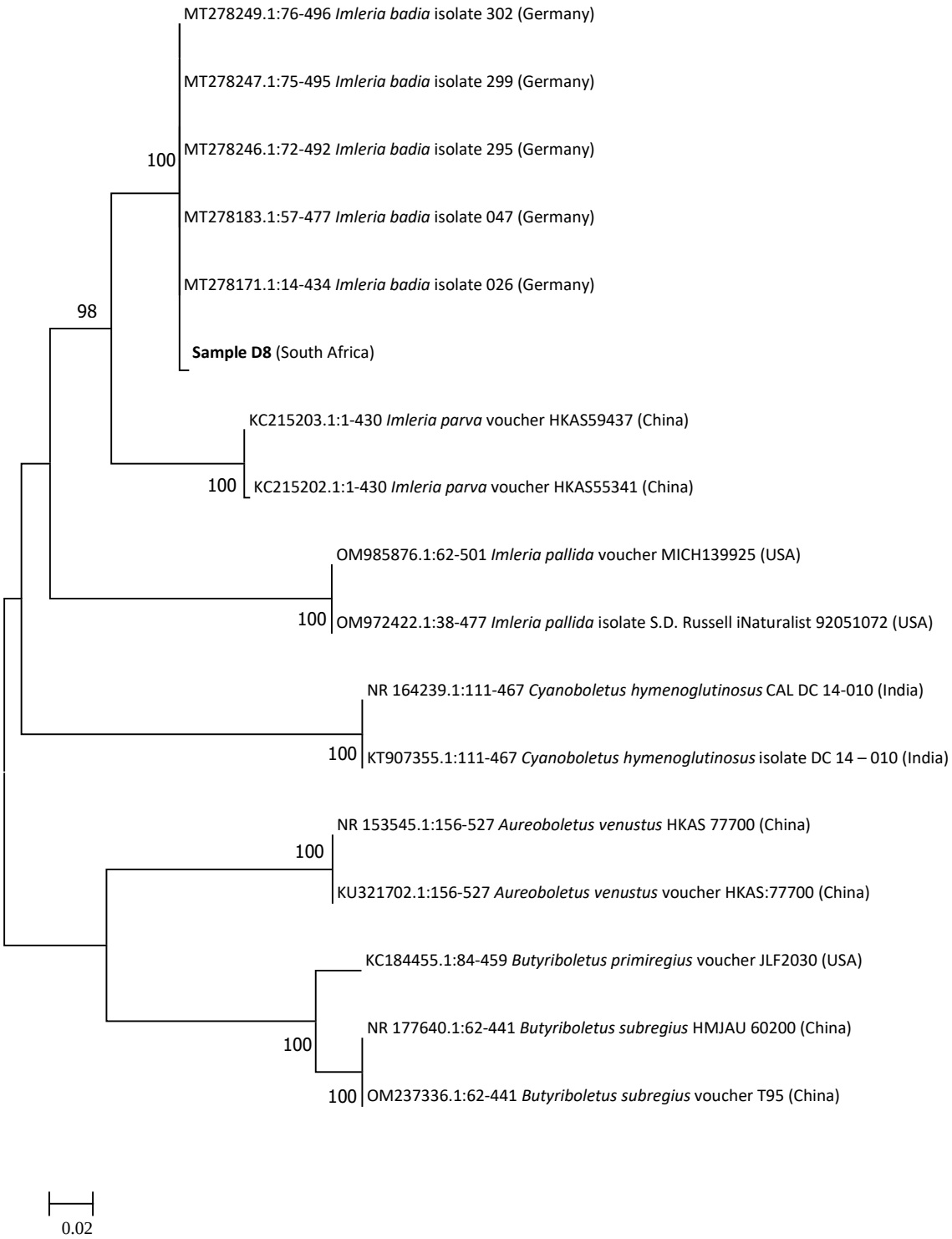



**Figure 4. 12** *Imleria badia* phylogram.

Unrooted phylogram of 17 internal transcriber region (ITS) sequences from 3 *Imleria* and other boletus species is based on Maximum Likelihood. The sequence characterized as D8 in bold was collected in this investigation. Values noted on the left of each group of sequences represent bootstrap supporting percentages (≥80 is shown).

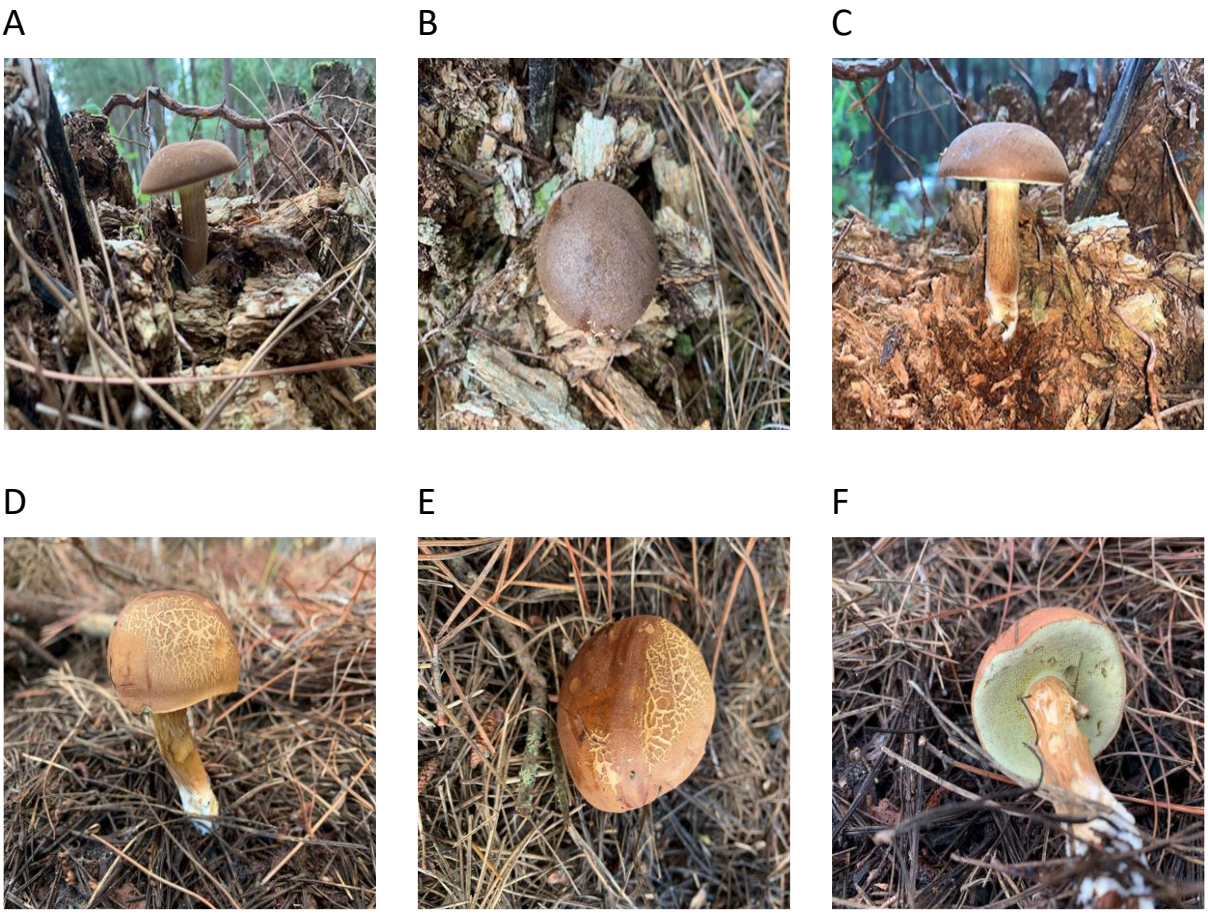



**Figure 4. 13** *Imleria badia* from the Tsitsikamma region.

A: Side view of fruiting body.; B: Top view of smooth polished cap.; C: Cylindrical, vertical dark brown lined stipe.; D: Side view of fruiting body.; E: Top view of cracked cap.; F: Olivaceous coloured pores, dressed around the stipe.

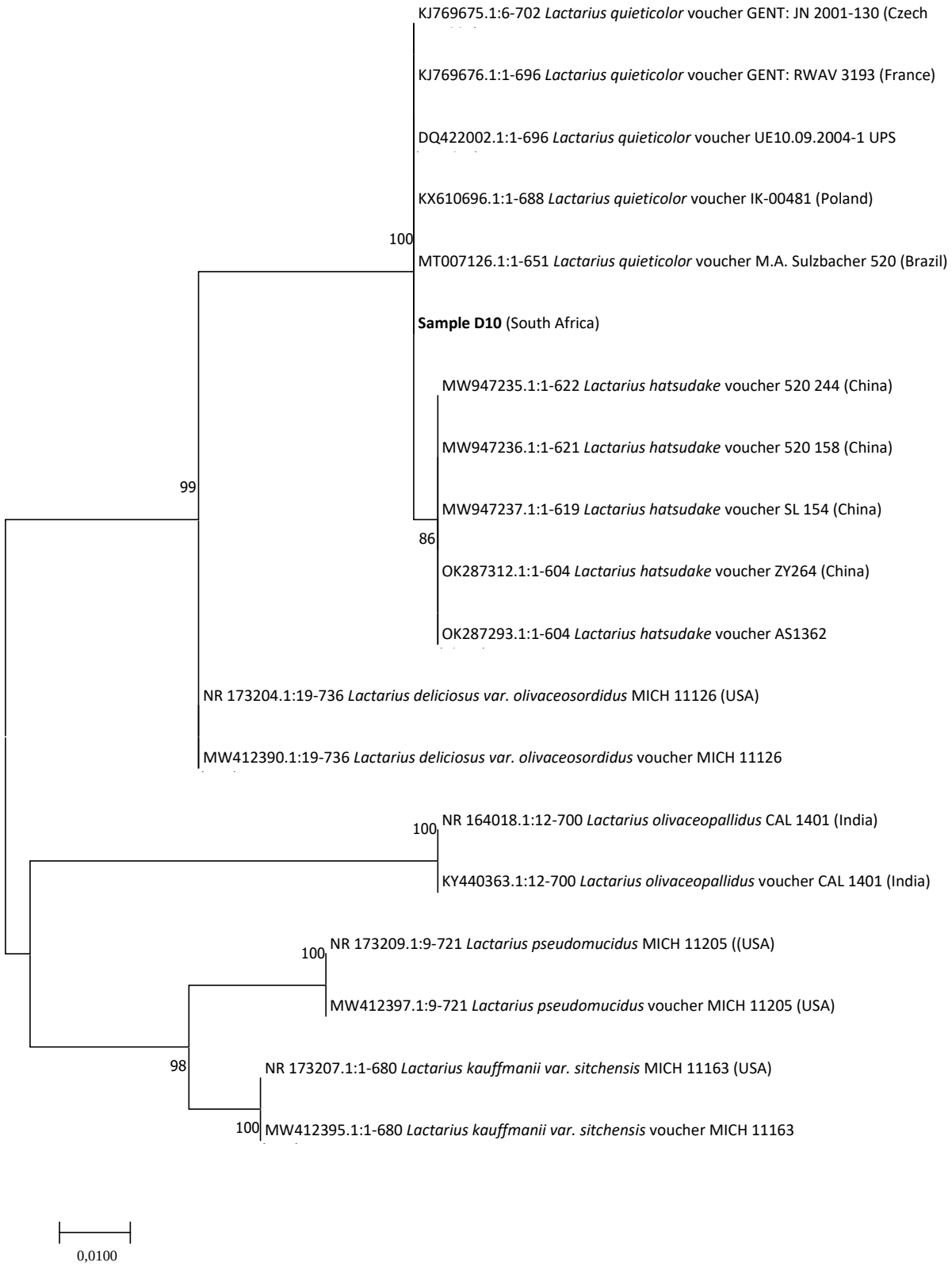



**Figure 4. 14** *Lactarius quieticolor* phylogram.

Unrooted phylogram of 19 internal transcriber region (ITS) sequence from 6 *Lactarius* species is based on Maximum Likelihood. The sequence labelled as Sample D10 in bold was collected in this study. Values observed on the left of each group of sequences represent bootstrap support percentages (≥80 values are shown).

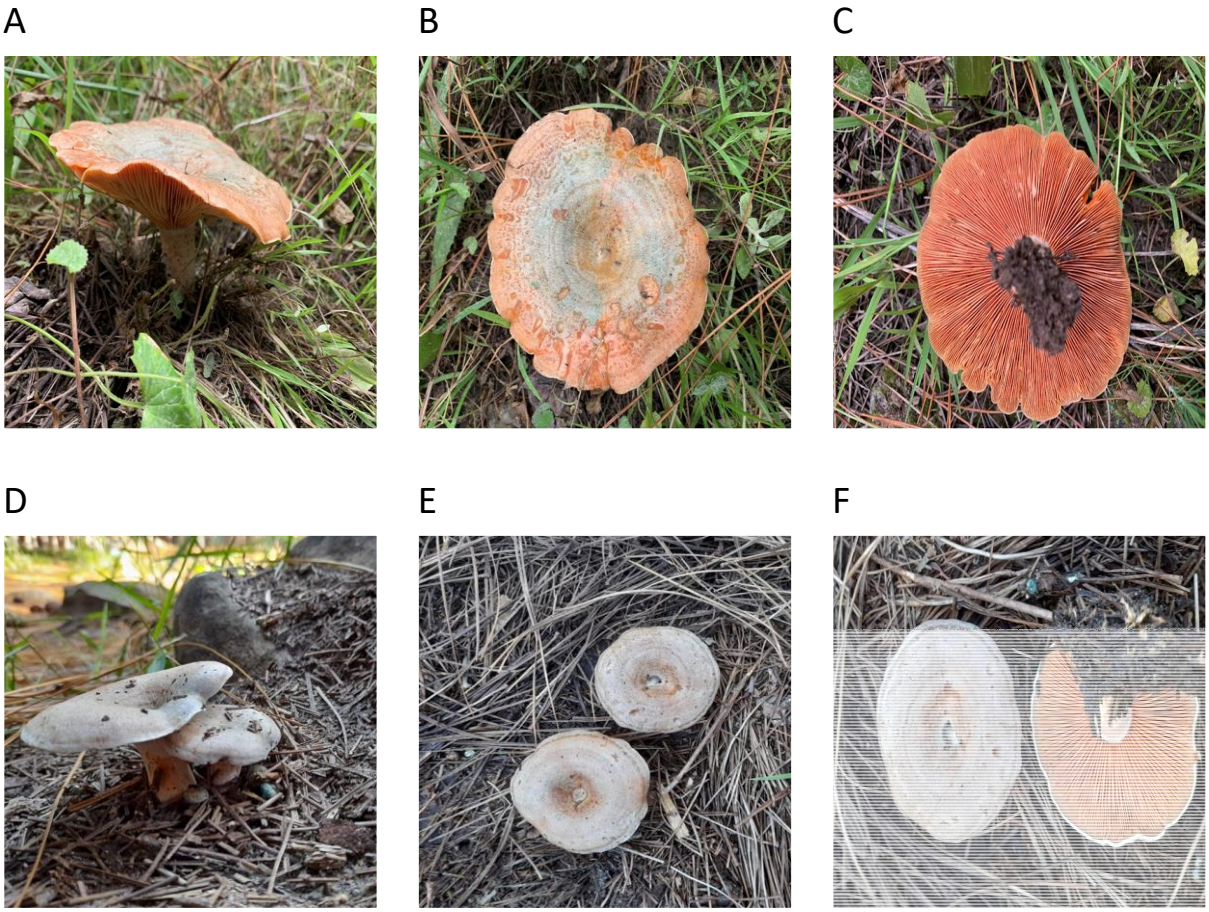



**Figure 4. 15** Various *Lactarius* sp. from the Tsitsikamma region.

*Lactarius quieticolor* A: Side view of the fruiting body, decurrent gill attachment, funnel-shape cap.; B: Top view of the cap, greenish concentric zones.; C: Gills underneath, crowded and unequal lengths. D-F: *Lactarius delisciosus* D: Side view of the fruiting bodies.; E: Top view of the caps, tan coloured concentric zones .; F: Top view of the cap (left) and gills underneath (right).

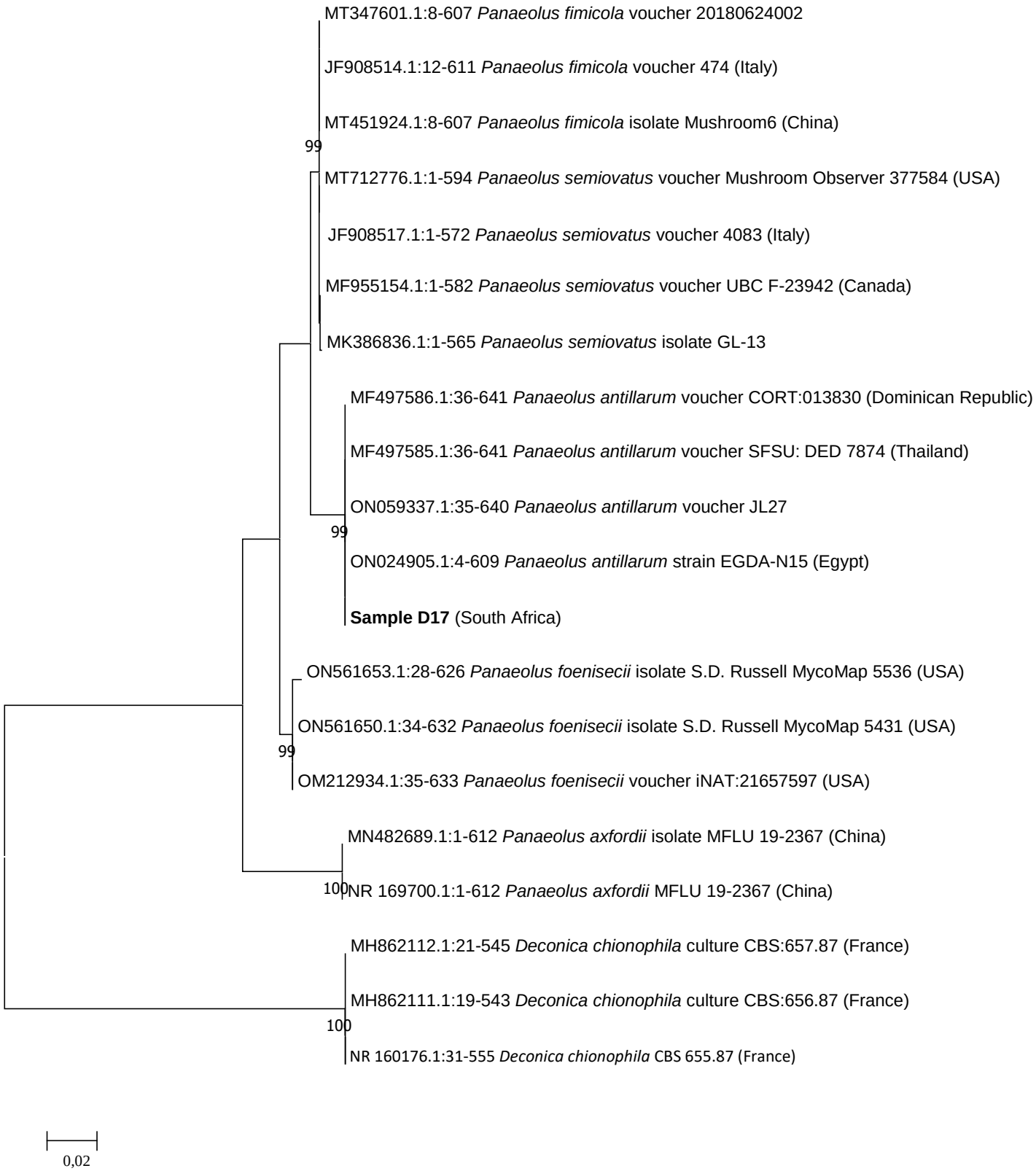



**Figure 4. 16** *Panaeolus antillarum* phylogram.

Unrooted phylogram of 20 internal transcriber region (ITS) sequence from 5 *Panaeolus* and one *Decomica* species is based on Maximum Likelihood. The sequence labelled as Sample D17 in bold was collected in this study. Values seen on the left of each group of sequences represent supporting bootstrap percentages (≥80 values are shown).

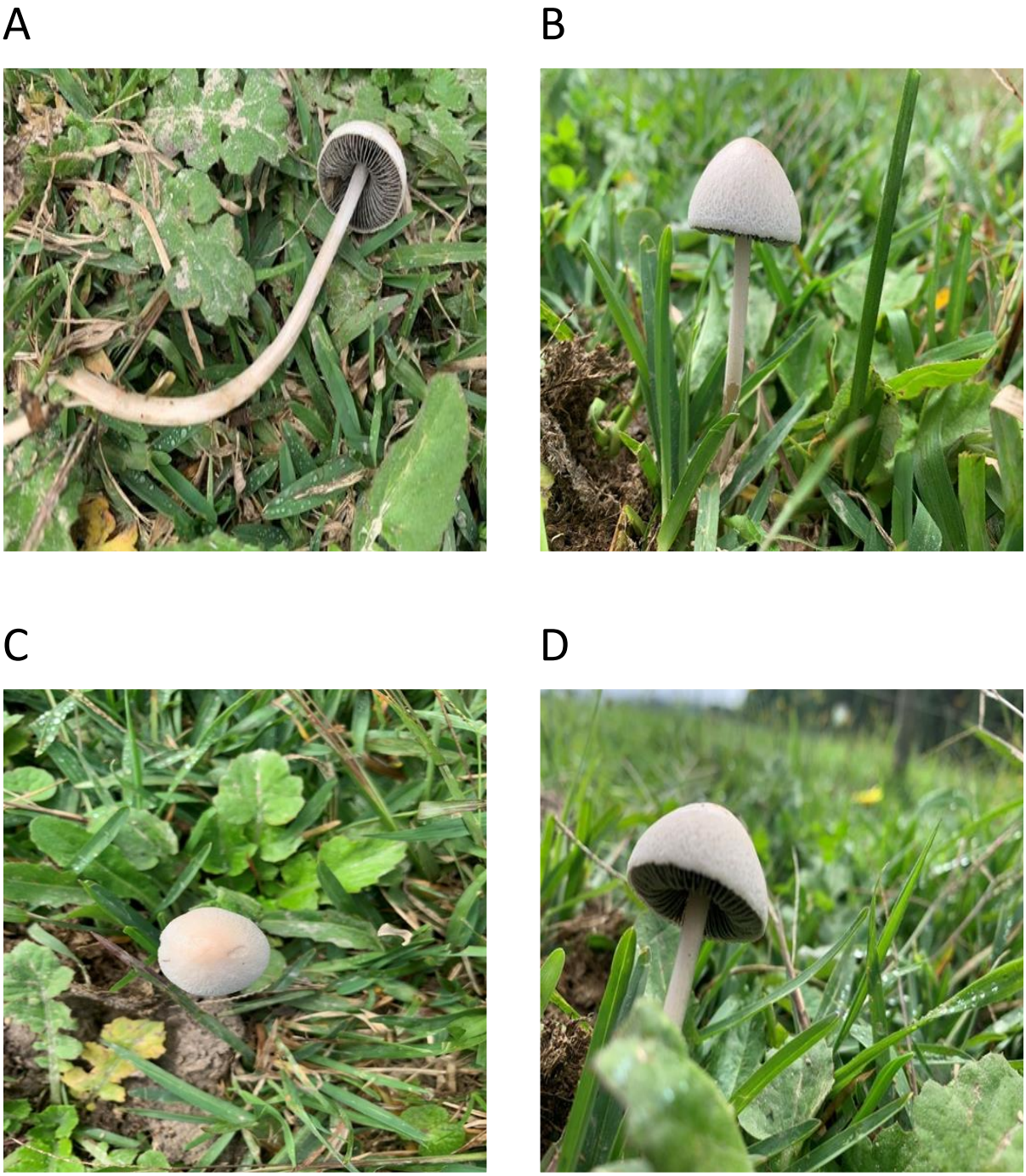



**Figure 4. 17** *Panaeolus antillarum* from the Tsitsikamma region.

A: Entire fruiting body, long slender stipe.; B: Side view of the fruiting body, growing inside dung.; C: Top view of the cap.; D: Side view of gills, dark grey, adnate to adnexed attached to the stipe.


**Figure 4. 18** Various *Russula* sp. from the Tsitsikamma region.

*Russula caerulea* A: Top view of the cap, central depression.; B: Side view of the fruiting body, gills protruding slightly beyond the margin of the cap.; C: Gills of the fruiting body, crowded and adnexed attached. D-F: *Russula capensis* D: Top view of the cap.; E: Side view of the fruiting body.; F: Gills of the fruiting body. G-I: *Russula sardonia* G: Side view of the fruiting body.; H: Top view of the cap.; I: Gills, crowded and stipe, flushed.

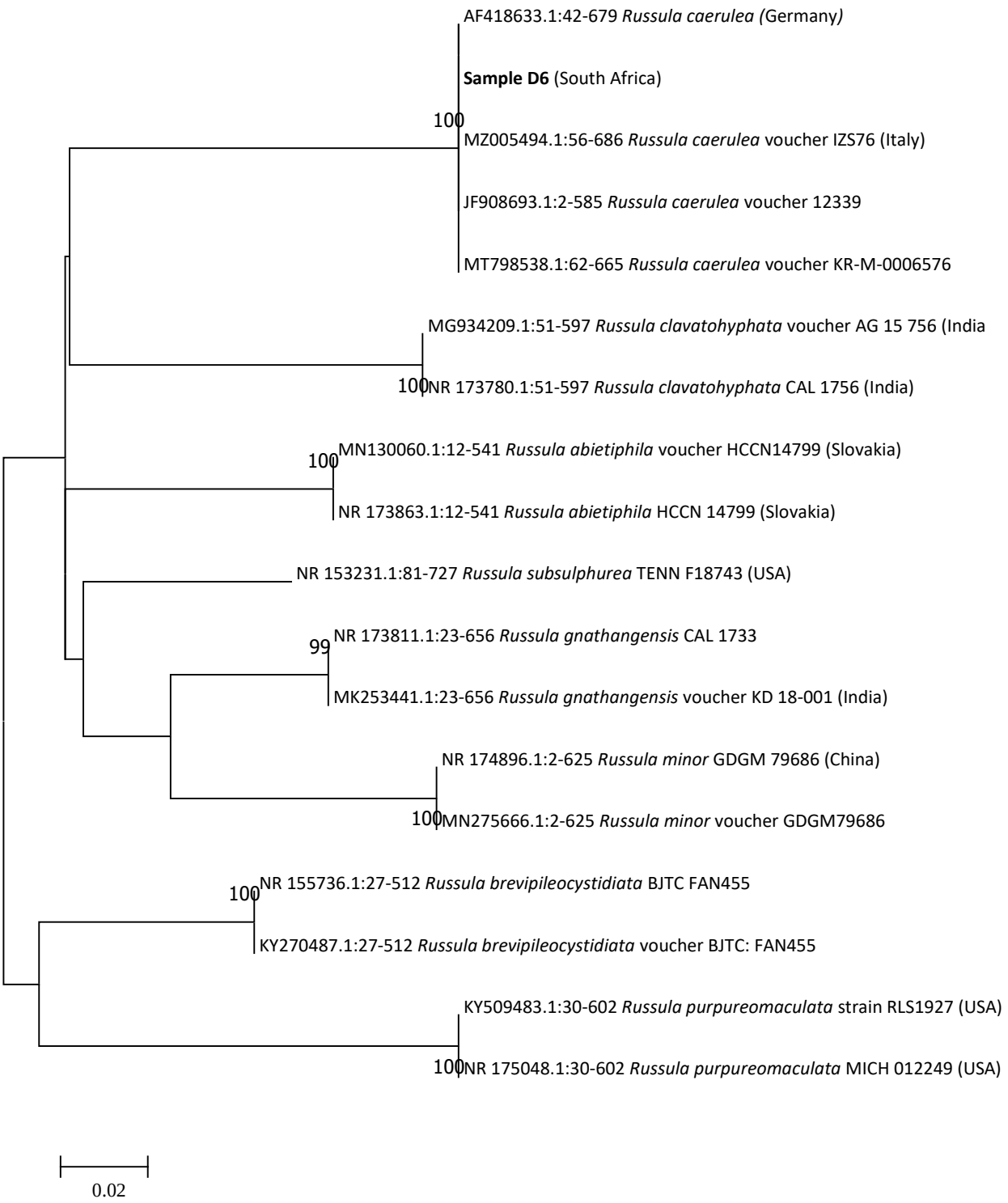



**Figure 4. 19** *Russula caerulea* phylogram.

Unrooted phylogram of 18 internal transcriber region (ITS) sequence from 8 *Russula* species is based on Maximum Likelihood. The sequence labelled as Sample D6 highlighted in bold was gathered in this study. Values noted on the left of each group of sequences represent bootstrap confirming percentages (≥80 values are shown).

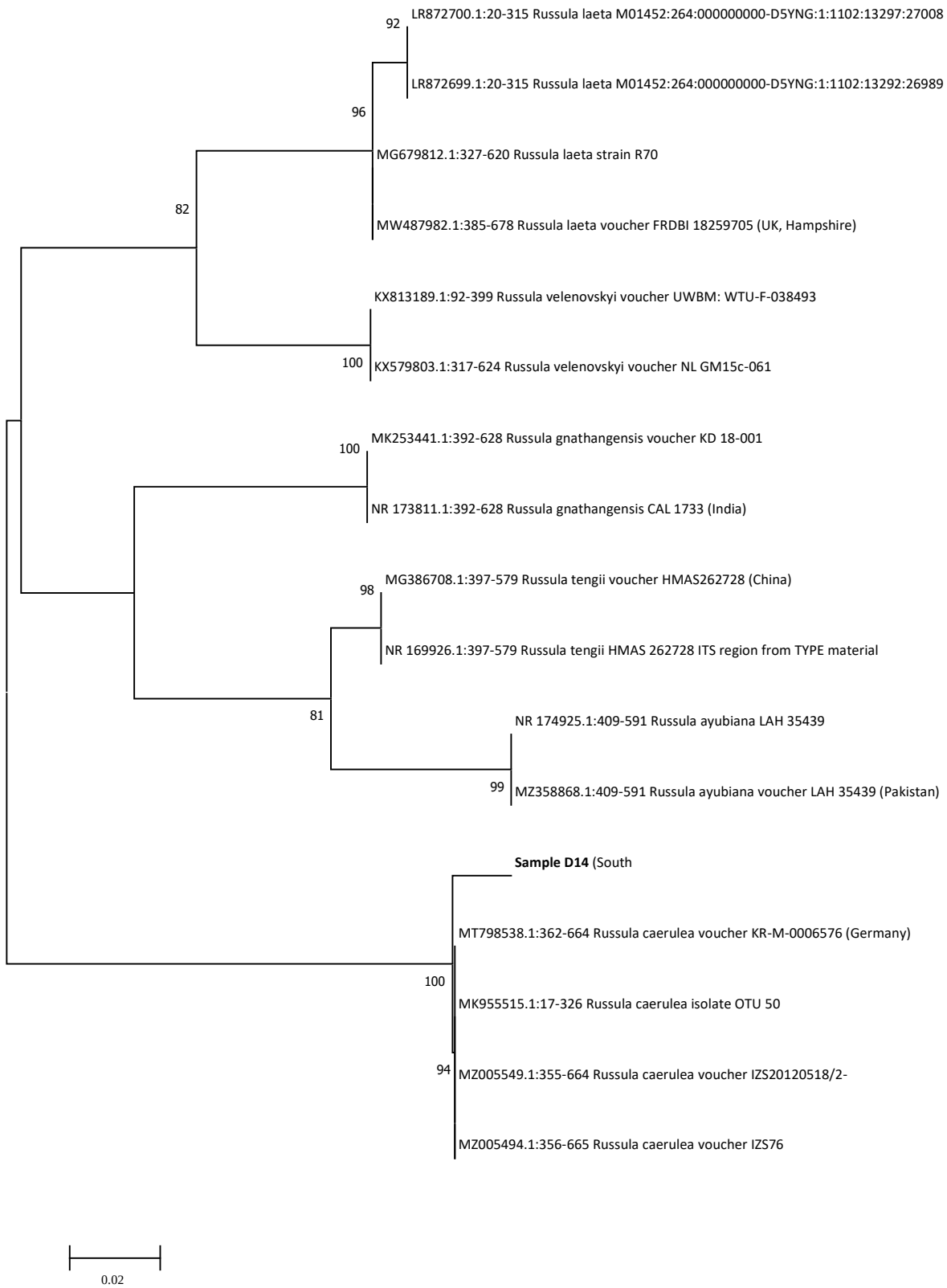



**Figure 4. 20** *Russula capensis* phylogram.

Unrooted phylogram of 17 internal transcriber region (ITS) sequence from 6 *Russula* species is based on Maximum Likelihood. The sequence labelled as Sample D14 in bold was gathered in this study. Values seen on the left of each group of sequences indicate bootstrap supporting percentages (≥80 values are shown).

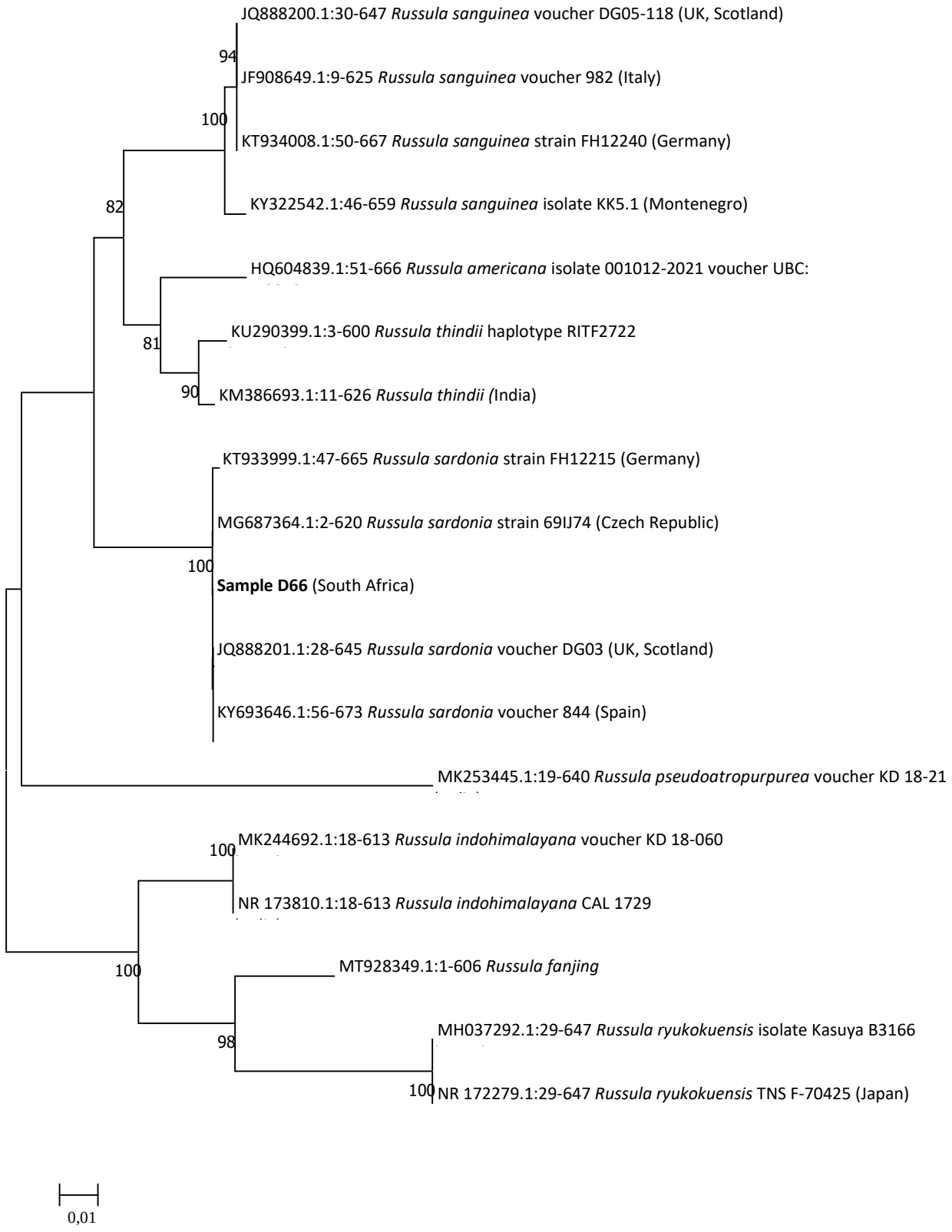



**Figure 4. 21** *Russula sordonia* phylogram.

FUnrooted phylogram of 18 internal transcriber region (ITS) sequence from 8 *Russula* species is based on Maximum Likelihood. The sequence labelled as Sample D66 in bold was collected in this study. Values observed on the left of each group of sequences represent bootstrap supporting percentages (≥80 values are shown).

# CHAPTER 5 – CONCLUSION

Previous studies documenting macro fungi from plantations within South Africa have reported various species that where probably introduced with their exotic host, in many cases *Pinus* and *Eucalyptus* spp. (Goldman & Gryzenhout 2019). These species included plant pathogens that cause white rot and mushrooms that form intricate beneficial relationships with hosts such as mycorrhiza (Dames et al., 1999; De Koker et al., 2000; Hawley et al., 2008; Hawley & Dames, 2004; Musvuugwa, 2014; Tchoumi et al., 2020). The biodiversity of macro fungi has not been well documented and remains largely unstudied in South Africa, not only based on morphological documentation but especially based on molecular identification of recent DNA sequence based phylogenies. In fact, a limited amount of these macro fungi within the country have been sequenced, regardless of their prominence in the environment.

The Tsitsikamma region is well known for its indigenous forests but also has cultivated pine plantations. In fact, these timber focussed *Pinus* plantations developed from the first ever established estates in 1883 near Knysna, South Africa (Van Der Zel & Brink, 1980). The area is a noteworthy environmental niche with a diverse array of invasive plant growth and important native biomes to the country, and various landscape types (Baard & Kraaij, 2014; Kraaij, Cowling, & Van Wilgen, 2013). Comprehending that various fungal species behave in a host specific way (Chen et al., 2018), it is assumed to find a copious amount of mushrooms preferring coniferous and hardwood plantation trees. Identities verified with up to date DNA sequence data, will be significant for conservation efforts in the area protecting the indigenous vegetation with their own cohort of associated fungi, and management of the pine plantation areas, since most of these mushrooms form mycorrhizal relationships with host trees.

The aims of this study was to collect macro fungi from the non-native pine plantations in the Tsitsikamma region, and to identify them by means of morphological observations and DNA sequencing. The data that was generated will be used to identify areas to focus on more in future, and will be useful to build future documentation true species identities of mushrooms associated with these alien plants. It will aid future studies to determine if macro fungi from these alien plants can grow in pristine native areas.

Chapters 1 and 2 summarised challenges to identify macro fungi, which represents a Kingdom of their own that is hyper-diverse in form and function. Large numbers of macro fungi still need to be characterized, despite a wide array of uses for humans and mushrooms being integral parts of any ecological system. The degree to which macro fungi have been studied in South Africa have been summarised, especially focusing on the Tsitsikamma region, which forms the focus area of this study. The unique landscape and importance of the region was also summarised.

As part of the collection and morphological identification of the collected samples, a system was developed to aid field and morphological observations. In addition to available field guides and relevant morphological identification found in literature, the compiled overall morphological characterise table for macro fungal species identification aimed to be a more user friendly, compacted a quantifiable way of documenting key observational characteristics. This information can then be used alongside pervious documented information for species identification. The simple but impactful and informative table will be easily understood by uninformed users, enthusiastic civilian scientist and experts alike. It provides the means of infield compact documentation each aspect of morphology as objectively as possible.

Results from this study revealed a diversity of mushrooms from a relatively small number of samples, mostly ectomycorrhizal beneficial to the pines. Various species of the important ectomycorrhizal genera *Amanita, Russula* and *Lactarius* was found. Moreover, in each of these genera, which include well known species from pines from across South Africa, one first report for South Africa was found, respectively. Of these, *Amanita morrisii* looked morphologically similar to *Amanita phalloides* and *Amanita pantherina*, but this study represents the first report for this vulnerable species outside of North America. The first reports of *Russula capensis* and *Lactarius quieticolor* was also unexpected because they resembled other species known from South Africa closely. For the remainder of species from these genera, the study confirmed their identities with DNA sequence comparisons.

Among the single species reports, noteworthy finds included the coprophilous *Panaeolus antillarum*, also a first official report for South Africa. Other species included edible and medicinal species such as *Imleria badia*, and the enticing and conspicuous *Gymnopilus junonius*. Again, confirmation of their identities based on DNA sequence comparisons are

incredibly useful since it confirmed the identities of these mushrooms. However, one specimen appeared to represent novel *Chlorophyllum*, to be described in future.

*R. capensis* was first described from South Africa and is not known from any other area of the world. It would thus appear that this species could be native, but it is not known from any native plant from South Africa. Our results showed that a specimen of *R. capensis* grouped with sequences represent *Russula caerulea*, which is known mostly from Europe. It will be necessary in future to use additional gene regions to determine if this species truly is the same species as *R. caerulea*, since ITS is known to not always have enough differences to differentiate between species. Microscopic comparisons will also be needed to compare specimens representing these two species.

Correct identification is vital, for example due to the high level of morphological plasticity expressed between various species within a single genus e.g. *Amanita rubescens*, *A. pantherina* and *A. morrisii.* Wrongful identification has led to confusion between species members. The misidentification of species can lead to the regretful consumption of poisonous mushrooms that may lead to unpleasant symptoms such as nausea, gastro-intestinal distress and vomiting as well as even deadly poisonous fungal species that can lead to unconsciousness, organ failure and even death when left untreated. For long morphological identification alone has been sufficient to distinguish between various fungal species, but clearly molecular techniques have greatly aided the correct identification of various mushrooms to species level. The combination of both morphological and molecular identification should be considered the ideal for true identification and characterisation of specimens, and will greatly aid in the discovery of more first reports and novel species for South Africa.

The lack of formalized documentation of macrofungal species and current information refinement on various source platforms for South Africa contribute to the confusion regarding identification of macro fungi within the country. Accurate identification of morphological similar fungal species is needed for the further reliable exploration of fungal specimens for their underlying scientific, economic, pharmaceutical applications and edibility status (Panda & Tayung, 2016). Single names linked to a single species without dispute, following rules and standards set out in the IUCN is essential (Schoch et al., 2014). Lastly, the information from

this study will contribute to future studies of the mushrooms present in, and beneficial to, plantations, and serves as a valuable comparison to studies in native areas.

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# APPENDICES

## APPENDIX 1 – PROJECT APPROVAL

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
NATURAL AND AGRICULTURAL SCIENCES
NATUUR- EN LANDBOUWETENSKAPPE

**DECISION LETTER: DEPARTMENT OF GENETICS RESEARCH COMMITTEE**

To: **M Herselman**
Department of Genetics, University of the Free State.

From: The Chairperson: Department of Genetics Research Committee.
Date: 23/06/2021

**Project Number**: Res 26/2021
**Project Title:** Checklist of macrofungi in the Tsitsikamma area, South Africa.

This is to confirm that your project has been considered by the Departmental Research Committee. The decision is as follows:

| | | |
|---|---|---|
| **a** | The full proposal have been approved without modification. | √ |
| **b** | A resubmission of the full application is required for approval. The required conditions for approval is attached in a separate document | |
| **c** | The application is rejected, based on the reasons outlined in the attached list. | |

**Note**: It is the responsibility of the Principle Investigator to notify the Department of Genetics Research Committee if the project or title changes along with the date of the Faculty meeting where the title change has been approved. Documentation relevant to the study such as permits and ethics approvals should be submitted to the Department of Genetics Research Committee, within a month after it has been obtained.

Yours Sincerely,

R. Rebello

_____

R Rebello: The Chairperson: Department of Genetics Research Committee

_____

JP Grobler: HOD Department of Genetics

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# APPENDIX 2 – COMPLETED INFIELD MORPHOLOGY TABLES (Highlighted in BOLD)

## *2.1 AMANITA MORRISII*

| | | |
|---|---|---|
| Specimen Number: | D5 | |
| Species Identification: | *Amanita morrisii* | |
| Section 1: Cap and Hymenium | Cap: Structure supported on the stipe or stalk. | Colour: **Dark brown to dark grey** |
| | | Shape: (Bell-Shaped/**Broadly Convex**/Broadly Umbonate – Wavy /Conical/**Convex**/Deeply Depressed/**Depressed** - Central part of the Cap sunken /Egg-Shaped/Funnel Shaped/Globose – spherical or globe-shaped /Hemispherical/Plane/Flat/Umbonate) |
| | | Surface Texture: (Dry/Hairy/Scaly/**Smooth**/Sticky) |
| | | Structures: (Scales/**Warts** – remnants of the universal veil)-**White to grey** |
| | Hymenium (**Gills**/Tubes): The layer of fertile cells that produce the spores. | Colour: **White** |
| | | Margins: The connective area of the cap and gills - **Non-striated** |
| | | Surface: (Appendiculate/Crenate – edged with rounded teeth/Crisped – finely wavy/**Entire – smooth and more even** /Lobate – lobate /Undulate – Wavy) |
| | | Sectional: (Acute/**Decurved**/Incurved – curved or rolled inward toward the stipe (margin) /Inrolled – rolled up on the side next to the stipe, when young (margin) /Obtuse/**Plane – flat** /Upturned) |
| | | Type Attachment: (**Free**/Adnexed/**Adnate**/Notched/**Notched Decurrant Tooth**/Seceding/Decurrent – Down Stipe/Depressed) |
| | | Length: (**Close** – spaced close together, between crowded and distant/Crowded – arranged extremely close together – full appearance/Distant – spaced far apart/Fanned/**Full and Intermediated**/Forked) |
| Section 2: Stipe and Structures | Stipe: Stem or stalk. | Colour: **White to pale grey** |
| | | Shape: (Bulbous base/Club-shaped/**Cylindrical**/Rooting base/**Tapering Down**/Tapering towards base/Tapering Upward) |
| | | Type: (**Central** – stipe attached at the centre of the cap /Excentric – stipe not centrally attached to the cap /Lateral – at the side) |
| | Structures | Ring: A band of tissue encircling the stem |
| | | (Absent/**Present**) |
| | | Position on the stipe: (**Top**/Middle/Bottom) |
| | | Volva: Cup – like structure remains of the universal veil around the base of the stipe - **irregular grey to brown patches** |
| | | (**Absent**/**Present**) - sample dependant |
| Section 3: Habitat/Substarte and Additional Information | Habitat/Substarte: The surface or material, such as soil or bark, to which the fungus is attached or on which it grows. | **In soil**/On tree/Other: |
| | | Soil: (Sandy/Muddy/Manure/Grass/**Forest**) |
| | | Tree: (Dead/Fallen/Decaying/Alive) |
| | | Surrounding Environment: (**Forest**/Pasture/Grass/Other)-**Pine** |
| | Bruising/Bleeding/Staining | Colour: |
| | | Action: (Touching/Cutting/Damaged) |
| | Odour/Smell | Similar: |
| Similar species: | *Amanita pantherina* | |

## 2.2 AMANITA MUSCARIA

| | | |
|---|---|---|
| Specimen Number: | D18 | |
| Species Identification: | *Amanita muscaria* | |
| Section 1: Cap and Hymenium | Cap: Structure supported on the stipe or stalk. | Colour: **Dark red** |
| | | Shape: (Bell-Shaped/Broadly Convex/Broadly Umbonate – Wavy /Conical/Convex/Deeply Depressed/Depressed - Central part of the Cap sunken /Egg-Shaped/Funnel Shaped/Globose – spherical or globe-shaped /**Hemispherical**/Plane/**Flat**/Umbonate) |
| | | Surface Texture: (Dry/Hairy/Scaly/**Smooth**/**Sticky**) |
| | | Structures: (Scales/**Warts** – remnants of the universal veil)-**White** |
| | Hymenium (**Gills**/Tubes): The layer of fertile cells that produce the spores. | Colour: **White** |
| | | Margins: The connective area of the cap and gills - **Striated** |
| | | Surface: (Appendiculate/Crenate – edged with rounded teeth/Crisped – finely wavy/**Entire – smooth and more even** /Lobate – lobate /Undulate – Wavy) |
| | | Sectional: (Acute/**Decurved**/Incurved – curved or rolled inward toward the stipe (margin) /Inrolled – rolled up on the side next to the stipe, when young (margin) /Obtuse/Plane – flat /Upturned) |
| | | Type Attachment: (**Free**/Adnexed/Adnate/Notched/Notched Decurrant Tooth/Seceding/Decurrent – Down Stipe/Depressed) |
| | | Length: (**Close** – spaced close together, between crowded and distant/**Crowded** – arranged extremely close together – full appearance/Distant – spaced far appart/Fanned/**Full and Intermediated**/Forked) |
| Section 2: Stipe and Structures | Stipe: Stem or stalk. | Colour: **White** |
| | | Shape: (**Bulbous base**/Club-shaped/**Cylindrical**/Rooting base/Tapering Down/**Tapering towards base**/Tapering Upward) |
| | | Type: (**Central** – stipe attached at the centre of the cap /Excentric – stipe not centrally attached to the cap /Lateral – at the side) |
| | Structures | Ring: A band of tissue encircling the stem - **membraneous and skirt-like** |
| | | (Absent/**Present**) |
| | | Position on the stipe: (**Top**/Middle/Bottom) |
| | | Volva: Cup – like structure remains of the universal veil around the base of the stipe |
| | | **(Absent**/Present) |
| Section 3: Habitat/Substarte and Additional Information | Habitat/Substarte: The surface or material, such as soil or bark, to which the fungus is attached or on which it grows. | **In soil**/On tree/Other: |
| | | Soil: (Sandy/Muddy/Manure/Grass/**Forest**) |
| | | Tree: (Dead/Fallen/Decaying/Alive) |
| | | Surrounding Environment: (**Forest**/Pasture/Grass/Other)-**Pine and Oak** |
| | Bruising/Bleeding/Staining | Colour: |
| | | Action: (Touching/Cutting/Damaged) |
| | Odour/Smell | Similar: |
| Similar species: | *Amanita caeasarea* | |

## 2.3 AMANITA PANTHERINA

| | | |
|---|---|---|
| Specimen Number: | D61 | |
| Species Identification: | *Amanita pantherina* | |
| Section 1: Cap and Hymenium | Cap: Structure supported on the stipe or stalk. | Colour: **Pale greyish brown** |
| | | Shape: (Bell-Shaped/Broadly Convex/Broadly Umbonate – Wavy /Conical/Convex/Deeply Depressed/Depressed - Central part of the Cap sunken /Egg-Shaped/Funnel Shaped/Globose – spherical or globe-shaped /**Hemispherical**/Plane/**Flat**/Umbonate) |
| | | Surface Texture: (Dry/Hairy/Scaly/**Smooth**/Sticky) |
| | | Structures: (Scales/**Warts** – remnants of the universal veil)-**White** |
| | Hymenium (**Gills**/Tubes): The layer of fertile cells that produce the spores. | Colour: **White** |
| | | Margins: The connective area of the cap and gills - **Striated** |
| | | Surface: (Appendiculate/Crenate – edged with rounded teeth/Crisped – finely wavy/**Entire – smooth and more even** /Lobate – lobate /Undulate – Wavy) |
| | | Sectional: (Acute/Decurved/Incurved – curved or rolled inward toward the stipe (margin) /Inrolled – rolled up on the side next to the stipe, when young (margin) /Obtuse/Plane – flat /**Upturned**) |
| | | Type Attachment: (**Free**/Adnexed/Adnate/Notched/Notched Decurrant Tooth/Seceding/Decurrent – Down Stipe/Depressed) |
| | | Length: (Close – spaced close together, between crowded and distant/**Crowded** – arranged extremely close together – full appearance/Distant – spaced far apart/Fanned/Full and Intermediated/Forked) |
| Section 2: Stipe and Structures | Stipe: Stem or stalk. | Colour: **White** |
| | | Shape: (**Bulbous base**/Club-shaped/Cylindrical/Rooting base/Tapering Down/**Tapering towards base**/Tapering Upward) |
| | | Type: (**Central** – stipe attached at the centre of the cap /Excentric – stipe not centrally attached to the cap /Lateral – at the side) |
| | Structures | Ring: A band of tissue encircling the stem - **membraneous, white** |
| | | (Absent/**Present**) |
| | | Position on the stipe: (**Top**/Middle/Bottom) |
| | | Volva: Cup – like structure remains of the universal veil around the base of the stipe - **concentric warty ring around base** |
| | | (Absent/**Present**) |
| Section 3: Habitat/Substarte and Additional Information | Habitat/Substarte: The surface or material, such as soil or bark, to which the fungus is attached or on which it grows. | **In soil**/On tree/Other: |
| | | Soil: (Sandy/Muddy/Manure/Grass/**Forest**) |
| | | Tree: (Dead/Fallen/Decaying/Alive) |
| | | Surrounding Environment: (**Forest**/Pasture/Grass/Other)-**Pine** |
| | Bruising/Bleeding/Staining | Colour: |
| | | Action: (Touching/Cutting/Damaged) |
| | Odour/Smell | Similar: |
| Similar species: | *Amanita excelsa*, *Amanita rubescens* | |

## 2.4 AMANITA RUBESCENS

| | | |
|---|---|---|
| Specimen Number: | D32 | |
| Species Identification: | *Amanita rubescens* | |
| Section 1: Cap and Hymenium | Cap: Structure supported on the stipe or stalk. | Colour: **Red-brown to rose brown** |
| | | Shape: (Bell-Shaped/Broadly Convex/Broadly Umbonate – Wavy /Conical/Convex/Deeply Depressed/Depressed - Central part of the Cap sunken /Egg-Shaped/Funnel Shaped/Globose – spherical or globe-shaped /**Hemispherical**/Plane/**Flat**/Umbonate) |
| | | Surface Texture: (Dry/Hairy/Scaly/**Smooth**/Sticky) |
| | | Structures: (Scales/**Warts** – remnants of the universal veil)-**White** |
| | Hymenium (**Gills**/Tubes): The layer of fertile cells that produce the spores. | Colour: **White** |
| | | Margins: The connective area of the cap and gills - **Faintly striated** |
| | | Surface: (Appendiculate/Crenate – edged with rounded teeth/Crisped – finely wavy/**Entire – smooth and more even** /Lobate – lobate /Undulate – Wavy) |
| | | Sectional: (Acute/**Decurved**/Incurved – curved or rolled inward toward the stipe (margin) /Inrolled – rolled up on the side next to the stipe, when young (margin) /Obtuse/Plane – flat /Upturned) |
| | | Type Attachment: (**Free**/Adnexed/Adnate/Notched/Notched Decurrant Tooth/Seceding/Decurrent – Down Stipe/Depressed) |
| | | Length: (Close – spaced close together, between crowded and distant/**Crowded** – arranged extremely close together – full appearance/Distant – spaced far appart/Fanned/**Full and Intermediated**/Forked) |
| Section 2: Stipe and Structures | Stipe: Stem or stalk. | Colour: **White** |
| | | Shape: (**Bulbous base**/Club-shaped/Cylindrical/Rooting base/Tapering Down/Tapering towards base/Tapering Upward) |
| | | Type: (**Central** – stipe attached at the centre of the cap /Excentric – stipe not centrally attached to the cap /Lateral – at the side) |
| | Structures | Ring: A band of tissue encircling the stem - **membraneous, white but stains red** |
| | | (Absent/**Present**) |
| | | Position on the stipe: (**Top**/Middle/Bottom) |
| | | Volva: Cup – like structure remains of the universal veil around the base of the stipe - **concentric warty ring** |
| | | (Absent/**Present**) |
| Section 3: Habitat/Substarte and Additional Information | Habitat/Substarte: The surface or material, such as soil or bark, to which the fungus is attached or on which it grows. | **In soil**/On tree/Other: |
| | | Soil: (Sandy/Muddy/Manure/Grass/**Forest**) |
| | | Tree: (Dead/Fallen/Decaying/Alive) |
| | | Surrounding Environment: (**Forest**/Pasture/Grass/Other)-**Pine** |
| | **Bruising**/Bleeding/Staining | Colour: **Blush red** |
| | | Action: (**Touching**/**Cutting**/**Damaged**) |
| | Odour/Smell | Similar: |
| Similar species: | *Amanita excels*, *Amanita pantherina* | |

## 2.5 *CHLOROPHYLLUM* SP.

| | | |
|---|---|---|
| Specimen Number: | D2 | |
| Species Identification: | *Chlorophyllum sp.* | |
| Section 1: Cap and Hymenium | Cap: Structure supported on the stipe or stalk. | Colour: **White** |
| | | Shape: (Bell-Shaped/Broadly Convex/Broadly Umbonate – Wavy /Conical/**Convex**/Deeply Depressed/**Depressed** - Central part of the Cap sunken /Egg-Shaped/Funnel Shaped/**Globose** – spherical or globe-shaped /**Hemispherical**/Plane/Flat/Umbonate) |
| | | Surface Texture: (Dry/Hairy/**Scaly**/Smooth/Sticky) |
| | | Structures: (**Scales**/Warts – remnants of the universal veil) - **amber-brown** |
| | Hymenium (**Gills**/Tubes): The layer of fertile cells that produce the spores. | Colour: **Greyish to olive green** |
| | | Margins: The connective area of the cap and gills - **Striated** |
| | | Surface: (Appendiculate/Crenate – edged with rounded teeth/Crisped – finely wavy/**Entire – smooth and more even** /Lobate – lobate /Undulate – Wavy) |
| | | Sectional: (Acute/Decurved/**Incurved** – curved or rolled inward toward the stipe (margin) /Inrolled – rolled up on the side next to the stipe, when young (margin) /Obtuse/Plane – flat /Upturned) |
| | | Type Attachment: (**Free**/Adnexed/Adnate/Notched/Notched Decurrant Tooth/Seceding/Decurrent – Down Stipe/Depressed) |
| | | Length: (Close – spaced close together, between crowded and distant/**Crowded** – arranged extremely close together – full appearance/Distant – spaced far appart/Fanned/**Full and Intermediated**/Forked) |
| Section 2: Stipe and Structures | Stipe: Stem or stalk. | Colour: Concolorous |
| | | Shape: (Bulbous base/Club-shaped/Cylindrical/Rooting base/Tapering Down/Tapering towards base/**Tapering Upward**) - smooth |
| | | Type: (**Central** – stipe attached at the centre of the cap /Excentric – stipe not centrally attached to the cap /Lateral – at the side) |
| | Structures | Ring: A band of tissue encircling the stem |
| | | (Absent/**Present**) |
| | | Position on the stipe: (**Top**/**Middle**/Bottom) |
| | | Volva: Cup – like structure remains of the universal veil around the base of the stipe |
| | | (**Absent**/Present) |
| Section 3: Habitat/Substarte and Additional Information | Habitat/Substarte: The surface or material, such as soil or bark, to which the fungus is attached or on which it grows. | In soil/On tree/Other: **Lawns** |
| | | Soil: (Sandy/Muddy/Manure/**Grass**/Forest) |
| | | Tree: (Dead/Fallen/Decaying/Alive) |
| | | Surrounding Environment: (Forest/**Pasture**/Grass/Other) |
| | Bruising/Bleeding/Staining | Colour: |
| | | Action: (Touching/Cutting/Damaged) |
| | Odour/Smell | Similar: |
| Similar species: | *Chlorophyllum molybdites* | |

53 *2.6 CLITOPILUS PRUNULUS*

54

| | | |
|---|---|---|
| Specimen Number: | D19 | |
| Species Identification: | *Clitopilus prunulus* | |
| Section 1: Cap and Hymenium | Cap: Structure supported on the stipe or stalk. | Colour: **White to grey-brown** |
| | | Shape: (Bell-Shaped/Broadly Convex/Broadly Umbonate – Wavy /Conical/**Convex**/Deeply Depressed/Depressed - Central part of the Cap sunken /Egg-Shaped/Funnel Shaped/Globose – spherical or globe-shaped /Hemispherical/Plane/Flat/Umbonate) |
| | | Surface Texture: (Dry/Hairy/Scaly/**Smooth**/Sticky) - **velvety** |
| | | Structures: (Scales/Warts – remnants of the universal veil) |
| | Hymenium (**Gills**/Tubes): The layer of fertile cells that produce the spores. | Colour: **White to light pink** |
| | | Margins: The connective area of the cap and gills |
| | | Surface: (Appendiculate/Crenate – edged with rounded teeth/Crisped – finely wavy/Entire – smooth and more even /Lobate – lobate /**Undulate – Wavy**) |
| | | Sectional: (Acute/Decurved/**Incurved** – curved or rolled inward toward the stipe (margin) /Inrolled – rolled up on the side next to the stipe, when young (margin) /Obtuse/Plane – flat /Upturned) |
| | | Type Attachment: (Free/Adnexed/Adnate/Notched/Notched Decurrant Tooth/Seceding/**Decurrent** – Down Stipe/Depressed) |
| | | Length: (Close – spaced close together, between crowded and distant/**Crowded** – arranged extremely close together – full appearance/Distant – spaced far appart/Fanned/Full and Intermediated/Forked) |
| Section 2: Stipe and Structures | Stipe: Stem or stalk. | Colour: **Concolorous** |
| | | Shape: (Bulbous base/Club-shaped/**Cylindrical**/Rooting base/Tapering Down/Tapering towards base/Tapering Upward) - smooth |
| | | Type: (**Central** – stipe attached at the centre of the cap /Excentric – stipe not centrally attached to the cap /Lateral – at the side) |
| | Structures | Ring: A band of tissue encircling the stem |
| | | (**Absent**/Present) |
| | | Position on the stipe: (Top/Middle/Bottom) |
| | | Volva: Cup – like structure remains of the universal veil around the base of the stipe |
| | | (**Absent**/Present) |
| Section 3: Habitat/Substarte and Additional Information | Habitat/Substarte: The surface or material, such as soil or bark, to which the fungus is attached or on which it grows. | In soil/On tree/Other: Lawns |
| | | Soil: (Sandy/**Muddy**/Manure/Grass/Forest) |
| | | Tree: (Dead/Fallen/Decaying/Alive) |
| | | Surrounding Environment: (**Forest**/Pasture/Grass/Other) |
| | Bruising/Bleeding/Staining | Colour: |
| | | Action: (Touching/Cutting/Damaged) |
| | Odour/Smell | Similar: |
| Similar species: | | |

## 2.7 GYMNOPILUS JUNONIUS

| | | |
|---|---|---|
| Specimen Number: | D8 | |
| Species Identification: | *Gymnopilus junonius* | |
| Section 1: Cap and Hymenium | Cap: Structure supported on the stipe or stalk. | Colour: **Orange** |
| | | Shape: (Bell-Shaped/Broadly Convex/Broadly Umbonate – Wavy /Conical/**Convex**/Deeply Depressed/Depressed - Central part of the Cap sunken /Egg-Shaped/Funnel Shaped/Globose – spherical or globe-shaped /Hemispherical/Plane/Flat/**Umbonate**) |
| | | Surface Texture: (**Dry**/Hairy/Scaly/**Smooth**/Sticky) |
| | | Structures: (Scales/Warts – remnants of the universal veil) |
| | Hymenium (**Gills**/Tubes): The layer of fertile cells that produce the spores. | Colour: **Yellow to rust brown** |
| | | Margins: The connective area of the cap and gills |
| | | Surface: (Appendiculate/Crenate – edged with rounded teeth/Crisped – finely wavy/**Entire – smooth and more even** /Lobate – lobate /Undulate – Wavy) |
| | | Sectional: (Acute/Decurved/Incurved – curved or rolled inward toward the stipe (margin) **/Inrolled** – rolled up on the side next to the stipe, when young (margin) /Obtuse/Plane – flat /Upturned) |
| | | Type Attachment: (Free/**Adnexed**/Adnate/Notched/Notched Decurrant Tooth/Seceding/Decurrent – Down Stipe/Depressed) |
| | | Length: (Close – spaced close together, between crowded and distant/**Crowded** – arranged extremely close together – full appearance/Distant – spaced far appart/Fanned/Full and Intermediated/Forked) - small and rounded |
| Section 2: Stipe and Structures | Stipe: Stem or stalk. | Colour: **Twany yellow to orange** |
| | | Shape: (Bulbous base/Club-shaped/**Cylindrical**/Rooting base/Tapering Down/Tapering towards base/Tapering Upward) - **thick** |
| | | Type: (**Central** – stipe attached at the centre of the cap /Excentric – stipe not centrally attached to the cap /Lateral – at the side) |
| | Structures | Ring: A band of tissue encircling the stem |
| | | (**Absent**/Present) |
| | | Position on the stipe: (Top/Middle/Bottom) |
| | | Volva: Cup – like structure remains of the universal veil around the base of the stipe |
| | | (**Absent**/Present) |
| Section 3: Habitat/Substarte and Additional Information | Habitat/Substarte: The surface or material, such as soil or bark, to which the fungus is attached or on which it grows. | In soil/**On tree**/Other: |
| | | Soil: (Sandy/Muddy/Manure/Grass/Forest) |
| | | Tree: (Dead/**Fallen**/**Decaying**/Alive) - **Unhealthy** |
| | | Surrounding Environment: (**Forest**/Pasture/Grass/Other) |
| | **Bruising**/Bleeding/Staining | Colour: **Warm orange to red** |
| | | Action: (**Touching**/Cutting/Damaged) |
| | Odour/Smell | Similar: **Mild pleasant** |
| Similar species: | **Other Gymnopilus species** | |

## 2.8 IMLERIA BADIA

| | | |
|---|---|---|
| Specimen Number: | D8 | |
| Species Identification: | *Imleria badia* | |
| Section 1: Cap and Hymenium | Cap: Structure supported on the stipe or stalk. | Colour: **Brown to brick-red** |
| | | Shape: (Bell-Shaped/Broadly Convex/Broadly Umbonate – Wavy /Conical/**Convex**/Deeply Depressed/Depressed - Central part of the Cap sunken /Egg-Shaped/Funnel Shaped/Globose – spherical or globe-shaped /Hemispherical/Plane/Flat/Umbonate) |
| | | Surface Texture: (Dry/Hairy/Scaly/**Smooth**/Sticky) - **polished** |
| | | Structures: (Scales/Warts – remnants of the universal veil) |
| | Hymenium (Gills/**Tubes**): The layer of fertile cells that produce the spores. | Colour: **White to olivaceous** |
| | | Margins: The connective area of the cap and gills |
| | | Surface: (Appendiculate/Crenate – edged with rounded teeth/Crisped – finely wavy/**Entire – smooth and more even** /Lobate – lobate /Undulate – Wavy) |
| | | Sectional: (Acute/Decurved/**Incurved** – curved or rolled inward toward the stipe (margin) /Inrolled – rolled up on the side next to the stipe, when young (margin) /Obtuse/Plane – flat /Upturned) |
| | | Type Attachment: (Free/Adnexed/Adnate/Notched/Notched Decurrant Tooth/Seceding/Decurrent – Down Stipe/**Depressed**) |
| | | Length: (Close – spaced close together, between crowded and distant/Crowded – arranged extremely close together – full appearance/Distant – spaced far appart/Fanned/Full and Intermediated/Forked) - **small and rounded** |
| Section 2: Stipe and Structures | Stipe: Stem or stalk. | Colour: **Concolorous** |
| | | Shape: (Bulbous base/Club-shaped/**Cylindrical**/Rooting base/Tapering Down/Tapering towards base/Tapering Upward) - smooth |
| | | Type: (**Central** – stipe attached at the centre of the cap /Excentric – stipe not centrally attached to the cap /Lateral – at the side) |
| | Structures | Ring: A band of tissue encircling the stem |
| | | (**Absent**/Present) |
| | | Position on the stipe: (Top/Middle/Bottom) |
| | | Volva: Cup – like structure remains of the universal veil around the base of the stipe |
| | | (**Absent**/Present) |
| Section 3: Habitat/Substarte and Additional Information | Habitat/Substarte: The surface or material, such as soil or bark, to which the fungus is attached or on which it grows. | **In soil**/On tree/Other: |
| | | Soil: (Sandy/Muddy/Manure/Grass/**Forest**) |
| | | Tree: (Dead/Fallen/Decaying/Alive) |
| | | Surrounding Environment: (**Forest**/Pasture/Grass/Other) |
| | **Bruising**/Bleeding/Staining | Colour: Blue |
| | | Action: (**Touching**/Cutting/**Damaged**) |
| | Odour/Smell | Similar: **Mild mushroom** |
| Similar species: | Other bolete species | |

## 2.9 LACTARIUS QUITECOLOR

| | | |
|---|---|---|
| Specimen Number: | D10 | |
| Species Identification: | *Lactarius quieticolor* | |
| Section 1: Cap and Hymenium | Cap: Structure supported on the stipe or stalk. | Colour: **Orange to warm red, tan to green concentric zones** |
| | | Shape: (Bell-Shaped/Broadly Convex/Broadly Umbonate – Wavy /Conical/Convex/Deeply Depressed/**Depressed** - Central part of the Cap sunken /Egg-Shaped/**Funnel Shaped**/Globose – spherical or globe-shaped /Hemispherical/Plane/Flat/Umbonate) |
| | | Surface Texture: (Dry/Hairy/Scaly/**Smooth**/**Sticky**) |
| | | Structures: (Scales/Warts – remnants of the universal veil) - **adhering pine needles** |
| | Hymenium (**Gills**/Tubes): The layer of fertile cells that produce the spores. | Colour: **Orange to red** |
| | | Margins: The connective area of the cap and gills |
| | | Surface: (Appendiculate/Crenate – edged with rounded teeth/Crisped – finely wavy/**Entire – smooth and more even** /Lobate – lobate /Undulate – Wavy) |
| | | Sectional: (Acute/Decurved/Incurved – curved or rolled inward toward the stipe (margin) /**Inrolled** – rolled up on the side next to the stipe, when young (margin) /Obtuse/Plane – flat /Upturned) |
| | | Type Attachment: (Free/Adnexed/Adnate/Notched/Notched Decurrant Tooth/Seceding/**Decurrent** – Down Stipe/Depressed) |
| | | Length: (Close – spaced close together, between crowded and distant/**Crowded** – arranged extremely close together – full appearance/Distant – spaced far appart/Fanned/**Full and Intermediated**/Forked) |
| Section 2: Stipe and Structures | Stipe: Stem or stalk. | Colour: **Concolorous** |
| | | Shape: (Bulbous base/Club-shaped/**Cylindrical**/Rooting base/Tapering Down/Tapering towards base/Tapering Upward) - **smooth** |
| | | Type: (**Central** – stipe attached at the centre of the cap /Excentric – stipe not centrally attached to the cap /Lateral – at the side) |
| | Structures | Ring: A band of tissue encircling the stem |
| | | (**Absent**/Present) |
| | | Position on the stipe: (Top/Middle/Bottom) |
| | | Volva: Cup – like structure remains of the universal veil around the base of the stipe |
| | | (**Absent**/Present) |
| Section 3: Habitat/Substarte and Additional Information | Habitat/Substarte: The surface or material, such as soil or bark, to which the fungus is attached or on which it grows. | **In soil**/On tree/Other: |
| | | Soil: (**Sandy**/Muddy/Manure/Grass/**Forest**) |
| | | Tree: (Dead/Fallen/Decaying/Alive) |
| | | Surrounding Environment: (**Forest**/Pasture/Grass/Other)-**Pine** |
| | Bruising/**Bleeding**/**Staining** | Colour: **Dark orange to red** |
| | | Action: (Touching/**Cutting**/**Damaged**) |
| | Odour/Smell | Similar: |
| Similar species: | *Lactarius deliciosus* | |

64 *2.10 PANAEOLUS ANTILLARIUM*

65

| | | |
|---|---|---|
| Specimen Number: | D17 | |
| Species Identification: | *Panaeolus antillarum* | |
| Section 1: Cap and Hymenium | Cap: Structure supported on the stipe or stalk. | Colour: **Pale to light brown** |
| | | Shape: (Bell-Shaped/Broadly Convex/Broadly Umbonate – Wavy /**Conical**/**Convex**/Deeply Depressed/Depressed - Central part of the Cap sunken /Egg-Shaped/Funnel Shaped/Globose – spherical or globe-shaped /Hemispherical/Plane/Flat/Umbonate) |
| | | Surface Texture: (Dry/Hairy/Scaly/**Smooth**/Sticky) |
| | | Structures: (Scales/Warts – remnants of the universal veil) |
| | Hymenium (**Gills**/Tubes): The layer of fertile cells that produce the spores. | Colour: **Greyish black** |
| | | Margins: The connective area of the cap and gills - **regular and nonstriated** |
| | | Surface: (Appendiculate/Crenate – edged with rounded teeth/Crisped – finely wavy/**Entire – smooth and more even** /Lobate – lobate /Undulate – Wavy) |
| | | Sectional: (Acute/**Decurved**/Incurved – curved or rolled inward toward the stipe (margin) /Inrolled – rolled up on the side next to the stipe, when young (margin) /Obtuse/Plane – flat /Upturned) |
| | | Type Attachment: (Free/**Adnexed**/**Adnate**/Notched/Notched Decurrant Tooth/Seceding/Decurrent – Down Stipe/Depressed) |
| | | Length: (Close – spaced close together, between crowded and distant/**Crowded** – arranged extremely close together – full appearance/Distant – spaced far appart/Fanned/**Full and Intermediated**/Forked) |
| Section 2: Stipe and Structures | Stipe: Stem or stalk. | Colour: **Light brown** |
| | | Shape: (Bulbous base/Club-shaped/**Cylindrical**/Rooting base/Tapering Down/Tapering towards base/Tapering Upward) - **long and slender** |
| | | Type: (**Central** – stipe attached at the centre of the cap /Excentric – stipe not centrally attached to the cap /Lateral – at the side) |
| | Structures | Ring: A band of tissue encircling the stem |
| | | (**Absent**/Present) |
| | | Position on the stipe: (Top/Middle/Bottom) |
| | | Volva: Cup – like structure remains of the universal veil around the base of the stipe |
| | | (**Absent**/Present) |
| Section 3: Habitat/Substarte and Additional Information | Habitat/Substarte: The surface or material, such as soil or bark, to which the fungus is attached or on which it grows. | **In soil**/On tree/Other: |
| | | Soil: (Sandy/Muddy/**Manure**/Grass/Forest) |
| | | Tree: (Dead/Fallen/Decaying/Alive) |
| | | Surrounding Environment: (Forest/**Pasture**/**Grass**/Other) |
| | Bruising/Bleeding/Staining | Colour: |
| | | Action: (Touching/Cutting/Damaged) |
| | Odour/Smell | Similar: |
| Similar species: | *Panaeolus subbalteatus, Panaeolina foenisecii* | |

## 2.11 RUSSULA CAERULEA

| | | |
|---|---|---|
| Specimen Number: | D6 | |
| Species Identification: | *Russula caerulea* | |
| Section 1: Cap and Hymenium | Cap: Structure supported on the stipe or stalk. | Colour: **Reddish purple** |
| | | Shape: (Bell-Shaped/Broadly Convex/Broadly Umbonate – Wavy /Conical/**Convex**/Deeply Depressed/Depressed - Central part of the Cap sunken /Egg-Shaped/Funnel Shaped/Globose – spherical or globe-shaped /Hemispherical/Plane/Flat/**Umbonate**) |
| | | Surface Texture: (Dry/Hairy/Scaly/**Smooth**/**Sticky**) |
| | | Structures: (Scales/Warts – remnants of the universal veil) - **adhering pine needles** |
| | Hymenium (**Gills**/Tubes): The layer of fertile cells that produce the spores. | Colour: **White to pale cream** |
| | | Margins: The connective area of the cap and gills - **protruding** |
| | | Surface: (Appendiculate/Crenate – edged with rounded teeth/Crisped – finely wavy/**Entire – smooth and more even** /Lobate – lobate /Undulate – Wavy) |
| | | Sectional: (Acute/Decurved/Incurved – curved or rolled inward toward the stipe (margin) /Inrolled – rolled up on the side next to the stipe, when young (margin) /Obtuse/Plane – flat /**Upturned**) |
| | | Type Attachment: (Free/**Adnexed**/Adnate/Notched/Notched Decurrant Tooth/Seceding/Decurrent – Down Stipe/Depressed) |
| | | Length: (Close – spaced close together, between crowded and distant/**Crowded** – arranged extremely close together – full appearance/Distant – spaced far appart/Fanned/Full and Intermediated/Forked) |
| Section 2: Stipe and Structures | Stipe: Stem or stalk. | Colour: **White** |
| | | Shape: (Bulbous base/Club-shaped/Cylindrical/Rooting base/Tapering Down/Tapering towards base/**Tapering Upward**) - **thick** |
| | | Type: (**Central** – stipe attached at the centre of the cap /Excentric – stipe not centrally attached to the cap /Lateral – at the side) |
| | Structures | Ring: A band of tissue encircling the stem |
| | | (**Absent**/Present) |
| | | Position on the stipe: (Top/Middle/Bottom) |
| | | Volva: Cup – like structure remains of the universal veil around the base of the stipe |
| | | (**Absent**/Present) |
| Section 3: Habitat/Substarte and Additional Information | Habitat/Substarte: The surface or material, such as soil or bark, to which the fungus is attached or on which it grows. | **In soil**/On tree/Other: |
| | | Soil: (Sandy/Muddy/Manure/Grass/**Forest**) |
| | | Tree: (Dead/Fallen/Decaying/Alive) |
| | | Surrounding Environment: (**Forest**/Pasture/Grass/Other)-**Pine** |
| | Bruising/Bleeding/Staining | Colour: |
| | | Action: (Touching/Cutting/Damaged) |
| | Odour/Smell | Similar: |
| Similar species: | *Russula capensis* | |

## 2.12 RUSSULA CAPENSIS

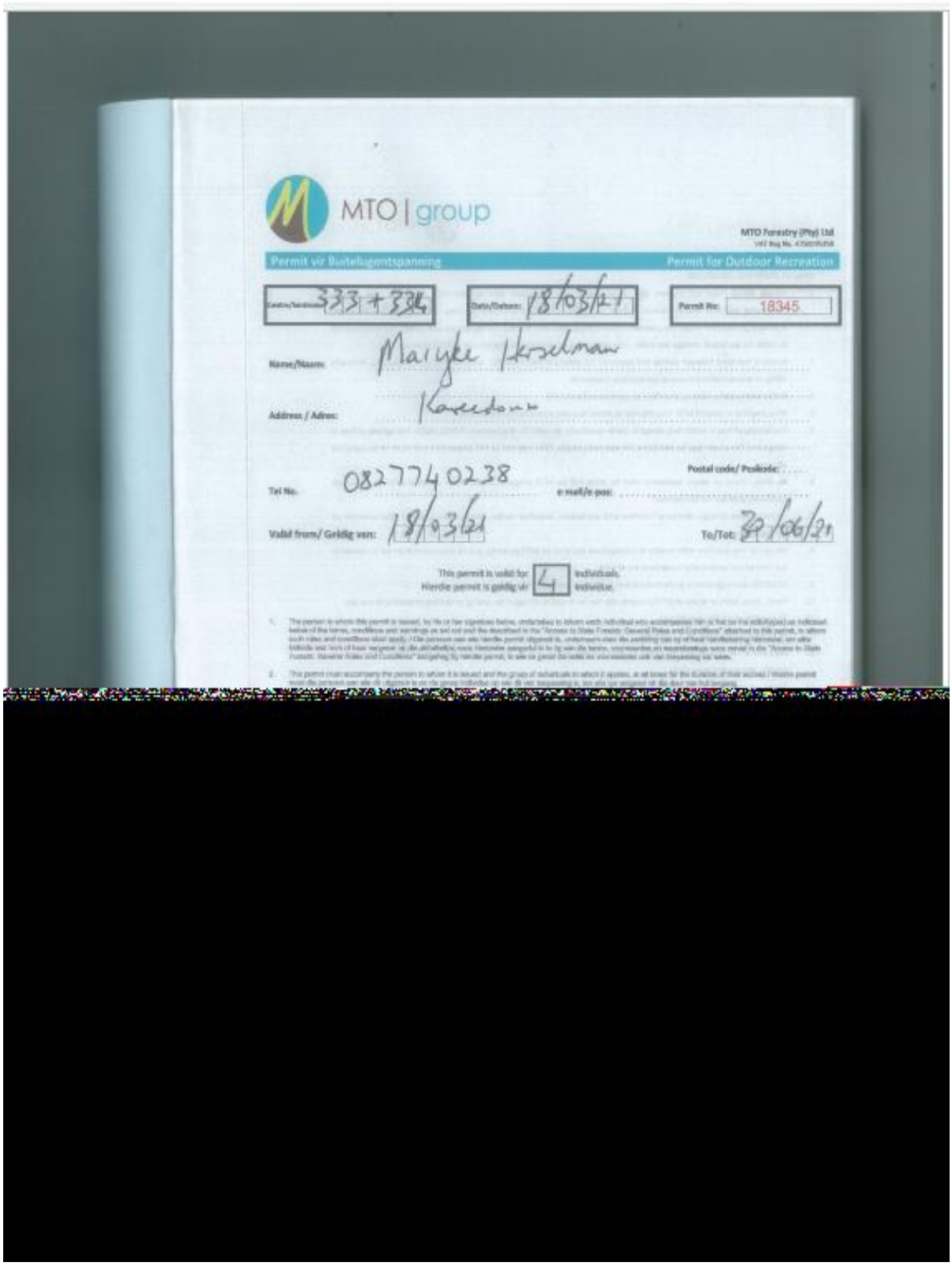
| | | |
|---|---|---|
| Specimen Number: | D14 | |
| Species Identification: | *Russula capensis* | |
| Section 1: Cap and Hymenium | Cap: Structure supported on the stipe or stalk. | Colour: **Reddish purple** |
| | | Shape: (Bell-Shaped/Broadly Convex/Broadly Umbonate – Wavy /Conical/**Convex**/Deeply Depressed/**Depressed** - Central part of the Cap sunken /Egg-Shaped/Funnel Shaped/Globose – spherical or globe-shaped /Hemispherical/Plane/Flat/Umbonate) |
| | | Surface Texture: (Dry/Hairy/Scaly/**Smooth**/**Sticky**) |
| | | Structures: (Scales/Warts – remnants of the universal veil) - **adhering pine needles** |
| | Hymenium (**Gills**/Tubes): The layer of fertile cells that produce the spores. | Colour: **Cream white to pale lemon yellow** |
| | | Margins: The connective area of the cap and gills - **protruding** |
| | | Surface: (Appendiculate/Crenate – edged with rounded teeth/Crisped – finely wavy/**Entire – smooth and more even** /Lobate – lobate /Undulate – Wavy) |
| | | Sectional: (Acute/**Decurved**/Incurved – curved or rolled inward toward the stipe (margin) /Inrolled – rolled up on the side next to the stipe, when young (margin) /Obtuse/**Plane – flat** /**Upturned**) |
| | | Type Attachment: (Free/**Adnexed**/Adnate/Notched/Notched Decurrant Tooth/Seceding/Decurrent – Down Stipe/Depressed) |
| | | Length: (Close – spaced close together, between crowded and distant/**Crowded** – arranged extremely close together – full appearance/Distant – spaced far appart/Fanned/Full and Intermediated/Forked) |
| Section 2: Stipe and Structures | Stipe: Stem or stalk. | Colour: **White** |
| | | Shape: (Bulbous base/Club-shaped/Cylindrical/Rooting base/Tapering Down/Tapering towards base/**Tapering Upward**) - **thick** |
| | | Type: (**Central** – stipe attached at the centre of the cap /Excentric – stipe not centrally attached to the cap /Lateral – at the side) |
| | Structures | Ring: A band of tissue encircling the stem |
| | | (**Absent**/Present) |
| | | Position on the stipe: (Top/Middle/Bottom) |
| | | Volva: Cup – like structure remains of the universal veil around the base of the stipe |
| | | (**Absent**/Present) |
| Section 3: Habitat/Substarte and Additional Information | Habitat/Substarte: The surface or material, such as soil or bark, to which the fungus is attached or on which it grows. | **In soil**/On tree/Other: |
| | | Soil: (Sandy/Muddy/Manure/Grass/**Forest**) |
| | | Tree: (Dead/Fallen/Decaying/Alive) |
| | | Surrounding Environment: (**Forest**/Pasture/Grass/Other)-**Pine** |
| | Bruising/Bleeding/Staining | Colour: |
| | | Action: (Touching/Cutting/Damaged) |
| | Odour/Smell | Similar: |
| Similar species: | *Russula caerulea* | |

## 2.13 *RUSSULA SARDONIA*

| | | |
|---|---|---|
| Specimen Number: | D66 | |
| Species Identification: | *Russula sardonia* | |
| Section 1: Cap and Hymenium | Cap: Structure supported on the stipe or stalk. | Colour: **Reddish pink** |
| | | Shape: (Bell-Shaped/Broadly **Convex**/Broadly Umbonate – Wavy /Conical/Convex/Deeply Depressed/Depressed - Central part of the Cap sunken /Egg-Shaped/Funnel Shaped/Globose – spherical or globe-shaped /Hemispherical/Plane/Flat/Umbonate) |
| | | Surface Texture: (Dry/Hairy/Scaly/**Smooth**/**Sticky**) |
| | | Structures: (Scales/Warts – remnants of the universal veil) |
| | Hymenium (**Gills**/Tubes): The layer of fertile cells that produce the spores. | Colour: **Cream white to pale lemon** |
| | | Margins: The connective area of the cap and gills - **protruding** |
| | | Surface: (Appendiculate/Crenate – edged with rounded teeth/Crisped – finely wavy/Entire – smooth and more even /Lobate – lobate /**Undulate – Wavy**) |
| | | Sectional: (Acute/Decurved/Incurved – curved or rolled inward toward the stipe (margin) /**Inrolled** – rolled up on the side next to the stipe, when young (margin) /Obtuse/Plane – flat /Upturned) |
| | | Type Attachment: (Free/**Adnexed**/Adnate/Notched/Notched Decurrant Tooth/Seceding/Decurrent – Down Stipe/Depressed) |
| | | Length: (Close – spaced close together, between crowded and distant/**Crowded** – arranged extremely close together – full appearance/Distant – spaced far appart/Fanned/Full and Intermediated/Forked) |
| Section 2: Stipe and Structures | Stipe: Stem or stalk. | Colour: **White flushed pink** |
| | | Shape: (Bulbous base/Club-shaped/Cylindrical/Rooting base/Tapering Down/Tapering towards base/**Tapering Upward**) - **thick** |
| | | Type: (**Central** – stipe attached at the centre of the cap /Excentric – stipe not centrally attached to the cap /Lateral – at the side) |
| | Structures | Ring: A band of tissue encircling the stem |
| | | (**Absent**/Present) |
| | | Position on the stipe: (Top/Middle/Bottom) |
| | | Volva: Cup – like structure remains of the universal veil around the base of the stipe |
| | | (**Absent**/Present) |
| Section 3: Habitat/Substarte and Additional Information | Habitat/Substarte: The surface or material, such as soil or bark, to which the fungus is attached or on which it grows. | **In soil**/On tree/Other: |
| | | Soil: (Sandy/Muddy/Manure/Grass/**Forest**) |
| | | Tree: (Dead/Fallen/Decaying/Alive) |
| | | Surrounding Environment: (**Forest**/Pasture/Grass/Other)-**Pine** |
| | Bruising/Bleeding/Staining | Colour: |
| | | Action: (Touching/Cutting/Damaged) |
| | Odour/Smell | Similar: Fairly pungent |
| Similar species: | *Russula xerampelina* | |

# APPENDIX 3 – SAMPLING PERMITS

## PERMIT FOR OUTDOOR RECREATION (PERMIT NO: 18345)



MTO | group

MTO Forestry (Pty) Ltd

Permit vir Buitelugontspanning | Permit for Outdoor Recreation

Permit No: 18345

Name/Naam: Mariyke Hendriksman

Address / Adres: Kareedouw

Tel No: 0827740238

Postal code/ Poskode:

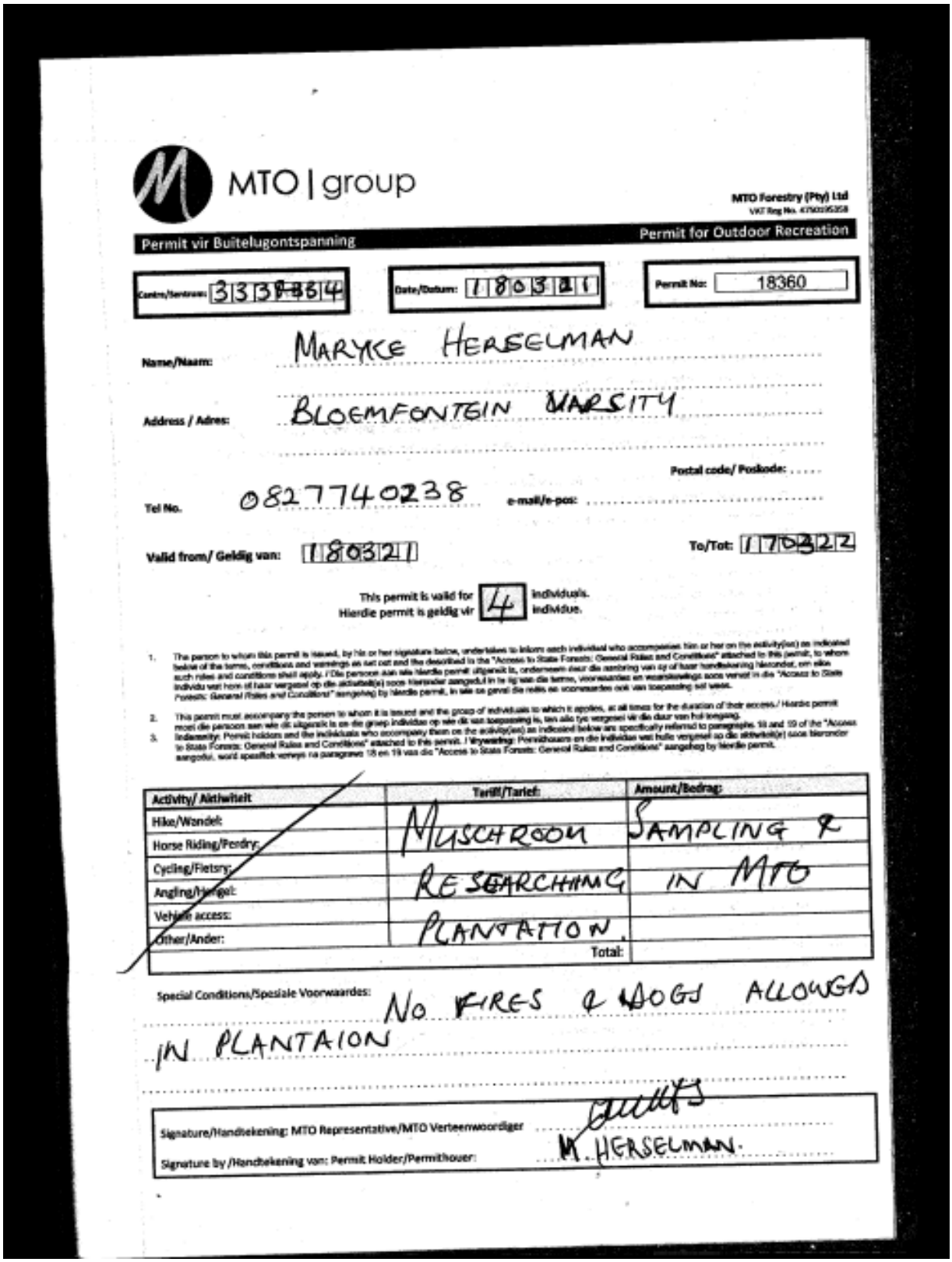
e mail/e-pos:

Valid from/ Geldig van: 18/03/21

To/Tot: 30/06/21

This permit is valid for: 4 individuals.
Hierdie permit is geldig vir individue.

78 PERMIT FOR OUTDOOR RECREATION (PERMIT NO: 18360)
79



MTO | group

MTO Forestry (Pty) Ltd
VAT Reg No. 4750235358

Permit vir Buitelugontspanning — Permit for Outdoor Recreation

Centre/Sentrum: 3339864 | Date/Datum: 180321 | Permit No: 18360

Name/Naam: MARYKE HERSELMAN

Address / Adres: BLOEMFONTEIN VARSITY

Postal code/ Poskode:

Tel No. 0827740238 e-mail/e-pos:

Valid from/ Geldig van: 180321 To/Tot: 170322

This permit is valid for 4 individuals.
Hierdie permit is geldig vir individue.

1. The person to whom this permit is issued, by his or her signature below, undertakes to inform each individual who accompanies him or her on the activity(ies) as indicated below of the terms, conditions and warnings as set out and the described in the "Access to State Forests: General Rules and Conditions" attached to this permit, to whom such rules and conditions shall apply. / Die persoon aan wie hierdie permit uitgereik is, onderneem deur die aanbring van sy of haar handtekening hieronder, om elke individu wat hom of haar vergesel op die aktiwiteit(e) soos hieronder aangedui in te lig van die terme, voorwaardes en waarskuwings soos vervat in die "Access to State Forests: General Rules and Conditions" aangeheg by hierdie permit, in wie se geval die reëls en voorwaardes ook van toepassing sal wees.
2. This permit must accompany the person to whom it is issued and the group of individuals to which it applies, at all times for the duration of their access./ Hierdie permit moet die persoon aan wie dit uitgereik is en die groep individue op wie dit van toepassing is, ten alle tye vergesel vir die duur van hul toegang.
3. **Indemnity:** Permit holders and the individuals who accompany them on the activity(ies) as indicated below are specifically referred to paragraphs 18 and 19 of the "Access to State Forests: General Rules and Conditions" attached to this permit. / **Vrywaring:** Permithouers en die individue wat hulle vergesel op die aktiwiteit(e) soos hieronder aangedui, word spesifiek verwys na paragrawe 18 en 19 van die "Access to State Forests: General Rules and Conditions" aangeheg by hierdie permit.

| Activity/ Aktiwiteit | Tariff/Tarief: | Amount/Bedrag: |
|---|---|---|
| Hike/Wandel: | MUSCHROOM | SAMPLING & |
| Horse Riding/Perdry: | | |
| Cycling/Fietsry: | RESEARCHING | IN MTO |
| Angling/Hengel: | | |
| Vehicle access: | PLANTATION. | |
| Other/Ander: | | |
| | Total: | |

Special Conditions/Spesiale Voorwaardes: No FIRES & DOGS ALLOWED IN PLANTAION

Signature/Handtekening: MTO Representative/MTO Verteenwoordiger

Signature by /Handtekening van: Permit Holder/Permithouer: M. HERSELMAN.