DESCRIPTION OF NOVEL SPECIES OF PSYCHEDELIC MUSHROOMS FROM SOUTHERN AFRICA

By

Onalerona Maloka

Thesis submitted in accordance with the requirements for the degree

Master of Science in Genetics

MSc Genetics

in the

Department of Genetics

Faculty of Natural and Agricultural Sciences

University of the Free State

Bloemfontein, South Africa

November 2022

Environmental & Biosafety Research Ethics Committee

Student Number: 2014153551

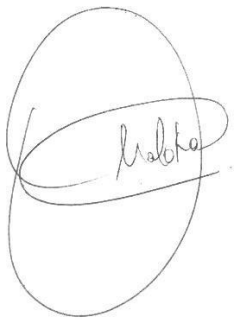
Ethical Clearance Number: UFS-ESD2018/0004

Main Supervisor: Marieka Gryzenhout, Ph. D.

Co – supervisor: Soumya Ghosh, Ph. D.

DECLARATION

“I, Onalerona Orefilempho Maloka, declare that the thesis hereby submitted by me for the degree of Master of Science in Genetics at the University of the Free State, is my own independent work and has not previously been submitted by me to another university or faculty. I furthermore, cede copyright of the thesis in favour of the University of the Free State”.



SIGNED BY: ONALERONA MALOKA – 30 NOVEMBER 2022

DEDICATION

This is dedicated to Matshidiso Gladys Maloka, my mother in heaven. Thank you for the sacrifices that enabled me to reach this dream, you made this all possible, I wish you could have seen me make it. A special appreciation to Masabata Talitha Modisaesi, my grandmother for being my everything.

Showing gratitude to my father and little brother for the endless support, Kea leboha. Palesa, Boitumelo and the rest of my family, words cannot begin to express what I feel in my heart.

ACKNOWLEDGEMENT

To my main supervisor at the University of the Free State (UFS), Dr Marieka Gryzenhout, for her guidance and encouragement throughout this study. Thank you for introducing e to the world of mycology and shaping me as a scientist.

Thank you to my co-supervisor, Dr Soumya Ghosh, for helping me perfect laboratory techniques. I'm a better scientist because of that.

Philippians 1:6 "*For I am confident of this very thing, that He who began a good work among you will complete it by the day of Christ Jesus".*

Thank You Lord Jesus for favour and new mercies every morning. This is the year that I am becoming who You meant me to be.

Table of Contents

ABSTRACT

The chemical compound psilocybin, responsible for causing hallucinations, is found in mushroom species of genera such as *Gymnopilus*, *Panaeolus*, *Pluteus*, and *Psilocybe*. Psilocybin also has a number of psychiatric and medical applications. Psilocybin-producing mushrooms have a wide distribution in South Africa and other parts of the world, but the biodiversity of these fungi is poorly known in South Africa. This study focused on the species identification of two sets of collections of *Psilocybe*, one from Lesotho and the other from Pretoria, based on morphology and different DNA sequence phylogenetic markers. A multi-locus phylogeny was constructed using the Internal Transcribed Spacer (ITS), RNA Polymerase II (RPB 1), and Translational Elongation Factor 1α (TEF-1α) gene regions. More than one marker was used to confirm identifications, and by combining the sequences, to also obtain better statistical support for groupings. By using the additional genes besides ITS, the usefulness of these additional markers to identify *Psilocybe* species was also investigated. Results showed that the two collections of samples were unique and different from each other based on all of the genes, except for the RPB 1 region that was found wanting. Although the TEF-1α was found sufficiently variable to also distinguish species similar to the ITS region, a relatively small number of species have been sequenced up to date. The distinct grouping of the two collections was confirmed by a number of macro- and micromorphological characteristics, and described as *Psilocybe malotiensis* prov. nom. and *Psilocybe orontawuli prov. nom.* respectively. Results from this study represent an important breakthrough where Southern African samples can now be sequenced and compared with specimens from elsewhere and should illustrate the presence of numerous novel species occurring in this region.

Keywords: *Psilocybe*, South Africa, psilocybin, ITS, TEF, RPB

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

List of terminology

Diversity

Taxonomists

Fungi

Ecotourism

Ecosystems

Ethnomycology

Ethnobiology

Organisms

Hallucinogenic

Neurotropic

Psilocybin: Chemical form (4-phosphoryloxy-N, N-dimethyltryptamine)

Psilocin: Chemical form (4-hydroxy-N, N-dimethyltryptamine)

Psychedelics

Serotonergic

Psilocin

Psychotropic

Agonists or partial-agonists

Psychoactive

phylogeny

Haploid

Paralogous genes

Phylogenic

Phylogenetic

List of Abbreviations

LSD: in lysergic acid diethylamide

B.C: Before Christ

B.C.E: Before Common Era

GPCSR: Genealogical Concordance Phylogenetic Species Recognition

rRNA: ribosomal Ribonucleic acid

RPB 1: RNA Polymerase
RPB 2: RNA Polymerase

SSU: Small Subunit ribosomal gene

LSU: Large Subunit ribosomal gene

ITS: Internal Transcribed Spacer

TEF-1α: Translational Elongation Factor 1α

DNA: Deoxyribonucleic acid

TAE: Tris-acetate-EDTA

TE: Tris-EDTA

Taq: Thermus aquaticus

PCR: Polymerase Chain Reaction

BLAST:

Basic Local Alignment

SI units

°C Degree Celsius

% Percentage

µl: Microlite

g: Gram

g/L: Gram per litre

Min minutes

ml: Millitre

mmol/L: Millimoles per litre

mM:

Millimolar

kg: Kilogram

nm: newton meter

Tm: Melting temperature

Clades

Electrophoresis

Morphological Chromatograms

Contigs

Polyphyletic Anecdotal Genera/genus

Alkaloids

Agaricales Genome

Tubulin

β-tubulin

eukaryotes

Search Tool

MEGA: Molecular Evolutionary Genetics Analysis

MAFFT: Multiple Sequence Alignment program

KOH: Potassium hydroxide

DMSO: Dimethyl Sulphoxide

NCBI: National Centre for Biotechnology Information

Exo-SAP-IT: Exonuclease 1 and Shrimp Alkaline Phosphatase

EDTA: Ethylenediamine tetra acetic acid

bp: base pair

et: alia (et al.)

CHAPTER 1 Introduction

1.1 Biodiversity of fungi

Fungi species are primary decomposers of organic material and contributes to 15% of the resources for species in environmental cycles of life (Olsen, Choffnes, Relman, and Pray, 2011; Keyhani, 2011). Even in the most tropical regions that are relatively diverse, an insignificant amount of fungi is described (Hawksworth 2001; Hawksworth and Lücking 2017; Kinge *et al*., 2020). This is a result of research on fungal biodiversity occurring over long periods, without producing definitive results or publishing and the lack of taxonomists (Mueller *et al*., 2007).

To date, the diversity of fungi in Southern Africa is not thoroughly investigated (Crous *et al*., 2006; Gryzenhout *et al*., 2010, 2012; Kinge *et al*., 2020). Approximately 36% (Crous *et al.,* 2006) of the macrofungi that are found in South Africa were recorded by Doidge (1950) and Eicker and Baxter (1999) but reflects on only a portion of the country's biodiversity (Crous *et al*., 2006). In 2006, only an estimate 780 new species of fungi had been discovered, out of a total estimate of 171 500 species (Crous *et al*., 2006). However, there are not many publications that update the research done by Crous et al. (2006). Furthermore, the lack of knowledge for the South African biodiversity makes it challenging to regulate fungal bio-exploration and research (Kinge *et al*., 2020). In order to help with this, Gryzenhout (2015) proposed that citizen scientists should be more included when studying the mycology in South Africa. With the focus on ecotourism, the protection and conservation of Southern Africa's fauna and flora, the time has come to dedicate financial resources and research to the preservation of fungi, and to study their role in ecosystems foundations (Crous *et al*., 2006).
Ethnomycology illustrates the cultural importance and historical relevance of the use of macrofungi in human life (Azeem, Hakeem and Ali, 2020). Ethnomycological surveys contribute to our understanding of the various local macrofungi practices and are essential in improving applications (Azeem, Hakeem and Ali, 2020; Osarenkhoe, John and Theophilus, 2020). Despite the study of organisms in ethnic use of cultures (ethnobiology), the ethnological understanding of mushrooms and their scientific documentation is a more recent development (Albuquerque and Alves, 2016). The recognition of mushrooms and the use as a source of food, medicine, revenue for smaller businesses in Africa, and the sociological impacts are challenged by the slow momentum of studies in ethnomycology (Osarenkhoe, John and Theophilus, 2020).

1.2 Hallucinogenic mushrooms

Hallucinogenic mushrooms have been referred to by many names, but the most accepted definition is the term ‘neurotropic’ because it describes the mushroom's influence on the central nervous system (Guzmán, Allen and Gartz, 1997; Schifano *et al.*, 2019). There are various types of hallucinogenic mushrooms, which are classified into four groups according to the compounds that they produce (Guzmán, Allen and Gartz, 1997). The first category contains species with psilocybin and their related derivates, and this includes species from the genera *Psilocybe*, *Gymnopilus*, *Panaeolus*, *Copelandia*, *Inocybe*, *Pluteus*, *Hypholoma*, *Panaeolina*, *Conocybe*, *Gerronema*, *Mycena*, *Agrocybe*, and *Galerina* (Guzmán, Allen and Gartz, 1997). The second category is dominated by *Amanita* species that contain ibotenic acid, including *A. regalis*, *A. muscaria,* and *A. pantherina* (Guzmán, Allen and Gartz, 1997). Ergot fungi are included in the third category, with species of *Cordyceps* and *Claviceps*, and with the most notable species *Claviceps purpurea*, which is a fungus that is high in lysergic acid diethylamide (LSD) (Guzmán, Allen and Gartz, 1997; Carod Artal, 2003). *Russula,* Gasteromycetes, and *Boletus* were among the species that classified as belonging to the last category and this is because of the lack of chemical analysis and the notion that they were considered to be suitable for spiritual ceremonies (Guzmán, Allen and Gartz, 1997).

Psilocybin-containing mushrooms are classified in the order Agaricales, and these psychedelics contain more than 300 identified species, across over 15 genera (Guzmán, Allen and Gartz, 1997; Strauss *et al.*, 2022). These mushrooms contain two main hallucinogenic active elements of the tyramine type, which are psilocybin (4-phosphoryloxy-N, N-dimethyltryptamine) and the derivative that is dephosphorylated psilocin (4-hydroxy-N, N-dimethyltryptamine) (Stamets, 1996; Musshoff, Madea and Beike, 2000; Cowan and Elkins, 2018; Dhanasekaran *et al.*, 2020). When metabolized, psilocybin, which is the main serotonergic compound in psychedelic mushrooms is rapidly dephosphorylated into psilocin (Passie *et al.*, 2002; Tsujikawa *et al.*, 2003). Although mushrooms that contain psilocybin are used recreationally because of their hallucinogenic properties (Fricke, Blei and Hoffmeister, 2017), numerous other beneficial applications also exist, such as the therapy that treat anxiety, depression, the addiction of alcohol and for the management of chronic pain (Krebs and Johansen,

2013; Lin, Lee and Yang, 2014; Carhart-Harris *et al.*, 2016; Castellanos *et al.*, 2020; Strauss *et al.*, 2022). These compounds have only relatively recently become characterized despite the ancient use of included these mushrooms.

1.3 The uses of psilocybin and derivatives

Throughout time, American, African, and European cultures regarded mushrooms that contain psilocybin as sacred and have adopted them for spiritual practices (Froese, Guzmán and Guzmán-Dávalos, 2016; Fricke, Blei and Hoffmeister, 2017). Historical murals support that many indigenous communities used these psychotropic mushrooms for their mind-manifestation and hallucinogenic powers (Nkadimeng, Nabatanzi, Steinmann and Eloff, 2020). In one of the post-paleolithic murals dated between 9000 – 7000 Before Common Era (B.C.E), human figures may be seen running while holding mushrooms that are connected using with the indications of lines that move in a direction towards their brains, possibly showing the effect that these hallucinogenic mushrooms have on their subconscious (Froese, Guzmán and Guzmán-Dávalos, 2016).

The ethnic groups in America, such as the Mexican Indians, considered these mushrooms sacred and have thought mythologically that hallucinogenic mushrooms were mediators with God. Moreso, the Nahum Aztecs perceived the mushrooms to be teonanacatl, which means the flesh of God (Azeem, Hakeem and Ali, 2020).

Psilocybin and its relatives are multiple serotonin receptor agonists or partial-agonists (Dhanasekaran *et al*., 2020), and therefore have novel pharmacology applications in the medical and psychiatric fields, such as replacement of antidepressant drugs that are currently available (Passie *et al.*, 2002; Ramírez-Cruz *et al.*, 2013; Carhart-Harris *et al.*, 2016; de Mattos-Shipley *et al.*, 2016). In recent clinical trials, psychedelic mushrooms were shown to decrease anxiety in patients with progressive cancer, has treated obsessive-compulsive disorders, depressive episodes, and other addictive behaviours such as nicotine addiction (Carhart-Harris *et al*., 2016; Fricke, Blei and Hoffmeister, 2017). These results were maintained in participants months after one or two doses were administered (Carhart-Harris *et al*., 2016; Fricke, Blei and Hoffmeister, 2017).

1.4 Psilocybin-containing genera

Fungi that have the psilocybin alkaloids include *Panaeolus* and *Psilocybe* (Stamets, 1996; Strauss *et al.*, 2022). Other genera with psilocybin alkaloids include *Gymnopilus* and *Pluteus* (Guzmán, Allen and Gartz, 1997; Bustillos *et al.*, 2014; Strauss *et al.*, 2022). The most well-known and well recognized genus from these hallucinogenic mushrooms are *Psilocybe*, because of their proven ethnomycology use, current applications and extensive use. This genus is also known for its widespread geographic occurrence, with approximately 150 known species across the world (Schifano *et al.*, 2019; Strauss *et al.,* 2022). *Psilocybe* was once a polyphyletic genus that is now composed of two genera, namely *Psilocybe* and *Deconica* (Moncalvo *et al.*, 2002; Matheny *et al.*, 2006; Ramírez-cruz *et al.*, 2013; Strauss *et al.*, 2022), with *Psilocybe* applied to the group of hallucinogenic mushroom species while *Deconica* is now used for species without psychoactive properties (Redhead *et al.*, 2007; Norvell *et al*., 2010; Gúzman, 2012; Ramírez-Cruz *et al.*, 2013; de Mattos-Shipley *et al.*, 2016; Strauss *et al.*, 2022). This proposed separation of the genus by Redhead et al. (2007) was further accepted through the International Nomenclature Committee for Fungi (Norvell *et al.,* 2010; Ramírez-Cruz *et al.*, 2013). *Psilocybe cubensis* is the best-known species with traits (Cowan and Elkins, 2017) and forms part of the group of mushrooms that are not difficult to cultivate (Kirsten and Bernardi, 2010).

1.5 The identification of fungal species

The identification of fungi is a fundamental step, and species are observed based on morphological systems that are constructed on established methods. Identification is vital in conservation biology, ecology and fungal application research because all biological features relating to a given species in an ecosystem can only be accounted with an unambiguous identifier such as a species name (Begerow *et al.*, 2010). While molecular information is currently applied for fungal phylogeny and systematics, morphological classification based on the Botanical Code of Nomenclature (Turland *et al*., 2017) is still required for accurate characterization and naming of a species. Identification, however, cannot rely solely on morphological characteristics because in some cases morphological characteristics are scarce and plastic, thus lacking discriminatory power to distinguish species. Fungi's di-morphic and pleomorphic life cycles also often hinder accurate morphological identification and allocation to a

species level, such as that in yeast-mycelial transitions (Begerow *et al.*, 2010). Dried, shredded, or otherwise processed fungi may not be readily morphologically identifiable and therefore require cultivation or molecular identification (Berruti *et al.*, 2014).

The Genealogical Concordance Phylogenetic Species Recognition (GPCSR) concept originated in response to rapid growing use of phylogenies that more accurately represents closely related species. This has led researchers to investigate the resolution power of multiple genes; instead of one (Fisher *et al.*, 2000; Taylor *et al.*, 2000). The reason GPCSR works effectively in fungi is that most of the fungal species appear to be haploid, having fewer paralogous genes thereby making it easier to analyze using several gene regions (Taylor *et al.*, 2000). In addition to this research, Hofstetter et al. (2007) used multi-locus phylogenetic studies to investigate the contrast between ribosomal Ribonucleic acid (rRNA)-coding genes and protein-coding genes and resolved phylogenetic relationships within *Lecanoromycetes* species (Miadlikowska *et al.*, 2014). The results revealed that the most suitable for the molecular systematics of *Lecanoromycetes* were RNA Polymerase II (RPB 1), and RNA Polymerase II (RPB 2), the second largest subunit (Hofstetter *et al.*, 2007; Miadlikowska *et al.*, 2014). More studies resembling the one of Hofstetter *et al.* (2007) are required to determine the most suitable region for other fungal groups.

The Internal Transcribed Spacer (ITS) region is deemed to be the DNA barcoding region that is acceptable for the identification of fungi at a species-level identification, and as a result has been sequenced for the majority of mushroom studies (Schoch *et al.*, 2012; Raja *et al.*, 2017). ITS was selected as the primary barcode marker and this was based on the easy use and accuracy it possesses, rather than a high level of resolution (Schoch *et al*., 2012; Stefani, Jones and May, 2014). However, published data observed that the ITS region unaided is not always enough, even though it is the most suitable candidate to be classified as primary fungal DNA barcode marker (Schoch *et al*., 2012; Stefani, Jones and May, 2014).

For taxonomic groups that have a low ITS sequence divergence, several genes, including RNA Polymerase II (RPB 1), Large Subunit ribosomal gene (LSU), Small Subunit ribosomal gene (SSU), and Translational Elongation Factor 1α (TEF-1α) have been selected and identified as possible secondary fungal barcode markers (Stefani,

Jones and May, 2014). Additionally, TEF-1α region is suggested to be the secondary universal fungal barcode for suitable for all fungi species (Schoch *et al.*, 2012; Stielow *et al.*, 2015). Other genes that are used to identify psilocybin-producing mushrooms include RPB 1, RPB 2, LSU and SSU (Ueno, Urano and Suzuki, 2003; Matheny, 2005; Redhead *et al.*, 2007; Schoch *et al.*, 2012, 2014; Raja *et al.*, 2017; Lee *et al.*, 2020; Ramírez-Cruz *et al.*, 2020; Zhang *et al.*, 2021). However, the genetic diversity of the fungal kingdom, makes it implausible for a single-marker barcode system to identify each specimen or culture at a species level (Schoch *et al*., 2012). It is therefore important to examine multiple genes as a method for advanced species identification.

Problem Statement:

The biodiversity of fungi in South Africa is poorly studied (Gryzenhout 2012, 2015; Kinge et al., 2020). This includes the biodiversity of psilocybin- containing species. Furthermore, the extent that psychedelic mushrooms are used in South African practices are not well documented, including their legal and illegal use. We therefore, need better and more standardized methods to identify these mushrooms as well as more research to contribute to existing databases. To this point, it is unknown precisely which species we have of genera that are known producers of these psychedelic compounds. However, this information is required to accurately identify species from South Africa as well as to identify illegal magic mushroom contraband, employing a standardized method for detection.

A number of psilocybin-containing species are known to occur in South Africa (Kinge *et al.,* 2020; Gryzenhout 2021), such as species from *Panaeolus*, *Panaeolina*, *Inocybe, Pluteus*, *Gymnopilus*, and *Psilocybe* (Guzmán, Allen and Gartz, 1997). None of these species, however, have been characterized based on published DNA sequence data. A previous study by Strauss (2022) showed that based on DNA sequence data of the ITS region, a number of possible new species may exist in South Africa. Furthermore, using only the ITS region presented, some inconclusive results were obtained in some cases, and the inclusion of more gene regions may clarify these relationships and confirm the status of some specimens as new species. No such other gene regions have thus far been sequenced for South African specimens.

Aim

This project aims to characterize two sets of samples of *Psilocybe* obtained from Pretoria, South Africa, and Lesotho. This was based on morphological examination, and DNA sequence comparisons. In order to improve species resolutions, secondary DNA markers

rather than the using the only the ITS marker was included. This will include the RPB 1 and TEF-1α regions that have been sequenced for *Psilocybe* species in previous studies (Zhang *et al.*, 2021). Additionally, since the samples were suspected to represent new species, the samples will be described using morphological methods.

Objectives

Morphological characterization (measurements, descriptions, and illustrations) will be done on the novel species following nomenclatural procedures. DNA sequence data of of the ITS genomic regions of the rRNA regions are also generated, as well as for the housekeeping genes translation elongation factor (TEF-1α) and a region of the RNA polymerase II gene (RPB 1). An additional objective of this study is to investigate the the contrast in results between sequence data of the different gene regions.

Hypothesis

Ultimately, novel species of psychedelic mushrooms exist in South Africa that need to be discovered and described. We investigated this in the current study, and the hypothesis that secondary genes have the potential as barcode markers to strengthen a database and increase species resolution for characterizing novel *Psilocybe* species. Consequently, using multiple genes will provide a more accurate and reliable approach to identify and differentiate psychedelic mushrooms than using only ITS, which has resolution limitations.

Expected outcomes

Two new Psilocybe species are expected to be described from the thesis. The discovery of new species from Southern Africa will advance the relevant research and preserve the the natural resources and biodiversity of South Africa. Morphological studies will reveal differentiating characteristics to distinguish the novel species from South Africa. Furthermore, the various DNA sequence markers will show that the two putative novel species are indeed unique. Findings will show that sequencing multiple markers will will improve identification.

The structure of this dissertation

Research on psychedelics has advanced significantly since the first descriptions in Mexico by Heim (1958), Heim and Hofmann (1958), Hofmann et al. (1958). Unfortunately, the biodiversity of psilocybin-containing mushrooms in South Africa is

223 poorly documented. Majority of the species discovery is documented in unpublished
224 articles or are found on mycology websites and only a few research studies have
225 reported on the existing mushroom diversity (Crous *et al*., 2006; Gryzenhout *et al*.
226 2012, 2015, 2020; Kinge *et al*., 2020). There are over 150 *Psilocybe* species all over
227 the world, including *Psilocybe semilanceata* and *P. cubensis* (Redhead *et al*., 2007;

Norvell *et al*., 2010). In this study, Chapter 1 provides a brief background and introduces the current challenges associated with the identification of psychedelic mushrooms. This chapter outlines the structure of this dissertation including the aims and expected outcomes of this study. The importance and subsequent contribution of this research to South Africa's diversity is also indicated. The aim, objectives, hypothesis and expected outcomes of this thesis are included in this chapter.

In Chapter 2, the literature review briefly highlights the history of psychedelic mushrooms and the subsequent impact of their discovery on modern-day applications. The hallucinogenic mushrooms containing psilocybin in their respective genera was discussed more in this chapter. Key mushroom descriptions as well as known South African psilocybin-producing mushrooms are summarized and this will help citizen mycologists across South Africa to identify the mushrooms in their surrounding environments and areas of interest. Different molecular genes were examined in the literature review to establish their potential as DNA barcode markers, their use in studies, and related limitations. The use of multiple genes for differentiating species will also be included in this chapter.
Many potential medicinal applications exist for psilocybin-assisted therapy especially in neurological conditions and more studies should investigate this naturally occurring substance (Strauss *et al*., 2022c). From what is known about these important fungi, it has become apparent that more species have to be described using new and improved identification methods. This research will benefit South Africa significantly, by finding alternative identification techniques, revealing the great and vast diversity that the country has, and developing possible therapies from locally produced mushrooms.

Chapter 3 discusses the methodology followed. DNA was extracted using a published, student- friendly extraction method for mushrooms. This method of genomic DNA extraction is quick and easy to use and provides optimal results using dried mushroom

samples. The Internal Transcribed Spacer (ITS), Translational Elongation Factor 1α (TEF-1α), and the RNA Polymerase II (RPB 1) were sequenced to identify *Psilocybe* samples from different locations. Phylogenetic studies were done using the three molecular markers and a multiple-gene phylogeny was constructed for the *Psilocybe*. The different genes produced high-quality genomic DNA and allowed for unique DNA sequence comparisons. The morphological traits of the different mushroom samples provide an overview on the different characteristics that make each individual specimen diverse.

In Chapter 4, the findings of this research are revealed. DNA sequence-based identifications showed that the Lesotho and other species are two distinct species, that don't group with any other known and sequenced *Psilocybe* species. They are also distinguishable based on unique morphological characteristics and are described with the provisional names *Psilocybe malotiensis* and *Psilocybe orontawuli*. Whereas the Lesotho species is currently only known from the Lesotho area, the other species is known from Pretoria and Cape Town, indicating that it could possibly be more widespread.

Reference

Azeem, U., Hakeem, K.R. and Ali, M., 2020. Fungi For Human Health. *Current Knowledge and Future Perspectives*.

Begerow, D., Nilsson, H., Unterseher, M. and Maier, W., 2010. Current state and perspectives of fungal DNA barcoding and rapid identification procedures. *Applied microbiology and biotechnology*, 87, pp.99-108.

Berruti, A., Amoroso, V., Gallo, F., Bertaglia, V., Simoncini, E., Pedersini, R., Ferrari, L., Bottini, A., Bruzzi, P. and Sormani, M.P., 2014. Pathologic complete response as a potential surrogate for the clinical outcome in patients with breast cancer after neoadjuvant therapy: a meta-regression of 29 randomized prospective studies. *Journal of clinical oncology*, 32(34), pp.3883-3891.

Bustillos, R.G., Dulay, R.M.R., Kalaw, S.P. and Reyes, R.G., 2014. Optimization of culture conditions for mycelial growth and basidiocarp production of Philippine strains of *Panaeolus antillarium* and *Panaeolus cyanescens*. *Mycosphere*, 5(3), pp.398-404.

Carhart-Harris, R.L., Bolstridge, M., Rucker, J., Day, C.M., Erritzoe, D., Kaelen, M., Bloomfield, M., Rickard, J.A., Forbes, B., Feilding, A. and Taylor, D., 2016. Psilocybin with psychological support for treatment-resistant depression: an open-label feasibility study. *The Lancet Psychiatry*, 3(7), pp.619-627.

Carod Arta, F.J., 2003. Neurological syndromes associated with the ingestion of plants and fungi with a toxic component (II). Hallucinogenic fungi and plants, mycotoxins and medicinal herbs. *Revista de Neurologia*, 36(10), pp.951-960.

Castellanos, J.P., Woolley, C., Bruno, K.A., Zeidan, F., Halberstadt, A. and Furnish, T., 2020. Chronic pain and psychedelics: a review and proposed mechanism of action. *Regional Anesthesia & Pain Medicine*, 45(7), pp.486-494.

Cowan, A.F. and Elkins, K.M., 2018. Detection and Identification of *Psilocybe cubensis* DNA Using a Real-Time Polymerase Chain Reaction High Resolution Melt Assay. *Journal of forensic sciences*, 63(5), pp.1500-1505.

Crous, P.W., Rong, I.H., Wood, A., Lee, S., Glen, H., Botha, W., Slippers, B., de Beer, W.Z., Wingfield, M.J. and Hawksworth, D.L., 2006. How many species of fungi are there at the tip of Africa?. *Studies in mycology*, 55, pp.13-33.

de Mattos-Shipley, K.M., Ford, K.L., Alberti, F., Banks, A.M., Bailey, A.M. and Foster, G.D., 2016. The good, the bad and the tasty: the many roles of mushrooms. *Studies in mycology*, 85(1), pp.125-157.

Dhanasekaran, D., Latha, S., Suganya, P., Panneerselvam, A., Kumar, T.S., Alharbi, N.S., Arunachalam, C., Alharbi, S.A. and Thajuddin, N., 2020. Taxonomic identification and bioactive compounds characterization of *Psilocybe cubensis* DPT1 to probe its antibacterial and mosquito larvicidal competency. *Microbial pathogenesis*, 143, p.104138.

Eicker, A. and Baxter, A.P., 1999. An historical overview of Southern African systematic mycology. *Transactions of the Royal Society of South Africa*, 54(1), pp.5-19.

Fisher, M.C., Koenig, G., White, T.J. and Taylor, J.W., 2000. A test for concordance between the multilocus genealogies of genes and microsatellites in the pathogenic fungus *Coccidioides immitis*. *Molecular Biology and Evolution*, *17*(8), pp.1164-1174. of genes and microsatellites in the pathogenic fungus *Coccidioides immitis*. *Molecular Biology and Evolution*, 17(8), pp. 1164–1174.

Fricke, J., Blei, F. and Hoffmeister, D., 2017. Enzymatic synthesis of psilocybin. *Angewandte Chemie International Edition*, 56(40), pp.12352-12355.

Froese, T., Guzmán, G. and Guzmán-Dávalos, L., 2016. On the origin of the genus *Psilocybe* and its potential ritual use in ancient Africa and Europe. *Economic Botany*, 70, pp.103-114.

Gryzenhout, M., Roets, F. and Villiers, R.D., 2010. Fungal conservation in Africa. *Mycologia Balcanica*, 7(1), pp.53-58.

Gryzenhout, M., Jefwa, J.M. and Yorou, N.S., 2012. The status of mycology in Africa: A document to promote awareness. *IMA fungus*, 3(1), pp.99-102.A document to promote awareness. *IMA fungus*, 3(1), pp.99–102.

Gryzenhout, M., 2015. The need to engage with citizen scientists to study the rich fungal biodiversity in South Africa. *IMA Fungus*, 6, pp. A58-A64.

328 Guzmán, G., 2012. New taxonomical and ethnomycological observations on *Psilocybe*
329 *ss* (fungi, basidiomycota, agaricomycetidae, agaricales, strophariaceae) from Mexico,
330 Africa and Spain. *Acta botánica mexicana*, (100), pp.79-106.

331 Guzmán, G., Allen, J.W. and Gartz, J., 1998. A worldwide geographical distribution
332 of the neurotropic fungi, an analysis and discussion. *Ann. Mus. Civ. Rovereto*, 14,
333 pp.189-280.

334 Hawksworth, D.L., 2001. The magnitude of fungal diversity: the 1,5 million species
335 estimate revisited. *Mycological research*, 105(12), pp.1422-1432.

336 Hawksworth, D.L. and Lücking, R., 2017. Fungal diversity revisited: 2.2 to 3.8 million
337 species. *Microbiology spectrum*, 5(4), pp.5-4.

338 Heim, R., 1958. Diagnose latine du *Psilocybe* Wassonii Heim, espèce hallucinogène
339 des Aztèques. *Revue de Mycologie (paris)*, 23, pp.119-120.

340 Heim, R. and Wasson, R.G., 1958. Les champignons hallucinogènes du Mexique
341 –Etudes ethnologiques, taxinomiques, biologiques, physiologiques et chimiques.
342 *Archives du Muséum national d'Histoire naturelle, 7ème série*, 6(1), pp.1-445.

343 Hofmann, A., Heim, R., Brack, A. and Kobel, H.A.N.S., 1958. Psilocybin, ein
344 psychotroper Wirkstoff aus dem mexikanischen Rauschpilz *Psilocybe mexicana*
345 Heim. *Experientia*, 14, pp.107-109.

346 Hofstetter, V., Miadlikowska, J., Kauff, F. and Lutzoni, F., 2007. Phylogenetic comparison
347 of protein-coding versus ribosomal RNA-coding sequence data: a case study of the
348 *Lecanoromycetes* (Ascomycota). *Molecular phylogenetics and evolution*, 44(1),
349 pp.412-426.

350 Keyhani, N.O., 2011. Fungal genomes and beyond. *Fungal Genome Biology*, 1, p. e101.

351 Kinge, T.R., Goldman, G., Jacobs, A., Ndiritu, G.G. and Gryzenhout, M., 2020. A first
352 checklist of macrofungi for South Africa. *MycoKeys*, 63, 1-48.

353 Kirsten, T.B. and Bernardi, M.M., 2010. Acute toxicity of *Psilocybe cubensis* (Ear.) Sing.,
354 *Strophariaceae*, aqueous extract in mice. *Revista Brasileira de Farmacognosia*, 20, pp.
355 397-402.

Krebs, T.S. and Johansen, P.Ø., 2013. Psychedelics and mental health: a population study. *PloS one*, 8(8), p. e63972.

Lee, T., Na, H.Y., Kim, K.M., Lee, H.S., Park, S.H., Choe, J.Y., Choi, K.C. and Byeon, S.J., 2020. Mycobiome analysis in fungal Infected formalin-fixed and paraffin-embedded tissues for identification of pathogenic fungi: A pilot study. *bioRxiv*, pp.2020-04.

Lin, S.H., Lee, L.T. and Yang, Y.K., 2014. Serotonin and mental disorders: a concise review on molecular neuroimaging evidence. *Clinical Psychopharmacology and Neuroscience*, 12(3), p.196–202.

Matheny, P.B., 2005. Improving phylogenetic inference of mushrooms with RPB1 and RPB2 nucleotide sequences (*Inocybe*; Agaricales). *Molecular phylogenetics and evolution*, 35(1), pp.1-20.

Matheny, P.B., Curtis, J.M., Hofstetter, V., Aime, M.C., Moncalvo, J.M., Ge, Z.W., Yang, Z.L., Slot, J.C., Ammirati, J.F., Baroni, T.J. and Bougher, N.L., 2006. Major clades of Agaricales: a multilocus phylogenetic overview. *Mycologia*, 98(6), pp.982-995.

Miadlikowska, J., Kauff, F., Högnabba, F., Oliver, J.C., Molnár, K., Fraker, E., Gaya, E., Hafellner, J., Hofstetter, V., Gueidan, C. and Otálora, M.A., 2014. A multigene phylogenetic synthesis for the class *Lecanoromycetes* (Ascomycota): 1307 fungi representing 1139 infrageneric taxa, 317 genera and 66 families. *Molecular Phylogenetics and Evolution*, 79, pp.132-168.

Moncalvo, J.M., Vilgalys, R., Redhead, S.A., Johnson, J.E., James, T.Y., Aime, M.C., Hofstetter, V., Verduin, S.J., Larsson, E., Baroni, T.J. and Thorn, R.G., 2002. One hundred and seventeen clades of euagarics. *Molecular phylogenetics and evolution*, 23(3), pp.357-400.

Mueller, G.M., Schmit, J.P., Leacock, P.R., Buyck, B., Cifuentes, J., Desjardin, D.E., Halling, R.E., Hjortstam, K., Iturriaga, T., Larsson, K.H. and Lodge, D.J., 2007. Global diversity and distribution of macrofungi. *Biodiversity and conservation*, 16, pp.37-48.

Musshoff, F., Madea, B. and Beike, J., 2000. Hallucinogenic mushrooms on the German market—simple instructions for examination and identification. *Forensic science international*, 113(1-3), pp.389-395.

Nkadimeng, S.M., Nabatanzi, A., Steinmann, C.M. and Eloff, J.N., 2020. Phytochemical, cytotoxicity, antioxidant and anti-inflammatory effects of *Psilocybe natalensis* magic mushroom. *Plants*, 9(9), p.1127.

Norvell, L.L., Hawksworth, D.L., Petersen, R.H. and Redhead, S.A., 2010. IMC9 Edinburgh nomenclature sessions. *IMA fungus*, 1(2), pp.143-147.

Olsen, L., Choffnes, E.R., Relman, D.A. and Pray, L., 2011. Fungal diseases: an emerging threat to human, animal and plant health. Workshop summary. *National Academies Press*.

Osarenkhoe, O., John, O. and Theophilus, D., 2014. Ethnomycological conspectus of West African mushrooms: An Awareness Document. *Advances in Microbiology,* 04(01), pp. 39–54.

Passie, T., Seifert, J., Schneider, U. and Emrich, H.M., 2002. The pharmacology of psilocybin. *Addiction biology*, 7(4), pp.357-364.

Raja, H.A., Miller, A.N., Pearce, C.J. and Oberlies, N.H., 2017. Fungal identification using molecular tools: a primer for the natural products research community. *Journal of natural products*, 80(3), pp.756-770.

Ramírez-Cruz, V., Guzmán, G., Villalobos-Arámbula, A.R., Rodríguez, A., Matheny, P.B., Sánchez-García, M. and Guzmán-Dávalos, L., 2013. Phylogenetic inference and trait evolution of the psychedelic mushroom genus *Psilocybe* sensu lato (Agaricales). *Botany*, 91(9), pp.573-591.

Ramírez-Cruz, V., Cortés-Pérez, A., Borovička, J., Villalobos-Arámbula, A.R., Matheny, P.B. and Guzmán-Dávalos, L., 2019. *Deconica cokeriana* (Agaricales, Strophariaceae), a new combination. *Mycoscience*, 61(2), pp.95-100.

Redhead, S.A., Moncalvo, J.M., Vilgalys, R., Matheny, P.B., Guzmán-Dávalos, L. and Guzmán, G., 2007. (1757) Proposal to conserve the name *Psilocybe* (Basidiomycota) with a conserved type. *Taxon*, 56(1), pp.255-257.

Schifano, F., Napoletano, F., Chiappini, S., Orsolini, L., Guirguis, A., Corkery, J.M., Bonaccorso, S., Ricciardi, A., Scherbaum, N. and Vento, A., 2019. Correction to: New Psychoactive Substances (NPS), Psychedelic Experiences and Dissociation: Clinical and Clinical Pharmacological Issues. *Current Addiction Reports*, 6, pp.153-154.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., Fungal Barcoding Consortium, Fungal Barcoding Consortium Author List, Bolchacova, E. and Voigt, K., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the national academy of Sciences*, 109(16), pp.6241-6246.

Schoch, C.L., Robbertse, B., Robert, V., Vu, D., Cardinali, G., Irinyi, L., Meyer, W., Nilsson, R.H., Hughes, K., Miller, A.N. and Kirk, P.M., 2014. Finding needles in hay-stacks: linking scientific names, reference specimens and molecular data for Fungi. *Database*, 2014, p. bau061.

Stamets, P., 1996. Psilocybin mushrooms of the world: an identification guide. *Ten Speed Press*. p. 245.

Stefani, F.O., Jones, R.H. and May, T.W., 2014. Concordance of seven gene genealogies compared to phenotypic data reveals multiple cryptic species in Australian dermocyboid *Cortinarius* (Agaricales). *Molecular Phylogenetics and Evolution*, 71, pp.249-260.

Stielow, J.B., Levesque, C.A., Seifert, K.A., Meyer, W., Irinyi, L., Smits, D., Renfurm, R. G.J.M.V., Verkley, G.J.M., Groenewald, M., Chaduli, D. and Lomascolo, A., 2015. One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes. *Persoonia-Molecular Phylogeny and Evolution of Fungi*, 35(1), pp.242-263.

Strauss, D., Ghosh, S., Murray, Z. and Gryzenhout, M., 2022a/b. An overview on the taxonomy, phylogenetics and ecology of the psychedelic genera *Psilocybe*, *Panaeolus*, *Pluteus* and *Gymnopilus*. *Frontiers in Forests and Global Change*, 5, p.813998.

Strauss, D., Ghosh, S., Murray, Z. and Gryzenhout, M., 2022c. Psilocybin containing mushrooms: a rapidly developing biotechnology industry in the psychiatry, biomedical and nutraceutical fields. *3 Biotech*, 12(12), p.339.

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S. and Fisher, M.C., 2000. Phylogenetic species recognition and species concepts in fungi. *Fungal genetics and biology*, 31(1), pp.21-32.

Tsujikawa, K., Kanamori, T., Iwata, Y., Ohmae, Y., Sugita, R., Inoue, H. and Kishi, T., 2003. Morphological and chemical analysis of magic mushrooms in Japan. *Forensic science international*, 138(1-3), pp.85-90.

446 Turland, N.J. and Wiersema, J.H., 2017. Synopsis of Proposals on Nomenclature-
447 Shenzhen 2017: A review of the proposals concerning the International Code of
448 Nomenclature for algae, fungi, and plants submitted to the XIX International Botanical
449 Congress. *Taxon*, 66(1), pp.217-274.

450 Ueno, R., Urano, N. and Suzuki, M., 2003. Phylogeny of the non-photosynthetic green
451 micro-algal genus *Prototheca* (*Trebouxiophyceae*, Chlorophyta) and related taxa inferred
452 from SSU and LSU ribosomal DNA partial sequence data. *FEMS microbiology letters*, 223
453 (2), pp.275-280.

454 Zhang, X., Yu, H., Yang, Q., Wang, Z., Xia, R., Chen, C., Qu, Y., Tan, R., Shi, Y., Xiang,
455 P. and Zhang, S., 2021. A forensic detection method for hallucinogenic mushrooms via
456 High-Resolution Melting (HRM) analysis. *Genes*, 12(2), p.199.

457 Zhang, X., Yu, H., Wang, Z., Yang, Q., Xia, R., Qu, Y., Tao, R., Shi, Y., Xiang, P., Zhang,
458 S. and Li, C., 2022. Multi-locus identification of *Psilocybe cubensis* by high-resolution melting
459 (HRM). *Forensic Sciences Research*, 7(3), pp.490-497.

CHAPTER 2. Literature Review

2.1 The discovery of magic mushrooms

The earliest reference of psychedelic mushrooms were produced from Mexico in the 16th century (Van Court *et al.*, 2022). The report described a type of mushroom used during spiritual ceremonies, that induced visions and caused intoxication (Van Court *et al.*, 2022).
The Mexican Indian ethnic groups in America considered hallucinogenic mushrooms sacred and have thought mythologically that these mushrooms were mediators with God (Gry, Andersson and Kristinsson, 2009; Azeem, Hakeem and Ali, 2020). The Nahum Aztecs as well as the Chichimecas, perceived small mushrooms growing in pastures to be "teonanacatl", which means the flesh of God (Gry, Andersson and Kristinsson, 2009; Azeem, Hakeem and Ali, 2020). Recent publications document the rise in popularity in the recreational use of psychedelic substances among young adults (Krebs and Johansen, 2013; Yockey and King, 2021; Strauss et al., 2022c). Research on the popularity and use of these psychedelics can inform mental health interventions and related policies and other applications (Yockey and King, 2021). However, the history of these psychedelic substances must first be understood.

Even though the origins of this practice includes using psychedelic mushrooms date back to 1500 B.C (Before Christ) (Wasson, 1957), it was only introduced and documented in 1453, in the work of Andrés de Olmos; "Antigüedades Mexicanas" from 1453 (Gry, Andersson and Kristinsson, 2009). In 1939, the first "teonanácatl" samples were identified as *Panaeolus campanulatus var. sphinctrinus* by Dr. David Linder, and it was determined that the shamans used more than one species of hallucinogenic mushrooms for spiritual ceremonies (Gry, Andersson and Kristinsson, 2009). The mushrooms included species from the genera *Psilocybe,* such as *Psilocybe caerulescens*, and *Psilocybe cubensis*, as well as *Panaeolus,* such as *Panaeolus acuminatus*, and *Panaeolus campanulatus var. sphinctrinus* (Gry, Andersson and Kristinsson, 2009). Roger Heim's work (1958) supported by his fellow colleagues, Heim and Hofmann (1958), Hofmann et al. (1958) discovered hallucinogenic active elements such as aeruginacin, baeocystin, psilocin, psilocybin, and norbaeocystin in the species *Inocybe aeruginascens*, *Psilocybe mexicana*, and *Psilocybe baeocystis* (Gry, Andersson and Kristinsson, 2009). This resulted in the identification of the same elements in different mushrooms throughout the world (Gry, Andersson and Kristinsson, 2009). The discovery of hallucinogens and their prevalence in the current social climate have led to interest in the benefits that these natural substances have. The use and applications of hallucinogens will be outlined in this section.

2.1.1 Psilocybin application in ancient spiritual practices

The psychedelic mushrooms most significant to these practices are those from the genus *Psilocybe*, which includes species such as *P. aztecorum*, *P. cubensis*, *P. caerulescens*, *P. zapotecorum*, and *P. mexicana* (Van Court *et al.*, 2022). Under the guidance of a shaman or spiritual guides, individuals would consume these *Psilocybe* mushrooms and experience neurological alterations such as synesthesia and hallucinations, resulting in a disconnection of the body and soul (Van Court *et al.*, 2022). It is believed that the medicinal properties produced by the mushrooms enabled the consumer to alter their perceptions of the condition they were seeking to be healed from and cure themselves (Wasson, 1957; Strauss *et al.*, 2022c; Van Court *et al*., 2022). Participants of these rituals would also be able to embark on a journey of self-introspection or would receive visions of missing loved ones in order to locate them (Van Court *et al.*, 2022). Indigenous communities continue to use *Psilocybe* mushrooms as a remedy for rheumatism, a pain killer for pain associated with stomach pain and toothaches, and a treatment for anxiety (Van Court *et al.*, 2022).

2.1.2 Modern applications for psilocybin

The scientific breakthrough of psilocybin identification and synthesis by Agurell and Nilsson (1968) ushered in the start of psilocybin research (Nichols, 2020). Subsequently, between 1960 and 1980, there were over 100 reports about psilocybin written and published in the literature (Nichols, 2020; Strauss *et al.*, 2022c). These reports included anecdotal accounts of human use as well as biochemical and analytical studies (Nichols, 2020; Strauss *et al.*, 2022c). Subsequent studies included those of Krebs and Johansen (2013), that analyzed six clinical studies conducted between 1966 and 1970, that were aimed at treating alcohol addiction, and revealed substantial recovery of patients six months after treatment (Strauss *et al.*, 2022c). For terminally sick cancer patients with anxiety, Grob et al. (2011) used psilocybin treatment, which resulted in an improved overall mood in patients that lasted over six months (Strauss *et al.*, 2022c). Another example, Rucker et al. (2016), reported that 79% of patients had decreased depression-related symptoms after psilocybin treatment in 19 studies conducted between 1949 and 1973 (Strauss *et al.*, 2022c). However, these studies were not controlled studies (Nichols, 2020; Strauss *et al.*, 2022c), a fundemental requirement for research ethics. The upside to this was because there were no reported deaths from the conducted trials (Metzner, 2005), other upcoming researchers were able to amend and adapt their research based on these favorable outcomes.

More recently, Garcia-Romeu et al. (2014) targeted tobacco addiction by using psilocybin treatment, and 80% of patients reduced or stopped smoking completely within six months after the treatment (Strauss *et al.*, 2022c). Bogenschutz et al. (2015) treated alcohol addiction using psilocybin and instantly observed improved behavior in patients after the treatment (Strauss *et al.*, 2022c). Other ailments that used psilocybin include mental health disorders (Carhart-Harris *et al.*, 2016; Strauss *et al.*, 2022c), chronic pain and cluster headaches (Castellanos *et al.*, 2020; Strauss *et al.*, 2022c), obsessive-compulsive disorder (Reiff *et al.*, 2020; Strauss *et al.*, 2022c), post-traumatic stress disorder (Chi and Gold, 2020; Strauss *et al.*, 2022c) among many others.

2.2. Psilocybin-containing genera

Psychedelics can be found in nature or can be produced from natural compounds, therefore forming a broad group of different substances such as Ayahuasca, lysergic acid (LSD), ketamine, peyote, mescaline, 3,4-methylenedioxymethamphetamine (MDMA), and psilocybin, just to name a few (Krebs and Johansen, 2013; Hase *et al.*, 2022; Strauss *et al.*, 2022c).

Psychedelic mushrooms are the most popularly used hallucinogen and can be easily cultivated (Strauss *et al.*, 2022a). Although the majority of these mushrooms were discovered in central America, these mushrooms occur worldwide (Gry, Andersson and Kristinsson, 2009; Strauss *et al.*, 2022c). Some of the psychedelic mushrooms include *Panaeolus*, *Pluteus*, *Psilocybe*, *Inocybe*, and *Gymnopilus*, which are known to have psilocybin, however not all psychedelic mushrooms contain this compound (Guzmán, Allen and Gartz, 1997; Musshoff, Madea and Beike, 2000; Guzmán 2008; Awan *et al.*, 2018; Strauss *et al.*, 2022a, 2022c). Other mushrooms include *Amanita*, *Conocybe*, *Copelandia*, *Cordyceps*, *Dictyophora*, *Pholiotina*, *Psathyrella*, and *Stropharia* (Guzmán, 2008; Strauss *et al.*, 2022a, 2022c). The four major psilocybin-producing genera *Psilocybe, Panaeolus*, *Gymnopilus* and *Pluteus* will be discussed in this section.

2.2.1 Psilocybe

Of all the hallucinogenic mushroom genera, *Psilocybe* is one of the most well-known, with an estimated 150 species occurring all over the world with the exception of Antarctica (Borovička *et al.*, 2011; Dhanasekaran *et al.*, 2020; Strauss *et al.*, 2022a). *Psilocybe* is a member of the agaric family Strophariaceae (Noordeloos, 1995; Boekhout *et al.*, 2002; Guzmán and Cortez, 2005). *Psilocybe* species contain the hallucinogenic alkaloids psilocybin, psilocin, and

baeocystin, and the concentration levels of these alkaloids differ between species (Beug and Bigwood, 1981; Strauss *et al.*, 2022a).

Psilocybe mushrooms are regularly found in substrates such as soil, dung, wood, and mosses (Stamets, 1996; Strauss *et al.*, 2022a). The ecology of species is described by two categories (Borovička *et al.*, 2011; Strauss *et al.*, 2022a). In one group, the species grow on the debris of plants in locations such as gardens, parks and forests, and in the other group the species grow on dung, in pastures or meadows (Borovička *et al.*, 2011; Strauss *et al.*, 2022a). *Psilocybe cubensis*, the most commonly known species can be seen growing on cattle faeces or surrounding areas (Ramírez-Cruz *et al.*, 2013).

'Little brown mushrooms' (LBMs) is a common term coined to describe the morphology of *Psilocybe* mushrooms (Li, Yuan and Liang, 2014; Strauss *et al.*, 2022b). These mushrooms have a viscid cap when moist, a dark to purplish black coloured spores and a dark purple-brown spore print (Melgarejo-Estrada *et al.*, 2020; Strauss *et al.*, 2022a). Several other distinguishing traits that make these mushrooms easy to identify include mycenoid or collyboid sections, gelatinous pellicle that can separate and white coloured gill edges (Stamets, 1996; Ramírez-Cruz *et al.*, 2013; Strauss *et al.*, 2022a). *Psilocybe* was classified into sixteen subgenera based on the following criteria; the morphology of the fruiting bodies, the thickness of the spore walls, the shape of the spores, and whether or not it has cheilocystidia and pluerocystidia (Guzmán, 1978; Strauss *et al.*, 2022a).

2.2.2 Panaeolus

Panaeolus, a genus with an estimate of 77 species, also forms part of the hallucinogenic group (Bustillos *et al.*, 2014; Silva-Filho, Seger and Cortez, 2019). Richard Evans Schultes, an American biologist, mistakenly identified *Panaeolus sphinctrinus* as one of the used mushrooms among the indigenous people of Mexico (Davis, 1996), but this erroneous reporting has subsequently been rectified (Guzmán-Dávalos *et al.*, 2008). The family relationship of this genus remains to be resolved (Strauss *et al.*, 2022a) and was once thought to belong to the Coprinaceae, however, upon further investigation it was assigned to the Strophariaceae mainly because of the presence of chrysocystidia and the color of the basidiospores (Strauss *et al.*, 2022a). Some publications recognize *Panaeolus* as part of the Psathyrellaceae family (Kaur, Kaur and Malik, 2014), while others recognize *Panaeolus* as forming part of the Bolbitiaceae family (Ajana *et al.*, 2020). In an effort to overcome this challenge, He et al. (2019) and Wijayawardene et al. (2020) assigned *Panaeolus* to the taxonomic category

Incertae sedis, which is designated for taxa having larger relationships that are undefined or unknown (Strauss *et al.*, 2022a). Recent DNA sequence results, however, assigned the genus to the Galeropsidaceae family (Kalichman, Kirk and Matheny, 2020; Strauss *et al.*, 2022a).

This genus contains high levels of psilocin and psilocybin, with the commonly known species, *Panaeolus cyanescens*, having higher levels than *Psilocybe* (Musshoff, Madea and Beike, 2000; Maruyama *et al.*, 2003; Kaur, Kaur and Malik, 2014; Strauss *et al.*, 2022a). *Panaeolus* can be coprophilic with species found in manure heaps or livestock dung such as that of horses, cows, buffalos, or elephant dung (Ediriweera, 2015; Wang and Tzean, 2015). For example, four species in the *Panaeolus antillarum* group were isolated from wild elephant dung in Thailand's Khao Yai National Park (Desjardin and Perry, 2017). Non-coprophilic *Panaeolus* species such as *Panaeolus bisporus* grow on grassy lawns and in pastures, fruiting when the environment is moist (Stamets, 1996; Senn-Irlet, Nyffenegger and Brenneisen, 1999; Kaur, Kaur and Malik, 2014)

Panaeolus are also termed LBMs and can range in colour from grey to brown to black and have a bell-shaped or conical cap, gills that are attached, long stalks, and a brown to purple to black coloured spore print (Stamets, 1996; He *et al.*, 2019; Strauss *et al.*, 2022a). *Panaeolina foenisecii* have distinguishing features such as a convex-to-bell-shaped cap, and their gills develop into a mottled darker brown color as spore mature (Goldman and Gryzenhout, 2019). *P. foenisecii was* previously classified in the genus *Panaeolus* but is now a separated group because of microscopic features (Goldman and Gryzenhout, 2019).

2.2.3 Gymnopilus

Gymnopilus includes over 200 species classified in the Agaricales as either in the Strophariaceae (1980) or Cortinariaceae (1986) (Guzmán-Dávalos *et al.*, 2003; Holec, 2005). However, *Gymnopilus* is not linked to the above mentioned families, as it is has become part of a separate clade termed "Gymnopilae" (Matheny *et al.*, 2006; Campi *et al.*, 2021), a member of the Hymenogastraceae family (Kirk et al. 2008; Campi et al., 2021). The The psychoactive compounds baeocystin, psilocin and psilocybin can be found in *Gymnopilus* (Gartz, 1989; Gry, Andersson and Kristinsson, 2009; Strauss *et al.*, 2022a).

This genus has an abundant number of xylophagous species, species feeding on or in wood, (Guzmán-Dávalos and Ovrebo, 2001; Colavolpe and Albertó, 2014; Campi *et al.*, 2021).

Gymnopilus species are distinguished by the presence yellow fruiting bodies as well as lamellae, ferruginous spore print, and the absence of a spore germ pore (Colavolpe and and Albertó, 2014; Campi *et al.*, 2021).

2.2.4 Pluteus

Pluteus is a very species-rich genus and geographically widespread with more than 500 species reported worldwide and forms part of the family *Pluteaceae* (Malysheva, Malysheva and Justo, 2016; Strauss *et al.*, 2022c). Molecular evidence supports the traditional division of *Pluteus* into three groups (*Pluteus, Celluloderma,* and *Hispidoderma*) (Justo *et al.*, 2010; Strauss *et al.*, 2022c). *Pluteus* contains psilocybin-producing species, including *Pluteus atricapillus*, *Pluteus nigroviridis*, *Pluteus cyanopus*, *Pluteus villosus, Pluteus glaucus*, *Pluteus salicinus* (Saupe,1981; Strauss *et al.*, 2022a).

The genus *Pluteus* is very prevalent in forested habitats and decaying wood (Menolli *et al.*, 2014). This genus inhabits sandy regions, humus, and on living plant tissue and is typified by *P. cervinus*, which is comprised of saprobic agaricoid fungi described for growing most commonly on decayed wood (Menolli and Capelari, 2010; Lezzi *et al.*, 2014).

Pluteus has a pilepellis as a cutis and metuloid pleurocystidia (Justo *et al.*, 2010). The other two sections are without metuloid pleurocystidia (Justo *et al.*, 2010). *Hispidoderma* pileipellis is comprised of elements made longer spatially as a cutis, one of either a trichoderm or a hymeniderm, and the *Celluloderma* pileipellis is comprised of clavate, short or spheropedunculate elements formed as a hymeniderm, with the transitions to an epithelium (Justo *et al.*, 2010).

2.3 Psilocybin-containing species from South Africa

A number of hallucinogenic mushrooms occur in South Africa. These mushrooms include species of *Psilocybe, Panaeolus, Gymnopilus* and *Pluteus* found across all provinces of South Africa and are summarized (Table 1).

The table was compiled from the Gryzenhout (2021) guide for South African mushrooms The information in table was supplemented by Kinge et al., (2020)'s documentation of psilocybin-producing mushrooms from South Africa based on fungorium and relevant literature as well as online resources and websites such as www. themycologyblog.com and the database of the National Collection of Fungi of South Africa. Studies have not been done in South Africa to confirm if these mushrooms are indeed hallucinogenic or not, and to verify the psilocybin and psilocin levels in the mushrooms.

2.4. Morphological identification

All fungi that have primary structures such as basidia and cystidia on gills are referred to as mushrooms (Tsujikawa *et al*., 2003). These fungal organisms can live above or beneath the surface of the ground (Elkhateeb *et al.*, 2021), may vary in size and can be extracted from their natural habitat (Cho, Myint and Khin, 2010). In the taxonomy of fungi, the shape, occurrence of stipe and spore size of mushrooms are characteristics frequently used to identify species (Parmasto & Parmasto 1987; Kauserud, Tsujikawa *et al*., 2003; Colman and Ryvarden, 2008). The nutritional mode and the rot type of mushrooms are vital components in identification (Kauserud, Colman and Ryvarden, 2008). The variation between mushrooms are influenced by factors such as the developmental stages of the species, the availability of key macronutrients essential for growth in the soil such as nitrogen and phosphorous and finally weather conditions (Tsujikawa *et al.*, 2003; Zhang *et et al.*, 2017).

The traditional approach to identifying mushrooms relies solely on the combination of morphological and ecological characteristics. Analysis of fruiting bodies, biological and ecological characteristics, as well as cultivation of fungi on different media to distinguish between mycelia of various species (Gadd, Watkinson and Dyer, 2007; Ao, Deb and Rao, 2020) for identification. This information is then compared against the description of other species using the principles and methods of taxonomy (Gadd, Watkinson and Dyer, 2007; Ao, Deb and Rao, 2020).

Identification of fungi to the species level presents several difficulties because of the great diversity within and between fungal species and could be ineffective taking into account species similarities (Tekpinar and Kalmer, 2019). The taxonomy of these species rely on three principles; classification, nomenclature and systematics (Gadd, Watkinson and Dyer, 2007). The current state of fungal taxonomy is in turmoil because many known species have similar variants and numerous undescribed species exist (Gadd, Watkinson and Dyer, 2007). Mycologists are integrating the traditional methods centred on morphological and ecological traits into currently available methods that use molecular traits (Gadd, Watkinson and Dyer, 2007). Ultimately, it is important to establish reliable and accurate identification techniques that overcome the inaccuracies and errors associated with using traditional methods (Ao, Deb and Rao, 2020). Additionally, the most frequent misleading causes for misidentification of mushroom species is the similarity in morphology and colour of these species (Ao, Deb and Rao, 2020). Furthermore, these approaches ultimately can take a long time to complete (Appiah, Agyare and Luo, 2017; Ao, Deb and Rao, 2020).

2.5. DNA Sequence comparisons

To prevent misidentification, a reliable identification system had to be developed, and in instances where morphological traits ultimately failed, molecular data provided a method to distinguish species phylogeny and taxonomy (Mutanen *et al.*, 2015; Ghorbani, Saeedi and De Boer, 2017; Maharachchikumbura *et al.*, 2021). There are different molecular techniques that are used for identification, including Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and real time PCR (q PCR) (Ben Abda *et al.*, 2011). DNA sequencing comparisons are also commonly used and are informative, especially for unknown samples difficult to identify. These comparisons provide parameters for precision and collectively include a valid taxonomic name, sampling details, accession information, verifiable raw sequence reads and data (Fajarningsih, 2016). Data from molecular systematics, primarily based on ribosomal DNA sequences, have revealed previously unknown natural in fungi (Lee, Cole and Linacre, 2000; Moncalvo *et al.*, 2002).

At present, DNA sequences represented by distinct and different regions of the genome have progressed into molecular data for species identification, exceeding the limits reached by traditional methods of identification (Tekpinar and Kalmer, 2019). Ultimately, to achieve species identification in fungi, molecular markers are used to target DNA regions that are aligned to sequences in the database to locate matching sequences, and this is followed by phylogenetic studies to determine which clades will define the species in question (Tekpinar and Kalmer, 2019). Further discussion of the different genes used for fungal species identification can be found in this section.

2.5.1 rRNA genes used for identification of fungi

Nuclear ribosomal RNA (rRNA) regions are used for fungal identification because of their ability to align to conserved primer sites without the redundancy of the third codon, and also maintain a high sequence variability aimed at species level identification of fungal organisms (Porras-Alfaro *et al.*, 2014). These rRNA regions are often used as molecular markers. These include the large subunit (LSU) gene (Gollotte, Van Tuinen and Atkinson, 2004; Pivato *et al.*, 2007; Rosendahl, McGee and Morton, 2009; Stockinger, Krüger and S chüßler, 2010), the small subunit (SSU) gene (Helgason, Fitter and Young, 1999; Wubet *et al.*, 2006; Lee, Lee and Young, 2008; Stockinger, Krüger and Schüßler, 2010; Nadimi, 2014), and the internal transcribed spacer (ITS) region (Wubet *et al.*, 2004; Hempel, Renker and Buscot, 2007; Stockinger, Krüger and Schüßler, 2010).

2.5.1.1 The Internal Transcribed Spacer (ITS) region

The ITS region originates in the fungal DNA as multiple copies and demonstrates intraspecies similarity and interspecies variation (Tisserat, N.A., Hulbert, S.H. and Sauer, K.M., 1994; Ueng *et al.*, 1998). The base pair length varies between 450 and 750 bp (Op De Beeck *et al.*, 2014). Extensive research studies have used the ITS region as a DNA barcode, and the related sequences are documented in public online databases such as Genbank and EMBL as a reference (Samson *et al*., 2010; Fajarningsih, 2016). According to a quantitative analysis of 244 studies that were published between the period of 1998 and 2003, 66% of those publications contained ITS sequence data, and 34% of the published phylogenetic hypotheses were solely based on ITS sequences (Álvarez and Wendel, 2003). Early studies of White et al. (1990) provided primers for amplifying ITS sequences samples for the majority of fungal and plant species and were set in the way so that ITS sequence data could be easily accessible. The ITS region is polymorphic, and can therefore distinguish between sequences at the species level (Samson *et al*., 2010; Stockinger, Krüger and Schüßler, 2010; Schoch *et al.*, 2012; Fajarningsih, 2016).

There are a number of short comings associated with the use of these genome regions. The occurrence of polymorphism within sequences from various taxonomic groups, on the contrary, can also complicate identification (Aanen, Kuyper and Hoekstra, 2001; Nilsson *et al.*, 2008; Tekpinar and Kalmer, 2019). The ITS region may not always distinguish between species, especially cryptic species and consists of multiple, possibly variable, copies in the genome (Schoch *et al.*, 2012; Stielow *et al.*, 2015; Magray *et al.*, 2019). This region is not effective for differentiating many fungal species that are closely related, particularly when they have been cloned and have difficulties with intraspecific variation (Kiss, 2012). Additionally,

the resolution for more complex taxonomic level fungal relationships for ITS is low in quality (Seifert, 2009; Begerow *et al.*, 2010; Schoch *et al.*, 2012; Stielow *et al.*, 2015).

Several molecular genetic mechanisms have implications for the accuracy of ITS sequences which could result in incorrect phylogenetic inference (Álvarez and Wendel, 2003). Homoplasy is a similarity that arises from independent evolution rather than inheritance at the hierarchical level and is identified by a lack of alignment with other traits in a phylogenetic study (Wake, Wake and Specht, 2011). Homoplasy affects whole genomes in the same way that it affects nucleotide sequences (Gaertig *et al.*, 2013). Homoplasy is more elevated in the ITS region than in any other DNA sequence data sets as a result of mutations, misalignment, sequencing errors, or all three of the factors combined (Álvarez and Wendel, 2003).

ITS sequences are able to undergo a process known as concerted evolution, a process in which nucleotide sequences evolve together rather than individually (Álvarez and Wendel, 2003). This occurs when the variation between repeated copies in the sequence becomes subjected to mechanisms such as gene conversion or unusual crossing over. As a consequence, the genetic variation within the genome is reduced, thus affecting the outcomes of phylogenetic reconstruction (Álvarez and Wendel, 2003; Small, Cronn and Wendel, 2004). In contrast, single-copy nuclear genes that are biparentally inherited are not subject to concerted evolution and have gene variants that minimize alignment ambiguity and improve the outcomes of homologous analysis (Bailey and Doyle, 1999; Cronn, Small and Wendel, 1999; Bortiri *et al.*, 2002; Sang, 2002; Álvarez and Wendel, 2003; Bailey *et al.*, 2003; Senchina *et al.*, 2003). Additionally, compared to ITS data results, single-copy nuclear genes exhibit decreased homoplasy (Álvarez and Wendel, 2003). Due to these factors, Álvarez and Wendel (2003) proposed using multiple or more single-copy nuclear loci (Cronn *et al.*, 2002; Marcela, 2021).

2.5.1.2 Small subunit (SSU)

The SSU region which is positioned directly upstream to the ITS region, evolves more gradually than the related ITS and LSU regions, which results in reduced variance across examined species (Mitchell and Zuccaro, 2006; Tekpinar and Kalmer, 2019). In phylogenetics, the SSU is mostly used at higher taxonomic classifications as it contains less hypervariable domains in fungi, contrary to the homolog (16S), which is generally used to identify bacteria species (Schoch *et al.*, 2012).

However, for phylogenetic analyses of Basidiomycota, the SSU region is used in conjunction with other nuclear molecular DNA or protein-coding regions (Skrede *et al.*, 2011; Zhao *et al.*,

2017; Tekpinar and Kalmer, 2019). Still, there are few studies that identified Basidiomycota species using the SSU region (Tekpinar and Kalmer, 2019). In a study, Swann and Taylor (1993) characterized Basidiomycota using the SSU region for the reason that the SSU region limited resolution in the 5.8S rRNA sequence, which was their region of interest (Tekpinar and Kalmer, 2019). The SSU region is an excellent region for detecting conserved regions at the genus or higher taxonomic level because of the previously mentioned slow rate of molecular evolution (White *et al*., 1990; Wu *et al.*, 2003; Tekpinar and Kalmer, 2019).

The region is restricted to classifying fungi at a lower taxonomic level and the variation present is insufficient for differentiation at the species level (White *et al*., 1990; Wu *et al.*, 2003; Cole *et al.*, 2014; Tekpinar and Kalmer, 2019). Regardless of the above stated, there is still a lack of SSU sequence accuracy in database entries making it a less optimal marker, and this needs to be an area that is focused on (Tekpinar and Kalmer, 2019). Additionally, the validity of SSU sequence deposits in the current database is low, rendering it a less suitable marker (Tekpinar and Kalmer, 2019). This should therefore be a specific area of focus in research using this marker.

2.5.1.3 Large subunit (LSU)

The LSU region, which is positioned immediately downstream of the ITS region, is generally used to address the shortcomings in fungal phylogenetics (Tekpinar and Kalmer, 2019). Similarly, relationships between basidiomycetes at the genus and family level (Bruns, Lee and Taylor, 1990; Koziak, Kei and Thorn, 2007) are best resolved by this gene (Fell *et al.*, 2000; Moncalvo *et al.*, 2000, 2002; Wei and Oberwinkler, 2001). The two hypervariable domains of LSU; D1, and D2, present in fungi are the regions of interest for research (Raja *et al.*, 2017; Tekpinar and Kalmer, 2019). This is demonstrated by Porras-Alfaro et al. (2014), indicating that longer sequences provide higher discriminatory power than shorter length sequences for both the ITS and LSU regions (Tekpinar and Kalmer, 2019). This research further supports that LSU is the best conservative molecular marker for species identification in fungal groups (Fell *et al.*, 2000; Moncalvo *et al.*, 2000, 2002; Wei and Oberwinkler, 2001; Matheny *et al.*, 2007; Zhao, Luo and Zhuang, 2011; Schoch *et al.*, 2012; Tekpinar and Kalmer, 2019).

Although there are no complications related to the process of alignment, amplification, editing, or sequencing when using the LSU region (Schoch *et al.*, 2012; Tekpinar and Kalmer, 2019), issues such as low-quality data and variable sequence length are still encountered in LSU databases. The limitation associated with the LSU gene would be that it discriminates between

802 species (Schoch *et al.*, 2012). Even though rRNA genes are mostly used in molecular
803 systematic investigations of fungi, some fungal taxa may exhibit poor resolution results
804 from SSU and LSU (Tekpinar and Kalmer, 2019).

805 2.5.2 Other genes that identify fungi

806 Housekeeping genes are regions found in a genome and are highly conserved (Zhang and Li,
807 2004). These regions are fundamental to the existence of a cell and can be studied across the
808 target organism by designing PCR primers complementary to nucleotide sequences to amplify
809 more variable intervening regions (Bustin, 2002; Andersen, Jensen and Ørntoft, 2004;
810 Brunner, Yakovlev and Strauss, 2004; Dheda *et al.*, 2004; Makhzani and Frey, 2014).
811 Housekeeping genes that are widely used include α-tubulin and β-tubulin genes, actin (ACT),
812 glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and protein-coding genes such as
813 translocation elongation factor-1 (TEF1-α) and the RNA polymerase II (RPB 1) (Goidin *et al.*,
814 2001; Kim *et al.*, 2003; Radonić *et al.*, 2004; Siaut *et al.*, 2007; Makhzani and Frey, 2014).
815 Additional candidate DNA barcoding markers beside the ITS regions are referred to as
816 secondary barcode markers (Stefani, Jones and May, 2014). This was established to assist
817 in the resolution of taxonomic groups of species that ITS has failed to resolve (Brazee *et al.*,
818 2011; Gazis, Rehner and Chaverri, 2011; Stefani, Jones and May, 2014). In order to become
819 a secondary identification marker, a prospective gene has to meet the following criteria: the
820 gene is required to be easy to amplify and be capable of differentiating between closely related
821 species (Visagie *et al.*, 2014). Additionally, it is also fundamental that species in every genus
822 be represented in the reference dataset (Visagie *et al.*, 2014).

823 Protein-coding genes are commonly used for species identification and provide a useful tool
824 to complement the phylogenetic analysis of rRNA regions for the resolution of relationships at
825 different taxonomic levels. The key benefit of using protein-coding genes is that these genes
826 exist in the fungal genome as a single copy, eliminating the potential challenges associated
827 with paralogs (Tekpinar and Kalmer, 2019). The introduction of the use of protein-coding
828 genes use for establishing significant phylogenetic relationships has been demonstrated by
829 many studies (Liu, Whelen and Hall, 1999; Brandon Matheny *et al.*, 2002; Tanabe *et al.*, 2004;
830 Tekpinar and Kalmer, 2019).

831 2.5.2.1 The RNA polymerase II (RPB 1)

832 The RPB 1 is useful because it has a universal copy and it has a slow sequence divergence
833 rate (Schoch *et al*., 2012). In order to investigate fungi at lower taxonomic levels, the upper
834 end of the RPB1 gene and the region between the 6th and 7th conserved domains of the RPB
835 2 genes were used because they contain a substantial amount of parsimony-informative sites
836 (Liu, Whelen and Hall, 1999; Brandon Matheny *et al.*, 2002; Tanabe, Watanabe and Sugiyama,

2002). RPB1 became a valuable marker based on the genes ability to be easily amplified (Tanabe *et al.*, 2004; Tekpinar and Kalmer, 2019) and was used to identify and describe species in the genus *Inocybe* and resolve *Inocybe* species phylogeny (Matheny, 2005). Additionally, RPB1 together with RPB2 were used together with LSU and SSU to examine the phylogeny of various genera of Ascomycota (Brandon Matheny *et al.*, 2002; Reeb, Lutzoni and and Roux, 2004; Tanabe *et al.*, 2004; Frøslev, Matheny and Hibbett, 2005; Matheny, 2005; Matheny *et al.*, 2007; Schoch *et al.*, 2012; O'Donnell *et al.*, 2013; Dokianakis *et al.*, 2018; Zhang *et al.*, 2021).

An incorrect copy number in the genome these genes may cause a problem because it affects PCR and sequencing successes (Tekpinar and Kalmer, 2019). Even though most fungal species have only one copy of the RPB1 and RPB2 genes, *Chytridiomycota* and *Zygomycota* indicated contradicting results by demonstrating two RPB1 genes with slightly different sequences and two comparable RPB2 copies (Liu, Hodson and Hall, 2006; Tekpinar and Kalmer, 2019). The RPB1 and RPB2 genes are capable of completely resolving species, but cannot provide a complete solution (Tekpinar and Kalmer, 2019). The biggest challenges these genes present include negative amplification results during PCR cycles, sequencing difficulties, and alignment issues (Tekpinar and Kalmer, 2019). Additionally, the performance of PCR reactions and sequencing results are affected by the genes genome copy number (Tekpinar and Kalmer, 2019). These complications restrict RPB1 and RPB2 from serving as universal barcodes (Tekpinar and Kalmer, 2019).

2.5.2.2 Translation elongation factor 1-α

The TEF1-α genes are used for more species-specific identification databases. The aminoacyl-tRNA coupling mechanism to ribosomes is mediated by the expression translation elongation factor EF1 protein, a signal encoded by the translational elongation factor 1-alpha gene (Triana-Alonso, Chakraburtty and Nierhaus, 1995; Zhao, Luo and Zhuang, 2011). Although primers often vary between genera because of the significant level of sequence polymorphism between them, the TEF1-α genes was taken into consideration as a possible candidate for a molecular marker (O'Donnell, 2000; Mirhendi *et al.*, 2015). Research from Stielow et al. (2015) suggested primers EF1-1018F and EF1-1620R as secondary universal DNA barcode primer pair for fungi. These findings revealed the promising results of using the phylogenetic approach that relies on TEF1-α sequences as an identification tool for differentiating and classifying *Armillaria* mushroom species (Zhao, Luo and Zhuang, 2011). In the investigation to find the best molecular marker, factors such as PCR amplification, and the variance existing between and among species were taken into consideration (Zhao, Luo and Zhuang, 2011). Whilst sequencing and PCR amplification of the TEF1-α gene was not particularly successful, the inter-specific distances were greater than the intra-specific distances (Zhao, Luo and Zhuang, 2011; Tekpinar and Kalmer, 2019).

2.5.2.3 β-tubulin

β-tubulin from the tubulin family develops in almost all eukaryotes and is made up of highly conserved proteins (Keeling and Doolittle, 1996) making this gene an excellent candidate for phylogenetic analysis, particularly for predicting species-level phylogenies (Einax & Voigt 2003). Tubulin proteins are the major parts of microtubules, which play important functions in eukaryotic cellular activities including shape preservation, cell division, intracellular transport, and the motility of the cell (Yang, Jan and Komatsu, 2007; Tekpinar and Kalmer, 2019). β-tubulin (BenA), was used to identify fungal species in the *Penicillium* genus (Schoch *et al.*, 2012; Tekpinar and Kalmer, 2019; Visagie *et al.*, 2021) and is the entrusted marker of identification for the *Penicillium* genus (Visagie *et al.*, 2014).

Despite the preceding statement, it needs to be considered that *Penicillium* has large ambiguousy aligned sites that could potentially have a negative effect on phylogeny (Visagie *et al.*, 2014). Furthermore, the outcome of studies for other *Penicillium* species shows discrepancies that require cloning to be executed, and consequently, more research is required to determine the efficiency of the gene as a molecular marker (Schoch *et al.*, 2012; Visagie *et al.*, 2014; Stielow *et al.*, 2015).

2.6. Using multiple genes for species delimitation

The use of single-copy markers for mushrooms for extensive research is still limited, and the information known has made small advancements for biodiversity. However, these markers are now incorporated more and more into multi-locus studies. In 2007, it was reported that 82% of phylogenetic trees were based on single-locus datasets (Kauff, Cox and Lutzoni, 2007). The reason for this low production of multigene phylogenetic analyses for a large and diverse set of taxa is because of the uncoordinated nature of data sequencing (Kauff, Cox and Lutzoni, 2007).

The integration of two or more molecular markers gives way to more accurate identification results (Schoch *et al.*, 2012; He *et al.*, 2022). Various studies on mushrooms have used data from more than one gene. For example, the use of multigene phylogenies has made a way for the taxonomy of the genus *Agaricus* to be well described (He *et al.*, 2022). Mentioned below are research breakthroughs that support the resolution obtained by multigene findings.

2.6.1 Multi-locus identification of fungi

One of the biggest in-depth multi-locus phylogenies to date is that of James et al. (2006), which was made up of some of the commonly used genes including the 5.8S rRNA, 18S rRNA,

and the 28S rRNA as well as the protein-coding genes RPB 1 and RPB 2, and the TEF1-α. This study recognized that the phylogenetic resolution between fungal basal groups and their relative relationships to the phyla Ascomycota and Basidiomycota, is important in the overall knowledge of the sequence of events preceding the evolution and colonization of land ecosystems (James *et al.*, 2006). The results of this multi-locus phylogeny further revealed that approximately four individual disruptions led to the loss of the cellular structure that is responsible for motility, i.e. the flagella that occurred throughout the evolution of the Kingdom Fungi (James *et al.*, 2006; Stielow *et al.*, 2015). However, the lack of standardized sets of procedures for acquiring and sequencing data creates obstacles in the development of large-scale multi-locus phylogenetic trees across a wide variety of species, accounts for the lack of studies similar to the one seen in James *et al.*, 2006.

By using multi-locus phylogeny comprised of RPB 1, RPB 2, SSU, and ITS markers, wild mushrooms that was found in local markets of India were able to be positively identified into the correct species description, for example *Tricholoma viridiolivaceum* and *Laccaria vinaceoavellanea* (Khaund and Joshi, 2014). Other noteworthy multi-locus studies included the multi-locus sequence analyses (MLSA) of the basidiomycetous yeast *Papiliotrema flavescens* using ITS and LSU sequences that highlighted the significant shortcomings of the two markers, while somehow still confirming the presence of two new cryptic species within the group (He *et al.*, 2022).

2.6.2 Multi-locus identification of psilocybin - producing mushrooms

There are many publications that are focused on psilocybin-producing mushrooms, and most of them support the use of multiple genes. These reports suggest that integrating nuclear rRNA genes with protein-coding genes, i.e., including genes such as β-tubulin, RPB 1, and RPB2 in multi-genes, will increase the identification potential and the number ot the branches in phylogenetic trees (Hansen, LoBuglio and Pfister, 2005; Hofstetter *et al.*, 2007; Tang, Jeewon and Hyde, 2007; Raja *et al.*, 2011). Thus far, the prevalent multi-locus studies include those of the LSU, RPB 1 (Böttcher *et al.*, 2022) and TEF1-α in addition to the ITS regions, for the the identification of *Psilocybe cubensis* (Raja *et al.*, 2017; Zhang *et al.*, 2021). These studies, however, only included a limited number of species (Raja *et al.*, 2017; Zhang *et al.*, 2021). Remarkably, magic mushrooms were identified from Japanese local markets using LSU and ITS, which was significant because they were in dry powders or capsules (Maruyama *et al.*, 2006). This method was done because it is highly challenging to determine the species of these mushroom solely based on morphological description (Maruyama *et al.*, 2006).

Other psilocybin-producing mushrooms have also been successfully identified using multiple genes. The diversity present in the ITS region significantly led to the positive identification of the new species *Gymnopilus swaticus* through the use of the LSU and ITS regions (Schoch *et al.*, 2012; Khan *et al.*, 2017; He *et al.*, 2022; Strauss *et al.*, 2022). The use of the molecular markers SSU, LSU, and ITS regions enabled the identification of *Pluteaceae* division in the direction of *Celluloderma*, *Hispidoderma*, and *Pluteus* (Justo *et al.*, 2011). Furthermore, the combination of the TEF1-α genes and the Internal Transcribed region effectively identified *Pluteus hubregtseorum* (Ševcikova *et al*., 2021).

2.6.3 South African multi-gene studies

In South African studies, there have been important developments inclusive of morphological and multi-locus phylogenetic identification. According to a recent study, eight different species of the genus *Ganoderma* were identified from all over the country, with two novel species discovered, by using β-tubulin, ITS, and TEF1-α (Tchotet Tchoumi *et al.*, 2019). Now 13 species of the South African *Ganoderma* population are known, including the two new reports *Ganoderma* cf. *cupreum* and *Ganoderma* cf. *resinaceum* (Tchotet Tchoumi *et al.*, 2019). From the above-mentioned, we now know the importance and impact that multigene studies can have. An in-depth multigene phylogeny for South African mushroom species is critically needed to understand the existing phylogenetic relationships, eliminate cryptic species and the complexities associated with these species as well as discover novel species (Goldman and Gryzenhout, 2019; Gryzenhout, 2021).

2.6.4 Limitations of using multi-locus genes

Unfortunately, several studies showed that combining more than one gene region may not always provide clear answers. For example, combining ITS and LSU with the aim of a pairwise comparison to separate taxa for *Mrakia gelida*, *Solicoccozyma aeria*, *Saitozyma podzolica*, and *Vishniacozyma victoriae* did not produce the best results (He *et al.*, 2022). When the results from this study were compared with sequences from publicly available resources, closely related species could not be differentiated (Yurkov et al. 2015; He *et al.*, 2022). To achieve effective analysis of multiple loci, the addition of protein-coding genes in conjunction with the precursive ITS and LSU may provide more resolution power, and the most appropriate gene or genes for the particular taxonomic group must be investigated first.

2.7. DNA Barcoding

Identification and classification completely rely on DNA barcoding, which is the use of DNA sequences of several standardized genetic markers (Schoch *et al.*, 2012; Stielow *et al.*, 2015;

Crous *et al.*, 2021). DNA barcoding plays an important role in identification in research related to biotechnology, conservation biology, ecology as well as evolutionary biology (Schoch *et al.*, 2012). Up to this point, DNA barcoding has proven to be an extremely beneficial tool for minimizing ambiguity in species identification (Schoch *et al.*, 2012). DNA barcoding requires standardized sequences to classify species using suitable primers that are relevant to the largest taxonomic group (Schoch *et al.*, 2012; Stielow *et al.*, 2015; Magray *et al.*, 2019). It is quite a powerful tool for species identification and discovery (Schoch *et al.*, 2012).

DNA barcoding depends on reliable reference libraries for which unknown specimens can be compared (Sun *et al.*, 2016). Therefore, by comparing new data against previously published sequences, the taxonomic reliability of the DNA barcodes is established (Sun *et al.*, 2016). To evaluate DNA barcoding as one of the mechanisms for biodiversity investigation, a classification system that predicts the species present in the specimen sample from the sequence data is required and simultaneously assigns taxonomic names independently from the previous taxonomic species assignments (Barcaccia, Lucchin and Cassandro, 2016; Sun *et al.*, 2016).

The DNA barcoding initiative has significantly improved the strategy for achieving sequence accuracy by establishing a benchmark that each considered molecular marker must meet. These parameters for precision collectively included a valid taxonomic name, sampling details, and accession information, verifiable raw sequence reads and data (Fajarningsih, 2016).

The All Fungi Barcoding meeting first acknowledged the ITS region as the best candidate for the barcoding of all the Fungal Kingdom in 2007 (Rossman 2007; Tekpinar and Kalmer, 2019). Following this, Schoch et al. (2012) reported that the ITS region was the official barcode for fungi (Begerow *et al.*, 2010; Bellemain, 2010; Stielow *et al.*, 2015; Acar, Dizkirici and Kalmer, 2017; Beker *et al.*, 2018; Vu *et al.*, 2019; Crous *et al.*, 2021) because it can be effortlessly amplified and has a big enough barcode gap (Schoch *et al.*, 2012; Tekpinar and Kalmer, 2019) Using the ITS region as a DNA barcode for fungal identification has numerous advantages, including high sequence diversity, a high number of copies per cell, conserved primer sites, and a large database of sequences (Tekpinar and Kalmer, 2019). It is fundamental to have genes that can accurately identify species, and for this reason other regions are considered. TEF1-α region is proposed as the secondary universal DNA barcode primer pair for fungi (Stielow *et al.*, 2015).

2.8 Conclusion

Psilocybin-producing mushroom species of the world are increasingly studied because of the numerous medical and psychiatric applications that can be developed from them. These mushrooms are also commonly used recreationally, but also have traditional use in many cultures of the world. However, their biodiversity in many regions is however, also is poorly studied and numerous novel species await discovery and description.

It is important to point out that morphology-derived identifications done by citizen mycologists or in scientifically executed publications, are not always reliable. In addition to morphology, the use of DNA sequence markers will be crucial for accurately identifying specimens, including those that are dry, incomplete or in the form of powder. Furthermore, it will assist in clarifying any taxonomic or misidentification problems. It is possible to include these different genes in novel research to improve identifications because sequences for genes other than ITS for these fungi are already available.

References

Aanen, D.K., Kuyper, T.W. and Hoekstra, R.F., 2001. A widely distributed ITS polymorphism within a biological species of the ectomycorrhizal fungus *Hebeloma velutipes*. *Mycological Research*, 105(3), pp.284-290.

Acar, I., Dizkirici, A. and Kalmer, A. and Uzun, Y., 2017. Phylogenetic relationships and taxonomical positions of two new records *Melanoleuca* species from Hakkari province, Turkey. *Biological Diversity and Conservation*, 10(3), pp.58-93.

Agurell, S. and Nilsson, J.L., 1968. A biosynthetic sequence from tryptophan to psilocybin. *Tetrahedron Letters*, 9, pp.1063-1064.

Agurell, S. and Nilsson, J.G.L., 1968. Biosynthesis of psilocybin. *Acta Chem. Scand*, 22(4).

Ajana, M., El Kholfy, S., Ouabbou, A., Nmichi, A., Ouazzani Touhami, A., Benkirane, R. and Douira, A., Study of *Panaeolus semiovatus* (Sowerby) S. Lundell and Nannf., (1938), A coprophilous fungus, *Plant Archives*, 20(1), pp. 1856–1858.

Álvarez, I.J.F.W. and Wendel, J.F., 2003. Ribosomal ITS sequences and plant phylogenetic inference. *Molecular phylogenetics and evolution*, 29(3), pp.417-434.

Andersen, C.L., Jensen, J.L. and Ørntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer research*, 64(15), pp.5245-5250.

Ao, T., Deb, C.R. and Rao, S.R., 2020. Molecular strategies for identification and characterization of some wild edible mushrooms of Nagaland, India. *Molecular Biology Reports,* 47(1), *pp.621-630.*

Appiah, T., Agyare, C. and Luo, Y., 2017. Molecular identification of some Ghanaian mushrooms using internal transcribed spacer regions, *Molecular Biology*, 06(03), pp. 3–7.

Azeem, U., Hakeem, K.R. and Ali, M., 2020. Fungi for Human Health. *Springer.*

Bailey, C.D., Carr, T.G., Harris, S.A. and Hughes, C.E., 2003. Characterization of *and* angiosperm nrDNA polymorphism, paralogy, and pseudogenes. *Molecular phylogenetics evolution*, 29(3), pp.435-455.

Bailey, C.D. and Doyle, J.J., 1999. Potential phylogenetic utility of the low-copy nuclear gene pistillata in dicotyledonous plants: comparison to nrDNA ITS and trnL intron in *Sphaerocardamum* and other *Brassicaceae*. *Molecular phylogenetics and evolution*, 13(1), pp.20-30.

Barcaccia, G., Lucchin, M. and Cassandro, M., 2015. DNA barcoding as a molecular tool to track down mislabeling and food piracy. *Diversity*, 8(1), p.2.

Begerow, D., Nilsson, H., Unterseher, M. and Maier, W., 2010. Current state and perspectives of fungal DNA barcoding and rapid identification procedures. *Applied microbiology and biotechnology*, 87, pp.99-108.

Beker, H.J., Eberhardt, U., Schütz, N. and Gulden, G., 2018. A review of the genus *Hebeloma* in Svalbard. *Mycoscience*, 59(4), pp.303-309.

Bellemain, E., Carlsen, T., Brochmann, C., Coissac, E., Taberlet, P. and Kauserud, H., 2010. ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. *BMC microbiology*, 10, pp.1-9.

Ben Abda, I., De Monbrison, F., Bousslimi, N., Aoun, K., Bouratbine, A. and Picot, S., 2011. Advantages and limits of real-time PCR assay and PCR-restriction fragment length *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 105(1), pp.17-22.

Beug, M. W. and Bigwood, J., 1981. Quantitative analysis of psilocybin and psilocin and *Psilocybe baecystis* (Singer and Smith) by high-performance liquid chromatography and by thin-layer chromatography. *Journal of Chromatography A*, 207(3), pp. 379–385.

Boekhout, T., Stalpers, J., Verduin, S.J., Rademaker, J. and Noordeloos, M.E., 2002. Experimental taxonomic studies in *Psilocybe* sect. *Psilocybe*. *Mycological research*, 106(11), pp.1251-1261.

Bogenschutz, M.P., Forcehimes, A.A., Pommy, J.A., Wilcox, C.E., Barbosa, P.C. and Strassman, R.J., 2015. Psilocybin-assisted treatment for alcohol dependence: a proof-

of-concept study. *Journal of psychopharmacology*, 29(3), pp.289-299.

Borovička, J., Noordeloos, M.E., Gryndler, M. and Oborník, M., 2011. Molecular phylogeny of *Psilocybe cyanescens* complex in Europe, with reference to the position of the secotioid *Weraroa novae-zelandiae*. *Mycological Progress*, 10, pp.149-155.

Bortiri, E., Oh, S.H., Gao, F.Y. and Potter, D., 2002. The phylogenetic utility of nucleotide sequences of sorbitol 6-phosphate dehydrogenase in *Prunus* (Rosaceae). *American Journal of Botany*, 89(10), pp.1697-1708.

Böttcher, R., Schmidts, I., Nitschko, V., Duric, P. and Förstemann, K., 2022. RNA polymerase II is recruited to DNA double-strand breaks for dilncRNA transcription in in *Drosophila*. *RNA biology*, 19(1), pp.68-77.

Brazee, N.J., Hulvey, J.P. and Wick, R.L., 2011. Evaluation of partial tef1, rpb2, and nLSU sequences for identification of isolates representing *Armillaria calvescens* and *Armillaria gallica* from northeastern North America. *Fungal Biology*, 115(8), pp.741-749.

Brunner, A.M., Yakovlev, I.A. and Strauss, S.H., 2004. Validating internal controls for quantitative plant gene expression studies. *BMC plant biology,* 4(1), pp.1-7.

Bustillos, R.G., Dulay, R.M.R., Kalaw, S.P. and Reyes, R.G., 2014. Optimization of culture conditions for mycelial growth and basidiocarp production of Philippine strains of *Panaeolus antillarium* and *Panaeolus cyanescens*. *Mycosphere*, 5(3), pp.398-404.

Bustin, S.A., 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of molecular endocrinology*, 29, pp.23-39.

Campi, M., Maubet, Y., Grassi, E., Niveiro, N. and Guzmán-Dávalos, L., 2021. First contribution to the genus *Gymnopilus* (Agaricales, Strophariaceae) in Paraguay. *Rodriguésia*, 72.

Carhart-Harris, R.L., Bolstridge, M., Rucker, J., Day, C.M., Erritzoe, D., Kaelen, M., Bloomfield, M., Rickard, J.A., Forbes, B., Feilding, A. and Taylor, D., 2016. Psilocybin with psychological support for treatment-resistant depression: an open-label feasibility study. *The Lancet Psychiatry*, 3(7), pp.619-627.

Castellanos, J.P., Woolley, C., Bruno, K.A., Zeidan, F., Halberstadt, A. and Furnish, T., 2020. Chronic pain and psychedelics: a review and proposed mechanism of action. *Regional Anesthesia & Pain Medicine*, 45(7), pp.486-494.

Chi, T. and Gold, J.A., 2020. A review of emerging therapeutic potential of psychedelic drugs in the treatment of psychiatric illnesses. *Journal of the Neurological Sciences*, 411, p.116715.

Cho, N.L., Myint, T. and Khin, M.T., 2020. Some wild edible mushrooms growing in Yinmabin village, Thazi Township. In *3rd Myanmar Korea Conf. Res. J.*(Vol. 3, pp. 585-593).

Colavolpe, M.B. and Albertó, E., 2014. Cultivation requirements and substrate degradation of the edible mushroom *Gymnopilus pampeanus* - A novel species for mushroom cultivation. *Scientia Horticulturae*, 180, pp.161-166.

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras- for Alfaro, A., Kuske, C.R. and Tiedje, J.M., 2014. Ribosomal Database Project: data and tools high throughput rRNA analysis. *Nucleic acids research*, 42(D1), pp.D633-D642.

Cronn, R.C., Small, R.L., Haselkorn, T. and Wendel, J.F., 2002. Rapid diversification of the cotton genus (*Gossypium*: Malvaceae) revealed by analysis of sixteen nuclear and chloroplast genes. *American journal of botany*, 89(4), pp.707-725.

Cronn, R.C., Small, R.L. and Wendel, J.F., 1999. Duplicated genes evolve independently after polyploid formation in cotton. *Proceedings of the National Academy of Sciences,* 96(25), pp.14406-14411.

Crous, P.W., Rossman, A.Y., Aime, M.C., Allen, W.C., Burgess, T., Groenewald, J.Z. and Castlebury, L.A., 2021. Names of phytopathogenic fungi: a practical guide. *Phytopathology,* 111(9), pp.1500-1508.

Desjardin, D. E. and Perry, B. A., 2017. *Panaeolus antillarum* (Basidiomycota, Psathyrellaceae) from wild elephant dung in Thailand. *Current Research in Environmental and Applied Mycology*, 7(4), pp. 275–281.

1122 Dhanasekaran, D., Latha, S., Suganya, P., Panneerselvam, A., Kumar, T.S., Alharbi, N.S.,
1123 Arunachalam, C., Alharbi, S.A. and Thajuddin, N., 2020. Taxonomic identification and

bioactive compounds characterization of *Psilocybe cubensis* DPT1 to probe its antibacterial and mosquito larvicidal competency. *Microbial pathogenesis*, 143, p.104138.

Dheda, K., Huggett, J.F., Bustin, S.A., Johnson, M.A., Rook, G. and Zumla, A., 2004. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques*, 37(1), pp.112-119.

Dokianakis, E., Tsirigotakis, N., Christodoulou, V., Poulakakis, N. and Antoniou, M., 2018. Identification of wild-caught phlebotomine sand flies from Crete and Cyprus using DNA barcoding. *Parasites & vectors*, 11(1), pp.1-9.

Ediriweera, S., Wijesundera, R., Nanayakkara, C. and Weerasena, J., 2015. First report of *Panaeolus sphinctrinus* and *Panaeolus foenisecii* (Psathyrellaceae, Agaricales) on elephant dung from Sri Lanka. *Frontiers in Environmental Microbiology*, 1, pp.19-23.

Elkhateeb, W., Thomas, P., Elnahas, M. and Daba, G., 2021. Hypogeous and epigeous epigeous mushrooms in human health. In *Advances in Macrofungi* (pp. 7-19). CRC Press.

Fajarningsih, N.D., 2016. Internal Transcribed Spacer (ITS) as DNA barcoding to identify fungal species: a review. *Squalen Bulletin of Marine and Fisheries Postharvest and Biotechnology*, 11(2), pp.37-44.

Fell, J.W., Boekhout, T., Fonseca, A., Scorzetti, G. and Statzell-Tallman, A., 2000. Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *International journal of systematic and evolutionary microbiology*, 50(3), pp.1351-1371.

Frøslev, T. G., Matheny, P. B. and Hibbett, D. S., 2005. Lower level relationships in the mushroom genus *Cortinarius* (Basidiomycota, Agaricales): A comparison of RPB1, RPB2, and ITS phylogenies. *Molecular Phylogenetics and Evolution*, 37(2), pp. 602–618.

Gadd, G., Watkinson, S.C. and Dyer, P.S. eds., 2007. *Fungi in the Environment* (Vol. 25). Cambridge University Press.

Gaertig, J., Wloga, D., Vasudevan, K.K., Guha, M. and Dentler, W., 2013. Discovery and functional evaluation of ciliary proteins in Tetrahymena thermophila. In *Methods in enzymology* (Vol. 525, pp. 265-284). Academic Press.

Garcia-Romeu, A., R Griffiths, R. and W Johnson, M., 2014. Psilocybin-occasioned mystical experiences in the treatment of tobacco addiction. *Current drug abuse reviews*, 7(3), pp.157-

164.

Gazis, R., Rehner, S. and Chaverri, P., 2011. Species delimitation in fungal endophyte

diversity studies and its implications in ecological and biogeographic inferences. *Molecular Ecology*, 20(14), pp.3001-3013.

Ghorbani, A., Saeedi, Y. and de Boer, H.J., 2017. Unidentifiable by morphology: DNA barcoding of plant material in local markets in Iran. *PloS one*, 12(4), p. e0175722.

Goidin, D., Mamessier, A., Staquet, M.J., Schmitt, D. and Berthier-Vergnes, O., 2001. genes Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and β-actin as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. *Analytical biochemistry*, 295(1), pp.17-21.

Gollotte, A., Van Tuinen, D. and Atkinson, D., 2004. Diversity of arbuscular mycorrhizal fungi colonising roots of the grass species *Agrostis capillaris* and *Lolium perenne* in a field experiment', *Mycorrhiza*, 14(2), pp. 111–117.

Grob, C.S., Danforth, A.L., Chopra, G.S., Hagerty, M., McKay, C.R., Halberstadt, A.L. and Greer, G.R., 2011. Pilot study of psilocybin treatment for anxiety in patients with advanced-stage cancer. *Archives of general psychiatry*, 68(1), pp.71-78.

Gry, J., Andersson, C. and Kristinsson, J., 2009. *Occurrence and use of hallucinogenic mushrooms containing psilocybin alkaloids*. Nordic Council of Ministers.

Guzmán-Dávalos, L., Mueller, G.M., Cifuentes, J., Miller, A.N. and Santerre, A., 2003. Traditional infrageneric classification of *Gymnopilus* is not supported by ribosomal DNA sequence data. *Mycologia*, 95(6), pp.1204-1214.

Guzmán-Dávalos, L., Contu, M., Ortega, A., Vizzini, A., Herrera, M., Ovrebo, C., Rodríguez, A., Villalobos-Arámbula, A.R., Palomera, V., Vargas, G. and Santerre, A., 2008. New morphological and molecular data on *Gymnopilus purpureosquamulosus* and its phylogenetic relationships among similar species. *Sydowia*, 60(1), pp.41-56.

Guzmán, G., 1978. Variation, distribution, ethnomycological data and relationships of *Psilocybe aztecorum*, a Mexican hallucinogenic mushroom. *Mycologia*, 70(2), pp.385-396.

Guzmán, G., Allen, J. W. and Gartz, J., 1997. A Worldwide geographical distribution of the Neurotropic fungi, an analysis and discussion. *Africa*, 14(1939), pp. 1–107.

1182 Guzman, G. and Cortez, V.G., 2005. A new species of *Psilocybe* (Agaricales,
1183 Strophariaceae) from southern Brazil. *Mycotaxon*, 93, pp.95-98.

1184 Hansen, K., LoBuglio, K.F. and Pfister, D.H., 2005. Evolutionary relationships of the cup-
1185 fungus genus *Peziza* and *Pezizaceae* inferred from multiple nuclear genes: RPB2, β-tubulin,
1186 and LSU rDNA', *Molecular Phylogenetics and Evolution*, 36(1), pp. 1–23.

1187 Hase, A., Erdmann, M., Limbach, V. and Hasler, G., 2022. Analysis of recreational
1188 psychedelic substance use experiences classified by substance. *Psychopharmacology*,
1189 pp.1-17.

1190 He, M.Q., Zhao, R.L., Hyde, K.D., Begerow, D., Kemler, M., Yurkov, A., McKenzie, E.H.,
1191 Raspe, O., Kakishima, M., Sanchez-Ramirez, S. and Vellinga, E.C., 2019. Notes, outline
1192 and divergence times of Basidiomycota. *Fungal diversity*, 99, pp.105-367.

1193 He, M.Q., Zhao, R.L., Liu, D.M., Denchev, T.T., Begerow, D., Yurkov, A., Kemler, M.,
1194 Millanes, A.M., Wedin, M., McTaggart, A.R. and Shivas, R.G., 2022. Species diversity of
1195 Basidiomycota. *Fungal diversity*, 114(1), pp.281-325.

1196 Helgason, T., Fitter, A.H. and Young, J.P.W., 1999. Molecular diversity of arbuscular
1196 mycorrhizal fungi colonising *Hyacinthoides non-scripta* (bluebell) in a seminatural woodland.
1197 *Molecular ecology*, 8(4), pp.659-666.

1198 Hempel, S., Renker, C. and Buscot, F., 2007. Differences in the species composition of
1199 arbuscular mycorrhizal fungi in spore, root and soil communities in a grassland
1200 ecosystem. *Environmental Microbiology*, 9(8), pp.1930-1938.

1201 Hofstetter, V., Miadlikowska, J., Kauff, F. and Lutzoni, F., 2007. Phylogenetic comparison of
1202 protein-coding versus ribosomal RNA-coding sequence data: a case study of the
1203 *Lecanoromycetes* (Ascomycota). *Molecular phylogenetics and evolution*, 44(1), pp.412-426.

1204 Holec, J., 2005. *The genus Gymnopilus (Fungi, Agaricales) in the Czech Republic with*
1205 *respect to collections from other European countries*. Acta Musei Nationalis Pragae.

1206 James, T.Y., Kauff, F., Schoch, C.L., Matheny, P.B., Hofstetter, V., Cox, C.J., Celio, G., early
1207 Gueidan, C., Fraker, E., Miadlikowska, J. and Lumbsch, H.T., 2006. Reconstructing the
1208 evolution of Fungi using a six-gene phylogeny. *Nature*, 443(7113), pp.818-822.

1209 Justo, A., Vizzini, A., Minnis, A.M., Menolli Jr, N., Capelari, M., Rodríguez, O., Malysheva, E.,

1210 Contu, M., Ghignone, S. and Hibbett, D.S., 2011. Phylogeny of the Pluteaceae (Agaricales,
1211 Basidiomycota): taxonomy and character evolution. *Fungal Biology*, 115(1), pp.1-20.

1212 Kalichman, J., Kirk, P.M. and Matheny, P.B., 2020. A compendium of generic names of
1213 agarics and Agaricales. *Taxon*, 69(3), pp.425-447.

1214 Kauff, F., Cox, C.J. and Lutzoni, F., 2007. Wasabi: an automated sequence processing
1215 system for multigene phylogenies. *Systematic Biology*, 56(3), pp.523-531.

1216 Kaur, M., Kaur, H. and Malik, N.A., 2014. Genus *Panaeolus*: new records from India. *Journal*
1217 *on New Biological Reports*, 3(1), pp.52-59.

1218 Kauserud, H., Colman, J.E. and Ryvarden, L., 2008. Relationship between basidiospore size,
1219 shape and life history characteristics: a comparison of polypores. *fungal ecology*, 1(1), pp.19-
1220 23.

1221 Khan, J., Kiran, M., Jabeen, S., Sher, H. and Khalid, A.N., 2017. *Gymnopilus penetrans* and
1222 *G. swaticus* sp. nov. (Agaricomycota: Hymenogastraceae); a new record and a new species
1223 from northwest Pakistan. *Phytotaxa*, 312(1), pp.60-70.

1224 Khaund, P. and Joshi, S.R., 2014. DNA barcoding of wild edible mushrooms consumed by
1225 the ethnic tribes of India. *Gene*, 550(1), pp.123-130.

1226 Kim, B.R., Nam, H.Y., Kim, S.U., Kim, S.I. and Chang, Y.J., 2003. Normalization of reverse
1227 transcription quantitative-PCR with housekeeping genes in rice. *Biotechnology letters*, 25,
1228 pp.1869-1872.

1229 Kiss, L., 2012. Limits of nuclear ribosomal DNA internal transcribed spacer (ITS) sequences
1230 as species barcodes for Fungi. *Proceedings of the National Academy of Sciences*, 109(27),
1231 pp.E1811-E1811.

1232 Koziak, A.T., Cheng, K.C. and Thorn, R.G., 2007. Phylogenetic analyses of *Nematoctonus*
1233 and *Hohenbuehelia* (Pleurotaceae). *Botany*, *85*(8), pp.762-773.

1234 Krebs, T.S. and Johansen, P.Ø., 2013. Psychedelics and mental health: a population
1235 study. *PloS one*, 8(8), p.e63972.

1236 Lee, J.C.I., Cole, M. and Linacre, A., 2000. Identification of hallucinogenic fungi from the
1237 genera *Psilocybe* and *Panaeolus* by amplified fragment length polymorphism.
1238 *Electrophoresis: An International Journal*, 21(8), pp.1484-1487.

1239 Lee, J., Lee, S. and Young, J.P.W., 2008. Improved PCR primers for the detection and
1240 identification of arbuscular mycorrhizal fungi. *FEMS microbiology ecology*, 65(2), pp.339-349.

1241 Lezzi, T., Vizzini, A., Ercole, E., Migliozzi, V. and Justo, A., 2014. Phylogenetic and
1242 morphological comparison of *Pluteus variabilicolor* and *P. castri* (Basidiomycota,
1243 Agaricales). *Ima fungus*, 5(2), pp.415-423.

1244 Li, Y.K., Yuan, Y. and Liang, J.F., 2014. Morphological and molecular evidence for a
1245 new species of *Psilocybe* from southern China. *Mycotaxon*, 129(2), pp.21-222.

1246 Liu, Y.J., Hodson, M.C. and Hall, B.D., 2006. Loss of the flagellum happened only once in
1247 the fungal lineage: phylogenetic structure of kingdom Fungi inferred from RNA polymerase II
1248 subunit genes. *BMC Evolutionary Biology*, 6, pp.1-13.

1249 Liu, Y.J., Whelen, S. and Hall, B.D., 1999. Phylogenetic relationships among ascomycetes:
1250 evidence from an RNA polymerse II subunit. *Molecular biology and evolution*, 16(12),
1251 pp.1799-1808.

1252 Magray, A.R., Lone, S.A., Ganai, B.A., Ahmad, F., Dar, G.J., Dar, J.S. and Rehman, S.,
1253 2019. Comprehensive, classical and molecular characterization methods of *Saprolegnia*
1254 (Oomycota; Stramnipila), an important fungal pathogen of fish. *Fungal Biology*
1255 *Reviews*, 33(3-4), pp.166-179.

1256 Maharachchikumbura, S.S., Wanasinghe, D.N., Cheewangkoon, R. and Al-Sadi, A.M., 2021.
1257 Uncovering the hidden taxonomic diversity of fungi in Oman. *Fungal Diversity*, 106, pp.229-
1258 268.

1259 Makhzani, A. and Frey, B., 2014, September. A winner-take-all method for training sparse
1260 convolutional autoencoders. In *NIPS deep learning workshop*.

1261 Malysheva, E.F., Malysheva, V.F. and Justo, A., 2016. Observations on *Pluteus*
1262 (Pluteaceae) diversity in South Siberia, Russia: morphological and molecular
1263 data. *Mycological Progress*, 15, pp.861-882.

Marcela, D., 2021. Universidad Autónoma de Querétaro Facultad de Química Evaluación de la bioaccesibilidad de proteínas de tortillas obtenidas a partir de harina de sorgo (Sorghum bicolor L. Moench) nixtamalizada por calentamiento óhmico Tesis Que como parte de los requi.

Maruyama, T., Yokoyama, K., Makino, Y. and Goda, Y., 2003. Phylogenetic relationship of psychoactive fungi based on the rRNA gene for a large subunit and their identification using the TaqMan assay. *Chemical and pharmaceutical bulletin*, 51(6), pp.710-714.

Maruyama, T., Kawahara, N., Yokoyama, K., Makino, Y., Fukiharu, T. and Goda, Y., 2006. Phylogenetic relationship of psychoactive fungi based on rRNA gene for a large subunit and their identification using the TaqMan assay (II). *Forensic science international*, 163(1-2), pp.51-58.

Matheny, P.B., 2005. Improving phylogenetic inference of mushrooms with RPB1 and RPB2 nucleotide sequences (*Inocybe*; Agaricales). *Molecular phylogenetics and evolution*, 35(1), pp.1-20.

Matheny, P.B., Liu, Y.J., Ammirati, J.F. and Hall, B.D., 2002. Using RPB1 sequences to improve phylogenetic inference among mushrooms (*Inocybe*, Agaricales). *American Journal of Botany*, 89(4), pp.688-698.

Matheny, P.B., Curtis, J.M., Hofstetter, V., Aime, M.C., Moncalvo, J.M., Ge, Z.W., Yang, Z.L., Slot, J.C., Ammirati, J.F., Baroni, T.J. and Bougher, N.L., 2006. Major clades of Agaricales: a multilocus phylogenetic overview. *Mycologia*, 98(6), pp.982-995.

Matheny, P.B., Wang, Z., Binder, M., Curtis, J.M., Lim, Y.W., Nilsson, R.H., Hughes, K.W., Hofstetter, V., Ammirati, J.F., Schoch, C.L. and Langer, E., 2007. Contributions of rpb2 and tef1 to the phylogeny of mushrooms and allies (Basidiomycota, Fungi). *Molecular phylogenetics and evolution*, 43(2), pp.430-451.

Melgarejo-Estrada, E., Suarez, M.E., Rocabado, D., Maillard, O. and Lechner, B.E., 2020. Checklist of Bolivian Agaricales: 1. Species with dark and pink spore prints. *Mycotaxon*, 134(4), p.739.

Menolli, N., Justo, A., Arrillaga, P., Pradeep, C.K., Minnis, A.M. and Capelari, M., 2014. Taxonomy and phylogeny of *Pluteus glaucotinctus sensu lato* Agaricales, Basidiomycota, a multicontinental species complex. *Phytotax*, 188(2), pp.78-90.

Menolli Jr, N. and Capelari, M., 2010. Notes on *Pluteus* (Pluteaceae, Agaricales) from Brazil including two new species and a new record. *Mycologia*, 102(3), pp.697-707.

Metzner, R. ed., 2005. *Sacred mushroom of visions: Teonanacatl: a sourcebook on the psilocybin mushroom*. Simon and Schuster.

Mirhendi, H., Makimura, K., de Hoog, G.S., Rezaei-Matehkolaei, A., Najafzadeh, M.J., Umeda, Y. and Ahmadi, B., 2015. Translation elongation factor 1-α gene as a potential taxonomic and identification marker in dermatophytes. *Medical mycology*, 53(3), pp.215-224.

Mitchell, J.I. and Zuccaro, A., 2006. Sequences, the environment and fungi. *Mycologist*, 20(2), pp.62-74.

Moncalvo, J.M., Lutzoni, F.M., Rehner, S.A., Johnson, J. and Vilgalys, R., 2000. Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. *Systematic biology*, 49(2), pp.278-305.

Moncalvo, J.M., Vilgalys, R., Redhead, S.A., Johnson, J.E., James, T.Y., Aime, M.C., Hofstetter, V., Verduin, S.J., Larsson, E., Baroni, T.J. and Thorn, R.G., 2002. One hundred and seventeen clades of euagarics. *Molecular phylogenetics and evolution,* 23(3), pp.357-400.

Musshoff, F., Madea, B. and Beike, J., 2000. Hallucinogenic mushrooms on the German market—simple instructions for examination and identification. *Forensic science international*, 113(1-3), pp.389-395.

Mutanen, M., Kekkonen, M., Prosser, S.W., Hebert, P.D. and Kaila, L., 2015. One species in eight: DNA barcodes from type specimens resolve a taxonomic quagmire. *Molecular Ecology Resources*, 15(4), pp.967-984.

Nadimi, M., 2015. Comparative mitochondrial genomics toward understanding genetics and evolution of arbuscular mycorrhizal fungi.

Nichols, D.E., 2020. Psilocybin: From ancient magic to modern medicine. *The Journal of antibiotics*, 73(10), pp.679-686.

Nilsson, R.H., Kristiansson, E., Ryberg, M., Hallenberg, N. and Larsson, K.H., 2008. Intraspecific ITS variability in the kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification. *Evolutionary bioinformatics*, 4, pp.EBO-S653.

Nkadimeng, S.M., Nabatanzi, A., Steinmann, C.M. and Eloff, J.N., 2020. Phytochemical, cytotoxicity, antioxidant and anti-inflammatory effects of *Psilocybe natalensis* magic mushroom. *Plants*, 9(9), p.1127.

Noordeloos, M.E., 1995. Notulae ad floram agaricinam Neerlandicam—XXIII. *Psilocybe* and *Pholiota*. *Persoonia-Molecular Phylogeny and Evolution of Fungi*, 16(1), pp.127-129.

O'Donnell, K., 2000. Molecular phylogeny of the Nectria haematococca-*Fusarium solani* species complex. *Mycologia*, 92(5), pp.919-938.

1332 O’Donnell, K., Rooney, A.P., Proctor, R.H., Brown, D.W., McCormick, S.P., Ward, T.J.,

Frandsen, R.J., Lysøe, E., Rehner, S.A., Aoki, T. and Robert, V.A., 2013. Phylogenetic analyses of RPB1 and RPB2 support a middle Cretaceous origin for a clade comprising all - agriculturally and medically important *Fusaria*. Fungal Genetics and Biology, 52, pp.20-31.

Paul, S., 1996. Psilocybin mushrooms of the world: An identification guide. Berkeley.

Pivato, B., Mazurier, S., Lemanceau, P., Siblot, S., Berta, G., Mougel, C. and Van Tuinen, D., 2007. Medicago species affect the community composition of arbuscular mycorrhizal fungi associated with roots. *New Phytologist,* 176(1), pp.197-210.

Porras-Alfaro, A., Liu, K.L., Kuske, C.R. and Xie, G., 2014. From genus to phylum: large-subunit and internal transcribed spacer rRNA operon regions show similar classification accuracies influenced by database composition. *Applied and environmental microbiology*, 80(3), pp.829-840.

Radonić, A., Thulke, S., Mackay, I.M., Landt, O., Siegert, W. and Nitsche, A., 2004. Guideline to reference gene selection for quantitative real-time PCR. *Biochemical and biophysical research communications*, 313(4), pp.856-862.

Raja, H., Schoch, C.L., Hustad, V., Shearer, C. and Miller, A., 2011. Testing the phylogenetic utility of MCM7 in the Ascomycota. *MycoKeys*, 1, pp.63-94.

Raja, H.A., Miller, A.N., Pearce, C.J. and Oberlies, N.H., 2017. Fungal identification using molecular tools: a primer for the natural products research community. *Journal of natural products*, 80(3), pp.756-770.

Ramírez-Cruz, V., Guzmán, G., Villalobos-Arámbula, A.R., Rodríguez, A., Matheny, P.B., Sánchez-García, M. and Guzmán-Dávalos, L., 2013. Phylogenetic inference and trait evolution of the psychedelic mushroom genus *Psilocybe* sensu lato (Agaricales). *Botany*, 91(9), pp.573-591.

Reeb, V., Lutzoni, F. and Roux, C., 2004. Contribution of RPB2 to multilocus phylogenetic studies of the *Euascomycetes* (Pezizomycotina, Fungi) with special emphasis on the lichen-forming *Acarosporaceae* and evolution of polyspory. *Molecular phylogenetics and evolution*, 32(3), pp.1036-1060.

Reiff, C.M., Richman, E.E., Nemeroff, C.B., Carpenter, L.L., Widge, A.S., Rodriguez, C.I., Kalin, N.H., McDonald, W.M. and Work Group on Biomarkers and Novel Treatments, a Division of the American Psychiatric Association Council of Research, 2020. Psychedelics and psychedelic-assisted psychotherapy. *American Journal of Psychiatry*, 177(5), pp.391-410.

Rosendahl, S., Mcgee, P. and Morton, J.B., 2009. Lack of global population genetic range differentiation in the arbuscular mycorrhizal fungus *Glomus mosseae* suggests a recent expansion which may have coincided with the spread of agriculture. *Molecular ecology,* 18(20), pp.4316-4329.

Rucker, J.J., Jelen, L.A., Flynn, S., Frowde, K.D. and Young, A.H., 2016. Psychedelics in the treatment of unipolar mood disorders: a systematic review. *Journal of Psychopharmacology*, 30(12), pp.1220-1229.

Sang, T., 2002. Utility of low-copy nuclear gene sequences in plant phylogenetics. *Critical reviews in Biochemistry and molecular biology*, 37(3), pp.121-147.

Saupe, S.G., 1981. Occurrence of psilocybin/psilocin in *Pluteus salicinus* (Pluteaceae). *Mycologia,* 73(4), pp.781-784*.*

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., Fungal Barcoding Consortium, Fungal Barcoding Consortium Author List, Bolchacova, E. and Voigt, K., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the national academy of Sciences*, 109(16), pp.6241-6246.

Seifert, K.A., 2009. Progress towards DNA barcoding of fungi. *Molecular ecology resources*, 9, pp.83-89.

Senchina, D.S., Alvarez, I., Cronn, R.C., Liu, B., Rong, J., Noyes, R.D., Paterson, A.H., Wing, R.A., Wilkins, T.A. and Wendel, J.F., 2003. Rate variation among nuclear genes and the age of polyploidy in *Gossypium*. *Molecular biology and evolution*, 20(4), pp.633-643.

Senn-Irlet, B., Nyffenegger, A. and Brenneisen, R., 1999. *Panaeolus bisporus*—an adventitious fungus in central Europe, rich in psilocin. *Mycologist*, 13(4), pp.176-179.

Siaut, M., Heijde, M., Mangogna, M., Montsant, A., Coesel, S., Allen, A., Manfredonia, A., Falciatore, A. and Bowler, C., 2007. Molecular toolbox for studying diatom biology in

Phaeodactylum tricornutum. *Gene*, 406(1-2), pp.23-35.

Silva-Filho, A.G.S., Seger, C. and Cortez, V.G., 2019. *Panaeolus* (Agaricales) from Western Paraná state, South Brazil, with a description of a new species, *Panaeolus sylvaticus. Edinburgh Journal of Botany,* 76(2), pp.297-309.

Skrede, I., Engh, I.B., Binder, M., Carlsen, T., Kauserud, H. and Bendiksby, M., 2011. Evolutionary history of *Serpulaceae* (Basidiomycota): molecular phylogeny, historical biogeography and evidence for a single transition of nutritional mode. *BMC Evolutionary Biology*, 11(1), pp.1-13.

Small, R.L., Cronn, R.C. and Wendel, J.F., 2004. Use of nuclear genes for phylogeny reconstruction in plants. *Australian Systematic Botany*, 17(2), pp.145-170.

Stamets, P. (1996) 'Psilocybin mushrooms of the world: an identification guide'. Ten Speed Press, p. 245.

Stefani, F.O., Jones, R.H. and May, T.W., 2014. Concordance of seven gene genealogies compared to phenotypic data reveals multiple cryptic species in Australian dermocyboid *Cortinarius* (Agaricales). *Molecular Phylogenetics and Evolution,* 71*,* pp.249-260.

Stielow, J.B., Levesque, C.A., Seifert, K.A., Meyer, W., Irinyi, L., Smits, D., Renfurm, R.G.J.M.V., Verkley, G.J.M., Groenewald, M., Chaduli, D. and Lomascolo, A., 2015. One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes. *Persoonia-Molecular Phylogeny and Evolution of Fungi*, 35(1), pp.242-263.

Stockinger, H., Krüger, M. and Schüßler, A., 2010. DNA barcoding of arbuscular mycorrhizal fungi. *New Phytologist*, 187(2), pp.461-474.

Strauss, D., Ghosh, S., Murray, Z. and Gryzenhout, M., 2022a. An overview on the taxonomy, phylogenetics and ecology of the psychedelic genera *Psilocybe*, *Panaeolus*, *Pluteus* and *Gymnopilu*s. *Frontiers in Forests and Global Change*, 5, p.813998.

Strauss, D., Ghosh, S., Murray, Z. and Gryzenhout, M., 2022c. Psilocybin containing mushrooms: a rapidly developing biotechnology industry in the psychiatry, biomedical and nutraceutical fields. *3 Biotech*, 12(12), p.339.

Sun, S.E., Li, Q.I., Kong, L., Yu, H., Zheng, X., Yu, R., Dai, L., Sun, Y., Chen, J., Liu, J. and Ni, L., 2016. DNA barcoding reveal patterns of species diversity among northwestern Pacific

molluscs. *Scientific Reports*, 6(1), p.33367.

Tanabe, Y., Saikawa, M., Watanabe, M.M. and Sugiyama, J., 2004. Molecular phylogeny of *Zygomycota* based on EF-1α and RPB1 sequences: limitations and utility of alternative markers to rDNA. *Molecular phylogenetics and evolution*, 30(2), pp.438-449.

Tanabe, Y., Watanabe, M.M. and Sugiyama, J., 2002. Are *Microsporidia* really related to Fungi? A reappraisal based on additional gene sequences from basal fungi. *Mycological Research*, 106(12), pp.1380-1391.

Tang, A.M., Jeewon, R. and Hyde, K.D., 2007. Phylogenetic utility of protein (RPB2, β-tubulin) and ribosomal (LSU, SSU) gene sequences in the systematics of *Sordariomycetes* (Ascomycota, Fungi). *Antonie van Leeuwenhoek*, 91, pp.327-349.

Tchotet Tchoumi, J.M., Coetzee, M.P.A., Rajchenberg, M. and Roux, J., 2019. Taxonomy and species diversity of *Ganoderma* species in the Garden Route National Park of South Africa inferred from morphology and multilocus phylogenies. *Mycologia*, 111(5), pp.730-747.

Tekpinar, A.D. and Kalmer, A., 2019. Utility of various molecular markers in fungal identification and phylogeny. *Nova Hedwigia*, 109(1-2), pp.187-224.

Tisserat, N.A., Hulbert, S.H. and Sauer, K.M., 1994. Selective amplification of rDNA internal transcribed spacer regions to detect *Ophiosphaerella korrae* and *O. herpotricha*. *DNA probes*, 12, p.17.

Triana-Alonso, F.J., Chakraburtty, K. and Nierhaus, K.H., 1995. The Elongation Factor 3 Is Unique in Higher Fungi and Essential for Protein Biosynthesis Is an E Site Factor (∗). *Journey of Biological Chemistry*, 270(35), pp.20473-20478.

Tsujikawa, K., Kanamori, T., Iwata, Y., Ohmae, Y., Sugita, R., Inoue, H. and Kishi, T., 2003. Morphological and chemical analysis of magic mushrooms in Japan. *Forensic science international*, 138(1-3), pp.85-90.

Ueng, P.P., Subramaniam, K., Chen, W., Arseniuk, E., Wang, L., Cheung, A.M., Hoffmann, G.M. and Bergstrom, G.C., 1998. Intraspecific genetic variation of *Stagonospora avenae* and its differentiation from *S. nodorum*. *Mycological Research*, 102(5), pp.607-614.

1446 Van Court, R.C., Wiseman, M.S., Meyer, K.W., Ballhorn, D.J., Amses, K.R., Slot, J.C.,
1447 Dentinger, B.T.M., Garibay-Orijel, R. and Uehling, J.K., 2022. Diversity, biology, and history
1448 of psilocybin-containing fungi: Suggestions for research and technological
1449 development. *Fungal Biology*, 126(4), pp.308-319.

1450 Visagie, C.M., Houbraken, J., Frisvad, J.C., Hong, S.B., Klaassen, C.H.W., Perrone, G.,
1451 Seifert, K.A., Varga, J., Yaguchi, T. and Samson, R.A., 2014. Identification and nomenclature
1452 of the genus *Penicillium*. *Studies in mycology*, 78(1), pp.343-371.

1453 Visagie, C.M., Frisvad, J.C., Houbraken, J., Visagie, A., Samson, R.A. and Jacobs, K., 2021.
1454 A re-evaluation of *Penicillium* section *Canescentia*, including the description of five new
1455 species. *Persoonia-Molecular Phylogeny and Evolution of Fungi*, 46(1), pp.163-187.

1456 Vu, D., Groenewald, M., De Vries, M., Gehrmann, T., Stielow, B., Eberhardt, U., Al-Hatmi
1457 A., Groenewald, J.Z., Cardinali, G., Houbraken, J. and Boekhout, T., 2019. Large-scale
1458 generation and analysis of filamentous fungal DNA barcodes boosts coverage for kingdom
1459 fungi and reveals thresholds for fungal species and higher taxon delimitation. *Studies in*
1460 *mycology*, 92(1), pp.135-154.

1461 Wake, D.B., Wake, M.H. and Specht, C.D., 2011. Homoplasy: from detecting pattern to
1462 determining process and mechanism of evolution. *Science*, 331(6020), pp.1032-1035.

1463 Wang, Y. W. and Tzean, S. S., 2015. Dung-associated, potentially hallucinogenic
1464 mushrooms from Taiwan. *Taiwania*, 60(4), pp. 160–168. doi: 10.6165/tai.2015.60.160.

1465 Wasson, R.G., 1957. Seeking the magic mushroom. *Life*, 42(19), pp.100-120.

1466 Kirk, P.M., Cannon, P.F., Minter, D.W. and Stalpers, J.A., 2008. *Dictionary of the Fungi.*
1467 *Wallingford, UK: CABI* (p. 599). ISBN 978-0-85199-826-8.

1468 Wei, M. and Oberwinkler, F., 2001. Phylogenetic relationships in *Auriculariales* and related
1469 groups–hypotheses derived from nuclear ribosomal DNA sequences. *Mycological Research,*
1470 105(4), pp.403-415.

1471 Wu, Z., Tsumura, Y., Blomquist, G. and Wang, X.R., 2003. 18S rRNA gene variation among
1472 common airborne fungi, and development of specific oligonucleotide probes for the detection
1473 of fungal isolates. *Applied and environmental microbiology*, 69(9), pp.5389-5397.

1474 Wubet, T., Wei, M., Kottke, I., Teketay, D. and Oberwinkler, F., 2004. Molecular diversity of

arbuscular mycorrhizal fungi in *Prunus africana*, an endangered medicinal tree species in dry Afromontane forests of Ethiopia. *New Phytologist*, 161(2), pp.517-528.

Wubet, T., Wei, M., Kottke, I., Teketay, D. and Oberwinkler, F., 2006. Phylogenetic analysis of nuclear small subunit rDNA sequences suggests that the endangered African Pencil

Cedar, *Juniperus procera*, is associated with distinct members of *Glomeraceae*. *Mycological Research*, 110(9), pp.1059-1069.

Yang, G., Jan, A. and Komatsu, S., 2007. Functional analysis of OsTUB8, an anther-specific β-tubulin in rice. *Plant science*, 172(4), pp.832-838.

Yockey, A. and King, K., 2021. Use of psilocybin ("mushrooms") among US adults: 2015–2018. *Journal of Psychedelic Studies*, 5(1), pp.17-21.

Yurkov, A.M., Sannino, C. and Turchetti, B., 2020. *Mrakia fibulata* sp. nov., a psychrotolerant yeast from temperate and cold habitats. *Antonie van Leeuwenhoek*, 113(4), pp.499-510.

Zhang, J., Wang, P., Fang, L., Zhang, Q.A., Yan, C. and Chen, J., 2017. Isolation and on characterization of phosphate-solubilizing bacteria from mushroom residues and their effect tomato plant growth promotion. *Polish Journal of Microbiology*, 66(1), pp.57-65.

Zhang, L. and Li, W.H., 2004. Mammalian housekeeping genes evolve more slowly than tissue-specific genes. *Molecular biology and evolution*, 21(2), pp.236-239.

Zhang, X., Yu, H., Wang, Z., Yang, Q., Xia, R., Qu, Y., Tao, R., Shi, Y., Xiang, P., Zhang, S. and Li, C., 2022. Multi-locus identification of *Psilocybe cubensis* by high-resolution melting (HRM). *Forensic Sciences Research*, 7(3), pp.490-497.

Zhao, P., Luo, J. and Zhuang, W.Y., 2011. Practice towards DNA barcoding of the *Nectriaceous* fungi. *Fungal Diversity*, 46, pp.183-191.

Zhao, R.L., Li, G.J., Sánchez-Ramírez, S., Stata, M., Yang, Z.L., Wu, G., Dai, Y.C., He, S.H., Cui, B.K., Zhou, J.L. and Wu, F., 2017. A six-gene phylogenetic overview of Basidiomycota and allied phyla with estimated divergence times of higher taxa and a phyloproteomics perspective. *Fungal Diversity,* 84, pp.43-74.

CHAPTER 3 Methods and Materials

3.1 Sample Collection

Samples (dried psychedelic mushroom species) were received from experienced citizen scientists, in the year 2021 from various locations in South Africa (Table 2). The dried fungorium samples were stored at room temperature in the Fungorium of Marieka Gryzenhout, Genetics Department, University of the Free State. These specimens will be deposited in the National Fungorium of South Africa, Agricultural Research Council, Pretoria.

3.2 DNA extraction

The genomic DNA extraction was performed in accordance with Strauss et al. (2021). The dried fungal specimens received from citizen mycologist collection were cut into small pieces and inserted into 1.5mL Eppendorf tubes. These small pieces of mushroom specimen were pulverized in a tissue lyser (©QIAGEN, TissueLyserll, Germany) using metal balls. A lysis buffer solution comprised of Tris (100mM)-EDTA (ethylenediaminetetracetic acid) (10 mM) - SDS (Sodium Dodecyl Sulfate) (2%) was prepared, and from this solution, an amount of 900 µL was aliquoted to 40 mg of each mushroom specimen. The solution was briefly mixed by inversion, and the cells opened and released the DNA. Thereafter, the lysis buffer mixture was incubated at 65°C for 30 min. This solution was centrifuged at the speed of 13,000 rpm for the period of 5 min at 4°C yielding in the formation of a supernatant. This formation separated the supernatant from the cellular debris, the supernatant was slowly pipetted out and the cellular debris was discarded. This step was repeated in order to reduce the cellular debris present. To purify the solution, 600 µL of chloroform:isoamyl (24:1) was added to the supernatant, and this was followed by a centrifugation stage at a speed of 13,000 rpm for 5 min at 4°C. The aqueous phase that contains the DNA was cautiously pipetted out. This process was performed twice to minimize the amount of cellular debris as well as to remove the PCR inhibiting chemicals in the solution.

Lastly, the recovered DNA was precipitated with refrigerated 100% ethanol and centrifuged using a speed of 16000 rpm for 30 min at 4°C. Consequently, the resultant DNA pellet was spun down, dried, and thereafter re-suspended in 40µL of warm nuclease-free water. Once the extraction process was successively concluded, the DNA was then preserved and stored at a temperature of - 20°C until the next step.

3.3 Quantification of DNA

For the qualitative analysis, the DNA samples were stained using Gel-Red nucleic acid gel stain (ThermoFisher Scientific, USA) and using gel electrophoresis (Cleaver Scientific Ltd, UK) in a 1% agarose gel, at 100 Volt, 400 Ma at 45 min and this was immersed in 1X TAE (Tris-EDTA (ethylenediaminetetraacetic acid)). The DNA was visualized using a gel doc (Vacutec, Roosevelt Park, South Africa). Subsequently, for quantitative analysis, the DNA concentrations of the extracted samples were quantified employing the NanoDrop® Spectrophotometer ND-1000 (ThermoFisher). Using the absorbance range 260/280 nm, the overall purity of the isolated DNA was determined. The DNA concentrations were standardized by dilution of 1:10. The Nanodrop measurements were repeated twice, and the averages were recorded.

3.4 PCR amplification

Three target regions of the genome were used to amplify DNA, using the process of Polymerase Chain Reaction (PCR). The target regions were; TEF1-α, RPB 1, and the ITS, ITS-5.8S region of the ribosomal operon. The primer information is listed (Table 3). Two different sets of the ITS primers were used; namely ITS1 (5'- TCCGTAGGTG AACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (20bp) (White *et al*., 1990) and for DNA that did not amplify using the previously mentioned primers, an alternative primer set, ITS1-F_KY02 (5'- TAG AGG AAG TAAAAG TCG TAA -3') (Toju *et al*., 2012) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (20bp) (White *et al*., 1990) was used. The ITS region, which is given the term of the primary barcode marker for fungi, this region has the greatest likelihood of accurate identification results for the widest range of fungal species (Schoch *et al.*, 2012), making these primers the optimal selection for the objectives of this study. For the protein coding regions, the TEF1-α and the RPB 1 regions were used, mainly because the RPB 1 had previously been used for the identification of *Inocybe* and Agaricales

(Zhang *et al.*, 2021) and the TEF1-α gene successfully targeted and identified *Psilocybe* species in the study from Cowan and Elkins (2018).

The selection of the TEF1-α and RBP 1 primer pairs for this current study was based on successful amplification of hallucinogenic mushrooms, including *Psilocybe* as mentioned in Zhang et al*.* (2021). The primers used for TEF1- α were TEF PC-EF-F (5'-FTTCATCAAGAAGGTCGGTTAC-3') (21bp) and TEF PC-EF-R: (5'- TCTCCGTGCCATCCAG-3') (16bp) with an expected base pair size of 67 bp. For the RBP 1 region, the primers that were used were RBP 1 PC-R1-F (5'- CTC TACTCGTTTCGCACCC-3') and RBP 1 PC-R1-R (5'-CGCACTCCTCGTTCAGC-3') (Zhang *et al.*, 2021) with an expected base pair size of 246 bp.

A 25 µL PCR reaction contained 1µL of template DNA (≈ 100-200ng), 1.25µL each for the forward and the reverse primers (10 µM), 12.5µL One Taq® 2X MM w/standard buffer (New England BioLabs, Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa) and 9µL nuclease free water (Ghosh *et al.*, 2021; Strauss *et al.*, 2021). The thermal cycling conditions used for the ITS PCR included the following: an initial phase of denaturation set at a temperature of 94°C for 2 min 30 sec, the second phase of denaturation at 40 cycles set at a temperature of 94°C for 30 sec, followed by an annealing step at 54°C for 30 sec, subsequently an initial extension step at 72°C for 40 sec. The last extension step was done at a final temperature of 72°C for a period of 10 min. This procedure was executed using the BioRad T100 Thermal Cycler (BIORAD, Johannesburg, South Africa). PCR products were visualized on 1% agarose gels. The same conditions were applied for the RPB 1 PCR and for TEF1-α PCR. PCR products were visualized on 1% agarose gels.

3.5 DNA Sequencing

PCR amplicons were purified using an ExoSAP-IT Express PCR Product Clean-up kit. For sequencing, the PCR products were marked using BigDye™ Terminator v3.1 Cycle Sequencing Kit including 2 µL each of the following reagents; the BigDye buffer, the forward and reverse primers (5 µM) of every molecular marks, sterile water, and lastly the purified PCR product was added. The thermal cycling conditions used for sequencing PCR included the following: an initial phase of denaturation set at a temperature of 96°C for 1 minute, the second phase of denaturation at 34 cycles set at a temperature of 96°C for 30 sec, followed by an annealing step at 52°C for 30 sec,

subsequently an initial extension step at 60°C for 4 min. The last extension step was done at a final temperature of 60°C for 3 min. This procedure was once again performed using the BioRad T100 Thermal Cycler. Sequencing was performed using a Genetic analyzer ABI3500 (Applied Biosystems, South Africa) available at the Genetics Department of the University of the Free State.

3.6 Phylogenetic analysis

The chromatograms of different molecular markers including ITS, TEF1-α and RPB 1 were edited using Geneious Prime® 2022.2 (Dotmatics, Boston, Massachusetts) software, and consensus sequence alignments of forward and reverse sequences were generated. The assembled sequences were used to generate datasets based on sequences that were retrieved from GenBank, the nucleotide database of NCBI (National Centre for Biotechnology Information) (Benson *et al.*, 2013) using BLAST (Basic Local Alignment Search Tool). For each sample, contigs were assembled and combined. The BLAST results were compared against existing sequences available on GenBank. The first 50-60 sequences were selected based on the percentage identity above 90% and limited to uncultured and environmental sample sequences. Two searches were performed, one with sequences limited to type material and the second was unlimited to type material sequences. The selected sequences confirmed that each of the collected sequences represented species from the genera *Psilocybe* and therefore only *Psilocybe* sequences were chosen for further analysis.

From the downloaded sequences, a dataset for each sample was built using the Molecular Evolutionary Genetics Analysis (MEGA) software 7.0 (Katoh *et al.*, 2002; Wang *et al.*, 2022) and subsequently aligned using the online server of the Multiple Sequence Alignment program (MAFFT) (Katoh *et al.*, 2002; Hunter, Glen and McDougal, 2016) with the selection of alignment to leave the gappy regions. The downloaded sequences formed a complete dataset, and the missing data from the beginning and ends of the dataset were filled with n. The construction of phylogenetic trees was done using MEGA and the phylogenetic analysis on the maximum-likelihood and the best fit model feature. Other selections included the test of phylogeny set on the bootstrap method, and the number of bootstrap replications set to 1000. Finally, the tree displayed branch length were set to hide values shorter than 80.

3.7 Morphological characterization

In the field, mushrooms were photographed, and morphological characteristics such as the size, shape, color and any other possible features of the mushroom were recorded.

Microscopic characteristics and slides were prepared and observed from dried material under a DM500 Leica compound, with Leica ICC50W digital camera and LasEZ software (Leica Microsystems DM LB) in 3% potassium hydroxide (KOH) and 10% lactic acid. Measurements at × 1000 magnification of all features were taken on the available material, including 50 measurements of basidospores and cystidia. Measurements were presented in the following format: (minimum)(average – standard deviation) - (average + standard deviation) (-max). Colour annotations were done using the HSC colour plates for Mycology (http://website.nbm-mnb.ca/mycologyweb pages/EssaysOnFungi/Collecting_mushrooms_for_scientific_study/Illustrations/HSV_ plates_for_mycology.pdf, accessed 27 Nov 2022).

References

Benson, D.A., Cavanaugh, M. and Clark, K., 2013. Genbank.-*Nucleic Acids Research*, 41 (D1): D36-D42.

Cowan, A.F. and Elkins, K.M., 2018. Detection and Identification of *Psilocybe cubensis* DNA Using a Real-Time Polymerase Chain Reaction High Resolution Melt Assay. *Journal of forensic sciences*, 63(5), pp.1500-1505.

Ghosh, S., Nomasonto, M., Mkhize, T.H. and Gryzenhout, M., 2021. A “pocket-friendly” Dimethyl Sulphoxide (DMSO) technique for mushroom genomic DNA extraction suitable for DNA-based identifications. *Microbial Biosystems*, 6(1), pp.66-74.

Hunter, S., Glen, M. and McDougal, R., 2016. Molecular tools for differentiating *Cyclaneusma minus* morphotypes and assessing their distribution in *Pinus radiate* forests in New Zealand. *New Zealand Journal of Forestry Science*, 46(1), pp.1-10.

Katoh, K., Misawa, K., Kuma, K.I. and Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic acids research*, 30(14), pp.3059-3066.

Matheny, P.B., Liu, Y.J., Ammirati, J.F. and Hall, B.D., 2002. Using RPB1 sequences to improve phylogenetic inference among mushrooms (*Inocybe*, Agaricales). American Journal of Botany, 89(4), pp.688-698

Strauss, D., Ghosh, S., Murray, Z. and Gryzenhout, M., 2021. Genomic DNA extraction from minimal amount of dried mushroom samples. *Microbial Biosystems,* 6(1), pp. 49–54.

Wang, S., Jiang, N., Jiang, L., Zhuang, Q., Chen, Q., Hou, G., Xiao, Z., Zhao, R., Li, Y., Zhao, C. and Zhang, F., 2022. Establishment and application of a quadruple real-time RT-PCR for detecting avian metapneumovirus. *Plos one*, 17(6), p. e0270708.

Zhang, X., Yu, H., Yang, Q., Wang, Z., Xia, R., Chen, C., Qu, Y., Tan, R., Shi, Y., Xiang, P. and Zhang, S., 2021. A forensic detection method for hallucinogenic mushrooms via High-Resolution Melting (HRM) analysis. *Genes*, 12(2), p.199.

Zhang, X., Yu, H., Yang, Q., Wang, Z., Xia, R., Chen, C., Qu, Y., Tan, R., Shi, Y., Xiang, P. and Zhang, S., 2021. A forensic detection method for hallucinogenic mushrooms via High-Resolution Melting (HRM) analysis. *Genes*, 12(2), p.199.

Chapter 4 Results and Discussion

4.1 Phylogenetic Analysis

The three molecular markers used in this phylogenetic analysis, namely TEF1-α, ITS, and RPB 1, were analysed individually and in a combined dataset, in order to provide the maximum amount of information (Zhang *et al.*, 2021). All the phylogenetic trees from this analysis produced different topologies. This was because not all of the species represented in the ITS tree, which is the most sequenced gene region across species and specimens for the *Psilocybe* genus, had corresponding TEF1-α and RPB 1 gene sequences (Table 4). Additionally, the species and specimens that were made available for the TEF1-α and RPB 1 genes also did not correspond. As a result, the TEF1-α and RPB 1 datasets did not include all the species from the ITS tree, and the different combinations of the species. Subsequently, this skewed the results of the phylogenetic analyses, and further indicated different closest relatives based on the species that were available in the datasets. This made comparing the various datasets against each other challenging. Lastly, in some cases, the RPB 1 gene region could not distinguish between species, and split the same species due to a single base sequence difference. However, the overall phylogenetic analyses for the samples from Lesotho and Pretoria, respectively, showed that these samples grouped separately, and confirmed the discovery of two new species. Furthermore, the two *Psilocybe* samples from Pretoria were shown to be genetically similar.

The ITS analysis formed the foundation of the results because it is the primary marker for *Psilocybe* species, and is also defined as the primary DNA barcode of fungi (Schoch *et al.*, 2012). Therefore, the ITS dataset was the largest and included the most species. For this study, 101 sequences were examined. The phylogenetic tree (Fig. 4.1) showed that the *Psilocybe* sequences were divided into two major clades, with the Southern African samples grouping within the first clade and grouping separately from each other within that clade. Sample JL, collected from Lesotho, grouped distinctly, but was closely related to a sequence labeled as *Psilocybe subaeruginosa* voucher Mushroom Observer 128946 (MH488731.1), which was collected from South Africa, Cape Town, Constantia by Alan Rockefellar, a citizen mycologist.

The Pretoria samples LP 225.1 and LP227.1 (Fig.4.1) were grouped together. Another sequence from a previous, unpublished study and collected from the same area LP 62, was added, and was shown to be almost identical to the sequences of LP 225.1 and LP227.1, at a bootstrap value of 99%. Two sequences labelled as *Psilocybe ovoideocystidiata* from the

United States of America, namely sample TVR13 (OL757580.1) which was identified in Missouri, St. Louis County, and voucher MushroomObserver.org/238931 (MK214734.1) that was identified in the Big Gunpowder Falls Trail, Baltimore Co., Maryland, formed a distinct but closely related sister group to LP 225.1 and LP 227.1. However, other *P. ovoideocystidiata* sequences could be found in various other places in the phylogenetic tree, indicating that the former sequences could be misidentified.

Numerous studies have used TEF1-α with effectiveness as a molecular marker (O'Donnell, 2000; Mirhendi *et al.*, 2015). Subsequently, Stielow et al. (2015) proposed that this gene should be the secondary universal DNA barcode. The TEF1-α dataset had 33 sequences, and the results confirmed that the groupings of the other genes that the Lesotho and Pretoria samples had, grouped separately from each other (Fig. 4.2). None of the samples grouped with any other known species, and in fact, the Lesotho sample formed closely related sister groups (bootstrap support 100%), with the Pretoria samples that are grouped together with a bootstrap value of 100%. The closest related sequence was labelled as *Psilocybe chuxiongensis* voucher IFRD_414-011 (KF586476.1), a species that was first identified in China and is closely related to *Psilocybe cubensis*, but with significant morphological characteristics differentiating between the two species (Ma *et al.*, 2014). However, the two available sequences of *P. chuxiongensis* grouped separately from each other in the analyses, with the second sequence forming a sister clade with *P. cubensis*.

RPB 1 gene could potentially be a powerful molecular marker based on the genes ability to be easily amplified, and has been used to resolve the phylogeny of many species (Tanabe *et al*., 2004; Matheny, 2005; Tekpinar and Kalmer, 2019), making identification of species much easier. This region RPB 1 had the least number of sequences in the dataset, including 18 sequences. The phylogenetic results (Fig. 4.3) once again divided the Southern African samples from this study. However, the RPB 1 sequences could not always distinguish between known species of *Psilocybe*, and in some cases sequences for some species grouped separately from each other, for example *P. thaiduplicatocystidiata* and *P. thaiaerugineomaculans.* This had an impact on the grouping of the sample JL that was identical to two *P. cubensis* sequences. The first *P. cubensis* sequence RHP5203 (TENN) (KC669345.1) is from Costa Rica and the second was labelled as *P. cubensis* voucher, V. Ramirez-Cruz 87 (XAL)*(*KC669344.1) from Mexico*. P. cubensis* is one of the best known *Psilocybe* species and is found in subtropical and tropical environments (Ramírez-Cruz *et al.*, 2013). The grouping that formed in this case of the sample JL, occurred even though, the very same sample grouped separately from *P. cubensis* sequences in the ITS and TEF1-α datasets.

The Pretoria samples grouped separately from each other based on a single base difference. Sample LP 225.1 was identical to *P. thaiduplicatocystidiata* from Thailand voucher S.C. Karunarathna NTS120 Isotype (XAL) (KC669362.1) and *P. thaiaerugineomaculans* from Thailand voucher S.C. Karunarathna NTS121 Holotype (XAL) (KC669361.1), at a bootstrap value of 100%. LP227.1 branched independently due to the single base sequence difference between the two samples. However, this was mirrored by other sequences of *P. thaiduplicatocystidiata* and *P. thaiaerugineomaculans* that also grouped separately from other representatives based on such minor sequence differences. These species are indigenous to the tropical environments of Thailand (Ramírez-Cruz *et al.*, 2013). It thus appears that RPB 1 is not an ideal gene region for *Psilocybe*, and it was compounded by the fact that there are also few sequences available. An accurate identification for the Southern African samples could thus not be derived from this gene region.

The combined dataset was only compiled from sequences of the ITS and TEF1-α region, since too few RPB sequences were available, which would have reduced the number of species that could be included significantly. The combined dataset was represented by 16 sequences (Table 4), and the results from the analysis (Fig.4.4) showed a robust and well-resolved phylogeny with a 100% bootstrap support for all the groups.

The Pretoria and Lesotho samples still grouped separately from each other and none of the samples grouped with known species. The Lesotho sample formed a closely related sister group with two sequences labelled as *Psilocybe chuxiongensis,* IFRD_414-011, and IFRD_414-010. It is noteworthy that the Pretoria samples grouped together once again independently.

It was difficult to determine which gene is best suited for *Psilocybe* identification because the RPB 1 and TEF1-α databases only contained a limited dataset of sequences. However, results showed that RPB 1 is most likely not a suitable gene for identification since it lacks resolution between known species. The TEF1-α sequences provided more sequence differences, but the gene use is limited given that it is still not sequenced representatively across species of *Psilocybe*. Another challenge detected for all gene regions is that species appear to be polyphyletic due to minor sequence differences between samples within species. This could possibly represent misidentifications or could possibly confirm that the species are indeed polyphyletic.

Sequencing standards should be set for *Psilocybe* and more genes could possibly be investigated to aid ITS sequences. Other genes used in previous studies include LSU (Fell *et al.*, 2000; Moncalvo *et al.*, 2000; Wei and Oberwinkler, 2001; Matheny *et al.*, 2007; Zhao,

Luo and Zhuang, 2011; Schoch *et al.*, 2012; Tekpinar and Kalmer, 2019), and SSU (White *et al.*, 1990; Wu *et al.*, 2003; Tekpinar and Kalmer, 2019). However, these genes are more conserved than ITS. Additionally, few species types are represented in sequencing efforts, which makes it more difficult to distinguish between true species identities and misidentifications. It is anticipated that numerous new species occur in areas that were previously understudied, such as Africa, should be described soon.

4.2 Taxonomy

Specimens for the two *Psilocybe* species that grouped separately and on their own based on multi-gene phylogenetic analyses, were found to differ greatly based on macro- and micromorphology. These features also distinguished the two species from other morphologically similar species, especially those known form South Africa. The descriptions for these two species as well as the provided names will follow in this section.

***Psilocybe orontawuli* Gryzenh., Maloka & Popich, *nom. prov.* Figures 4.5a-f, and 4.6.**

Diagnosis: *Pileus:* umbonate with broadly umbonate, papillate apex, buff becoming brown, margin striate, hygrophanous; subdecurrent lamellae; sheathed annulus; non-gutullate elliptical to ovoid to citriniform basidiospores guttulated, elliptical to ovoid to citriniform on cylindrical basidia; *Pleurocystidia:* clavate to obovoid to oval; cheilocystidia obovoid to spathulate to lecythiform to mucronate.

Mushrooms growing in soil, grouped. *Pileus:* 18-23 mm in diam. as dried, umbonate, top view orbicular, apex broadly umbonate and slightly papillate. *Colour:* buff (hue 50, saturation 100, value 100) when young becoming brown (hue 50, saturation 100, value 60) when older, margin striate, hygrophanous, smooth; dark purple-brown spore deposit sometimes found. *Lamellae:* subdecurrent, close with intermediate lengths, margin smooth, mottled. *Stipe:* central, equal, sheathing central annulus, beige (hue 50, saturation 30, value 100), smooth, with concentric fibrils. *Odour:* unknown. *Basidiospores:* (8.5-)9-10.5(-11) x (5-)5.5-7(-7.5) µm, thick-walled, elliptical to ovoid to citriniform, brown (hue 50, saturation 100, value 60), non- guttulate, occasional germpore at apex, apiculus truncated. *Basidia:* (18.5-)19.5-22.5(-23.5) x (5.5-)6-7.5(-8) µm, 4-spored, cylindrical to having a slight broadened base, hyaline, thin-walld, sterigmata (2.5-)3-4.5(-5) µm long, widened base.

Pleurocystidia: (11.5-)12.5-22(-24) x (6.5-)7-11(-12.5) μm, clavate to obovoid to oval, hyaline, thin-walled. *Cheilocystidia:* (12-)12.5-15.5(-15.5) x (6.5-)7-8 μm, ovoid to spathulate to lecythiform to mucronate, hyaline, thin-walled, unbranched, often with apices slightly thickened. *Stipitipellis:* unknown. *Pileipellis:* tri-layered. *Clamp connections:* unknown. *Psilocybin content*: Unknown.

Type: SOUTH AFRICA, Pretoria, Gauteng Province, 2018, L. Popich, holotype LP227.

Etymology: Orontawuli (Xhosa language) - traditional hut with thatched roof

Habitat and distribution: Gauteng province, South Africa.

Additional specimen examined: SOUTH AFRICA, Pretoria, Gauteng Province, 2018, L. Popich, holotype LP225

***Psilocybe malotiensis* Gryzenh., Maloka & MacGillivray, nom. prov. Figures 4.5g-m, and 4.7.**

Diagnosis: *Pileus:* narrowly parabolic to narrowly conical, rounded to papillted apex, straw coloured, margin incurved, rarely hygrophanous; adnexed lamellae, no annulus, stipe with bulbous base; guttulate elliptical to subovate to citriniform basidiospores; subclavate basidia is mostly nonsymmetrical; *Pleurocystidia*: clavate to ovoid to slightly mucronate; cheilocystidia subclavate to ventricose-rostrate to capitulate to lecythiform to mucronate; pileipellis with dermatocystidia.

Mushrooms growing in grasslands, single to scattered. *Pileus:* (5-)4-17(-22) mm long in diameter, (3-)1-11(-12) mm wide as dried, narrowly parabolic to narrowly conical, top view orbicular, apex rounded to umbonate to papillate. *Colour*: dark straw (hue 50, saturation 50, value 100) when young paling to straw (hue 50, saturation 20, value 100) with age,margin even, appendiculate when very young, incurved when mature, rarely hygrophanous, smooth, bluish veins when old. *Lamellae:* to adnexed, subdistanced close, margin smooth. *Stipe:* (8-)13-44(-82) mm long, (0.5-)0.1-2.1(-3) mm wide,central, equal with abrupt bulbous base, (0.5)-2-9(-10) × (0.5-)2.1-10(-9) mm, white (hue 50, saturation 0, value 100) to light straw (hue 50, saturation 5, value 100), no annulus. *Odour:* unknown. *Basidiospores:* (12.5-)13-14 x (8-)8.5-9(-9.5) μm, hick- walled, elliptical to subovate to citriniform, mostly nonsymmetrical,

brown (hue 50, saturation 00, value 60), guttulate, occasional germpore at apex, apiculus truncated, no appendage to slight. *Basidia:* (23-)24-26.8(-28) x (12-)13-13.5(-14.5) μm, 4-spored, clavate, hyaline, thin-walled, sterigmata 2 μm. *Pleurocystidia:* (11-)12-18(-22) x (6.5-)7.5-10(-12.5) μm, clavate to ovoid to slightly mucronate, hyaline, thin-walled. *Cheilocystidia:* (18.5-)20-22(-24) x (8.4)10-15(-19.6) μm, subclavate to ventricose- rostrate to capitulate to lecythiform to mucronate, hyaline, thin-walled, unbranched. *Stipitipellis:* parallelocutic, single layered. *Pileipellis:* tri-layered, dermatocystidia present. *Clamp connections* present. *Psilocybin content*: reputed to be active.

Type: LESOTHO, Kolojane, Berea Province, 17 January 2021, J. MacGillivray, holotype LM.

Etymology: The Maloti mountains form the highland region of the Kingdom of Lesotho, where the mushrooms were found; -ensis (Latin) refers to location.

Habitat and distribution: Grasslands and pastures in the Maloti mountains, Lesotho.

4.2.2 Taxonomy

Currently, only five species of *Psilocybe* (Table 2.1) have been formally reported from South Africa (Kinge *et al*., 2020; Gryzenhout, 2021). These include *P. cubensis, P. subaeruginascens*, *P. cylindrispora*, *P. coprophila* (currently *Deconia coprophila*) and the only locally described species, *P. natalensis*. None of these species resembles *P. orontawuli* and *P. malotiensis* morphologically. The remote occurrence of *P. malotiensis* makes it unlikely that this species originated from outside the country, or represents a species that was introduced from elsewhere. This species was grouped closely to the sequences generated by Alan Rockefellar for a sample from Constantia in Cape Town. However, the record (128946) in the Mushroom Observer website (https://mushroomobserver.org/128946) showed mushrooms with convex to flattened caps, which are vastly different from the closed caps of *P. malotiensis*.

The samples representing *P. orontawuli* were grouping very closely, yet still distinctly, from ITS sequences of *P. ovoideocystidiata*, a species described from the United States of America (Gaines, 2007). However, *P. ovoideocystidiata* has adnate gills and basidiospores roughly 8 x 6 μm (Gaines, 2007), while *P. orontawuli* has subdecurrent

gills, with longer basidiospores (8.5-)9-10.5(-11) x (5-)5.5-7(-7.5) μm. Besides these features, mushrooms of this species looked similar to those of Mushroom Observer record 238931.

References

Fell, J.W., Boekhout, T., Fonseca, A., Scorzetti, G. and Statzell-Tallman, A., 2000. Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *International journal of systematic and evolutionary microbiology*, 50(3), pp.1351-1371.

Ma, T., 2014. *Psilocybe chuxiongensis*, a new bluing species from subtropical China. *Phytotaxa*, 156(4), pp.211-220.

Matheny, P.B., Wang, Z., Binder, M., Curtis, J.M., Lim, Y.W., Nilsson, R.H., Hughes, K.W., Hofstetter, V., Ammirati, J.F., Schoch, C.L. and Langer, E., 2007. Contributions of rpb2 and tef1 to the phylogeny of mushrooms and allies (Basidiomycota, Fungi). *Molecular phylogenetics and evolution*, 43(2), pp.430-451.

Moncalvo, J.M., Lutzoni, F.M., Rehner, S.A., Johnson, J. and Vilgalys, R., 2000. Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. *Systematic biology*, 49(2), pp.278-305.

O'Donnell, K., Rooney, A.P., Proctor, R.H., Brown, D.W., McCormick, S.P., Ward, T.J., Frandsen, R.J., Lysøe, E., Rehner, S.A., Aoki, T. and Robert, V.A., 2013. Phylogenetic analyses of RPB1 and RPB2 support a middle Cretaceous origin for a clade comprising all agriculturally and medically important *Fusaria*. *Fungal Genetics and Biology*, 52, pp.20-31.

Ramírez-Cruz, V., Guzmán, G., Villalobos-Arámbula, A.R., Rodríguez, A., Matheny, P.B., Sánchez-García, M. and Guzmán-Dávalos, L., 2013. Phylogenetic inference and trait evolution of the psychedelic mushroom genus *Psilocybe* sensu lato (Agaricales). *Botany*, 91(9), pp.573-591.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., Fungal Barcoding Consortium, Fungal Barcoding Consortium Author List, Bolchacova, E. and Voigt, K., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the national academy of Sciences*, 109(16), pp.6241-6246.

Stielow, J.B., Levesque, C.A., Seifert, K.A., Meyer, W., Irinyi, L., Smits, D., Renfurm, R.G.J.M.V., Verkley, G.J.M., Groenewald, M., Chaduli, D. and Lomascolo, A., 2015.

1901 One fungus, which genes? Development and assessment of universal primers for
1902 potential secondary fungal DNA barcodes. *Persoonia-Molecular Phylogeny and*
1903 *Evolution of Fungi*, 35(1), pp.242-263.

1904 Tanabe, Y., Saikawa, M., Watanabe, M.M. and Sugiyama, J., 2004. Molecular
1905 phylogeny of Zygomycota based on EF-1α and RPB1 sequences: limitations and utility
1906 of alternative markers to rDNA. *Molecular phylogenetics and evolution*, 30(2), pp.438-
1907 449.

1908 Tekpinar, A.D. and Kalmer, A., 2019. Utility of various molecular markers in fungal
1909 identification and phylogeny. *Nova Hedwigia*, 109(1-2), pp.187-224.

1910 Wei, M. and Oberwinkler, F., 2001. Phylogenetic relationships in Auriculariales and
1911 related groups–hypotheses derived from nuclear ribosomal DNA sequences.
1912 *Mycological Research*, 105(4), pp.403-415.

1913 White, T.J., Bruns, T., Lee, S.J.W.T. and Taylor, J., 1990. Amplification and direct
1914 sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a*
1915 *guide to methods and applications*, 18(1), pp.315-322.

1916 Wu, Z., Tsumura, Y., Blomquist, G. and Wang, X.R., 2003. 18S rRNA gene variation
1917 among common airborne fungi, and development of specific oligonucleotide probes for
1918 the detection of fungal isolates. *Applied and environmental microbiology*, 69(9), pp.
1919 5389-5397.

1920 Zhang, X., Yu, H., Wang, Z., Yang, Q., Xia, R., Qu, Y., Tao, R., Shi, Y., Xiang, P., Zhang, S.
1921 and Li, C., 2022. Multi-locus identification of Psilocybe cubensis by high-resolution melting
1922 (HRM). *Forensic Sciences Research*, 7(3), pp.490-497.

1923 Zhao, P., Luo, J. and Zhuang, W.Y., 2011. Practice towards DNA barcoding of the
1924 nectriaceous fungi. *Fungal Diversity*, 46, pp.183-191.

CHAPTER 5 CONCLUSION

It is important to investigate hallucinogenic fungi that contain psilocybin because of the therapeutic application potential. This study could serve as a guide to better-identify these fungi in South Africa. The robust multiple-gene phylogeny supported the distinctive morphological features of the two collections of Southern African samples, despite the many limitations encountered. The study now sets a standard to describe other specimens from South Africa that might be new to characterize the biodiversity of these important psychedelic species. It will also aid the identification of known species not expected to occur in South Africa.

Novel species occur in Southern Africa and continue to be discovered. All that is required to support these findings are publications documenting and describing the diversity present in this region. Additionally, relevant taxonomic monographs are necessary for each of the psilocybin-producing genera, and future research should focus on this. This will significantly aid in the regulation of these fungi for recreational use, (which is still illegal in South Africa), and the possible cultivation of native species since interest in growing these psychedelic mushrooms is rapidly escalating across the world (Strauss *et al*., 2022c).

Appendices

Appendix A List of Tables

Table 1

Table 1: List of South African psilocybin-producing mushrooms

Genus	Species	Geographical location	Reference
Psilocybe	*Psilocybe coprophila*	South Africa	Kinge *et al*., 2020
	Psilocybe cubensis	Eastern Cape, on the KwaZulu-Natal coastline, Mpumalanga, Limpopo	Gryzenhout, 2021
	Psilocybe subaeruginascens	Gauteng, KwaZulu-Natal	Gryzenhout, 2021
	Psilocybe natalensis	Gauteng,Pretoria, KwaZulu-Natal, Western Cape	Gryzenhout, 2021 Kinge *et al*., 2020, Nkadimeng *et al.*, 2020
	Psilocybe cylindrispora	South Africa	Kinge *et al*., 2020
Panaeolus	*Panaeolus cyanescens*	KwaZulu-Natal, Mpumalanga	Gryzenhout, 2021
	Panaeolus subbalteatus	Gauteng, Pretoria	Goldman and Gryzenhout, 2019
	Panaeolus papilionaceus	Widespread across South Africa	Gryzenhout, 2021 Kinge *et al*., 2020
	Panaeolus caliginosus	South Africa	Kinge *et al*., 2020
	Panaeolus fimicoloides	South Africa	Kinge *et al*., 2020
	Panaeolus fimicola	South Africa	Kinge *et al*., 2020
	Panaeolus retitugus	South Africa	Kinge *et al*., 2020
	Panaeolus semivatus	South Africa	Kinge *et al*., 2020
	Panaeolus semiovatus f. exannulatus	South Africa	Kinge *et al*., 2020 Pearson, 1950
	Panaeolus solidipes	South Africa	Kinge *et al*., 2020
	Panaeolus sphinctrinus	South Africa	Kinge *et al*., 2020
	Panaeolus subbalteatus	South Africa	Kinge *et al*., 2020
	Panaeolus campanulatus	South Africa	Van der Westhuizen and Eicker 1988, Kinge *et al.,* 2020

Pluteus	*Pluteus salicinus*	Gauteng, Pretoria	Goldman and Gryzenhout, 2019 Kinge *et al*., 2020
	Pluteus romellii	Gauteng, Pretoria	Goldman and Gryzenhout, 2019 Kinge *et al*., 2020
	Pluteus atromarginatus	South Africa	Kinge *et al*., 2020
	Pluteus pellitus	South Africa	Kinge *et al*., 2020
	Pluteus semibulbosus	South Africa	Kinge *et al*., 2020
	Pluteus thomsonii	South Africa	Kinge *et al*., 2020
Gymnopilus	*Gymnopilus junonius*	Western Cape and Gauteng	Goldman and Gryzenhout, 2019, Gryzenhout, 2021, Kinge *et al*., 2020
	Gymnopilus purpureosquamulosus	Eastern Cape, Western Cape, Gauteng, Mpumalanga KwaZulu-Natal, Limpopo	Gryzenhout, 2021
	Gymnopilus subearlei	KwaZulu-Natal	Gryzenhout, 2021
	Gymnopilus penetrans	South Africa	Kinge *et al*., 2020
	Gymnopilus sapineus	South Africa	Kinge *et al*., 2020
	Gymnopilus hydridus	South Africa	Kinge *et al*., 2020

Table 2

Table 2: Species used in this study

Number	Specimen code	Genus	Collector	Country
1.	JL	*PSILOCYBE*	JON	Lesotho
2.	LP 225.1	*PSILOCYBE*	LIZ PAPICH	South Africa, Pretoria
3.	LP 227.1	*PSILOCYBE*	LIZ PAPICH	South Africa, Pretoria

Table 3

Table 3: Primers used for amplifying DNA barcoding of species.

PRIMER	**PRIMER SEQUENCE**	**SIZE**	**AMP SIZE**	**MELTING TEMPERATURE**	**REFERENCE**
ITS 1	5'-TCCGTAGGTGAACCTGCGG-3'	19bp	500 – 800 bp	54.0	White *et al.*, 1990 Romanelli *et al.*, 2014
ITS 4	5'-TCCTCCGCTTATTGATATGC-3	20bp	500 – 800 bp	54.0	White *et al.*, 1990
ITS1-F_KY02	5'- TAG AGG AAG TAA AAG TCG TAA -3'	20bp	200 - 1,000 bp	54.0	Toju *et al.,* 2012
ITS 4	5'-TCCTCCGCTTATTGATATGC-3	20bp	500 – 800 bp	54.0	White *et al.*, 1990
RPB PC-R1-F	5'-CTCTACTCGTTTCGCACCC-3'	19bp	246 bp	54.0	Zhang *et al.,* 2021
RPB PC-R1-R	5'-CGCACTCCTCGTTCAGC-3'	17bp	246 bp	54.0	Zhang *et al.,* 2021
TEF1 PC-EF-F	5'-FTTCATCAAGAAGGTCGGTTAC-3'	21bp	67 bp	54.0	Zhang *et al*., 2021
TEF1 PC-EF-R	5'-TCTCCGTGCCATCCAG-3'	16bp	67 bp	54.0	Zhang *et al*., 2021

Table 4

Table 4: Specimens of *Psilocybe* and outgroups used in this study.

Species	Voucher Number/ Isolate	**Location**	**ITS**	**TEF**
Psilocybe allenii	PRM 899876 Specimen voucher PRM 899876 Specimen voucher PRM 899876 isolate P26	USA	NR 119821.2 HE994440.1	HE994455.1
Psilocybe allenii	Specimen voucher PRM 899877 Specimen voucher PRM 899877 isolate 40	USA	HE994441.1	HE994456.1
Psilocybe cyanescens	PRM 901481 Voucher PRM 901481	Germany	NR 111478.1	GU565158.1
Psilocybe cyanescens	Voucher PRM 902040	Belgium	GU565176.1	GU565161.1
Psilocybe chuxiongensis	IFRD 414-011	China	NR 156572.1	KF586476.1
Psilocybe chuxiongensis	Voucher IFRD414-010	China	KF586470.1	KF586472.1
Psilocybe cubensis	Specimen voucher PRM: 909585	Czech Republic	HF912352.1	HF912338.1
Psilocybe cubensis	Voucher MFUL10-0335 18S ribosomal RNA gene partial sequence	Thailand	KF586478.1	KF586480.1
Psilocybe serbica	Isolate PRM 934302 basilicata	Italy	MF958471.1	MF958455.1
Psilocybe serbica	Isolate Cacialli Panchetti	Italy	MF958472.1	MF958456.1
Psilocybe serbica	Isolate 031022-25 legit C. Lavorato	Italy	MF958473.1	MF958457.1
Psilocybe azurescens	Voucher PRM 901020 18S small subunit ribosomal RNA gene partial sequence	USA: Oregon	GU565173.1	GU565159.1

Appendix B List of Figures

Figure 4.1

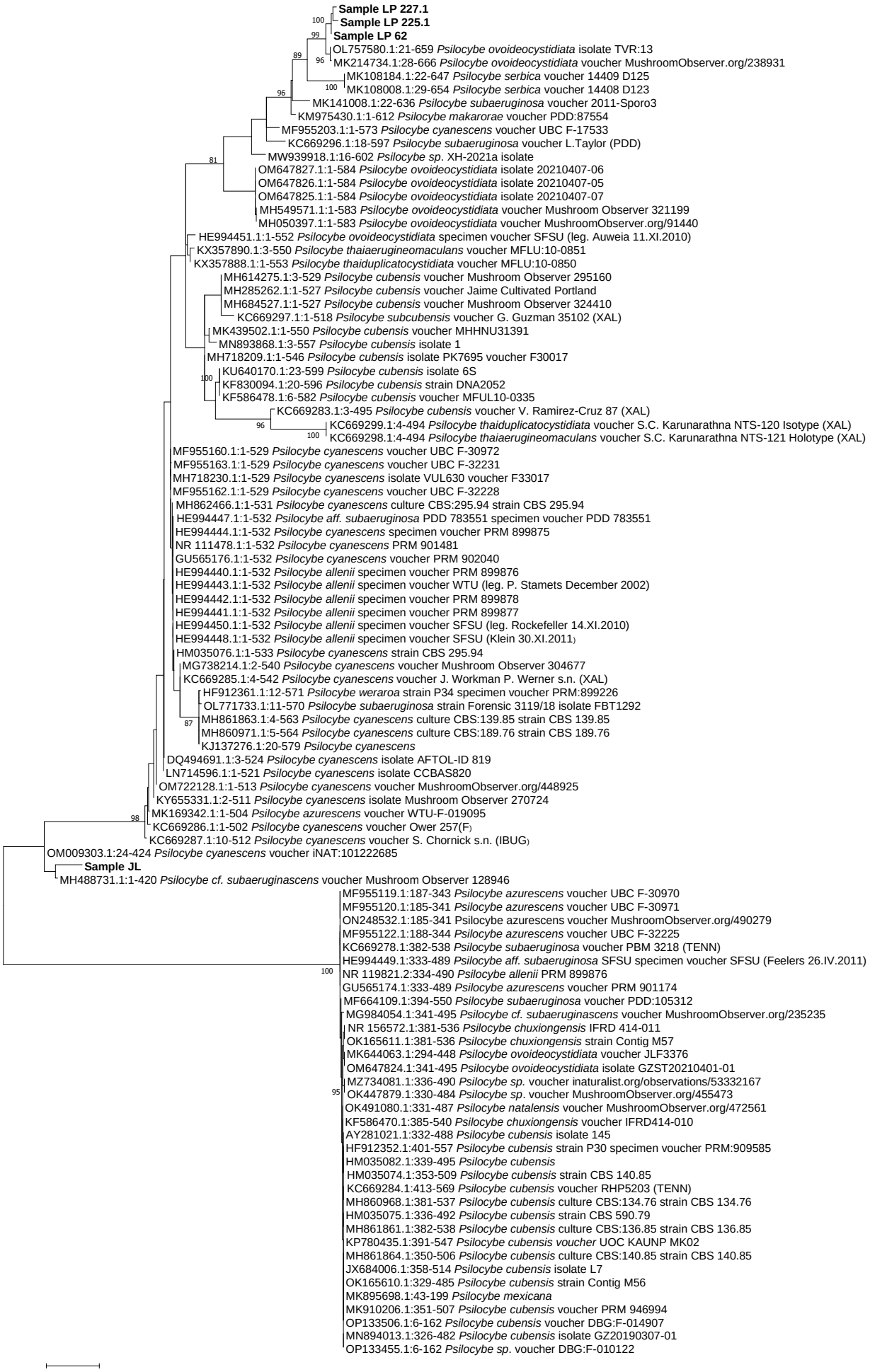
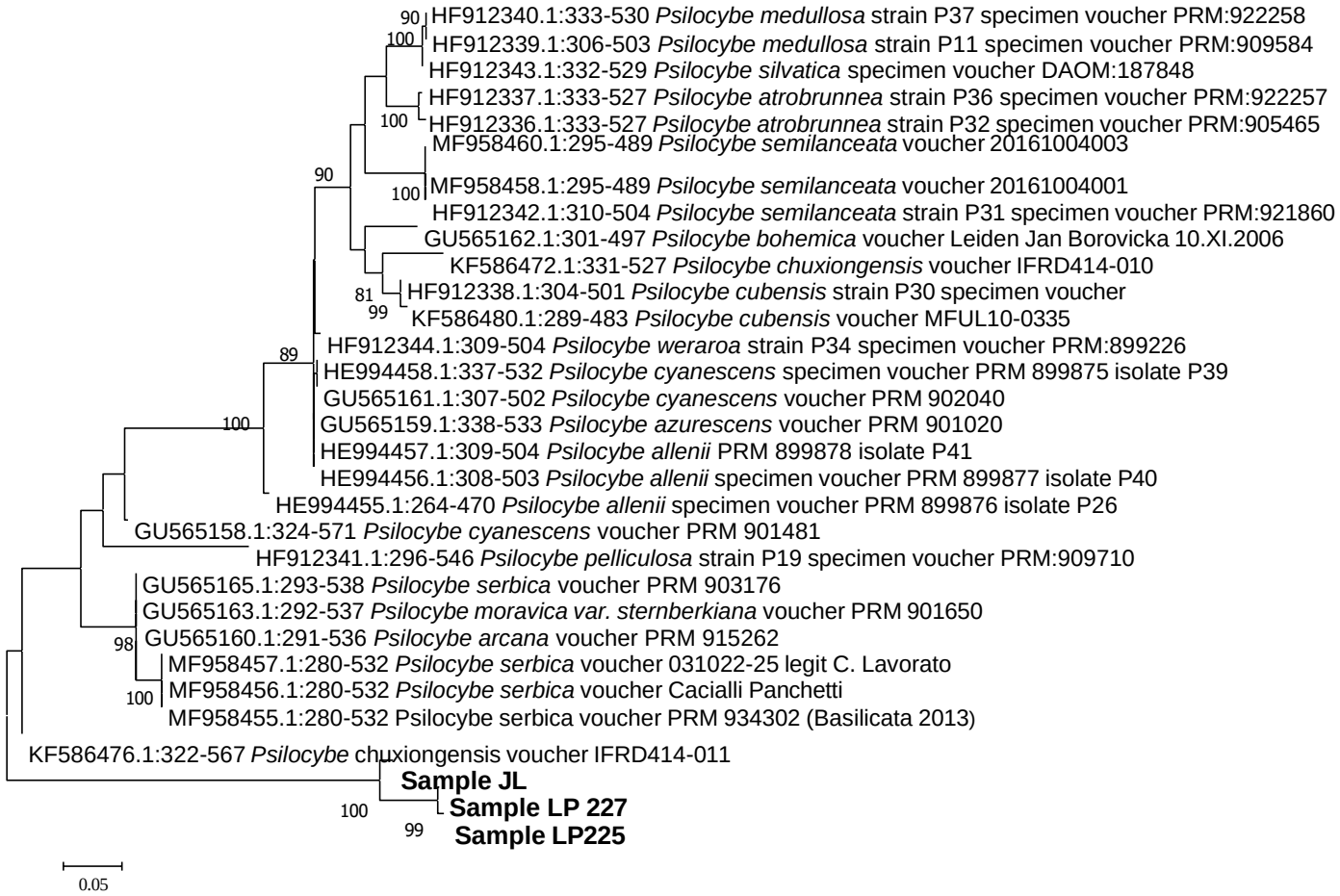

Sample LP 227.1
Sample LP 225.1
Sample LP 62
OL757580.1:21-659 *Psilocybe ovoideocystidiata* isolate TVR:13
MK214734.1:28-666 *Psilocybe ovoideocystidiata* voucher MushroomObserver.org/238931
MK108184.1:22-647 *Psilocybe serbica* voucher 14409 D125
MK108008.1:29-654 *Psilocybe serbica* voucher 14408 D123
MK141008.1:22-636 *Psilocybe subaeruginosa* voucher 2011-Sporo3
KM975430.1:1-612 *Psilocybe makarorae* voucher PDD:87554
MF955203.1:1-573 *Psilocybe cyanescens* voucher UBC F-17533
KC669296.1:18-597 *Psilocybe subaeruginosa* voucher L.Taylor (PDD)
MW939918.1:16-602 *Psilocybe sp*. XH-2021a isolate
OM647827.1:1-584 *Psilocybe ovoideocystidiata* isolate 20210407-06
OM647826.1:1-584 *Psilocybe ovoideocystidiata* isolate 20210407-05
OM647825.1:1-584 *Psilocybe ovoideocystidiata* isolate 20210407-07
MH549571.1:1-583 *Psilocybe ovoideocystidiata* voucher Mushroom Observer 321199
MH050397.1:1-583 *Psilocybe ovoideocystidiata* voucher MushroomObserver.org/91440
HE994451.1:1-552 *Psilocybe ovoideocystidiata* specimen voucher SFSU (leg. Auweia 11.XI.2010)
KX357890.1:3-550 *Psilocybe thaiaerugineomaculans* voucher MFLU:10-0851
KX357888.1:1-553 *Psilocybe thaiduplicatocystidiata* voucher MFLU:10-0850
MH614275.1:3-529 *Psilocybe cubensis* voucher Mushroom Observer 295160
MH285262.1:1-527 *Psilocybe cubensis* voucher Jaime Cultivated Portland
MH684527.1:1-527 *Psilocybe cubensis* voucher Mushroom Observer 324410
KC669297.1:1-518 *Psilocybe subcubensis* voucher G. Guzman 35102 (XAL)
MK439502.1:1-550 *Psilocybe cubensis* voucher MHHNU31391
MN893868.1:3-557 *Psilocybe cubensis* isolate 1
MH718209.1:1-546 *Psilocybe cubensis* isolate PK7695 voucher F30017
KU640170.1:23-599 *Psilocybe cubensis* isolate 6S
KF830094.1:20-596 *Psilocybe cubensis* strain DNA2052
KF586478.1:6-582 *Psilocybe cubensis* voucher MFUL10-0335
KC669283.1:3-495 *Psilocybe cubensis* voucher V. Ramirez-Cruz 87 (XAL)
KC669299.1:4-494 *Psilocybe thaiduplicatocystidiata* voucher S.C. Karunarathna NTS-120 Isotype (XAL)
KC669298.1:4-494 *Psilocybe thaiaerugineomaculans* voucher S.C. Karunarathna NTS-121 Holotype (XAL)
MF955160.1:1-529 *Psilocybe cyanescens* voucher UBC F-30972
MF955163.1:1-529 *Psilocybe cyanescens* voucher UBC F-32231
MH718230.1:1-529 *Psilocybe cyanescens* isolate VUL630 voucher F33017
MF955162.1:1-529 *Psilocybe cyanescens* voucher UBC F-32228
MH862466.1:1-531 *Psilocybe cyanescens* culture CBS:295.94 strain CBS 295.94
HE994447.1:1-532 *Psilocybe aff. subaeruginosa* PDD 783551 specimen voucher PDD 783551
HE994444.1:1-532 *Psilocybe cyanescens* specimen voucher PRM 899875
NR 111478.1:1-532 *Psilocybe cyanescens* PRM 901481
GU565176.1:1-532 *Psilocybe cyanescens* voucher PRM 902040
HE994440.1:1-532 *Psilocybe allenii* specimen voucher PRM 899876
HE994443.1:1-532 *Psilocybe allenii* specimen voucher WTU (leg. P. Stamets December 2002)
HE994442.1:1-532 *Psilocybe allenii* specimen voucher PRM 899878
HE994441.1:1-532 *Psilocybe allenii* specimen voucher PRM 899877
HE994450.1:1-532 *Psilocybe allenii* specimen voucher SFSU (leg. Rockefeller 14.XI.2010)
HE994448.1:1-532 *Psilocybe allenii* specimen voucher SFSU (Klein 30.XI.2011)
HM035076.1:1-533 *Psilocybe cyanescens* strain CBS 295.94
MG738214.1:2-540 *Psilocybe cyanescens* voucher Mushroom Observer 304677
KC669285.1:4-542 *Psilocybe cyanescens* voucher J. Workman P. Werner s.n. (XAL)
HF912361.1:12-571 *Psilocybe weraroa* strain P34 specimen voucher PRM:899226
OL771733.1:11-570 *Psilocybe subaeruginosa* strain Forensic 3119/18 isolate FBT1292
MH861863.1:4-563 *Psilocybe cyanescens* culture CBS:139.85 strain CBS 139.85
MH860971.1:5-564 *Psilocybe cyanescens* culture CBS:189.76 strain CBS 189.76
KJ137276.1:20-579 *Psilocybe cyanescens*
DQ494691.1:3-524 *Psilocybe cyanescens* isolate AFTOL-ID 819
LN714596.1:1-521 *Psilocybe cyanescens* isolate CCBAS820
OM722128.1:1-513 *Psilocybe cyanescens* voucher MushroomObserver.org/448925
KY655331.1:2-511 *Psilocybe cyanescens* isolate Mushroom Observer 270724
MK169342.1:1-504 *Psilocybe azurescens* voucher WTU-F-019095
KC669286.1:1-502 *Psilocybe cyanescens* voucher Ower 257(F)
KC669287.1:10-512 *Psilocybe cyanescens* voucher S. Chornick s.n. (IBUG)
OM009303.1:24-424 *Psilocybe cyanescens* voucher iNAT:101222685
Sample JL
MH488731.1:1-420 *Psilocybe cf. subaeruginascens* voucher Mushroom Observer 128946
MF955119.1:187-343 *Psilocybe azurescens* voucher UBC F-30970
MF955120.1:185-341 *Psilocybe azurescens* voucher UBC F-30971
ON248532.1:185-341 Psilocybe azurescens voucher MushroomObserver.org/490279
MF955122.1:188-344 *Psilocybe azurescens* voucher UBC F-32225
KC669278.1:382-538 *Psilocybe subaeruginosa* voucher PBM 3218 (TENN)
HE994449.1:333-489 *Psilocybe aff. subaeruginosa* SFSU specimen voucher SFSU (Feelers 26.IV.2011)
NR 119821.2:334-490 *Psilocybe allenii* PRM 899876
GU565174.1:333-489 *Psilocybe azurescens* voucher PRM 901174
MF664109.1:394-550 *Psilocybe subaeruginosa* voucher PDD:105312
MG984054.1:341-495 *Psilocybe cf. subaeruginascens* voucher MushroomObserver.org/235235
NR 156572.1:381-536 *Psilocybe chuxiongensis* IFRD 414-011
OK165611.1:381-536 *Psilocybe chuxiongensis* strain Contig M57
MK644063.1:294-448 *Psilocybe ovoideocystidiata* voucher JLF3376
OM647824.1:341-495 *Psilocybe ovoideocystidiata* isolate GZST20210401-01
MZ734081.1:336-490 *Psilocybe sp.* voucher inaturalist.org/observations/53332167
OK447879.1:330-484 *Psilocybe sp*. voucher MushroomObserver.org/455473
OK491080.1:331-487 *Psilocybe natalensis* voucher MushroomObserver.org/472561
KF586470.1:385-540 *Psilocybe chuxiongensis* voucher IFRD414-010
AY281021.1:332-488 *Psilocybe cubensis* isolate 145
HF912352.1:401-557 *Psilocybe cubensis* strain P30 specimen voucher PRM:909585
HM035082.1:339-495 *Psilocybe cubensis*
HM035074.1:353-509 *Psilocybe cubensis* strain CBS 140.85
KC669284.1:413-569 *Psilocybe cubensis* voucher RHP5203 (TENN)
MH860968.1:381-537 *Psilocybe cubensis* culture CBS:134.76 strain CBS 134.76
HM035075.1:336-492 *Psilocybe cubensis* strain CBS 590.79
MH861861.1:382-538 *Psilocybe cubensis* culture CBS:136.85 strain CBS 136.85
KP780435.1:391-547 *Psilocybe cubensis voucher* UOC KAUNP MK02
MH861864.1:350-506 *Psilocybe cubensis* culture CBS:140.85 strain CBS 140.85
JX684006.1:358-514 *Psilocybe cubensis* isolate L7
OK165610.1:329-485 *Psilocybe cubensis* strain Contig M56
MK895698.1:43-199 *Psilocybe mexicana*
MK910206.1:351-507 *Psilocybe cubensis* voucher PRM 946994
OP133506.1:6-162 *Psilocybe cubensis* voucher DBG:F-014907
MN894013.1:326-482 *Psilocybe cubensis* isolate GZ20190307-01
OP133455.1:6-162 *Psilocybe sp*. voucher DBG:F-010122
100
99
89
96
100
96
81
100
96
100
87
98
100
95
0,1

Figure 4.1: Unrooted phylogenetic tree (phylogram) representing a Maximum Likelihood analysis for Psilocybe species based on DNA sequences of the Internal Transcribed region.

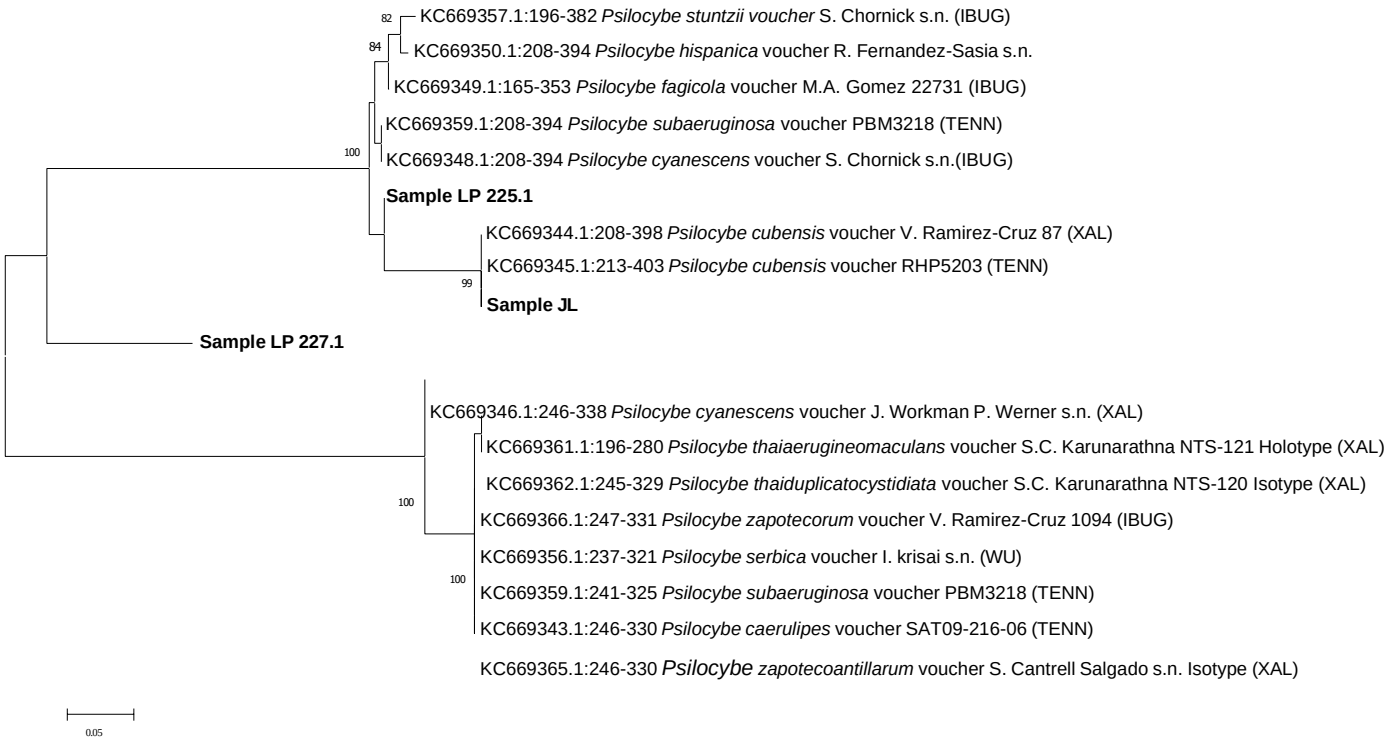
Figure 4.2



The bootstrap values are indicated on the branch

Figure 4.2: Phylogenetic tree (phylogram) representing a Maximum Likelihood analysis for *Psilocybe* species based on the translation elongation factor 1-alpha region. The bootstrap values are indicated on the branch.

Figure 4.3

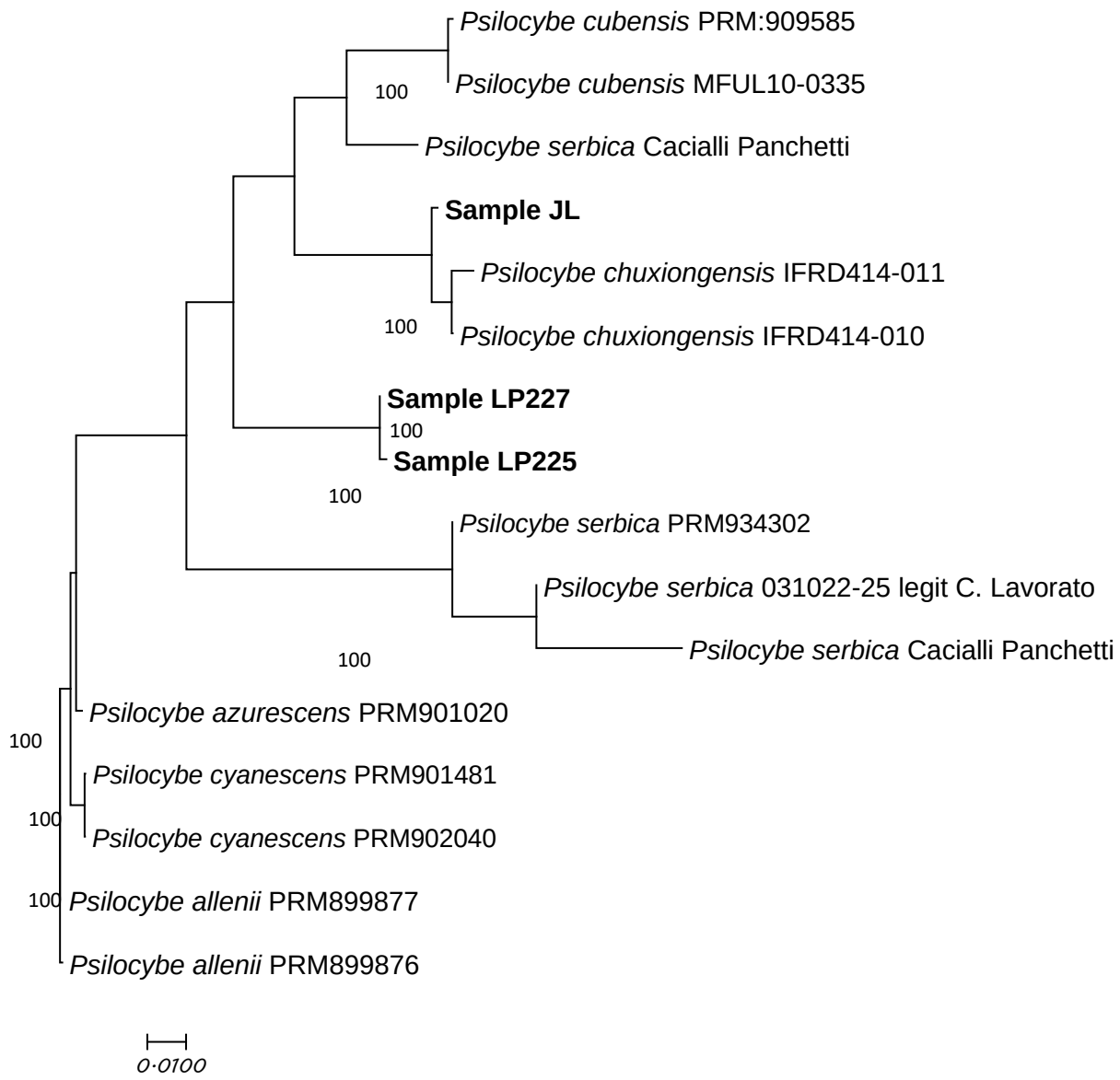


7 The bootstrap values are indicated on the branch

8 Figure 4.3: Phylogenetic tree (phylogram) representing a Maximum Likelihood analysis for *Psilocybe*

9 species based on the the RNA polymerase II (RPB-1) region.

Figure 4.4


Psilocybe cubensis PRM:909585
Psilocybe cubensis MFUL10-0335
100
Psilocybe serbica Cacialli Panchetti
Sample JL
Psilocybe chuxiongensis IFRD414-011
100
Psilocybe chuxiongensis IFRD414-010
Sample LP227
100
Sample LP225
100
Psilocybe serbica PRM934302
Psilocybe serbica 031022-25 legit C. Lavorato
Psilocybe serbica Cacialli Panchetti
100
Psilocybe azurescens PRM901020
100
Psilocybe cyanescens PRM901481
100
Psilocybe cyanescens PRM902040
100
Psilocybe allenii PRM899877
Psilocybe allenii PRM899876
0·0100

The bootstrap values are indicated on the branch

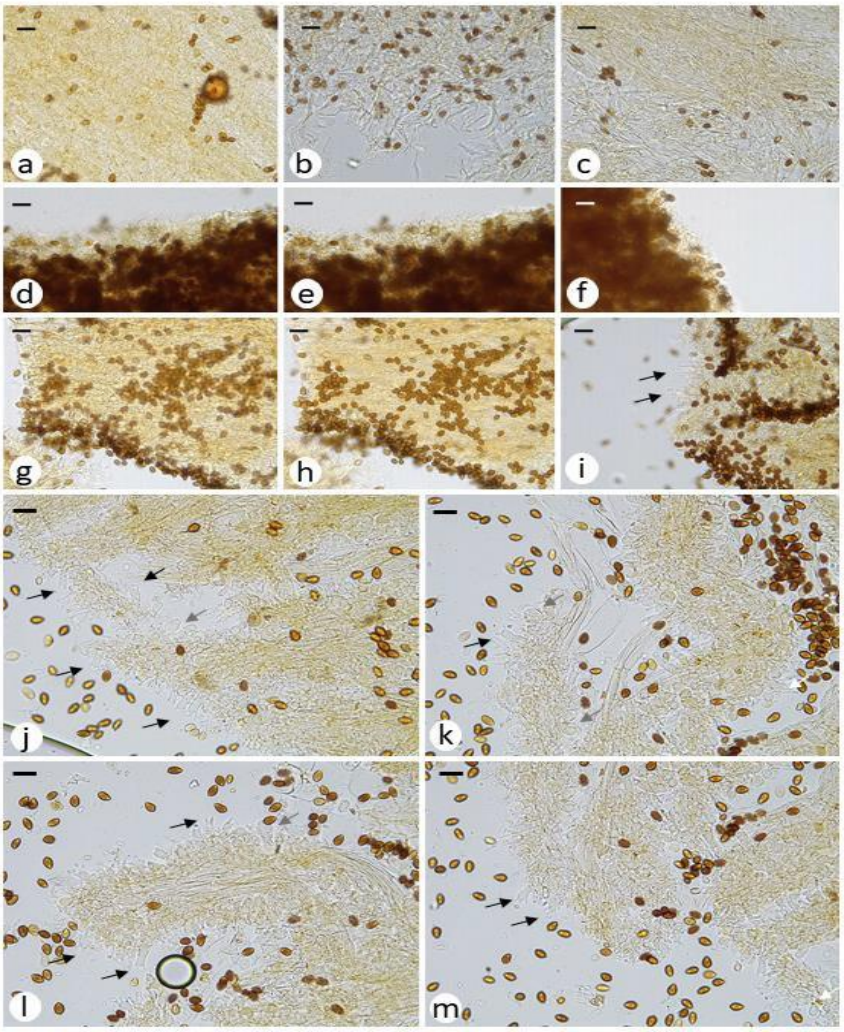
Figure 4.4: Phylogenetic tree (phylogram) representing a Maximum Likelihood analysis for *Psilocybe* species based on the combined regions of the Translation elongation factor 1-alpha, Internal Transcribed region, RNA polymerase II (RPB-1).

Figure 4.5

14 Figure 4.5 (a-f). *Psilocybe orontawuli*. Spore deposit indicated by black arrow. (g-m).

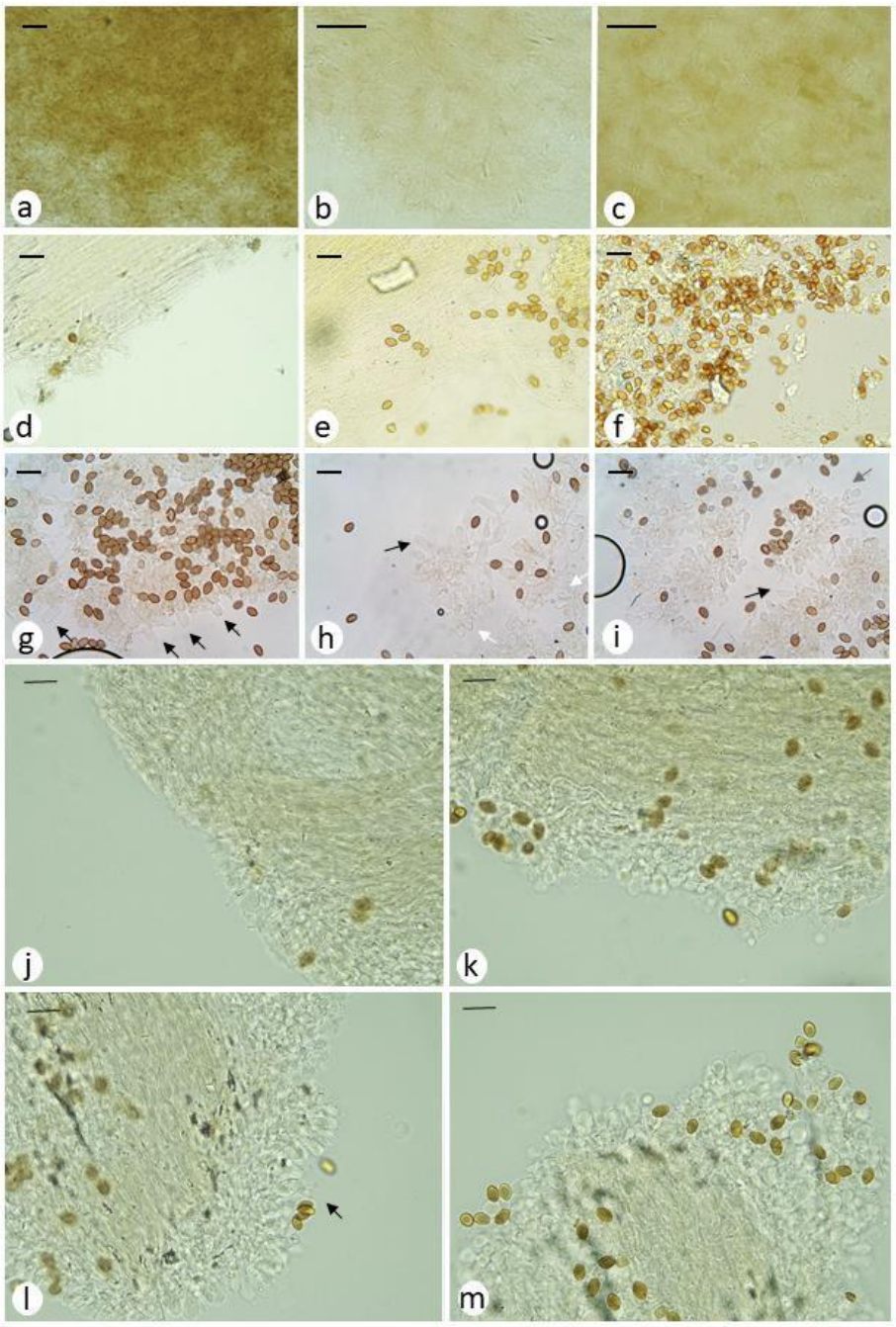
15 *Psilocybe malotiens*

Figure 4.6



16 Fig. 4.6 Micrographs of *Psilocybe orontawuli*. (a-c) Pileipellis. (d-f) Cheilocystidia.
17 (g) Hymenphoral trama. (h-i) Basidiospores. (j-m) Basidia (black arrows) and
18 pleurocystidia (grey arrows). Scale bars 20 µm.

Figure 4.7



19 Fig. 4.7 Micrographs of *Psilocybe malotiensis.* (a-c) Pileipellis. (d) Stipitipellis. (e-f)
20 Basidiospores. (g-i) Basidia (black arrows) and pleurocystidia (grey arrows). (j-m)
21 Cheilocystidia. Scale bars 20 µm.